PRECLINICAL MODELLING OF BREAST CANCER DRUG RESPONSES

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This thesis is submitted for the degree of Doctor of Philosophy
Declaration

This thesis is the result of my own work and includes nothing which is the outcome of work done in collaboration except as declared in the acknowledgements and specified in the text.

This thesis is the result of work carried out at the Cancer Research UK Cambridge Institute between October 2016 and September 2020. It is not substantially the same as any that I have submitted or is being concurrently submitted for a degree or diploma or other qualification at the University of Cambridge or any other University or similar institution.

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Preclinical Modelling of Breast Cancer Drug Responses

Abigail Elizabeth Shea

Breast cancer is a highly heterogeneous disease, exhibiting both inter- and intra-tumour heterogeneity at genomic and phenotypic levels. This remains a key limitation in the treatment of the disease. While recent advances have dramatically improved our understanding and treatment strategies for specific subgroups of patients, those with triple negative breast cancer remain limited to few therapeutic options, due to a lack of actionable targets. Developments in our understanding of the DNA damage response in breast cancer have unveiled an unprecedented opportunity to treat this subgroup of patients, who commonly harbour alterations in related pathways. Accelerated by the discovery of breast cancer predisposition genes *BRCA1* and *BRCA2*, a number of compounds which target the DNA damage response are entering preclinical drug development. However, the success of new therapies is dependent upon preclinical models which mirror clinical drug responses and accurately capture the heterogeneity of this complex disease. Patient-derived tumour xenografts (PDTXs) have been found to recapitulate the main genomic and histological features of breast cancer and reflect the diversity of drug responses observed in the clinic. This thesis outlines the key findings on the development of an avatar co-clinical trial platform to assess the concordance with clinical drug responses on a matched patient/PDTX basis. However, as with many approved therapies, an understanding of the dynamics of drug response and the evolutionary principles underpinning the disease remain at the forefront of biological interest and are essential to prevent, or anticipate, the development of drug resistance. In order to approach this question, we leveraged the preclinical platform and used single cell technologies to shed light on the dynamics of drug response and mechanisms of drug tolerance, to drugs targeting the DNA damage response in breast cancer.
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# Table of Contents

Chapter 1: Introduction ................................................................................................................. 1

1.1 Breast cancer ......................................................................................................................... 1
    1.1.1 Breast cancer heterogeneity ....................................................................................... 3
    1.1.2 Patient stratification in breast cancer ........................................................................ 5
    1.1.3 Treatment strategies in breast cancer ........................................................................... 7
    1.1.4 Triple negative breast cancer ..................................................................................... 10

1.2 The DNA damage response ................................................................................................. 12
    1.2.1 DNA damage response mechanisms .......................................................................... 13
    1.2.2 Alterations in the DNA damage response ................................................................. 16
    1.2.3 Targeting the DNA damage response in breast cancer ........................................... 18

1.3 Preclinical models of breast cancer .................................................................................... 20
    1.3.1 Historical approaches to tumour modelling ............................................................... 20
    1.3.2 Patient derived tumour xenografts .......................................................................... 23
    1.3.3 Breast cancer PDTXs ............................................................................................... 26
    1.3.4 Using PDTXs in translational science ..................................................................... 28

1.4 Outlook and scope of the thesis ......................................................................................... 31

1.5 Research Aims ...................................................................................................................... 32

Chapter 2: Materials and Methods .......................................................................................... 33

2.1 PDTX in vivo studies ........................................................................................................... 33
    2.1.1 Establishment and passage of PDTX models ............................................................ 33
    2.1.2 In vivo pharmacology studies .................................................................................... 34
    2.1.3 In vivo trial statistical considerations and analysis .................................................. 35

2.2 Cell culture assays .............................................................................................................. 36
    2.2.1 Generation and culture of PDTCs .............................................................................. 36
    2.2.2 Cell line tissue culture .............................................................................................. 36
    2.2.3 Cell line serum deprivation ..................................................................................... 37
    2.2.4 Cell quantification using a haemocytometer ............................................................. 37
    2.2.5 Cell quantification using Vi-CELL ........................................................................... 38
    2.2.6 siRNA ....................................................................................................................... 38
## 2.3 Drug dosing experiments and analysis ........................................ 40
2.3.1 High throughput drug screening of PDTCs ..................................... 40
2.3.2 High throughput drug screening of cell lines ................................... 41
2.3.3 Analysis of high-throughput drug screening and growth dynamics ........ 42
2.3.4 Drug dosing time course experiments of PDTC ................................. 44
2.3.5 Drug dosing time course experiments of cell lines ............................. 44

## 2.4 Protein analysis ............................................................................. 45
2.4.1 PDTX tissue fixation and paraffin embedding .................................... 45
2.4.2 Immunohistochemistry .................................................................. 45
2.4.3 Tissue microarray construction ....................................................... 48
2.4.4 Protein extraction from tissue samples ............................................ 49
2.4.5 Protein extraction from cultured cell lines ....................................... 49
2.4.6 Protein extraction from cultured PDTCs .......................................... 50
2.4.7 Protein quantification (BCA assay) ................................................... 50
2.4.8 Western blot ................................................................................... 51
2.4.9 ELISA for quantification of PAR ....................................................... 54
2.4.10 LC-MS/MS .................................................................................... 55
2.4.11 Flow cytometry ............................................................................. 57
2.4.12 Mass cytometry experiments ......................................................... 60
2.4.13 Mass cytometry analysis ............................................................... 64

## 2.5 Genomics ....................................................................................... 65
2.5.1 Nucleic acid extraction .................................................................... 65
2.5.2 Nucleic acid quantification .............................................................. 65
2.5.3 Assessing RNA quality control ....................................................... 66
2.5.4 Short tandem repeat analysis .......................................................... 66
2.5.5 RNA-sequencing library preparation ............................................. 66
2.5.6 RNA-sequencing analysis ............................................................... 67
2.5.7 Exome sequencing library preparation ......................................... 68
2.5.8 Exome sequencing analysis ............................................................. 69
2.5.9 Single cell RNA-sequencing experiments ........................................ 70
2.5.10 Single cell RNA-sequencing analysis ............................................ 72
2.5.11 Analysis of primary breast cancer samples .................................... 73
2.5.12 De novo motif search .................................................................... 74
Chapter 3: Development of a co-clinical trial platform............................. 76

3.1 Introduction ................................................................................................. 76
3.2 Aims ............................................................................................................. 80
3.3 Results and discussion................................................................................ 81
  3.3.1 The development of the PARTNER PDTX cohort ............................ 81
  3.3.2 Quality control pipeline of the PDTXs ............................................. 85
  3.3.3 Exploring characteristics of the PARTNER PDTX cohort .......... 93
  3.3.4 Preservation of genomic features through passaging .................. 100
  3.3.5 Establishment of a co-clinical trial ................................................. 105
  3.3.6 Optimisation of the co-clinical trial dose schedule ........................ 112
  3.3.7 An optimised co-clinical trial platform ........................................ 116
3.4 Conclusions ............................................................................................... 123

Chapter 4: Modelling drug responses to DDR compounds \textit{ex vivo}......... 124

4.1 Introduction ................................................................................................. 124
4.2 Aims ........................................................................................................... 128
4.3 Results and discussion................................................................................ 129
  4.3.1 Development of a drug screening platform for DDR-targeting compounds .... 129
  4.3.2 Comparing drug responses between TNBC PDTXs ............................ 135
  4.3.3 Drug response profiles following \textit{in vivo} treatment ......................... 143
  4.3.4 Leveraging \textit{ex vivo} drug screen data to design new treatment strategies .... 157
4.4 Conclusions ............................................................................................... 162

Chapter 5: The dynamics of drug response to PARP inhibition ............... 163

5.1 Introduction ................................................................................................. 163
5.2 Aims ........................................................................................................... 169
5.3 Results and discussion................................................................................ 170
  5.3.1 Relating genomic features to PARP inhibitor responses .................. 170
  5.3.2 Identifying functional changes upon PARP inhibition ...................... 175
  5.3.3 Investigating known mechanisms of resistance ............................... 178
  5.3.4 Exploring genomic changes............................................................... 187
5.3.5 Global shifts in the transcriptomic landscape.......................................................... 195
5.3.6 Transcriptomic population dynamics using scRNA-sequencing.............................. 206
5.3.7 Interrogating phenotypic populations using mass cytometry .................................. 217
5.3.8 Targeting a drug tolerant phenotype........................................................................ 235
5.3.9 Working towards a mechanism of drug tolerance..................................................... 242

5.4 Conclusions .................................................................................................................. 257

Chapter 6: Conclusions and clinical relevance .............................................................. 258

6.1 Clinical relevance and context ..................................................................................... 258
6.2 Aligning PDTX models with the clinical setting......................................................... 259
6.3 Leveraging the PDTX platform to study mechanisms of drug response 262

References .......................................................................................................................... 265
List of Figures

Chapter 1
1.1.1 – Tumour heterogeneity and clonal evolution 4
1.2.1 – DNA damage and mechanisms of repair 15
1.3.3 – PDTXs and PDTCs retain the molecular features of the originating tumour 27

Chapter 2
2.2.4 – Haemocytometer cell counting grid 38
2.4.11 – Flow cytometry gating strategy 59
2.4.13 – Mass cytometry gating strategy 64
2.5.9.1 – Generation of GEMs from single cell suspensions 71
2.5.9.2 – Chromium Single Cell 3’ library structure 71

Chapter 3
3.1 – PARTNER clinical trial 78
3.3.1 – PDTX cohort reflects the diversity of the clinical cohort 82
3.3.2.1 – Quality control pipeline for PDTX models 86
3.3.2.2 – Potential issues during PDTX engraftment 88
3.3.2.3 – STR profiling of PDTX models 89
3.3.2.4 – Quality control IHC of co-clinical trial cohort 91
3.3.2.5 – Clinical biomarker IHC of co-clinical trial cohort 92
3.3.3.1 – Genomic features of co-clinical trial cohort 94
3.3.3.2 – TP53 and BRCA1 expression in co-clinical trial cohort 96
3.3.3.3 – HRD in the co-clinical trial cohort 98
3.3.4.1 – Correlation of SNVs using multi-region sequencing 101
3.3.4.2 – Correlation of SNVs between biological replicates 103
3.3.4.3 – Correlation of SNVs between passages 104
3.3.5.1 – Co-clinical trial design 106
3.3.5.2 – PDTX avatar responses in a co-clinical trial 108
3.3.5.3 – PDTX in vivo growth dynamics 109
3.3.5.4 – Exploring parallel drug responses in vivo 111
3.3.6.1 – Drug tolerability experiments 113
3.3.6.2 – Target engagement experiments 115
3.3.7.1 – Optimised co-clinical trial design 117
3.3.7.2 – PDTX responses to an optimised co-clinical trial 119
3.3.7.2 – Adopting a PDTX response criteria

Chapter 4

4.1 – Development of an integrated experimental framework
4.1.1 – Development of an ex vivo drug screening platform
4.1.2 – Optimisation of drug screening conditions
4.1.3 – Correlation of drug sensitivities between experiments
4.2.1 – PDTX growth dynamics
4.2.2 – Comparing drug responses between PDTX models
4.2.3 – Exploring shapes of dose response curves
4.2.4 – Differential drug responses between PDTX models
4.3.1.1 – Development of an ex vivo drug screening platform
4.3.1.2 – Optimisation of drug screening conditions
4.3.1.3 – Correlation of drug sensitivities between experiments
4.3.2.1 – PDTX growth dynamics
4.3.2.2 – Comparing drug responses between PDTX models
4.3.2.3 – Exploring shapes of dose response curves
4.3.2.4 – Differential drug responses between PDTX models
4.3.3.1 – Experimental design of drug screen following in vivo treatment
4.3.3.2 – Effects of in vivo treatment on PDTC growth dynamics
4.3.3.3 – Drug response profiles after in vivo treatment
4.3.3.4 – Changes in drug response profiles after in vivo treatment
4.3.3.5 – CTO-specific changes are conserved across passages
4.3.3.6 – Changes in drug responses after olaparib treatment in vivo
4.3.3.7 – Drug response profiles after AZD1775 treatment in vivo
4.3.3.8 – AZD1775 treated samples exhibit decreased sensitivity
4.3.4.1 – Exploring SNVs involved in DDR-processes in model 1006
4.3.4.2 – Testing sequential therapy with CT and PARP inhibitors

Chapter 5

5.3.1.1 – Studying drug response dynamics to olaparib
5.3.1.2 – Differential PDTX drug responses to olaparib
5.3.2 – Changes in drug responses after olaparib treatment in vivo
5.3.3.1 – Effects of olaparib on models 1141
5.3.3.2 – Effects of olaparib on models 1022
5.3.3.3 – Genomics alterations of 1006 and 1040 after olaparib treatment
5.3.3.4 – Exploring resistance mechanisms in models 1006 and 1040
5.3.4.1 – Exploring SNVs involved in DDR-processes in model 1006
5.3.4.2 – Changes in VAF of patient mutations
5.3.4.3 – Emergent and depleted SNVs
5.3.4.4 – Treatment effects on the mutational landscape
5.3.5.1 – Olaparib causes a global transcriptomic shift
5.3.5.2 – Olaparib causes permanent gene expression changes
5.3.5.3 – Differential expression and GSEA analysis
5.3.5.4 – Olaparib effects on DNA repair signalling
5.3.5.5 – Olaparib effects on mesenchymal phenotypes
5.3.6.1 – Experimental design of single cell RNA-sequencing
5.3.6.2 – single cell RNA-sequencing reveals transcriptomic populations
5.3.6.3 – Gene set enrichment to characterise transcriptomic populations
5.3.6.4 – Drug-specific transcriptomic population changes
5.3.7.1 – Experimental design of mass cytometry
5.3.7.2 – Mass cytometry gating and barcode quality control
5.3.7.3 – Mass cytometry reference sample quality control
5.3.7.4 – Extended mass cytometry quality control measures
5.3.7.5 – Exploring drug-specific changes using FlowSOM
5.3.7.6 – Features of FlowSOM metaclusters
5.3.7.7 – Olaparib causes phenotypic population changes
5.3.7.8 – FlowSOM reveals permanent phenotypic changes
5.3.7.9 – Validation of mass cytometry findings
5.3.8.1 – Exploring alternative hypotheses regarding olaparib-induced changes
5.3.8.2 – Olaparib and salinomycin exhibit synergy in model 1006
5.3.8.3 – Further exploring olaparib and salinomycin synergy
5.3.8.4 – Carboplatin and salinomycin do not exhibit synergy
5.3.9.1 – Olaparib induced expression changes in p-c-Jun
5.3.9.2 – Motif analysis to identify candidate transcription factors
5.3.9.3 – Expression of KLF4 and MTF1 in PDTX models
5.3.9.4 – Olaparib alters transcription factor expression in cell lines
5.3.9.5 – Optimisation of olaparib drug screen in cell lines
5.3.9.6 – Pilot siRNA experiment to knock down KLF4 and MTF1
5.3.9.7 – Growth dynamics of cell lines following siRNA knockdown
5.3.9.8 – KLF4/MTF1 knockdown does not alter olaparib response

Chapter 6
6.1 – Preclinical modelling of breast cancer drug responses
## List of Tables

### Chapter 2

<table>
<thead>
<tr>
<th>Section</th>
<th>Table Title</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.2.1</td>
<td>PDTC complete growth media</td>
<td>36</td>
</tr>
<tr>
<td>2.3.1.1</td>
<td>Drug panel for high throughput drug screening</td>
<td>40</td>
</tr>
<tr>
<td>2.3.1.2</td>
<td>Drug dosing schedule</td>
<td>41</td>
</tr>
<tr>
<td>2.4.2.1</td>
<td>Optimised conditions for IHC</td>
<td>47</td>
</tr>
<tr>
<td>2.4.2.2</td>
<td>Primary antibodies for IHC</td>
<td>48</td>
</tr>
<tr>
<td>2.4.2.3</td>
<td>Post primary/secondary antibodies for IHC</td>
<td>48</td>
</tr>
<tr>
<td>2.4.4</td>
<td>PDTX tumour lysis buffer recipe (western blot)</td>
<td>49</td>
</tr>
<tr>
<td>2.4.5</td>
<td>RIPA lysis buffer recipe</td>
<td>50</td>
</tr>
<tr>
<td>2.4.8.1</td>
<td>Western blot antibodies</td>
<td>53</td>
</tr>
<tr>
<td>2.4.8.2</td>
<td>Western blot protocols</td>
<td>53</td>
</tr>
<tr>
<td>2.4.8.3</td>
<td>Western blot buffers</td>
<td>54</td>
</tr>
<tr>
<td>2.4.9</td>
<td>ELISA lysis buffer</td>
<td>55</td>
</tr>
<tr>
<td>2.4.10.1</td>
<td>LC gradient profile (paclitaxel and olaparib)</td>
<td>56</td>
</tr>
<tr>
<td>2.4.10.2</td>
<td>LC gradient profile (carboplatin)</td>
<td>56</td>
</tr>
<tr>
<td>2.4.11</td>
<td>Flow cytometry antibody panel</td>
<td>59</td>
</tr>
<tr>
<td>2.4.12</td>
<td>Mass cytometry antibody panel</td>
<td>63</td>
</tr>
</tbody>
</table>

### Chapter 3

<table>
<thead>
<tr>
<th>Section</th>
<th>Table Title</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>3.3.1</td>
<td>Clinical information of PDTXs in the co-clinical trial cohort</td>
<td>84</td>
</tr>
</tbody>
</table>

### Chapter 4

<table>
<thead>
<tr>
<th>Section</th>
<th>Table Title</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>4.3.2</td>
<td>DDR targeted drug panel</td>
<td>136</td>
</tr>
</tbody>
</table>
Abbreviations

A
ADP: Adenosine diphosphate
ATM: Ataxia-telangiectasia-mutated
ATR: ATM- and Rad3-related
AUC: Area under the curve

B
BCA: Bicinchoninic acid
BER: Base excision repair
BET: Bromodomain and extra-terminal motif
BRCA1: Breast cancer 1
BRCA2: Breast cancer 2
BSA: Bovine serum albumin

C
CEP17: Chromosome 17 centromeric enumeration probe
CNA: Copy number aberration
CNV: Copy number variant
CPM: Counts per million
CSB: Cell staining buffer
CT: Chemotherapy cohort
CTC: Circulating tumour cell
CTG: CellTiter-Glo® 3D
CTO: Chemotherapy with olaparib

D
DAB: 3,3’-Diaminobenzidine
DDR: DNA damage response
DMSO: Dimethyl sulfoxide
DNA-PK: DNA-dependent protein kinase
DSB: Double strand breaks

E
EBV: Epstein Bar Virus
ECL: Enhanced chemiluminescence
EGFR: Epidermal growth factor receptor
EMT: Epithelial to mesenchymal transition
ER: Oestrogen receptor
ERBB2: Erb-B2 Receptor Tyrosine Kinase 2
ESR1: Oestrogen Receptor 1

F
FBS: Foetal bovine serum

G
G1-phase: First growth phase
G2-phase: Second growth phase
γ-H2AX: Histone H2AX phosphorylation at serine 139
GEM: Gel Bead-In-Emulsions
GEMM: Genetically engineered mouse model
GSEA: Gene set enrichment analysis

H
HER2: Human epidermal growth factor 2
HR: Homologous recombination
HRD: Homologous recombination deficiency
HILIC: Hydrophilic interaction liquid chromatography conditions
HPLC: High performance liquid chromatography
HRP: Horseradish peroxidase
IC50: Half maximal inhibitory concentration
IGV: Integrative Genomics Viewer
IHC: Immunohistochemistry
IntClust: Integrative Cluster

KLF4: Kruppel-like factor 4

LC-MS/MS: Liquid chromatography and tandem mass spectrometry
LOH: Loss of heterozygosity

M-phase: Mitosis phases
MDS: Multi-dimensional scaling plot
MET: Mesenchymal to epithelial transition
MMR: Mismatch repair
MMTV: Mouse mammary tumour virus
MST: Minimum spanning tree
MTF1: Metal regulatory transcription factor 1
mTOR: Mammalian target of rapamycin

NAD: Nicotinamide adenine dinucleotide
NBF: Neutral buffered formalin
NER: Nucleotide excision repair
NGS: Next generation sequencing
NHEJ: Non homologous end joining
NICE: National Institute for Health Care Excellence
NSG: NOD.Cg-Prkdc<sup>scid</sup> Il2rg<sup>tm1Wjl</sup>/SzJ
NST: Invasive carcinoma of no special type

PAR: Poly adenosine diphosphate ribose
PARG: PAR glycohydrolase
PARP: PAR polymerase
PBCP: Personalised breast cancer programme
PBMC: Peripheral-blood mononuclear cell
PBS: Phosphate buffered saline
PCA: Principal component analysis
pCR: Pathological complete response
PCR: Polymerase chain reaction
PD-L1: Programmed death-ligand 1
PDTC: Patient-derived tumour cells
PDTX: Patient-derived tumour xenograft
PFA: Paraforma formaldehyde
PI3K: Phosphoinositide 3-kinase
PIKKs: Phosphoinositide 3-kinase-related kinases
PR: Progesterone receptor
PT: Post-treated sample

qPCR: Quantitative polymerase chain reaction

RCB: Residual cancer burden
RIN<sup>e</sup>: RNA Integrity Number
RLU: Relative light units
ROS: Reactive oxygen species
RSR: Replication stress response

SDS: Sodium dodecyl sulphate
SDS-PAGE: SDS polyacrylamide gel electrophoresis
siRNA: Small interfering RNA
SNP: Single nucleotide polymorphism
SNV: Single nucleotide variants
S-phase: Synthesis phase
SSB: Single strand breaks
ssDNA: Single stranded DNA
STR: Short tandem repeat

T
T: Treated sample
TIL: Tumour infiltrating lymphocyte
TLS: Translesion synthesis
TMA: Tissue microarray
TMM: Trimmed mean of M-values
TNBC: Triple negative breast cancer
tSNE: t-Distributed Stochastic Neighbour Embedding

U
UMI: Unique Molecular Identifier
UMAP: Uniform manifold approximation and projection
UV: Ultraviolet

V
VAF: Variant allelic frequency

W
WGS: Whole genome sequencing
Chapter 1: Introduction

1.1 Breast cancer

Though many perceive cancer to be a disease of the modern age, accounts are dispersed through virtually every period of recorded history. The Edwin Smith Surgical Papyrus chronicled eight cases of tumours or ulceration involving the breast. Dating back to the time of Pharaoh Djoser (~3000 B.C.), this account is considered by many to be the earliest written historical document of breast cancer. Reflective of the impact this disease would have over the next five millennia, the author of this text made clear that no treatments could succeed in preventing its inevitable fatality (1-3).

The Greek physician, Hippocrates, attributed cases in breast cancer to his Humoral Theory of Medicine, first coining the term *karkinos*, meaning *crab*, in around 460 B.C. Describing the most grievous cases, *karkinoma*, was later translated to *cancer* in Latin, by the Roman physician Celsus. In around 200 A.D., Galen used the word *onkos* (or *onco*) to describe tumours, which translates from Greek into *swelling, mass or load*. Like Hippocrates, Galen believed breast cancer was a humoral disease; this theory prevailed throughout the Middle Ages (1-3).
With the genesis of modern medicine came new theories regarding breast cancer. In the 17th and 18th centuries, the leading opinion was that breast cancer was a lymphatic disease and it was around this time that physicians started to advocate for surgical resection of breast tumours. In the 18th century Henri Le Dran described breast cancer, not as a systemic disease, but a local affliction, the progression of which occurs in stages; indeed, this is still recognised today (2, 3).

The 19th century brought fundamental changes to our understanding and practices in the treatment of breast cancer. Notably, pathologists Johannes Muller and Rudolph Virchow observed that tumours are composed of cells. This underpinned a major shift in focus. Muller postulated that cancers develop from the blastema between normal tissues (opposing the lymphatic theory) and later Virchow made the pivotal observation that cancers are derived from normal cells. Radical mastectomy was first proposed by William Halsted, which continued into the 20th century. In addition, Thomas Beatson indicated, for first time, that some breast cancers are hormone-dependent (4), which, a hundred years later, led to practice-changing discoveries into anti-hormonal therapies (5).

In the 20th century, the development of radiotherapy, chemotherapy, mammography, the dawn of statistically-driven clinical trials and molecular stratification have shaped the way we now treat breast cancer. Today, there are approximately 55,000 new breast cancer cases each year in the UK. While 10-year survival currently stands at 80%, breast cancer remains one of the leading causes of cancer related deaths in women in the UK (6, 7). For these reasons, the dynamics of disease progression, the mechanisms by which breast cancers evade therapeutic intervention and the evolutionary principles underpinning the disease remain at the forefront of biological interest.
1.1.1 Breast cancer heterogeneity

Breast cancers are heterogeneous entities, exhibiting both inter- and intra-tumour heterogeneity at genomic, epigenomic, transcriptomic and phenotypic levels. This is reflected in the diversity of interpatient drug responses observed in the clinic and is the biggest challenge in the management of the disease.

Extensive efforts have been made to stratify patients into molecular subgroups, to enhance our understanding of the disease and to aid in patient stratification. In 2000, Perou et al. classified breast cancers into four intrinsic subtypes based on gene expression patterns: oestrogen receptor (ER)-positive luminal-like, basal-like, human epidermal growth factor two (HER2)-positive and normal breast (8). Later luminal-like was further divided into luminal A and luminal B with distinct gene expression profiles and prognoses (9, 10). More recently, breast cancer taxonomy was redefined and validated as at least 10 subtypes, or Integrative Clusters (IntClust), which shed light on the inter-tumour heterogeneity, using copy number profiling, gene expression and somatic mutations (11-15) and reflect differences in long-term survival (16).

Breast cancers also display heterogeneity within an individual tumour, which can promote disease progression and facilitate the development of drug resistance (17). This diversity is composed of both genetic and epigenetic elements, which collectively give rise to a heterogeneous phenotype. Accordingly, tumours are subject to evolutionary principles.

The initiation and progression of cancers are dependent on genomic driver events which either activate oncogenic pathways or inactivate tumour suppressors. Accumulation of genetic alterations continues throughout the lifetime of a tumour; indeed, one of the hallmarks of cancer is genetic instability and this fuels genetic diversity (18, 19). This was described in 1958 by evolutionary biologist Julian Huxley, as “genetic inhomogeneity” in cancer (20). First postulated by Nowell in
1976 (21), laws of Darwinian evolution can be applied to cancer, termed \textit{clonal evolution}; genetic diversity yields certain clones with a higher propensity to survive the selective pressures of the cancer ecosystem, whether that be cancer therapies or the site of metastasis (figure 1.1.1).

\textbf{Figure 1.1.1 Tumour heterogeneity and clonal evolution.} Tumours exhibit both inter- and intra-tumour heterogeneity. Intra-tumour heterogeneity is the substrate for clonal evolution, whereby genetic and epigenetic factors yield clones with different abilities to survive selection pressures.

However, epigenetic factors can also govern the phenotype of tumour cells and since selection is driven by phenotypes, heritable epigenetic diversity can also act as the substrate for clonal evolution. Even within the conceptual framework of cancer stem cells, whereby intra-tumour heterogeneity is based on epigenetic-driven differentiation hierarchies, phenotypic selection can occur by Darwinian principles (22, 23).
The biology of intra-tumour heterogeneity has inspired extensive study for many decades. Next generation sequencing and, to an even greater extent, single cell technologies, have vastly improved our understanding in breast cancer (24-29). Direct mechanisms of clonal dynamics and cancer evolution patterns are difficult to delineate and are likely to coexist but it is clear that a combination of genetic, epigenetic and proteomic modalities is essential to fully elucidate this (30-32).

1.1.2 Patient stratification in breast cancer

Clinically, patient stratification relies on several factors, including histological subtype, grade, stage and clinical biomarkers. Treatment strategies are then elected based on an integrated view of these elements.

The World Health Organisation has defined 19 major histological subtypes of breast carcinoma. The most common, invasive carcinoma of no special type (NST, previously denoted as invasive ductal carcinoma) encompasses 70-75% of cases. Lobular carcinomas account for 10-14% of breast cancers and the remaining are defined as carcinomas of special type (17 subtypes including tubular, mucinous and micropapillary). These are identified based on specific morphological features (33, 34).

Histological grade of a tumour reflects the degree of differentiation and proliferation. Based on the Elston- and Ellis-modified Scarff-Bloom-Richardson system, the histological grade is composed of three features: the percentage of cells with tubule formation, the degree of nuclear pleomorphism and mitotic count. Each feature is scored and the final grade (G1, G2, G3) is determined by summation of the individual scores. Originally outlined in 1991, Elston and Ellis demonstrated tumour grade had very strong prognostic value; in a cohort of 1831 patients, patients with G1 tumours had significantly better chances of survival compared to those with G2 or G3 (35).
The prognostic stage of breast cancer indicates the degree of disease progression and can be reported in two ways: TNM or numbered staging systems. Briefly, TNM refers to tumour (T), node (N) and metastasis (M), each of which is sub-classified and conveyed to the patient. T is reported as TX (for which the tumour size cannot be assessed), Tis (indicating ductal carcinoma in situ, DCIS) or T1-4 based on the size of the primary tumour. Similarly, NX refers to an inability to assess the degree of lymph node involvement and N0-N3 is based on the level of metastasis to the lymph nodes. For metastasis, M0 accounts for no distant metastasis, cMo(i+) indicates that there is no detectable metastatic tumour but cancer cells are present in the blood, bone marrow or distant lymph nodes and M1 refers to distant metastasis. This was first outlined by Pierre Denoix in the 1940s and 1950s, and has since been revised many times by the American Joint Committee on Cancer (36). An alternative scoring system exists (I-IV), which broadly concatenates the results of the TNM scoring. This assigns stage I to early breast cancer, which remains local or has progressed only to the lymph nodes close to the breast, up to stage IV which denotes advanced cancer with metastatic spread.

With regard to biomarkers, patient stratification in breast cancer relies heavily on the expression of HER2 and hormone receptors: ER and progesterone receptor (PR). Treatment strategies are largely dependent on this and approximately 15% of patients are negative for all three markers, termed triple negative breast cancer (TNBC). 70% of invasive breast cancers are ER positive and most of these also express PR (37). Both ER and PR status is determined by immunohistochemistry (IHC), and is reported using an Allred score (38). This approach is on a scale of zero to eight and is the sum of two parameters: the percentage of positively stained cells (zero to five) and the levels of intensity (zero to three). In the UK, a percentage of hormone receptor-positive cells is also given and cases with greater than one percent cells are considered positive (39).

Erb-B2 Receptor Tyrosine Kinase 2 (ERBB2) encodes HER2 and is amplified in approximately 13-15% of breast cancer patients. This amplification leads to the
activation of the HER2 pathway (34). Patient samples are tested to identify levels of HER2 expression, which is reported with a score of 0 to 3+ (40). The score is first determined by IHC, based on the intensity and number of positive cells. Scores of 0 or 1+ are determined to be negative, and 3+ are positive. Tumours found to be 2+ are referred to a second diagnostic test, usually in situ hybridisation, which is used to confirm gene amplification using probes. Current recommendations mandate a dual probe approach, which identifies the ratio of copies of ERBB2 to chromosome 17 (using chromosome 17 centromeric enumeration probe, CEP17). A gene amplification is accepted if the ratio of ERBB2:CEP17 is equal to or greater than two and/or if the mean ERBB2 gene copy is equal to or greater than six (40).

Within the context of clinical trials, other biomarkers are commonly used to aid patient stratification, including Ki67, to mark proliferation, and the presence of tumour infiltrating lymphocytes (TILs). Recent initiatives, including the Personalised Breast Cancer Programme (PBCP) in Cambridge, offer whole genome sequencing (WGS) and RNA-sequencing to breast cancer patients. Not only is this developing an enormous data resource for future scientific endeavours but it also identifies actionable targets for individual patients in real time. This unveils the possibility of implementing a molecular stratification system that faithfully reflects the inter-tumour heterogeneity of breast cancer in routine clinical practice.

1.1.3 Treatment strategies in breast cancer

The treatment strategies for breast cancer depend heavily on the histological subtype, grade, stage and clinical biomarkers. In early breast cancer, this commonly consists of surgery, radiotherapy and/or pharmacological intervention. While vast efforts continue in the development of new therapies, particularly for specific patient cohorts, there is also momentum to reduce over-treating in clinical practice. This can have important long-term implications for patients with less aggressive subtypes. However, this relies on more accurate patient stratification, an understanding of the tumour evolution and identification of biomarkers for drug
resistance. Along with the standard of care treatment strategies, some of these de-escalation measures are outlined below.

Surgery has always been, and still is, the major treatment for early breast cancer. Historically, radical mastectomies were popularised as the leading strategy for surgical intervention, however current surgical practices seek to use breast conservation surgery (lumpectomy) as the standard of care (41, 42). The current recommendations for this adopt a no ink on tumour approach, whereby tumours should not reach the margins of the excised tissue, dramatically reducing the need for re-excision (43). Some patients may require a mastectomy but in most cases breast reconstruction surgery can be considered. Indeed, de-escalation efforts have also been implemented into lymph node resection following successful clinical trials (44). Today, the standard procedure is to first biopsy the sentinel node (located by injecting a tracer material) and to only perform axillary surgery in patients who have detectable cancer cells in the lymph nodes (41).

Radiation therapy is commonplace in the treatment of early breast cancer, both in the cases of breast conserving surgery and mastectomy, usually in the adjuvant (post-surgery) setting (41). The cancer-killing mechanisms induced by radiotherapy are thought to be twofold: DNA damage and induction of an abscopal effect. The latter, originally coined by R. H. Mole in 1953 (45), refers to therapeutic benefits noted at metastases distant to the site of radiation. This is thought to be triggered by the induction of an antigen-specific anti-tumour immune response (46). Similar to surgical procedures, efforts have been undertaken to de-escalate radiotherapy, including partial breast irradiation (47, 48) and hypofractionated radiotherapy (49, 50). The primary aim of this is to reduce side effects whilst maintaining the clinical benefit to the patient.

The type of pharmacological intervention in the treatment of breast cancer relies on the expression of HER2 and hormone receptors. Current approved pharmacological
agents can broadly be divided into: chemotherapy, endocrine and HER2-targeted therapies.

The use of chemotherapy depends on the disease subtype and can be given prior to surgery (neoadjuvant), which acts to shrink the tumour to aid in full surgical resection, or post-surgery (adjuvant). Aligning with radiotherapy, chemotherapy broadly acts by inducing DNA damage, which can have synergistic effects in cancers with inherent genome instability or defects in DNA repair pathways (discussed later). For TNBC, standard of care usually includes a taxane and anthracycline combination, with the use of platinum agents in some cases (41).

The application of neoadjuvant chemotherapy has gained some traction in recent years. Not only does this aid in surgery, response can be highly indicative of prognosis. Particularly in patients with TNBC, pathological complete response (pCR) at surgery following neoadjuvant chemotherapy is highly correlated with survival (51). Although TNBC patients have higher frequencies of pCR than other subtypes, those that have residual disease at surgery have significantly shorter event-free survival and overall survival; hazard ratios were found to be 0.24 and 0.16 respectively in favour of pCR (51).

Since Beatson’s first accounts of hormone-dependency in breast cancer (4), multiple therapies have been developed for patients with ER and/or PR-positive tumours. These patients can benefit from endocrine therapies, particularly those targeting the ER pathway. ER is encoded by Oestrogen Receptor 1 (ESR1) and is a ligand-activated transcription factor. ER targeting drugs include tamoxifen (which binds to and inhibits ER) or aromatase inhibitors (which blocks the synthesis of oestrogen), both of which can be given in the adjuvant setting for at least five years (52).

For patients with ERBB2-amplified tumours (HER2 overexpression), the development and approval of trastuzumab (marketed as Herceptin®) has
dramatically improved survival. Trastuzumab is an anti-HER2 recombinant humanised monoclonal antibody. In 2002, it was approved by the UK’s National Institute for Health Care Excellence (NICE) for use in late stage HER2-positive breast cancer (53) and in 2006 for early HER2-positive breast cancer (54). Standard of care for these patients includes adjuvant trastuzumab for up to one year or in combination with chemotherapy and pertuzumab (another HER2-targeting antibody) in the neoadjuvant setting (41, 55).

For patients with TNBC, pharmacological treatment strategies in the UK are limited to chemotherapy and it is, in part, due to a lack of actionable targets that these patients have worse prognoses than other molecular subtypes (56). In 2019, approval was granted in the USA and Europe for the use of atezolizumab (marketed as Tecentriq®) for treatment of programmed death-ligand 1 (PD-L1)-positive TNBC (57-59). Atezolizumab is a monoclonal antibody and is the first immunotherapy approved to treat breast cancer. However, it is not yet recommended by NICE for use in the UK and only benefits a subset of TNBC patients.

Given the lack of targeted treatments, standard of care for TNBC currently consists of neoadjuvant chemotherapy followed by surgery and adjuvant radiotherapy (41). However, extensive efforts are underway to further characterise the aetiology of these tumours and thus identify potential avenues for therapeutic intervention.

1.1.4 Triple negative breast cancer

Encompassing approximately 15% of breast cancer patients (26), TNBC is characterised by a histological lack of ER, PR and HER2. Patients with TNBC have a worse prognosis than other subtypes (56) and have been shown to have increased likelihood of distant recurrence and death within 5 years of diagnosis (60) compared to other breast cancers. This is largely due to an aggressive phenotype, lack of therapeutic options and, although representing a small proportion of patients, it is a
phenotypically diverse population. As such, extensive efforts have been undertaken to stratify TNBCs by molecular characteristics and to gain insights into their aetiology to identify clinically-targetable features.

TNBC can be divided into two subgroups based on expression of basal cytokeratins (CK5/6) and epidermal growth factor receptor (EGFR); these subgroups have been shown to have distinct behaviours and prognoses (61, 62). When classifying tumours by the intrinsic subtypes (8, 9), the vast majority (81%) of TNBC patients are defined as basal, with the remaining patients divided between HER2-enriched (10%), normal-like (5%), luminal B (3%) and luminal A (1%) (63). In 2011, Lehmann et al. sought to further dissect the molecular features of TNBCs. The authors identified six distinct subgroups based on gene expression: two basal-like (BL1, BL2) subtypes, an immunomodulatory, a mesenchymal, a mesenchymal stem-like and a luminal androgen receptor subtype (64). These were enriched for distinct features (e.g. elevated cell cycle and DNA damage response gene expression in BL1 and growth factor signalling in BL2). Survival metrics (including relapse-free survival) were found to be significantly different between these subtypes and a subsequent study revealed a significant association between TNBC subtype and response to neoadjuvant chemotherapy (65).

Within the integrative cluster breast cancer classification system (11), 80% of TNBCs are classified into IntClust4 or IntClust10. IntClust10 is heavily enriched for TNBCs and exhibits high genomic instability. Approximately 26% of TNBCs belong to IntClust4, which is associated with extensive lymphocytic infiltration and a favourable outcome. These subgroups were shown to have different clinical outcomes and Rueda et al. later demonstrated that they exhibit substantial differences in their recurrence trajectories (16). These data reflect the heterogeneous nature of TNBC, exemplifying the complexity in its treatment.

Many research groups have sought to characterise the mutational landscape of TNBC. Shah et al. performed a comprehensive analysis into the clonal and
mutational evolution of these tumours (26); TP53 was found to be the most frequently mutated gene (62% of basal TNBCs and 43% of non-basal TNBCs). Frequent mutations were also identified in PIK3CA (10%), USH2A (9%), MYO3A (9%), PTEN (8%) and RB1 (8). Another group described that 80% of basal tumours had TP53 mutations and again revealed a high degree of overlap between the TNBC and basal classification (66).

The immune system is thought to play a key role in TNBC and basal tumours. TNBCs have been shown to have higher number of TILs (67) and higher PD-L1 mRNA (68, 69) and protein (70, 71) expression than other breast cancer subtypes. As mentioned previously, atezolizumab has been approved for treatment of PD-L1-positive TNBC (57-59). However, the pattern of immune infiltration is thought to be highly heterogeneous between patients and as such only a subset of TNBC patients can benefit from PD-L1-targeting immunotherapies. Therefore, there requires further efforts to develop new treatment strategies for these patients.

A potential therapeutic target being explored is the cellular response to DNA damage. TNBCs are characterised by high levels of genomic instability. In addition, these tumours commonly display alterations in DNA repair signalling pathways, including an increased incidence of mutations in breast cancer genes 1 and 2 (BRCA1/2) (~10% of TNBC patients) (10, 66, 72, 73). This offers an unprecedented opportunity to explore new avenues for therapeutic intervention for these patients.

1.2 The DNA damage response

The DNA damage response (DDR) is defined as a network of cellular pathways which sense, signal and repair lesions occurring in the DNA of cells during growth, development and replication (74). A normal cell receives tens of thousands of lesions each day and, in the large part, these are repaired efficiently and with high fidelity (75). However, cancer cells often have alterations in the DDR and replication stress response (RSR) making them more susceptible to DNA damage,
and thus more dependent on remaining repair pathways. A classic strategy to target cancer cells, employed by chemotherapy and radiotherapy, is to cause excess DNA damage which, if left unrepaired, can lead to cell cycle arrest and ultimately cell death. Cancer cells generally have increased rates of proliferation compared to non-cancerous cells which exposes a vulnerability to DNA damage. Indeed, genome instability is a hallmark of cancer (18, 19) and so by targeting the mechanisms by which cancer cells cope, it provides new avenues which can be exploited in the treatment of the disease.

1.2.1 DNA damage response mechanisms

Exogenous and endogenous factors contribute to the levels and types of DNA damage a cell experiences, including reactive oxygen species (ROS) generated during normal metabolism or inflammation, replication stress, ultraviolet (UV) radiation or by chemotherapy agents (76). Multiple distinct mechanisms exist to repair DNA damage and the choice largely depends on the type of lesion and the cell cycle stage at which the damage occurs.

DNA lesions can broadly be divided into: base mismatches, DNA adducts, single stranded breaks (SSBs) and double stranded breaks (DSBs), each of which can be repaired by distinct mechanisms (77) (figure 1.2.1). Base mismatches include substitutions, insertions and deletions. These can occur during replication or as a result of alkylating agents and can be repaired by mismatch repair (MMR). Bulky DNA adducts can arise from environmental mutagens (e.g. UV radiation) or chemotherapy (e.g. platinum agents) and are repaired by nucleotide excision repair (NER). SSBs are the most common type of DNA damage, which can be induced in tumours through the use of alkylating agents and radiotherapy. SSBs are repaired through base excision repair (BER), which involves poly adenosine diphosphate ribose polymerase (PARP); PARP can be therapeutically targeted in breast cancer.
DSBs are the most toxic type of DNA damage to a cell and can result in loss of large chromosomal regions. These occur through ionising radiation, ROS, topoisomerase inhibitors or as a consequence of unrepaired SSBs present during replication. The two main mechanisms by which DSBs can be repaired are homologous recombination (HR) or non-homologous end joining (NHEJ). HR requires the presence of a sister chromatid to use as a template and consequently its utility is limited to the synthesis (S) or the second growth phase (G2) of the cell cycle. Conversely, NHEJ, though more error-prone, can be employed at any stage of the cell cycle. In mammalian cells, NHEJ is the primary repair pathway, whilst HR is essential in the repair of DSBs which arise from stalled replication forks (76, 78).

NHEJ involves the direct ligation of DNA strands at the site of the DSB, which can lead to DNA rearrangements. DSBs are first sensed by the Ku70/80 heterodimer, which recruits DNA-dependent protein kinase catalytic subunit (DNA-PKcs), forming the DNA-PK complex. This leads to the recruitment of key NHEJ factors, including nucleases (to trim ends), polymerases (to fill ends) and ligation machinery. Following minimal end-processing, DNA ends are re-ligated by the XRCC4-Ligase IV complex (75, 76, 79).

Conversely, in HR the first step is end resection, which is performed by endo- and exonucleases including MRE11, CtIP, DNA2 and EXO1. This creates 3-prime single stranded DNA (ssDNA) overhangs, onto which RPA binds to protect the ssDNA from degradation and remove any secondary structures. The recombinase protein RAD51 is then loaded onto the RPA-coated ssDNA to form a nucleoprotein complex. RAD51 invades the homologous sequence in the sister chromatid, using it as a template for DNA synthesis and accurate repair. Both BRCA1 and BRCA2 (mutations in which confer breast cancer predisposition) are crucial for HR. BRCA1 is important in the initial steps and acts as a scaffold to recruit and organise the necessary proteins to the site of repair; BRCA2, along with PALB2, loads RAD51 onto the ssDNA (78, 80).
Figure 1.2.1 DNA damage and mechanisms of repair. Diverse types of DNA damage can occur through exogeneous and endogenous factors, which are repaired by different repair pathways (top panel). DSBs are repaired by HR or NHEJ (bottom panel).

The DDR signalling pathways are facilitated by three phosphoinositide 3-kinase (PI3K)-related kinases (PIKKs): ataxia-telangiectasia-mutated (ATM), ATM- and Rad3-related (ATR) and the aforementioned DNA-PKcs. While these have distinct roles and each govern aspects of the global DDR, they have similar domain organisations and common structural features. In addition, there is vast cross-talk between the kinases, which share certain substrates, have overlapping functions and can largely compensate for each other (81).

ATM is critical for orchestration of the cellular response to DSBs and acts to amplify the signal. Upon the induction of a DSB, the MRN complex (composed of...
MRE11, RAD50 and NBS1) binds to the chromatin. ATM is recruited by NBS1, and phosphorylates a wide variety of targets, including the histone H2AX at serine 139 (referred to as γ-H2AX), originally reported by Rogakou et al. in 1998 (82). This serves as a platform to recruit other DDR factors. Indeed, γ-H2AX is the principal marker used in laboratory experiments to identify DSBs.

Conversely, ATR is the key regulator in the repair of SSBs, the RSR and is essential for cell proliferation. ATR is recruited to extended lengths of SSBs coated in RPA, via ATRIP. The key function is to activate CHK1 and the subsequent signalling pathway leads to slowing or arrest of the cell cycle. This provides time to either repair the DNA damage or activate senescence or apoptosis pathways. The major role of DNA-PKcs is to promote the repair of DSBs by NHEJ (76, 81), described above.

The DDR and RSR are complex networks, the global orchestration of which requires a myriad of factors. While in a normal cell, this is accomplished efficiently and with limited errors, cancer cells frequently display alterations in these networks.

1.2.2 Alterations in the DNA damage response

Malignant tumours, including breast cancers, commonly have alterations in components of the DDR and RSR, which can contribute to the accumulation of DNA damage in cancer. While repair pathways can have largely compensatory roles, alterations lead to a high dependency on remaining repair pathways.

One of the most commonly mutated genes in breast cancer is TP53, which encodes the p53 transcription factor. This is involved in regulation of the cell cycle and orchestrates many DDR mechanisms (83). TP53 is described as a tumour suppressor gene, the loss of which is a major driver in cancer. TP53 was found to be mutated 35.4% of breast cancers (15, 66), and TNBC/basal-like tumours are
particularly enriched for *TP53* mutations. As described before, 80% of basal-like cancers display *TP53* mutations and these are mostly nonsense and frame shifts (66). In the presence of DNA damage, p53 can halt the cell cycle at the first growth phase (G1), can trigger apoptosis and has roles in BER, NER, HR and NHEJ, among others (84). Loss of p53 eliminates a critical damage sensor, making cancer cells more reliant on other pathways.

*BRCA1* and *BRCA2* genes were discovered in 1994 and 1995 respectively (85-89) as hereditary genes which can predispose patients to breast and ovarian cancer. This unveiled new avenues for the targeting of the DDR. Germline mutations in *BRCA1* and *BRCA2* are associated with a cumulative risk of developing breast cancer of 72% and 69% respectively by the age of 80 (90). In a cohort of 1824 TNBC patients, unselected for family history of breast cancer or ovarian cancer, 11.2% were found to have deleterious germline mutations in *BRCA1* (8.5%) or *BRCA2* (2.7%) (73). Tumours from patients with germline alterations in *BRCA1* or *BRCA2* commonly lose the wildtype allele of the gene. This can occur through somatic mutations, copy number aberrations (CNAs) or inactivation by promoter hypermethylation, leading to an absence of functional protein and homologous recombination deficiency (HRD) (91). Since HR is necessary for the repair of lesions which stall replication forks and/or cause DSBs, HRD can dramatically impair the repair capacity of the cell.

Germline mutations in other genes involved in DDR are also associated with an increased risk of breast cancer, e.g. *PALB2*, *BRIP1*, *ATM* (92-95). Somatic mutations in these genes, among others, have the ability to dysregulate HR even in the absence of *BRCA1/2* mutations, a phenotype termed *BRCA*nness (96, 97). Functional biomarkers and computational approaches are now being developed to predict HRD in tumours. Examples of this include IHC detection of nuclear RAD51 foci (98-100) or analysis of genomic scars by an array-based comparative genomic hybridisation (101-104), single nucleotide polymorphism (SNP) genotyping (105-
or mutational signatures (109-111). This offers the potential to leverage therapies targeted towards tumours with HRD in the wider context of breast cancer.

1.2.3 Targeting the DNA damage response in breast cancer

Classic cancer treatments have relied on generating excess DNA damage, which preferentially target cancer cells over normal cells due to the increased proliferation rate of cancers. However, when breast cancers exhibit deficiencies in DNA repair pathways, it also provides opportunities for targeted therapies. This has potential for more potent cancer-killing, whilst ensuring cancer cell selectivity.

A prevailing concept in the study of DDR targeting is synthetic lethality. Originally described in *Drosophila melanogaster* in the early 20th century, the term was coined in 1946 by Theodore Dobzhansky (112-114). Synthetic lethality was defined as a type of genetic interaction where the co-occurrence of two genetic events results in organismal or cellular death but either genetic event alone does not. Although originally described for loss-of-function mutations, other types of perturbations can yield synthetic lethal relationships, including gene overexpression or chemical compounds (115).

Perhaps the most well-known DDR-targeting agents to date which illustrates synthetic lethality are inhibitors of PARP, a major factor in the repair of SSBs. In 2005, two studies were published showing that BRCA1 or BRCA2 dysfunction profoundly sensitises cells to PARP inhibitors (116, 117). Crucially, Farmer et al. showed that this sensitivity was specific to homozygous mutants (BRCA<sup>−/−</sup>) and not heterozygous mutants (BRCA<sup>+/−</sup>). This suggested that in the context of BRCA mutations in cancer, there would be selective killing of cancer cells which had lost both copies but sparing of the normal cells which maintained one wildtype allele (116).
At this time, the model proposed by the authors was related to the role of PARP in repair of SSBs. Both groups hypothesised that in the absence of PARP, if unrepaired SSBs encountered a replication fork, they may generate DSBs. Ordinarily these would be repaired by HR but in the context of HRD, these unrepaired lesions would lead to genomic instability, cell cycle arrest and apoptosis. Even if repaired by more error-prone mechanisms, these would cause large numbers of aberrations and lead to loss of cell viability. However, in recent years the mechanism of action of PARP inhibitors is thought to be more complex. This is likely to be a combination of unrepaired SSBs leading to DSBs and physical trapping of the inactivated PARP onto the chromatin (118, 119). When the latter occurs during replication, it is thought to lead to stalling or collapsing of the replication fork, resulting in the generation of more deleterious DSBs, for which HR is required (120).

Following success in preclinical and clinical trials (121-123), the PARP inhibitor olaparib (marketed as Lynparza®) was approved for treatment of metastatic breast cancers with germline BRCA1/2 mutations in the US and Europe in 2018 and 2019 respectively (124, 125). Ongoing are a number of clinical trials to expand the use of olaparib to wider contexts. The PARTNER clinical trial, a multisite trial established in Cambridge, explores the use of olaparib in the neoadjuvant setting, for patients with BRCA1/2 mutations and/or TNBC in combination with chemotherapy (paclitaxel and carboplatin) (126).

In addition to PARP inhibitors, a number of other compounds and combinations are in preclinical and clinical development targeting the DDR and RSR (77). These include inhibitors of the main kinases regulating the DDR (ATR, ATM and DNA-PKcs) (81, 127) as well as WEE1, which controls the cell cycle checkpoint between the G2 and mitosis (M) phases (128).

By inhibiting factors which preserve the delicate balance of genome stability and proliferation, it is possible to leverage biological weaknesses which are specific to
cancer cells. However, the translation of this accumulation of knowledge to clinically-relevant therapies is crucially dependent on the availability of accurate preclinical models.

1.3 Preclinical models of breast cancer

In order to delineate the complexity of breast cancer and leverage this knowledge clinically, appropriate preclinical models are paramount. As discussed previously, breast cancers are highly heterogeneous entities at inter- and intra-tumour levels, in both the genotype and phenotype, and this must be captured in the modalities one uses to study the disease.

1.3.1 Historical approaches to tumour modelling

Historically, and remaining today, human cancer-derived immortalised cell lines are the most widely used preclinical model to study cancer. The first cultured cancer cell line, HeLa, was derived from cervical cancer cells in 1951 by Gey and colleagues at John Hopkins University and aptly took its name from Henrietta Lacks from whom the cells were derived (129, 130). Originally adopting a roller-tube cell culture technique, today HeLa cells and other immortalised cell lines are commonly grown in plastic cell culture flasks as either adherent or suspension cell cultures and cryopreserved to maintain the integrity of the originating tumour.

In addition, non-tumour cells can be used to create immortalised cell lines. While these cells ordinarily have a limited capacity for replication and ultimately senesce, these can be modified by either infection with viruses to make the cells express oncoproteins (e.g. T-antigen from the simian virus 40 or E6/E7 from the human papilloma virus) or by modifying the cells to express human telomerase reverse transcriptase protein. Both methods immortalise the cell lines for continued growth in culture (131). Today, depositories exist, including American Type Culture
Collection which maintain and stock cell lines, and enormous efforts have been made to characterise human cancer cell lines in multiple dimensions (132-139).

Despite the applicability of cancer cell lines in the study of the disease and screening of anticancer drug sensitivities, they have received vast criticism for many decades. The first instance of this began in the 1960s when scientists started to report the cross-contamination of distinct cell lines. This was first evident with inter-species contamination (140, 141) and later vast intra-species cross-contamination was identified using isoenzyme analysis (142) and karyotype and chromosome band analysis (143-148). Specifically, to breast cancer, Nelson-Rees and colleagues revealed both inter- and intra-species cross-contamination of cell lines in 1977 (146).

The establishment and propagation of cell lines present a magnitude of bottlenecks for the cell population and therefore the resulting cell line may exhibit a limited resemblance to the tumour from which it originated (149). This can have profound consequences in the translation of preclinical to clinical drug development. This concept, along with genomic evolution and drift of cell lines once in culture, remains a concern today, exemplified in a publication by Todd Golub’s group (150). The authors uncovered cell line genetic diversification which can lead to considerable phenotypic differences, including drug responses, demonstrating the ways in which these limitations can impact our understanding of cancer biology and the development of therapeutics.

However, despite these recognised shortcomings, immortalised cancer cell lines remain the most commonly used preclinical model of cancer, due to their wide accessibility, ease of use and suitability for molecular manipulation. In addition, cell lines can be implanted into mice and used for in vivo studies. Mouse-derived cell lines can be implanted into immunoprophicient mice (termed allografts) and human-derived cells into immunodeficient mice (termed xenografts) to prevent the elicitation of an immune response. Today, a number of techniques can be applied
to assess the extent of cell line contamination and genetic drift, including amplification of minisatellite region DNA (151, 152) or short tandem repeat (STR) profiling (153) but it is widely recognised that there is a need for more complex model systems in the study of the disease.

In addition to cell lines, genetically engineered mouse models (GEMMs) play an important role in the study of tumour initiation and progression. In these mouse models, tumours arise *de novo* and can be used to study the early stages of the disease. They are thought to more closely mimic intra-tumour heterogeneity than cell lines and can be used to elucidate interactions between cancer cells and the tumour microenvironment.

Research into the development and use of GEMMs began in the 1980s; the first breast cancer GEMM was developed by Leder and Stewart in 1984 (154, 155). Leder et al. created a transgenic mouse model which spontaneously developed mammary tumours by expressing c-myc gene driven by the mouse mammary tumour virus (MMTV) promoter/enhancer. In a later publication, the same group generated a GEMM with a higher propensity to develop tumours and accelerated tumorigenesis by crossing separate strains of transgenic mice which carried either the v-Ha-ras or the c-myc gene driven the MMTV promoter/enhancer (156, 157). Today, a variety of GEMMs have been generated and are commonly used to study tumour evolution and the genetic contributors to cancer. One of the major benefits of GEMMs over human cell-derived models is the ability to use immunoproliferent mice. Today, these remain the main class of preclinical model compatible with the study and development of immunotherapies.

However, they are not without their shortfalls. Although GEMMs accurately recapitulate inherited cancer syndromes, one of the key limitations is that the induced alteration usually occurs in all cells within the animal; this does not well model the emergence of spontaneous mutations in the tumour cell of origin. More sophisticated GEMMs have now been developed which enable the conditional
inactivation of tumour suppressor genes or activation on oncogenes in specified cells or tissues and/or at a specific time. This is commonly achieved using exogenous chemicals or viruses, which allows for more flexibility and control over the tumorigenesis (158).

Another key limitation is that GEMMs are primarily generated by manipulation of one or (at most) a few genes and so they cannot recapitulate tumorigenesis which occurs from an accumulation of genetic events. Furthermore, the use of artificial promoters mean they do not always mimic the phenotype observed clinically and the study of late stage and/or metastatic disease is somewhat limited. GEMMs do not capture the full genetic and epigenetic complexity found in the human tumour cell population and it is for these reasons that the use of other preclinical models is gaining popularity.

1.3.2 Patient derived tumour xenografts

Patient-derived tumour xenografts (PDTXs) are mouse models which involve the transplantation of fresh human cancer samples into immunodeficient mice. These are horizontally expanded or cryopreserved to create a biobank of material for use in preclinical science and drug discovery.

Although different laboratories have adopted slightly different protocols, the overall principles remain consistent (159). Samples to be engrafted can be solid tumours (either primary or metastatic samples collected by surgery or biopsy procedures), fluid drained from malignant ascites or pleural effusions, or circulating tumour cells (CTCs) enriched from peripheral blood. Solid tumours can be implanted as tumour fragments or single cell suspensions, either alone or coated in Matrigel. Matrigel provides a matrix and growth factors which are thought to aid engraftment. Implantations are commonly performed under the skin on the flank of the mouse (subcutaneous) or at the site of tumour origin (orthotopic). These factors can contribute to the engraftment efficiency of the PDTX platform and may affect
the characteristics of the generated PDTX, however few studies have objectively compared the implantation protocols and resulting xenografts.

PDTXs are widely considered to be the preclinical models which most faithfully recapitulate both the intra-tumour heterogeneity and inter-tumour diversity of the human cancers. However, the generation of PDTX models require mice to be severely immunocompromised to prevent the elicitation of an immune response. The most commonly used strain is NOD.Cg-Prkdc<sup>scid</sup>Il2rg<sup>tm1Wjl</sup>/SzJ (NSG<sup>®</sup>) (160, 161). In these mice, mature B and T cells are absent, as well as natural killer cells and complement; dendritic cells and macrophages are defective (162). These factors severely compromise the mouse immune system, preventing a host immune response against the graft.

While necessary for the generation of PDTXs, the use of immunodeficient mice is contentious and is a recognised shortcoming of the models. The immune system is known to play a crucial in both tumour progression and drug responses and so the lack of elements of the immune system is likely to alter the functional characteristics of the tumour. In addition, the capacity to study immunotherapies is currently very limited in these models. Many groups are attempting to reconstitute the human immune system in immunodeficient mice, by co-engraftment of immune components and in some cases this can be patient-matched (163). This would not only improve the cancer modelling but enable the study of tumour-immune interactions. The aim of humanised mice is being explored in multiple ways, including the engraftment of haematopoietic stem and progenitor cells or peripheral-blood mononuclear cells (PBMCs). One key barrier to the humanisation of PDTX mice is the rapid onset of graft versus host disease in which the grafted immune cells mount an immune response against the murine host tissue and so experiments are limited to short time frames.

Tumour development and progression is thought to be governed by both malignant cancer cells and the interactions with the tumour microenvironment and these
interactions can contribute to the intra-tumour heterogeneity in cancer (164). The tumour microenvironment is composed of infiltrating immune cells, the blood and lymphatic vascular networks, stromal fibroblasts and the extracellular matrix. Through engraftment of PDTXs, these human components are lost and are thought to be replaced by murine equivalents, evident through histological analysis. While it is clear that tumours retain the ability to recruit accessory cells and form vascular networks, more analysis is required to determine how this impacts the functional characteristics of the tumour.

Another area of contention is the extent to which PDTX generation selects for cellular clones and the genomic evolution of the tumour through passaging. Similar to cell lines, the generation of PDTXs presents bottlenecks for the tumour which can impact the clonal dynamics. This is of particular concern for subcutaneous PDTXs, where cellular interactions and mechanical constraints may differ from the native environment.

In 2015, it was reported using deep-genome and single-cell sequencing methods that the abundance of mutational clusters can change dramatically through PDTX generation and propagation (165). This was found to occur at varying degrees, from extreme selective engraftment of minor clones to moderate polyclonal engraftment, as well as ongoing clonal dynamics through serial passaging. Our group performed an analysis of the clonal dynamics upon engraftment and serial passaging on 104 samples from 22 models using PyClone (166). This revealed some clonal selection upon initial engraftment (i.e. between the patient sample and first passage) but that this was minimal through serial transplantation. Further analysis into the engraftment-associated dynamics strongly suggested that most of the clonal dynamics within xenografts are not associated with known driver genes.

A more recent publication reported rapid accumulation of CNAs during PDTX generation and passaging (167). Genomic evolution was more rapid early on in the generation of the tumour and the rate decreased at later passages. This group
claimed that the dynamics of clonal evolution between PDTX generation and passaging are distinct from those observed in patients between primary and recurrent/metastatic tumours. This is perhaps not surprising given the distinct selection pressures separating clinical and PDTX tumours. In contrast to PDTX tumours for example, patients may receive multiple lines of therapy and can present a diverse array of multi-organ metastases, captured in the dataset between primary and advanced disease samples. Indeed, this publication used cohort comparisons and did not interrogate matched patient and PDTX tumour evolution. PDTX cohorts are often enriched for more aggressive subtypes, making cohort analysis invalid. While this raised interesting and important concerns about the clonal compositions of PDTXs, it did not convincingly identify divergent evolutionary trajectories. It is therefore clear that further examination is required on a matched patient/PDTX basis, to study underlying evolutionary principles. Despite their downfalls, PDTXs nevertheless offer the most accurate representation of human cancer and their utility is increasing in the study of breast cancer.

1.3.3 Breast cancer PDTXs

Given the heterogeneity and genomic complexity of breast cancers, PDTX models provide the ideal tool to study the disease at high resolution. Many groups worldwide have generated biobanks of breast cancer PDTX models (165, 168-176), which have been utilised extensively for studying breast cancer biology, including mechanisms of drug resistance (177-180) and metastasis (169, 181). Models have been derived from multiple sample types, including of primary breast tumours, metastatic samples and CTCs. While the engraftment protocols vary by group, including the site of implantation (e.g. subcutaneous/flank, mammary fat pad) and the mouse strain (e.g. nude, NSG), these models have been shown to faithfully recapitulate the main features of the originating tumours.

Our group has established and rigorously characterised a large biobank of breast cancer PDTXs, published in 2016 (166). Models were derived from primary breast
cancer samples, metastatic biopsies (brain, skin, liver, bone, axilla, lymph nodes) and liquid metastases (e.g. pleural effusions and ascites). This biobank represents most breast cancer clinical and molecular subtypes, with a slight enrichment for TNBC and more aggressive subtypes of ER-positive breast cancer. This is in line with that observed by others (168) and is likely a consequence of the engraftment process.

**Figure 1.3.3 PDTX models and PDTCs retain the molecular features of the originating tumour.** Examples of copy number plots, scatter plots of methylated CpGs (from reduced representation bisulphite sequencing), pathway activation scores, variant allelic frequencies of single nucleotide variants (SNVs) and mutations profiles, all of originating tumour sample, PDTX and PDTC. Figure adapted from (166).
Our group demonstrated that the PDTX models reflect the molecular and morphological features of the originating tumour (figure 1.3.3). PDTX samples were characterised using exome sequencing for somatic mutations, shallow WGS for copy number profiling, microarray analysis of the transcriptome and reduced-representation bisulphite sequencing for methylation. These features were found to be mostly preserved from the patient sample, into an established PDTX and through serial passaging. In addition, the group pioneered the short-term culture of dissociated PDTX fragments, or patient-derived tumour cells (PDTCs), for high-throughput drug screening. These short-term cultures were shown to maintain the molecular features of the PDTX and reflect in vivo drug responses.

PDTXs are widely considered to be the preclinical models which most faithfully recapitulate both the intra-tumour heterogeneity and inter-tumour diversity of the human cancers. In addition to their use in basic biology and interrogating molecular mechanisms, PDTX models are leading the way in preclinical drug development and translational science.

1.3.4 Using PDTXs in translational science

PDTXs sit at the forefront of translational biology and preclinical drug discovery, moving towards the realm of personalised cancer therapy. Approximately 85% of preclinical compounds entering oncology clinical trials fail to demonstrate sufficient safety and efficacy to gain regulatory approval (182) and this is largely due to inadequate predictive value of preclinical models used in the past. PDTXs have been shown to reflect the diversity of inter-patient drug responses observed in the clinic, which provides opportunities to accelerate the drug development process.

Bruna and Rueda et al. (166) pioneered the use of short-term cultures of PDTCs for high-throughput drug screening and this presents vast implications for academic and pharmaceutical companies alike. This high-throughput principle could be adopted to screen large numbers of compounds in a select few models with specific
genomic compositions, or to screen a large cohort with a specific compound of interest, mimicking population studies.

In a similar vein, PDTX biobanks can be used as a proxy for patient populations as part of *in vivo* clinical trials. This approach was adopted by Gao et al. in 2015 (183), who established around 1000 PDTX models and performed *in vivo* compound screens using a 1 x 1 x1 experimental design, meaning one animal per model per treatment (first outlined by Migliardi et al. (184)). In this setting, PDTXs were used to assess responses to 62 treatments across six indications; the focus here was on the population response, not that of the individuals and therefore exemplifies the potential for using PDTXs on a large scale or as a surrogate for human clinical trials.

More focused *in vivo* trials using PDTXs can be performed to study specific drugs, targets or cohorts, exemplified in colorectal cancer by Bertotti et al. (185). This group produced a cohort of 85 PDTXs from metastatic colorectal cancer patients, termed *xenopatients*. The study demonstrated that xenopatients responded to the anti-EGFR antibody cetuximab with rates and extents analogous to the clinic and could be prospectively stratified as responders or non-responders on the basis of several predictive biomarkers. The authors then implemented the platform to identify and test new molecular biomarkers of resistance and alternative therapeutic targets. This illustrates the use of PDTXs in both population-level studies and hypothesis-driven science.

PDTXs can also be used for biomarker development, both *in vivo* and *in vitro*, to explore dynamics of response, mechanisms of drug resistance, tolerance and disease relapse (177-180, 186, 187). By recapitulating the intra-tumour heterogeneity of human cancer to a finer degree than any other preclinical models, PDTXs can be used to shed light on changing clonal compositions upon therapeutic pressure.
More recent studies have explored the use of PDTXs as avatars, in which a matched patient/PDTX framework is established, termed a co-clinical trial. In this setting, mice are enrolled in parallel treatment strategies to their matched patient in an attempt to emulate the clinical response. Initial studies have shown that PDTXs faithfully recapitulate the clinical responses of the patients from which they were derived (168, 169, 188). One study, published in 2017, screened PDTXs established for 92 patients with various solid cancers against the same 129 treatments that were administered clinically. This demonstrated that PDTXs replicate clinical outcomes even when patients undergo several additional cycles of therapy over time (188).

While in its infancy, PDTX avatars have the potential to guide clinical decision making and clinical trials have been initiated with avatars included in research arms. However, a key limitation of using PDTXs in this way is the delay caused by engraftment and expansion of the model. The only feasible way this can be addressed, and indeed the approach taken by the EXPLORE trial, is by generating the PDTX while the patient receives standard of care or another clinical trial treatment schedule (189). Only then if the patient relapses do they receive the PDTX-based treatment strategy. A few early studies have shown feasibility in the clinical practice, particularly in the advanced setting (190, 191).

While this has huge implications in the field of precision medicine, PDTX-based clinical trials come with their own ethical implications (192). Possibly a more feasible way to use PDTX models in routine clinical practice could be to guide clinical decision making based on drug response data of an established PDTX model with similar characteristics. Biopsies could be molecularly characterised and compared with available PDTX collections (159). In addition, it remains extremely difficult to initiate clinical trials on novel compounds in the curative setting, so PDTXs could be utilised in this context to accelerate the drug development process.

While the use of PDTXs in both basic and translational science is increasing exponentially, there is a greater need than ever for standardisation of experimental
and analysis procedures, as well as data sharing. A recent paper tested the robustness of PDTX studies and issued standard guidelines for experimental procedures and informatic pipelines (193). Additionally, PDTX consortiums, such as NCI PDXNet and the EurOPDX, have been established to help in data and model sharing, as well as harbour international collaboration in the field. These initiatives are paving the way for next-generation tumour modelling and anticipatory precision cancer medicine.

1.4 Outlook and scope of the thesis

Throughout history, breast cancer has been at the forefront of scientific interest and research endeavours, due to its prevalent, perilous and largely indiscriminate nature. While recent scientific discoveries have revolutionised the ways in which we treat certain subgroups of patients, it is clear there remains an unmet clinical need for those with TNBC. The field of DDR offers new opportunities for targeted therapies, which could benefit these patients. However, drug development is dependent upon preclinical models which accurately reflect clinical drug responses and mirror the evolutionary principles underpinning this complex disease. Only through advancing our understanding of cancer biology and leveraging models which accurately recapitulate the complexity of breast cancer, can we hope to succeed in improving outcome for these patients.

This thesis is composed of two central experimental themes. The first, encompassing chapters 3 and 4, comprises experiments aimed at developing an integrated in vivo and ex vivo experimental framework in which PDTX models are aligned with the clinical setting. In the form of a co-clinical trial using PDTXs as avatars, we hope that this will pave the way for future uses of PDTXs in anticipatory breast cancer medicine. The second theme, described in chapter 5, revolves around leveraging this unique platform to tease out mechanisms of drug response. With this, we hope to better understand how clinically relevant treatment schedules in early breast cancer impact a tumour's evolutionary trajectory.
1.5 Research Aims

1. To develop and optimise a PDTX co-clinical trial alongside a neoadjuvant clinical trial for triple negative breast cancer patients.

2. To develop an *ex vivo* high throughput drug screening platform to study functional evolutionary trajectories of tumours upon alternative therapeutic pressures.

3. To leverage the PDTX platform to explore the dynamics of drug response to PARP inhibitors.
Chapter 2: Materials and Methods

2.1 PDTX in vivo studies

2.1.1 Establishment and passage of PDTX models

The procedures involved in the establishment and propagation of the PDTX biobank was published by our group in 2016 (166). PDTX tumour implantation, volume measurements and maintenance of the biobank was performed by the Preclinical Genomics Core at the Cancer Research UK Cambridge Institute animal facility according to local regulations and procedures. Briefly, primary or metastatic breast cancer tissue fragments were obtained from consenting patients at Addenbrooke’s hospital by surgical resection or tissue biopsies. Tissue fragments were embedded in Matrigel (Corning, 354230) and implanted subcutaneously into the flank of severely immunocompromised female NSG mice. Mice were routinely monitored for health concerns. Tumour volumes were measured weekly by calliper using the following formula, whereby \( L, W \) and \( V \) represent tumour length, width and volume respectively.

\[
V = (L \times W^2) / 2.
\]

Mice were humanely sacrificed when tumour volumes reached 1500 mm\(^3\). Fragments from each PDTX tumour were cryopreserved in foetal bovine serum (FBS) (ThermoFisher, A3840401) with 10% dimethyl sulfoxide (DMSO) (Sigma,
Tumour fragments were also flash frozen in liquid nitrogen and fixed in 10% neutral buffered formalin (NBF) (CellPath, BAF-6000-08A) and where necessary tumour fragments were horizontally expanded by serially implanting tumour fragments in multiple hosts. To rescue a PDTX model, cryopreserved tissue fragments were thawed in a 37°C water bath, washed twice in RPMI-1640 (ThermoFisher, 21875-034) and embedded in Matrigel for subcutaneously implantation.

### 2.1.2 *In vivo* pharmacology studies

PDTX tumours were horizontally expanded into multiple hosts. At the appropriate point (either a defined time after implantation or based on tumour volume), mice were enrolled into treatment cohorts and dosed according to the allocated treatment schedules. Paclitaxel (Selleckchem, S1150) was formulated in 1:1 Ethanol:Kolliphor (Sigma, C5135-500G) and diluted prior to use in Vetivex® saline. Carboplatin (Selleckchem, S1215) was formulated in sterile water. Both paclitaxel and carboplatin were administered intravenously and on days of co-administration, carboplatin was dosed first followed by paclitaxel four hours later. Olaparib was formulated in 30% Kleptose/10% DMSO (Sigma, D2650) in water and AZD1775 was formulated in 0.05% methylcellulose. Olaparib, AZD1775, Kleptose and methylcellulose were acquired from AstraZeneca through a collaboration. Both were administered by oral gavage and on days of co-administration, olaparib was administered first, followed by AZD1775 six hours later. Mice were carefully monitored for adverse effects and tumours were measured weekly with callipers. Mice were humanely sacrificed at the defined endpoint (either immediately after treatment or when the tumour reached 1500 mm³). Terminal cardiac bleeds and blood was collected into lithium heparin tubes. Plasma was separated for pharmacokinetic analysis by centrifugation at 500 xg for 10 minutes at 4°C and stored at -80°C. Drug dosing and volume measurements was performed by the Preclinical Genomics Core at the Cancer Research UK Cambridge Institute animal facility.
2.1.3 *In vivo* trial statistical considerations and analysis

In specific experiments outlined in the main text, mice were assigned into cohorts using a stratified randomisation approach which aims to evenly distribute initial tumour volume sizes. To achieve this, tumour volumes from a group of mice are ranked from low to high and then assigned to a cohort using a spiral approach (e.g. 1-3, 3-1, 1-3… for three cohorts).

The *in vivo* trials were first analysed by comparing tumour volumes at defined time points, area under the curve (AUC) and regression coefficient following log$_2$ transformation of tumour volume. AUC was calculated using Prism (v8.1.0), which computes the AUC using the trapezoid rule. In the instance that one cohort reached limits before the end of treatment, the AUC was calculated for all cohorts of that PDTX model up to this point. The regression coefficient (slope) was calculated by linear regression, following log$_2$ transformation of the tumour volume over time up to the end of treatment. All parameters were compared between cohorts using two-tailed, unpaired Welch’s t-test, not assuming equal variance.

The *in vivo* trials were also analysed using an adapted PDTX response classification, based on RECIST criteria. Percentage change in tumour volume ($\Delta$Vol$_t$) from baseline ($V_0$) was computed for each mouse at each time point ($t$) using the following formula as described previously (183):

$$\Delta\text{Vol}_t = \left(\frac{V_t - V_0}{V_0}\right) \times 100$$

At the end of treatment, avatar mice were classified as having progressive disease (PD, > 20% increase), stable disease (SD, 20% increase to 30% decrease), partial response (PR, >30% decrease) or compete response (CR, 100% decrease, no measurable tumour).
2.2 Cell culture assays

2.2.1 Generation and culture of PDTCs

Enzymatic and mechanical methods were applied to dissociate PDTX tumour fragments into single cell suspensions (patient-derived tumour cells, PDTCs) using the Tumour Dissociation Kit (Miltenyi, 130-095-929) following the manufacturer’s recommendations for medium tumours. Cryopreserved tumour fragments were thawed quickly in a 37°C water bath. Tumour fragments were added to gentleMACS C tubes (Miltenyi, 130-093-237) with 10 mL of RPMI-1640 (ThermoFisher, 21875-034) and centrifuged at 300 xg for 3 minutes. Media was aspirated and 5 mL of tumour dissociation mix was added. Tumours were mechanically disrupted using the gentleMACS Dissociator (Miltenyi, 130-093-235). Following dissociation, cell suspensions were filtered through a 40 μm filter, centrifuged at 300 xg for 7 minutes and resuspended in the appropriate volume of PDTC culture media (table 2.2.1).

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Final concentration</th>
<th>Supplier</th>
<th>Catalogue number</th>
</tr>
</thead>
<tbody>
<tr>
<td>RPMI-1640</td>
<td></td>
<td>ThermoFisher</td>
<td>201875-034</td>
</tr>
<tr>
<td>B27 (serum-free)</td>
<td>1X</td>
<td>ThermoFisher</td>
<td>17504001</td>
</tr>
<tr>
<td>EGF</td>
<td>20 ng/ml</td>
<td>Peprotech</td>
<td>AF-100-15-1MG</td>
</tr>
<tr>
<td>FGFβ</td>
<td>20 ng/ml</td>
<td>Gibco</td>
<td>PHG0023</td>
</tr>
<tr>
<td>Penicillin-Streptomycin</td>
<td>50 U/ml</td>
<td>ThermoFisher</td>
<td>15070063</td>
</tr>
<tr>
<td>Gentamicin</td>
<td>5 μg/ml</td>
<td>ThermoFisher</td>
<td>15750060</td>
</tr>
</tbody>
</table>

2.2.2 Cell line tissue culture

Cell lines were obtained from laboratory stocks, stored in liquid nitrogen and were subject to routine short tandem repeat (STR) analysis and mycoplasma testing. Cell lines were grown in adherent culture conditions and were passaged every four days or at 80% confluency by trypsinisation. To detach adherent cells, tissue culture flasks were washed once with phosphate buffered saline (PBS) and incubated with 0.05% trypsin (ThermoFisher, 25300054) for 5 minutes at 37°C. Growth media
containing serum was added to inactivate trypsin and detached cells were reseeded at the correct concentration in new tissue culture flasks. After use, cells were cryopreserved in FBS (ThermoFisher, A3840401)/10% DMSO (Sigma, D2650) in liquid nitrogen for long-term storage. HCC1954 and MDA-MB-231 cells were cultured in RPMI-1640 (ThermoFisher, 201875-034)/10% FBS. MDA-MB-468 cells were cultured in DMEM (ThermoFisher, 41966-029)/10% FBS. SUM149 and SUM159 cells were cultured in Ham’s F12 media (ThermoFisher, 11765-054), supplemented with 5 µg/mL insulin (ThermoFisher, 12585-014), 1 µg/mL hydrocortisone (Stem Cell, 07925) and 5% FBS.

2.2.3 Cell line serum deprivation

As a positive control for signalling activation, serum-deprived cell lines were utilised. To achieve this HCC1954 cells were cultured in culture media (containing serum) to 70% confluency in tissue culture flasks. Cells were washed with PBS and replaced with culture media (without serum). Cells were serum-deprived for 24 hours and then harvested using trypsin as described previously. Cell pellets were washed once with ice-cold PBS and lysed as described in 2.4.5.

2.2.4 Cell quantification using a haemocytometer

Cell suspensions were mixed at a one to one ratio with 0.4% trypan blue (final concentration 0.2%). 15 µL of cell suspension was added to a haemocytometer slide with a cover slip. The total numbers of cells were counted in each large outer corner square (figure 2.2.4, indicated in blue), employing a system whereby cells are only counted if they are within a square or on the bottom or left edge. Dead cells (indicated by trypan blue staining) were also counted to get an estimation of viability. The mean was calculated to estimate the number of cells in each large square, which represents 0.1mm³ or 1 X 10⁻⁴ mL. The cell number was multiplied by 10⁴ to calculated cells/mL.
2.2.5 Cell quantification using Vi-CELL

Vi-CELL XR Cell Viability Analyser was used to assess viability and concentrations of cell lines and PDTCs. This is an automated hemocytometer which performs trypan blue dye exclusion of dead cells, by delivering samples to a flow cell and using a digital camera to image. Cell suspensions were diluted in duplicate, and 500 µL were added to Vi-CELL sample vials (Beckman, 383721). Vials were loaded onto the carrousel and were analysed using the settings for all cells.

2.2.6 siRNA

Cell lines were cultured to 80% confluency and harvested using trypsin as described in 2.2.2. Cells were plated at 0.3 X 10^6 cells/well of 6-well plates in 2 mL of cell culture media and were cultured for 24 hours at 37°C prior to transfection. ON-TARGETplus SMARTpool human MTF1 (Dharmacon, L-020078-00-0005) and ON-TARGETplus SMARTpool human KLF4 (Dharmacon, L-005089-00-0005) were used as test siRNAs and ON-TARGETplus non-targeting control pool (Dharmacon, D-001810-10-05) was used to control for transfection and other experimental procedures. To prepare the siRNA mixes, 5 nmol was briefly centrifuged and resuspended in 250 µL of RNase-free water to 20 µM. The solution was mixed by pipetting up and down and then placed on an orbital mixer for 30
minutes at room temperature. The stock concentration of 20 µM was confirmed using Nanodrop (as described in 2.5.2) and aliquoted to avoid freeze-thaw cycles. 20 µM siRNA stocks were diluted 80x to 0.25 µM in serum-free media (appropriate media for the cell line) and incubated at room temperature for 5 minutes. Dharmafect 4 transfection reagent (Dharmacon, T-2004-01) was diluted 50x in serum-free media and incubated at room temperature for 5 minutes. Diluted siRNA and transfection reagents were mixed at a one to one ratio and incubated for 20 minutes at room temperature. Media was removed from plate wells containing cells and replaced with 1.6 mL of cell culture media (containing serum). 400 µL of the appropriate transfection mix (1:1 of diluted siRNA and transfection reagent) was added to each well. Untreated control wells were replaced with 2 mL of cell culture media (containing serum). The final concentration in each well of siRNA was 25 nM with 4 µL of transfection reagent. For wells which were treated with both KLF4 and MTF1 siRNAs, a final concentration of 25 nM of each siRNA was used with 4 µL of transfection reagent. Cells were cultured for 6 hours post-transfection and then cell culture media was replaced.

Cells were harvested for protein analysis at day 0 (day of transfection) and 48, 72 and 144 hours post-transfection. Cells were harvested for drug screening 24 hours post-transfection. To harvest cells, media was removed and wells were washed with 1 mL of PBS. 0.5 mL of trypsin was added to each well and incubated for 5 minutes at 37°C. 1 mL of growth media was added to inactivate trypsin. Cell suspensions were transferred to an Eppendorf, centrifuged at 300 xg for 5 minutes and media was aspirated. For protein analysis, cell pellets were washed once with ice-cold PBS and lysed as described in 2.4.5. A western blot was then performed as described in 2.4.8. For drug screening, cell pellets were resuspended in 1 mL of complete media and cell numbers were counted using trypan blue and a haemocytometer as described in 2.2.4. Cells were plated in 384-well plates at a 500 cells/well in 50 uL (1 X 10^4 cells/mL) and a drug screening was performed as described in 2.3.2.
2.3 Drug dosing experiments and analysis

2.3.1 High throughput drug screening of PDTCs

Cryopreserved PDTX tumour fragments were dissociated into single cell suspensions as described in 2.2.1 and were resuspended in PDTC complete media (table 2.2.1). Cell concentrations were determined by Vi-CELL and diluted to the appropriate concentration for screening. Where possible, cell concentrations were normalised across samples within a given experiment (between 1-3 X 10^6 cells/mL). Cells were plated using 50 µL of cell suspension/media per well of a 384-well cell culture plate (Greiner, 781090) using a multidrop dispenser. Cells were cultured for 24 hours at 37°C prior to dosing. Stock drug concentrations were prepared according to table 2.3.1.1 and stored long-term in a desiccator. Prior to dosing, working solutions were prepared at the stock concentration (10 or 1 mM) and using a 100x dilution (0.1 or 0.01 mM) in the appropriate solvent and plated in drug plates (Labcyte, P-05525).

<table>
<thead>
<tr>
<th>Drug</th>
<th>Supplier</th>
<th>Catalogue number</th>
<th>Solvent</th>
<th>Stock Concentration (mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Paclitaxel</td>
<td>Selleckchem</td>
<td>S1150</td>
<td>DMSO</td>
<td>10</td>
</tr>
<tr>
<td>Carboplatin</td>
<td>Selleckchem</td>
<td>S1215</td>
<td>Water</td>
<td>10</td>
</tr>
<tr>
<td>Epirubicin</td>
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<td>S1223</td>
<td>DMSO</td>
<td>10</td>
</tr>
<tr>
<td>Olaparib</td>
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<td>S1060</td>
<td>DMSO</td>
<td>10</td>
</tr>
<tr>
<td>BMN-673</td>
<td>Selleckchem</td>
<td>S7048</td>
<td>DMSO</td>
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</tr>
<tr>
<td>AZD1775</td>
<td>Selleckchem</td>
<td>S1525</td>
<td>DMSO</td>
<td>10</td>
</tr>
<tr>
<td>AZD7648</td>
<td>Astrazeneca</td>
<td>Collaboration</td>
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<td>10</td>
</tr>
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<td>10</td>
</tr>
<tr>
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<td>Selleckchem</td>
<td>S2783</td>
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<td>10</td>
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<tr>
<td>JQ1</td>
<td>Selleckchem</td>
<td>S7110</td>
<td>DMSO</td>
<td>10</td>
</tr>
</tbody>
</table>

Cells were dosed using a 7-dose schedule of 0.01, 0.03, 0.1, 0.3, 1, 3, 10 µM, apart from ATM inhibitor AZD0156 due to solubility, which was dosed 0.001, 0.003,
0.01, 0.03, 0.1, 0.3, 1 µM. Cells were dosed using the Echo Acoustic Liquid Handler instrument following table 2.3.1.2 and each well was backfilled with solvent to 150 nL to ensure all wells received the same volume. Media-only wells were used as positive controls (no cells – representative of 100% death) and negative control wells (containing cells) were dosed with 150 nL DMSO (Sigma, D2650).

<table>
<thead>
<tr>
<th>Final concentration (µm)</th>
<th>Stock concentration (µm)</th>
<th>Drug volume (µm)</th>
<th>Solvent volume (µm)</th>
<th>Total volume dosed (µL)</th>
</tr>
</thead>
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<tr>
<td>10</td>
<td>10</td>
<td>50</td>
<td>100</td>
<td>150</td>
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<tr>
<td>0.1</td>
<td>0.1</td>
<td>50</td>
<td>100</td>
<td>150</td>
</tr>
<tr>
<td>0.03</td>
<td>0.1</td>
<td>15</td>
<td>135</td>
<td>150</td>
</tr>
<tr>
<td>0.01</td>
<td>0.1</td>
<td>5</td>
<td>145</td>
<td>150</td>
</tr>
</tbody>
</table>

Cell viability was measured using CellTiterGlo® 3D (CTG) (Promega, G9683) on day 0 (day of dosing) and a fixed time point after (3-14 days). CTG was added to cells using a multidrop dispenser and incubated at room temperature shaking for 30 minutes. Luminescence was measured using the PHERAstar multi-plate reader.

2.3.2 High throughput drug screening of cell lines

Cell lines were cultured to 80% confluency and harvested using trypsin as described in 2.2.2. Cells were counted using Vi-CELL and plated at 500 cells/well in 50 µL of media (1 X 10^4 cells/mL), in a 384-well cell culture plate (Greiner, 781090). Cells were cultured for 24 hours at 37°C prior to dosing. Cells were dosed with Olaparib or Epirubicin using a 7-dose schedule of 0.01, 0.03, 0.1, 0.3, 1, 3, 10 µM, using the Echo Acoustic Liquid Handler as described in 2.3.1. Media-only and DMSO-dosed wells were used as positive and negative controls respectively, representing 100% death and basal growth. Cell viability was measured using using
CellTiterGlo® 3D (CTG) (Promega, G9683) on day 0 (day of dosing) and a fixed
time point after (48, 72 or 96 hours). CTG was added to cells using a multidrop
dispenser and incubated at room temperature shaking for 30 minutes.
Luminescence was measured using the PHERAcstar multi-plate reader.

2.3.3 Analysis of high-throughput drug screening and growth
dynamics

Drug responses were normalised within a given plate and sample, using the mean
values of background as positive controls (media-only wells with no cells,
representative of 100% response) and solvent as negative controls (basal growth,
representative of 0% response). Drug responses were calculated using the formula:

\[
Viability(\%) = \frac{(\text{Luminescence} - \text{background})}{(\text{Solvent} - \text{background})} \times 100
\]

\[
\text{Response(\%)} = 100 - \text{Viability(\%)}
\]

Non-parametric isotonic regression using the R function isoreg was used to fit the
set of technical replicates of a given drug response for a given sample, as described
in (166). The area under the curve, AUC, was computed on the model fits using the
package flux (v0.3-0), which uses the trapezoid rule. The half maximal inhibitory
concentration (IC50) concentration was predicted by fitting a smooth line to the
isotonic regression line. To estimate variability of the AUC and IC50 values,
isotonic regression curves were fit to each of the technical replicates individually,
computing AUC and IC50 values and producing error bars for the overall estimates
based on standard deviation.

Nine theoretical dose response curves were established. Of these, seven were based
on from those previously published (166) but adapted to reflect the seven dosage
points, and three were developed new for this project. Each response was classified
into the theoretical curves by a minimum squares approach.
The change in AUC between two samples (1 and 2) and the statistical significance was calculated, first by subtracting AUC$_2$ from AUC$_1$. A combined standard deviation value was calculated by taking the square root of the sum of the two variance values and 95% confidence intervals were identified. The change in AUC was considered to be non-significant if the confidence intervals spanned 0.

$$\text{Combined SD}_{(1,2)} = \sqrt{\text{Var}_1 + \text{Var}_2}.$$  
$$\text{Upper interval} = (\text{AUC}_1 - \text{AUC}_2) + (1.96 * SD_{(1,2)})$$  
$$\text{Lower interval} = (\text{AUC}_1 - \text{AUC}_2) - (1.96 * SD_{(1,2)})$$

For combination drug screening, data was normalised to positive and negative controls as described above. Synergistic effects were measured using the Bliss model, which compares the observed response under a given combination of two drugs and concentrations, and the predicted response under a model of independence (195). Within each combination experiment, single-agent controls were included and responses were fitted using an isotonic regression as described above. Based on these, predicted responses were calculated using the following formula, where $R_A$ is the response at a given concentration of drug A and $R_B$ is the response at a given concentration of drug B.

$$R_{\text{predicted}} = R_A + R_B - R_A R_B$$
This was then compared to observed responses ($R_{\text{observed}}$), which were fitted using a bivariate isotonic fit on the drug concentration obtained from the R package isotonic.pen (v1.0). The synergistic ‘score’ was calculated by subtracting $R_{\text{predicted}}$ from $R_{\text{observed}}$. A combination was considered synergistic if $R_{\text{observed}} > R_{\text{predicted}}$ and antagonistic if $R_{\text{observed}} < R_{\text{predicted}}$. This is based on methods described by Bruna and Rueda et al. (166).

The growth dynamics of cultured cells were also explored in parallel. Net luminescence was calculated by subtracting the mean value of media-only wells from the luminescence of a well of interest. *Ex vivo* growth was defined as net luminescence at time (t) divided by net luminescence at time (0). To explore exponential growth, this was log$_2$ transformed.

### 2.3.4 Drug dosing time course experiments of PDTC

Cryopreserved PDTX tumour fragments were dissociated into single cell suspensions following 2.2.1 and were resuspended in PDTC complete media (table 2.2.1). Cell concentrations were determined by Vi-CELL and were diluted to 2 X $10^6$ cells/mL. Cells were plated in Ultra-low attachment 6 well plates (Costar, 3471) with 2 mL/well. Cells were dosed with the appropriate drug/concentration 24 hours after plating by adding the drug directly into cell culture media. After a fixed time after dosing as determined by the time course, cells were harvested. Cell suspensions were transferred to an Eppendorf, centrifuged at 300 xg for 5 minutes and media was aspirated. Cell pellets were washed once with ice cold PBS and lysed as described in 2.4.6. A western blot was then performed as described in 2.4.8.

### 2.3.5 Drug dosing time course experiments of cell lines

Cell lines were cultured in tissue culture flasks in the appropriate growth media until 80% confluent. Cells were harvested using trypsin and plated in 10 cm petri dishes at 2.2 X $10^6$ cells/dish in 12 mL of growth media. Cells were dosed with the
appropriate drug/concentration 48 hours after plating by adding the drug directly into cell culture media. After a fixed time after dosing as determined by the time course, cells were harvested. To harvest cells, plates were washed with 6 mL of ice-cold PBS. 1 mL of PBS was added, adherent cells were scraped and transferred to an Eppendorf. Cell suspensions were centrifuged at 300 xg for 5 minutes and supernatant was aspirated. Cell pellets were lysed as described in 2.4.5. A western blot was then performed as described in 2.4.8.

2.4 Protein analysis

2.4.1 PDTX tissue fixation and paraffin embedding

All fixation and paraffin embedding was performed by the Histopathology core facility at the Cancer Research UK Cambridge Institute. Immediately after harvesting, PDTX tumour fragments were fixed in 10% NBF for 24 hours at room temperature. NBF was replaced with 70% ethanol prior to embedding, for a maximum of 5 days. Fixed tumour samples were embedded into paraffin wax blocks using the ASP300 processor (Leicor).

2.4.2 Immunohistochemistry

All immunohistochemistry (IHC) was performed by the Histopathology core facility at the Cancer Research UK Cambridge Institute, with the exception of HER2 staining which was performed at the Department of Histopathology at Addenbrooke’s Hospital. Briefly, this involved sectioning, deparaffinisation, antigen retrieval, immunohistochemistry and scanning. Conditions for each antibody staining were optimised by the Histopathology core facility.

Paraffin-embedded tissue blocks were sectioned (3µm). Deparaffinisation and rehydration was performed prior to IHC using the automated ST5020 multistainer system (Leica). This involved two 10-minute immersions in xylene, followed by
two 5-minute immersions in 100% ethanol and one 5-minute immersion in 70% ethanol. Antigen retrieval was conducted using either heat-induced or enzymatic methods and specific conditions are outlined in table 2.4.2.1. Heat-induced antigen retrieval (using either sodium citrate or Tris-EDTA) was performed at 100°C. The enzymatic method utilised Bond enzyme concentrate (Leica, AR9551) with a proteolytic enzyme concentration of 101.8µg/mL for EGFR staining and was performed at 37°C.

All IHC was conducted on the Polymer Refine Detection System (Leica, DS9800) using the automated Bond-III platform. The target-specific procedures are outlined in table 2.4.2.1. Primary antibodies were used to locate the target of interest (table 2.4.2.2). A post-primary rabbit anti-mouse IgG antibody (i in table 2.4.2.3) was used to localise mouse primary antibodies. This was followed by staining with a secondary anti-rabbit IgG antibody conjugated to a horseradish peroxidase (HRP)-polymer complex. The antibody staining was visualised using chromogenic detection, in which the HRP enzyme conjugated to the secondary antibody cleaved the 3,3’-Diaminobenzidine (DAB) substrate to produce a brown precipitate at the location of the target protein. The post-primary and secondary HRP-polymer antibodies were supplied with the Polymer Refine Detection System (Leica, DS9800). IHC staining and detection was conducted following the manufacturer’s recommendations aside from the instances outlined below.

For instances in which the primary antibody was raised in alternative species (e.g. the rat antibody reactive against mouse CD45), an alternative antibody was used (ii in table 2.4.2.3) prior to the secondary anti-rabbit HRP-polymer. For staining in which the primary antibody was raised in rabbit (e.g. reactive against mouse Ki67 and human CD49F), the secondary anti-rabbit HRP-polymer was used directly without the need for post-primary staining. Additional DAB enhancer (Leica, AR9432) was used to intensify staining for all antibodies except ER and PR. For the antibody reactive against mouse Ki67, a mouse-on-mouse blocking reagent
(Vector, MKB-2213) was used and an isotype specific post-primary antibody was found to be optimal (iii in table 2.4.2.3).

Post-IHC de-hydration and clearing was performed using the automated ST5020 (Leica). Sections were mounted using the CV5030 (Leica) and slides were scanned using the Aperio AT2 scanner (Leica) at 20x with a resolution of 0.5µm/pixel.

HER2 staining was performed at Addenbrooke’s Hospital using the PATHWAY rabbit anti-HER2 monoclonal antibody (Ventana, 790-2991) and the iView DAB Detection Kit (Ventana, 760-091).

<table>
<thead>
<tr>
<th>Table 2.4.2.1 Optimised conditions for IHC</th>
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<td>Target antigen</td>
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<tr>
<td>-----------------</td>
</tr>
<tr>
<td>Ki67</td>
</tr>
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<td>CD45</td>
</tr>
<tr>
<td>CD45</td>
</tr>
<tr>
<td>CD45</td>
</tr>
<tr>
<td>CD20</td>
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<td>AE1/AE3</td>
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<td>ESA</td>
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<tr>
<td>ER</td>
</tr>
<tr>
<td>PR</td>
</tr>
<tr>
<td>P53</td>
</tr>
<tr>
<td>Vimentin</td>
</tr>
<tr>
<td>EGFR</td>
</tr>
<tr>
<td>CD49F</td>
</tr>
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</table>
2.4.2.2 Primary antibodies for IHC

<table>
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<tr>
<th>Target antigen</th>
<th>Target species</th>
<th>Raised species</th>
<th>Catalogue number</th>
<th>Supplier</th>
<th>Concentration/Dilution</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ki67</td>
<td>Mouse</td>
<td>Rabbit</td>
<td>IHC-00375</td>
<td>Bethyl Labs</td>
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</tr>
<tr>
<td>CD45</td>
<td>Mouse</td>
<td>Rat</td>
<td>Ab25386</td>
<td>Abcam</td>
<td>5 µg/mL</td>
</tr>
<tr>
<td>Ki67</td>
<td>Human</td>
<td>Mouse</td>
<td>M7240</td>
<td>Dako</td>
<td>1:400</td>
</tr>
<tr>
<td>CD45</td>
<td>Human</td>
<td>Mouse</td>
<td>M0701</td>
<td>Dako</td>
<td>1.5 µg/mL</td>
</tr>
<tr>
<td>CD20</td>
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<td>Mouse</td>
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<td>0.95 µg/mL</td>
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<tr>
<td>AE1/AE3</td>
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<td>Mouse</td>
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<td>Dako</td>
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<tr>
<td>ESA</td>
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<td>Mouse</td>
<td>NCL-L-ESA</td>
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<td>Mouse</td>
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<td>Mouse</td>
<td>M7001</td>
<td>Dako</td>
<td>1:1000</td>
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<td>Vimentin</td>
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<td>Mouse</td>
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<tr>
<td>EGFR</td>
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<td>Mouse</td>
<td>28-0005</td>
<td>Zymed</td>
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</tr>
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<td>CD49F</td>
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<td>Rabbit</td>
<td>HPA012696</td>
<td>Atlas</td>
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2.4.2.3 Post-primary/secondary antibodies for IHC

<table>
<thead>
<tr>
<th>No.</th>
<th>Target</th>
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<th>Catalogue number</th>
<th>Supplier</th>
<th>Concentration/Dilution</th>
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<tbody>
<tr>
<td>i</td>
<td>Anti-Mouse IgG</td>
<td>Rabbit</td>
<td>Supplied with DS9800</td>
<td>Leica</td>
<td>As provided</td>
</tr>
<tr>
<td>ii</td>
<td>Anti-rat</td>
<td>Rabbit</td>
<td>A110-322A</td>
<td>Bethyl Labs</td>
<td>1:250</td>
</tr>
<tr>
<td>iii</td>
<td>Anti-Mouse IgG1</td>
<td>Rabbit</td>
<td>ab125913</td>
<td>Abcam</td>
<td>1:1500</td>
</tr>
</tbody>
</table>

2.4.3 Tissue microarray construction

All IHC and histology was performed by the histopathology core facility at the Cancer Research UK Cambridge Institute. A tissue microarray (TMA) was constructed for the co-clinical trial cohort, which consisted of 0.6 mm diameter cores from (where possible) multiple passages of each model, two mice per passage and two cores per mouse. Internal controls consisted of two independent PDTX models which grew as mouse tumours and two which grew as human B-cell lymphomas, with multiple cores from each. In addition, since the majority of the cohort are TNBC, one ER+/HER2+ and one ER+/HER2- model were included as positive controls for biomarker staining.
2.4.4 Protein extraction from tissue samples

Flash frozen cryopreserved PDTX tumour fragments were used for protein extraction. Tumours fragments were added to an Eppendorf containing 50 µL of PDTX tumour lysis buffer (table 2.4.4) with protease inhibitors (ThermoFisher, 1861279) and phosphatase inhibitors (ThermoFisher, 78427). To homogenise, a 5mm stainless steel bead (QIAGEN, 69989) was added to the Eppendorf and the QIAGEN tissue lysis instrument was run for 60 seconds at a frequency of 30/s. A further 50 µL of lysis buffer was added and homogenised tissue was incubated on ice for 30 minutes. Protein-containing supernatants were collected following centrifugation at 21,000 xg for 30 minutes at 4°C. Protein lysates were quantified using Pierce BCA protein assay kit as described in 2.4.7.

Table 2.4.4 PDTX tumour lysis buffer recipe (western blot)

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Final concentration</th>
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<td>Sodium chloride (NaCl)</td>
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</tr>
<tr>
<td>TritonX-100</td>
<td>1 %</td>
</tr>
<tr>
<td>EDTA</td>
<td>5 mM</td>
</tr>
<tr>
<td>Sodium fluoride (NaF)</td>
<td>50 mM</td>
</tr>
<tr>
<td>β-Glycerophosphate</td>
<td>25 mM</td>
</tr>
<tr>
<td>Tris-HCl (pH 7.5)</td>
<td>50 mM</td>
</tr>
</tbody>
</table>

2.4.5 Protein extraction from cultured cell lines

Cultured cell lines were harvested using trypsin from 6-well plates, 10 cm petri dishes or tissue culture flasks. As described, during harvesting, serum-containing complete media was added to inactivate trypsin and cell pellets were washed with ice cold PBS. RIPA lysis buffer (table 2.4.5) containing protease inhibitors (ThermoFisher, 1861279) and phosphatase inhibitors (ThermoFisher, 78427) was added to cell pellets (approximately 100 uL lysis buffer per 1 X 10⁶ cells). Cell pellets were vortexed and incubated on ice for 30 minutes. Protein-containing supernatants were collected following centrifugation at 21,000 xg for 30 minutes at
4°C. Protein lysates were quantified using Pierce BCA protein assay kit as described in 2.4.7.

**Table 2.4.5 RIPA lysis buffer recipe**

<table>
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<th>Reagent</th>
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<tr>
<td>TritonX-100</td>
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<tr>
<td>Sodium deoxycholate</td>
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<tr>
<td>Sodium dodecyl sulphate (SDS)</td>
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<tr>
<td>Tris pH 8.0</td>
<td>50 mM</td>
</tr>
</tbody>
</table>

**2.4.6 Protein extraction from cultured PDTCs**

Cultured PDTCs were harvested as described previously. PDTX tumour lysis buffer (table 2.4.4) containing protease inhibitors (ThermoFisher, 78438) and phosphatase inhibitors (ThermoFisher, 78427) was added to cell pellets (approximately 5 uL lysis buffer per 1 X 10^6 cells). Cell pellets were vortexed and incubated on ice for 30 minutes. Protein-containing supernatants were collected following centrifugation at 21xg for 30 minutes at 4°C. Protein lysates were quantified using Pierce BCA protein assay kit as described in 2.4.7.

**2.4.7 Protein quantification (BCA assay)**

Bicinchoninic acid (BCA) assay standards were made by diluting Bovine Serum Albumin (BSA) (ThermoFisher, 23209) in lysis buffer (PDTX lysis buffer or RIPA buffer). BCA protein assay reagent B was BCA working solution was made to 50:1 of BCA protein assay reagent A (ThermoFisher, 23228) and reagent B (ThermoFisher, 1859078). Standards 5 μL of standard or protein of interest was added in duplicate to a 96-well round bottom assay plate (Costar, 3788) and incubated for 30 minutes at 37°C. The absorbance was read at 562nm and protein concentration was calculated using a calibration curve.
2.4.8 Western blot

Protein lysates were prepared and quantified at described above. Protein mixes were prepared so as to load an equal amount of protein per sample (30-50 μg). Loading buffer (ThermoFisher, NP0007) and reducing reagent (ThermoFisher, NP0009) were added at 4x and 10x dilutions respectively and lysis buffer (to match the sample) was used to normalise volumes. Protein mixes were incubated at 70°C to denature the proteins and placed on ice. Proteins were loaded along with a protein ladder (Bio-Rad, 161-0373) onto the appropriate precast polyacrylamide gel as described in table 2.4.8.2. Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) was performed using running buffer as described in table 2.4.8.2. MOPS, MES and Tris-Acetate running buffers were prepared at 20x dilution in deionised water. Gels were run for 30 minutes at 60V and then 90 minutes at 120V. Proteins were transferred to the membrane using either dry or wet transfer methods. For a dry transfer, pre-assembled dry transfer stacks, iBlot 2 nitrocellulose Stacks (ThermoFisher, IB23001/2) were used at the appropriate transfer time as described in table 2.4.8.2. For a wet transfer, low-fluorescence PVDF transfer membranes (Bio-Rad, 1620260) were soaked in 100% methanol for 10 minutes to activate. The proteins were transferred for 2 hours at 110V. Transfer buffer was prepared by diluting NuPAGE transfer buffer (ThermoFisher, NP00061) 20x in deionised water with 10% final concentration of methanol.

After transfer, proteins were visualised with Ponceau S solution (Sigma, P7170), membranes were trimmed to the appropriate size and washed thoroughly with water. Membranes were blocked for 1 hour shaking at room temperature in blocking buffer (table 2.4.8.3). Membranes were incubated overnight at 4°C with primary antibody, diluted in blocking buffer to the appropriate concentration as described in table 2.4.8.1. Membranes were washed three times in washing buffer (10 minutes per wash) shaking at room temperature. Membranes were incubated for 1 hour shaking at room temperature with secondary antibodies reactive against the species of the primary antibody. Secondary antibodies were diluted in blocking
buffer to the appropriate concentration as described in table 2.4.8.1. Membranes were washed three times. Enhanced chemiluminescence (ECL) was used to visualise antibody binding using secondary antibodies bound to horseradish peroxidate (HRP). As such, ECL reagents 1 and 2 were mixed at 1:1 ratio and added to membranes for a 1 minute incubation. Membranes were developed in a dark room by exposing x-ray films to the emitted light. Membranes were washed three times, and subsequent primary antibody staining was performed following the same protocol.

In the instances of low protein expression, Super Signal West Femto chemiluminescent reagent (ThermoFisher, 10391544) was used to develop membranes using secondary antibodies conjugated to HRP. In some cases, fluorescent labelled secondary antibodies were alternatively used and visualised using the LI-COR membrane scanner. This was adopted for high abundance proteins, e.g. β-actin. In the instances of low protein lysate concentration, Bolt™ gels were utilised instead of NuPAGE™, which allows for greater loading volumes.

In the instances of sequential primary antibody staining for proteins of the same molecular weight, membranes were stripped by incubating with stripping buffer at 50°C for 30 minutes shaking. Afterwards, membranes were washed thoroughly with water and blocked for 1 hour shaking at room temperature in blocking buffer. Subsequent antibody staining was performed as above.
Materials and Methods

Table 2.4.8.1 Western blot antibodies

<table>
<thead>
<tr>
<th>Target</th>
<th>Clone</th>
<th>Supplier</th>
<th>Host</th>
<th>Dilution</th>
<th>Catalogue number</th>
<th>Protocol</th>
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</thead>
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<td>Cell Signaling Technology</td>
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<td>2197</td>
<td>1</td>
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<tr>
<td>p-ATM (Ser1981)</td>
<td>D6H9</td>
<td>Cell Signaling Technology</td>
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<td>Sigma-Aldrich</td>
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<tr>
<td>p-e-Jun (Ser63)</td>
<td>KM-1</td>
<td>Santa Cruz Biotechnology</td>
<td>Mouse</td>
<td>1:1000</td>
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<tr>
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(IRDye 680Rd)

Table 2.4.8.2 Western blot protocols

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<th>Transfer time</th>
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<td>ATM p-Chk2</td>
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<td>110V 2 hours</td>
</tr>
<tr>
<td>2</td>
<td>Medium molecular weight</td>
<td>p-e-Jun E-Cadherin Vimentin KLF4 MTF1</td>
<td>NuPAGE™ 4-12% BisTris (NP0322, ThermoFisher) Bolt™ 4-12% BisTris (NP00102, ThermoFisher)</td>
<td>MOPS (NP000102, ThermoFisher)</td>
<td>Dry</td>
<td>20V 1 min 23 V 4 min 25 V 2 min (7 min total)</td>
</tr>
<tr>
<td>3</td>
<td>Low molecular weight</td>
<td>γ-H2AX</td>
<td>NuPAGE™ 4-12% BisTris (NP0321, ThermoFisher)</td>
<td>MES (NP002, ThermoFisher)</td>
<td>Dry</td>
<td>20V 1 min 23 V 3 min 20sec 25 V 1 min 40 sec (6 min total)</td>
</tr>
</tbody>
</table>
### Materials and Methods

#### Table 2.4.8.3 Western blot buffers

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Component</th>
<th>Volume/ Mass</th>
<th>Final concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wash buffer</td>
<td>TBS</td>
<td>1000 mL</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Tween-20</td>
<td>1 mL</td>
<td>0.1%</td>
</tr>
<tr>
<td>Blocking buffer</td>
<td>TBS</td>
<td>1000 mL</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Tween-20</td>
<td>1 mL</td>
<td>0.1%</td>
</tr>
<tr>
<td></td>
<td>Powdered milk</td>
<td>50g</td>
<td>5% (w/v)</td>
</tr>
<tr>
<td>ECL 1</td>
<td>dH₂O</td>
<td>18 mL</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Luminol</td>
<td>200 µL of 250 mM (A-8511, Sigma)</td>
<td>2.5 mM</td>
</tr>
<tr>
<td></td>
<td>P. Coumaric acid</td>
<td>88 µL of 90 mM (C-9008, Sigma)</td>
<td>0.4 mM</td>
</tr>
<tr>
<td></td>
<td>Tris-HCl pH 8</td>
<td>2 mL of 1 M</td>
<td>100 mM</td>
</tr>
<tr>
<td>ECL 2</td>
<td>dH₂O</td>
<td>18 mL</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Tris-HCl pH 8</td>
<td>2 mL of 1 M</td>
<td>100 mM</td>
</tr>
<tr>
<td></td>
<td>H₂O₂</td>
<td>12 µL of 30% 31642, Sigma)</td>
<td>0.018%</td>
</tr>
<tr>
<td>Stripping buffer</td>
<td>dH₂O</td>
<td>368.75 mL</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Tris-HCl pH 8</td>
<td>31.25 mL of 1 M</td>
<td>62.5 mM</td>
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<tr>
<td></td>
<td>SDS</td>
<td>100 mL of 10%</td>
<td>2%</td>
</tr>
<tr>
<td></td>
<td>β-mercaptoethanol</td>
<td>144 µL</td>
<td>0.03%</td>
</tr>
</tbody>
</table>

#### 2.4.9 ELISA for quantification of PAR

Flash frozen PDTX samples were homogenised in 1 mL of ELISA lysis buffer (table 2.4.9), using a 5mm stainless steel bead (QIAGEN, 69989) and the QIAGEN tissue lysis instrument was run for 45 seconds at a frequency of 30/s. Homogenised tissue was incubated on ice for 15 minutes and SDS was added to 1% final concentration. The sample was vortexed, incubated at 100°C for 5 minutes and snap-cooled on ice for 1 minute. Protein-containing supernatants were collected following centrifugation at 10,000 xg for 10 minutes at 4°C. Protein lysates were quantified using Pierce BCA protein assay kit as described in 2.4.7.

The HT PARP in vivo Pharmacodynamic Assay II (Trevigen, 4520-096-K) was used to quantify levels of PAR according to the standard manufacturer’s instructions using 1500 ng of protein per well. Internal controls (Jurkats cells) were
used in all experiments to measure drift. Standards, internal controls and samples of interest were analysed in triplicate. Chemiluminescence was measured using the PHERAAstar multi-plate reader. The net Relative Light Unit (RLU) values were calculated by subtracting the mean background luminescence (without PAR) from the RLU values. The net RLU values of the standards were plotted as a function of PAR values (pg/mL). The trend line was set to intercept 0. The formula of the calibration line was used to calculate PAR concentration of each sample from the net RLU values. The standard deviation was calculated from the replicate PAR concentrations.

<table>
<thead>
<tr>
<th>Table 2.4.9 ELISA lysis buffer</th>
</tr>
</thead>
<tbody>
<tr>
<td>Reagent</td>
</tr>
<tr>
<td>Lysis reagent</td>
</tr>
<tr>
<td>PMSF in ethanol</td>
</tr>
<tr>
<td>Halt protease inhibitor</td>
</tr>
</tbody>
</table>

2.4.10 LC-MS/MS

For pharmacokinetic analysis, liquid chromatography and tandem mass spectrometry (LC-MS/MS) was optimised and performed by the Pharmacokinetics and Bioanalytics core facility at the CRUK Cambridge Institute. Briefly, pre-processing of plasma samples involved precipitation of proteins using 1% acetic acid in acetonitrile and removal of phospholipids and proteins using Hybrid-SPE-PPT plates. Samples were evaporated to dryness at 45°C and reconstituted in 50:50 methanol/water (for analysis of paclitaxel and olaparib) or acetonitrile:200 mM ammonium acetate at 95:5 (for analysis of carboplatin). High performance liquid chromatography (HPLC) was used to separate components of the plasma (Accela Pump, Thermo Scientific). For paclitaxel and olaparib, reverse-phase columns were used to separate components by polarity (Phenomenex Luna C18, µm, 100X2.1 mm) whereby hydrocarbon 18 stationary phase interacts strongly with molecules of low polarity and weakly with those of high polarity. A gradient mobile phase
was used to elute components: mobile phases A and B were 0.1% formic acid in water and 0.1% formic acid in acetonitrile respectively. A flow rate of 0.3 mL/min was used for 7 minutes, with the gradient profile in table 2.4.10.1.

**Table 2.4.10.1 Liquid chromatography gradient profile (paclitaxel and olaparib)**

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>% A</th>
<th>% B</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>70</td>
<td>30</td>
</tr>
<tr>
<td>3.0</td>
<td>20</td>
<td>80</td>
</tr>
<tr>
<td>5.5</td>
<td>20</td>
<td>80</td>
</tr>
<tr>
<td>5.51</td>
<td>70</td>
<td>30</td>
</tr>
<tr>
<td>7</td>
<td>Stop</td>
<td></td>
</tr>
</tbody>
</table>

For carboplatin, an amine column was used (ACE Excel 1.7 NH2, 100 X 2.1 mm) under hydrophilic interaction liquid chromatography (HILIC) conditions. In this approach, the amine-coated silica stationary phase has high polarity and is more suited for polar analytes, such as carboplatin, which have low retention in reverse-phase chromatography. A gradient mobile phase was employed; mobile phases A and B were 10 mM ammonium formate and acetonitrile: 200 mM ammonium formate at 95:5. A flow rate of 0.4 mL/min was used for 7 minutes with the gradient profile in table 2.4.10.2.

**Table 2.4.10.2 Liquid chromatography gradient profile (carboplatin)**

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>% A</th>
<th>% B</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>20</td>
<td>80</td>
</tr>
<tr>
<td>2.5</td>
<td>50</td>
<td>50</td>
</tr>
<tr>
<td>5.0</td>
<td>50</td>
<td>50</td>
</tr>
<tr>
<td>5.51</td>
<td>20</td>
<td>80</td>
</tr>
<tr>
<td>7</td>
<td>Stop</td>
<td></td>
</tr>
</tbody>
</table>

For all three analytes, eluted components were then analysed by tandem mass spectrometry using ThermoScientific TSQ Vantage spectrometer. An electrospray ionisation method was adopted to nebulise, desolvate and ionise the effluent from the liquid chromatography. A triple quadrupole model was used to separate and detect ions based on mass to charge (m/z) ratio. Concentrations were quantified using a calibration curve and all samples were analysed with isotope labelled
2.4.11 Flow cytometry

In order to phenotype the PDTX model AB861 as a B-cell lymphoma, flow cytometry was performed. A breast cancer cell line (MDA-MB-231) and healthy donor peripheral mononuclear cells (PBMCs) were used as breast cancer and immune cell controls respectively.

Cryopreserved PDTX tumour fragments of model AB861 were dissociated into single cell suspensions following 2.2.1 and were resuspended in PDTC complete media (table 2.2.1). MDA-MB-231 cells were cultured to 80% confluency and harvested using trypsin (as described in 2.2.2). Healthy donor PBMCs were isolated from leucocyte cones obtained from NHS Blood and Transplant bank under the appropriate ethical approval (REC 08/H0308/178). PBMCs were isolated by Dr Brent O’Carrigan from the Caldas group. Briefly, this involved washing cone contents with PBS, separation over density gradient medium Lymphoprep (StemCell Technologies, 07811), red cell lysis with ACK lysis buffer (ThermoFisher, A1049201) and a final PBS wash. Isolated PBMCs were cryopreserved in FBS (LifeTech, 10500064) supplemented with 10% DMSO (Sigma, D2650) until use, then thawed at 37°C.

The subsequent antibody staining was performed in collaboration with Dr Brent O’Carrigan in the Caldas Group. An unstained control of each sample was also prepared, on which all downstream steps (except antibody and viability dye staining) were performed in parallel. PBMCs were used as a viability dye control so were processed in parallel but antibody staining was omitted. Compensation beads were used as single colour controls (described below).
Staining buffer was prepared with PBS, 0.5% BSA (Sigma, A9647), 0.05% Sodium Azide (Sigma, S2002) and sterile filtered. 2 X 10^6 cells per sample were resuspended in 100 µL of staining buffer in a round bottom 96-well plate (Costar, 3879). Cells were centrifuged at 300 xg for 5 minutes at room temperature, supernatant was discarded and cells were resuspended in 100 µL of staining buffer. Fc receptors were blocked using TruStain human (Biolegend, 422302) and mouse (Biolegend, 101319) FC blocking solutions, both at 1:100 dilution in staining buffer for 10 minutes at room temperature. Cells were centrifuged at 300 xg for 5 minutes at 4°C and the supernatant were discarded. Cells were resuspended in 100 µL of staining buffer containing the antibodies as outlined in table 2.4.11, and incubated on ice for 30 minutes. Cells were washed twice with 200 µL of staining buffer and centrifuged at 300 xg for 5 minutes at room temperature.

Red blood cell lysis buffer (Biolegend, 420301) was prepared 1:10 in deionised water. 150 µL of red blood cell lysis solution was added to samples, incubated for 5 minutes at room temperature and cells were washed twice with staining buffer, as above. To stain for viability, cells were washed once with PBS and resuspended in 100 µL of Fixable Viability Dye eFluor780 (eBioscience, 65-0865-18), diluted 1:1000 in PBS. Cells were incubated with the viability dye for 15 minutes at 4°C, then washed twice with staining buffer.

Cells were fixed in 50 uL of 4% paraformaldehyde solution (PFA) in PBS for 15 minutes at room temperature. To quench the fixation reaction, 150 µL of staining buffer was added, cells were centrifuged at 600 xg for 5 minutes. Cells were resuspended in 100 µL of staining buffer.

UltraComp eBeads compensation beads (ThermoFisher, 01-2222-42) were used as single colour controls. One drop of compensation beads was added to three tubes so as to assign one tube to each antibody and mixed vigorously by vortexing. 1 µL of antibody was added to a tube and mixed well. Beads were incubated for 30
minutes at 4°C in the dark. 2 mL of staining buffer was added to each tube and centrifuged at 300 xg for 3 minutes. The supernatant was discarded and 200 µL of staining buffer was added to each tube.

Cell suspensions were filtered prior to running. Samples and compensation beads were run on the BD LSR II flow cytometer by Dr Brent O’Carrigan from the Caldas Group. Approximately 100,000 events were acquired per sample.

Table 2.4.11 Flow cytometry antibody panel

<table>
<thead>
<tr>
<th>Target</th>
<th>Reactivity</th>
<th>Fluorophore</th>
<th>Clone</th>
<th>Catalogue number</th>
<th>Supplier</th>
<th>Dilution</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD45</td>
<td>Human</td>
<td>PE-Cy7</td>
<td>HI30</td>
<td>25-0459-42</td>
<td>ThermoFisher</td>
<td>5uL/test</td>
</tr>
<tr>
<td>CD3</td>
<td>Human</td>
<td>BV510</td>
<td>UCHT1</td>
<td>563546</td>
<td>BD</td>
<td>1:200</td>
</tr>
<tr>
<td>CD19</td>
<td>Human</td>
<td>BV711</td>
<td>HCD56</td>
<td>318304</td>
<td>Biolegend</td>
<td>1:200</td>
</tr>
</tbody>
</table>

Flow cytometry data was analysed in FlowJo (v10.6.1). Fluorescent channel compensation data was obtained from the BD LSR II flow cytometer and imported into FlowJo. Sequential gating was performed on acquired events, as cells, single cells, live cells and CD45-positive cells based on the scatter profiles and protein expression (compared to unstained controls), as shown in figure 2.4.11.

Figure 2.4.11 Flow cytometry gating strategy
2.4.12 Mass cytometry experiments

Cryopreserved PDTX tumour fragments were dissociated as described in 2.2.1, resuspended in 10 mL of PDTC media (table 2.2.1.) and immediately placed on ice. Cell concentrations were measured using the Vi-CELL and 2.5 X 10^6 cells per sample were resuspended in 1 mL of complete media. Intercalator Rhodium 103Rh (Fluidigm, 201103), a live-dead exclusion marker, was added to the cell suspension at 1:1000 (final concentration 2 µM) and incubated for 15 minutes at 37°C.

1 mL of Maxpar Cell Staining Buffer (CSB) (Fluidigm, 201068) was added to cell suspensions, centrifuged at 300 xg for 3 minutes at room temperature and the supernatant was discarded. 2% PFA fixation buffer was prepared by diluting 4% PFA (Thermo Scientific, 28908) in 1X Maxpar PBS (Fluidigm, 201058). Cell pellets were resuspended in 200 µL of 2% PFA solution and incubated for 10 minutes at room temperature. 1 mL of CSB was added to cell suspensions, centrifuged at 700 xg for 3 minutes and room temperature and supernatant was discarded. Cell pellets were resuspended in 100 µL of CSB solution.

1X Maxpar Barcode Perm Buffer was prepared by diluting 1 mL of 10x stock (Fluidigm, 201057) into 9 mL of Maxpar PBS. Cell suspensions were diluted into 0.5 mL of 1X Maxpar Barcode Permeabilisation Buffer, centrifuged at 700 xg for 3 minutes at room temperature and the supernatant was discarded. Cell pellets were resuspended with 0.2 mL of 1X MaxPar Barcode Perm Buffer. Cell-ID 20-Plex Pd Barcoding Kit (Fluidigm, 201060) was used to allow sample multiplexing, using a combination of six Palladium isotopes (^{102Pd}, ^{104Pd}, ^{105Pd}, ^{106Pd}, ^{108Pd}, ^{110Pd}). Sample barcodes were prepared by adding 100 µL of 1X Maxpar Barcode Perm Buffer to each barcode. 50 µL of each Pd barcode was added to the respective samples, mixed gently by pipetting and incubated for 30 minutes at room temperature. 1 mL of CSB was added to samples, centrifuged at 700 xg for 3 minutes at room temperature and supernatant was discarded. Cell pellets were resuspended in 100 µL of CSB. Samples labelled with unique barcodes were pooled.
into a common Eppendorf. Samples were centrifuged at 700 xg for 3 minutes at room temperature and the supernatant was discarded. Cell pellets were resuspended in 50 µL of CSB.

Extracellular antibody mix was prepared fresh in CSB at described in table 2.4.12. All antibodies were titrated and validated by Dimitra Georgopoulou in the Caldas Group (publication in review). 50 µL of extracellular antibody mix was added to each 50 µL cell suspension, mixed gently by pipetting and incubated for 30 minutes at room temperature. 1 mL of CSB was added to samples, centrifuged at 700 xg for 3 minutes at room temperature and supernatant was discarded. The wash step was repeated twice. Cell pellets were resuspended in 50 µL of CSB.

Cell suspensions were incubated on ice for 5 minutes. 0.25 mL of 100% ice cold methanol (Thermo Scientific, 10245850) was added dropwise to cell suspensions and incubated on ice for 15 minutes. 1 mL of CSB was added to samples, centrifuged at 800 xg for 3 minutes at room temperature and supernatant was discarded. The wash step was repeated. Cell pellets were resuspended in 50 µL of CSB. Intracellular antibody mix was prepared fresh in CSB at described in table 2.4.12. 50 µL of intracellular antibody mix was added to 50 µL cell suspension, mixed gently by pipetting and incubated for 30 minutes at room temperature. 1 mL of CSB was added to samples, centrifuged at 700 xg for 3 minutes at room temperature and supernatant was discarded. The wash step was repeated. Cell pellets were resuspended in 50 µL of CSB.

Secondary antibody mix was prepared fresh in CSB at described in table 2.4.12. 50 µL of secondary antibody mix was added to 50 µL cell suspension, mixed gently by pipetting and incubated for 20 minutes at room temperature. 1 mL of CSB was added to samples, centrifuged at 700 xg for 3 minutes at room temperature and supernatant was discarded. The wash step was repeated three times. Intercalator Iridium (191Ir, 193Ir), an intact single cell inclusion marker was prepared by diluting
Materials and Methods

Cell-ID Intercalator Ir stock (Fluidigm, 201192) 1:1000 in Maxpar Fix and Perm Buffer (Fluidigm, 201067) and mixed by vortex. Cell pellets were resuspended in 0.3 mL of Intercalator Iridium solution and incubated overnight at 4°C.

1 mL of CSB was added to cell suspensions, centrifuged at 700 xg for 3 minutes at room temperature and the supernatant was discarded. The wash step was repeated. Cell pellets were resuspended with 1 mL of Maxpar water (Fluidigm, 201069), centrifuged at 800 xg for 3 minutes at room temperature and the supernatant was discarded. The wash step was repeated. Cell pellets were resuspended with 1 mL of Maxpar water and diluted to approximately 0.5 X 10^6 cells/mL based on initial cell concentrations. Cells were filtered through a 70 µm cell strainer. EQ four element calibration beads (^{140/142}Ce, ^{151/153}Eu, ^{165}Ho, ^{175}Lu) (Fluidigm, 201078) was added to the sample at a volume of 10% the final volume for running.

Samples were run using the Helios mass cytometer by the Flow Cytometry core facility at the Cancer Research UK Cambridge Institute. Briefly, the cytometer was calibrated 2 hours prior to the sample run following the manufacturer’s recommendations. Samples were run as per batches of 1.5 mL in 45 minutes at a concentration of 0.5 X 10^6 cells/mL for 150-300 events/second.
### Table 2.4.12 Mass cytometry antibody panel

<table>
<thead>
<tr>
<th>Target</th>
<th>Reactivity</th>
<th>Metal/Fluorophore</th>
<th>Clone</th>
<th>Catalogue number</th>
<th>Supplier</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Extracellular</strong></td>
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<td></td>
</tr>
<tr>
<td>MHC Class I</td>
<td>Mouse</td>
<td>FITC</td>
<td>28-8-6</td>
<td>114606</td>
<td>BioLegend</td>
</tr>
<tr>
<td>CD31</td>
<td>Mouse</td>
<td>APC</td>
<td>390</td>
<td>17-0311-80</td>
<td>ThermoFisher</td>
</tr>
<tr>
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<td>APA5</td>
<td>135907</td>
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<tr>
<td>CD45</td>
<td>Mouse</td>
<td>147Sm</td>
<td>30-F11</td>
<td>3147003B</td>
<td>Fluidigm</td>
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<td>CD298</td>
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<td>LNH-94</td>
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<tr>
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<td>9C4</td>
<td>3141006</td>
<td>Fluidigm</td>
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<tr>
<td>HER2</td>
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<td>C51</td>
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<td>GNS-1</td>
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<tr>
<td>Cleaved caspase 3</td>
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<td>172Yb</td>
<td>5A1E</td>
<td>3172023</td>
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<td>p-Rb (S807/S811)</td>
<td>Human</td>
<td>166Er</td>
<td>J112-906</td>
<td>3166011</td>
<td>Fluidigm</td>
</tr>
<tr>
<td>p-Histone H3 (S28)</td>
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<td>HTA28</td>
<td>3175012A</td>
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<td>p38 (T180/Y182)</td>
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<td>156Gd</td>
<td>D3F9</td>
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<td>p-S6 (S240/S244)</td>
<td>Cross</td>
<td>142Nd</td>
<td>D57.2.2E</td>
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</tr>
<tr>
<td>p-4E-BP1 (T37/T46)</td>
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<td>236B4</td>
<td>3149005</td>
<td>Fluidigm</td>
</tr>
<tr>
<td>p-AKT (S473)</td>
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<td>152Sm</td>
<td>D9E</td>
<td>3152005</td>
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<td>p-Erk1/2 (T202/Y204)</td>
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<td>p-BAD (S112)</td>
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</tr>
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<td>p-CREB (S133)</td>
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<td>87G3</td>
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<td>p-c-Jun (S63)</td>
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<td>173Yb</td>
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<tr>
<td><strong>Secondary</strong></td>
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</table>
2.4.13 Mass cytometry analysis

Raw data was first analysed using FlowJo (v10.6.1) to gate live single cells as shown in figure 2.4.13. First, to distinguish intact cells from calibration beads, $^{191}$Ir-positive and $^{140}$Ce-negative events were gated. Second, single cells were identified using $^{191}$Ir (x axis) and event length (y axis). Third, live cells were gated as $^{103}$Rh-negative. At this stage, three samples were removed due to low cell numbers and <50% live cells.

![Mass cytometry gating strategy.](image)

Downstream analysis was performed in the Cytobank web tool (196). Data from all channels were transformed using the hyperbolic arcsinh function. ViSNE plots were created using markers of interest as described in the main text and event counts were down-sampled to the lowest sample for each analysis. For ViSNE analysis, Cytobank default settings were utilised: 1000 iterations, 30 perplexity, 0.5 theta. Human cells were manually gated using a combination of human-reactive antibodies against CD298 and EpCAM, and mouse-reactive antibodies against MHC class I and CD45. Human cells were further gated for the CD298+/EpCAM+ population, which was used for FlowSOM clustering. FlowSOM clustering was performed using hierarchical consensus clustering method, 7 metaclusters, 100 clusters, 10 iterations and normalised scales.
2.5 Genomics

2.5.1 Nucleic acid extraction

Nucleic acids (DNA and RNA) were extracted from PDTX samples using the AllPrep DNA/RNA kit (QIAGEN, 80204). First, flash frozen PDTX samples were homogenised in 1 mL of RLT buffer (containing β-mercaptoethanol), using a 5mm stainless steel bead (QIAGEN, 69989) and the QIAGEN tissue lysis instrument was run for 45 seconds at a frequency of 30/s. Due to excess, homogenised tissue in RLT was divided into aliquots and stored at -80° for future use. Typically, 300 µL of homogenised tissue was used for the nucleic acid extraction following the manufacturer’s instructions. DNA was eluted into EB buffer (10 mM Tris-Cl, pH 8.5) and RNA was eluted into water.

2.5.2 Nucleic acid quantification

Nucleic acids (DNA and RNA) were first quantified by NanoDrop 2000 UV spectrophotometer using the solvent as a blank. The ratio of absorbance at 260 nm and 280 nm (260/280) was used to assess the purity of DNA or RNA. Pure DNA and RNA samples would be expected to produce 260/280 values of 1.8 and 2.0 respectively; values much lower than this could indicate contamination of protein, phenol or other contaminants that absorb at 280 nm. The 260/230 ratio was also used to measure nucleic acid purity with pure nucleic acids commonly producing values of 2.0-2.2

Next, nucleic acids were quantified using the Invitrogen Qubit Fluorometric Quantitation assays, which utilise specific dyes that emit fluorescence when bound to DNA, RNA or protein. Depending on the required throughput, Qubit or Quant-iT quantification assay kits were used and fluorescence values were read using a Qubit Fluorometer or the PHERAstar respectively. All assay kits were used according to manufacturer’s instructions.
2.5.3 Assessing RNA quality control

Prior to next generation sequencing (NGS), RNA quality was determined using an automated electrophoresis system, TapeStation (Agilent). The RNA ScreenTape Assay was used following the manufacturer’s recommendations and analysed on the 4200 TapeStation system. Briefly, 1 µL (between 25-500 ng/µL) of each sample or RNA Ladder (Agilent, 5067-5578) was mixed with 5 µL of RNA Sample Buffer (Agilent, 5067-5577) by vortexing. The samples and ladder were denatured at 72°C for 3 minutes then incubates on ice. Samples were loaded to the Agilent 4200 TapeStation instrument with the RNA ScreenTape (Agilent, 5067-5630) and analysed using the 4200 TapeStation Controller Software. RNA quality was analysed using the RNA Integrity Number (RINe) score, which was determined by the software based on the shape of the electropherogram. The RINe was calculated on a scale of 1 to 10, with a high RINe score indicating highly intact RNA and a low RINe score indicating a strongly degraded RNA sample.

2.5.4 Short tandem repeat analysis

Tumours were profiled for repetitions of short tandem repeats (STR). DNA was extracted from PDTX tumours as described in 2.5.1 Nucleic acid extraction. STR profiling was performed by the Research Instrumentation core facility at the Cancer Research UK Cambridge Institute using a panel of 16 STR loci, as well as a mouse-specific marker. Where possible, passage 0 was analysed by STR to create a profile against which later passages were compared to analyse genomic drift and cell contamination.

2.5.5 RNA-sequencing library preparation

RNA was extracted from flash frozen PDTX samples, quantified using NanoDrop and Qubit and the quality was assessed using TapeStation (as described in sections 2.5.1-3). Prior to sequencing, RNA stocks were normalised based on RNA concentration and, where possible, a randomised plate layout was adopted. RNA-
sequencing library preparation was performed by the Genomics core facility at Cancer Research UK Cambridge Institute. Illumina TruSeq stranded mRNA protocol was used following the manufacturer’s recommendations (Illumina, 20020595). Briefly, Poly-A containing mRNA molecules were purified using magnetic beads with attached poly-T oligos and fragmented. First strand cDNA was synthesised using reverse transcriptase and random primers, followed by second strand cDNA synthesis. 3’ ends were adenylated and adapters were ligated. For all samples, the high-throughput workflow was used and so dual-index adapters were ligated. DNA fragments were enriched by polymerase chain reaction (PCR). Libraries were quantified by quantitative PCR (qPCR) and subject to quality control measures by TapeStation (Agilent). Successful libraries were normalised based on concentration and were pooled. Libraries were sequenced on either HiSeq 4000 or NovaSeq 6000 platforms, using either single end 50 reads or paired end 50 reads, with an aim of achieving approximately 15 x10^6 reads/sample.

2.5.6 RNA-sequencing analysis

Pre-processing and alignment were performed by Dr Yaniv Eyal-Lubling from the Caldas Group to produce a count matrix. All downstream analysis (including normalisation, differential expression and gene set enrichment analysis was performed by myself). Analysis was performed using R (v3.6.0).

Prior to alignment, sequencing quality was enforced using Trim Galore (v0.4.2). As described in (197), reads were aligned to a combined human (hg19) and mouse (mm10) reference genome using STAR (v2.5.2b) (198, 199). Counts were assigned to genes using featureCounts (v1.5.2), whereby the alignment score is used to distinguish reads as being sourced from human or mouse (200). Raw read counts were analysed using EdgeR package (v3.26.8) (201, 202) following the workflow described in (203). Lowly expressed genes were filtered out and then counts were normalised by library size using the trimmed mean of M-values (TMM) method.
The data was explored using a multi-dimensional scaling (MDS) plot on which differences between expression profiles were visualised in two dimensions. Heatmaps were produced by the package ComplexHeatmap (v2.0.0). TMM-normalised log2-transformed counts per million (Log2CPM) values were scaled by row (gene) and displayed as a z score. Top variable genes were identified by ranking genes by variance. Top variable and strongly expressed genes were identified by finding the top 5000 genes by variance and the top 5000 genes by mean expression, and then identifying the genes which were common between both lists. To find the top genes which differed between groups of samples, the mean normalised expression values of one group was subtracted from the mean values of the other group, and the genes with the greatest differences were identified. All hierarchical clustering was performed using Euclidean distances as a measure of dissimilarity.

Each in vivo trial was sequenced separately and so a design matrix was created to reflect trial cohort and sampling (treated or post-treated) time point. Using a generalised linear model, dispersions were estimated using the Cox-Reid profile adjusted likelihood method. Differential expression was tested using the quasi-likelihood F-test and genes were considered differentially expressed if the p value was lower than 0.05. Gene set enrichment analysis (GSEA) was performed using fgsea package (v1.10.1), using the Hallmark signatures from the Molecular Signatures Database (v7.1) (204, 205). Significant gene sets were identified if the adjusted p value was lower than 0.05 and were plotted using ggplot2 (v3.3.2). Box plots were created using Prism (v8.1.0) and displayed Log2CPM values. Statistical significance between log2CPM values of different samples were calculated using two-tailed unpaired t-tests in Prism.

2.5.7 Exome sequencing library preparation

DNA was extracted from flash frozen PDTX samples and quantified using NanoDrop and Qubit (as described in sections 2.5.1-3). Prior to sequencing, DNA stocks were normalised based on DNA concentration. Exome sequencing library
preparation was performed by the Genomics core facility at Cancer Research UK Cambridge Institute. Nextera Flex for Enrichment protocol was used following the manufacturer’s recommendations (Illumina, 20025524). Briefly, genomic DNA was fragmented and tagged with DNA adaptor sequences using bead-linked transposomes. Following clean-up, adapter-tagged DNA was amplified by PCR, adding index 1 (i7) and index 2 (i5) adapters, and sequences required for sequencing cluster generation. Libraries were quantified and pooled. Double stranded DNA libraries were denatured and exon-specific biotinylated capture probes were hybridised to target regions of interest. Hybridised probes and targeted library fragments were captured. The enriched library was amplified by PCR. The quantity and quality of the enriched libraries were determined by Qubit and TapeStation respectively. Enriched libraries were sequenced on the NovaSeq 6000 using paired end 100 reads, aiming for approximately 100x coverage.

2.5.8 Exome sequencing analysis

Exome sequencing alignment, mutation calling and copy number analysis was performed by Dr Ai Nagano from the Caldas Group. Raw sequencing reads were aligned to a combined human hg19 and mouse mm10 reference genome as described in (197). Single nucleotide variant (SNV) mutation calling was performed using Mutect2 (206), CaVEMan (207), MuSE (208) and Mpileup (209) and SNVs were called if there was a consensus by two or more methods. To distinguish somatic from germline mutations, the patient-matched normal sample (blood) was obtained from the Personalised Breast Cancer Programme (PBCP). Since the PBCP sample was sequenced by WGS, mutations were also compared to a panel of normals developed by Dr Maurizio Callari and Dr Oscar Rueda from the Caldas Group to remove technical artefacts. Copy number analysis was performed by Ai Nagano using the CopywriteR R package (210, 211).

After initial SNV mutation calling, synonymous or non-coding mutations were removed from the analysis. Manual filtering steps were applied for each sample,
removing mutations with variant allelic frequency (VAF) <0.05, depth <15 or number of altered reads <5. All mutations were manually inspected in Integrative Genomics Viewer (IGV, v2.8.2) and mutations which fulfilled just one filtering criteria were considered on a case-by-case basis (e.g. VAF <0.05 but depth >15 and altered reads >5). For direct comparisons between samples, mutations were removed in depth <15 in any sample.

Heatmaps were produced using the package ComplexHeatmap (v2.0.0) in R version 3.6.0 and hierarchical clustering was performed using Euclidean distance as a measure of dissimilarity. When comparing mutations following in vivo treatment, emergent mutations were defined as mutations which were present in no untreated mice (or regions therein) but were detected in any one treated/post-treated sample with a VAF greater than 0.05. Depleted mutations were defined as mutations which were present in multiple untreated samples but no treated/post-treated samples. Functional implications of mutations were determined using the Cancer Genome Interpreter online tool (212).

### 2.5.9 Single cell RNA-sequencing experiments

Cryopreserved PDTX tumour fragments were dissociated as described in 2.2.1, resuspended in 3mL PDTC media (table 2.2.1) and immediately placed on ice. Cells were counted using Vi-Cell. An input cell count of 10,000 cells in 34 µL was desired but based on previous experiments cell suspensions were prepared to account for 50% loss. As such, cell suspensions were diluted to 0.45 X 10^6 cells/mL in PDTC media. From this point, all downstream processing was performed by the Genomics Core Facility at the CRUK Cambridge Institute according to the Chromium Single Cell 3’ Reagent Kits v2 User Manual (213). Samples were processed using the Chromium Single Cell 3’ Library and Gel Bead Kit v2 (10x Genomics, PN-120237) and Chromium Single Cell A Chip Kit (10x Genomics, PN-120236). Briefly, this involved the generation of Gel Bead-In-Emulsions (GEMs),
reverse transcriptase reaction including barcoding, sample clean-up, amplification of second strand of cDNA, library construction and sequencing.

Figure 2.5.9.1 Generation of GEMs from single cell suspensions.

A GEM was produced for each individual cell within a sample. Barcoded, full-length cDNA was generated from poly-adenylated mRNA molecules using primers containing: an Illumina R1 sequence (read 1 sequencing primer), a 16 nucleotide 10x barcode, a 10 nucleotide Unique Molecular Identifier (UMI) and a poly-DT primer sequence. During library preparation, enzymatic fragmentation and size selection were used to optimise cDNA amplicon size. P5, P7, a sample index and R2 (read 2 primer sequence) were added via end repair, A-tailing, adaptor ligation and PCR. As such, the final construct (figure 2.5.9.2) contained a UMI to identify the molecule, a 10x barcode to identify the cell and a sample index to identify the sample.

Figure 2.5.9.2. Chromium Single Cell 3’ library structure.

Libraries were sequenced using paired end sequencing with single indexing. Read 1 contained the Single Cell 3’ 16 nucleotide 10x barcode and 10 nucleotide UMI. Read 2 was used to sequence the cDNA fragment. Sample index sequences were
incorporated as the i7 index read (Chromium i7 Multiplex Kit, PN-120262, 10x Genomics). Libraries were sequenced using HiSeq 2500 and NovaSeq 6000 platforms, using the recommended number of cycles: 26 cycles (read 1), 8 cycles (i7 index), 0 cycles (i5 index), 98 cycles (read 2), aiming for 50,000 reads per cell.

2.5.10 Single cell RNA-sequencing analysis

All single cell RNA-sequencing analysis was performed by Dr Alistair Martin from the Caldas Group. CellRanger Pipeline (214) was used to process Chromium single cell RNA-sequencing output and the R package Seurat (215, 216) was used for downstream analysis. The standard workflow was utilised as described in (217, 218), unless otherwise outlined below.

Briefly, raw base call files generated by Illumina sequencers were demultiplexed into FASTQ files. Counts were aligned to human or mouse genomes to generate a UMI count matrix. Cells were filtered based on number of unique genes per cell (filter out those with <200). Cells were also filtered based on the percentage of mitochondrial genes (>25% and >35% for batch 1 and 2 respectively). The mitochondrial gene threshold was increased dramatically from the recommended threshold of 5%.

Next, data was normalised and scaled. The gene expression measurements of each cell were normalised by the total expression, multiplied by a scale factor (10,000 default) and then log-transformed. Highly variable genes were identified (2000 per dataset) using standardised variance, which considered the mean-variance relationship of each gene. To integrate multiple samples, anchors were identified, which represent two cells (one from each dataset) predicted to originate from a common biological state (216). Using the identified anchors, multiple datasets were integrated and the following workflow was performed on all cells together.
Data was subject to linear transformation (scaling), which shifted the expression of each gene so the mean expression across all cells was 0 and the variance across all cells was 1. Linear dimensional reduction was performed (principal component analysis, PCA). Clustering was performed by first constructing a K-nearest neighbour graph based on the Euclidean distance in PCA space, and then finding local neighbours. For clustering, the first 50 principal components were used as input. A uniform manifold approximation and projection (UMAP) was created to visualise the linear reduction data and subsequent clustering. This is a non-linear dimensional reduction technique and was used for visualisation purposes only.

Differentially expressed genes were identified for each cluster (cluster biomarkers). Gene set enrichment was used to identify significantly enriched gene sets for each cluster using the Hallmark gene set from Molecular Signatures Database. The significance was determined using a Fisher test, and a Benjamini-Hochberg correction was applied for multiple testing. To identify whether the proportion of cells in each cluster was significantly different between conditions, an independent binomial model was fit to each cluster using the experimental condition as a factor. In short, this tested whether a cluster had more or fewer cells in an experimental condition than one would expect given the fractions in the cluster overall. The coefficient and 95% confidence intervals associated with each cluster/condition were extracted (using the Wald method) and p values were calculated, correcting for multiple testing (Benjamini-Hochberg). All cells were scored using G1/S and G2/M gene sets as described in (219). Briefly, this score was generated by taking the sum of the normalised gene expression values per gene set for each cell.

2.5.11 Analysis of primary breast cancer samples

Primary breast cancer patient samples and matched normal tissue samples (blood) were analysed as part of the Personalised Breast Cancer Programme (PBCP) at Addenbrooke’s Hospital. All genomic data from patient samples described in this
thesis was shared as part of this initiative and all analysis was performed by the scientists involved, led by Dr Oscar Rueda.

Germline and somatic copy number and mutational data was sequenced by WGS. The contribution of mutational signatures was estimated using the R package deconstructSigs (220) by Chris Boursnell. HRD-LOH and HRDetect scores were generated using the HRDetect_pipeline function from the R package signature.tools.lib (221, 222) by Dr Kate Eason. Percentage tumour content was estimated using Canvas (223), RNA-sequencing was conducted on breast cancer samples to obtain gene expression data. This was normalised for batch effects, library size and gene size. Integrative clusters and PAM50 classifications were determined using custom scrips published previously by our group (14) and the R package iC10 by Raquel Manzano Garcia.

2.5.12 De novo motif search

As described in 2.5.6, bulk RNA-sequencing analysis was performed using EdgeR package and workflow (v3.26.8) (201, 202). Using a p-value threshold of 0.05, differentially expressed genes were identified between model 1006 untreated and olaparib (treated) samples, and between untreated and olaparib (post-treated) samples. Differentially expressed genes common between both lists were identified. Using the R package biomaRt (v2.40.5) (224, 225), the sequences 5000 base pairs upstream of the gene flanks were retrieved for each common differentially expressed gene.

The MEME-ChIP tool (226) on the MEME Suite (v5.1.1) webpage (227) was used to identify novel DNA-binding motifs and analyse them for similarity to known binding motifs. The default settings were selected apart from those outlined below. Classic enrichment mode was used, in which one set of sequences was provided and the significance values of the discovered motifs were determined based on their enrichment relative to a random model. A 1st order background model was selected,
which normalised for biased distribution dimers (e.g. GC content) in the DNA sequences. The expected motif site distribution was set at zero or one occurrence per sequence, since we reasoned that some motifs may be missing from some sequences. Ten motifs were discovered with minimum and maximum widths of 6 and 15 respectively.

MEME was used to discover motifs enriched in multiple DNA sequences (228). The statistical significance of discovered motifs was reported using an E-value, which was based on its log likelihood ratio, width, sites, the background letter frequencies and the size of the training set. This represents an estimate of the expected number of motifs with the given log likelihood ratio, same width and site counts, that one would expect in a similarly sized set of random sequences. Discovered motifs with an E-value of <0.05 were considered significant.

TOMTOM was used as a motif comparison tool (229), in which significant discovered motifs were compared to known DNA binding motifs of transcription factors using target databases: jolma2013, JASPAR2018_CORE_vertebrates_non-redundant, uniprobe_mouse. The significance of this was reported as a p-value, which represents the probability that a random motif of the same width as the target would have an optimal alignment to the discovered motif with a match score as good or better than the target. A p-value of <0.05 was considered significant.
Chapter 3: Development of a co-clinical trial platform

3.1 Introduction

Patient-derived tumour xenografts (PDTXs) are largely considered to be the preclinical models which bear the greatest resemblance to the complexity of cancer and mimic the diversity of clinical drug responses. For these reasons, they are commonly used in experimental cancer biology. However, their future utility in clinical medicine is reliant on assessing the extent to which PDTX models mirror clinical drug responses, cancer evolutionary trajectories on a matched patient basis and their potential predictive value.

A number of publications have emerged in recent years and reveal great promise in using PDTX models in this way (230). A large-scale examination of parallel drug responses was performed by Izumchenko et al. in multiple cancer types, including breast cancer (188). In this publication, 129 drug responses were tested across 92 PDTX models and the authors demonstrated that PDTXs can reproduce both positive and negative clinical outcomes. Across all cancer types, this study revealed concordance with clinical drug responses in 112/129 cases tested, and 9/10 of breast cancer cases. Crucially they described multiple examples in which PDTXs retain
therapeutic accuracy over time, including after multiple treatments in the patient. PDTX models generated from early resections were able to predict drug responses to both first-line and subsequent therapies employed clinically. Although none of these described cases were in breast cancer, it suggests PDTXs established early in the disease course may have clinical utility when patients present with relapsed or recurrent disease.

Other studies have reported similar data from matched PDTX and clinical drug responses. Specific to breast cancer, Marangoni et al. reported a drug response concordance in 5 of 7 cases tested (168). Similarly, Zhang et al. examined parallel drug responses in 11 PDTX models for 13 treatment schedules and response was mirrored in 12/13 cases (169). Overall, these data support the use of PDTXs as avatar models in breast cancer. However, studies to date have been limited to anecdotal analyses and have not systematically interrogated the concordance of drug responses between PDTX models and patients enrolled in a controlled clinical trial with mirrored treatment schedules. There remains a need to thoroughly examine the extent to which PDTX models can be used as avatars in breast cancer, to test both first-line and subsequent treatment strategies.

The Caldas laboratory previously developed a breast cancer PDTX biobank which was deeply characterised at molecular and phenotypic levels. These models were found to faithfully recapitulate the main features of the tumours from which they originated and preserve these features over multiple passages (166). This thesis chapter aims to expand on the PDTX biobank developed by the group to establish a co-clinical trial platform. The contents of this chapter include the establishment, quality control and characterisation of the cohort, the optimisation of the co-clinical trial design, analyses and pharmacokinetic experiments.

The PDTX co-clinical trial was established alongside the PARTNER trial at Addenbrooke’s hospital, led by Dr Jean Abraham (figure 3.1). As described
Figure 3.1 PARTNER clinical trial. Patients with TNBC and/or germline BRCA1/2 mutations were enrolled in the PARTNER clinical trial. Research and control treatment schedules included four cycles of neoadjuvant chemotherapy (paclitaxel and carboplatin) with and without olaparib respectively. Both trial arm treatment schedules were followed by three cycles of anthracycline based chemotherapy and then surgery. The clinical outcome was defined as pCR or non-pCR at surgery.
previously, a co-clinical trial is a patient/PDTX framework in which PDTX models are treated with matched treatment schedules to the patient from whom they were derived. Co-clinical trials are being adopted to both emulate and predict clinical drug responses. The PARTNER trial is an ongoing clinical trial in the neoadjuvant setting. Patients with triple negative breast cancer (TNBC) and/or germline BRCA1/2 mutations are enrolled into either the research or control arms and receive four cycles of carboplatin and paclitaxel, with or without olaparib respectively. All patients then receive three cycles of anthracycline-based chemotherapy and the primary endpoint is pathological complete response (pCR) at surgery. The majority of PDTX models in this cohort were generated from primary biopsies taken prior to the commencement of treatment, though midpoint and surgical samples were also implanted where possible.

This clinical trial has a number of features which made it fitting for the parallel co-clinical trial. First, there remains an unmet clinical need to improve treatment options this patient group. TNBC remains the subset of patients with the worst survival outcome (56), largely due to a lack of therapeutic targets. However, these patients commonly harbour alterations in the DNA damage response (DDR), including mutations in BRCA1/2, and so treatment with olaparib offers new opportunities for therapeutic intervention.

Second, the tumours from patients enrolled in this trial are treatment-naïve. PDTX models have been subject to criticism surrounding genomic evolution and the existence of distinct human and mouse evolutionary trajectories remains elusive. One of the main bottlenecks in tumour evolution is therapeutic pressure and so when patients receive multiple lines of therapy prior and subsequent to the development of the matched PDTX model, it becomes difficult to delineate the drivers of evolution. By ensuring all PDTX models in this cohort are treatment naïve, it provides an ideal framework to study both time- and drug-dependent evolutionary trajectories. To this end, longitudinal sample collection from the clinical trial will enable the investigation of human and murine-specific evolution.
Third, this clinical trial sits in the neoadjuvant, curative setting. This provides a short time-frame after which a drug response outcome can be defined and so presents an ideal scenario to mirror in the mouse. In addition, the curative setting unveils an interesting opportunity for PDTX models to accelerate the drug development process. It remains exceptionally difficult to trial new compounds in this setting, even in the cases of strong preclinical data. However, non-pCR at surgery after neoadjuvant chemotherapy strongly correlates with poor outcome (51), so there remains an urgent need for new compounds and combinations for these patients. By experimentally testing the alternative clinical trial arm to that which the patient received, as well as other clinically relevant therapeutic strategies, it offers the potential to assess the most efficacious treatments for these patients. Moreover, we can examine the impact of different therapies on long-term cancer evolutionary patterns in the context of early breast cancer.

### 3.2 Aims

1. To develop and perform quality control measures on a PDTX cohort from patients enrolled in the PARTNER clinical trial
2. To characterise the PDTX cohort using genomics and histopathology
3. To develop and optimise the experimental and analytical framework of a PDTX co-clinical trial platform
3.3 Results and discussion

3.3.1 The development of the PARTNER PDTX cohort

A cohort of PDTX models was established from patients enrolled in the PARTNER clinical trial. As described by Bruna and Rueda et al. (166), fresh breast cancer tissue samples were coated in Matrigel and implanted subcutaneously into the flank of female NSG mice. This was typically performed within one hour of resection from the patient. 67 independent samples were implanted and, where possible, each tumour sample was implanted into two mice. Of the 67 samples, 22 were successfully engrafted (33%), meaning the tumour grew in the mouse to size limits (figure 3.3.1a). Of these, 14 were successfully established (21% of implanted), meaning the tumour was passaged into multiple mice which grew to size limits. Of the 67 implanted samples, 57 were pre-treatment biopsies, three were midpoint biopsies, three were surgical samples and four were relapsed/metastatic samples (figure 3.3.1b). Samples from multiple time points were implanted from eight patients but were established from just one patient, 1059.

A number of parameters were assessed to determine whether the engraftment process enriched for clinical characteristics, including tumour type, tumour grade, percentage tumour infiltrating lymphocytes (TILs) and molecular biomarkers (figure 3.3.1c). No single parameter was found to be dramatically enriched in the PDTXs compared to the clinical cohort, indicating that the PDTX cohort represents the diversity observed in the clinic. Although a small number of medullary and apocrine tumours were implanted, most established PDTX models were from ductal tumours and one was metaplastic. Interestingly, there was no association between the percentage of tumour infiltrating lymphocytes (TILs) in the originating sample with engraftment rate, despite the immunocompromised environment of the NSG mouse. The majority of PDTX models in the final cohort were ER, PR and HER2 negative, IntClust10 and had a basal PAM50 classification, as expected considering the enrolment criteria.
Figure 3.3.1 The engraftment process does not enrich for any clinical features and the PDTX cohort reflects the diversity of the clinical cohort. a) Number of PDTX models implanted, engrafted and successfully established from the PARTNER clinical trial. b) Types of samples implanted as PDTX models (left) and eight patients from whom multiple samples were implanted. c) Pie charts depicting the clinical features of the patient tumours from the PARTNER clinical trial, which were implanted and established as PDTX models. NST: No special type. TILs: tumour infiltrating lymphocytes.
Of the 14 established PDTX models from patients enrolled in the PARTNER clinical trial, eight models were selected to develop the co-clinical trial platform (table 3.3.1). This cohort contained two patients with germline *BRCA1* mutations, all had basal PAM50 gene expression profiles and they represented a mixture of IntClust 4ER-, 9 and 10. Six patients had TNBC, one was weakly positive for ER and one was weakly positive for PR. Clinically, five of the patients received the control arm of the clinical trial (chemotherapy: CT); of these three had pCR at surgery and two had non-pCR. Three patients received the research arm (chemotherapy and olaparib: CTO), with one presenting with pCR and two having residual disease at surgery. This cohort was selected to reflect all possible combinations of clinical drug responses (both trial arms and both outcomes).
Table 3.3.1: Clinical information of the PDTX models in the co-clinical trial cohort.
NST: non-special type. pCR: pathological complete response. CT: chemotherapy (control) trial arm. CTO: chemotherapy and olaparib (research) trial arm.

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- Development of a co-clinical trial platform
3.3.2 Quality control pipeline of the PDTXs

Despite their utility in translational science, it is important to perform quality control checks on PDTX models. A robust quality control pipeline has been established (figure 3.3.2.1a) to ensure the models, and specifically the tumours from individual mice used in each experiment, are of human epithelial cell origin. The first step is short tandem repeat (STR) profiling. This involves amplification of repeat regions of the genome and quantification of the number of repeats. A panel of 15 STR loci and one sex type marker (AMEL) are used to create a PDTX model fingerprint of an early passage, against which later passages are compared (figure 3.3.2.1b). This helps to identify genomic drift and possible cell contamination from other models. In addition, since the loci are specific to human cells, a lack of amplification in these loci can identify tumours of mouse origin.

The development of murine tumours has been described many times in immunocompromised mice. A recent publication reported a prevalence of murine tumours during PDTX generation and propagation of 3.2% (231). This group observed that after passaging tumours for up to four generations, at least one mouse tumour was detected from 24 of the 54 PDTX-lines (24 of 761 mice). These tumours were identified as lymphomas (most commonly), mammary tumours, osteosarcomas and hemangiosarcomas. While the development of lymphomas in NOD-SCID mice, particularly of thymic origin, has been reported frequently in the literature (232-234), NSG mice were thought to be more resistant (160). However, Moyer et al. compared implantation of primary tissue into NOD-SCID and NSG mice and observed similar frequencies of lymphomas between strains (231).

In our PDTX platform, we observe the development of mouse tumours, which can be either CD45-positive or CD45-negative by immunohistochemistry (IHC) (representative images in figure 3.3.2.2a). Interestingly, mouse tumours commonly, though not exclusively, develop at the site of implantation, leading to the compelling hypothesis that the tumour implantation may predispose the mouse to
Figure 3.3.2.1 Quality control pipeline for PDTX models. a) Flow chart outlining the quality control steps prior to the initiation of an experiment. b) STR loci panel for confirmation of human origin and detection of genomic drift. c) Immunohistochemistry panel for confirmation of human epithelial origin.
Development of a co-clinical trial platform

tumorigenesis. In some cases, within a single passage, both human PDTX and murine tumours can develop in sister mice, which emphasises the importance of regular and robust quality control measures. To date, we have not identified any specific tumour characteristics which predispose the host mouse to the development of murine tumours but this requires further analysis.

The next quality control step involves IHC and a seven-antibody panel has been established (figures 3.3.2.1c and 3.3.2.2a). This is used to identify tumours of both mouse and human immune cell origin. We and others have observed a small number of human immune cell-derived tumours during the development of the PDTX biobank (235-239). These are identified as CD45-positive and are most commonly of B-cell origin (CD19/CD20-positive), though a very small number of T-cell lymphomas have also been identified.

The prevalence of this in the generation of PDTX models varies throughout the literature (reported between 11-52%) but these tumours are typically faster growing and commonly present with metastatic lesions in the lymph nodes. An example of IHC staining of a B-cell lymphoma can be seen in figure 3.3.2.2a. Using flow cytometry, a B-cell lymphoma (AB861) was analysed along with a human breast cancer cell line, MDA-MB-231, and healthy donor peripheral-blood mononuclear cells (PBMCs) (figure 3.3.2.2b). This revealed CD45 expression levels to be similar to the PBMCs. Within the CD45-positive population of AB861, cells were CD19-positive and CD3-negative indicating B-cell, rather than T-cell, origin.

The B-cell lymphomas identified in our biobank have been found to be associated with Epstein Barr virus (EBV). In our laboratory, this has been confirmed by alignment of sequencing data to the EBV genome and fluorescent in situ hybridisation (data not shown), consistent with the literature. A large proportion of the human population is infected with EBV, which is associated with a number of malignancies but in the large part this is controlled by the human immune system. The immunocompromised environment of the host NSG makes them susceptible to
Figure 3.3.2.2 Examples of potential issues which can arise during PDTX engraftment. a) IHC displaying protein expression of tumours from mouse origin, along with human tumours from immune cell and epithelial cell origin. b) Flow cytometry data demonstrated that AB861 was of B-cell origin (CD45+, CD19+, CD3-).
Figure 3.3.2.3 STR profiling of PDTX models used in the co-clinical trial demonstrated human cell origin and lack of genomic drift from passage 0.

* Indicates three STR peaks detected at this loci. One mouse of PAR1006-x3 presented three peaks at 5, 10, 11 for Penta E. PAR1141-x0 presented three peaks at 11, 13, 14 for Penta D. Grey box indicates STR loci which were not tested.
the development of EBV-associated tumours which arise from infected immune cells present in the implanted human tissue.

In addition to EBV-associated B-cell lymphomas, Bondarenko et al. reported the development of an EBV-negative T-cell tumours during PDTX generation. These were identified as CD45-positive, CD19/20-negative and CD3-positive (239). This is consistent with observations in our biobank. Taken together, these findings emphasise the importance of routine quality control checks prior to any in vivo or ex vivo experiments and alongside in vivo propagation of the PDTX biobank.

Quality control checks were performed on the PDTX models in the co-clinical trial cohort. STR profiling results are shown as a heatmap in figure 3.3.2.3. This demonstrated that the models were of human cell origin and displayed limited genomic drift over multiple passages. It should be noted that at both S8S1179 and FGA loci, model 1022 displayed heterozygous peaks at the first passage (x0) but later passages were homozygous. This could suggest a degree of genomic drift but since all other loci remain stable through passaging, it was not deemed a concern. Additionally, 1006-x3 and 1141-x0 presented with three peaks at loci Penta E and Penta D respectively. With regard to 1006-x3, this could indicate cell contamination but since the other loci were consistent between passages, it is more a likely to be a technical artefact. Since just one passage of 1141 was analysed, it is difficult to delineate whether this indicates duplication of the loci due to genomic instability, cell contamination or a technical artefact.

The PDTX co-clinical trial cohort was also subject to quality control checks by IHC (figure 3.3.2.4) which confirmed that they were of human, epithelial cell origin. In addition, staining with ER, PR and HER2 demonstrated that the PDTX models retained the biomarker expression of the clinical sample (figure 3.3.2.5). This staining was performed in a tissue microarray (TMA) containing an ER/PR/HER2-positive PDTX model (STG195), a human B-cell lymphoma and a murine tumour as internal controls.
Figure 3.3.2.4 Quality control IHC demonstrates that PDTX models used in the co-clinical trial were of epithelial cell origin.
Figure 3.3.2.5 Clinical biomarker IHC demonstrated that PDTX tumours retain the biomarker expression of the clinical sample from which they were derived.
3.3.3 Exploring characteristics of the PARTNER PDTX cohort

The molecular features of the co-clinical trial cohort were investigated. All patients from whom the PDTX models were derived were enrolled in the Personalised Breast Cancer Programme (PBCP) at Addenbrooke’s Hospital. In this initiative, the patient’s normal and tumour samples were analysed by whole genome sequencing (WGS) and RNA-sequencing to gain insights into genomic alterations and gene expression.

Figure 3.3.3.1 summarises the genomic features of the patient tumours from which the PDTX models were derived. Two patients, 1006 and 1040 were found to have germline alterations in BRCA1 (figure 3.3.3.1a). 1006 has a germline copy number variant (CNV) detected in the normal sample and a somatic copy number aberration (CNA) which led to the loss of the other allele in the tumour (figure 3.3.3.1d) As such, the patient was shown to have very low expression of BRCA1 at the RNA level compared to other models in the PBCP cohort (figure 3.3.3.2b,d).

Patient 1040 has a germline pathogenic mutation in BRCA1: c.4327C>T (p.Arg1443Ter) (figure 3.3.3.1a). This is a single nucleotide variant (SNV) stop gain mutation, which encodes a truncated, non-functional protein (240). In the tumour, we observed loss of heterozygosity (LOH) with loss of the wildtype allele by a CNA (figure 3.3.3.1d). In the patient tumour, we observed low BRCA1 gene expression at the RNA-level relative to the PBCP cohort (figure 3.3.3.2b,d) but this was substantially higher than in 1006. This is in line with the notion that the mutation produces a non-functional protein with some residual expression at the RNA level.

All tumours harboured somatic mutations in TP53. This is in line with previous reports which described that 80% of basal cancers display TP53 mutations, most of which are stop gains and frameshifts (66). To test whether these mutations had functional consequences, we explored the gene expression levels of TP53 in the
Figure 3.3.3.1 Genomic features of the patient tumours in PARTNER co-clinical trial cohort. a) Germline alterations in \textit{BRCA1}. b) Tumour content (%) of patient tumour samples. c) Somatic mutations d) Copy number aberrations, as detected by log$_2$ copy number (corrected for ploidy). Amplification is defined as a copy number of greater than 2X ploidy i.e. in a 2n tumour, amplification is >4 copies of a gene.
Development of a co-clinical trial platform

patient samples. RNA-sequencing was performed and analysed by the scientists involved in PBCP initiative and was shared as part of the collaboration. Figure 3.3.3.2a displays the normalised RNA expression of seven of the eight patient tumours and figure 3.3.3.2c shows this relative to all samples in the PBCP cohort. It should be noted that the patient RNA sequencing data was not available for 1221.

To validate these findings, we performed IHC on the matched PDTX tumours (figure 3.3.3.2e). Both models 1006 and 1045 (which had the highest RNA expression levels) expressed p53 protein. Models 1040, 1022 and 1053 (lowest RNA expression) did not express the protein. Interestingly, while models 1008 and 1141 had similar gene expression levels, 1141 expressed p53 protein but 1008 did not. There are two explanations for this. First, the TP53 mutation in 1008 may prevent the translation of RNA into protein. Alternatively, this could be a technical artefact. The patient sample of tumour 1008 was found to have low tumour content (figure 3.3.3.1b) and so it is plausible that the TP53 RNA expression was from normal cell contamination. Further analysis would be required to validate this; PDTX tumours have almost 100% cellularity and so could be used for validation.

In addition, mutations in a number of other known oncogenes and tumour suppressor genes were detected, including BRAF (1006), PTEN (1022, 1053, 1121) and RB1 (1221). We investigated the copy number profiles of these tumours and identified gene amplification of MYC on chromosome 8 in three models (1045, 1053 and 1141). This is a common feature of IntClust 9, into which 1053 and 1141 were classified (241). No patients displayed amplification of ERBB2, leading to HER2 overexpression; this is in line with the clinical classifications of the tumour histology, which was preserved in the PDTX tumours (figure 3.3.2.5).

Even in the absence of mutations in BRCA1 and BRCA2, it is becoming clear that alterations in other genes have the ability to dysregulate homologous recombination (HR). This could sensitisise tumours to DDR-targeting compounds, such as olaparib, and is termed BRCAness (96, 97). A number of computations tools have been
Figure 3.3.3.2 Heterogeneous expression of TP53 and BRCA1 was observed between patient tumour samples at the RNA and and PDTX samples at the protein level. a-d) Normalised RNA expression of TP53 (a,c) and BRCA1 (b,d) of patient tumour samples, displayed as a bar chart (a,b) or relative to all patients in the PBCP cohort. e) IHC staining of PDTX samples for p53.
developed to predict HR deficiency (HRD). Figure 3.3.3.3 displays the contribution of each mutational signature (a), HRD-LOH score (b) and HRDetect score (c). These were computed by the scientists involved in the PBCP initiative.

Mutational signatures are characteristic patterns of mutation types within a tumour genome, which can be used to infer mutational processes (242). These are physiological readouts of the types of DNA damage which have occurred as a result of exogenous and endogenous DNA damaging agents and DNA repair processes during tumorigenesis. Signature 3 is associated with inactivating mutations of \textit{BRCA1} and \textit{BRCA2} and can be used to infer HRD (109, 243). Signature 3 is characterised by a fairly uniform distribution of mutations across all possible 96 base substitution types, as well as elevated numbers of large deletions (3 to 50 base pairs) with overlapping microhomology at breakpoint junctions. The latter may reflect the requirement to repair DSBs by alternative mechanisms, such as NHEJ, in HRD tumours. In addition, but to a lesser extent, signature 8 is thought to be associated with an absence of BRCA1 and BRCA2 function (243).

Interestingly, some recent studies have focussed efforts on exploring the aetiology of tumours with high levels of signature 3 but with intact BRCA1/2. Polak et al. undertook a comprehensive analysis into the links between signature 3 and other genetic and epigenetic events in HR-related genes. The authors identified a number of alterations in other components of HR, which yield the same characteristic mutational signature, including epigenetic silencing of \textit{RAD51C} and germline mutations in \textit{PALB2}, but interestingly not variants in \textit{ATM}, \textit{CHEK2} or \textit{NBN} (244).

The HRD-LOH score (figure 3.3.3.3b) was developed by Abkevich et al. in 2012 (107). This study examined the association between genomic patterns of LOH and HRD. They found that the number of long LOH regions (>15 Mb but less than the whole chromosome) was significantly higher in tumours with deficient BRCA1/2; the number of these regions within a tumour genome defines the HRD-LOH score. Analysis of this score in our cohort revealed the highest to be 1006 and 1022.
Computational approaches can be used to reveal HRD in patient tumour samples. a) Contribution of mutational signatures. b) HRD-LOH score. c) HRDetect score.
The development of HRDetect score took these observations a step further (111). This publication identified six mutational signatures predictive of BRCA1/2 deficiency: base substitution signatures 3 and 8, microhomology-mediated indels, rearrangement signatures 3 and 5, and HRD-LOH score (discussed above). The authors then developed a weighted model which was used to discriminate BRCA1/2 deficient from proficient tumours using a threshold of 0.7. When this tool was applied to the co-clinical trial cohort (figure 3.3.3.2c), 1006, 1022, 1040 and 1053 were above the threshold.

As discussed previously and depicted in figure 3.3.3.1, 1006 and 1040 both have absent or non-functional *BRCA1*, which accounts for the high levels of signature 3, HRDetect score and, for 1006, HRD-LOH score. The low HRD-LOH score for 1040 could be attributed to the low percentage of tumour cells in the sample (figure 3.3.3.1b). Interestingly, although no germline or somatic mutations were detected, 1053 was found to have very low *BRCA1* gene expression (figure 3.3.3.2b,d), the HRDetect score was greater than the threshold of 0.7 and the tumour had a high contribution from signature 3. It is possible that *BRCA1* was inactivated in this tumour by promoter methylation. To fully characterise this model, it would be necessary to experimentally test this.

While no *BRCA1/2* pathogenic mutations were detected for 1022, the HRDetect score exceeded the indicated threshold and the HRD-LOH score was the highest of all tumours in the cohort. In addition, gene expression of *BRCA1* was low and at similar level as 1040 (figure 3.3.3.2b,d). Further analysis of this tumour uncovered a germline missense variant in *BRCA1* (c.5019G>A p.Met1673Ils), though this has previously been classified as benign (245). Interestingly, in this tumour there was a *BRCA1* copy number of 3 with LOH, indicating loss of one allele and triplication of the other. We observed that the germline variant was not present in the triplicated allele of the tumour, supporting the notion that it was not pathogenic. Although further analysis is required to fully understand the mechanisms by which gene expression is reduced in this tumour and to explain the HRD phenotype, a likely
hypothesis is that promoter methylation occurred in the wildtype allele prior to triplication.

This patient also harbours germline mutations in *ATM* (c.5497-8T>C) and *BRIP1* (c.584T>C p.Leu195Pro), but both mutations have been annotated as ‘benign’, indicating lack of disease association (246, 247). Possible contributors to this HRD phenotype are germline deleterious mutations in *FANCA* and *PMS1*, and a structural variant in *RAD51B*, all of which have roles in DNA repair. Although these were not indicated in the study by Polak et al., it would be conceivable that alterations in these genes may give rise an HRD phenotype.

### 3.3.4 Preservation of genomic features through passaging

We next performed an in-depth analysis of one PDTX model, 1006, exploring the genomic features of two passages, with multiple mice per passage and multiple regions per mouse.

Multi-region sequencing revealed that genomic alterations are highly consistent between multiple regions of the same tumour, illustrated in figure 3.3.4.1. Focusing our analysis on coding single nucleotide variants (SNV) (including non-synonymous or stop-gains) we observed that a high percentage of mutations were common between all three regions of the same tumour (89 and 80%). We observed a high correlation between the variant allele frequencies (VAF) of the individual regions (Pearson correlation coefficient, r, values of 0.95-0.99 and p<0.0001). Crucially, all 50 somatic mutations detected in the patient sample were common to all regions of both mice. When comparing the mutations to a list of cancer driver genes; three were detected in these samples (*BRAF, TP53, CASP8*). Importantly, all were identified in the patient sample and were present in all regions of both mice. Consistent with previous data, this suggests a low degree of spatial heterogeneity at the mutational level and that horizontal expansion of PDTX tumours should not select for mutations present in specific spatial regions of the tumour.
Multi-region sequencing approach

Tumour

Mouse 1
Region 1
Region 2
Region 3

Mouse
Region 1
Region 2
Region 3

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Patient SNVs common between all regions 50/50

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<tr>
<td>Unique to one region</td>
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Patient SNVs common between all regions 50/50

r value calculated using Pearson correlation.

Figure 3.3.4.1 Multi-region sequencing revealed a high correlation in SNVs between multiple regions of the same tumour. a) Multi-region sequencing approach. b) Venn diagram showing number of coding SNVs (non-synonymous or stop gains) shared between regions of the same tumour, for two biological replicates. c) Table showing number and percentage of SNVs shared between multiple regions of the same tumour. d) Correlation plot comparing VAFs of SNVs in multiple regions of the same tumour. r value calculated using Pearson correlation.
We then sought to identify the proportion of mutations which are common between mice within a given passage (figure 3.3.4.2). When analysing the mice for which we had multiple regions (passage 1, mice 1 and 2), mutations were called if present in one or more regions. Within passage 1, only 15% of mutations were unique to one mouse, and 23% for passage 2. 50/50 and 49/50 patient mutations were common between all mice of passage 1 and 2 respectively and, crucially, mutations detected in the cancer driver genes (BRAF, TP53, CASP8) were common between all mice in both passages. Within both passages, we observed significant correlations between the VAFs of different mice (p <0.0001).

When analysing the proportion of mutations shared between passages, we adopted two approaches, depicted in figure 3.3.4.3. When considering all mutations present in each passage, in any region of any mouse, 66% of mutations were shared between passages 1 and 2 (50/50 patient mutations). However, when considering only mutations common between all mice within a given passage, 78% of mutations were shared between passages (49/50 patient mutations). Importantly, the mutations within the known driver cancer driver genes (BRAF, TP53, CASP8) were common to both passages.

This in-depth analysis sheds light on the mutational landscape the PDTX tumours and illustrates that the main features are preserved through serial passaging.
Figure 3.3.4.2 SNVs are highly preserved between biological replicates within the same passage. a) Venn diagram showing number of coding SNVs (non-synonymous or stop gains) shared between biological replicates (different mice) within a given passage. b) Table showing number and percentage of SNVs shared between mice within a given passage. c) Correlation plot comparing VAFs of SNVs in different mice. r value calculated using Pearson correlation.
Figure 3.3.4.3 SNVs are highly preserved from the patient to the PDTX and through passaging. Venn diagrams showing number of mutations preserved through passaging. (top panel) all mutations detected in any mice in passages 1 and 2. (bottom panel) mutations common between all mice within a given passage.
3.3.5 Establishment of a co-clinical trial

After establishment of the PDTX models and quality control measures, a proof-of-concept co-clinical trial was performed, as illustrated in figure 3.3.5.1a. One tumour per model was horizontally expanded into 30 mice. After two weeks, mice were randomly enrolled into trial cohorts (n=5 per cohort) and treatment commenced. Mice were treated for 77 days (11 weeks). Immediately post-treatment, two mice per cohort were humanely culled and the tumours were excised. These tumour samples were representative of the patient’s surgical sample and were denoted as treated. Three mice per cohort continued to grow off treatment until size limits. These were used to represent relapsed cancer samples and were denoted as post-treated. The untreated cohort grew until size limits, to depict the basal growth and evolutionary trajectory of the tumour in the absence of therapeutic pressure.

As depicted in figure 3.3.5.1b, the initial trial design encompassed two aspects. First, to assess the degree to which the PDTXs reflect clinical drug responses, mice were given treatment schedules to mirror both clinical trial arms; denoted as the co-clinical trial cohorts. These included the avatar (same as patient) and alternative clinical trial arms. Mice were treated with carboplatin and paclitaxel chemotherapy agents, with or without olaparib (PARP inhibitor), denoted as CTO and CT respectively. Four cycles of treatment were given, in line with the clinical treatment schedule (figure 3.3.5.1c). Paclitaxel was administered weekly, carboplatin every three weeks and olaparib was administered on days 3-14 of each cycle. Paclitaxel and carboplatin were administered intravenously and olaparib was administered orally, to mirror with the clinical administration routes.

In the second aspect of the trial design, the platform was leveraged to explore the use of DDR-targeting drugs olaparib and AZD1775 (WEE1 inhibitor) in the neoadjuvant setting and to shed light on the dynamics of drug response/resistance to these compounds. A comprehensive analysis was performed on these samples, which is described in subsequent chapters.
Development of a co-clinical trial platform

Figure 3.3.5.1 Co-clinical trial design. a) Experimental design. b) Co-clinical and exploratory trial cohorts. c) Treatment schedule of the co-clinical trial. d) PDTX models used in co-clinical trial. CT: chemotherapy. CTO: chemotherapy and olaparib. pCR: pathological complete response. Non-pCR: non-pathological complete response.
Initially, six models were selected to reflect all possible clinical scenarios (figure 3.3.5.1d). Figure 3.3.5.2a displays the growth curves of the untreated and avatar arms of the co-clinical trials over 77 days of treatment. Four patients received CT clinically and two received CTO. Three patients had pCR at surgery and three had non-pCR. PDTX models 1022 and 1006 experienced dramatic growth inhibition between the untreated and avatar arms, whereas the other models did not.

A series of parameters were used to compare between the untreated and avatar arms of each model (depicted in figure 3.3.5.2b and supplementary figure 2). Models 1022 and 1006 showed significant differences in tumour volumes after 2 cycles (42 days) and at the end of treatment (77 days). Significant differences were also observed between the area under the curve (AUC) of the growth curve and the regression coefficient following log₂ transformation of tumour volume and linear regression analysis. This is in line with the clinical drug response; both patients and matched PDTX models responded to their respective therapies. Conversely, the three models derived from patients who received non-pCR did not show significant differences in any of the four parameters, between the untreated and avatar arms.

However, the patient from whom 1053 was derived received a complete clinical response but the PDTX model did not show a significant difference between the untreated and avatar arms. Indeed, the treated cohort showed non-significant but higher values in all parameters. We observed that 1053 was slower growing than models 1022 and 1006. Accordingly, we investigated the growth dynamics of all models in the study, to assess their suitability to the trial design.

Figure 3.3.5.3 depicts the raw (a) and log₂ transformed (b) growth curves of the six models used in the co-clinical trial. This clearly illustrates the inter-tumour heterogeneity in growth rate but interestingly this did not correlate with clinical drug response. Doubling time of the tumour volume ranged from 11 days (1022) to 45 days (1008). Figure 3.3.5.3c shows the tumour volume at the end of treatment (77 days) and the six models can broadly be divided into fast- and slow-growing
Figure 3.3.5.2 PDTX avatar responses in a co-clinical trial. a) Growth curves of mice untreated (black) and treated with the same treatment schedule as the matched patient (red for CT, green for CTO) for 11 weeks (77 days). b) Plot indicating significance values and log_{10}(fold change) of treated mice compared to untreated mice over multiple parameters. Regression coefficient calculated by linear regression of log_{2}(tumour volume). p values calculated using unpaired Welch’s t-test (unequal variance) comparing untreated to treated mice.
Figure 3.3.5.3 PDTX models display exponential growth in vivo and can be divided into fast-growing and slow-growing models. a) Tumour growth curves of untreated mice from fast (top panel) and slow (lower panel) growing models. b) Linear regression of log₂(tumour volume) of untreated mice from fast (top panel) and slow (lower panel) growing models. c) Tumour volume of untreated cohort at the end of treatment course (11 weeks, 77 days). d) Regression coefficient calculated by linear regression of log₂(tumour volume). Bar charts show mean values. Error bars show standard deviation.
models. The original rationale to commence treatment at a fixed point after implantation was two-fold. First, this most closely mirrors the clinical scenario; treatment should commence as soon as feasible without waiting for a defined tumour volume. Second, if PDTXs are to be used in routine clinical practice, one of the main limitations is likely to be the time-frame required to generate the models and perform experimentation. Consequently, it reasoned sensible to start the *in vivo* trial as soon as possible.

Based on this data, we set a threshold of 0.05 regression coefficient for $\log_2$(tumour volume), which represents a doubling time of 20 days, for suitability to this trial design (figure 3.3.5.3d). For other models, we hypothesised that in order to observe treatment-mediated volume changes, treatment must be administered within a tumour-dependent time frame. To achieve this, treatment should commence at a defined tumour volume, rather than a defined time point. This alternative experimental design also has further advantages, as it enables tumour regression to be observed. As such, the change in tumour volume can be calculated and avatar mice can be analysed using criteria comparable to RECIST.

As discussed previously, in addition to the avatar and alternative clinical trial arms, the PDTX models were treated with other compounds and combinations. This was part of an exploratory study to interrogate the dynamics of drug response both on treatment and following drug withdrawal. Figure 3.3.5.4 shows growth curves for models 1040 and 1022 treated with various treatment strategies *in vivo*. This is discussed in subsequent chapters but exemplifies the power of the co-clinical trial platform, which can be leveraged in the study of the disease and as an experimental framework to increase the number of compounds a given tumour receives. This could have profound impacts on the future use of PDTXs in anticipatory cancer medicine.
The co-clinical trial platform can be used to explore parallel drug responses in vivo using clinical and experimental compounds and combinations.
3.3.6 Optimisation of the co-clinical trial dose schedule

Despite observing significant differences between the untreated and avatar cohorts of PDTX models from patients who received a clinical pCR, no mice from these models experienced a complete response i.e. no measurable tumour after treatment. We reasoned that this may be due to the administered doses and so performed some optimisation experiments.

Due to a lack of preclinical data using these compounds in combination, the doses used in the co-clinical trials described in section 3.3.5 were adapted from the human doses relative to reference body mass ratios (human 60kg, mouse 20g). The human carboplatin dose (AUC5) was converted to a total human dose of 480 mg using the Calvert equation (248) using an average glomerular filtration rate of 71 mL/min. This was calculated based on an average 50 year old female with serum creatinine levels of 0.9 mg/dL using the Cockroft-Gault equation (249). 480 mg total dose was converted to 8 mg/kg human dose, equivalent to 0.16 mg for a mouse. The human paclitaxel dose of 80 mg/m² was converted to 2.16 mg/kg using a reference body surface area correction factor of 37 kg/m². This translated to a dose of 0.04 mg per average mouse. However, based on previous tolerability data on NSGs in our group, 0.07 mg was administered. The olaparib dose in the avatar cohort (CTO) was converted from a 150 mg human dose (2.5 mg/kg) to a 0.05 mg mouse dose based on body mass. For the olaparib monotherapy cohort, a higher dose was used based on previous literature (1 mg or 50 mg/kg) (250).

Following the initial co-clinical trials, we tested the tolerability of increased doses of each compound in line with other preclinical studies (250, 251). Using a small sample size per cohort (n=2 of non-tumour bearing mice), tolerability was tested using two doses of each drug (figure 3.3.6.1a). Mice were carefully monitored for adverse effects (e.g. body weight changes) but even the combination of the three highest doses (paclitaxel 0.14 mg, carboplatin 0.8 mg, olaparib 1 mg) was tolerated. Liquid chromatography and tandem mass spectrometry (LC- MS/MS) was used to


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Figure 3.3.6.1 *In vivo* experiment demonstrates tolerability of higher drug concentrations in combination and all three drugs were detected in the plasma.

a) Experimental design, with *n*=2 per cohort. b) Concentrations of drugs detected in the plasma of mice analysed by LC-MS/MS. Plasma taken 2 hr, 6 hr and 0.5 hr after dosing with paclitaxel, carboplatin and olaparib respectively.
detect the concentrations of the drugs in the plasma of mice culled two hours, six hours and 30 minutes after administration with paclitaxel, carboplatin and olaparib respectively. Indeed, at all concentrations the three drugs could be detected in the plasma. An outstanding question is whether the plasma concentrations are comparable to the clinical pharmacokinetics of these compounds and it would be beneficial to test this using patient samples from the PARTNER clinical trial.

We next aimed to assess the degree of target engagement of the PARP inhibitor, olaparib. An ELISA was used to measure levels of poly adenosine diphosphate ribose (PAR) in the tumour samples of PDTX model 1040 treated with either the CTO combination or the olaparib monotherapy, which had olaparib doses of 0.05 mg and 1 mg respectively. Tumour samples were collected two hours after the final dose of olaparib. The net relative light units (RLU) were calculated, which reflects the detected luminescence minus background luminescence. A calibration curve was generated to calculate the concentration of PAR (pg/mL) (figure 3.3.6.2a). Jurkat samples of known PAR concentration were used as internal controls to measure drift between experiments. Low, medium and high Jurkat controls had known concentrations of 20-60, 150-300 and 750-1150 pg/mL respectively and we detected concentrations of 42, 217 and 934 pg/mL, with low variability between technical replicates (figure 3.3.6.2b).

Figure 3.3.6.2c shows the calculated PAR concentrations between untreated mice (n=5), and mice treated with CTO or olaparib monotherapy (both n=2). We observed a large degree of variability between biological replicates in the untreated cohort (PAR concentration 57-1539 pg/mL), with a mean value of 690 pg/mL. The CTO and monotherapy cohorts had mean concentrations of 242 and 21 pg/mL respectively. To validate the results, an independent experiment was performed using the Jurkat controls and n=2 samples per cohort; a high correlation was observed between the PAR concentrations detected in each experiment (figure 3.3.6.1e). While the large variability between the untreated samples and the small sample size made it difficult to draw conclusions, the mean PAR concentration of
Figure 3.3.6.2 Concentrations of PAR following olaparib treatment to assess target engagement, detected by ELISA. a) Calibration curve for the quantification of PAR. b) PAR concentration of internal controls. Bar shows mean value and error bars show standard deviation. c) Concentration of PAR (mg/mL) in the tumour samples of untreated mice (n=5) and mice treated with CTO (n=2) and olaparib monotherapy (n=2). Each dot represents mean of n=3 technical replicates per sample. Line indicates mean value per cohort. d) Two independent ELISA experiments showed high correlation in PAR concentrations. Correlation coefficient, r, calculated using Pearson correlation.
both the CTO and monotherapy cohorts were lower than in the untreated mice. The higher dose in the monotherapy cohort compared to CTO is reflected in the PAR levels. While it is clear further analysis is required to better understand the variability between the untreated cohort and the experiment should be expanded using another model, these findings are in line with published data. Using the same dose as in the monotherapy cohort (1 mg) and harvesting the tumour after two hours, Lallo et al. observed a decrease in levels of PAR from approximately 500 pg/mL to negligible levels, as detected using an ELISA (250).

### 3.3.7 An optimised co-clinical trial platform

Based on the observations from the proof-of-concept co-clinical trials, as well as pharmacokinetic and target engagement analyses, a number of optimisation steps were implemented (figure 3.3.7.1a). The proof-of-concept and optimised trial designs are denoted as *protocols 1* and *2* respectively.

First, it was decided to commence treatment when the average tumour volume per model reached 200 mm$^3$. Mice were allocated into treatment cohorts using a stratified randomisation approach which aimed to evenly distribute initial tumour volume sizes among cohorts. We reasoned that this would be more suitable for models which had slower and more variable growth rates, one of the key limitations of protocol 1. In addition, this allowed tumour regression to be observed and the data could be analysed using criteria similar to RECIST.

Surgical resection was implemented on tumours following treatment. Where possible, this was performed on two mice per cohort. These tumours were harvested as *treated* samples, as in protocol 1, but by surgical resection instead of culling the mice. Since all patients enrolled in this clinical trial underwent surgery, we felt this more accurately recapitulated relapse which may follow treatment. Based on the pharmacokinetic and target engagement analysis, doses of paclitaxel, carboplatin
Figure 3.3.7.1 Optimised co-clinical trial design. a) Experimental design of proof-of-concept (protocol 1) and optimised (protocol 2) co-clinical trial strategies. b) PDTX models used in optimised co-clinical trial. c) Co-clinical trial cohorts.
and olaparib were increased to 0.14 mg (7 mg/kg), 0.8 mg (40 mg/kg) and 1 mg (50 mg/kg) respectively.

Using the optimised trial design, denoted as protocol 2, the untreated, avatar and alternative co-clinical trial arms were repeated using four PDTX models (figure 3.3.7.1b). The intention is to expand this to all eight PDTX models in the PARTNER co-clinical trial cohort, to reflect the diversity of drug responses observed in the clinic.

This data was first analysed using the same criteria as protocol 1 (figure 3.3.7.2): tumour volume midway through treatment, (2 cycles, 42 days), at the end of treatment (77 days) and AUC of the growth curve. We observed tumour regression for some models and thus not all tumour growth curves were exponential. Consequently, we omitted the analysis of the regression coefficient.

As with protocol 1, we observed significant differences in the tumour volumes (mid and after treatment) and AUC between treated and avatar cohorts of model 1022; clinically, this patient received pCR. However, we also observed significant differences between untreated and avatar cohorts across all parameters for model 1221 and in tumour volume at the end of treatment for model 1040; these patients did not receive pCR. While 1221 was not used in protocol 1, the discrepancy between the two protocols for 1040 is likely due to the change in the administered dose. The inconsistency between the clinical and PDTX drug responses led us to adopt a new analytical approach.

Figure 3.3.7.3 displays the results of the avatar cohorts from the co-clinical trial for all models tested using protocol 2. A PDTX response criteria was implemented, adapted from RECIST (figure 3.3.7.3a). A clinical response of pCR corresponded to a PDTX complete response. A clinical non-pCR (incorporating residual cancer burden scores of RCB 1-3) was reflected in PDTX responses of partial response, stable disease or progressive disease.
**Figure 3.3.7.2 PDTX responses in an optimised co-clinical trial.** a) Growth curves of mice untreated (black) and treated with the same treatment schedule as the matched patient (red for CT, green for CTO) for 11 weeks (77 days) (b-d) Bar charts showing tumour volume midway through treatment (42 days, (b)), at the end of treatment (77 days (c)), and AUC of tumour growth curves over the course of treatment (77 days). Bar charts display mean with error bars depicting standard deviation. p values calculated using unpaired Welch’s t-test (unequal variance) comparing untreated to treated mice. Significance: * <0.05, ** <0.01, *** <0.001, **** <0.0001.
Percentage change in tumour volume (ΔVol) from baseline (V₀) was computed for each mouse at each time point (t) using the formula: ΔVol = ((Vₜ – V₀) / V₀) X 100 as described previously (183). Figure 3.3.7.3b shows the average percentage change in tumour volume at the end of treatment for the avatar cohorts of each PDTX model. Figure 3.3.7.3c illustrates the PDTX drug responses of individual mice in each avatar cohort; grey bars reflect mice that either started treatment when the tumour was not measurable (models 1022, 1040, 1221) or were sacrificed before the end of treatment (1141). Figure 3.3.7.3d shows the ΔVol over time for models with complete response (left – 1022) and progressive disease (right - 1141). Figure 3.3.7.3e depicts a waterfall plot of individual mice within each cohort. Crucially, all mice from patients who received non-pCR had PDTX responses classified as progressive disease, stable disease or partial response, and all mice from patients who had a pCR exhibited PDTX drug responses of complete response.

While these data are highly encouraging and support the use of PDTX models as avatars, there are a number of limitations which must not be overlooked. First, these experiments were limited to a small number of PDTX models. While this is inevitable, given the engraftment rate, the quality control measures which are essential before embarking on the trials and the scale of the experiments, there is no doubt that more models are needed before it can be concluded that PDTXs fully recapitulate the clinical drug responses observed in the matched patient. To that end, using the optimised trial design, only one model, 1022, received a clinical pCR and so it is essential to test this in a larger sample size. One of the key limitations of trials using protocol 1 was the lack of concordance in slow growing models, including 1053. As such, it is critical to perform the optimised protocol 2 using this model.

Another limitation is the variability between biological replicates within a given cohort, which prompts the question of the appropriate sample size within a treatment cohort. For some models (for example 1040 and 1221) we observed different drug responses between replicates (depicted in figure 3.3.7.3c). Although
Figure 3.3.7.3 PDTX responses in an optimised co-clinical trial, using a drug response classification system adapted from RECIST a) PDTX response classification b) Average percentage change in tumour volume (ΔVol) at the end of treatment from baseline. Bar chart shows mean +/- standard deviation. c) PDTX responses of individual mice in each cohort. d) Examples of complete response (left) and progressive disease (right). e) Waterfall plot of ΔVol of individual mice in the co-clinical trial. Bar colour indicates clinical drug response.
all mice were shown to be concordant with the patient, it is not unreasonable to deliberate that some mice may fall within unmatched response criteria. It is therefore essential that we have enough replicates within a given cohort to ascertain a PDTX drug response with high confidence, from which we can determine the concordance with that of the matched patient. It is also possible, that the number of replicates required may be patient-dependent; some models may exhibit higher variability between replicates and thus require more statistical power. This could be determined during the engraftment and propagation stages.

We implemented a PDTX response criteria based on RECIST which requires a measurable initial tumour volume. Although the PDTX tumour growth rate is very consistent between biological replicates, we often see differences in the growth lag time due to technical factors, such as different sizes of implanted tumour fragments. In protocol 2, mice were assigned to treatment cohorts using a stratified randomisation approach, which aimed to evenly distribute initial tumour volumes among cohorts. While we felt this was the most appropriate method, it sometimes meant that within a given cohort some mice were not at a measurable volume when treatment commenced (illustrated by the grey panels in figure 3.3.7.3c). As such, these mice could not be assigned a PDTX drug response classification. Perhaps the most appropriate way to assess PDTX drug responses is a combination between the analyses used in protocols 1 and 2, and a suitable analytical method should be adapted when data is available from more models.

While acknowledging the limitations of this study, we believe this represents a novel and elegant demonstration of the clinical power of PDTX models. Aligned with an ongoing clinical trial in a difficult-to-treat patient cohort, we demonstrated remarkable concordance in drug responses, particularly in highly proliferative tumours. We have developed an experimental framework (including robust quality control measures) which could be extrapolated to wider patient cohorts in breast cancer, among other cancer types. In addition, testing the alternative clinical trial arm to that which the patient received and other treatment strategies offers the
potential to leverage this platform as an anticipatory clinical tool, to prospectively assess the most efficacious treatments for patients.

3.4 Conclusions

Here we present findings on the development of a PDTX cohort from tumour samples of patients enrolled in an ongoing neoadjuvant clinical trial. These models were subject to robust quality control measures and molecular characterisation. Thorough genomic and histological analyses revealed high preservation of the key features of the tumours from the patient to the PDTX and through multiple rounds of passaging. We then developed and optimised a co-clinical trial design which can be used to explore the extent to which PDTX models recapitulate clinical drug responses to standard of care chemotherapy and a targeted agent on a matched patient-PDTX basis, using mirrored treatment schedules. We implemented a PDTX drug response classification based on RECIST criteria and found concordance with clinical drug responses in 4/4 models tested. Here we present a preclinical experimental framework which has profound implications for the future use of PDTX models in clinical practice and anticipatory cancer medicine.
Chapter 4: Modelling drug responses to DDR compounds ex vivo

4.1 Introduction

Patient-derived tumour xenografts (PDTXs) are widely considered to be the preclinical model which bear the greatest resemblance to the tumours from which they originated at molecular and histological levels. As such, they have strong utility in translational and basic science alike, aiding in pharmacological studies and providing insights into patterns of tumour evolution.

However, animal studies are expensive and labour-intensive to perform, especially on a large scale. Therefore, there is incentive to develop tools which allow PDTXs to be leveraged to perform larger scale experiments ex vivo, while retaining the features which make them advantageous over classical model systems e.g. cell lines.

Our group previously pioneered the use of dissociated PDTX cells (namely patient-derived tumour cells, PDTCs) for ex vivo high-throughput drug screening experiments, adopting a similar approach to that described in cell lines (133) and organoids (252). Using short-term cultures, these were found to retain the molecular
features of the PDTX tumour, measured using gene expression, pathway activation, single nucleotide variants (SNVs), copy number aberrations (CNA) and DNA methylation. In addition, the vast majority (82.5%) of drug responses observed \textit{ex vivo} were recapitulated when tested \textit{in vivo} (166).

As discussed previously, we have established a co-clinical trial platform, in which tumour fragments are obtained from patients enrolled in an ongoing clinical trial, PARTNER, prior to the commencement of treatment. These were engrafted into mice as PDTX models, horizontally expanded to create a living biobank of replicate tumours and then treated \textit{in vivo} with different treatment schedules. Treatment schedules included both clinical trial arms (chemotherapy with and without olaparib, CT and CTO respectively), olaparib and AZD1775 as monotherapies and in combination (figure 4.1). As described before, olaparib and AZD1775 are inhibitors of PARP and WEE1 respectively. Biological replicates within a given cohort were collected at two times points. Two mice per cohort were collected immediately after treatment (denoted as \textit{treated} samples) and three were left to continue growing off-treatment to size limits (denoted as \textit{post-treated}). These act as model systems for residual disease following neoadjuvant treatment and relapsed disease respectively and allow one to explore the drug response dynamics both on treatment and when treatment is withdrawn.

Leveraging a robust high-throughput drug screening method would dramatically expand the capabilities of the co-clinical trial platform, for both personalised cancer therapy and exploratory science. First, on a matched patient-PDTX basis, \textit{ex vivo} drug screening would increase the number of drugs a given patient’s tumour receives. This could be either in the treatment-naïve setting (alongside \textit{in vivo} drug testing) or following one or multiple rounds of \textit{in vivo} treatment. Regarding the latter, the \textit{in vivo} treated or post-treated samples could be used to aid in clinical decision-making for patients who have residual disease at surgery and/or relapsed disease respectively (figure 4.1). To use the PDTXs in this way, there needs to be a thorough examination of whether previous lines of treatment affect drug responses.
Figure 4.1: Development of an integrated experimental framework. Encompassing both in vivo and ex vivo treatment, this experimental framework enables one to explore the impact of alternative treatment schedules on drug sensitivity profiles.
To this end, it remains elusive whether PDTXs generated from early resections can predict clinical drug responses to second lines of therapy (and beyond) or whether tumours need to be exposed to parallel treatment schedules in the mouse. Izumchenko et al. eluded to idea that PDTXs retain their therapeutic accuracy over time but did not test this in breast cancer (188). Here we are able to explore whether in vivo treatment impacts the drug sensitivity profiles of PDTXs.

Second, the high-throughput drug screening platform could aid in the acceleration of drug discovery. Requiring relatively few cells, the drug screening platform can be used either to screen multiple models for a limited number of compounds or a few PDTX models with specific characteristics (for example known clinical responses or molecular features) for a large number of compounds. In addition, combination experiments can reveal novel combination therapies which could be efficacious for patients.

The high-throughput drug screening platform can also be used to gain mechanistic insights into the effects of parallel in vivo drug treatment. While genomics and transcriptomic analyses provide a magnitude of information about the features of a given tumour at a given time point, the evolution of that tumour is governed by its phenotype. Clinically, an understanding of this is imperative to facilitate the treatment and ultimate eradication of the cancer. As such, we reasoned that exploring the functional characteristics of a tumour following in vivo treatment could provide mechanistic information about how that given treatment acts to evolve the tumour. Indeed, the study of both treated and post-treated samples enable one to investigate the dynamics of the drug response, drug resistance mechanisms and whether these are transient or reversible phenomena after a drug holiday. If this is successful, we also reason that the high-throughput drug screening platform could be used to design next-generation in vivo studies to test the efficacy of sequential therapies based on preclinical data from models which faithfully recapitulate the complexity of breast cancer.
Patients enrolled on the PARTNER clinical trial present with triple negative breast cancer (TNBC) and/or germline BRCA1/2 mutations. TNBC patients have limited therapeutic options and do not benefit from other targeted therapies approved for use in breast cancer e.g. ER- or HER2-targeting compounds. However, these patients often harbour alterations in the DNA damage response (DDR) and a number of DDR-targeting compounds are entering preclinical and clinical drug development. Olaparib, a PARP inhibitor, is the test compound of the PARTNER clinical trial and has been found to have a synthetically lethal relationship with BRCA1/2, due to the roles of BRCA1/2 in homologous recombination (HR). Other preclinical compounds in the drug development process include inhibitors of WEE1, ATR, ATM, DNA-PK and CHK1/2, which all have roles in the DDR, replication stress response (RSR) and maintaining the delicate balance of genomic instability and cell viability in a tumour cell. Here we aimed to develop an ex vivo platform suitable to test these novel compounds in PDTX models and leverage this platform to characterise drug responses to DDR compounds following in vivo treatment.

4.2 Aims

1. To develop and optimise an ex vivo high-throughput drug screening platform suitable for DDR-targeting compounds
2. To compare drug responses between TNBC PDTX models within the PARTNER co-clinical trial cohort
3. To study the impact of parallel in vivo treatment on the drug sensitivity profiles of PDTX models
4. To leverage the platform to design new therapeutic strategies.
4.3 Results and discussion

4.3.1 Development of a drug screening platform for DDR-targeting compounds

The ex vivo high-throughput drug screening workflow was adapted from Bruna and Rueda et al. (2016) (166) and is depicted in figure 4.3.1.1a. PDTX tumours were grown in vivo, divided into tumour fragments and cryopreserved. To generate PDTCs, these were dissociated using a combination of enzymatic and mechanical dissociation procedures and plated into a 384-well plate (figure 4.3.1.1b). After 24 hours these were dosed with drug compounds. Cell viability was measured using the commercial reagent CellTiter-Glo® 3D (CTG) at day 0 (day of dosing) and then a fixed point afterwards (3-14 days).

A cell plating and dosing layout (figures 4.3.1.1b, d) was optimised whereby media-only wells act as positive controls (no cells, representative of 100% response) and solvent-only wells (usually DMSO) as negative controls (basal growth, representative of 0% response). A dosing layout was optimised to exclude the outer edges, include a 7-dose pattern (spanning 0.01-10 μM in half log_{10} increments) and to include three technical replicates of each drug and dose within a given plate. This plate layout was suitable for either one sample (plated rows C-N) with 12 drugs or two samples (plated rows C-H and I-N) with six drugs. Each technical replicate occupied a different position within the plate which minimised technical variability.

For the analysis, drug responses were normalised to positive and negative controls within a given plate to calculate a value of response (%) for each drug and each concentration. These were plotted on a dose response curve (figure 4.3.1.1c) and drug responses were reported as area under the curve (AUC) or half maximal inhibitory concentration (IC50), representing the estimated concentration at which drug response was 50%.
Figure 4.3.1.1: The development of an *ex vivo* high-throughput drug screening platform to model drug responses. a) Schematic of *ex vivo* drug screening experimental procedures. b) Plate layout of dissociated PDTCs. c) Measurements of drug response using dose response curves. d) Dosing strategy of PDTCs.
The first optimisation step was determining the time point at which drug responses should be measured for DDR-targeting compounds. The protocol published by our group described the measurement of drug response seven days after dosing (166). However, pilot experiments (depicted in figure 4.3.1.2a-c) revealed that at this time point olaparib showed little response in PDTX models which we knew responded to the same compound \textit{in vivo} (e.g. model 1040 depicted in figure 4.3.1.2c). Since the response for 1040 did not reach 50\% over any of the concentrations tested, it was not possible to calculate the IC50. However, when we measured response at day 14, the dose response curve increased to such a level which enabled IC50 estimation. In addition, the drug responses for other compounds also increased dramatically between seven and 14 days. We reasoned that since PDTX models have different \textit{ex vivo} growth rates and DDR-targeting compounds rely on active cell cycling and the accumulation of DNA damage, a longer time frame was appropriate.

The next optimisation step was the concentration at which cells were plated. While it would be beneficial to perform a thorough investigation of the growth dynamics of the PDTX models, and indeed this is ongoing in our group, we were hesitant to optimise the experimental conditions on a model-specific basis. We reasoned that the possible clinical utility of this platform relied on ease of use and should be suitable for a large number of models which have dramatically different growth dynamics both \textit{in vivo} and \textit{ex vivo}. As such, we performed a small study to investigate the growth dynamics of model 1022 over 14 days at four different concentrations (figure 4.3.1.2d).

It should be noted from here on in, \textit{ex vivo} growth is defined as net luminescence at day\((t)\) divided by net luminescence at day\((0)\). The commercial reagent CTG was used, which lyses cells and generates a luminescence signal proportional to the amount of ATP present. This is used to indicate the number of live cells in a culture. Net luminescence represents detected luminescence minus background (media-only wells). To assess exponential growth, \textit{ex vivo} growth was log\(_2\) transformed.
Figure 4.3.1.2: Optimisation of drug screening conditions for DDR-targeting compounds. AUC (a), IC50 (b) and example dose response curves (c) of PDTCs incubated for seven and 14 days with drug compounds. Error bars show 95% confidence intervals. d) Growth dynamics of PAR1022 PDTCs seeded at different cell concentrations, illustrated by log2-transformation of *ex vivo* growth (net luminescence, / net luminescence). Luminescence was generated by CTG reagents, and was proportional to the amount of ATP, indicating live cells. Plot shows mean value and error bars show standard deviation.
At all concentrations tested, the PDTCs had positive growth at day seven and 14. While at day 14 the growth started to plateau, this occurred in all concentrations tested. From this we reasoned that, where possible, within a given experiment, it was desirable to maintain a consistent cell number but that within the range tested (1-4 X 10^6 cells/mL) the plating concentration did not dramatically affect the growth rate. Indeed, since the growth dynamics are likely to be a biological feature, both in treatment naïve tumours and following in vivo treatment, this will be an important aspect of data interpretation.

We next performed quality control steps to test the reproducibility of drug responses. We observed a high correlation between technical replicates within a given plate (figure 4.3.1.3a). We then examined the degree to which drug responses were conserved within each model, presented as AUC and IC50 (figure 4.3.1.3b, c). We performed drug screens using different spatial regions of a PDTX tumour from the same mouse in independent experiments and found a high correlation in AUC and IC50 values. In addition, we revealed a high correlation between AUC and IC50 values from PDTX tumours from different mice tested in independent experiments, as well as mice from different passages. Taken together, this indicates that the drug responses are highly conserved between technical and biological replicates and between in vivo passages, in line with the data previously published by our group (166).
Figure 4.3.1.3: Drug sensitivities are highly conserved between independent experiments, mice within a given passage and between passages. a) Correlation plots of calculated response of replicate wells within a given experiment for PAR1006 (left), PAR1040 (middle), PAR1022 (right). b-c) Correlation plots to show calculated AUC (b) and IC50 (c) values. Left – different spatial regions from the same mouse tested in independent experiments. Middle - different mice within the same passage tested in independent experiments. Right – mice from different passages tested in independent experiments. r value indicates Pearson correlation.
4.3.2 Comparing drug responses between TNBC PDTXs

Following optimisation of the experimental protocol, we performed a DDR-focussed high-throughput drug screen on treatment naïve PDTX tumours from the PARTNER co-clinical trial cohort. The rationale for this was twofold. First, we wanted to compare growth dynamics and drug responses between PDTX models with similar clinical characteristics. Second, we sought to optimise the analysis workflow for the drug screen for these compounds.

Three models were selected which were found to have a *BRCAness* phenotype: 1006, 1040, 1022. As discussed previously, 1006 and 1040 have germline *BRCA1* alterations and 1022 was found to have high levels of signature 3, HRD score and HRDetect scores, indicating homologous recombination deficiency (HRD). We reasoned that these models would have measurable responses to DDR-targeting compounds and so could be used to optimise the analysis workflow. In addition, they had the highest *in vivo* growth rates of the PDTX models in the co-clinical trial cohort, so we predicted they would perform well in the *ex vivo* experiments.

A drug panel was developed (table 4.3.2), composed of three chemotherapy agents (including those tested in the PARTNER clinical trial), two PARP inhibitors (olaparib and BMN-673) and other DDR-targeting compounds. The panel also included inhibitors of mammalian target of rapamycin (mTOR) and bromodomain and extra-terminal motif (BET) proteins. The PI3K/AKT/mTOR pathway is thought to be involved in the development of chemoresistance in TNBC and offer potential avenues for therapeutic targeting (253). JQ1 (BET inhibitor) is a candidate compound for TNBC (254) and has recently been found to have a synergistic relationship with several DNA-damaging agents, including paclitaxel (255). In addition, deletion of DNA-repair genes (e.g. *BRAC1* and *BRCA2*) was found to sensitise TNBC to JQ1 and BET inhibition suppresses DNA repair, sensitising HR-proficient tumours to PARP inhibitors (256). As such, these compounds were considered relevant to the study of drug responses in TNBC.
Table 4.3.2: DDR targeted drug panel.

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<tr>
<td>Paclitaxel</td>
<td>Chemotherapy (taxane)</td>
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<tr>
<td>Carboplatin</td>
<td>Chemotherapy (platinum)</td>
</tr>
<tr>
<td>Epirubicin</td>
<td>Chemotherapy (anthracycline)</td>
</tr>
<tr>
<td>Olaparib</td>
<td>PARP inhibitor</td>
</tr>
<tr>
<td>BMN-673</td>
<td>PARP inhibitor</td>
</tr>
<tr>
<td>AZD1775</td>
<td>WEE1 inhibitor</td>
</tr>
<tr>
<td>AZD7648</td>
<td>DNA-PK inhibitor</td>
</tr>
<tr>
<td>AZD6738</td>
<td>ATR inhibitor</td>
</tr>
<tr>
<td>AZD0156</td>
<td>ATM inhibitor</td>
</tr>
<tr>
<td>AZD7762</td>
<td>CHEK1/2 inhibitor</td>
</tr>
<tr>
<td>AZD8055</td>
<td>mTOR1/2 inhibitor</td>
</tr>
<tr>
<td>JQ1</td>
<td>BET inhibitor</td>
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We performed a DDR-targeted high-throughput drug screen as described above, using a 14-day endpoint in models 1006, 1040, 1022. We first examined the \textit{ex vivo} growth of PDTCs after 14 days. While normalised to the same seeding concentration, we observed that the three PDTX models had dramatically different luminescence values at day 0 (24 hours after plating) (figure 4.3.2.1a). The luminescent signal did not correlate with the percentage viability at plating, as determined by Vi-Cell (figure 4.3.2.1d) and so this discrepancy is likely to be a model-specific feature (e.g. different metabolic rates, sensitivity to lysis) or Vi-CELL technical limitations.

For all three models, the net luminescence values increased over 14 days, indicating an increase in the number of viable cells (figure 4.3.2.1c). To further delineate this, \textit{ex vivo} growth was calculated as described before (net luminescence\textsubscript{t} / net luminescence\textsubscript{0}) and this was log\textsubscript{2} transformed (figure 4.3.2.1b). Interestingly, we observed differences in \textit{ex vivo} growth between the three models over 14 days; 1022 was the most highly proliferative and 1006 was the least. We next assessed whether \textit{ex vivo} growth dynamics correlated with that observed \textit{in vivo}. Figure 4.3.2.1e shows the \textit{in vivo} growth curves (raw tumour volumes and following log\textsubscript{2} transformation). Using the regression coefficient (slope) of the log\textsubscript{2}-transformed graph to indicate growth rate, we observed that model 1022 had the highest growth \textit{in vivo} and 1006 had the lowest, in line with our \textit{ex vivo} data. While thorough analyses of the growth dynamics of these models would be both interesting and important to understand the biology of the tumours, this is a first indication that \textit{ex vivo} growth may correlate to some degree with that observed \textit{in vivo}.

We next compared the drug responses between the PDTX models by calculating AUC and IC\textsubscript{50} as previously described (166). The data had low variability between replicates and, with the exception of epirubicin and BMN-673, the dose response curves were within range to calculate IC\textsubscript{50} values. Overall the models responded very similarly to the compounds, as expected given the clinical characteristics. However, some subtle differences could be identified (figure 4.3.2.2). We observed
**Figure 4.3.2.1.** PDTXs and PDTCs exhibit model-specific growth dynamics. a-d) Growth dynamics of PDTCs *ex vivo*. e-f) Growth dynamics of PDTXs *in vivo*. a) Day 0 luminescence values of PDTCs, indicating number of viable cells 24 hours after plating. b) Log2-transformation of *ex vivo* growth (net luminescence / net luminescence0) at day 14. c) Net luminescence values at day 0 and day 14. d) Viability of PDTCs at the time of plating determined by Vi-CELL. e) *In vivo* growth curve (top) and log2-transformation of tumour volume (bottom). e) Regression coefficient (slope) of log2(tumour volume) illustrating *in vivo* PDTX growth rate. Bar charts show mean and error bars present standard deviation.
Figure 4.3.2.2: High-throughput drug screening platform can be used to compare drug responses between PTDX models. AUC (a) and IC50 (b) values of each PTDX model for the drugs tested. Error bars show 95% confidence intervals. c) Table showing drug targets of the compounds used in the drug screen.
that model 1040 has a significantly higher AUC for BMN-673 (PARP inhibitor) and AZD0156 (ATM inhibitor) than the other models. Model 1006 has significantly lower AUC for AZD7762 (CHK1/2 inhibitor) than the other models and 1040 has a higher AUC for AZD8055 (mTOR inhibitor) than 1022. Interestingly, though many of these compounds rely on the cell cycle, the AUC and IC50 values did not correlate with the growth; 1022 which had the highest growth did not consistently have the highest or lowest AUC values.

While the AUC and IC50 can provide insights into drug responses, we observed cases in which compounds produced the same AUC values but dramatically different shapes of the dose response curves. Our group previously developed eight theoretical curves based on the percentage of dead cells at five dosage points, into which experimental dose response curves can be classified. Here, this framework was adapted for seven doses, the 14-day time point and the drug panel (figure 4.3.2.3a, b). Nine theoretical curves were developed and revealed additional differences in drug responses to DDR compounds (figure 4.3.2.3c, d). The AUC values of BMN-673 and paclitaxel were very similar for model 1040 but the dose response curves were classified as different shapes. A similar scenario was observed in model 1022 for the ATM and ATR inhibitors, AZD0156 and AZD6738 respectively.

This analytical framework also revealed additional differences between PDTX models. Figure 4.3.2.4 illustrates examples of heterogeneous drug responses observed in our system. We noted cases in which drug responses had similar AUC values but different shaped curves (figure 4.3.2.4b), different AUC values but the same shaped curve (figure 4.3.2.4c) and differences in both AUC and dose response curves between PDTX models (figure 4.3.2.4d). Indeed, we also observed cases in which drug responses were very similar between models (figure 4.3.2.4a).

Taken together, these data demonstrate that the high-throughput drug screening platform was suitable to assess PDTX drug responses to DDR-targeting compounds.
Figure 4.3.2.3. Analysis of dose response curve shapes can offer an additional layer of information. Theoretical shapes (a) and examples of real dose response curves which are classified into the corresponding shapes (b). c) Examples of cases in which different drugs produce the same AUC value but have different dose response curves. Error bars and shaded areas show 95% confidence intervals. d) Shapes of the dose response curves for each of the drugs and PDTX models tested.
Figure 4.3.2.4: The high-throughput drug screening platform reveals differential drug response profiles of PDTX models with similar clinical characteristics. We observe examples drugs in which models have the same AUC and dose response shapes (a), same AUC but different shapes (b), different AUC but the same shape (c) and different AUC and shape (d). Error bars ad shaded areas show 95% confidence intervals.
ex vivo. While it would have been advantageous to test a PDTX model with different clinical characteristics, we were able to reveal subtle differences in drug response profiles between models with similar genomic and clinical features. This exemplifies the power of using an ex vivo drug screening approach to tease out drug response dynamics both between independent tumours and between compounds.

4.3.3 Drug response profiles following in vivo treatment

We next aimed to leverage the ex vivo drug screening platform to explore the impact of in vivo treatment on the drug response profiles of PDTX models (figure 4.3.3.1). This was achieved using four models (1006, 1040, 1022, 1141). As described previously, cohorts of PDTXs were treated in vivo with multiple drugs in parallel as part of a co-clinical trial. Tumours were harvested immediately following treatment (denoted as treated samples) and following drug withdrawal (post-treated). For the purpose of this experiment, post-treated samples were analysed for a number of reasons.

First, we were interested in whether a short course of treatment (77 days) could impact the long-term functional characteristics of the tumour i.e. persistent or permanent effects. We hypothesised that any functional changes observed immediately after treatment may result from cell cycle changes or other features which, while intriguing, may not affect the long-term evolutionary trajectory of the tumour. Any permanent changes (e.g. due to the emergence of mutations) should be identifiable even after a drug holiday.

Second, TNBC patients have higher frequencies of pathological complete response (pCR) at surgery following neoadjuvant chemotherapy than other breast cancer subtypes (51). However, if these patients relapse they have limited therapeutic options. Since the post-treated samples model relapsed disease, we felt there was a benefit in exploring drug responses to possible next-line therapies. Furthermore, a likely limitation in using PDTXs in routine clinical medicine is the timeframe
Modelling drug responses to DDR compounds *ex vivo*

Figure 4.3.3.1: Experimental design using the high-throughput drug screen platform to explore the impact of *in vivo* treatment on drug response profiles. Four PDTX models (PAR1006, PAR1040, PAR1022, PAR1141) were horizontally expanded and treated *in vivo* with different treatment schedules. Following treatment, tumours grew to size limits off-treatment and harvested as *post-treated* samples. One mouse per cohort was dissociated and used for drug screen.
required to generate and test PDTX models. As such, their greatest utility is likely to be in relapsed disease.

Third, the majority of the *in vivo* treatments were efficacious and so there was limited material immediately after treatment. As such, it was decided to reserve this material for a more target approach to answer specific biological questions. In addition, the use of post-treated samples meant that the tumours were size matched with the untreated cohort. We hypothesised that the tumour volume when harvested may affect the functional characteristics (e.g. growth dynamics, levels of necrosis). By size-matching the cohorts, we aimed to reduce this variability.

It should be noted that for model 1006, the *in vivo* trial was divided into two parts. Passage 1 (x1) was treated with the following treatments: CTO, olaparib, AZD1775, olaparib and AZD1775 combination. Passage 2 (x2) was treated with: CT or CTO. For both, untreated controls were included. For this *ex vivo* experiment, both passages 1 and 2 were analysed, along with their matched untreated controls.

We first explored the *ex vivo* growth dynamics. Figure 4.3.3.2a depicts the log₂ transformation of *ex vivo* growth at day 14 for each sample. As described previously, *ex vivo* growth was defined as net luminescence at day(t) divided by net luminescence at day(0). Luminescence was generated using the reagent CTG and indicated the number of viable cells in a culture.

In line with our previous experiment, the untreated samples from 1040 and 1022 proliferated over 14 days; again, *ex vivo* growth was highest in 1022. However, for model 1006 we observed a small decrease in luminescence over 14 days. This data provides just a snapshot into the numbers of viable PDTCs at a fixed time point and so we cannot delineate the temporal dynamics (i.e. whether PDTCs proliferated and then died, or their whether growth was static). Experiments are ongoing in our group to explore alternative reagents to CTG. In particular it would be preferential to use reagents which can be measured over multiple time points (rather than an
Figure 4.3.3.2: *In vivo* treatment causes both model- and treatment-specific changes in PDTC growth dynamics. a) Log$_2$-transformation of *ex vivo* growth (net luminescence, $\ell_n$) after 14 days. Each plot displays an *in vivo* trial. b) Log$_2$(*ex vivo* growth) of untreated and Olap+AZD1775 cohorts for each model. c) Log$_2$(*ex vivo* growth) of untreated and CT cohorts for each model. All bar charts show mean +/- standard deviation.
endpoint assay). This would enable deep characterisation of the PDTC growth dynamics. Although model 1141 was not tested previously, we observed positive growth over 14 days.

Interestingly, we observed differences in *ex vivo* growth between PDTX models which had been treated *in vivo*. Although we observed both patient- and drug-specific changes in growth dynamics, we were able to identify some patterns consistent across multiple models (figure 4.3.3.2b-c). We observed in all four models that *in vivo* treatment with the olaparib and AZD1775 (WEE1 inhibitor) combination had a negative impact on growth. This preliminary data suggests that short term treatment may affect a tumour’s long-term growth trajectory. What is particularly striking is that for 3/4 models, we observed negative growth over 14 days (for 1022, this was only sample for which luminescence declined). As described previously, these tumour samples were harvested from the mouse at the *post-treated* time point i.e. at size limits, following a period of drug withdrawal. During this time off treatment, all tumours resumed proliferation, demonstrating that (at least *in vivo*) they are capable of growth. The data here reveals a novel and interesting finding. It is possible that treatment regimens impact the ability of a tumour to proliferate in an *ex vivo* system and may reveal changes in its functional characteristics. While these findings need to be further explored, they pose interesting questions about the effect of short-term treatment on long-term growth characteristics of breast cancers, which could be targeted therapeutically.

Indeed, we also observed the opposite phenomenon, in which *in vivo* treatment with CT had a positive impact on the growth dynamics of PDTCs (figure 4.3.3.2c). Again, this needs to be explored further but could suggest that chemotherapy treatment enriches for a more aggressive, proliferative phenotype. An interesting line of analysis would be to compare the *in vivo* growth rate of the CT cohort after drug withdrawal; we hypothesise that the growth rate would be higher than in the untreated counterpart. While acknowledging the limitations, these data demonstrate
Figure 4.3.3.3: The high-throughput drug screening platform reveals changes in drug response profiles following in vivo treatment. Ex vivo drug responses from mice from PAR1040 (a,c,e) and PAR1022 (b,d,f) enrolled in different in vivo cohorts, presented as AUC (a-b), IC50 (c-d) and shapes of the growth curve (e-f) as defined using theoretical curves. Error bars show 95% confidence intervals.
the potential utility of exploring effects of parallel drug treatment on a tumour’s functional characteristics.

We next investigated the *ex vivo* drug responses. For each model, multiple samples were analysed which had been treated *in vivo* with different treatments. AUC, IC50 and dose response curve shapes are displayed in figure 4.3.3.3 and supplementary figure 3. We observed low variability between technical replicates, indicated by the error bars (95% confidence intervals) and the dose response curves were mostly within range to calculate IC50.

We observed that in some cases, prior *in vivo* treatment did not change the AUC, IC50 or shape of the dose response curve, for example JQ1 in model 1040. In other cases, *in vivo* treatment had significant effects on the dose response curves, for example the response of model 1040 to BMN-673. In this model, *in vivo* treatment with CT significantly increased sensitivity to BMN-673, depicted by increased AUC and decreased IC50 values. Conversely, *in vivo* treatment with olaparib decreased sensitivity to BMN-673. The latter finding was observed in all three *in vivo* cohorts treated with olaparib: CTO, olaparib monotherapy, olaparib and AZD1775 combination. This is perhaps unsurprising, considering BMN-673 is also a PARP inhibitor. It stands to reason that the cells which grew following *in vivo* treatment would have decreased sensitivity to the same class of compound. This was a reassuring finding which exemplifies the robustness of the platform.

To further explore the effects of *in vivo* treatments, the AUC change was calculated by subtracting the AUC of the treatment cohorts from the AUC of the untreated cohort for each drug and each model. These changes are depicted as a heatmap in figure 4.3.3.4. We observed significant changes which were both model- and drug-specific, indicating that the mechanisms by which tumours respond to a specific drug are likely be heterogeneous between patients.
Figure 4.3.3.4: The high-throughput drug screening platform reveals both model- and cohort-specific changes in drug sensitivity following in vivo treatment. AUC change is defined as the calculated AUC for mice enrolled in an in vivo treatment cohort and sampled at the post-treated (PT) time point minus AUC for mice enrolled in the in vivo untreated (UT) cohort. Only significant changes are shown in colour.
Figure 4.3.3.5: Functional changes caused by CTO treatment are conserved between PDTX passages. a) Experimental design of in vivo treatment of PAR1006 across two passages. b) Heatmap indicating AUC change (AUC of CTO post-treated sample minus untreated AUC) of PAR1006 passages 1 and 2. Dot indicates significant change. c) Examples of dose response curves of cases of significant concordance (BMN-673 and AZD7648), no significant change in either (AZD6748) and significance disagreement (Epirubicin) between passages. Shaded areas show 95% confidence intervals.
Before attempting to identify patterns in the data, we first assessed the degree to which drug response changes were preserved across parallel experiments. The purpose of this was to determine if these changes were a direct consequence of a specific drug treatment or simply biological variability between tumours in sister mice, independent of the treatment cohort. To test this in a robust way, we investigated two passages of model 1006, both treated \textit{in vivo} with CTO. We then compared drug responses to their untreated counterpart (figure 4.3.3.5a). Of the 12 drugs, we observed concordance in the direction of change in 75\% of cases (9/12 drugs), illustrating both increases and decreases in sensitivity (figure 4.3.3.5b, c). Of these, 4/12 showed statistical concordance (significant change in both or neither passages) and 5/12 without statistical concordance (one passage showed a significant change). Non-significant disagreement was observed for 2/12 drugs, for which the direction of change differed by passage but only for one was the change significant. 1/12 drugs showed statistical disagreement, whereby \textit{in vivo} treatment with CTO caused a significant increase and decrease in sensitivity to epirubicin in passages 1 and 2 respectively. Overall, these data suggest that that the changes in drug response are consistent between passages and reflect functional changes which occur as a consequence of \textit{in vivo} treatment.

We next sought to identify patterns of functional changes which were consistent between multiple models and could be validated to explore novel mechanisms of drug response. We observed that following \textit{in vivo} treatment with olaparib monotherapy, two models (1006 and 1040) showed significant decreases in sensitivity to multiple DDR-targeting compounds (figure 4.3.3.6a-c). This suggested a global reorganisation of the DDR. However, models 1022 and 1141 showed little or no changes in sensitivity to these drugs. This demonstrated heterogenous patterns of tumour evolution at the phenotypic level.

To investigate this further, we analysed the \textit{in vivo} drug responses of the four models to olaparib (figure 4.3.3.6d). As described previously, models 1006 and 1040 both harbour germline alterations in \textit{BRCA1} (homozygous copy number loss
Figure 4.3.3.6: Olaparib treatment in vivo causes model-specific changes in drug response. a) Heatmap showing AUC change (AUC of olaparib post-treated sample minus untreated) for DDR-targeting compounds. Dot indicates significant change. b) AUC change between untreated and olaparib post-treated samples. Error bars show 95% confidence intervals. c) Ex vivo dose response curves for AZD6738 (top panel) and AZD0156 (bottom panel), comparing untreated and olaparib post-treated tumours. Shaded areas show 95% confidence intervals. d) Growth curves of models PAR1006, PAR1040, PAR1022 and PAR1141 treated in vivo with olaparib. Dotted line indicates end of treatment. e) Table of drug names and targeted.

<table>
<thead>
<tr>
<th>Name</th>
<th>Target</th>
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<tbody>
<tr>
<td>AZD1775</td>
<td>WEE1</td>
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<tr>
<td>AZD7648</td>
<td>DNA-PK</td>
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<tr>
<td>AZD6738</td>
<td>ATR</td>
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<tr>
<td>AZD0156</td>
<td>ATM</td>
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<tr>
<td>AZD7762</td>
<td>CHK1/2</td>
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for 1006 and pathogenic mutation with LOH for 1040). Model 1022 also exhibits an HRD phenotype with high signature 3, HRD-LOH and HRDetect scores. In line with this, all three models responded to olaparib in vivo and to a similar degree. Model 1141 is HR proficient and as such did not respond to olaparib in vivo. This was a particularly interesting result because it demonstrated that although tumours may exhibit similar responses to a treatment strategy, the functional characteristics of the relapsed tumours can differ. Although limited by the number of models we tested, it was striking that both tumours for which we observed a DDR reorganisation had genomic alterations in BRCA1. As such, it is not unreasonable to hypothesise that this may be a contributing factor. Indeed, this could have important consequences for the future use of DDR-targeting compounds after olaparib treatment. These findings triggered a thorough investigation of the dynamics of drug response to olaparib, which is outlined in chapter 5.

We then investigated the effects of in vivo drug treatment with AZD1775 (WEE1 inhibitor) on subsequent sensitivity to the same compound (figure 4.3.3.7-8). We observed that in vivo treatment did not change the drug sensitivity of the post-treated samples in models 1006, 1040 and 1141 (figure 4.3.3.7a-b). In model 1022 the sensitivity was slightly but significantly increased compared to the untreated. This was an interesting finding because one would assume that tumour cells which regrew following drug treatment would have intrinsic or acquired features which conferred resistance. As such, we reasoned that tumours would have decreased sensitivity to same compound with which they were previously treated. The fact that we did not observe such resistance led us to explore this further.

We analysed the in vivo drug responses of these models to AZD1775 and indeed all models responded well, with dramatic growth inhibition (figure 4.3.3.7c). However, upon drug withdrawal, all tumours resumed proliferation, supporting the concept that viable cells remained upon therapeutic pressure. This led us to hypothesise that the features which enabled cells to survive on treatment may be reversible.
Figure 4.3.3.7: AZD1775 post-treated tumours have comparable AZD1775 drug responses to untreated tumours when tested *ex vivo*. a) AUC change (AUC of AZD1775 post-treated sample minus untreated) for *ex vivo* response to AZD1775. Error bars show 95% confidence intervals. b) *Ex vivo* dose response curves for AZD1775, comparing untreated and AZD1775 post-treated tumours. Shaded areas show 95% confidence intervals. c) Growth curves of models PAR1006, PAR1040, PAR1022 and PAR1141 treated *in vivo* with AZD1775. Dotted line indicates end of treatment.
Figure 4.3.3.8: Tumours harvested immediately after in vivo AZD1775 treatment exhibit decreased sensitivity to AZD1775 when tested ex vivo. a) Growth curves of model PAR1006 treated in vivo with AZD1775. Dotted line indicates end of treatment. Circle indicates the samples which were used for the ex vivo drug screens. b) AUC change (AUC of AZD1775 treated or post-treated sample, minus untreated) for ex vivo response to AZD1775. c) IC50 values of untreated, treated and post-treated samples for ex vivo response to AZD1775. Error bars show 95% confidence intervals. d) Ex vivo dose response curve of AZD1775 showing untreated, AZD1775 (T) and AZD1775 (PT) samples. Shaded areas show 95% confidence intervals.
To further investigate this, we tested the drug sensitivity of the treated sample (immediately after treatment) from model 1006. Figure 4.3.3.8a shows the *in vivo* growth curves of this model treated with AZD1775 and the specific samples used to test drug sensitivity are indicated. Interestingly, in the sample which was harvested immediately after treatment, we observed a significant decrease in sensitivity, as shown by the change in AUC and IC50 values (figure 4.3.3.8b, c). This suggests that, as expected, immediately after treatment tumours have some degree of resistance but when the drug is withdrawn, the drug sensitivity reverts back to the untreated state. The concept of a drug holiday effect for AZD1775 has previously been reported in leukaemia cell lines. After the generation of cell line strains resistant to AZD1775, cell regained sensitivity when cultured in drug-free media (257). Reversible drug tolerance is an intriguing line of thought with important clinical implications. While this extends beyond the scope of this thesis, it would be very interesting to perform a comprehensive analysis of the drug response dynamics to AZD1775.

### 4.3.4 Leveraging *ex vivo* drug screen data to design new treatment strategies

The aim of PARTNER clinical trial is to test the efficacy of the chemotherapy and olaparib combination therapy in the neoadjuvant setting. However, an outstanding biological question with great clinical relevance is the differential response and long-term evolutionary trajectories of tumours treated with combination versus sequential therapy. The *ex vivo* drug screen revealed a significant increase in sensitivity to PARP inhibitors (olaparib and BMN-673) in multiple models following *in vivo* treatment with CT (figure 4.3.4.1a, c). In addition, we observed differential sensitivities to chemotherapy drugs (paclitaxel and carboplatin) after treatment with olaparib in *vivo*, though this was model-specific (figure 4.3.4.1b, d).

There have been initial reports in the literature to suggest that the treatment sequence of chemotherapy and olaparib may have functional implications. Marques
Figure 4.3.4.1: In vivo treatment with chemotherapy affects the ex vivo responses to PARP inhibitors and vice versa a) AUC change (AUC of CT post-treated minus untreated) for BMN-673 and olaparib. b) AUC change (AUC of olaparib post-treated minus untreated) for paclitaxel and carboplatin. Error bars show 95% confidence intervals. c) Ex vivo dose response curves for olaparib and BMN-673, comparing untreated and CT post-treated tumours from PAR1040 and PAR1141. d) Ex vivo dose response curves for paclitaxel and carboplatin, comparing untreated and olaparib post-treated tumours for PAR1040 and PAR1006. Shaded areas show 95% confidence intervals. e) Log2 counts per million (log2 CPM) of PARP1 in PDTX tumours detected by RNA-sequencing. Statistical significance determined using a Welch’s t-test not assuming equal variance. ** p=0.0021
et al. screened 313 patients with ovarian cancer undergoing neoadjuvant chemotherapy and evaluated PARP1 protein expression by IHC and western blot. Although 60% of patients did not express PARP1, the authors revealed a decrease in intratumoural PARP1 following chemotherapy. This was shown using both cohort analysis (chemo-naïve vs post-chemotherapy patients) and in matched samples before and after treatment (258). Crucially, these patients received carboplatin and paclitaxel, which mirrored the PARTNER clinical and co-clinical trials. The same group then published in vitro findings which demonstrated that exposure to PARP inhibitors prior to chemotherapy sensitised cell lines to lower doses of chemotherapy. This suggested that pre-treatment with PARP inhibitors followed by chemotherapy was more efficient at growth inhibition and the induction of apoptosis than vice versa (259).

While the authors acknowledged the limitations of their study, the concepts described were thought-provoking and were rationale to further explore this in our model system. We performed bulk RNA-sequencing analysis on the PDTX samples treated with CT in vivo. We did not observe a decrease in levels of PARP1 (figure 4.3.4.1e); indeed, for model 1006 we observed a significant increase in the post-treated samples compared to untreated. This suggested that the mechanisms in our system were distinct from that those described in the literature.

Expanding on this preliminary data, we designed an in vivo preclinical trial to test the efficacy of sequential therapy (figure 4.3.4.2a). This trial design encompasses two stages. First, mice will be treated with CT or olaparib in vivo using clinically-relevant doses and treatment schedules to match the co-clinical trial design (denoted as first-line therapy). As described previously, tumour samples will be cryopreserved immediately after treatment (treated) and at size limits (post-treated). These cryopreserved samples will then be re-implanted into new mice.

Denoted as second-line, these mice will be used test the efficacy of CT and olaparib using the same doses and treatment schedule as in the first instance. This will
Figure 4.3.4.2: Experimental design of an in vivo trial to explore the efficacy of sequential therapy for chemotherapy and olaparib. a) In vivo trial design. b) First-line drug responses of PAR1040 to chemotherapy (CT) and olaparib. c) Tumour volumes at the end of treatment. Bar chart shows mean +/- standard deviation. p value calculated using unpaired t test. ** p=0.0017
explore whether pre-treatment affects the efficacy to second-line therapy. In addition, by testing the treated samples, we hope to explore the effect of a drug holiday on subsequent sensitivity to the same compound.

This preclinical trial is currently ongoing using model 1040 and first-line treatment was performed as part of the co-clinical trial described in chapter 3. This model was selected for a number of reasons. Following first-line chemotherapy treatment, we observed significant increases in *ex vivo* sensitivity to both PARP inhibitors (BMN-673 and olaparib). We also observed a significant decrease in sensitivity to paclitaxel following *in vivo* treatment with olaparib. While these data oppose the published theories, we were interested in validating them *in vivo*.

Second, this tumour did not respond to chemotherapy as a first-line treatment (figure 4.3.4.2b, c). As such, it was deemed to be an appropriate model to test whether pre-treatment with olaparib is effective at sensitising the tumour. As described previously, although more TNBC patients respond to neoadjuvant chemotherapy than other subtypes, those that do not respond have limited therapeutic options and, for these patients, there is unmet clinical need. Indeed, this was the case for the patient from whom this model was derived. After receiving non-pCR to neoadjuvant chemotherapy, the patient received olaparib in the adjuvant setting, to which she responded for some time and then progressed. This matched clinical data provides a unique opportunity to compare the evolutionary trajectory in the PDTX and the patient.

Taken together, these data pose some interesting hypotheses regarding transient and permanent functional effects of treatment. As outlined previously, our platform provides a unique opportunity to study this in a controlled setting. An added advantage of our system is that the PDTXs are treatment-naïve, removing an important caveat in many model systems. One outstanding question is whether PDTX models retain their therapeutic accuracy over time, i.e. whether PDTXs generated from early resections can predict clinical drug responses to subsequent
lines of therapy or whether PDTX tumours need to be treated in parallel. While we do observe drug response changes following in vivo treatment, these are generally subtle and the extent to which this translates to in vivo differences remains to be determined. It would be useful to test this in matched patient-PDTX cases. In the ideal setting, one would test the treatment-naive PDTX for subsequent lines of therapy administered clinically and ascertain the degree of drug response concordance. It would then be useful to treat PDTXs with the sequential lines of therapy administered to the patient and assess if this affects the drug response concordance. Furthermore, one could engraft longitudinal samples (i.e. pre-treatment and relapsed disease) and test the changes in drug response, both in vivo and ex vivo. This would help to further interrogate the functional and genomic evolutionary trajectories between the patient and the PDTX.

4.4 Conclusions

Here we present the development and optimisation of a high-throughput drug screening platform for testing novel compounds targeting the DDR. Using dissociated PDTCs, this dramatically expands the capabilities of the co-clinical trial platform to increase the number compounds a given tumour receives, shed light on mechanisms of drug response and develop new therapeutic strategies. We leveraged this platform to explore changes in drug sensitivity following in vivo treatment with both clinically-relevant and novel compounds. We identified patient- and treatment-specific changes, including a global shift in response to DDR-targeting drugs following olaparib treatment and a drug holiday effect for AZD1775. This inspired a thorough investigation into the dynamics of drug response to olaparib, which is explored in the following chapter. In addition, we drew on findings from the ex vivo drug screen to design a preclinical trial and explore the efficacy of sequential treatment with chemotherapy and olaparib. This could have far-reaching clinical implications and exemplifies the power of integrating both in vivo and ex vivo strategies to explore phenotypic evolution under alternative therapeutic pressure.
Chapter 5: The dynamics of drug response to PARP inhibition

5.1 Introduction

Major strides have been made in understanding the biological mechanisms underlying the initiation and progression of breast cancer, leading to the identification of molecular targets involved in its aetiology. As such, there have been developments in targeted cancer therapeutics and, with major successes in clinical trials, these have revolutionised cancer treatment for large numbers of patients. To reflect this, breast cancer survival has increased dramatically in the past few decades, with a ten-year survival increasing from 40% in 1971 to 78% in 2010 (260).

However, there is a subset of patients for whom there remains an unmet clinical need, due to an aggressive phenotype and lack of actionable targets. Treatment options for triple negative breast cancer (TNBC) patients remain limited to chemotherapy and radiotherapy but recent advances in our understanding of the roles of the DNA damage response (DDR) pathways in breast cancer offers new avenues for therapeutic intervention.
Accelerated by the discovery of breast cancer predisposition genes, *BRCA1* and *BRCA2* (85-89), a number of targeted agents are entering preclinical and clinical development. To date, the most successful of these is olaparib, which is approved for treatment of metastatic breast cancers with *BRCA1/2* mutations in both the US and Europe (124, 125). Olaparib is an inhibitor of poly(ADP-ribose) polymerase 1 (PARP1) and PARP2, abundant nuclear proteins which have roles in DNA repair pathways and the maintenance of genomic stability. The primary function of PARP1 (among other PARP proteins) is to catalyse the polymerisation of poly(ADP-ribose) (PAR) units, resulting in their post-translational attachment to itself and target proteins, known as (auto)PARylation (261). This contributes to the functions of PARP1 in DNA repair of single-stranded breaks, double stranded breaks and stabilisation of the DNA replication forks. Of note, upon generation of single stranded DNA breaks, PARP1 is rapidly recruited and binds to the damage. It then uses nicotinamide adenine dinucleotide (NAD+) for autoPARylation, which acts to relax the chromatin and recruit repair proteins to the site of damage.

PARP inhibitors act by two mechanisms. First, they prevent NAD+ binding, inhibiting the catalytic activity of the enzymes. This leads to the accumulation of unrepaired single strand breaks, among other types of damage. Secondly, cumulative autoPARylation causes the dissociation of PARP1 from DNA and so by inhibiting NAD+ binding, PARP1/2 proteins become trapped on the chromatin. This is thought to contribute to stalled replication forks and subsequent DNA damage. Both mechanisms lead to the accumulation of double stranded breaks, the repair of which occurs via homologous recombination (HR) (120).

Both *BRCA1* and *BRCA2* have essential roles in HR and, as such, olaparib has been found to have a synthetically lethal relationship with these proteins (116, 117). As discussed in previous chapters, olaparib shows great promise in the early breast cancer setting, for patients with non-functional *BRCA1* or *BRCA2* and, more broadly, TNBC patients, many of whom exhibit a *BRCaanness* phenotype (96, 97).
However, as with all cancer therapies, drug resistance mechanisms sit at the forefront of clinical interest as these have the potential to confound the future use of these agents. Drug resistance mechanisms can broadly be divided into pre-existing and acquired, with the latter referring to changes in the genotype or the phenotype of a tumour during treatment which results in it no longer being susceptible to killing by that agent. Indeed, a wide range of resistance mechanisms have been identified for olaparib and other PARP inhibitors.

Perhaps the most frequently observed mechanism by which cells are able to resist killing is by re-expression of BRCA1/2 or other genes contributing to a BRCAness phenotype, all of which lead to a restoration of HR repair capacity. In cases of single nucleotide variants or insertions/deletions which lead to frame-shifts, secondary mutations can occur to restore the open reading frame and thus protein activity. Other secondary mutations can include reversions of the wildtype sequence and mutations restoring the wildtype amino acid. These has been described many times in therapy-resistant BRCA1/2-mutated breast and ovarian tumours (262-266). In the instances of HR deficiency caused by gene promoter hypermethylation, demethylation can restore gene expression and protein function. This has been shown for BRCA1 methylation in PDTXs (178) and ovarian tumour samples in the context of chemotherapy resistance (267).

Other mechanisms have been found to restore HR function, even when BRCA-deficiency is retained. Loss of factors involved in non-homologous end joining (NHEJ) has been shown to lead to BRCA1-independent HR. This includes 53BP1 (268-270), RIF1 (271-273), REV7 (274, 275) and the Shieldin complex (276-281) and is thought from result from their roles in limiting end resection, promoting NHEJ. In their absence, BRCA1-independent HR is preferential and PARP inhibitor resistance occurs. Alterations in a number of other components of the DNA damage and replication stress responses have been found to mediate PARP inhibitor resistance, including DYNLL1, ATMIN, EZH2, PTIP and SLFN11 (78).
Other mechanisms of resistance have been identified, involving the target directly. One group performed a comprehensive study into the impact of PARP1 mutations on PARP inhibitor sensitivity (282). The authors found that although loss of PARP1 expression in tumours with a complete loss of BRCA1 was synthetically lethal, it could be tolerated in cases where there was some residual BRCA1 function. This was demonstrated in two human BRCA1-hypomorphic cell lines; PARP inhibitor resistance resulted from mutations which ablated PARP1 expression. Interestingly, they also identified PARP1 mutations which conferred resistance by altering the DNA binding potential. While maintaining PARP1 expression, these mutants prevented, or reduced, the ability of PARP inhibitors to trap PARP1 on the chromatin at the site of DNA damage. An example of this was identified in a patient with de novo resistance to olaparib.

Mutations in PAR glycohydrolase (PARG) have been found to confer resistance to PARP inhibitors in BRCA2-mutant cell lines. PARG is involved in the degradation of PAR chains. As such, PARG depletion was found to restore PAR formation, partially rescue PARP1 signalling and decrease sensitivity to PARP inhibitors (283).

Another possible mechanism of resistance involves upregulation of drug efflux pumps. A number of publications have reported an upregulation of Abcb1a (encoding MDR1/P-gp) and Abcb1b in models of PARP inhibitor resistance. It has also been reported that MDR1 inhibitors can re-sensitise the tumours to PARP inhibition (284, 285).

While there is no doubt of the clinical relevance of these isolated mutations and well-defined resistance mechanisms, there is likely to be a wide range of responses observed in the clinical setting. Over recent years, there has been a myriad of publications relating to alternative means by which cells are able to evade therapy. Driven by observations that acquired resistance emerges in the absence of new mutations, we are able to learn from other biological systems. In 1944, Bigger et al.
reported in *The Lancet* that a population of bacteria within a genetically homogenous culture of *Staphylococcus aureus* survived prolonged exposure to penicillin, coining the term ‘persisters’. Later this minor population was found to have a transient tolerance to antibiotics which was attributed to phenotypic switching and constituted slower growing, largely-quiescent cells (286, 287). This concept was leveraged by Sharma et al., who identified a chromatin-mediated reversible drug-tolerant state in lung cancer cell lines, which demonstrated greater than 100-fold reduced drug sensitivity. This phenotype is transiently acquired and relinquished at low frequency by individual cells in a population (288).

This has led to wide ranging hypotheses regarding the reversible nature of drug tolerance and therefore the clinical impact of a drug holiday. Shaffer et al. approached this using Luria-Delbruck fluctuation analysis in melanoma. The authors described transcriptional variability at a single cell level which involved high levels of resistance markers in a very small percentage of cells. They then described a multi-stage process by which epigenetic reprogramming causes these rare cells in transient transcriptional states to become stably resistant when therapeutic pressure was applied (289, 290).

Bell et al. explored a similar concept in acute myeloid leukaemia, for which up to 40% of patients show acquired resistance without new non-synonymous coding mutations. The authors described a stable non-genetic resistance mechanism to BET inhibitors caused by adaptive transcription plasticity which, unlike models of drug persistence, cannot be overcome by a drug holiday. This was accompanied by widespread transcriptional changes and phenotypic alterations, to an immature cancer stem cell like state. The authors also reported that drug sensitivity could be restored by active cellular reprogramming and this was achieved using epigenetic drugs via new enhancer formation (186).

These mechanisms may not be entirely conflicting and are likely to coexist to some degree, particularly regarding the genetic and non-genetic basis of drug resistance
The dynamics of drug response to PARP inhibition and tolerance (291). A recent publication described adaptive mutability in drug-tolerant persister cells (292, 293). Again, drawing on knowledge of antibiotic resistance in bacteria, Russo et al. challenged the idea that all drug resistant genetic mutants are present in the originating tumour mass. In colorectal cancer, the authors described a down-regulation of mismatch repair and HR genes, as well as an up-regulation of error-prone polymerases in persister cells tolerant to EGFR/BRAF inhibitors, activating stress-induced mutagenic mechanisms. They proposed that this increased DNA damage and mutability act as a transient strategy to boost genetic diversity and aid in the tumour’s ability to evade therapeutic pressure.

The exact mechanisms underlying drug resistance, tolerance and persistence are complex. It is probable that these are cancer-type, drug and even patient-specific and it is likely that multiple mechanisms will coexist. What is clear is that to fully elucidate the mechanisms involved in any response to therapeutic pressure, it is essential to adopt a combination of genomic, transcriptomic and phenotypic modalities. Furthermore, the complex diversity within a population of cells, both at genomic and phenotypic levels, means that single cell analytical methods are indispensable. The use of complex model systems is also important to explore these biological processes. Regardless of the leading hypotheses, the dynamics of drug response are reliant on cellular diversity. As such, it is integral that preclinical models are adopted which fully recapitulate the intra-tumour heterogeneity of the originating cancer.

We propose that our preclinical platform provides the ideal framework to study such mechanisms in response to PARP inhibition. Using a combination of in vivo and ex vivo experimentation on PDTX models, multi-omic analysis (both bulk and at a single cell resolution) and genetic manipulation using basic model systems, we hope to shed light on the dynamics of drug response to olaparib. This will be in the context of early breast cancer, with the aim of better understanding how short treatment schedules of targeted therapy can impact the future evolutionary trajectory of a tumour.
5.2 Aims

1. Identify whether the genomic composition of PDTX tumours reflect drug responses to PARP inhibitors
2. Investigate whether known mechanisms of PARP inhibitor resistance can be identified following treatment with clinically-relevant schedules
3. In the absence of known resistance mechanisms, interrogate the dynamics of drug response using multi-omic modalities
4. Explore whether PARP inhibition leads to new therapeutic vulnerabilities which could be targeted clinically.
5.3 Results and discussion

5.3.1 Relating genomic features to PARP inhibitor responses

In order to explore the dynamics of drug response to olaparib, we adopted the experimental design outlined in figure 5.3.1.1a. Four PDTX models from our co-clinical trial cohort were selected based on their genomic features relevant in the study of olaparib. These models were derived from patients enrolled on the PARTNER clinical trial and exhibited a range of clinical responses to the trial arms they received, as described in previous chapters.

Model 1006 has both germline and somatic $BRCA1$ copy number (CN) losses, does not express $BRCA1$ at the RNA level, has high HRD-LOH and HRDetect scores, indicating HR deficiency (HRD) (figure 5.3.1.1c-f). Model 1040 harbours a germline pathogenic mutation in $BRCA1$ with loss of heterozygosity (LOH) in the tumour. While this tumour has some residual RNA expression of $BRCA1$ (albeit low), the mutation is known to produce a truncated, non-functional version of the protein, leading to HRD (245). This is reflected in the HRDetect score, which is above the threshold of 0.7, indicated by the authors to suggest an HRD phenotype (111). Interestingly, this tumour exhibits a low HRD-LOH score but this can be attributed to the low cellularity of the tumour in the patient sample (figure 5.3.1.1d).

Model 1022 has a germline benign missense variant in $BRCA1$ but this is not maintained in the tumour. This patient has a somatic CN gain (CN=3) with LOH, suggesting one allele was lost and the other was triplicated. Interestingly, this tumour also has high HRD-LOH and HRDetect scores. RNA expression of $BRCA1$ was shown to be low compared to the PBCP cohort and at a similar level to model 1040 (figure 5.3.1.1c). Although this would need to be validated experimentally, it is likely that at least one of the $BRCA1$ alleles exhibits promoter hypermethylation.

Model 1141 does not exhibit any germline alterations in $BRCA1$ but a somatic CN gain (CN=4). To reflect this, the HRDetect is lower than the indicated threshold of
The dynamics of drug response to PARP inhibition

Figure 5.3.1.1 Experimental design and PDTX models used to study the dynamics of drug response to olaparib. a) Experimental design of in vivo trial. b) Genomic features of BRCA1 in the four PDTX models selected for this study. c) Normalised expression of BRCA1 in the patient tumour samples relative to other samples in PBCP cohort. d) Tumour content (%) of the patient tumour samples. e) HRD-LOH score of the patient tumour samples. f) HRDetect scores of the patient tumour samples. Threshold of 0.7 HRDetect score indicates HRD phenotype.
0.7, suggesting HR proficiency. None of the tumours exhibited any pathogenic BRCA2 alterations.

This patient cohort was selected to reflect a variety of means by which HRD can occur in patients. Model 1141 was used as a control against which to compare drug responses, as it presents with functional HR. Interestingly, this patient received chemotherapy and olaparib combination clinically and did not respond. This was mirrored in the PDTX co-clinical trial (outlined in previous chapters) and so was included to represent a tumour known not to respond to the test compound.

After robust quality measures, these PDTX tumours were passaged into multiple mice and treated in vivo with olaparib (along with other compounds in parallel, as discussed in previous chapters). Although the clinical trial involved chemotherapy and olaparib as a combination, we decided for the purpose of studying drug response mechanisms it was preferential to use olaparib as a monotherapy. We reasoned this would also allow direct comparisons to previous studies of olaparib resistance. Any findings would then be validated using a combination approach.

In line with the clinical trial, mice were treated with olaparib for 77 days, using doses and treatment schedules to mirror those published by others (250). Traditional in vitro and in vivo studies to identify drug resistance mechanisms have involved long-term continuous treatment until tumour cells acquire features which allow them to resume growth or propagate cells which exhibit pre-existing resistance. The extent to which this translates to the clinical setting is limited. Particularly in early breast cancer, treatment schedules are likely to be shorter with a defined endpoint; for neoadjuvant treatment, this is surgery. In order to study drug response dynamics in a way that is relevant to the patients in our trial cohort, it was essential to use treatment schedules which mirror those adopted clinically.

As discussed, hypotheses have been proposed about the extent to which drug tolerance mechanisms are transient and reversible, or permanent. Reversible
mechanisms lead to the compelling hypothesis that patients could benefit from a drug holiday, responding again to treatments to which they had previously responded. An example of this in our own work was seen in chapter 4; immediately following in vivo treatment with the WEE1 inhibitor, AZD1775, dissociated cells had decreased sensitivity to the same compound. However, after drug withdrawal, sensitivity increased to a similar level as the untreated tumour (figure 4.3.3.8). Although this strategy is rarely implemented clinically, it would have strong implications for patients. To study this, we incorporated a drug holiday into our experimental design. Five mice were used per cohort and mice treated with olaparib were humanely culled at two time points. Immediately post-treatment, two tumours per cohort were samples, denoted as treated (T). Three mice per cohort were left to continue growing off treatment until tumour size limits (1500 mm³), denoted as post-treated (PT). All untreated mice were culled at size limits and so were size-matched with the post-treated tumours.

Figure 5.3.1.2 illustrates the in vivo and ex vivo drug responses of the four models to olaparib. As expected, based on the genomic features, all models except 1141 responded very well to olaparib in vivo, showing a significant difference in tumour volume at the end of treatment (figure 5.3.1.2a, b). This is also reflected in the ex vivo drug responses (figure 5.3.1.2c), as determined using the drug screening platform described in chapter 4. Using a threshold of greater than 0.2 area under the curve (AUC) to indicate a positive response (based on previous experience in our group), only model 1141 did not respond. Although models 1006 and 1040 show genomic alterations in BRCA1, whereas 1022 has a suspected epigenetic mechanism of gene suppression, we observe similar responses to olaparib. This is in line with previous reports that BRCA-methylated tumours have comparable olaparib responses as BRCA-mutated tumours in breast cancer PDTXs (178).

We then sought to analyse the impact of in vivo treatment on the long-term growth trajectory. In all three models which responded (1006, 1040, 1022), exponential tumour growth resumed when treatment was removed. To assess whether the
Figure 5.3.1.2 TNBC PDTX models exhibit differential responses to olaparib.
a) In vivo growth curves of the four models treated with olaparib (purple) or untreated controls (black). Dotted line indicates end of treatment. b) Tumour volume at the end of treatment (77 days). Statistical significance determined using unpaired two-tailed Welch’s t-test (unequal variance). c) Ex vivo drug responses to olaparib. Error bars show 95% confidence intervals. d) Log₂ transformation and linear regression of growth curves of untreated tumours (from day 0) and olaparib post-treatment (from day 77). e) Regression coefficient of log₂ transformed growth curves as in (d). Statistical significance determined using unpaired two-tailed t-test. * p<0.05, ** p<0.01, *** p<0.001, **** p<0.0001
growth rate following treatment differed to the untreated tumours, we log$_2$-transformed the growth curves (from day 0 for untreated and from day 77 for post-treated). We performed linear regression and used the regression coefficient (slope) to indicate growth rate (figure 5.3.1.2d, e). We observed a small but significant decrease in growth rate following treatment in models 1006 and 1040. This indicates that although tumours resume growth when the drug is withdrawn, olaparib does have some impact on the long-term growth trajectory. That said, it is clear that viable cells remain during treatment and once treatment is removed, they rapidly revert back to an actively proliferative state.

These data demonstrate that using both in vivo and ex vivo drug screening methods, TNBC PDTX models exhibit differential drug responses to olaparib and this correlates with HR deficiency. The samples obtained from these in vivo trials provided the material to perform the subsequent experiments outlined in this chapter.

5.3.2 Identifying functional changes upon PARP inhibition

As described in chapter 4, we performed a high-throughput drug screening experiment on untreated and post-treated samples, for a number of treatment cohorts. Following olaparib treatment, we observed two patterns of drug response changes (figure 5.3.2). Models 1006 and 1040 displayed significant decreases in the area under the curve (AUC) values for a number of compounds targeting the DDR, including ATM, ATR and CHK1/2 inhibitors, indicating reduced sensitivity. This suggests a global reorganisation of the repair pathways. Interestingly, we did not observe this for model 1022, in line with 1141 which did not respond to olaparib in vivo. This led us to hypothesise that while tumours may seem to have comparable drug responses, they may exhibit distinct molecular mechanisms underlying drug responses.
Figure 5.3.2 Olaparib treatment in vivo causes model-specific changes in drug response profiles. The change in AUC of olaparib post-treated tumours compared to untreated tumours for DDR-targeting compounds, as identified by ex vivo drug screening experiments. Error bars show 95% confidence intervals. Drug name on y-axis and drug target in parentheses.
Interestingly, following *in vivo* treatment with olaparib, we did not observe major changes in the *ex vivo* drug responses to olaparib or BMN-673 (another PARP inhibitor) (figure 5.3.2). This could be due to a number of factors. First, we tested post-treated tumours which had experienced a drug holiday. As demonstrated with the WEE1 inhibitor, it is plausible that drug resistance or tolerance is a reversible phenomenon and following a period of time off-treatment, tumours revert back to a drug-sensitive state. To address this, as with the WEE1 inhibitor, it would be important to test the tumours sampled immediately following treatment.

Second, when acting as a monotherapy, PARP inhibitors rely on cells accumulating DNA damage as a result of a decreased repair capacity and PARP trapping on the chromatin. For this to occur, cells need to be in an actively cycling state. As described in chapter 4, we do not always observe active cell cycling of tumours *ex vivo* and sometimes growth is limited. This could account for the lack of changes in response to the PARP inhibitors. To conclusively address this, it would be essential to passage treated and post-treated tumours and assess *in vivo* whether tumours have decreased sensitivity to olaparib.

An alternative explanation is that some drug responses may not be captured in our *ex vivo* system. While our group previously reported that the vast majority (82.5%) of drug responses observed *ex vivo* were recapitulated when tested *in vivo*, stromal compartments may play a crucial role in drug responses to some compounds. Again, this should be validated *in vivo*.

While acknowledging the limitations, it is clear that we observe functional changes in the tumours following treatment *in vivo*. It is also evident that PDTX models exhibit distinct response mechanisms, even when *in vivo* responses are comparable. To further delineate this, we chose to concentrate on exploring drug response changes through treatment trajectories using the *in vivo* sample sets.
5.3.3 Investigating known mechanisms of resistance

We next sought to identify known mechanisms of PARP inhibitor resistance in the PDTX models treated *in vivo* with olaparib. As a negative control, we first analysed model 1141 by RNA-sequencing. As demonstrated in figure 5.3.3.1a, this model did not respond to olaparib *in vivo*. Since this model was slow-growing, no samples were taken immediately following treatment but all at the post-treated sampling point. We performed bulk RNA-sequencing on all ten mice (n=5 per cohort). When analysed using a multi-dimensional scaling (MDS) plot (figure 5.3.3.1b), we did not observe any clustering of samples by cohort. Similarly, hierarchical clustering of samples using expression of all genes revealed no clear separation between cohorts (figure 5.3.3.1c). This indicated that there was no global response to olaparib at the transcriptomic level.

As described previously, the high-throughput drug screening did not reveal any major functional changes (figure 5.3.3.1d). However, we did observe a minor decrease in sensitivity to AZD2014, an mTOR inhibitor. To assess this further, we used a targeted approach to look at RNA expression levels for members of the mTOR pathway. This did not reveal any significant differences between cohorts (figure 5.3.3.1e). It should be noted that this analysis is not normalised for gene length and so should only be used to assess differences between samples (i.e. not relative expression between genes). From this we concluded that olaparib treatment did not cause major changes in the transcriptomic landscape or functional characteristics of 1141. This can be explained by the tumour’s pre-existing resistance to PARP inhibitors, due to HR proficiency.

We next performed a similar analytical approach in model 1022 (figure 5.3.3.2). While this model responded very well to olaparib *in vivo*, we did not observe any major functional changes by the *ex vivo* drug screening platform. Again, immediately following treatment, tumour volumes in the olaparib cohort were too
Figure 5.3.3.1 Olaparib treatment did not cause major changes in the transcriptional landscape or functional characteristics of model 1141. a) In vivo growth curves of model 1141 untreated (black) and olaparib (purple) cohorts. Dotted line indicates end of treatment (77 days). b) MDS plot of gene expression profiles of untreated and olaparib post-treated tumours. c) Heatmap displaying all genes of model 1141 untreated and olaparib post-treated tumours. Hierarchical clustering using Euclidean distance. d) Changes in ex vivo drug responses following olaparib treatment in vivo. Error bars show 95% confidence intervals. e) Normalised gene expression (TMM normalised log₂ CPM) of components of the mTOR pathway. Statistical significance tested using two tailed unpaired t-test. All were non-significant.
small and so all tumours were left to continue growing off treatment until size limits.

Using an MDS plot and hierarchical clustering (figure 5.3.3.2b, c), we sought to explore whether olaparib treatment caused a global transcriptomic shift. Interestingly, while all untreated samples clustered together, the olaparib post-treated samples had a more variable transcriptomic profile. This exemplified that at bulk resolution, we did not observe a major transcriptomic change which was uniform between all biological replicates.

We next performed differential expression analysis to determine whether any individual genes were significantly different between cohorts (figure 5.3.3.2d). Remarkably just one gene fell above the p value threshold of 0.05. BRCA1 was found to be differentially expressed with significantly higher expression in the olaparib cohort than untreated. This was found to have a log fold change of 8.73 and a false discovery rate of 0.00165. We then examined the normalised gene expression levels in all other 1022 cohorts treated in vivo (figure 5.3.3.2e). Gene expression was higher in all five biological replicates in the cohort treated with olaparib as a monotherapy. Interestingly, in the cohort treated with equivalent concentrations of olaparib in addition to the WEE1 inhibitor (AZD1775), we observed increased BRCA1 expression in two of five mice. This suggests that in some cases the addition of a second compound interferes with resistance mechanisms. We did not observe significant differences in the chemotherapy and olaparib combination (CTO) cohort, which can be attributed to the 20 times lower dose of olaparib.

Although not validated, we had previously hypothesised that the low BRCA1 gene expression in this model was due to promoter methylation. As such, we propose that olaparib treatment caused promoter demethylation and the tumour underwent re-expression of BRCA1. This has been reported in PDTX models in the literature (178). While this needs to be validated at both epigenetic and protein levels, this is
Figure 5.3.3.2 Olaparib treatment causes an increase in *BRCA1* expression in model 1022. a) *In vivo* growth curves of model 1022 untreated (black) and olaparib (purple) cohorts. Dotted line indicates end of treatment (77 days). b) MDS plot of gene expression profiles of untreated and olaparib post-treated tumours. c) Heatmap displaying all genes of model 1022 untreated and olaparib post-treated tumours. Hierarchical clustering using Euclidean distance. d) Differential expression analysis reveals *BRCA1* as the only differentially expressed gene between untreated and olaparib post-treated tumours. e) Normalised gene expression (TMM normalised log₂CPM) of *BRCA1* in model 1022 treated *in vivo* with different compounds. Statistical significance tested using two tailed unpaired t-test. **** p < 0.0001
the first example of a traditional PARP inhibitor resistance mechanism in our model system.

We next sought to identify known resistance mechanisms in models for which HRD was caused by BRCA1 genomic alterations. As described previously, 1006 does not express BRCA1 at the RNA level due to both germline and somatic CN losses in BRCA1 (CN of 0 in the tumour). Model 1040 has a pathogenic germline mutation (c.4327C>T, p.Arg1443Ter) with LOH in the tumour which, while BRCA1 expression is retained at the RNA level, encodes a non-functional truncated version of the protein (245). As expected, both models responded very well to olaparib in vivo with significantly lower tumour volumes at the end of treatment. For both models, two tumours were collected immediately after treatment (treated, T) and three were left to grow off treatment to size limits (post-treated, PT), which were size-matched with the untreated cohort (figure 5.3.3.3a, b).

We did not observe any significant differences in RNA expression of BRCA1 for either model (figure 5.3.3.3c, d) indicating that there was not a reversion of the genomic features accounting for the HR deficiency. To confirm this, we performed exome sequencing on the treated and post-treated samples. For 1006, CN analysis revealed that the homozygous CN loss was retained in all mice treated with olaparib (figure 5.3.3.3d). Similarly, for model 1040 the homozygous pathogenic mutation was retained in all mice and no secondary mutations were identified in BRCA1 (figure 5.3.3.3f).

To elucidate further whether known resistance mechanisms could be at play in our system, we explored the gene expression levels of known resistance markers (figure 5.3.3.4). Again, for the purpose of this analysis, expression was not normalised for gene length and so should not be used to compare between genes (only between samples). Since we harvested just two tumours at the treated sampling point, statistical significance was determined between the untreated and post-treated tumours only.
Figure 5.3.3.3 Models 1006 and 1040 retain the genomic alterations in BRCA1 following olaparib treatment. In vivo growth curves of models 1006 (a) and 1040 (b) untreated (black) and olaparib (purple) cohorts. Dotted line indicates end of treatment (77 days). Normalised gene expression (TMM normalised log2 CPM) of BRCA1 in models 1006 (c) and 1040 (e) Statistical significance tested using two tailed unpaired t-test. Both were non-significant. d) Integrative genomics viewer (IGV) to demonstrate that model 1006 olaparib treated and post-treated tumours retain the homozygous BRCA1 copy number loss observed in the untreated tumour. f) IGV to show the pathogenic mutation in 1040 was retained in mice treated with olaparib.
First, we explored a number of components involved in DDR rewiring, including NHEJ factors TP53BP1, RIF1, REV7 and the Shieldin complex. Loss of these factors is thought to confer resistance by restoring HR function in the absence of BRCA1 but we did not observe a significant decrease in either model. Interestingly, there was a significant increase in RIF1 in model 1006 but the implications of this are unclear. Alterations in other components of the DNA damage and replication stress responses have been found to mediate PARP inhibitor resistance, including loss of DYNLL1, ATMIN, EZH2, PTIP and SLFN11 but the mechanisms are not so well understood. In model 1006, we observe an increase in ATMIN and a decrease in both DYNLL1 and EZH2, but these minor decreases were deemed unlikely to be sufficient to confer resistance to olaparib in our system.

We observed a significant increase in BRCA2 expression for 1006, which could confer a small degree of resistance but since this is not the mechanism by which HR deficiency occurs, it is unlikely to be the full story. Elevated RAD51 is thought to confer sensitivity to PARP inhibitors, however we observed a decrease in expression in 1006. Other known mechanisms of resistance result from alterations in PARP or PARG. We did not observe any significant changes in expression in either model. Similarly, we also did not observe any changes in the drug efflux pumps, ABCB1 or ABCG2.

Interestingly, one report found that in BRCA1-deficient PDTXs, almost all PARP inhibitor resistant tumours exhibited reduced expression of SHLD1, SHLD2, TP53BP1 and/or PARP1 (277). Based on their findings, Dev et al. made the striking hypothesis that even within a given cohort of biological replicates, alternative molecular alterations converging in the same pathway (loss of Shieldin activity) could arise due to the polyclonal nature of tumours.

While we did not observe any significant differences in expression of SHLD1, SHLD2, TP53BP1 or PARP1, this led us to hypothesise that within a given cohort, tumours from different mice may exhibit distinct resistance mechanisms. To
Figure 5.3.3.4 RNA-sequencing does not reveal any known resistance mechanisms consistent between biological replicates of models 1006 and 1040 treated with olaparib. Normalised gene expression (TMM normalised log$_2$CPM) of known PARP inhibitor resistance markers in model 1006 (a) and 1040 (b) treated in vivo with olaparib. Statistical significance tested between untreated and post-treated tumours using two-tailed unpaired t-test. * p<0.05, ** p<0.01, *** p<0.001, **** p<0.0001 c) Heatmap depicting gene expression levels of individual mice (rows) within each cohort.
address this, figure 5.3.3.4c displays the expression levels of known resistance genes for individual mice within each cohort. While we observed some inter-mouse heterogeneity for model 1006, just one tumour exhibited a dramatic reduction in expression of SHLD1. This marked finding revealed that even within a cohort of biological replicates with comparable drug responses and growth phenotypes, there exists heterogeneity in the mechanisms of response. For model 1040, we observed a greater degree of inter-mouse variability but did not observe any dramatic differences in expression which could account for response to olaparib.

Taken together, these data reveal that in our experimental framework, we were able to identify known resistance mechanisms. Remarkably, in a clinically-relevant time frame, model 1022 exhibited a re-expression of BRCA1 in all biological replicates and we hypothesise that this was due to promoter demethylation.

However, we have also demonstrated that the means by which tumours are able to survive upon therapeutic pressure and repopulate a tumour when treatment is withdrawn cannot always be explained by known resistance mechanisms. This could be due to the intrinsic experimental difference between current strategies and our clinically relevant approach. Interestingly, for model 1006 we observed a uniform response between biological replicates in terms of growth inhibition and the trajectory by which tumours regrow after treatment is removed. However, we observed an isolated resistance mechanism (loss of SHLD1) in one mouse. This led us to the compelling hypothesis that multiple mechanisms of response may coexist and that even in the cases of identifiable resistance mechanisms, this may be an incomplete picture.
5.3.4 Exploring genomic changes

The central dogma in the study of drug resistance and tumour progression is one of Darwinian evolution. Individual cells or genomic clones within a heterogeneous population will, by chance, have a higher propensity to survive the selection pressures of the cancer ecosystem. Drawing on our findings that even in the absence of known resistance mechanisms, tumours are able to survive treatment with olaparib and subsequently resume growth at a near-comparable rate to an untreated counterpart, we reasoned that tumours may have acquired or enriched for genomic features which aid in this response.

To study this, we performed exome sequencing on model 1006 which had been treated with olaparib as described previously. We adopted a multi-region sequencing approach, using three regions from tumours of two mice per cohort (figure 5.3.4.1a). For the olaparib cohort, two mice from the post-treated sampling point were subject to multi-region sequencing. All other tumours in both cohorts were sequenced using one tumour fragment per mouse.

As described previously, bulk RNA-sequencing revealed only minor changes in expression levels of known PARP inhibitor resistance genes. However, reports in the literature have identified PARP1 mutations which confer drug resistance by altering the DNA binding potential (282). While maintaining PARP1 expression, these mutants prevented, or reduced, the ability of PARP inhibitors to trap PARP1 on the chromatin at the site of DNA damage. An example of this was also identified in a patient with de novo resistance to olaparib. To qualify whether this could be observed in our system, single nucleotide variants (SNVs) were called using the exome sequencing data. No mutations were identified in PARP1 in any of the samples analysed and indeed neither were any mutations in the genes known to be involved in PARP inhibitor resistance (described in section 5.3.3).
Figure 5.3.4.1 No relevant SNVs involved in DDR-processes were detected in model 1006 which could contribute to olaparib responses. 
a) Multi-region sequencing approach used for exome sequencing. b) Venn diagram showing overlap between detected SNVs and genes in the GO Term gene set 0006281 (DNA Repair). c) VAFs of mutations in TP53 and RECQL4. For each gene, left plot depicts VAFs between cohorts and right depicts VAFs between multiple regions of the same mouse.
We then explored whether any mutations could be identified which were known to be involved in the DDR processes. We compared called SNVs to those in the GO Term gene set 0006281 (DNA Repair) and identified mutations in TP53 and RECQL4 (figure 5.3.4.1b). We then examined the variant allele frequency (VAF) in each individual tumour fragment. TP53 mutation was present at a VAF of 1 in all analysed regions of all samples, whereas the mutation in RECQL4 was present in just one untreated mouse at a very low frequency (figure 5.3.4.1c). As such, it was deemed unlikely that these would contribute to the drug response phenotype.

Next, we sought to identify changes in VAF of mutations detected in the patient tumour (figure 5.3.4.2). As described in chapter 3, the patient’s primary tumour sample was sequenced by whole genome sequencing as part of the Personalised Breast Cancer Programme. Here we reasoned that while some degree of evolution may result from implanting and passaging the PDTX tumour, it is more likely that mutations preserved between the patient and the PDTX would have functional implications. Figure 5.3.4.2a displays a heatmap of VAFs in the patient sample and the PDTX untreated and olaparib cohorts, with genes ordered by log fold change between untreated and olaparib tumours. Interestingly, hierarchical clustering separated the tumours by cohort, indicating that we do observe some treatment-specific genomic changes. However, when the statistical significance was tested between the VAFs of untreated and post-treated tumours, only four genes were significant: mutations in SPTBN5, SLC25A14 decreased following treatment and mutations in NYO5A and PIAS1 increased. It should be noted that the significance could not be tested in the treated tumours because of insufficient replicates, however the individual VAFs detected in each tumour fragment can be seen in figure 5.3.4.2b-e.

We then explored the functional implications of these mutations using the Cancer Genome Interpreter. All four significant mutations were predicted passengers and were not present in driver genes. As such, we concluded that while treatment appeared to cause some genomic selection, it was unlikely to fully explain the
Figure 5.3.4.2: Exploring changes in VAFs of mutations detected in the patient tumour sample. a) Heatmap showing the VAFs of mutations detected in the patient sample. Hierarchical clustering was performed using Euclidean distances. Statistical significance determined using an unpaired t-test between untreated and olaparib post-treated tumours. Genes ordered by log fold change between untreated and olaparib tumours (treated and post-treated combined) b-e) Mutations which were found to be significantly enriched (b, c) or depleted (d, e) in the olaparib cohort compared to untreated. For each gene, left plot depicts VAFs between cohorts and right depicts VAFs between multiple regions of the same mouse.
response dynamics we observed. Crucially, mutations detected in known oncogenes (*BRAF, TRIP10, ACTG2*), tumour suppressor genes (*TP53, CASP8*), and those found to be cancer predisposition mutations (*TP53*) or predicted drivers (*ACTG2*) had very consistent VAFs between conditions.

Next, we sought to identify any new mutations which emerged or were depleted upon treatment with olaparib, with a focus on coding, non-synonymous or stop-gain mutations. Figure 5.3.4.3a shows the mean VAF in untreated and olaparib treated (left) and untreated and olaparib post-treated (right) tumours. Emergent mutations (highlighted in blue) were defined as mutations which were present in no untreated mice (or regions therein) but were detected in any one treated/post-treated sample with a VAF greater than 0.05. Depleted mutations (highlighted in red) were defined as mutations which were present in multiple untreated samples but no treated/post-treated samples.

The heatmap in figure 5.3.4.3b shows the VAF of the mutations in each biological replicate and the functional annotation of each mutation as determined using the Cancer Genome Interpreter. Interestingly, we do observe mutations in known driver genes and tumour suppressor genes. However, with the exception of two, the majority of these are predicted passengers. In an olaparib post-treated tumour an emergent mutation in TRRAP (tumour driver) was observed (VAF 0.25) which was classified as a predicted driver. TRRAP complexes have been shown to be involved in HR repair of double strand DNA breaks by chromatin modification and remodelling. One study showed TRRAP-deficient cells exhibited a reduction in 53BP1 foci formation upon irradiation and a decrease in accumulation of BRCA1 and RAD51 repair molecules (294). While mutations in this gene have not yet been implicated in PARP inhibitor resistance, it would be interesting to explore this interaction further. In addition, a predicted driver mutation was observed in *PTGS1* (tumour suppressor gene) in the same tumour. However, the VAF was very low (0.02) and so was unlikely to contribute to the observed response phenotype.
Depleted in samples, correlation post synonymous/stop treatment

Figure 5.3.4.3: Emergent and depleted SNVs can be observed following olaparib treatment in vivo. a) Correlation plots showing average VAFs of coding, non-synonymous/stop gain mutations in untreated and olaparib treated/T (left) and olaparib post-treated/PT (right) cohorts. Correlation coefficient, $r$, depicting Pearson correlation. Depleted mutations (red) defined as those present in one or more untreated samples, but no olaparib T/PT samples. Emergent mutations (blue) defined as present in one or more olaparib T/PT samples but no untreated. b) Heatmap showing VAF of depleted and emergent mutations in each sample.
As described previously, one hypothesis is that within a given cohort of biological replicates, alternative resistance mechanisms could arise due to the polyclonal nature of tumours, but converge in a related pathway. To assess this, the emergent and depleted mutations were analysed using gene ontology analysis to identify any common pathways or processes. However, no significant gene sets were identified. Another interesting finding was that mutations in SLC30A7 were present at low frequencies in 4/5 treated/post-treated but no untreated samples. Two different mutations were identified (p.Q307P and p.H208D), but further analysis of this revealed that they are likely passengers and the gene is not of clinical significance.

To conclude our analysis of the genomic contribution to drug response, we examined the overall genomic architecture of the tumour (figure 5.3.4.4). Focussing our attention first on all detected SNVs (left, figure 5.3.4.4a) and then coding non-synonymous or stop gains (right, figure 5.3.4.4b), we performed hierarchical clustering. Using all SNVs, we observed no clear separation by cohort. When analysing coding SNVs, tumours separated to some degree by cohort but this was not exact and there were outliers on both sides. Using stringent criteria for mutation-calling (VAF>0.05, depth >15, number of altered reads >5), we assessed the number of mutations in each sample of each cohort (figure 5.3.4.4c-f). When considering all SNVs, we observed a significant decrease in the olaparib post-treated tumours compared to the untreated tumours (148 and 130 respectively). However, there was no significant differences between the numbers of coding (non-synonymous/stop gain) mutations.

Taken together, these data indicate that olaparib treatment in vivo causes some minor genomic changes, with enrichment and depletion of pre-existing mutations. However, the majority of these changes appear to be passengers and the contribution of this towards the drug response phenotype is likely to be minimal. We do not observe any known resistance mechanisms at the mutational level, nor do we observe any clear genomic changes consistent between biological replicates which could fully explain the dynamics of drug response. Having performed a
Figure 5.3.4.4 Analysis of SNVs do not reveal any major treatment-specific changes in the mutational landscape. Heatmaps showing VAFs of all SNVs (a) and coding, non-synonymous/stop gain SNVs (b). Clustering analysis performed using Euclidean distances. c-f) Frequency of all SNVs (c, e) or coding, non-synonymous/stop gain SNVs (d, f) averaged across treatment groups (c, d) or across multiple regions of the same tumour for two mice per cohort (e, f). Mutations called in VAF >0.05, number of altered reads >5 and depth >15. Statistical significance tested using two tailed unpaired t-test. * p<0.05
comprehensive genomic analysis using multi-region sequencing with no clear result, this led us to hypothesise that the root of the response dynamics is likely to be non-genetic.

5.3.5 Global shifts in the transcriptomic landscape

To further explore the dynamics of drug response to olaparib, we performed a comprehensive gene expression analysis using model 1006 using bulk RNA-sequencing. As with exome sequencing, we adopted a multi-region sequencing approach, analysing three regions per mouse for two untreated and two olaparib post-treated tumours (figure 5.3.5.1a). For all other mice, one region per tumour was sequenced.

We first analysed all samples using an MDS plot and observed a separation by cohort (figure 5.3.5.1b). Hierarchical clustering using the gene expression levels of all genes revealed some separation by model but this was not perfect (figure 5.3.5.1c). However, reassuringly, for three of the four tumours for which multiple regions were sequenced, the different regions clustered together. We then performed hierarchical clustering using the top ~250 strongly expressed and variable genes (figure 5.3.5.1d). This was achieved by first identifying the top 5000 genes by variance and the top 5000 genes by mean expression and identifying those common between the two lists (~250). Interestingly, we observed a perfect separation by cohort, with all multiple regions clustering together. These data revealed that in model 1006, olaparib treatment in vivo resulted in a global transcriptomic shift. This is in contrast to the genomic data, in which one sample per cohort separated from the other biological replicates when analysing SNV VAFs (figure 5.3.4.4). This demonstrates that the transcriptomic profiles of the PDTX tumours as a result of olaparib treatment are independent of the genomic architecture.
Clustering analysis reveals a global transcriptomic shift as a result of olaparib treatment. a) Multi-region sequencing approach used for bulk RNA sequencing. b) MDS plot reveals a separation between treatment groups. Heatmaps showing z score (scaled by row) of all genes (c) and top 250 strong and variable genes (d). Clustering analysis performed using Euclidean distances. Multiple regions from the same tumour are indicated.
Figure 5.3.5.2: Olaparib treatment causes persistent gene expression changes.
Heatmap depicting z scores of top 50 genes which differ between untreated and olaparib treated samples. Top 20 genes identified by subtracting the mean normalised expression values of the treated tumours from the untreated and ranking the gene expression differences by absolute value. Hierarchical clustering analysis performed using Euclidean distances.
One of the critical questions regarding drug resistance and tolerance mechanisms is the transient/reversible or permanent nature of the phenotypic changes. This has profound implications for the clinical use of compounds and determines whether a drug-holiday treatment strategy could benefit patients. To explore this using gene expression data, we identified the top 50 genes which differed between the untreated and olaparib treated samples (harvested immediately after treatment). This was achieved by subtracting the mean normalised expression values of the treated tumours from the untreated and identifying the genes with the greatest gene expression differences (figure 5.3.5.2). We then performed hierarchical clustering to determine with which samples the post-treated (following a drug holiday) clustered. Interestingly, the post-treated samples clustered perfectly with the treated, indicating that the gene expression changes are largely permanent. This was also attempted with a larger number of genes (up to 200) and the result was consistent (data not shown).

We then explored whether any of the gene expression changes were reversed. We performed a statistical analysis between the gene expression levels of untreated and post-treated tumours for the top 50 genes as in figure 5.3.5.2. As expected, the vast majority of these were significantly different, but 7/50 genes were non-significant between the untreated and post-treated tumours. This was striking because, although it is clear that the post-treated tumours exhibit a phenotype more comparable to the treated tumours, this is not a complete picture and we do observe a small degree of reversion.

Differential expression analysis between the untreated and post-treated tumours revealed a large number of differentially expressed genes (figure 5.3.5.3a). However, we were unable to perform differential expression between the untreated and treated tumours due to lack of replicates. Gene set enrichment analysis (GSEA) was performed between the untreated and post-treated tumours using the Hallmark gene sets from the Molecular Signatures Database. The top 10 significant gene sets
Figure 5.3.5.3: Differential expression and GSEA enrichment analysis for model 1006. a) Differential expression analysis reveals a large number of differentially expressed genes. b) Top 10 significant gene sets by normalised enrichment score, identified by gene set enrichment analysis using the Hallmark gene sets. Enrichment plots and top 30 genes contributing to the leading edge for DNA Repair (c) and Epithelial Mesenchymal Transition (d) gene sets.
The dynamics of drug response to PARP inhibition

(by adjusted p-value) are displayed in figure 5.3.5.3b. This revealed some striking
findings.

First, we observed a significant negative enrichment of the DNA Repair gene set,
with a normalised enrichment score of -2.15 and an adjusted p-value of 0.00482.
Figure 5.3.5.3c shows a heatmap of the top 30 genes contributing to the leading
edge of the GSEA analysis and hierarchical clustering separated the samples by
cohort, with the exception of one untreated sample clustering with the olaparib
samples. This finding was particularly interesting, since it linked with the functional
changes we observed in the drug screening experiment for both models 1006 and
1040. As described previously, we observed a significant decrease in sensitivity to
a number of compounds targeting the DDR, including ATM, ATR, CHK1/2
inhibitors (figure 5.3.5.4a, b).

To explore this further, phosphorylation levels of ATM and CHK2 were measured
in the PDTX tumour samples by western blot (figure 5.3.5.4c). Upon DNA damage,
ATM autophosphorylates at Ser1981, indicating activation. ATM then
phosphorylates CHK2 at Thr68, leading to a DNA repair signalling cascade (81).
In line with the drug screening findings, we observed a decrease in p-ATM and p-
CHK2 in the post-treated samples compared to the untreated, suggesting lower
activity through these pathways. In the treated samples, we observed an increase in
p-ATM, which can be attributed to a higher level of DNA damage. This can be seen
figure 5.3.5.4d, in which levels of γ-H2AX are considerably higher in the treated
sample than the untreated or post-treated. Interestingly, we then explored gene
expression levels of ATM and CHK2, along with ATR and CHK1, which were also
implicated by the drug screening experiment (figure 5.3.5.4e). We observed a
significant increase ATM and ATR RNA expression but a decrease in both CHK1
and CHK2. These findings indicate that the decrease in p-ATM is independent of
RNA expression levels. The decreased CHK2 expression explains the lower p-
CHK1 observed in the western blot. Additionally, we also tested the protein levels
The dynamics of drug response to PARP inhibition

Figure 5.3.5.4 Olaparib treatment alters the expression levels of DNA repair genes and DDR-signalling, which has functional implications for the tumour. a-b) Changes in drug sensitivity to DDR compounds following olaparib treatment, as determined by ex vivo drug screening. Error bars show 95% confidence intervals. c) Western blot showing levels of p-ATM and p-Chk2 in model 1006 treated in vivo with olaparib or AZD1775. d) Western blot showing levels of γ-H2AX in 1006 samples. e) Normalised gene expression (TMM normalised log₂(CPM)) in model 1006 treated in vivo with olaparib. Statistical significance tested using two tailed unpaired t-test. * p<0.05, ** p<0.01. f) Differential expressed DDR genes between untreated and olaparib post-treated samples.
in samples treated with AZD1775 (figure 5.3.5.4c) and it should be noted that the same pattern of expression was observed.

Leading on from this, we then sought to explore if any other genes involved in the DDR processes were differentially expressed between the untreated and post-treated tumours. Figure 5.3.5.4f displays genes relating to each repair pathway which are differentially expressed between the untreated and post-treated samples. The first striking finding was that the majority of differentially expressed DDR genes were downregulated. While this could be regarded as counter-intuitive, it mirrors that described by Russo et al. in a recent publication (292). This group reported a down-regulation of mismatch repair (MMR) and HR genes, as well as an up-regulation of error-prone polymerases in persister cells tolerant to EGFR/BRAF inhibitors. The authors proposed a stress-induced mutagenic mechanism to increase DNA damage, mutability and microsatellite instability as a transient strategy to increase genetic diversity. We also observed, like Russo et al., an upregulation of the error prone DNA polymerase, Polκ and a downregulation of three subunits of high-fidelity DNA polymerase Polɛ (POLE2, POLE3, POLE4), though interestingly not the catalytic subunit (encoded by POLE).

Russo et al. tested whether the molecular marks of the proposed therapy-induced mutagenesis could be detected in the genome using exome sequencing. However, they revealed that there was no change in the overall mutational burden between parental, persister or drug-resistant populations. This is line with our findings described previously, in which we did not observe an overall increase in the number of mutations following treatment, indeed we saw a small but significant decrease (figure 5.3.4.4). However, the authors then focussed specifically on microsatellite regions, in which MMR deficiency can be observed. In line with this hypothesis, the authors reported increased genetic instability in these regions in cells resistant to targeted agents. Although here we have not adopted such a targeted approach, it may be interesting to explore the microsatellite regions using the exome sequencing data to determine if we observe the same.
The findings here were particularly striking. Russo et al. discussed the possibility that the downregulation of key effectors in DNA repair, such as MMR and HR, exposes a vulnerability which could be exploited clinically using PARP inhibitors. Indeed, the fact that we observe similar phenomena as a result of PARP inhibition, it seems unlikely that this would be the case and is a remarkably novel finding. One crucial difference between these data is the reversibility. Russo et al. indicated that the increased mutability is a transient method to boost genetic diversity, but that this is later reversed to avoid the accumulation of deleterious mutations. Here we observe a permanent shift in the DNA repair genes, indicating a similar but distinct mechanism.

Returning to the GSEA analysis, the most significant positively enriched gene set was *Epithelial to Mesenchymal Transition*, with a normalised enrichment score of 1.88 and an adjusted p-value of 0.00193. Figure 5.3.5.3d shows a heatmap of the top 30 genes contributing to the leading edge of the GSEA analysis and hierarchical clustering separated the samples by cohort. This led us to hypothesise that olaparib treatment *in vivo* may contribute to a change in the characteristics of the tumour, towards a less differentiated, mesenchymal-like phenotype.

The contribution of epithelial to mesenchymal transition (EMT) to drug resistance mechanisms has been long described in the literature as a means by which cells evade therapy. Most recently, within the conceptual framework of permanent non-genetic drug response mechanisms, Bell et al. revealed that treatment caused widespread transcriptional changes and phenotypic alterations to an immature cancer stem cell like state in acute myeloid leukaemia (186). Crucially, using single cell RNA-sequencing of serial bone marrow samples during treatment, this group described that the transcriptional programme associated with resistance was observed in the residual cells at the time of best clinical response. They also reported that even once therapy was withdrawn, the malignant cells which survived the therapeutic challenge did not revert to the transcriptional state of the pre-therapy population. Indeed, they identified that the transcriptional profile was similar to that
of human leukaemia stem cells. Although the authors report this in a very different setting, the described mechanism has striking comparisons to our findings. Like Bell et al., we observe that even when tumours respond exceptionally well to treatment, the residual cells have adopted a phenotype which remains when treatment is withdrawn. In addition, that phenotype is enriched for mesenchymal markers.

To explore this more deeply, we identified individual genes related to EMT which were differentially expressed between the untreated and post-treated samples (figure 5.3.5.5a). This clearly revealed a mesenchymal-like phenotype consistent among all biological replicates. A western blot revealed a decrease in expression of E-Cadherin and an increase in expression of vimentin at the protein level, characteristic of EMT-like processes (figure 5.3.5.5b). Interestingly, we observe a similar phenotype for model 1006 treated with an alternative compound (AZD1775), but did not observe this for model 1040. This suggests that the dynamics by which cells respond to therapeutic challenge are patient- rather than compound- specific.

E-Cadherin and vimentin were selected for the western blot as characteristic EMT markers. However, the result was intriguing, since neither of these genes were differentially expressed at the RNA level. This led us to hypothesise that in an untreated tumour, different spatial regions may express EMT markers to differing degrees. We reasoned that treatment may select for or induce a phenotype which already exists in certain regions. To address this, immunohistochemistry (IHC) was performed for vimentin. In the untreated sample, we observed regions of both high and low expression but following treatment with olaparib, the tissue was predominately vimentin\textsuperscript{HIGH}. This finding, along with vast publications to support it, suggests that within a heterogenous population one may observe a wide diversity of phenotypes and thus drug responses. As such, we decided that to fully elucidate the mechanisms involved, single cell analyses would be instrumental.
Figure 5.3.5.5 Model 1006 adopted a mesenchymal-like phenotype following treatment with olaparib. a) Normalised gene expression (TMM normalised log2 CPM) in model 1006 treated in vivo with olaparib. All genes were found to be differentially expressed between untreated and post-treated samples. b) Western blot displaying levels of E-cadherin and vimentin in models 1006 and 1040 treated in vivo with olaparib or AZD1775. c) IHC for vimentin in model 1006 treated with olaparib. Two biological replicates of each (rows).

The dynamics of drug response to PARP inhibition
Taken together, this gene expression data revealed some thought-provoking and novel findings. We observed a global transcriptomic shift caused by treatment with olaparib and this change was largely conserved when treatment was withdrawn. Crucially, this appeared to be independent of changes at the genomic level. We note two key findings. First, we observed a decrease in gene expression of a number of genes involved in DNA repair pathways, which has functional implications for the tumour. These permanent changes could have profound effects to a patient’s response to next-line therapies. Second, tumours appeared to adopt a mesenchymal-like phenotype which persisted when treatment is withdrawn. These data are in line with previous publications but is a clear and elegant demonstration that distinct mechanisms of response can co-exist within the same system.

5.3.6 Transcriptomic population dynamics using scRNA-sequencing

To further explore the response dynamics, we utilised single cell RNA-sequencing in model 1006 treated in vivo with olaparib. As described previously, tumour samples were collected at two time points: immediately after treatment (treated) and at size limits (post-treated) (figure 5.3.6.1a). Where possible, two biological replicates were analysed per condition. In addition to olaparib, one biological replicate was also sequenced at each time point from tumours treated with AZD1775 to assess whether transcriptomic changes were drug-specific. Cryopreserved tumour fragments were dissociated and single cell RNA-sequencing was performed in two runs, using fragments from the same mouse (3846) as reference samples between runs (figure 5.3.6.1b).

Single cell analysis was performed by Dr Alistair Martin in the Caldas Group and the results were interpreted in collaboration. Briefly, transcripts were aligned to the human or mouse genome. After quality control checks, an average of 1117 human cells were analysed per sample (8939 total human cells). Human tumour cells from all samples were analysed together to identify common populations. This was
The dynamics of drug response to PARP inhibition

Figure 5.3.6.1 Experimental design for single cell RNA-sequencing a) Schematic showing samples analysed by single cell RNA-sequencing. b) Table showing sequencing runs with mouse 3846 (*) used as a reference sample to control between batches.
achieved by identifying anchors between samples. Figure 5.3.6.2a shows a non-linear dimensional reduction UMAP of all cells within the dataset. Using an unsupervised graph-based clustering, six populations were identified. Differential expression was used to identify markers which defined each cluster. Figure 5.3.6.2b depicts the normalised gene expression levels of cluster marker genes.

We next explored the expression of cell cycle genes. We adopted an approach described by Tirosh et al. (219), which involved scoring the expression of cell cycle phase-specific gene signatures in each individual cell. These signatures were previously shown to reflect G1/S or G2/M phases by synchronisation (295) and single cell sequencing experiments (296). Tirosh et al. described that phase-specific signatures were highly expressed in a subset of malignant cells, distinguishing cycling cells from non-cycling. Figure 5.3.6.2c depicts an estimation of the cell cycle states of PDTX cells on the basis of the G1/S and G2/M gene set scores. Figure 5.3.6.2d shows the gene set score of each cell by cluster, revealing that clusters 2 and 3 represent cycling cells which predominate in G1/S and G2/M cell cycle phases respectively. While Tirosh et al. described that these signatures were sufficient to determine high-cycling cells, the previous publication by Macosko et al. also included gene sets for other cell cycle phases (S, M and M/G1). To build on this analysis further, it would be interesting to determine whether these other cell cycle phases aid in defining the transcriptomic clusters.

To further elucidate the features which define each cluster, we performed gene set enrichment analysis using the Hallmark gene sets from the Molecular Signatures Database. Figure 5.3.6.3a shows the top five gene sets by enrichment score for each cluster, found to have an adjusted p value of greater than 0.05 using a Fisher test. By manually examining this data and the cluster marker genes, we defined each transcriptomic population (figure 5.3.6.3b).

Cluster 0 was defined by epithelial-like markers, exhibiting high expression of cytokeratins and claudins, and negative enrichment for gene sets relating to EMT.
Figure 5.3.6.2 Single cell RNA-sequencing reveals transcriptomic populations.  
a) UMAP displaying all analysed cells from model 1006. Colour indicates transcriptomic cluster. b) Marker genes identified as defining features of each cluster. c) Dot plot showing G1/S and G2/M scores of each cell, coloured by cluster. d) Box plot showing G1/S and G2/M for each cluster.
On the converse, cluster 2 was defined by positive enrichment for EMT markers (e.g. vimentin) and EMT gene sets. Interestingly, this cluster was also positively enriched for E2F targets and DNA repair, as well as cells in the G1/S cell cycle phase. Cluster 1 was defined by immediate early-activation transcription factors (e.g. FOS, JUN, ATF3, IER2, EGR1) which was also reflected in significant enrichment of the gene set TNFA SIGNALING VIA NFKB.

Cells residing in cluster 3 were predominantly in the G2/M cell cycle phase, which was also reflected in the gene set enrichment (G2M CHECKPOINT). When defining cluster 4, we noticed significant enrichment for genes relating to both hypoxia (e.g. CA9, ADM, ANGPTL4) and MHC class I antigen presentation (e.g. HLA-A, HLA-B, B2M). This was a striking finding given the immunocompromised host environment and there is conflicting evidence in the literature about the contribution of hypoxia to antigen presentation (297-300). However, these data suggest a co-expression of related genes, which indicates that hypoxia may augment MHC class I antigen presentation in our system.

The smallest population, cluster 5, was enriched for IGFBP2, a gene associated in the literature with mechanisms of drug resistance. Other marker genes included TFF3, HES6, ASCL2, ANXA1 and SPDEF, all of which have also been associated with drug resistance and/or cancer progression. While this population constitutes a minor proportion of all samples, it could have important implications for the drug responses of the tumour.

While these populations were identified using an unbiased data-driven approach, they have remarkable similarities to those described by Savage et al. (301). The authors performed single cell RNA-sequencing on TNBC PDTX models. Like us, they identified a mesenchymal/stem cluster, which had significantly elevated expression of mesenchymal genes (e.g. VIM) and stem cell-like genes (e.g. ITGA6/CD49F). They also identified a basal-like cluster with elevated expression of cytokeratins. Similar to our cluster 3, they defined a ‘proliferative’ population.
Gene set enrichment can aid in defining features of transcriptomic clusters. a) Top five Hallmark gene sets (Molecular Signature Database) by enrichment score for each cluster. Colour indicates adjusted p value as determined by a Fisher test. b) Table outlining the defining features of each transcriptomic cluster.
which was enriched for cell cycle genes and contained the majority of cells scoring highly for the G2/M phase. Interestingly, they also identified an antigen presentation cluster (marked by genes similar to our cluster 4) but these did not co-express hypoxia-associated genes. Instead, they described that their mesenchymal/stem-like cluster was enriched for hypoxia-induced transcripts. Studying this in the context of EGFR-targeted drugs, the authors then went on to describe an EGFR-dependent tumour initiating program in breast cancer.

We then explored the proportion of cells in each cluster following treatment with olaparib (figure 5.3.6.4). We first analysed the proportions of clusters between reference samples of each run (figure 5.3.6.4a) which revealed remarkable concordance. This was reassuring that on a technical level, we observed little variability between sequencing runs. However, to further reduce this technical variability, we compared the cluster proportions with the untreated samples in each run (figure 5.3.6.4b). Within the first run, we observed some heterogeneity between biological replicates (e.g. cluster 2 between untreated samples) but were able to identify some general trends (e.g. a decrease of cluster 0 in both post-treated samples). Within the second sequencing run, we identified a dramatic increase in cluster 1 in the olaparib treated tumour.

We then decided to analyse the treatment-specific changes across multiple replicates and runs (figure 5.3.6.4c), using statistical analyses which took into consideration replicate samples. From this, we were able to identify transient, permanent and post-treatment transcriptomic changes. As a control, we also identified differences following AZD1775 treatment (figure 5.3.6.4d). Although this extends beyond the scope of this thesis, it should be noted that the population changes described below are drug-specific and alternative population changes were seen in response to AZD1775.

First, the most striking finding was a permanent decrease in cluster 0. Defined as an epithelial-like population, this was in line with the bulk RNA-sequencing data,
The dynamics of drug response to PARP inhibition

Figure 5.3.6.4 Single cell RNA-sequencing reveals drug-specific population changes. a) Percentage of cells in each cluster between reference samples (untreated mouse 3846) between sequencing runs. b) Percentage of cells in each cluster for samples in run 1 (left) and run 2 (right). The effect of olaparib (c) and AZD1775 (d) on the proportions of each cluster. Statistical analysis performed by fitting an independent binomial model. Coefficient and confidence intervals were extracted and p values were computed correcting for multiple testing (Bejamini-Hochberg).

* p<0.05, ** p<0.01, *** p<0.001, **** p<0.0001
which revealed a significant decrease in epithelial markers. Interestingly, a number of genes which defined this population (KRT81, CLDN3) were found to be differentially expressed at the bulk-RNA level (figure 5.3.5.5a).

We observed a significant increase in cluster 1 in both the olaparib treated and post-treated samples, but with lower statistical significance between untreated and post-treated tumours. While this demonstrates that the transcriptomic population persists following treatment, it suggests a small degree of reversion, in line with the findings from bulk RNA-sequencing. This population was defined by immediately early-response genes (e.g. FOS, JUN), which implicates these transcription factors in the tumour’s response (albeit short-term) to therapeutic pressure.

In addition, we observed a post-treatment increase in cluster 2, which is defined as a mesenchymal stem-like population (vimentin\textsuperscript{HIGH}). This was a surprising finding, given the bulk RNA-sequencing data. As described previously, when all bulk RNA samples were clustered using the Hallmark EMT gene set, treated and post-treated samples clustered together (separately from untreated) (figure 5.3.5.3d). This single cell observation that the EMT-like population is enriched only in the post-treated samples suggested an otherwise unappreciated phenomenon. However, further analysis revealed that many of the cluster 2 marker genes are also expressed in cluster 1. It is plausible that clusters 1 and 2 contain cells in similar phenotypic states but with cluster 2 enriched for immediate early-response genes. After treatment is withdrawn and these transcription factors are no longer as active, cells may retain this mesenchymal-like phenotype but in the absence of immediate-early response genes. Alternatively, this may represent two mesenchymal populations with distinct transcriptional programmes, which could not be detected at the bulk RNA level.

We observed transient decreases in both cluster 3 (hypoxia/antigen presentation) and cluster 4 (proliferative, G2/M). A rational explanation for the decrease in cluster 3 may simply relate to the size of the tumour. The untreated and post-treated
samples were both collected when the tumours reached size limits, which is likely to impose a small degree of hypoxia. However, the treated samples were collected at a smaller volume. While this may not be the full story, it offers a valid explanation for this finding.

The decreased proportion of highly-proliferative cells was striking. One of the leading hypotheses regarding drug tolerance mechanisms is the persistence of a small population of slower growing, largely-quiescent cells which are able to repopulate a tumour following withdrawal of the drug (288). What is unclear from this analysis and requires further investigation is the proliferative state of all cells. It would be interesting to explore whether all treated cells have a reduced proliferation rate compared to the untreated.

Interestingly, we observe a stepwise increase in the proportion of cells in cluster 5, with significance between the untreated and post-treated samples. This population is defined by high expression of resistance markers (e.g. IGFBP2). This has similarities to the mechanisms described by Shaffer et al., who report semi-coordinated transcription of a number of resistance markers at high levels in a very small percentage of cells. Mediated by JUN/AP-1, the authors describe that this transient transcriptional state is converted to a stably resistant state by epigenetic reprogramming upon the addition of a drug. While this current analysis does not reveal a clear resistance ‘mechanism’ present in this minor population which dominates the transcriptional landscape after treatment, these cells may constitute a pre-resistant population with the potential to proliferate and take over if the tumour was treated with continuous therapeutic pressure.

However, there is a caveat of the analysis which should not go unrecognised. In order to analyse multiple datasets, the analysis workflow involves identifying anchors to integrate the samples and identify common populations. As a feature of this, major transcriptomic shifts may be masked. This was clear in the interpretation of the data, since we observed a dramatic transcriptomic shift in the bulk RNA-
sequencing data but only minor population changes at a single cell resolution. To further elucidate whether we observe a similar phenomenon to Shaffer et al., we explored the bulk RNA-sequencing data further. Shaffer et al. identified 13 resistance markers which were expressed at very high levels at low frequency in an untreated cell population. We sought to explore whether these were differentially expressed in our post-treated samples using bulk RNA-sequencing. Indeed, five were significantly higher following olaparib treatment (EGFR, FGFR1, LOXL2, WNT5A, AXL), indicating that treatment causes an enrichment or phenotypic change involving these resistance markers. Focussing back on the single cell data, we then explored whether these five markers are in fact present at a low frequency in the untreated tumours. We observed high expression of these genes in subsets of cells (data not shown) but these cells did not reside in any specific cluster, nor was any cluster significantly enriched for these genes.

It should be noted that this analysis focussed on the human compartment. A feature of using PDTX models is that the non-cell autonomous compartment (including stroma, infiltrating immune cells, vasculature) are rapidly replaced by mouse cells upon transplanting the primary tissue. Alignment of transcripts to separate species’ genomes allows easy interrogation of these components and the impact of treatment. While this extends beyond the scope of this thesis, it offers an interesting opportunity to study the complex interaction between the cell autonomous and non-cell autonomous tumour compartments.

Taken together, these data reveal some interesting findings. First, we were able to identify heterogeneous transcriptomic populations within a tumour sample, defined by cell type markers, cell cycle phases and signalling activation. Second, the proportions of cells residing in each transcriptomic state altered in a drug-specific manner. Specific to olaparib, we observed transient changes which reversed when treatment was withdrawn, including an increase in cells expressing high levels of immediate early response genes and a decrease in a highly proliferative population. We also observe persistent changes, including a decrease in an epithelial population.
and an increase in mesenchymal/stem-like cells. Finally, we observed a step-wise increase in a population high for known drug resistance markers, which could constitute a pre-resistant cell state, as described in the literature. This demonstrates the wide-ranging benefits in deciphering drug response mechanisms at a single cell resolution.

5.3.7 Interrogating phenotypic populations using mass cytometry

To expand on our findings and to validate the phenotypic population changes identified by single cell RNA-sequencing, we utilised a novel single-cell proteomics-based approach. Mass cytometry, or Cytometry with Time-of-Flight (CyTOF), is a multi-parametric methodology which combines flow cytometry with time of flight mass spectrometry (302). By employing heavy-metal conjugated antibodies (rather than fluorophores), this technique enables high-throughput analyses of single cell suspensions with up to 40 markers. This technique, along with its imaging counterpart (imaging mass cytometry) has had major successes in recent years, shedding light on immune and tumour cell landscapes in many cancer types (30, 31, 303-308).

Our group have developed a breast-cancer specific CyTOF antibody panel and optimised its use for breast cancer PDTXs (publication in review). The experiments outlined below utilise this optimised panel and methodology. The development and validation of the panel was performed exclusively by Dr Dimitra Georgopoulou from the Caldas Group and is beyond the scope of this thesis.

The optimised methodology involved the dissociation of cryopreserved tumour fragments, sample barcoding using a combination of heavy metals and cell staining (figure 5.3.7.1). To study the dynamics of drug response upon parallel treatment strategies, tumour samples from multiple trial arms were analysed, including (where possible) both treated and post-treated samples. Unfortunately, due to
The dynamics of drug response to PARP inhibition

1. Tumour dissociation

2. Barcoding and pooling

3. Cell staining

Figure 5.3.7.1 Experimental design and antibody panel for mass cytometry in model 1006. Model 1006 was treated in vivo with multiple compounds, over two passages. Samples were analysed by mass cytometry and stained in two pools using a reference sample to compare between batches. Cell staining was performed using an antibody panel spanning human tumour, mouse stroma, cell cycle/apoptosis and oncogenic signalling markers.
constraints with tissue availability, no untreated samples within the first passage of model 1006 (PAR1006-x1) were available. To alleviate this, we also analysed samples from passage two (untreated, CT and CTO trial arms). We reasoned that since mice enrolled in the CTO arm were present in both trials, this could be used to control between passages. In addition, we had bulk RNA-sequencing data from all samples in both passages, which could be used to validate any findings. To test this in advance of the experiment, we performed hierarchical clustering of bulk RNA-sequencing data from the untreated samples (both passages) and olaparib samples (passage 1). This demonstrated that untreated samples from passages 1 and 2 clustered together (separately from the olaparib cohort) (supplementary figure 4).

In addition, previous experiments from our group has revealed remarkable concordance in cellular phenotypes identified by mass cytometry between passages of the same PDTX (publication in review).

Two barcoding pools were used, with ten samples per pool. In the cases of two biological replicates, one was assigned to each pool to minimise batch effects. In addition, a reference sample from a different PDTX model (AB555M) was used, for which the same dissociated cell suspension was separated into two, with one assigned to each pool. This ER-positive model (AB555M) was also chosen as an internal control to identify model-specific differences in luminal markers. The antibody panel was broadly divided into: human tumour compartment, mouse stromal compartment, oncogenic signalling activation and cell cycle and apoptosis. Figure 5.3.7.2 depicts the pre-processing and barcoding quality control measures in the mass cytometry workflow. Figure 5.3.7.2a outlines the raw data gating strategy. First, intact cells were identified based on positive expression of the intercalator Iridium ($^{191}\text{Ir}$) and negative expression of $^{140}\text{Ce}$. $^{140}\text{Ce}$ is one of the elements in the EQ four element calibration beads (309). Second, in order to identify single cells, events with exceptionally high expression of $^{191}\text{Ir}$ were removed as doublets. Third, live cells were identified by negative expression of Rhodium ($^{103}\text{Rh}$), a live-dead exclusion marker.
Figure 5.3.7.2 Mass cytometry gating and barcoding quality control. a) Gating strategy to identify cells, single cells and live cells. b) Sample barcode reference (308). c) Heatmap showing median values of palladium isotopes (barcodes) in each sample. d) Histogram showing expression of palladium isotopes in pool 1.
Figure 5.3.7.2b depicts the expected expression of the six palladium isotopes ($^{102}$Pd, $^{104}$Pd, $^{105}$Pd, $^{106}$Pd, $^{108}$Pd, $^{110}$Pd) used for barcoding ten samples for each pool (310). In figure 5.3.7.2c, a heatmap shows the median values of the isotopes in each sample, also displayed as a histogram for pool 1 in figure 5.3.7.2d. It should be noted that as part of the quality control measures, three samples were removed. Sample 16 was removed due to very low cell count and samples 1 and 19 were removed because they were composed of <50% alive cells.

We next explored whether any batch effects could be observed between pools (figure 5.3.7.3a, b). To achieve this, the two reference samples (AB555) were analysed using a viSNE plot. This is an analytic tool which allows one to map high-dimensional cytometry data onto two dimensions (311). This is based on the t-Distributed Stochastic Neighbour Embedding (tSNE) algorithm and as such single cells are displayed in a two-dimensional dot plot using tSNE1 and tSNE2 as the x- and y-axes respectively, with each dot representing a single cell. Figures 5.3.7.3a and b shows the distribution of cells from the two reference samples in a viSNE plot. We observed a clear overlap of cells from different pools, which demonstrated no obvious batch effect.

We then sought to explore whether samples from different PDTX models cluster separately. Using the two reference samples (AB555) and two untreated PAR1006 samples, we observed a clear separation in phenotypic space using viSNE analysis (figure 5.3.7.3c). In addition, when we displayed the expression of ER on the viSNE plot, we observe strong positive expression in AB555 cells and negative expression in PAR1006 (figure 5.3.7.3d). This is in line with both the clinical classification of these tumours and the tissue-level staining as determined by IHC (figure 5.4.7.3e). Taken together, these data reveal that on a technical level the experiment was successful. We observed efficient barcoding, no obvious batch effects between staining pools and a clear separation in phenotypic space between PDTX models.
Figure 5.3.7.3 Mass cytometry reference sample quality control measures. viSNE displaying AB555 reference samples to compare between pools, overlaid (a) and individual (b). Down-sampled to 10,000 cells/sample for each sample. c) viSNE displaying two AB555 reference samples and two biological replicates of PAR1006 (all down-sampled to 10,000 events) per sample. d) Expression of ER overlaid on viSNE plot as in (c). e) IHC showing tissue protein expression of ER in PAR1006 and AB555 tumours.
Further quality control measures prior to mass cytometry data interpretation. a) All human PARP1006 samples were clustered using viSNE (downsampled to 25,000 cells/sample). b) Cells from biological replicates (untreated) resided in a similar phenotypic space. c) Expression of human (EpCAM, CD298) and mouse (MHC class I, CD45) overlaid on the viSNE as in (a). d) Human and mouse populations can be clustered using expression of mouse/human-specific markers. e) Correlation between median values of human gate in mass cytometry and bulk RNA-sequencing, identified between matched mouse samples.
After quality control measures, we analysed all PAR1006 samples by viSNE analysis (figure 5.3.7.4a). Events were down-sampled to that of the lowest sample (25,000 events/sample). Only PAR1006 samples were used to generate the ViSNE and therefore markers (ER, CK8/18, HER2) not expressed in this TNBC model were excluded (supplementary figure S5). The resulting viSNE showed some interesting features, with samples occupying different phenotypic spaces. Reassuringly, the two untreated biological replicates (figure 5.3.7.4b) exhibited a remarkably similar phenotype by viSNE.

We then sought to explore the human and mouse compartments. In line with manuscript in review from our group, a combination of antibodies reactive against human CD298 and EpCAM were used to identify human cells. CD298 is a subunit (β3) of the sodium/potassium-transporting ATPase, is essential for the cell and thus is broadly expressed (312, 313). In addition, EpCAM is commonly expressed in epithelial breast cancer cells and so using a combination of the two markers is optimal to isolate all human cells. By contrast, mouse-specific antibodies against MHC class I and CD45 are used to identify mouse cells. Manual gating was using to distinguish human and mouse populations (figure 5.3.7.4d). Within the mouse population, we observe CD45-positive and -negative cells, in line with what we expected.

As a further quality control measure, we sought to identify whether the overall protein expression values correlated with bulk RNA-sequencing levels. To achieve this, we identified seven human tumour markers which we expected to have diverse expression between samples (EpCAM, CD44, VE-Cadherin, CD49F, EGFR, p53, Vimentin). We then identified the correlation between the median expression value obtained by mass cytometry and the RNA expression level (log2CPM) on a matched mouse basis. For all but one marker we observed a significant correlation between expression values (figure 5.3.7.4e and supplementary figure 6). The only marker for which we did not observe a significant correlation was vimentin (r=0.4247). This has previously been identified by both IHC and single cell RNA-sequencing.
Figure 5.3.7.5 FlowSOM clustering reveals drug-specific changes in phenotypic populations. a) Schematic displaying steps involved in FlowSOM clustering. b) Heatmap showing events counts of cells from each sample by metacluster. b) ViSNE plots of untreated and CT tumours with cells coloured by metacluster. c) Minimum spanning trees of untreated and CT tumours.
to have profound intra-tumour heterogeneity. As such, this further exemplifies the value of analysing these samples at a single cell resolution.

We next sought to identify phenotypic populations. To achieve this, we first gated for human cells as described above. We then gated for CD298+/EpCAM+ cells as we reasoned that cells with double negative expression for these markers were likely to be unstained which may bias clustering (figure 5.3.7.5a). To identify populations of cells, we adopted a FlowSOM clustering method (314). FlowSOM is an algorithm in which cells are clustered based on selected channels. Clusters are then assigned into metaclusters which reflect different phenotypic populations, and visualised using a minimum spanning tree (MST).

We performed a number of different iterations of FlowSOM clustering using different markers, altering the number of clusters and metaclusters in order to identify phenotypically diverse populations. Our optimised clustering was performed using only cell-type markers expressed in our samples (EpCAM, CD44, VE-Cadherin, CD49F, EGFR, Vimentin) with 100 clusters and 7 metaclusters. Along with ER, HER2 and CK8/18, CD24 was also omitted because it was found to have low expression compared to model AB555 (supplementary figure 5).

Figures 5.3.7.5b-d show the proportion of cells in each cluster by sample. As expected, we observe very similar compositions among the two untreated biological replicates. We also observe highly similar phenotypic populations in biological replicates treated with CT. As described above, this experiment included samples derived from two passages of model 1006. CTO tumours were analysed from both passages and we sought to use these to compare between passages. We observed some degree of variability between CTO tumours in passages 1 and 2. The most striking of this was a high proportion of cells in metacluster 6 from passage 2 and low proportion from passage 1. However, it should be noted that the absence of metacluster 6 was not a passage-specific phenomenon; indeed, we also observed this in CT tumours from passage 2. In addition, the heterogeneity we observe
**Figure 5.3.7.6 Exploring features of FlowSOM metaclusters.** a) Heatmap showing median expression of each marker by metacluster. b) Representative histogram (sample 15) showing expression of markers used in FlowSOM clustering by metacluster.
between CTO replicates is as great as between biological replicates of AZD1775. Taken together, these data suggest that the untreated tumours reflect the basal phenotypic composition of this tumour in the absence of therapeutic challenge which can be extrapolated between passages, though any findings should be validated.

These data demonstrate some interesting patterns in response. We observed an increased abundance of cells in metacluster 4 regardless of the drug, suggesting a uniform response to therapeutic challenge. We also observe some drug-specific effects, e.g. a decrease of metacluster 6 following CT treatment. Other population changes occurred across multiple but not all treatment groups (e.g. increase in metacluster 1), suggesting patterns of behaviour which could be used to associate drug classes. The variability between biological replicates, exclusive to the treated samples, suggests a degree of stochasticity in the evolutionary trajectory of tumours. Despite near-identical phenotypic diversity in the untreated tumour, the same therapeutic challenge could drive tumour evolution through alternative paths.

We next explored the markers which defined each metacluster (figure 5.3.7.6a). As described above, clustering was performed using only cell-type markers but the clusters also exhibited diverse expression of cell cycle/apoptosis and signalling markers. Of the seven metaclusters, three expressed vimentin and four did not. Interestingly, in general the vimentin\textsuperscript{HIGH} metaclusters expressed higher levels of signalling proteins, as well as Ki67 and pRb, suggesting a greater proliferative state. We observe two EpCAM\textsuperscript{LOW} metaclusters and five EpCAM\textsuperscript{HIGH}. Generally, the expression of EGFR, VE-Cadherin and CD49F followed a similar pattern; metaclusters with high expression of one marker usually also had high expression of the others, and vice versa. These data demonstrated heterogeneous phenotypic populations and FlowSOM clustering enabled one to tease out this information and the impact of therapeutic pressure.
Focussing our attention back on the impact of olaparib on the phenotypic landscape of the tumours, we explored the untreated and olaparib treated/post-treated samples (figure 5.3.7.7). Using viSNE analysis, we observed remarkable concordance between biological replicates (figure 5.3.7.7a, b). When the expression of markers was overlaid on the viSNE plots, we observed heterogeneous vimentin expression in both the untreated and olaparib samples. This reflects what has previously been identified at the transcriptomic and histological levels. However, we observed a dramatic increase in expression of EGFR, VE-Cadherin and CD49F, visible in both vimentin$^{\text{HIGH}}$ and vimentin$^{\text{LOW}}$ regions (figure 5.3.7.7c).

To further elucidate this, we explored the proportions of FlowSOM metaclusters (figure 5.3.7.8). The untreated tumours were dominated by cells in metaclusters 2 and 6. These were vimentin$^{\text{LOW}}$/CD44$^{\text{MID}}$ and vimentin$^{\text{HIGH}}$/CD44$^{\text{HIGH}}$ regions respectively, both with high expression of EpCAM, low expression of EGFR and VE-Cadherin, and medium CD49F expression. The first striking finding after treatment was that the phenotypic population changes were consistent between the treated and post-treated tumours. This indicates that the phenotypic changes/enrichment were permanent and cells did not revert back to a pre-treated phenotype when the drug was withdrawn. Second, the tumours were more phenotypically diverse after treatment, composed of cells in a greater number of metaclusters. We observed a decrease in metacluster 2 and a dramatic increase in metacluster 4. These differ in expression of EGFR, VE-cadherin, CD49F and CD44, which are all more highly expressed by cells in metacluster 4. We also observed a decrease in metacluster 6 and an increase in 7, again representing higher expression of EGFR, VE-cadherin and CD49F after treatment. The expression of CD44 was lower in metacluster 7, suggesting no overall change in this marker. Interestingly, metaclusters 4 and 7 express higher levels of p-c-Jun than 2 and 6, which, like single cell RNA-sequencing, implicates the AP1 transcription factors and downstream signalling in the response to olaparib. However, there is crucial distinction between
Figure 5.3.7.7 Olaparib treatment causes phenotypic population changes. a-b) ViSNE plots showing distribution of untreated, olaparib (treated) and olaparib (post-treated) samples, overlaid (a) and individual (b). c) Expression of cell type markers overlaid on the viSNE plots.
Figure 5.3.7.8 FlowSOM reveals permanent phenotypic population changes by olaparib treatment. a) Heatmap showing events counts of cells from each sample by metacluster. b) Minimum spanning tree of one biological replicate from each condition. Inner circle density represents event counts per cluster. Outer circle colour represents metacluster. c) Expression of markers in each cluster (colour of circles). Metaclusters 4 and 7 (enriched after olaparib treatment) are indicated with dotted lines. d) Expression of vimentin in each cluster in untreated, olaparib (treated) and olaparib (post-treated) samples, showing both Vimentin\textsuperscript{HIGH} and Vimentin\textsuperscript{LOW} clusters are present in all conditions.
these two data sets. By mass cytometry, the population changes were permanent, persisting when the drug with withdrawn. By single cell RNA-sequencing, we observed a reversion in the population enriched for the AP1/immediately early response transcriptional programme. This suggests distinct dynamics at the transcriptional and phenotypic levels.

To validate these findings, we explored the gene expression levels of these marker genes (figure 5.3.7.9a). We first checked whether the gene expression reflected our protein-level observations in the samples which were analysed by mass cytometry (untreated from passage 2 and olaparib tumours from passage 1). Gene expression was significantly higher for EGFR and VE-cadherin but interestingly not for CD49F (though this displayed wider intra-tumour variation). Likewise, we observed no changes in EpCAM and Vimentin gene expression levels, but CD44 was significantly lower after treatment. These demonstrated that overall the gene expression levels reflected the proteomics determined by mass cytometry, but that the single cell analysis provided additional resolution. We then explored whether the findings were consistent between passages 1 and 2. Indeed, we observed significant increases in EGFR, VE-Cadherin and CD49F following olaparib treatment within passage 1. We observed no significant differences in expression of Vimentin and CD44, but did observe a significant decrease in gene expression of EpCAM. Overall, these data validated our mass cytometry findings and show consistency between untreated samples from multiple passages.

We then validated these findings using IHC (figure 5.3.7.9b). As expected, we observed a dramatic increase in expression of EGFR and CD49F, in line with mass cytometry and bulk RNA sequencing. We also sought to validate the previous bulk RNA-sequencing findings which showed a decrease in a number cytokeratins. In line with this, IHC staining using a pan-cytokeratin antibody revealed a dramatic decrease in expression.
Figure 5.3.7.9 Mass cytometry findings were validated using bulk RNA-sequencing and IHC. a) Normalised gene expression (TMM normalised log2CPM) of genes implicated by mass cytometry as variable (left) and consistent (right) following in vivo with olaparib. Statistical significance tested between untreated (passages 1 or 2) and post-treated tumours using two-tailed unpaired t-test. * p<0.05, ** p<0.01, *** p<0.001, **** p<0.0001. b) IHC for EGFR, CD49F and pan-cytokeratin in model 1006 treated with olaparib.
Taken together, these data pose interesting hypotheses. As discussed previously, a recent publication described rare cell populations expressing high levels of resistance markers prior to drug exposure; indeed, we observed a similar phenomenon using single cell RNA-sequencing. The authors reported that after treatment, resistant cells expressed these markers at more uniformly high levels (289). Interestingly, EGFR was one of the key markers implicated in this mechanism. When analysing the viSNE plot of untreated samples (figure 5.3.7.7c), we noted a similar observation. Prior to treatment, rare cell populations expressed high levels of EGFR, and indeed the same cells also expressed higher levels of VE-Cadherin and CD49F. After treatment, we observed an increased frequency of similar phenotypic populations; EGFR was also differentially expressed at a bulk gene expression level.

In another publication, authors uncovered an EGFR-enriched stem-like population using single cell technologies in breast cancer PDTX models (301). EGFR was found to be highly expressed in cells enriched for a mesenchymal/stem-like signature and flow-sorted EGFR\textsuperscript{HIGH} populations had enhanced with tumour initiating capacity. Like us, this group identified a mesenchymal/stem like population by single cell RNA-sequencing. Remarkably, the key markers they found to define this population were EGFR and ITGA6 (encoding CD49F). This has profound similarities to our system, and is also in line with our bulk RNA-sequencing findings, in which tumours were enriched for EMT signatures after treatment with olaparib.

Shaffer et al. indicated that their drug-induced epigenetic reprogramming was partially mediated by the activity of transcription factors JUN and/or AP-1 and TEAD (289). In our system, mass cytometry revealed high expression of p-c-Jun in the emergent phenotypic populations (particularly those high for EGFR/CD49F/VE-Cadherin) after olaparib treatment, implicating c-Jun in phenotypic switching. This is in line with previous findings in melanoma, in which resistance to BRAF/MEK inhibitors was associated with a high abundance of c-Jun
and characteristics of a mesenchymal-like phenotype (315). Expanding on these associations, the authors then demonstrated that overexpressing c-Jun in drug-naïve melanoma cells induced an EMT-like phenotypic switch which was similar to that observed following BRAF inhibition. They concluded that c-Jun is a key mediator of the mesenchymal-like phenotype associated with resistance, and so it stands to reason that c-Jun/AP1 may be involved in the response dynamics in our system. Again, this is in line with the transient increase in expression of immediate early response genes (\textit{JUN, FOS, ATF3, IER2, EGR1}) we observed by single cell RNA-sequencing.

Based on bulk RNAsequencing data, single cell RNA-sequencing and mass cytometry, we propose a non-genetic mechanism whereby olaparib treatment causes a phenotypic switch and/or enrichment of a pre-existing population. We have demonstrated at a single cell resolution that emergent populations are enriched for mesenchymal/stem-like markers, exhibit high AP1/JUN signalling and have validated these findings using multiple modalities.

\section*{5.3.8 Targeting a drug tolerant phenotype}

Drawing on our findings from bulk gene expression, single cell RNA-sequencing and mass cytometry, we propose a non-genetic mechanism of drug tolerance. The previous data relied on assessing tumours which had been treated \textit{in vivo} and represented a fixed time point in the trajectory of the drug response. As such, it was difficult to delineate whether the observed differences were due to a phenotypic change induced as a consequence of olaparib treatment or an enrichment of a pre-existing population. Based on this, we have formed two fundamental hypotheses (figure 5.3.8.1a).

To assess this experimentally, we used dissociated treatment-naïve cells from the model 1006. Cells were treated \textit{ex vivo} with olaparib, using a concentration previously identified as IC50 for this model (figure 5.3.8.1b). Cells were then
Figure 5.3.8.1 Exploring phenotypic switching or enrichment of pre-existing populations. a) Two alternative hypotheses underlying the observed changes in phenotypic populations. b) Dose response of model 1006 to olaparib. IC50 calculated to be ~3 uM. c) Experimental design of time course to identify phenotypic population changes ex vivo. d) Western plot showing vimentin expression of model 1006 during time course of ex vivo olaparib treatment.
collected at multiple time points after treatment to assess expression changes and to identify the timepoint at which this occurs (figure 5.3.8.1e). Vimentin was used as a key EMT marker. Although this was not one of the principles markers identified in the mass cytometry analysis as representing the induced or enriched population, previous experiments had revealed an increase in expression following olaparib treatment in vivo which could be successfully tested using a western blot (figure 5.3.5.5b) and vimentin expression was a key marker in our single cell RNA-sequencing data. Using two replicates per time point, we identified a dramatic increase in vimentin expression after 4 hours, indicating the timepoint at which the phenotypic change occurred or enrichment could be identified.

To experimentally test our hypotheses, we drew on findings that the potassium ionophore compound salinomycin selectively targets cells which have been induced through an EMT (316). This compound was identified using a high-throughput compound screen on mesenchymally transdifferentiated breast epithelial cells and was found to reduce the proportion of cancer stem cells by more than 100-fold compared to paclitaxel. We reasoned that if olaparib induced a phenotypic change towards a mesenchymal stem cell-like phenotype, this could be selectively targeted with salinomycin.

We performed a combination drug screen in which cells were first treated with different concentrations of olaparib. Using the time point determined in figure 5.3.8.1, cells were dosed with salinomycin 4 hours after olaparib treatment. Three technical replicates were included for each of the 56 dose combinations (solvent control and 0.01 uM to 10 uM in seven dose increments for each drug). Synergistic effects were measured using the Bliss model, which compares the observed response under a given combination of two drugs to the predicted response under a model of independence. Remarkably, we observed a large degree of synergy for model 1006 (figure 5.3.8.2). However, we did not observe synergy (and indeed some degree of antagonism) for model 1040, which have previously been shown to lack the EMT-like processes. This demonstrated that in some cases, olaparib
Figure 5.3.8.2 Sequential treatment with olaparib and salinomycin demonstrates synergistic cancer killing in model 1006. a) Experimental design of ex vivo sequential drug screen. b) Heatmap of synergy score for models 1006 and 1040. c) Heatmap of observed responses (%) for models 1006 and 1040. d) Dose response curves of olaparib upon different concentrations of the salinomycin and vice versa. e) Area under the curve (AUC) of olaparib upon different concentrations of salinomycin and vice versa.
induces a phenotypic change towards a mesenchymal stem-like state, which can be selectively targeted using salinomycin.

To assess whether this phenomenon could be extrapolated to a wider context, we repeated the experiment with 1022 and 1141 (figure 5.3.8.3a). As described previously, 1141 did not exhibit a BRCA1ness phenotype and thus did not respond to olaparib in vivo. Conversely, 1022 exhibited a clear resistance mechanism to olaparib, in which BRCA1 was re-expressed at the RNA level following in vivo treatment. Here we report that although we did not observe the same phenotypic change as with model 1006, olaparib and salinomycin show a synergistic relationship in these tumours. This is a remarkable finding, as it demonstrates the generalisability of the result and shows the potential clinical uses of salinomycin following olaparib treatment in the wider context of breast cancer.

We then sought to explore whether the same findings could be observed if olaparib and salinomycin were treated in combination (figure 5.3.8.3b). As before, for model 1040 we did not observe synergy. For models 1006 and 1022, we observed synergy but to a lesser degree as with sequential treatment. Model 1141 exhibited synergy for the sequential treatment but not in combination. This was particularly interesting because it was further convincing evidence that salinomycin selectively targets a population induced by olaparib treatment, rather that the two drugs targeting independent populations within a heterogenous tumour.

Using mass cytometry, we previously identified that the phenotypic change induced by olaparib could be observed following treatment with other compounds, which suggested it was patient- rather than compound-specific. To assess this, we performed a combination experiment with salinomycin and carboplatin (figure 5.3.8.4). Indeed, no synergy was observed for either model 1006 nor 1040. This demonstrated that the phenotypic population which was targeted by salinomycin was induced specifically by olaparib and not any therapeutic challenge.
Figure 5.3.8.3 Salinomycin and olaparib synergy can be observed in other PDTX models and in combination. a) Experimental design, heatmap of synergy score and of observed responses (%) of olaparib and salinomycin sequential treatment for models 1022 and 1141. b) Experimental design, heatmap of synergy score and of observed responses (%) of olaparib and salinomycin combination treatment for models 1006, 1040, 1022 and 1141.
Figure 5.3.8.4 Salinomycin and carboplatin do not exhibit a synergistic relationship. a) Experimental design of salinomycin and carboplatin sequential treatment b) Heatmap of synergy score of salinomycin and carboplatin combination treatment for models 1006 and 1040. c) Heatmap of observed responses (%) of salinomycin and carboplatin treatment for models 1006 and 1040.
5.3.9 Working towards a mechanism of drug tolerance

We next sought to explore potential mechanisms which contribute to the phenotypic change. A recent publication reported that breast cancer endocrine resistance is associated with enhanced phenotypic plasticity, driven by global enhancer reprogramming (317). This group described resistance-induced gene expression changes with remarkable similarities to those we describe in response to olaparib treatment (downregulation of epithelial differentiation markers and upregulation of mesenchymal invasive markers, including EGFR). Mechanistically, the authors explored the contribution of transcription factor activity and demonstrated that the enhancer reprogramming was driven by differential high-order assemblies of transcription factors, particularly between ERα and GATA3/AP1.

We hypothesised that the phenotypic change observed following olaparib treatment may be caused by differential transcription factor activity. Based on our observations using single cell RNA-sequencing and mass cytometry, we implicated c-Jun (and as such, AP1 transcription factors) as a major player in phenotypic switching. To explore this further, we performed a western blot on model 1006 treated ex vivo with olaparib and/or salinomycin (figure 5.3.9.1). We observed a bimodal pattern of p-c-Jun activation, with an increase at both 1 hour and 8 hours after treatment with olaparib. Interestingly, the increase at 8 hours was partially alleviated with the addition of salinomycin. While more convincing evidence is required to delineate the molecular mechanism (for example knock out of cJun), this supports its involvement in our system.

To build on these findings, we adopted an unbiased data-driven approach to further explore transcription factor involvement. While c-Jun was implicated, we hypothesised that other transcription factors may also be involved (either by inducing phenotypic plasticity or maintaining the phenotype). Using the bulk RNA-sequencing data, we identified differentially expressed genes between the untreated and olaparib treated, and between untreated and post-treated samples. Although the
Figure 5.3.9.1 Olaparib induces expression changes in p-c-Jun. a) Experimental design of time course after olaparib and salinomycin treatment to identify signalling changes. b) Western plot showing p-c-Jun expression of model 1006 during time course of *ex vivo* olaparib and salinomycin treatment.
single cell RNA-sequencing analysis revealed some transient gene expression changes, we focussed our efforts on identifying drivers of a permanent shift. As such, we identified common differentially expressed genes between the untreated and the two time points (260 up and 247 down) (figure 5.3.9.2a). We performed a de novo motif search in the regions 5kb upstream of all differentially expressed genes to identify any common motifs. We then compared significantly enriched motifs to known transcription factor binding motifs (figure 5.3.9.2b).

The same analysis was performed using only upregulated genes (260 common). Of the two analyses, four transcription factors were identified as significant in both: KLF4, MTF1, ONECUT and ZNF384. Based on these data and a literature search, KLF4 and MTF1 were chosen as candidate transcription factors to drive the observed phenotypic changes (figure 5.3.9.2c-d).

Kruppel-like factor 4 (KLF4) is an evolutionarily conserved zinc finger-containing transcription factor, which regulates diverse cellular processes including cell growth, proliferation and differentiation (318). In 2006, KLF4 was demonstrated to be one of four factors which can be used to generate pluripotent stem cells from differentiated fibroblast cells in culture (319). It has been implicated in human cancers as both a tumour suppressor and an oncogene, with its roles greatly dependent on cellular context.

The contribution of KLF4 to EMT or the reversal (MET) is disputed. One publication reported KLF4 to be highly expressed in more than 70% of breast cancers and required for the maintenance of breast cancer stem cells (320). In addition, the authors demonstrated that knockdown of KLF4 inhibited migration and invasion of breast cancer cells, arguing for a positive connection between KLF4 and EMT.

However, other reports have been conflicting. One group demonstrated that KLF4 silencing led to alterations indicative of EMT, including loss of E-cadherin protein
**Figure 5.3.9.2 De novo motif analysis identified KLF4 and MTF1 as candidate transcription factors.** a) Differentially expressed genes identified by bulk RNA-sequencing between untreated and treated, and untreated and post-treated tumours. Common genes (260 up, 247 down) were identified. b) Top five significantly enriched motifs identified using MEME tool. E-value provides an estimate of statistical significance of identified motif. 'Sites' refers to the number of 5kb regions contributing to the motif. 'Matches' depicts number of known motifs with a significant match to identified motif. c-d) Known transcription factor binding motif (top) and identified motif from 5kb upstream of differentially expressed genes (bottom) for KLF4 (c) and MTF1 (d).
Another report described KLF4 as a major repressor of EMT. Shown to have reduced expression during TGFβ-induced EMT, experiments revealed KLF4 to be essential for the maintenance of epithelial differentiation and that its downregulation was required to induce a mesenchymal phenotype (322). A number of EMT-related genes were reported as direct KLF4 target genes, including E-cadherin, N-cadherin, vimentin and Jnk1, the latter of which was described to play a central role in mediating KLF4-controlled EMT, migration and apoptosis. This was particularly striking since Jnk1 phosphorylates and regulates the activity and expression of Jun proteins, and thus has central roles in AP1 transcription factor activity (323). Indeed, Jnk1 phosphorylates c-Jun at Ser63 (as shown in figure 5.3.9.1b). KLF4 has been shown to have a dual function as both a transcriptional activator and repressor. While the direct mechanisms remain elusive, it is clear that KLF4 has a fundamental role in processes involved in phenotypic plasticity and tumour progression.

Metal regulatory transcription factor 1 (MTF1), also known as MRE-binding transcription factor 1, is a zinc finger protein involved in the cellular adaptation to various stress conditions, including exposure to heavy metals (324). Knockout of MTF1 in ovarian cancer cells was shown to inhibit EMT, upregulating epithelial markers E-cadherin and cytokeratin 7, and downregulating mesenchymal markers Snai2 and B-catenin (325). Interestingly this knockout also upregulated expression of KLF1 and the authors posited that MTF1 may function as a transcriptional repressor of KLF4. Furthermore, MTF1 has been found to be significantly elevated in many human cancer types, including breast cancer (326). Taken together, these two transcription factors appeared to be interesting candidates to explore further as drivers of phenotypic plasticity in our system.

We first explored the expression levels of KLF4 and MTF1 in our samples (figure 5.3.9.3). Using a western blot to measure the protein level, all models expressed MTF1 but interestingly 1006 did not express KLF4. This was a surprising result but we reasoned that while the protein level may be too low to identify on a western
Figure 5.3.9.3 Expression levels of KLF4 and MTF1 in PDTX models. a) Western blot showing KLF4 and MTF1 expression in untreated PDTX tumours. b) KLF4 and MTF1 gene expression model 1006 untreated, olaparib treated (T) and post-treated (PT) samples. Statistical significance tested using two tailed unpaired t-test. * p < 0.05
The dynamics of drug response to PARP inhibition

blot, it may retain some functional significance. In addition, the western blot was performed on untreated samples and when we interrogated KLF4 RNA expression levels in those treated with olaparib (figure 5.3.9.3b), we observed a significant increase in the post-treated sample. As such, we reasoned that there was value in continuing to explore both genes.

To interrogate the molecular mechanisms involved, we utilised immortalised cell lines. While PDTXs have wide ranging advantages over basic model systems, they are expensive to propagate and are more technically challenging to use. As such, it was decided that in order to gain mechanistic insights, it was advantageous to use cell lines which have been well characterised and can easily be harboured for genetic manipulation. However, since we had observed heterogeneous drug response dynamics between PDTX models, it was essential that we selected the correct model. We identified five breast cancer cell lines which could potentially be used (figure 5.3.9.4a). Four represented TNBC and one (HCC1954) was HER2 positive. One cell line (SUM149) possessed a BRCA1 exon 11 frameshift mutation (2288delT) with LOH (327). As such, SUM149 cells do not express the full length BRAC1 protein but a hypomorphic splice variant with some residual function (328, 329).

Using the same olaparib dose which had previously been identified as IC50 for the PDTX model 1006 (3 uM), cells were treated and protein was collected as various time points afterwards (figure 5.3.9.4c). A western blot was used to identify changes in expression of KLF4 and MTF1 in each cell line (figure 5.3.9.4d) and we identified both cell line- and transcription factor-specific dynamics. For HCC1954, we observed decreasing expression of both factors over the time course. A similar pattern was observed for KLF4 in MDA-MB-468 and MTF1 in SUM149. After 1 hr, we observed an increase in expression of both transcription factors for MDA-MB-231 cells and KLF4 in SUM149, suggesting early transcription factor activity following treatment. For SUM159, we observed increased expression of both factors at 4 hours, which was sustained at 8 hours for KLF4. While these data
Figure 5.3.9.4 Identifying transcription factor expression changes upon olaparib treatment. a) Characteristics of possible cell lines to use to explore molecular mechanisms involved in olaparib response. b) Dose response of model 1006 to olaparib. IC50 calculated to be ~3 uM. c) Experimental design of time course to identify transcription factor expression changes upon olaparib treatment. d) Western blot to show changes in transcription factor (KLF4, MTF1) expression over a time course of olaparib treatment.
demonstrate that the dynamics of transcription factor activity is complex and heterogenous between cell lines, it reveals that expression of both KLF4 and MTF1 are altered in response to olaparib treatment. This was reassuring evidence that these factors may be implicated.

We next explored the response of these cell lines to olaparib (figure 5.3.9.5) We reasoned that in order to identify whether the transcription factors altered the response to or phenotypic changes caused by olaparib, it was essential to use cell lines which had a basal level of sensitivity to the compound. We also wanted to identify the time frame of the experiment which would allow this response to be captured. To achieve this, we performed an *in vitro* drug screen on the cell lines and measured cell viability after 48, 72 and 96 hours (figure 5.3.9.5a). As expected, owing to the *BRCA1* mutation, SUM149 cells responded to olaparib and this could be identified after 72 hours. The next best responder was MDA-MB-468 and using the cut-off of AUC 0.2 to indicate a positive response, this was observed only at 96 hours (figure 5.3.9.5b). We aimed to measure the drug responses when cells were in an exponential growth phase (figure 5.3.9.5d-e) and had achieved at least two replications over the course of the experiment (figure 5.3.9.5c). For both cell lines, the 96 hour time point fit these criteria.

In order to test whether KLF4 or MTF1 are involved in olaparib response, we employed a small interfering RNA (siRNA) approach to induce short-term silencing of each transcription factor. As a preliminary experiment, we first tested whether the gene silencing was effective and could be retained over the timeframe required for a drug screen (figure 5.3.9.6a). In the method we proposed, cells were to be transfected, dosed with the compound after 48 hours and cell viability would be measured after 144 hours (96 hours post dosing, as determined in figure 5.3.9.5). The western blots in figure 5.3.9.6b and c show the protein expression levels of the transcription factors over this time frame. In both cell lines, the gene silencing was very effective. For MDA-MB-468, some KLF4 expression could be observed at 144 hours (the endpoint of the drug screen) but minimal in the other time points.
Figure 5.3.9.5 Optimisation of olaparib drug screen using breast cancer cell lines.
a) Dose response curves to olaparib in breast cancer cell lines, with viability measured 48, 72 and 96 hours after dosing. b) Area under the curve (AUC) of dose response curves for breast cancer cell lines at 48, 72 and 96 hours after dosing. c) Number of growth replications of breast cancer cell lines after 48, 72 and 96 hours. d) Net luminescence of breast cancer cell lines over 96 hours. e) Log₂ transformation of luminescence at each time point (Lₜ) divided by luminescence at 0 hours (L₀).
The dynamics of drug response to PARP inhibition

Figure 5.3.9.6 Pilot siRNA experiment to test gene silencing in breast cancer cell lines. a) Proposed experimental design to test olaparib drug responses of breast cancer cell lines following siRNA gene silencing, and corresponding sampling time points for pilot siRNA experiment to test duration of gene silencing. b-c) Western blots showing expression of KLF4 and MTF1 over 144 hours, following siRNA gene silencing in SUM149 (b) and MDA-MB-468 (c) cell lines.
For MTF1, some residual expression could be seen at all time points. While it may be useful to optimise these conditions further, for the purpose of this exploratory experiment, we deemed this result sufficient. For SUM149, the knockdown was extremely effective and retained at all time points.

We next sought to explore whether knockdown of KLF4 and/or MTF1 altered the in vitro response to olaparib. To achieve this, cells were transfected with non-targeting, KLF4 and/or MTF1 siRNAs using two technical replicates for each condition (figure 5.3.9.7a). Protein levels were measured 48 hours after transfection (time of dosing) (figure 5.3.9.7b). Although there was some residual protein expression, the knockdown was effective. Interestingly, for both cell lines KLF4 knockdown increased expression of MTF1. On the converse, MTF1 knockdown increased KLF4 expression. This same interaction has been reported in ovarian cancer cells, in which KLF4 expression was upregulated in MTF1 knockout cells compared to controls (325). The authors indicated a molecular mechanism underlying MTF1-mediated EMT in which MTF1 transcriptionally repressed KLF4 expression. While our data support this, we also observe a co-dependent relationship in which knockout of either transcription factors increases the expression of the other.

We explored whether gene suppression altered the growth rate over 96 hours. We observed no changes in growth rate as a result of knockdown of either KLF4 or MTF1 (figure 5.3.9.7c, d) consistent between biological replicates. In line with previous experiments, cells proliferated in an exponential manner throughout the course of the experiment and achieved approximately three replications over 96 hours for all conditions.

We next sought to determine the effect of gene suppression on sensitivity to olaparib (figure 5.3.9.8). Based on our previous findings, we hypothesised that KLF4 and/or MTF1 transcription factor activity may alter the transcriptional
Figure 5.3.9.6 Pilot siRNA experiment to test gene silencing in breast cancer cell lines. a) Experimental design to test olaparib drug responses of breast cancer cell lines following siRNA gene silencing, and corresponding sampling time points for western blot analysis. b-c) Western blots showing expression of KLF4 and MTF1 48 hours after siRNA transfection in SUM149 (b) and MDA-MB-468 (c). d) Net luminescence (top) and Log2 fold change of luminescence (V) at each time point divided by time 0 (bottom), over 96 hours for SUM149 (left) and MDA-MB-468 (right). E) Number of growth replications after 96 hours.
Figure 5.3.9.8 Gene expression silencing of KLF4 and/or MTF1 does not dramatically impact olaparib sensitivity in breast cancer cell lines. a-b) Dose response curves of cell populations following siRNA gene silencing of KLF4 and/or MTF1 in MDA-MB-468 (a) and SUM149 (b) cells. c-d) Area under the curve (AUC) of dose response curves following siRNA gene silencing as in (a or b) for MDA-MB-468 (c) and SUM149 (d) cells.
landscape of cells following treatment with olaparib in order to aid in a cell’s ability to evade therapy. As such, we posited that by silencing gene expression of these transcription factors, we would observe an increase in drug sensitivity to olaparib. Figure 5.3.9.8 depicts the results of a drug screening experiment of MDA-MB-468 and SUM149 to olaparib following siRNA gene expression silencing. For MDA-MB-468 we observed a small decrease in sensitivity to olaparib in all siRNA conditions. While this minor observed effect may be due to a low basal response to olaparib and thus masking of phenotypic changes, it was not convincing evidence that these transcription factors are implicated in the dynamics of drug response. Similarly, we observed no changes in drug sensitivity in SUM149 cell after siRNA gene silencing consistent between replicates.

It is plausible that KLF4 and MTF1 are implicated in the dynamics of drug response but that their silencing does not directly impact drug sensitivity. To explore this in a more robust manner, it would be important to perform a gene expression analysis on these cell lines following olaparib treatment to determine if a similar transcriptomic shift can be observed as in the PDTX model 1006. If that is the case, it would be interesting to identify if this transcriptomic shift is suppressed or reduced by silencing of KLF4 and/or MTF1. An anticancer drug candidate (LOR-253) has demonstrated potent anti-tumour efficacy in many cancer types and is in preclinical development. This compound is a small molecular inhibitor of MTF1, which also acts by inducing KLF4 (330). If these transcription factors were found to have roles in olaparib response, it would offer an interesting opportunity to target the upstream regulators of phenotypic changes involved in drug response and disease relapse.

Our previous data demonstrated that c-Jun signalling/AP1 transcription factor activity is involved in a tumour’s response to olaparib. However, the timeframe at which we observed increased expression of p-c-Jun by western blot was not compatible with this acting as the primary driver of the phenotypic changes we observed in PDTX models treated in vivo. As such, we adopted an unbiased data-
driven approach to identify transcription factors, which yielded KLF4 and MTF1 as interesting candidates. While it is clear that further experiments are essential to determine the contribution of these transcription factors to a cell’s ability to respond to olaparib, these data do not disprove their involvement in our system and there is benefit in exploring this further.

5.4 Conclusions

In this chapter, we aimed to explore the dynamics of drug response to PARP inhibitors. In line with previous publications, we demonstrated that the genomic composition of PDTX tumours impacts their response; tumours with a BRCAness phenotype responded to a higher degree. We reported that in a clinically relevant time frame, traditional drug resistance mechanisms can be identified in breast cancer PDTX models. However, we also revealed that in many cases tumour cells are able to survive on treatment and resume growth after drug withdrawal in the absence this. Using multiple modalities (including at a single cell resolution), we described the emergence of phenotypic populations enriched for mesenchymal/stem-like markers, exhibiting high AP1/cJun signalling. We propose a non-genetic mechanism by which olaparib is able to induce a phenotypic switch which aids in a tumour’s response to evade therapeutic challenge. Crucially, we demonstrated that this emergent population can to be selectively targeted with salinomycin, offering potential new combination therapeutic strategies in breast cancer.
Chapter 6: Conclusions and clinical relevance

6.1 Clinical relevance and context

Breast cancer is one of the leading causes of cancer related deaths in women in the UK (6). Encompassing approximately 15% of breast cancer patients, triple negative breast cancer (TNBC) remains the subtype with the worst prognosis (56), due to an aggressive phenotype and limited therapeutic options. However, TNBC patients commonly harbour alterations in components of DNA damage response (DDR) pathways, which unveils an opportunity for therapeutic intervention. The most well-known examples of this are alterations in breast cancer predisposition genes, BRCA1 and BRCA2, which confer enhanced sensitivity to PARP inhibitors, including olaparib. As such, clinical trials are underway to assess the efficacy of olaparib in early breast cancer, for TNBC patients and those harbouring BRCA1/2 alterations (126).

However, as with all targeted therapies, drug resistance and disease relapse sit at the forefront of biological interest and have the potential to confound clinical utility of effective compounds. An understanding of the dynamics of drug response and
the mechanisms by which tumours resist or evade killing is paramount. Broad ranging mechanisms have been described to contribute to PARP inhibitor resistance but a comprehensive assessment of this relies on leveraging preclinical models which reflect the complexity of human breast cancer and experimental strategies which mimic the clinical scenario.

Patient-derived tumour xenografts (PDTXs) have been shown to retain the intratumour heterogeneity, genomic and histological features of the originating tumours. While the benefits of PDTX models in translational science are abundant, their use in clinical medicine remains in its infancy. This project developed and optimised an integrated experimental framework aligning PDTX models with the clinical setting. This enabled us to interrogate the dynamics of drug response and disease relapse following PARP inhibitor treatment at high resolution in TNBC.

6.2 Aligning PDTX models with the clinical setting

The first aspect of this project was the development of an integrated experimental framework aligning PDTX models with the clinical setting. Encompassing both in vivo and ex vivo aspects, described in chapter 3 and chapter 4 respectively, these data exemplified the potential power of using PDTX models in anticipatory cancer medicine.

In chapter 3, we presented the development and optimisation of a co-clinical trial. This was aligned with an ongoing neoadjuvant clinical trial at Addenbrooke’s hospital, in which patients with TNBC and/or BRCA1/2 mutations were treated with chemotherapy with and without the PARP inhibitor, olaparib. We revealed remarkable concordance between drug responses of patients and their corresponding PDTX models. While this is not the first-time concordant drug responses have been reported (168, 169, 188), it represents a novel and robust approach to assess the extent to which PDTXs recapitulate clinical drug responses in a controlled clinical trial setting.
Figure 6.1 Preclinical modelling of breast cancer drug responses
In addition to the patient-matched treatment schedule, we tested the alternative clinical trial arm, as well as a number of experimental compounds and combinations \textit{in vivo}. \textit{Chapter 4} described the optimisation of a high-throughput drug screening platform (previously pioneered by our group (166)) for DDR compounds. Our group previously identified that the vast majority (82.5\%) of drug responses observed \textit{ex vivo} were recapitulated when tested \textit{in vivo}. As such, we reasoned that these would provide the ideal tools to test potential treatment strategies for patients.

\textit{Chapter 4} also described the impact of \textit{in vivo} treatment on subsequent \textit{ex vivo} drug sensitivities. This approach was adopted to test the consequences of first-line therapy on a tumour’s functional phenotype. We observed both patient- and drug-specific dynamics, in which pre-treatment with compound $X$ affected a tumour’s response to compound $Y$. We noted some interesting observations spanning multiple models, including the potential efficacy of sequential PARP inhibitor and chemotherapy treatment, which inspired new preclinical trials.

We envisage that these data provide the foundation upon which PDTX models could one day directly impact clinical decision making. A patient is able to receive just one treatment strategy at any one time, which may result in complete response, partial response or disease progression. Additionally, if a patient does respond, they may later present with relapsed disease. PDTXs and the corresponding PDTCs offer the capabilities to dramatically expand the number of compounds a given tumour receives and could prospectively anticipate the most efficacious treatment strategies for patients. While it is unlikely that PDTX models are going impact early breast cancer, it could gain traction for patients who present with relapsed disease or residual tumour burden following treatment. Indeed, a clinical trial is currently recruiting patients using this approach in mantle cell lymphoma (189) and early studies have shown feasibility in clinical practice (190, 191).
6.3 Leveraging the PDTX platform to study mechanisms of drug response

In TNBC and breast cancers with a BRCAness phenotype, PARP inhibitors offer a potential avenue of therapeutic intervention. However, drug resistance and relapse remain obstacles in the treatment of the disease and multiple mechanisms of PARP inhibitor resistance have been reported in the literature. In chapter 5, we leveraged our PDTX platform and a clinically relevant treatment schedule to tease out the molecular mechanisms involved in a tumour’s response to the PARP inhibitor olaparib.

In line with previous publications, we observed that the genomic composition and BRCA status impacts a tumour’s response to olaparib. Indeed, we tested this in four models: three BRCAness tumours with different aetiologies and one with functioning homologous recombination. In one model, we observed an example of a traditional PARP inhibitor resistance mechanism, in which BRCA1 was re-expressed in 5/5 mice treated with olaparib. This was a PDTX which we hypothesised had suppressed BRCA1 expression via epigenetic mechanisms and was a remarkable finding, given the relatively short time frame of treatment.

However, we also observed instances in which tumour cells survived on treatment with rapid regrowth upon drug withdrawal, in the absence of any traditional resistance mechanisms. Focussing our attention on one PDTX model, we performed a robust analysis of the mutational landscape, which uncovered little convincing evidence of a genetic mechanism. Using multiple modalities and at a single cell resolution, we observed the emergence of a phenotypic population enriched for mesenchymal/stem-like markers, exhibiting high AP1/cJun signalling. We revealed that this emergent population could be selectively targeted using salinomycin, unveiling a new therapeutic strategy for relapsed disease in TNBC.
While this chapter was in the context of DDR targeting in early TNBC, we noted some interesting observations which are relevant to the wider cancer field. First, we identified traditional resistance mechanisms using clinically-relevant time frames. We observed that diverse resistance mechanisms can co-exist within the same system; biological replicates of the same PDTX exhibited distinct mechanisms of response and multiple mechanisms were identified within the same tumour sample. Therefore, in order to delineate the fine details, it is paramount that complex model systems are adopted.

We identified that the phenotypic evolution which resulted from drug treatment was a targetable phenomenon. Based on this, we postulate that in order to successfully eradicate a tumour, one must either target the mechanisms by which the tumour evolves upon the selection pressures of the cancer ecosystem or target the output of the evolutionary trajectory. We demonstrated that salinomycin can selectively target the induced phenotypic population which aids in a tumour’s ability to resist treatment with olaparib. As such, it stands to reason that salinomycin could either be used as a therapeutic strategy to treat relapsed breast cancer or in combination as a first-line therapy to impede the tumour’s ability to evolve and thus survive.

Indeed, this thesis demonstrates that only through leveraging preclinical models which accurately recapitulate the heterogeneity of human breast cancer and adopting analytic tools which resolve such complexity, can we hope to succeed in improving outcome for these patients. We hope this project and the work outlined in this thesis has gone a small way to develop tools which could aid in the future treatment of this complex disease and increase our understanding of the evolutionary principles that underpin its progression.
References


-265-


54. NICE. Trastuzumab for the adjuvant treatment of early-stage HER2-positive breast cancer. 2006.


References


References


References


the PARP inhibitor AZD2281 alone and in combination with platinum drugs. Proc Natl Acad Sci U S A. 2008;105(44):17079-84.


Supplementary Figure 1: Gating strategy of AB861 samples by flow cytometry.
**Supplementary figure 2 PDTX responses in a co-clinical trial setting.**

Tumour volume of untreated and treated mice midway through treatment (6 weeks, (a)) and at the end of treatment (11 weeks, (b)). c) Area under the curve of tumour growth curves over the course of treatment (11 weeks). d) Regression coefficient calculated by linear regression of log₂(tumour volume). Bar charts showing mean ± standard deviation of untreated and treated values. (left) PDTX models from patients who received pCR at surgery. (right) PDTXs from patients who received non-pCR. P values calculated using unpaired Welsh’s t-test (unequal variance) comparing untreated to treated mice. Significance: *<0.05, **<0.01, ***<0.001, ****<0.0001.
Supplementary figure 3: AUC, IC50 and dose response curve shapes for *in vivo* treatment cohorts for PAR1006 and PAR1141.
Supplementary figure 4: Hierarchical clustering of bulk RNA-sequencing data to compare untreated samples from passages 1 and 2 with olaparib samples from passage 1.
Supplementary figure 5: Markers were not used for FlowSOM because of the molecular subtype and expression in PAR1006.
Supplementary figure 6: Correlation between bulk RNA-seq and median CyTOF values (human gate)
## Supplementary table 1. Metadata of IHC

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## Supplementary table 2. Metadata of STR profiling

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### Supplementary Table 3. Metadata of optimisation drug screens.

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**Supplementary table 4.** Metadata of *ex vivo* drug screens on co-clinical trial samples.

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**Supplementary table 5.** Metadata of single cell RNA-sequencing.

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### Supplementary table 6. Metadata of CyTOF samples.

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<td>20</td>
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