Modelling therapy resistance in *BRCA1/2* mutant cancers

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Abstract

Although PARP inhibitors target *BRCA1* or *BRCA2* mutant tumour cells, drug resistance is a problem. PARP inhibitor resistance is sometimes associated with the presence of secondary or “revertant” mutations in *BRCA1* or *BRCA2*. Whether secondary mutant tumour cells are selected for in a Darwinian fashion by treatment is unclear. Furthermore, how PARP inhibitor resistance might be therapeutically targeted is also poorly understood. Using CRISPR-mutagenesis, we generated isogenic tumour cell models with secondary *BRCA1* or *BRCA2* mutations. Using these in heterogeneous *in vitro* culture or *in vivo* xenograft experiments where the clonal composition of tumour cell populations in response to therapy was monitored, we established that PARP inhibitor or platinum salt exposure selects for secondary mutant clones in a Darwinian fashion, with the periodicity of PARP inhibitor administration and the pre-treatment frequency of secondary mutant tumour cells influencing the eventual clonal composition of the tumour cell population. In xenograft studies the presence of secondary mutant cells in tumours impaired the therapeutic effect of a clinical PARP inhibitor. However, we found that both PARP inhibitor sensitive and PARP inhibitor resistant *BRCA2* mutant tumour cells were sensitive to AZD-1775, a WEE1 kinase inhibitor. In mice carrying heterogeneous tumours, AZD-1775 delivered a greater therapeutic benefit than olaparib treatment. This suggests that despite the restoration of some *BRCA1* or *BRCA2* gene function in “revertant” tumour cells, vulnerabilities still exist that could be therapeutically exploited.

Introduction

Heterozygous germ-line mutations in the *BRCA1* or *BRCA2* tumour suppressor genes strongly predispose to cancers of the breast, ovary, pancreas, and prostate (1,2). BRCA1 and BRCA2 are involved in homologous recombination (HR), a process used to repair DNA double-strand breaks (DSBs), and other DNA lesions that impair replication forks (3-6). Extensive preclinical and clinical data has established that loss of *BRCA1* or *BRCA2* function is associated with sensitivity to small molecule PARP inhibitors (7). Recently, the PARP inhibitor (PARPi) Lynparza (olaparib/AZD2281 – KuDOS/AstraZeneca) was approved for the treatment of platinum-responsive, *BRCA1* or *BRCA2* mutant high-grade serous ovarian carcinomas (HGSOC) (8).

Despite a number of profound and sustained anti-tumour responses in patients treated with PARP inhibitors, drug resistance limits the overall effectiveness of these agents (9-12). A number of mechanisms of PARP inhibitor resistance have been identified, including upregulation of PgP drug transporters, loss of 53BP1 or REV7 function, or secondary “revertant” mutations within the *BRCA1* or *BRCA2* genes themselves (13,14). These secondary *BRCA* gene mutations restore *BRCA1* or *-2* open reading frames and encode proteins that have partial function (13-16). In some *BRCA2* mutant patients, initial clinical responses to PARPi are seen, followed by the emergence of profoundly PARPi resistant lesions (13). The gradual emergence of PARPi resistance during treatment has led to the hypothesis that PARPi treatment might provide a Darwinian selective pressure effect,

where a secondary mutant clone has a selective advantage over non-secondary mutant tumour clones, once PARPi treatment is applied (7,17). Although this hypothesis has not as yet been tested, if such a Darwinian process did exist, a secondary mutant clone might be expected to gradually dominate a tumour cell population over the course of PARPi therapy. To date, only one approach for targeting tumour cell clones with secondary *BRCA1/2* mutations has been proposed, namely the use of thiopurines (18). The wide application of thiopurines in the treatment of cancer has been limited by safety concerns (18), suggesting that additional therapeutic approaches for targeting secondary *BRCA1/2* mutant tumour cells might also be required.

We set out to assess, both *in vitro* and *in vivo*, whether tumour cells with secondary *BRCA1 or -2* gene mutations are selected for by PARPi treatment in a Darwinian fashion. To do this, we used CRISPR-Cas9 mediated gene targeting in *BRCA1* or *BRCA2* mutant tumour cells to generate daughter clones with secondary mutations. By mixing these secondary mutant clones with parental tumour cells in *in vitro* co-cultures or *in vivo* tumour xenografts, we established that PARPi treatment can select for secondary mutant clones in a Darwinian fashion. Using these same systems, we also found that exposure to a clinical WEE1 kinase inhibitor (AZD-1775) minimized the selection of secondary mutant tumour cells, targeting parental and secondary mutant cells to a similar extent, whilst having minimal effects on non-tumour cells.

Materials and Methods

Cell lines

CAPAN1 and SUM149 cells were obtained from American Type Tissue Collection. DLD1-*BRCA2*$^{WT/WT}$ and DLD1-*BRCA2*$^{-/-}$ cells were purchased from Horizon Discovery Inc. All cells were cultured according to the supplier's instructions. All cells were STR typed to confirm identity and verified to be mycoplasma-free prior to the study.

Small molecule inhibitors

Olaparib, talazoparib, and AZD-1775 were obtained from Selleck Chemicals. Inhibitor stock solutions were prepared in DMSO and stored in aliquots at minus 20°C. Inhibitors were added to cell cultures so that final DMSO concentrations were constant at 1% (v/v).

CRISPR-generated PARPi-resistant secondary mutant cell lines

CAPAN1.B2.S* and SUM149.B1.S* were generated from CAPAN1 and SUM149 parental tumour cell lines. Parental lines were transiently transfected with 5 μg of gRNA (described below) and 5 μg of a Cas9 pMA-T expression vector (GE Healthcare) using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. Twenty-four hours later, cells were re-plated into 15 cm dishes at 2000 cells/dish and exposed to 100 nM talazoparib for two weeks after which clones were cultured in talazoparib free media until visible. Clones were manually isolated, and re-plated into 96-well plates for expansion. DNA from clones was isolated using DNeasy Blood and Tissue Kit

(Qiagen) and PCR amplified using *BRCA1* or *BRCA2* primers described below. PCR products were subcloned using TOPO TA Cloning Kit, with pCR2.1-TOPO (Invitrogen). Sanger sequencing confirmed secondary *BRCA1* or *BRCA2* mutations from 20 subcloned *E.coli* colony sequences per cell line.

gRNA (in pMA-T vector): *BRCA2*, 5′-GAGCAAGGGAAAATCTGTCC-3′ and *BRCA1*, 5′-CCAAAGATCTCATGTTAAG-3′. The *BRCA2* gRNA contains the *c.6174delT* mutation specific to the CAPAN1 cell line.

Primers for PCR amplification were: *BRCA1*, 5′-TGCTTTCAAAACGAAAGCTG-3′, 5′-ACCCAGAGTGGGCAGAGAA-3′; *BRCA2*, 5′-CTGTCAGTTCATCATCTTCC-3′, 5′-ATGCAGCCATTAAATTGTCC-3′.

Confocal microscopy

Cells on glass coverslips were exposed to 5 Gy IR using an X-ray machine and then fixed and stained 5 hours later as described previously (19). Nuclei were stained using DAPI diluted in PBS (1:10,000 v/v), RAD51 using the Abcam (ab137323) antibody and γH2Ax using the Millipore (05-636) antibody. At least 100 cells were assessed per coverslip. Cells scoring positive had > 5 foci per nucleus.

Immunoprecipitation (IP) and Western Blotting

IP and western blotting were performed as described previously (15,20). Antibodies targeting phospho-CDC(Y15) (#4539), PARP1 (#9542), γ-H2AX (#9718) (all Cell Signalling Technology), and tubulin (#T6074, Sigma) were employed in western blots.

Cell-based Assays

Short-term drug exposure and clonogenic assays were performed as described previously (21). In brief, cells were seeded either into 384-well or 6-well plates at a concentration of 500 to 2,000 cells per well. After 24 hours, cells were exposed to olaparib, talazoparib, or AZD-1775. For short-term drug exposure, cell viability was assessed after five days of drug exposure using CellTiter-Glo Luminescent Cell Viability Assay (Promega) as per the manufacturer's instructions. For clonogenic assays, drug was replenished every three days for up to 14 days, at which point colonies were fixed with TCA and stained with sulforhodamine B. Colonies were counted and surviving fractions calculated by normalizing colony counts to colony numbers in vehicle-treated wells. Survival curves were plotted using a four-parameter logistic regression curve fit as described in (22).

***In vitro* co-culture drug exposure assays**

Cells were plated in a fixed starting ratio of secondary mutant:parental cells in either 24 well or 6 well plates, or T75 flasks and exposed to either olaparib, talazoparib, or AZD-1775 for 14 or 21 days. In “constant drug exposure” experiments, media containing drug was replenished every three days. In “intermittent drug exposure” experiments, cells were exposed to media

containing drug for 24 hours after which, media was removed, cells were washed using PBS and then cells were re-cultured in media without drug for 48 hours, at which point cells were "refed" with media containing drugs as before.

DLD1 drug exposure assay

DLD1-*BRCA2*^{WT}-GFP and DLD1- *BRCA2*$^{-/-}$-RFP cells were plated at one of the following ratios: 1:1, 1:10, 1:100, or 1:1000. Twenty-four hours later, cells were exposed to DMSO, olaparib, or talazoparib. Aliquots of cell populations were analysed by flow cytometry, every 3-4 days, (LSR II, Beckman-Coulter) for GFP and RFP cell populations. Drug was replenished every three days.

Droplet digital PCR (ddPCR) Assays

Cells were pelleted and genomic DNA was extracted using the DNeasy Blood and Tissue Kit (Qiagen) following the manufacturer's instructions. DNA concentration was measured using Qubit broad range detection kit (Invitrogen). Restriction digestion was performed with *Eco*RI (BD Biosciences) and final working dilutions were made at 5 μg/uL per sample. DNA reaction mixtures were performed as described previously (23).

Primers and probes were as follows:

CAPAN1

ddPCR PROBE: VIC-CTGGACAGATTTTC,

FORWARD PRIMER: 5'- TCTCATCTGCAAATACTTGTGGGATT-3'

REVERSE PRIMER: 5'- TTGTCTTGCGTTTTGTAATGAAGCA-3'

CAPAN1.B2.S*

ddPCR PROBE: 6FAM- CTGATACCTGATTTTC

FORWARD PRIMER: 5'- TCTCATCTGCAAATACTTGTGGGATT -3'

REVERSE PRIMER: 5'- TTGTCTTGCGTTTTGTAATGAAGCA -3'

SUM149

ddPCR PROBE: VIC- TTTGTCAACCTAGCCTTCCA

FORWARD PRIMER: 5'- TGACAGCGATACTTTCCCAGA -3'

REVERSE PRIMER: 5'- GAGATCTTTGGGGTCTTCAGC -3'

SUM149.B1.S*

ddPCR PROBE: 6FAM-ACCAGGTGCATTTGTTAACTTCA

FORWARD PRIMER: 5'- TGACAGCGATACTTTCCCAGA -3'

REVERSE PRIMER: 5'- GCAAAACCCTTTCTCCACTTACT -3'

AZD-1775 sensitivity assessment

One hundred forty-six cancer cell lines were profiled as described previously (21,24). In brief, cells were plated at a density of 250 or 500 cells per well. Twenty-four hours later, media containing WEE1 inhibitor was added to adherent cells. After five days of drug exposure, cell viability was measured using CellTitre-Glo (Promega). Luminescence data was log2 transformed and centered according to the plate median value. Surviving fractions were calculated relative to the DMSO-exposed control wells to generate AUC data.

Xenograft experiments

Female BALB/c nude mice aged 4-6 weeks and 15-22 g in weight (Charles River Laboratories) were inoculated subcutaneously with $5x10^6$ tumour cells

into the right flank. When tumours reached 100 mm^3, six animals from each cohort were sacrificed as sentinels to enable estimation of parental and secondary mutant tumour cell frequencies prior to treatment. Remaining animals were randomized into different treatment arms (n=6) as described in the main text. Mice were weighed once weekly; tumours were measured twice weekly. When tumours reached 1500 mm^3, tumours were harvested and half of the tissue was formalin fixed, the other half was snap frozen in liquid nitrogen for DNA isolation.

Cell cycle analysis

Asynchronously growing CAPAN1 and CAPAN1.B2.S* cells were plated in 10 cm dishes ($5x10^5$ cells/plate) and treated with 1 µM AZD-1775 or DMSO for 72 hours. The cells were pulse labelled with 30 µM BrdU (Sigma, B5002) for 1 hour prior to collection. Cells were harvested using trypsin, washed with PBS, fixed in cold 70% ethanol and stored at -20°C overnight. Samples were washed with 2 M NaCl/0.5% Triton X-100 and incubated for 30 minutes at room temperature. Cell pellets were resuspended in 0.1 M sodium tetraborate for 2 minutes and subsequent cell pellets were incubated at room temperature for 1 hour in anti-BrdU antibody (BD Biosciences, 347580) diluted in 0.5% TWEEN-20/1%BSA/PBS (1 µg antibody per 1 million cells). Sample pellets were washed in PBS/1% BSA. Cells were then incubated for 30 minutes at room temperature with goat anti-mouse IgG FITC antibody (Sigma, F0257) diluted in 0.5% TWEEN-20/1%BSA/PBS (1 µg antibody per 1 million cells). Cells were pelleted and resuspended in PBS containing 10 µg/ml RNaseA

(Sigma, R4642) and 20 µg/ml propidium iodide (Sigma, P-4170) and incubated at room temperature for 30 minutes. Cell cycle analysis was performed on a FACS LSR II and analysed using FlowJo software (FlowJo, USA).

Results

Generation of PARP inhibitor-resistant models harbouring secondary *BRCA1 or BRCA2* mutations

We used CRISPR-Cas9 mediated gene targeting to generate novel tumour cell models with secondary mutations in either *BRCA1* or *BRCA2* and then used these in *in vitro* and *in vivo* co-culture systems to assess the clonal evolution of tumour cell populations in response to therapy (Figure 1A). To generate these models we used two PARP inhibitor sensitive tumour cell lines, the pancreatic ductal adenocarcinoma tumour cell line, CAPAN1 (*BRCA2* mutation *c.6174delT*, p.S1982fs*22) (13,15,25), and the breast tumour cell line SUM149 (*BRCA1* mutant *c.2288delT,* p.N723fsX13) (26). We designed specific CRISPR guide RNA (gRNA) expression constructs targeting PAM (protospacer adjacent motifs) sequences close to either the *BRCA2 c.6174delT* mutation in CAPAN1 cells or the *BRCA1 c.2288delT* mutation in SUM149 cells, and transiently transfected these into cells, alongside a Cas9 expression construct. We reasoned that the error-prone repair of DSBs in these *BRCA*-gene defective tumour cell lines would in some cells cause frameshift secondary mutations in either *BRCA1* or *BRCA2* that restored the open reading frame. In a CAPAN1-derived daughter cell clone,

CAPAN1.B2.S*, we identified a five base pair (bp) *BRCA2* secondary mutation, *c.[6174delT;6182del5]*, in addition to the parental *c.6174delT* mutation (*c.6174delT* : *c.[6174delT;6182del5]* allele ratio of 3:2) (Figure 1B, Table 1). The *BRCA2 c.[6174delT;6182del5]* secondary mutation in CAPAN1.B2.S* was predicted to restore the open reading frame of the gene and to encode a 3612 amino acid (aa) BRCA2 protein (Figure 1C), confirmed by western blotting (Supplementary Figure 1A). The secondary mutation in CAPAN1.B2.S* was associated with olaparib and also talazoparib resistance (Figure 1D and Supplementary Figure 1B, ANOVA $p<0.0001$) and the ability to form nuclear RAD51 foci in response to ionising radiation (Student's t-test $p<0.001$, Figure 1E, 1F, Supplementary Figure 1C), a biomarker of functional DNA repair by BRCA2. The CAPAN1.B2.S* clone also exhibited a similar doubling time to the parental CAPAN1 clone, of 2.5 days (Supplementary Figure 1D). Using the same approach, we also identified other CAPAN1 daughter clones with secondary mutations, including CAPAN1.B2.S*2, which had three different *BRCA2* alleles (*BRCA2 c.[6174delT;6185del5], BRCA2 c.[6174delT;6183delG],* and *BRCA2 c.[6174delT;6184delTC])* and CAPAN1.B2.S*3, which also had three different *BRCA2* alleles *(BRCA2 c.[6174delT;6183del6], BRCA2 c.[6174delT;6183del5],* and *BRCA2 c.[6174delT;6185del3])* (Supplementary Table 1).

Using a similar approach in SUM149 cells, we identified SUM149.B1.S*, a daughter clone which possessed a secondary mutation (an 80-bp *BRCA1* deletion, *c.[2288delT;2293del80])*, as well as the parental *c.2288delT* mutation (*c.2288delT : c.[2288delT;2293del80]* alleles in a 1:2 ratio) (Figure

1G, Table 1)). This secondary mutation was predicted to restore the open reading frame in the parental *BRCA1 c.2288delT* allele and to encode an 1836 aa BRCA1 protein (Figure 1H, Supplementary Figure 2A). SUM149.B1.S* exhibited both olaparib and talazoparib resistance (Figure 1I, Supplementary Figure 2B, ANOVA $p<0.0001$), and restoration of RAD51 nuclear localisation in response to DNA damage (Figure 1J, Supplementary Figure 2C, Supplementary Figure 2D, $p<0.01$, Student's t test). SUM149 and SUM149.B1.S* cells exhibited similar proliferation rates (Supplementary Figure 2E).

Darwinian selection of *BRCA1- or BRCA2*-proficient clones by PARPi treatment *in vitro*

To assess whether a Darwinian selective process might operate in the case of PARPi resistance, we mixed parental and secondary mutant tumour cells in *in vitro* co-cultures (i.e. CAPAN1 parental with CAPAN1.B2.S* secondary mutant cells, or SUM149 with SUM149.B1.S* cells) and then exposed the co-cultures to two different *BRCA*-gene selective drugs, olaparib or the platinum salt cisplatin (Figure 2A). We then monitored the relative frequency of each clone in response to drug exposure using droplet digital PCR (ddPCR) (27). To do this, we used duplex PCR reactions that included fluorophore-labelled digital PCR probes that were complementary to either parental or secondary mutant alleles (Supplementary Table 2, see methods). In pilot experiments where we mixed CAPAN1 and CAPAN1B2.S* cells in 1:1, 1:10, 1:100 ratios, we were able to accurately detect these different ratios using the ddPCR assay (Supplementary Figure 3). Using this ddPCR approach, we assessed whether

olaparib or cisplatin exposure would preferentially select for the secondary mutant clones in either CAPAN1 plus CAPAN1B2.S* co-cultures or SUM149 plus SUM149.B1.S* co-cultures. In these experiments we exposed co-cultures to either: (i) DMSO (the drug vehicle), (ii) constant exposure to olaparib (drug replenished every three days), (iii) intermittent exposure to olaparib (where drug was applied for 24 hours then washed out and replenished with media not containing drug for 48 hours), (iv) constant exposure to cisplatin (drug replenished every three days), or (v) intermittent 24 hour pulses of cisplatin (where drug was applied for 24 hours then washed out and replenished with media not containing drug for 48 hours) (Figure 2A). As expected, in both CAPAN1 and SUM149 co-cultures, constant exposure to either olaparib or cisplatin caused a greater reduction in the total tumour cell population size than intermittent drug exposure (Figure 2B). For example, in the CAPAN1 co-culture, 37% of the cell population survived after 14 days when exposed to constant olaparib, compared to 55% when intermittent drug exposure was used (Figure 2B). Despite these reductions in population size, both olaparib and cisplatin exposure caused an increase in the relative frequency of the secondary mutant clones compared to the parental clone (Figure 2C), effects replicated when mixed cultures were exposed to a chemically distinct PARP inhibitor, talazoparib (Supplementary Figure 4A). We found that the enrichment in the secondary mutant clones compared to the parental clones was most profound when cultures were constantly exposed to either olaparib or cisplatin, compared to intermittent drug exposures (Figure 2C). In cultures exposed to the drug vehicle, the proportion of CAPAN1.B2.S* and SUM149.B1.S* in DMSO exposed cultures remained

the same throughout the experiment (1:20 secondary mutant:parental clone, Supplementary Figure 4B). We therefore concluded that whilst constant drug exposure elicited a more profound reduction in the size of the tumour cell population, it did enrich for secondary mutant clones.

We then assessed whether the initial frequency of a secondary mutant clone in a tumour cell population might influence the time taken for this clone to dominate the population when it was exposed to the selective pressure of PARP inhibitor therapy. To do this, we generated CAPAN1.B2.S* : CAPAN1 mixed *in vitro* cultures with 1:1, 1:20, and 1:40 clone ratios and then exposed these to olaparib. We then estimated the temporal evolution of the culture in response to drug treatment by using ddPCR to measure CAPAN1.B2.S* : CAPAN1 ratios over time (Figure 2D). We found that in each culture, olaparib exposure caused an increase in the frequency of the secondary mutant clone compared to DMSO exposed cultures, with the fraction of CAPAN1.B2.S* cells in each PARPi exposed culture gradually increasing over time (Figure 2D). We also found that the initial frequency of the secondary mutant clone prior to drug treatment influenced the ability of the secondary mutant clone to eventually dominate the population (i.e. >75% of the cell population) once cells were exposed to PARP inhibitor, as might be expected of a Darwinian process (Figure 2D, compare 1:1, 1:20 and 1:40 ratio cultures).

We also used a different model system, isogenic DLD1 tumour cell lines with or without targeted mutations in *BRCA2* (DLD1.*BRCA2*$^{WT/WT}$ and DLD1.*BRCA2*$^{-/-}$ (28,29), to validate these observations. We labelled

DLD1.*BRCA2*$^{WT/WT}$ cells with a green fluorescent protein (GFP) and DLD1.*BRCA2*$^{-/-}$ cells with a red fluorescent protein (RFP) marker to enable detection and monitoring of co-culture populations via FACS (Supplementary Figure 5A). We found that in the absence of drug exposure, the DLD1.*BRCA2*$^{WT/WT}$ cells exhibited a selective advantage over DLD1.*BRCA2*$^{-/-}$ cells, as previously shown (28) (Supplemental Figure 5B), and that these cells exhibit more than a 10-fold difference in olaparib sensitivity (Figure 3A). We then mixed DLD1.*BRCA2*$^{WT/WT}$ cells into DLD1.*BRCA2*$^{-/-}$ cells *in vitro* at starting ratios of 1:1, 1:10, 1:100 and 1:1000, exposed these co-cultures to either olaparib or talazoparib, and monitored the temporal evolution of the population in response to PARPi (Supplementary Figure 5A). Similar to the CAPAN1 and SUM149 isogenic models, we observed that olaparib and talazoparib both selected for DLD1.*BRCA2*$^{WT/WT}$ cells over DLD1.*BRCA2*$^{-/-}$ cells in a Darwinian fashion (Figure 3B). For example, both olaparib and talazoparib exposure resulted in a 3-fold increase in DLD1.*BRCA2*$^{WT/WT}$ cells compared to the DMSO exposed cell population after 13 days of drug exposure (Figure 3B). Additionally, we noticed that the time taken for the DLD1.*BRCA2*$^{WT/WT}$ clone to reach clonal dominance was less in cell populations that had higher starting proportion of DLD1.*BRCA2*$^{WT/WT}$ cells (Figure 3C, D, E), as observed in the CAPAN1 co-culture model.

Darwinian selection of secondary mutant tumour cells also operates *in vivo*

We also assessed whether a Darwinian process influenced the *in vivo* response to PARPi treatment. To do this, we generated cohorts of mice

bearing subcutaneous xenografts consisting of a mixture of CAPAN1 parental and CAPAN1.B2.S* secondary mutant tumour cells (Figure 4A). We found that inoculating $5x10^6$ tumour cells at a 1:1 CAPAN1:CAPAN1.B2.S* ratio reproducibly generated 100 mm^3 xenografts 10 days after innoculation, where each clone was present in equal proportion (Figure 4B). When tumours reached 100 mm^3, tumour bearing mice were randomised into the following treatment cohorts to assess the selective pressure of PARPi treatment *in vivo*: (i) olaparib (50 mg/kg) administered once daily, (ii) olaparib (50 mg/kg) administered every other day, (iii) olaparib (50 mg/kg) administered twice a week on days 1 and 4, (iv) drug vehicle administered daily. In addition sentinel mice were sacrificed prior to treatment so that the CAPAN1:CAPAN1.B2.S* ratio in tumours prior to therapy could be confirmed (Figure 4A, Supplementary Figure 6A). We found that 50 mg/kg olaparib treatment, administered daily, every other day, or twice weekly, though well-tolerated, did not decrease tumour growth compared to the vehicle ($p > 0.05$ ANOVA for tumour volume in each olaparib treatment cohort *vs.* vehicle, Supplementary Figure 6B, Supplementary Figure 6C). We hypothesized that the absence of overall anti-tumour efficacy in this particular case might be due to failure to inhibit the PARPi secondary mutant clone in xenografts. To test this, we isolated tumour DNA from olaparib treated mice (after 28 days treatment) and assessed the relative ratio of parental *vs.* secondary mutant clones by ddPCR. In mice that received drug vehicle alone, the ratio of parental *vs.* secondary mutant clones remained unchanged at 50 % (data not shown). However, in mice that received olaparib treatment, the relative frequency of CAPAN1.B2.S* cells increased in response to therapy (Figure 4C). This

increase in CAPAN1.B2.S* frequency, in preference to the parental clone, was dependent upon the periodicity of PARPi administration, e.g. daily administration of olaparib caused the greatest increase in CAPAN1.B2.S* enrichment, followed by every other day treatment and then bi-weekly administration (Figure 4C, 4D). This suggested that PARPi administration also selected for secondary mutant tumour cell clones *in vivo* and that the degree of secondary mutant clone selection was related to the extent of selective pressure applied.

AZD-1775, a WEE1 kinase inhibitor, targets both parental and secondary BRCA mutant clones *in vitro* and *in vivo*

The co-culture model systems described above allowed us to establish that PARPi resistance, when driven by secondary mutations in *BRCA1* or *BRCA2*, can operate along Darwinian principles. We also assessed whether we could identify therapeutic vulnerabilities that would allow targeting of both parental and secondary mutant tumour cell clones as a means to minimise the impact of secondary mutation. We assessed whether small molecule WEE1 cell cycle checkpoint kinase inhibitors (WEE1i) (30) might have utility in this regard. We focused on WEE1 inhibitors for a number of reasons. WEE1 prevents premature mitotic entry by phosphorylating and inhibiting cyclin-dependent kinases such as Cyclin Dependent Kinase 1 (CDK1) (31,32). This activity is particularly critical in tumour cells with p53 pathway defects; p53 defects often co-occur with *BRCA* mutations, and although secondary mutations in *BRCA1/2* drive PARPi resistance, resistant tumours and cell lines remain p53 mutant (13). CAPAN1.B2.S* and SUM149.B1.S* clones retained the p53

mutations present in CAPAN1 and SUM149 parental tumour cell clones (Supplementary Figure 7, Supplementary Figure 8). We also found that in an analysis of *in vitro* sensitivity to the clinical WEE1 kinase inhibitor, AZD-1775, in a panel of tumour cell lines, CAPAN1.B2.S* and SUM149.B1.S* were amongst the most sensitive of 146 lines profiled (Figure 5A). We confirmed this AZD-1775 sensitivity in subsequent clonogenic survival experiments and found that, when compared to non-tumour breast epithelial cell lines (MCF10A and MCF12A), both CAPAN1 and SUM149-derived secondary mutant tumour cell clones retained profound sensitivity to AZD-1775 seen in parental tumour cells (average 22 fold difference in AUC, $p < 0.0001$ versus MCF10A or MCF12A, ANOVA, Figure 5B). We confirmed these observations using co-culture systems and found that at SF_{50} concentrations (concentration required to inhibit 50% of cells) of either olaparib or AZD-1775, olaparib exposure increased the relative frequency of the secondary mutant clones, but AZD-1775 did not (Figure 5C). This observation was confirmed when we used ddPCR to monitor the frequency of the secondary mutant clone over time in co-cultures exposed to AZD-1775 (Figure 5D). We also observed that parental and secondary mutant SUM149 and CAPAN1 clones were sensitive to additional small molecule cell cycle checkpoint inhibitors including PF-477736, a CHK1 inhibitor (33), and VX-970, an ATR inhibitor (34) when compared to non-tumour epithelial cells (Supplementary Figure 9A, Supplementary Figure 9B). This suggested that even when partial BRCA1 or BRCA2 protein function was restored by secondary mutation, vulnerability to small molecule inhibitors that target cell cycle checkpoints still existed. These effects did not appear to represent a relatively non-specific sensitivity to

cytotoxic agents in the parental and secondary mutant tumour cells, as these did not display an overtly distinct level of sensitivity to paclitaxel, capecitabine or gemcitabine when compared to MCF10 or MCF12A cells (Supplementary Figure 9C-F).

Previous studies have shown that WEE1 inhibitors cause tumour cell cytotoxicity by reducing the extent of CDC2 phosphorylation at Y15 (35). We found that in both CAPAN1 and CAPAN1.B2.S* cells, AZD-1775 exposure caused a decrease in CDC2 Y15 phosphorylation, an effect that was enhanced with prolonged drug exposure (Figure 5E). We noted that AZD-1775 exposure caused an increase in H2AX phosphorylation (γH2AX), a biomarker of DNA damage, in both CAPAN1 and secondary mutant CAPAN1.B2.S* cells (Figure 5E). This increase in γH2AX was commensurate with an increase in PARP cleavage, a measure of apoptosis (Figure 5E). Using FACS profiling, we found that AZD-1775 exposure had a very similar effect on cell cycle fractions in both CAPAN1 and CAPAN1.B2.S* cells, both of which demonstrated a profound reduction in the fraction of cells in active S-phase, with a commensurate increase in the proportion of cells in non-replicating S (Supplementary Figure 10). In CAPAN1 cells, AZD-1775 exposure caused a reduction in the active S-phase fraction from 25.9 % to 3.4 % (with a 3.9 to 52.1 % increase in non-replicating S phase), whilst CAPAN1.B2.S* cells showed a reduction in active S from 27.8 % to 4.2 % (with a 3.3 to 51.4 % increase in non-replicating S). These observations were reminiscent of those seen in H3K36me3-deficient cells, where WEE1 inhibition also caused a severe reduction in the active S phase fraction (36).

This suggested that WEE1 inhibition targeted CAPAN1 cells in S–phase, regardless of whether *BRCA2* was dysfunctional (as in CAPAN1) or somewhat reconstituted by the presence of a secondary *BRCA2* mutation (as in CAPAN1.B2.S*).

To investigate whether WEE1 inhibitor sensitivity in PARPi sensitive and resistant clones also operated *in vivo*, we assessed the effect of AZD-1775 treatment on mice bearing mixed CAPAN1/CAPAN1.B2.S* xenografts (each clone present at a 1:1 ratio, Figure 5F). Mice with established tumours were treated with either AZD-1775, olaparib or drug vehicle. Sentinel mice sacrificed prior to treatment showed the CAPAN1:CAPAN1.B2.S* ratio in tumours prior to therapy was 1:1 (Figure 5G). We used the time taken for tumours to reach 1500 mm^3 as a surrogate measure of survival (Figure 5H) and found that whilst olaparib treatment had minimal benefit (p=0.86, Log ranked Mantel-Cox test compared to vehicle), AZD-1775 treatment led to a significant survival benefit (p=0.011 Log ranked Mantel-Cox test compared to olaparib) (Figure 5I). Consistent with these observations, ddPCR analysis of tumours at the end of treatment showed that olaparib therapy caused a relative enrichment in the frequency of the secondary mutant clone (p = 0.058 compared to vehicle, Student's t test) whilst AZD-1775 did not (p = 0.43, compared to vehicle, Student's t test) (Figure 5J).

Discussion

In this study we used CRISPR-generated *BRCA1* or *BRCA2* secondary mutant daughter clones alongside isogenic parental cell lines to demonstrate

that PARPi exposure selects for secondary mutant clones in a Darwinian manner, both *in vitro* and *in vivo*. We found that the extent of selection for secondary mutant clones was influenced by the frequency of drug administration. In mice bearing tumours comprised of an equal proportion of *BRCA2* mutant and *BRCA2* secondary mutant tumour cells, olaparib had minimal effects on tumour growth but did preferentially select for the secondary mutant daughter clone over the parental tumour cell. It would be reasonable to infer that high frequencies of secondary mutant cells hinder the therapeutic effectiveness of PARP inhibitors. We also found that a WEE1 inhibitor, AZD-1775, had a greater therapeutic effect on mixed parental/secondary mutant tumours than olaparib. This example suggests that therapeutic vulnerabilities might still exist in tumours that have a high frequency of secondary mutant clones. Our data also suggest that secondary mutant and parental tumour cells also show sensitivity to other cell cycle/DNA damage repair inhibitors, including CHK1 and ATR inhibitors (Supplementary Figure 9). It seems possible that whilst secondary *BRCA1* or *BRCA2* gene mutations restore some HR function, these are unlikely to reverse the complex set of genomic rearrangements, aneuploidy and p53 mutations found in *BRCA1* or *BRCA2* mutant tumours prior to treatment (37). We hypothesise that it is these latter characteristics that sensitise tumour cells to drugs such as WEE1 inhibitors, perhaps explaining why AZD-1775 targeted both parental and secondary mutant clones. This hypothesis remains to be tested, but the observation that secondary mutant tumour cells are sensitive to AZD-1775 raises the possibility that therapeutic vulnerabilities still exist in PARPi resistant tumours.

In clinical studies, the maximum tolerated dose (MTD) for single-agent AZD-1775 was identified as 225 mg twice per day orally over 2.5 days per week for 2 weeks per 21-day cycle, a dosing regime sufficient to elicit a number of anti-tumour responses (38). In our *in vivo* studies (Figure 5) we used 30 mg/kg AZD-1775 twice-daily treatments for the entire duration of the study (150 days). This treatment approach was well tolerated in mice and based on prior mouse-based experiments using this WEE1 inhibitor (39). Nevertheless, it is possible that a similar constant dosing approach may not be well-tolerated in humans. Subsequent work might assess the potential of using intermittent WEE1 inhibitor dosing schedules to assess whether these also elicit a survival benefit in experiments similar to those shown in Figure 5.

One implication of this work is that the detection of secondary *BRCA1* or *BRCA2* mutations in patients could be important in influencing the choice of therapy. At present, secondary mutations in *BRCA1* or *BRCA2* can be detected by Sanger DNA sequencing (14-16) or by targeted DNA capture and deep sequencing (13). Circulating tumour DNA and circulating tumour cells might also display some of the secondary *BRCA1* or *BRCA2* mutations found in solid tumours. Detecting secondary mutations in such liquid biopsies might allow the early emergence of secondary mutations to be identified as a biomarker predicting the eventual clinical manifestation of PARPi resistance. One avenue we will now explore is to utilise the *in vivo* system we have described here to assess this possibility. A key quality of the model systems described here is that they allow the construction of co-cultures and

xenografts where the frequency and identity of secondary mutants is known. This will hopefully facilitate experiments that aim to examine further principles that govern clonal evolution and influence drug resistance in *BRCA1* or *BRCA2* mutant cancers. Alongside these models, we also note that the first patient derived xenograft tumours (PDX) with PARPi resistance-causing mutations have been recently described (40). These provide another system in which to assess how the clonal structure of tumours evolve in response to therapy. The combined use of engineered systems, such as that described here, alongside PDX systems will be critical in establishing what factors determine the response to treatment, and importantly, what therapeutic approaches could be taken to minimise the impact of secondary *BRCA1/2* gene mutations.

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References

1. Domchek SM, Armstrong K, Weber BL. Clinical management of BRCA1 and BRCA2 mutation carriers. *Nat Clin Pract Oncol* **2006**;3:2-3.
2. Alexandrov LB, Nik-Zainal S, Wedge DC, Aparicio SA, Behjati S, Biankin AV*, et al.* Signatures of mutational processes in human cancer. *Nature* **2013**;500:415-21.
3. Tutt A, Bertwistle D, Valentine J, Gabriel A, Swift S, Ross G*, et al.* Mutation in Brca2 stimulates error-prone homology-directed repair of DNA double-strand breaks occurring between repeated sequences. *EMBO J* **2001**;20:4704-16.
4. Moynahan ME, Chiu JW, Koller BH, Jasin M. Brca1 controls homology-directed DNA repair. *Mol Cell* **1999**;4:511-8.
5. Johnson RD, Liu N, Jasin M. Mammalian XRCC2 promotes the repair of DNA double-strand breaks by homologous recombination. *Nature* **1999**;401:397-9.
6. Arnaudeau C, Lundin C, Helleday T. DNA double-strand breaks associated with replication forks are predominantly repaired by homologous recombination involving an exchange mechanism in mammalian cells. *J Mol Biol* **2001**;307:1235-45.
7. Lord CJ, Tutt AN, Ashworth A. Synthetic lethality and cancer therapy: lessons learned from the development of PARP inhibitors. *Annu Rev Med* **2015**;66:455-70.
8. Ledermann J, Harter P, Gourley C, Friedlander M, Vergote I, Rustin G*, et al.* Olaparib maintenance therapy in patients with platinum-sensitive relapsed serous ovarian cancer: a preplanned retrospective analysis of

outcomes by BRCA status in a randomised phase 2 trial. *Lancet Oncol* **2014**;15:852-61.

9. Ledermann J, Harter P, Gourley C, Friedlander M, Vergote I, Rustin G*, et al.* Olaparib maintenance therapy in platinum-sensitive relapsed ovarian cancer. *N Engl J Med* **2012**;366:1382-92.
10. Audeh MW, Carmichael J, Penson RT, Friedlander M, Powell B, Bell-McGuinn KM*, et al.* Oral poly(ADP-ribose) polymerase inhibitor olaparib in patients with BRCA1 or BRCA2 mutations and recurrent ovarian cancer: a proof-of-concept trial. *Lancet* **2010**;376:245-51.
11. Tutt A, Robson M, Garber JE, Domchek SM, Audeh MW, Weitzel JN*, et al.* Oral poly(ADP-ribose) polymerase inhibitor olaparib in patients with BRCA1 or BRCA2 mutations and advanced breast cancer: a proof-of-concept trial. *Lancet* **2010**;376:235-44.
12. Lord CJ, Ashworth A. Mechanisms of resistance to therapies targeting BRCA-mutant cancers. *Nat Med* **2013**;19:1381-8.
13. Barber LJ, Sandhu S, Chen L, Campbell J, Kozarewa I, Fenwick K*, et al.* Secondary mutations in BRCA2 associated with clinical resistance to a PARP inhibitor. *J Pathol* **2013**;229:422-9.
14. Norquist B, Wurz KA, Pennil CC, Garcia R, Gross J, Sakai W*, et al.* Secondary somatic mutations restoring BRCA1/2 predict chemotherapy resistance in hereditary ovarian carcinomas. *J Clin Oncol* **2011**;29:3008-15.
15. Edwards SL, Brough R, Lord CJ, Natrajan R, Vatcheva R, Levine DA*, et al.* Resistance to therapy caused by intragenic deletion in BRCA2. *Nature* **2008**;451:1111-5.

16. Sakai W, Swisher EM, Jacquemont C, Chandramohan KV, Couch FJ, Langdon SP*, et al.* Functional restoration of BRCA2 protein by secondary BRCA2 mutations in BRCA2-mutated ovarian carcinoma. *Cancer Res* **2009**;69:6381-6.

17. Greaves M, Maley CC. Clonal evolution in cancer. *Nature* **2012**;481:306-13.

18. Issaeva N, Thomas HD, Djureinovic T, Jaspers JE, Stoimenov I, Kyle S*, et al.* 6-thioguanine selectively kills BRCA2-defective tumors and overcomes PARP inhibitor resistance. *Cancer Res* **2010**;70:6268-76.

19. Shen Y, Rehman FL, Feng Y, Boshuizen J, Bajrami I, Elliott R*, et al.* BMN 673, a Novel and Highly Potent PARP1/2 Inhibitor for the Treatment of Human Cancers with DNA Repair Deficiency. *Clin Cancer Res* **2013**;19:5003-15.

20. Hill SJ, Clark AP, Silver DP, Livingston DM. BRCA1 pathway function in basal-like breast cancer cells. *Mol Cell Biol* **2014**;34:3828-42.

21. Miller RE, Brough R, Bajrami I, Williamson CT, McDade S, Campbell J*, et al.* Synthetic Lethal Targeting of ARID1A-Mutant Ovarian Clear Cell Tumors with Dasatinib. *Mol Cancer Ther* **2016**;15:1472-84.

22. Farmer H, McCabe N, Lord CJ, Tutt AN, Johnson DA, Richardson TB*, et al.* Targeting the DNA repair defect in BRCA mutant cells as a therapeutic strategy. *Nature* **2005**;434:917-21.

23. Pender A, Garcia-Murillas I, Rana S, Cutts RJ, Kelly G, Fenwick K*, et al.* Efficient Genotyping of KRAS Mutant Non-Small Cell Lung Cancer Using a Multiplexed Droplet Digital PCR Approach. *PLoS One* **2015**;10:e0139074.

24. Campbell J, Ryan CJ, Brough R, Bajrami I, Pemberton HN, Chong IY*, et al.* Large-Scale Profiling of Kinase Dependencies in Cancer Cell Lines. *CellReports* **2016**;14:2490-501.

25. Goggins M, Schutte M, Lu J, Moskaluk CA, Weinstein CL, Petersen GM*, et al.* Germline BRCA2 gene mutations in patients with apparently sporadic pancreatic carcinomas. *Cancer Res* **1996**;56:5360-4.

26. Elstrodt F, Hollestelle A, Nagel JH, Gorin M, Wasielewski M, van den Ouweland A*, et al.* BRCA1 mutation analysis of 41 human breast cancer cell lines reveals three new deleterious mutants. *Cancer Res* **2006**;66:41-5.

27. Hindson CM, Chevillet JR, Briggs HA, Gallichotte EN, Ruf IK, Hindson BJ*, et al.* Absolute quantification by droplet digital PCR versus analog real-time PCR. *Nat Methods* **2013**;10:1003-5.

28. Hucl T, Rago C, Gallmeier E, Brody JR, Gorospe M, Kern SE. A syngeneic variance library for functional annotation of human variation: application to BRCA2. *Cancer Res* **2008**;68:5023-30.

29. Zimmer J, Tacconi EM, Folio C, Badie S, Porru M, Klare K*, et al.* Targeting BRCA1 and BRCA2 Deficiencies with G-Quadruplex-Interacting Compounds. *Mol Cell* **2016**;61:449-60.

30. Hirai H, Iwasawa Y, Okada M, Arai T, Nishibata T, Kobayashi M*, et al.* Small-molecule inhibition of Wee1 kinase by MK-1775 selectively sensitizes p53-deficient tumor cells to DNA-damaging agents. *Mol Cancer Ther* **2009**;8:2992-3000.

31. Molinari M. Cell cycle checkpoints and their inactivation in human cancer. *Cell Prolif* **2000**;33:261-74.

32. Liu Q, Guntuku S, Cui XS, Matsuoka S, Cortez D, Tamai K*, et al.* Chk1 is an essential kinase that is regulated by Atr and required for the G(2)/M DNA damage checkpoint. *Genes Dev* **2000**;14:1448-59.

33. Blasina A, Hallin J, Chen E, Arango ME, Kraynov E, Register J*, et al.* Breaching the DNA damage checkpoint via PF-00477736, a novel small-molecule inhibitor of checkpoint kinase 1. *Mol Cancer Ther* **2008**;7:2394-404.

34. Hall AB, Newsome D, Wang Y, Boucher DM, Eustace B, Gu Y*, et al.* Potentiation of tumor responses to DNA damaging therapy by the selective ATR inhibitor VX-970. *Oncotarget* **2014**;5:5674-85.

35. Do K, Doroshow JH, Kummar S. Wee1 kinase as a target for cancer therapy. *Cell Cycle* **2013**;12:3159-64.

36. Pfister Sophia X, Markkanen E, Jiang Y, Sarkar S, Woodcock M, Orlando G*, et al.* Inhibiting WEE1 Selectively Kills Histone H3K36me3-Deficient Cancers by dNTP Starvation. *Cancer Cell* **2015**;28:557-68.

37. Nik-Zainal S, Davies H, Staaf J, Ramakrishna M, Glodzik D, Zou X*, et al.* Landscape of somatic mutations in 560 breast cancer whole-genome sequences. *Nature* **2016**;534:47-54.

38. Do K, Wilsker D, Ji J, Zlott J, Freshwater T, Kinders RJ*, et al.* Phase I Study of Single-Agent AZD1775 (MK-1775), a Wee1 Kinase Inhibitor, in Patients With Refractory Solid Tumors. *J Clin Oncol* **2015**;33:3409-15.

39. Aarts M, Sharpe R, Garcia-Murillas I, Gevensleben H, Hurd MS, Shumway SD*, et al.* Forced mitotic entry of S-phase cells as a

therapeutic strategy induced by inhibition of WEE1. *Cancer Discov* **2012**;2:524-39.

40. Ter Brugge P, Kristel P, van der Burg E, Boon U, de Maaker M, Lips E, *et al.* Mechanisms of Therapy Resistance in Patient-Derived Xenograft Models of BRCA1-Deficient Breast Cancer. *J Natl Cancer Inst* **2016**;108.

Figure and Table legends

Figure 1. Characterization of *BRCA1* and *BRCA2* secondary mutant, PARP-inhibitor resistant clones.

A. Schematic showing experimental design. SUM149 and CAPAN1 cells were transfected with Cas9 and CRISPR gRNA expression constructs targeting *BRCA1* or *BRCA2,* respectively, to induce DSB and subsequently create a secondary *BRCA1 or BRCA2* mutation reinstating the open reading frame.

B. DNA sequence for CAPAN1.B2.S* showing 5 bp deletion in *BRCA2*. PAM sequence is underlined in blue.

C. Predicted BRCA2 protein structure for CAPAN1.B2.S*. The predicted amino acid length is shown.

D. Dose-response survival curves for CAPAN1.B2.S* (red) and CAPAN1 parental cell lines exposed to olaparib ($P < 0.0001$, ANOVA). Error bars represent SEM (standard error of the mean) from triplicate experiments.

E. Representative images for nuclear RAD51 foci formation in CAPAN1 and CAPAN1.B2.S* cells following IR exposure. Scale bar = 10 μm.

F. Bar chart illustrating quantitation of nuclear RAD51 foci. Cells containing more than five foci were counted as positive. Mean ± SEM for three independent experiments are shown. p values were calculated using Student's t test.

G. DNA sequence for SUM149.B1.S* showing 80 bp deletion in *BRCA1*. PAM sequence is underlined in blue.

H. Predicted BRCA1 protein structure for SUM149.B1.S*. The predicted amino acid length is shown.

I. Dose-response survival curves for SUM149.B1.S* (green) and SUM149 parental cells exposed to olaparib (P <0.0001, ANOVA). Error bars represent SEM from triplicate experiments.

J. Bar chart illustrating quantitation of nuclear RAD51 foci. Cells containing more than five foci were counted as positive. Mean ± SEM (standard error of the mean) for three independent experiments are shown. p values were calculated using Student's t test.

Figure 2. Olaparib exposure induces Darwinian selection favouring secondary BRCA mutants *in vitro*.

A. Experimental schematic. Secondary mutant and parental cells were mixed at a 1:20 ratio and then exposed to DMSO, olaparib, or cisplatin. After drug exposure, populations were analysed for surviving fraction and secondary mutant:parental proportions using ddPCR.

B. Bar graph illustrating the effect of drug exposure on population surviving fraction in either CAPAN1.B2.S*:CAPAN1 or SUM149.B1.S*:SUM149 co-cultures.

C. Bar graph illustrating the increase in secondary mutant clone frequency following 14 days of drug exposure.

D. Graphs showing the frequency of CAPAN1B2*S cells in CAPAN1/CAPAN1B2* co-cultures exposed to 500 nM olaparib. Clone frequency was estimated by ddPCR at the time points shown. Error bars represent SEM from three independent measurements.

Figure 3. PARPi-induced selectivity operates in BRCA2 isogenic DLD1 tumour cells.

A. Dose-response curves illustrating 6-well clonogenic survival data for BRCA2 isogenic cells, DLD1.*BRCA2*$^{WT/WT}$ and DLD1.*BRCA2*$^{-/-}$, exposed to olaparib over 14 days. Error bars represent SEM from triplicate experiments.

B. Bar graph illustrating the increase in DLD1.*BRCA2*$^{WT/WT}$ frequency in a 1:100 starting DLD1.*BRCA2*$^{WT/WT}$:DLD1.*BRCA2*$^{-/-}$ ratio co-culture following 13 days of drug exposure.

C. Bar graph showing starting ratio of DLD1.*BRCA2*$^{-/-}$ to DLD1.*BRCA2*$^{WT/WT}$ influences time (days) for DLD1.*BRCA2*$^{WT/WT}$ cells to reach clonal dominance (75% of cell population).

D-E. Graphs showing time required for DLD1.*BRCA2*$^{WT/WT}$-GFP to reach clonal dominance (75%, dotted line) when exposed to either 25 nM or 100 nM of olaparib **(D)** or 10 nM of talazoparib **(E)** in DLD1.*BRCA2*$^{WT/WT}$:DLD1.*BRCA2*$^{-/-}$ co-cultures.

Figure 4. PARPi-induced Darwinian selection of BRCA proficient tumour cells *in vivo.*

A. Experimental schematic of mixed CAPAN1:CAPAN1.B2.S* xenografts treated with olaparib.

B. Bar chart illustrating CAPAN-1 (white) and CAPAN1.B2.S* (red) cell frequency in tumour xenografts prior to drug treatment. Values shown for each animal were derived from three tumour sections with mean ± SEM shown.

C. Bar graph illustrating the increase in secondary mutant clone frequency in tumours following 28-day olaparib treatment (n=6, mean ± SEM shown). p values were calculated by Student's t test.

D. Correlation between fold increase in the frequency of the secondary mutant clone and the periodicity of olaparib administration.

Figure 5. PARPi-resistant, secondary mutant clones and parental tumour cells are sensitive to AZD-1775 *in vitro* and *in vivo*.

A. Waterfall plot comparing AUC values collated from a five day exposure to AZD-1775 from 146 cancer cell lines.

B. Dose-response survival curves illustrating 6-well clonogenic survival data in CAPAN1, CAPAN1.B2.S*, SUM149, SUM149.B1.S*, MCF-10A and MCF-12A cells exposed to AZD-1775.

C. Bar graph illustrating the increase in secondary mutant clone frequency following 14 days of drug exposure.

D. Graph showing the frequency of CAPAN1B2*S cells in CAPAN1/CAPAN1B2* co-cultures exposed to AZD-1775. Clone frequency was estimated by ddPCR and the time points shown. Error bars represent SEM from three independent measurements. This experiment was conducted alongside the experiment described in Figure 2D; to allow comparison, the response to olaparib and DMSO exposure from Figure 2D is re-plotted here.

E. Western blot for CAPAN1 and CAPAN1.B2.S* cells lysates probed for pCDC2(Y15), γ-H2AX (a DNA damage marker), and cleaved PARP1 (a marker of apoptosis). Tubulin was used as a loading control.

F. Experimental schematic of mixed CAPAN1:CAPAN1.B2.S* xenografts treated with olaparib or AZD-1775.

G. Bar chart illustrating CAPAN-1 (white) to CAPAN-1.B2.S* (red) clone ratio in in tumour xenografts prior to drug treatment. Values shown from six sentinel animals with mean ± SEM shown.

H. Tumour volume plotted against length of treatment for individual xenografts comprised of CAPAN1:CAPAN1.B2.S* mixed tumour cells over 150 days (n=18 total, n=6 in each cohort).

I. Survival curves using maximum tumour size (1500 mm^3) as a surrogate for survival from the experiment shown in E.

J. Bar chart showing proportion of CAPAN1.B2.S* tumour cells following treatment from the experiment shown in E (n=6, mean ± SEM). P values were calculated by Student's t test.

Supplementary Figure 1.

A. Western blot of lysates with anti-BRCA2 antibodies show almost full length BRCA2 is present in CAPAN1.B2.S*.

B. Dose-response survival curves for talazoparib for CAPAN1.B2.S* (red) compared to the parental cell line ($P < 0.0001$, ANOVA). Error bars represent SEM from triplicate experiments.

C. Bar chart illustrating quantitation of nuclear γ-H2AX foci. Cells containing more than five foci were counted as positive. Mean ± SEM (standard error of the mean) for three independent experiments are shown. p values were calculated using Student's t test.

D. Fold change in cell count plotted against time in CAPAN1 and CAPAN1.B2.S* show isogenic cell lines have similar growth rates. Error bars represent SEM from triplicate experiments.

Supplementary Figure 2.

A. IP followed by western blotting of lysates with anti-BRCA1 antibodies showing re-expression of near full-length BRCA1 in SUM149.B1.S*. B, BRCA1 IP; IgG, control IP.

B. Dose-response survival curves for talazoparib for SUM149.B1.S* (green) compared to the parental cell line (P <0.0001, ANOVA). Error bars represent SEM from triplicate experiments.

C. Representative images for nuclear RAD51 foci formation in SUM149 and SUM149.B1.S* cells following IR exposure. Scale bar = 10 μm.

D. Bar chart illustrating quantitation of nuclear γ-H2AX foci. Cells containing more than five foci were counted as positive. Mean ± SEM (standard error of the mean) for three independent experiments are shown. p values were calculated using Student's t test.

E. Fold change in cell count plotted against time in SUM149 and SUM149.B1.S* show isogenic cell lines growth rates. Error bars represent SEM from triplicate experiments.

Supplementary Figure 3. Sensitive of ddPCR assay can detect up to 1:100 secondary mutant to parental tumour cell events.

ddPCR plots showing droplet populations observed for 1:2, 1:10, or 1:100 starting ratios of secondary mutant:parental tumour cells with either

CAPAN1.B2.S* (blue, FAM amplitude) or CAPAN1 (green, VIC amplitude). Key: Black drops- empty droplets, blue- CAPAN1.B2.S* DNA FAM positive droplets, green- CAPAN1 DNA VIC positive droplets.

Supplementary Figure 4. Olaparib and talazoparib select for secondary mutant tumour cells.

A. CAPAN1/ CAPAN1.B2.S* or SUM149/ SUM149.B1.S* mixed cultures (1:10 secondary mutant:parental cell ratio) were exposed to either either 1 μM olaparib or 0.1 μM talazoparib for 21 days. The frequency of secondary mutant cells was monitored by ddPCR. Graphs show the frequency of secondary mutant tumour cells in the population over time.

B. No fitness discrepancy between parental and secondary mutant clone observed for mixed CAPAN-1 or SUM149 co-cultures. Bar graphs showing day 1 and day 14 DMSO exposed controls for both CAPAN1 and SUM149 mixed secondary mutant:parental tumour cell populations.

Supplementary Figure 5. DLD1.BRCA2$^{WT/WT}$ tumour cells have a fitness advantage over DLD1.BRCA2$^{-/-}$ cells *in vitro*.

A. Experimental schematic for evaluating mixed cell populations consisting of DLD1.*BRCA2*$^{WT/WT}$-GFP and DLD1.*BRCA2*$^{-/-}$-RFP cells at different starting ratios (1:1, 1:10, 1:100, 1:1000) over 40 days. Cells were exposed to either olaparib or talazoparib at a low and high dose and periodically monitored by FACS analysis.

B. Graph showing temporal evaluation of DLD1.*BRCA2*$^{WT/WT}$ tumour cell population within mixed DLD1.*BRCA2*$^{WT/WT}$-GFP:DLD1.*BRCA2*$^{-/-}$-RFP co-cultures over 40 days.

Supplementary Figure 6. Olaparib has little efficacy in mixed CAPAN-1 xenografts.

A. Bar chart illustrating CAPAN-1 (white) to CAPAN-1.B2.S* (red) ratio in day 0 (pretreatment control) xenografts (n=6, mean ± SEM).

B. Tumour response in mixed CAPAN1:CAPAN1.B2.S* xenografts treated for 28 days with 1) vehicle, 2) olaparib – 50 mg/kg (daily), 3) olaparib – 50 mg/kg (every-other-day), and 4) olaparib – 50 mg/kg (2x/weekly) (n=6, mean ± SEM).

C. Tolerability of olaparib treatment *in vivo* over 28 day exposure (n=6, mean ± SEM).

Supplementary Figure 7. Exome sequencing of CAPAN-1.B2.S* show retention of TP53 mutations.

A-D. Exome sequencing confirms CAPAN1.B2.S* cells retained same *TP53* mutation **(A)** and variants **(B-D)** as observed in the CAPAN1 parental cell line.

Supplementary Figure 8. Exome sequencing of SUM149.B1.S* show retention of TP53 mutation.

A. Exome sequencing confirms SUM149.B1.S* cells retained the same *TP53* mutation as observed in the SUM149 parental cell line.

Supplementary Figure 9. BRCA-proficient and -deficient cells exhibit sensitivity to additional DNA damaging agents.

A-B. Five day dose-response survival curves for **(A)** PF-477736, **(B)** VX-970, **(C)** Gemcitabine, **(D)** Paclitaxel, **(E)** Doxorubicin and **(F)** Vinorelbine for CAPAN1, CAPAN1.B2.S*, SUM149 or SUM149.B1.S* compared to MCF-10A and MCF-12A cell lines. Error bars represent SEM from triplicate experiments.

Supplementary Figure 10. AZD-1775 causes an active S phase reduction in both CAPAN1 and CAPAN-1.B2.S* cells.

BrdU and propidium iodide (PI) FACS profiling plots are shown with the fraction of cells in each cell cycle phase indicated. CAPAN1 and CAPAN1.B2.S* cells were exposed to 1 µM AZD-1775, or DMSO for 72 hours. Following this, the cell cycle distribution of the cells was assayed by BrdU/PI FACS analysis as shown.