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Materials and Methods

Cell lines

The following breast cancer cell lines BT549, HCC38, HCC1143, MDA-MB-436; the immortalised mammary epithelial cell line MCF10A and the immortalised retinal pigment epithelial cell line RPE-1 were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA). The breast cancer cell line SUM159 was obtained from Asterand (Royston, UK). All cell lines were grown in 5% CO₂ at 37°C. BT549 was grown in DMEM Medium (Gibco, ThermoFisher Scientific, Waltham, MA, USA) supplemented with 10% fetal bovine serum (FBS) (Gibco, ThermoFisher Scientific, Waltham, MA, USA) and Recombinant Human Insulin (10µg/mL; Sigma-Aldrich, St. Louis, MO, USA). HCC38 and HCC1143 were grown in RPMI-1640 Medium (Gibco, ThermoFisher Scientific, Waltham, MA, USA) supplemented with 10% fetal bovine serum (FBS) (Gibco, ThermoFisher Scientific, Waltham, MA, USA). MDA-MB-436 was grown in RPMI-1640 Medium (Gibco, ThermoFisher Scientific, Waltham, MA, USA) supplemented with 10% fetal bovine serum (FBS) (Gibco, ThermoFisher Scientific, Waltham, MA, USA) and Recombinant Human Insulin (10µg/mL; Sigma-Aldrich, St. Louis, MO, USA). MCF10A was grown in DMEM/F-12 (Gibco, ThermoFisher Scientific, Waltham, MA, USA) supplemented with 5% Horse Serum (Gibco, ThermoFisher Scientific, Waltham, MA, USA), EGF (20ng/mL; Peprotech, London, UK), Hydrocortisone (0.5mg/mL; Sigma-Aldrich, St. Louis, MO, USA), Cholera Toxin (100ng/mL; Sigma-Aldrich, St. Louis, MO, USA), and Recombinant Human Insulin (0.1mg/mL; Sigma-Aldrich, St. Louis, MO, USA). SUM159 cells were grown in Ham’s F-12 Nutrient Mixture (Gibco, ThermoFisher Scientific, Waltham, MA, USA) supplemented with 5% FBS (Gibco, ThermoFisher Scientific, Waltham, MA, USA), HEPES (10nM; Sigma-Aldrich, St.
Louis, MO, USA), Hydrocortisone (1µg/mL; Sigma-Aldrich, St. Louis, MO, USA), and Recombinant Human Insulin (5µg/mL; Sigma-Aldrich, St. Louis, MO, USA). Each media was prepared according to the supplier’s recommendations. All cell lines were grown in the presence of Penicillin-Streptomycin (Gibco, Thermo Fisher Scientific, Waltham, MA, USA) at a final concentration of 100 U/mL. Doxycycline hyclate (Sigma-Aldrich, St. Louis, MO, USA) was added to cells at a final concentration of 0.125mg/mL, 0.25mg/mL and 0.5mg/mL for SUM159, RPE1 and MCF10A cells, respectively. STR validated cell line stocks were used and all cell lines used were within 30 passages of this STR typing. All cells were routinely tested for mycoplasma.

**Lentiviral p20 HORMAD1 and GFP construct generation**

HORMAD1 or GFP cDNAs were cloned into the pInducer20 lentivirus expression vector (a gift from Stephen Elledge, Addgene plasmid # 44012) according to a protocol described previously (1). Lentiviral particles were produced in HEK293T cells by co-transfection of the relevant pINDUCER20 plasmids with the pMD2.G (Addgene plasmid # 12259) and psPAX2 (Addgene plasmid # 12260) packaging vectors, which were a gift from Didier Trono. Virus-containing cell culture media was collected for downstream transduction of cell lines and the virus titre was estimated in SUM159 using serial dilution of viral supernatants and selection with 0.5mg/ml Geneticin (Gibco, Thermo Fisher Scientific, Waltham, MA, USA).

**Generation of isogenic and clonal cell systems**

Parental (heterogenous) SUM159 cells were isolated in 96 well plates using serial dilutions to obtain a final density of 1 cell/well. Colony formation was monitored by light microscopy and single-cell colonies were expanded to generate “parental clones”. Parental clones were plated into 6-well plates, infected with lentiviral vectors at a multiplicity of infection (MOI) of 1. After two days, selection was initiated by adding 0.5
mg/mL of Geneticin (Gibco, Thermo Fisher Scientific, Waltham, MA, USA) and continued for at least three days. Single-cell clones were derived for a second time in order to achieve isogenic clonally-derived cells, with doxycycline-inducible HORMAD1 expression. Clones with doxycycline-inducible HORMAD1 were selected using western blotting, whilst GFP-expressing clones were selected using a flow cytometry. The same process was performed to obtain MCF10A and RPE1 cell lines, but without the initial selection of single cell clones.

**Primary RNA Interference Screening**

Cells from a SUM159 parental clone and a clonally-derived HORMAD1-expressing SUM159 sub-line, were reverse transfected with a Dharmacon SMARTpool siRNA library (Dharmacon, Lafayette, CO, USA) targeting a total of 1280 genes. This included: 720 genes encoding the kinome and kinase-related genes, 80 tumour suppressor genes, and 480 genes featured in the Cancer Gene Census (Table S1), as previously described (2). siRNA oligos were arrayed in 384-well plates which included the positive control (siPLK1; Dharmacon, Lafayette, CO, USA) and multiple non-targeting controls (siCON1 and siCON2; Dharmacon, Lafayette, CO, USA and AllStar; QIAGEN, Hilden, Germany). 20nM of siRNA and 0.05μL of transfection reagent RNAiMAX (Invitrogen, Thermo Fisher Scientific, Waltham, MA, USA) were used per well. Screens were carried out in triplicate. Twenty-four hours post-transfection, cells were treated with doxycycline (0.5 μg/ml) for induction of HORMAD1 expression, or vehicle (DMSO; Sigma-Aldrich, St. Louis, MO, USA). Cell viability was measured 5 days post-transfection using CellTiter-Glo (Promega, Madison, WI, USA).

Data processing and quality control analysis were conducted using the cellHTS2 R package as described previously (3, 4). Drug Effect was calculated as follows: $x_{DE} = (x_{doxycycline} - x_{DMSO})$. Where: $x_{DE}$ = drug effect for siRNA $x$, $x_{doxycycline} = \log_2$ plate-
centred luminescence value for siRNA $x$ in the doxycycline arm, $x_{DMSO} = \log_2$ plate-centred luminescence value for siRNA $x$ in the DMSO arm.

(DE)-Z scores were then calculated using the formula: $DE\; Z-score = \frac{x_{DE} - \mu_{DE}}{\sigma_{DE}}$. Where: $x_{DE} =$ Drug Effect for siRNA $x$, $\mu_{DE} =$ Median Drug Effect for entire library, $\sigma_{DE} =$Median Absolute Deviation of Drug Effects across entire library. This methodology was described previously (5). For subsequent interrogation, we selected genes with a DE-Z score of less than -3, indicating a HORMAD1/doxycycline induced gene sensitivity. We removed genes which also had a DE-Z of less than -2 in the parental cell line. We also removed inherently toxic genes (Z score of less than -3).

**Single oligo siRNA deconvolution RNA interference validation**

Secondary validation experiments were performed with the same methodology as the primary screen but using sets of four individual targeting siRNAs (Dharmacon, Lafayette, CO, USA), as well as siRNA SMARTpools (Dharmacon, Lafayette, CO, USA) utilised in the primary screen. Data were analysed and presented using the normalised percentage inhibition, $NPI= \frac{x-c_-}{c_+ - c_-} \times 100$. Where: $x =$ raw luminescence value for siRNA $x$, $c_- =$ average raw luminescence value for non-targeting siRNA control (siALLSTAR), $c_+ =$ average raw luminescence value for targeting siRNA control (siPLK1), as described previously (6). Data from triplicate wells of a single replicate are shown.

**Validation of gene knockdown by RT-qPCR**

Gene knockdown following treatment with each siRNA was determined by RT-qPCR. Briefly, total RNA was isolated using the RNeasy Mini kit (QIAGEN, Hilden, Germany) for 6-well plate transfections, and the MagMAX-96 Total RNA Isolation Kit (Ambion, Thermo Fisher Scientific, Waltham, MA, USA) for 96-well plate transfections and cDNA generated with the High-Capacity cDNA Reverse Transcription kit (Applied
Biosystems, Foster City, CA, USA). Reverse-transcription quantitative PCR (RT-qPCR) was performed using PowerUp SYBR Green Master Mix (Applied Biosystems, Foster City, CA, USA), on a 7900 HT Fast Real-Time PCR system RT-PCR (Applied Biosystems, Foster City, CA, USA). Primer oligos were purchased from Integrated DNA Technologies (Coralville, IA, USA). Primer sequences are shown in Table S6.

**Long-term siRNA-based viability assays**

For long-term cell viability assays in SUM159 cells, cells were treated with doxycycline (0.125 μg/ml) for 72 hours to induce HORMAD1 expression. Cells were then plated in 96-well plates (600 cells/well) and forward transfected with 25nM AllStar non-targeting siRNA (QIAGEN, Hilden, Germany) or targeting siRNAs (Dharmacon, Lafayette, CO, USA), using 0.1μL RNAiMax (Invitrogen, Thermo Fisher Scientific, Waltham, MA, USA) in a total volume of 100μL. Twenty-four hours after transfection, cells were passaged to 6-well plates in triplicate and grown for 11 days in the presence of doxycycline (0.125 μg/ml). Media containing fresh doxycycline was replaced every 72 hours. Cell viability was measured using CellTiter-Blue (Promega, Madison, WI, USA). Data are presented as surviving fraction, relative to mock-transfected cells. For clonogenic survival assays, SUM159, MCF10A and RPE-1 cells were treated with doxycycline (0.125 μg/ml, 0.5 μg/ml and 0.25 μg/ml for SUM159, MCF10A and RPE1, respectively) for 72 hours, plated in 96-well plates (700 cells/well and 400 cells/well respectively), forward transfected and passaged as described above. Cells were fixed with 10% trichloroacetic acid (Thermo Fisher Scientific, Waltham, MA, USA) and stained with sulphorhodamine B (Sigma-Aldrich, St. Louis, MO, USA). Colonies were counted manually. All cell-based assays were performed in triplicate wells and data shown is representative of 2 or more replicates.
siRNA mediated knockdown experiments in HORMAD1 positive cell lines

BT549, HCC38, HCC1143, MDA-MB-436 cells were plated in 96-well plates (1000 cells/well) and forward transfected with 25nM AllStar non-targeting siRNA (QIAGEN, Hilden, Germany) or targeting siRNAs (Dharmacon, Lafayette, CO, USA), using 0.1μL RNAiMax (Invitrogen, Thermo Fisher Scientific, Waltham, MA, USA) in a total volume of 100μL. Cell viability was estimated 7 days post transfection using CellTiter-Glo (Promega, Madison, WI, USA). Data were analysed and presented as normalised percentage inhibition (NPI).

CRISPR-Cas9-based viability assays

pLentiCRISPR-mCherry was a gift from Beat Bornhauser (Addgene plasmid # 75161, (7)). Edit-R crRNA sequences targeting POLH were custom-designed using CRISPR v4.2 software (TEFOR) (8). Target sequences of each crRNA are listed in Table S7. Stable Cas9-expressing cell lines were generated by lentivirus-mediated transduction of the pLentiCRISPR-mCherry construct, followed by FACS sorting of mCherry-positive cells using a BD FACSaria II flow cytometer (BD Biosciences, San Jose, CA, USA). For SUM159 experiments, cells were plated in 24-well plates and forward transfected with 10nM of Edit-R synthetic crRNA (Dharmacon, Lafayette, CO, USA) and 10nM of tracrRNA (Dharmacon, Lafayette, CO, USA) in a 1:1 ratio using 0.5μL RNAiMAX (Invitrogen, Thermo Fisher Scientific, Waltham, MA, USA), in a total volume of 500μL. After 24 hours, cells were passaged to 6-well plates in triplicate and grown for 11 days in the presence of doxycycline (0.125 μg/ml). Cell viability was measured using CellTiter-Blue (Promega, Madison, WI, USA). Data are presented as surviving fraction, relative to mock-transfected cells. For experiments in HCC38 and BT549, cells were plated in 96-well plates (1000 cells/well) and forward transfected with 10nM of Edit-R synthetic crRNA and 10nM of tracrRNA (Dharmacon, Lafayette, CO, USA)
using 0.05μL RNAiMAX (Invitrogen, Thermo Fisher Scientific, Waltham, MA, USA), in a total volume of 50μL. Cell proliferation was assessed by Incucyte (Sartorius, Göttingen, Germany) time-lapse within-incubator microscopy (4x zoom, 1 image per day) over a 6-day period, using the Cas9-mCherry signal as a marker of total nuclei number. Data are presented as the relative number of nuclei, normalised to mock-transfected cells. Assays were performed in triplicate wells and data shown is representative of 2 or more replicates.

**Clonogenic dose-response survival assays**

The ATR inhibitors VE-821, VX-970/M6620 (berzosertib), AZ20 and AZD6738 (ceralasertib) were purchased from Selleck Chemicals (Houston, TX, USA). For clonogenic dose-response survival assays, cells were seeded in 6-well plates at a density of 200 to 300 cells per well and continuously exposed to drug 16 hours after seeding, for 14 days. Media containing fresh drug was replaced every 72 hours. Cells were fixed with 10% trichloroacetic acid and stained with 0.4% sulphorhodamine B (SRB) (Sigma-Aldrich, St. Louis, MO, USA) in 1% acetic acid. SRB was solubilised with 10 mmol/L Tris-base (Sigma-Aldrich, St. Louis, MO, USA) and plates were read at 490 nm using a Wallac Victor 1420 multilabel counter (Perkin-Elmer, Waltham, MA, USA). Assays were performed in triplicate wells and data shown is representative of 2 or more replicates.

**Western blot analysis**

Cell lysates were prepared using 2% sodium dodecyl sulphate (SDS) (Invitrogen, Thermo Fisher Scientific, Waltham, MA, USA) and subsequently sonicated (2 cycles of 5 seconds, 80% amplitude). Cell lysates equivalent to 15-20μg of protein were diluted in 4x Laemmlı sample buffer (277.8 mM Tris-HCl, pH6.8, 44.4% (v/v) glycerol, 4.4% SDS and 0.02% bromophenol blue), boiled for 5 minutes at 95°C and resolved
by SDS-PAGE gel electrophoresis. Proteins were transferred onto a nitrocellulose membrane using western blotting. Membranes were then blocked in 5% milk/TBS-T (20mM Tris, 150 mM NaCl, 0.1% Tween 20), and probed with primary antibody overnight at 4°C (diluted in 5% milk/TBS-T). The following primary antibodies were used in this study: HORMAD1 (1:500, HPA037850, Sigma-Aldrich, St. Louis, MO, USA), POLH (1:500, 13848, Cell Signaling, Danvers, MA, USA), Actin (1:30000, A5316, Sigma-Aldrich, St. Louis, MO, USA), Tubulin (1:5000, T5168, Sigma-Aldrich, St. Louis, MO, USA), Vinculin (1:5000, V9131, Sigma-Aldrich, St. Louis, MO, USA). Following primary antibody incubation, membranes were washed 4x with TBS-T for 5 minutes at room temperature, and then incubated with a secondary antibody (raised against the species of the primary antibody) conjugated to HRP. Membranes were washed a further 4 times for 5 minutes at room temperature before imaging using electrochemiluminescence (ECL) (GE Healthcare, Chicago, IL, USA).

**Immunofluorescence analysis of γH2AX**

Clonally-derived HORMAD1-inducible SUM159 cells were treated with doxycycline (0.125 μg/ml) for 48 hours, to induce HORMAD1 expression. Cells were then grown on Borosilicate Glass 22mm square coverslips (Fisher Scientific, Leicestershire, UK) for 24 hours in the presence of doxycycline (0.125 μg/ml) (72 hours total). Cells were fixed in 4% (v/v) PFA (Thermo Fisher Scientific, Waltham, MA, USA) and permeabilised with 0.2% (v/v) Triton X-100 (Sigma-Aldrich, St. Louis, MO, USA) in PBS for 10 minutes at room temperature. Coverslips were incubated with DNase I (Sigma-Aldrich, St. Louis, MO, USA) diluted to 1000 units/ml in PBS, for 2 hours in a humidified chamber at 37°C. Cells were immunostained with primary antibody against γH2AX (05-636, Merck Millipore, Burlington, MA, USA) diluted 1:1000 in IFF buffer (1% bovine serum albumin, 2% foetal calf serum in PBS). Cells were washed 3x with
PBS, incubated with fluorescently labelled anti-mouse Alexa Fluor® 555 (Invitrogen, Thermo Fisher Scientific, Waltham, MA, USA) secondary antibody for 1 hour at room temperature, washed another 3x with PBS, and then counterstained with 1µg/mL DAPI (Invitrogen, Thermo Fisher Scientific, Waltham, MA, USA). Coverslips were mounted onto microscope slides using Vectashield antifade mounting medium (Vector Laboratories Inc., Burlingame, CA, USA) and imaged at 100x on a Nikon A1 confocal microscope (Nikon, Tokyo, Japan). Data was quantified as percentage of DAPI-stained nuclei with >5 γH2AX foci. Approximately 150 cells were scored per condition per replicate and 4 replicates were performed.

**Aberrant nuclear structure analysis**

Clonally-derived HORMAD1(or GFP)-inducible SUM159 cells were passaged in continuous presence of doxycycline (0.125 µg/ml) for 8 weeks. Cells were then seeded on Borosilicate Glass 22mm coverslips (Fisher Scientific, Leicestershire, UK) and grown for 24 hours. Cells were fixed in 4% (v/v) PFA (Thermo Fisher Scientific, Waltham, MA, USA), permeabilised in Triton X-100 (0.2%) and stained with 1µg/mL DAPI (Thermo Fisher Scientific, Waltham, MA, USA). Slides were analysed by eye at 100x on a Zeiss fluorescent microscope (Carl Zeiss, Oberkochen, Germany). Approximately 150 cells were scored for aberrant nuclear structures (micronuclei, nuclear buds and anaphase bridges) per condition, per replicate and 4 replicates were performed.

**STRING Protein Network analysis**

STRING Protein Network Analysis (9) was carried out by importing the list of 63 candidate genes into the multiple proteins search tab and selecting *Homo sapiens* as the organism. MCL clustering with inflation parameter of 3 was used as the clustering method. For network settings, the following settings were used: 1) meaning of network
edges: evidence; 2) active interaction sources: textmining, experiments, databases, co-expression, neighborhood, gene fusion and co-occurrence; 3) minimum required interaction score: medium confidence (0.400); 4) network display mode: interactive svg.

**Statistical analysis**

All data are presented as the mean with standard deviation, from 3 replicate experiments. All pair-wise statistical analysis was performed using parametric t-tests, unless otherwise stated.
Supplementary Figures

Supplementary Figure Legends

Fig. S1: Doxycycline-inducible HORMAD1 expression drives genomic instability. A Western blot analysis of doxycycline-induced HORMAD1 expression in two clonally-derived HORMAD1-inducible SUM159 cell lines. Asterisk refers to a non-specific band. B Western blot analysis of doxycycline-induced HORMAD1 expression in isogenic cell line models compared expression in the HORMAD1 positive cell line MDAMB436. C Scatter dot plot displaying increased percentage of cells with >5 γH2AX foci per DAPI-stained nucleus, measured after acute expression of HORMAD1 (72 hours). Approximately 150 cells were analysed per condition. Each dot represents a single biological replicate (n= 4). p values represent Fisher’s exact test. D Representative immunofluorescence images of γH2AX foci. E Scatter dot plot displaying increased percentage of aberrant nuclear structures (micronuclei, nuclear buds, nucleoplasmic bridges) per DAPI-stained nucleus measured 8 weeks after continuous expression of HORMAD1. Approximately 150 cells were analysed per condition. Each dot represents a single biological replicate (n= 4). p values represent Fisher’s exact test. F Representative immunofluorescence images of aberrant nuclear structures. G Flow cytometry analysis of doxycycline-induced GFP expression in two clonally-derived GFP-inducible SUM159 cell lines. H Scatter dot plot displaying no significant changes in the percentage of aberrant nuclear structures (micronuclei, nuclear buds, nucleoplasmic bridges) per DAPI-stained nucleus measured 8 weeks after continuous expression of GFP. Approximately 150 cells were analysed per condition. Each dot represents a single biological replicate (n= 4). p values represent Fisher’s exact test.
**Fig. S2: RNAi screen quality control data.** A-B Representative Z’ factor analysis showing the distribution of positive (siPLK1) and negative control (siCON1, siCON2, ALLSTAR) Z-score values. A Z’ factor value >0.3 indicates a good separation of positive and negative control Z-score values. C Boxplot illustrating the distribution of Z-scores of positive and negative control siRNAs for each technical replicate in the clonally-derived HORMAD1-inducible SUM159 doxycycline experimental arm. Boxes represent individual Z-scores and error bars represent the SD. D Representative Spearman’s correlation analysis of Z-scores from replicate 1 and 2 in the clonally-derived HORMAD1-inducible SUM159 doxycycline experimental arm ($r^2= 0.96$).

**Fig. S3: Fourteen DDR-associated gene candidates identified in the primary screen.** A-N Bar plots displaying Z-scores (grey) and Drug Effect (DE) Z-scores (red= clonally-derived HORMAD1-inducible SUM159, black= parental SUM159) for the 14 DDR-associated gene candidates identified in the primary screen. siRNAs that significantly impaired cell viability following HORMAD1 expression were identified on the basis of criteria discussed in text.

**Fig. S4: Validation of siRNA gene silencing effects by RT-qPCR analysis.** A-E Bar plot displaying the percentage of mRNA expression of the indicated gene, following transfection of clonally-derived HORMAD1-inducible SUM159 with an siRNA SMARTpool or 4 individual siRNAs targeting: A ATR; B BRIP1; C POLH; D TDP1; E XRCC1. Non-targeting siRNA (siALLSTAR) was used as a control. Gene silencing effects were evaluated 48 hours post-transfection by RT-qPCR, using primers specific towards the respective gene and ACTB as a normalisation control. Error bars indicate SD of the mean ($n= 3$) and $p$ values represent the Student’s t test.

**Fig. S5: Deconvolution RNAi validation of 14 gene candidates.** A-J Bar plots displaying normalised percentage inhibition (NPI) of clonally-derived control GFP-
inducible SUM159 cells (+DOX/+GFP vs. -DOX/-GFP) or parental SUM159 cells, transfected with siRNA SMARTpool or four individual siRNAs targeting A-B ATR; C-D BRIP1; E-F POLH; G-H TDP1; I-J XRCC1 and exposed to GFP expression or doxycycline treatment for 4 days. Non-targeting (siALLSTAR) and targeting (siPLK1) siRNAs were used as normalisation controls. Fourteen DDR-related gene candidates were further evaluated in secondary deconvolution experiments. ATR, BRIP1, POLH, TDP1 and XRCC1 met the validation criteria discussed in text. Error bars indicate SD from mean effects, p values represent multiple Student t tests (p= ***< 0.0001, p= **< 0.001, p= *<0.05).

**Fig. S6: Generation of clonally-derived HORMAD1-inducible MCF10A and RPE1 cell lines.** A-B Western blot analysis of doxycycline-induced HORMAD1 expression in A clonally-derived HORMAD1-inducible MCF10A and B clonally-derived HORMAD1-inducible RPE1. C-D Cell growth curves displaying reduced cell number of C MCF10A cells and D RPE1 cells, following acute expression of HORMAD1 (48 hours). Cell number was normalised relative to T0 counts. Error bars indicate SD from mean effects (n= 3), p values represent multiple Student t tests. E and F Comparison of induced HORMAD1 expression to that of endogenous levels in MDAMB436.

**Fig. S7: HORMAD1-associated genetic dependencies in HORMAD1 positive cell lines.** Candidate HORMAD1 genetic dependencies were knocked down using siRNA in four HORMAD1 positive cell lines. A-C and G-H Show effect of knockdown on cell viability 7 days post transfection. Data is presented as normalised percent inhibition (NPI). Data from triplicate wells are shown and error bars indicate SD from mean, p values represent Student t tests. D-F and I-J Show knockdown achieved by siRNA (NT black and siRNA red) in each cell line. Knockdown was measured by RT-qPCR
and normalised to *ACTB*. Data from triplicate qPCR reactions are shown and error bars indicate SD from mean.

**Fig. S8: HORMAD1 expression does not sensitise cells to ATR inhibitors. A-D** Clonogenic dose-response survival curves displaying no significant changes in cell survival of HORMAD1-inducible SUM159 cells (+DOX/+HORMAD1 vs. -DOX/-HORMAD1) exposed to increasing concentrations of **A** VE-821, **B** VX970, **C** AZ20 and **D** AZD6738 for 14 days. Error bars indicate SD from mean effects ($n=3$), $p$ values represent two-way repeated measures ANOVA. **E-F** Clonogenic dose-response survival curves displaying no significant changes in cell survival of **E** HORMAD1-inducible MCF10A cells (+DOX/+HORMAD1 vs. -DOX/-HORMAD1) and **F** HORMAD1-inducible RPE1 cells (+DOX/+HORMAD1 vs. -DOX/-HORMAD1) exposed to increasing concentrations of AZD6738 for 14 days. Error bars indicate SD from mean effects ($n=3$), $p$ values represent two-way repeated measures ANOVA.

**Fig. S9: Validation of HORMAD1-driven POLH dependency in 21.5-weeks HORMAD1-expressing SUM159 cells.**

**A** Bar plot displaying reduced surviving fractions of clonally-derived HORMAD1-inducible SUM159 cells (+DOX/+HORMAD1 vs. -DOX/-HORMAD1) previously exposed to HORMAD1 expression for 20 weeks, transfected with siRNAs targeting *POLH* and exposed to HORMAD1 expression for 11 additional days. Non-targeting (siALLSTAR) and targeting (siPLK1) siRNAs were used as transfection controls and surviving fractions calculated from mock-transfected cells. Error bars indicate SD from mean effects ($n=3$), $p$ values represent multiple Student t tests ($p=**<0.001$, $p=***<0.0001$, $p=\times<0.001$, $p=\times<0.05$). **B** Bar plot displaying the percentage of *POLH* mRNA expression following siRNA-mediated depletion of *POLH* described in **A**, measured by
RT-qPCR and normalised to ACTB.

**Fig. S10: Candidate hits lacking HORMAD1-driven selectivity in SUM159, MCF10A and RPE1 cells.**

**A** Bar plot displaying no significant changes in colony counts of SUM159 cells (+DOX/+HORMAD1 vs. -DOX/-HORMAD1) transfected with siRNA SMARTpools targeting POLK, POLI and REV1. Non-targeting (siALLSTAR) siRNA was used as normalisation control. Error bars indicate SD from mean effects \((n=3)\), \(p\) values represent multiple Student t tests. **B** Representative colony images from experiment A. **C** Bar plot displaying the percentage of POLK, POLI and REV1 mRNA expression following siRNA-mediated gene knockdown for experiments described in A, measured by RT-qPCR and normalised to ACTB. **D** Bar plot displaying no significant changes in colony counts of MCF10A cells (+DOX/+HORMAD1 vs. -DOX/-HORMAD1) transfected with siRNA SMARTpools targeting POLI and REV3L. Non-targeting (siALLSTAR) siRNA was used as normalisation control. Error bars indicate SD from mean effects \((n=3)\), \(p\) values represent multiple Student t tests. **E** Representative colony images from experiment D. **F** Bar plot displaying the percentage of POLI and REV3L mRNA expression following siRNA-mediated gene knockdown for experiments described in D, measured by RT-qPCR and normalised to ACTB. **G** Bar plot displaying no significant changes in colony counts of RPE1 cells (+DOX/+HORMAD1 vs. -DOX/-HORMAD1) transfected with siRNA SMARTpools targeting POLI and REV1. Non-targeting (siALLSTAR) siRNA was used as normalisation control. Error bars indicate SD from mean effects \((n=3)\), \(p\) values represent multiple Student t tests. **H** Representative colony images from experiment G. **I** Bar plot displaying the percentage of POLI and REV1 mRNA expression following siRNA-mediated gene knockdown for experiments described in G, measured by RT-qPCR and normalised to ACTB.
**Fig. S11:** HORMAD1-associated TLS genetic dependencies in HORMAD1 positive cell lines. HORMAD1-associated genetic dependencies in HORMAD1 positive cell lines. Candidate HORMAD1 genetic dependencies were knocked down using siRNA in four HORMAD1 positive cell lines. **A-B and E-F** Show effect of knockdown on cell viability 7 days post transfection. Data is presented as normalised percent inhibition (NPI). Data from triplicate wells are shown and error bars indicate SD from mean, *p* values represent multiple Student t tests. **C-D and G-H** Show knockdown achieved by siRNA in each cell line (NT black and siRNA red). Knockdown was measured by RT-qPCR and normalised to ACTB. Data from triplicate qPCR reactions are shown and error bars indicate SD from mean.

**Fig. S12 – Fig. S17:** Uncropped scans of western blots.
Fig. S1

A

|          | SUM159 | H1-clone 1 | H1-clone 2 |
|----------|--------|------------|------------|
| DOX (HORMAD1) | - | + | - | + |
| HORMAD1 Non-specific | | | | |
| Actin | | | | |

B

|          | SUM159 | H1-clone 1 |
|----------|--------|------------|
| DOX (HORMAD1) | - | + |
| HORMAD1 | | |
| β-ACTIN | | |

C

\[ \gamma H2AX \text{ foci} \]

\[ \% \text{ cells with } > 5 \gamma H2AX \text{ foci} \]

\[ p < 0.01 \]

D

[Images of DAPI, γH2AX, and merge]

E

Aberrant Nuclear Structures

\[ \% \text{ aberrant nuclear structures} \]

\[ p < 0.001 \]

F

[Images of Micronucleus, Nuclear Bud, Nucleoplasmic Bridge]

G

[Images of fluorescence with D.OX (GFP) and +D.OX (GFP)]

H

Aberrant Nuclear Structures

\[ \% \text{ aberrant nuclear structures} \]

\[ p = \text{ns} \]
**Fig. S2**

**A**

![Graph A](image1)

**B**

![Graph B](image2)

**C**

![Graph C](image3)

**D**

![Graph D](image4)

Transfection of the siRNA library (1280 siRNA pools, arrayed in 384-well plates) with Doxycycline (500 ng/ml) and measurement of cell viability with CellTiter-Glo. Optimisation of transfection conditions over 5 days. Overnight treatment with Doxycycline.

**A) H1-clone 1 + DOX replicate 1 vs. replicate 2**

**B) Z-score and Quality Control analysis**

**C) H1-clone 1 + DOX control siRNA Z scores**

**D) H1-clone 1 + DOX replicate 1 vs. replicate 2**

Spearman = 0.9650
Fig. S3

A) XRCC1

B) TDP1

C) NTHL1

D) DCLRE1A

E) PTEN

F) MSH4

G) BRIP1

H) PNKP

I) ATR

J) FANCC

K) ERCC4

L) POLH

M) TOPBP1

N) NEK1
Fig. S4
Fig. S5

A  SUM159 GFP-clone 1 ATR

B  SUM159 parental clone ATR

C  SUM159 GFP-clone 1 BRIP1

D  SUM159 parental clone BRIP1

E  SUM159 GFP-clone 1 POLH

F  SUM159 parental clone POLH

**Legend:**
- **DMO:** DMSO (-GFP)
- **DOX:** DOX (+GFP)

**Notes:**
- NS
- *
Fig. S5
continued

G
SUM159 GFP-clone 1 TDP1

H
SUM159 parental clone TDP1

I
SUM159 GFP-clone 1 XRCC1

J
SUM159 parental clone XRCC1
Fig. S6

A

B

C

D

E

F

MCF10A

RPE1

DOX

- +

DOX

- +

Actin

Actin

-40

-40

HORMAD1

HORMAD1

-50

-50

Relative cell number (fold change from 0 hours)

Time (Hours)

Relative cell number (fold change from 0 hours)

Time (Hours)

MCF10A

RPE1

HORMAD1

HORMAD1

-50kDa

-40kDa

-50kDa

-40kDa

HORMAD1

β-ACTIN

HORMAD1

β-ACTIN

HA tagged

Endogenous

HA tagged

Endogenous

MW (KDa)

MW (KDa)

-50kDa

-40kDa

-50kDa

-40kDa
Fig. S7

A. ATR

B. BRIP1

C. TDP1

D. % ATR expression

E. % BRIP1 expression

F. % TDP1 expression

G. POLH

H. XRCC1

I. % POLH expression

J. % XRCC1 expression

siALLSTAR  siATR

siALLSTAR  siBRIP1

siALLSTAR  siTDP1

siALLSTAR  siTDP1

siALLSTAR  siPOLH

siALLSTAR  siXRCC1

p < 0.0001
Fig. S9

A

H1-clone 1 week 20 - siRNA POLH depletion 11-day survival assay

B

p<0.0001

surviving fraction

% POLH expression

- HORMAD1

+ HORMAD1

p<0.0001

- HORMAD1

+ HORMAD1

p<0.0001

- HORMAD1

+ HORMAD1

p<0.0001

- HORMAD1

+ HORMAD1

p<0.0001

- HORMAD1

+ HORMAD1

p<0.0001

- HORMAD1

+ HORMAD1
Fig. S10

A. SUM159

B. SUM159

C. % POLK expression

D. MCF10A

E. MCF10A

F. % POLK expression

Data from Fig. S10
Fig. S10 continued

G

RPE1

H

RPE1

I

surviving fraction

\( G \)

\( H \)

\( I \)

\( \text{siALLSTAR} \)

\( \text{siPOLI} \)

\( \text{siREV1} \)

\( \text{DOX (HORMAD1)} \)

\( \text{DOX (HORMAD1)} \)

\( \text{DOX (HORMAD1)} \)

\( p = 0.001 \)

\( p < 0.01 \)

\( \text{siALLSTAR} \)

\( \text{siPOLI} \)

\( \text{siREV1 pool} \)

\( \text{siPOLI pool} \)

\( \text{siREV1 pool} \)

\( \text{siPOLI pool} \)

\( \text{siREV1 pool} \)

\( \text{siPOLI pool} \)

\( \text{siREV1 pool} \)
Fig. S11

A

B

C

D

E

F

G

H

Normalised percent inhibition

Normalised percent inhibition

% POLK expression

% REV1 expression

% REV3L expression

% REV7 expression

MDAMB436  HCC38  BT549  HCC1143

MDAMB436  HCC38  BT549  HCC1143

MDAMB436  HCC38  BT549  HCC1143

MDAMB436  HCC38  BT549  HCC1143

MDAMB436  HCC38  BT549  HCC1143

MDAMB436  HCC38  BT549  HCC1143

% TDP1 expression

% TDP1 expression

% TDP1 expression

% TDP1 expression

siALLSTAR  siPOLK

siALLSTAR  siREV1

siALLSTAR  siTDP1 pool

siALLSTAR  siTDP1 pool

siALLSTAR  siTDP1 pool

siALLSTAR  siTDP1 pool

siALLSTAR  siTDP1 pool

siALLSTAR  siTDP1 pool

p < 0.0001

p < 0.0001

p < 0.0001

p < 0.0001

p < 0.0001

p < 0.0001
Fig. S12

A

SUM159 H1-clone 1

MDAMB436

DOX (HORMAD1)

-  +

SF 1B region in figure

Anti-HORMAD1

SUM159 H1-clone 1

MDAMB436

DOX (HORMAD1)

-  +

SF 1B region in figure

Anti-Actin

MDAMB436

SUM159 H1-clone 1
Fig. S13

DOX (HORMAD1)

| DOX (HORMAD1) | Cas9+ | mock | crRNA3 | + | - | + | - | + | - | + |
|---------------|-------|------|--------|---|---|---|---|---|---|---|

Anti-POLH

50

Anti-Tubulin

75

DOX (HORMAD1)

| DOX (HORMAD1) | Cas9+ | mock | crRNA6 | + | - | crRNA7 | + | - |
|---------------|-------|------|--------|---|---|--------|---|---|

Anti-POLH

50

Anti-Tubulin

75
**Fig. S14**

**HCC38**

| Condition   | crRNA3 | crRNA4 | crRNA5 |
|-------------|--------|--------|--------|
| Mock        |        |        |        |
| POLH        |        |        |        |
| POLH        |        |        |        |
| POLH        |        |        |        |
| Cas9+       |        |        |        |

Region in figure

**Anti-POLH**

Region in figure

**Anti-HORMAD1**

Region in figure

**Anti-Vinculin**
Fig. S15

BT549

|      | Mock | POLH | POLH | POLH | POLH | crRNA3 | crRNA4 | crRNA5 |
|------|------|------|------|------|------|--------|--------|--------|

Anti-POLH

Anti-HORMAD1

Anti-Vinculin

Region in figure

75

50

100
Fig. S17

Anti-HORMAD1

MW (Kda)
- 50kDa

SF 6F region in figure  SF 6E region in figure

Anti-Actin

MW (Kda)
- 40kDa

SF 6F region in figure  SF 6E region in figure
Supplementary References

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