Supplementary data

E-cadherin/ROS1 inhibitor synthetic lethality in breast cancer

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Supplementary Figure Legends

Supplementary Figure 1. Western blot showing E-cadherin expression in parental MCF7 cells ($MCF7^{parental}$) and the *CDH1* CRISPR-Cas9 mutagenised $MCF7^{B04}$ and $MCF7^{B05}$ clones.

Supplementary Figure 2. Plot of median difference in Z-scores ("Delta Z score") for each siRNA in the library of data presented in Fig.1E. Delta Z scores calculated by: (median Z score in $MCF7^{A02}$ cells - median Z score in $MCF7^{Parental}$ cells). siRNA designed to target MET, ALK, AXL or KDR are highlighted.

Supplementary Figure 3. ROS1 expression is upregulated in E-cadherin defective cells. Western blot showing expression of ROS1, MET and ALK in $MCF7^{Parental}$ and $MCF7^{A02}$ cells. ACTIN expression is used as loading control.

Supplementary Figure 4. A, Heatmap representation of supervised clustering of siRNA Z-scores. Breast tumour cell lines were clustered according to E-cadherin expression status, and differential effects between E-cadherin defective and wild-type groups were identified using median permutation tests. Statistically significant effects ($p<0.05$) are shown. **B,** Waterfall and box/whiskers plot of CDC45BPA, PLK4, MAPKAPK3, GSK3A and ROS1 siRNA Z-scores across the breast tumour cell line panel. E-cadherin defective cells are annotated in blue, whereas E-cadherin wild-type cells are annotated in black. In the box/whiskers plot, $p<0.05$ between E-cadherin defective and wild-type groups, using the median permutation test.

Supplementary Figure 5. Box/whiskers plots of MET, ALK, and ROS1 siRNA Z-scores in a panel of non-isogenic breast tumour cell line models. Annotated in blue, E-cadherin defective cells, annotated in black, E-cadherin wild-type cells. **p<0.05 between E-cadherin defective and wild-type groups, median permutation test.

Supplementary Figure 6. **Genetic dependencies for E-cadherin deficiency in pan cancer cell line models. A,** Scatter plot illustrating *CDH1* mRNA expression levels in 69 pan cancer tumour cell lines from cosmic (left) and CCLE (right) dataset (1). **B,** Heatmap representation of a supervised clustering of siRNA Z-scores. Pan cancer tumour cell lines were clustered according to *CDH1* status and differential effects between *CDH1* defective and wild type groups identified using the median permutation test. Statistically significant effects ($p<0.05$) are shown. **C,** box/whiskers plot of ROS1 siRNA Z-scores across the Pan cancer tumour cell line panel. Annotated in blue, *CDH1* defective cells, annotated in black, *CDH1* wild-type cells. In the box/whiskers plot, $p<0.05$ between *CDH1* defective and wild-type groups, using the median permutation test.

Supplementary Figure 7. Validation of ROS1 synthetic lethality in E-cadherin defective breast tumour cell line models. A-E, Normalised Percentage Inhibition NPI data from individual and pooled ROS1 siRNA effects in breast cancer cell line panel with/without E-cadherin expression where n=6 (E-cadherin defective) and n=6 (E-cadherin wild-type), $*p<0.05$ measured by Student's t-test.

Supplementary Figure 8. ROS1 inhibitor sensitivity in E-cadherin defective cells is independent of mesenchymal or epithelial status. Foretinib dose-response survival curves in E-cadherin wild type SUM149 cells that have undergone EMT (2), E-cadherin defective BT549 and CAL120 cells that have undergone EMT (2), E-cadherin defective SKBR3 and SUM44 cells that have not undergone EMT (2), exposed to foretinib for six days. Error bars represent SEM from three independent experiments. Dose-response in *SLC34A2-ROS1* translocation-positive HCC78 cells is shown as a positive control.

Supplementary Figure 9. Expression of MET, ALK, AKT and ERK in a panel of breast tumour cell lines. A, Western blot illustrating expression of MET, ALK, pAKT, AKT, pERK, ERK, E-cadherin in a panel of 23 breast tumour cell line models. ACTIN expression is used as loading control. Cell line names are colour-coded according to the presence of *CDH1* gene mutations, gene deletion events or *CDH1* promoter hypermethylation events. Waterfall plots illustrating quantification of MET **B** ALK **C** pAKT **D** AKT **E** ERK **F** pERK **G** expression normalized to ACTIN loading control using ImageJ. In blue are breast tumour cell lines classified as E-cadherin defective and in black are cell lines classified as E-cadherin wild type.

Supplementary Figure 10. ROS1 and pROS1 expression in a panel of breast tumour cell lines. A, Western blot illustrating expression of ROS1, pROS1 and E-cadherin in a panel of 23 breast tumour cell line models cells. ACTIN expression is used as loading control. Cell line names are colour-coded according to the presence of *CDH1* gene mutations, gene deletion events or *CDH1* promoter hypermethylation events. Waterfall plots illustrating quantification of ROS1 **B** and pROS1 **C** expression normalized to ACTIN loading control using ImageJ. In blue are breast tumour cell lines classified as E-cadherin defective and in black are cell lines classified as E-cadherin wild type.

Supplementary Figure 11. *De novo* endocrine resistant E-cadherin defective ILC breast tumour cells are sensitive to ROS1 inhibitors *in vitro* and *in vivo*. A, Box whisker plots illustrating foretinib or crizotinib sensitivity in 14 breast tumour cell lines, defined by log_2 area under the curve (AUC) values. ** *p* value = 0.0025, Student's t-test, * *p* value = 0.016 Student's t-test. Log_2 area under the curve (AUC) values for *de novo* endocrine resistant MDAMB134IV cells are indicated with a black arrow. **B**, and **C,** Therapeutic response to foretinib (B) or crizotinib (C) in mice

bearing established E-cadherin defective MDAMB134VI xenografted tumours. After tumours had established, animals were treated with either foretinib or crizotinib (25 mg/kg every other day) for the duration of the experiment. Relative tumour volumes after the initiation of treatment are shown. Error bars indicate SEM. n for each cohort = 10 $p < 0.001$, ANOVA for vehicle vs. crizotinib or foretinib treatment. **D**, Immunohistochemistry images of tumours extracted from animals in (C) at the end of crizotinib treatment. Representative images of H&E, Ki67 and cleaved Caspase 3 are shown (magnification = 20x). Scale bar represents 100μm.

Supplementary Figure 12. Synthetic lethality of ROS1 inhibition in E-cadherin deficient gastric tumour cell lines. A, Western blot illustrating E-cadherin expression in 6 gastric tumour cell lines. Cell line names are colour-coded according to the presence of *CDH1* gene mutations or *CDH1* promoter hypermethylation events. Uncropped western blot images are shown in Supplementary Fig. S20. **B**, waterfall and box/whiskers plot of log SF_{50} for 7 day foretinib/crizotinib survival curves carried out in a panel of breast and gastric tumour cell lines with/without E-cadherin expression. Error bars represent SEM from three independent experiments. Annotated in blue, E-cadherin deficient cells, were selective to foretinib/crizotinib in comparison to the proficient cohort, in black, ($p<0.05$, Student t-test). **C**, Left: scatter plot illustrating *CDH1* mRNA expression levels in gastric tumour cell lines from the CCLE dataset (1). Tumour cell lines were classified as "*CDH1* defective" or "*CDH1* wild type" based on a threshold of 7 nominal units of expression. Right: Crizotinib sensitivity (as defined by concentration in μM that causes 50 % inhibition, SF_{50}) in the same gastric tumour cell line panel * $p <0.05$ Student's t test (3).

Supplementary Figure 13. **E-cadherin defective cells exposed to foretinib are characterized by an apoptotic response**. Western blot illustrating PARP cleavage in E-cadherin defective MCF10A $CDH1^{-/-}$ cells exposed to 1μM foretinib.

Supplementary Figure 14. A, Quantifications of the length in mitosis in E-cadherin defective MCF7^{A02} and MCF7^{Parental} cells exposed to foretinib or vehicle control for a 24 hour period. Statistical significance was determined using the Student's *t*-test, ****p<0.0001. **B,** Quantifications of the length in mitosis in E-cadherin defective MCF7^{A02} and MCF7^{Parental} cells exposed to crizotinib or vehicle control for a 24 hour period. Statistical significance was determined using the Student's *t*-test, ****p<0.0001.

Supplementary Figure 15. ROS1 inhibition in E-cadherin defective cells is characterized by cytokinesis defects. Time lapse microscopy images illustrating cell division in E-cadherin defective MCF7^{A02} cells exposed to vehicle undergoing normal cytokinesis **A,** foretinib with lagging chromosome leading to failed cytokinesis **B,** foretinib characterized by membrane oscillation followed by regression of cleavage furrow **C**, crizotinib with lagging chromosome followed by membrane oscillation leading to failed cytokinesis **D**, crizotinib characterized by membrane oscillation leading to failed cytokinesis **E**. MCF7^{A02} cells were first transfected with a mCherry-H2B plasmid, FACS sorted for mCherry-H2B to facilitate DNA visualization, and then exposed to foretinib, crizotinib or vehicle for a 24-hour period. Scale bar, 10 μm.

Supplementary Figure 16. A, Immunofluorescence for p120 and MKLP1 in anaphase/telophase MCF7^{A02} and MCF7^{parental} cells exposed to 0.5μM crizotinib, foretinib or vehicle control. Reduced accumulation of p120 and co-localization on the equatorial cortex. Scale bar, 10 μm. **B,** p120 phosphorylation is altered in E-cadherin defective cells. Western blot showing phospho-p120 (Tyr228 and Ser320) in MCF10A CDH1$^{+/+}$ and MCF10A CDH1$^{-/-}$ cells, exposed to foretinib for 5 hours.

Phosphorylation of γH2AX is used as a positive control demonstrating DNA damage in E-cadherin defective cells.

Supplementary Figure 17. A, Western blot illustrating CDC42BPA silencing caused by four different CDC42BPA siRNAs (1, 2, 3, 4 and the SMARTpool) compared to two different non-targeting siRNAs (siCONT1, siCONT2). Uncropped western blot images are shown in Supplementary Fig. S20. **B,** Bar chart illustrating cell inhibition caused by CDC42BPA siRNAs in MCF10A $CDH1^{+/+}$ and MCF10A $CDH1^{-/-}$ cells. NPI = normalized percentage inhibition (compared to siCONT (NPI=1) and siPLK1 (NPI=0)). Error bars represent standard error of the mean (SEM) from three independent experiments. **C,** Normalised Percentage Inhibition NPI data from individual and pooled CDC42BPA siRNA effects in breast cancer cell line panel with/without E-cadherin expression where n=6 (E-cadherin defective) and n=6 (E-cadherin wild-type), *p<0.05 measured by Student's t-test. **D,** Surviving fraction data from $MCF7^{A02}$ and $MCF7^{parental}$ cell lines exposed to 10μM chelerythrine for six continuous days, at which point cell viability was assessed. ****p<0.0001 Student's t-test as shown.

Supplementary Figure 18. *In vivo* E-cadherin/foretinib synthetic lethality in an E-cadherin defective isogenic model. A, Mice bearing luciferase-labeled $MCF7^{A02}$ xenografts, were treated as indicated. Each data point represents the mean increase in tumor volume after the instigation of treatment and error bars represent SEM, where n for each cohort = 10 animals. (***p=0.0002, ANOVA for vehicle vs. foretinib). **B,** Box/whisker plot representation of relative tumour volume at day 41 of treatment for the same mice as in (A) (***p<0.001, Students t-test). **C**, Mice bearing luciferase labeled $MCF7^{Parental}$ xenografts, were treated as indicated. Each data point represents the mean increase in tumor volume after the instigation of treatment and error bars represent SEM, where n for each cohort = 10 animals. (p=0.6845, ANOVA for vehicle

vs. foretinib). **D,** Box/whisker plot representation of relative tumour volume at day 41 of treatment for the same mice as in (C) (p=ns, Students t-test). **E,** Effect of foretinib in an E-cadherin defective isogenic model. Full body bioluminescence images illustrating tumour burden in mice bearing luciferase-labeled $MCF7^{Parental}$ (A) and $MCF7^{A02}$ (B) xenografts, before or after treatment with foretinib. Representative BLI flux in-life images of mice show strong anti-tumour effect of foretinib in the E-cadherin defective *vs.* the E-cadherin wild type cohort.

Supplementary Figure 19. pROS1, ROS1 and MET expression in foretinb treated E-cadherin defective PDX xenografts. Therapeutic response to foretinib treatment in mice bearing BCM2665 PDX. BCM2665 was transplanted into 19 recipient mice; once tumours had established, animals were treated over a 47-day period with either drug vehicle (n=11), or foretinib (25 mg/kg every other day, n= 8). Immunohistochemistry images of tumours extracted from animals at the end of foretinib treatment. Representative images of H&E and pROS1 **A**, ROS1 **B** and MET **C** are shown (magnification = 20x). Scale bar represents 100μm.

Supplementary Figure 20. Uncropped westerns from main and supplementary figures.

Supplementary Materials and Methods

RNAi and small-molecule synthetic lethal screens

Cell lines were transfected with SMARTpool siRNAs, using Lipofectamine 2000 (Invitrogen) transfection reagent. The bespoke siRNA library targeting >1000 human protein kinase genes and genes in the Cancer Gene Census was purchased from Dharmacon. Each well in this library contained a SMARTpool of four distinct siRNA species targeting different sequences of the target transcript. Each plate was supplemented with siCONTROL (24 wells, (Dharmacon)) or siPLK1 (8 wells ((Dharmacon)), which served as controls. Cells were cultured for six days after transfection, at which point cell viability was estimated by use of Cell-Titre Glo (Promega). Luminescent readings from each 384 well were $\log_2$ transformed, centred according to plate median effects and then Z-score standardised according to the library median effect and the median absolute deviation. In total we used data from three biological replicate screens in the final analysis.

For small-molecule screens, we used an in-house curated drug library containing 80 compounds present at eight different concentrations (0.5, 1, 5, 10, 50, 100, 500 and 1000 nM) as listed in Supplementary Table S9. 250 cells were seeded in each well of a series of 384 well plates. Twenty-four hours later, cells were exposed to small-molecules and then continuously cultured for six days at which point cell viability was estimated using Cell-Titre Glo (Promega).

REVEALER analysis

REVEALER analysis was performed as described in (4). In the first instance, we used REVEALER for *de novo* discovery without a seed (4), using ROS1 siRNA Z scores as the target sensitivity values and the mutational status of 23 cancer driver genes (those mutated in >2 breast tumour cell lines) and the E-cadherin status of breast tumour cell lines shown in Fig 2B as molecular features.

Cell survival and apoptosis assays

For clonogenic survival assays exponentially growing cells were seeded in six-well plates at a concentration of 1000–2000 cells per well. Cells were continuously exposed to drug with media and drug replaced every 72h. After 15 days, cells were fixed and stained with sulforhodamine-B (Sigma-Aldrich).

Short-term survival assays were performed in 96-well plates. For measurement of sensitivity to drugs, cells were seeded in 96-well plates at a concentration of 1000-2000 cells per well. 24 hours post seeding, drug treatment was initiated and cells were continuously exposed to the drug with media and drug replenished 48 hours post initial treatment. After seven days, cell viability was estimated using Cell-Titre Glo (Promega).

The ApoTox Glo assay (Promega) was used to assess caspase 3/7 activity as per the manufacturer's instructions. Cells were plated in 96 well plates at the density of 5000 cells per well. 24 hours post seeding, drug treatment was initiated and maintained for 48 hours prior to caspase 3/7 analysis.

Cell sorting

Sorting of cells took place on a BD FACSAria II SORP flow cytometer (BD Biosciences, USA) equipped with a 488 nm blue laser, a 532 nm green laser, a 561 nm yellow laser, a 633 nm red laser and a 404 nm violet laser. mCherry was measured with 595 LP 610/20 BP. It is operated by BDFACSDiva software.

***In vivo* assessment of foretinib and crizotinib efficacy**

MDA-MD-134VI and MCF7 isogenic *in vivo* studies were carried out by Crown biosciences. For the *in vivo* study using MDA-MD-134VI tumour cells, 6-8 weeks

female NOD SCID mice were implanted with estrogen pellets (17b-estradiol, 0.18 mg/ 60 days, Innovative Research of America, Sarasota, Florida, USA) at the left flank one day before the tumour inoculation. Each mouse was than inoculated subcutaneously at the right flank region with MDA-MB-134VI tumour cells (1 x 10^7) in 0.2 ml of PBS (1:1 matrigel) for tumour development. The treatments were initiated when the mean tumour size reached approximately 100-150 mm^3.

For the *in vivo* study using MCF7 CRISPR isogenic tumour cells, first luciferase labelled cell lines were generate (Crown Biosciences). Briefly, 5-8 weeks female athymic nude (Hsd:AthymicNude-*Foxn1*^{nu}) mice were implanted with estrogen pellets (17b-estradiol, 0.18 mg/ 60 days, Innovative Research of America, Sarasota, Florida, USA) at the left flank one day before the tumour inoculation. Each mouse was than inoculated orthotopically into the lower left-side 2nd mammary fat pad with MCF-7 (ICR) lux and MCF-7 E-cad (ICR) lux cell tumour cells (5 x 10^6) in 0.1ml of RPMI:Matrigel (1:1 matrigel) for tumour development. The treatments were initiated when the mean tumour size reached approximately 100-200 mm^3. Disease progression for each group was also measured by capturing full body bioluminescence (BLI) counts, by injecting mice with 150mg/kg D-Luciferin 15 minutes prior to imaging. 10 minutes following administration of D-Luciferin mice were anaesthetised and placed into the imaging chamber (Spectrum CT) and imaged for luminescence (*ventral view*; up to 5 mice laid alongside each other in cage order). For all *in vivo* studies mice were dosed with the test agents every other day until the end of the study. Foretinib was dissolved in DMSO, aliquoted, and stored at −80 °C. Before use, aliquots were further diluted in 1% hydroxypropylmethylcellulose/0.2% SDS. Crizotinib was reconstituted in 1% hydroxypropylmethylcellulose/ 0.2% SDS, aliquoted, and stored at −80 °C. All *in vivo* modelling was performed in accordance with the United Kingdom Home Office Animals (Scientific Procedures) Act 1986, Home Office Project Licence PPL 70/7317 and CBUK SOPs. Tumours were FFPE

fixed and slides were stained with H&E, or incubated with antibodies against Ki-67 and cleaved caspase-3.

References

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