Tumor Evolution and Resistance in Response to BET Inhibitor Combination Therapies in Triple-Negative Breast Cancer

The Harvard community has made this article openly available. Please share how this access benefits you. Your story matters

<table>
<thead>
<tr>
<th>Citation</th>
<th>Ge, Jennifer Yawei. 2019. Tumor Evolution and Resistance in Response to BET Inhibitor Combination Therapies in Triple-Negative Breast Cancer. Doctoral dissertation, Harvard University, Graduate School of Arts &amp; Sciences.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Citable link</td>
<td><a href="http://nrs.harvard.edu/urn-3:HUL.InstRepos:42029636">http://nrs.harvard.edu/urn-3:HUL.InstRepos:42029636</a></td>
</tr>
<tr>
<td>Terms of Use</td>
<td>This article was downloaded from Harvard University’s DASH repository, and is made available under the terms and conditions applicable to Other Posted Material, as set forth at <a href="http://nrs.harvard.edu/urn-3:HUL.InstRepos:dash.current.terms-of-use#LAA">http://nrs.harvard.edu/urn-3:HUL.InstRepos:dash.current.terms-of-use#LAA</a></td>
</tr>
</tbody>
</table>
Tumor evolution and resistance in response to BET inhibitor combination therapies in triple-negative breast cancer

A dissertation presented

by

Jennifer Yawei Ge

to

The Division of Medical Sciences

in partial fulfillment of the requirements for the degree of Doctor of Philosophy in the subject of Biological and Biomedical Sciences

Harvard University
Cambridge, Massachusetts

May 2019


Tumor evolution and resistance in response to BET inhibitor combination therapies in triple-negative breast cancer

Abstract

Triple-negative breast cancer (TNBC) is an aggressive subtype of breast cancer that has limited treatment options beyond chemotherapy. In preclinical studies, inhibitors of BET family proteins have been found to have efficacy, but emergence of resistance remains a problem. TNBC has been shown to contain considerable intratumor heterogeneity, which serves as the substrate for tumor evolution and poses a challenge to achieving durable responses, especially with monotherapies. Therefore, we aimed to identify combination therapies that would extend the effectiveness of BET inhibition and to understand their effects on intratumor heterogeneity, as well as mechanisms of resistance.

In order to rationally design combination therapies, we performed CRISPR/Cas9 and small molecule screens to identify synergistic targets for the prototypical BET inhibitor JQ1. We followed up on numerous hits in in vitro synergy studies and in vivo studies on cell-line and patient-derived xenografts. We found that targeting the cell cycle was generally efficacious with BET inhibition, and we chose palbociclib, a CDK4/6 inhibitor, and paclitaxel, a microtubule targeting chemotherapy, for further study.

We used DNA barcoding to track the changes in intratumor heterogeneity during treatment with palbociclib and paclitaxel combined with JQ1. We found that the combination treatments exerted the strongest evolutionary pressure, as they selected for the smallest number of subclones. Although we detected a pre-existing RB1-mutant subpopulation that expanded in response to JQ1+palbociclib, mathematical modeling and genomic profiling
suggested high rates of *de novo* acquired resistance and increased phenotypic heterogeneity in the resistant population, suggesting multiple mechanisms of resistance.

Lastly, we found that prolonged treatment with JQ1 and palbociclib produced resistant cells that were tetraploid. Using fluorescent reporters, we investigated the dynamics and mechanisms of tetraploidization and found that JQ1 and palbociclib directly induce tetraploidy through disruption of mitosis and cytokinesis.

In summary, we have identified novel combination therapies for TNBC and investigated their impact on tumor evolution, as well as their previously unrecognized effects on cell division. Our findings provide rationale for further preclinical and clinical study of combined BET and CDK4/6 inhibition and suggest new vulnerabilities in resistant cells that may inform second-line treatments following progressive disease.
# Table of Contents

Abstract .......................................................................................................................... iii  
Table of Contents ........................................................................................................... v  
List of Figures ............................................................................................................... vi  
List of Tables ................................................................................................................. vii  
Acknowledgements ....................................................................................................... viii  
Attributions .................................................................................................................... x  
Chapter 1: Introduction ................................................................................................. 1  
  Epidemiology and classification of breast cancer ......................................................... 2  
  Biology and treatment of triple-negative breast cancer .................................................. 3  
  BET family proteins as a novel epigenetic target ............................................................ 8  
  Intratumor heterogeneity, tumor evolution, and treatment resistance ....................... 12  
    Models of tumor evolution ......................................................................................... 14  
    Sources of intratumor heterogeneity ......................................................................... 16  
    Clinical implications .................................................................................................. 17  
  Overview of dissertation ............................................................................................. 18  
Chapter 2: Genetic screens identify synergistic BET inhibitor combinations .............. 28  
Chapter 3: Population dynamics in BET inhibitor combination therapies ................... 70  
Chapter 4: Combined BET and CDK4/6 inhibition induces mitotic failure and tetraploidy…… 108  
Chapter 5: Conclusion ................................................................................................. 129  
  Overview ...................................................................................................................... 130  
  Major findings and implications ............................................................................... 131  
  Conclusions and future directions ......................................................................... 133  
Appendix A: Experimental compounds ....................................................................... 138  
Appendix B: Mathematical modeling ............................................................................ 143
List of Figures

Figure 1.1: Clinical and biological characteristics of TNBC. .......................................................... 4
Figure 1.2: Function of BET proteins. .............................................................................................. 9
Figure 1.3: Response and resistance to BET inhibitors in TNBC. ................................................. 11
Figure 1.4: Clonal evolution and intratumor heterogeneity in cancer. .................................... 13
Figure 2.1: CRISPR/Cas9 screen. .................................................................................................. 33
Figure 2.2: Small molecule screen. ............................................................................................... 35
Figure 2.3: Synergy studies with various compounds combined with JQ1 in SUM159, SUM149, and their derived JQ1-resistant lines. ................................................................. 38
Figure 2.4: Synergy studies with JQ1 and palbociclib in a panel of TNBC cell lines. ............ 40
Figure 2.5: Effects of JQ1 combinations on cell-line and patient-derived xenografts 41
Figure 2.6: Histology of xenografts treated with BET inhibitor combinations. .......... 43
Figure 2.7: Effects of JQ1 combinations with palbociclib and paclitaxel on SUM159 in vitro. .... 45
Figure 2.8: RNA-seq of xenografts treated with JQ1, palbociclib, and paclitaxel combinations. 46
Figure 2.9: Differentially expressed cell cycle genes in xenografts treated with JQ1, palbociclib, and their combination. ................................................................. 48
Figure 2.10: Single cell RNA-seq of xenografts treated with JQ1 and paclitaxel. ............... 52
Figure 2.11: Differentially expressed genes between clusters of tumor cells in xenografts treated with JQ1 and paclitaxel. ................................................................. 53
Figure 2.12: Differentially expressed genes between clusters of mouse immune cells in xenografts treated with JQ1 and paclitaxel. ................................................................. 54
Figure 2.13: Optimal drug schedule varies between cell lines .................................................... 56
Figure 3.1: Tumor diversity in xenografts after JQ1 combination treatments. ........................ 75
Figure 3.2: Population diversity in SUM159 cells during in vitro selection with JQ1 combination treatments. ................................................................. 77
Figure 3.3: Clonal expansion of cells selected with JQ1 combinations. .................................... 80
Figure 3.4: Proportions of shared barcodes between replicates. .................................................. 81
Figure 3.5: Design of the mathematical model of barcode evolution during treatment. ....... 84
Figure 3.6: Comparison of Shannon indices of simulated vs. experimental data. ................. 86
Figure 3.7: Comparison of proportion of shared barcodes in simulations of JQ1 treatment vs. experimental data. ................................................................. 88
Figure 3.8: Comparison of proportion of shared barcodes in simulations of palbociclib treatment vs. experimental data. ................................................................. 89
Figure 3.9: RB1 loss is one mechanism of resistance to JQ1+palbociclib. .............................. 90
Figure 3.10: RNA-seq of cells following selection in JQ1 and palbociclib. ......................... 92
Figure 3.11: Single cell RNA-seq reveals increased heterogeneity in JQ1+palbociclib post-selection populations. ........................................................................... 94
Figure 4.1: Cells that develop resistance to JQ1 and palbociclib are tetraploid. ............... 112
Figure 4.2: Combined BET and CDK4/6 inhibition directly induce tetraploidy. .............. 115
Figure 4.3: Tetraploidy is not induced by cell fusion or by selection of pre-existing tetraploid cells. ........................................................................... 117
Figure 4.4: Live imaging of cells following treatment with JQ1 and palbociclib. ............... 119
Figure 4.5: JQ1 and palbociclib disrupt expression of cell cycle genes. ......................... 121
Supplementary Figure A.1: Pharmacokinetics of selected compounds tested in vivo. 142
Supplementary Figure B.1: Proportions of shared barcodes between replicates in simulated data. ........................................................................... 149
List of Tables

Table 3.1: Growth and death parameters used in the mathematical model of barcode selection ................................................................. 85
Supplementary Table A.1: Compounds tested in \textit{in vitro} synergy studies .................................................. 139
Supplementary Table A.2: JQ1 and palbociclib concentrations tested in TNBC panel .......... 140
Supplementary Table A.3: Compounds tested \textit{in vivo} ......................................................................................... 141
Acknowledgements

My first thank you has to go to Nelly and Franziska for their constant mentorship and support, both academically and personally, throughout my PhD. They put their confidence in me from day 1 (maybe too much confidence!) and encouraged me to pursue a project that challenged and motivated me to become the best scientist I can be. Their dedication to research that will make a difference to patient care has been so inspiring to me, as is their dedication to the success and well being of all their trainees. They are two of the kindest and most brilliant people I know, and I am so honored to have been able do my training with both of them.

Thank you to all the members of the Polyak and Michor labs, who have been so supportive and amazing to work with. I have learned so much from you guys, and you have all contributed so much to this project. Thank you especially to Michalina for teaching me everything when I first joined the lab; Laura and Anne for the great times playing Pokemon Go and HQ Trivia and for sharing their snacks; Shaokun, for starting the project on BET inhibitors and doing all work that led us to the combinations; and Bojana, for our marathon cake decorating sessions. Thank you also to Carlos, Robert, Masa, Natalie, Doris, Anthony, Ekram, Sungjin, Ece, Ben, Anushree, Kat, and Nick for all the great conversations over lunch and coffee, Jimmy Fund Walks, bike rides, and housewarming (or bathroom-warming) parties. I feel so lucky to have been able to come to work with you guys every day, and 4 years really does go by fast.

Thank you to Myles, David, and Cathy for your mentorship on my DAC. I know you are all very busy and important (and hard to schedule) people, and I so appreciate the time you took to guide this project. Thank you to Loren, Amy, Yi, Jen, and Lisa in the MD-PhD office for everything you guys do to support us, from organizing events to helping with fellowship applications, to providing chocolate, and raising enough money so I don’t have to pay for the rest of med school! I am so grateful that you guys are always looking out for us. Thank you also
to my HST and MD-PhD class, Rick and Patty, as well as Kate, Tucker, Danny, and everyone else in the BBS office.

Thank you to all my past mentors, Bill Thompson, Martha Bulyk, David Rand, Jim Mossman, and Zhijin Wu, for everything they taught me that prepared me so well for grad school and for their enthusiasm and support that encouraged me to keep pursuing science. Thank you also to Jay Bradner and Jun Qi, the people who invented JQ1, one of the first small molecule BET inhibitors. Jay probably doesn’t remember this, but he actually did my HST interview almost 7 years ago. I was terrified that day and was pretty sure that the interview was not going to go well, but Jay was so kind, and we had a great conversation. Of course I had no idea who he was at the time or that I would end up studying his drug, so it was serendipitous that I was able to cross paths with him at that point, and without him I would literally not be here today.

A huge thank you to my dog Arnold, who I adopted 4 years ago and who has been with me for nearly all of grad school. He is the happiest and most easy-going dog, his tail is always wagging, and he is just the best doggy a girl could ask for. He has taught me to take it easy, relax, and have fun no matter where life takes you. I also have to thank Arnold for introducing me to all the people and dog friends I have made. Thank you, Seth and Huddie; Cora and Seamus; Kelsey, Ian, and ChaCha; Dan and Tesla; Max, Havens, Rigby, and Baby Ivy; Claire and Bilbo; Heesun and Keewi; Hieu, Angie, and Baby Royce; and Claire, Amanda, and Maggie, for helping me start off each day right at Dog Party, for being the best trivia team on Mission Hill, for the movie nights and cook outs, and of course for taking care of Arnold when I got stuck in lab. You guys have been as big a part of my grad school experience as anyone. Thoughts and prayers for Ike and Little Jerry.

Most important of all, thank you to my parents and grandparents. Without their love, their time, and their support of my education, I would not be lucky enough to be where I am today. Thank you!!
Attributions

Chapter 1:
J.Y.G. wrote the chapter.

Chapter 2:
S.S. performed the CRISPR/Cas9 screen, which was analyzed by H.W. and B.W. with supervision from X.S.L. I.H. and J.E. performed the small molecule screen and analysis with supervision from J.B. J.Y.G. performed in vitro proliferation, synergy, and flow cytometry studies. J.Y.G. and S.S. performed xenograft studies. J.Y.G. and S.S. performed extracted RNA for bulk RNA-seq. A.L. prepared the single cell RNA-seq libraries with supervision from K.W. J.Y.G. performed analysis on RNA-seq data. K.P. and F.M. supervised the overall study with help from M.B.

Chapter 3:
J.Y.G. performed all cell culture experiments, DNA barcode libraries preparations, and RNA extractions, as well as barcode and RNA-seq analyses. S.S. performed the lentiviral infection of the DNA barcode library. Y.K. and G.A.H. performed the digital droplet PCR with supervision from C.P. A.F. created the \textit{RB1} knockout line with supervision from P.S. J.Y.G. and T.O.M. designed the mathematical model, and J.Y.G. performed the computer simulations. A.L. performed single cell RNA-seq library preparation with supervision from K.W. A.T. performed the exome sequencing and data analysis. J.Q. provided the JQ1 compound. K.P. and F.M. supervised the overall study with help from M.B.

Chapter 4:
J.Y.G. performed all cell culture experiments, FACS experiments, and data analyses. J.Y.G. and A.T. performed lentiviral infections of cell lines. M.K. created the pQC-mCherry plasmid and
performed the live imaging with supervision from D.P. J.Q. provided the JQ1 compound. K.P. and F.M. supervised the overall study with help from M.B. and D.P.

*Chapter 5:*

J.Y.G. wrote the chapter.
Chapter 1:

Introduction
**Epidemiology and classification of breast cancer**

Breast cancer is the most commonly diagnosed cancer amongst women worldwide and the second leading cause of cancer deaths for women in the United States\(^1,2\). In 2019, there will be an estimated 269,000 new cases of invasive breast cancer and 63,000 new cases of non-invasive breast cancer among women in the U.S., as well as 2,700 cases of invasive breast cancer in men\(^2\). In addition, about 42,000 U.S. women are expected to die from breast cancer in 2019\(^2\). Overall, about 1 in 8 women will develop invasive breast cancer in her lifetime\(^2\).

Breast cancer is not one disease but a group of biologically defined subtypes. Clinically, it is classified according to three biomarkers: the hormone receptors for estrogen (ER) and progesterone (PR), and human epidermal growth factor receptor 2 (HER2). The presence or absence of staining for these receptors by immunohistochemistry (IHC) defines three clinical subtypes: ER or hormone receptor-positive (ER/PR\(^+\)), HER2-positive (HER2\(^+\)), and triple-negative (ER\(^-\), PR\(^-\), HER2\(^-\))\(^3\). Breast cancer has also been classified into four molecular subtypes based on gene expression profiling: luminal A and luminal B, which primarily comprise ER\(^+\) breast cancers; HER2-enriched, which correlate with clinically ER\(^-\), PR\(^-\), HER2\(^+\) cancers; and basal, which are generally triple-negative\(^3,4\). However, the definitions of these molecular subtypes are not universally agreed upon, and thus they are not used in clinical practice.

The subtypes of breast cancer have important implications for therapy and prognosis. ER-positive breast cancers are the most common, comprising approximately 80% of all breast cancers\(^3,5,6\). Tumors of this subtype are dependent on signaling from the estrogen receptor for proliferation and can be inhibited by blocking the receptor or blocking estrogen production. Thus, these patients are candidates for endocrine therapy, with or without chemotherapy, along with local treatment of disease with surgery or radiation\(^7\). Likewise, patients with cancers that overexpress HER2 (about 20% of breast cancers\(^3,5,6\)) benefit from therapies that block the HER2 receptor\(^7\).
However, 15-20% of breast cancers lack expression of all three receptors and thus are categorized as triple-negative breast cancer (TNBC)\textsuperscript{3,5,6}. Because TNBCs do not express ER or HER2, the drugs that have been developed to target those receptors cannot be used, and chemotherapy remains the only approved pharmacologic option\textsuperscript{8}. TNBC has a higher incidence in young African American and Hispanic women and is associated with more aggressive, higher-grade disease and poorer prognosis than other subtypes of breast cancer, with a 5-year survival of 79%\textsuperscript{5,6,8-10} (Figure 1.1A). Compared with other subtypes, there is a high risk of early recurrence, and the difference in survival rates is greatest within the first 2 years following diagnosis (although, TNBC does have a lower rate of late recurrence)\textsuperscript{9}. Thus, the limited treatment options and overall poor survival in TNBC represents a significant unmet medical need.

**Biology and treatment of triple-negative breast cancer**

Because TNBC is an ill-defined classification, being only distinguished by the absence of markers, it is a highly heterogeneous disease. This inter-tumor heterogeneity poses a significant challenge to treatment, as it hinders identification of a common effective target and development of a therapy that is successful for all cases. Therefore, there have been considerable efforts to better understand the underlying drivers and biological characteristics of the disease.

The advent of next-generation sequencing has enabled comprehensive molecular profiling and spurred the search for recurrent mutations in each cancer type that can be targeted. In 2012, Shah et al. and Curtis et al. reported on the genomic and transcriptomic landscapes in triple-negative, basal, and other types of breast cancer\textsuperscript{11,12}. They observed a wide range of the extent of the mutational burden among TNBC tumors that is distinct from other subtypes\textsuperscript{11,12}. By far the most common mutation was \textit{TP53} (62%), but \textit{PTEN}, \textit{RB1}, \textit{PIK3CA}, \textit{USH2A}, and \textit{MYO3A} were also among the top mutated genes, albeit each at a prevalence of
Figure 1.1: Clinical and biological characteristics of TNBC. 
(A) Overall survival by subtype after a diagnosis of breast cancer. Adapted from Onitilo et al. 2009\textsuperscript{5}. (B) Heterogeneous features observed between TNBCs. Adapted from Metzger-Filho et al. 2012\textsuperscript{13}.
10% or less. However, only 20% of cases contained potentially “actionable” mutations, i.e. mutations for which there were available targeted therapies\textsuperscript{12}. Using an integrated pathways approach, basal tumors were found to be generally enriched in transcriptional changes involving cell cycle, DNA repair, and apoptosis, with many sharing dysregulation of p53, \textit{PTEN}, \textit{PIK3CA} pathways\textsuperscript{11,12}. However, they also varied by additional involvement of other pathways, including chromatin remodeling, ERBB signaling, integrin signaling, and WNT/cadherin\textsuperscript{12}.

Further study on large TNBC cohorts yielded more insight into the heterogeneity and possible sub-subtypes of TNBC. In their landmark study, Lehmann et al. reanalyzed previously published gene expression data from various cohorts, totaling 587 TNBC cases, and identified 6 clusters: two basal-like (BL1 and BL2), immunomodulatory (IM), mesenchymal (M), mesenchymal stem-like (MSL) and luminal androgen receptor (AR)\textsuperscript{14}. Subsequently, Burstein et al. performed analysis on 198 TNBC cases, which they clustered into 4 subtypes: luminal androgen receptor (LAR), mesenchymal (MES), basal-like immunosuppressed (BLIS), and basal-like immune-activated (BLIA)\textsuperscript{15}. These studies generally agreed on the luminal androgen and mesenchymal types, but there was discordance between the two basal-like groups from each study, while Lehmann’s immunomodulatory group classified as either mesenchymal or basal-like immune-activated\textsuperscript{15}. These proposed subtypes were found to possess differential pathway expression, drug sensitivities, and prognoses, suggesting new treatment targets and better personalization of therapy.

One important characteristic that has long been known of TNBC is its deficiency in DNA damage repair and genomic instability. Of those women with germline \textit{BRCA1} mutations, 75% develop triple-negative disease\textsuperscript{10}. Furthermore, although the majority of TNBCs do not arise from germline \textit{BRCA1/2} mutations (termed sporadic TNBC), many still possess a phenotype of DNA damage repair deficiency or “BRCAness,” which may be due to epigenetic modifications of \textit{BRCA1} or aberrations in other members of the pathway\textsuperscript{13}. Mutations in other DNA repair genes, such as \textit{PALB2}, \textit{FANCM}, and \textit{RAD51}, have been implicated in TNBC carcinogenesis as well\textsuperscript{16}. 
The DNA damage pathways are most correlated with Lehmann’s basal-like 1 and 2 subtypes\textsuperscript{14}. Thus, it was hypothesized that TNBCs would have increased sensitivity to platinum-based chemotherapies, which crosslink and damage the DNA, leading to cell death in the absence of repair. Based on clinical trials that have shown high response rates to carboplatin in \textit{BRCA1/2} mutated tumors, platinum agents are considered appropriate first-line therapies for metastatic disease in carriers\textsuperscript{17,18}. However, the benefit has not been shown for patients with high “BRCAness” scores but without a germline \textit{BRCA1/2} mutation\textsuperscript{18}. PARP inhibitors have also received much attention as another way to target DNA repair and are now approved for germline \textit{BRCA1/2}-mutant breast cancer. Cells deficient in homologous recombination repair due to loss of \textit{BRCA1/2} are rendered dependent on PARP proteins for single-strand DNA repair\textsuperscript{19}. Recently, two clinical trials have demonstrated improved progression-free survival (PFS) in metastatic breast cancer with a germline \textit{BRCA} mutation\textsuperscript{20,21}. However, cells can potentially become resistant through restoration of BRCA function with reversion of the mutation or epigenetic silencing\textsuperscript{19}.

Another distinctive characteristic of TNBC is the immune response. Compared to other subtypes, TNBCs express higher levels of PD-L1 and are more immunogenic, possibly due to their increased genome instability and mutation rates, which can generate more neoantigens\textsuperscript{22}. However, there exists heterogeneity in the amount of immune activation. Numerous studies have now demonstrated that expression of immune response genes and amount of tumor-infiltrating lymphocytes (TIL) are associated with better responses to chemotherapy and better outcomes\textsuperscript{22}. These findings suggest that TNBC or a subset of TNBCs could have a favorable response to anti-PD-1 or PD-L1 immunotherapy. The recently published phase III IMpassion130 trial tested the addition of atezolizumab to nab-paclitaxel in metastatic TNBC, and among patients with PD-L1\textsuperscript{+} tumors, atezolizumab prolonged overall survival from 15.5 to 25.0 months\textsuperscript{23}. Treatment with other checkpoint inhibitors, combination therapies, as well as other modalities of immunotherapy are actively being investigated.
About 30% of TNBCs express the androgen receptor (AR), making them possible candidates for anti-androgen therapy. Lehmann’s AR subtype was found to be the most distinct from the other TNBC subtypes, as these were mostly luminal rather than basal. Two phase II clinical trials have shown clinical benefit rates (CBR) of AR antagonists of 19% and 33% in AR+, ER/PR- metastatic disease. Additional phase II and III studies are ongoing.

Other avenues for therapy in TNBC have also been explored. The luminal androgen receptor subtype was found to have frequent PIK3CA mutations, and cell lines of this subtype were sensitive to inhibition of the PI3K/mTOR pathway. The mTOR inhibitor everolimus has been tested in two phase II TNBC trials as an addition to chemotherapy in the neoadjuvant setting; however, it did not increase response rates while it did increase toxicity. In addition, EGFR overexpression is often a feature of TNBC, but trials of the EGFR monoclonal antibody cetuximab as monotherapy and combined with chemotherapy have not demonstrated efficacy. Finally, studies have not shown any benefit from addition of the anti-angiogenic agent bevacizumab.

The defining characteristics of TNBC are also the ways in which it is heterogeneous, thus making treatment especially challenging (Figure 1.1B). Some targeted therapies in clinical development show some efficacy, but benefits to PFS have so far been modest, on the order of months. There is currently a lack of any validated biomarkers in clinical practice that could indicate even a subset of TNBC patients who are more likely to respond. In addition, these targeted therapies have generally been studied in the metastatic setting, while no new drugs have been approved for non-metastatic TNBC. Yet, non-metastatic TNBC has a high risk of early recurrence following neoadjuvant or adjuvant chemotherapy. Thus, new therapies are still sorely needed.
BET family proteins as a novel epigenetic target

In recent years, epigenetic regulators have emerged as novel dependencies and therapeutic targets in cancer. Among those that have received much attention are the bromodomain and extra-terminal domain (BET) family of proteins, which include BRD2, BRD3, BRD4, and BRDT. BET proteins contain two bromodomain motifs that recognize acetylated lysine residues on histone tails and transcription factors and thus function as epigenetic readers\textsuperscript{28,29}. BET proteins occupy promoters and enhancers and, through their extra-terminal (ET) and C-terminal domains (CTD), recruit various transcriptional regulatory complexes, including chromatin modifiers, transcription factors, and transcriptional co-activators (Figure 1.2A)\textsuperscript{29}. For instance, BRD4 specifically is known to recruit P-TEFb to the chromatin, which phosphorylates RNA polymerase II and thereby promotes transcriptional elongation (Figure 1.2A)\textsuperscript{30}. Thus, BET proteins couple the histone acetylation mark, which is associated with open chromatin and activation, to transcription.

Functionally, BET proteins are involved in the control of cell cycle as well as cellular differentiation (Figure 1.2B). BRD2 associates with the E2F transcription factors which are central to the G1/S phase transition\textsuperscript{31}, and BRD4 is necessary for the mitotic spindle checkpoint by regulating the expression of Aurora B\textsuperscript{32}. BRD2 and BRD4 also remain bound to chromatin during mitosis and prime a set of genes for rapid transcription upon completion of cytokinesis\textsuperscript{33}. In addition, BET proteins bind to a number of transcription factors to act as co-regulators of several lineage-specific transcriptional programs, including GATA1 for hematopoiesis, NF-κB for inflammatory cytokines, PPARγ for adipogenesis, and MyoD for myogenesis\textsuperscript{29,34}. However, their cell-type specific roles are not completely understood.

In several cancer types like multiple myeloma, leukemia, and lymphoma, BET proteins have been shown to drive transcription of key oncogenes such as MYC and BCL2 by localizing to enhancers\textsuperscript{35-39}, and in the rare cancer NUT midline carcinoma, BRD4 is even mutated
Figure 1.2: Function of BET proteins.
(A) BET proteins such as BRD4 recognize acetylated residues on histones and transcription factors and recruit chromatin modifiers and transcriptional co-activators to the chromatin, including the Mediator complex, JMJD6, and P-TEFb. Adapted from Shi and Vakoc, 2014. (B) Various functional networks are co-regulated by BET proteins through interactions with transcription factors and other proteins. Adapted from Belkina and Denis, 2012.
itself to form a proto-oncogene\textsuperscript{40}. The role of BET proteins may be especially important in large regulatory regions called super-enhancers, which contain a high density of enhancers and are enriched for transcriptional co-activators and histone marks\textsuperscript{41}. It has been proposed that cancer cells have enrichment of super-enhancers at oncogenes and that these super-enhancers are particularly sensitive to BRD4 inhibition, giving BRD4 some degree of specificity for malignant cells\textsuperscript{35,39}. Therefore, the BET family of proteins is an attractive target for therapy as they are critical in a variety of cancers to the function of oncogenes, which have otherwise been regarded as undruggable.

Recently, several small molecule inhibitors have been developed, including JQ1, I-BET, and OTX015, that block the binding of BET proteins to the chromatin, thereby inhibiting oncogene expression and cell proliferation\textsuperscript{36,42-44}. The prototypical JQ1 has been widely studied in NUT midline carcinoma, multiple myeloma, leukemias, lymphomas, prostate cancer, ovarian cancer, and lung cancer, to name a few, and is able to induce a range of effects such as differentiation, growth arrest, senescence, apoptosis, and anti-tumor immunity\textsuperscript{36,37,42,45-47}. There are currently about a dozen active clinical trials of BET inhibitors in the U.S., including phase I and II studies in hematologic and solid cancers, not to mention one phase III in coronary artery disease. In completed early phase trials, the most commonly reported toxicities have been thrombocytopenia, diarrhea, fatigue, and anorexia\textsuperscript{48-50}.

Previously, our group and others have demonstrated the efficacy of BET inhibitors in TNBC\textsuperscript{51,52}. In 2016, Shu et al. tested JQ1 and other BET inhibitors in a panel of breast cancer lines and found that sensitivity to BET inhibition was correlated with the basal subtype (Figure 1.3A)\textsuperscript{51}. JQ1 also inhibited tumor growth in SUM159, MDA-MB-231, and patient-derived xenografts (Figure 1.3B)\textsuperscript{51}. JQ1 was found to displace BRD4 from the chromatin, disrupting expression of super-enhancer associated genes (Figure 1.3C) and thus causing G1 arrest and apoptosis\textsuperscript{51}. However, cells rapidly developed resistance to JQ1, and derived resistant lines
Figure 1.3: Response and resistance to BET inhibitors in TNBC.
(A) Heat map of IC50s for various BET inhibitors and inactive analogues in a panel of breast cancer cell lines. (B) Tumor weights of cell-line (SUM159 and MDA-MB-231) and patient-derived (IDC50X and EL12-58) xenografts following treatment with JQ1 for 2 weeks. (C) Top super-enhancer associated genes targeted by JQ1. (D) Models of resistance to BET inhibitors. In resistant TNBC, BRD4 activates transcription in the presence of BET inhibitors through bromodomain-independent binding to the chromatin through MED1, following phosphorylation by activated CK2. Adapted from Shu et al. 201651 and Settleman, 201653.
gained super-enhancers such as BCL-xL but were still dependent on BRD4 binding to the chromatin\textsuperscript{51}. BRD4 was found to employ indirect, bromodomain-independent binding to the chromatin through MED1 (Figure 1.3D)\textsuperscript{51}. Because of the development of resistance, effective combination therapies must be developed in order to prevent, or at least delay, resistance and to extend the efficacy of BET inhibition.

**Intratumor heterogeneity, tumor evolution, and treatment resistance**

The emergence of resistance in tumors stems from their intratumor genetic and phenotypic heterogeneity, which provide the substrate for tumor evolution\textsuperscript{54}. A single tumor may contain multiple subclones with differential sensitivities to treatment so that when therapy is given, sensitive cells are eliminated, allowing for expansion of resistant clones which are responsible for recurrence and disease progression. Thus, cancer cell populations are subject to the same evolutionary forces that act on populations of organisms. In 1976, Peter Nowell first proposed the clonal evolution model of cancer, which postulated that cancers arise and develop through the stepwise selection of subclones with variants that confer growth advantages and biological properties necessary at each stage of tumor progression\textsuperscript{55}.

Thus, evolution shapes not only the tumor’s development of resistance but also all other aspects of tumor progression (Figure 1.4A). The classic model of tumorigenesis involves the sequential accumulation of mutations, as was famously described by Fearon and Vogelstein in 1990, where they linked alterations in specific oncogenes and tumor suppressor genes to the stages of colorectal cancer\textsuperscript{56}. Initiation of treatment strongly influences the direction of the tumor’s evolution, as resistant cells that were either a pre-existing subpopulation or acquired resistance over the course of treatment gain a growth advantage and lead to relapsed disease. Lastly, in the formation of distant metastases, alterations are selected for that allow cells to successfully complete the multiple steps of the metastatic cascade, including local invasion,
Figure 1.4: Clonal evolution and intratumor heterogeneity in cancer. (A) Clonal evolution over the course of tumor progression, from initiation, diagnosis, and treatment, to relapse and metastasis. Adapted from Yates and Campbell, 2012. (B) Models of tumor evolution. Adapted from Davis et al. 2017. (C) Examples of cell-intrinsic and cell-extrinsic sources of heterogeneity. Adapted from McGranahan and Swanton, 2017.
intravasation, survival in the circulation, extravasation, and colonization in the foreign microenvironment. This explains the increased aggressiveness of tumor cells at metastatic sites, as well as the heterogeneity between primary and metastatic lesions and between metastatic lesions at separate sites. It is important to emphasize that fitness is always context-dependent and relative fitness can change depending on the tumor site, as well as with treatment.

Models of tumor evolution

Several models of tumor evolution have been proposed to describe the initiation and progression of cancer (Figure 1.4B). A mechanism of stepwise accumulation of mutations in tumorigenesis suggests a linear model of evolution, where rare subclones that develop advantageous alterations successively outcompete all other cells in clonal sweeps. This model would imply that tumors are largely homogenous, arising from a single common ancestor cell that contained all of the necessary mutations for malignant transformation. In contrast, in branching evolution, multiple subclones are able to expand simultaneously and coexist within the tumor, leading to intratumor heterogeneity. All cells though may still share common “trunk” mutations, which are derived from their common ancestor and are thought to be early driver events. An extension of branching evolution is neutral evolution, where no clones have any selective advantage and subclones and expand or go extinct due to genetic drift. Thus, under neutral evolution, tumors would have extensive intratumor heterogeneity. Finally, a newer model of punctuated evolution has been proposed in which, rather than a gradual accumulation of mutations, tumors go through short periods of rapid mutagenesis that produce large-scale aberrations and tremendous heterogeneity. This may be followed by expansion of only a few clones.

Numerous multi-regional sequencing studies have demonstrated extensive heterogeneity in many cancer types, supporting a model of branching evolution. Gerlinger et al.
performed exome sequencing on multiple primary and metastatic sites in renal cell carcinoma patients and reconstructed a branched phylogenetic relationship between the regions. Anderson et al. used fluorescence in situ hybridization (FISH) to analyze 30 cases of acute lymphoblastic leukemia, the majority of which were found to have complex branching architecture. Thus, assessment of clonal architecture does not necessarily require high-throughput sequencing and could also be done with few loci. In TNBCs, Shah et al. found a wide range of clonal frequencies, with some tumors containing only a few clones, while others exhibited more extensive clonal evolution. Nevertheless, basal tumors on average still had a greater number of clones than non-basal tumors. They also found that known drivers typically had the highest clonal frequencies, indicating that they are early events. Such studies have also been performed in glioblastoma, chronic lymphocytic leukemia, and lung adenocarcinoma.

However, the mode of evolution may vary across different cancers. Williams et al. found that about one-third of the tumors they analyzed across cancer types had a distribution of mutation frequencies consistent with their model of neutral evolution. However, Tarabichi et al. argued that this model used inappropriate simplifying assumptions about ploidy and stochasticity of cell division and that the model was not sufficient to detect weak selection. Sottoriva et al. proposed a related “Big Bang” model of colorectal cancer development, in which most of the observed intratumor heterogeneity is generated early on during initial tumor formation in a period of punctuated evolution, which is then followed by largely neutral clonal expansion. Evidence of punctuated evolution has been reported in other studies as well, such as in prostate tumors that had signatures of chained chromosomal rearrangements that likely occurred together and in breast tumors that had one or two clonal aneuploid populations that diverged from the diploid population early on. Mechanisms for these rapid large-scale changes include chromoplexy, in which multiple chromosomes break and are rearranged into a new configuration, as well as chromothripsis, which is found in 2-3% of all cancers and
involves rearrangement of one or a few chromosomes that have shattered into tens to hundreds of fragments.\(^7\)

In order to achieve successful treatment of cancer, it is critical to understand how tumors evolve to develop resistance to existing therapies, as well as to new drugs in development. Numerous resistance mutations have now been documented for various targeted agents, including the T790M mutation that confers resistance to EGFR inhibitors\(^7\), secondary mutations in ALK that confer resistance to ALK inhibitors\(^7\), and secondary KIT mutations that confer resistance to imatinib\(^7\). Such mutations have frequently been found to be pre-existing in the tumor as a minor subclone even before the initiation of treatment\(^7\). The alternative route is de novo acquired resistance. Although the absence of variants in sequencing may not rule out pre-existing resistance, since the mutation may occur at such a low frequency as to be undetectable, there is nevertheless evidence for acquired resistance through deep sequencing and dilution cloning studies.\(^7\) In addition, certain chemotherapies are thought to increase genomic instability and the mutation rate, which may accelerate de novo acquisition of resistance. The cause of resistance may not be genetic though but rather epigenetic. This lends itself to yet another model of drug resistance, in which cells that are in a drug-tolerant, potentially transient state are first selected for, and stable genetic resistance is later acquired within this population.\(^8\)

**Sources of intratumor heterogeneity**

There are multiple reasons why heterogeneity develops within a tumor and why it is maintained (Figure 1.4C). First, cell intrinsic mechanisms include the increased genomic instability and elevated somatic mutation rates seen in many tumors, which can be due to mutations in mismatch or double-strand break repair genes or deficiencies in chromosomal segregation.\(^6\) Basal breast cancers have significantly higher clonality than non-basal types\(^1\), and TNBC has been found to have a 13 times higher mutation rate than normal cells, with a
subset that can be distinguished by its genomic instability\textsuperscript{14,84}. In addition, epigenetic heterogeneity, such as cell-to-cell variation in DNA methylation and histone modifications, contribute to heterogeneity in transcription and phenotypic cell state\textsuperscript{54}. Cancer cells are thought to have increased transcriptional stochasticity and plasticity\textsuperscript{86}. On the other hand, cell extrinsic mechanisms involving aspects of the tumor microenvironment may select for different clones in different niches due to variations in contact with immune and stromal cells, extracellular matrix, levels of hypoxia, and drug delivery\textsuperscript{54}. Furthermore, clonal cooperation may support the maintenance of heterogeneity if, for example, a subset of cells secretes factors that drive proliferation of all other cells, and thus the drivers may not necessarily be the dominant clone in the population\textsuperscript{86}.

\textit{Clinical implications}

Heterogeneity is a feature of virtually all cancers\textsuperscript{87} and must be considered in drug development, diagnosis, risk stratification, and choice of therapy. Intratumor heterogeneity poses a significant challenge to the development of targeted therapies, as not all cells within the population may depend on the same driver gene, and subclones may possess resistance mutations or bypass mechanisms. Unfortunately, this means that there is almost never a durable response to these agents, which limits their benefits, especially as monotherapies, to overall survival\textsuperscript{60}. Targeting trunk mutations, i.e. mutations that occur early in tumor development and are likely to be present in a majority of cells, is an attractive strategy; however, these early drivers may not always be the dominant clone\textsuperscript{12} or be responsible for driving the tumor at later stages and may not even be “druggable.” During diagnosis and disease management, assessment of heterogeneity may be predictive of treatment outcome and inform the choice of therapy, as clonality is associated with prognosis\textsuperscript{87,88}. However, a single biopsy may not reflect the heterogeneity of the whole tumor, and even with multi-region sampling, important subclones may be missed or simply be too rare for detection. Finally, potential clonal
divergence in metastases presents an even greater problem for biopsy and treatment of late-stage disease.

Numerous open questions remain that warrant further study. There is a need to better understand the changes in heterogeneity in response to treatment, in order to anticipate resistance and plan management of recurrent and metastatic disease. Does long-term therapy induce convergent or divergent evolution, and does the tumor undergo increased branching evolution resulting in polyclonal resistance? The question of whether resistance is pre-existing or acquired may point to the mode of resistance to be expected, as well as whether therapy should be administered aggressively to eliminate persister pools or metronomically to delay selection for resistant subclones\textsuperscript{89}. In clinical practice, monitoring changes in heterogeneity remains an issue, as biopsies may underestimate heterogeneity, and repeated biopsies of solid tumors are usually not feasible. Circulating tumor cells and DNA are being investigated as methods for liquid biopsies, but further study is needed to assess their accuracy and clinical relevance\textsuperscript{90}. Lastly, is evolution itself targetable, and can proteins such as epigenetic modifiers be modulated in order to limit tumor stochasticity\textsuperscript{91,92}?

**Overview of dissertation**

TNBC is an aggressive subtype of breast cancer that currently has limited treatment options beyond chemotherapy. Numerous characteristics distinguish it from other types of breast cancer, both in terms of its underlying biology and its clinical behavior. Yet, the considerable amount of heterogeneity seen both between and within TNBC tumors has made it especially challenging to develop successful treatments. Inhibitors of BET proteins, such as the prototypical JQ1, have recently been shown to be effective in preclinical models of TNBC. However, the rapid development of resistance necessitates the identification of combination therapies, in order to achieve better responses, and investigation of their effects on intratumor heterogeneity, in order to understand how resistance develops.
In Chapter 2, we performed CRISPR/Cas9 and small molecule screens in order to rationally design combination therapies involving JQ1. We identified a number of hits that we validated with in vitro synergy studies and in vivo studies on cell-line and patient-derived xenografts. We found that targeting the cell cycle was generally an effective strategy in combination with BET inhibition, and we chose palbociclib, a CDK4/6 inhibitor, and paclitaxel, a microtubule-inhibiting chemotherapy, for further study. The combinations induced cell cycle arrest and modest increases in apoptosis.

In Chapter 3, we used DNA barcoding to study the changes in clonality in cell populations during treatment with JQ1, palbociclib, and paclitaxel alone and in combinations. We observed that short-term in vivo treatments maintained tumor diversity due to predominantly cytostatic effects. We then characterized the population dynamics with long-term in vitro treatments, in which we found that the combination treatments reduced the population diversity most and therefore exerted the strongest selective pressure. We used mathematical modeling, exome sequencing, RNA-seq, and single cell RNA-seq to investigate the evolutionary path and resulting mechanisms of resistance to JQ1 and palbociclib. We found that although a small subpopulation of RB1-mutant cells were selected for, the cells also had high rates of de novo acquired resistance, and there was increased phenotypic heterogeneity in the post-treatment population, suggesting multiple mechanisms of resistance.

In Chapter 4, we found that cells resistant to JQ1, palbociclib, and the combination became tetraploid after long-term selection. We used fluorescent reporter cells to demonstrate that this tetraploidization is induced rapidly upon initiation of treatment via mitotic errors, including chromosomal segregation deficiency, mitotic slippage, and failure of cytokinesis, rather than cell fusions or selection of pre-existing tetraploid cells.

In summary, we have identified novel combination therapies for TNBC and investigated their impact on tumor evolution, possible mechanisms of resistance, and previously unrecognized effects on cell division. TNBC represents a significant unmet medical need for
which new treatments are urgently needed, and our findings provide rationale for further preclinical and clinical development of BET inhibition combined with CDK4/6 inhibition. In addition, our findings suggest new vulnerabilities in resistant cells that may inform second-line treatments and management of progressive disease.
REFERENCES


Roche-Lestienne, C. et al. Several types of mutations of the Abl gene can be found in chronic myeloid leukemia patients resistant to STI571, and they can pre-exist to the onset of treatment. *Blood* **100**, 1014-1018, doi:10.1182/blood.V100.3.1014 (2002).


Chapter 2:

Genetic screens identify synergistic BET inhibitor combinations
Jennifer Y. Ge\(^1\)\(^,\)\(^2\)\(^,\)\(^6\)\(^,\)\(^7\), Shaokun Shu\(^1\), Hua-Jun Wu\(^2\)\(^,\)\(^10\)\(^,\)\(^11\), Isaac Harris\(^8\)\(^,\)\(^9\), Jennifer Endress\(^8\)\(^,\)\(^9\), Xintao Qiu\(^1\)\(^,\)\(^4\), Adrienne Luoma\(^3\), Binbin Wang\(^2\), Kai W. Wucherpfennig\(^3\)\(^,\)\(^9\), Joan Brugge\(^6\)\(^,\)\(^9\), X. Shirley Liu\(^2\)\(^,\)\(^4\)\(^,\)\(^12\), Myles Brown\(^1\)\(^,\)\(^4\)\(^,\)\(^7\)\(^,\)\(^9\), Franziska Michor\(^2\)\(^,\)\(^5\)\(^,\)\(^9\)\(^,\)\(^10\)\(^,\)\(^11\)\(^,\)\(^12\), and Kornelia Polyak\(^1\)\(^,\)\(^4\)\(^,\)\(^5\)\(^,\)\(^7\)\(^,\)\(^9\)\(^,\)\(^12\)

Departments of \(^1\)Medical Oncology, \(^2\)Data Sciences, and \(^3\)Cancer Immunology and Virology, and Centers for \(^4\)Functional Cancer Epigenetics and \(^5\)Cancer Evolution, Dana-Farber Cancer Institute, Boston, MA 02215, USA. \(^6\)Harvard-MIT Division of Health Sciences and Technology, Departments of \(^7\)Medicine and \(^8\)Cell Biology, and \(^9\)Ludwig Center at Harvard, Harvard Medical School, Boston, MA 02115, USA. \(^10\)Department of Biostatistics, Harvard T. H. Chan School of Public Health, Boston, MA 02115, USA. \(^11\)Department of Stem Cell and Regenerative Biology, Harvard University, Cambridge, MA 02138, USA. \(^12\)The Eli and Edythe L. Broad Institute of MIT and Harvard, Cambridge, MA 02142, USA.

Portions of this chapter are part of manuscripts in preparation as:


ABSTRACT

BET family proteins are transcriptional regulators that are involved in the activation of oncogenes in cancer. We previously demonstrated the anti-proliferative activity of small molecule inhibitors of BET in triple-negative breast cancer. However, the rapid development of resistance requires the identification of effective combination therapies. Here, we used CRISPR/Cas9 and small molecule inhibitor screens to systematically identify synergistic and resistance interactions with BET inhibition. We found that targeting cell cycle, transcriptional activation, ubiquitin-proteasomal degradation, anti-apoptotic pathways, and DNA damage could synergize with BET inhibition and potentially overcome resistance. We validated several hits in vitro and in vivo and used RNA-seq to further characterize tumor response to BET inhibition with JQ1, combined with CDK4/6 inhibition with palbociclib and microtubule inhibition with paclitaxel. Palbociclib and JQ1 were broadly synergistic and downregulated cell cycle pathways to achieve significant growth arrest. Our results indicate novel targeted combination therapies involving BET inhibitors for TNBC.

INTRODUCTION

Bromodomain and extra-terminal (BET) family proteins (BRD2, BRD3, BRD4, and BRDT) are regulators of transcription that function as epigenetic readers\(^1\). Specifically, BET proteins recognize acetylated lysine residues on histone tails, which are associated with open chromatin and transcriptional activation, and recruit transcription factors, chromatin modifiers, and other transcriptional co-activators to the chromatin\(^1,2\). In several cancer types, including multiple myeloma, leukemia, and lymphoma, BET proteins have been shown to drive transcription of key oncogenes such as MYC and BCL2 by localizing to super-enhancers that drive their expression\(^2-5\), and in the rare cancer NUT midline carcinoma, BRD4 is even mutated itself to form a proto-oncogene\(^6\). Hence, BET proteins are critical to the function of oncogenic drivers in a variety of cancers and are an attractive anti-cancer target. Recently, several small molecule
inhibitors have been developed, including the prototypical JQ1, iBET151, and OTX015, that block the binding of BET proteins to the chromatin, inhibiting expression of these oncogenes and thus cell proliferation\textsuperscript{7-10}. BET inhibitors have therefore received much interest as a promising new therapeutic strategy to selectively target oncogenes that have been otherwise regarded as “undruggable.”

Previously, we and others have demonstrated the efficacy of BET inhibitors in triple-negative breast cancer (TNBC), an aggressive subtype of breast cancer that currently lacks options for targeted treatment\textsuperscript{11,12}. However, cells can rapidly develop resistance to these drugs via multiple different mechanisms, including bromodomain-independent chromatin binding of BRD4 through MED1 in TNBC\textsuperscript{12} and transcriptional activation through β-catenin in acute myeloid leukemia\textsuperscript{13,14}. Therefore, effective combination therapies must be developed that can extend the efficacy of BET inhibitors and delay or prevent resistance.

Genetic screens are powerful tools that have helped to elucidate the functions of many genes and identify ones that affect phenotypes of interest. By perturbing large numbers of genes at a time using chemical mutagenesis, transposons, small molecule compounds, or RNA interference (RNAi) and then looking for alterations in phenotype, genetic screens have been instrumental in annotating the genome, identifying epistatic interactions, and finding novel drivers and dependencies in cancer\textsuperscript{15-19}. The advantage of such forward genetics approaches is that they are unbiased and can cover the whole genome. The recent development of the CRISPR/Cas9 system, in which the Cas9 endonuclease is targeted to cleave DNA at specific sites using short guide RNAs (sgRNAs), has allowed for systematic and precise deletion of any gene in the genome\textsuperscript{20,21}. CRISPR/Cas9 screens are thus able to generate knockout lines in a high-throughput way using pooled sgRNAs, which can then be recovered after selection by sequencing\textsuperscript{22,23}. This method has been useful in identifying targets that contribute to drug resistance and synthetic lethal interactions\textsuperscript{24-26}. 
Thus, in order to rationally find drug candidates that may be effective in combination with BET inhibition, we performed CRISPR/Cas9 and small molecule screens to find new dependencies in TNBC cells in the presence of the BET inhibitor JQ1. We then followed up on numerous hits by assessing for synergy in vitro and efficacy in vivo. We chose two of these drugs, palbociclib and paclitaxel, for further study of their effects on cell viability and gene expression, to begin to understand their mechanisms of action.

RESULTS

CRISPR/Cas9 screen identifies cell cycle and transcriptional activation as synergistic targets with BET inhibition

To identify synthetic lethal targets and mechanisms of resistance of BET inhibitors, we began by performing a genome-wide CRISPR/Cas9 knockout screen. We used a library targeting 18,460 genes in TNBC cell lines SUM159 and SUM149 treated with JQ1 or DMSO, as well as in their JQ1-resistant derivatives SUM159R and SUM149R. Across both parental cell lines, sgRNAs that became depleted after JQ1 treatment included those targeting G1/S cell cycle progression (CDK4, SKP2), tumorigenic transcriptional regulators (BRD2, EP300, CREBBP, NELFB), and DNA damage repair (PRKDC), indicating that they became more essential under BET inhibition and are synergistic (Figure 2.1A-B). Conversely, sgRNAs that targeted G1/S cell cycle inhibition genes (CDKN1A, NF2, JUNB) and tumor suppressive transcriptional regulators (ARID1A, TCEB3) were enriched, indicating that they contribute to JQ1 resistance (Figure 2.1A-B). In addition, other cell cycle regulators (MYC, CCND1) and transcriptional activators including components of the Mediator complex (MED23, MED24, MED30) were synergistic hits in SUM159, while cell cycle and transcription-related tumor suppressors (RB1, BRD7) were resistance hits (Figure 2.1A). In SUM149, deletion of CSNK2B, a subunit of CK2 which phosphorylates and stabilizes BRD4\textsuperscript{27}, was synergistic with JQ1, while deletion of genes known
Figure 2.1: CRISPR/Cas9 screen.
Top hit gene deletions that are significantly depleted (blue) and enriched (red) in JQ1-treated vs. untreated SUM159 (A), SUM149 (B), SUM159R (C), and SUM149R (D) cells. Genes are ranked by $p$-value. Dotted lines represent $p < 0.001$ cutoff for significance.
to function in ubiquitination of BRD4 (SPOP, CUL3) and promote proteasomal degradation and made cells more resistant (Figure 2.1B). Various proteasomal subunit genes were also synergistic hits in both SUM159 and SUM149 (PSMA3, PSMA8, PSMD1 in SUM159, Figure 2.1A, and PSMA5, PSMD7 in SUM149, Figure 2.1B).

In the JQ1-resistant cells SUM159R and SUM149R, genes involved in transcription by RNA polymerase II were enriched among the top synergistic hits (GTF2H1, GTF2H5, MED19, MED24, MED26, TAF6 in SUM159R, Figure 2.1C, and CREBBP, DR1, GTF2F2, GTF2H5, TAF5, TFAP4 in SUM149R, Figure 2.1D). This implies that these cells still rely on BRD4/RNA polymerase II complex function, which is consistent with our prior data demonstrating the dependency of JQ1-resistant cells on BRD4. Notably, CDK4 and BRD2 were top synergy hits in all four cell lines, suggesting that their deletion not only sensitizes cells to BET inhibitors but also overcomes resistance (Figure 2.1).

Small molecule screen identifies cell cycle progression, DNA damage, and apoptosis pathways among new sensitivities in BET inhibitor resistance

To further identify targets to overcome BET inhibitor resistance, we performed a cellular viability screen in SUM159, SUM159R, SUM149, and SUM149R, using two small molecule libraries totaling 475 compounds targeting a range of pathways (Figure 2.2A). To identify potentially synergistic compounds, we compared the differences in sensitivity between the derived JQ1-resistant lines and their parental lines and looked for drugs that the JQ1-resistant lines were more sensitive to than the parental lines. In SUM159, signaling pathway targets were most frequent among the top hits, including VEGFR, EGFR, BCR-ABL, MEK, and PI3K (Figure 2.2B). On the other hand, the top hits in SUM149 included DNA damage agents (topoisomerase, DNA synthesis, microtubule), cell cycle inhibitors (CDK4/6, PLK, Aurora kinase), histone deacetylase (HDAC) inhibitors, and HSP90 inhibitors (Figure 2.2C). As expected, both SUM159R and
Figure 2.2: Small molecule screen. 
(A) Pathways targeted by each compound library. Differences in sensitivities to compounds screened between parental and derived JQ1-resistant SUM159 (B) and SUM149 (C) cell lines. Dotted lines represent cutoff of 10% difference. Drugs are grouped by pathway. Targets that are represented in several top hits are indicated. AUC, area under the curve.
Figure 2.2 (continued)

A
Selective Library
144 compounds

Anti-Cancer Library
386 Compounds

B
SUM159
Proteotoxic
Cytoskeleton
Epigenetics
Cell Cycle
JAK/STAT
Apoptosis
Immunology
Metabolism
DNA Damage
Others
Ubiquitin
Wnt
Protein Tyrosine Kinase
MAPK
Endocrinology & Hormones
PI3K/Akt/mTOR
TGF-beta/Smad

C
SUM149
TGF-beta/Smad
Ubiquitin
Others
Wnt
Epigenetics
PI3K/Akt/mTOR
Endocrinology & Hormones
Protein Tyrosine Kinase
MAPK
Metabolism
Apoptosis
Immunology
JAK/STAT
DNA Damage
Cytoskeleton
Cell Cycle
Proteotoxic
SUM149R were more resistant to compounds targeting BET proteins than SUM159 and SUM149, respectively, which served as a positive control (Figure 2B-C). Inhibitors of the anti-apoptotic protein BCL2, was a hit that was shared between both lines (Figure 2B-C), consistent with our previous findings that expression of anti-apoptotic factors contributes to JQ1 resistance. Topoisomerase inhibitors were also among the hits for both SUM159 and SUM149 (Figure 2B-C), which may reflect the sensitivity to DNA damage and transcription that we found in the CRISPR screen. Furthermore, both SUM159R and SUM149R shared increased sensitivity to inhibitors of CDK9, a component of P-TEFb, as well as several other non-specific CDK inhibitors, and SUM149R was also more sensitive to a CDK4/6-specific inhibitor. Together with our CRISPR screen findings, these results indicate that cell cycle progression, transcriptional activation, and DNA damage repair in general become more essential in JQ1 resistance.

Validation of screen hits with in vitro synergy studies

In order to validate the results of the CRISPR/Cas9 and small molecule screens, we followed up on several of the top hits from each screen by testing for synergy with JQ1. We prioritized drugs that were already FDA-approved to facilitate translatability of our results. Thus, we selected a CDK4/6 inhibitor (palbociclib), DNA damage agents (gemcitabine, doxorubicin), microtubule inhibitors (paclitaxel, vincristine, and eribulin), and an HSP90 inhibitor (NVP-HSP990). These drugs were tested over a range of concentrations in combination with various concentrations of JQ1 in SUM159 and SUM149, as well as the resistant lines.

We found that in general, most of these drugs tended to be more synergistic in the resistant lines compared with in the sensitive parental lines, in which they were additive or antagonistic (Figure 2.3). This supports our hypothesis that the pathways we selected become more essential in resistant cells. These combinations may thus be favorable in delaying
Figure 2.3: Synergy studies with various compounds combined with JQ1 in SUM159, SUM149, and their derived JQ1-resistant lines. Normalized isobolograms show interaction effects between JQ1 and candidate compounds. Points represent various combinations of concentrations. Diagonal line represents additivity, below the line synergistic, and above the line antagonistic. Drug targets are indicated.
progression, as they would select against resistant cells. Unexpectedly though, most of the compounds tested were antagonistic in SUM149. This could reflect a decreased sensitivity to DNA damage in these cells, which have a mutation in BRCA1, when co-treatment with JQ1 slows down their rate of proliferation.

Notably, the most synergistic drug combination was JQ1 and palbociclib. Palbociclib was found to synergize with JQ1 over all concentrations tested across all four cell lines, in addition to being more synergistic in the resistant lines compared to the parental lines (Figure 2.3). We further validated this combination in an expanded panel of 8 additional TNBC cell lines. JQ1 and palbociclib were strongly synergistic in 6 of the 8 lines (Figure 2.4), suggesting that the combination is broadly effective in TNBC. Taken together, these results imply that targeting the cell cycle is a generally efficacious strategy in combination with BET inhibition.

**In vivo validation of BET inhibitor combinations**

Next, we sought to assess the efficacy of several of these candidate combination therapies *in vivo* using orthotopic mammary fat pad xenografts in immunodeficient (NOG) mice. We chose to test palbociclib, paclitaxel, NVP-HSP990, and eribulin in combination with JQ1 in SUM159 and/or SUM159R xenografts (Figure 2.5). We found that neither eribulin nor NVP-HSP990 were effective alone or combined with JQ1 in these models (Figure 2.5E-F). We thus chose to focus on palbociclib and paclitaxel. We further tested these drugs along with JQ1 in a patient-derived xenograft (PDX), IDC50X, which came from a patient with an aggressive metaplastic TNBC. We found in all three xenografts tested that the JQ1+palbociclib essentially halted tumor growth, limiting tumor size significantly more than either of the single agents (Figure 2.5A,C-D). In addition, in SUM159R, neither JQ1 nor palbociclib alone had a significant effect on tumor size, but they did when used together (Figure 2.5C), indicating that the combination could overcome resistance to either drug alone. Thus, we confirmed the synergy between CDK4/6 and BET
Figure 2.4: Synergy studies with JQ1 and palbociclib in a panel of TNBC cell lines. Normalized isobolograms show interaction effects between JQ1 and palbociclib. Points represent various combinations of concentrations. Diagonal line represents additivity, below the line synergistic, above the line antagonistic.
Figure 2.5: Effects of JQ1 combinations on cell-line and patient-derived xenografts.
Tumor sizes of SUM159 (A, B) and SUM159R xenografts (C) following 2 weeks of treatment with JQ1, palbociclib, paclitaxel, or combinations, as well as patient-derived IDC50X xenografts following 8 days of treatment (D). Tumor sizes of SUM159 xenografts following 2 weeks of treatment with JQ1, eribulin, or the combination (E) and SUM159R xenografts following 2 weeks of treatment with JQ1, paclitaxel, NVP-HSP990, or combinations (F).
inhibition both in vitro and in vivo. On the other hand, JQ1+paclitaxel inhibited tumor growth slightly more than either drug alone in SUM159 and SUM159R, although the difference was not statistically significant (Figure 2.5B-C,F). However, JQ1 and paclitaxel were antagonistic in the IDC50X PDX, as the combination was significantly worse than JQ1 alone (Figure 2.5D). The lack of a significant benefit from the addition of paclitaxel, though, could have been due to missed doses from the added toxicity of the combination.

Interestingly, hematoxylin and eosin (H&E) staining of the post-treatment xenografts showed that treated cells had strikingly altered morphology compared to untreated tumors. In response to palbociclib and JQ1+palbociclib, cells in SUM159, SUM159R, and IDC50X xenografts became enlarged with decreased nuclear-cytoplasmic ratio as well as multiple irregular hypochromatic nuclei (Figure 2.6A). These features can be seen to some extent in tumors treated with JQ1+paclitaxel as well (Figure 2.6A). These morphologic changes are consistent with cellular senescence, with reorganization of the chromatin.

**Palbociclib and paclitaxel combined with JQ1 induce significant cell cycle arrest and some apoptosis**

To further understand the mechanisms behind the drugs’ effects, we asked whether they were influencing tumor growth by inhibiting proliferation or by increasing apoptosis. Thus, we performed immunofluorescence on post-treatment SUM159 xenografts for cyclin D1, phospho-histone H3, and cleaved caspase 3. We found that tumors treated with palbociclib and paclitaxel alone or in combination with JQ1 had increased numbers of cells staining positive for cyclin D1 and phospho-histone H3, respectively, but little change in the amount of cleaved caspase 3 (Figure 2.6B). Thus, palbociclib and paclitaxel primarily arrested cells in G1 and M phase, respectively, without inducing a significant amount of apoptosis. A caveat though is that these tumors were collected following two weeks of treatment, so that any cell death that occurs in sensitive cells upon initiation of treatment would no longer be detectable.
Figure 2.6: Histology of xenografts treated with BET inhibitor combinations. 
(A) Hematoxylin and eosin (H&E) staining of SUM159 and SUM159R xenografts following 2 weeks of treatment and patient-derived xenograft IDC50X following 8 days of treatment. 
(B) Immunofluorescence staining of SUM159 xenografts for cleaved caspase 3, pHistone H3, and cyclin D1. Scale bars represent 50 µm.
Thus, to more thoroughly examine the response to treatment, we treated SUM159 cells in culture with JQ1, palbociclib, and paclitaxel, alone and in combinations. We found that both JQ1+palbociclib and JQ1+paclitaxel inhibited growth significantly more than any of the three drugs alone (Figure 2.7A). The cell morphology was again noticeably altered, with cells becoming enlarged following treatment with JQ1 and palbociclib and even more so with the combination, as well as more apoptotic cells following treatment with JQ1+paclitaxel (Figure 2.7B). Flow cytometry analysis for cell cycle confirmed that both JQ1 and palbociclib arrested cells in G1 phase, and furthermore there was an even higher G1 fraction in cells treated with the combination compared with either alone (Figure 2.7C). Apoptosis levels were also increased in both combination treatments, particularly with JQ1+paclitaxel, while each single treatment only had a minimal effect (Figure 2.7D). Thus, we concluded that palbociclib and paclitaxel combined with JQ1 induce significant cell cycle arrest with moderate increases in apoptosis rates.

**RNA-seq shows downregulation of cell cycle genes in JQ1 and palbociclib**

In order to gain a more comprehensive understanding of the response of the tumors to these therapies, we performed RNA-seq on the post-treatment SUM159, SUM159R, and IDC50X xenografts. We found that JQ1 treatment in SUM159 tumors induced downregulation of genes involved in multiple cell cycle pathways, including S, G2/M, and M phase, as well as spindle microtubule genes (Figure 2.8). Although we did not observe alterations of these pathways in the other xenograft models, this may have simply been due to the lower dose of JQ1 used in those studies, leading to more subtle differences in levels of gene expression. Treatment with palbociclib and JQ1+palbociclib induced substantial downregulation of genes involved in all phases of the cell cycle, as well as spindle microtubule genes (Figure 2.8A). Furthermore, a subset of cell cycle genes, namely ones involved in mitosis, was expressed at an even lower level in the combination-treated tumors compared with palbociclib-treated tumors (Figure 2.9).
Figure 2.7: Effects of JQ1 combinations with palbociclib and paclitaxel on SUM159 in vitro.

(A) Growth curves of SUM159 cells treated with JQ1, palbociclib, and paclitaxel, alone and in combinations. (B) Brightfield images of treated SUM159 cells. Scale bars represent 100 µm. (C) Proportion cells in each phase of the cell cycle by propidium iodide staining following 24 hours of treatment. (D) Proportion of early apoptotic (annexin V+/PI-) and late apoptotic (annexin V+/PI+) cells following 3 days of treatment.
Figure 2.8: RNA-seq of xenografts treated with JQ1, palbociclib, and paclitaxel combinations.
Heat maps show differentially expressed process networks in SUM159 and SUM159R xenografts and patient-derived xenograft IDC50X, treated with JQ1, palbociclib, and the combination compared with vehicle (A) and treated with JQ1, paclitaxel, and the combination compared with vehicle (B).
Figure 2.8 (continued)

A

Endoplasmic reticulum stress pathway
Anti-apoptosis mediated by estrogen
Anti-apoptosis mediated by NF-kB
Apoptotic nucleus
Cell-matrix interactions
Integrin-mediated cell-matrix adhesion
Platelet-endothelium-leukocyte interactions
Integrin phosphorylation
Integrin priming
Cytokines
Cell junctions
Actin filaments
Cytoskeletal microtubules
Regulation of cytoskeleton rearrangement
Intermediate filaments
Spindle microtubules
G1-S intercellular regulation
G1-S growth factor regulation
Positive regulation of cell proliferation
G2/M
Meiosis
G1-S
G2-M
Mitosis
Core
S phase
Core
MMR repair
BER-NER repair
DSB repair
Checkpoint
Phagocytosis
Th17-derived cytokines
TCR signaling
Phagosome in antigen presentation
Antigen presentation
IL-10 anti-inflammatory response
IL-2 signaling
IL-6 signaling
Compliment system
Histamine signaling
Innate inflammatory response
IL-4 signaling
Interferon signaling
NF signaling
Response to unfolded proteins
ER and cytoplasm
Folding in normal condition
Protein folding nucleus
ECM remodeling
Connective tissue degradation
Proteolysis in cell cycle and apoptosis
Ubiquitin-proteasomal proteolysis
Progestosterone signaling
WNT signaling
ESR1 nuclear pathway
Gonadotropin regulation
BMP-TGF-β signaling
NOTCH signaling
Androgen receptor signaling cross-talk
ERBB-family signaling
Androgen receptor nuclear signaling
Blood vessel morphogenesis
Regulation of angiogenesis
Regulation of EMT
Hedgehog signaling
Nuclear receptors transcrptional regulation
Transcription by RNA polymerase II
Chromatin modification
mRNA processing
Regulation of initiation
Elongation-Termination
Translation initiation

B

TAX JQ1+TAX

Anti-apoptosis mediated by PI3K/AKT
Apoptotic nucleus
Anti-apoptosis mediated by MAPK and JAK/STAT
Anti-apoptosis mediated by estrogen
Death Domain receptors & caspases in apoptosis
Cell-matrix interactions
Platelet-endothelium-leukocyte interactions
Integrin-mediated cell-matrix adhesion
Attractive and repulsive receptors
Intermediate filaments
Actin filaments
Cytoskeletal microtubules
Spindle microtubules
Negative regulation of cell proliferation
G1-S intercellular regulation
G1-S growth factor regulation
Positive regulation of cell proliferation
Meiosis
S phase
Mitosis
Core
G2-M
Checkpoint
Phagocytosis
IL-6 signaling
BCR pathway
Th17-derived cytokines
Kallikrein-kinin system
IgE signaling
Protein C signaling
IL-4 signaling
IL-10 anti-inflammatory response
Complement system
IL-12,15,18 signaling
IL-2 signaling
Antihistamine signaling
IL-9 signaling
Histamine signaling
ECM remodeling
Nitric oxide signaling
ESR1-nuclear pathway
WNT signaling
NOTCH signaling
ERBB-family signaling
TGF-β, GDF and Activin signaling
ESR2 pathway
Oxidation signaling
Gonadotropin regulation
BMP-TGF-β signaling
Androgen receptor signaling cross-talk
Progestosterone signaling
Regulation of EMT
Hedgehog signaling
Blood vessel morphogenesis
Regulation of angiogenesis
Nuclear receptors transcrptional regulation

SUM159R
SUM159R
SUM159R
IDC56X
IDC56X
IDC56X

SUM159R
SUM159R
SUM159R
IDC56X
IDC56X
IDC56X

<10
5
0
>10
log2(p-value)

47
Figure 2.9: Differentially expressed cell cycle genes in xenografts treated with JQ1, palbociclib, and their combination.
Heat map shows log2 fold changes of differentially expressed cell cycle genes in SUM159 and SUM159R xenografts and patient-derived xenograft IDC50X, treated with JQ1, palbociclib, and the combination, compared with vehicle.
Figure 2.9 (continued)
We observed the same effects in the IDC50X xenografts (Figure 2.8A and Figure 2.9). Thus, we confirmed that the combined effects of JQ1 and palbociclib on the cell cycle was greater than that of either drug alone, consistent with the enhanced efficacy of the combination treatment.

However, there was no change to cell cycle pathway expression in SUM159R following palbociclib treatment (Figure 2.8A). Indeed, palbociclib alone did not have a significant effect on tumor size (Figure 2.5C). Interestingly, this tumor had increased expression of cyclin E (CCNE1), which was not observed in the other two xenografts (Figure 2.9). This suggests that cyclin E overexpression is capable of conferring resistance to palbociclib. Nevertheless, the combination treatment did result in significantly smaller tumors compared with control despite cyclin E overexpression (Figure 2.5C), further highlighting the synergistic interaction between the two drugs, as well as the ability to overcome resistance to both JQ1 and palbociclib alone.

In contrast, we found very few differentially expressed genes in xenografts treated with paclitaxel alone or in combination with JQ1 (Figure 2.8B). This could be due in part to resistance arising to paclitaxel, as there was no longer a difference in size between SUM159 tumors that were untreated and treated with paclitaxel at 2 weeks (Figure 2.5B). IDC50X, though, had decreased expression of spindle microtubule genes but increased expression of signaling pathway genes associated with G1/S progression. This could explain the resistance to paclitaxel treatment seen in the xenograft and in the patient from whom the line was derived, as well as the antagonistic effect of paclitaxel with JQ1 in this tumor.

Overall, we found relatively few differentially expressed genes and pathways in our RNA-seq data. These results could reflect the substantial amount of heterogeneity seen in the response to treatment both between different tumors and between different regions of the same tumor. Thus, changes in gene expression in these samples may not be readily detectable with few replicates and with bulk RNA-seq.
Single cell RNA-seq reveals heterogeneity in tumor and stromal cells treated with JQ1 and paclitaxel

We then performed single cell RNA-seq to ask whether there was indeed intratumor heterogeneity in the response to treatment. We analyzed one tumor each from the vehicle, JQ1, paclitaxel, and JQ1+paclitaxel treatment groups. We found that tumor cells formed four clusters (Figure 2.10A). The first cluster had increased expression of protein synthesis genes, namely ribosomal proteins, and was comprised entirely of untreated tumor cells (Figure 2.10A and Figure 2.11). Thus, JQ1 and paclitaxel treatment both inhibit this tumorigenic phenotype. The second cluster exhibited gene expression consistent with a senescence-associated secretory phenotype (SASP) or an epithelial-to-mesenchymal transition (EMT) phenotype and was enriched amongst paclitaxel-treated cells (Figure 2.10A and Figure 2.11). The last two clusters were differentiated by cell cycle, and the JQ1+paclitaxel combination treated tumor had the fewest dividing (S/G2/M) cells (Figure 2.10A and Figure 2.11), consistent with a smaller tumor size. Thus, while paclitaxel alone can promote SASP or an EMT phenotype, this could potentially be prevented with the addition of BET inhibitors.

We also analyzed the infiltrating mouse cells separately and found that this population consisted of host immune cells, comprising two small populations of erythrocytes and macrophages and the rest all neutrophils (Figure 2.10B and Figure 2.12). Interestingly, the neutrophils formed four different phenotypic clusters that also differed in their proportions between different treatments (Figure 2.10B and Figure 2.12). The tumor treated with paclitaxel alone had a higher proportion of neutrophils expressing interferon response genes, which could potentially have been recruited by senescent tumor cells, and a smaller proportion of chemotactic neutrophils (Figure 2.10B and Figure 2.12), which could be an effect of paclitaxel’s inhibition of microtubules. Moreover, tumors treated with the JQ1+paclitaxel combination had increased numbers of phagocytic neutrophils (Figure 2.10B and Figure 2.12). Thus, though
Figure 2.10: Single cell RNA-seq of xenografts treated with JQ1 and paclitaxel. t-SNE plots of human cancer cells (A) and mouse immune cells (B) in tumors, colored by cluster (left) and by treatment group (right).
Figure 2.11: Differentially expressed genes between clusters of tumor cells in xenografts treated with JQ1 and paclitaxel.
Figure 2.12: Differentially expressed genes between clusters of mouse immune cells in xenografts treated with JQ1 and paclitaxel.
these were immunocompromised mice lacking adaptive immune cells, the outcome could still be influenced by the altered phenotypes of neutrophils as a result of treatment.

**Drug schedule affects treatment outcome**

Lastly, we investigated whether altering the order of drug administration would affect treatment outcomes. Thus, we treated SUM159 and SUM149 parental cells and SUM159R and SUM149R JQ1-resistant derivatives with JQ1 and palbociclib or paclitaxel, sequentially for 1 week each in either order, or concurrently for 1 week followed by vehicle for 1 week. We found in all cell lines that JQ1 followed by palbociclib was superior to palbociclib followed by JQ1; however, upfront combination was superior to either sequential schedules in SUM159 and SUM149R (Figure 2.13A). With sequential treatment of JQ1 and paclitaxel, we found that JQ1 followed by paclitaxel was superior in the SUM159 and SUM149 parental cells but the reverse was true in the resistant lines (Figure 2.13B). In all cases, though, upfront combination of JQ1+paclitaxel was equally or more effective than the better sequential therapy (Figure 2.13B). We observed the same result with JQ1 and paclitaxel when we treated mice with SUM159 and SUM159R xenografts (Figure 2.13C). These data may reflect selection for or modulation of sensitivity to the second drug, as well as the increased cytotoxic effect of JQ1+paclitaxel, where upfront treatment with the most effect therapy is best, compared with JQ1+palbociclib, where prolonged inhibition of proliferation is beneficial.

**DISCUSSION**

In this study, we identified potential combination therapies involving BET inhibitors using high-throughput CRISPR and small molecule inhibitor screens. We found that inhibition of cell cycle was especially synergistic with BET inhibition, as well as targeting DNA damage and transcription. We chose several targets, including CDK4/6, DNA damage, microtubule, and
Figure 2.13: Optimal drug schedule varies between cell lines. Growth curves of SUM159, SUM159R, SUM149, and SUM149R cell lines treated with JQ1 and palbociclib (A) and JQ1 and paclitaxel (B) sequentially in either order or concomitantly. (C) Tumor weights of SUM159 and SUM159R xenografts following 2 weeks of treatment with JQ1 and paclitaxel sequentially in either order or concomitantly. (continued)
Figure 2.13 (continued)

A

B

SUM159

SUM159R

SUM149

SUM149R

Cell number

Cell number

Day

Day

B

SUM159

SUM159R

SUM149

SUM149R

Cell number

Cell number

Day

Day

C

SUM159

SUM159R

Tumor weight (g)

Tumor weight (g)

VEH

JQ1->TAX

TAX->JQ1

JQ1+TAX=>VEH

DMSO

JQ1->PAL

PAL->JQ1

JQ1+PAL=>DMSO

DMSO

TAX->JQ1

JQ1+TAX=>DMSO

ns

ns

***

***

**

**
HSP90, to validate both in vitro and in vivo and chose palbociclib, a CDK4/6 inhibitor, and paclitaxel, a microtubule-inhibiting chemotherapy, for further study. We found that these drugs combined with JQ1 could more effectively induce cell cycle arrest and apoptosis compared to any of the single agents.

Palbociclib and JQ1 were remarkably synergistic across several models, and our flow cytometry and RNA-seq data showed evidence of a deeper cell cycle arrest with the combination treatment compared to either drug alone. Previous studies have reported that CDK4/6 inhibitor combined with BET inhibition can overcome resistance to other drugs in mantle cell lymphoma\(^3\) and luminal breast cancer\(^4\). Notably, JQ1 and palbociclib were effective even in cell lines that were relatively resistant to JQ1 or palbociclib alone, e.g. SUM149R and SUM159R. Alterations in RB1 and cyclin E are known to modulate sensitivity to CDK4/6 inhibitors\(^3,4\), and interestingly, SUM159R, which was resistant to palbociclib compared with the parental SUM159 line, had increased expression of cyclin E. Furthermore, JQ1 and palbociclib were synergistic even in cell lines that were RB1-null. This could indicate that the efficacy of palbociclib with JQ1 may not depend only on the canonical cyclin D1-CDK4/6-Rb pathway. In our CRISPR screen, we found that genes involved in control of cell cycle and mitotic progression in general were among the top hits that sensitized cells to JQ1. Previous studies have also reported synergy between JQ1 and CDK1 and CDK2\(^5,6\). These synergistic drug interactions reflect an established role of BRD4 during mitotic progression and exit\(^7,8\).

Other studies have also found synergy between BET inhibition and anti-microtubule drugs like paclitaxel and vincristine\(^9,10\). In our TNBC models that we tested, we found that the effects of JQ1 and paclitaxel were not consistent across all cell lines. The combination did increase apoptosis in SUM159 in vitro but it was antagonistic in SUM149 in vitro as well as in IDC50X in vivo. The patient from whom this xenograft was derived had an extremely aggressive disease and was heavily pre-treated, failing several regimens including paclitaxel. Thus, the sensitivity to the JQ1 and paclitaxel combination may depend on the characteristics of the
tumor, such as its DNA damage response, and may perhaps be associated with TNBC subtype\textsuperscript{41}.

In our single cell RNA-seq study, we identified four distinct clusters of neutrophils, the proportions of which were affected by paclitaxel treatment. This could be a direct effect of the paclitaxel treatment on neutrophils or an indirect effect through the tumor microenvironment, such as from secreted factors by a subpopulation of senescent tumor cells. Neutrophils have been relatively understudied in the area of immuno-oncology, but other papers have identified different neutrophil phenotypes, including polarization into pro- and anti-tumor neutrophils\textsuperscript{42-44}. A caveat is that we used immunocompromised mice here, which lack T- and B-cells, in order to grow human tumor lines in an \textit{in vivo} model. Thus, further study is required in immunocompetent models to elucidate the significance of these neutrophil types.

Lastly, we found that altering drug schedule affected the outcome to BET inhibitor combinations, which may be due to selection for certain clones with differential drug sensitivity or multi-drug resistance, or alteration of the cell state or cell plasticity. The question of drug schedule is often overlooked in clinical trials, and it is often unclear how combination therapies should be administered. However, some studies have shown that the schedule of administration of combination therapies can indeed affect sensitivity and outcome\textsuperscript{45-47}. We found that the JQ1 and paclitaxel combination should be given upfront, and this result has clear implications for clinical practice. Since paclitaxel is a common front-line chemotherapy for TNBC, this suggests that the potential benefit from adding JQ1 would be abolished after acquiring paclitaxel resistance. Further study of scheduling in these as well as other combination therapies in general is warranted, in order to understand the principles underlying the schedule effect and for schedule optimization.

Our findings have direct translational impact and clinical significance. TNBC is an aggressive disease, associated with younger age and worse prognosis than other subtypes of breast cancer, and novel targeted therapies to treat this disease are still lacking. CDK4/6
inhibitors have been approved for advanced ER$^+$ breast cancer but are thought to be ineffective in basal-like breast cancers\textsuperscript{48}. However, we have found that palbociclib greatly improved the sensitivity to JQ1 and thus may be useful in TNBC. Our study provides rationale for further preclinical and clinical investigation of this combination.

**METHODS**

**Cell lines:** SUM159 and SUM149 breast cancer cells were obtained from Steve Ethier (University of Michigan) and cultured in 50% DMEM/F12 and 50% Human Mammary Epithelial Cell Growth Medium with 10% FBS. JQ1-resistant derived SUM159R and SUM149R lines were previously described\textsuperscript{12} and also cultured in the presence of 20 $\mu$M and 10 $\mu$M JQ1, respectively. CAL-51 and CAL-120 cells were obtained from DSMZ and cultured in DMEM with 20% and 10% FBS, respectively. HCC3153 and HCC38 cells were obtained from Adi Gazdar and ATCC, respectively, and cultured in RPMI with 10% FBS. Hs578-T cells were obtained from ATCC and cultured in DMEM with 10% FBS and 10 $\mu$g/mL insulin. MDA-MB-231 and MDA-MB-436 cells were obtained from ATCC and cultured in McCoy's Medium with 10% FBS, as well as 10 $\mu$g/mL insulin for MDA-MB-436. PMC42 cells were obtained from Richard Whitehead and cultured in RPMI with 1% FBS, 1 $\mu$g/mL insulin, and 10 nM hydrocortisone. 1% Pen Strep (Thermo Fisher Scientific) was also added to all media. PDX IDC50 was derived from a primary tumor of highly invasive metaplastic TNBC resistant to chemotherapy and radiation therapy and propagated in immunodeficient mice.

**CRISPR/Cas9 screen:** 100 million SUM159, SUM159R, SUM149, and SUM149R cells were infected with the pooled lentiviral GeCKO v2 library at a multiplicity of infection of 0.3. After 3 days of puromycin selection, half of the surviving cells were collected as pre-treatment control samples, and the rest of the cells were cultured for an additional 5 passages with or without different concentrations of JQ1 treatment. PCR was performed on genomic DNA to construct
the sequencing library. Each library was sequenced at 30-40 million reads to achieve 300X coverage on average over the CRISPR library.

**Small molecule screen:** SUM159 and SUM159R cells were seeded in 384-well plates at a seeding density of 500 cells/well and SUM149 and SUM149R cells at a seeding density of 250 cells/well. Assay robustness was determined by obtaining a Z' factor for each cell line using vincristine (1 µM) as a cytotoxic agent. The Z' factor for cell lines were as follows: SUM149 = 0.35; SUM149R = 0.56; SUM159 = 0.60; SUM159R = 0.38. For the screen, cells were seeded in 30 µL at their respective cell densities. After 24 hours, compounds from the drug library plates were pin-transferred (100 nL) onto the cells and 20 µL of fresh media was added. After 72 hours, plates were washed with PBS and fixed/stained using a 4% formaldehyde (Sigma-Aldrich) and 5 µg/mL bisBenzimide H 33342 trihydrochloride (Sigma-Aldrich) solution diluted in PBS. Cell numbers were determined by imaging plates and quantifying nuclei using the Acumen Cellista (TTP Labtech). Data were analyzed using R and Prism. The differential drug sensitivity between the resistant and parental cells was calculated as the difference between the areas under the dose-response curves (AUC).

**Compounds:** Palbociclib was obtained from MedChemExpress. Paclitaxel, doxorubicin, and eribulin were obtained from the Dana-Farber Cancer Institute Research Pharmacy. Vincristine and gemcitabine were obtained from Sigma-Aldrich. NVP-HSP990 was obtained from Selleckchem. See Appendix A for concentrations tested.

**Synergy assays:** Synergy studies were performed in 384-well plates (Corning). SUM159 cells were seeded at a density of 200 cells/well, SUM159R and SUM149 at a density of 500 cells/well, and SUM149R at a density of 1000 cells/well, in 50 µL of media. The following day, drugs were pin-transferred into the wells from a 500X concentrated drug plate made in a 384-well plate (Greiner Bio-One) using the JANUS Automated Workstation (Perkin Elmer). Five concentrations for each drug were chosen between 20% and 80% inhibition, and half doses were used for combinations, with 4 replicate wells for each concentration of each drug alone.
and 8 replicates of each concentration pair. After 3 days, cells were stained with 5 µg/mL Hoechst 33342 (Sigma-Aldrich) in PBS, and nuclei were imaged and counted using the Celigo Imaging Cytometer (Nexcelom).

**Xenograft studies:** Animal studies were performed according to protocol 11-023, approved by the Dana-Farber Cancer Institute Animal Care and Use Committee. Studies of JQ1, palbociclib, and paclitaxel combinations on SUM159R and IDC50X were performed by the Dana-Farber Cancer Institute Lurie Family Imaging Center.

Cells were injected orthotopically into the mammary fat pads of 6-week-old female NOD.Cg-Prkdc<sup>scid</sup>Il2rg<sup>tm1Sug</sup>/JicTac (NOG) mice (Taconic). 2 million SUM159 or SUM159R cells or 200,000 IDC50X cells were injected into each fat pad in 25 µL of DMEM/F12 and 25 µL of Matrigel (Corning). Once tumors became palpable, mice were randomized with 5 mice per group and treated for up to 2 weeks. 1:9 DMSO in hydroxypropyl-β-cyclodextrin (Sigma-Aldrich) was used as vehicle and administered daily by intraperitoneal (i.p.) injection. JQ1 was dosed at 30-50 mg/kg daily i.p., palbociclib at 75 mg/kg daily by gavage, paclitaxel at 10 mg/kg twice weekly i.p., eribulin at 0.25-0.5 mg/kg twice weekly i.p., and NVP-HSP990 at 0.5 mg/kg daily in drinking water. For drug schedule studies, mice were treated with vehicle for 2 weeks, with JQ1 for 1 week followed by paclitaxel for 1 week, paclitaxel for 1 week followed by JQ1 for 1 week, or JQ1+paclitaxel for 1 week of followed by vehicle for 1 week.

Mouse body weights and caliper measurements of tumor size were recorded every 3 days. After completion of treatment, mice were euthanized, and tumors were dissected and formalin-fixed and paraffin embedded (FFPE) or flash frozen for further study.

**Proliferation assays:** For all proliferation studies, JQ1 was dosed at 100 nM, palbociclib at 160 nM, and paclitaxel at 0.6 nM. Plates were imaged daily or twice daily and cells were counted using the Celigo Imaging Cytometer (Nexcelom). Media was exchanged for fresh media with drugs every 3-4 days. Cell nuclei were also counted at the endpoint using fluorescence imaging of Hoechst 33342 stained cells.
Flow cytometry analysis: For cell cycle analysis, cells were fixed overnight in 70% ethanol and then stained in 20 µg/mL propidium iodide (Thermo Fisher Scientific) with 0.2 mg/mL PureLink RNase A (Thermo Fisher Scientific) in 0.1% Triton X-100 (Sigma-Aldrich) for 30 minutes. For analysis of apoptosis, cells were stained with using the Alexa Fluor® 488 Annexin V/Dead Cell Apoptosis Kit as directed (Thermo Fisher Scientific). Fluorescence intensities were acquired on an LSRFortessa cytometer (BD Biosciences). Data were analyzed using FlowJo.

Histology and immunofluorescence: Xenografts chunks in FFPE were cut into slides and stained with hematoxylin and eosin (H&E) by the Brigham and Women’s Hospital Pathology Core Facility. For immunofluorescence, unstained slides were deparaffinized, and antigen retrieval was performed in Dako Target Retrieval Solution pH 9 (Agilent) for 30 minutes in a steamer. Slides were blocked for 10 minutes with 10% goat serum and then stained with anti-cyclin D1 primary antibody (1:50, Abcam, ab134175) at 4°C overnight, followed by staining with anti-rabbit secondary antibody (1:100, Thermo Fisher Scientific, A21245) for 30 minutes at room temperature. Slides were then incubated with primary antibodies against cleaved caspase 3 (1:100, Cell Signaling Technology, CST 9661) and phospho-histone H3 (1:400, Abcam, ab5176), labeled with Zenon™ Alexa Fluor™ 488 and 555 Rabbit IgG Labeling Kits, respectively (Thermo Fisher Scientific, Z25304 and Z25305), for 1 hour at room temperature. Slides were imaged on a Leica SP5X laser scanning confocal microscope.

RNA extraction: Frozen tumor chunks were pulverized using the Covaris CP02 Tissue Pulverizer. Tissues were homogenized by passing the sample through a 23g needle. RNA was extracted from xenografts using the AllPrep DNA/RNA Mini Kit (Qiagen). Isolated RNA was treated with the RNase-Free DNase Set (Qiagen). RNA was prepared from duplicate samples.

Bulk RNA-sequencing: Bulk RNA-seq libraries were prepared from total RNA by the Dana-Farber Cancer Institute Molecular Biology Core Facilities (MBCF) using the Illumina TruSeq Stranded mRNA Library Prep Kit and 16-18 samples were multiplexed per lane for NGS.
**Single cell RNA-sequencing**: Fresh tumors were finely chopped and dissociated to single cells in DMEM/F12 with 2 mg/mL collagenase IV (Worthington), 2 mg/mL hyaluronidase (Sigma-Aldrich), and 2 mg/mL bovine serum albumin (Sigma-Aldrich) for 1.5 hours at 37˚C. Dissociated cells were then washed twice in PBS with 0.04% RNase-free BSA (New England BioLabs). Cells were then diluted to 700 cells/µL and filtered through a 35 µm nylon mesh prior to library preparation. Single cell RNA-seq libraries were prepared using the Chromium Single Cell 3’ Library & Gel Bead Kit v2 and Single Cell A Chip (10X Genomics). Samples were multiplexed 8 per lane for NGS. Sequencing was performed by MBCF.

**Genomic data analysis**: Sequence reads from the CRISPR/Cas9 screen libraries were analyzed using MAGeCK\(^{49}\) to obtain the read counts for each sgRNA. Control sgRNAs were used to normalize the data. The MAGeCK TEST algorithm was used to compare treatment with control samples to determine the significantly enriched and depleted sgRNAs and genes, with a cutoff of \(p < 0.001\). Libraries from different doses of JQ1 treatment in the same cell line were tested separately and merged together by choosing the lowest \(p\)-value. If a gene was enriched in one library but depleted in the other, the gene was set as unchanged. MageckFlute\(^{50}\) was used to visualize the data.

RNA-seq reads were aligned to the hg19 build of the human genome using STAR\(^{51}\) and counted using HTSeq-count\(^{52}\). Reads were also mapped to the mm10 build of the mouse genome, and those reads that mapped better to the mouse genome than human genome were filtered out. Libraries were then normalized for sequencing depth, and differential genes were determined using DESeq2\(^{53}\). Differential gene lists were analyzed for enriched pathways and process networks using MetaCore.

Single cell RNA-seq reads were aligned to the hg19 build of the human genome and the mm10 build of the mouse genome and counted using CellRanger (10X Genomics). Mouse and human cells were analyzed separately. Using Seurat\(^{54}\), cells with a high level of mitochondrial genes or a high or low number of detected genes were filtered out. Gene counts were log-
normalized and scaled, and the number of mitochondrial and UMI s were regressed out. The top principal components were then calculated and used to perform clustering and t-Distributed Stochastic Neighbor Embedding (t-SNE). Differential genes for each cluster were identified, and differential gene lists were analyzed for enriched pathways and process networks using MetaCore.
REFERENCES


39 Liu, P. Y. et al. The BET bromodomain inhibitor exerts the most potent synergistic anticancer effects with quinone-containing compounds and anti-microtubule drugs. *Oncotarget* **7** (2016).


Chapter 3:

Population dynamics in BET inhibitor combination therapies
Jennifer Y. Ge\textsuperscript{1,2,10,11}, Shaokun Shu\textsuperscript{1,11}, Yanan Kuang\textsuperscript{1,6}, Adrienne Luoma\textsuperscript{3}, Thomas O. McDonald\textsuperscript{2,7,14,15}, Grace A. Heavey\textsuperscript{1,6}, Anne Fassl\textsuperscript{4,12}, Cloud Paweletz\textsuperscript{1,6}, Piotr Sicinski\textsuperscript{4,12}, Kai W. Wucherpfennig\textsuperscript{3,13}, Jun Qi\textsuperscript{4}, Myles Brown\textsuperscript{1,8,11,13}, Aaron Thorner\textsuperscript{9}, David Pellman\textsuperscript{6,7,13}, Franziska Michor\textsuperscript{2,7,13,14,15,16}, and Kornelia Polyak\textsuperscript{1,7,8,11,13,16}

Departments of \textsuperscript{1}Medical Oncology, \textsuperscript{2}Data Sciences, \textsuperscript{3}Cancer Immunology and Virology, \textsuperscript{4}Cancer Biology, and \textsuperscript{5}Pediatric Oncology, \textsuperscript{6}Belfer Institute of Applied Cancer Science, and Centers for \textsuperscript{7}Cancer Evolution, \textsuperscript{8}Functional Cancer Epigenetics, and \textsuperscript{9}Cancer Genome Discovery, Dana-Farber Cancer Institute, Boston, MA 02215, USA. \textsuperscript{10}Harvard-MIT Division of Health Sciences and Technology, Departments of \textsuperscript{11}Medicine and \textsuperscript{12}Genetics, and \textsuperscript{13}Ludwig Center at Harvard, Harvard Medical School, Boston, MA 02115, USA. \textsuperscript{14}Department of Biostatistics, Harvard T. H. Chan School of Public Health, Boston, MA 02115, USA. \textsuperscript{15}Department of Stem Cell and Regenerative Biology, Harvard University, Cambridge, MA 02138, USA. \textsuperscript{16}The Eli and Edythe L. Broad Institute of MIT and Harvard, Cambridge, MA 02142, USA.

Manuscript in preparation:

**ABSTRACT**

Intratumor heterogeneity poses a major challenge to curing cancer and is responsible for treatment resistance and disease progression through tumor evolution. The effects of various treatments on the direction of evolution, though, are not well studied. Here, we used lentiviral DNA barcoding and mathematical modeling to investigate clonal dynamics during treatment selection with BET inhibitor therapy combined with CDK4/6 or microtubule inhibition in triple-negative breast cancer. Furthermore, we used exome, bulk RNA, and single cell RNA sequencing to profile the genomic and transcriptomic changes in heterogeneity in the post-selection resistant cells. We found that the combination therapies strongly selected for a subset of the initial clones. We identified pre-existing *RB1* mutations as a potential mechanism of resistance to the BET+CDK4/6 combination but otherwise found that resistance was more likely to be acquired *de novo* through transcriptomic changes, leading to multiple mechanisms of resistance and increased heterogeneity after treatment. Our findings provide insight into how resistance can arise to novel BET inhibitor combination therapies in TNBC and inform the management of progressive disease.

**INTRODUCTION**

Heterogeneity is an intrinsic feature of tumors that fuels their evolution and development\(^1\)\(^-\)\(^2\). At every stage of cancer progression, evolution selects for the fittest cells, and clones with growth advantages conferred by genetic or epigenetic alterations expand and result in more aggressive disease. In tumor initiation, clones with an accumulation of mutations that allow for uninhibited growth form the primary tumor mass; during treatment, cells with resistance to drug therapy lead to disease relapse; and in late stage disease, cells able to complete the metastatic cascade seed distant metastases\(^3\)\(^-\)\(^5\). This intratumor heterogeneity makes cancer especially hard to treat and must be considered in the development of new therapies. However, there have been few studies that have investigated the effects of treatment on tumor diversity,
and the impact of heterogeneity on treatment outcome is frequently not considered. It is critical to understand how the selective pressures of different drugs act on tumor cell populations, in order to better prevent resistance and manage progressive disease.

Recently, inhibition of bromodomain and extra-terminal (BET) proteins has received much attention as a novel targeted therapy in several types of cancer. BET proteins function as epigenetic readers of acetylation on histones and transcription factors and regulate transcription by recruiting transcriptional co-activators, chromatin modifiers, and additional transcription factors to the chromatin\textsuperscript{6}. In cancer, they have been shown to be especially important to the expression of oncogenes like \textit{MYC} and \textit{BCL2}, which may be especially sensitive to BET inhibition\textsuperscript{7,8}. Therefore, several small molecules, including JQ1, iBET151, and OTX015, have been under investigation in preclinical and clinical studies\textsuperscript{9-13} and show promise as a way to target oncogenes which have hitherto been regarded as “undruggable.” It has been found though that cells can develop resistance through a number of mechanisms, such as indirect binding to the chromatin through MED1\textsuperscript{12,14}. We previously found with genetic screens that palbociclib, a CDK4/6 inhibitor, and paclitaxel, a microtubule-inhibiting chemotherapy, may be effective in combinations with BET inhibitors and thus may help prevent resistance. However, the evolution of tumors undergoing selection with these compounds, that is, how heterogeneity changes in the population during treatment and whether resistance is pre-existing or acquired \textit{de novo}, has never been studied.

In order to investigate population dynamics, lineage-tracing techniques must be employed to quantify subclonal populations and track the fates of individual cells. Pulse-chase methods, multicolor fluorescent reporters, and lentiviral integration site mapping have been used to trace and detect division of cells\textsuperscript{15,16}. Recently, methods of DNA barcoding have been developed, where cells are labeled by viral infection of unique DNA sequences, which are integrated into the genome and copied to all daughter cells\textsuperscript{17,18}. Population structure can then be recovered following expansion or selection using barcode sequencing. One advantage is that
DNA barcoding has much higher complexity than previous methods, and many more clones, including rare clones, can be detected in a high-throughput way. These methods have been particularly useful in studying cellular differentiation and evolution of drug resistance\textsuperscript{17,19,20}.

Here, we use the ClonTracer DNA barcode library\textsuperscript{17} to trace the clonal architecture of triple-negative breast cancer (TNBC) cells during treatment with JQ1 \textit{in vivo} and \textit{in vitro}. We develop a mathematical to simulate barcode selection to infer the rates of pre-existing and acquired resistance. Furthermore, we profiled and assessed heterogeneity in post-treatment cells using exome and single cell RNA-sequencing.

\textbf{RESULTS}

\textit{In vivo} differences in post-treatment heterogeneity reflect cytostatic effect of JQ1 combination therapies

We previously produced xenografts with SUM159 cells, which had been barcoded with the ClonTracer library, in immunodeficient NOG mice and treated them with JQ1, palbociclib, and paclitaxel, alone and in combinations (Chapter 2). After one or two weeks of treatment, we collected the tumors and prepared libraries for sequencing from pre- and post-treatment samples. In order to quantify the overall amount of diversity, we used the Shannon index, a measure that is commonly used in population ecology (Equation 3.1). To our surprise, tumors treated with the combination therapies tended to have higher diversity than with either drug alone, which also tended to have higher diversity than those treated with vehicle (Figure 3.1A). Furthermore, tumors that were untreated or treated with single agents had a shift in their barcode frequency distributions towards fewer barcodes making up a larger proportion of the population (Figure 3.1B). This result suggests that these drugs had a primarily cytostatic effect \textit{in vivo}, where the combination treatments inhibited the growth of all cells in the starting population and thus maintained the initial tumor diversity, whereas subclones with the fastest
Figure 3.1: Tumor diversity in xenografts after JQ1 combination treatments. 

(A) Shannon index of diversity in tumors before treatment and after treatment with vehicle, JQ1, palbocicilib, paclitaxel, and combinations. 
(B) Cumulative distribution of barcode frequencies of ranked barcodes in tumors before and after 2 weeks of treatment. 
(C) Scatterplots of Shannon indices vs. tumor weights of xenografts before or after 1 or 2 weeks of treatment. 
(D) Venn diagram of the number of shared barcodes between xenografts prior to treatment.
growth rates quickly expanded and became dominant in tumors when treatment was less effective or not given. Moreover, the Shannon index was negatively correlated with tumor weight for untreated tumors, which is consistent with selection for the fittest clones in fast growing tumors (Figure 3.1C).

**Long-term treatment with JQ1 combinations selects for both clones with pre-existing and acquired resistance**

Next, we wanted to ask whether the treatments would select for the same or different clones across multiple replicates in each group, which would suggest pre-existing or acquired resistance, respectively. However, we did not see resistance arise *in vivo* within a two-week treatment period. In addition, we were not able to compare the amount of shared barcodes between different xenograft replicates because we found that the tumors that developed had mostly unique and very few shared barcodes (Figure 3.1D). This indicates that there was already selection for different clones that would graft in individual mice prior to starting treatment and thus they were not comparable. Therefore, we passaged the ClonTracer barcoded SUM159 cells *in vitro* in the presence of JQ1, palbociclib, paclitaxel, JQ1+paclitaxel, or JQ1+palbociclib for up to 18 passages. This allowed us to examine how clonality changes over time with a longer-term treatment and during development of resistance.

We observed that the growth rate of treated cells initially slowed, particularly in combination-treated groups, but then increased again after several passages to nearly the same rate as the DMSO controls, suggesting a population bottleneck due to treatment selection (Figure 3.2A-B). Interestingly, one replicate treated with JQ1+palbociclib died out at passage 9 and was not able to recover from this bottleneck. We then performed barcode sequencing on several passages throughout the treatment course. We found that, with each passage, the diversity decreased for all treatment groups, as expected due to loss of barcodes from random
Figure 3.2: Population diversity in SUM159 cells during *in vitro* selection with JQ1 combination treatments.

Growth rates during treatment with JQ1 combined with palbociclib (A) and paclitaxel (B). Shannon indices of barcode diversity during passaging in JQ1 and palbociclib (C) or JQ1 and paclitaxel (D). Cumulative frequencies of ranked barcodes at various passages of treatment with JQ1 and palbociclib (E) and JQ1 and paclitaxel (F). Number of barcodes making up the top 50% of the cell population at the last passage of JQ1 and palbociclib (G) and JQ1 paclitaxel (H).
Figure 3.2 (continued)
sampling, but the diversity fell more in JQ1, paclitaxel, and palbociclib treated samples compared with DMSO and the most in combination-treated samples (Figure 3.2C-D). Cell populations treated with JQ1+palbociclib and JQ1+paclitaxel also had the fastest shifts in their barcode frequency distributions towards fewer barcodes representing more of the population, compared with those treated with single agents or DMSO (Figure 3.2E-F). By the last passage, only 2 and 13 barcodes on average made up the top 50% of the populations, respectively, (Figure 3.2G-H). In particular, the Shannon index fell the fastest initially with JQ1+palbociclib and then plateaued, indicating that this treatment generated the strongest selection for resistant clones (Figure 3.2C).

We then compared the barcodes that were selected between replicates to see whether resistance was likely to be pre-existing or acquired de novo. We expected that clones with pre-existing resistance would be shared amongst multiple replicates, whereas those that acquired resistance during treatment would be unique to individual replicates. We found that following treatment selection in JQ1 or palbociclib, most of the barcodes were unique to individual replicates but a few were shared amongst the top barcodes (Figure 3.3-Figure 3.4), suggesting that treatment selects for both clones with pre-existing resistance and clones that had acquired resistance. However, in the JQ1+palbociclib and JQ1+paclitaxel selected populations, there were more barcodes that were unique rather than shared between replicates, particularly among the top barcodes (Figure 3.4). The fact that the same barcodes were not selected for to the same extent between replicates suggests that resistance to the combination treatments is rare in the initial population and is more likely to be acquired. Interestingly, the JQ1+palbociclib replicate that died out had a very similar barcode composition to one of the replicates that continued to proliferate (Figure 3.3A). We hypothesized that this was due to an acquired phenotype allowing for escape from growth arrest.
Figure 3.3: Clonal expansion of cells selected with JQ1 combinations. Plots show frequencies of top barcodes (those representing at least 0.5% of the population at any passage) in cell populations over the course of treatment with JQ1 and palbociclib (A) and JQ1 and paclitaxel (B). Colors represent unique barcodes, gray background represents all other barcodes in the population, and each plot represents one replicate.
Figure 3.4: Proportions of shared barcodes between replicates.
Plots show proportions of top barcodes (x-axis) that are unique, shared between 2 replicates, or shared between all 3 replicates, at the last passage of cells treated with JQ1 and palbociclib (A) and with JQ1 and paclitaxel (B).
Figure 3.4 (continued)

A

<table>
<thead>
<tr>
<th></th>
<th>DMSO</th>
<th>JQ1</th>
<th>PAL</th>
<th>JQ1+PAL</th>
</tr>
</thead>
<tbody>
<tr>
<td>Proportion</td>
<td><img src="image1.png" alt="Graph" /></td>
<td><img src="image2.png" alt="Graph" /></td>
<td><img src="image3.png" alt="Graph" /></td>
<td><img src="image4.png" alt="Graph" /></td>
</tr>
</tbody>
</table>

Top barcodes

B

<table>
<thead>
<tr>
<th></th>
<th>DMSO</th>
<th>JQ1</th>
<th>TAX</th>
<th>JQ1+TAX</th>
</tr>
</thead>
<tbody>
<tr>
<td>Proportion</td>
<td><img src="image5.png" alt="Graph" /></td>
<td><img src="image6.png" alt="Graph" /></td>
<td><img src="image7.png" alt="Graph" /></td>
<td><img src="image8.png" alt="Graph" /></td>
</tr>
</tbody>
</table>

Top barcodes
Mathematical modeling is consistent with a high level of acquired resistance to JQ1 and palbociclib

In order to computationally test how much resistance is pre-existing vs. acquired, we designed a mathematical model to simulate the barcode selection experiments to see what rates of resistance were consistent with our experimental data. We used a two-type birth-death process model with sensitive and resistant cells, with a proportion of the initial barcodes $\rho$ having pre-existing resistance (Figure 3.5). Sensitive and resistant cells have their own birth rates ($b_s$ and $b_r$) and death rates ($d_s$ and $d_r$). At each division, a sensitive cell can acquire resistance by giving rise to a resistant daughter cell, at a transition rate $\mu$. The growth rates for each cell type were experimentally measured, using lines derived from the post-treatment pools as the resistant cell types, while death rates were estimated from previous flow cytometry analyses of apoptosis (Table 3.1, Figure 2.7D). We used the distributions of the sequenced barcodes in pre-treatment samples to estimate the initial barcode complexity and frequencies. Replicate plates were sampled from this initial pool, and proliferation and passaging were simulated for 18 passages, as we did in the in vitro experiments, for a range of parameters for $\rho$ and $\mu$. We evaluated the simulation results by comparing them with the experimental data using their Shannon indices of diversity and proportions of shared barcodes between replicates. (See Methods and Appendix B for details of model design.)

We ran iterations of the simulation for $\rho$ ranging from $1\times10^{-1}$ to $1\times10^{-6}$ and for $\mu$ ranging from $1\times10^{-2}$ to $1\times10^{-6}$, focusing on the JQ1 and palbociclib treatments. We found that several parameter combinations fit the Shannon indices of the experimental data for both drugs, including high rates of $\rho$ and/or high rates of $\mu$ (Figure 3.6). However, a comparison of the proportion of shared barcodes between replicates in the simulations vs. the data narrowed down the parameter search space (Supplementary Figure B.1). Notably, only a high transition rate could match the high proportion of unique barcodes that we observed experimentally. The best
Figure 3.5: Design of the mathematical model of barcode evolution during treatment. See Methods for model details.
### Table 3.1: Growth and death parameters used in the mathematical model of barcode selection

<table>
<thead>
<tr>
<th></th>
<th>Growth rates (day⁻¹)</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>DMSO</td>
<td>JQ1</td>
<td>PAL</td>
</tr>
<tr>
<td>Parental</td>
<td>0.8186</td>
<td>0.4707</td>
<td>0.3696</td>
</tr>
<tr>
<td>JQ1-R</td>
<td>0.8842</td>
<td>0.7473</td>
<td></td>
</tr>
<tr>
<td>PAL-R</td>
<td>0.6081</td>
<td></td>
<td>0.5231</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th></th>
<th>Death rates (day⁻¹)</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>DMSO</td>
<td>JQ1</td>
<td>PAL</td>
</tr>
<tr>
<td>Parental</td>
<td>0.0238</td>
<td>0.0319</td>
<td>0.0223</td>
</tr>
</tbody>
</table>
Figure 3.6: Comparison of Shannon indices of simulated vs. experimental data. Each plot shows changes in diversity for a given value of $\rho$ for various values of $\mu$ in simulations of passaging in JQ1 (A) and palbociclib (B).
fit parameters for JQ1 were $\rho = 1 \times 10^{-5}$ and $\mu = 5 \times 10^{-2}$ (Figure 3.7), and the best fit for palbociclib was $\rho = 2.5 \times 10^{-3}$ and $\mu = 5 \times 10^{-2}$ (Figure 3.8). Therefore, the simulated results were consistent with a high level of acquired resistance to both JQ1 and palbociclib.

**RB1 loss is one possible mechanism of resistance to JQ1+palbociclib**

In order to see if any genetic mutations could contribute to treatment resistance, we performed exome sequencing of pre- and post-selection cells. We detected a nonsense mutation in RB1 (E864*) in the JQ1+palbociclib-selected cells, at an allele frequency of 27%. This was not detected with exome sequencing in any of the other cell populations. However, we used digital droplet PCR (ddPCR) to determine if the mutation was in a rare pre-existing clone in the pre-treatment population, as well as if it was in any of the other post-treatment groups. We found that indeed this RB1 mutation was present in the pre-treatment pool, at a frequency of approximately 1 in 100,000 (Figure 3.9A). We also found the mutation in some replicates treated with JQ1 or palbociclib alone, but it either remained at approximately the same frequency or expanded to at most 0.05% in one palbociclib replicate (Figure 3.9A). Interestingly, the E864* mutation was much more highly selected by JQ1+palbociclib in one replicate (24.1% by ddPCR), while it only expanded to 0.7% in the other replicate (Figure 3.9A). Thus, we concluded that Rb loss is not necessary for resistance to JQ1+palbociclib but is one possible mechanism.

To validate whether Rb is functionally relevant to JQ1 and palbociclib resistance, we deleted RB1 in SUM159 cells using CRISPR (Figure 3.9B) and treated them with the drug combination. RB1 deleted cells became resistant to JQ1+palbociclib (Figure 3.9C), and the two drugs were no longer synergistic but merely additive (Figure 3.9D).
Figure 3.7: Comparison of proportion of shared barcodes in simulations of JQ1 treatment vs. experimental data.

(A) Distributions of proportions of shared barcodes for various values of \( \rho \) and \( \mu \) over 5 iterations of the simulation compared with experimental data. (B) Likelihoods of the experimental data given the distributions produced by the simulation for various values of \( \rho \) and \( \mu \).
Figure 3.8: Comparison of proportion of shared barcodes in simulations of palbociclib treatment vs. experimental data.

(A) Distributions of proportions of shared barcodes for various values of $\rho$ and $\mu$ over 5 iterations of the simulation compared with experimental data. (B) Likelihoods of the experimental data given the distributions produced by the simulation for various values of $\rho$ and $\mu$. 

89
Figure 3.9: RB1 loss is one mechanism of resistance to JQ1+palbociclib.  
(A) Frequency of RB1 E864* mutation in pre-treatment and post-selection cell populations.  
(B) Western blot of pRb in wild type and RB1 deleted SUM159 cells.  
(C) Growth curves of wild type and RB1 deleted SUM159 cells treated with DMSO or 77 nM JQ1 and 611 nM palbociclib.  
(D) Normalized isobolograms show interaction effects between JQ1 and palbociclib for wild type and RB1 deleted cells. Points represent various combinations of concentrations. Diagonal line represents additivity, below the line synergistic, above the line antagonistic.
**G1/S pathways are upregulated in JQ1+palbociclib resistant cells**

We then asked how gene expression changed after selection with each drug treatment; thus, we performed RNA-seq on the post-selection resistant cells. Notably, we found that G1/S pathways were upregulated in JQ1- and JQ1+palbociclib-selected cells compared to DMSO-treated cells (Figure 3.10A). In particular, cyclin E expression was increased in JQ1+palbociclib-selected cells compared with JQ1-selected cells and in JQ1 compared with DMSO (Figure 3.10B). *CDKN1A* (p21) and *RB1* were also decreased in the combination compared with JQ1 resistant cells (Figure 3.10B). In contrast, we previously found the opposite, that all cell cycle pathways including G1/S were downregulated in JQ1 and palbociclib treatment (Figure 2.8). This suggests that genes involved in the G1/S transition are important to escaping JQ1+palbociclib-induced growth arrest. In addition, Myc and BCL-xL were also more highly expressed in the combination, compared to JQ1 alone, compared to DMSO (Figure 3.10C), consistent with our previous work that identified them as gained super-enhancers in the derived JQ1-resistant line SUM159R12. Furthermore, a set of DNA replication (*ORC2, ORC5, MCM8, TOP1, WRN*), chromosome segregation (*ANAPC13, ANAPC2, ZWILCH*), and transcriptional activation (*CDK7*) genes was upregulated in combination-resistant cells, which was not observed in cells resistant to either agent alone (Figure 3.10B), indicating that there is increased sensitivity to DNA transcription and instability in JQ1+palbociclib resistance.

**Single cell RNA-seq reveals increased heterogeneity in JQ1+palbociclib**

Lastly, we performed single cell RNA-seq to examine whether there was heterogeneity in the response to any of the treatments. Using *t*-distributed Stochastic Neighbor Embedding (*t*-SNE), we found that the cells primarily clustered by treatment group (Figure 3.11A) and that within treatment groups, cells formed only one or two clusters (Figure 3.11B), which were distinguished by cell cycle phase (data not shown). However, JQ1+palbociclib-resistant cells
Figure 3.10: RNA-seq of cells following selection in JQ1 and palbociclib.
Heat maps show differentially expressed (A) process networks, (B) genes in the cell cycle core network, and (C) genes MYC and BCL2L1 (BCL-xL), in SUM159 cells following 18 passages in JQ1, palbociclib, and the combination, compared with DMSO.
Figure 3.10 (continued)
Figure 3.11: Single cell RNA-seq reveals increased heterogeneity in JQ1+palbociclib post-selection populations.

(A) t-SNE plots of cells from the pre- and post-selection populations by single cell RNA-seq, colored by cluster (left) and treatment group (right). Each point represents one single cell. (B) Separate t-SNE plots of cells of each treatment group. Each point represents one single cell. (C) Heat map of differentially expressed genes in each cluster of the JQ1+palbociclib-resistant population. Each column represents one single cell.
Figure 3.11 (continued)

A

B

C

Expression

HBE1
KRT7
TLP1
JUNB
IL6
KRT8
IL18
HRAS
CCND3
MKI67
HSP90AB1
TUBB
MMP1
RAC1
CCND1
CDC20
AURKA
CDK4
TLP3
H2AFJ
COL1A1
FN1
VIM
RHOA
EGR1
SPARC

cluster 1  cluster 2  cluster 3  cluster 4
formed four distinct clusters, which were not related to cell cycle (Figure 3.11B-C). Two of these clusters expressed genes consistent with a senescence-associated secretory phenotype (SASP, e.g., $H2AFJ$, $COL1A1$, $FN1$, $TIMP3$, $IL6$, $IL18$), while the third cluster had increased expression of $CCND1$ and $CDK4$, and the fourth cluster had increased expression of $CCND3$ (Figure 3.11C). These distinct phenotypes indicate that cells can respond differently to the JQ1+palbociclib combination and that there may be multiple mechanisms of resistance or clonal cooperation that drives the resistance.

**DISCUSSION**

Here, we used DNA barcode sequencing and mathematical modeling to investigate the clonal dynamics during treatment with BET inhibitor combinations. We discovered that there was significant selection for a small subset of clones, particularly with combination treatments. Furthermore, the clones selected differed greatly between replicates, especially in combination treatments, and this was consistent with a high rate of de novo acquired resistance to these therapies. Using genomic and transcriptomic profiling, we found that cells upregulated genes involved in the G1/S transition and downregulated genes involved in cell cycle inhibition to allow for escape from growth arrest. One potential mechanism was loss of Rb, but this was not found in the majority of the cells, and thus there is heterogeneity in the mechanism of resistance.

To our knowledge, this is the first study examining the evolution of cell populations during treatment with JQ1 or the JQ1+palbociclib combination. The selection for Rb-mutant clones was due to a nonsense mutation, E864*, which has previously been reported in human tumor samples and is inferred to be pathogenic$^{21,22}$. Rb loss is a well-known mechanism of resistance to palbociclib in ER$^+$ breast cancer$^{23,24}$ and was also a hit in our CRISPR screen that made cells more resistant to JQ1 (Chapter 2). We found that Rb loss could confer resistance to the JQ1+palbociclib combination as well, but the mutation was not clonal. In addition, some Rb-null lines that we previously tested were still sensitive to the JQ1+palbociclib combination.
(Chapter 2). Thus loss of Rb is neither necessary nor sufficient for resistance to JQ1+palbociclib. We did not find exome mutations in other cell cycle genes, and other mutations that arose in the resistant cells are of unknown significance.

The high rate of acquired resistance compared with pre-existing resistance that we found suggests epigenetic rather than genetic mechanisms, which would be expected to be related to the mutation rate of DNA replication. Acquired resistance also corresponds with the cell-to-cell transcriptomic variation that we saw in single cell RNA-seq, since each surviving clone would have had to develop resistance independently. Thus, resistance seems to be largely mediated by transcriptomic changes. We found that our post-selection SUM159 cells had upregulation of G1/S pathways, in contrast to downregulation of all cell cycle pathways as we previously saw (Chapter 2). Cyclin E overexpression has been found to induce resistance to palbociclib\(^24\), and we also found that SUM159R cells, which are relatively resistant to palbociclib as a monotherapy, had upregulation of cyclin E when treated with palbociclib (Chapter 2). Notably, cyclin E was not differentially expressed in any cluster in our single cell RNA-seq data.

The increased phenotypic heterogeneity we saw with single cell RNA-seq after long-term selection in the JQ1+palbociclib combination suggests that there are multiple mechanisms of resistance. The gene expression pattern seen in two of the clusters was consistent with SASP. Thus, clonal cooperation, through these secreted factors, may contribute to growth of the rest of the population. Indeed, minor subpopulations have been shown to be capable of driving tumor growth through such non-cell-autonomous mechanisms\(^25\). Polyclonal resistance could pose a challenge to treatment, but if senescent cells prove to be supporting the growth of other cells, they could possibly be targeted to trigger tumor collapse.

With our methods, we were not able to determine which phenotypic cluster corresponded with which barcode or which barcode or cluster corresponded with the \(RB1\)-mutant clone, as single cell RNA-seq libraries were not sequenced deep enough to observe the mutation. However, various groups are developing methods of expressed barcodes\(^26\), as well as
reporter systems that would allow for isolation of cells from the initial pool with barcodes of interest. These new methods would help to refine our understanding of how genetic vs. transcriptomic heterogeneity is involved in resistance.

In summary, we have investigated the impact of BET inhibitor therapy and combination therapies on tumor evolution. These drugs may provide possibilities for new treatments for TNBC. Our results allow us to anticipate the emergence of resistance following treatment and inform the management of progressive disease.

METHODS

**Cell lines**: SUM159 breast cancer cells were obtained from Steve Ethier (University of Michigan) and cultured in 50% DMEM/F12 and 50% Human Mammary Epithelial Cell Growth Medium with 10% FBS and 1% PenStrep (Thermo Fisher Scientific).

**DNA barcoding**: The high-complexity ClonTracer barcode library was a gift from Frank Stegmeier (Novartis). SUM159 cells were barcoded by lentiviral infection using 8 µg/mL polybrene (Millipore). After 24 hours of incubation with virus, infected cells were selected with 2 µg/mL puromycin. To ensure that the majority of cells were labeled with a single barcode per cell, we used a target m.o.i. of approximately 0.1, corresponding to 10% infectivity after puromycin selection. Infected cell populations were expanded in culture for the minimal amount of time to obtain a sufficient number of cells to set up replicate experiments.

**CRISPR knockout**: CRISPR guides targeting *RB1* were designed using CRISPOR\(^\text{27}\): (1) TCCTGAGGAGGACCCAGAGC, (2) CGGTGGCGGCCGTTTTTCGG, (3) GGACAGGTTTGTGCGAAAT. Guides were cloned in lentiCRISPRv2 as previously described\(^\text{28}\). For virus production HEK293T cells were transfected with the respective lentiCRISPRv2-plasmid (empty vector served as control) and lentiviral envelope (VSVG) and packaging (Δ8.9) plasmids using Polyfect (Qiagen). Transfection medium was changed the next day. Medium with virus particles was collected 48 hours later and passed through a 0.45 µm
syringe filter followed by virus concentration using Amicon Ultra-15 100kDa centrifugal columns. Concentrated virus was added to 8x10^4 SUM159 cells together with 10 µg/mL Polybrene (Millipore). To achieve efficient RB1 knockout, virus from all three guides was used on the same cells. The same amount of virus carrying empty lentiCRISPRv2 was used for control cells. 48 hours post-infection, medium was changed and cells were placed under puromycin selection (3 µg/mL) for 6 days.

**Compounds:** Palbociclib was obtained from MedChemExpress. Paclitaxel was obtained from the Dana-Farber Cancer Institute Research Pharmacy. 

**Xenograft studies:** *In vivo* studies were previously performed by bilateral orthotopic mammary fat pad injection of 2 million barcoded SUM159 into NOD.Cg-Prkdc<sup>scid</sup>/Il2rg<sup>tm1Sug</sup>/JicTac (NOG) mice (Taconic). Once tumors were palpable, mice were treated for up to 2 weeks with vehicle (1:9 DMSO in hydroxypropyl-β-cyclodextrin), JQ1 (30-50 mg/kg daily intraperitoneal), palbociclib (75 mg/kg daily by gavage), paclitaxel (10 mg/kg twice weekly intraperitoneal), JQ1 and paclitaxel, or JQ1 and palbociclib.

**Clonal selection with therapies *in vitro***: Barcoded SUM159 cells were grown in DMSO, JQ1 (100 nM), paclitaxel (0.6 nM), palbociclib (160 nM), JQ1+paclitaxel, or JQ1+palbociclib, in triplicates. Cells were initially plated in 10 cm plates with 2 million cells per plate and then split 1:4 when approximately 80% confluent. Cells were grown for up to 18 passages.

**DNA/RNA extraction:** DNA and RNA were extracted from cultured cells and xenografts using the AllPrep DNA/RNA Mini Kit (Qiagen). Viably frozen cells from the last treatment passage were thawed and treated for 2-3 additional passages before DNA/RNA extraction. Frozen tumors were pulverized using the Covaris CP02 Tissue Pulverizer. Tissues or cells were homogenized by passing the sample through a 23g needle. Isolated RNA was treated with the RNase-Free DNase Set (Qiagen). RNA was prepared from duplicate samples. The QIAamp DNA Maxi and Mini Kits (Qiagen) were also used to extract DNA from those samples on which we did not perform RNA-seq.
Barcode sequencing: PCR was used to amplify barcodes and introduce Illumina adaptors along with a 5 bp index sequence for multiplexing as described\textsuperscript{17}. 2 µg of genomic DNA was used as template, and 15-16 samples were multiplexed for NGS. PCR products were run on 0.8% agarose NGS E-Gels (Invitrogen) to verify the correct library size, and bands were cut out and purified using the MinElute Gel Extraction Kit (Qiagen).

RNA-sequencing: Bulk RNA-seq libraries were prepared from total RNA by the Dana-Farber Cancer Institute Molecular Biology Core Facilities (MBCF) using the Illumina TruSeq Stranded mRNA Library Prep Kit and 16-18 samples were multiplexed per lane for NGS. For single cell RNA-seq, equal numbers of cells from triplicates were combined and washed twice in PBS with 0.04% RNase-free BSA (New England BioLabs). Cells were then diluted to 700 cells/µL and filtered through a 35 µm nylon mesh prior to library preparation. Single cell RNA-seq libraries were prepared using the Chromium Single Cell 3’ Library & Gel Bead Kit v2 and Single Cell A Chip (10X Genomics). Samples were multiplexed 8 per lane for NGS. MBCF performed all sequencing.

Whole exome sequencing: Libraries were prepared from equal amounts of pooled genomic DNA from triplicates by the Dana-Farber Cancer Institute Center for Cancer Genome Discovery (CCGD). Prior to library preparation, DNA was fragmented to 250 bp (Covaris sonication) and further purified using Agentcourt AMPure XP beads. Size-selected DNA was ligated to sequencing adaptors with sample-specific barcodes using the Kapa Hyper kit. Libraries were pooled and sequenced on an Illumina MiSeq to estimate the library DNA concentration based on the number of reads per sample. Libraries were pooled and captured using SureSelect Human All Exon v5 in 7 x 3-plex and 1 x 2-plex. Captures were performed using the Agilent SureSelect Hybrid Capture kit. Captures were pooled together and sequenced on 4 lanes of the Illumina HiSeq 3000.

Digital droplet PCR: Taqman primer/probe mix was custom-made by Life Technologies. The allele-specific MGB probes were labeled with either VIC or FAM at the 5’ end and a
nonfluorescent quencher (NFQ) at the 3' end. The forward primer sequence was 5'-ACAGCGACCGTGCTGCTC-3', reverse primer sequence was 5'- TTCAGTGGTTAGGAGGTTGC-3', wild-type (E864) probe sequence was 5'-VIC-AAGAAGTGCTGAAGGAA-MGB-NFQ-3', and mutant (E864*) probe sequence was 5'-FAM-AAAAGAAGTGCTTAAGGAA-MGB-NFQ-3'.

dPCR cycling conditions were: 95°C for 10 minutes, 40 cycles of 94°C for 30 seconds and 57°C for 60 seconds, 10°C forever. The reaction mixture (25 uL) included ddPCR™ Supermix for Probes (Bio-Rad), custom-made Taqman primer/probe mix, and appropriate DNA templates. Droplets were generated on the Automated Droplet Generator (Bio-Rad). Reactions were cycled on a thermocycler and were read on the QX200 Droplet Reader (Bio-Rad). Data analysis was performed with QuantaSoft (Bio-Rad).

**Barcode analysis:** Barcode sequencing reads were demultiplexed and filtered for reads with 30 bp length, containing an Illumina adaptor, matching the barcode pattern (alternating A/T and C/G), having a Phred quality score of at least 10 for all base pairs and an average Phred score of 30. For each sample, barcodes that had only 1 read were also filtered out. The Shannon index was used to quantify barcode diversity

\[
H = - \sum_i p_i \ln p_i
\]

where \(p_i\) is the frequency for barcode \(i\). Because of the high complexity of the barcode library, we limited our analysis to barcodes that were observed in at least one sequencing library of the plates being compared, to ensure that we were comparing barcodes that were present in the initial seeded pools. To compare replicates within treatment groups, we considered the intersection between replicates of the unions of barcodes observed across all passages sequenced. When comparing between treatment groups, we used the intersection of barcodes seen in all replicate plates in all treatments in at least one passage (\(n = 13,248\) for JQ1+palbociclib experiment and 16,330 for JQ1+paclitaxel experiment).
**Genomic data analysis:** RNA-seq reads were aligned to the hg19 build of the human genome using STAR\(^{29}\) and counted using HTSeq-count\(^{30}\). Libraries were then normalized for sequencing depth, and differential genes were determined using DESeq2\(^{31}\). Differential gene lists were analyzed for enriched pathways and process networks using MetaCore.

Single cell RNA-seq reads were aligned to the hg19 build of the human genome and counted using CellRanger (10X Genomics). Using Seurat\(^{32}\), cells with a high level of mitochondrial genes or a high or low number of detected genes were filtered out. Gene counts were log-normalized and scaled, and the number of mitochondrial and UMIs were regressed out. The top principal components were then calculated and used to perform clustering and \(t\)-Distributed Stochastic Neighbor Embedding (\(t\)-SNE). Differential genes for each cluster were identified, and differential gene lists were analyzed for enriched pathways and process networks using MetaCore.

Exome data were analyzed by the Dana-Farber Cancer Institute CCGD. Reads were demultiplexed and aligned to the reference sequence b37 edition from the Human Genome Reference Consortium using Picard\(^{33}\). Mutation analysis for single nucleotide variants (SNV) was performed using MuTect v1.1.4\(^{34}\) and annotated by Variant Effect Predictor (VEP)\(^{35}\). After initial identification of SNVs and indels by MuTect and GATK respectively, the variants were annotated using OncoAnnotate to determine what genes are impacted and their effect on the amino acid sequence.

Copy number variants were identified using RobustCNV, an algorithm in development by the CCGD. Briefly, RobustCNV relies on localized changes in mapping depth of sequenced reads in order to identify changes in copy number. Observed values are normalized against the mapping depth in a panel of normals (PON) sampled with the same capture bait set. Normalized coverage data are then segmented using Circular Binary Segmentation\(^{36}\) with the DNAcopy Bioconductor package. Finally, segments are assigned gain, loss, or normal-copy calls using a
cutoff derived from the within-segment standard deviation and a tuning parameter set based on comparisons to array-CGH calls in separate validation experiments.

**Mathematical modeling:** A two-type birth-death process with sensitive and resistant types with their own birth and death parameters was used to model the selection of clones during treatment with JQ1 or palbociclib. A proportion of the barcodes $\rho$ are initially resistant, and sensitive cells mutate into resistant cells at a rate $\mu$. Birth and death rates for each cell type of each clone were sampled from a log normal distribution centered around experimentally determined rates and were heritable to all daughter cells. The average growth rates were measured in proliferation assays with pre-treatment and resistant lines derived from single cell clones. The average death rates were estimated from flow cytometry data of apoptosis after treatment with JQ1 or palbociclib. The barcode complexity was estimated using the total number of unique barcodes seen in all barcode libraries, and the initial barcode distribution was estimated using 3 pre-treatment barcode libraries. Replicate plates were sampled from this initial population to simulate seeding. Mutation and expansion were simulated for 18 passages using the Binomial-Negative Binomial algorithm\textsuperscript{37,38}. See Appendix B for complete methods.

Simulation results were compared with experimental data using the Shannon index (Equation 3.1) and using the proportion of overlapping barcodes. The simulation was run for a range of parameters for $\rho$ and $\mu$. A wide range from $\rho = 1 \times 10^{-1}$ to $1 \times 10^{-6}$ and from $\mu = 1 \times 10^{-2}$ to $1 \times 10^{-6}$ was first sampled for both JQ1 and palbociclib selection with one simulation run for each parameter combination. A smaller range that most closely matched the experimental data was then chosen and sampled, with 5 simulation runs for each parameter combination. To determine the best-fit parameters, we calculated a likelihood for each parameter set using the distribution of proportion of shared barcodes over the 5 runs. This was computed as the sum of the likelihoods of observing the proportion of shared barcodes between 1, 2, and 3 replicates, assuming a normal distribution with mean and variance seen over the 5
simulations, for each number of top barcodes in each replicate, summed over the 3 experimental replicates.
REFERENCES


Picard: A set of command line tools (in Java) for manipulating high-throughput sequencing (HTS) data and formats such as SAM/BAM/CRAM and VCF, <http://broadinstitute.github.io/picard/> (2019).


Chapter 4:

Combined BET and CDK4/6 inhibition induces mitotic failure and tetraploidy
Jennifer Y. Ge\textsuperscript{1,2,7,8}, Anne Trinh\textsuperscript{1,8}, Mijung Kwon\textsuperscript{3}, Jun Qi\textsuperscript{4}, Myles Brown\textsuperscript{1,5,8,9}, David Pellman\textsuperscript{3,6,9}, Franziska Michor\textsuperscript{2,6,9,10,11,12}, and Kornelia Polyak\textsuperscript{1,5,6,8,9,12}

Departments of \textsuperscript{1}Medical Oncology, \textsuperscript{2}Data Sciences, \textsuperscript{3}Pediatric Oncology, and \textsuperscript{4}Cancer Biology, and Centers for \textsuperscript{5}Functional Cancer Epigenetics and \textsuperscript{6}Cancer Evolution, Dana-Farber Cancer Institute, Boston, MA 02215, USA. \textsuperscript{7}Harvard-MIT Division of Health Sciences and Technology, Department of \textsuperscript{8}Medicine, and \textsuperscript{9}Ludwig Center at Harvard, Harvard Medical School, Boston, MA 02115, USA. \textsuperscript{10}Department of Biostatistics, Harvard T. H. Chan School of Public Health, Boston, MA 02115, USA. \textsuperscript{11}Department of Stem Cell and Regenerative Biology, Harvard University, Cambridge, MA 02138, USA. \textsuperscript{12}The Eli and Edythe L. Broad Institute of MIT and Harvard, Cambridge, MA 02142, USA.

Manuscript in preparation:

ABSTRACT

Tetraploidy is a common feature in cancer that can be an intermediate step towards aneuploidy and genomic instability, thus accelerating the rate of tumor evolution. Here, we found that triple-negative breast cancer cells that had acquired resistance to combination treatment with the BET inhibitor JQ1 and the CDK4/6 inhibitor palbociclib had undergone tetraploidization. We explored the mechanisms and dynamics of this tetraploidization using flow cytometry and live imaging analysis of cells with fluorescent tags and cell cycle reporters. We determined that genome doubling occurs rapidly following treatment with JQ1+palbociclib as a result of mitosis and cytokinesis failure. Furthermore, we found that JQ1 and palbociclib can both individually induce tetraploidy through disruption of expression of cell cycle and spindle microtubule genes. Our results indicate novel mechanisms of action and resistance to BET and CDK4/6 targeted therapies and suggest new vulnerabilities in resistant cells.

INTRODUCTION

Genomic instability is a common hallmark of cancer that can allow cells to increase their proliferation and gain new functions, through acquisition of genetic alterations. Tumors commonly exhibit not only mutations of single genes but also variation in whole chromosome copy number. Aneuploidy can be found in 88% of all cancers, with 17% of the genome amplified and 16% deleted in a typical cancer. One possible mechanism that has been proposed for the development of aneuploidy is tetraploidization of diploid cells. Whole genome doubling occurs in nearly one-third of cancers and can arise by mitotic slippage, cytokinesis failure, and cell fusion. Aneuploidy and tetraploidy can both inhibit and promote tumorigenesis. Cells may undergo cell cycle arrest and apoptosis as a result abnormal copy number and increased cellular stress. On the other hand, aneuploidy can be advantageous to cells, by altering levels expression of key genes or allowing for increased genetic diversification. Moreover, genome doubling in cancer has been found to be associated with poor prognosis.
Many common anti-cancer drugs are aimed at further increasing the mutation rate in order to elicit an apoptotic response. Cells that are deficient in DNA repair in particular can have increased sensitivity to DNA-damaging chemotherapies\(^9\). However, cells may not undergo apoptosis and instead develop tolerance to aneuploidy. For example, paclitaxel, a chemotherapeutic agent that targets the microtubule, can induce tetraploidy and aneuploidy following prolonged arrest and mitotic slippage\(^10\). Therefore, increasing genomic instability may be somewhat of a double-edged sword that can produce tetraploid cells, which may be able to undergo more rapid evolution and be more drug-resistant\(^11\).

In our previous work, we have been studying BET and CDK4/6 inhibition as a novel combination therapy in triple-negative breast cancer (TNBC), an aggressive subtype of breast cancer for which there are limited treatment options. We found that the combination of JQ1 and palbociclib was able to effectively halt proliferation in TNBC cell lines by inducing cell cycle arrest, in addition to some increase in apoptosis. We also developed lines resistant to each drug alone and to the combination by growing cells in the presence of each drug for multiple passages. Here, we show that these resistant lines are near-tetraploid and that combined BET and CDK4/6 inhibition rapidly induces tetraploidy through mitotic errors. In order to quantify the extent of this tetraploidization and investigate its dynamics, we used fluorescent labels and cell cycle reports with flow cytometry and live imaging analysis, in order to elucidate the drugs' effects on cell division.

RESULTS

**JQ1 and palbociclib-resistant cells are near-tetraploid**

We used flow cytometry analysis to quantify ploidy in our derived resistant SUM159 cells, which were previously selected in JQ1, palbociclib, or the combination (Chapter 3). We found that all three populations had an increased fraction of cells that were approximately 4n, as well as gain of a small peak near 8n (Figure 4.1A). This change was most significant in the JQ1+palbociclib-
Figure 4.1: Cells that develop resistance to JQ1 and palbociclib are tetraploid. 
(A) Histograms of DNA content by flow cytometry in propidium iodide (PI) stained post-selection SUM159 cells. Representative karyotypes of cells passaged in JQ1 (B), palbociclib (C), JQ1+palbociclib (D), and DMSO (E). Arrows indicate clonal (black) or non-clonal (white) structural abnormalities.
selected cells, which no longer had any 2n cells, indicating that they were all aneuploid (Figure 4.1A). Interestingly, the near-4n peaks in the resistant populations were centered at slightly less than 4n and were broader than the peaks in the DMSO-treated samples. These genomes are likely to have arisen through tetraploidization followed by chromosomal losses in subsequent mitoses, causing heterogeneity in chromosomal copy numbers. Thus, this would support the hypothesis that tetraploidy is an unstable intermediate that promotes further chromosomal instability.

We confirmed the ploidy findings by karyotyping, which showed that JQ1, palbociclib, and JQ1+palbociclib-selected cells all had 4 copies of most chromosomes, with some chromosomes undergoing further losses or gains to 3-6 copies (Figure 4.1B-D). There was also much cell-to-cell variability in copy number, as well as in structural abnormalities (data not shown), further supporting our hypothesis of increased genomic instability following tetraploidization. On the other hand, cells passaged in DMSO were primarily near diploid with only a loss of chromosome 13 and translocations on chromosomes 1, 9, and 14, which were clonal (Figure 4.1E). However, a minority of the cells (3 out of 20 counted) was also found to be tetraploid with heterogeneity in chromosomal copy number (Figure 4.1E and data not shown). This indicates that this cell line exhibits some degree of genomic instability and undergoes spontaneous tetraploidization.

**Tetraploidization is induced by JQ1 and palbociclib treatment**

We thus asked whether this tetraploidy was induced by the JQ1 and palbociclib treatments directly or whether pre-existing tetraploid clones were more resistant to treatment and thus underwent clonal expansion. In order to quantify the proportion of tetraploid cells and distinguish them from 4n diploid cells in G2/M phase, we labeled SUM159 cells with a fluorescence ubiquitination cell cycle indicator (FUCCI) and then again used flow cytometry to assess DNA content in only the G1 fraction. We found that within 7 days, cells treated with JQ1+palbociclib
nearly all had a DNA content of more than 2n (Figure 4.2A). We also observed a significant increase in DNA content after JQ1 and palbociclib treatment in other TNBC cell lines, SUM149 and CAL-51 (Figure 4.2B-C), and in normal breast epithelial cells MCF10A (Figure 4.2D), as well as with another CDK4/6 inhibitor, ribociclib, combined with JQ1 (Figure 4.2E). Therefore, the induction of tetraploidy was not specific to the SUM159 cell line or to palbociclib.

Furthermore, immunofluorescence staining on treated cells revealed changes in their nuclei and overall morphology. After 3 days of treatment with JQ1, palbociclib, or both, cells became much larger with more cytoplasmic area (Figure 4.2F). In addition, some cells were binucleated, which was particularly common in those treated with both JQ1 and palbociclib (Figure 4.2F). This would suggest that they arose through either cell-cell fusions or through mitotic exit without cytokinesis.

**Tetraploidy is not caused by cell fusion**

In order to test whether SUM159 cells undergo spontaneous fusions to form tetraploids, we labeled them with either GFP or RFP and mixed them together in co-culture. We then examined the population for the presence of yellow (GFP⁺/RFP⁺) cells, which must have arisen through fusions of red and green cells. We did indeed find fused cells at a low but detectable rate (Figure 4.3A). However, the fraction of double-positive cells remained relatively unchanged after 7 days of treatment. JQ1-treated cells had a small increase in the GFP⁺/RFP⁺ fraction from 0.5% to 1%, but JQ1+palbociclib-treated cells actually had a decrease in the double-positive fraction (Figure 4.3B). Thus, we ruled out cell-cell fusion as a major mechanism for tetraploidization.

**Pre-existing tetraploid cells are not more drug-resistant**

To see whether tetraploid cells are inherently more drug-resistant, we used fluorescence-
Figure 4.2: Combined BET and CDK4/6 inhibition directly induce tetraploidy. Histograms of DNA content by flow cytometry in Hoechst-stained FUCCI-labeled G1 SUM159 (A), SUM149 (B), CAL-51 (C), and MCF10A (D) cells following treatment with JQ1 and palbociclib and in G1 SUM159 cells (E) following treatment with JQ1 and ribociclib, for 7 days. Tables indicate proportions of cells in the tetraploid gate. (F) Immunofluorescence staining of α-tubulin and phospho-histone H3 with DAPI in SUM159, SUM149, CAL-51, and MCF10A cells following 3 days of treatments. Scale bars represent 50 µm.
Figure 4.2 (continued)

A. SUM159
- Treatment: DMSO 6.11, JQ1 44.0, PAL 23.1, JQ1+PAL 69.0

B. SUM149
- Treatment: DMSO 12.0, JQ1 62.2, PAL 68.9, JQ1+PAL 90.4

C. CAL-51
- Treatment: DMSO 4.28, JQ1 19.1, PAL 6.56, JQ1+PAL 26.5

D. MCF10A
- Treatment: DMSO 1.74, JQ1 3.78, PAL 94.0, JQ1+PAL 92.7

E. SUM159
- Treatment: DMSO 4.21, JQ1 11.2, RIB (200 nM) 6.35, RIB (500 nM) 7.34, JQ1+RIB (200 nM) 27.0, JQ1+RIB (500 nM) 69.7

F. DAPI α-tubulin phospho-histone H3

116
Figure 4.3: Tetraploidy is not induced by cell fusion or by selection of pre-existing tetraploid cells. (A) GFP⁺/RFP⁺ (yellow) SUM159 cells (arrowheads) created by spontaneous fusions of single-color cells in co-culture. (B) Frequency of fused GFP⁺/RFP⁺ cells by flow cytometry after 7 days of co-culture and treatment with DMSO, JQ1, palbociclib, or JQ1+palbociclib. (C) Dose response curves for JQ1 and palbociclib in diploid cells (parental and FUCCI) and sorted cells enriched for tetraploids (FUCCI-tet and GFP/RFP-tet). (D) Normalized isobolograms show interaction effects between JQ1 and palbociclib for diploid and tetraploid-enriched cells. Points represent various combinations of concentrations. Diagonal lines represent additivity, below the line synergistic, above the line antagonistic.
activated cell sorting (FACS) to enrich for spontaneously occurring tetraploid cells by sorting for the FUCCI-labeled G1 4n cells and for the GFP+/RFP+ cells. However, these cells did not have any differences in sensitivity to JQ1 or palbociclib compared with the unsorted FUCCI-labeled or parental unlabeled lines (Figure 4.3C). Furthermore, the combination of JQ1 and palbociclib was still equally synergistic in the tetraploid-enriched cells over a range of concentrations for both drugs (Figure 4.3D). Therefore, the rapid shift in DNA content in cells during treatment is not due to selection of pre-existing tetraploid cells.

**JQ1 and palbociclib induce errors in mitosis**

To further investigate the mechanism and dynamics of tetraploidization, we performed live cell imaging for two days on SUM159 cells with fluorescently labeled histone H2B and plasma membrane. We observed that, in addition to prolongation of cell cycle and decreased division rate, there were a variety of mitotic errors in cells, which began within a few hours after adding JQ1, palbociclib, or the combination. Notably, JQ1 and palbociclib both caused errors in chromosomal segregation, where cells further divided their chromosomes following anaphase, forming two or more nuclei in daughter cells (Figure 4.4A). These micronuclei occasionally merged again in the following cell division but other times remained separate. In cells treated with JQ1 or the combination, there was also marked prolongation of the length of mitosis, with failure to initiate or progress through the phases of mitosis, particularly in JQ1+palbociclib treatment (Figure 4.4B). At times, this concluded with mitotic slippage or lack of cytokinesis (Figure 4.4B). In addition, there was a modest increase in cell death, particularly in the combination-treated cells, consistent with previously observed increases in apoptotic rates by flow cytometry (Figure 2.7D). Cell fusion events were infrequent but observed in one cell each treated with JQ1 and with JQ1+palbociclib (Figure 4.4B), again consistent with flow cytometry results (Figure 4.3). Thus, we concluded that JQ1 and palbociclib are able to induce
Figure 4.4: Live imaging of cells following treatment with JQ1 and palbociclib. (A) Representative images of mitoses in cells with fluorescently labeled H2B and plasma membrane and treated with DMSO, JQ1, palbociclib, and JQ1+palbociclib. Numbers indicate hours following start of treatment. (B) Cell cycle phase lengths for individual cells during time-lapse imaging period, with abnormal chromosomal segregation, apoptosis, and fusion events marked. Time indicates number of hours from start of the first observed mitosis for each cell.
tetraploidization through numerous possible errors in mitosis, which indicates that BET proteins, CDK4, and CDK6 are involved in multiple stages of cell division.

**JQ1 and palbociclib disrupt expression of mitosis genes**

Since BET proteins are transcriptional regulators and BRD4 is known to be involved in the transcription of genes necessary for mitotic exit\(^{12}\), we hypothesized that JQ1 generates errors in mitosis through dysregulation of mitosis genes. Thus, we reanalyzed our previously collected RNA-seq data from SUM159 xenografts treated with JQ1 and palbociclib (Chapter 2) and SUM159 cells that had undergone selection in JQ1 and palbociclib (Chapter 3) to ask if there were relative changes between treated vs. untreated cells in their levels of transcription of cell cycle genes. We used Spearman’s rho to calculate the correlation between ranks of expression of genes in gene lists from cell cycle related process networks. In the xenografts, we found that palbociclib alone and in combination with JQ1 decreased Spearman’s rho for multiple process networks, particularly G2/M and spindle microtubule, more than compared with a random gene list (Figure 4.5A). In the post-selection cells which had acquired resistance, there was also a decrease in Spearman’s rho for G1/S genes after treatment with JQ1, palbociclib, or both, consistent with our findings that this pathway contributes to drug resistance, but there were also changes in spindle microtubule genes (Figure 4.5B). This suggests that JQ1 and palbociclib have effects on multiple phases of the cell cycle, particularly chromosomal segregation, which can lead to chromosomal instability in cell division.

**DISCUSSION**

Here, we found that, much to our surprise, the entire population of JQ1+palbociclib double resistant cells that we previously generated (Chapter 3) was near-tetraploid. We determined that tetraploidy was induced very rapidly upon adding the combination treatment
Figure 4.5: JQ1 and palbociclib disrupt expression of cell cycle genes.
Spearman’s rank correlation coefficient (rho) for expression of cell cycle genes in treated SUM159 xenografts (A) and in SUM159 cells following in vitro selection (B) compared to vehicle. Spearman’s rho is calculated using cell cycle process network gene lists from MetaCore, comparing replicates within treatment groups (black) and between samples treated with JQ1 (red), palbociclib (blue), and JQ1+palbociclib (purple) compared with vehicle. A random list of 150 genes is used as a control.
through major errors in mitosis. Furthermore, JQ1 and palbociclib both disrupted expression of mitosis genes and also individually induced tetraploidy, though at a lower rate.

Our work is consistent with previous findings that BET inhibition can lead to multinucleation in TNBC, through direct suppression of Aurora kinase gene expression\textsuperscript{13}, but tetraploidy induced by CDK4/6 inhibition has never been reported. We observed a significant proportion of cells that were near tetraploid in the post-palbociclib selection population, as well as segregation errors in cells treated with palbociclib alone, albeit to a lesser extent than with the JQ1+palbociclib combination. Thus, we propose that tetraploidy-induced arrest through mitotic failure is a novel mechanism of action of CDK4/6 inhibition. Activity of the cyclin D/CDK4/6 complex is known to be involved in programmed polyploidy in megakaryocytes and duplication of centrosomes\textsuperscript{14,15}. We also find here that palbociclib broadly affects the relative levels of transcription of cell cycle and microtubule genes. Whether or not tetraploidy occurs in patient tumors following palbociclib treatment requires investigation by analyzing clinical trial samples for numbers of centromeres, centrosomes, and mitotic spindle defects.

We observed that the tetraploidy phenotype is induced very early upon treatment with JQ1+palbociclib and is followed by arrest. Evidence suggests that arrest following tetraploidization can be due to damage to the spindle, cytoskeleton, or DNA and is p53 dependent\textsuperscript{6}. However, our resistant cells were eventually able to escape this arrest and continue proliferating at nearly the same rate as parental untreated cells (Figure 3.2A). Cyclin D1 and D2 have been both been reported to mediate tolerance to genome doubling\textsuperscript{16,17}, and cyclin D2 is upregulated in our models in response to palbociclib (Figure 2.9). Furthermore, in our resistant lines selected in JQ1+palbociclib, we found Cyclin D1 and D3 to be upregulated in separate subpopulations (Figure 3.11). We also previously found overexpression of other genes involved in DNA stability and replication, as well as chromosomal segregation (Figure 2.9). However, the decision of cells to arrest, continue with cell cycle, or undergo apoptosis following JQ1+palbociclib-induced tetraploidization requires further study. Interestingly, Myc, a known
target of BRD4, is upregulated in our resistant cells (Figure 3.10C) but has been found to influence mitotic cell fate in the direction of death in mitosis over slippage\textsuperscript{18}. The lack of apoptosis in our cells may be due to the fact that Bcl-xL is simultaneously upregulated (Figure 3.10C), which tips the scale in favor of survival.

Whole genome doubling is observed in approximately one-third of human cancers, and tetraploidy and aneuploidy have been associated with poor prognosis\textsuperscript{5,8}. Tetraploidy in patient samples has been associated with mutations in \textit{TP53}, amplification of \textit{CCNE1}, and \textit{RB1} loss, suggesting that it is propagated by defects in G1 arrest as seen in our SUM159 model\textsuperscript{5}. Furthermore, it has been proposed that tetraploidy leads to increased tolerance to DNA damage and chromosomal instability and thus accelerates the rate of tumor evolution\textsuperscript{6,8}. This could result in increased heterogeneity in relapsed disease following JQ1+palbociclib treatment, which would pose a challenge to subsequent clinical management. However, the increased cellular stress imposed by tetraploidy may sensitize them to unique vulnerabilities, such as centrosome and genomic instability or proteoxic and metabolic stress\textsuperscript{19-21}.

In summary, we have shown that the combination of JQ1 and palbociclib rapidly induce errors in mitosis and tetraploidization, contributing to the drugs’ combined ability to induce deep cell cycle arrest. We also report a novel mechanism of action for CDK4/6 inhibition. Though tetraploidy may increased the rate of evolution and accelerate tumor progression, it may also expose new vulnerabilities that can be targeted. Thus, understanding the role of tetraploidy in cell cycle arrest and mechanisms of adaptation to tetraploidy will inform the treatment and management of progressive disease following BET and CDK4/6 targeted treatments.

**METHODS**

**Cell culture:** SUM159 and SUM149 breast cancer cells were obtained from Steve Ethier (University of Michigan) and cultured in 50% DMEM/F12 and 50% Human Mammary Epithelial Cell Growth Medium with 10% FBS. SUM159 cells resistant to JQ1, palbociclib, and JQ1 and
palbociclib were previously derived by growing them in DMSO, JQ1 (100 nM), palbociclib (160 nM), or JQ1 and palbociclib for 18 passages. CAL-51 cells were obtained from DSMZ and cultured in DMEM with 20% FBS. MCF10A cells were obtained from ATCC and cultured in DMEM/F12 with 5% horse serum, 10 µg/mL insulin, 20 ng/mL EGF, 0.5 µg/mL hydrocortisone, and 0.1 µg/mL cholera toxin. 1% Pen Strep (Thermo Fisher Scientific) was also added to all media.

**Lentiviral reporter constructs:** SUM159, SUM149, CAL-51, and MCF10A cells were labeled with FUCCI (pBOB-EF1-FastFUCCI-Puro, AddGene). SUM159 cells were also labeled with GFP (pEGFP-N1, Clontech), RFP (pDsRed-Monomer-C1, Clontech), or GFP-H2B followed by mCherry-plasma membrane (modified from pQC-TdTomato-IX, Addgene). For all virus production, 293FT cells were transfected with plasmid, TransIT-293 Reagent Transfection Reagent (Mirus Bio), and Ready-to-Use Packaging Plasmid Mix (Cellecta). Transfection media was changed the next day. Medium with virus particles was collected 2-3 days later and passed through a 0.45 µm syringe filter followed by virus concentration using Lenti-X Concentrator (Takara) as directed. Concentrated virus was added to a 10 cm dish of 70% confluent cells with 8 µg/mL Polybrene (Millipore). FastFUCCI-labeled cells were selected with 1 µg/mL puromycin and expanded for 6-9 days and then sorted by FACS for GFP+ or mCherry+ cells. GFP- and RFP-SUM159 cells were sorted for positive cells. H2B-SUM159 cells were first sorted for GFPhigh cells, which were expanded and infected with mCherry-plasma membrane. GFP+/mCherrylow cells were sorted, expanded, and then sorted again for GFP+/mCherry+ cells. Tetraploid-enriched lines were generated by staining cells with 10 µg/mL Hoechst 33342 (Sigma-Aldrich) and sorting for GFP+/RFP+/Hoechsthigh cells from the FUCCI-SUM159 cell population and for RFP+/GFP+ cells from co-cultured RFP-SUM159 and GFP-SUM159 cells.

**Compounds:** Palbociclib was obtained from MedChemExpress. Ribociclib, RO-3306, and rapamycin were obtained from Selleckchem. For all studies, cells were treated with JQ1 and palbociclib at their IC50 concentrations. SUM159 cells were treated with ribociclib at 200 or 500
nM. For G2 synchronization, SUM159 and SUM149 cells were treated overnight with 3 µM RO-3006.

**Flow cytometry analysis:** For ploidy analysis, post-selection cells were fixed overnight in 70% ethanol and then stained in 20 µg/mL propidium iodide (Thermo Fisher Scientific) with 0.2 mg/mL PureLink RNase A (Thermo Fisher Scientific) in 0.1% Triton X-100 (Sigma-Aldrich) for 30 minutes. Propidium iodide fluorescence intensity was acquired on an LSRFortessa cytometer (BD Biosciences). For tetraploidy and fusion studies following drug treatments, FUCCI-labeled cells and GFP- and RFP-SUM159 cells were stained with 10 µg/mL Hoechst 33342 (Sigma-Aldrich). Hoechst, GFP, and mCherry or RFP fluorescence intensity were acquired on the flow cytometer. Flow cytometry data were analyzed using FlowJo.

**Karyotyping:** Karyotyping was performed by the Brigham and Women’s Hospital CytoGenomics Core. 20 metaphases were counted from each treatment sample and 5-6 cells were karyotyped.

**Immunofluorescence:** Cells were grown on glass cover slips. After treatment with experimental compounds, cells were fixed in methanol for 5 minutes, then washed with PBS, and blocked with 10% goat serum for 1 hour. Cells were stained with primary antibodies against phospho-histone H3 (1:400, Abcam, ab5176) and α-tubulin (1:100, Sigma-Aldrich, T9026) at 4°C overnight, followed by staining with anti-rabbit (1:100, Thermo Fisher Scientific, A11008) and anti-mouse IgG1 (1:100, Thermo Fisher Scientific, A21125) secondary antibodies for 1 hour at room temperature. Slides were imaged on a Leica SP5X laser scanning confocal microscope.

**Live cell imaging:** GFP-H2B/mCherry-plasma membrane SUM159 cells were plated in 24 well µ-plates (ibidi) with 20,000 cells per well. Starting the following day, images were collected every 10 minutes for 48 hours from 10-15 positions in each well, immediately after addition of fresh media with DMSO, JQ1, palbociclib, or JQ1 and palbociclib. Images were acquired using a Nikon Ti inverted microscope equipped with a Yokogawa CSU-X1 spinning disk confocal head, Spectral Applied Precision LMM-5 with AOTF, a Hamamatsu ORCA ER cooled CCD camera.
and the Nikon Perfect Focus System. Images were acquired from Andor brightfield, FITC, and Cy3 channels from 4 z steps of 0.5 µm, using a 20X objective. An Okolab cage incubator was used to maintain samples at 37°C and 5% humidified CO₂. Image acquisition was controlled with MetaMorph (Molecular Devices). For data analysis, about 25 cells were selected for each treatment condition from the images at the first time point, and the lengths of each phase of the cell cycle were recorded for the duration of the live imaging period. At each division, only one daughter cell was selected for analysis.

**Dose response and synergy assays:** Dose response and synergy studies were performed in 384-well plates (Corning). SUM159 cells were seeded at a density of 200 cells/well in 50 µL of media. The following day, drugs were pin-transferred into the wells from a 500X concentrated drug plate made in a 384-well plate (Greiner Bio-One) using the JANUS Automated Workstation (Perkin Elmer). For dose response curves, ten serial dilutions were chosen, with 4 replicate wells for each concentration. For synergy studies, five concentrations for each drug were chosen between 20% and 80% inhibition, and half doses were used for combinations, with 4 replicate wells for each concentration of each drug alone and 8 replicates of each concentration pair. After 3 days, cells were stained with 5 µg/mL Hoechst 33342 (Sigma-Aldrich) in PBS, and nuclei were imaged and counted using the Celigo Imaging Cytometer (Nexcelom). To quantify the level of drug response for dose response curves, we used the normalized growth rate inhibition²².

**RNA-seq analysis:** RNA-seq data were previously generated from SUM159 cells (Chapter 2 and Chapter 3). Total RNA was extracted using the AllPrep DNA/RNA Mini Kit (Qiagen), and libraries were prepared and sequenced with the Illumina TruSeq Stranded mRNA Library Prep Kit by the Dana-Farber Cancer Institute Molecular Biology Core Facilities (MBCF). Reads were aligned to the hg19 build of the human genome using STAR²³ and counted using HTSeq-count²⁴, then normalized and tested for differential expression using DESeq²⁵.
REFERENCES


Chapter 5:

Conclusion
Overview

Triple-negative breast cancer (TNBC) is an aggressive subtype of breast cancer that is defined by absence of expression of the estrogen receptor (ER), progesterone receptor (PR), and HER2. It is associated with younger age, increased aggressiveness, and poorer survival than other types of breast cancer, and furthermore it has limited options for therapy. Therefore, TNBC represents a significant unmet medical need and new therapies are urgently needed. However, TNBC is a complex disease with considerable heterogeneity in both its biological characteristics and clinical behavior, thus making it difficult to find effective treatments. Though progress has been made in understanding potential vulnerabilities, attempts to target them, such as with DNA damage agents, inhibitors of signal transduction, and immune checkpoint blockade, have been met with only limited success.

Inhibition of bromodomain and extra-terminal (BET) proteins is emerging as a novel strategy to target the cancer epigenome. BET proteins, including BRD2, BRD3, BRD4, and BRDT function as transcriptional regulators that recognize acetylation marks on histones and transcription factors and recruit other factors to carry out transcriptional activation and chromatin modification. In cancer, they have been reported to be particularly important to the transcription of oncogenes through their activity at super-enhancers, thereby driving tumor proliferation. Numerous groups have reported activity of small molecule inhibitors of BET in numerous types of cancer, both hematologic and solid. In our previous work, we found that BET inhibitors including JQ1 are particularly effective in TNBC, displacing BRD4 from the chromatin and decreasing expression of specific oncogenes. However, TNBC cells were able to develop resistance to JQ1 through bromodomain-independent binding to the chromatin through MED1. Therefore, combination therapies are needed to prevent and overcome resistance to BET inhibition.

The development of resistance, as well as other phases of tumor progression, is thought to arise through cancer evolution, that is, Darwinian selection for the fittest clones within the
tumor population\textsuperscript{17,18}. From the expansion of proliferative clones during tumor initiation, to the emergence of resistant clones during treatment, to the seeding of metastasis-enabled clones, cancer is an ever-evolving disease, fueled by the tremendous amount of heterogeneity within the tumor\textsuperscript{19,20}. This heterogeneity stems from the intrinsic genomic instability and transcriptomic plasticity of cancer cells, as well as variation in the tumor microenvironment\textsuperscript{19,20}. Because of this cell-to-cell variability, full eradication of a tumor is exceedingly challenging, and thus, heterogeneity has important clinical implications, including accurate tissue diagnosis, selection of treatment regimens, prognosis, and management of relapsed disease\textsuperscript{20}. Therefore, a better understanding is needed of the effects of any given anti-cancer treatment on intratumor heterogeneity and how it affects the direction of evolution of a tumor.

In this dissertation, we have identified and tested potential combination therapies involving BET inhibitors in TNBC models and selected two candidates, palbociclib, a CDK4/6 inhibitor and paclitaxel, a microtubule-inhibiting chemotherapy, for further study of clonal dynamics and mechanisms of action and resistance.

**Major findings and implications**

In Chapter 2, we used genetic screens as an unbiased way to discover novel synthetic lethal interactions and mechanisms of resistance to BET inhibition. The major findings were:

- Targeting cell cycle, DNA damage, transcriptional activation, and ubiquitin-proteasomal degradation is synergistic with BET inhibition.
- Palbociclib combined with JQ1 inhibits proliferation significantly better than either drug alone both \textit{in vitro} and \textit{in vivo}, as does paclitaxel in certain models.
- Palbociclib and paclitaxel combined with JQ1 primarily induce cell cycle arrest by downregulation of cell cycle pathways but also moderately increase apoptosis.
• Altering the schedule of administration of JQ1, palbociclib, and paclitaxel affects treatment outcome.

Therefore, we identified novel synergistic drug interactions with BET inhibition and began to understand their mechanisms of action, which form the basis of promising new therapeutic strategies for TNBC.

In Chapter 3, we used DNA barcoding, mathematical modeling, and genomic analyses to gain a comprehensive understanding of the effects of palbociclib and paclitaxel combined with JQ1 on tumor clonality and the genomic landscape. The major findings were:

• The combination of JQ1+palbociclib and JQ1+paclitaxel primarily have a cytostatic effect \textit{in vivo} but exert the strongest selective pressure \textit{in vitro} compared with the single agents.

• The rate of \textit{de novo} acquired resistance to JQ1 and palbociclib is much higher than the rate of pre-existing resistance.

• Overexpression of G1/S pathways, including but not limited to \textit{RB1} loss, is one potential mechanism of resistance to JQ1+palbociclib.

• JQ1+palbociclib treatment induces increased heterogeneity in the post-selection tumors, suggesting multiple mechanisms of resistance.

Thus, we gained an understanding of the characteristics of cells resistant to JQ1, palbociclib, and how resistance is acquired, which informs the management of relapsed disease.

In Chapter 4, we found that all the cells that emerged following selection in JQ1+palbociclib, as well as a large proportion of those selected by each drug alone, became near-tetraploid, and we investigated the mechanism by which these drugs induce tetraploidy. The major findings were:

• Tetraploidy is directly induced by JQ1 and palbociclib treatment, rather than selection for pre-existing tetraploid cells.
• JQ1 and palbociclib induce major errors in mitotic progression, chromosomal segregation, and cytokinesis, which can induce cell cycle arrest.

• JQ1 and palbociclib disrupt expression of genes involved in mitosis.

Thus, we identified tetraploidization as a previously unrecognized mechanism of action of CDK4/6 inhibitors and as a mechanism of cell cycle arrest in response to combined targeting of BET and CDK4/6. Furthermore, cells must gain adaptation to aneuploidy in order to become resistant, which may expose new vulnerabilities in relapsed disease after development of resistance to the combination.

Conclusions and future directions
In this dissertation, we found novel combination targeted therapies for TNBC and sought to gain a comprehensive understanding of the overall response of tumors to these drugs, the effect on genetic and phenotypic heterogeneity, and possible mechanisms of resistance. Our work has also generated several more important questions for future study.

First, as with all targeted therapies, biomarkers must be identified and validated that can predict patient who will have a favorable response. Our results indicated that JQ1 and paclitaxel could be effective in some cases, as in our SUM159 and SUM159R models, but they were antagonistic in others, including in SUM149 and IDC50X. It is unclear if this might be due to variable sensitivity to DNA damage or alterations in phenotypes of immune cells or if response could be associated with TNBC subtype. Moreover, while RB1 is known to be a mechanism of resistance to palbociclib\(^2\) and we did find that it could confer sensitivity to the JQ1+palbociclib combination, we also observed that JQ1 and palbociclib were synergistic in some lines that were Rb-null. Thus, the mechanism of synergy could be also due to other pathways, such as tetraploidy-induced arrest or ubiquitin-proteasomal degradation.

Second, although we were able to investigate changes in clonal, genetic, and phenotypic heterogeneity separately, we lacked a method to integrate these findings. Here, we used
mathematical modeling as a way to simulate possible outcomes in order to infer whether clones are selected because they acquire resistance. However, expressed barcode methods, where single cell RNA sequencing is linked to a cell-specific barcode\textsuperscript{22} would allow us to more directly observe the changes in transcription in separate lineages that might lead to resistance. In addition, methods being developed to retrieve specific clones of interest from the initial cell population would allow us to characterize particular clones in the pre-treatment pool that eventually developed into the resistant population. These studies would help refine our understanding of cellular heterogeneity and plasticity and establish whether resistance is truly acquired through transcriptomic changes or selected from pre-existing clones with common transcriptional profiles.

Lastly, tetraploidization as a novel mechanism of action for BET and CDK4/6 inhibition must be explored. Further study is needed to validate this effect, especially in samples of patient tumors that have been treated with palbociclib. The mechanism of arrest following tetraploidy is not fully understood, nor the mechanism of cell fate decisions to arrest and persist or undergo apoptosis. Though in our flow cytometry and live imaging data, we did see some increase in apoptosis from the JQ1+palbociclib combination treatment, particularly following initiation of treatment, most cells survived, and fully resistant cells capable of escape growth arrest presumably arise from this pool. The efficacy of the JQ1+palbociclib combination would be further improved if we could tip the scale towards apoptosis to induce not just a cytostatic effect but also a cytotoxic effect. Moreover, we saw some evidence that the tolerance to aneuploidy may be dependent on DNA stability genes, and such vulnerabilities should be investigated to understand how they could be exploited to manage resistant disease.

In conclusion, although these topics remain to be better understood, we believe that our findings have significant translational impact. The efficacy of JQ1 and palbociclib, as well as JQ1 and paclitaxel, in our preclinical models provide rationale for further study in order to extend the efficacy and overcome resistance to BET inhibition and to develop them into novel therapies
for TNBC, a disease that is lacking in effective treatment options. Our results provide the first insight into their mechanisms of action and the eventual outcomes to be expected in the cancer evolutionary process.
REFERENCES


Appendix A:

Experimental compounds
**Supplementary Table A.1: Compounds tested in *in vitro* synergy studies**

<table>
<thead>
<tr>
<th>Compound</th>
<th>Target</th>
<th>Cell line IC50 (concentration range tested in combinations)</th>
<th>SUM159</th>
<th>SUM159R</th>
<th>SUM149</th>
<th>SUM149R</th>
</tr>
</thead>
<tbody>
<tr>
<td>JQ1</td>
<td>BET</td>
<td></td>
<td>100 nM</td>
<td>3.9 µM</td>
<td>49 nM</td>
<td>11 µM</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>(45 - 220 nM)</td>
<td>(500 nM - 30 µM)</td>
<td>(19 - 130 nM)</td>
<td>(7.1 - 18 µM)</td>
</tr>
<tr>
<td>Palbociclib</td>
<td>CDK4/6</td>
<td></td>
<td>160 nM</td>
<td>2.9 µM</td>
<td>1.5 µM</td>
<td>16 µM</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>(18 nM - 1.5 µM)</td>
<td>(33 nM - 260 µM)</td>
<td>(61 nM - 40 µM)</td>
<td>(3.2 - 79 µM)</td>
</tr>
<tr>
<td>Paclitaxel</td>
<td>Microtubule</td>
<td></td>
<td>0.6 nM</td>
<td>1.0 nM</td>
<td>1.3 nM</td>
<td>1.2 nM</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>(0.20 - 1.7 nM)</td>
<td>(0.30 - 3.3 nM)</td>
<td>(0.87 - 2.0 nM)</td>
<td>(0.6 - 2.3 nM)</td>
</tr>
<tr>
<td>Eribulin</td>
<td>Microtubule</td>
<td></td>
<td>0.18 nM</td>
<td>0.26 nM</td>
<td>0.11 nM</td>
<td>0.15 nM</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>(0.042 - 0.74 nM)</td>
<td>(0.083 - 0.82 nM)</td>
<td>(0.0088 - 1.4 nM)</td>
<td>(0.014 - 1.5 nM)</td>
</tr>
<tr>
<td>Vincristine</td>
<td>Microtubule</td>
<td></td>
<td>2.8 nM</td>
<td>1.0 nM</td>
<td>1.3 nM</td>
<td>0.70 nM</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>(0.77 - 3.1 nM)</td>
<td>(0.37 - 2.6 nM)</td>
<td>(0.92 - 2.0 nM)</td>
<td>(0.26 - 1.8 nM)</td>
</tr>
<tr>
<td>Doxorubicin</td>
<td>DNA damage</td>
<td></td>
<td>15 nM</td>
<td>7.0 nM</td>
<td>13 nM</td>
<td>11 nM</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>(10 - 21 nM)</td>
<td>(3.3 - 14 nM)</td>
<td>(5.5 - 29 nM)</td>
<td>(4.6 - 24 nM)</td>
</tr>
<tr>
<td>Gemcitabine</td>
<td>DNA damage</td>
<td></td>
<td>1.2 nM</td>
<td>29 nM</td>
<td>1.3 nM</td>
<td>1.3 nM</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>(0.86 - 3.0 nM)</td>
<td>(5.3 - 150 nM)</td>
<td>(0.50 - 3.5 nM)</td>
<td>(0.80 - 2.1 nM)</td>
</tr>
<tr>
<td>NVP-HSP990</td>
<td>HSP90</td>
<td></td>
<td>11 nM</td>
<td>8.0 nM</td>
<td>16 nM</td>
<td>12 nM</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>(4.1 - 14.3 nM)</td>
<td>(3.0 - 21 nM)</td>
<td>(7.6 - 19 nM)</td>
<td>(8.1 - 17 nM)</td>
</tr>
</tbody>
</table>
### Supplementary Table A.2: JQ1 and palbociclib concentrations tested in TNBC panel

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Doubling time (days)</th>
<th>Mutation status</th>
<th>IC50 (concentration range tested in combinations)</th>
<th>JQ1</th>
<th>Palbociclib</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>TP53</td>
<td>RB1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CAL-51</td>
<td>1.0</td>
<td>wt</td>
<td>wt</td>
<td>900 nM</td>
<td>250 nM</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>(360 nM - 2.2 µM)</td>
<td></td>
</tr>
<tr>
<td>CAL-120</td>
<td>1.7</td>
<td>wt</td>
<td>wt (intron mutation)</td>
<td>320 nM (250 nM - 1.5 µM)</td>
<td>11 µM</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>(1.8 - 40 µM)</td>
<td></td>
</tr>
<tr>
<td>HCC38</td>
<td>2.0</td>
<td>homozygous missense + del</td>
<td>210 nM (120 - 370 nM)</td>
<td>470 nM (61 nM - 40 µM)</td>
<td></td>
</tr>
<tr>
<td>HCC3153</td>
<td>1.5</td>
<td>wt</td>
<td>wt</td>
<td>460 nM (120 - 900 nM)</td>
<td>6.3 µM</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>(3.1 - 10 µM)</td>
<td></td>
</tr>
<tr>
<td>Hs578T</td>
<td>1.6</td>
<td>homozygous missense</td>
<td>100 nM (45 - 220 nM)</td>
<td>600 nM</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>(18 nM - 1.5 µM)</td>
<td></td>
</tr>
<tr>
<td>MDA-MB-231</td>
<td>1.0</td>
<td>homozygous missense</td>
<td>290 nM (120 - 900 nM)</td>
<td>410 nM</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>del</td>
<td>(50 nM - 3.3 µM)</td>
<td></td>
</tr>
<tr>
<td>MDA-MB-436</td>
<td>1.6</td>
<td>frameshift</td>
<td>homozygous frameshift</td>
<td>560 nM</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>(360 nM - 2.2 µM)</td>
<td>4.0 µM</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>(3.1 - 10 µM)</td>
<td></td>
</tr>
<tr>
<td>PMC42</td>
<td>1.3</td>
<td>wt</td>
<td>wt</td>
<td>3.7 µM (510 nM - 30 µM)</td>
<td>75 nM</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>(50 nM - 10 µM)</td>
<td></td>
</tr>
<tr>
<td>SUM149</td>
<td>1.1</td>
<td>missense</td>
<td>49 nM (19 - 130 nM)</td>
<td>1.5 µM</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>(61 nM - 40 µM)</td>
<td></td>
</tr>
<tr>
<td>SUM159</td>
<td>0.7</td>
<td>insertion</td>
<td>100 nM (45 - 220 nM)</td>
<td>160 nM</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>(18 nM - 1.5 µM)</td>
<td></td>
</tr>
</tbody>
</table>

*wt, wild-type; del, deletion*
Supplementary Table A.3: Compounds tested *in vivo*

<table>
<thead>
<tr>
<th>Compound</th>
<th>Target</th>
<th>Cell line dose, schedule, and route</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>SUM159</td>
</tr>
<tr>
<td></td>
<td></td>
<td>SUM159R</td>
</tr>
<tr>
<td></td>
<td></td>
<td>IDC50X</td>
</tr>
<tr>
<td>JQ1</td>
<td>BET</td>
<td>30 - 50 mg/kg daily i.p.</td>
</tr>
<tr>
<td>Palbociclib</td>
<td>CDK4/6</td>
<td>75 mg/kg daily p.o. (oral gavage)</td>
</tr>
<tr>
<td></td>
<td>Palbociclib</td>
<td>75 mg/kg daily p.o. (oral gavage)</td>
</tr>
<tr>
<td>Paclitaxel</td>
<td>Microtubule</td>
<td>10 mg/kg twice weekly i.p.</td>
</tr>
<tr>
<td>NVP-HSP990</td>
<td>HSP90</td>
<td>0.5 mg/kg daily p.o. (in drinking water)</td>
</tr>
<tr>
<td>Eribulin</td>
<td>Microtubule</td>
<td>0.25 - 0.5 mg/kg twice weekly i.p.</td>
</tr>
</tbody>
</table>

i.p., intraperitoneal; p.o., by mouth (per os)
Supplementary Figure A.1: Pharmacokinetics of selected compounds tested in vivo
Appendix B:

Mathematical modeling
Supplementary simulation methods

We used a birth-death process to model the selection of clones, using two cell types: sensitive cells, with an initial proportion of $1 - \rho$, and resistant cells, with an initial proportion $\rho$. Sensitive and resistant cells have their own birth rates ($b_s$ and $b_r$) and death rates ($d_s$ and $d_r$), which are log normally distributed. The means of the growth rates ($\lambda_s = b_s - d_s$ and $\lambda_r = b_r - d_r$) were measured from proliferation assays of pre-treatment cells and resistant lines, which comprised 10 single cell clones from the last passage of each of the 3 replicates, derived from growing sparsely plated cells in the presence of JQ1 or palbociclib. The growth rate of the pre-treatment cells was assumed to be a combination of the growth rates of both sensitive and resistant cells.

Therefore, the sensitive cell growth rate is a function of $\rho$:

$$X(t) = \rho X(0)e^{\lambda_s t} + (1 - \rho)X(0)e^{\lambda_r t}$$

$$\lambda_s = \frac{1}{t} \log \frac{X(t) - \rho X(0)e^{\lambda_r t}}{(1 - \rho)X(0)}$$

where $X(t)$ is the number of total cells at time $t$. Death rates were estimated from flow cytometry data of percent of apoptotic cells after treatment with JQ1 or palbociclib in pre-treatment cells as follows. Since the total number of cells at time $t$ is $X(t) = X(0)e^{\lambda t}$, the number of cells that will die in the next infinitesimal time step is

$$dX(t) = dX(0)e^{\lambda t}$$

so the total number of cells that have died up to time $t$ is

$$D(t) = \int_0^t dX(0)e^{\lambda t} \, dt = \frac{dX(0)}{\lambda}(e^{\lambda t} - 1)$$

Therefore, the fraction of dead cells by flow cytometry at time $t$ is

$$\frac{D(t)}{X(t) + D(t)} = \frac{d(e^{\lambda t} - 1)}{e^{\lambda t}(\lambda + d) - d}$$

The death rate of resistant cells was assumed to be the same as untreated sensitive cells. Birth rates were thus calculated as $b_s = \lambda_s + d_s$ and $b_r = \lambda_r + d_r$. 
The initial barcode distribution was determined from the sequencing results. Across all libraries sequenced, we observed 903,900 unique barcodes. Thus, we estimated the complexity of the library to be 1 million. We used the barcode frequency observed in the pre-treatment libraries to estimate the initial barcode distribution. In 3 libraries, we observed 294,844 unique barcodes. Therefore, these frequencies were used for the top 294,844 barcodes, while the remaining barcodes were assumed to have frequencies less than the minimum observed frequency and were drawn from an exponential distribution. For each barcode, we sampled a birth and death rate from a log normal distribution centered around the experimentally derived parameters and with a variance 10% of the means. These birth and death rates were heritable to all daughter cells. To simulate plating, we sampled 3 replicate plates of 2 million cells each from a starting pool of 700 million cells with this distribution.

To simulate growth and mutation during passaging, we used the Binomial-Negative Binomial (BNB) algorithm by Mather et al.\textsuperscript{1} For resistant cells, which do not undergo further mutation, expansion is simulated for one time step $\Delta t$ as the sum of $m \sim B(n_0, 1 - \alpha)$ and $n \sim NB(n, 1 - \beta)$, where

$$\alpha = \frac{d e^{(b-d)\Delta t - d}}{b e^{(b-d)\Delta t - d}} \quad \text{and} \quad \beta = \frac{b e^{(b-d)\Delta t - b}}{b e^{(b-d)\Delta t - d}} \quad (B.5)$$

according to Durrett, 2014\textsuperscript{2}. The binomially distributed variable $m$ simulates the number of starting cells that do not go extinct within $\Delta t$, while the negative binomially distribution variable $n$ simulates the proliferation of those $n$ cells.

For sensitive cells, the birth rate of is adjusted for mutation, which occur at rate $\mu$, so that the adjusted birth rate is $b^* = b(1 - \mu)$ and the mutation rate is $\mu^* = \mu b$. In each time step, we sampled $r \sim U(0,1)$ for each sensitive barcode to sample the next mutation time

$$t_m = \frac{1}{R} \log \left( \frac{\frac{1}{n_0}(R - W + 2b^*) - W - R + 2d}{\frac{1}{n_0}(-R - W + 2b^*) - W + R + 2d} \right) \quad (B.6)$$

and the next extinction time.
\[ t_e = \frac{1}{R} \log \left( \frac{W - R + 2dR^{1/n_0}}{W + R - 2dR^{1/n_0}} \right) \]  

where \( R = \sqrt{(b^*-d)^2 + (2b^* + 2d + \mu^*)\mu^*} \), \( W = b^* + d + \mu^* \), and \( n_0 \) is the starting number of sensitive cells of that barcode. Thus, barcodes that go extinct have

\[ r < \left( \frac{R - W + 2d}{R + W - 2b^*} \right)^{n_0} \]

Barcodes that go extinct within the time step, i.e. \( t_e < \Delta t \), are replaced with a count of 0. We used \( \Delta t = 1 \) day, since in the experiment, cells were checked daily and split if they reached 80% confluence. Expansion of barcodes that did not mutate within the time step, i.e. \( t_m > \Delta t \), is simulated according to equations (B.5), with modifications such that \( n \neq 0 \). For the remainder of barcodes that do undergo mutation, we looped through each mutation event in order. For each barcode, the expansion of sensitive cells of that barcode is simulated up to the mutation time \( t_m \) as the sum of \( \tilde{m} \sim B(n_0 - 1, 1 - p_E(t_m)/p_M(t_m)) \), 1, and \( \tilde{n} \sim NB(\tilde{m} + 2, p_B(t_m)) \), where

\[ p_M(t_m) = \frac{RC(t_m) + 2dS(t_m) - WS(t_m)}{RC(t_m) - 2b^*S(t_m) + WS(t_m)} \]
\[ p_E(t_m) = \frac{d(1-p_M(t_m))}{W - d - b^*p_M(t_m)} \]
\[ p_B(t_m) = \frac{b^*p_E(t_m)}{d} \]

and where \( C(t) = \cosh \frac{Rt}{2} \) and \( S(t) = \sinh \frac{Rt}{2} \). One resistant cell of that barcode is added, and its expansion for the rest of the time step is simulated according to equations (B.5). A new mutation time is then simulated for that barcode according to equations (B.6)-(B.8). Expansion of other barcodes up to \( t_m \) is simulated according to equations (B.5), with modifications such that \( n \neq 0 \). These steps are repeated until all next mutation times are beyond \( \Delta t \).

The simulation is allowed to run for another \( \Delta t \), until the total number of cells reaches a number corresponding to 80% confluence (5 million). A split of 1:4 is then simulated by sampling down to \( \frac{1}{4} \) of the population. If the current passage is a passage for which we
performed barcode sequencing in the experiment, the other ¾ of the cells are outputted for diversity analysis. The entire BNB algorithm is repeated for a total of 18 passages.
REFERENCES


Supplementary Figure B.1: Proportions of shared barcodes between replicates in simulated data.
Plots show proportion of shared barcodes between replicates in initial simulations over a wide range of parameters for JQ1 (A) and palbociclib (B) treatment.