A STUDY OF SINGLE-STRANDED DNA GAPS IN THE RESPONSE TO REPLICATION STRESS AND SYNTHETIC LETHALITY

A Dissertation Presented
By
KE CONG

Submitted to the Faculty of the
Morningside Graduate School of Biomedical Sciences at UMass Chan Medical School in partial fulfillment of the requirements for the degree of
DOCTOR OF PHILOSOPHY

JANUARY 3, 2022

CANCER BIOLOGY PROGRAM
A STUDY OF SINGLE-STRANDED DNA GAPS IN THE RESPONSE TO
REPLICATION STRESS AND SYNTHETIC LETHALITY

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KE CONG

This work was undertaken in the Morningside Graduate School of Biomedical Sciences Cancer Biology Program

Under the mentorship of

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JANUARY 3, 2022
I would first express my sincere gratitude to my mentor, Dr. Sharon Cantor, for giving me the opportunity to join her team and grow as a scientist. I am so fortunate to learn not only from her passion for research, but also from her perseverance for life. Her thought and vision inspire me to broaden my horizons. Sharon guides me with extraordinary patience and always encourages me whenever I encounter difficulties. I genuinely appreciate for all her training and care, which motivate me to keep improving all the way along my PhD career.

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ABSTRACT

Mutations in the hereditary breast/ovarian cancer genes BRCA1/2 were shown to be synthetic lethal with poly(ADP-ribose) polymerase inhibitors (PARPi). This toxicity is assumed to derive from PARPi-induced DNA double strand breaks (DSBs) that necessitate BRCA function in homologous recombination (HR) and/or fork protection (FP). However, PARPi accelerates replication forks. While high-speed replication could cause DSBs, the finding that PARPi leads to single-stranded DNA (ssDNA) gaps/nicks suggests replication gaps could also or alone be the cause of synthetic lethality.

Here, we demonstrate that PARPi toxicity derives from replication gaps. Isogenic cells deficient in BRCA1 or the BRCA1-associated FANCJ, with common DNA repair defects in HR and FP, exhibit opposite responses to PARPi. Deficiency in FANCJ, a helicase also mutated in hereditary breast/ovarian cancer and Fanconi anemia, causes aberrant accumulation of fork remodeling factor HLTF and limits unrestrained DNA synthesis with ssDNA gaps. Thus, we predict replication gaps as a distinguishing factor and further uncouple HR, FP and fork speed from PARPi response. BRCA-deficient cells display excessive gaps that are diminished upon resistance, restored upon re-sensitization and when targeted augment synthetic lethality with PARPi. Furthermore, we define the source of gaps to defects in Okazaki fragment processing (OFP). Unchallenged BRCA1-deficient cells have elevated poly(ADP-ribose) and chromatin-associated PARP1 but aberrantly low XRCC1
indicating a defective backup OFP pathway. Remarkably, 53BP1 loss resuscitates OFP by restoring XRCC1-LIG3 that suppresses the sensitivity of BRCA1-deficient cells to drugs targeting OFP or generating gaps. Collectively, our study highlights unprotected lagging strand gaps as a determinant of synthetic lethality, providing a new paradigm and biomarker for PARPi toxicity.
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<tr>
<td>53BP1</td>
<td>p53-binding protein 1</td>
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<tr>
<td>Abs</td>
<td>Antibodies</td>
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<tr>
<td>APH</td>
<td>Aphidicolin</td>
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<tr>
<td>ATM</td>
<td>Ataxia-telangiectasia mutated</td>
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<tr>
<td>ATP</td>
<td>Adenosine triphosphate</td>
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<td>ATR</td>
<td>ATM and Rad3-related</td>
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<td>ATRi</td>
<td>ATR inhibitor</td>
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<td>ATRIP</td>
<td>ATR-interacting protein</td>
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<td>BACH1</td>
<td>BRCA1 interacting C-terminal helicase</td>
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<td>BER</td>
<td>Base excision repair</td>
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<td>BIR</td>
<td>Break-induced replication</td>
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<td>BLM</td>
<td>Bloom syndrome protein</td>
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<td>BRCA</td>
<td>Breast cancer susceptibility genes</td>
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<td>BrdU</td>
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<td>CPT</td>
<td>Camptothecin</td>
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<tr>
<td>CRISPR-</td>
<td>Clustered regularly interspaced short palindromic repeats</td>
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<tr>
<td>CtIP-</td>
<td>C-terminal-binding protein interacting protein</td>
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<tr>
<td>DKO-</td>
<td>Double knockout</td>
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<td>DNA-</td>
<td>Deoxyribonucleic acid</td>
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<td>DNA2-</td>
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<td>dNTP-</td>
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<td>DTT-</td>
<td>Dithiothreitol</td>
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<td>EdU-</td>
<td>5-ethynyl-2'-deoxyuridine</td>
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<td>EM-</td>
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<td>EXO1-</td>
<td>Exonuclease 1</td>
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<td>FA-</td>
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<td>FP-</td>
<td>Fork protection</td>
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<td>G4-</td>
<td>Guanine- or G-quadruplex</td>
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<td>GFP-</td>
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<td>GNL1-</td>
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<td>hTERT-</td>
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<td>HDR-</td>
<td>Homology-directed repair</td>
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<td>Reactive oxygen species</td>
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<td>RPA-</td>
<td>Replication Protein A</td>
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<td>RPA inhibitor</td>
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<td>WCEs-</td>
<td>Whole-cell extracts</td>
</tr>
<tr>
<td>WRN-</td>
<td>Werner syndrome ATP-dependent helicase</td>
</tr>
<tr>
<td>WT-</td>
<td>Wild-type</td>
</tr>
<tr>
<td>XRCC1-</td>
<td>X-ray repair cross-complementing protein 1</td>
</tr>
<tr>
<td>ZRANB3-</td>
<td>Zinc finger Ran-binding domain-containing protein 3</td>
</tr>
</tbody>
</table>
Chapter I introduces the background of my thesis work.

Chapter II includes the study that has been published as:


Chapter III includes the study that has been published as:


Chapter IV discusses my conclusions, final perspectives and future directions.

The contributions of Chapter II and III are acknowledged at the end of each chapter.
I also contributed the following publications but do not include them in this dissertation:


CHAPTER I Introduction

Preface

Figure 1.3, 1.4 and their legends are taken from the graphic abstracts of my published articles:


1.1 Replication stress and single-stranded DNA (ssDNA) gaps

1.1.1 Basics about replication stress and its links with cancer

Maintaining replication and genomic integrity is essential for cell proliferation. However, DNA replication encounters obstacles, lesions and damage from different origins, which induce replication stress and undermine cell fitness. Therefore, cells have evolved sophisticated responses to deal with these interruptions and to replicate DNA accurately and efficiently. In the absence of appropriate control and repair, impaired DNA will accumulate, thereby posing a serious threat to cell proliferation.

Replication stress can be triggered by factors from various sources. It is well-known that exogenous agents, including ultraviolet (UV) light, ionizing radiation (IR), X-rays, as well as numerous genotoxic chemicals, could damage genome. During the S phase, lesions resulted from such stress will render the replication prone to errors. Importantly, the DNA synthesis process is also a critical cause of replication stress. When lacking external toxic events, damaged DNA accumulate even during normal DNA replication. Such endogenous stressors include reactive oxygen species (ROS), reduction of DNA nucleotide pool, stochastic misincorporation of ribonucleotides, DNA sequences difficult for replication, topological hindrance, dysregulated chromatin compaction and/or conflicted transcription process.

Although there is not yet a specified or unified definition of this phenomenon, replication stress is widely acknowledged as an issue threatening genome stability and triggering certain responses. When DNA replication is perturbed, the S phase of cell cycle is not completed timely and precisely. These
Perturbations lead to altered replication fork dynamics, which is an immediate response to replication stress\(^5\). When DNA polymerases replicate damaged DNA, fork progression will slow down, temporarily stall or even reverse the direction (Figure 1.1). This intermediate state allows polymerases to cease their activity and relevant pathways to repair the lesions. If DNA replication fails to continue or restart, the replisome complex may become disassociated and forks may collapse. Collapsed forks usually undergo fork degradation, which is mediated by exo- and/or endonuclease DNA cleavage and cause double-stranded DNA breaks (DSBs)\(^3,5\) (Figure 1.1).

**Figure 1.1:** Schematic of replication fork intermediates during replication stress.
Here is a brief summary of some representative intermediates of replication forks. Upon encountering DNA lesions and/or replication stress, forks could slow down, stall (A) or reverse the direction (B) in response to stress. DNA synthesis could resume or restart (C) if forks are stabilized and protected under coordination (D). However, if the forks are not well-protected (E), nucleases will degrade replication forks (F) and cause fork collapse (G).

Replication stress is also a feature often observed in cancer\(^6\textsuperscript{-8}\). Cancer is likely to initiate from overwhelmed replication stress. For example, mutated replication factors, such as cell division cycle 6 (CDC6)\(^9\) and/or alternative DNA polymerases\(^10\), can alter cell cycle progression and promote tumorigenesis because of inaccurate replication. The activation of oncogenes and/or deregulation of tumor suppressors can generate DNA damage and replication stress\(^11,12\). Notably, MYC-activated cancer cells have a greater tendency towards gaining ROS, which further exacerbate this stress\(^13\). Additionally, distinct from differentiated cells that rarely or never divide, cancer cells generate stress due to the mitotic pressure from increased DNA replication and cell proliferation. Permanent proliferative stimuli can affect processivity of replication forks, induce frequent origin firing or re-replication, and cause deregulated DNA synthesis. Subsequently, an inadequate source of deoxynucleoside triphosphates (dNTPs) or other nutrients will increase replication stress and genomic instability\(^14,15\).

Since evergrowing evidence has shown the relationships between replication stress and cancer\(^16\), it is essential for researchers to further understand the complexity and hallmarks of replication stress.
1.1.2 Genome-wide ssDNA gaps arise under replication stress

Clear cellular markers that characterize replication stress will help to study related response processes in cancer. But the investigation is difficult due to the lack of certain DNA lesions or structures displaying it. However, replication stress often leads to accumulating stretches of single-stranded DNA (ssDNA). Exogenous and endogenous genotoxic agents can damage DNA, inducing hundreds of thousands of lesions per day in mammalian genomes\textsuperscript{17,18}. The majority of such lesions are ssDNA break\textsuperscript{19}, which are more frequent than their conversions to dangerous DSBs. The sources of ssDNA can range from metabolic attack from ROS to spontaneous DNA decay\textsuperscript{20}.

Perturbed fork dynamics during replication stress can contribute to gap formation. First, replication fork slowing and/or stalling can generate ssDNA gaps by specific uncoupling between DNA helicases and the replisome complex, not originating from primary DSBs. When replication forks and polymerases are stalled by physical barriers, the replicative minichromosome maintenance (MCM) helicase frequently continues moving downstream the parental DNA. After unwinding a few hundred base pairs along the replication fork, MCM helicase then expose ssDNA from this helicase–polymerase uncoupling\textsuperscript{21}. Next, DNA replication process is often rapidly regulated and tightly organized by potential inherent property of stalled replisomes, in spite of encountering various damage. In this scenario, in response to tolerating potential obstacles, replisome complex can uncouple the fork movement and bypass the problematic region by directly repriming DNA synthesis. Consequently, stretches of ssDNA gaps could also form on nascent DNA by
such 'skipping' of lesions behind the fork\textsuperscript{22} (Figure 1.2). These daughter-strand gaps then convert into duplex DNA in a post-replicative manner. Of note, cancer cells may undergo more stress due to dysregulated coordination of replication. Collectively, the influence on replisome movement and fork dynamics induce increased formation of ssDNA stretches.

![Diagram showing replication fork and ssDNA gap formation](image)

**Figure 1.2: Schematic of ssDNA gap formation during replication stress.** Perturbed fork dynamics during replication stress could lead to ssDNA gap formation. Gaps could form by the uncoupling of replisome complex at or near the fork (indicated by the stop sign). Gaps could also derive from lesion bypass or skipping behind the fork (indicated by the red stars on both leading and lagging strands).

The persistence of ssDNA, functioning as primary source of replication stress, triggers cellular responses that facilitate the fork machinery to overcome replication stress. Once ssDNA gaps are exposed, they are bound by Replication Protein A (RPA) and recruit the serine/threonine protein kinase ataxia-telangiectasia mutated (ATM)- and rad3-related (ATR) via ATR-interacting protein (ATRIP)\textsuperscript{23,24}. As one of the central checkpoint kinases, ATR activates subsequent events that involve the phosphorylation of RPA, checkpoint kinase 1 (CHK1) and histone variant H2AX. These signaling cascades modulate replication fork dynamics and are usually defined as
“replication stress response”. Together with the intermediates of replication forks under stress such as fork slowing, stalling and other altered fork dynamics (Figure 1.1), they could act as a set of markers of replication stress\textsuperscript{25}.

Notably, many of these response mechanisms actually reduce replication stress. This is because they facilitate replication completion and promote DNA repair by reducing global replication. Heightened replication stress response allows cells to tolerate higher level of stress. Whereas loss of replication stress response in fork dynamics and checkpoint signaling usually leads to cellular transformation and cancer predisposition. Cancer treatment thus deliberately takes advantage of this balance by semi-selectively targeting replication stress in cancer.

1.1.3 Replication stress is exploited for cancer therapy

Enhanced proliferative stimuli and sustained mitogenic signaling can generate replication stress, if not suppressed by checkpoints and repair mechanisms. In fact, lack of such control mechanisms for DNA replication is often a characterized phenomenon of cancer cells, either due to loss of tumor suppressors or oncogene override during malignant transformation\textsuperscript{16}. Cancer therapy thus may be similar as halting a running car without a brake. One option is to find an alternative stop method. While another is to increase the speed, get rid of additional control systems and overheat the motors. Although this is destructive, similar approach will also exhaust cancer to a stressful and unsustainable level, thereby triggering breakage and stop.
Conventional replication stress-inducing agents were primarily identified from serendipitous findings of tumor death following applications rather than invented on purpose\textsuperscript{26}. Chemotherapy regimens usually enhance replication stress within cancer cells by directly introducing lesions in template DNA and/or by interfering normal DNA replication and cell division. As a result of increased proliferative rate or impaired checkpoint response, cancers become more sensitive than normal cells to the genotoxic stress from these DNA-damaging drugs, such as DNA-reactive agents (including alkylating agents and platinum compounds), antimetabolites, topoisomerase poisons, etc\textsuperscript{26} Therefore, aiming at DNA is commonly assumed as the therapeutic target to attenuate cancer proliferation. Not surprisingly, despite using these conventional genotoxic chemotherapies for decades, their application is still limited. The Achilles Heel could be their associated toxicities to normal proliferative cells such as gut epithelium and bone marrow\textsuperscript{27}. Another limitation impairing long-term efficacy is the development of drug resistance.

Alternatively, therapeutic effects can be achieved by increasing stress above the threshold\textsuperscript{28,29}. In contrast to traditional concept, combining conventional chemotherapeutic agents with those that impair replication stress response is capable of inducing catastrophic replication stress\textsuperscript{30}. For example, pushing tumor cells through cell cycles by further depressing their checkpoints is beneficial for promoting cancer cell death. Therefore, drugs are increasingly being designed to exploit replication stress\textsuperscript{31}. Along these lines, a key will be to enhance replication stress in a targeted manner. In fact, the reliance of highly proliferating cancer to replication stress response suggests that manipulating
key biological transitions and responses will exert the cytotoxic effects\textsuperscript{32}. Some targets include interrupting S phase entry, stalling replication forks, separating replisome complex at collapsed forks and promoting premature entry into mitosis. Therefore, targeting related signaling pathways that govern these checkpoints provides the means of eliminating cancer cells by exacerbating the loss of proliferation-control processes\textsuperscript{28,29}.

Exploiting replication stress could also be highlighted by the lack of efficient DNA repair systems for genomic instability in cancer\textsuperscript{33-35}. It has been known that genes recurrently altered in tumor cells function in DNA repair pathways, which are also referred to as caretakers\textsuperscript{36}. One of the most frequently mutated genes is \textit{TP53}, which encodes p53 tumor suppressor protein\textsuperscript{37}. In fact, p53 protein is well-known for its roles in DNA damage response\textsuperscript{38,39} and the induction of cell cycle arrest, senescence, or apoptosis\textsuperscript{40}. Loss of this gene could thus explain why most cancer cells fail to undergo programmed cell death. In addition, syndromes with general DNA repair deficiency also result in increased incidence of corresponding cancer\textsuperscript{3}. Especially, DNA repair proteins mutated in cancer have been identified as the underlying genetic alterations across all major subtypes of cancer\textsuperscript{41}. Without disturbing normal cells, combination therapies that potentiate the replication stress may harness the tumor-specific mutations due to the defective DNA damage response. Collectively, the unprecedented insights into DNA repair deficiency during S phase and replication stress have laid the foundation for drug manipulation and precision medicine treatments.
1.2 Roles of BRCA-Fanconi anemia (BRCA-FA) pathway in replication stress and fork plasticity

1.2.1 Beyond break repair: BRCA-FA pathway in replication stress response

Deficiency in DNA repair pathways raises the likelihood of various malignant neoplasms, including breast and ovarian cancers that are among the leading causes of cancer-related deaths in women. Breast cancer susceptibility genes type 1 and 2, *BRCA1* and *BRCA2* (BRCA1/2 or BRCA), are mutated in hereditary breast and ovarian cancer\(^{42,43}\). Loss of these tumor suppressors is associated with a lack of efficient homology-directed repair (HDR) repair and subsequent genomic instability\(^{44,48}\). As critical components of homologous recombination (HR), BRCA1/2 proteins regulate multiple steps of DSB repair to preserve genome integrity, including the nucleolytic resection of breaks and the loading of central recombinase factor RAD51 on processed DSB ends\(^{49-52}\).

In fact, emerging evidence has shown that BRCA1/2 proteins not only repair DSBs, but also suppress replication stress. For instance, despite functional HR and checkpoints, mutant BRCA1 in primary human cells remains defective in dampening replication stress\(^{53}\). Additionally, BRCA1 repairs UV-induced lesions that trigger genotoxic stress\(^{54}\). Furthermore, exposure to the cellular metabolite aldehyde induces BRCA2 haploinsufficiency and renders BRCA2 heterozygous cells to potentiated replication stress\(^{55}\). Mutations in the partner and localizer of BRCA2 (PALB2), the key molecular adaptor for BRCA1 recruiting BRCA2-RAD51 machinery in HR pathway, also result in aberrant replication stress response\(^{56,57}\). Moreover, RAD51 mediates template switch,
strand transfer and/or other recombinational mechanisms to fill post-replicative gaps, which further inhibit replication stress\textsuperscript{58-60}.

Defective BRCA proteins also causes other pathological consequences more than cancer. Biallelic inactivation of BRCA genes leads to a rare and genetically heterogeneous syndrome Fanconi anemia (FA). FA patients typically present with developmental abnormalities, progressive bone marrow failure and predisposition to cancer\textsuperscript{61,62}. To date, mutations in at least 22 distinct genes, from Fanconi anemia complementation group A (\textit{FANCA}) to \textit{FANCW}, have been found linked with this disease\textsuperscript{62-64}. Although BRCA genes are initially recognized by their cancer predisposition, the designation of \textit{BRCA2} as \textit{FANCD1}\textsuperscript{65} and \textit{BRCA1} as \textit{FANCS}\textsuperscript{66} among FA genes establishes the notion of BRCA-FA pathway. Furthermore, functioning in an orchestrated manner, all the FA proteins interact together to repair a certain form of DNA damage interstrand crosslinks (ICLs)\textsuperscript{62}. Cells from FA patient thus exhibit exquisite sensitivity to agents that generate ICLs such as mitomycin C (MMC) and cisplatin\textsuperscript{67}.

Given that fixing ICL involves organized detecting and removing actions, studies of BRCA-FA pathway have been focusing on the intermediate steps of ICL-induced break repair in S phase\textsuperscript{63}. Typically, ICL is first recognized by \textit{FANCM} and then recruits the FA core complex comprised of 8 FA (\textit{FANCA}, \textit{FANCA}, \textit{FANCB}, \textit{FANCC}, \textit{FANCE}, \textit{FANCF}, \textit{FANCG}, \textit{FANCL} and \textit{FANCM}) and other FA-associated proteins\textsuperscript{62,67}. The FA core complex contains a E3 ubiquitin ligase (\textit{FANCL}) and monoubiquitinates the \textit{FANCD2/FANCI} heterodimer\textsuperscript{68}. This monoubiquitinated heterodimer can then localize to the damage sites and
coordinate with downstream FA proteins such as BRCA2/FANCD1, BRCA1/FANCS, PALB2/FANCN, RAD51/FANCR, FANCJ and so forth, to perform the ICL repair process mainly via HR\textsuperscript{62,63,69}.

However, broader roles independent of ICL repair, such as regulating mitosis and stabilizing common fragile sites, have emerged for BRCA-FA pathway in preserving genomic integrity\textsuperscript{70,71}. Therefore, it is necessary to study BRCA-FA pathway beyond break repair. Although fork slowing or stalling might not directly sensitize BRCA-FA-deficient cells, recent advances have elucidated how BRCA-FA pathway regulates fork dynamics. These findings provide a novel perspective for understanding disordered replication stress response in BRCA-FA diseases beyond DNA break repair\textsuperscript{72}.

1.2.2 Players for regulating fork dynamics: fork protection and remodeling

An initial link between BRCA-FA factors and replication stress response was established by BRCA2 in stabilizing stalled forks formed following the treatment of hydroxyurea (HU)\textsuperscript{73}. HU is a ribonucleotide reductase inhibitor that causes replication stress through decrease of deoxyribonucleotide generation\textsuperscript{74,75}. More recent reports have demonstrated that forks stalled by HU undergo pathological nucleolytic resection in cells deficient in BRCA1/2 and other components of the BRCA-FA pathway\textsuperscript{76-78}. Mechanistically, BRCA1/2 prevent nascent DNA at stalled replication forks from meiotic recombination 11 homolog (MRE11)-mediated resection through deposition of RAD51 onto exposed ssDNA\textsuperscript{79-81}. During replication stress response, fork processing should be properly coordinated for initial slowing, transient pause, and subsequent
restart upon stress release. Indeed, several BRCA-FA proteins, including RAD51, RAD51 paralogs, FANCl, FANCM, FANCD2, FANCD2/FANCI-associated nuclease 1 (FAN1) and the FANCD2-FAN1 interaction, also regulate slowing and resuming mechanisms in response to stress\textsuperscript{82-87}. Thus, replication machinery is orchestrated by BRCA-FA pathway and displays remarkable plasticity when encountering replication interference.

During these response transactions when replication forks experience stress, one well-recognized structure that aids DNA repair and ensures smooth bypass of damage for replication is fork reversal\textsuperscript{88,89}. Replications stress causes forks to reverse their course with re-annealing of the intact parental duplex and annealing of the nascent synthesized DNA strands. This fork reversal structure, a so-called “chicken foot”, converts a standard three-way replication fork into a four-way junction (Figure 1.1B). Although long considered as a pathological consequence of fork interference, fork reversal is now appreciated for fine-tuning of fork speed and facilitating recruitment of restart factors, such as RecQ-like helicase (RECQ1) and DNA replication helicase/nuclease 2 (DNA2) / Werner syndrome ATP-dependent helicase (WRN)\textsuperscript{90-92}. Many enzymes, known as fork remodelers, have displayed fork reversal activity, including DNA translocases RAD54, FANCM, helicase-like transcription factor (HLTF), SWI/SNF-related, matrix-associated, actin-dependent regulator of chromatin, subfamily A-like 1 (SMARCAL1) and zinc finger Ran-binding domain-containing protein 3 (ZRANB3) and DNA helicases WRN, Bloom syndrome protein (BLM) and F-box helicase 1 (FBH1)\textsuperscript{93-102}. In addition, the pivotal HR factor RAD51 joins the list of remodelers and regulates
fork uncoupling and reversal as a global response to various genotoxic agents\textsuperscript{103}. In particular, fork remodelers from sucrose nonfermenting 2 (SNF2) family, such as HLTF, ZRANB3, and SMARCAL1, can act via similar mechanisms of action\textsuperscript{104}. While how each player impacts fork dynamics individually in BRCA-FA deficient background still requires further investigation.

Meanwhile, fork reversal also plays a prerequisite role in fork degradation. This structure of reversed fork is an excellent substrate for recruiting various nucleases (Figure 1.1E), including aforementioned MRE11, as well as C-terminal-binding protein interacting protein (CtIP), Exonuclease 1 (EXO1) and DNA2, which mediate short or long end resections\textsuperscript{105}. Despite the fact that excessive fork degradation may eventually end up with fork collapse and genomic instability, limited processing of the reversed forks is still favorable for mitigating replication stress and fork restart in BRCA1/2-deficient cells\textsuperscript{106,107}. If reversed forks lack effective protection from BRCA-FA pathway, these replication intermediates will result in unrestricted degradation. And this extensive fork degradation has been associated with increased genomic instability and chemotherapeutic sensitivity\textsuperscript{77,78,108-111}. Correspondingly, in the absence of BRCA-FA proteins, rewired fork protection is able to confer chemoresistance independent of HR\textsuperscript{112-115}. In view of the complexity and the diversity of BRCA-FA pathway, the relative contributions of specific factors to the alterations of fork dynamics await detailed analysis.

The advent of appropriate assays was indispensable for monitoring replication fork progression and fork degradation upon the exposure to replication-stalling agents. Single-molecule DNA fiber analysis is a central
technique in gaining mechanistic insights for perturbations of fork dynamics\textsuperscript{116-120}. But one critical limitation of this tool is the restriction of resolution to few kilobases per micrometer. Thus, to detect ssDNA gaps in hundred-nucleotide or shorter scale, high resolution method such as electron microscopy (EM)\textsuperscript{121}, or employment of ssDNA-specific enzyme S1 nuclease combined with DNA fiber approach are needed for ongoing forks\textsuperscript{122,123}. Small ssDNA gaps frequently act as replicative intermediates of stress response mechanisms. For example, ssDNA gaps because of checkpoint defects and faulty gap-filling mechanisms, can recruit RAD51 to promote fork reversal and restart in response to stress\textsuperscript{79,103,124,125}. Therefore, it is imperative to further explore the relationships between ssDNA gaps and BRCA-FA pathway.

1.2.3 Role of FANCJ in replication stress response

Among the downstream DNA-repair proteins in BRCA-FA pathway, FANCJ, also known as BRCA1 interacting helicase 1 (BRIP1) / BRCA1 interacting C-terminal helicase (BACH1), was first discovered by its direct and functional interaction with BRCA1\textsuperscript{126}. Acting as helicase, translocase, ATPase and important in the repair of ICL, FANCJ plays pivotal roles in BRCA-FA pathway and tumor suppression\textsuperscript{127-133}. Similar to BRCA1/2 and other downstream FA proteins, FANCJ contributes to HR that repairs DSBs caused by DNA damage including ICLs, UV or ionizing radiation\textsuperscript{134-136}. Particularly, our previous study discovered that the FANCJ interaction with mismatch repair (MMR) protein, MutL homolog 1 (MLH1), is essential to resolve ICL, in contrast to the FANCJ-
BRCA1 binding that is dispensable in ICL correction\textsuperscript{137,138}. Together, FANCJ is one of the critical factors implicated in sensing DNA damage and fixing DSBs.

However, emerging studies have uncovered additional roles for FANCJ more than damage repair, extending its functions to replication stress response. Initially, FANCJ is found functioning in regulation of timely replication progression\textsuperscript{139}. Another clue is that FANCJ-deficient cells display sensitivity to the replication stress-inducing agents HU and the polymerase inhibitor aphidicolin (APH)\textsuperscript{140,141}. Independent of the FA core complex, FANCJ stills promotes the restart of APH-stalled replication forks\textsuperscript{142}. Additionally, multiple interactions of FANCJ also contribute to its roles in replication stress response. For instance, disrupting the FANCJ-MLH1 interaction gives rise to a pronounced delay in S phase restart following APH-induced replication stress\textsuperscript{143}. FANCJ acts with Topoisomerase Binding Protein 1 (TopBP1) to ensure replication checkpoint control and fork integrity\textsuperscript{144}. Importantly, FANCJ interacts and colocalizes with ssDNA-binding protein RPA in nuclear foci upon DNA damage or replication stress in human cells\textsuperscript{145}. In support of the association with replication fork, FANCJ exists at forks in a replication-dependent manner\textsuperscript{146}. Aside from unwinding duplex DNA structures\textsuperscript{147}, FANCJ resolves guanine- or G-quadruplex (G4) DNA secondary substrates derived from guanine-rich DNA sequences embedded in different regions of the genome and suppresses G4-induced DNA damage, replication stress and genomic instability\textsuperscript{148-152}. Thus, FANCJ might function in multiple replication processes in light of its interaction with G4 DNA\textsuperscript{153,154}. 
Collectively, FANCJ is also a critical regulator of the response to replication stress. In contrast to numerous reports about the impact of BRCA1/2 deficiency in replication stress, more roles of FANCJ in fork dynamics still need to be investigated.

1.3 PARP inhibitor (PARPi) is synthetic lethal with BRCA deficiency

1.3.1 PARPi: discoveries, target therapies and resistance mechanisms

To cure cancers with BRCA-FA mutations, genotoxic chemotherapy that exploits the DNA repair defects has been applied clinically for decades. Since most genotoxic chemotherapy drugs damage cancer and non-malignant, proliferating cells together, targeted therapy and selective killing have long been pursued by oncology researchers. In 2005, remarkable discovery showed that cells with defective BRCA genes were highly toxic to the inhibition of poly(ADP-ribose) (PAR) polymerase (PARP). This synthetic lethal interaction or synthetic lethality, achieved from the concurrent perturbation of two complementary pathways, then attracted abundant attention due to the prospect of new generation for cancer therapies. PARP inhibitors (PARPis) are thus generated for pharmacological inhibition and clinical use in multiple indications, including breast and ovarian cancers.

This PARPi synthetic lethality found in BRCA deficiency is attributed to the defects in HR and further coupled with loss of replication fork protection, both of which results in wide-spread accumulation of unrepaired DNA DSBs. Mechanistically, PARP1, the major protein in PARP family, identifies DNA damage such as ssDNA breaks and one-ended DSBs.
Recognition of DNA lesions activates PARP1 catalytic activity, which lead to auto-poly(ADP-ribosyl)ation (PARylation) and induces DNA repair processes. However, PARP inhibition specifically traps PARP1 on ssDNA intermediate and interrupt the downstream processes, thereby causing DNA damage that is generally fixed by BRCA1/2- and RAD51-dependent HR. Therefore, in HR-defective cancer cells because of disruptive mutations in BRCA or other FA genes, inhibiting PARP1 induces synthetically toxic accumulation of DNA damage, collapse of replication forks, and cell lethality.

Despite the high efficacy and initial response of PARPi in HR-deficient tumors, resistance is unfortunately acquired via different cellular mechanisms. Similar to other pharmacological resistance, one common mechanism is the upregulation of multidrug resistance pumps to efflux PARPi. Drug efflux transporters, such as ATP-binding cassette and P-glycoprotein (also called ABCB1), might mediate PARPi resistance in BRCA-deficient cancers. In contrast, another mechanism to engender resistance is to affect the PARPi target. For example, either PARP1 mutations, which abrogate PARP1 trapping on DNA or loss of PAR glycohydrolase (PARG), will confer resistance to PARPi treatment.

In terms of BRCA-deficient background, most cancers gain resistance by restoring HR repair. Secondary or reversion mutations of BRCA genes caused by selection pressure is usually a critical way to rescue PARPi sensitivity. In fact, this resistance mechanism has been similarly observed in patients receiving platinum chemotherapy and other DNA-damaging agents.
Alternatively, hypomorphic BRCA activity\textsuperscript{191} or mutations in other HR genes\textsuperscript{192,193} could also restore PARPi resistance in tumors.

To reactivate HR, loss of resection inhibition is a specific means to bypass the BRCA1 deficiency. One well-known example is the loss of p53-binding protein 1 (53BP1). Given that 53BP1 regulates the non-homologous end-joining (NHEJ) pathway, its loss can thus reverse the HR deficiency in BRCA1-defective cells\textsuperscript{194-196} and promote PARPi resistance\textsuperscript{197,198}. Accordingly, other landmark reports, such as blocking 53BP1 localization to DSBs\textsuperscript{199}, disrupting Shieldin protein complex that protect DSB ends\textsuperscript{200-204}, or increasing DSB end resection\textsuperscript{205} unveil related mechanisms to render BRCA1-deficient tumors resistant to PARPi.

BRCA1/2 proteins are also required for fork stability in response to DNA damage and replication stress. Accordingly, BRCA-deficient cancer cells might restore fork protection to alleviate PARPi sensitivity. Indeed, restricting nuclease recruitment\textsuperscript{112,114} and loss of fork remodelers\textsuperscript{111} can both contribute to PARPi resistance. Moreover, many complex interactions are uncovered to rewire fork protection in different manners\textsuperscript{72,206}. Another factor involved is Schlafen Family Member 11 (SLFN11), which is recruited to forks upon replication stress\textsuperscript{207}. Loss of SLFN11 protects nascent DNA\textsuperscript{208} and impairs prolonged cell cycle arrest\textsuperscript{209,210}, thereby decreasing PARPi toxicity. Notably, most factors involved in these pathways do not ameliorate HR in BRCA deficiency. This phenomenon not only suggests the significance of fork stability and replication progression, but also shows that PARPi resistance can arise from mechanisms other than repairing breaks by HR.
Even though all these results point to potential limitations of PARPi, gaining further understanding of PARPi resistance could provide important information about PARP1 function in replication stress response and cancer. Additional exploration for PARP activity itself might provide necessary knowledge to better manipulate and leverage PARPi treatment in the clinic.

1.3.2 PARP activity in DNA replication and regulating fork dynamics.

In fact, PARP1 has been observed for its association with replicated chromatin and its enhanced activity in nascent replication fork more than three decades ago. While perhaps due to its abundance across chromatin, PARP1 does not display enrichment at forks. Instead, the PAR catabolic enzyme PARG enriches at forks by interacting with replication machinery protein, proliferating cell nuclear antigen (PCNA). Conceivably, these findings about PARG imply that PARylation is important and tightly regulated during replication. PARP1, together with HR, also plays a role in fork slowing following replication stress. Since loss of other ssDNA break repair proteins does not influence fork progression, studies discover a direct function of PARP activity in modulating replication fork reversal upon genotoxin treatment. Although earlier papers do not illustrate PARP activity in unchallenged fork dynamics, a recent report unveils the impact of PARPi in replication fork progression.

Then how to understand PARPi synthetic lethality with BRCA deficiency based on PARP activity? It was previously believed that PARP activity is potentially initiated due to stochastic ssDNA from attack of ROS, base
excision repair (BER) intermediates, dysregulated topoisomerases or other DNA damage\textsuperscript{20}. Thus, PARP activity is depicted to maintain replication fork integrity via its role in ssDNA break repair with HR\textsuperscript{223}. However, recent reports indicate that PARP1 can indeed function at the replication fork, promoting Okazaki fragment maturation\textsuperscript{222,224,225}. These findings about the main origin of PARP activity in S phase provide another possibility for PARP1-trapped lesions, which can fundamentally cause the synthetic lethality in HR-deficient cancers. Possibly, during the same or following S phase, unligated Okazaki fragments can either convert into DSBs and/or collapsed replication forks as conventional mechanism model expects. Alternatively, this discontinuous strand can directly push the genotoxic stress towards catastrophe. If the PARP-dependent repair cannot seal the unligated Okazaki fragments, the accumulation of aberrant ssDNA gaps from these unprocessed lagging strand would induce genome instability and thus impair cell fitness in HR-defective cells.

1.3.3 Understandings of PARPi synthetic lethality via lagging strand gaps

Lagging strand synthesis is stepwise and strictly controlled via various factors\textsuperscript{226,227}. Different from continuous leading strand synthesis, lagging strand replication requires successive repriming by the DNA polymerase alpha (Pol \(\alpha\))–primase complex. DNA fragments, i.e., Okazaki fragments, are synthesized by DNA polymerase delta (Pol \(\delta\)) progressively. A key component involved in replication machinery is the homotrimeric ring-shaped protein PCNA, which encircles and slides along DNA strands\textsuperscript{228}. During DNA synthesis, PCNA interacts with the replicative polymerases, nuclease and ligases to enhances
their processivities on each strand\textsuperscript{229}. Mechanistically, when Pol $\delta$ arrives at the end of a nascent Okazaki fragment, it creates a 5$'$ flap structure by displacing the 5$'$ end of the preceding fragment. This displaced 5$'$ flap is cleaved by nucleases and generates a ligatable nick. Flap endonuclease 1 (FEN1) plays a major role in processing the flaps, with additional contribution from DNA2 and EXO1\textsuperscript{230-232}. To complete lagging strand synthesis, DNA ligases then join these Okazaki fragments. Defective Okazaki fragment ligation leads to accumulating a massive amount of ssDNA nicks/gaps on genome (Figure 1.2). Thus, processing of Okazaki fragments by DNA nucleases and ligases must be coordinated reliably to preserve genome integrity.

As the major DNA ligase active in replication, DNA ligase I (LIG1) localizes at replication foci and joins Okazaki fragments as a main ligase\textsuperscript{233}. However, LIG1 is dispensable for cell viability \textsuperscript{234,235}, indicating the presence of a compensatory pathway apart from LIG1 mechanism. DNA ligase III (LIG3), another important ligase in eukaryotes and vertebrates, functions together with scaffold protein X-ray repair cross-complementing protein 1 (XRCC1) as a complex during BER, nucleotide excision repair (NER), and ssDNA break repair\textsuperscript{236,237}. Indeed, studies have showed that LIG3 can compensate and ligate Okazaki fragments when LIG1 is absent\textsuperscript{238,239}.

During the repair processes mentioned above, PARP1 recruits XRCC1 and downstream LIG3 and DNA polymerase beta (Pol $\beta$). During DNA replication, inhibiting canonical LIG1 and FEN1 activates PARP1 and enhances PARylation at lagging strand\textsuperscript{222,224}. PARP1 thus serves as a sensor of single-strand breaks/gaps from unligated Okazaki fragments in unperturbed cells.
Along these lines, PARPi might result in a large number of ssDNA gaps behind the replication fork, mainly from Okazaki fragment processing (OFP) defects.

These findings further change the conventional view about how PARPi causes synthetic lethality in BRCA-deficient cancers. In fact, HR pathway mediated by BRCA1/2 has been found essential in preventing replication-associated gaps\textsuperscript{103,240,241}. Suggesting the source of gaps, ssDNA that remain behind the fork recruit BRCA1 together with BRCA2 and RAD51. These proteins are then involved in HR-based repair of the gaps using the intact daughter strand as template\textsuperscript{242}. Without functional BRCA1/2, RAD51-dependent HR is not feasible, and the substitute error-prone DNA repair machinery can lead to genomic rearrangements and cell lethality.

Previous evidence also implicates that loss of BRCA pathway triggers higher PARP activity and impairs the OFP\textsuperscript{243}, although the detailed mechanisms still remain unclear. Accordingly, discontinuous lagging strand synthesis with ssDNA gaps may not be resolved by HR-mediated gap repair in BRCA1/2-deficient cancers. Therefore, it is possible that aberrant accumulation of such lesions, when exceeds the threshold of Okazaki fragment ligation, underpins the PARPi hypersensitivity.

1.4 Challenges for the dogma of PARPi synthetic lethality

1.4.1 Inconsistencies of chemotherapy response call into question the DSB repair model

In face of the alternative mechanisms, could DSB repair model still act as a determinant factor for genotoxic therapy response? Indeed, the concept that
unrepaired DSBs sensitize BRCA-deficient cancers lacking efficient HR function is thought to underly the mainstream therapeutic strategies. The ssDNA breaks, nicks and/or gaps induced by damage or replication stress are presumed to be converted to DSBs during replication. Moreover, degradation or collapse of replication fork is recently proposed to be a key precursor of breaks in response to genotoxic chemotherapy. However, the framework of DSB repair usually needs compromised explanations to fit the conventional dogma, and the relationships between PARPi response and genetic background also vary in distinct cell systems. For example, restoration of fork protection alone could render BRCA2-deficient cells resistant to PARPi\textsuperscript{112}. While in the report where BRCA2 was initially found to protect replication forks, cells prone to fork degradation showed similar resistance to PARPi\textsuperscript{77}. Although the fork protection model may not establish a tight relation with PARPi response, HR, as the crucial repair function, is still believed to be the determinant. Nevertheless, outlier cases again exist. A FA patient cell line with the heterozygous RAD51 T131P mutant is proficient in HR. But it indeed exhibits characteristic sensitivity to PARPi and platinum linked with chromosomal aberrations and instability\textsuperscript{244}. Furthermore, HR deficiency is sometimes dispensable for PARPi toxicity in the clinic\textsuperscript{245}, suggesting that lesions different from DSBs or genomic instability could be overlooked or even directly contribute to cell death.

Since even one single unrepaired DSB is able to drive cellular death in eukaryotes, this lesion is usually assumed as the most cytotoxic DNA damage\textsuperscript{246}. Extensive DSBs generated by genotoxic agents were initially
detected in treated cells through the technique called pulsed field gel electrophoresis\textsuperscript{247-250}. Especially, BRCA proteins mediate HDR that repairs DSBs\textsuperscript{251}, consistent with DSBs as the essential lesions that cause the hypersensitivity in BRCA deficiency. Therefore, causal relationship is established for the susceptibility of BRCA-deficient cells to genotoxins, as well as PARPi, that generate these breaks. However, there is no straightforward evidence that PARPi gives rise to massive DSBs directly. Given that the synthetic lethality between PARPi and BRCA deficiency is so obvious, it is also puzzling that how the inhibition of a ssDNA repair protein induces such a high sensitivity in HR deficiency. Similarly, genotoxic agents used for BRCA cancers, such as cisplatin\textsuperscript{252} and camptothecin\textsuperscript{253}, only create primary lesions like adducts and/or crosslinks. They are distinct from nuclease or IR that form DSBs in a direct manner\textsuperscript{254}. Along with all the considerations above, the conventional thinking of synthetic lethality of PARPi in BRCA deficiency may need to be reconsidered. A novel and comprehensive framework could reduce the complexity of DSB repair model and elucidates the mechanism of PARPi better.

1.4.2 Implications for novel paradigms of ssDNA in chemotherapy response

One possible explanation that impairs the importance of BRCA-FA pathway at the replication fork is that cells might lack sufficient stalled or reversed forks for replication fork degradation during different situations. Indeed, fork stalling is not a frequent replication process, and the replisome machinery is proficient in bypassing DNA lesions induced by various genotoxins\textsuperscript{255-257}. This bypass
event in daughter strands will cause ssDNA gaps that need subsequent repair by translesion synthesis (TLS) and HR. And these gaps bound by RPA mainly originate in the wake of replication forks rather than repair foci for collapsed forks and/or DSBs. Such findings thus question the stalled-fork theory and reflect a model of discontinuous replication with multiple repriming events. New evidence further proves that, after multiple doses of cisplatin treatment, adaptive response of fork repriming mediated by primase and DNA directed polymerase (PRIMPOL) promotes ssDNA gap formation. In addition, Oxygen starvation and/or HU-induced metabolic disruptions can trigger ROS to accumulate daughter-strand gaps behind the replication forks. It is noteworthy that hypoxia impairs HDR with no replication arrest, which again demonstrates fork stalling is unnecessary in response to certain type of stress.

If unrepaired gaps embed within nascent DNA replication forks, could they disrupt genome stability even in the absence of conversion to DSBs? Indeed, gaps or ssDNA breaks can disassemble the replisome and result in DSBs. However, there are clues supporting the cytotoxicity of ssDNA gaps. During HU-induced replication stress, BRCA1/2-deficient cells have been found to produce unrestrained replication tracts, which contain ssDNA gaps. Additionally, multiple separation-of-function models indicate that such replication gaps underlie chemotherapy response of cisplatin, and DSBs could instead derive from apoptosis.

Notably, ssDNA gaps associate with many DNA transactions. Accumulating ssDNA gaps mostly characterize DNA replication stress and even DNA damage. Thus, for further illuminating the cellular response of PARP
inhibition in BRCA deficiency, it is significant to revisit the source of replication gaps stemming from PARPi. It is possible that BRCA-deficient cells are prone to ssDNA gaps rather than spontaneous DSBs during unperturbed DNA synthesis. Another possibility is that PARPi targets replication gap suppression instead of DSB repair. Therefore, the comparison between canonical HR repair/fork protection and ssDNA gap suppression will provide new insights towards the essential mechanism of synthetic lethality. Perhaps replication gaps are the key to understand current enigmatic resistance of PARPi.

1.5 Rationale and scope for the thesis

Breast and ovarian cancers, especially those deficient in BRCA-FA genes, endanger women worldwide. For decades, scientists and clinicians have been studying the mechanisms of tumorigenesis, cancer evolution, as well as novel therapeutic approaches. Replication stress is widely appreciated as a principal driver for genomic instability and tumor development. To study the pivotal regulation of replication stress response, researchers focus the spotlight mainly on BRCA1/2 due to their dominant roles in HR. In contrast, the function of FANCJ, a BRCA1-associated DNA helicase that is also mutated in hereditary breast/ovarian cancer and FA, remains elusive in replication stress response. FANCJ specifically acts in multiple facets of DNA damage response and lesion repair. Therefore, the investigation of how FANCJ deficiency disrupts fork dynamics could highlight its special importance in replication stress response and disease pathogenesis.
For the first part of thesis research in Chapter II, we explored the function of FANCJ at the replication fork (Figure 1.3). The replisome or proteins that associate with replication forks were investigated in the presence or absence of FANCJ via an unbiased proteomics method. The fork remodeler HLTF was found enriched at replication forks in FANCJ-deficient cells. Employing DNA fiber analysis, we uncovered that FANCJ protects forks by inhibiting HLTF, the fork reversal protein that normally slows DNA replication. On the contrary, HLTF-mediated fork reversal prevented the unrestrained and discontinuous replication caused by FANCJ. Notably, ssDNA gaps also formed during unrestrained DNA synthesis. Thus, FANCJ and HLTF played opposing roles at replication fork. Loss of both might lead to a dead-end response to replication stress. This mechanism indicates that cells maintain a tight control of fork dynamics to attenuate replication stress and preserve genome integrity (Figure 1.3).
Figure 1.3: Opposing roles of FANCJ and HLTF at forks during replication stress.
We find that loss of FANCJ enhances the replisome association of HLTF. HLTF depletion suppresses fork degradation in FANCJ-deficient cells, and FANCJ depletion suppresses aberrant fork elongation in HLTF-deficient cells. However, the combined loss of HLTF and FANCJ causes severe replication stress. Highlights of Chapter II:
- FANCJ suppresses the accumulation of HLTF in replisomes (top)
- HLTF promotes fork degradation in FANCJ-deficient cells (middle)
- FANCJ promotes fork elongation, ssDNA gaps, and degradation in HLTF-deficient cells (middle)
- Combined loss of FANCJ and HLTF enhances replication stress (bottom)

To further combat against BRCA-mutated cancers, oncologists found PARPi as a promising cure. The concept of synthetic lethality enlightens the following studies of PARPi and other anticancer drugs. However, it is still puzzling that BRCA cancers sometimes evade PARPi cytotoxicity due to the resistance frequently happened in the clinic. A better understanding of the synthetic lethal interaction becomes crucial for developing more effective anticancer therapeutics. Despite the expanding resistance mechanisms, unfortunately, how BRCA cancers overcome PARPi toxicity is still an open-ended question. In terms of target therapy and personalized medicine, potential biomarkers that characterize PARPi-sensitive cells are also of great clinical significance. Here, in contrast to conventional frameworks of HR and fork protection, replication-associated ssDNA gaps are proposed as novel paradigms underlying synthetic lethality between PARPi and BRCA deficiency.

For the second part of thesis research in Chapter III, we investigated whether inducing genome-wide replication gaps essentially drives synthetic lethality (Figure 1.4). In accordance with this hypothesis, we found that cells with BRCA1/2- or RAD51-deficiency displayed excessive replication gaps
following PARPi treatment. PARPi-induced gaps were suppressed along with \textit{de novo} and/or genetic resistance and restored with re-sensitization. Whereas HR deficiency or fork degradation lacked this correlation with PARPi sensitivity. In particular, cells deficient in BRCA1 or FANCJ presented similar HR and fork protection defects but responded differently to PARPi. Moreover, agents enhancing unprotected gaps through inhibiting RPA uniquely sensitized BRCA1-deficient cells and enhanced PARPi toxicity.

We further discovered that replication gaps could be caused by OFP defects. Specifically, in unchallenged BRCA1-deficient cells, PARP1 appeared trapped in chromatin and PAR level was elevated with replication. We found that 53BP1 deletion in BRCA1 deficiency, in addition to HR restoration, also restored XRCC1-LIG3 as a backup pathway that resuscitates OFP and rescued the sensitivity of drugs targeting lagging strand. An important implication here is that synthetic lethality stems from OFP defects and its restoration confers PARPi resistance (Figure 1.4).
**Figure 1.4: Replication gaps are a key determinant of synthetic lethality.**

We report that replication gaps couple with PARPi sensitivity, whereas defects in DNA repair, fork protection, or fork speed can be uncoupled, implicating gaps as the sensitizing lesion. Correspondingly, gap suppression fully aligns with resistance including cells with BRCA1 and 53BP1 deficiency that regain Okazaki fragment processing. Highlights of Chapter III:

- PARP inhibitor (PARPi) sensitivity aligns with extent of replication gap formation
- Targeting gaps resensitizes and augments PARPi synthetic lethality
- Gaps in BRCA1-deficient cells are due to Okazaki fragment processing (OFP) defects
- OFP defects in BRCA1-deficient cells are rescued by 53BP1 loss

Collectively, both of these studies highlight replication gaps as a distinctive factor in replication stress and synthetic lethality between PARP inhibitor and BRCA deficiency. These findings will pave the way for discovering novel biomarkers and therapeutic targets for BRCA cancers.
CHAPTER II

BRCA-FA protein FANCJ mediates unrestrained replication with gaps during replication stress

Preface

Chapter II is adapted almost verbatim from my previously published study\textsuperscript{268}:

2.1 Abstract
The DNA helicase FANCJ is mutated in hereditary breast and ovarian cancer and Fanconi anemia (FA). Nevertheless, how loss of FANCJ translates to disease pathogenesis remains unclear. We addressed this question by analyzing proteins associated with replication forks in cells with or without FANCJ. We demonstrate that FANCJ-knockout (FANCJ-K/O) cells have alterations in the replisome that are consistent with enhanced replication stress, including an aberrant accumulation of the fork remodeling factor helicase-like transcription factor (HLTF). Correspondingly, HLTF contributes to fork degradation in FANCJ-K/O cells. Unexpectedly, the unrestrained DNA synthesis that characterizes HLTF-deficient cells is FANCJ dependent and correlates with S1 nuclease sensitivity and fork degradation. These results suggest that FANCJ and HLTF promote replication fork integrity, in part by counteracting each other to keep fork remodeling and elongation in check. Indicating one protein compensates for loss of the other, loss of both HLTF and FANCJ causes a more severe replication stress response.

2.2 Introduction
Preserving genome integrity is absolutely essential for cell survival and to prevent disease. BRCA1 and BRCA2 are tumor suppressors with central functions in the DNA damage response that preserve genome integrity. In double-strand break repair, they mediate distinct steps of homology-directed repair (HDR). Genome preservation functions for BRCA1 and BRCA2 also involve roles in the replication stress response, which enables cells to cope with
perturbations to replication. When forks stall, BRCA1 and BRCA2 protect nascent DNA from degradation. In BRCA1- and BRCA2-deficient cells, MRE11-dependent nucleolytic processing of reversed forks leads to fork degradation\textsuperscript{77,78}. Preventing fork reversal through depletion of fork remodelers such as SMARCAL1, ZRANB3, or helicase-like transcription factor (HLTF) restores fork protection to BRCA1 and BRCA2-deficient cells and in some cases improves resistance to stress-inducing agents\textsuperscript{72,108,109,111}.

Given this understanding, it is proposed that perturbations in the replication stress response along with defects in DNA repair underlie BRCA-Fanconi anemia (FA) pathway maladies. Indeed, hereditary breast and ovarian cancer cells as well as cells from FA patients have proliferation defects. In conjunction with sources of endogenous replication stress, especially in rapidly dividing cells, FA cells may ultimately lose proliferation capacity and develop anemia or bone marrow failure as found in FA\textsuperscript{63}. Loss of the BRCA-FA pathway could also elevate replication stress. However, the underlying cause of exacerbated replication stress aside from elevated DNA damage responses in FA cells remains unclear, because little is known about how the BRCA-FA pathway contributes to the replisome function.

The BRCA-associated FANCJ DNA helicase is mutated in hereditary breast and ovarian cancer as well as in FA\textsuperscript{128,129,269}. Although experimental analyses have focused largely on FANCJ function in response to genotoxic agents, it is clear that FANCJ is needed for endogenous replication problems as well. For example, knockdown of FANCJ causes increased DNA damage in otherwise unperturbed S phase cells\textsuperscript{139}. The endogenous source of replication stress is
unknown but could be unusual DNA structures that have a propensity to form at stalled forks. In support of this point, along with induction of γ-H2AX and slower growth, FANCJ-deficient cells display microsatellite instability. FANCJ could counteract replication perturbations as it travels with the elongating replication fork.

Here, we used DNA fiber analysis to uncover a function for FANCJ in fork protection. Through an unbiased proteomics approach, we also identify proteins that associate with replication forks in an FANCJ-dependent manner. We present evidence that FANCJ limits fork degradation by suppressing HLTF, which normally slows and remodels DNA replication forks. In addition, we find that HLTF fork remodeling limits permissive replication mediated by FANCJ. We propose that FANCJ and HLTF participate in a general surveillance mechanism by counteracting each other to maintain unperturbed DNA replication. In response to stress, these opposing activities are critical for replication forks to have dynamic response.

2.3 Results
2.3.1 FANCJ is required for fork protection
To determine if FANCJ functions in fork protection, we measured replication-fork elongation using DNA fiber spreading analysis. First, we generated human FANCJ-knockout (FANCJ-K/O) 293T cells using clustered regularly interspaced short palindromic repeats (CRISPR)-CRISPR associated protein 9 (Cas9) technology (Figure 2.1A). FANCJ loss generated the expected sensitivity to mitomycin C (MMC), and complementation with FANCJWT
elevated MMC resistance (Figure 2.2A). Next, fork degradation was assessed by measuring the ratio of 5-chloro-2'-deoxyuridine (CldU) to 5-iodo-2'-deoxyuridine (IdU) tract lengths following sequential pulses with IdU and CldU prior to hydroxyurea (HU) treatment. FANCJ-K/O cells had significantly greater fork degradation than control cells with FANCJ (Figure 2.1B). In these assay conditions, fork protection was restored by inhibition of the nuclease MRE11 with mirin \textsuperscript{272} (Figure 2.1B). As with FANCJ-K/O 293T cells, we found that FANCJ-null Fanconi anemia complementation group J (FA-J) patient fibroblasts had significant fork degradation. Both fork degradation and MMC sensitivity were suppressed by complementation with FANCJ\textsuperscript{WT}, but not the catalytic FANCJ K52R mutant FANCJ\textsuperscript{K52R} (Figures 2.1C, 2.1D, and 2.2B). Based on a complementary assay in which IdU tract lengths were measured only when also labeled with CldU, which was incorporated concurrently with the HU treatment assay \textsuperscript{110}, we could conclude that the enhanced degradation in 293T or FA-J cells without FANCJ or its catalytic activity was not due to premature termination of replication and/or unintentional breaks (Figures 2.2C and 2.2D). Furthermore, using this assay, we detected fork degradation in CRISPR-Cas9-generated FANCJ-K/O U2OS cells. The MMC sensitivity of FANCJ-K/O U2OS cells was suppressed by re-expression of FANCJ\textsuperscript{WT}, but not vector (Figure 2.2E), validating functional loss of FANCJ. A similar level of fork degradation was also observed upon depletion of BRCA2 \textsuperscript{77,110} (Figure 2.2F). In summary, these findings are consistent with the idea that FANCJ and its helicase activity are required to protect forks from MRE11-dependent degradation.
Figure 2.1: FANCJ and its helicase activity protect nascent DNA at replication forks from MRE11 dependent degradation.

(A) Western blot analysis with the indicated antibodies (Abs) of lysates from control and FANCJ K/O 293T cells. (B) Schematic, representative images and quantification of quantification of CldU/IdU ratio after HU treatment with or without mirin (50 µM). (C) Western blot analysis with indicated Abs of lysates from FANCJ null FA-J cells complemented with vector (V), wild-type (WT) or a catalytically inactive FANCJ (K52R) mutant. (D) Schematic, representative images and quantification of CldU/IdU ratio after HU treatment. Each dot represents one fiber. For each analysis, at least 200 fibers are quantified from two independent experiments. Red bars represent the median. Statistical analysis according to two-tailed Mann-Whitney test; ****, P < 0.0001. Scale bar, 10µm.
Figure 2.2: FANCJ and its helicase activity protect nascent DNA at replication forks in different models. Related to Figure 2.1.

(A) Western blot analysis with the indicated antibodies (Abs) of lysates from FANCJ K/O, control 293T cells, or FANCJ K/O cells transfected with vector (V) or wild-type FANCJ (WT). Cell survival assay with indicated cells under increasing concentrations of MMC. (B) Cell survival assay with FA-J cells complemented with V, WT or K52R under increasing concentrations of MMC. (C) Schematic, representative images, and quantification of IdU tract length in FANCJ K/O and control 293T cells. (D) Representative images, and quantification of IdU tract length in FANCJ null FA-J cells complemented as noted. For (C) and (D), at least 100 fibers are quantified for each. (E) Western blot analysis with the indicated Abs of lysates from FANCJ K/O, control U2OS cells, or FANCJ K/O cells transfected with V or WT. Cell survival assay with indicated cells under increasing concentrations of MMC. (F) Western blot analysis with the indicated Abs of lysates from FANCJ K/O and control U2OS cells expressing shRNA against BRCA2 or NSC. Quantification of IdU tract length in FANCJ K/O and control U2OS cells with NSC or BRCA2 shRNA. At least 200 fibers are quantified from two independent experiments. Red bars represent the median. Statistical analysis according to two-tailed Mann-Whitney test; ****, P < 0.0001; *, P<0.05.
2.3.2 Identification of proteins at replication forks in unchallenged FANCJ-K/O cells

To mechanistically explain the role of FANCJ in replication, we sought to analyze how FANCJ loss altered the composition of the replisome. Isolation of proteins on nascent DNA (iPOND) has revealed the accumulation of FA proteins at active replication forks including FANCJ\textsuperscript{213,271}. We detected mild, but reproducible, FANCJ association with replication forks (Figure 2.3A). Upon thymidine chase, FANCJ association was lost, along with other replisome components including proliferating cell nuclear antigen (PCNA), confirming the presence of FANCJ only at the active replication fork (Figure 2.3A). Despite a growth defect and higher levels of $\gamma$-H2AX (Figures 2.4A and 2.4B) consistent with endogenous DNA damage, unchallenged FANCJ-K/O 293T cells had similar median length of CldU (Figure 2.4C) and precipitated similar amounts of PCNA and histone H2B by iPOND compared to control 293T cells (Figure 2.3B). Thus, iPOND in FANCJ-K/O and control 293T cells is a tractable system to address how FANCJ contributes to the composition of the replisome.

To quantitatively compare the composition of replication-fork-associated factors between FANCJ-proficient and deficient cells, we combined iPOND and stable isotope labeling by amino acids in cell culture (SILAC). Following successful incorporation of isotopes, cells “light” (FANCJ K/O) or “heavy” (control) were labeled for 10 min with 5-ethynyl-2′-deoxyuridine (EdU). EdU was similarly incorporated within a 10-min pulse (Figure 2.4D). Equal cell numbers were combined and processed together for coupling to biotin azide and precipitation of DNA-bound proteins for mass spectrometry (MS) analyses.
(Figure 2.3C). For each protein identified, we plotted the light versus heavy ratio and p value. Proteins found after an EdU pulse labeling and thymidine chase (Table 2.1) were excluded from the analysis to ensure that only proteins at replicating forks were compared. We successfully isolated active replication forks, as illustrated by the majority of high-confidence proteins from iPOND-MS with low p values and multiple peptide identifications that are also known replisome components. According to the iPOND-SILAC ratios, the majority of proteins quantitated from the two cell populations did not substantially change between FANCJ-K/O and control cells (Figure 2.3D).

While the majority of proteins that are directly involved in DNA synthesis accumulate similarly on nascent DNA in FANCJ-K/O cells (Table 2.2), we did observe changes of several factors known to function in genomic stability, DNA damage repair, and the replication stress response. In particular, we found reductions in the splicing-associated factor THOC2 and the chromatin architecture factors KDM1A and SMARCA1. Other proteins that were reduced included phosphoglycerate kinase (PGK1), of which deficiency causes anemia; KIF4A, which is a protein that promotes homologous recombination (HR) and binds BRCA2; and KDM1A/LSD1 (lysine-specific histone demethylase 1A), which functions in the 53BP1 DNA damage response. The factors most enriched included HLTF, which functions in replication fork remodeling, and GNL3 (nucleostemin) and PSPC1, which function in the DNA damage response. In addition, FANCJ-K/O cells were enriched for PSIP1 and CDCA7L, both of which form a complex and function
in DNA end resection and HR. Thus, even under nonchallenged conditions, iPOND revealed that FANCJ contributes to the composition of the replisome.

Figure 2.3: Isolation of proteins on nascent DNA (iPOND) from FANCJ K/O and control 293T cells.

(A) Western blot analysis with the indicated Abs of input or iPOND samples following non-click, click or Thymidine chase from 293T cells. (B) Western blot analysis with indicated Abs of input or iPOND samples from control and FANCJ K/O 293T cells. (C) Schematic representation of SILAC IPOND technique. (D) Volcano plots of the p-values versus the log2 protein abundance differences between purified replisomes of 293T control (heavy) and FANCJ K/O (light) cells. Significantly enriched and reduced proteins are highlighted in red and blue, respectively. p-values are calculated from the data of three biological repeats. (E) Table shows Light/Heavy ratio in chromatin fractions for indicated proteins. (F) Western blot analysis with the indicated Abs of WCE and chromatin preparations. (G) Western blot analysis with the indicated Abs of cells transfected with vector (V) or wild-type FANCJ (WT). Asterisk indicates nonspecific band.
Figure 2.4. FANCJ loss in 293T cells does not measurably alter replication fork progression in unchallenged conditions but HLTF is enriched in FANCJ K/O cells with or without HU. Related to Figure 2.3.

(A) Cell growth assay with FANCJ K/O or control 293T cells plated at low density and counted as indicated. (B) Quantification of γ-H2AX intensity in FANCJ K/O or control 293T cells. Statistical analysis according to two-tailed Mann-Whitney test; ****, P < 0.0001. (C) Schematic and quantification of CldU tract length in FANCJ K/O and control 293T cells. The intensity was measured in at least 150 cells for each. (D) Cells were labeled with EdU and carried out with Click-iT EdU imaging kit. A representative image is shown. The percent of cells with EdU foci was quantified and graphed. Data represent mean ± s.d. from three independent experiments. (E) 293T cells were treated with 3mM HU for 2hr or left untreated. Chromatin fractions were analyzed by immunoblotting for the indicated proteins. (F) FA-J cells complemented with WT or V were collected and fractionated, chromatin fractions were analyzed by immunoblotting for the indicated proteins.
Table 2.1: Table of proteins found in Thymidine chase. Related to Figure 2.3D. Proteins in red were also found significant in iPOND.

<table>
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<tr>
<th>HELLS</th>
<th>EEF1A1</th>
<th>CROCC</th>
<th>ZNF90</th>
<th>HNRNPA8</th>
<th>RAN</th>
<th>RPSA</th>
<th>HSPA1B</th>
<th>DDX3</th>
<th>HIST1H2AJ</th>
<th>SUMO3</th>
<th>HP1BP3</th>
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<td>HNRNPA</td>
<td>HMGB1</td>
<td>LUBB</td>
<td>ILF2</td>
<td>NONO</td>
<td>H2AFV</td>
<td>PPIA</td>
<td>RCC1</td>
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<td>HIST1H1C</td>
<td>EIR4A1</td>
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<td>H2AFZ</td>
<td>HNRNPK</td>
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<td>PSIP1</td>
<td>H3FD</td>
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<td>NCL</td>
<td>HIST1H4A</td>
<td>HIST2H3P2</td>
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<td>SUPT16H</td>
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Table 2.2: Raw data of iPOND-MS experiments. Related to Figure 2.3D. Top 40 decreased or increased proteins, including HLTF marked as red.

<table>
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<th>Uniprot Entry</th>
<th>Entry Name</th>
<th>Increased</th>
<th>log2Ratio</th>
<th>Uniprot Entry</th>
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<td>P00558</td>
<td>PGK1_HUMAN</td>
<td>SARPN</td>
<td>1.99268579</td>
<td>P28979</td>
<td>SARPN_HUMAN</td>
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<td>Q05239</td>
<td>KIF4A_HUMAN</td>
<td>HLTF</td>
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<tr>
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<td>Q8NN27</td>
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<td>PSQC1</td>
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2.3.3 HLTF is enriched in the chromatin of FANCJ-K/O cells

We focused on HLTF because of its role in regulating fork dynamics. HLTF is a fork remodeler that restrains DNA replication in response to stress and promotes fork reversal\textsuperscript{99}. Moreover, similar to the iPOND results, SILAC-chromatin-MS revealed that HLTF was enriched in the chromatin fraction of FANCJ-K/O 293T cells (Figure 2.3E). Furthermore, immunobLOTS validated
enriched HLTF in chromatin of FANCJ-K/O cells and showed slight enrichment in whole-cell extracts (WCEs) as compared to SMARCA1, which was reduced in both WCE and chromatin (Figure 2.3F). HLTF levels did not change and remained enriched in FANCJ-K/O 293T cells upon treatment with HU (Figure 2.4E), suggesting that HLTF enrichment was due to FANCJ deficiency as opposed to DNA damage in FANCJ-K/O cells. The chromatin of FA-J patient immortalized fibroblast cells complemented with vector also trended toward enriched HLTF as compared to FA-J cells complemented with FANCJWT (Figure 2.4F), suggesting that these findings were independent of cell type. HLTF chromatin enrichment was also suppressed by transfection of FANCJ-K/O cells with FANCJWT as compared to vector (Figure 2.3G). Collectively, these findings are consistent with FANCJ counteracting HLTF chromatin enrichment in a cell-type-independent manner.

2.3.4 HLTF contributes to fork degradation in FANCJ-K/O cells

Depletion of fork remodelers such as HLTF restores fork protection to BRCA1- and BRCA2-deficient cells108,109,111. To determine the contribution of HLTF to fork degradation in FANCJ-K/O cells, we measured the ratio of CldU to IdU tract lengths following HU treatment in FANCJ-K/O cells with or without HLTF depletion. HLTF depletion in either control or FANCJ-K/O 293T cells did not alter the tract lengths in unchallenged conditions (Figures 2.5A, 2.6A, and 2.6B). After HU treatment, while HLTF depletion did not alter fork degradation in control cells, HLTF depletion did significantly reduce fork degradation in
FANCJ-K/O 293T cells (Figure 2.5B), consistent with HLTF contributing to fork degradation in this background.

Given that HLTF depletion leads to unrestrained replication during stress\textsuperscript{99}, we considered that this phenotype could generate longer tract lengths and therefore mask fork degradation. To mitigate this possible issue, we extended the duration of stress before tract lengths were measured. FANCD2-depleted cells also fail to slow in response to stress, but with the extension of low-dose HU, forks degrade\textsuperscript{77,86}. As with short-term, high-dose HU, FANCJ-K/O 293T cells underwent enhanced degradation as compared to control cells upon the extension of low-dose HU (Figures 2.5C and 2.6C). Again, nascent-strand degradation in FANCJ-K/O 293T cells was suppressed by HLTF depletion with either of two distinct small hairpin RNAs (shRNAs), suggesting that enriched HLTF contributes to fork degradation in FANCJ-K/O 293T cells (Figures 2.5C and 2.6C). Notably, however, during extended replication stress, HLTF depletion, similar to FANCJ-K/O cells, had a significant degradation of nascent DNA (Figures 2.5C and 2.6C). In agreement, similar findings were found in U2OS cells (Figures 2.6D and 2.6E). Collectively, these findings indicate that FANCJ and HLTF are critical to protect nascent DNA during prolonged replication stress and that loss of either factor leads to fork degradation, which depends on the other factor.
Figure 2.5: HLTF contributes to fork degradation in FANCJ K/O cells and FANCJ contributes to fork degradation in HLTF depleted cells following prolonged stress.

(A) Western blot analysis with the indicated Abs of lysates from control and FANCJ K/O 293T cells expressing shRNA against HLTF or NSC. (B) Schematic, representative images and quantification of CldU/IdU ratio after HU treatment. (C) Schematic, representative images and quantification of CldU tract length. Each dot represents one fiber; at least 200 fibers are quantified from two independent experiments. Red bars represent the median. Statistical analysis according to two-tailed Mann-Whitney test; ****, P < 0.0001
2.3.5 HTLF supports the recovery of FANCJ-K/O cells from HU-induced stress

We sought to address the relationship between FANCJ and HTLF with respect to cell growth and survival following replication stress. Both control or FANCJ-K/O U2OS cells exhibited similar reductions in growth kinetics with HTLF...
depletion (Figures 2.7A and 2.8A). There was a significant gain in MMC resistance with HLTF depletion in FANCJ-K/O cells (Figure 2.7B). By comparison, there was a significant loss in HU resistance in HLTF-depleted FANCJ-K/O cells. Indeed, HLTF depletion highly sensitized FANCJ-K/O cells to HU, whereas HLTF depletion in control cells enhanced resistance to HU (Figure 2.7B). Significant sensitivity to HU was also found in FANCJ-null FA-J cells upon HLTF depletion (Figure 2.8B), further suggesting that loss of FANCJ and HLTF was detrimental for recovery from nucleotide depletion. To further evaluate replication stress, we queried the level of $\gamma$-H2AX found in EdU-positive cells as a marker of replication-associated breaks\textsuperscript{214}. As compared to control cells, FANCJ-K/O cells exhibit increased levels of replication stress. Moreover, the $\gamma$-H2AX/EdU ratio in FANCJ-K/O cells was significantly enhanced upon HLTF depletion (Figure 2.7C). Increased replication stress was also detected in HLTF-depleted FANCJ-K/O cells in a modified alkaline comet assay. Newly synthesized BrdU-positive DNA appears as a halo-like structure of “loose DNA” when replication is discontinuous due to stress\textsuperscript{287,288} (Figure 2.7D). Collectively, these data reveal that the combined loss of FANCJ and HLTF elevates replication stress following HU treatment.

To assess whether fork degradation in FANCJ-K/O cells is suppressed by loss of a distinct remodeler, we depleted SMARCAL1 using shRNA reagents (Figure 2.8C). FANCJ-K/O U2OS cells were more sensitive to SMARCAL1 depletion than control cells in unchallenged conditions. Indeed, with one of the shRNA reagents that had the greatest SMARCAL1 depletion (as observed in FANCJ-proficient cells), the survival of FANCJ-K/O U2OS cells was
dramatically reduced (Figure 2.8C). However, SMARCAL1 depletion with either of three shRNA reagents in FANCJ-K/O 293T cells did not dramatically reduce viability. Therefore, we tested whether fork protection was enhanced (Figure 2.8D). SMARCAL1 depletion enhanced fork protection in FANCJ-K/O 293T cells (Figure 2.8E). These findings further suggest that the reversed fork substrate is important for degradation in FANCJ-deficient cells.

**Figure 2.7: HLTF counteracts replication stress in FANCJ K/O cells.**
(A) Western blot analysis with the indicated Abs of lysates from control and FANCJ K/O U2OS cells and when expressing shRNA against HLTF or NSC. (B) Cell survival assays with FANCJ K/O and control U2OS cells expressing shRNA against HLTF or NSC under increasing concentrations of MMC or HU. Data represent the mean percent ± s.d. of survival from three independent experiments. (C) γ-H2AX/EdU ratio in FANCJ K/O and control U2OS cells expressing shRNA against HLTF or NSC following treatment with HU. At least 200 cells were measured for each. The assay was completed in triplicate for each shRNA. (D) Quantification of the percent of comet tail DNA in FANCJ K/O and control 293T cells expressing shRNA against HLTF or NSC following treatment with HU and images of comet tail formation. 100 cells were measured.
for each. Statistical analysis according to two-tailed Mann-Whitney test; ****, P < 0.0001; *, P<0.05.

Figure 2.8: Growth dynamics and fork degradation in FANCJ K/O U2OS, FA-J, or 293T K/O cells upon HLTF or SMARCAL1 depletion. Related to Figure 2.7.
(A) FANCJ K/O and control U2OS cells expressing shRNA against HLTF or NSC were plated at low density, then collected and counted at indicated time. (B) Western blot analysis with the indicated Abs of lysates from FA-J cells expressing shRNA against HLTF or NSC. Cell survival assays with FANCJ null FA-J cells expressing shRNA against HLTF or NSC under increasing concentrations of HU. Data represent the mean percent ± s.d. of survival from three independent experiments. (C) Western blot analysis with the indicated Abs of lysates from control U2OS cells. Live cell imaging of FANCJ K/O and control U2OS cells expressing shRNA (a) or (b) reagent to SMARCAL1 or NSC following puromycin selection. (D) Western blot analysis with the indicated Abs of lysates from FANCJ K/O and control 293T cells. (E) Schematic and quantification of CldU/IdU ratio after HU treatment. At least 100 fibers are quantified for each. Each dot represents one fiber; red bars represent the median. Statistical analysis according to two-tailed Mann-Whitney test; ****, P < 0.0001.
2.3.6 FANCJ is required for unrestrained replication that generates S1-nuclease-sensitive tracts in HLTF-deficient cells

The replication stress induction and heightened HU sensitivity in HLTF depleted FANCJ-K/O cells predicted that the unrestrained replication during stress that characterizes HLTF depletion was prohibited in FANCJ-K/O cells. To test this idea, we first confirmed that HLTF depletion, as previously achieved in HCT116 cells, maintained fork speed despite the presence of HU (Figures 2.9A and 2.9B). We also confirmed that HCT116 cells with or without FANCJ and HLTF depletion did not have significant differences in tract lengths in unchallenged conditions (Figure 2.10A). We observed a reduction in fork progression in cells deficient in FANCJ, indicating that FANCJ is not required for restraining replication elongation in HU (Figures 2.9A, 2.9B, 2.10B, and 2.10C). Notably, HLTF depletion in these FANCJ-deficient cells (FANCJ-depleted HCT116 or FANCJ-deleted 293T and U2OS) did not generate the previously noted unrestrained replication found in control cells (Figures 2.9A, 2.9B, 2.10B, and 2.10C). These findings indicate that HLTF deficiency causes permissive replication that is dependent on FANCJ.

Unrestrained replication is associated with single-stranded DNA (ssDNA) gap formation. To detect gaps not directly observable in DNA fiber assays, we treated nuclei with the S1 nuclease after the second pulse (C1dU) and before spreading the DNA onto the glass slide (Figure 2.9C). When gaps are present, ssDNA regions were nicked by the nuclease, generating shorter C1dU tracts. In the absence of the S1 nuclease, HLTF-depleted HCT116 cells had longer C1dU fibers than control cells and therefore a higher C1dU/IdU ratio, as
expected. However, after the addition of S1 nuclease, only HLTF-deficient cells presented a decrease in the CldU fiber lengths, which significant decreased the CldU/IdU ratio (Figures 2.9C and 2.10D). The effect of the S1 nuclease was similar in HLTF-depleted 293T cells (Figure 2.10D). These data indicate that in HLTF-deficient cells, ssDNA regions accumulate in DNA tracts exposed to HU. In FANCJ-deficient cells, the addition of S1 nuclease with control or HLTF depletion had no effect on the CldU/IdU ratio (Figure 2.9C). Therefore, ssDNA gaps were generated upon exposure to HU in HLTF-deficient cells in a manner dependent on FANCJ.

Figure 2.9: FANCJ contributes to unrestrained replication and S1 nuclease sensitivity in HLTF depleted cells. (A) Western blot analysis with the indicated Abs of lysates from HCT116 cells expressing shRNA against FANCJ, HLTF, or NSC. (B) Schematic, representative images and quantification of CldU tract length during HU treatment. (C) Schematic and quantification of CldU/IdU ratio in the indicated...
shRNA expressing HCT116 cells with or without S1 nuclease incubation. Each dot represents one fiber; at least 200 fibers are quantified from two independent experiments. Red bars represent the median. Statistical analysis according to two-tailed Mann-Whitney test; ****, P < 0.0001 (D) Model of opposing roles of FANCJ and HLTF at forks.

Figure 2.10. HLTF and FANCJ loss do not alter fork progression in unchallenged conditions and FANCJ contributes to unrestrained replication in HLTF depleted cells that leads to S1 nuclease sensitivity. Related to Figure 2.9.

(A) Schematic and quantification of CldU tract length in HCT116 untreated cells with shRNA FANCJ. (B) Schematic, representative images and quantification of CldU tract length during HU treatment in the indicated shRNA expressing U2OS cells. (C) Schematic, representative images and quantification of CldU tract length during HU treatment in the indicated shRNA expressing 293T cells. (D) Schematic and quantification of CldU to IdU ratio in the indicated shRNA expressing 293T cells, with or without S1 nuclease incubation. Each dot represents one fiber. At least 100 fibers are quantified for each. Red bars represent the median. Statistical analysis according to two-tailed Mann-Whitney test; ****, P < 0.0001. (E) Schematic and representative images of chromosome spreads from HU- treated HCT116 cells expressing the indicated shRNAs.
2.4 Discussion

In this study, we report that FANCJ loss significantly alters the replisome. We utilize iPOND and quantitative proteomics to show that among other changes, the remodeling factor HLTF is enriched in FANCJ-K/O cells. We find that following replication stress induced by HU, FANCJ-K/O cells have shorter replication tracts, and this loss of nascent DNA is dependent on HLTF. These findings are consistent with FANCJ promoting fork protection by countering HLTF fork reversal. However, depletion of HLTF and restoration of fork protection while improving MMC resistance dramatically sensitizes FANCJ-K/O cells to HU. These findings indicate that HLTF-dependent fork degradation is beneficial to the restoration of replication in FANCJ-K/O cells. We also report that failure to slow replication in response to stress that characterizes HLTF-depleted cells\(^99\) corresponds with nascent DNA being not only S1 nuclease sensitive but also vulnerable to degradation, outcomes that are dependent on FANCJ. Collectively, these findings indicate that a dynamic response to replication stress requires both FANCJ and HLTF. Loss of one or the other skews the response to enhance fork degradation, whereas loss of both proteins generates a toxic “dead end” replication stress response (Figure 2.9D).

The finding that fork degradation is observed in HLTF-depleted cells during prolonged stress, but not during short-term stress, could reflect that unrestrained replication initially conceals degradation. This is reminiscent of the finding that FANCD2-depleted cells fail to slow in response to stress, but forks degrade under long-term stress\(^86\). A defect in fork reversal has been proposed to account for the effect of HLTF depletion on fork progression\(^99\). Rather than
slowing and reversing upon stress, the unrestrained DNA synthesis could skip over difficult-to-replicate regions, generating “bad-quality” replication with gaps that serve as entry points for nucleases that degrade nascent DNA. We show that unrestrained replication in HLTF-deficient cells correlates with S1 nuclease sensitivity suggesting formation of ssDNA gaps. FANCJ-dependent ssDNA induction could drive senescence and chromosomal aberrations in HLTF-deficient cells. This idea, however, is at odds with the role of FANCJ combating senescence by localizing BRCA1 to chromatin\textsuperscript{290}. Moreover, we did not detect chromosomal aberrations in HLTF-deficient cells in response to HU (Figure 2.10E), suggesting that conditions that are sufficient to induce gap formation and fork degradation may not be sufficient to induce gross genomic instability detectable in metaphase spreads. Nevertheless, the finding that unrestrained replication, gap formation, and fork degradation in HLTF-deficient cells are dependent on FANCJ suggests these phenotypes are functionally linked. To limit replication during stress and prevent genomic instability, HLTF may regress the replication fork by annealing the stalled nascent strand to the undamaged newly synthesized strand\textsuperscript{95,99,289}. In addition, with its remodeling or translocase activity, HLTF may displace FANCJ at stalled replication forks to limit replication.

Our findings raise interesting questions about the role of FANCJ in regulating HLTF chromatin localization and function in the cell and how FANCJ may function together with other components of post-replication repair to restrict fork reversal proteins. Fork remodelers have distinct substrates. Replication of G4s, repeat regions, and other natural barriers present in
heterochromatin that tend to form secondary structures can slow or stall replication and reverse replication forks. The propensity for these natural triggers of replication fork reversal to form in FANCJ-K/O cells could lead to greater replication stress, induction of HLTTF, and dependence on it for recovery. Therefore, despite extensive resection, FANCJ-K/O cells are able to restart stalled forks, which may reflect HLTTF-dependent fork restart pathways contributing to cell survival. Likewise, BRCA2 mutant cells are dependent on MUS81 for survival and restart following HU treatment\textsuperscript{114}. It is also possible that the survival of FANCJ-K/O cells following HU could be linked to HLTTF because of nonreplication functions in cell cycle regulation, sister chromatid cohesion, or chromosome condensation\textsuperscript{291}.

Although enriched HLTTF provides fitness benefits during HU stress, the failure to regulate HLTTF fork reversal activity under conditions when FANCJ is absent or mutated could compromise the genome. HLTTF fork reversal activity could protect the replication fork by limiting ssDNA accumulation. However, in the absence of FANCJ, overactive HLTTF-dependent fork remodeling and resection may be detrimental to recovery from interstrand crosslink (ICL)-induced lesions that require a series of distinct processing events for replication to restart. This provides an explanation for why HLTTF depletion enhances MMC resistance in FANCJ-K/O cells. An overabundance of HLTTF in FANCJ-K/O mice could contribute to the subfertility, germ cell attrition, and hypersensitivity to replication inhibitors\textsuperscript{270,292}. Problems restoring replication due to enriched HLTTF could contribute to the defects in proliferation and self-renewal that led to a G2/M arrest and the exhaustion of bone marrow cells in FA. Problematic
replication caused by an overabundance of replication-fork-slowing factors could also be a driving force in transformation. FA patients that do not succumb to bone marrow failure often develop leukemia and other cancers. The finding that HLTF is both disrupted and amplified in cancer suggests that the consequence of loss of HLTF and unrestrained replication are comparable to enriched HLTF and unrestrained replication fork reversal.

We propose that FANCJ serves to limit HLTF-dependent reversed forks that are extensively degraded by MRE11. If true, FANCJ loss should magnify the degradation in cells lacking BRCA1 or BRCA2 in which MRE11 is unrestricted. The depletion of BRCA2 in control or FANCJ-K/O U2OS cells did not provide much evidence for distinct roles in fork protection. However, to understand the relationship between FANCJ, BRCA2, and other fork-protection proteins, additional experiments, including electron microscopy, will be critical. It is possible that despite enriched HLTF, fork remodeling is compromised in FANCJ-null cells because other fork remodelers are deficient. For example, FANCJ promotes RPA chromatin loading\textsuperscript{144}, and RPA recruits SMARCAL1 to stalled forks\textsuperscript{144,293}; therefore, SMARCAL1 may not be maintained at forks as robustly in FANCJ-K/O cells. The reduced viability upon loss of SMARCAL1 in FANCJ-K/O U2OS cells suggests that residual SMARCAL1 at forks may be critical for viability, perhaps for telomere maintenance. In contrast, SMARCAL1 depletion in FANCJ-K/O 293T cells did not dramatically reduce viability and enhanced fork protection. While these findings suggest that the reversed fork structure is degraded in FANCJ-deficient cells, fork degradation could be
furthered by aberrantly activated MRE11 activity given that FANCJ interacts with MRE11 and inhibits its exonuclease activity\textsuperscript{134}.

In summary, we show that FANCJ associates specifically with nascent DNA in cells, consistent with other reports\textsuperscript{213,271}. These findings suggest that FANCJ travels with the replication fork, which would enable it to respond rapidly to DNA damage or replication stress. Characterization of the replisome in FANCJ-K/O cells revealed that the fork reversal factor HLTF is elevated and causative in replication fork degradation associated with FANCJ deficiency. FANCJ joins the growing list of FA proteins that function beyond ICL repair in the replication stress response. Similar to BRCA1, BRCA2, FANCD2, FANCA, and FANCM\textsuperscript{77,78,294}, FANCJ also protects nascent DNA at stalled forks under stressful conditions. In addition, we report that FANCJ is causative in unrestrained replication, gap formation, and fork degradation associated with HLTF deficiency. Whether this insight provides tools for targeting FANCJ- or HLTF-associated cancer remains to be determined.

2.5 Materials and Methods

\textit{Cell Lines}

293T and U2OS cell lines were grown in DMEM supplemented with 10% fetal bovine serum and penicillin and streptomycin (100 U/mL each). FA-J (EUFA30-F) was immortalized with human telomerase reverse transcriptase (hTERT)\textsuperscript{137}, and HCT116 cells were cultured as previously described\textsuperscript{128,289}.

\textit{shRNA}
FANCJ-K/O and control 293T, U2OS, HCT116 cells were infected with pLK 0.1 vectors containing shRNAs against non-silencing control (NSC) or one of three shRNAs against HLTF: (A) mature antisense sequence 5′-TTTGTGATGATAACTTCTTGCA-3′, (B) 5′-TAAGAAGGTAAGTATGGCAAC-3′, and as described with changes shown. The shRNAs against SMARCAL1 include (a) mature antisense sequence 5′-AAACACTGCAATGTTCCGC-3′, (b) 5′-TTGTGTCAGCATTAGATGAGC-3′, (c) 5′-ATGAGTTGGGTTAGCAAAGGG-3′. shRNAs were obtained from the University of Massachusetts Medical School (UMMS) shRNA core facility. HCT116 cells were infected with FSIPPW vectors containing shRNAs against NSC or FANCJ. Stable shRNA cell lines were selected with puromycin (0.5–1 µg/mL) or G418 (300–600 mg/mL).

**CRISPR-Cas9 K/O Generation**

The CRISPR-sgRNA (single guide RNA) construct was generated ligating the sgRNA sequence targeting two different sites on exon 2 of FANCJ (5′-GGTCTGAATATACAATTGGTG-3′ [guide 1] or 3′-TCATCATAGCAAGCTGT-5′ [guide 2]) onto the pX330 plasmid containing the Cas9 gene (Addgene catalog number 42230). The target sequence was bioinformatically designed to minimize off-target effects. To assess the specificity of the CRISPR endonuclease activity, a GFP reporter construct was generated ligating the M427 vector with the same sgRNA sequence used previously. Briefly, 293T or U2OS cells were transfected with both the reporter and the CRISPR constructs. 48 hr after transfection, GFP+ cells were sorted using the FACS Aria II (BD Biosciences) and subsequently seeded at one cell per well in 96-well
plates. Clones were analyzed for FANCJ protein levels, and those without expression of the protein were genotyped following PCR using forward primer (5′-CATTACCACAATCCTATGGG-3′) and reverse primer (5′-CTGGAAAGCTGGTTTACTC-3′). The exon 2 ATG start site and remaining sequence is similar to the native sequence, but in K/O clone 1, there is a 1-nt insertion (bold), and in K/O clone 2, there is a deletion of 2 nt before the protospacer adjacent motif (PAM) site (underlined) of guide 1 (italics). WT: ATGTCTTCAATGTGGTCTGAATACATTTGGGGGTGAAGATTT; K/O clone 1: ATGTCTTCA TTTGTGGTCTGAATACATTGGGGGTGAAGATTT; K/O clone 2: ATGTCTTCA-GTGGTC TGAATACATTGGGGTGAAGATTT.

**Immunoblotting and Abs**

Cells were harvested, lysed, and processed for western blot analysis as described previously using 150mM NETN lysis buffer (20 mM Tris [pH 8.0], 150 mM NaCl, 1 mM EDTA, 0.5% NP-40, 1 mM phenylmethylsulfonyl fluoride, 10 mg/mL leupeptin, and 10 mg/mL aprotinin). For cell fractionation, we isolated cytoplasmic and soluble nuclear fractions with the NE-PER Kit (Thermo Scientific) according to the manufacturer’s protocol; to isolate the chromatin fraction, the insoluble pellet was resuspended in RIPA buffer and sonicated in a BioRuptor according to the manufacturer’s protocol (high power, 15 min, 30s on and 30s off at 4°C). Proteins were separated using SDS-PAGE and electrotransferred to nitrocellulose membranes. Membranes were blocked in 5% milk PBS-Tween and incubated with primary antibody for 1 hr. Abs for western blot analysis included anti-PCNA (Abcam), anti-H2B (Cell Signaling
Technology), anti-β-actin (Sigma), anti-FANCJ (E67), anti-HLTF (Abcam), anti-SMARCA1 (Abcam), and anti-HLTF (Santa Cruz Biotechnology). Membranes were washed, incubated with horseradish-peroxidase-linked secondary antibodies (Amersham), and detected by chemiluminescence (Amersham).

Viability Assays

Cells were seeded onto 96-well plates (500 cells per well, performed in triplicate for each experiment) and incubated overnight. The next day, cells were treated with increasing doses of MMC for 1 hr in serum-free media or HU and maintained in complete media for 5 days. Percentage survival was measured photometrically using a CellTiter-Glo viability assay (Promega) in a microplate reader (Beckman Coulter DTX 880 Multimode Detector). For the growth assay, cells were seeded onto 12-well plates and counted at the indicated times using a hemocytometer.

Immunofluorescence

Immunofluorescence was performed as described previously\textsuperscript{126}. Cells were grown on coverslips. The next day, cells were fixed and permeabilized. After incubation with primary antibodies against γ-H2AX (Millipore), cells were washed and then incubated with secondary antibody. After washing, coverslips were mounted onto glass slides using Vectashield mounting medium containing DAPI (Vector Laboratories). For EdU labeling, cells were left untreated or treated with 0.5 mM HU for 3 hr and released at 1 hr. EdU labeling was carried
out with a Click-iT EdU imaging kit (Invitrogen) according to the manufacturer's instructions.

**DNA Fiber Assays**

To directly visualize replication fork dynamics, we established single-molecular-DNA fiber analysis. In this assay, progressing replication forks in cells were labeled by sequential incorporation of two different nucleotide analogs, IdU (50 µM) and CldU (50 µM), into nascent DNA strands for the indicated time and conditions. After nucleotide analogs were incorporated in vivo, the cells were collected, washed, spotted (2.5 µL of 10^5 cells/mL PBS cell suspension), and lysed on positively charged microscope slides (Globe Scientific, 1358W) by 7.5 µL spreading buffer (0.5% SDS, 200 mM Tris-HCl [pH 7.4], and 50 mM EDTA) for 8 min at room temperature. For experiments with the ssDNA-specific endonuclease S1, after the CldU pulse, cells were treated with CSK100 buffer (100 mM NaCl, 10 mM MOPS, 3 mM MgCl2 [pH 7.2], 300 mM sucrose, and 0.5% Triton X-100) for 10 min at room temperature, then incubated with S1 nuclease buffer (30 mM sodium acetate [pH 4.6], 10 mM zinc acetate, 5% glycerol, and 50 mM NaCl) with or without 20 U/mL S1 nuclease (Invitrogen, 18001-016) for 30 min at 37°C. The cells were then scraped in PBS + 0.1% BSA and centrifuged at 7,000 rpm for 5 min at 4°C. Cell pellets were resuspended at ~1,500 cells/mL and lysed with lysis solution on slides. Individual DNA fibers were released and spread by tilting the slides at 45 degrees. After air-drying, fibers were fixed by 3:1 methanol/acetic acid at room temperature for 3 min. After air-drying again, fibers were rehydrated in PBS,
denatured with 2.5 M HCl for 30 min, washed with PBS, and blocked with blocking buffer (3% BSA and 0.1% Triton in PBS) for 1 hr. Next, slides were incubated for 2.5 hr with primary antibodies for (IdU:1:100, mouse monoclonal anti-BrdU, Becton Dickinson 347580; CldU: 1:100, rat monoclonal anti-BrdU, Abcam 6326) diluted in blocking buffer, washed several times in PBS, and then incubated with secondary antibodies (IdU:1:200, goat anti-mouse, Alexa 488; CldU: 1:200, goat anti-rat, Alexa Fluor 594) in blocking buffer for 1 hr. After washing and air-drying, slides were mounted with Prolong (Invitrogen, P36930). Finally, visualization of green and/or red signals by fluorescence microscopy (Axioplan 2 imaging, Zeiss) provided information about the active replication directionality at the single-molecule level. Representative cropped images are shown on a black backdrop.

**iPOND**

iPOND was performed as described previously\textsuperscript{213,296}. The click reaction was completed in 2 hr. Capture of DNA-protein complexes utilized streptavidin-coupled C1 magnabeads for 1 hr. Beads were washed with lysis buffer (1% SDS in 50 mM Tris [pH 8.0]), low-salt buffer (1% Triton X-100, 20 mM Tris [pH 8.0], 2 mM EDTA, and 150 mM NaCl), high-salt buffer (1% Triton X-100, 20 mM Tris [pH 8.0], 2 mM EDTA, and 500 mM NaCl), and lithium chloride wash buffer (100 mM Tris [pH 8.0], 500 mM LiCl, and 1% Igepal) and then twice in lysis buffer. For most experimental samples, $4 \times 10^8$ HEK293T cells were used; light and heavy labeled cells were mixed 1:1 prior to the click reaction.
iPOND samples were separated by SDS-PAGE. Gel regions above and below the streptavidin band were excised and treated with 45 mM DTT for 30 min, and available cysteine residues were carbamidomethylated with 100 mM iodoacetamide for 45 min. After destaining the gel pieces with 50% acetonitrile (MeCN) in 25 mM ammonium bicarbonate, proteins were digested with trypsin (Promega) in 25 mM ammonium bicarbonate at 37°C. Peptides were extracted by gel dehydration (60% MeCN, 0.1% trifluoroacetic acid [TFA]), vacuum dried, and reconstituted in 0.1% formic acid.

**SILAC and Liquid Chromatography Tandem MS**

2 µL tryptic digests was analyzed on the Thermo Q-Exactive mass spectrometer coupled to an EASY-nLC system (Thermo Fisher). Peptides were separated on a fused silica capillary (12 cm x 100 µm ID) packed with Halo C18 (2.7 µm particle size, 90 nm pore size; Michrom Bioresources) at a flow rate of 300 nL/min. Peptides were introduced into the mass spectrometer via a nanospray ionization source at a spray voltage of 2.2 kV. Mass spectrometry data were acquired in a data-dependent top-10 mode, and the lock mass function was activated (m/z, 371.1012; use lock masses, best; lock mass injection, full MS). Full scans were acquired from m/z 350 to 1,600 at 70,000 resolution (automatic gain control [AGC] target, 1e6; maximum ion time [max IT], 100 ms; profile mode). Resolution for dd-MS2 spectra was set to 17,500 (AGC target: 1e5) with a maximum ion injection time of 50 ms. The normalized collision energy was 27 eV. A gradient of 0 to 40% acetonitrile (0.1% FA) over 55 min was applied.
**MS Data Analysis**

SILAC experiments were performed in triplicate and analyzed in MaxQuant using the requantify option with two unique peptides required to identify a protein. The peptide search was performed with the UniProt Human Proteome AUP000005640 that includes both canonical protein sequences and isoforms. The search parameters permitted a 10 ppm precursor MS tolerance and a 0.02 Da MS/MS tolerance. Carboxymethylation of cysteines was set up as fixed modifications, and oxidation of methionine (M), SILAC labeling ($^{13}\text{C}_6^{15}\text{N}_2$) at lysine, and ($^{13}\text{C}_6^{15}\text{N}_4$) at arginine were allowed as variable modifications. Up to two missed tryptic cleavages of peptides were considered with the false-discovery rate set to 1% at the peptide level. The SILAC ratio for each identified peptide was calculated by the quantitation node. Known contaminants and reverse hits were removed prior to statistical analysis. Low-scoring proteins were automatically removed by MaxQuant and did not receive a ratio value. As a quality control, we confirmed that the isotopic labels were fully incorporated and observed that heavy lysine +8 and heavy arginine +10 isotopic labels were incorporated at greater than 95% in 95% of all peptides. For statistical analysis, we recorded the light-to-heavy (L/H) ratio for each identified protein from MaxQuant, which compares the area of the heavy and light isotopic peaks for each identified peptide. These ratios were log2 transformed, sorted by rank, and plotted in a rank order plot versus log2 ratio. In addition, for proteins identified in at least two experiments, we determined p values by the *limma* statistical analysis package for R Bioconductor and plotted the data in a volcano
plot of log2 L/H ratio versus -log10 p value to identify statistically significant changes in the proteome with a substantial log2 fold.

**Alkaline BrdU Comet Assay**

The alkaline BrdU comet assay was performed as described previously and following treatment with 4 mM HU for 2 hr.

**Chromosome Spreads**

Chromosome spreads were prepared as previously described. Briefly, cells were treated with HU as indicated. Cells were washed and recovered in fresh media for 8 hr before Colcemid arrest (1:200, KaryoMAX, 4 hr). Cells were harvested by trypsinization, incubated in 75 mM KCl for 18 m at 37°C and fixed in a 3:1 methanol/acetic acid solution. The fixed suspension was dropped onto slides to obtain chromosome spreads and mounted with VectaShield mounting medium with DAPI (Vector Laboratories) and visualized using fluorescence microscopy (Axioplan 2 imaging, Zeiss).

**Statistical Methods**

Statistical differences in DNA fiber assays, immunofluorescence, and the alkaline BrdU comet assay were determined using a two-tailed Mann-Whitney test. Statistical analysis was performed using GraphPad Prism (Version 7.0). MS data analysis is described above. In all cases, *p < 0.05, **p < 0.01, ***p < 0.001, and ****p < 0.0001.
Data and Software Availability

Raw images and data have been deposited in Mendeley Data and are available at https://data.mendeley.com/datasets/zjtbfxhkg3/1.

2.6 Acknowledgements

Sharon Cantor designed the experiments and wrote the manuscript with the help from Ke Cong. Ke Cong performed the majority of experiments collaborating with Min Peng (co-first author of the paper), as well as other members in Cantor lab. Min Peng mainly contributed to the iPOND experiments in Figures 2.3, 2.4 and Tables 2.1, 2.2, with the help from Bin Deng and Lihua Julie Zhu. Min also contributed to Western blot analysis, viability assays and immunofluorescence. Monika Morocz, Lili Hegedus and Lajos Haracska contributed to the alkaline BrdU comet assay in Figure 2.7.

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CHAPTER III

Replication gaps as a key determinant of PARP inhibitor synthetic lethality with BRCA deficiency

Preface

Chapter III is adapted almost verbatim from my previously published study:297

3.1 Abstract
Mutations in BRCA1 or BRCA2 (BRCA) is synthetic lethal with poly(ADP-ribose) polymerase inhibitors (PARPi). Lethality is thought to derive from DNA double-stranded breaks (DSBs) necessitating BRCA function in homologous recombination (HR) and/or fork protection (FP). Here, we report instead that toxicity derives from replication gaps. BRCA1- or FANCJ-deficient cells, with common repair defects but distinct PARPi responses, reveal gaps as a distinguishing factor. We further uncouple HR, FP, and fork speed from PARPi response. Instead, gaps characterize BRCA-deficient cells, are diminished upon resistance, restored upon resensitization, and, when exposed, augment PARPi toxicity. Unchallenged BRCA1-deficient cells have elevated poly(ADP-ribose) and chromatin-associated PARP1, but aberrantly low XRCC1 consistent with defects in backup Okazaki fragment processing (OFP). 53BP1 loss resuscitates OFP by restoring XRCC1-LIG3 that suppresses the sensitivity of BRCA1-deficient cells to drugs targeting OFP or generating gaps. We highlight gaps as a determinant of PARPi toxicity changing the paradigm for synthetic lethal interactions.

3.2 Introduction
Since the landmark finding that poly(ADP-ribose) polymerase inhibition (PARPi) is synthetic lethal with deficiency in the hereditary breast cancer genes BRCA1 and BRCA2 (BRCA), the primary sensitizing lesion has been attributed to DNA double-stranded breaks (DSBs)\textsuperscript{155,156}. PARPi has significant anticancer activity in cancers harboring defects in BRCA or other genes required for the repair of
DSBs by homologous recombination (HR)\textsuperscript{44}. BRCA proteins also function in fork protection (FP), which limits excessive nucleolytic degradation of stalled replication forks. In their absence, stalled forks are expected to collapse into DSBs\textsuperscript{73,77-79}. Correspondingly, the PARPi olaparib is believed to trap PARP1 on DNA and interfere with DNA replication and promote fork collapse\textsuperscript{168,169,298,299}. Thus, PARPi is considered especially toxic in BRCA-deficient cells because forks collapse into DSBs that are not repaired due to HR defects. The observation that restoration of HR and/or FP is associated with chemoresistance in BRCA-deficient cancer is consistent with this DSB model of therapy response\textsuperscript{112,181,188,194,195}.

More recently, the stalled fork model was confronted with the observation that the PARPi olaparib did not slow or block DNA replication, but rather accelerated DNA replication\textsuperscript{222}. This unrestrained replication was consistent with the role of PARP1 in promoting replication-fork reversal, a mechanism by which replication forks slow, pause, and reverse direction when confronted with replication obstacles\textsuperscript{91,125,220-222,255,300-302}. To fit the DSB framework of PARPi toxicity, it was proposed that unrestrained replication leads to DSB formation\textsuperscript{222,303}. Thus, while the proposed mechanism by which PARPi cause DSBs has evolved, the underlying concept that DSBs drive synthetic lethality in BRCA-deficient cancers has remained largely unchallenged.

While current models emphasize DSBs as the sensitizing lesions of PARPi, single-stranded DNA (ssDNA) breaks or gaps are commonly associated with agents inducing replication stress and were historically considered in genotoxic chemotherapy response\textsuperscript{304,305}. Likewise, PARPi causes an accumulation of
single-strand breaks (SSBs), nicks, and gaps\textsuperscript{225,306}. The source of the gaps could range from loss of a diverse set of PARP1 functions such as ssDNA break repair or Okazaki fragment processing (OFP)\textsuperscript{91,224,300,301}. Functionally, PARP1 recruits proteins to ssDNA by its ability to synthesize protein-conjugated polymers of ADP-ribose or PAR. Incompletely processed Okazaki fragments that escape processing by canonical FEN1 and LIG1 are bound by PARP1, and PAR signaling is activated. PARP1 then recruits X-ray repair cross-complementing protein 1, XRCC1, to complete OFP\textsuperscript{224,307,308}. Thus, PARPi is predicted to disrupt OFP and generate lagging strand gaps\textsuperscript{225}. In conventional models, nicks/gaps or PARPi trapped proteins are proposed to be converted to DSBs during DNA replication and sensitize BRCA-deficient cells lacking DSB repair.

However, several lines of evidence suggest that PARPi toxicity could stem from sources distinct from the eventual induction of DSBs. For example, PARPi sensitizes cells deficient or mutated in OFP factors such as flap endonuclease I (FEN1), LIG1, XRCC1, or proliferating cell nuclear antigen (PCNA)\textsuperscript{170,224,309-311}, implying that combined replication gaps are insurmountable and lead to cell death. Intriguingly, and further raising lagging strand gaps as a cause of toxicity, FEN1 loss is synthetic lethal in BRCA-Fanconi anemia (FA)-deficient cells\textsuperscript{312-314}. In addition, PARPi is synthetic lethal, with loss of genes having no corresponding defect in HR or FP\textsuperscript{170,315}, and HR deficiency is not required for PARPi response in the clinic\textsuperscript{245}, indicating that lesions distinct from DSBs could drive cell death. Notably, aside from HR and FP, BRCA proteins function in preventing replication-associated gaps\textsuperscript{77,79,103,109,240,241,316-318}. In particular, the
central recombination protein RAD51 functions in replication gap suppression (RGS) in a manner that is separated for its role in HR or post-replication gap filling\textsuperscript{109,318}. Replication gaps could be the basis for under-replication in BRCA-deficient cells that provides opportunities for synthetic lethal interactions, with loss of genes functioning during or in the resolution of replication\textsuperscript{107,319-321}.

We also noted that DSB framework requires concessions because HR and FP vary in their relation to PARPi response in several cell models\textsuperscript{72,77,108,109,244,322}. Here, we considered that immediate induction of widespread replication gaps could drive synthetic lethality. Consistent with this interpretation, we report that BRCA-RAD51-deficient cells accumulate excessive gaps in response to PARPi. Our findings indicate that gaps result in PARPi-induced sensitivity due to OFP defects that exhaust replication protein A (RPA) pools. Correspondingly, BRCA1-deficient cells are vulnerable to the inhibition of OFP and ssDNA binding by RPA that exposes gaps. OFP defects in BRCA1-deficient cells are rescued by the loss of 53BP1, indicating that lagging strand gaps are a critical determinant of PARPi synthetic lethality and should be considered to be a biomarker of PARPi response.

3.3 Results

3.3.1 The length of nascent DNA following PARPi is aberrantly enhanced in PARPi-sensitive BRCA1-deficient cells and hyper-restrained in FANCJ-deficient cells that are not PARPi sensitive

To explore the connection between replication dynamics and PARPi response, we analyzed replication fork progression following PARPi treatment. We
performed DNA fiber assays that monitor fork dynamics by incorporating nucleoside analogs into newly synthesized DNA strands, which can then be fluorescently labeled. We used human retinal pigmented epithelial (RPE1-human telomerase reverse transcriptase [hTERT], \(TP53^{-/-}\); herein, RPE1 control) cells and confirmed that BRCA1 deficiency (BRCA1 knockout [K/O] RPE1-hTERT, \(TP53^{-/-}\) cells) caused sensitivity to PARPi and the DNA crosslinking agent cisplatin (Figures 3.1A and 3.1B). Next, we verified that cells displayed longer dual-labeled replication tracts (5-iodo-2'-deoxyuridine [IdU] and 5-chloro-2'-deoxyuridine [CldU]) when treated at 10 \(\mu\)M PARPi for 2 h\(^{222}\) (Figure 3.1C). Moreover, we confirmed that BRCA1-deficient cells have greater fork asymmetry that is reduced by PARPi\(^{222}\) (Figure 3.2A).

The total length of nascent DNA tracts following PARPi was not reported to be different between BRCA1-deficient and - proficient cells at 24 h post-10 \(\mu\)M PARPi\(^{222}\). However, we found that BRCA1-deficient cells had significantly longer replication tracks at 2 h post-10 \(\mu\)M PARPi (Figure 3.1C), suggesting that the initial response to PARPi is distinct when BRCA1 is absent. To decipher whether aberrant lengthening of nascent DNA following in the immediate aftermath of PARPi reflects cell sensitivity, we analyzed DNA fibers at several time points post-0.5 \(\mu\)M PARPi, a dose in which BRCA1 K/O cells are more sensitive than controls (Figure 3.1B). We observed longer DNA tracts in BRCA1 K/O cells as compared to control cells at all time points before 24 h (Figure 3.1D). These findings suggest that fork lengthening within a few hours of PARPi could distinguish sensitive versus resistant lines.
We previously found that the aberrant fork lengthening during replication stress was dependent on the FANCJ (BACH1/BRIP1) helicase. In most respects, however, FANCJ phenocopies BRCA1 with functions not only in the suppression of hereditary breast/ovarian cancer and FA but also in promoting HR and FP. Thus, we analyzed the response of FANCJ-deficient (FANCJ K/O RPE1-hTERT, TP53−/−) cells to PARPi. In striking contrast to BRCA1 K/O, we found that FANCJ K/O cells, along with U2OS and 293T FANCJ K/O cell lines, were not sensitive to PARPi, while, as expected, they were sensitive to cisplatin, mitomycin C, or the topoisomerase inhibitor camptothecin (CPT) (Figures 3.1A, 3.1B, and 3.2B–3.2D). In contrast to BRCA1 K/O cells, FANCJ K/O cells did not show DNA tract lengthening at 2 h post-10 μM PARPi (Figure 3.1C), suggesting that FANCJ is required for fork lengthening and sensitivity following PARPi. These findings also highlighted that not all HR- and FP-deficient lines are sensitive to PARPi.
Figure 3.1: PARPi-induced fork lengthening and ssDNA gaps are greater in BRCA1-deficient but not in FANCJ-deficient cells.

(A) Western blot analysis with the indicated antibodies of lysates from Control, FANCJ K/O and BRCA1 K/O RPE1 cells. (B) Cell survival assays for indicated cells under increasing concentrations of olaparib or cisplatin. Data represent the mean percent ± SD of survival for each dot. (C) Schematic and quantification of the DNA fiber assays for the length of dual-color tracts in indicated cells following olaparib treatment (10 μM, 2 h). (D) DNA fiber assays for the length of dual-color tracts in indicated cells following olaparib treatment (0.5 μM) at different periods of time. (E) DNA fiber assays for IdU tracts with or without S1 nuclease incubation in indicated cells following olaparib treatment (10 μM, 2 h). For (C)-(E), each dot represents one fiber; at least 200 fibers are quantified from two biological independent experiments (n=2). Red bars
represent the median ± interquartile range. All statistical analysis according to Kruskal-Wallis test followed by Dunn’s test. **p < 0.01, ****p < 0.0001, ns: not significant. (F) Schematic and quantification of mean ssDNA intensity for indicated cells following CldU pre-labeling and olaparib release in 0.5 μM, 2 h with EdU added at the last 20 min. At least 200 cells are quantified from n=2. Dashed lines indicate a cut-off intensity level (at 0.01) for all cell lines. Cells with intensity higher than that are calculated for percentages. EdU and ssDNA are measured by arbitrary units. (G) Model illustrating gap formation following PARPi treatment in Control, BRCA1 K/O and FANCJ K/O RPE1 cells.
Figure 3.2: PARPi-induced fork lengthening and ssDNA gaps are enhanced in BRCA1-deficient cells and suppressed in FANCJ-deficient cells. Related to Figure 3.1.

(A) Schematic and quantification of the ratio of IdU/CldU in Control and BRCA1 K/O RPE1 cells following olaparib treatment (10 μM, 2 h). (B) Cell survival assays for Control, FANCJ K/O and BRCA1 K/O RPE1 cells under increasing concentrations of camptothecin (CPT). Data represent the mean percent ± SD of survival for each dot. (C) and (D) Western blot analysis with the indicated antibodies of lysates from Control, FANCJ K/O U2OS cells (C) and 293T cells (D). Cell survival assays for indicated cells under increasing concentrations of olaparib and mitomycin C (MMC). Data represent the mean percent ± SD of survival for each dot. (E) Schematic and quantification of the length of dual-color tracts with or without S1 nuclease incubation in BRCA1 K/O RPE1 cells following olaparib treatment (0.5 μM, 24 h). For (A), (B) and (F), each dot represents one fiber; at least 200 fibers are quantified from two biological independent experiments (n=2). Red bars represent the median. Statistical analysis according to Kruskal-Wallis test followed by Dunn’s test. All p values are described in Statistical methods. (F) Up left: Treatment strategy and scheme for the non-denaturing immunofluorescence of mean ssDNA intensity per nucleus. Bottom: Representative images of ssDNA immunofluorescence for indicated cells following treatment. Scale bars, 50μm. Up right: Quantification of mean ssDNA intensity for indicated cells following CldU pre-
labeling and olaparib release in 10 μM, 2 h. At least 300 cells are quantified from n=3. Red bars represent the median ± interquartile range, a.u. for arbitrary units. All statistical analysis according to Kruskal-Wallis test followed by Dunn’s test. ****p < 0.0001, ns: not significant.

3.3.2 Replication-associated ssDNA gaps are enhanced in BRCA1-deficient cells and hyper-restrained in FANCJ-deficient cells

We hypothesized that fork lengthening in response to PARPi leads to the formation of replication gaps that are exacerbated in BRCA-deficient cells due to their aberrant fork-lengthening phenotype that correlates with gap formation\(^86,103,240,268\). To determine whether gaps form in the vicinity of the accelerated replication forks following PARPi treatment, cells were labeled with IdU/CldU and incubated with the S1 nuclease to digest ssDNA regions\(^122,123\). If nascent ssDNA regions are within the labeled replication tracts, then S1 nuclease will cut and shorten the visible IdU/CldU replication tracts. Following 2 h PARPi (10 μM), conditions under which lengthening is readily observed in both the control and BRCA1 K/O cells (Figure 3.1C), we observed that S1 nuclease treatment also reduced replication tract lengths (Figure 3.1E). Following the lower-dose 0.5 μM PARPi, in which tracts lengthened in BRCA1 K/O cells, tracts also shortened with S1 nuclease treatment (Figure 3.2E). By contrast, in FANCJ K/O cells, tracts did not lengthen or display S1 nuclease sensitivity even following high-dose 10 μM PARPi (Figure 3.1E), consistent with our previous finding that unrestrained replication and ssDNA gap formation is dependent on FANCJ and that when fork lengthening is not detected, neither are gaps\(^240,268\). Moreover, these findings suggest that PARPi sensitivity correlates with initial lengthening and/or gap formation.
If gaps indicate PARPi sensitivity and RGS indicates PARPi resistance, then it will be critical to readily detect them. To assess whether genome-wide immunoreactive ssDNA gaps could be observed immediately following PARPi, cells were pre-labeled with nucleoside analogs, and non-denaturing immunofluorescence (IF) was performed\textsuperscript{324}. Following 10 µM PARPi that lengthens replication tracts in both control and BRCA1 K/O cells (Figure 3.1C), we observed a positive signal for ssDNA, as compared to FANCJ K/O cells that did not score positive (Figure 3.2F). Following 0.5 µM PARPi for 2 h that lengthens replication tracts in BRCA1 K/O cells but not controls (Figure 3.1D), it was evident that BRCA1 K/O cells had more replicating cells with elevated ssDNA intensity (Figure 3.1F). These findings suggest that replication-associated ssDNA can be detected by IF. Moreover, PARPi sensitivity could derive from either excessive replication fork speed and/or ssDNA gaps that are elevated in BRCA1 K/O cells as compared to control and are avoided in FANCJ K/O cells that are not PARPi sensitive (Figure 3.1G).

3.3.3 Fork lengthening alone does not underlie synthetic lethality caused by PARPi

To understand whether lengthening or gaps confer PARPi toxicity, we sought to uncouple them. One way to accelerate replication is to deplete the cell-cycle regulator p21, which when combined with PARPi, further lengthens replication tracts\textsuperscript{222}, a finding we confirmed in both RPE1 and U2OS cells (Figures 3.3A, 3.3B, 3.4A, and 3.4B). Notably, the lengthening of tracts in p21-depleted RPE1 or U2OS cells did not enhance PARPi sensitivity (Figures 3.3C and 3.4C). By
contrast, p21 depletion significantly decreased ssDNA formation (Figures 3.3D and 3.4D), consistent with previous observations. These findings suggest that fork lengthening following PARPi treatment can occur without a significant induction of ssDNA or sensitivity and that gaps rather than speed could also underlie toxicity (Figure 3.3E).

Figure 3.3: PARPi-induced fork acceleration and sensitivity can be uncoupled.
(A) Western blot analysis with the indicated antibodies of lysates from RPE1 cells expressing shRNA against non-silencing control (NSC), p21(A) and p21(B). (B) Schematic and quantification of the length of CldU tracts in indicated RPE1 cells following olaparib treatment (10 μM, 2 h). Each dot represents one fiber; at least 200 fibers are quantified from two biological independent experiments (n=2). (C) Cell survival assays for indicated RPE1 cells under increasing concentrations of olaparib. Data represent the mean percent ± SD of survival for each dot. (D) Schematic and quantification of mean ssDNA intensity for indicated RPE1 cells following CldU pre-labeling and olaparib release (10 μM, 2 h). At least 200 cells are quantified from n=2. Red bars represent the median ± interquartile range, a.u. for arbitrary units. Statistical analysis according to Kruskal-Wallis test followed by Dunn’s test. **p < 0.01, ****p < 0.0001. (E) Model for fork acceleration without gaps following PARPi treatment in p21-proficient and -deficient background.
Figure 3.4: PARPi-induced fork lengthening and sensitivity are uncoupled. Related to Figure 3.3.

(A) Western blot analysis with the indicated antibodies of lysates from U2OS cells expressing shRNA against non-silencing control (NSC), p21(A) and p21(B). (B) Schematic and quantification of the length of CldU tracts in indicated U2OS cells following olaparib treatment (10 μM, 2 h). Each dot represents one fiber; at least 200 fibers are quantified from two biological independent experiments (n=2). (C) Cell survival assays for indicated U2OS cells under increasing concentrations of olaparib. Data represent the mean percent ± SD of survival for each dot. (D) Schematic and quantification of mean ssDNA intensity for indicated U2OS cells following CldU pre-labeling and olaparib release (10 μM, 2 h). At least 200 cells are quantified from n=2. Red bars represent the median ± interquartile range. Statistical analysis according to Kruskal-Wallis test followed by Dunn’s test. All p values are described in Statistical methods.

3.3.4 Gap suppression correlates with PARPi resistance and resensitization restores gaps

If a gap toxicity threshold is fundamental to PARPi sensitivity, then RGS should confer resistance. To test this hypothesis, we analyzed the BRCA1-deficient
mouse ovarian tumor cell line BR5 and the PARPi-resistant derived cell line BR5-R1 with an unknown mechanism of resistance\textsuperscript{326}. We confirmed PARPi and cisplatin sensitivity of BR5 compared to BRCA1-proficient T2 tumor cells\textsuperscript{326} (Figures 3.5A and 3.6A). At 10 \( \mu \)M PARPi, a concentration at which replication tract lengthening is readily detected in control cells (Figure 3.1C), replication tract lengths were increased in the BR5 cells and BRCA1-proficient T2 cells, but not in the PARPi resistant BR5-R1 cells (Figure 3.6B). Moreover, the PARPi-resistant BR5-R1 cells did not show an increase in PARPi-induced gaps compared to the PARPi-sensitive BR5 cells (Figure 3.5B). Thus, similar to the FANCJ K/O cells, BR5-R1 are sensitive to cisplatin (Figure 3.6A) but resistant to PARPi by a mechanism that includes both fork restraint and RGS.

To further query the relationship between gaps and PARPi toxicity, we analyzed the effect of an ataxia telangiectasia and Rad3-related or ATR inhibitor (ATRi), VE-821, shown to restore PARPi sensitivity to PARPi-resistant BR5-R1 cells\textsuperscript{326}. We observed that along with PARPi sensitivity, co-incubation with the ATRi generated widespread gap induction (Figures 3.5C and 3.5D), raising the possibility that ATRi synergizes with PARPi due to gap induction.

\textit{De novo} PARPi resistance in BRCA1- or BRCA2-deficient cancers is linked to BRCA reversion mutations that reinstate reading frames and restore BRCA function\textsuperscript{181,188,327}. To identify whether fork restraint and RGS were also achieved by genetic reversion, we analyzed the BRCA2 mutant PEO1 ovarian cancer cell line and the derived BRCA2 reversion cell clone C4-2\textsuperscript{328}. We found that the C4-2 clone had not only gained PARPi resistance but also displayed fork restraint and reduced gap induction (Figures 3.6C–3.6E). These findings
suggest that both BRCA1 and BRCA2 are important for restraining replication and preventing gaps in response to PARPi and that resistance by either de novo or reversion mutation correlates with RGS.

Restored FP alone has been proposed to confer chemoresistance in BRCA-deficient cancer cells\textsuperscript{112}. In particular, depletion of the chromatin remodeler chromodomain helicase DNA binding protein 4 (CHD4) in BRCA2-deficient cells enhances PARPi resistance and restores FP\textsuperscript{112,113}. Notably, along with PARPi resistance, we found that CHD4 depletion suppressed gaps following PARPi treatment (Figures 3.6F–3.6H), suggesting that RGS also contributes to PARPi resistance in this model. Restored FP and RGS could be linked, in that one is required for the other. However, this does not appear to be the case. The (BRCA2 S3291A) mutant that is defective in FP\textsuperscript{77} is by contrast proficient in RGS and confers PARPi resistance to BRCA2-deficient Chinese hamster V-C8 cells similar to BRCA2 wild type (WT) (Figures 3.6I–3.6K). These findings further uncouple FP from therapy response\textsuperscript{77} and reveal in another BRCA2-deficient and -proficient model that RGS correlates with PARPi resistance.

Restored HR alone has also been proposed to confer chemoresistance in BRCA-deficient cells. In particular, HR is restored in BRCA1-deficient cells by loss of DNA end resection factors, such as 53BP1\textsuperscript{194,195}. We confirmed that in BRCA1 K/O, deletion of 53BP1 (DKO) enhances PARPi resistance\textsuperscript{203} (Figures 3.5E and 3.6L) and, remarkably, observed significant RGS (Figure 3.5F). Correspondingly, we found that 53BP1 depletion in the BRCA1-deficient cancer
cell line, BR5, enhanced PARPi resistance and RGS (Figures 3.6M and 3.6N). Thus, 53BP1 loss restores both HR\textsuperscript{194,195} and RGS as found here.

To consider the clinical relevance of gaps further, we analyzed PARPi-sensitive versus PARPi-resistant BRCA1 mutant patient-derived xenograft (PDX) that gained PARPi resistance upon serial passage in mice. Tumor samples that were PARPi sensitive showed significant PARPi-induced lengthening of tracts (Figure 3.5G). Consistent with this lengthening reflecting replication with gaps, elongated tracts dramatically shortened upon S1-nuclease treatment (Figure 3.5G). In contrast, PARPi-resistant tumor samples did not show PARPi-induced lengthening (but showed shortening), and tracts were not shortened by S1 nuclease cleavage (Figure 3.5G), indicating that replication gaps were not present. Collectively, these models demonstrate that replication-restraint defects and gaps characterize BRCA deficiency in human and mouse cancer cell lines as well as human tumors, and that restored fork restraint and RGS are associated with both known and \textit{de novo} mechanisms of PARPi resistance. Moreover, ATRi serves as a tool to resensitize and restore gaps (Figure 3.5H).
Figure 3.5: Gap suppression correlates with PARPi resistance and resensitization restores gaps.
(A) Cell survival assays for T2, BR5 and BR5-R1 (BR5-derived PARPi resistant cells) cells under increasing concentrations of olaparib. (B) Schematic and quantification of mean ssDNA intensity for BR5 and BR5-R1 cells following CldU pre-labeling and olaparib release (10 μM, 2 h). (C) Cell survival assays for BR5-R1 cells under increasing concentrations of olaparib with or without ATR inhibitor (VE-821, 1μM). (D) Quantification of mean ssDNA intensity for BR5-R1 cells following CldU pre-labeling and olaparib release (10μM, 2h), with or without ATR inhibitor (VE-821, 1μM). (E) Cell survival assays for indicated RPE1 cells under increasing concentrations of olaparib. For (A), (C) and (E), Data represent the mean percent ± SD of survival for each dot. (F) Quantification of mean ssDNA intensity for indicated cell lines following CldU pre-labeling and olaparib release (10μM, 2 h). For (B), (D), and (F), at least 200 cells are quantified from 2 biological independent experiments. (G) Schematic and quantification of the length of CldU tracts with or without S1 nuclease incubation in indicated PDX samples and olaparib treatment (10μM, 2 h). Each dot represents one fiber; at least 150 fibers are quantified for each sample independently. Red bars represent the median ± interquartile range. Statistical analysis according to Kruskal-Wallis test followed by Dunn’s test. **p < 0.01, ****p < 0.0001, ns: not significant. (H) Model indicating that gap suppression predicts resistance in response to PARPi in both de novo and engineered backgrounds.
Figure 3.6: Survival, fork lengthening, and gap suppression in known and de novo models of PARPi resistance. Related to Figure 3.5.

(A) Cell survival assays for T2, BR5 and BR5-R1 cells under increasing concentrations of cisplatin. Data represent the mean percent ± SD of survival for each dot. (B) Schematic and quantification of the length of IdU tracts in T2,
BR5 and BR5-R1 following olaparib treatment (10 μM, 2 h). Each dot represents one fiber; at least 200 fibers are quantified from two biological independent experiments (n=2). (C) Cell survival assays for C4-2 and PEO1 cells under increasing concentrations of olaparib. (D) Schematic and quantification of the length of dual-labeled tracts in PEO1 and C4-2 cells following olaparib treatment (1 μM, 4 h). Each dot represents one fiber, at least 100 fibers are quantified for each. (E) Schematic and quantification of mean ssDNA intensity for indicated cell lines following CldU pre-labeling and olaparib release (1 μM, 4 h). (F) Western blot analysis with the indicated antibodies of lysates from C4-2 NSC, PEO1 NSC and PEO1 shCHD4 cells. (G) Cell survival assays for C4-2 NSC, PEO1 NSC and PEO1 shCHD4 cells under increasing concentrations of olaparib. (H) Schematic and quantification of mean ssDNA intensity for PEO1 cells expressing shRNA against NSC and CHD4 following CldU pre-labeling and olaparib release (1 μM, 4 h). (I) Western blot analysis with the indicated antibodies of lysates from V-C8, V-C8+BRCA2 and V-C8+BRCA2 S3291A cells. (J) Cell survival assays for V-C8, V-C8+BRCA2 and V-C8+BRCA2 S3291A cells under increasing concentrations of olaparib. (K) Schematic and quantification of mean ssDNA intensity for V-C8 and indicated cells following CldU pre-labeling and olaparib release (1 μM, 4 h). (L) Western blot analysis with the indicated antibodies of lysates from Control, BRCA1 K/O and BRCA1/53BP1 K/O RPE1 cells. (M) Western blot analysis for T2 and BR5 cells expressing shRNA against NSC, 53BP1(A) and 53BP1(B), and cell survival assays for indicated cells under increasing concentrations of olaparib. (N) Schematic and quantification of mean ssDNA intensity for BR5 cells expressing shRNA against NSC, 53BP1(A) and 53BP1(B) following CldU pre-labeling and olaparib release (10 μM, 2 h). All red bars represent the median ± interquartile range. For (E), (H), (K) and (N), at least 200 cells are quantified from n=2. Statistical analysis according to Kruskal-Wallis test followed by Dunn’s test. All p values are described in Statistical methods.

3.3.5 Gaps predict PARPi sensitivity in HR- and FP-proficient cells

PARPi is thought to lead to an accumulation of DSBs that are not effectively repaired in BRCA-deficient cells. However, PARPi also sensitizes HR-proficient cells, suggesting a distinct phenomenon such as gaps could confer PARPi sensitivity. To assess the role of gaps, we sought to identify a model system in which the BRCA pathway was compromised for RGS, but HR and FP were intact. The FA patient fibroblast line with one mutant RAD51 allele (RAD51-T131P) is sensitive to cisplatin or PARPi, but unexpectedly is HR
 proficient, leading to the proposal that the FP defect underlies the sensitivity\textsuperscript{108,109,244,322} (Figure 3.7A). Conversion of the mutant RAD51 allele to WT enhanced PARPi resistance as expected and restored RGS (Figures 3.7B and 3.7C), consistent with the function of RAD51 in RGS\textsuperscript{79,103,316,318,330}, and that either the restored RGS and/or FP conferred the greater resistance. By comparison, deletion of the mutant allele (–/WT) provides only partial resistance and RGS as compared to the parental (Figures 3.8A and 3.8B), suggesting that two RAD51 alleles are essential for the restoration of RGS and/or FP. As reported, we found that FP is restored by the depletion of RADX, a negative regulator of RAD51\textsuperscript{331} (Figures 3.8C and 3.8D). Given that HR and FP are presumed to confer PARPi resistance, it was surprising that restored FP in the FA or BRCA1-deficient cells did not alter PARPi sensitivity. Given that gaps remained, it suggested that gaps do not derive from degraded forks and that gaps could mediate the PARPi sensitivity in this model (Figures 3.7A, 3.7D, 3.7E, and 3.8C–3.8F).
Figure 3.7: PARPi-induces gaps and sensitizes cells regardless of HR and fork protection (FP) proficiency.

(A) Left: Is fork degradation the cause for gaps and PARPi sensitivity in HR proficient cells? Right: Model indicating that PARPi-induced gaps still sensitize cells when fork protection (FP) is restored by depleting RADX. (B) Cell survival assays for patient fibroblasts (RA2630) RAD51 T131P (T131P/WT) and RAD51 double-allele CRISPR corrected (WT/WT) cells under increasing concentrations of olaparib. (C) Schematic and quantification of mean ssDNA intensity for indicated cell lines in (B) following CldU pre-labeling and olaparib release (10 μM, 2 h). (D) Cell survival assays for indicated cells under increasing concentrations of olaparib. For all cell survival assays, data represent the mean percent ± SD of survival for each dot. (E) Quantification of mean ssDNA intensity for indicated cell lines following CldU pre-labeling and olaparib release (10 μM, 2 h). For (C) and (E), at least 200 cells are quantified from two biological independent experiments. All red bars represent the median ± interquartile range. All statistical analysis according to Kruskal-Wallis test followed by Dunn’s test. ****p < 0.0001, ns: not significant.
Figure 3.8: Gaps predict PARPi sensitivity in HR- and FP-proficient cells. Related to Figure 3.7.

(A) Cell survival assays for patient fibroblasts (RA2630) RAD51 T131P (T131P/WT) and RAD51 single- allele CRISPR deleted cells (-/WT) under increasing concentrations of olaparib. (B) Quantification of mean ssDNA intensity for indicated cell lines in (A) following CldU pre-labeling and olaparib release (10 μM, 2 h), at least 200 cells are quantified from from two independent biological experiments (n=2). (C) Western blot analysis with the indicated antibodies of lysates from patient fibroblasts (RA2630) RAD51 T131P cells and BRCA1 K/O RPE1 cells expressing shRNA against non-silencing control (NSC) and RADX. (D) Schematic and quantification of the CldU/IdU ratio after 3 mM HU treatment for 5 h in indicated cells, at least 200 fibers are quantified from n=2. (E) Schematic and quantification of mean ssDNA intensity for indicated cell lines following CldU pre-labeling and olaparib release (10 μM, 2 h), at least 100 cells are quantified from n=2. All red bars represent the median ± interquartile range. Statistical analysis according to Kruskal-Wallis test followed by Dunn’s test. All p values are described in Statistical methods. (F) Cell survival assays for indicated cell lines under increasing concentrations of olaparib. For all cell survival assays, data represent the mean percent ± SD of survival for each dot.

3.3.6 Targeting RPA augments synthetic lethality between PARPi and BRCA1 deficiency

The increased replication gaps in BRCA-deficient cells could render the single-stranded binding protein RPA essential for viability. To address this idea, we
used single-molecule localization microscopy (STORM) for direct visualization and quantification of ssDNA-bound RPA based on individual replication assemblies (5-ethynyl-2′-deoxyuridine-positive [EdU+], mini-chromosome maintenance complex component 6-positive [MCM6+], and PCNA+ sites). To measure the amounts of incorporated RPA within individual replisomes, we performed unbiased correlation-based image analyses in control, BRCA1−, and FANCJ-depleted osteosarcoma cell line (U2OS) cells following PARPi treatment (Figures 3.9A, 3.9B, and 3.10A). At 10 µM PARPi, we observed that the average RPA fork density was enhanced in the BRCA1-depleted and control cells, as compared to FANCJ-depleted U2OS cells (Figure 3.9B), which is consistent with BRCA1 being required to suppress gaps and FANCJ being required to make gaps.

To test the hypothesis that PARPi is synthetic lethal with BRCA1 deficiency due to the generation of gaps that exceed levels that can be protected by RPA, we addressed whether following PARPi, the BRCA1 K/O cells more readily exhausted the pool of RPA. Following PARPi, the RPE1 cell lines gained chromatin bound (CB)-RPA that correlated with an increase in γ-H2AX, most notably in BRCA1 K/O cells (Figures 3.9C and 3.10B). Consistent with an enhanced reliance on RPA to protect ssDNA, we also observed that RPA depletion reduced the survival of BRCA1 K/O cells to PARPi as compared to the other RPE1 cells (Figures 3.9D and 3.10C). When RPA subunits were instead overexpressed, PARPi resistance was uniquely elevated in BRCA1 K/O cells (Figure 3.9E). These results suggest that RPA is a critical buffer of PARPi toxicity in BRCA1-deficient cells. Consistent with this premise, a small
molecule (RPAi, compound NERx-329) that blocks RPA-ssDNA interaction displayed single-agent activity in the BRCA1 K/O cells (Figures 3.9F and 3.10D–3.10F). Furthermore, at a sublethal dose, the RPAi augmented synthetic lethality between BRCA1 K/O and PARPi while having little impact on the control or FANCJ K/O RPE1 cells (Figures 3.9G, 3.10F, and 3.10G). These findings further implicate replication gaps in PARPi synthetic lethality and indicate that targeting the RPA protection of gaps magnifies synthetic lethality in BRCA1-deficient cells.

Gaps converted to DSBs could underlie the killing of BRCA-deficient cells such as when gaps either interface with a second round of replication or are digested by nucleases. Alternatively, DSBs could derive from other sources such as apoptosis. To address this idea, we identified a dose of PARPi that BRCA1 K/O cells uniquely activated apoptosis as measured by induction of PARP1 and caspase-3 cleavage and verified suppression by apoptosis inhibitors, Z-VAD-FMK, or emricasan (Figures 3.10H and 3.10I). We observed that the inhibition of apoptosis not only elevated the resistance of BRCA1 K/O but also suppressed PARPi-induced DSBs (Figures 3.10J and 3.10K), suggesting that apoptosis contributes to the loss of cell viability and DSB formation in BRCA1-deficient cells.
Figure 3.9: PARPi synthetic lethality in BRCA1-deficient cells is linked to RPA exhaustion and can be augmented by targeting RPA.

(A) Western blot analysis with the indicated antibodies of lysates from U2OS cells expressing siRNA against non-silencing control, FANCJ and BRCA1. (B) Schematic and quantification of average RPA density at each replication fork in STORM analysis for indicated cells and treatment (10 μM, 2 h Olaparib). At least 40 single cells are quantified from two biological independent experiments (n=2) respectively for each group, every dot represents the pair localized RPA density from one nucleus. All red bars represent the median ± interquartile range. Statistical analysis according to Kruskal-Wallis test followed by Dunn’s test. (C) Control, BRCA1 K/O and FANCJ K/O RPE1 cells are treated with Olaparib (10 μM) at indicated time and stained for CB-γH2AX/RPA. Dashed lines indicate maximum γH2AX levels in untreated Control cells. Cells higher than those are marked as red and calculated for percentages. Each dot represents one cell; cells (n=1500 ± 300) are collected from n=2. (D) Western blot analysis with the indicated antibodies of lysates from Control and BRCA1 K/O RPE1 cells expressing siRNA against RPA in increasing doses (0, 0.025nM, 0.05nM), and cell survival assays for cells above under increasing concentrations of olaparib. (E) Western blot analysis for indicated antibodies in Control and BRCA1 K/O RPE1 cells with negative mock transfection (-) and over-expression of superRPA cDNA (sRPA shows higher bands in immunoblots due to P2A tag), and cell survival assays for cells above under
increasing concentrations of olaparib. For (D) and (E), data represent the mean percent ± SD of survival for each dot. (F) Clonogenic assays for Control and BRCA1 K/O cells treated with RPA inhibitor (RPAi, NERx-329) under indicated doses. Mean survival percentages of n=4 with SEM are collected for all cells. (G) Clonogenic assays for indicated cells under increasing olaparib with sublethal dose of RPA inhibitor (RPAi, NERx-329). Mean survival percentages of n=3 with SEM are collected for all cells. Statistical analysis according to t test. All p values are described in quantification and statistical analysis.
Figure 3.10: PARPi synthetic lethality in BRCA1-deficient cells is augmented by targeting RPA and corresponds with apoptosis and DSB formation. Related to Figure 3.9.

(A) Sample image for STORM analysis with staining of RPA70: white, EdU: red, MCM6: green, PCNA: blue. Scale bar, 3000nm. (B) Bar graphs show percentages of RPA positive cells for different cells and treatments in Figure 5C. (C) Western blot analysis and cell survival assays for FANCJ K/O cells expressing indicated siRPA under increasing concentrations of olaparib. (D) Cell survival assays for indicated cell lines with sensitivity normalized to untreated respectively at different concentrations of RPA inhibitor (0.3, 0.4, 0.5 μM for NERx-329). For cell survival assays, data represent the mean percent ± SD of survival for each bar. (E) Sensitivity of BRCA1-deficient UWB1 and complemented ovarian cancer cell lines to RPA inhibitor NERx-329. Data represent the mean percent ± SD of survival for each dot. (F) Representative images and quantification for clonogenic assays with indicated cells treated with RPA inhibitor (RPAi, NERx-329) under indicated doses. Mean survival percentages of four biological independent experiments (n=4) with SEM are collected for all cells. (G) Representative images and quantification for clonogenic assays for indicated cells under increasing olaparib with sublethal dose of RPA inhibitor (RPAi, NERx-329). Mean survival percentages of n=3 with SEM are collected for all cells. (H) Apoptosis is induced in BRCA1 K/O cells by 72h post-PARPi (Olaparib, 50μM) and CPT (positive control, 1μM) as noted by cleavage of PARP1 and Caspase-3 by Western blot analysis. (I) Apoptosis proteins in (H) are suppressed by apoptosis inhibitors Z-VAD-FMK (50μM) or Emricasan (50μM) with 2h pretreatment and maintained for the duration of the experiment (72h). (J) Inhibition of apoptosis with Emricasan (50μM) enhances cell survival of BRCA1 K/O cells treated with PARPi (50μM). (K) Pulsed field capillary electrophoresis analysis (left) and quantitation (right) for fragments of genome DNA (gDNA) after PARPi treatment in BRCA1 K/O cells reveal DNA fragmentation is suppressed by Emricasan (50μM). Left: X-axis: DNA sizes (base pair), Y-axis: Relative fluorescence units (RFU); purple numbers embedded indicate the sizes for each peak.

3.3.7 BRCA1-deficient cells have OFP defects

PARPi is synthetic lethal with loss of genes functioning in OFP and PARP1 functions in OFP\(^{224,314}\). To address the possibility that PARPi is synthetic lethal with BRCA1 deficiency due to combined OFP defects, we sought to analyze endogenous PAR polymerization, which when elevated in unperturbed cells, suggests a lagging strand problem\(^{224}\). We verified FEN1 depletion in conjunction with the PAR catabolism by PAR glycohydrolase inhibitor
elevated PAR levels as also observed following treatment with methyl methanesulfonate (MMS), a drug that induces ssDNA gaps (Figures 3.11A and 3.11B). While following these perturbations, PAR was similarly elevated in the distinct RPE1 cell lines (Figures 3.11C and 3.11D), and we found that they were distinct in unchallenged conditions. Specifically, as compared to control cells or FANCJ K/O cells that displayed remarkably low PAR levels, BRCA1 K/O cells had higher PAR levels (Figure 3.12A), suggesting that the source of PAR was not from DNA base damage but from an OFP defect.

To further assess whether OFP defects were the source of S phase PAR in BRCA1 K/O cells, we used emetine, an inhibitor of DNA replication that prevents the formation of OFs. As found previously, emetine completely suppressed the appearance of S phase PAR in control and FEN1-depleted cells, as well as in BRCA1 K/O cells (Figures 3.11E and 3.11F), consistent with PAR requiring lagging strand synthesis. In summary, these findings strongly implicate unligated OFs are the source of gaps and cause of S phase PARylation in BRCA1 K/O cells.

Next, we examined whether PAR mirrored gap induction in the PARPi-sensitive and -resistant models. PAR was significantly lower in the de novo PARPi-resistant BR5-R1 cells as compared to PARPi-sensitive BR5 cells (Figure 3.12B). Moreover, PARPi-resistant BRCA1 and 53BP1 DKO cells displayed significantly lower PAR levels than PARPi-sensitive BRCA1 K/O cells (Figure 3.12C). Furthermore, PARPi-resistant corrected FA cells (WT/WT) had dramatically reduced PAR levels compared to the parental cells (T131P/WT) (Figure 3.12D). In PARPi-sensitive V-C8 cells, PAR was also suppressed, with
BRCA2 complementation (Figure 3.12E) akin to previous findings. These results suggest that OFP defects are corrected in cells that gain PARPi resistance.

Figure 3.11: Poly(ADP-ribose) validation for Okazaki fragment processing defects. Related to Figure 3.12.

(A) Top: Representative images of immunofluorescence for shFEN1 Control, BRCA1 K/O and FANCJ K/O RPE1 cells following PARG inhibitor (10 μM) and EdU for 20min, stained for poly (ADP-ribose) (PAR) and EdU. Scale bars, 50μm. Bottom: Immunofluorescence images for Control, BRCA1 K/O and FANCJ K/O RPE1 cells following MMS (0.2mg/ml) and EdU for 20min, stained for poly (ADP-ribose) (PAR) and EdU. Scale bars, 50μm. (B) Western blot analysis with the indicated antibodies of lysates from Control, FANCJ K/O and BRCA1 K/O RPE1 cells expressing shRNA against non-silencing control (NSC) and FEN1. (C) Quantification of relative PAR intensity across indicated cells incubated with PARG inhibitor (10 μM) or MMS (0.2 mg/ml) for 20min. Average values of five biological independent experiments (n=5) with SEM are calculated for all interphase cells. (D) Quantification of immunofluorescence for...
shFEN1 Control, BRCA1 K/O and FANCJ K/O RPE1 cells following PARG inhibitor (10 μM) and EdU for 20 min, stained for poly (ADP-ribose) (PAR) and EdU. Dashed lines indicate maximum PAR level in untreated Control cells. Cells higher than those are calculated for percentages respectively. Each dot represents one cell; at least 2000 cells are collected from n=5. (E) Quantification of relative PAR intensity across indicated Control cells incubated with or not with emetine (10 μM, 1 h), with or without PARG inhibitor (10 μM) and EdU added during the final 20 min. Average values of n=3 with SEM are calculated for all interphase cells. (F) Quantification of relative PAR intensity for Control and BRCA1 K/O RPE1 cells incubated with or without emetine (10 μM, 1 h), with PARG inhibitor (10 μM) and EdU added during the final 20 min. Average values of n=5 with SEM are calculated for all interphase cells. For (C), (E) and (F), at least 200 cells are measured for each experiment. Statistical analysis according to t test, all p values are described in Statistical methods.

Figure 3.12: PARPi-sensitive cells display high PAR reversed upon PARPi resistance.
(A) Top: Representative images of immunofluorescence for Control, BRCA1 K/O and FANCJ K/O RPE1 cells stained for poly (ADP-ribose) (PAR) and EdU. Cells were incubated without (top) or with (bottom) PARG inhibitor (10 μM) for poly (ADP-ribose) (PAR) and EdU for 20 min to detect DNA synthesis. Scale
bars, 50\(\mu\)m. Bottom: Scatterplot of indicated cells for mean PAR and EdU intensity per nucleus after incubation with EdU with or without PARG inhibitor (10 \(\mu\)M, 20 min, using DMSO as control). Dashed lines indicate maximum PAR level in untreated Control cells. Each dot represents one cell; at least 500 cells are collected for each from three biological independent experiments (n=3). (B) Scatterplot of mean PAR and EdU intensity for BR5 and BR5-R1 cells. Cells are incubated with EdU with or without PARG inhibitor (10 \(\mu\)M, 20 min) for indicated groups. At least 2000 cells are collected from n=3. Dashed lines indicate maximum PAR level in untreated BR5-R1 cells. (C) Scatterplot of PAR and EdU for BRCA1 and BRCA1/53BP1 K/O cells. Cells are incubated same as above for indicated groups. At least 1200 cells are collected from n=3. Dashed lines indicate maximum PAR level in untreated BRCA1 K/O cells. (D) Scatterplot of PAR and EdU for patient fibroblasts (RA2630) RAD51 T131P (T131P/WT) and RAD51 double-allele CRISPR corrected (WT/WT) cells. Cells are treated same as above. At least 2000 cells are collected from n=3. Dashed lines indicate maximum PAR level in untreated WT/WT cells. (E) Scatterplot of PAR and EdU for V-C8 and V-C8+BRCA2 cells. Cells are treated same as above. At least 1200 cells are collected from n=2. Dashed lines indicate maximum PAR level in untreated V-C8+BRCA2 cells. For all of the above, cells higher than those lines are calculated for percentages, respectively.

3.3.8 53BP1 deletion restores lagging strand synthesis in BRCA1 K/O cells via alternative OFP

To decipher the OFP defect in BRCA1 K/O cells, we considered that FEN1 depletion induced similar PAR levels with or without BRCA1 (Figures S6A–S6D), consistent with intact canonical OFP in BRCA1 K/O cells. Thus, we analyzed the chromatin accumulation of PARP1 that functions in backup OFP. As compared to controls, we observed that CB-PARP1 levels were increased in BRCA1 K/O cells (Figure 3.13A), consistent with the higher PAR. In fact, the PARP1 “trapping” in untreated BRCA1 K/O cells was similar to control cells treated with PARPi (Figure 3.13A), indicating that PARP1 was aberrantly CB in BRCA1 K/O cells. Lagging strand defects generate gaps that trigger PARP1 activation that attempts to rectify the problem by recruiting ssDNA binding protein XRCC1\textsuperscript{163,300,339-341}. However, unlike PARP1, XRCC1 was aberrantly
low in BRCA1 K/O cells, with its reduction primarily in EdU+ cells (Figures 3.13B and 3.13C). BRCA1 K/O cells also had a reduced EdU intensity in EdU+ cells and elevated 53BP1 nuclear foci (Figures 3.13D, 3.13E, and 3.14A), suggesting that replication was less robust and/or under-replicated342-345. These data suggest that in BRCA1 K/O cells, PARP1 fails to effectively recruit XRCC1 to engage backup lagging strand synthesis308.

Could 53BP1 interfere with the function of PARP1 in lagging strand synthesis? In accordance with the reduced PAR (Figure 3.12C), we observed that 53BP1 deletion in the BRCA1 K/O cells reduced PARP1 chromatin association (Figures 3.13A and 3.13B). Notably, XRCC1, LIG3 (Figures 3.13B and 3.13C), and replication were instead elevated (Figure 3.13D), suggesting that backup OFP was in place239,308,346. Similarly, 53BP1 depletion suppressed PAR and elevated XRCC1 and LIG3 in BRCA1-deficient BR5 cells, indicating that restored alternative OFP was not specific to the RPE1 cells (Figures 3.14B and 3.14C). Consistent with a switch in the mechanism of OFP, control cells had a greater PAR induction than DKO cells following depletion of the canonical OFP factors FEN1 or LIG1, whereas DKO had a greater PAR induction following depletion of the alternative OFP factor LIG3 (Figures 3.13F, 3.14D, and 3.14E). We also observed that 53BP1 deletion increased the resistance of BRCA1 K/O cells to inhibitors of FEN1 (FEN1i) or ligases (ligase I/III/IV i) as well as to the gap-inducing drug MMS79,336,337. These findings suggest that as compared to the DKO cells and control cells that have intact XRCC1-LIG3 backup, the BRCA1 K/O cells are more vulnerable to the titration of a pan-ligase inhibitor or loss of canonical OFP factor FEN1 (Figure 3.13G). Our data suggest
that BRCA1 deficiency activates PARP1 but fails to stimulate XRCC1-LIG3 as a backup pathway for OF ligation unless 53BP1 is deleted (Figure 3.13H).

**Figure 3.13:** Okazaki fragment processing (OFP) defects in BRCA1-deficient cells are suppressed by 53BP1 depletion. (A) Quantification of chromatin bound PARP1 (CB-PARP1) for Control, BRCA1 K/O and BRCA1/53BP1 K/O (labeled as Con, DKO in figure, same as follows) RPE1 cells with or without treatment (0.5 μM, 2 h Olaparib), with EdU added to the final 20min. For quantification, EdU+ cells were gated according to positive EdU incorporation. (B) Western blot analysis for indicated antibodies in chromatin extraction for Control, BRCA1 K/O and BRCA1/53BP1 K/O RPE1 cells. (C) Quantification of CB-XRCC1 for untreated Control, BRCA1 K/O and BRCA1/53BP1 K/O RPE1 cells with EdU incubated for 20min. For quantification, EdU+ cells were gated according to positive EdU incorporation. (D) Quantification of mean EdU intensity from EdU+ cells. EdU was incubated for 20min in indicated cells. For (A), (C) and (D), at least 600 cells are quantified from n=3. Bars represent the median ± interquartile range. All statistical analysis according to Kruskal-Wallis test followed by Dunn’s test. ****p < 0.0001. (E) Immunofluorescence showing 53BP1 chromatin foci in untreated Control and BRCA1 K/O RPE1 cells. Cells ≥ 1 foci per nucleus were measured. At least 150 cells were quantified from n=3. Statistical analysis according to Mann-Whitney test. ****p < 0.0001. Scale bar, 10μm. (F) Average percentages of PAR positive (PAR+) over total for indicated cells after PARG inhibitor (10 μM, 20 min) and EdU incubation. For quantification, PAR positive cells were gated by
maximum PAR level in Control NSC cells for each experiment. Average values of n=4 with SEM are calculated. *p < 0.05. (G) Cell survival assays for indicated cells under increasing concentrations of MMS, FEN1 inhibitor (FEN1i) and Ligase I/III/IV inhibitor (Ligase I/III/IV i). Data represent the mean percent ± SD of survival for each dot. (H) Model summarizing the function of BRCA1 and 53BP1 in regulating lagging strand synthesis, and their interactions with the backup OFP pathway.

**Figure 3.14:** Analysis for 53BP1 depletion and alternative OFP. Related to Figure 3.13.

(A) Representative images (20x) for 53BP1 chromatin foci in untreated Control and BRCA1 K/O RPE1 cells. More cells with ≥ 1 foci can be observed in BRCA1 K/O cells. Scale bar, 50μm. (B) Mean PAR and EdU intensity per nucleus for indicated cells were measured after PARG inhibitor (10 μM, 20min) and EdU incubation. For quantification, PAR positive cells were gated by maximum PAR level of untreated BR5 cells in Figure 6B. Each dot represents one cell; at least
800 cells are collected from two biological independent experiments (n=2) for each. (C) Left: Western blot analysis for indicated antibodies in chromatin extraction from untreated BR5 cells expressing shRNA against NSC, 53BP1(A) and 53BP1(B). Right: Quantification of CB-XRCC1 for untreated BR5 cells expressing shRNA against NSC, 53BP1(A) and 53BP1(B) with EdU incubated for 20min. For quantification, EdU+ cells were gated according to positive EdU incorporation. At least 600 cells are quantified from n=2 for each. Bars represent the median ± interquartile range. Statistical analysis according to Kruskal-Wallis test followed by Dunn’s test. ****p < 0.0001. (D) Western blot analysis in Control and BRCA1/53BP1 K/O (DKO) RPE1 cells expressing shRNA against NSC, FEN1, LIG1 and LIG3. (E) Mean PAR and EdU intensity per nucleus for indicated cells were measured after PARG inhibitor (10 μM, 20min) and EdU incubation. For quantification, PAR positive cells were gated by mean maximum PAR level in Control NSC cells. Each dot represents one cell; at least 800 cells are collected from n=4 for each.

3.4 Discussion

As clinical interventions for PARPi resistance are lacking, understanding the sensitizing lesion is of critical importance. The DSB framework was logical given the synthetic lethality between PARPi and BRCA-deficient cells that are defective in HR and FP, functions that fix and limit DSBs. However, this DSB model does not fully align with cell models and clinical outcomes. Furthermore, suppression and repair of replication-associated ssDNA gaps is also a function of the BRCA and PARP1 pathways\textsuperscript{224,240,317}. Thus, we considered whether ssDNA gaps deriving from the combined loss of PARP1 and BRCA instead caused synthetic lethality. Consistent with the gap model, we observe that RGS underlies acquired PARPi resistance and resensitization restores gaps. We also reveal that replication gaps in BRCA1-deficient cells result from defects in OFP and that 53BP1 deletion reverses this defect by engaging a backup lagging strand ligation pathway mediated by PARP1-XRCC1-LIG3\textsuperscript{224,239,346}. Accordingly, DKO cells gain XRCC1 and LIG3 as well as resistance to inhibitors of PARP1, FEN1, pan-ligases, or the methylating agent MMS. Significantly,
LIG3 depletion in DKO cells restores PAR as well as PARPi sensitivity, an outcome linked to widespread gap induction, but not DSBs\textsuperscript{347}. Clinical relevance is also supported as gaps accumulate more excessively in BRCA-deficient immortalized cells, cancer cell lines, and patient tumors as compared with BRCA-proficient controls. Notably, BRCA1-deficient cells also show sensitivity to RPA inhibitors that augments synthetic lethality with PARPi. We, therefore, propose that PARPi kills BRCA1-deficient cells due to compounded lagging strand gaps that exhaust RPA pools and that gap accumulation is the root cause of therapy sensitivity and “BRCAness”\textsuperscript{240}.

Our data also highlight that PARPi sensitivity does not stem from a speed threshold, but rather a gap threshold that exceeds RPA pools. As reported, we find that PARPi lengthens replication tracts\textsuperscript{222} that are significantly longer in BRCA-deficient cells. However, replication tracts are discontinuous. In contrast, hyper-lengthened tracts are continuous in p21-depleted cells that are not PARPi sensitive, implicating gaps as the toxic factor. Lengthening without gaps could be mediated by translesion synthesis (TLS). p21 is a TLS inhibitor\textsuperscript{348}, and enhanced TLS in BRCA2-deficient cells confers resistance\textsuperscript{113,349}. Moreover, TLS suppresses gaps induced by chemotherapy or oncogenes\textsuperscript{349} and counters gaps in yeast\textsuperscript{258}. When not avoided by TLS and exposed by drugs, gaps and RPA exhaustion could contribute to outcomes of replication stress\textsuperscript{107,321,333,350}. Correspondingly, modulating the gap threshold via RPA depletion or inhibition enhanced the sensitivity of BRCA1-deficient cells to PARPi, whereas RPA overexpression limited this sensitivity. Again, RPA loss could expose gaps that convert to DSBs; however, our findings suggest that a significant number of
PARPi-induced DSBs stem from apoptosis and not directly from PARPi, raising the possibility that DSBs are not the primary killing event. It remains unclear whether DSBs not inhibited by apoptosis stem directly from PARPi and are cell lethal or rather result from the partial inhibition of apoptosis or other technical issues. A full kinetic analysis will also be critical to decipher the relationship between these events but suggest that genotoxin-induced DSBs derive in large part from apoptosis²⁴⁰,³⁵¹,³⁵².

Our gap model is in line with recent reports revealing that gaps forming in BRCA-deficient cells are independent of DSBs²⁴¹. While it is possible that eventual PARPi-induced fork degradation, collapse, and/or DSBs contribute to the toxicity in BRCA-deficient cells, we present a series of separation-of-function models that indicate a greater prediction between the immediate induction of gaps and PARPi toxicity than loss of either HR or FP (Table 3.1). An important comparison revealing gaps as a discriminating factor is between BRCA1 and FANCJ. Both are mutated in hereditary breast/ovarian cancer and FA, with their loss disrupting both HR and FP¹²⁸,¹³⁵,²⁶⁸, but unlike BRCA1 loss, FANCJ loss does not sensitize to PARPi or generate gaps or PAR in unchallenged cells. Either FANCJ is not required for processing PARPi-induced DSBs or DSBs are not the sensitizing lesion. Logically, if DSBs were the sensitizing lesion, then HR-proficient cells should repair DSBs and show PARPi resistance. However, the HR-proficient FA cell line RAD51-T131P is sensitive, albeit not hypersensitive, possibly because of its slow growth or proficiency in HR and/or post-replication gap repair that is distinct from RGS²⁴⁴,³¹⁸. However,
the sensitivity does not change even when FP is restored by RADX depletion, indicating that HR and FP are not sufficient to confer PARPi resistance.

Table 3.1: PARPi response in different genetic backgrounds. Related to Figures 3.1, 3.2 and 3.5-3.8.

<table>
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<tr>
<th>Cell line</th>
<th>HR*</th>
<th>FP*</th>
<th>GS*</th>
<th>PARPi response</th>
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<td>+</td>
<td>+</td>
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<td>BRCA1 and RADX deficient</td>
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* HR: homologous recombination, FP: fork protection, GS: gap suppression

Table 3.1: PARPi response in different genetic backgrounds. Related to Figures 3.1, 3.2 and 3.5-3.8.

The concept that synthetic lethality derives from a distinct lesion is further supported by the range of synthetic lethal combinations unrelated to DSB repair\textsuperscript{224,314,315,353,354}. Moreover, the synthetic lethality between PARPi and XRCC1, FEN1 and BRCA deficiency, or a PCNA OFP mutant and BRCA1 deficiency also implicates gaps due to OFP defects as the sensitizing lesion\textsuperscript{170,225,311-313}. Even within the classical paradigm of HR deficiency conferring PARPi response, synthetic lethality includes widespread gap induction\textsuperscript{355,356} that at the very least indicates that gaps are a biomarker of response. Interestingly, not only does the BRCA-RAD51 pathway function in RGS but it also functions in post-replication gap filling\textsuperscript{79,86,240,241,317,318}. Thus,
distinct BRCA functions could facilitate the removal of PARP1, process unligated OFs, or fill gaps in post-replication.

Gaps from OFP defects could lead to the appearance of unrestrained replication. Similar to loss of OFP such as FEN1, LIG1, PCNA, or PARP1, loss of BRCA-RAD51 pathway proteins is associated with unrestrained replication. Mechanistically, it is possible that lagging strand gaps interfere with nascent strand annealing reactions that slow and remodel replication forks. Correspondingly, in FANCJ-deficient cells, there is an absence of lagging strand gaps possibly due to parental DNA forming G4s, and replication is hyper-restrained.

Our studies also have important implications for how 53BP1 deletion rescues BRCA1 deficiency from PARPi sensitivity as well as embryonic lethality. 53BP1 functions in both unperturbed and perturbed replication. Dual deficiency in replication and BRCA1 expression could enhance 53BP1 in chromatin because replication dilutes its recruiting histone mark that in turn favors BRCA1 binding. In BRCA1 K/O cells, 53BP1 could interfere with XRCC1 recruitment or its attempts to process the lagging strand. Notably, loss of 53BP1 leads to gap formation, providing a platform for PARP1-XRCC1 binding. Alternatively, given that 53BP1 exists in a complex with DNA polymerase α (Polα), 53BP1 deletion could in part rescue replication in BRCA1 K/O cells by liberating a sequestered pool of Polα. If this scenario is correct, then inhibitors of Polα would resensitize PARPi-resistant BRCA1/53BP1-deficient cells. Conceivably, lagging strand gaps in BRCA-RAD51-deficient cells cause the vulnerability to cytotoxic nucleotides, trigger
sister chromatid exchange reactions\textsuperscript{362}, and/or induce chromatid-type aberrations\textsuperscript{363}.

In summary, our findings establish a model to help further develop targeted treatment with PARPi, such that combined therapies maximize exposed lagging strand gaps. It will be crucial to consider whether gaps are the genetic vulnerability resensitizing PARPi-resistant cells\textsuperscript{180,364,365} or whether they underlie combinations that synergize with PARPi\textsuperscript{299,353,365-367}. The rationale for limiting PARPi to HR-defective cancers is in question because of significant clinical benefit across ovarian cancer patients regardless of BRCA status\textsuperscript{245,368}. Moreover, a key future question will be whether, in a range of synthetic lethality models, gaps confer toxicity, RGS confers chemoresistance, and targeting RGS pathways will maximize the efficacy of genotoxic chemotherapy. These findings highlight the importance of considering ssDNA gaps as a critical biomarker and determinant of PARPi synthetic lethality.

\textit{Limitations of the study}

Based on RGS being closely coupled with therapy response, whereas HR and FP in several cases do not correlate, our findings support a ssDNA gap framework for PARPi synthetic lethality. However, our findings do not differentiate whether toxicity reflects the number, size, and/or persistence of ssDNA gaps. Short-term gaps promote an adaptive response to tolerate genotoxic chemotherapy\textsuperscript{263}. Ideally, ssDNA sequencing or direct detection tools will facilitate the defining of a replication gap threshold underlying PARPi toxicity. Moreover, although HR-proficient cells display PARPi sensitivity,
suggesting that DSBs are not required, the conversion of gaps to DSBs cannot be ruled out as a contributing factor. Furthermore, our study does not provide visual insight with microscopy toward the relationship between replication gaps, DSBs, and/or stalled forks that in yeast were found to be physically distinct entities.258

3.5 Materials and Methods

**Cell lines and gene editing**

Human RPE1-hTERT cell lines were grown in DMEM+GlutaMAX-I (GIBCO, 10569) supplemented with 10% FBS and 1% Pen Strep (100 U/ml). U2OS, 293T, PEO1, C4-2 and V-C8 derived cell lines were grown in DMEM (GIBCO, 11965) supplemented with 10% fetal bovine serum (FBS) and 1% Pen Strep (100 U/ml). T2, BR5, BR5-R1 cell lines were cultured in DMEM (CORNING cellgro, 15-017-CV) with 10% FBS, penicillin and streptomycin (100 U/ml each), and 1% L-glutamine. The resistant cell line BR5-R1 was maintained in 1 μM olaparib. FA Patient fibroblasts (RA2630 T131P, CRISPR corrected clone 3-39 ΔMUT/WT or −/WT, and double-allele CRISPR corrected clone WT/WT) were cultured in DMEM (GIBCO, 11965) supplemented with 10% FBS, 1% Pen Strep (100 U/ml), 1% GlutaMAX-I, 1% MEM NEAA and 1% Sodium pyruvate.244 UWB1 and complemented cells were maintained in MEGM Bullet Kit (Lonza, CC-3150): RPMI-1640 (CORNING cellgro, 10-040-CV) 1:1 with 3% FBS and 1% Pen Strep. All the cell lines were cultured at 37°C, 5% CO2. The generation of RPE1-hTERT TP53−/− BRCA1 K/O and BRCA1/53BP1 double K/O Cas9 cells were described elsewhere.203 FANCJ gene knockout in RPE1-hTERT
TP53 Cas9 cells was introduced by using two synthesized gRNAs (gRNA #1: GGGTCGAGGAAGGTAACGG, gRNA #2: GGCAATCACC-ACACCTTTCA) and the protocol from IDT technology. For generating FANCJ gene knockout in RPE1-hTERT TP53 Cas9 cells, 10 µL tracrRNA (100 µM) and 10 µL 20 nt crRNA (100 µM) were annealed in 80 µL nuclease free duplex buffer (IDT#11-05-01-03) to form a 10 µM gRNA solution. The crRNAs were designed using CRISPR design tools of Benchling. In brief, 100,000 cells were seeded out the day before transfection in 12-well dish in 1 mL medium. 30 minutes prior to transfection the medium was replaced with 750 µL of medium. The 3 µL of 10 µM for each gRNA was added to optiMEM (Life Technologies) to final volume of 125 µL and incubated briefly at RT. Then 6 µL Lipofectamine RNAiMAX (Invitrogen) was added and supplemented to a total volume of 250 µL with optiMEM. Mixture was incubated at RT for 20 min before adding to the cells. The next day the medium was changed, and 2 days after transfection the cells were serial diluted in 96 well plates to obtain single clones. 2 weeks later, the cell clones were passages for new 96 wells, and screened for full loss of both BRIP1 alleles using PCR approach (PCR primer set flanking 50 end (PCR#78: TTCCATTGGATGCGAAGT, PCR#79: CGCTCAAGGGAGG-TAAGGATAG), and one primer set flanking the full-length gene (PCR#78 + PCR#43, CCACAACACGTGGGATT). Successful K/O clones were validated by WB. U2OS FANCJ K/O and 293T FANCJ K/O cells were generated and maintained as previously described268.

Chemicals
The following drugs were used in the course of this study: PARP inhibitor olaparib (AZD-2281, SelleckChem), cisplatin (Sigma-Aldrich), camptothecin (Sigma-Aldrich), ATR inhibitor (VE-821, SelleckChem), PARG inhibitor (Tocris, PDD 0017273; 5952), Emetine (MCE, HY-B1479B), Methyl methansulfonate (MMS, Sigma-Aldrich 129925), RPA inhibitor (NERx Biosciences, NERx-329), FEN1 inhibitor (LNT1, Tocris 6510), Ligase I/III/IV inhibitor (L189, MedChemExpress HY-15588), Z-VAD-FMK (SelleckChem S7023), Emricasan (SelleckChem S7775). Reagents including 5-chloro-2′-deoxyuridine (CldU) and 5-iodo-2′-deoxyuridine (IdU) were obtained from Sigma-Aldrich. Concentration and duration of treatment are indicated in the corresponding figures and sections.

Immunoblotting and antibodies

Cells were harvested, lysed and processed for western blot analysis as described previously using 150mM NETN lysis buffer (20mM Tris (pH 8.0), 150mM NaCl, 1mM EDTA, 0.5% NP-40, 1mM phenylmethylsulfonyl fluoride, 10mg/ml leupeptin, 10mg/ml aprotinin). Chromatin fractionation was performed by subjecting cells to extraction with 0.1% Triton X-100 followed by 10min sonication (medium intensity for 30s on /30s off by Diagenode bioruptor) in RIPA buffer (Cold Spring Harbor Protocol). Proteins were separated using SDS–PAGE and electro-transferred to nitrocellulose membranes. Membranes were blocked in 5% not fat dry milk (NFDM) phosphate-buffered saline (PBS)/Tween 20 and incubated with primary antibodies for overnight at 4°C. Antibodies for western blot analysis included anti-β-actin (Sigma A5441), anti-
FANCJ (E67), anti-BRCA1 (Cell Signaling Technology 9010), anti-p21 (Cell Signaling Technology 2947), anti-CHD4 (Abcam ab70469), anti-BRCA2 (Abcam ab123491), anti-53BP1 (Novus Biological NB100-304), anti-RADX (CXorf57, Abcam ab228707), anti-RPA70/RPA1 (Cell Signaling Technology 2267), anti-RPA32/RPA2 (Abcam ab2175), anti-FEN1 (Abcam ab109132), anti-PARP1 (Abcam ab227244), anti-XRCC1 (Abcam ab134056), anti-LIG1 (Santa Cruz sc-271678), anti-LIG3 (GeneTex GTX70143) and anti-H2B (Cell Signaling Technology 8135), anti-Cleaved Caspase-3 (Cell Signaling Technology 9661) and anti-PARP (Cleaved PARP1, Cell Signaling Technology 9542). Membranes were washed, incubated with corresponding horseradish peroxidase-linked secondary antibodies (Amersham, GE Healthcare for rabbit; Thermo Fisher Scientific for mouse) for 1h at room temperature (RT) and detected by chemiluminescence imaging system (Bio-Rad).

Plasmids and RNA interference

cDNAs for human RPA subunits (superRPA) were generated by Toledo lab^{333}. Plasmid transfections were performed with X-tremeGENE HP DNA Transfection Reagent (Roche 6366244001). U2OS cells were reverse transfected using RNAi-MAX transfection reagent (Life Technologies 13778150) and siRNA targeting FANCJ/BRIP1 (QIAGEN SI03110723), BRCA1 (QIAGEN SI00299495), or scrambled negative control (ORIGENE SR30004) in 6-well plates for 48 h before super-resolution microscopy analysis. RPE1 cells were reverse transfected using Lipofectamine RNAiMAX (Invitrogen P/N 56532) with siRNA targeting RPA1 (Dharmacon D-015749-04-0005). Stably transduced
cells were generated by infection with pLKO.1 vectors containing shRNAs against non-silencing control (NSC) or one of the shRNAs against corresponding genes:

- p21(CDKN1A) includes (A) 5′-TAAGGCAGAAGATGAGCG-3′, (B) 5′-AAAGTCGAAGTTCCATCGCTC-3′;
- 53BP1 (TP53BP1) includes (A) 5′-AAACCAGTAAGACCAAGTATC-3′, (B) 5′-AATCAATACTAATCAGCCTG-3′;
- CHD4 includes 5′-AATTCATAGGATGTCAGCAG-3′;
- RADX (CXorf57) includes 5′-ATTTCAGAATACATCTTCAG-3′;
- FEN1 includes 5′-TACTCTCAGTACAGTCCAGTCC-3′;
- LIG1 includes 5′-TTCACGGACTCGAATAAACCG-3′;
- LIG3 includes 5′-AATGTAGTCCTTAAAGTGGGC-3′.

The information was obtained from Dharmaco website (https://horizondiscovery.com), and the shRNAs were obtained from the University of Massachusetts Medical School (UMMS) shRNA core facility. Cells were selected by puromycin for 3-5 days before experiments were carried out.

**Immunofluorescence and microscopy**

**Single-stranded DNA (ssDNA)**

Cells were grown on coverslips in 10 μM CldU for 48 h before released into the indicated treatment in figures without CldU. After treatment, cells were washed with PBS and pre-extracted by 0.5% Triton X-100 made in phosphate-buffered saline (PBS) on ice. Cells were then fixed using 4% formaldehyde for 15 min at room temperature (RT), and then permeabilized by 0.5% Triton X-100 in PBS
again. Permeabilized cells were then incubated with primary antibodies against CldU (Abcam 6326) at 37°C for 1h. Cells were washed and incubated with secondary antibodies (Alexa Fluor 594) for 1h at RT. EdU labeling was performed using Click-iT EdU Alexa Fluor 488 Imaging Kit (Invitrogen, C10337) according to the manufacturer’s instructions. After washing, coverslips were mounted onto glass slides using VECTASHIELD mounting medium containing DAPI (Vector Laboratories).

*Poly(ADP-ribose)*

Cells cultured on coverslips were fixed with 4% formaldehyde in PBS for 10 min at RT and subsequently permeabilized by a 5 min incubation in ice-cold methanol/aceton solution (1:1). After blocking the cells with 10% fetal calf serum for 30 min (alternatively add 3% BSA), coverslips were incubated with the primary antibody (anti-PAR Polyclonal antibody, Trevigen 4336-BPC-100) at 37°C for 1h. Followed by PBS washing, cells were then incubated with the appropriate fluorescently labeled secondary antibody for 1h at RT. EdU labeling was performed as above. Coverslips were then washed, stained with DAPI (1 μg/ml in PBS, 30 min) and mounted using VECTASHIELD as above.

*Chromatin bound proteins*

Cells on coverslips were plated on ice for 0.5-1 min before pre-extracted by ice-cold PBS+0.5% Triton for 5 min. Then, cells were fixed by 3% paraformaldehyde/2% sucrose for 10 minutes at RT. Cells were washed twice with PBS-T (0.01% Tween) and incubated with primary antibodies (RPA70/RPA1 Ab, Cell Signaling Technology #2267; anti-phospho-Histone-H2A.X/γ-H2AX Ser139, Millipore 05-636 clone JBW301; anti-PARP1, Abcam
ab227244; anti-XRCC1, Abcam ab134056; anti-53BP1, Novus Biological, NB100-304) in filtered DMEM + 10% FBS (alternatively add 3% BSA) at 37°C for 1h. After 3x PBS-T washing, coverslips were incubated with appropriate secondary antibodies (in DMEM + 10% FBS) and DAPI. Finally, after washing with PBS-T (x3), coverslips were mounted with Prolong (Invitrogen, P36930). For all assays above, images were collected by fluorescence microscopy (Axioplan 2 imaging and Axio Observer, Zeiss) at a constant exposure time in each experiment. Representative images were processed by ImageJ software. Mean intensity of immunofluorescence for each nucleus were measured with Cell Profiler software version 3.1.5 from Broad Institute.

**DNA fiber assay and S1 nuclease analysis**

These assays were performed similarly as previously described in Chapter II²⁶⁸. Cells were labeled by sequential incorporation of two different nucleoside analogs, IdU and CldU, into nascent DNA strands for the indicated time and conditions. After nucleoside analogs were incorporated in vivo, the cells were collected, washed, spotted, and lysed on positively charged microscope slides by 7.5 µL spreading buffer for 8 min at room temperature. For experiments with the ssDNA-specific endonuclease S1, after the CldU pulse, cells were treated with CSK100 buffer for 10 min at room temperature, then incubated with S1 nuclease buffer with or without 20 U/mL S1 nuclease (Invitrogen, 18001-016) for 30 min at 37°C. The cells were then scraped in PBS + 0.1% BSA and centrifuged at 7,000 rpm for 5 min at 4°C. Cell pellets were resuspended at 1,500 cells/mL and lysed with lysis solution on slides. Individual DNA fibers
were released and spread by tilting the slides at 45 degrees. After air-drying, fibers were fixed by 3:1 methanol/acetic acid at room temperature for 3 min. After air-drying again, fibers were rehydrated in PBS, denatured with 2.5 M HCl for 30 min, washed with PBS, and blocked with blocking buffer (PBS + 0.1% Triton + 3%BSA) for 1 hr. Next, slides were incubated for 2.5 hr with primary antibodies for (IdU, Becton Dickinson 347580; CldU, Abcam 6326) diluted in blocking buffer, washed several times in PBS, and then incubated with secondary antibodies (IdU, goat anti-mouse, Alexa Fluor 488; CldU, goat anti-rat, Alexa Fluor 594) in blocking buffer for 1 hr. After washing and air-drying, slides were mounted with Prolong (Invitrogen, P36930). Finally, visualization of green and/or red signals (measure at least 100 fibers for each experiment) by fluorescence microscopy (Axioplan 2 imaging, Zeiss) provided information about the active replication directionality at the single-molecule level.

Viability assays
For cell survival assays, cells were seeded onto 96-well plates (300-500 cells per well, performed in biological triplicates for each experiment group) and incubated overnight. For patient fibroblasts, cells are seeded onto 6-well plates (5,000 cells per well). Clonogenic assays as mentioned were seeded onto 6-cm dishes (500-800 cells per dish) and performed at least in three biological independent experiments for each group. The next day, cells were treated with increasing doses of drugs as indicated in corresponding figures and maintained in complete media for 5 to 7 days for 96-well, 10-12 days for clonogenic assay. Camptothecin (CPT) and methyl methanesulfonate (MMS) were treated for 1
hour and then replaced by fresh complete media. Percentage survival was measured either photometrically using a CellTiter-Glo 2.0 viability assay (Promega) or CCK-8 (Dojindo) in a microplate reader (Beckman Coulter DTX 880 Multimode Detector) or by manual cell counting after methanol/0.5% crystal violet staining.

**STORM analysis**

For super resolution imaging experiments, cells were trypsinized and seeded on glass coverslips in six-well plates in low density. siRNA transfection, drug treatment, and EdU incorporation were performed directly on cells on coverslips. We used an optimized pre-extraction and fixation protocol for our immunofluorescence experiments in order to clearly visualize chromatin bound nuclear fraction of cells and to minimize nonspecific antibody labeling from the cytoplasm and non-chromatin bound proteins that could significantly increase noise for image analysis. Cells were permeabilized with 0.5% Triton X-100 in ice-cold CSK buffer (10 mM HEPES, 300 mM Sucrose, 100 mM NaCl, 3 mM MgCl₂, and 0.5% Triton X-100, pH = 7.4) in RT for 10 minutes and fixed with 4% paraformaldehyde (Electron Microscopy Sciences 15714) in RT for 30 minutes. Following fixation, cells were washed twice with PBS and blocked with blocking buffer (2% glycine, 2% BSA, 0.2% gelatin, and 50 mM NH₄Cl in PBS). For nascent DNA detection, cells were pulse-labeled with 10 µM EdU (ThermoFisher A10044), a thymidine analog, 15 minutes before permeabilization and fixation so that it would be incorporated into nascent DNA during replication in S phase cells. After fixation, EdU was tagged with Alexa
Fluor 647 picolyl azide through click reaction (Click-iT chemistry, ThermoFisher, C10640). The cells were blocked with blocking buffer at least overnight at 4°C. Before imaging, the samples were stained with primary antibodies against rb-RPA70 (Abcam ab79398), rb-MCM6 (conjugated to AF568, Abcam ab211916), and ms-PCNA (Santa cruz sc-56) in blocking buffer for 1h at RT, then secondary antibodies (goat anti-mouse AF488, Invitrogen A11029; goat anti-rabbit AF 750, Invitrogen A21039) in blocking buffer for 30 minutes at RT. Super resolution imaging and other related processes were described before.\textsuperscript{369}

\textit{PDX studies}

Triple-negative breast cancer patient-derived xenograft (PDX), PNX017, was from a patient with a hemizygous germline BRCA1 mutation (1105_1106insTC); the wild-type BRCA1 allele was lost in the tumor, following a Loss of Heterozygosity model. PNX017 was derived at Fox Chase Cancer Center under IRB and IACUC approved protocols. PDX tumors were grown in NOD.Cg-Prkdc\textsuperscript{scid}Il2rg\textsuperscript{tm1Wjl}/SzJ (NSG) mice. Resistant tumors were obtained from mice after sensitive tumors progressed on serial treatments of PARP inhibitors (rucaparib, 150mg/kg, 2x daily, 5 days, 2 days off, 5 days). The tumors were harvested at approximately 500 mm\textsuperscript{3} and dissociated in 0.2% collagenase, 0.33 mg/ml dispase solution for 3h at 37°C. The dissociated cells were maintained at 37°C in RPMI1640 + 10% FBS and used for DNA fiber assays within 24h of tumor extraction. DNA fiber assay and S1 nuclease analysis were performed as described above.
**DNA pulsed field capillary electrophoresis**

BRCA1 K/O RPE1 cells were assayed for DSBs by pulsed field capillary electrophoresis with the Agilent Femto Pulse systems as previously described. Briefly, 200,000 cells were plated per 10 cm plate and allowed to adhere overnight. Subsequently, the media was aspirated and treated with appropriate volume of complete medium containing 50 µM olaparib in order to match the molar ratio of drug to cells found in the 96-well CellTiter-Glo assay; cells treated with apoptotic inhibitor were pretreated for 2h with 50 µM Emricasan (SelleckChem, S7775) and maintained for the duration of the experiment, and untreated cells received DMSO as control. At 96h, all attached and floated cells were collected, then washed, and high molecular weight genomic DNA (gDNA) was isolated with the FiberPrep Genomic DNA Extraction Kit (Genomic Vision, EXT-001) according to the manufacturer’s instructions; the final digestion step was supplemented with an additional 5 µL of beta-agarase to ensure full digestion of the agarose plug (New England Biolabs, M0392). Genomic DNA was analyzed on the Femto Pulse in the 3h mode for gDNA and large fragments according to the manufacturer’s instructions. The retention time of gDNA and DNA fragments was experimentally determined (intact gDNA observed as signal above 8,000 s of capillary retention time, and DNA fragmentation observed in the 3,000 s to 8,000 s window of capillary retention time). The percentages of signal from fragments and genomic DNA were measured by ProSize Data Analysis Software and plotted by Graph Pad Prism.
Quantification and statistical analysis

Statistical differences in DNA fiber assays and immunofluorescence intensity were determined by nonparametric Kruskal-Wallis test followed by Dunn’s test for multiple comparisons in non-Gaussian populations. Two group comparisons were determined using two-tailed Mann-Whitney test. Statistical differences in viability assays with small sample sizes were determined by unpaired t test. Statistical analysis was performed using GraphPad Prism (Version 7.0). In all cases, ns: not significant (p > 0.05), *p < 0.05, **p < 0.01, ***p < 0.001, and ****p < 0.0001.

Data and code availability

Unprocessed blots, gels, and microscopy images are available on Mendeley Data at https://doi.org/10.17632/g96fktpb58.1. Raw Data for STORM analysis will be made available by the corresponding author upon request.

3.6 Acknowledgements

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CHAPTER IV

Summary, discussion and future directions
4.1 Summary of results and conclusions

Disruptions to DNA replication cause replication stress, which slows the cell cycle and induces a complex response. This replication stress response protects genome integrity while also promoting the recovery of replication. The response is often disrupted in cancer upon loss of the hereditary breast cancer genes *BRCA1* and *BRCA2* (BRCA or BRCA1/2), which are also mutated in Fanconi anemia (FA), a rare genomic instability disease. Although the BRCA proteins have multifaceted roles, functions are largely undefined for the BRCA1-associated DNA helicase FANCJ in the replication stress response. Additionally, how FANCJ deficiency translates to hereditary breast/ovarian cancer and FA remains poorly understood. Therefore, the first goal of my thesis was to provide new insights towards the specific functions of FANCJ at replication fork in response to replication stress.

In the study of Chapter II, replisome proteins were analyzed in the presence or absence of FANCJ through iPOND and quantitative proteomics. Particularly, fork remodeler HLTF accumulated aberrantly at the fork in FANCJ-deficient cells. Following HU-induced replication stress, fork degradation was facilitated by HLTF in FANCJ knockout cells. Consistent with the roles of BRCA proteins, FANCJ protected replication forks by counteracting HLTF-mediated fork reversal. Interestingly, the unrestrained fork elongation caused by HLTF deficiency also depended on FANCJ and is associated with ssDNA gaps. The cells deficient in either one or both factors showed distinct responses to MMC and HU. Generally, these outcomes implied that FANCJ and HLTF play
opposing roles in maintaining fork integrity, restraining DNA synthesis, and coordinating replication stress response.

The study of ssDNA gaps and replication stress response also suggests the investigation to another important family of proteins, PARPs, which are specifically targeted for synthetic lethality in BRCA-FA mutant tumors. Unfortunately, despite the development of PARPi as a promising clinical intervention, resistance eventually develops. Although BRCA-mediated HR and fork protection are rationalized to be the essential mediators of the response, this break repair function does not correlate thoroughly with the outcomes of PARPi treatment. Recently, evidence from our work suggests that dysregulated fork dynamics could lead to ssDNA gaps that sensitize cells. In Chapter II, we have shown that unrestrained replication due to a fork remodeling defect generates ssDNA gaps. Since BRCA1/2 and PARP1 also restrict replication-associated ssDNA gaps, such essential lesions have been taken into considerations as the cause of chemotherapy sensitivity in BRCA-deficient cells. Thus, the second goal of my thesis was to explore the role of ssDNA gaps in driving the synthetic lethality between PARPi and BRCA deficiency.

Based on the research of Chapter III, PARPi could cause accelerated replication associated with ssDNA gaps that are more abundant in BRCA-deficient cells and tumors. In contrast, replication gaps induced by PARPi were suppressed in PARPi-resistant FANCJ-deficient cells. Similarly, replication gaps were restricted in either de novo or engineered cells that showed PARPi resistance in BRCA1 deficiency. Regardless of HR and fork stability status, cells with different genetic background consistently illustrated that gap
accumulation predicted PARPi sensitivity and gap suppression predicted resistance. To separate fork acceleration from gaps, we also indicated that PARPi-induced hyper-lengthened forks in p21-deficient cells did not form gaps or cause PARPi sensitivity. Moreover, manipulating the level of ssDNA binding protein RPA could either augment or rescue PARPi sensitivity in BRCA1-deficient cells, further suggesting gaps as the determinant factor. Collectively, these results disclose that replication gaps correlate with PARPi response more directly than HR deficiency or fork degradation.

To explore the source of replication gaps, we found that similar to PARP1, BRCA1 functioned in Okazaki fragment processing (OFP). Cells deficient in BRCA1 resemble cells with excessive PARP1 “trapping” after PARPi treatment. Correspondingly, PAR was elevated unless lagging strand synthesis was inhibited. Despite high PARP activity, chromatin bound XRCC1 and LIG3 were diminished, suggesting that defects in backup lagging strand processing caused gaps in BRCA1 deficiency. Strikingly, 53BP1 loss that restores HR and confers PARPi resistance in BRCA1-deficient cells, mediated a switch to alternative OFP with XRCC1 and LIG3. Further linking lagging strand gaps to PARPi response, such defects of OFP in BRCA1-deficient cell lines and tumors were also reversed upon PARPi resistance along with reduced PAR levels. Together, our data indicate that BRCA1 functions in a non-redundant manner with PARP1 in OFP; loss of these functions could underlie synthetic lethality; and restoring OFP in BRCA1-deficient cells by 53BP1 loss could confer PARPi resistance in tumors.
In summary, according to all the findings above, we propose that the alteration of replication gaps is regulated distinctly by FANCJ and BRCA1/2 upon various replication stress. Moreover, we suggest that replication gaps from lagging strand act as the fundamental cause of synthetic lethality between PARPi and BRCA deficiency. We believe this unprecedented understanding of BRCA-FA pathway in the context of a gap repair model will shed light on FA pathogenesis and cancer prevention and provide insights to discover novel potential therapeutic targets. Our work will bring broader implications into the DNA repair field.

4.2 Final perspectives and concerns

4.2.1 Limitations of the replication gap study

In spite of various results above supporting our hypothesis of replication gaps, we indeed note that there are limitations that potentially weaken our conclusions. Below, we highlight several restricted aspects of this study and point out alternative experiments for further exploration.

First, DSBs could still come from ssDNA conversion and function as a contributing factor to sensitivity, even though HR-proficient cells could be sensitive to PARPi and our DSB-related results showed that PARPi-induced DSBs derive largely from apoptosis (Figure 3.10). Accordingly, a recent report indicates that PARPi generates DSBs from ssDNA gaps following multiple cell cycles. Thus, it is significant to confirm whether DSB formation is causative or rather derives from cell death. In addition to using genotoxins and apoptosis inhibitors specifically, we should also monitor the DSB formation following
different dose and time courses of PARPi or other genotoxin treatments. PARPis with various potency of PARP-trapping should be considered as well\textsuperscript{371}. Further assays are needed to determine the detailed characterization of DSB induction, such as time interval, abundance and possible molecular markers. In terms of examining breaks in cells, one basic method for quantifying DNA single- or double-stranded breaks could be comet assay\textsuperscript{372,373}, as we used in Chapter II. This alternative experiment recognizes the head of the comet as a spherical mass of undamaged DNA, and the damaged DNA or loops around breaks stream out from the head as a tail in gel electrophoresis. Moreover, to verify the apoptotic DSBs, we could employ the apoptosis-detecting TUNEL (terminal deoxynucleotidyl transferase dUTP nick end labeling) assay\textsuperscript{374} together with \textit{in situ} ligation of tags eliminating the DNA damage from other situations\textsuperscript{375}. This combined approach might assist us to identify the source of DNA fragmentation more unambiguously.

Next, it is also necessary to upgrade the assays that detect ssDNA gaps. Technique limitations still exist in differentiation between ssDNA and DSBs. As a result, researchers often overlook the possibility that ssDNA gap by itself could be a direct genotoxic lesion induced by PARPi or other chemotherapy. An example of such experimental limitations is found in the fact that ssDNA gaps might be detectable only as a very early read-out or within a limited time window. Often, \textit{in vitro} studies with different drugs are done over prolonged period of time such that initial ssDNA gaps are converted to DSBs. The dynamic processing of replication intermediates also challenges the comprehensive characterization of ssDNA gaps. A snapshot of ssDNA gaps can be achieved
by EM, which can “freeze” replicating forks and allow the direct visualization of ssDNA on normal and reversed forks\textsuperscript{121}. EM is capable of further differentiating whether certain genotoxicity reflects the number, size, position and persistence of replication gaps, and achieve a more thorough view of gap formation when combined with DNA fiber analysis for fork dynamics. Moreover, the fiber assay can also be employed to measure the lengths of replication gaps. In particular, they can be measured by single molecule analysis of resection tracks (SMART) assay\textsuperscript{376-378}. This method harnesses the exposed epitope of pre-pulsed thymidine analogue in DNA without denaturation to detect ssDNA gaps and/or resection. To improve the application of this experiment, it will be helpful to develop more specific ssDNA antibodies in the future.

Additionally, exploring the details of ssDNA gaps also depends on cutting-edge technologies that emerge recently. For instance, a novel next-generation sequencing tool that captures ssDNA breaks through genome-wide ligation of 3′-hydroxy ends followed by sequencing (GLOE-Seq) could map Okazaki fragments and the distribution of ssDNA gaps\textsuperscript{379}. Possibly, the combination with other DSB sequencing techniques will reveal more biases and asymmetries when comparing ssDNA break and DSB\textsuperscript{380}. Furthermore, other than light microscopy and EM, molecular combing could also be directly visualized by atomic force microscopy\textsuperscript{381}. This method is easily prepared and determines DNA surface topography, which is even able to examine single nucleotide mismatches and ssDNA binding proteins\textsuperscript{382,383}.

Ideally, all these approaches for ssDNA gaps will allow us to better define such critical intermediates of replication and repair. Future development of
additional reliable tools will facilitate the detection of gap threshold underlying PARPi and other genotoxin toxicity from bench to bedside.

4.2.2 Roles of FANCJ in mediating PARPi sensitivity

In this dissertation, the direct comparison between isogenic cells deficient in the hereditary breast/ovarian cancer genes, FANCJ or BRCA1, with common defects in HR and FP, highlights gap formation as a distinguishing factor to PARPi-sensitive or -resistant cells. Since OFP defects act as the source of gaps in BRCA-RAD51 deficiency as showed in Chapter III, the underlying mechanisms about how FANCJ mediates toxicity and regulates lagging strand synthesis await further investigation.

One possible explanation is that FANCJ facilitates the exposure of replication gaps. As previously found, FANCJ could maintain replication despite stress by unwinding aberrant duplex DNA regions or secondary DNA structures such as guanine-quadruplex (G4) structures that interfere with fork progression. In FANCJ-deficient cells, G4s are worth considering as the mediator of gap suppression due to the requirement of FANCJ for stabilizing and unfolding these structures. As the fork remodeler HLTF enriches at fork with FANCJ deficiency (Chapter II), there is a propensity for the replication barriers that trigger fork reversal to form. Indeed, it remains unknown about whether FANCJ interacts with HLTF or how FANCJ regulates HLTF and even other factors for replication fork reversal. Nevertheless, G4s and/or other endogenous causes of fork remodeling in FANCJ deficiency could enhance the frequency of difficult-to-replicate sequences that then require HLTF-mediated
fork reversal to resume replication. Disrupted fork progression consequently restricts the formation of exposed gaps. In addition, PARPi further disturbs the fork dynamics in FANCJ-deficient cells. Accordingly, G4s may not be properly stabilized or accessed for re-priming, and replication may not reprime or form gaps in response to PARPi. Correspondingly, if G4s accumulate at parental DNA of lagging strands\textsuperscript{384}, FANCJ loss could exacerbate the absence of gaps and highly restrain fork progression. Therefore, the PARPi toxicity is likely avoided or at least suppressed in FANCJ-deficient cells.

However, what is the mechanism that potentially supports this hypothesis? According to our previous findings illustrating how FANCJ responds to replication stress and ICL lesion\textsuperscript{143}, the lagging strand synthesis might rely on FANCJ and its interaction with the MMR protein MLH1. Perhaps when mobilized by MLH1, FANCJ displaces upstream MMR complexes that would otherwise “hide” ssDNA into secondary structures such as G4s. Notably, the exposed ssDNA is fundamental for PARP1 activation that coordinates the backup OFP and the restart of stalled replication forks. Consistent with this function, cells lacking FANCJ or its MLH1 interaction, might have a dramatic reduction in backup lagging strand synthesis and replication restart following stress. Thus, secondary structures at lagging strand could be an important source of replication stress, and the exposure of ssDNA here may require FANCJ and its MLH1 interaction.
4.2.3 BRCAness reconsideration: DSBs or ssDNA gaps

To explore the distinct roles of replication ssDNA gaps, we also utilized multiple separation-of-function models. Additional experiments should be investigated for whether there are residual HR function or repair defects that cannot be detected. However, depending on the evidence of PARPi response in our gap study, one key question needs to be reconsidered is that: what is the essential DNA lesion that underlies the BRCA defects?

For decades, the toxicity and repair of DSBs has been at the forefront of cancer biology. This focus is mainly based on the identification of DSB repair (DSBR) function for the hereditary breast cancer genes BRCA1 and BRCA2. BRCA proteins are required for faithfully fixing DSBs by RAD51-dependent HR. Correspondingly, tumors deficient in the BRCA genes are highly sensitive to genotoxins commonly thought to generate DSBs such as PARPi and other chemotherapy agents. Bringing the DSBR model of chemotherapy response full circle, chemoresistance is associated with restored HR, such as through BRCA reversion mutations. The essential roles of BRCA proteins including RAD51 in proliferation was thought to stem from DSBs being induced during cellular metabolism. Failure to repair endogenous DSBs could explain the development of tumor and FA associated with bone marrow failure and other developmental defects. Expanded roles for BRCA proteins in DNA replication and overcoming replication stress also maintained the centrality of DSBs and DSBR. The BRCA proteins protect nascent DNA from degradation by nucleases such as MRE11 when DNA replication forks stall, and therefore limit forks from collapsing into DSBs. Collectively, roles in repairing and preventing
DSBs supports the framework that DSBs are the toxic lesions sensitizing cells deficient in the BRCA and other FA genes. Accordingly, BRCA-mutant tumors and -deficient cell lines share similar molecular characteristics, which are termed “BRCAness”. However, our recent results call the framework of BRCAness linked to DSBR into question\textsuperscript{240,385}. Interestingly, we highlight a function that could single-handedly counter the effectiveness of chemotherapy distinct from the DSB prevention and repair of the BRCA-FA pathway.

Notably, when replication forks are under stress, BRCA1, BRCA2, RAD51 along with other FA proteins restrain DNA replication and prevent ssDNA gap formation\textsuperscript{240}. Replication gaps also develop following treatment of cells with replication stress inducing drugs such as PARPi or cisplatin. These gaps are found in genomic regions different from DSBs or stalled forks and instead exist in the wake of DNA replication forks as replication “jumps”, reprimes or transverses around replication blocks. Together with the findings in this dissertation, we further demonstrated this BRCA-RAD51 ssDNA gap suppression function and provided evidence of a paradigm changing framework that ssDNA gaps are the toxic lesion induced by genotoxins that sensitize cells deficient in BRCA genes.

Given that gap accumulation more accurately predicts the response of genotoxins compared to DSB dogma, the ssDNA gap model should alternatively be considered for genotoxic chemotherapy and even “BRCAness”. For example, the failure to promote continuous DNA replication and mobilize subsequent gap filling pathways could trigger many cellular perturbations, such as the under-replication, G2/M checkpoint arrest, micronuclei and immune
response associated with BRCA-deficiency. Conceivably, these ssDNA lesions could underlie and exacerbate the failure of rapidly replicating bone marrow in FA patients. Recently, a study of early steps in tumorigenesis also indicates that patient cells with BRCA2 haploinsufficiency display a deregulated checkpoint during replication stress. This might phenocopy our findings about unrestricted fork progression interspersed by ssDNA gap forming. Along with the elevated DNA damage and attenuated apoptosis there, we speculate that the accumulation and tolerization of ssDNA gaps may act as an initiating event that ultimately destabilizes the genome upon BRCA deficiency in precancerous tissue. In fact, BRCA proteins repairs daughter-strand gaps induced by metabolic assaults, further implicating replication gaps as the underlying lesions in BRCA-mutant cancer.

Therefore, our ssDNA gap framework not only challenges the classical DSB model, but also proposes that ssDNA gaps are the fundamental biomarker of BRCAness. Conceivably, combined gap-inducing chemotherapies will provide the most effective treatment in BRCA-mutant cancer.

4.2.4 Mechanistic implications for PARPi resistance in BRCA1/53BP1 double deficiency

Theoretically, the vulnerability of BRCA1 deficiency to PARPi-induced cell death has been attributed largely to HR defects. However, our results demonstrate that synthetic lethality between BRCA1 deficiency and PARPi could instead be linked to OFP defects (Chapter III). Interestingly, we reveal that the rescue of BRCA1 deficient cells by 53BP1 loss restores OFP by a
compensatory pathway mediated by LIG3 and XRCC1. Such evidence indicates that reevaluation is necessary to assess the mechanism of the PARPi resistance in this HR-restored situation.

Our findings suggest that in BRCA1-deficient cells, canonical OFP factors such as LIG1 and/or FEN1 are disrupted during normal DNA replication, leading to impaired Okazaki fragment joining. Consequently, PARP1 is activated in an attempt to correct the lagging strand problem. PARP1 appears trapped in chromatin and PAR levels are elevated. However, this PARP1 rescue plan is foiled by the aberrant accumulation of 53BP1 in BRCA1-deficient cells. As such, deletion of 53BP1 serves to resuscitate OFP. Along these lines, 53BP1 loss enables PARP1-dependent poly-ADP ribosylation (PARylation) to mediate LIG3-XRCC1 chromatin recruitment. Collectively, our data indicate that BRCA1 functions in a non-redundant manner with PARP1 in OFP so that BRCA1 deficiency heightens dependence on OFP factors such as PARP1 or LIG1. Besides, this work may implicate that without BRCA1, PARPi resistance as well as the embryonic viability requires restored OFP.

Nevertheless, one of conundrums lies in revealing details of the underlying mechanism for how BRCA1 and 53BP1 impact OFP and downstream pathways. The switch to alternative OFP by 53BP1 loss is likely downstream of PARP activation by unligated Okazaki fragments. Since PAR is already elevated in BRCA1-deficient cells (Figure 3.12), subsequent PAR signaling steps for recruiting XRCC1 is probably disturbed. Conceivably, 53BP1 might bind PARylated proteins that would otherwise recruit XRCC1. There is a chance that 53BP1 blocks LIG3-XRCC1 chromatin association perhaps because 53BP1
binds histones\textsuperscript{199,388}. For example, Histone H3 was recently found to be a key substrate for ADP-ribosylation by PARP1 and histone PARylation factor 1 (HPF1), when LIG1 is deficient. Consistent with OFP pathway being relevant, the depletion of PARP1 or HPF1 attenuated LIG3-XRCC1 function in backup joining of Okazaki fragments\textsuperscript{346}. Thus, we venture to propose that in BRCA1 deficient cells, the backup OFP could be reactivated through histone recruitment upon 53BP1 loss.

The DSB formation should also be revisited in this scenario. Indeed, latest evidence does not show immediate formation of DSBs in PARPi re-sensitization of BRCA1 and 53BP1 dual-deficiency by LIG3 depletion\textsuperscript{347}. This study implies that although the HR gets restored, it might not be a major contributor to resistant mechanisms. In other words, BRCA1 and 53BP1 double deficient cells might depend more on gap suppression to prevent breaks rather than HR to repair damage-induced breaks. For further investigation of this hypothesis, directly perturbing HR in this BRCA1/53BP1 double deficient background could provide more insights.

4.3 Future directions: exploiting lagging strand gaps for synthetic lethality

Features that distinguish cancer from non-cancer cells allow for selective targeting. Identifying common vulnerability and biomarkers shared by cancer will guide the development of targeted therapy. If “BRCAness” arises from gaps in aberrant lagging strand DNA synthesis rather than a defect in DSB repair, the potential of PARPi targeting may expand to a larger range of vulnerable tumors\textsuperscript{389}. Similarly, if restored HR shows minimal importance in known PARPi-
resistant models, novel strategies to overcome such resistance could rely on modulating OFP factors as we discovered above.

Indeed, more than challenging conventional understandings, our findings of gap model further provide guidance on developing treatments that will act synergistically with PARPi. Gaps from discontinuous lagging strand, as activators recruiting PARP1 and downstream repair proteins, could be increased to potentiate PARPi cytotoxicity. It is of note that PARPi shows clinical benefit for ovarian cancer patients beyond those exhibiting BRCA deficiency; and such evidence has extended the applications of PARPi regardless of HR status\textsuperscript{368}. Compared to other PARPi-based combination treatments, novel strategies that utilize this newly reframed mechanism of action about how PARPi works might provide unprecedented improvements in patient response and prognosis. Therefore, it is significant to consider exploiting these unligated lagging strand gaps for augmenting synthetic lethality. This could be combined therapies that synergize with PARPi or enhance the gap vulnerability to re-sensitize PARPi resistant cells. Here, we will review and discuss recent rationales about the PARP activity and PARPi in lagging strand synthesis with respect to their mechanisms, current available targets, predictive biomarkers of response and resistance, and combinational strategies discovered or waiting to unfold.
4.3.1 Pushing replication stress to the limit by gap accumulation at lagging strand

During undisturbed replication, forks display relatively little ssDNA or do so rapidly with a lagging strand bias due to unligated Okazaki fragments. The size of most daughter-strand gaps suggests that they derives from unfinished Okazaki fragments\textsuperscript{22}. Under replication stress, it is also probable that ssDNA gaps accumulate more on lagging strand. Conceivably, this notion leads to the speculation that OFP is prevented by these lesions. Given the physiological occurrence of gaps at discontinuous lagging strand synthesis, ssDNA gaps in this area could thus be exacerbated and act as vulnerable targets for genotoxic chemotherapy.

Indeed, replication fork uncoupling induced by inhibition of POLA1 (the DNA primase subunit of the Pol α complex) generates ssDNA gaps on lagging strand\textsuperscript{390}. Massive ssDNA formation, a pathological hallmark of replication stress, can further exhaust the RPA pool and cause replication catastrophe\textsuperscript{333}. Besides, PARPi can induce ssDNA gaps, which recruit RPA binding on chromatin and protecting gaps. Our evidence demonstrated that PARPi increased chromatin bound RPA and induced enhanced synthetic lethality when RPA pool is depleted or inhibited in BRCA1-deficient cells\textsuperscript{297}. PARPi resistance can be induced by overexpressing RPA subunits in BRCA deficiency background (Figure 3.9), further emphasizing the importance of gap protection in PARPi resistance. Most interestingly, inhibition of apoptosis not only elevated the resistance of BRCA1-deficient cells, but also suppressed PARPi-induced DSBs (Figure 3.10). These results suggested that apoptosis could contribute to
the source of DSBs in BRCA1 deficiency. Possibly, cells undergoing apoptosis are committed to dying, so these DSBs are not causing their death. While gap accumulation beyond a threshold might more directly predicts cell fate in response to genotoxicity. Targeting Pol α, for instance, could be another candidate tool to kill or re-sensitize BRCA-mutant cancers.

Based on available evidence, it is reasonable to anticipate that designing drugs to harness gaps from lagging strand could augment PARPi synthetic lethality. In fact, there are clues showing that unligated Okazaki fragments lead to PARPi-sensitivity in BRCA deficiency. Cells expressing PCNA K164R are deficient in S phase PCNA mono-ubiquitination, resulting in perturbed Okazaki fragment maturation and an accumulation of gaps due to impaired PCNA unloading on lagging strand\(^ {311}\). This defective OFP also exhibits DNA2-mediated nascent DNA degradation, which expands the gaps in the lagging strand. This mutant with impaired OFP and diminished gap suppression render cells susceptible to BRCA1/2 deficiency and even more sensitive when combined with PARPi. Thus, direct disruption of lagging strand processing could generate ssDNA gaps at the lagging strand and increase the PARPi synthetic lethality in BRCA deficiency.

4.3.2 Targeting the canonical factors for Okazaki Fragment processing

Given the role of FEN1 in ensuring maturation of the Okazaki fragments during DNA replication, FEN1 inhibition leads to delayed processing and ligation of the lagging strand and consequently ssDNA gaps. In fact, loss of FEN1 function is synthetic lethal with BRCA deficiency\(^ {312-314}\), in line with what
we propose that PARPi synthetic lethality in BRCA deficiency may be explained by OFP defects. Specifically, stimulating PAR synthesis with the FEN1 inhibitor can recruit XRCC1 at sites of replication. It is in line with the function for PARP-dependent ssDNA break repair in OFP, as is the observation that FEN1 inhibitor causes hypersensitivity to PARP1/2- and/or XRCC1-deleted cells\textsuperscript{224}. Although clear or even opposite roles of FEN1 in BRCA1/2-mutated cancers remain to be elucidated\textsuperscript{391}, these studies support FEN1 as a potential drug target in cancer therapy. This direction underpins future discovery and development of novel FEN1 inhibitors with better pharmacokinetic and pharmacodynamic properties.

Other than FEN1, structure-specific nuclease DNA2 also play roles in removing the long 5′ RNA-DNA flaps that arise during OFP in difficult-to-replicate genomic regions\textsuperscript{392}. An effective and selective inhibitor of DNA2 can sensitize cells to DNA damage drugs and synergize with PARP inhibitors\textsuperscript{393}. It is conceivable that DNA2 inhibition could likewise kill BRCA1/2-deficient cancer cells.

Similarly, this idea of disrupting lagging strand synthesis coincides with the established sensitivity of LIG1-mutated cells to PARPi\textsuperscript{309,310}. To further target this major ligase in joining Okazaki fragments, Ligase I inhibitors show cytotoxicity by interfering with phosphodiester bond formation of the DNA ligation reaction\textsuperscript{394,395}. Moreover, such LIG1 inhibitors have also been found to sensitize BRCA2-deficient cancers, which again confirms the synthetic lethality by targeting lagging strand ligation\textsuperscript{396}. 
4.3.3 Preventing the backup pathway from taking place as therapeutic strategy

When discrete lagging strand synthesis exceeds the ability of canonical factors such as FEN1 and LIG1, the compensatory pathway via PARP and XRCC1 complex takes over to process and ligate the Okazaki fragments. To back up the major ligase, LIG3 has been suggested to be responsible for ligating Okazaki fragments alternatively when LIG1 is absent\textsuperscript{238,239}. LIG3 functions in repairing ssDNA breaks together with XRCC1 as a complex\textsuperscript{236,237}, which is recruited by PARP1. Conceivably, targeting the backup pathway could also potentiate the cytotoxicity induced by PARPi, or even re-sensitize those PARPi-resistant cells. Indeed, the inhibitor of ligase I and III not only increases the genotoxicity of DNA damaging agents at subtoxic concentrations\textsuperscript{394}, but also re-sensitizes therapy-resistant cancers synergistically with PARPi\textsuperscript{397}. In addition, Dias and colleagues identify LIG3 as a top target by genetic screen to revert the PARPi resistance with BRCA1 and 53BP1 dual deficiency\textsuperscript{347}. Indeed, this could be related to BER or other ssDNA break repair pathways, making it unclear if faulty processing of Okazaki fragment or other functions underlies the synthetic lethality. It is evident that LIG3 is a critical vulnerability because it mediates backup OFP. Supporting this, our results showed that LIG3 loss elevated PAR uniquely in BRCA1/53BP1 double-knockout cells (Figure 3.13), which exhibit resistance to drugs targeting canonical lagging strand synthesis. Collectively, impeding backup ligases could lead to compounded lagging strand gaps and assist PARPi in killing BRCA1/2-deficient cells.

Intriguingly, several other discoveries involved backup pathways further substantiate that PARPi toxicity could stem from ssDNA gap formation on
lagging strand. PARPi sensitizes cells deficient or mutated in OFP factors such as EXO1, XRCC1 and POLB, implying that combined replication gaps at lagging strand are insurmountable for cell death.

4.3.4 Other findings that implicate lagging strand defects and/or replication gaps for synthetic lethality

Most of the players in OFP also play roles in nucleotide or base excision repair (NER or BER) for ssDNA break, consistent with the assumption that faulty OFP may disrupt genome integrity and need repair pathways. In contrast, studies have revealed that HR deficiency is not necessary for clinical response with PARPi. Based on the complexity of these pathways, the source of PARPi synthetic lethality could be more than repair deficiency. Here, other implications for lagging strand defects and replication gaps are discussed.

In fact, PARPi hypersensitivity is observed with loss of genes having no corresponding defects in HR or fork protection. Cells deficient in ribonuclease H2 show PARPi hypersensitivity, which is caused by impaired ribonucleotide excision repair with topoisomerase cleavage and PARP trapping. Furthermore, it is worth considering that this synthetic lethality between RNASEH2 deletion and PARPi results from combined OFP defects, given that ribonuclease H2 also has the potential to remove RNA primers employed in lagging strand synthesis.

Fugger and colleagues found that inhibition of DNPH1, a protein that eliminates cytotoxic nucleotide hmdU, enhances the sensitivity of PARPi Olaparib. In the screen for Olaparib-sensitivity modulators, they also
uncovered OFP factors LIG3 and POLB, indicating that the lagging strand deficiency acts synergistically with PARPi sensitivity in HR-deficient background\textsuperscript{361}. Interestingly, HPF1 was among one of the top targets as well. Indeed, loss of HPF1 has been known to confer PARPi sensitivity\textsuperscript{399}, and it appears as top hit in another PARPi sensitivity screen\textsuperscript{355}. Most importantly, HPF1 is found to modulate the catalytic activity of PARP1 and promote backup pathway for OFP\textsuperscript{346,400}.

In the absence of BRCA1/2, another relevant pathway could be break-induced replication (BIR). BIR repairs single-ended DSBs and bypasses collapsed forks independently of HR. Accordingly, inactivation of RAD52, a core protein mediating BIR, has been found to be synthetic lethal with BRCA1/2-deficiency\textsuperscript{401,402}. Additionally, inhibiting PARP1 and RAD52 synergistically kill various BRCA-deficient tumors\textsuperscript{403}. Although researchers explain this phenomenon by the addiction to RAD52-dependent repair, another possibility is indicated by the fact that Rad52 in yeast functions in repairing incomplete lagging strand synthesis due to faulty OFP\textsuperscript{404}. Furthermore, RAD52 depletion in HR-intact cells also display PARP1 hyperactivation and sensitivity to PARPi\textsuperscript{243}, which further hint the roles of RAD52 in lagging strand synthesis.

Secondary DNA structure derived from the ssDNA by lagging strand defects provide targets to re-sensitize PARPi resistance. If the 5’ end of OFP is compromised, uncleaved flaps could form aberrant structure such as hairpins and G4 structures in DNA regions with repeated sequences. In the template lagging strand, if G4 structures embed between Okazaki fragments as replication barriers, there could be longer ssDNA gaps around the G4 sites. In
this scenario, G4 structures would induce greater replication stress and further disruptions to lagging strand synthesis\textsuperscript{384}. Targeting such G-rich structures in lagging-strand regions of telomeres, the G4 stabilizing ligand Pyridostatin (PDS) indeed kills cells with defective BRCA pathway and re-sensitize PARPi-resistant cells by exacerbating replication stress\textsuperscript{405}.

\textbf{Figure 4.1: Exploiting replication gaps for synthetic lethality.} (A) As opposed to making DNA double stranded breaks (represented by the axe), genotoxins/drugs should make gaps (represented by woodpecker). (B) The tumor gap vulnerability depends on defective lagging strand synthesis (canonical in green, the BRCA-RAD51 pathway in purple that links to backup in yellow), as well as re-priming activity and overall defects in gap repair and genome surveillance mechanisms.

Additionally, there is evidence that gaps derive not only from lagging strand, but also from leading strand (Figure 4.1). As reported, lagging-strand damage bypass and gap suppression requires post-replicative gap filling by TLS\textsuperscript{406}. Although the TLS pathway works on both strands and hardly distinguishes itself from leading strand, inhibition of TLS polymerase REV1 indeed induces ssDNA gaps and replication stress to sensitize cancer cells\textsuperscript{349}. 
In response to PARPi, ssDNA gaps also form on leading strand. Indeed, recent studies demonstrate that leading strand gaps are generated by the Primase and DNA-directed polymerase (PRIMPOL) repriming and robustly filled by post-replicative repair\textsuperscript{263,370,407-410} (Figure 4.1). Given that some results show that short-term gaps enhance an adaptive tolerance to chemotherapy\textsuperscript{263,407}, further studies should clarify that whether leading strand gaps contribute to PARPi sensitivity or resistance.

Together with all the evidence above (Figure 4.1), Okazaki fragment processing turns out to be a converging place for DNA replication, repair, and recombination proteins. Since these pathways ensure the replication of lagging strand in an accurate, timely and failsafe manner, selectively targeting key players can further implicate novel optimization and combinational strategy, which will pave the way for improving cancer management in the future.
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