MEDIZINISCHE UNIVERSITÄT WIEN

Dissecting the role of Polymerase θ in BRCA mutant cancers

Doctoral thesis at the Medical University of Vienna for obtaining the academic degree

Doctor of Philosophy

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Declaration

The majority of the work presented in this thesis was done in the laboratory of Joanna Loizou at the Research Center for Molecular Medicine (CeMM) or at the Center for Cancer Research of the Medical University of Vienna. Due to the dissolution of the Loizou group in June 2022, I joined the laboratory of Georg Winter, also at CeMM, where final experiments for the manuscript listed below were carried out.

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All sections of this thesis were written by the author, Anna Schrempf, with input from Joanna Loizou.

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Abstract

The DNA damage response is a double-edged sword in cancer treatment, as it prevents cancer development by safeguarding genomic integrity but also represents an excellent tool for cancer cells to survive under challenging conditions. The identification of DNA repair processes that become essential specifically in cancer cells has become a priority in cancer research and fuels the development of novel anti-cancer therapies. The first DNA damage response-targeted therapy to enter the clinics was PARP inhibition which is specifically toxic in cells with BRCA mutations, as often found in breast and ovarian cancer. A newly discovered target that also shares a synthetic lethal interaction with BRCA is polymerase theta (POLθ), an error-prone polymerase that was long thought to merely function as a back-up enzyme in the repair of DNA double-strand breaks. Using a small-molecule inhibitor of the POLθ polymerase domain in BRCA1 mutant cells, we sought to investigate the basis of this genetic interaction in more detail. We uncovered an unanticipated role of POLθ in the suppression of replication-associated single-stranded DNA (ssDNA) gaps on the lagging strand of replication. The excessive single-stranded DNA gap formation eventually impairs replication fork progression and causes replication stress. This impacts on cell cycle progression and causes hypersensitivity to ATR inhibition, a vulnerability that could potentially be exploited by combination therapy of ATR and POLθ inhibitors. Using a genome-wide CRISPR knock out screen, we identified that loss of NBN, a member of the MRN-complex, and of the cell cycle regulator CDK6, alleviates the effects of POLθ inhibition in the absence of BRCA1. The reduced amount of single stranded DNA gaps upon MRE11 inhibition could be explained by a role of the MRN-complex in nucleolytic processing of replication gaps that are excessively generated upon POLθ inhibition. Furthermore, CDK6 loss hindered cells from S-phase entry, the phase of the cell cycle in which cells are most sensitive to POLθ inhibition. Overall, we have shown that ssDNA gap formation, modulated by cell cycle progression and ssDNA processing mechanisms, underlies the synthetic lethality between BRCA1 and POLθ.

Zusammenfassung

Die DNA-Reparatur ist ein zweischneidiges Schwert in der Krebsbehandlung, da sie die Krebsentwicklung durch den Schutz der genomischen Integrität verhindert, aber auch ein hervorragendes Instrument für Krebszellen darstellt, um unter schwierigen Bedingungen zu überleben. Die Identifizierung von DNA-Reparaturprozessen, die speziell in Krebszellen von entscheidender Bedeutung sind, ist zu einer Priorität in der Krebsforschung geworden und treibt die Entwicklung neuer Krebstherapien voran. Die erste in der Klinik genutzte, auf DNA-Reparatur abzielende Therapie, war die PARP-Hemmung, die speziell für Zellen mit BRCA-Mutationen toxisch ist, wie sie häufig bei Brust- und Eierstockkrebs vorkommen. Ein neu entdeckter Angriffspunkt, der ebenfalls eine synthetische letale Interaktion mit BRCA aufweist, ist die Polymerase Theta (POLθ), eine fehleranfällige Polymerase, von der man lange Zeit annahm, dass sie lediglich als Reserveenzym bei der Reparatur von DNA-Doppelstrangbrüchen fungiert. Mit Hilfe eines niedermolekularen Inhibitors der POLθ-Polymerase-Domäne in BRCA1-Mutantenzellen wollten wir die Grundlagen dieser genetischen Interaktion genauer untersuchen. Wir entdeckten eine unerwartete Rolle von POLθ bei der Unterdrückung von Replikations-assoziierten einzelsträngigen DNA-Lücken auf dem diskontinuierlichen Strang der Replikation. Die übermäßige Bildung einzelsträngiger DNA-Lücken beeinträchtigt schließlich die Progression der Replikationsgabel und verursacht Replikationsstress. Dies wirkt sich auf die Progression des Zellzyklus aus und verursacht eine Überempfindlichkeit gegenüber der ATR-Hemmung, eine Schwachstelle, die möglicherweise durch eine Kombinationstherapie von ATR- und POLθ-Inhibitoren ausgenutzt werden könnte. Mithilfe eines genomweiten CRISPR-Screens haben wir festgestellt, dass der Verlust von NBN, einem Mitglied des MRN-Komplexes, und des Zellzyklusregulators CDK6 die Auswirkungen der POLθ-Hemmung in Abwesenheit von BRCA1 abschwächt. Die verringerte Last an einzelsträngiger DNA-Lücken bei MRE11-Hemmung könnte durch eine Rolle des MRN-Komplexes bei der nukleolytischen Verarbeitung von Replikationslücken erklärt werden, die durch POLθ-Hemmung übermäßig entstehen. Darüber hinaus hinderte der Verlust von CDK6 die Zellen am Eintritt in die S-Phase, in der sie am empfindlichsten auf POLq-Inhibition reagieren. Insgesamt haben wir gezeigt, dass die Bildung von Einzelstrang-DNA-Lücken, die durch die Zellzyklusprogression und einzelsträngige DNA-Lücken-Verarbeitungsmechanismen moduliert wird, der synthetischen Letalität zwischen BRCA1 und POLθ zugrunde liegt.

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List of Abbreviations

Abbreviation	Full name
γH2AX	Phosphorylated form of histone H2AX
AP site	Apurinic/apyrimidinic site
APE1/2	Apurinic/apyrimidinic Endodeoxyribonuclease 1
ASF1	Histone Chaperone Anti-Silencing Factor 1
ATM	Ataxia-Telengiectasia Mutated
ATR	Ataxia-Telengiectasia and Rad3-Related
ATRIP	Ataxia-Telengiectasia and Rad3-Related Interacting Protein
BLM	Bloom Syndrome RecQ Like Helicase
BRCA1	Breast Cancer Gene 1
BRCA2	Breast Cancer Gene 2
Cas	CRISPR-associated protein
CCNE1	Cyclin E1
CDC6	Cell Division Cycle 6
CDK	Cyclin-dependent kinase
CDT1	Chromatin Licensing and DNA Replication Factor 1
CHK	Checkpoint Kinase
CPD	Cyclobutene-Pyrimidine Dimer
CRISPR	Clustered Regularly Interspaced Short Palindromic Repeats
CRISPRa	CRISPR activation
CRISPRi	CRISPR interference
CSA/B	Cockyane Syndrome A/B
DDB2	Damage Specific DNA Binding Protein 2
DDR	DNA Damage Response
DNA	Deoxyribonucleic Acid
DNA2	DNA Replication Helicase/Nuclease 2
DNA-PKcs	DNA-Protein Kinase catalytic subunit
DNA2	DNA Replication Helicase/Nuclease 2
dNTP	Deoxyribonucleoside triphosphate
DSB	Double-strand break
dsDNA	Double-strand DNA
ERCC1	Excision Repair Cross-Complementation Group 1
EXO1	Exonuclease 1
FA	Fanconi anemia
FANC	Fanconi Anemia Complementation Group
HLTF	Helicase Like Transcription Factor
HR	Homologous Recombination
LIG1/3	Ligase 1/3
MCM2-7	Minichromosome Maintenance Protein 2-7
MGMT	O-6-Methylguanine-DNA-Methyltransferase
MLH1	MutL Homolog 1
MMEJ	Microhomology-mediated end joining
MRE11	MRE11 Homolog, Double Strand Break Repair Nuclease
MRN-complex	MRE11-RAD50-NBN complex
MSH2/6	MutS Homolog 2
MUS81	MUS81 Structure-specific Endonuclease Subunit
NBN	Nibrin
NHEJ	Non-homologous End Joining
ORC	Origin Recognition Complex
PCNA	Proliferating Cell Nuclear Antigen
PARP	Poly(ADP-Ribose)Polymerase
PARylation	Poly(ADP-Ribose)ylation
POLD3	DNA Polymerase Delta 3, accessory subunit
POLδ	Polymerase delta
POLε	Polymerase epsilon
POLι	Polymerase iota
POLκ	Polymerase kappa
POLν	Polymerase nu
POLθ	Polymerase theta
POLζ	Polymerase zeta
Pre-RC	Pre-Replication Complex
PrimPol	Primase and DNA directed Polymerase
RAD51	RAD51 recombinase
ROS	Reactive Oxygen Species
SHLD	Shieldin Complex Subunit 1

SMARCAL1	SWI/SNF related Matrix-associated Actin-dependent Regulator of Chromatin subfamily A like1
SSA	Single-Strand annealing
ssDNA	Single-stranded DNA
TCGA	The Cancer Genome Atlas
TMEJ	Polymerase Theta-Mediated End Joining
TOPBP1	DNA Topoisomerase II Binding Protein 1
TP53	Tumor Protein P53
WEE1	WEE1 G2 Checkpoint Kinase
WRN	WRN RecQ Like Helicase
XPC	Xeroderma Pigmentosum Complementation Group C
XRCC4	X-ray Repair Cross Complementing 4
ZRANB3	Zinc Finger RANBP2-type containing 3

1 Chapter 1: Introduction

1.1 Understanding cancer

1.1.1 What is cancer?

The American Cancer Society has defined cancer as a "group of diseases characterized by uncontrolled growth and spread of abnormal cells". In 2016, 1.2 million Europeans died from cancer which corresponds to 25.8% of all death cases (ec.europa.eu/Eurostat/statistics-explained/index.php?title=Cancer_statistics). Worldwide, cancer accounts to more than 10 million deaths yearly, highlighting cancer as a major global health burden (Howlader *et al*, 2019). Although cancer represents a group of more than 100 different disease types, they all share a common set of alterations. These hallmarks of cancer (Hanahan & Weinberg, 2000, 2011) are described in more detail below.

Sustaining proliferative signaling: One of the most obvious hallmarks is sustained proliferative signaling. Whereas normal cells depend on external signals to induce cell growth or division, cancer cells no longer require such external signals for constitutive expansion. Instead, cancer cells can produce growth factors themselves or stimulate neighboring cells for increased secretion. Alternatively, elevated expression of growth factor receptors on the cellular surface or alterations in signaling pathways downstream of such receptors, e.g. in the Ras pathway, can increase proliferative signaling (Cheng *et al*, 2008; Nih *et al*, 1987; Kinzler & Vogelstein, 1996).

Evading growth suppressors: Cancer cells must actively circumvent anti-proliferative signals that serve to ensure tissue homeostasis by limiting cell growth. Such signals usually converge on regulation of the cell cycle, e.g. by blocking the progression from G_1 to the replicative S phase. One central regulator is phosphorylated retinoblastoma associated protein (Rb1) which sequesters the transcription factor E2F, thereby blocking proliferation. Only when phosphorylation of Rb (pRb) is blocked, is E2F released to activate cell proliferation. Factors of the pRb signaling pathway, including Rb and Transforming Growth Factor β (TGFβ), which suppress Rb phosphorylation, are frequently mutated in cancer (Fynan & Reiss, 1993).

Activating invasion and metastasis: Cancers at advanced disease stages are often detected at distant sites of the body. This process, called metastasis, is caused by alterations in cell-to-cell adhesion, e.g. by loss of E-cadherin. Metastasis includes several steps: local invasion followed by entry into the blood or lymphatic system which supports distribution of cancer cells throughout the body and finally extravasation and formation of new micro-metastatic lesions that can grow into full tumors (Talmadge & Fidler, 2010).

Enabling replicative immortality: In non-neoplastic cells, the number of cellular divisions is limited by a natural barrier: the length of the chromosome-end protecting structure, called telomere (Blasco, 2005; Blackburn, 2001). Cancer cells have evolved sophisticated routes to overcome this barrier. One of them involves upregulation of a specialized DNA-polymerase, called telomerase, that adds more tandem hexanucleotide repeats to chromosome ends, therefore increasing telomere length.

Inducing angiogenesis: Increased cellular proliferation comes with an elevated requirement of nutrients and oxygen as well as the necessity to remove carbon dioxide and metabolic waste. To this end, cancer cells generate blood vessels in a process called angiogenesis (Hanahan & Folkman, 1996). A central player in angiogenesis is vascular endothelial growth factor A (VEGFA), a ligand which orchestrates blood vessel growth also during embryonic and postnatal development (Ferrara, 2009).

Resisting cell death: Apoptosis is a form of programmed cell death in which cells are disassembled and taken up by neighboring cells or professional phagocytic cells. In the apoptotic signaling pathway, upstream sensors and downstream effectors culminate on mitochondria that release cytochrome c to induce apoptosis (Green & Reed, 1998). Cancer cells commonly find ways to circumvent apoptotic signaling, e.g. by mutations in *TP53*, which elicits apoptosis by upregulating the expression of proapoptotic Bax in response to sensing DNA damage (Junttila & Evan, 2009).

Reprogramming energy metabolism: Increased cellular proliferation of cancer cells comes with alterations in cellular metabolism. The most well-known example is the Warburg effect which describes a switch in glucose metabolism of cancer cells. Instead of using the citric acid cycle and oxidative phosphorylation, cancer cells prefer glycolysis even under aerobic conditions. This is believed to be advantageous since glycolytic intermediates can be directly diverted into various biosynthetic pathways to generate nucleosides or amino acids (DeBerardinis & Chandel, 2020).

Avoiding immune destruction: The immune system represents a barrier to cancer formation by constantly monitoring and eliminating aberrant cells. Therefore, cancer cells with effective mechanisms to evade the immune system are selected for (Bindea *et al*, 2010). One such mechanism includes inhibiting the activation of infiltrating immune cells by secreting TGF-β or other immune-suppressive factors (Yang *et al*, 2010).

Tumor promoting inflammation: While the immune system provides a protective mechanism against cancer formation, it can also have an inverse effect. Accumulation of cells of the innate and adaptive immune system in tumors can provide a pro-tumorigenic microenvironment by secreting growth and survival factors, signaling molecules that facilitate angiogenesis, metastasis and invasion, as well as reactive oxygen species which support mutagenesis of cancer genomes (Grivennikov *et al*, 2010).

Genome instability and mutation: The altered behavior of cancer cells described above is based on genetic changes in neoplastic cells. During cancer development, genetic alterations (i.e. mutations) which confer a growth advantage over other cells in the tumor environment are selected, supporting the accumulation of mutations over time. Such an accumulation can be accelerated by alterations in pathways that act as caretakers of the genome, such as DNA-damage signaling factors (e.g. p53) and DNA repair pathways (Negrini *et al*, 2010). This is highlighted by the increased incidence of cancer formation in individuals with germline mutations in such pathways. One example is Lynch syndrome, in which mutations in DNA mismatch repair genes support the formation of hereditary non-polyposis colon cancer (Fishel *et al*, 1993).

1.1.2 Using the human genome to understand cancer

Technical advances in molecular biology techniques beginning in the 1940ies allowed the identification of mutations in genes that drive tumor growth, termed cancer driver genes. The first type of identified cancer driver genes were oncogenes. Oncogenes act in a dominant way, as loss of function of one allele of an oncogene, in some cases caused by a single point mutation, was shown to be sufficient for transformation (Reddy *et al*, 1982). This contrasts with tumor suppressor genes, such as *RB1*, which require both copies to be altered to allow transformation (Knudson, 1971). The understanding of the recessive nature of tumor suppressive genes formed the basis for understanding heritable cancers. Individuals with one inactivated allele of a tumor suppressor gene are predisposed to cancer as only one second hit is required to allow cellular transformation (Rahman, 2014). Following this finding, numerous oncogenes (e.g. *EGFR, BRAF, KRAS, MYC*) as well as tumor suppressors (e.g. *BRCA1, BRCA2, PTEN, RB1, ARID1A, TP53*) were identified with some occurring predominately in specific types of cancer (e.g. *BRCA* mutations in breast cancer) and others occurring in many cancer types (e.g. *TP53*) (**Figure 1**). The identification as well as mechanistic investigation of identified cancer driver genes has laid the foundation for early cancer diagnosis and tailored choice of treatment.

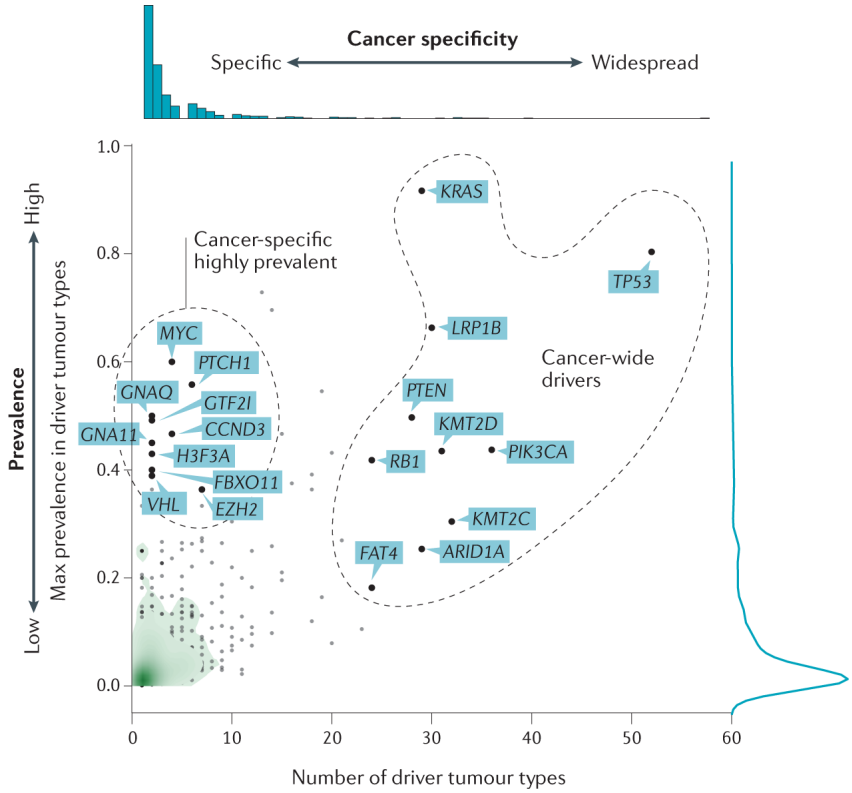

Figure 1: Cancer driver genes and their distribution among cancer types. Each dot represents one cancer driver gene. Driver genes that are shared among many cancers as well as cancer-type specific genes are highlighted. Figure reprinted with permission from Springer Nature (Martínez-Jiménez *et al*, 2020).

The introduction of next generation sequencing (NGS) technologies in the early 2000s (Goodwin *et al*, 2016) paved the way for large tumor sequencing initiatives around the world, including many projects under the umbrellas of The Cancer Genome Atlas (TCGA), the International Cancer Genome Consortium (ICGC) and more recently, The Pan-Cancer Analysis of Whole Genomes (PCAWG)) (Hudson *et al*, 2010; McLendon *et al*, 2008; Campbell *et al*, 2020). This has produced a huge amount of publicly available sequencing data of tumor samples, currently in the range of tens of thousands, which have been vital in pushing cancer research forward.

Leveraging these extensive datasets, the discovery that cancer cells acquire numerous mutations, of which only a few are expected to be the origin of tumor formation, came as a surprise (Vogelstein *et al*, 2013). Tumors typically harbor around two to eight cancer driver mutations whereas the remaining mutations have no impact on tumorigenesis and were therefore termed “passenger mutations”. In contrast, cancer driver mutations are under positive selection, giving rise to the concept of Darwinian evolution in tumorigenesis (Greaves & Maley, 2012). This was shown to occur in a multi-step process of random mutagenesis followed by positive selection of cells with a growth advantage over neighboring cells. This process can take decades with first cancer driver mutations occurring already in early stages of life, in some cases even *in utero* (Williams *et al*, 2022; Van Egeren *et al*, 2021). Mutations in cancer driver genes establish an at-risk mutant lineage which is predisposed to tumor

formation but does not always lead to cancer. This opens the question how many cells accumulate driver mutations in a human lifetime without ever turning cancerous as well as which combination of extrinsic and intrinsic factors is required to shift the balance towards cancer formation.

Traditionally, cancer research has focused on identifying mutations in individual genes followed by gene-specific mechanistic investigations. One theoretical framework that has transformed our understanding of cancer genomics, is the concept of mutational signatures (Nik-Zainal *et al*, 2012; Alexandrov *et al*, 2013; Koh *et al*, 2021). Instead of looking at individual mutations in cancer genes, mutational signatures allow the definition of mutational patterns within their sequence context. The identified patterns are a result of DNA damage and consecutive DNA repair pathways and can include base substitutions, small insertions or deletions, chromosome copy number alterations and genome rearrangements. This is especially valuable since the identification of mutational signatures can shed light on the etiology of cancer formation, e.g. external mutational agents such as tobacco smoke or deficiency of DNA repair pathways such as homologous recombination (HR) (Alexandrov *et al*, 2016; Polak *et al*, 2017).

Summarizing, the identification of cancer driver genes as well as large sequencing studies enabled by NGS have driven the field of cancer research forward. With the rapid accumulation of publicly available tumor sequencing data, one future challenge will be to close the knowledge gap between available data and mechanistic understanding of tumorigenesis. Theoretical frameworks like mutational signatures that define genome-wide patterns as a product of the cancer`s entire mutational history will be instrumental in closing this knowledge gap.

1.1.3 Our fight against cancer: traditional chemotherapy versus targeted cancer therapy

Although cases of cancer formation have been documented for hundreds of years, insights into the molecular mechanisms behind neoplastic transformation began to emerge only in the second half of the twentieth century. Until the 1950s, the only available cancer therapy was surgical resection of cancer tissue followed by introduction of radiation therapy in the 1960s. Both surgical and radiation therapy shared the same substantial disadvantage: they could not target metastatic disease. Only the introduction of drugs as chemotherapy, first documented in the 1940s with the use of nitrogen mustard, allowed reaching cancer cells in every organ (Gilman, 1963). Although in this first example of chemotherapeutic treatment, the non-Hodgkin's lymphoma progressed a few weeks after treatment, the concept of anti-cancer drugs that induce tumor regression was established. Within the next decades, cancer drug development transitioned from a government supported research effort into a multi-billion

dollar industry yielding numerous classes of chemotherapy and recently, also targeted therapies. Selected classes of chemotherapeutic agents are described below.
Alkylating agents: The first chemotherapeutic agent ever used, nitrogen mustard, belongs to this class of chemotherapeutics. Alkylating agents form covalent bonds with DNA that can cause DNA single-strand breaks, DNA double-strand breaks or crosslinks that ultimately result in cell death (Brookes & Lawley, 1961).

Platinum compounds: Platinum compounds, such as cisplatin and carboplatin, form covalent bonds with DNA to form intra-strand or inter-strand crosslinks upon intracellular activation.
Antimetabolites: These compounds closely resemble building blocks of DNA such as pyrimidines or purines. They inhibit tumor growth by incorporation into DNA or RNA and by inhibition of DNA synthesis enzymes. As they act in S-phase, the efficacy of these compounds is more schedule- than dose-dependent. One example is methotrexate, which inhibits dihydrofolate reductase, an enzyme in thymidine synthesis. Antimetabolites are still widely used for the treatment of solid tumors and hematological malignancies (Tiwari, 2012).

Anthracyclines: Anthracyclines were originally extracted from Streptomyces spp. One known example is doxorubicin which inhibits cancer cell growth by intercalating with DNA as well as inhibition of topoisomerase II.

Topoisomerase inhibitors: Topoisomerases are enzymes that release torsional stress of DNA by passing one DNA strand through a previously generated nick in the complementary strand (Wang, 2002). Generally, topoisomerase inhibitors generate DNA double-strand breaks that destabilize the genome.

A substantial drawback of chemotherapy is the inability to distinguish between cancer and normal cells, resulting in extensive acute and long-term adverse effects that can affect all organs of the body. Therefore, the field of cancer therapy is now moving towards targeted therapies that aim at functional nodes in signaling networks of oncogenic cells. The therapeutic targeting of functional nodes that are essential specifically in cancer cells, opens a therapeutic window that allows killing of cancer cells while leaving normal cells unharmed. Genetic alterations that confer tumor-specific vulnerabilities can be gain-of-function mutations in oncogenes (such as *HER2, BCR-ABL, EGFR*) or loss-of-function mutations in tumor suppressor genes (e.g. *TP53, RB1, PTEN*). Gain-of-function mutations in oncogenes have been intensively researched for the development of targeted therapeutics for cancers with so-called oncogene addiction (Weinstein, 2002). One example of oncogene addiction is the constitutively active ABL kinase in chromic myeloid leukemia, caused by the BCR-ABL fusion

gene. Cancers with inactivated tumor suppressor genes, on the other hand, are more difficult to target as such therapies would have to restore tumor suppressor function. Moreover, there are numerous cases of oncogenes that have been denoted “undruggable” based on their protein structure (e.g. H-Ras, K-Ras, Myc). In these cases, the concept of synthetic lethality, which was originally described in the fruit fly (Dobzhansky, 1946), is especially valuable as it allows indirect targeting of undruggable targets.

Synthetic lethality is a type of genetic interaction in which combined loss of two genes results in cell death whereas loss of each individual genes does not affect cellular viability. In the context of cancer treatment, cancer cells acquire mutations that are associated with a new vulnerability that can be exploited therapeutically (**Figure 2**). This phenomenon has also been termed non-oncogene addiction (Solimini *et al*, 2007).

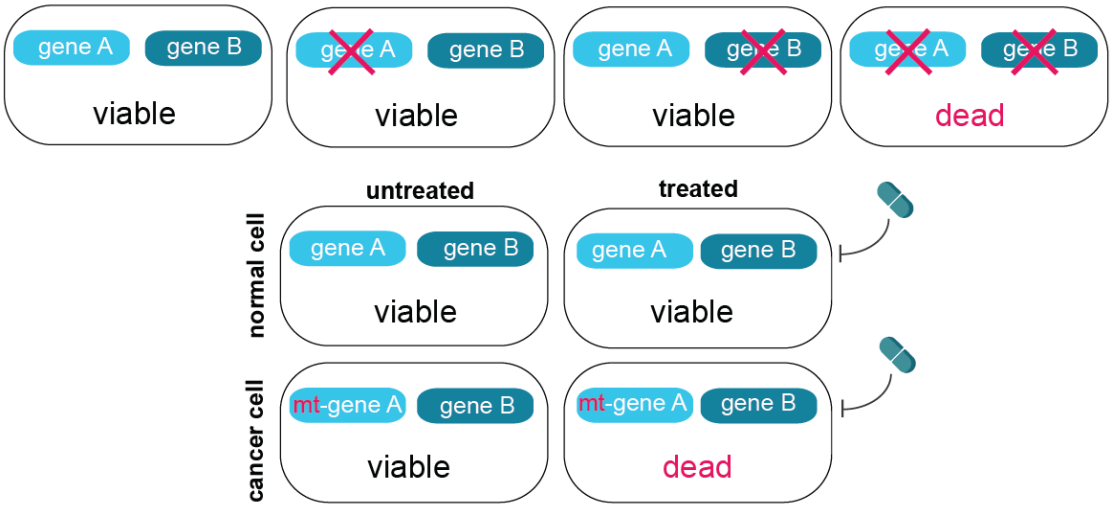

Figure 2: The concept of synthetic lethality and its use in cancer therapy. Top: the concept of synthetic lethality explained by two genes A and B. While loss of either gene A or gene B is compatible with cell viability, combined loss of gene A and B leads to cell death. Bottom: the concept of synthetic lethality applied to cancer therapy. Cancer cells are mutant in gene A, making them dependent on gene B. Therefore, drugging the protein encoded by gene B is specifically harmful to cancer cells.

The role model of a synthetic lethal interaction that is successfully exploited in cancer therapy is the interaction between BRCA1/2 and Poly(ADP-Ribose)-Polymerase (PARP) (Lord & Ashworth, 2017). Mutations in *BRCA1/2* are the most common genetic lesion associated with familial ovarian and breast cancer and sensitize cells to PARP inhibition. Several PARP inhibitors have been FDA-approved as single agent therapy of BRCA mutant breast cancer. BRCA1 and BRCA2 are not an exception. Defects in DNA repair genes are very common in cancer cells and facilitate the accumulation of genomic alterations. Therefore, the next chapter will focus on the DNA damage response and its implication for cancer and cancer therapy.

1.2 The DNA damage response: a double-edged sword in cancer protection and therapy

1.2.1 A brief overview of the DNA damage response

Cellular DNA is constantly exposed to a number of DNA damaging agents of exogenous (e.g. UV light, tobacco smoke, ionizing radiation (IR)) or endogenous (e.g. byproducts of metabolism such as reactive oxygen species (ROS) or dietary nitrosamines) origin. These generate DNA lesions such as base modifications and DNA single or double strand breaks that, if remain uncorrected, can lead to genomic instability, a hallmark of cancer. To avoid the accumulation of mutations, cells have developed a complex network that is responsible for the resolution of DNA damage, also termed the DNA damage response. Although there are numerous interconnected DNA repair pathways that are specialized on different types of DNA lesions, they share a common architecture (**Figure 3**). Sensor proteins such as the PI3 kinase (PI3K) ataxia telangiectasia mutated (ATM) initiate a signaling cascade that is further amplified by signal transducers. Finally, effector proteins, such as polymerases or ligases, are responsible for performing the repair steps. Beyond DNA repair, the DNA damage response directly influences other cellular processes such as apoptosis, cell cycle regulation and transcriptional state.

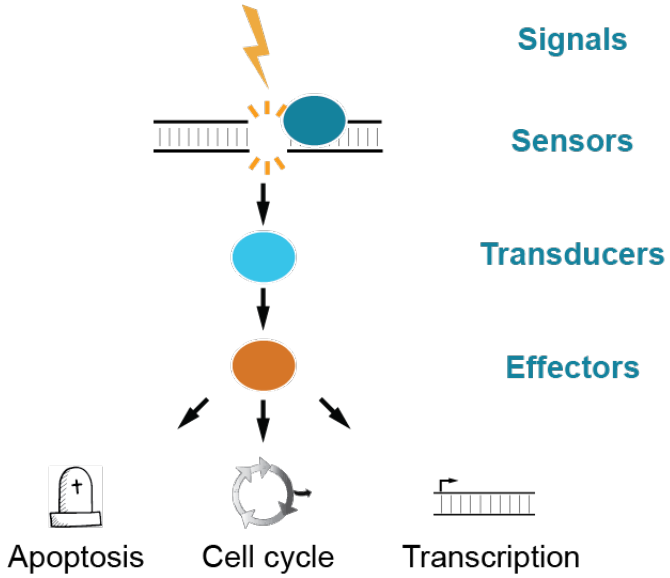

Figure 3: The general outline of DNA damage response pathways. Upon sensing of DNA damage, the signal is amplified by signal transducers that recruit effector proteins. These are involved in damage resolution as well as influencing other cellular processes such as apoptosis, cell cycle and transcription. Adapted from Zhou & Elledge, 2000.

Certain types of lesions, whether induced by endogenous or exogenous factors including therapeutic agents such as chemotherapeutic compounds, are repaired by specified DNA repair pathways (**Table 1**). In the following table, the main DNA repair pathways will be briefly outlined.

Repair pathway	Lesion	Endogenous sources	Exogenous sources
Direct repair	Alkylated bases	Dietary nitrosamines	Alkylating agents (e.g. temozolomide)
Base excision repair	Abasic sites, alkylated bases, deaminated bases, oxidative bases	Reactive oxygen species	Alkylating agents (e.g. temozolomide)
Nucleotide excision repair	Helix-distorting lesions		Cisplatin, UV-light
Mismatch repair	Mismatched nucleotides	Faulty replication	
Interstrand crosslink repair	Interstrand crosslinks	Acetaldehyde, formaldehyde	Mitomycin C, cisplatin, psoralens, nitrogen mustards
DNA double-strand break repair	Double-strand breaks	Replication fork collapse, V(D)J recombination, class switch recombination	Topoisomerase inhibitors, ionizing radiation, replication inhibitors

Table 1: The main DNA repair pathways are specialized to repair specific DNA lesions, that can be caused by endogenous or exogenous agents.

Direct repair

Direct repair corresponds to a direct reversal of the DNA lesion, the simplest form of DNA repair. Such directly reversible DNA lesions include base modifications, e.g. alkylation of the O^6 position of guanine. This type of alkylation can be induced by dietary nitrosamines or chemotherapeutic agents such as temozolomide. Since 6-oxo-guanine pairs with thymidine instead of cytosine, this type of base modification introduces G:C > A:C base substitutions and is considered as highly mutagenic. The enzyme 6-methylguanine DNA methyltransferase (MGMT) prevents this mutation by demethylating O^6-metyhlguanine lesions (Kaina *et al*, 2007).

Base excision repair

Base excision repair corrects small lesions that do not distort the helical structure of DNA (Dianov & Hübscher, 2013). This pathway is initiated by a DNA glycosylase that removes the damaged base, generating an abasic site, also called apurinic/apyrimidinic (AP) site. AP endonucleases such as APE1/APE2 then create a nick in the phosphodiester backbone. At this point, the pathway is divided into long patch or short patch base excision repair. Long patch base excision repair removes and re-synthesizes 2-13 nucleotides of one strand, whereas short patch base excision repair removes only a single nucleotide. DNA polymerases then fill the gap, thereby displacing the “old” DNA strand generating a DNA flap which is hydrolytically cleaved by flap endonucleases, such as flap endonuclease 1 (FEN1). In the last step, a DNA ligase forms a phosphodiester bond to join the newly synthesized DNA with the original DNA strand.

Nucleotide excision repair

Unlike base excision repair, nucleotide excision repair recognizes helix-distorting lesions of versatile origin such as UV-induced cyclobutene-pyrimidine dimers (CPDs), ROS-generated cyclopurines or intrastrand crosslinks (Nouspikel, 2009). Therefore, mutations in base excision repair genes predispose to skin cancer and confer hypersensitivity to UV and platinum agent therapy. Nucleotide excision repair can be divided into two subpathways, that differ in their lesion recognition step. Global genome nucleotide excision repair monitors the entire genome for helix distorting lesions whereas transcription coupled nucleotide excision repair is only initiated when RNA polymerase II is stalled during the elongation step of transcription. XPC, DDB and RAD23B are responsible for sensing DNA lesions in global genome nucleotide excision repair whereas the Cockayne syndrome group A and B proteins (CSA/CSB) are responsible for lesion recognition in transcription coupled nucleotide excision repair. The following steps are shared between both subpathways. XPG and XPF-ERCC1 incise the DNA strand a short stretch 5` and 3`of the DNA lesion, followed by resynthesis of the removed DNA stretch by polymerases δ (Polδ) and ε (Polε) and ligation by ligase 3 (LIG3).

Mismatch repair

When incorrect nucleotides are incorporated into the daughter strand during replication, mismatch repair is required for their removal. Lesion recognition is mediated by the MutSa complex, consisting of MutS homologs 2 and 6 (MSH2/MSH6) (short mismatches) or the MutSb complex, consisting of MutS homologs 2 and 3 (MSH2/MSH3) (long mismatches) (Li, 2008). In the next step, the heterodimer MutL, consisting of MutL homolog 1 (MLH1) and PMS2, is recruited. Exonuclease 1 (EXO1) then excises the mismatched DNA and DNA ligase I seals the nick.

Interstrand crosslink repair

Interstrand crosslinks are highly toxic as they represent barriers to replication and transcription. They are caused by external agents such as bifunctional alkylating agents, mitomycin C and platinum compounds or by endogenous agents, such as acetaldehyde and formaldehyde, byproducts of cellular metabolism (Rosado *et al*, 2011; Langevin *et al*, 2011). Cells in G0 or G1-phase use replication independent nucleotide excision repair to resolve interstrand crosslinks. The two nucleases XPF and ERCC1 excise the interstrand crosslink, followed by gap filling by translesion synthesis polymerases. During S-phase, interstrand crosslink repair requires the Fanconi anemia (FA) and HR pathways. FANCM senses lesions and recruits the FA core complex consisting of FANCA, FANCB, FANCC, FANCF, FANCG, FANCL, FAAP100, FAAP20 and FANCM itself (Clauson *et al*, 2013; Palovcak *et al*, 2017). FANCI and FANCD2 then form the ID complex, a necessary step for the recruitment of other repair proteins such as the XPF-ERCC1 complex that incises the interstrand crosslink, converting it into a DNA

double-strand break (Kottemann & Smogorzewska, 2013). While translesion repair fills the gap in one DNA duplex, HR resolves the DNA double-strand break in the other duplex.

DNA double-strand break (DSB) repair

DSBs are very toxic lesions that arise from exogenous or endogenous sources. Exogenous agents include ionizing radiation and topoisomerase inhibitors, whereas a common endogenous source is programmed DSB-generation in the immune system, such as during V(D)J recombination and class switch recombination (Bednarski & Sleckman, 2019). In addition, DSBs are generated when replication forks collide with unresolved DNA lesions resulting in fork collapse (Pfeiffer *et al*, 2000). As DSBs pose a threat to cellular survival, cells have developed several pathways for their resolution that are selected in a highly controlled manner (**Figure 4**). Non-homologous end joining (NHEJ) is a non-conservative pathway that acts during all phases of the cell cycle and joins DNA ends with minimal sequence loss (Difilippantonio *et al*, 2000). In contrast to NHEJ, there are three pathways that require resected DNA ends: Single-Strand Annealing (SSA), Homologous Recombination (HR) and Polymerase Theta-Mediated End Joining (TMEJ). SSA involves annealing of homologous repeat sequences flanking a DSB, causing deletion of the sequence between the repeats, and is therefore considered a mutagenic pathway. HR is a conservative pathway that acts predominantly during S and G2 phases of the cell cycle, when a sister chromatid is available. By using this homologous DNA strand as a template, HR allows precise repair of the DSB. TMEJ, on the other hand, is a non-conservative pathway that anneals DNA ends at regions of microhomology, followed by fill-in synthesis by the error-prone translesion synthesis enzyme polymerase theta (POLθ) (for more information on POLθ and translesion synthesis, see Sections 1.3.5 to 1.3.7).

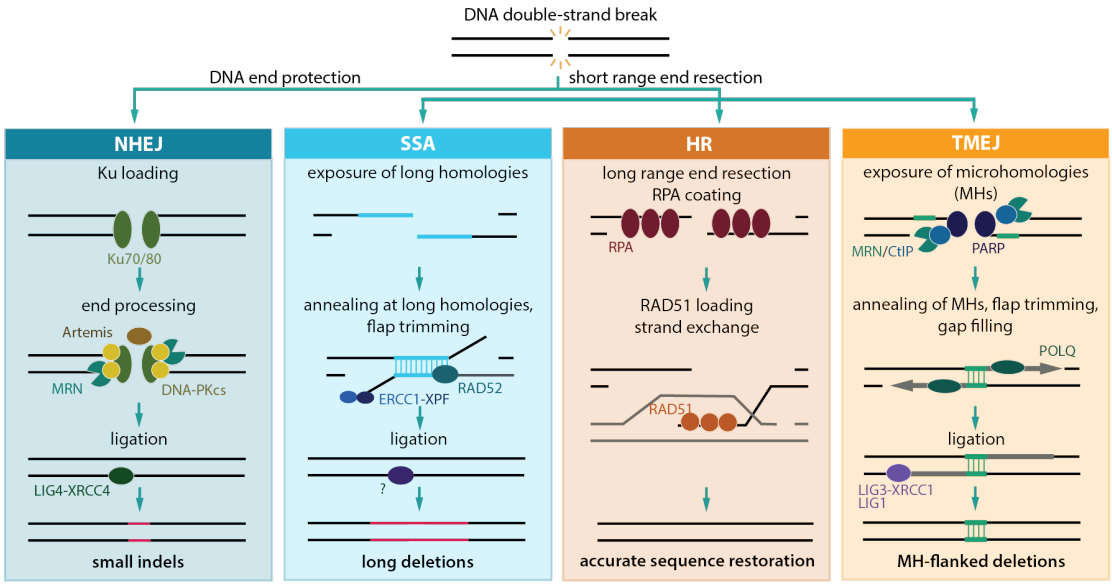

Figure 4: DNA DSBs can be repaired by four main pathways: Non-Homologous End Joining (NHEJ), Single-Strand Annealing (SSA), Homologous Recombination (HR) and Polymerase Theta-Mediated End Joining (TMEJ).

While HR is the most precise repair pathway, it depends on the availability of a homologous template. Furthermore, NHEJ might seem more conservative than TMEJ but requires compatible DNA ends. To consider these preferences and dependencies and avoid choosing unsuited pathways that may induce oncogenic transformation, cells have developed sophisticated mechanisms that guide repair pathway choice. Most of these mechanisms culminate on the regulation of end resection that determines the choice between NHEJ and the three pathways HR, SSA and TMEJ.

End resection occurs in two phases. First, limited end resection, also called end clipping, is catalyzed by the MRN-complex, exposing regions of microhomology that can guide TMEJ (Truong *et al*, 2013). This is followed by extensive resection driven by helicases and nucleases such as DNA replication helicase/nuclease 2 (DNA2), BLM RecQ like helicase (BLM), EXO1, RB binding protein 8 endonuclease (CtIP) and WRN RecQ like helicase (WRN) that uncover long stretches of single stranded DNA, committing the cell to the use of HR or SSA (Sturzenegger *et al*, 2014; Symington & Gautier, 2011).

The end resection machinery is strongly influenced by the cell cycle. Cyclin dependent kinases (CDKs) phosphorylate factors involved in end resection such CtIP or EXO1 (Yun & Hiom, 2009; Tomimatsu *et al*, 2014). Non-cycling cells, on the other hand, show decreased end resection with NHEJ as the dominant DSB repair pathway (Symington & Gautier, 2011). End resection is also regulated by accessory factors such as 53BP1 which blocks access of CtIP to DNA-ends, thereby channeling pathway choice towards NHEJ. Upon phosphorylation by ATM, 53BP1 recruits the Shieldin complex, consisting of SHL1, SHLD2, SHLD3 and REV7 that restrains resection, thereby antagonizing HR, TMEJ and SSA (Noordermeer *et al*, 2018; Dev *et al*, 2018; Gupta *et al*, 2018).

Non-Homologous End Joining: NHEJ starts with binding of Ku, a heterodimer of Ku70 and Ku80, to free DNA ends (Chang *et al*, 2017). Upon recruitment of the catalytic subunit of DNA protein kinase (DNA-PKcs), a synaptic complex of two DNA-PKcs molecules bound to two DNA ends is formed. Autophosphorylation of DNA-PKcs initiates a conformational change that dissociates DNA-PK from DNA ends to allow access of other NHEJ factors. In the last step, LIG4, XRCC4 and XLF catalyze the ligation of DNA ends. Enzymes that are required for limited processing of DNA ends, in case of incompatible end structures, such as PNKP, polymerase μ and polymerase λ, require XRCC4 to grant access to DNA ends. This interaction ensures that ligases are in close proximity to DNA ends, thereby limiting processing of DNA ends and nucleotide loss during NHEJ (Budman *et al*, 2007)

Single-Strand Annealing: Following end resection by CtIP, flanking regions of homology, usually exceeding 200bp in mammals, are annealed by RAD52 (Ren *et al*, 2014). Non-

homologous single stranded DNA tails are removed by ERCC1-XPF followed by filling of DNA gaps and ligation of DNA ends by unknown players.

Homologous Recombination: After limited resection by the MRN-CtIP complex, extensive resection of more than 1kb requires EXO1, BLM and DNA2 (San Filippo *et al*, 2008). Exposed single stranded DNA is coated by replicaton protein A (RPA) to avoid formation of secondary structures (Sung & Klein, 2006). BRCA1 and BRCA2 then load RAD51 onto ssDNA, displacing RPA. RAD51 catalyzes homology search in the homologous sister chromatid, resulting in strand exchange. The invading 3' DNA end then primes DNA synthesis from the homologous sister chromatid, allowing precise repair.

Polymerase Theta-Mediated End Joining: TMEJ shares the first step of resection with SSA and HR. After limited end resection driven by the MRN-complex and coating of single-stranded DNA by RPA, instead of committing to long-range resection, short regions of microhomology are annealed (Ramsden *et al*, 2021). For this reason, this pathway has also been termed microhomology-mediated end joining (MMEJ) in the past. In contrast to SSA, regions of microhomology are shorter and annealing does not depend on RAD52. This is followed by removal of DNA flaps and fill-in synthesis by the error-prone translesion enzyme polymerase theta (POLθ), that has given the pathway its name. Finally, DNA ends are sealed by LIG3, XRCC1 and ligase 1 (LIG1).

1.2.2 The DDR protects from cancer

The most critical role of DNA repair is to protect from irreversible carcinogenesis. This is highlighted by the increased cancer predisposition in cases of germline mutations in key DDR players such as MSH1, TP53, BRCA1/2, BLM. Upon loss or attenuation of function of DDR factors, the genome can no longer be adequately protected from DNA damage of endogenous or exogenous sources, resulting in genomic instability, a hallmark of cancer.

1.2.3 Targeting the DDR in cancer

While the DNA damage response maintains genomic integrity to prevent cancer formation, it is also involved in the response to cancer therapy by processing therapeutically induced DNA lesions (**Table 1**). DSBs induced by radiotherapy or radiomimetics such as bleomycin, for instance, depend on DSB repair pathways such as non-homologous end joining or HR for repair (van de Kamp *et al*, 2021). Alkylated DNA, on the other hand, can be processed by base excision repair, nucleotide excision repair or by direct reversal of the DNA alkylation using the alkyltransferase O-6-methyguanine-DNA-methyltransferase (MGMT) (Sharma & Dianov, 2007; Sugasawa *et al*, 2001; Sedgwick, 2004). Since DNA repair pathways determine the efficacy of resolution of chemotherapy induced DNA lesions, the increased expression of DNA

repair factors can confer resistance to DNA damaging agents. Therefore, the DDR acts as a double-edged sword in cancer formation and therapeutic response.

There are two rationales behind targeting the DDR in cancer therapy. First, as described above, targeting the DDR might impair resolution of chemotherapeutically induced DNA lesions. Therefore, combinations of chemotherapeutic agents with compounds inhibiting DNA repair factors that are responsible for repairing the induced lesion are thought to produce increased sensitivity. One such example is the combination of the alkylating agent temozolomide and PARP inhibitors (Gill *et al*, 2015; Cao *et al*, 2019). PARP acts in base excision repair, which is known to process apurinic sites, a DNA lesion induced by temozolomide. Therefore, absence of PARP attenuates repair of temozolomide induced lesions, thereby increasing cellular sensitivity.

The second rationale behind targeting the DDR in cancer therapy is based on the concept of synthetic lethality, which was already introduced in Section 1.1.3. While the loss of function of DNA repair genes drives the tumorigenic phenotype, it also comes with deleterious effects on genome stability. As a result, cancer cells rely on compensatory pathways to allow cellular survival. This provides an excellent opportunity for anti-cancer therapy, as targeting such a compensatory pathway will be detrimental exclusively in cancer cells. One process that is frequently deregulated in cancer cells is the integrity of replication (Kotsantis *et al*, 2018). To allow continuous cell proliferation, irrespective of the generation of DNA lesions, cancer cells mutate oncogenes (e.g. *KRAS*), checkpoint genes (e.g. *TP53*) or overexpress cell cycle regulators (e.g *CCNE1, CCND2*) (Vousden & Lane, 2007; Kok *et al*, 2020). This leads to higher levels of endogenous replication-associated lesions compared to non-cancerous cells, also termed oncogene induced replication stress (Gaillard *et al*, 2015). However, increased replication stress confers dependance on replication stress signaling by the Ataxia telangiectasia and Rad3 related (ATR) and checkpoint kinase 1 (CHK1) kinases, a sensitivity that can be targeted with ATR and CHK1 inhibitors (Murga *et al*, 2011).

The promise of cancer-specific toxicity of DDR-targeted drugs has fueled efforts to identify and exploit DDR targets. In the following, the most promising DDR targets that are currently in clinical development, are discussed.

PARP: The PARP family contains 17 isoforms, of which PARP1 is the most well studied (Krishnakumar & Kraus, 2010). PARP1 is a multi-functional DNA damage sensor which acts in several DNA repair pathways such base excision repair, DNA DSB repair and single-strand break repair. Furthermore, PARP1 is involved in processes beyond DNA repair such as cellular differentiation, gene transcription, inflammation, mitosis, cell death and metabolism (Weaver & Yang, 2013; Bock & Chang, 2016). Upon recruitment to damaged DNA, PARP1 adds chains of poly-ADP-ribose (PAR) onto target proteins and on itself (auto-PARylation). These PAR-

chains can recruit other repair proteins, such as XRCC1, to facilitate timely repair as well as allow release of PARP1 from DNA (Caldecott, 2014). Numerous models have been proposed to explain the toxicity of PARP inhibitors in cells deficient of HR repair. It is known that PARP inhibitors trap PARP on DNA by preventing auto-PARylation (Helleday, 2011; Murai *et al*, 2012). Upon collision with a replication fork, the single-stranded break with trapped PARP1-protein is converted into a DSB which cannot be processed in HR-deficient cells, resulting in cellular lethality. More recently, PARP1 was shown to participate in physiological processing of the lagging DNA strand during replication (Vaitsiankova *et al*, 2022; Hanzlikova *et al*, 2018). This will be discussed in more detail in Section 2.

Four PARP inhibitors have been approved by the Food and Drug Administration (FDA), representing the first class of DDR-targeted compounds achieving FDA approval: olaparib (Lynparza, AstraZeneca), rucaparib (Clovis), niraparib (MK4827, Tesaro) and talazoparib (Pfizer).

DNA-PKcs: DNA-PKcs, a factor of the NHEJ pathway, is recruited to DSB ends by Ku. Upon DNA-PK dependent phosphorylation, Ku slides inwards on DNA, allowing access of other end processing factors to the DSB ends and initiating the next steps of NHEJ. When DNA-PKcs is inhibited, it remains bound to DSB ends and impedes the access of other DSB repair factors, thereby attenuating repair. DNA-PKcs inhibition hypersensitizes cells to ionizing radiation and topoisomerase inhibitors but has only shown a limited effect on its own, potentially because most endogenous DSBs are processed by HR during replication (Zhao *et al*, 2006). Sufficient selectivity over other phosphoinositide 3-kinases (PI3Ks) such ATR and ATM has posed a challenge to the development of DNA-PKcs inhibitors. Various DNA-PKcs inhibitors including nedisertib (Merck), AZD7648 (AstraZeneca) and VX-984 (Vertex Pharmaceuticals) are currently in clinical trials as monotherapies or in combination with radio- or chemotherapies (Zenke *et al*, 2020; Goldberg *et al*, 2020; Timme *et al*, 2018).

ATR: The kinase ATR is a major player in the replication stress response and in cell cycle regulation. Upon resection of DNA, for instance during the first steps of HR, single-stranded DNA bound RPA recruits ATR which in turn limits origin firing to prevent further ssDNA accumulation. Increased replication stress in cancer cells often comes with an increased dependency on ATR. Furthermore, many cancer cells have a de-regulated G_1-S checkpoint (e.g. by *TP53* or *ATM* mutations) and therefore depend on a functional G_2-M checkpoint which is mediated by ATR. Therefore, ATR is a valuable target in cancers with increased replication stress or harboring mutations in G_1 checkpoint genes such as ATM. Currently, four ATR inhibitors are in phase I and II clinical trials as monotherapy or in combination with chemo- or

radiotherapy: AZD6738 (AstraZeneca), berzosertib (Vertex Pharmaceuticals), M3344 (Merck) and BAY1895344 (Bayer).

ATM: The kinase ATM is an important player in DSB signaling and acts as a tumor suppressor which is frequently mutated in many cancer types (Landau & Wu, 2013; Beà *et al*, 2013). Preclinical studies have shown that loss of ATM sensitizes cells to ionizing radiation which has made ATM an attractive target for combination treatment (Taylor *et al*, 1975; Lavin, 2008). Currently, three ATM inhibitors are tested in clinical trials: AZD0156 (AstraZeneca), AZD1390 (AstraZeneca) and M354 (Merck).

CHK1: Similar to ATR, CHK1 participates in the replication stress response and the G_2-M checkpoint of the cell cycle, making it an ideal target in cancer cells with replication stress and cell cycle deregulation (McNeely *et al*, 2014). Currently, there are two CHK1 inhibitors in clinical trials. Prexasertib (Eli Lilly) is tested as monotherapy in platinum-resistant ovarian cancer whereas SRA737 (Sareum) is used for advanced solid tumors or Non-Hodgkin`s lymphoma.

WEE1: The WEE1 kinase works in parallel with CHK1 to regulate the G_2-M checkpoint of the cell cycle (Du *et al*, 2020). Similar to CHK1 inhibitors, WEE1 inhibition might override the G_2-M checkpoint, forcing cells into mitosis, irrespective of their DNA damage status, increasing genomic instability and cell death (Esposito *et al*, 2021). The WEE1 inhibitor AZD1775 (AstraZeneca) is currently in phase I and II clinical trials as monotherapy or in combination with chemotherapy, radiotherapy, PARP or immune checkpoint inhibitors.

1.2.4 Limitations and future perspectives of targeting the DDR in cancer therapy

The exquisite sensitivity of cancer cells to DDR-targeted therapies comes with limitations. First, clear biomarkers or sequencing-based methods to identify DDR mutations or pathway alterations, are required to identify patients that will benefit from a certain therapy. Second, the extensive crosstalk between DNA repair pathways allows functional buffering and can support the generation of treatment resistant cancer cells. However, cancer cells that acquire resistance mutations might become dependent on other pathways, which could be exploited in a second line therapy (**Figure**). Therefore, a thorough understanding of the DDR network and its compensatory interactions is required to (a) identify novel targets that are exploitable in cancer therapy and (b) pinpoint newly acquired dependencies resulting from resistance mechanisms, that can be targeted in second line therapies.

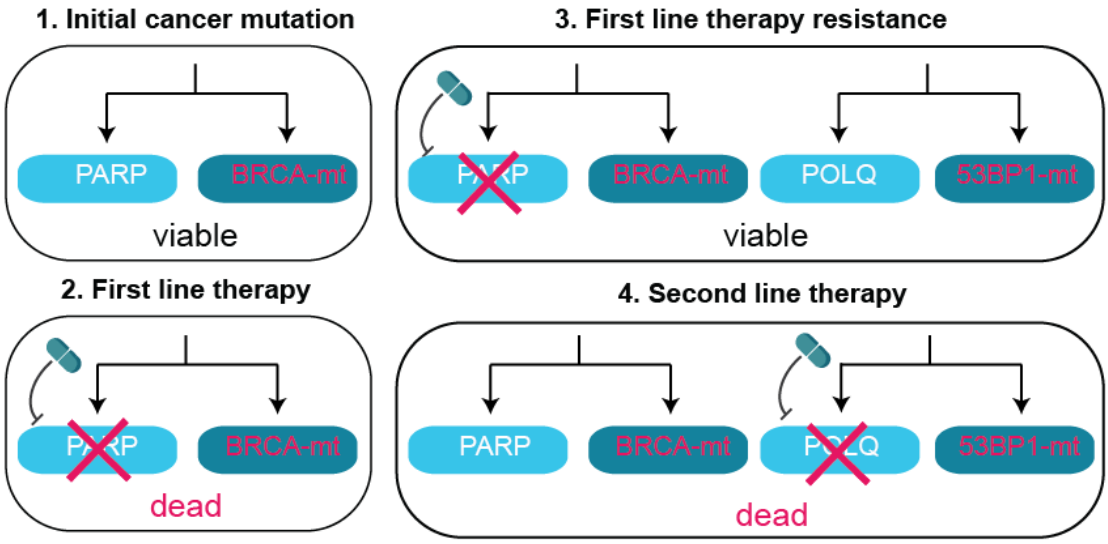

Figure 4: Resistance to first-line therapy is enabled by mutations that may confer sensitivity to second line therapies. This is exemplified by a hypothetical scenario in which a BRCA mutant cancer is treated with a PARP inhibitor (1-2). Mutations in the end joining factor 53BP1 release the dependency on PARP and result in treatment resistance (3). However, mutations in 53BP1 come with a dependency on polymerase theta (POLθ). Treatment with POLθ inhibitors target this dependency and elicit cellular death (4).

To compile such information, the use of high-throughput technologies such as genome scale CRISPR-screens as well as the public availability of the resulting data will be instrumental. Public databases such as the Cancer Dependency Map portal (www.depmap.org) or the Project Score (www.score.depmap.sanger.ac.uk) are the foundation for systematic identification of synthetic lethal interactions. Only recently, a novel synthetic lethal interaction between mismatch repair and the RecQ-family helicase WRN was identified leveraging information from publicly available datasets. In specific, WRN was shown to maintain DNA integrity in mismatch repair deficient cells characterized by microsatellite stability, making WRN an attractive target in microsatellite instable cancers such as colon, gastric, endometrial and ovarian cancers (Behan *et al*, 2019; Chan *et al*, 2019; Lieb *et al*, 2019).

1.3 Replication stress and ssDNA

1.3.1 Normal replication fork progression

During the S phase of the cell cycle, the genome needs to be duplicated to allow subsequent cellular division. To ensure that chromosomal duplication is only happening once, origins of replication are "licensed" in the G_1 phase of the cell cycle by assembly of a pre-replication (pre-RC) complex (Méchali, 2010) **(Figure 5)**. During this process, the replicative DNA helicase minichromosome maintenance complex 2-7 (MCM2-7) is loaded onto DNA by the six-subunit origin recognition complex (ORC). This process also requires cell division cycle 6 (CDC6) and chromatin licensing and DNA replication factor 1 (CDT1) which are subsequently released from

chromatin. Origin firing occurs upon entry into S-phase and is facilitated by cyclin dependent kinases (CDKs) and Dbf4-Cdc7 (DDK). Not all licensed origins are fired. Dormant origins may serve as a back-up in case of fork slowing or stalling (Ibarra *et al*, 2008). In the next step, the replicative helicase, the CDC45-MCMs-GINS (CMG) complex, opens the double helix and allows loading of two replisomes onto the replication bubble which will then travel in opposite directions. Polymerase α primes DNA-synthesis which is then extended by polymerase ε and δ on the leading and lagging strands, respectively. In eukaryotes, replication forks travel with an approximate speed of 1-2 kb per minute (Tuduri *et al*, 2010). Many factors such as cell type, chromatin context, DNA secondary structures and deoxyribonucleoside triphosphate (dNTP) and histone availability affect replication fork speed (Poli *et al*, 2012; Mejlvang *et al*, 2014; Mendez-Bermudez *et al*, 2018).

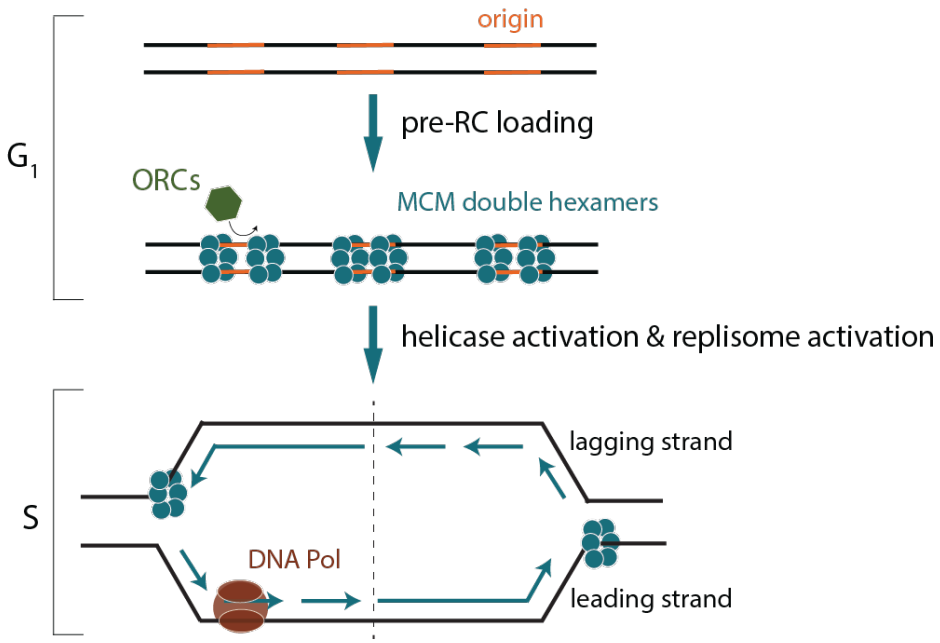

Figure 5: Normal replication fork progression. Modified from Técher & Pasero, 2021.

1.3.2 Replication stress and the replication stress response

Replication stress is defined as slowing or stalling of replication fork progression. Sources of replication stress include fragile sites which are regions of the genome that are intrinsically difficult to replicate, collisions between the replication and transcription machinery as well as oncogenic stress. Overexpression of oncogenes such as cyclin E increases origin firing and impairs replication fork progression (Kok *et al*, 2020; Llobet *et al*, 2020) which contributes to genomic instability. Therefore, replication stress is thought to be a major driver of early carcinogenesis (Bartkova *et al*, 2006b; Gorgoulis *et al*, 2005; Bartkova *et al*, 2006a). Stressed or stalled replication forks initiate a cascade of signaling events which are collectively termed the replication stress response. The overall aim of this DNA damage signaling network is to

slow down replication fork progression to allow the resolution of the replication stress causing lesion or the recovery of sufficient DNA building blocks. First, RPA binds to exposed ssDNA that is generated by uncoupling of the MCM helicase and the replisome (Byun *et al*, 2005). ATR interacting protein (ATRIP) and DNA topoisomerase 2 binding protein (TOPBP1) then facilitate binding of ATR, the center of the replication checkpoint signaling cascade, to RPA. Together with CHK1, ATR initiates a complex signaling cascade that impacts on various cellular processes including chromatin accessibility, cell cycle, fork reversal, origin firing and post-replicative repair **(Figure 6).**

Downstream targets of ATR include the histone variant H2AX as a first sensor of DNA damage (Ward & Chen, 2001) and the cell division cycle 25 (CDC25) phosphatase family that slows down cell cycle progression (Mailand *et al*, 2000; Falck *et al*, 2001; Zhao *et al*, 2002). Other targets of ATR, such as the WEE1 kinase, also impact on cell cycle progression (Beck *et al*, 2010; Elbæk *et al*, 2022). Both conserved RECQ like helicases WRN and BLM are also downstream effectors of ATR/CHK1 signaling that impact on replication fork progression (Ammazzalorso *et al*, 2010; Davies *et al*, 2004). Targeting of the MCM helicase by ATR has been shown to modulate its association with components of the FA pathway at stressed replication forks (Cortez *et al*, 2004; Hae *et al*, 2004). Several members of the FA pathway have also been shown to be targeted directly by the ATR/CHK1 kinases, including FANCI that is involved in regulating origin firing (Chen *et al*, 2015; Sobeck *et al*, 2009; Collins *et al*, 2009). Fork processing enzymes such as DNA2 and EXO1, which are involved in fork resection, as well as SMARCAL1, a known fork reversal enzyme, are also downstream effectors of replication checkpoint signaling (El-shemerly *et al*, 2008; Hu *et al*, 2012; Couch *et al*, 2013). Furthermore, ATR/CHK1 signaling affects HR-mediated repair of stalled replication forks, as shown by phosphorylation of BRCA1, PALB2 and RAD51 (Buisson *et al*, 2017; Tibbetts *et al*, 2000). Another process that is affected by the S phase checkpoint is chromatin accessibility: the activity of the histone chaperone anti-silencing factor 1 (ASF1) was shown to be indirectly modulated by CHK1 (Klimovskaia *et al*, 2014).

Taken together, the central signaling components of the replication checkpoint, ATR and CHK1, modulate the activity of numerous downstream effectors that are involved in several cellular processes with the aim to slow down replication fork and cell cycle progression.

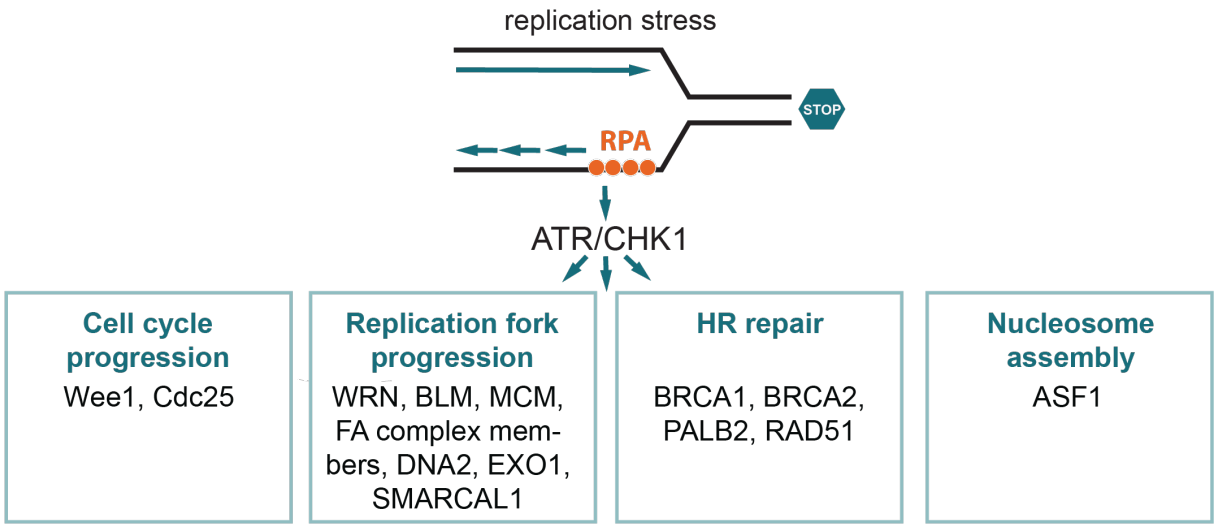

Figure 6: The replication stress response modifies numerous downstream cellular processes to ensure replication fork integrity. Box titles denote cellular processes and the individual targeted factors that are downstream of ATR/CHK1 kinases in response to replication stress.

If ATR/CHK1 signaling fails to limit the level of replication stress, stalled forks with exposed ssDNA accumulate which eventually exhaust the available nuclear pool of RPA. Consequently, ssDNA regions remain exposed and available to nucleolytic cleavage which results in fork breakage and genomic instability. This phenomenon is also termed replication catastrophe (Toledo *et al*, 2017) **(Figure 7).**

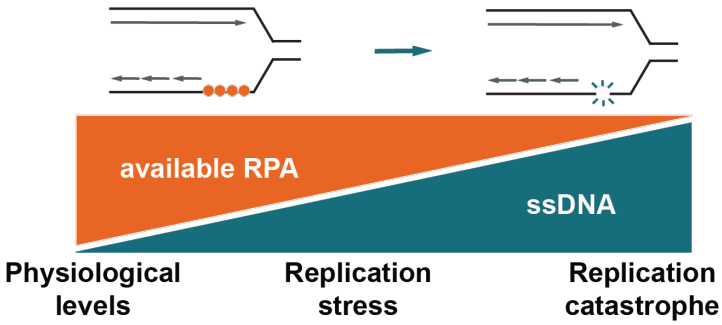

Figure 7: The nuclear pool of RPA determines the threshold of tolerable replication stress before ssDNA becomes exposed, ending in replication catastrophe.

1.3.3 Fork reversal

When the structure of a reversed fork was first visualized in budding yeast, it was thought to represent a terminally arrested fork (Sogo *et al*, 2002). After years of research, we have learned that fork reversal is not a form of irreversible fork arrest but represents an active process that enables repair and restart of stalled replication forks. During fork reversal, the parental strands re-anneal, bringing the newly synthesized daughter strands into a duplex (Neelsen & Lopes, 2015). This has two major advantages. (1) Any replication blocking lesion is now positioned in a DNA duplex which allows the use of a homologous template for error-free repair. (2) The replication fork is preserved in a more stable form until replication can

continue. Several factors including the SWI/SNF-related matrix-associated actin dependent regulator of chromatin subfamily A-like protein 1 (SMARCAL1), Zinc finger RANBP2-Type Containing 3 (ZRANB3), Helicase like transcription factor (HLTF) and RAD51 are involved in fork reversal **(Figure 8).** While SMARCAL1 binds to RPA which accumulates on ssDNA at stalled replication forks, ZRANB3 binds to PCNA upon its poly-ubiquitination by HLTF (Bhat *et al*, 2015; Bansbach *et al*, 2009; Ciccia *et al*, 2012). The known HR factor RAD51 can also promote fork reversal (Zellweger *et al*, 2015). The choice of fork reversal pathway is thought to depend on the structure of the replication intermediate (Kile *et al*, 2015; Bétous *et al*, 2013). Recent studies have shown that fork reversal is not completely abrogated upon depletion of both SMARCAL1 and ZRANB3, indicating that other factors promoting fork reversal exist (Taglialatela *et al*, 2017; Kolinjivadi *et al*, 2017).

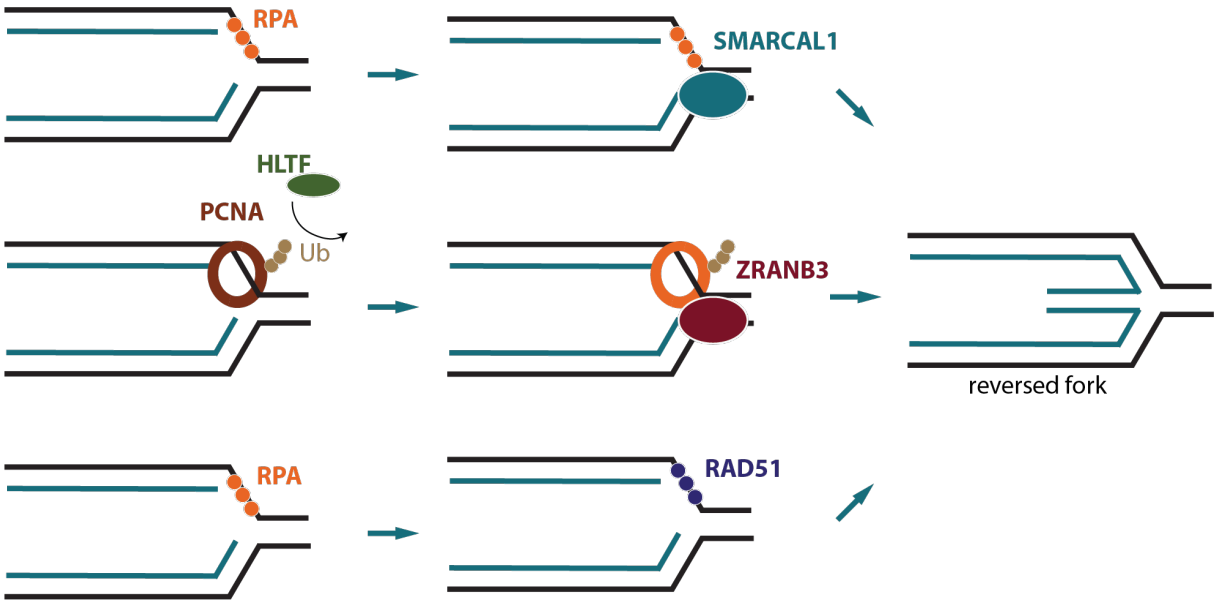

Figure 8: Three mechanisms of fork reversal. (Top) SMARCAL1 promotes fork reversal after binding to RPA. (Middle) ZRANB3 allows fork reversal after binding to PCNA which was poly-ubiquitinated by HLTF. (Bottom) RAD51 displaces RPA for fork reversal. Adapted from Quinet *et al*, 2017.

1.3.4 Fork protection

The annealed nascent strands in a reversed fork resemble a DSB and are therefore vulnerable entry points for nucleolytic degradation. Several factors of the HR pathway have been shown to protect reversed forks from nucleolytic attack, including BRCA1/2 and the recombinase RAD51 (Hashimoto *et al*, 2010). Indeed, extensive fork degradation was shown to be partially responsible for the sensitivity of BRCA deficient tumors to chemotherapeutic treatment. By loading RAD51 onto already formed regressed forks, BRCA1/2 ensure that the regressed arms are protected from MRE11 dependent resection (Schlacher *et al*, 2011; Ying *et al*, 2012) **(Figure 9).** After removal or bypass of the lesion, the fork can be restarted by the combined action of RECQ1 and WRN. In the absence of BRCA1/2, CtIP initiates resection by MRE11

and EXO1 (Lemaçon *et al*, 2017). This is followed by either extensive nucleolytic degradation behind the fork junction of fork restart, catalyzed by MUS81 structure-specific endonuclease subunit (MUS81) and DNA polymerase delta 3 accessory subunit (POLD3).

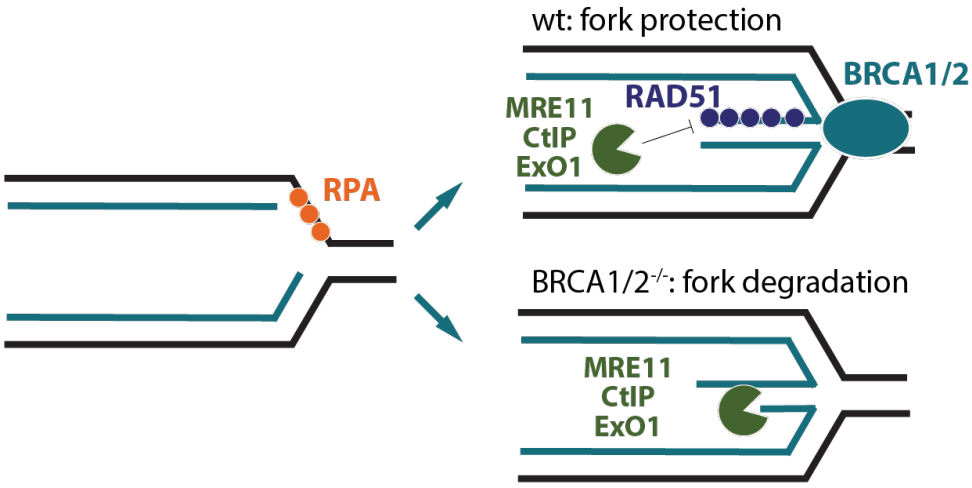

Figure 9: BRCA1/2 and RAD51 protect the reversed fork from nucleolytic degradation. Adapted from Quinet *et al*, 2017.

1.3.5 ssDNA and post-replicative repair

During normal fork progression, only a limited amount of ssDNA is continually generated on the lagging strand. However, if the replication fork faces an obstacle on the leading strand, uncoupling of the replisome and the helicase can expose larger regions of ssDNA. The unique enzyme Primase and DNA-directed Polymerase (PrimPol) can then initiate replication downstream of obstacles and extend its own DNA or RNA primers, leaving a ssDNA gap behind (García-Gómez *et al*, 2013; Wan *et al*, 2013). Obstacles on the lagging strand that interfere with the progression of replicative polymerases but not of the helicase, usually do not affect overall replication fork progression. Okazaki fragments downstream of the obstacle will allow continuing replication past the lesion, but, like repriming on the leading strand, leave a ssDNA gap behind. Such ssDNA regions that are formed in the process of replication but are not necessarily located in the vicinity of a replication forks during their recognition and repair, are termed “replication gaps” or “daughter strand gaps” (Cong *et al*, 2021; Wong *et al*, 2021) **(Figure 10).** As mentioned above, all kinds of ssDNA with a minimum length of around 30bp, were shown to be, at least temporarily, bound by the ssDNA binding protein RPA (Bhat & Cortez, 2018; Kim *et al*, 1994).

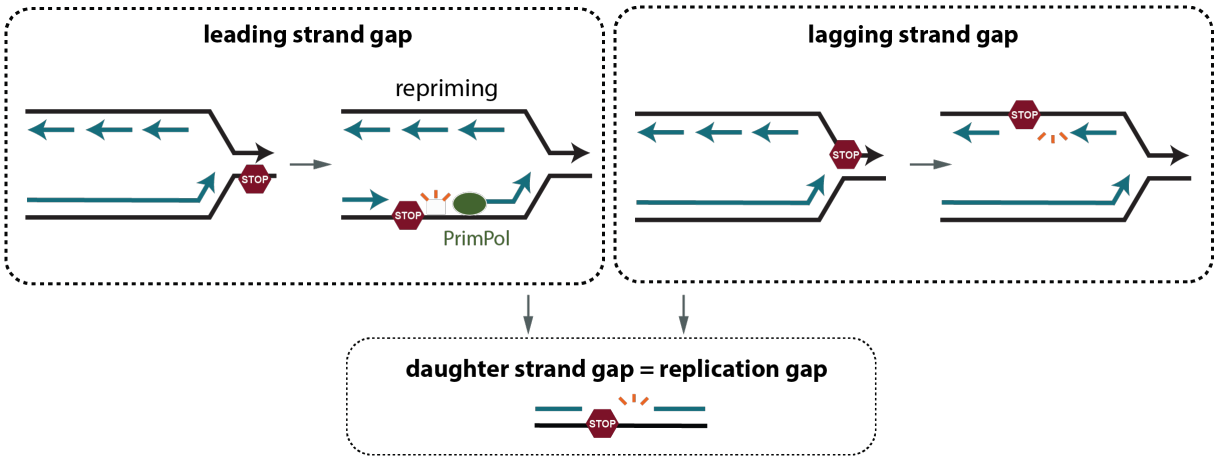

Figure 10: Replication fork obstacles on the leading and lagging strand of replication lead to the formation of daughter strand gaps, also called replication gaps.

Once a replication gap has been formed, there are two major pathways for post-replicative repair: template switching and translesion synthesis **(Figure 11).**

Template switching is initiated by post-translational modifications of PCNA, in specific SUMOylation and poly-ubiquitination (Hoege *et al*, 2002). First, the strand containing the DNA lesion anneals with the newly synthesized double stranded sister chromatid, forming a sister chromatin junction, like it is also found during HR (Giannattasio *et al*, 2014). This releases the newly synthesized undamaged strand which is used as a template to repair the lesion. Intrinsically, template switching is a precise repair mechanism as it does not introduce base modifications. However, it can also give rise to chromosomal rearrangements, when a non-sister chromatid is used as template (Branzei & Foiani, 2007).

Translesion synthesis is also initiated by post-translational modifications of PCNA, in specific mono-ubiquitination of lysine 164 (K164) by RAD18/RAD6 (Kannouche & Lehmann, 2004). This modification serves as a signal to switch between replicative and translesion polymerases, a highly conserved class of polymerases that are characterized by a shallow active site. While this allows non-native templates such as DNA lesions to enter the active site, it also comes at the cost of lower processivity and fidelity. Well known translesion polymerases are POLν, POLι, POLκ, REV1, POLθ, POLζ, POLη, each of which has its own specific substrate spectrum and properties. While translesion polymerases can replicate across a variety of DNA lesions including thymine glycols, intrastrand crosslinks and [6-4]photoproducts, replication gaps that are formed during replication stress, are also included in their substrate spectrum (Seki *et al*, 2004; Yoon *et al*, 2010; Belousova *et al*, 2010). Therefore, translesion synthesis is an important post-replicative DNA repair mechanism that mitigates the risk of replication catastrophe.

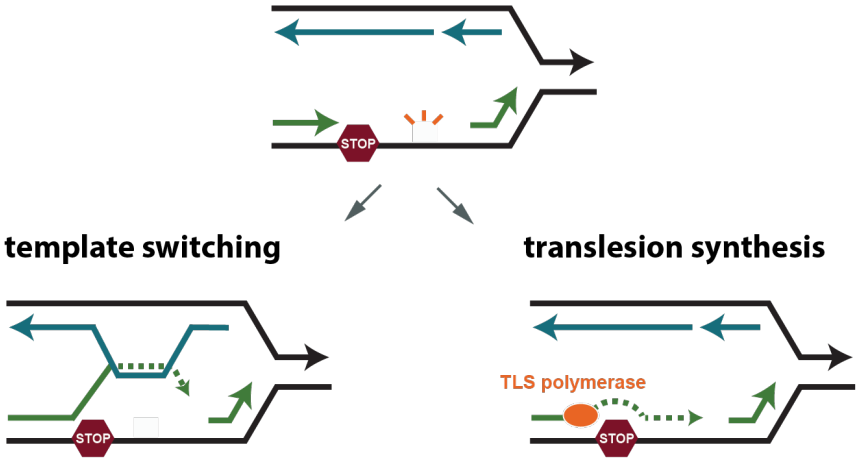

Figure 11: The two major pathways for post-replicative repair of daughter strand gaps are template switching and translesion synthesis. Adapted from Gao *et al*, 2017.

1.3.6 The translesion synthesis polymerase θ as a novel anti-cancer target: Prologue

A translesion synthesis polymerase that is currently attracting interest as a novel putative target in cancer therapy, is Polymerase θ (POLθ). POLθ has numerous functions within the DDR: it acts as a translesion polymerase but is also a central player in the DSB repair pathway TMEJ, as described in Section 1.2.1. Cells with mutations in the HR factors BRCA1 and BRCA2 are dependent on POLθ activity, making this protein an attractive target in HR deficient cancers (Ceccaldi *et al*, 2015; Mateos-Gomez *et al*, 2015). For this reason, numerous academic and industry groups are now developing compounds to specifically inhibit POLθ activity and the first clinical studies in BRCA mutant patients are ongoing (https://www.artios.com/press-release/artios-doses-first-patient-in-phase-1-2a-study-of-pol%ce%b8-inhibitor-art4215/). The following review article will focus on the diverse functions of POLθ within the DDR, its highly debated influence on genomic stability as well as the rationale behind targeting POLθ in cancer.

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Trends in Cancer

Review

Targeting the DNA Repair Enzyme Polymerase θ in Cancer Therapy

Anna Schrempf,[1,2] Jana Slyskova,[1,2,*] and Joanna I. Loizou[1,2,*]

Targeted cancer therapies represent a milestone towards personalized treatment as they function via inhibition of cancer-specific alterations. Polymerase θ (POLQ), an error-prone translesion polymerase, also involved in DNA double-strand break (DSB) repair, is often upregulated in cancer. POLQ is synthetic lethal with various DNA repair genes, including known cancer drivers such as *BRCA1/2*, making it essential in homologous recombination-deficient cancers. Thus, POLQ represents a promising target in cancer therapy and efforts for the development of POLQ inhibitors are actively underway with first clinical trials due to start in 2021. This review summarizes the journey of POLQ from a backup DNA repair enzyme to a promising therapeutic target for cancer treatment.

Highlights

POLQ is a versatile DNA repair enzyme that is central in TMEJ for the error-prone repair of DNA DSBs. POLQ also functions in other DNA repair pathways including base excision repair, interstrand crosslink repair, and DNA damage tolerance by translesion synthesis.

Cancer cells often acquire mutations in DNA repair genes, making them dependent on remaining DNA repair pathways. Dependence on TMEJ is characterized by an increased POLQ expression which is associated with poor patient prognosis.

Depletion of POLQ in POLQ-dependent cancers leads to synthetic lethality. This is well described for malignancies deficient in homologous recombination (e.g., due to mutations in *BRCA1* or *BRCA2*). Hence, the use of POLQ inhibitors might be a promising strategy for targeted cancer therapy.

POLQ: Exploiting a Cancer Vulnerability for Therapy

To increase efficiency and lower the burden of toxic side effects, a major goal of cancer therapy is to progress from a 'one-drug-fits-all' to an individualized treatment approach tailored to the tumor-specific molecular features. Two main targeted therapeutic strategies are currently utilized in cancer treatment, both exploiting cancer-specific vulnerabilities. In the first approach, therapeutic suppression of aberrantly upregulated oncogenes alleviates the growth advantage of cancer cells. The second approach is based on the phenomenon that genetic alterations acquired by tumor cells cause their dependency on other compensatory pathways, loss of which leads to **synthetic lethality** (see Glossary). Therefore, therapeutic inhibition of pathways that are synthetic lethal with a cancer-specific alteration evokes cellular death in tumor cells while leaving normal cells unharmed [1]. The recent advent of genome-wide genetic interaction studies has demonstrated the extensive number of synthetic lethal interactions in cancer, many of which can potentially be translated to targeted cancer therapies [2].

Cancer cells frequently acquire mutations in DNA repair genes and respond by rewiring their DNA repair network to utilize compensatory pathways for survival. Dependency on compensatory DNA repair pathways opens room for the development of cancer-specific small molecule inhibitors. A group of successful drugs that use this mode of action are poly(ADP-ribose) polymerase (PARP) inhibitors, approved for the treatment of BRCA-deficient cancers. The essentiality of PARP for cancer cells with loss-of-function mutations in BRCA1/2 is remarkable as such cancer cells are up to 1000 times more sensitive to PARP inhibitors than healthy cells [3,4]. Although challenges such as the acquisition of drug resistance need to be faced, the clinical success of inhibitory drugs targeting DNA repair enzymes is highly encouraging. In this context, the DNA-repair enzyme **polymerase θ** (POLQ) has received increasing attention. POLQ is upregulated in numerous cancers and its overexpression is associated with poor prognosis [5–9]. Moreover, synthetic lethal interactions between POLQ and multiple DNA repair genes, including factors involved in homologous recombination (such as BRCA1/2), have been identified [10–16]. For these reasons, POLQ inhibitors, currently in development in multiple biotech companies and laboratories, represent a promising cancer treatment strategy and are soon to be tested in clinical trials.

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In this review, we first focus on the unique protein structure that allows POLQ to fulfill its diverse roles. We further discuss conflicting evidence of whether POLQ suppresses or promotes genetic stability, given that it is an intrinsically error-prone DNA synthesis enzyme. Finally, we address why POLQ meets the criteria of a promising target in cancer therapy and summarize the state-of-the art in POLQ inhibitor development.

POLQ Structure and Function: A Versatile DNA Repair Enzyme with a Unique Domain Architecture

POLQ Is Central in POLQ-Mediated End Joining, a DNA Double-Strand Break Repair Pathway

POLQ is involved in the repair of DNA double-strand breaks (DSBs), the most cytotoxic type of DNA lesion. If unrepaired, DSBs can have deleterious consequences including genomic rearrangements and cell death. Therefore, a specialized network consisting of at least three pathways is responsible for their repair (Figure 1A). Most DSBs are repaired by **canonical nonhomologous end joining** (c-NHEJ), a pathway that directly religates DNA ends without extensive processing, by introducing small insertions and deletions at break sites [17]. In S and G2 phases of the cell cycle, when a sister chromatid is available, **homologous recombination** (HR) is favored as the only precise DSB repair pathway [18]. POLQ is involved in a third pathway [originally named alternative end joining (alt-EJ) or microhomology-mediated end joining (MMEJ)] that was later termed **polymerase theta-mediated end joining** (TMEJ) due to requirement of POLQ [19]. TMEJ is initiated by PARP1 recruitment to resected DNA-ends [20–22]. Upon activation by phosphorylated CtIP, 3′ overhangs are generated by helicases such as the MRE11–RAD50–NBS1 (MRN) complex. POLQ then binds to long single-stranded DNA (ssDNA) overhangs generated by 5′–3′ resection of DSBs and anneals sequences with 2–6 base pairs of microhomology to use them as primers for DNA synthesis [23–25]. The stabilized DNA ends are then ligated by LIG3–XRCC1 or LIG1 [26–28] (Figure 1A).

Repair by TMEJ is error prone and introduces characteristic sequence alterations, also called **mutational signatures** with two characteristic attributes. Firstly, since POLQ uses microhomologies for strand annealing and yet only a minority of DNA ends contain such regions, end resection is necessary to make microhomologies accessible. The end joining of resected DNA at microhomologous sequences may result in characteristic **microhomology-flanked deletions** [29]. Secondly, POLQ tends to abort template-dependent extension from an annealed microhomologous sequence and reanneal at secondary sequences. This results in short stretches of *de novo* DNA that resembles the sequence flanking the break, also called **templated insertions** [30]. Templated insertions can originate from the opposite strand (in *trans*) or from the same strand (in *cis*), when the protruding ssDNA snaps back on itself [24,31]. Most interestingly, templated insertions can be utilized to map genome-wide TMEJ activity and by doing so, TMEJ most likely contributes to a variety of loci mutated in human disorders, emphasizing TMEJ's role in the etiology of human diseases [30].

DNA DSB repair pathways are tightly regulated. In the G1 phase of the cell cycle, during which a sister chromatid for HR is unavailable, association of the highly abundant Ku-heterodimer and 53BP1 with free DNA ends inhibits end resection, thereby channeling repair towards c-NHEJ [32]. In G2 and S phases of the cell cycle, however, 53BP1 is removed from DNA ends by phosphorylated CtIP in complex with BRCA1 and MRN, thereby shifting the balance to favor HR. Since TMEJ and HR both require resected DNA ends, they directly compete with each other for the same substrate. POLQ appears to displace RAD51, a key HR factor, from ssDNA via a proposed RAD51-binding domain [12] and may also counteract RPA, another HR factor [33]. Furthermore, depletion of HR

Glossary

5′ deoxyribose phosphate lyase activity: the catalytic activity of cleaving the ribose phosphate linkage 5′ to an abasic site. Since dRP-lyase activity is usually preceded by a DNA-lyase that cleaves the ribose-phosphate linkage 3′ to the abasic site, dRP-lyase activity results in removal of the 5′ deoxyribose-5-phosphate at the abasic site.
Base excision repair: a repair pathway that is responsible for removing small, non-helix distorting base lesions such as alkylated, deaminated or oxidized bases.
Canonical non-homologous end joining: a DNA DSB repair pathway which, in contrast to HR, does not depend on a homologous repair template and joins the broken DNA ends after minimal modification.
Homologous recombination: an umbrella term for several pathways dedicated to the accurate repair of DNA DSBs using a homologous chromosome segment as a template.
Microhomology-flanked deletion: a characteristic scar that is introduced by TMEJ in DNA DSB repair. Since POLQ anneals sequences with microhomologies to prime DNA synthesis, the break point is characterized by a stretch of microhomology while the sequence that was originally between the microhomologies is lost.
Mutational signature: combinations of mutation types originating from the same mutational process, which can be endogenous (e.g., lack of a certain DNA repair pathway) or exogenous (e.g., exposure to UV light).
One-ended DNA DSB: a DNA DSB that only has one 5′ end and one 3′ end. Such a break is generated when DNA replication encounters a DNA single-strand break followed by replication fork collapse, or when the replication fork stalls and a nuclease cleaves one arm.
Polymerase θ: a DNA repair enzyme that acts in numerous DNA-repair pathways, most importantly in TMEJ. The only eukaryotic polymerase known to date that also contains a helicase domain.
Polymerase theta-mediated end joining: a DNA DSB repair pathway that depends on the activity of POLQ. This leaves a particular mutational signature that is characterized by microhomology-flanked deletions and/or templated insertions.

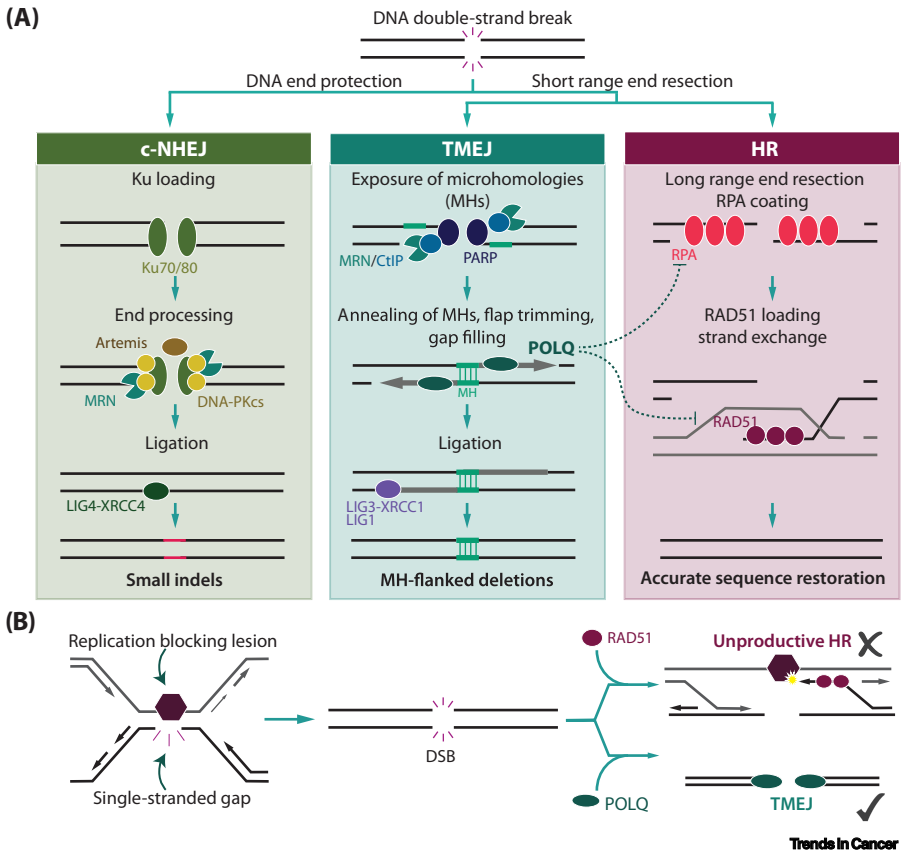

Figure 1. Roles of POLQ in DSB Repair. (A) DSBs can be repaired by three main pathways: c-NHEJ is characterized by DNA end protection by 53BP1 and Ku70/80 and DNA end processing by several factors including the MRN complex (MRE11–RAD50–NBS1) and Artemis. DNA-PKcs then recruits LIG4 with its scaffolding partner XRCC4 for ligation of processed ends. TMEJ and HR share the initial DNA end resection step. After recruitment of PARP, the MRN complex processes the DNA ends to generate 3′ overhangs. In TMEJ, POLQ anneals exposed sequences of microhomology, using them as a primer for DNA synthesis, followed by sealing of DNA ends by LIG3–XRCC1 or LIG1. In HR, the first short-range end-resection step is followed by long-range end resection and coating of 3′ single-stranded DNA with RPA. RAD51 then induces strand exchange using a homologous repair template for accurate restoration of the original DNA sequence. Competition between TMEJ and HR for resected DNA ends is highlighted by POLQ displacing RPA and RAD51 from ssDNA. (B) Certain types of DNA lesions depend on TMEJ for repair. Upon replication, replication-blocking lesions that are associated with regions of under-replicated DNA are converted into DSBs. Since the sister chromatid is unavailable as a repair template due to persistence of the replication blocking lesion, HR is unproductive, leaving TMEJ as the only remaining repair pathway available. Abbreviations: DNA-PKcs, DNA-dependent protein kinase catalytic subunit; DSB, DNA double-strand break; HR, homologous recombination; MH, microhomology; NHEJ, nonhomologous end joining; c-NHEJ, canonical NHEJ; PARP, poly(ADP-ribose)polymerase; POLQ, polymerase θ; TMEJ, polymerase theta-mediated end joining.

Synthetic lethality: the phenomenon that the combined loss of two genes causes cell death whereas the individual deficiency of either gene does not.

Templated insertion: a characteristic scar that is introduced by the action of TMEJ in DNA DSB repair. POLQ frequently aborts extension from one annealed sequence and reanneals at a secondary sequence to restart DNA synthesis, thereby generating small stretches that resemble the sequence around the DSB.

Terminal transferase: an enzyme that catalyzes the template-independent addition of nucleotides to the 3′ terminus of DNA.

Translesion synthesis polymerase: a specialized polymerase that can synthesize DNA opposite DNA lesions. The bypass of damaged DNA sites by translesion polymerases avoids stalling of replication forks.

proteins such as BRCA1, BRCA2, or RPA, increases the TMEJ-specific mutational signature suggesting that TMEJ factors are negatively regulated by HR factors [25].

Notably, due to its high mutagenicity, TMEJ has been considered merely a backup DNA repair pathway. However, it is becoming increasingly evident that TMEJ also functions in the presence of other DSB repair pathways and might be the only available pathway for specific types of DNA lesions [22]. Such lesions include collapsed replication forks with sister chromatids containing

replication-obstructing lesions (e.g., an interstrand crosslink) rendering them an unsuitable repair template for HR [34] (Figure 1B). In *Caenorhabditis elegans*, POLQ has been shown to be indispensable for repair of G4 quadruplex structures, thereby preventing genomic rearrangements at the expense of small deletions [19]. Future research is needed to assess the precise regulation of TMEJ and HR in order to identify the conditions and in particular the types of DNA lesions, that depend on TMEJ activity.

POLQ Is Involved in DNA Damage Tolerance and Repair Pathways beyond DSB Repair

Increasing evidence suggests that POLQ is involved in DNA damage tolerance and repair of lesions other than DSBs. POLQ can function as a **translesion synthesis polymerase** and thus incorporates nucleotides opposite apurinic/apyrimidinic sites, thymine glycols, and thymidine dimers [35–40]. In addition, POLQ has been shown to be important for replication and the repair of replication-associated lesions [41,42]. Depletion of POLQ results in decreased replication fork velocity and an increased amount of stalled replication forks upon treatment with hydroxyurea, a chemical used to induce replication fork stalling [12]. DNA single-strand breaks that are converted into DSBs upon encountering replication forks might also depend on repair by POLQ [11]. Furthermore, while POLQ appears to be essential for the repair of interstrand crosslinks (ICLs) in *Drosophila*, *Arabidopsis*, and *C. elegans* [43–45], most studies in mammalian systems demonstrate that POLQ is not required for this type of repair, potentially due to redundancy with other TLS polymerases [10,46]. A few exceptions have been reported: POLQ knockout (KO) mouse embryonic fibroblasts (MEFs) have been shown to be hypersensitive to mitomycin C, an ICL-inducing agent, and higher levels of micronuclei in response to mitomycin C were observed in POLQ mutant mice [15,47]. Finally, based on the presence of a weak **5′-deoxyribose phosphate lyase** activity in its polymerase domain, POLQ was suggested to act in **base excision repair** (BER) [48], although the extent of its involvement is a matter of debate [49–52]. In conclusion, POLQ is involved in multiple DNA repair pathways but deeper insights into both the variation between model organisms as well as the mechanistic function of POLQ in each pathway are lacking.

The Unique Domain Architecture of POLQ Enables Its Diverse Functions

POLQ encodes an A-family polymerase that contains both an N-terminal conserved superfamily 2 helicase domain and a C-terminal DNA polymerase domain, linked by an unstructured central region (Figure 2). As such, POLQ is the only eukaryotic polymerase known to date that contains a helicase domain. A coordinated interplay between all domains is necessary to allow for execution of POLQ activity [53] (Figure 2). The polymerase domain is responsible for DNA synthesis either using its **terminal transferase** or templated extension activity. Despite its low sequence conservation, the central domain appears to be important for regulating POLQ substrate selection. A mutant version of POLQ lacking its central domain can perform TMEJ on short ssDNA substrates (≤26 nucleotides) whereas full-length POLQ cannot [53]. Finally, the helicase domain contains both ATPase and helicase activity as well as proposed RAD51-binding motifs that may displace RAD51 from ssDNA in an ATPase-dependent manner [12,35,54–56]. This domain is required for performing TMEJ on longer ssDNA substrates since binding of the polymerase domain alone results in an unproductive snap-back mechanism [53]. In summary, POLQ contains a helicase domain capable of competing with HR for resected DNA-ends and a polymerase domain for strand annealing and extension, connected by a flexible central region.

POLQ and Genomic Stability: A Repair Enzyme That (De)stabilizes the Genome

Whether POLQ suppresses or promotes genomic instability is a matter of debate. Biochemical studies have shown that POLQ polymerase activity has low fidelity and its involvement in DSB repair frequently culminates in large deletions and templated insertions [29,30,57].

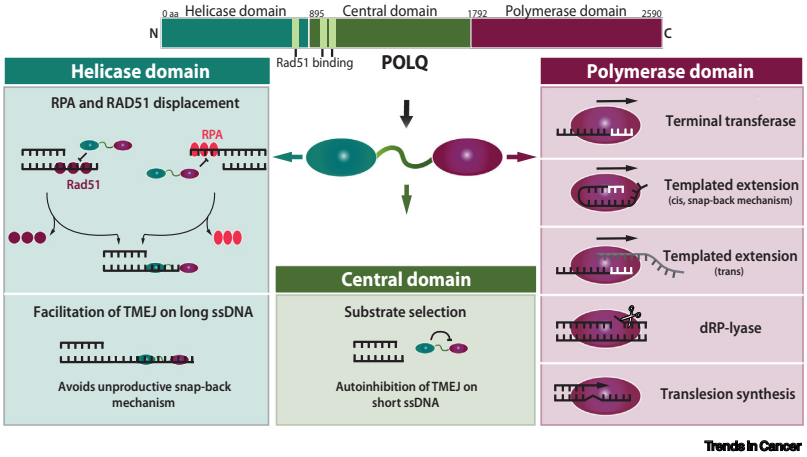

Figure 2. POLQ Has a Unique Domain Architecture Enabling Its Diverse Functions. POLQ consists of three domains: an N-terminal helicase domain that is linked to a C-terminal polymerase domain by an unstructured central region. Each domain fulfills specific functions that in combination contribute to the diverse functions of POLQ in DNA repair and damage tolerance pathways. The helicase domain (left panel) counteracts RPA and RAD51, thereby impeding repair by homologous recombination. In addition, helicase binding adjacent to the polymerase domain avoids an unproductive snap-back mechanism and therefore facilitates TMEJ on long ssDNA substrates. The central domain (middle panel) is vital for substrate selection as it autoinhibits POLQ activity on short ssDNA. The polymerase domain (right panel) of POLQ is a 'Swiss Army knife' in DNA repair: in TMEJ, it can function as a terminal transferase or catalyze templated extension from an annealed sequence using both the same strand snapped back on itself (in *cis*) or the other strand (in *trans*). In addition to double-strand break repair, the polymerase can function as a dRP-lyase in base excision repair and perform translesion synthesis opposite UV lesions. Abbreviations: dRP, 5′ deoxyribose phosphate; POLQ, polymerase θ; ssDNA, single stranded DNA; TMEJ, polymerase theta-mediated end joining.

Beyond *in vitro* systems, various studies performed in mouse and human systems have yielded conflicting findings both supporting and opposing its role as a guardian of genomic stability (Table 1). POLQ has been shown to protect genomic stability: its depletion increases DSB formation, exacerbates sensitivity to various genotoxic agents, and destabilizes replication forks [10,12,14,46,52,58]. Other studies, however, have reported that POLQ depletion decreases chromosomal translocations and UV-associated mutations and its overexpression increases DNA damage markers and impairs cell cycle progression [6,13,40]. Furthermore, POLQ overexpression in numerous cancer types including lung, bladder, ovarian, uterine, and breast cancer is associated with an increased mutation load and poor clinical outcome [5,6,12,59,60]. Along these lines, both a mutagenic effect of POLQ at the nucleotide level and a stabilizing effect at the chromosomal level have been described [40]. Here, POLQ was shown to be indispensable for mutagenic translesion synthesis opposite UV-induced lesions. However, upon UV exposure, POLQ-depleted cells acquired more chromosomal aberrations compared to wild-type (WT) cells, most likely as a consequence of reduced replication fork stability. Importantly, POLQ-deficient mice have an increased incidence of skin cancer, suggesting that POLQ promotes replication through UV-induced DNA lesions and therefore might prevent replication fork collapse. In the absence of translesion synthesis, unreplicated ssDNA might be converted into **one-ended double-strand breaks** and potentially chromosomal translocations, if not repaired properly [40]. This is in stark contrast to another study in which suppression of POLQ substantially decreased chromosomal translocations [13]. We hypothesize that the discrepancy between these two investigations originates from two main differences. (i) The protective effect of

Table 1. Does POLQ Promote or Suppress Genomic Stability?

POLQ status	Model system	Consequence	Refs
POLQ promotes genomic stability			
mut (S1932P, polymerase domain)	Mouse	- Increased spontaneous and radiation induced micronuclei in erythroblasts	[10,47]
KO	Mouse	- Sensitivity of clonal bone marrow stromal cells to IR and DSB-inducing agents (bleomycin, etoposide, ICRF-193 and camptothecin)	[46,51]
KO	Mouse embryonic fibroblasts	- Increased DSB formation - Reduced fork progression through UV lesions - Sensitivity to UV - Elevated sister chromatid exchanges - Elevated chromosomal aberrations - Elevated unreplicated ssDNA	[40]
KO	Mouse	- Increased incidence of skin cancer	[40]
KD	Human laryngeal cancer cell line (SQ20B), human cervical cancer cell line (HeLa)	- Sensitivity to IR - Increased IR-induced γH2AX foci	[52]
KO	Human bone osteosarcoma epithelial cells (U2OS)	- Sensitivity to cisplatin and IR	[14]
POLQ suppresses genomic stability			
Over-expression	Immortalized human lung fibroblasts (MRC5-SV)	- Accumulation in S-phase - Increased DNA damage markers (γH2AX, pCHK2) - Lower replication fork speed - Elevated chromosomal aberrations	[6]
KD	Mouse embryonic fibroblasts	- Decreased telomere fusions in the absence of the shelterin complex (Trf1/Trf2) and c-NHEJ factor Ku80	[13]
KD	Big blue mouse embryonic fibroblasts (BBMEFs)	- Reduced UV-induced mutations	[40]

HU, hydroxyurea; IR, ionizing radiation; KD, knockdown; mut, mutated.

POLQ most likely originates from its function in translesion synthesis which, although being intrinsically mutagenic, protects from cancer-driving chromosome rearrangements while the destabilizing effect likely stems from its role in TMEJ [13]. (ii) We speculate that the engagement of POLQ in the repair of one-ended and two-ended DSBs might differ substantially in its outcome.

POLQ is evolutionary conserved in metazoans and plants, illustrating its importance in genome stability. Despite its error-prone activity, repair by POLQ is often the safer option compared to other processes that act in its absence and potentially result in gross genomic aberrations. Understanding the role of POLQ in maintaining genome stability requires more in-depth studies and will provide more insight into whether POLQ-activity in various DNA repair and damage tolerance pathways is driving or protecting from tumorigenic progression.

POLQ and Cancer: A Novel Candidate for Targeted Cancer Therapy

POLQ Is Overexpressed in Cancer, Associated with a Characteristic Mutational Signature and Poor Prognosis

The overexpression of POLQ in a variety of malignancies, including those of colon, rectum, lung, stomach, breast, ovary and head and neck, sparked the interest in POLQ as a novel cancer target [5,6,8,9,12,61]. In breast and lung cancer, POLQ upregulation is linked to poor prognosis and shorter relapse-free survival of patients [7,9]; therefore, POLQ is included in a gene panel whose expression is used to predict cancer aggressiveness [62,63].

Whether POLQ overexpression is causative for cancer progression or occurs as a protective mechanism in genomically unstable cancer cells remains elusive. Since TMEJ activity is known to generate genomic translocations, it is intuitive to assume that POLQ upregulation contributes to carcinogenesis. Nonetheless, several arguments support a model in which POLQ expression is upregulated just after malignant transformation [12,64]. Based on several studies, we discuss two potential mechanisms that explain cancer-related POLQ upregulation. (i) The proliferative advantage of cells with elevated POLQ expression within the tumor might lead to their expansion in a Darwinian model. This model is supported by findings that high POLQ expression allows cancer cells to tolerate increased replication stress and might therefore increase tumor fitness [65]. (ii) POLQ expression might also be induced by a specific signaling mechanism. The depletion of HR genes was shown to increase POLQ expression and this could be reversed by complementation of HR factors, proposing a negative regulation of POLQ expression by the HR pathway [12]. As a direct link between HR deficiency and POLQ overexpression, depletion of BRCA1/2 is thought to upregulate FANCD2 which recruits POLQ to DNA lesions [64]. However, this is difficult to reconcile with the observation that FANCD2 and POLQ share a synthetic lethal relationship and further work is needed to clarify this interaction [12,66]. Furthermore, the tumor suppressor p53 influences POLQ expression, as shown by an up to 20-fold higher POLQ expression levels in *TP53* mutated cells compared to WT cells [67].

Another piece of evidence supporting HR-directed POLQ upregulation comes from the analysis of cancer genomes. POLQ-mediated repair translates into a particular mutational signature, which is increased in frequency in breast, ovarian, and pancreatic cancers, all associated with HR deficiency [68,69]. In addition, templated insertions, another feature of TMEJ activity, are more prevalent in genomes of breast cancer patients with *BRCA1/2* germline mutations [70]. Thus, it seems plausible that cancer cells lacking an intact HR pathway use POLQ-dependent repair as a compensatory mechanism to maintain genome stability.

POLQ Is Synthetic Lethal with Genes Frequently Mutated in Cancer

Depletion of POLQ in an HR-deficient background has been shown to impair cell viability, proposing a synthetic lethal relationship between POLQ and HR factors [10–13,66,71] (Table 2, depicting genes with a validated synthetic lethal or synthetic sick relationship with POLQ). Yet, the exact mechanism of the synthetic lethality between POLQ and HR factors is poorly characterized. We postulate two models to explain this; one focusing more on the role of POLQ in TMEJ (the pathway model) and one on its effect on RAD51 (the RAD51 model) (Figure 3). In the pathway model, an HR-deficient cancer relies on POLQ due to its activity in TMEJ. Continuous proliferation of cancer cells causes chronic replication stress and therefore an increased load of DSBs when collapsed replication forks are not resolved. While such DSBs would be repaired by HR in healthy cells, HR-deficient cancer cells depend on TMEJ for their repair. The observation that inhibitors of LIG3 and LIG1, both acting in TMEJ, synergize with PARP inhibitors in human breast cancer cell lines, supports this model [72]. In the RAD51 model, an HR-deficient cancer cell relies on POLQ due to its antirecombinase activity. Upon depletion of POLQ, the increased RAD51 activity in HR-deficient cells is cytotoxic by an unknown mechanism [12,71]. This model is supported by a series of sophisticated complementation studies in HR-deficient cells, where re-expression of POLQ lacking its RAD51-binding domain does not rescue cellular survival in POLQ-depleted, HR-deficient cancer cells to the extent of WT POLQ cells [12]. Furthermore, loss of RAD51 in a POLQ- and HR-deficient setting rescues cellular survival, suggesting that increased RAD51 activity is toxic to HR-deficient cells [12,71]. Yet, colony-formation assays of BRCA1-depleted cells lacking the RAD51-interaction domain of POLQ have shown that the interaction with RAD51 is dispensable for HR-deficient cells [33]. Both models potentially

Table 2. Validated POLQ Synthetic Lethal and Synthetic Sick Genes

POLQ synthetic lethal gene	Model system	Depletion of POLQ	Depletion of synthetic lethal gene	Double depletion phenotype reported	Refs
ATM	Mouse	LOF mutation (S1932P, polymerase domain)	LOF mutation	- Neonatal lethality - Growth retardation - Enhanced genomic instability	[10]
	Human ovarian cancer cell line (A2780)	KD	Inhibitor Ku55933	- Reduced cellular viability	[12]
ATR	Human bone osteosarcoma epithelial cells (U2OS)	KO	KD, inhibitor VE822	- Enhanced genomic instability - Reduced cellular viability	[11]
	Human breast cancer cell lines (BT-474, MDA-MB-436)	KO	Inhibitor VE822	- Reduced cellular viability	[11]
BRCA1	Mouse embryonic fibroblasts	KD	Cre-mediated KO	- Enhanced genomic instability - Reduced clonogenicity	[13]
	Human breast cancer cell lines (MCF7, HCC1937)	KD	LOF mutation	- Reduced clonogenicity	[13]
	Human breast cancer cell line (MDA-MB-436)	KD	LOF mutation	- Hypersensitivity to PARP-inhibitor rucaparib	[12]
	Human colon cancer cell line (HCT-116)	Inhibition	KD	- Reduced cellular viability	https://ir.ideayabio.com/news-events/presentations
	Genetically engineered mouse model	Inhibition	KO	- Reduced tumor growth	[71]
	Retinal pigmented epithelium cell line (RPE-1)	Inhibition	KO	- Reduced cellular viability - Hypersensitivity to PARP-inhibitor rucaparib	[71]
BRCA2	Mouse embryonic fibroblasts	KD	Cre-mediated KO	- Enhanced genomic instability - Reduced clonogenicity	[13]
	Human Fanconi anemia cell line (VU423)	KD	LOF mutation	- Increased chromosomal aberrations in response to MMC - Hypersensitivity to PARP inhibitor rucaparib	[12]
	Human lung cancer cell line (A549)	KD	KD	- Hypersensitivity to cisplatin and PARP-inhibitor BMN673	[66]
	Human colon cancer cell line (HCT-116)	Inhibition	KD	- Reduced cellular viability	https://ir.ideayabio.com/news-events/presentations
	Retinal pigmented epithelium cell line (RPE-1)	Inhibition	KO	- Reduced cellular viability	[71]
FANCD2	Xenotransplants of human ovarian cancer cell line (A2780)	KD	KD	- Hypersensitivity to cisplatin, MMC and PARP-inhibitor ABT-888 - Decreased tumor volume	[12]
	Mouse	KO	LOF mutation	- Most double mutants die neonatally - Congenital malformation - Premature death	[12]
	Mouse embryonic fibroblasts	KO	LOF mutation	- Hypersensitivity to PARP-inhibitor rucaparib	[12]
	Human lung cancer cell line (A549)	KD	KD	- Hypersensitivity to cisplatin and PARP-inhibitor BMN673	[66]

Table 2. (continued)

POLQ synthetic lethal gene	Model system	Depletion of POLQ	Depletion of synthetic lethal gene	Double depletion phenotype reported	Refs
Ku70	Mouse embryonic fibroblasts	KO	LOF mutation	- Reduced clonogenicity - Proliferative defect	[34]
RAD51C	Patient derived xenograft	Inhibition	Loss of expression	- Reduced tumor growth - Hypersensitivity to PARP-inhibitor olaparib	[71]
RAD52	Human bone osteosarcoma epithelial cells (U2OS)	LOF mutation (exon 16)	LOF mutation	- Reduced rate of replication fork progression	[14]
TP53BP1	Mouse embryonic fibroblasts	KO	LOF mutation	- Proliferative defect - Impaired cell cycle progression - Accumulation of non-productive HR-intermediates in S-phase	[15]

LOF, loss-of-function.

contribute to the synthetic lethal interaction between HR and POLQ: RAD51 channels repair pathway choice towards HR while POLQ counteracts this process by antagonizing RAD51. If HR is nonfunctional, diversion of pathway choice by RAD51 is detrimental and needs to be suppressed by POLQ. In the absence of RAD51, however, pathway choice is no longer diverted towards nonfunctional HR, making POLQ's antirecombinase activity dispensable.

Surprisingly, increasing evidence suggests that loss of POLQ can also be detrimental in the presence of a functional HR pathway, suggesting that HR is not able to fully compensate for TMEJ activity [15,65]. Poor c-NHEJ substrates that are also excluded from repair by HR; for example, collapsed replication forks with damaged sister chromatids might depend on TMEJ activity (Figure 1B). In line with this, high POLQ expression levels have been shown to protect from replication stress in the presence of functional HR, as shown by hypersensitivity to replication fork stalling agents upon POLQ depletion [11,65]. This holds promise for the use of POLQ inhibitors in HR-proficient cancers, particularly in combination with other drugs that exacerbate replication stress (e.g., ATR or topoisomerase inhibitors) [11].

In addition, POLQ has synthetic lethal interactions with genes beyond the HR pathway. A DNA damage response (DDR) focused CRISPR KO screen in POLQ-deficient MEFs, revealed that a surprisingly high number (45%) of the 309 analyzed murine DDR genes were synthetic lethal with POLQ [15]. The identified and validated POLQ-synthetic lethal genes function in numerous DDR pathways, including ICL repair, highlighted by hypersensitivity of POLQ KO MEFs to mitomycin C (MMC). Although it should be kept in mind that mouse cells are more prone to using TMEJ compared to human cells [73], this study positions POLQ at the center of a dense network of compensatory interactions that can be actively explored for expanding the set of cancers with POLQ dependency. In fact, some 30% of breast cancer cases in the Cancer Genome Atlas harbor mutations in POLQ synthetic lethal genes identified in this study, thereby significantly expanding the subset of POLQ-dependent cancers [15].

Development of POLQ Inhibitors

As POLQ activity is essential in HR-deficient cells, inhibition of POLQ is a promising cancer treatment strategy. The availability of crystal structures for both the helicase and the polymerase domain has been instrumental in the design of potent inhibitors [56,74]. Since both POLQ

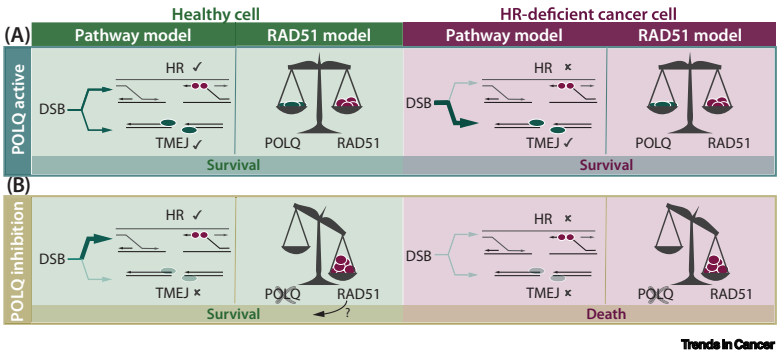

Figure 3. Two Models Explain POLQ Synthetic Lethality in HR-Deficient Cancers. (A) In the presence of functional POLQ, healthy cells can repair end-resected DSBs using both HR and TMEJ while HR-deficient cancer cells depend on POLQ for repair. (B) Upon POLQ inhibition, two models explain the hypersensitivity of HR-deficient cells: according to the pathway model, HR-deficient cancer cells have no remaining pathway for repair of end-resected DSBs resulting in cellular death. The RAD51 model, in contrast, suggests that increased RAD51 levels, caused by loss of RAD51 suppression by POLQ, drive synthetic lethality. It is unclear, however, why increased RAD51 levels are tolerated in the presence of HR. Abbreviations: DSB, DNA double-strand break; HR, homologous recombination; POLQ, polymerase θ; TMEJ, polymerase theta-mediated end-joining.

domains contain druggable sites, it remains elusive which domain is the preferred target [56]. BRCA1-depleted cells carrying inactivating mutations in the helicase or the polymerase domain show compromised growth compared to that of WT POLQ cells, suggesting that both enzymatic activities are essential in an HR-deficient background [33]. Complementation of POLQ lacking its helicase domain in HR-deficient cancers does not rescue viability to the extent of WT POLQ, proposing the helicase domain as an effective target site [12]. However, the helicase domain proved indispensable for translesion synthesis opposite UV lesions, which protects from the development of skin cancer in mice [40]. Further experiments are required to confirm this in human cells. The polymerase domain, however, is required to perform most of the TMEJ functions *in vitro*, arguing that targeting this domain will interfere with most of POLQ mediated functions in DSB repair [24]. In addition, both the polymerase and lyase enzymatic activities reside in a single nucleophilic residue within the polymerase domain, representing an enzymatic Achilles heel that might serve as an attractive target site given that the absence of both translesion synthesis as well as TMEJ activity of POLQ is not toxic to human cells [75].

To date, at least three independent biotech companies have invested into the development of POLQ inhibitors, starting with first clinical trials in 2021: IDEAYA Biosciences (San Francisco, USA), REPARE Therapeutics (Montreal, Canada) and Artios Pharma (Cambridge, UK). IDEAYA Biosciences introduced inhibitors with <10 nM potency directed against both helicase and polymerase domains while Artios Pharma's lead POLQ inhibitor program focuses on molecules targeting polymerase activity with another helicase inhibitor program in progress (https://ir.ideayabio.com/news-events/presentations). Numerous other companies have included POLQ in their pre-clinical research focused on synthetic lethality-based drug discovery. Recently, the antibiotic novobiocin has been identified to function as an inhibitor of POLQ helicase activity in an *in vitro* screen and as such being suppressive on HR-deficient cancer cell viability and tumor growth [71]. While treatment with this compound shows promising results both *in vitro* and *in vivo*, further work is required to investigate potential off-target effects, especially considering the high required drug doses.

Potential Use of POLQ Inhibitors in the Clinics

To ensure the clinical success of POLQ inhibitors, it is important to identify patient groups that would benefit from such an approach and synergistic drug combinations to potentiate the antiproliferative effect. Since POLQ dependency is best described in the context of HR deficiency, first clinical trials will most likely include patients with HR-deficient solid tumors (https://ir.ideayabio.com/news-events/presentations). Based on experimental data obtained *in vitro* and *in vivo*, patients harboring cancer-specific alterations in genes beyond the HR pathway might benefit from POLQ inhibitor treatment (Table 2). However, further research is needed to expand the repertoire of targetable synthetic lethal interactions of POLQ.

Novel POLQ inhibitors can be used as single agents or in combination with either classical chemotherapeutics or DNA repair inhibitors (Table 2) [12,66]. Despite the revolutionary efficacy of PARP inhibitors for the treatment of *BRCA*-mutated tumors, the clinical trial objective response rate is rarely above 50% and acquisition of drug resistance has been observed in most patients [76,77]. Depletion of POLQ via shRNA was shown to further sensitize HR-deficient cells to PARP inhibitors and combining PARP inhibition with POLQ inhibition also elevated antiproliferative effects as compared to each treatment alone (Table 2) [12,71] (https://ir.ideayabio.com/news-events/presentations). *BRCA* mutations in PARP-inhibitor-resistant cells often display a TMEJ-specific mutational signature, hence it is possible that POLQ might contribute to the acquisition of PARP inhibitor resistance [78]. In addition, resistance to PARP inhibitors can occur via loss of *53BP1*, a gene shown to be synthetic lethal with POLQ, thereby rendering these cells dependent on POLQ [15,79]. Therefore, using POLQ inhibitors in combination with PARP inhibitors, or as a second-line therapy, might prolong drug response and delay resistance acquisition [71,80]. It remains to be elucidated whether POLQ inhibition would also be beneficial for the treatment of cells that acquire PARP inhibitor resistance via other mechanisms, such as loss of the Shieldin complex [81].

Apart from PARP inhibitors, other drugs could potentially synergize with POLQ inhibitors and thus may be utilized independent of the HR functional status. Due to the involvement of POLQ in the resolution of replication associated lesions, POLQ-deficient cells are hypersensitive to the accumulation of DNA lesions at replication forks [11]. Consequently, combining POLQ inhibitors with ATR or topoisomerase inhibitors might represent a novel cancer treatment strategy. POLQ inhibitors might also synergize with traditional genotoxic agents as POLQ overexpression was identified as a resistance mechanism upon exposure of lung cancer cells to cisplatin [66]. Furthermore, p53-deficient cells use NHEJ and TMEJ to cope with therapy-induced DSBs. Therefore, POLQ inhibition reduces cellular viability after neocarzinostatin (a radiomimetic drug) treatment, especially in combination with DNA-PK inhibitors to suppress NHEJ [67]. Thus, POLQ inhibition might represent a synergistic treatment strategy also in HR-competent cancers, in combination with replication stress-inducing agents or in *TP53*-mutant cancers in combination with genotoxic agents and with NHEJ inhibitors.

Concluding Remarks and Future Perspectives

Recent work has highlighted the potential of POLQ as a novel target in the treatment of HR-deficient cancers and potentially also other cancer types. However, understanding the synthetic lethal environment of POLQ and its implications for cancer therapy represents an ongoing and important challenge for experimental and computational research (see Outstanding Questions). Learning about the individual contribution of each domain to POLQ function will not only provide more insight into POLQ biology but also aid potent inhibitor design, while minimizing toxic side effects. Furthermore, a topic of particular controversy is the effect of POLQ on genomic stability. It is unclear whether POLQ has a destabilizing effect on the genome due to its intrinsic

Outstanding Questions

Is TMEJ dispensable in cells that are proficient in other DSB repair pathways (such as c-NHEJ and HR) and if not, which types of DNA lesions depend on TMEJ?

Since both HR and TMEJ compete for resected DNA ends through several inhibitory interactions, which factors ultimately dictate pathway outcome?

What are the conditions that determine whether POLQ has a protective or a detrimental outcome on genome stability?

Does the repair outcome of TMEJ depend on whether it acts on one-ended DSBs (e.g., replication stress associated lesions) or two-ended DSBs (e.g., induced by endonucleases such as Cas9)?

Does POLQ overexpression act as a protective mechanism against cancer-associated genomic instability or is its overexpression causative for cancer progression?

Which mechanisms upregulate POLQ expression? Are POLQ overexpressing cancer cells selected in a Darwinian manner or do specific regulatory signaling mechanisms exist (or both)?

Which mechanisms cause cellular death upon depletion of POLQ in an HR-deficient background? Do HR-deficient cancers depend on POLQ due to its role in TMEJ (the pathway model; Figure 3) or due to its inhibitory effect on Rad51 (the RAD51 model; Figure 3)?

Does POLQ share synthetic lethal interactions with other genes other than those that function in HR that could be exploited for cancer therapy?

Which POLQ domain is the better drug target? Is it the polymerase domain which is nearly self-sufficient for most TMEJ functions or is it the helicase domain which contains important RAD51-inhibition binding sites?

Based on the observation that certain DNA lesions depend on TMEJ for repair, does inhibition of POLQ have toxic outcomes in healthy cells?

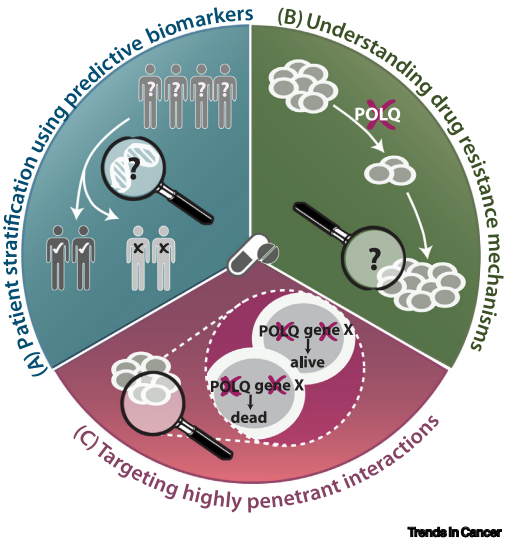

Figure 4. Clinical Challenges Associated with POLQ Inhibitors. To ensure clinical success of POLQ inhibitors and their safe and efficient applicability, three major areas of knowledge must be expanded. (A) Identification of patient biomarkers that predict outcomes of POLQ inhibition in particular tumor types. (B) Understanding of how cancer cells acquire resistance to POLQ inhibitors and how to address emerging resistance. (C) Identification of highly penetrant synthetic lethal interaction partners of POLQ to achieve sufficient efficiency despite tumor heterogeneity. Abbreviations: POLQ, polymerase θ.

What are predictive biomarkers that are indicative of the response to POLQ inhibition in cancer treatment? Can simple and scalable assays be developed to identify such biomarkers?

Which rational drug combinations with POLQ inhibitors can be used to prolong drug response and delay or prevent acquisition of treatment resistance?

What are the mechanisms of resistance to POLQ inhibitors and what are the second line therapies that could re-establish a treatment response?

mutagenicity or whether pathways involving POLQ are the only feasible repair option for numerous DNA lesions, including collapsed replication forks and G4 quadruplex structures.

Given that potent and specific POLQ inhibitors are moving into the clinics, a number of challenges remain and here much can be learnt from PARP inhibitors as approved drugs for DNA repair-targeted cancer therapy (Figure 4). A major challenge is the identification of predictive biomarkers, such as a common genomic or transcriptional signature, characterizing tumors that would respond to POLQ inhibition. Beyond using expensive next-generation sequencing strategies, the introduction of routine assays will simplify patient stratification. For PARP inhibitors, diagnostic tools have been introduced that quantify genomic signatures indicative of PARP inhibitor sensitivity.

Another hurdle common to most drugs used in cancer therapy is the acquisition of drug resistance. Apart from pharmacological resistance mechanisms shared between many drugs, such as upregulation of P-glycoprotein pumps, administration of POLQ inhibitors will most likely select for specific resistance mechanisms [82]. An anticipated resistance mechanism will be the restoration of HR, for example, by reversal mutations in BRCA, as has also been described for PARP inhibitors [78,83]. The identification of resistance mechanisms, as well as drug combinations for the treatment of resistant tumors, is key to adjusting cancer therapy in a timely manner.

For POLQ inhibitors being utilized as highly efficient chemotherapeutics, it is important to target highly penetrant synthetic lethal interactions; for example, using POLQ inhibitors in patients with mutations in genes that share a strong and highly penetrant synthetic lethal interaction with POLQ. Ideally, the synthetic lethal relationship between POLQ and the cancer-specific alteration (e.g., *BRCA2* mutation) is penetrant to an extent that tumor heterogeneity does not reduce the cancer-specific POLQ dependency. It remains to be seen whether the interactions between POLQ and its described synthetic lethal partners, such as HR factors, fulfil those criteria [84].

Finally, it is key to highlight that as small-molecule inhibitors targeting DNA repair enzymes, such as POLQ inhibitors, are emerging, a deeper mechanistic understanding of the rewiring of the DNA repair network will expand the repertoire of actionable therapeutic strategies to improve cancer treatment.

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References

1. Dobzhansky, T. (1946) Genetics of natural populations; recombination and variability in populations of Drosophila pseudoobscura. *Genetics* 31, 269–290
2. Han, K. *et al.* (2017) Synergistic drug combinations for cancer identified in a CRISPR screen for pairwise genetic interactions. *Nat. Biotechnol.* 35, 463–474
3. Farmer, H. *et al.* (2005) Targeting the DNA repair defect in BRCA mutant cells as a therapeutic strategy. *Nature* 434, 917–921
4. Bryant, H.E. *et al.* (2005) Specific killing of BRCA2-deficient tumours with inhibitors of poly(ADP-ribose) polymerase.[erratum appears in Nature. 2007 May 17;447(7142):346]. *Nature* 434, 913–917
5. Kawamura, K. *et al.* (2004) DNA polymerase θ is preferentially expressed in lymphoid tissues and upregulated in human cancers. *Int. J. Cancer* 109, 9–16
6. Lemée, F. *et al.* (2010) DNA polymerase θ up-regulation is associated with poor survival in breast cancer, perturbs DNA replication, and promotes genetic instability. *Proc. Natl. Acad. Sci. U. S. A.* 107, 13390–13395
7. Higgins, G.S. *et al.* (2010) Overexpression of POLQ confers a poor prognosis in early breast cancer patients. *Oncotarget* 1, 175–184
8. Lessa, R. *et al.* (2014) Adult height and head and neck cancer: a pooled analysis within the INHANCE Consortium. *Head Neck* 36, 1391
9. Allera-Moreau, C. *et al.* (2012) DNA replication stress response involving PLK1, CDC6, POLQ, RAD51 and CLASPIN upregulation prognoses the outcome of early/mid-stage non-small cell lung cancer patients. *Oncogenesis* 1, 1–10
10. Shima, N. *et al.* (2004) The mouse genomic instability mutation chaos1 is an allele of Polq that exhibits genetic interaction with Atm. *Mol. Cell. Biol.* 24, 10381–10389
11. Wang, Z. *et al.* (2019) DNA polymerase (POLQ) is important for repair of DNA double-strand breaks caused by fork collapse. *J. Biol. Chem.* 294, 3909–3919
12. Ceccaldi, R. *et al.* (2015) Homologous-recombination-deficient tumours are dependent on Polθ -mediated repair. *Nature* 518, 258–262
13. Mateos-Gomez, P.A. *et al.* (2015) Mammalian polymerase θ promotes alternative NHEJ and suppresses recombination. *Nature* 518, 254–257
14. Kelso, A.A. *et al.* (2019) Distinct roles of RAD52 and POLQ in chromosomal break repair and replication stress response. *PLoS Genet.* 15, 1–28
15. Feng, W. *et al.* (2019) Genetic determinants of cellular addiction to DNA polymerase theta. *Nat. Commun.* Published online September 19, 2019. https://doi.org/10.1038/s41467-019-12234-1
16. Mengwasser, K.E. *et al.* (2019) Genetic Screens Reveal FEN1 and APEX2 as BRCA2 Synthetic Lethal Targets. *Mol. Cell* 73, 885–899 e6
17. Chang, H.H.Y. *et al.* (2017) Non-homologous DNA end joining and alternative pathways to double-strand break repair. *Nat. Rev. Mol. Cell Biol.* 18, 495–506
18. Ranjha, L. *et al.* (2018) Main steps in DNA double-strand break repair: an introduction to homologous recombination and related processes. *Chromosoma* 127, 187–214
19. Koole, W. *et al.* (2014) A polymerase theta-dependent repair pathway suppresses extensive genomic instability at endogenous G4 DNA sites. *Nat. Commun.* 5, 1–10
20. Audebert, M. *et al.* (2004) Involvement of poly(ADP-ribose) polymerase-1 and XRCC1/DNA ligase III in an alternative route for DNA double-strand breaks rejoining. *J. Biol. Chem.* 279, 55117–55126
21. Wang, M. *et al.* (2006) PARP-1 and Ku compete for repair of DNA double strand breaks by distinct NHEJ pathways. *Nucleic Acids Res.* 34, 6170–6182
22. Truong, L.N. *et al.* (2013) Microhomology-mediated end joining and homologous recombination share the initial end resection step to repair DNA double-strand breaks in mammalian cells. *Proc. Natl. Acad. Sci. U. S. A.* 110, 7720–7725
23. Chan, S.H. *et al.* (2010) Dual roles for DNA polymerase theta in alternative end-joining repair of double-strand breaks in *Drosophila*. *PLoS Genet.* 6, 1–16
24. Kent, T. *et al.* (2015) Mechanism of microhomology-mediated end-joining promoted by human DNA polymerase θ. *Nat. Struct. Mol. Biol.* 22, 230–237
25. Ahrabi, S. *et al.* (2016) A role for human homologous recombination factors in suppressing microhomology-mediated end joining. *Nucleic Acids Res.* 44, 5743–5757
26. Simsek, D. *et al.* (2011) DNA ligase III promotes alternative non-homologous end-joining during chromosomal translocation formation. *PLoS Genet.* 7, 1–11
27. Liang, L. *et al.* (2008) Human DNA ligases I and III, but not ligase IV, are required for microhomology-mediated end joining of DNA double-strand breaks. *Nucleic Acids Res.* 36, 3297–3310
28. Lu, G. *et al.* (2016) Ligase I and ligase III mediate the DNA double-strand break ligation in alternative end-joining. *Proc. Natl. Acad. Sci. U. S. A.* 113, 1256–1260
29. Sallmyr, A. and Tomkinson, A.E. (2018) Repair of DNA double-strand breaks by mammalian alternative end-joining pathways. *J. Biol. Chem.* 293, 10536–10549
30. Schimmel, J. *et al.* (2019) Templated Insertions: A smoking gun for polymerase theta-mediated end joining. *Trends Genet.* 35, 632–644
31. Kent, T. *et al.* (2016) Polymerase θ is a robust terminal transferase that oscillates between three different mechanisms during end-joining. *Elife* 5, 1–25
32. Bunting, S.F. *et al.* (2010) 53BP1 inhibits homologous recombination in brca1-deficient cells by blocking resection of DNA breaks. *Cell* 141, 243–254
33. Mateos-Gomez, P.A. *et al.* (2017) The helicase domain of Polθ counteracts RPA to promote alt-NHEJ. *Nat. Struct. Mol. Biol.* 24, 1116–1123
34. Wyatt, D.W. *et al.* (2016) Essential roles for polymerase θ-mediated end joining in the repair of chromosome breaks. *Mol. Cell* 63, 662–673
35. Seki, M. *et al.* (2003) POLQ (Pol θ), a DNA polymerase and DNA-dependent ATPase in human cells. *Nucleic Acids Res.* 31, 6117–6126
36. Hogg, M. *et al.* (2004) Crystallographic snapshots of a replicative DNA polymerase encountering an abasic site. *EMBO J.* 23, 1483–1493
37. Hogg, M. *et al.* (2011) Lesion bypass activity of DNA polymerase θ (POLQ) is an intrinsic property of the pol domain and depends on unique sequence inserts. *J. Mol. Biol.* 405, 642–652

38. Kusumoto, R. *et al.* (2002) Translesion synthesis by human DNA polymerase η across thymine glycol lesions. *Biochemistry* 41, 6090–6099
39. Takata, K.I. *et al.* (2006) Human DNA polymerase N (POLN) is a low fidelity enzyme capable of error-free bypass of 5S-thymine glycol. *J. Biol. Chem.* 281, 23445–23455
40. Yoon, J.H. *et al.* (2019) Error-prone replication through UV Lesions by DNA polymerase θ protects against skin cancers. *Cell* 176, 1295–1309.e15
41. Alexander, J.L. *et al.* (2016) Multiple mechanisms contribute to double-strand break repair at rereplication forks in *Drosophila* follicle cells. *Proc. Natl. Acad. Sci. U. S. A.* 113, 13809–13814
42. Roerink, S.F. *et al.* (2014) Polymerase theta-mediated end joining of replication-associated DNA breaks in *C. elegans*. *Genome Res.* 24, 954–962
43. Beagan, K. *et al.* (2017) Drosophila DNA polymerase theta utilizes both helicase-like and polymerase domains during microhomology-mediated end joining and interstrand crosslink repair. *PLoS Genet.* 13, 1–19
44. Muzzini, D.M. *et al.* (2008) Caenorhabditis elegans POLQ-1 and HEL-308 function in two distinct DNA interstrand cross-link repair pathways. *DNA Repair (Amst)* 7, 941–950
45. Inagaki, S. *et al.* (2006) *Arabidopsis TEBICHI , with helicase and DNA polymerase domains, is required for regulated cell division and differentiation in meristems*. 18 pp. 879–892
46. Yousefzadeh, M.J. *et al.* (2014) Mechanism of suppression of chromosomal instability by DNA polymerase POLQ. *PLoS Genet.* 10, e1004654
47. Shima, N. *et al.* (2003) Phenotype-based identification of mouse chromosome instability mutants. *Genetics* 163, 1031–1040
48. Prasad, R. *et al.* (2009) Human DNA polymerase θ possesses 5′-dRP lyase activity and functions in single-nucleotide base excision repair *in vitro*. *Nucleic Acids Res.* 37, 1868–1877
49. Yoshimura, M. *et al.* (2006) Vertebrate POLQ and POLβ cooperate in base excision repair of oxidative DNA damage. *Mol. Cell* 24, 115–125
50. Ukai, A. *et al.* (2006) Role of DNA polymerase θ in tolerance of endogenous and exogenous DNA damage in mouse B cells. *Genes Cells* 11, 111–121
51. Arana, M.E. *et al.* (2008) Low-fidelity DNA synthesis by human DNA polymerase theta. *Nucleic Acids Res.* 36, 3847–3856
52. Higgins, G.S. *et al.* (2010) A small interfering RNA screen of genes involved in DNA repair identifies tumor-specific radiosensitization by POLQ knockdown. *Cancer Res.* 70, 2984–2993
53. Black, S.J. *et al.* (2019) Molecular basis of microhomology-mediated end-joining by purified full-length Polθ. *Nat. Commun.* 10 Published online September 27, 2019. https://doi.org/10.1038/s41467-019-12272-9
54. Büttner, K. *et al.* (2007) Structural basis for DNA duplex separation by a superfamily-2 helicase. *Nat. Struct. Mol. Biol.* 14, 647–652
55. Richards, J.D. *et al.* (2008) Structure of the DNA repair helicase Hel308 reveals DNA binding and autoinhibitory domains. *J. Biol. Chem.* 283, 5118–5126
56. Newman, J.A. *et al.* (2015) Structure of the helicase domain of DNA polymerase theta reveals a possible role in the microhomology-mediated end-joining pathway. *Structure* 23, 2319–2330
57. Seki, M. *et al.* (2004) High-efficiency bypass of DNA damage by human DNA polymerase Q. *EMBO J.* 23, 4484–4494
58. Goff, J.P. *et al.* (2009) Lack of DNA polymerase θ (POLQ) radiosensitizes bone marrow stromal cells *in vitro* and increases reticulocyte micronuclei after total-body irradiation. *Radiat. Res.* 172, 165–174
59. Shinmura, K. *et al.* (2019) POLQ overexpression is associated with an increased somatic mutation load and PLK4 overexpression in lung adenocarcinoma. *Cancers (Basel)* 11, 1–18
60. Bentley, J. *et al.* (2004) DNA double strand break repair in human bladder cancer is error prone and involves microhomology-associated end-joining. *Nucleic Acids Res.* 32, 5249–5259
61. Pillaire, M.J. *et al.* (2010) A DNA replication signature of progression and negative outcome in colorectal cancer. *Oncogene* 29, 876–887
62. Cazaux, C. and Hoffmann, J.-S. (2012) Signature for the diagnosis of cancer aggressiveness and genetic instability, WO 2012/156501 Al
63. Cazaux, C. and Hoffmann, J.-S. (2017) Signature for the diagnosis of lung cancer aggressiveness and genetic instability, US 9,90,556 B2
64. Kais, Z. *et al.* (2016) FANCD2 maintains fork stability in BRCA1/2-deficient tumors and promotes alternative end-joining DNA repair. *Cell Rep.* 15, 2488–2499
65. Goullet De Rugy, T. *et al.* (2016) Excess Polθ functions in response to replicative stress in homologous recombination-proficient cancer cells. *Biol. Open* 5, 1485–1492
66. Dai, C.H. *et al.* (2016) Co-inhibition of pol θ and HR genes efficiently synergize with cisplatin to suppress cisplatin-resistant lung cancer cells survival. *Oncotarget* 7, 65157–65170
67. Kumar, R.J. *et al.* (2020) Hyperactive end joining repair mediates resistance to DNA damaging therapy in p53- deficient cells. *bioRxiv*. Published online April 3, 2020. https://doi.org/10.1017/CBO9781107415324.004
68. Nik-Zainal, S. *et al.* (2016) Landscape of somatic mutations in 560 breast cancer whole-genome sequences. *Nature* 534, 47–54
69. Alexandrov, L.B. *et al.* (2013) Signatures of mutational processes in human cancer. *Nature* 500, 415–421
70. Carvajal-Garcia, J. *et al.* (2020) Mechanistic basis for microhomology identification and genome scarring by polymerase theta. *Proc. Natl. Acad. Sci. U. S. A.* 117, 8476–8485
71. Zhou, J. *et al.* (2020) Polymerase theta inhibition kills homologous recombination deficient tumors. *bioRxiv*. Published online May 26, 2020. https://doi.org/10.1101/2020.05.23.111658
72. Tobin, L.A. *et al.* (2012) Targeting abnormal DNA repair in therapy-resistant breast cancers. *Mol. Cancer Res.* 10, 96–107
73. Finnie, N.J. *et al.* (1995) DNA-dependent protein kinase activity is absent in xrs-6 cells: Implications for site-specific recombination and DNA double-strand break repair. *Proc. Natl. Acad. Sci. U. S. A.* 92, 320–324
74. Zahn, K.E. *et al.* (2015) Human DNA polymerase θ grasps the primer terminus to mediate DNA repair. *Nat. Struct. Mol. Biol.* 22, 304–311
75. Laverty, D.J. *et al.* (2018) Mechanistic insight through irreversible inhibition: DNA Polymerase θ uses a common active site for polymerase and lyase activities. *J. Am. Chem. Soc.* 140, 9034–9037
76. Livraghi, L. and Garber, J.E. (2015) PARP inhibitors in the management of breast cancer: current data and future prospects. *BMC Med.* 13, 1–16
77. Lord, C.J. and Ashworth, A. (2017) PARP inhibitors: synthetic lethality in the clinic. *Science (80-.)* 355, 62–75
78. Edwards, S.L. *et al.* (2008) Resistance to therapy caused by intragenic deletion in BRCA2. *Nature* 451, 1111–1115
79. Jaspers, J.E. *et al.* (2013) Loss of 53BP1 causes PARP inhibitor resistance in Brca1 -mutated mouse mammary tumors. *Cancer Disc.* 3, 68–81
80. Gogola, E. *et al.* (2019) Resistance to PARP inhibitors: lessons from preclinical models of BRCA-associated cancer. *Annu. Rev. Cancer Biol.* 3, 235–254
81. Dev, H. *et al.* (2018) Shieldin complex promotes DNA end-joining and counters homologous recombination in BRCA1-null cells. *Nat. Cell Biol.* 20, 954–965
82. Rottenberg, S. *et al.* (2008) High sensitivity of BRCA1-deficient mammary tumors to the PARP inhibitor AZD2281 alone and in combination with platinum drugs. *Proc. Natl. Acad. Sci. U. S. A.* 105, 17079–17084
83. Sakai, W. *et al.* (2008) Secondary mutations as a mechanism of cisplatin resistance in BRCA2-mutated cancers. *Nature* 451, 1116–1120
84. Ryan, C.J. *et al.* (2018) Synthetic lethality and cancer – penetrance as the major barrier. *Trends Cancer* 4, 671–683

2 Chapter 2: Aims of this thesis

The discovery of synthetic lethal interactions within the DDR has opened new avenues for DDR-targeted therapies. The clinically most advanced target is PARP which becomes essential in the absence of HR factors such as BRCA1/2 and is actively exploited in tumors carrying loss-of-function mutations in these genes (Lord & Ashworth, 2017). One common model explaining the dependency of BRCA1/2-mutant cells on PARP activity is based on the role of PARP in DNA single-strand break repair. Upon inhibition, PARP can no longer undergo auto-poly-(ADP-ribosyl)ation (PARylation) and remains bound to DNA. If an incoming replication fork collides with the trapped PARP enzyme, the single-strand break is converted into a DSB which cannot be processed in the absence of a functional HR pathway, therefore driving cell death.

Only recently, a completely novel role of PARP in replication fork stability was identified. PARP was shown to participate in replication by signaling single-stranded gaps that occur during Okazaki fragment generation on the lagging strand (Hanzlikova *et al*, 2018; Vaitsiankova *et al*, 2022). Upon PARP inhibition, replication gaps accumulate on the lagging strand which are bound by RPA to protect them from nucleolytic attack. However, if single-stranded DNA levels exhaust the nuclear RPA pool, unprotected single-stranded DNA is exposed to nucleolytic attack in a process called replication catastrophe (Cong *et al*, 2021). Considering that BRCA1/2 are involved in suppression of single-stranded DNA gaps, this newly discovered role of PARP might provide an alternative explanation for the hypersensitivity of BRCA mutant cells to PARP inhibition (Cong *et al*, 2021; Panzarino *et al*, 2021).

Unlike PARP inhibitors, the underlying mechanisms explaining the synthetic lethal relationship between BRCA1/2 and POLθ are only partially understood. As POLθ participates in DSB repair, one model proposes that DSBs in cells harboring mutations in BRCA1/2 depend on TMEJ for repair, which requires POLθ for microhomology annealing and gap filling. Considering that this enzyme also possesses translesion polymerase activity, POLθ could also be involved in gap filling of single-stranded gaps on the lagging strand during replication. In this study, we aim to:

1. Understand the molecular mechanisms underlying the synthetic lethality of POLθ loss in BRCA1 deficient cancer cells.
2. Identify modulators of POLθ inhibition in BRCA1 mutant cells that might be clinically relevant.

3 Chapter 3: Results

3.1 Prologue

In this study, we sought to identify the molecular mechanisms underlying the synthetic lethality of POLθ loss in BRCA1 deficient cells. To model POLθ loss, we used a patented compound that targets the polymerase domain of POLθ. We could recapitulate that POLθ loss confers sensitivity in cells carrying loss of function mutations in the HR factor BRCA1, using both an isogenic cellular model system of retinal pigment epithelial cells that are immortalized with human telomerase (hTERT-RPE1) as well as a triple negative breast cancer cell line (SUM149PT). Furthermore, we observed a profound increase of single-stranded DNA upon POLθ inhibition in the context of BRCA1 deficiency, supporting the hypothesis that POLθ is involved in filling of those gaps. The increased load of unfilled gaps results in replication fork stalling, as exemplified by increased replication fork asymmetry in DNA fiber assays. In addition, increased replication gaps drive a replication stress phenotype with elevated levels of chromatin-bound RPA and γH2AX, which can be exacerbated by combined treatment with an ATR inhibitor. Finally, a genome-wide CRISPR screen allowed identification of modulators of the BRCA1-POLθ genetic interaction, including members of the MRN-complex that are involved in single-stranded DNA processing, as well as cell cycle regulators, that modify entry into S-phase where POLθ inhibition is most toxic.

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3.2 PDF of the article

POLθ processes ssDNA gaps and promotes replication fork progression in BRCA1 deficient cells

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Summary

POLθ is an error prone DNA polymerase whose loss is synthetic lethal in cancer cells bearing *BRCA1/2* mutations. To investigate the basis of this genetic interaction, we utilize a small molecule inhibitor targeting the POLθ polymerase domain. We find that POLθ processes ssDNA gaps that emerge in the absence of BRCA1, thus promoting unperturbed replication fork progression and survival of BRCA1 mutant cells. To uncover suppressors of the functional interaction between POLθ and BRCA1, we perform a genome-scale CRISPR-Cas9 knockout screen and identify NBN, a component of the MRN complex, and cell cycle regulators such as CDK6. While the MRN complex nucleolytically processes ssDNA gaps, CDK6 drives cell cycle progression, thereby exacerbating replication stress, a defining feature of BRCA1 deficient cells that lack POLθ activity. Thus, ssDNA gap formation, modulated by cell cycle regulators and MRN complex activity, underlies the synthetic lethality between POLθ and BRCA1, an important insight for ongoing clinical trials with POLθ inhibitors.

Introduction

Mutations in breast cancer susceptibility proteins 1 and 2 (*BRCA1/2)* are the most common genetic predisposition associated with familial breast and ovarian cancer (Apostolou and Fostira, 2013). Thus, there is a growing interest in identifying synthetic lethal interactions of BRCA1/2 which can be exploited for targeted therapy, exemplified by the exquisite hypersensitivity of BRCA1/2 deficient cells to inhibitors of poly(ADP-ribose) polymerases (PARP). BRCA1/2 function in homologous recombination (HR), a DNA double-strand break (DSB) repair pathway that utilizes the sister chromatid for precise repair. BRCA1/2 are indispensable for replication fork protection, a function that is independent of DSB repair (Hashimoto et al., 2010; Chaudhuri et al., 2016). Specifically, these factors facilitate RAD51 loading onto nascent DNA, hence protecting stalled replication forks from collapse. In the absence of BRCA1/2, loss of RAD51 loading as well as unrestrained replication lead to the formation of single stranded DNA (ssDNA) (Hashimoto et al., 2010; Kolinjivadi et al., 2017; Taglialatela et al., 2017, 2021). Recent lines of research have shown that such ssDNA regions are fundamental to the hypersensitivity of BRCA1/2 deficient cells to PARP inhibition (Cong et al., 2021; Dias et al., 2021). This can be explained by a role of PARP1/2 in sensing of unprocessed Okazaki fragments that form during discontinuous replication of lagging strands (Hanzlikova et al., 2018). Excessive ssDNA formation in cells lacking both PARP1/2 and BRCA1/2 activity ultimately exhausts cellular replication protein A (RPA), causing cell death by replication catastrophe (Toledo et al., 2013; Cong et al., 2021; Panzarino et al., 2021). This suggests that replication gaps underlie BRCA deficiency and are fundamental to PARP inhibitor response (Cong et al., 2021; Dias et al., 2021; Panzarino et al., 2021).

Despite the clinical success of PARP inhibitors in the treatment of BRCA1/2 mutant cancers, the emergence of resistance is common, underlining the urgency to identify novel targets that can be exploited in resistant tumors (Lord and Ashworth, 2013). In this context, the error-prone DNA polymerase, Polymerase Theta (POLθ), has received considerable interest as a potential drug target (Ceccaldi et al., 2015; Mateos-Gomez et al., 2015). POLθ is a versatile enzyme which contains both a helicase and a polymerase domain. POLθ is involved in numerous DNA repair pathways including Polymerase Theta Mediated End Joining (TMEJ), an error-prone pathway that seals resected DNA DSB ends, introducing characteristic microhomology-flanked deletions (Koole et al., 2014). Beyond DSB repair, POLθ functions as a translesion synthesis polymerase, to process helix distorting lesions such as those generated by UV radiation (Yoon et al., 2019).

The genetic interaction between BRCA1 and POLθ is believed to be a result of their converging roles in DNA DSB repair (Ceccaldi et al., 2015; Mateos-Gomez et al., 2015). Resected DNA DSBs which cannot be processed by HR in the context of BRCA1/2 deficiency, are thought to be processed by POLθ-dependent TMEJ. The dependency on TMEJ in HR deficient cells has contributed to the interest in POLθ as an anti-cancer target (Schrempf et al., 2021). Recently, two first-in-class POLθ inhibitors (POLθi) with *in vivo* efficacy have been reported that potentially represent a valuable therapeutic approach for the treatment of BRCA1/2 deficient cancers (Zatreanu et al., 2021; Zhou et al., 2021). However, although ssDNA gaps are now considered to be the major lesions that drive cell death in BRCA1/2 deficient cells treated with PARP inhibitors or cisplatin, it remains unclear whether ssDNA formation contributes to the genetic interaction between BRCA1/2 and POLθ.

In this study, we show that loss of POLθ activity in the context of BRCA1 deficiency exposes ssDNA, which causes replication stress and deregulation of S-phase progression. In a genome-wide CRISPR-Cas9 knockout (ko) screen, we uncover modulators of the functional interaction between BRCA1 and POLθ including the MRN complex and the cycle regulator CDK6. We find that the MRN and CDK6 activity promote POLθi-induced DNA damage by two distinct cellular processes. Loss of MRN-complex activity suppresses nucleolytic processing of gaps whereas CDK6 loss reduces replication stress by diminishing entry into S-phase. Thus, we define a role for POLθ in suppressing the accumulation of ssDNA gaps, a hallmark that is fundamental to its synthetic lethality with BRCA1, and uncover molecular factors that modulate this interaction.

Results

POLθ processes ssDNA gaps generated in BRCA1 deficient cells

Although loss of POLθ activity is known to be synthetic lethal with BRCA1 deficiency, the basis of this genetic interaction is not fully understood (Ceccaldi et al., 2015; Mateos-Gomez et al., 2015). To address this question, we used a small-molecule inhibitor of POLθ (termed POLθi) based on the structures of a recently published POLθi patent family (Blencowe et al., 2020a) (**Supp Fig 1A**). Through computational docking, we show that the POLθi binds to an allosteric pocket in the thumb subdomain of POLθ (**Fig 1A**) and subsequently inhibits its polymerase activity (see accompanying manuscript). We tested the inhibitor using an isogenic pair of p53 mutant human TERT-immortalized retinal pigment epithelial (RPE1) cells deficient or proficient for BRCA1 (denoted RPE1 *BRCA1*$^{-/-}$ *TP53*$^{-/-}$ and RPE1 *TP53*$^{-/-}$, respectively) (**Supp Fig 1B-C**). As expected, the BRCA1 deficient cells were hypersensitive to increasing concentrations of POLθi compared to their BRCA1 wild type counterparts (**Fig 1B, Supp Fig 1D**). In addition, POLθi increased the sensitivity of RPE1 *TP53*$^{-/-}$ cells to the DSB-inducing agent etoposide in a dose-dependent manner, in line with POLθ functioning in DSB repair (**Supp Fig 1F**).

The accumulation of ssDNA gaps has been reported in BRCA1 deficient cells (Hanzlikova et al., 2018; Cong et al., 2021; Panzarino et al., 2021; Taglialatela et al., 2021). While it is known that PARP1 signals the presence of these gaps (Hanzlikova et al., 2018), the processing steps that lie downstream of this signaling are still under debate. To determine if loss of POLθ activity affects the processing of ssDNA gaps in BRCA1 deficient cells, we utilized the triple negative breast cancer cell line SUM149PT, which harbors a hemizygous BRCA1 frameshift mutation resulting in a partially defective BRCA1 protein and hypersensitivity to PARP and POLθ inhibition (**Supp Fig 1B-E**). To visualize ssDNA, cells were pre-labelled with the nucleoside analogue BrdU, which was detected by immunofluorescence under native conditions. When making the entire genome accessible to the BrdU antibody by denaturing the cells prior to immunofluorescence, the BrdU signal was dramatically increased, providing a quality control for robust integration of nucleoside analogue (**Supp Fig 1G**). Under native conditions, we observed elevated ssDNA levels following POLθi as quantified by an increase in BrdU foci (**Fig 1C-D**). The BrdU foci overlapped with chromatin-bound RPA32, another marker of ssDNA, supporting the specificity of the staining (**Fig 1C**). Cells with increased numbers of BrdU foci displayed an increased nuclear size, potentially suggesting an effect of increased ssDNA gap formation on cell cycle progression (**Supp Fig 1H-I**).

To understand whether POLθi induced ssDNA is generated at replication forks, we used a modified DNA fiber assay which includes S1 nuclease treatment. Cells were labelled with the nucleoside analogues CldU and IdU followed by incubation with S1 nuclease to digest regions of ssDNA (Quinet et al.,2017) (**Fig 1E**). Under vehicle treatment, S1 nuclease incubation did not affect the length of labelled tracks in RPE1 *TP53*$^{-/-}$ cells, whereas RPE1 *BRCA1*$^{-/-}$ *TP53*$^{-/-}$ cells displayed shortened tracks, supporting an increased ssDNA burden in untreated BRCA1 deficient cells (**Fig 1E-G**). POLθ inhibition led to a further decrease in track length specifically in BRCA1 deficient cells indicating increased formation of replication gaps upon loss of POLθ (**Fig 1G**). These results suggest that ssDNA gaps are processed by POLθ in the absence of BRCA1.

To address the consequences of unfilled ssDNA gaps on replication fork progression, we assessed fork dynamics under POLθ inhibition. Independently of BRCA1 status, inhibition of POLθ resulted in decreased fork speed, as previously described (Ceccaldi et al., 2015) (**Fig 1H-I**). Moreover, POLθ inhibition induced a significant decrease in the symmetry of IdU and CldU labeled tracks, specifically in BRCA1 deficient cells, indicating fork stalling (**Fig 1J**). Taken together, our findings reveal a novel function of POLθ in replication gap filling, which facilitates unperturbed replication fork progression in BRCA1 deficient cells.

POLQ inhibition induced replication stress is exacerbated in BRCA1 deficient cells

We next sought to identify the cellular consequences of impaired replication fork progression in the absence of POLθ. After treatment with replication stress-inducing agents, RPA has been shown to coat regions of ssDNA to protect it from nucleolytic attack. When RPA levels become limiting, unprotected ssDNA is converted to DSBs, which are signaled by γH2AX (Toledo et al., 2013). To determine if cells lacking POLθ activity exhibit elevated levels of replication stress, we quantified γH2AX as well as chromatin-bound RPA70 in RPE1 *TP53*$^{-/-}$, RPE1 *BRCA1*$^{-/-}$ *TP53*$^{-/-}$ and SUM149PT cells. POLθ inhibition increased the percentage of cells positive for markers of ssDNA and DSBs **(Fig2A-B, Supp Fig 2A-B),** suggesting that the compromised replication fork integrity in the absence of POLθ affects overall replication stress levels. Given the specific sensitivity of BRCA1 deficient cells to POLθ inhibition, we asked whether the observed replication stress is less tolerated in the absence of BRCA1. Indeed, RPE1 *BRCA1*$^{-/-}$ *TP53*$^{-/-}$ showed increased phosphorylation of RPA32 at serine 4/8, a known replication stress signal, at later time points after POLθ inhibition, indicating that replication

stress induced by POLθ inhibition is poorly resolved in BRCA1 deficient cells **(Fig 2C, Supp Fig 2C).**

We next determined whether increased ssDNA gaps and replication stress impact cell cycle progression of cells lacking POLθ activity. Thus, we synchronized RPE1 cells in the G2/M phase of the cell cycle using nocodazole, followed by release into medium containing DMSO or POLθi. At early time points after release, POLθ inhibition induced a delay of S-phase entry in both cell lines. However, while the cell cycle profile of POLθi and DMSO treated RPE1 *TP53*$^{-/-}$ is indistinguishable at 12 hours after release, POLθ inhibition causes a consistent delay of S-phase entry in BRCA1 deficient cells also at later time points **(Fig 2D).**

Considering the elevated levels of ssDNA and replication stress in BRCA1 deficient cells under POLθ inhibition, we determined whether POLθi treatment would synergize with replication stress inducing agents. Given that the kinase ATR functions to suppress replication stress by reducing origin firing, thereby limiting the formation of ssDNA, we tested POLθ in combination with the ATR inhibitor ceralasertib. Indeed, a sublethal dose of ceralasertib specifically sensitized RPE1 *BRCA1*$^{-/-}$ *TP53*$^{-/-}$ to POLθi **(Fig 2E)**. The above data suggest that lack of POLθ activity leads to replication stress, causing prolonged replication stress signaling and defects in cell cycle progression in BRCA1 deficient cells which can be exacerbated with ATR inhibitor co-treatment.

A genome-wide CRISPR-Cas9 ko screen identified modulators of the BRCA1-POLθ interaction

POLθ is an emerging drug target in BRCA1/2 deficient cancers (Schrempf et al., 2021). Therefore, understanding the cellular factors which impact on this genetic interaction might inform on clinically relevant drug modulators. To this end, we performed a genome-wide CRISPR-Cas9 ko screen in a cellular model of breast cancer, a cancer type which is included in ongoing clinical trials with POLθ inhibitors (*A Study of ART4215 for the Treatment of Advanced or Metastatic Solid Tumors*, ClinicalTrial.gov Identifier: NCT04991480). We transduced SUM149PT cells with a lentiviral pool encoding the genome-wide Toronto v3 guide RNA (gRNA) library (TKOv3) (Hart et al., 2017) and selected transduced cells with puromycin. To enrich for suppressors of drug response, transduced cells were exposed to 2-2.5μM of POLθi, a dose determined to kill 90% of the cell population (LD_{90}) or DMSO for 18 days **(Supp Fig 3A-B).** Next, we extracted genomic DNA with integrated gRNA sequences and used next generation sequencing (NGS) to identify gRNA abundances **(Fig 3A).** For quality control, we compared gRNA abundance between end (day 18 of treatment) and early time point samples

which were collected five days after puromycin selection. gRNAs targeting essential genes were depleted in the late time point of both DMSO and POLθi treated screens whereas control gRNAs such as those targeting olfactory receptor genes had no effect on cellular survival **(Supp Fig 3C-D).** To identify sensitizers of POLθi, we focused on gRNAs which are depleted in POLθi treated compared to DMSO treated samples. As expected, gRNAs targeting HR pathway factors, such as RAD51C, were among the top depleted hits (**Supp Fig 3E**). Moreover, gRNAs targeting several factors of the Fanconi Anemia pathway, which is involved in replication fork stabilization beyond its role in inter-strand crosslink repair, were also depleted (**Supp Fig 3E**).

To determine genes that facilitate the functional interaction between POLθ and BRCA1, we focused on enriched gRNAs (**Fig 3B**). This led to the identification of NBN, a component of the MRN complex reported to function in replication fork stability (Hashimoto et al., 2010). Interestingly, we saw an enrichment of cell cycle regulators, including CDK6, a target that is currently exploited in breast cancer therapy, through the use of CDK4/6 inhibitors (Fry et al., 2004). To annotate the top enriched biological processes among significant positive hits ($p < 0.005$), we used the DAVID GO-term analysis tool (Huang et al., 2009a, 2009b), identifying the terms 'Cell cycle' and 'Cell division' as top enriched terms **(Fig 3C).** In summary, an unbiased genome-wide CRISPR-Cas9 ko screen identified the MRN complex and cell cycle regulators as modulators of the genetic interaction between BRCA1 and POLθ.

Loss of activity of the MRN complex and CDK6 alleviate the functional interaction between BRCA1 and POLθ

To investigate the molecular mechanism by which loss of MRN complex activity suppresses the functional interaction between BRCA1 and POLθ, we compared the levels of chromatin-bound RPA upon single or combined inhibition of POLθ and MRE11. As described above, POLθ inhibition increased RPA chromatin binding and γH2AX levels in RPE1 *BRCA1*$^{-/-}$ *TP53*$^{-/-}$. However, concomitant treatment with mirin, a small-molecule inhibitor of MRE11 activity, suppressed RPA chromatin binding and decreased H2AX phosphorylation compared to POLθ inhibition alone (Dupré et al., 2008) (**Fig 4A**). These data suggest that the MRN complex cleaves and processes ssDNA, that accumulates in BRCA1 deficient cells lacking POLθ activity, thus promoting ssDNA accumulation, formation of DNA double-strand breaks and cell death. Thus, the MRN complex partially drives the toxicity of the functional interaction between BRCA1 and POLθ in the cellular replication stress response.

To further understand how loss of CDK6 activity alleviates the hypersensitivity of BRCA1 deficient cells to POLθ inhibition, we took advantage of the selective CDK4/6 inhibitor palbociclib (Fry et al., 2004). As expected, CDK4/6 inhibition efficiently increased the percentage of cells in the G1 phase of the cell cycle by delaying S-phase entry **(Fig 4B, Supp Fig 4A).** To address the effect of reduced S-phase entry on replication stress levels, we stained for chromatin-bound RPA32 and γH2AX. Compared to POLθi treatment alone, CDK4/6i co-treatment drastically alleviated replication stress, as shown by decreased RPA chromatin binding and nuclear γH2AX signal **(Fig 4C, Supp Fig 4B-C).** To further explore the genetic interaction between CDK6 and POLθ without affecting CDK4 activity, we generated stable ko cell lines of CDK6 in SUM149PT. To clarify whether the alleviated replication stress with loss of CDK6 activity also translates into increased viability, we compared proliferation of CDK6 ko and control SUM149PT in the presence of POLθi by Incucyte live-cell imaging. Indeed, CDK6 loss, induced by two independent gRNAs, increased proliferation of SUM149PT under POLθ inhibition compared to cells transduced with a control gRNA (**Fig 4D, Supp Fig 4D**). This rescue also held up in BRCA1 deficient RPE1 during long-term POLθi treatments with replenishing fresh compound every three days while not influencing the POLθi response of BRCA1 wildtype RPE1 cells (**Supp Fig 4E**). To assess the kinetics of CDK6-dependent alleviation of POLθi response, we utilized an inducible Cas9 expression system to target CDK6 and monitored growth of control and *BRCA1* deficient RPE cells under POLθi or DMSO treatment in a competitive growth assay. Whereas CDK6 loss had only a minor effect on POLθi response of RPE1 *TP53*$^{-/-}$ (**Supp Fig 4F**), it alleviated the toxicity of POLθi in RPE1 *BRCA1*$^{-/-}$ *TP53*$^{-/-}$, starting from 4 days after treatment (**Fig 4F)**. Overall, we have shown that the genetic interaction between BRCA1 and POLθ depends on ssDNA, a substrate for nucleolytic processing by the MRN complex, which is specifically formed in dividing cells, explaining why CDK6 loss provides a fitness advantage in response to POLθ inhibition.

Discussion

The occurrence of replication gaps across the genome has challenged the hypothesis that DSBs are the main drivers of cellular toxicity upon treatment with PARP inhibitors or genotoxic agents such as cisplatin (Cong et al., 2021; Panzarino et al., 2021). In this study, we propose that ssDNA gap formation contributes to the synthetic lethality between BRCA1 and POLθ. Using a small-molecule inhibitor targeting the POLθ polymerase domain, we directly visualized gaps by immunofluorescence and DNA fiber assays. Previous work has demonstrated an increase in BrdU foci in mouse embryonic fibroblasts lacking both BRCA1 and the end protection factor 53BP1, using another small-molecular inhibitor of POLθ (Zatreanu et al.,

2021). The elevated BrdU foci number was attributed to increased resection at DNA DSBs. Using S1 nuclease fiber assays, we show that POLθ inhibition in BRCA1 deficient cells results in elevated levels of ssDNA that arise, at least partially, from replication gaps. Given that the inhibitor used in this study targets the POLθ polymerase domain, we hypothesize that POLθ exerts its function in gap filling through utilizing its translesion synthesis activity (Hogg et al., 2011). In line with our study, loss of gap filling by inhibition of other translesion synthesis polymerases, for example by the REV1-Polζ inhibitor JH-RE-06, has been shown to expose ssDNA gaps as a cancer vulnerability (Nayak et al., 2020; Taglialatela et al., 2021). Moreover, inhibitors of translesion synthesis have been shown to synergize with other gap-inducing treatments such as ATR inhibition (Nayak et al., 2020). This is consistent with our finding that ATR inhibition synergizes with POLθ inhibition in BRCA1 deficient cells. However, it remains poorly understood why other TLS polymerases cannot fully compensate for POLθ loss in BRCA1 deficient cells. Potentially, POLθ is preferred over other TLS polymerases since it contains not only a polymerase domain capable of filling ssDNA gaps but also a helicase domain which could process flap structures that are generated during removal of the DNA-RNA primers of downstream Okazaki fragments (see accompanying manuscript).

Previously, PARP1 was shown to recruit POLθ to sites of DNA DSBs (Mateos-Gomez et al., 2015). Since PARP1 functions as a sensor of unligated Okazaki fragments on the lagging strand (Hanzlikova et al., 2018), we speculate that PARP1 might recruit POLθ to sites of ssDNA gaps. An alternative, not mutually exclusive, mode of recruitment could be through translesion synthesis signaling, as POLθ has been shown to bind ubiquitinated PCNA in UV-irradiated human fibroblasts (Yoon et al., 2019). Moreover, POLθ is known to displace RAD51 from ssDNA overhangs of resected DSBs, thereby determining the DSB pathway choice between HR and TMEJ (Ceccaldi et al., 2015). We hypothesize that this displacement might also function in the context of ssDNA gaps which are recognized by RAD51. Furthermore, the POLθ helicase domain was shown to preferentially unwind lagging strands of substrates resembling stalled replication forks (Ozdemir et al., 2018). We hypothesize that DNA unwinding by POLθ may allow access to ssDNA gaps formed on the lagging strand for subsequent gap filling. Taken together, we propose a hypothetical model in which POLθ is recruited by PARP1 or translesion synthesis signaling to function in ssDNA gap filling by combining helicase dependent unwinding of the lagging strand, RAD51 displacement and translesion synthesis activity.

Here, we show that lack of ssDNA processing by POLθ alters replication fork dynamics in BRCA1 deficient cells, leading to asymmetric fork progression and consecutive replication

stress. Previous studies have implicated POLθ in the response to replication stress induced by hydroxyurea as well as in the regulation of replication timing under unchallenged conditions (Lemée et al., 2010; Fernandez-Vidal et al., 2014; Wang et al., 2019). We reason that the endogenous functions of POLθ in replication progression become apparent in genetic backgrounds with increased steady-state replication stress, such as BRCA1 deficiency. Furthermore, POLθ has been shown to bind to the origin recognition complex bringing it in close physical proximity with replication forks, the sites of potential ssDNA formation (Fernandez-Vidal et al., 2014). Altogether, these findings suggest that tight regulation of POLθ activity with respect to replication is essential, both in wild type cells to ensure genome integrity and BRCA1 deficient cells where POLθ becomes essential for cell survival.

We identified NBN, a member of the MRN complex, as a suppressor of the genetic interaction between BRCA1 and POLθ. Given the known role of BRCA1/2 in limiting MRN complex activity, we hypothesize that the MRN complex destabilizes the genome by processing replication gaps which are formed in the context of POLθ inhibition, into DSBs (Hashimoto et al., 2010; Tirman et al., 2021) **(Fig 4G).** In support of this, we show that short-term inhibition of MRE11 activity alleviates replication stress in response to POLθ inhibition (see accompanying manuscript). Future studies will be necessary to address the downstream processing of ssDNA gaps in the absence of MRE11 and POLθ activity. Further to this, we identified an enrichment of cell cycle regulators as modulators of POLθi response in BRCA1 deficient cells. We show that CDK6 activity facilitates cell cycle progression thus exacerbating POLθi induced replication stress **(Fig 4G).** This can be suppressed by genetic loss of CDK6 or by inhibiting CDK4/6 activity with the small-molecule palbociclib, which has been FDA approved for the treatment of specific subtypes of breast cancer (Fry et al., 2004). Our results suggest that concomitant application of CDK6 and POLθ inhibitors might have antagonistic effects, an important insight when considering the future clinical use of POLθ inhibitors.

In conclusion, we leveraged single molecule approaches as well as high-throughput genomics to dissect the genetic interaction between POLθ and BRCA1, thus identifying their converging roles in maintaining replication fork stability as fundamental to their synthetic lethal relationship. Furthermore, the role of POLθ in ssDNA processing is conserved in BRCA2 deficient cells, strengthening the importance of POLθ function for genome stability in BRCA mutant cells (see accompanying manuscript). Our findings provide important insights into POLθ and BRCA1 biology which are especially valuable given that clinical trials with POLθ inhibitors are ongoing.

Limitations of the Study

Here, we report an increase in ssDNA gaps upon POLθ inhibition in a BRCA1 deficient background. However, we cannot comment on size and location of these gaps. Electron microscopy, which could provide more detailed insights into gap kinetics and position, is technically challenging to perform due to the extreme sensitivity of BRCA1 defective cells to POLθ inhibition, preventing the collection of sufficient amounts of intact genomic DNA. Another potential limitation of this study is the lack of a genetic model for POLθ loss. Since BRCA1 and POLθ share a lethal interaction, the generation of cell lines that are genetically deficient for both factors is challenging. Therefore, future studies using different POLθ inhibitors, potentially also targeting different enzymatic domains, will be necessary to fully characterize the underlying molecular mechanisms.

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Author Contributions

AS, SB and JIL conceptualized the study. AS, SB, EA-V, MR and JW curated data. DK and GT contributed to microscopy. AAM, AK and MW performed the *in silico* docking analysis. AS and SB performed analysis and visualization. GE, VC, GEW and JIL supervised and carried out project administration associated with the study. AS and SB with input from JIL wrote the original draft and all authors reviewed and edited the final manuscript. AS and JIL obtained funding.

Declaration of Interests

The authors declare no competing interests.

Figure Titles and Legends

Figure 1: POLθ processes ssDNA gaps generated in BRCA1 deficient cells. (A) Computational docking model of small-molecule POLθ inhibitor (POLθi) bound to the POLθ polymerase domain (PDB entry 4X0P) with a detailed view of the allosteric binding pocket in the thumb region. Residues in proximity of the ligand (below 4A) are shown in grey. Two polar contacts with Gly2122 and Arg2201 are indicated in orange lines. Visualized with PyMOL (Schrödinger, L., & DeLano, W. (2020). PyMOL. Retrieved from http://www.pymol.org/pymol) **(B)** Clonogenic survival assays for RPE1 *TP53*$^{-/-}$ and RPE1 *BRCA1*$^{-/-}$ *TP53*$^{-/-}$ cells treated with increasing concentrations of POLθi with medium being replaced every three days. Representative images are shown in Supp Fig 1D. Data represent mean ± SD of n=3. **(C)** Representative images of SUM149PT treated with DMSO or 5μM POLθi for 24 hours and stained for RPA32 and BrdU under native conditions. Nuclear DNA was counterstained with DAPI. Scale bar=5μm. **(D)** Quantification of BrdU foci number per cell treated as indicated in (C). Only cells with ≥3 foci were quantified. At least 100 BrdU positive cells were collected from n= 2. The median is indicated. p-values were calculated using unpaired t-test. **(E)** Top: Scheme of the CldU/IdU pulse-labeling protocol, followed by S1 nuclease treatment. Bottom: Representative images of DNA fibers of RPE1 *TP53*$^{-/-}$ and RPE1 *BRCA1*$^{-/-}$ *TP53*$^{-/-}$, exposed to DMSO or 5μM POLθi, with or without S1 nuclease treatment. **(F)** IdU track lengths in RPE1 *TP53*$^{-/-}$ cells with DMSO or POLθi, with and without S1 nuclease treatment. p-values were calculated using the Mann-Whitney test. Data represent mean ± SD. **(G)** Same as in (F) for RPE1 *BRCA1*$^{-/-}$ *TP53*$^{-/-}$. p-values were calculated using the Mann-Whitney test. Data represent mean ± SD. **(H)** Top: Scheme of the CldU/IdU pulse-labeling for DNA fiber assay. Bottom: Representative images of CldU and IdU stained DNA fibers of RPE1 *TP53-/-* and RPE1 *BRCA1*$^{-/-}$ *TP53*$^{-/-}$ with or without 5μM POLθi. **(I)** Fork speed of RPE1 *TP53/-*$^{-}$ and RPE *BRCA1*$^{-/-}$ *TP53/-*$^{-}$ cells with and without POLθi. At least 200 fibers were measured from n = 2. Data represent mean ± SD. p-values were calculated using the Mann-Whitney test. **(J)** Fork arrest in RPE1 *TP53*$^{-/--}$ and RPE *BRCA1*$^{-/-}$ *TP53*$^{-/-}$ cells treated with DMSO or POLθi. High values indicate high symmetry, so low levels of fork arrest. At least 200 fibers were measured from n = 2. Data represent mean ± SD. p-values were calculated using the Mann-Whitney test.

Figure 2: POLQ inhibition induced replication stress is exacerbated in BRCA1 deficient cells. (A) Representative images of RPE1 *TP53*$^{-/-}$ and RPE *BRCA1*$^{-/-}$ *TP53*$^{-/-}$ cells, treated with DMSO or 5μM POLθi for 24 hours, and stained for chromatin-bound RPA70 and γH2AX**.** Nuclear DNA was counterstained with DAPI. 192x magnification. Scale bar=10μm. **(B)** Quantification of mean intensity of chromatin-bound RPA32 and γH2AX in RPE1 *TP53*$^{-/-}$ and RPE *BRCA1*$^{-/-}$ *TP53*$^{-/-}$ cells treated as indicated in (A). Cells with a mean intensity higher than 800 a.u. for RPA70 and 10 000 a.u. for γH2AX are marked in cyan and calculated for percentages. Each dot represents 1 cell. 8800 cells were collected from n = 2. a.u., arbitrary units. **(C)** Quantification of mean intensity of phosphorylated RPA32 (pRPA) at Serine 4/8 in RPE *TP53*$^{-/-}$ and RPE1 *BRCA1*$^{-/-}$ *TP53*$^{-/-}$ treated for 48 hours with DMSO or 5μM POLθi. At least 3400 cells were collected from n=2. p-values were calculated with Kruskal-Wallis test. a.u. arbitrary units **(D)** (Top) Scheme of experimental setup and flow cytometry gating strategy. Cells were synchronized in G1 using a 16-hour nocodazole treatment, followed by release in medium with DMSO or 5μM POLθi and harvested at different time points after a 30-minute EdU chase. (Bottom) Percentage of early S phase cells at different time points after release from nocodazole synchronization in DMSO or 5μM POLθi containing medium. Data represent mean ± SD. At least 10 000 singlets were collected from an n = 2. p-values were calculated with unpaired t-test. **(E)** Confluence of RPE1 *TP53*$^{-/-}$ (G) and RPE1 *BRCA1*$^{-/-}$ *TP53*$^{-/-}$ (G) over 5 days of single or dual ATRi (500 nM)/POLθi (3μM) treatment, detected by Incucyte live-cell imaging. The data are a representative set of images of n=2.

Figure 3: A genome-wide CRISPR-Cas9 ko screen identified modulators of the BRCA1-POLθ genetic interaction. (A) Scheme of the experimental setup of the genome-wide CRISPR-Cas9 ko screen. SUM149PT cells were transduced with the TKO v3 gRNA library and puromycin was added after 1 day of recovery. After 7 days of antibiotic selection, cells were either treated with DMSO or 2-2.5μM POLθi over 18 days, followed by genomic DNA extraction and NGS sequencing for determining gRNA abundances. **(B)** Scatter plot of MAGeCK analysis of genome-wide CRISPR-Cas9 ko screen in POLθi treated SUM149PT normalized to DMSO. Only genes with log2(fold change) > 0 are shown. Cell cycle genes as well as genes of the MRN complex are highlighted in orange and cyan, respectively. **(C)** Top 10 GO-terms of genes targeted by enriched gRNAs (p-value < 0.005), identified using DAVID Bioinformatics Resources (Huang et al., 2009b, 2009a).

Figure 4: Loss of activity of the MRN complex and CDK6 alleviate the functional interaction between BRCA1 and POLθ. (A) Quantification of immunofluorescence showing chromatin-bound RPA32 and γH2AX mean intensity levels in RPE1 *BRCA1*$^{-/-}$ *TP53*$^{-/-}$ cells

treated for 6 hours with DMSO, 5 μM POLθi and/or 50μM MRE11i. At least 5000 cells were collected from n = 2. a.u., arbitrary units **(B)** RPE1 *BRCA1*$^{-/-}$ *TP53*$^{-/-}$ and SUM149PT were treated for 24 (RPE1) or 48 hours (SUM149PT) with DMSO, 5μM POLθi and/or 1μM CDK4/6i followed by a 30-minute EdU chase to label newly synthesized DNA. Data represent mean ± SD. At least 20 000 singlets were collected from an n = 2. Representative flow cytometry plots and gating strategy are shown in Supp Fig 4A. **(C)** Percentage of RPE1 *BRCA1*$^{-/-}$ *TP53*$^{-/-}$ cells positive (>1000 a.u.) for both γH2AX and chromatin-bound RPA32, after 24 hours of single or dual POLθi (5μM)/CDK4/6i(1μM) treatment. At least 500 cells were collected from n = 2. **(D)** Confluence of SUM149PT transduced with control or one of two independent CDK6-gRNAs with 5μM POLθi treatment normalized to DMSO, detected by Incucyte live-cell imaging. The corresponding proliferation curves for both DMSO and POLθi are shown in Supp Fig 4D. Data represent mean ± SD of 3 technical replicates and are a representative set of images of n=2. **(E)** Experimental setup of competitive growth assay. RPE1 *TP53*$^{-/-}$ and RPE1 *BRCA1*$^{-/-}$ *TP53*$^{-/-}$ cells were transduced with control or CDK6 gRNAs at an approximate multiplicity of infection (MOI) of 0.5 and seeded with Shield-1 to induce Cas9 expression in the presence or absence of POLθi two days later. Flow cytometry was used to measure the growth kinetics of ko (i.e. mVenus-positive) cells over time. **(F)** mVenus-positive cells under POLθi treatment normalized to DMSO over time, treated as indicated in (E). Data represent mean ± SD of 2 biological replicates. **(G)** Proposed model explaining modulatory effects of the MRN complex and CDK6 on the BRCA1-POLθ genetic interaction, as shown based on a replication fork in a BRCA1 deficient cell.

Tables with Titles and Legends

Methods and Methods

Plasmids

The Toronto human knockout pooled library (TKOv3) was a gift from Jason Moffat (University of Toronto, Canada Addgene # 90294). For virus production, the psPAX2 (a gift from Didier Trono, EPFL, Switzerland; Addgene plasmid # 12260) and VSV.G (a gift from Tannishtha Reya, UCSD, USA; Addgene plasmid # 14888) packaging plasmids were used. lentiCRISPR v2 was a gift from Feng Zhang (Addgene plasmid # 52961; http://n2t.net/addgene:52961 ; RRID:Addgene_52961). DD-Cas9-mVenus was a gift from Raffaella Sordella (Addgene plasmid # 90085; http://n2t.net/addgene:90085 ; RRID:Addgene_90085)

Competitive growth assay

RPE1 *TP53*$^{-/-}$ and RPE1 *BRCA1*$^{-/-}$ *TP53*$^{-/-}$ cells were transduced with DD-Cas9-mVenus encoding either a control or a CDK6 gRNA at an approximate MOI of 0.5. After two days of recovery, the percentage of mVenus-positive cells was measured by flow cytometry to determine initial mVenus levels (day 0) and cells were seeded into 12-well plates together with 200nM Shield-1 (Aobious, #AOB1848S) to induce gRNA expression and DMSO/3μM POLθi. To assess cellular growth kinetics, the percentage of mVenus-positive cells was monitored by flow cytometry every three to four days and normalized to day 0.

Mammalian cell culture

All cells were grown at 3% oxygen and 37°C and routinely checked for mycoplasma contamination. RPE1 *TP53*$^{-/-}$ and SUM149PT were kindly provided by Steve Jackson (Wellcome/Cancer Research UK Gurdon Institute, Cambridge, UK). RPE1 *BRCA1*$^{-/-}$ *TP53*$^{-/-}$ were kindly provided by Dan Durocher (Lunenfeld-Tanenbaum Research Institute, Toronto, Canada). RPE1 cell lines were grown in Gibco DMEM/F-12 with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin. SUM149PT were grown in Gibco Ham's F-12 with 5% FBS, 10 mM HEPES, 1 μg/ml hydrocortisone, 5 μg/ml insulin and 1% penicillin/streptomycin.

Generation of cell lines

For generating CDK6 ko cell lines, gRNAs were designed using https://www.vbc-score.org/ (Michlits et al., 2020) and cloned into the pLCV2 backbone, followed by virus production (same procedure as described for TKO v3 virus production) and transduction of cell lines. Transduced cells were selected with puromycin (1.5μg/ml for RPE1 *TP53*$^{-/-}$, 20μg/ml for RPE1 *BRCA1*$^{-/-}$ *TP53*$^{-/-}$ and 1μg/ml for SUM149PT) and efficiency of ko was determined using TIDE (Brinkman et al., 2014). Used gRNA sequences: CDK6#1: 5`-GAAGAACGGAGGCCGTTTCG-3, CDK6#2: 5`- GCTGGACTGGAGCAAGACTT-3` and control: 5`-CTCTTCGCTATTACGCCAGC-3`.

Cell growth assays

Dose response curves: Dose response curves were performed in 96-well plates by seeding 400 RPE1 *TP53*$^{-/-}$ or 500 RPE1 *BRCA1*$^{-/-}$ *TP53*$^{-/-}$ cells per well in technical triplicates. For POLθi-etoposide co-treatment dose response curves, etoposide serial dilutions were added one hour after starting POLθi treatment at indicated concentrations. After four (etoposide)/five (POLθi) days of treatment, cell viability was measured using Cell Titer-Glo (Promega).

Clonogenic survival assays: Clonogenic survival assays for POLθi and olaparib were performed in 6-well plates by seeding cells at low density (for SUM149PT 1000 cells, for RPE *TP53*$^{-/-}$ 200 cells and for RPE1 *BRCA1*$^{-/-}$ *TP53*$^{-/-}$ 200 cells per well). On the same day, serial dilutions of POLθi were added. Medium with fresh compound was renewed every three days. After nine and eleven days of treatment, for RPE and SUM149PT respectively, colonies were fixed in 3.7% formaldehyde in PBS and stained with crystal violet solution (0.1% (w/v), 10% EtOH). Colony area was quantified using the ColonyArea plugin in ImageJ (Guzmán et al., 2014) and normalized to DMSO.

Proliferation: Proliferation was assessed with an IncuCyte Live-Cell Analysis Imager (Sartorius). Cellular confluence was monitored over 5 days with images taken every 6 hours. Cells were seeded in 48-well plates (for SUM149PT 4000 cells, for RPE1 *TP53*$^{-/-}$ 1000 cells, for RPE *BRCA1*$^{-/-}$ *TP53*$^{-/-}$ 2000 cells per well) and drugs were added immediately after seeding.

Genome-wide CRISPR-Cas9 ko screen

Virus production: The Toronto Knockout (TKO) v3 CRISPR Library virus was produced in a one-production step. HEK-293T cells were seeded in 10cm dishes and transfected 24 hours later, with the TKO library plasmid pool, pVSVG and psPAX2 packaging plasmids, using polyethylenimine (PEI). 72 hours later, supernatant containing virus was harvested, centrifuged at 2000 rpm for 5 minutes to remove cell debris and stored at -80°C.

Screen setup: SUM149PT cells were infected with the lentiviral TKOv3 library at a MOI of 0.3 and puromycin-containing medium (0.5 ug/ml) was added the next day to select for transductants. As soon as untransduced control cells were dead (after 5 days of puromycin selection, referred to as “early time point”), 25 million cells were harvested for genomic DNA extraction. After 7 days of puromycin selection, cells were re-seeded with DMSO or a lethal dose (LD) LD90 concentration of POLθi (dynamically adjusted, 2-2.5μM) and sub-cultured every three days. After 18 days of treatment, 25 million cells per condition were harvested and genomic DNA was extracted using the QIAmp DNA Blood Mini Kit, according to the manufacturer’s protocol (referred to as “end time point”).

sgRNA amplification and sequencing: Genome-integrated gRNA sequences were amplified by PCR using NEBNext Ultra II Q5 Master Mix. A mixture of P5 forward primers with staggers from 1 to 8 bp and barcoded P7 reverse primers were added in a second round of PCR. The resulting PCR2 product was purified by size-exclusion using magnetic AMPure XP DNA beads (NEB), using a 1:0.95 followed by a 1:1.2 ratio clean-up. Barcoded samples were pooled and

sequenced on two flow cells of an Illumina NextSeq 2000 machine using 75 cycles single-read sequencing.

Screen analysis: gRNA sequences were retrieved by trimming all sequences 5' to the adapter sequence (5'-CGAAACACCG-3'). Bowtie (v 2.3.4) was used for alignment and gRNA count. Gene-level depletion scores were calculated using MAGeCK (Li et al., 2014). End time point samples were compared to early time point samples to analyze depletion of gRNAs targeting essential genes. To identify gene-drug interactions, the POLθi treated end point sample was compared to the respective DMSO control.

Immunoblotting

Cell extracts were prepared in RIPA lysis buffer (NEB), supplemented with protease inhibitors (Sigma) and phosphatase inhibitors (Sigma). Immunoblots were performed using standard procedures. Protein samples were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) 3-8% or 4-12% gradient gels (Invitrogen) and subsequently transferred onto PVDF membranes. The following antibodies were used in 5% milk: BRCA1 (1:1000, Santa Cruz, #sc-6954), α-Tubulin (1:1000, CellSignaling, #3873), RPA32 (1:1000, abcam, #ab2175), Vinculin (1:5000, Sigma-Aldrich, #MAB3574). Secondary antibodies (HRP-conjugated goat anti-mouse or anti-rabbit IgG, Jackson Immunochemicals) were used at a 1:5000 dilution. Immunoblots were imaged using a Curix60 (AGFA) table-top processor.

Compounds and inhibitors

POLθi (1-[6-[3-Methyl-4-[(3-methyltetrahydrofuran-3-yl)methoxy]phenyl]pyrazin-2-yl]-3-(6-methyl-3-pyridyl)urea) was custom synthesized by Enamine with 90% purity. MRE11i mirin (#HY-19959), CDK6i Palbociclib (PD 0332991, #HY-50767) and hydroxyurea (#HY-B0313) were purchased from MedChemExpress. Etoposide (VP-16, #S1225), PARPi olaparib (AZD2281, #S1060) and ATRi ceralasertib (AZD6738, #HY-19323) were purchased from SelleckChemicals. Chemicals were dissolved in DMSO, aliquoted and stored at −20 °C.

Flow cytometry

Cell Cycle Profile

For determining cell cycle profiles with EdU and DAPI, cells were harvested following a 30-minute incubation time with 10 μM EdU. After one wash in 0.5% BSA in PBS, cells were fixed in 2% paraformaldehyde in PBS for 20 minutes at room temperature. Cells were washed again in 1x saponin-based permeabilization and wash buffer (Click-iT™ EdU Cell Proliferation Kit for Imaging, Invitrogen) and stained using the Click-iT EdU Alexa Fluo 488 Picolyl Azide

Toolkit (Fisher Scientific, #15403493) according to the manufacturer's specifications. DNA was counterstained with DAPI. Cells were washed one more time with 1x saponin based permeabilization and wash buffer, before proceeding to flow cytometry using a BD LSR-Fortessa X-20. Gating and cell cycle analysis were performed using FlowJo (v10). For cell cycle analysis, 20 000 events in the Singlets gate were recorded.

Cell Cycle Synchronization

Cells were synchronized in mitosis with nocodazole (100 ng/ml, Sigma-Aldrich) for 16 hours. To release cells from the mitotic block, cells were washed with warm PBS1X and then complete medium with either DMSO or 5 μM POLθi was added to the wells. At the indicated time points, cells were harvested following a 30-minute incubation time with 10 μM EdU. Cells were prepared as described above for EdU staining.

Statistical analysis

Statistical tests were performed as indicated in the figure legends to determine statistical significance and were performed using GraphPad Prism (Version 9.0). In all cases, ns: not significant ($p > 0.05$), *: $p < 0.05$, **: $p < 0.01$, ***: $p < 0.001$ and ****: $p < 0.0001$.

Gene ontology-term analysis

DAVID Bioinformatics Resources (Huang et al., 2009a, 2009b) functional annotation online-tool was used for GO-term analysis of genes represented by the top enriched gRNAs ($p < 0.005$), using "Uniprot Biological Process" as functional annotation term. The gene list was treated as an unordered query.

Identification and scoring of a POLθi binding pocket in the POLθ polymerase domain

The chemical structures of POLθ inhibitors were extracted from two patents (Blencowe et al., 2020a, 2020b). In these patents, the inhibitory potency of 342 compounds was measured on the polymerase domain of POLθ. In the following, compounds of both patents will be referred to as POLθ inhibitors. The strongest inhibitor, 25A-90, is referred to in the singular (POLθ inhibitor). The thiazoleurea compounds (Blencowe et al., 2020b) are referred to as 24A-xxx followed by the ID number (e.g. 24A-116) and the heterocyclic substituted urea compounds (Blencowe et al., 2020a) as 25A-xxx (e.g. 25A-92). POLθ inhibitors for docking analysis were generated using PubChem Sketcher (Ihlenfeldt, Bolton, and Bryant 2009). The structure of the POLθ polymerase domain was obtained from the RCSB Protein Database (PDB entry 4X0P)(Zahn et al., 2015). Nucleotides, DNA, and solvent were deleted from the structure and the protein was prepared using Maestro (Schrödinger Release 2022-1: BioLuminate,

Schrödinger, LLC, New York, NY, 2021.) Three independent methods were used to identify possible binding pockets, namely blind docking with AutoDock Vina (Oleg TRott, 2009), binding site identification with SiteMap (Halgren, 2009) and the consensus pocket identification approach MetaPocket (Huang, 2009) AutoDock Vina 1.1 was used to perform the blind docking experiment in which buriedness was set to zero, exhaustiveness level to 8, and the number of binding modes to 9. The dimensions of the grid box were scaled to include the complete protein. Additionally, SiteMap and MetaPocket were used to identify potential binding sites. The results were analyzed using the SiteScore and size values calculated by SiteMap and the z-score calculated by MetaPocket. Binding hypotheses were further analyzed by their ability to generate reasonable hypotheses for the activity cliff pairs (25A-104, 25A-150), (25A-69, 25A-54), and (25A-213, 25A-90).

DNA fiber assay

Asynchronous cells were pulse labelled with 25 μM CldU (Sigma-Aldrich, #C6891) for 20 minutes, washed twice with warm PBS and then labeled with 250 μM IdU (Sigma-Aldrich, #I7125) for 20 minutes. POLθI (5μM) was added 90 minutes before the labeling and was present for the entire labeling time. Cells were trypsinized, counted and resuspended at a final concentration of 1-2x10^3 cells/μL. Two μL of cell suspension were lysed on a clean glass slide with 8 μl of MES lysis buffer (500 mM MES pH 5.6, 0.5% SDS, 50 mM EDTA, 100 mM NaCl) for 7 minutes, then the slide was tiled 15° to allow the DNA to spread. Slides were air dried for 30 minutes, fixed in freshly prepared acetic acid/methanol (1:3) for 10 min, air dried and store at 4°C overnight. Slides were rehydrated with PBS 1X for 5 minutes, DNA was denatured with 2.5 M HCl for 80 minutes, slides were washed several times with PBS and blocked in blocking solution (5% BSA, 0.2% Triton X-100 in PBS) for 20 minutes. Slides were incubated with primary antibody mix of anti-BrdU (abcam, #ab6326, 1:100) which recognizes CldU, and anti-BrdU (BD biosciences, #347580, 1:50) which recognizes IdU in blocking solution for 90 minutes at 37°C in a humid chamber. After incubation, slides were washed once with 0.1% Tween in PBS and twice with PBS for 3 minutes each. Slides were incubated with secondary antibody mix of donkey anti-mouse CY3 (Jackson ImmunoResearch, #715-165-150) and chicken anti-rat Alexa 488 (ThermoFisher, #A-21470) in blocking solution for 45 minutes at 37°C degrees in a humid chamber. Slides were washed 3 times in PBS, air dried, mounted in vectashield plus (Vector labs) and stored at 4°C until image acquisition. Images were acquired with an Olympus Upright BX61 fluorescence microscope with a 60X oil immersion objective 1.35 NA. According to the fiber density between 5 and 10 images were captured per condition and at least 200 fibers were measured using ImageJ software version 2.3.0/1.53f. For fork

speed experiments, the conversion factor used was 1 um = 2.59kb (Jackson and Pombo, 1998).

S1 nuclease assay

The S1 assay was conducted as previously described (Taglialatela et al., 2021). Briefly, cells were pulse labeled with 25 μM CldU (Sigma-Aldrich, #C6891) for 20 minutes, washed twice with warm PBS and then labeled with 250 μM IdU (Sigma-Aldrich, #I7125) for 40 minutes. POLθi (5μM) was added 90 minutes before labeling and was present during the whole labeling procedure. Cells were washed once with PBS and then permeabilized with CSK100 buffer (100 mM NaCl, 10 mM PIPES pH 6.8, 3mM MgCl2, 300 mM sucrose, Triton 0.5% X-100) for 10 minutes at room temperature. Exposed nuclei were washed once with S1 buffer (30 mM Sodium acetate pH 4, 2 mM Zinc sulphate, 50 mM NaCl, 5% glycerol) and then incubated with 10 U/mL of S1 nuclease (Sigma-Aldrich, #N5661) in S1 buffer for 20 minutes at 37°C. Nuclei were scrapped in 1 mL of PBS 0.1% BSA, centrifugated 5 minutes at 7000 rpm and resuspend in PBS to a final concentration of 1-2x103 nuclei/μL. DNA was spread and stained as is the DNA fiber assay.

Immunofluorescence and Imaging

ssDNA: Cells were grown on 12mm glass coverslips in 10 μM BrdU for 48 hours, followed by the indicated treatments as described. After treatment, cells were washed in PBS and pre-extracted with Pre-Extraction buffer (10mM PIPES, 100mM NaCl, 3mM MgCl2, 1mM EGTA, 0.5% Triton X-100 and 300mM Sucrose) for 10 minutes at 4°C, followed by Cytoskeleton Stripping Buffer B (10mM Tris pH 7.5, 10mM NaCl, 3mM MgCl2, 1% Tween20, 0.5% sodium deoxycholate) for additional 10 minutes at 4°C (O'sullivan et al., 2021). Cells were then washed in PBS, fixed using 2% formaldehyde for 15 minutes at room temperature and permeabilization was carried out for 10 minutes in 0.5% Triton X-100 in PBS. Cells were incubated 1 hour in blocking buffer and stained with primary antibodies against BrdU (Abcam, #ab6326) at 37°C for 1 hour. Cells were washed and incubated with secondary antibodies (Alexa Fluor 555) for 1 hour at room temperature. After washing, cells were incubated with DAPI (1 ug/ml) and mounted onto glass slides using DAKO Fluorescent Mounting Medium (Agilent Technologies S3023). Images were visualized by confocal microscopy (LSM-700 Zeiss) at a constant exposure time in each experiment. The number of BrdU foci per nucleus was measured with Cell Profiler software version 4.2.1 from the Broad Institute.

High content-microscopy: Cells were grown in 96-well optically-clear cyclic olefin bottom plates (PhenoPlate #6055300, Perkin Elmer), pre-extracted, fixed and permeabilized as

described above. Cells were labelled with primary antibodies (RPA70, 1:500, abcam, #ab79398, RPA 32/2, abcam, 1:500, #ab2175, phospho-RPA32 (Ser4,Ser8), 1:500, Bethyl Laboratories, #A300-245A, phospho-histone-H2A.X, 1:1000, Sigma-Aldrich, #05-636-I, anti-phospho-histone-H2A.X/γ-H2AX 20E3, 1:400, Cell Signaling, #9718) overnight at 4°C. Cells were washed three times in PBS-Tween 0.1% and incubated with appropriate secondary antibodies for 1 hour at room temperature. Automated multichannel wide-field microscopy for quantitative image-based cytometry (QIBC) was performed on an Olympus IXplore SpinSR inverted research microscope using 40X magnification (Olympus, Life Science Solutions). Images were analyzed with the Olympus ScanR Image Analysis Software (Olympus OSIS Life Science Solutions, version 3.3), a dynamic background correction was applied, and nuclei segmentation was performed using an edge-based object detection module based on the DAPI signal. Mean intensities and scatter plots were displayed using Python version 3.

Supplemental Information Titles and Legends

Supp Fig1: POLθ processes ssDNA gaps generated in BRCA1 deficient cells. (A) Chemical structure of POLθ inhibitor used in this study (Blencowe et al., 2020a). **(B)** Western Blot illustrating BRCA1 absence in SUM149PT, RPE *TP53*$^{-/-}$ and RPE1 *BRCA1*$^{-/-}$ *TP53*$^{-/-}$. Tubulin was used as a loading control. **(C)** Clonogenic survival assays of RPE1 *TP53*$^{-/-}$ and RPE1 *BRCA1*$^{-/-}$ *TP53*$^{-/-}$ with the PARP inhibitor olaparib to validate BRCA1 status. Colony area normalized to DMSO control after 9 (RPE1 cell lines) or 11 days (SUM149PT) is shown. Data represent mean ± SD of n=3. **(D)** Representative images of clonogenic survival assays of RPE1 *TP53*$^{-/-}$, RPE1 *BRCA1*$^{-/-}$ *TP53*$^{-/-}$ and SUM149PT with indicated doses of POLθi, as quantified in Fig 1B and Supp Fig 1E **(E)** Clonogenic survival assays of SUM149PT with increasing concentrations of POLθi. Representative images are shown in Supp Fig 1D. Data represent mean ± SD of n=3. **(F)** Dose response curves of RPE1 *TP53*$^{-/-}$ with etoposide, co-treated with DMSO or increasing doses of POLθi. Data represent mean ± SD of n=3. **(G)** Representative images (left) and quantification (right) of the denaturation control for BrdU immunofluorescence. Fixed samples were exposed to 2N HCl for one hour before staining and immunofluorescence. a.u., arbitrary units. **(H)** Representative images of SUM149PT cells after 24 hours of 5μM POLθi treatment showing increased nucleus size in cells with high BrdU foci number (≥3 foci, shown in red) compared to cells with low BrdU foci number (<3 foci, shown in blue). Scale bar = 5μm. **(I)** Quantification of nuclear area of SUM149PT cells treated with DMSO or POLθi grouped into cells with low numbers of BrdU foci (“low BrdU”, <3 foci) and cells with high numbers of BrdU foci (“high BrdU”, ≥3 foci). a.u., arbitrary units. p-values were calculated with unpaired t-test.

Supp Fig2: POLQ inhibition induced replication stress is exacerbated in BRCA1 deficient cells. (A) Representative images of SUM149PT cells, treated with DMSO or 5μM POLθi for 48h hours, and stained for chromatin-bound RPA70 and γH2AX**.** Nuclear DNA was counterstained with DAPI. 192x magnification. Scale bar=10μm. **(B)** Quantification of mean intensity of chromatin-bound RPA70 and γH2AX in SUM149PT cells treated as indicated in (A). Cells with a mean intensity higher than 1,000 a.u. for RPA70 and 10,000 for γH2AX are marked in cyan and used to calculate percentages. Each dot represents 1 cell. At least 5000 cells were analyzed from n = 2. a.u., arbitrary units **(C)** Quantification of mean intensity of pRPA(Ser4/8) of SUM149PT cells treated for 24 hours with DMSO or 5μM POLθi. At least 13 000 cells were analyzed. p-values were calculated with Kruskal-Wallis test. a.u. arbitrary units

Supp Fig3: A genome-wide CRISPR-Cas9 ko screen identified modulators of the BRCA1-POLθ genetic interaction (A) POLθi drug response assay with SUM149PT over 15 days to estimate the LD_{90} concentration with replenishing fresh drug every three days. Based on this assay, we selected a concentration of 2-2.5μM for the CRISPR-Cas9 ko screen. **(B)** Survival of POLθi-treated SUM149PT normalized to DMSO during genome-wide CRISPR-Cas9 ko screen. **(C-D)** Quality control of genome-wide CRISPR-Cas9 ko screen **(C)** Log2-fold changes of all genes, highlighting essential genes (in orange), as defined by (Hart et al., 2017), in DMSO and POLθi-treated cells. **(D)** Log2-fold changes of all genes, highlighting genes belonging to the olfactory receptor family (in cyan), as defined by the HGNC (HUGO Gene Nomenclature Committee, https://www.genenames.org/). **(E)** Scatter plot of MAGeCK analysis of genome-wide CRISPR-Cas9 ko screen in POLθi-treated SUM149PT normalized to DMSO. Only genes with log2(fold change) < 0 are shown, highlighting genes in the electron transport chain, Fanconi Anemia pathway, Homologous Recombination and DNA Damage Response Signaling.

Supp Fig4: Loss of activity of the MRN complex and CDK6 alleviate the functional interaction between BRCA1 and POLθ. (**A)** Representative flow cytometry plots of RPE1 *BRCA1*$^{-/-}$ *TP53*$^{-/-}$ treated with DMSO or 1μM CDK4/6i for 24 hours. **(B)** Representative images of RPE1 *BRCA1*$^{-/-}$ *TP53*$^{-/-}$ cells after 24 hours of single or dual POLθi (5μM)/CDK4/6i(1μM) treatment. **(C)** Percentage of SUM149PT cells after 48h of single or dual POLθi (5μM)/CDK6i(1μM) treatment, normalized to DMSO, that are positive for both γH2AX and chromatin-bound RPA32. At least 3500 cells were collected from n =2. **(D)** Confluence of control or CDK6 ko SUM149PT with DMSO or 5μM POLθi treatment, detected by Incucyte live-cell imaging. Survival normalized to DMSO is shown in Fig 4D. **(E)** Cell numbers of POLθi

treated RPE *TP53*$^{-/-}$ and RPE1 *BRCA1*$^{-/-}$ *TP53*$^{-/-}$ normalized to DMSO. Cells were counted and reseeded with fresh POLθi (3μM) every three days. **(F)** Competitive growth assay, as described in Fig 4F, for RPE1 *TP53*$^{-/-}$. Data represent mean ± SD of 2 technical replicates.

Supp Table1: MAGeCK analysis from CRISPR-Cas9 ko screen treating SUM149PT with POLθi

Results were generated using the software package MAGeCK, comparing SUM149PT after 18 days of POLθi versus DMSO treatment.

References

Apostolou, P., and Fostira, F. (2013). Hereditary breast cancer: The Era of new susceptibility genes. *Biomed Res. Int.* 2013. doi:10.1155/2013/747318.

Blencowe, P., Charles, M., Cridland, A., Edkwuru, T., Heald, R., Macdonald, E., et al. (2020a). Heterocyclic substituted ureas, for use against cancer.

Blencowe, P., Charles, M., Cridland, A., Edkwuru, T., Heald, R., Macdonald, E., et al. (2020b). Thiazolureas as anticancer agents.

Brinkman, E. K., Chen, T., Amendola, M., and Van Steensel, B. (2014). Easy quantitative assessment of genome editing by sequence trace decomposition. *Nucleic Acids Res.* 42. doi:10.1093/nar/gku936.

Ceccaldi, R., Liu, J. C., Amunugama, R., Hajdu, I., Primack, B., Petalcorin, M. I. R., et al. (2015). Homologous-recombination-deficient tumours are dependent on Polθ -mediated repair. *Nature* 518, 258–262. doi:10.1038/nature14184.

Chaudhuri, A. R., Callen, E., Ding, X., Gogola, E., Duarte, A. A., Lee, J. E., et al. (2016). Replication fork stability confers chemoresistance in BRCA-deficient cells. *Nature* 535, 382–387. doi:10.1038/nature18325.

Cong, K., Peng, M., Kousholt, A. N., Lee, W. T. C., Lee, S., Nayak, S., et al. (2021). Replication gaps are a key determinant of PARP inhibitor synthetic lethality with BRCA deficiency. *Mol. Cell* 81, 3128-3144.e7. doi:10.1016/j.molcel.2021.06.011.

Dias, M. P., Tripathi, V., Heijden, I. Van Der, Cong, K., Bhin, J., Gogola, E., et al. (2021). Loss of Nuclear DNA Ligase III Reverts PARP Inhibitor Resistance in BRCA1 / 53BP1 Double-deficient Cells by Exposing ssDNA Gaps. *Mol. Cell*, 1–17. doi:10.1016/j.molcel.2021.09.005.

Dupré, A., Boyer-Chatenet, L., Sattler, R. M., Modi, A. P., Lee, J. H., Nicolette, M. L., et al. (2008). A forward chemical genetic screen reveals an inhibitor of the Mre11-Rad50-Nbs1 complex. *Nat. Chem. Biol.* 4, 119–125. doi:10.1038/nchembio.63.

Fernandez-Vidal, A., Guitton-Sert, L., Cadoret, J. C., Drac, M., Schwob, E., Baldacci, G., et al. (2014). A role for DNA polymerase Ĵ in the timing of DNA replication. *Nat. Commun.* 5. doi:10.1038/ncomms5285.

Fry, D. W., Harvey, P. J., Keller, P. R., Elliott, W. L., Meade, M., Trachet, E., et al. (2004). Specific inhibition of cyclin-dependent kinase 4/6 by PD 0332991 and associated antitumor activity in human tumor xenografts. *Mol. Cancer Ther.* 3, 1427–1438. doi:10.1158/1535-7163.1427.3.11.

Halgren, T. A. (2009). Identifying and characterizing binding sites and assessing druggability. *J. Chem. Inf. Model.* 49, 377–389. doi:10.1021/ci800324m.

Hanzlikova, H., Kalasova, I., Demin, A. A., Pennicott, L. E., Cihlarova, Z., and Caldecott, K. W. (2018). The Importance of Poly(ADP-Ribose) Polymerase as a Sensor of Unligated Okazaki Fragments during DNA Replication. *Mol. Cell* 71, 319-331.e3. doi:10.1016/j.molcel.2018.06.004.

Hart, T., Tong, A. H. Y., Chan, K., Van Leeuwen, J., Seetharaman, A., Aregger, M., et al. (2017). Evaluation and design of genome-wide CRISPR/SpCas9 knockout screens. *G3 Genes, Genomes, Genet.* 7, 2719–2727. doi:10.1534/g3.117.041277.

Hashimoto, Y., Chaudhuri, A. R., Lopes, M., and Costanzo, V. (2010). Rad51 protects nascent DNA from Mre11-dependent degradation and promotes continuous DNA synthesis. *Nat. Struct. Mol. Biol.* 17, 1305–1311. doi:10.1038/nsmb.1927.

Hogg, M., Seki, M., Wood, R. D., Doublié, S., and Wallace, S. S. (2011). Lesion bypass activity of DNA polymerase θ (POLQ) is an intrinsic property of the pol domain and depends on unique sequence inserts. *J. Mol. Biol.* 405, 642–652. doi:10.1016/j.jmb.2010.10.041.

Huang, B. (2009). Metapocket: A meta approach to improve protein ligand binding site prediction. *Omi. A J. Integr. Biol.* 13, 325–330. doi:10.1089/omi.2009.0045.

Huang, D. W., Sherman, B. T., and Lempicki, R. A. (2009a). Bioinformatics enrichment tools: Paths toward the comprehensive functional analysis of large gene lists. *Nucleic Acids Res.* 37, 1–13. doi:10.1093/nar/gkn923.

Huang, D. W., Sherman, B. T., and Lempicki, R. A. (2009b). Systematic and integrative analysis of large gene lists using DAVID bioinformatics resources. *Nat. Protoc.* 4, 44–57. doi:10.1038/nprot.2008.211.

Jackson, D. A., and Pombo, A. (1998). Replicon clusters are stable units of chromosome structure: Evidence that nuclear organization contributes to the efficient activation and propagation of S phase in human cells. *J. Cell Biol.* 140, 1285–1295. doi:10.1083/jcb.140.6.1285.

Kolinjivadi, A. M., Sannino, V., De Antoni, A., Zadorozhny, K., Kilkenny, M., Técher, H., et al. (2017). Smarcal1-Mediated Fork Reversal Triggers Mre11-Dependent Degradation of Nascent DNA in the Absence of Brca2 and Stable Rad51 Nucleofilaments. *Mol. Cell* 67, 867-881.e7. doi:10.1016/j.molcel.2017.07.001.

Koole, W., Van Schendel, R., Karambelas, A. E., Van Heteren, J. T., Okihara, K. L., and Tijsterman, M. (2014). A polymerase theta-dependent repair pathway suppresses extensive genomic instability at endogenous G4 DNA sites. *Nat. Commun.* 5, 1–10. doi:10.1038/ncomms4216.

Lemée, F., Bergoglio, V., Fernandez-vidal, A., Machado-silva, A., and Pillaire, M. (2010). DNA polymerase θ up-regulation is associated with poor survival in breast cancer , perturbs DNA replication , and promotes genetic instability. *PNAS* 107. doi:10.1073/pnas.0910759107.

Lord, C. J., and Ashworth, A. (2013). Mechanisms of resistance to therapies targeting BRCA-mutant cancers. *Nat. Med.* 19, 1381–1388. doi:10.1038/nm.3369.

Mateos-Gomez, P. A., Gong, F., Nair, N., Miller, K. M., Lazzerini-Denchi, E., and Sfeir, A. (2015). Mammalian polymerase θ promotes alternative NHEJ and suppresses recombination. *Nature* 518, 254–257. doi:10.1038/nature14157.

Michlits, G., Jude, J., Hinterndorfer, M., de Almeida, M., Vainorius, G., Hubmann, M., et al. (2020). Multilayered VBC score predicts sgRNAs that efficiently generate loss-of-function alleles. *Nat. Methods* 17. doi:10.1038/s41592-020-0850-8.

Nayak, S., Calvo, J. A., Cong, K., Peng, M., Berthiaume, E., Jackson, J., et al. (2020). Inhibition of the translesion synthesis polymerase REV1 exploits replication gaps as a cancer vulnerability. *Sci. Adv.* 6. doi:10.1126/sciadv.aaz7808.

O'sullivan, J., Mersaoui, S. Y., Poirier, G., and Masson, J. Y. (2021). Assessment of global dna double-strand end resection using brdu-dna labeling coupled with cell cycle discrimination imaging. *J. Vis. Exp.* 2021, 1–15. doi:10.3791/62553.

Oleg TRott, A. J. O. (2009). AutoDock Vina: Improving the Speed and Accuracy of Docking with a New Scoring Function, Efficient Optimization, and Multithreading. *J. Comput. Chem.* 32, 174–182. doi:10.1002/jcc.

Ozdemir, A. Y., Rusanov, T., Kent, T., Siddique, L. A., and Pomerantz, R. T. (2018). Polymerase θ-helicase efficiently unwinds DNA and RNA-DNA hybrids. *J. Biol. Chem.* 293, 5259–5269. doi:10.1074/jbc.RA117.000565.

Panzarino, N. J., Krais, J. J., Cong, K., Peng, M., Mosqueda, M., Nayak, S. U., et al. (2021). Replication gaps underlie BRCA deficiency and therapy response. *Cancer Res.* 81, 1388–1397. doi:10.1158/0008-5472.CAN-20-1602.

Schrempf, A., Slyskova, J., and Loizou, J. I. (2021). Targeting the DNA Repair Enzyme Polymerase θ in Cancer Therapy. *Trends in Cancer* 7, 98–111. doi:10.1016/j.trecan.2020.09.007.

Taglialatela, A., Alvarez, S., Leuzzi, G., Sannino, V., Ranjha, L., Huang, J. W., et al. (2017). Restoration of Replication Fork Stability in BRCA1- and BRCA2-Deficient Cells by Inactivation of SNF2-Family Fork Remodelers. *Mol. Cell* 68, 414-430.e8. doi:10.1016/j.molcel.2017.09.036.

Taglialatela, A., Leuzzi, G., Sannino, V., Cuella-Martin, R., Huang, J.-W., Wu-Baer, F., et al. (2021). REV1-Polζ maintains the viability of homologous recombination-deficient cancer cells through mutagenic repair of PRIMPOL-dependent ssDNA gaps. *Mol. Cell*, 1–18. doi:10.1016/j.molcel.2021.08.016.

Tirman, S., Quinet, A., Wood, M., Meroni, A., Cybulla, E., Jackson, J., et al. (2021). Temporally distinct post-replicative repair mechanisms fill PRIMPOL-dependent ssDNA

gaps in human cells. *Mol. Cell* 81, 4026-4040.e8. doi:10.1016/j.molcel.2021.09.013.

Toledo, L. I., Altmeyer, M., Rask, M. B., Lukas, C., Larsen, D. H., Povlsen, L. K., et al. (2013). ATR prohibits replication catastrophe by preventing global exhaustion of RPA. *Cell* 155, 1088. doi:10.1016/j.cell.2013.10.043.

Wang, Z., Song, Y., Li, S., Kurian, S., Xiang, R., Chiba, T., et al. (2019). DNA polymerase (POLQ) is important for repair of DNA double-strand breaks caused by fork collapse. *J. Biol. Chem.* 294, 3909–3919. doi:10.1074/jbc.RA118.005188.

Yoon, J. H., McArthur, M. J., Park, J., Basu, D., Wakamiya, M., Prakash, L., et al. (2019). Error-Prone Replication through UV Lesions by DNA Polymerase θ Protects against Skin Cancers. *Cell* 176, 1295-1309.e15. doi:10.1016/j.cell.2019.01.023.

Zahn, K. E., Averill, A. M., Aller, P., Wood, R. D., and Doublié, S. (2015). Human DNA polymerase θ grasps the primer terminus to mediate DNA repair. *Nat. Struct. Mol. Biol.* 22, 304–311. doi:10.1038/nsmb.2993.

Zatreanu, D., Robinson, H. M. R., Alkhatib, O., Boursier, M., Finch, H., Geo, L., et al. (2021). Polθ inhibitors elicit BRCA-gene synthetic lethality and target PARP inhibitor resistance. *Nat. Commun.*, 1–15. doi:10.1038/s41467-021-23463-8.

Zhou, J., Gelot, C., Pantelidou, C., Li, A., Yücel, H., Davis, R. E., et al. (2021). A first-in-class polymerase theta inhibitor selectively targets homologous-recombination deficient tumors. *Nat. Cancer*. doi:10.1038/s43018-021-00203-x.

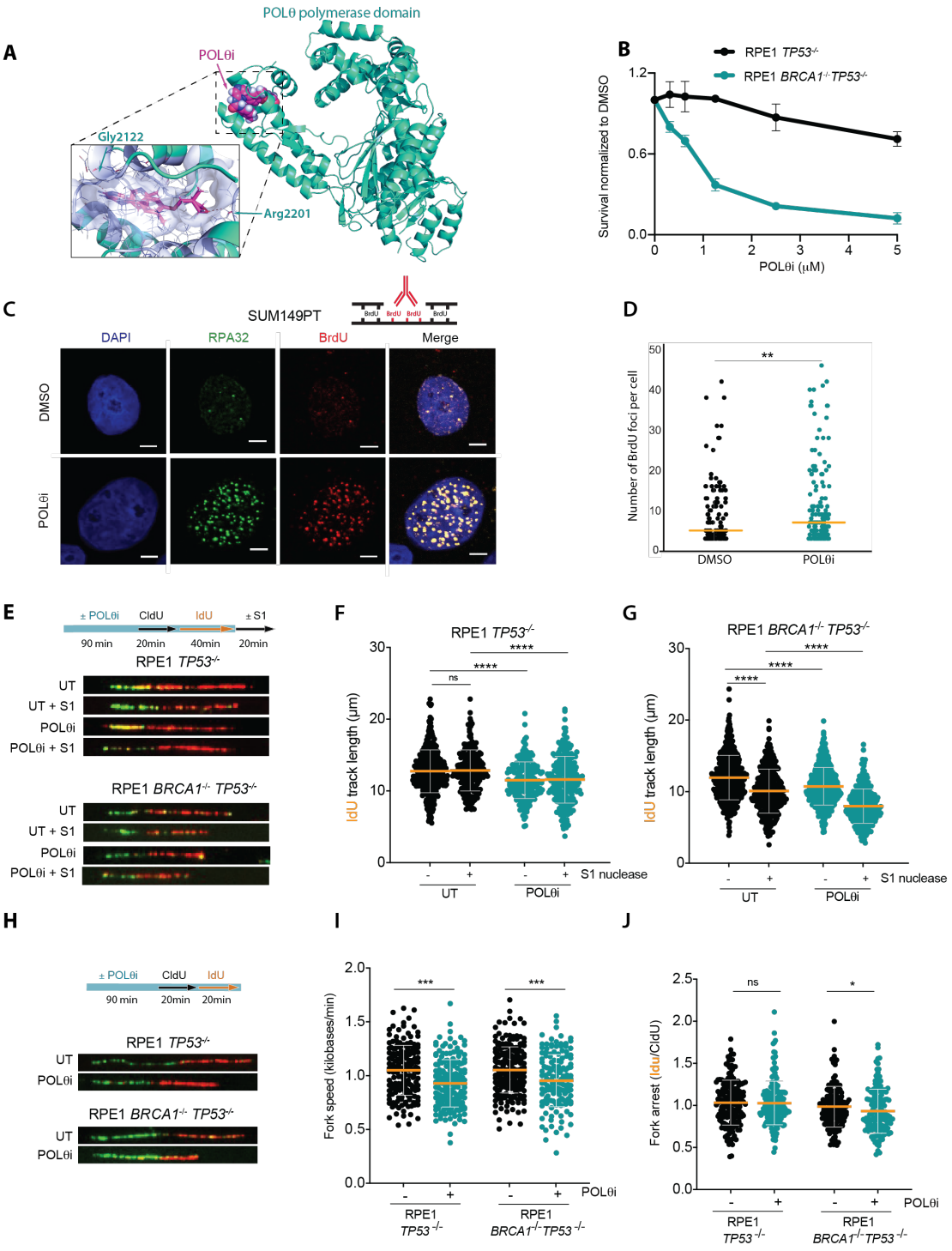


Fig1: POLθ processes ssDNA gaps generated in BRCA1 deficient cells.

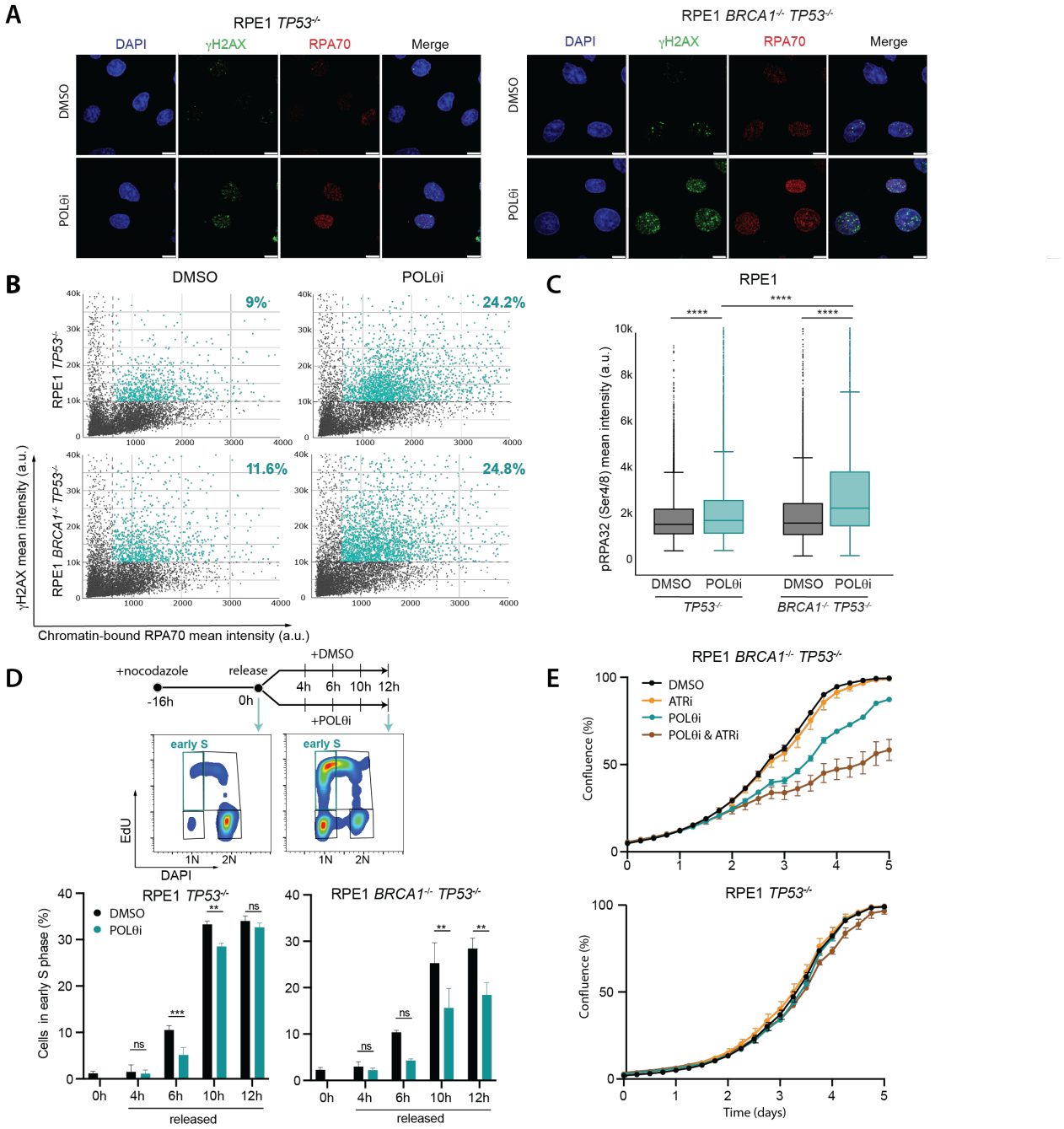


Fig2: POLθ inhibition induced replication stress is exacerbated in BRCA1 deficient cells.

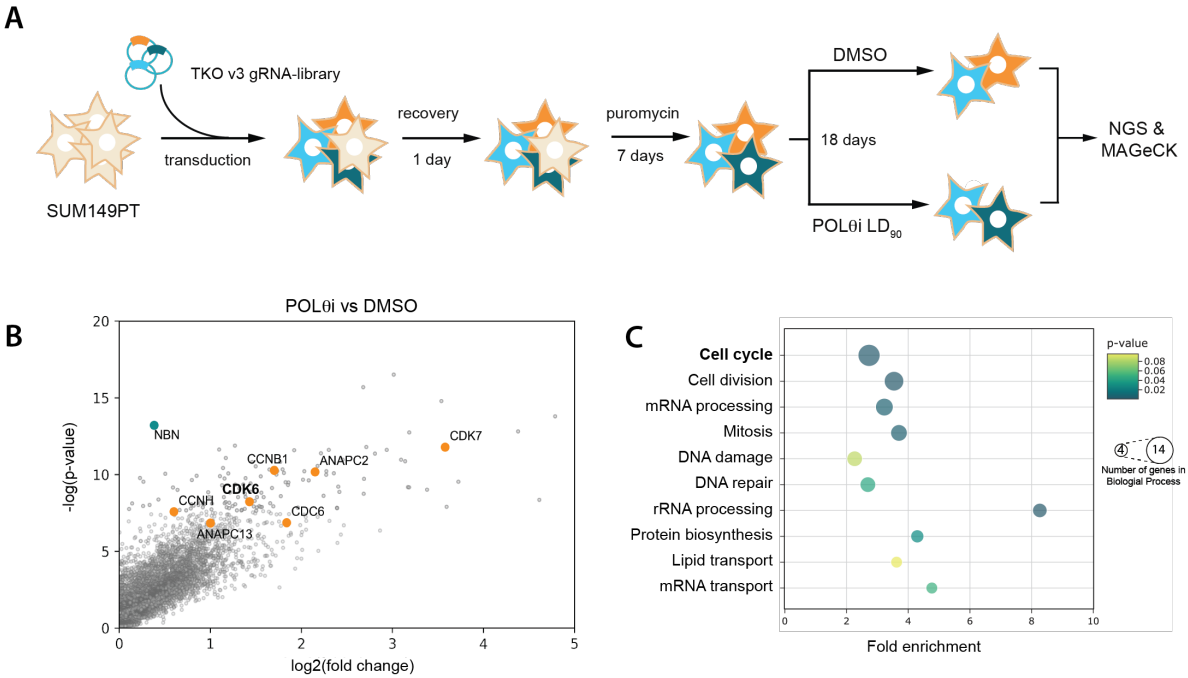


Fig3: A genome-wide CRISPR-Cas9 ko screen identified modulators of the BRCA1-POLθ genetic interaction.

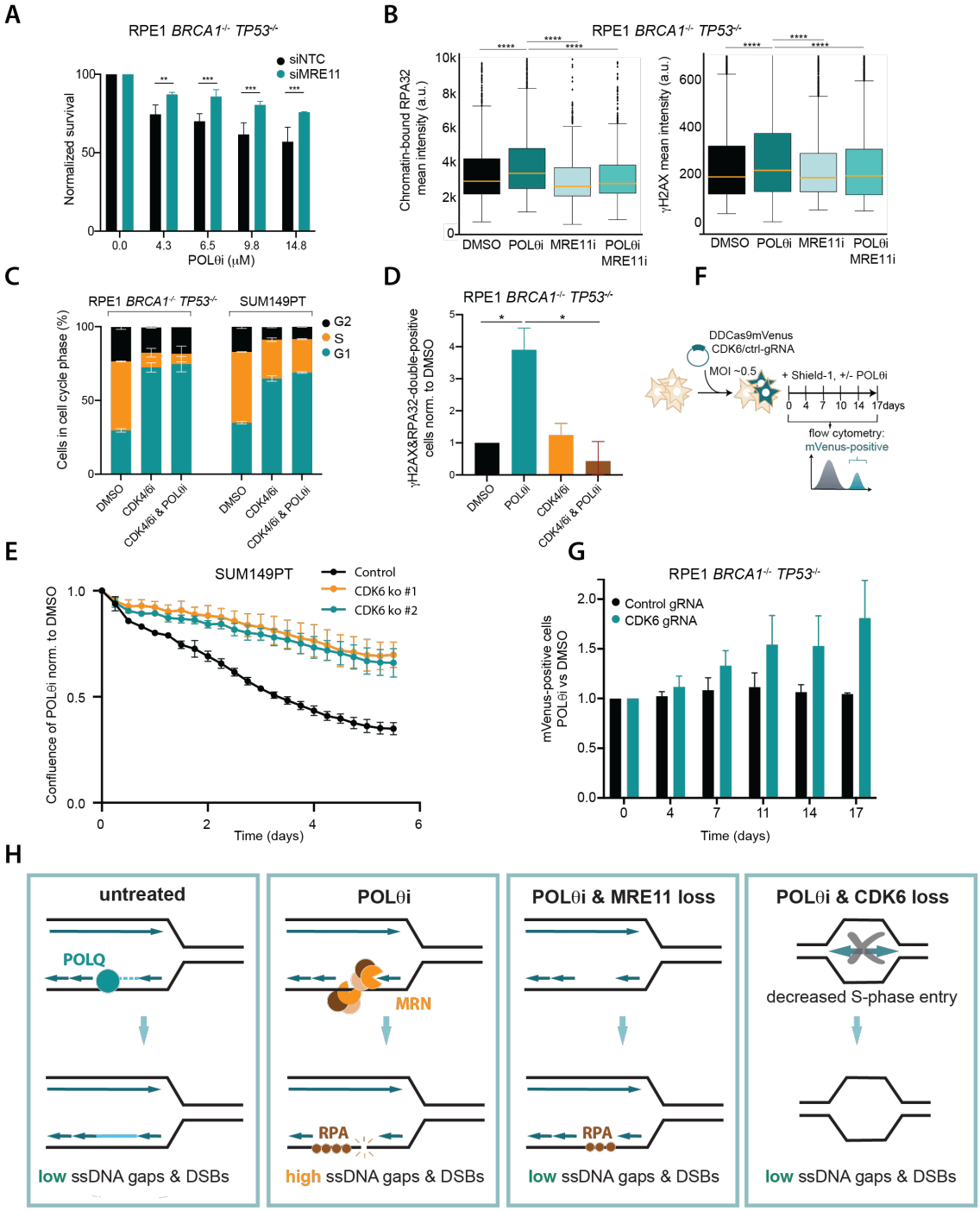
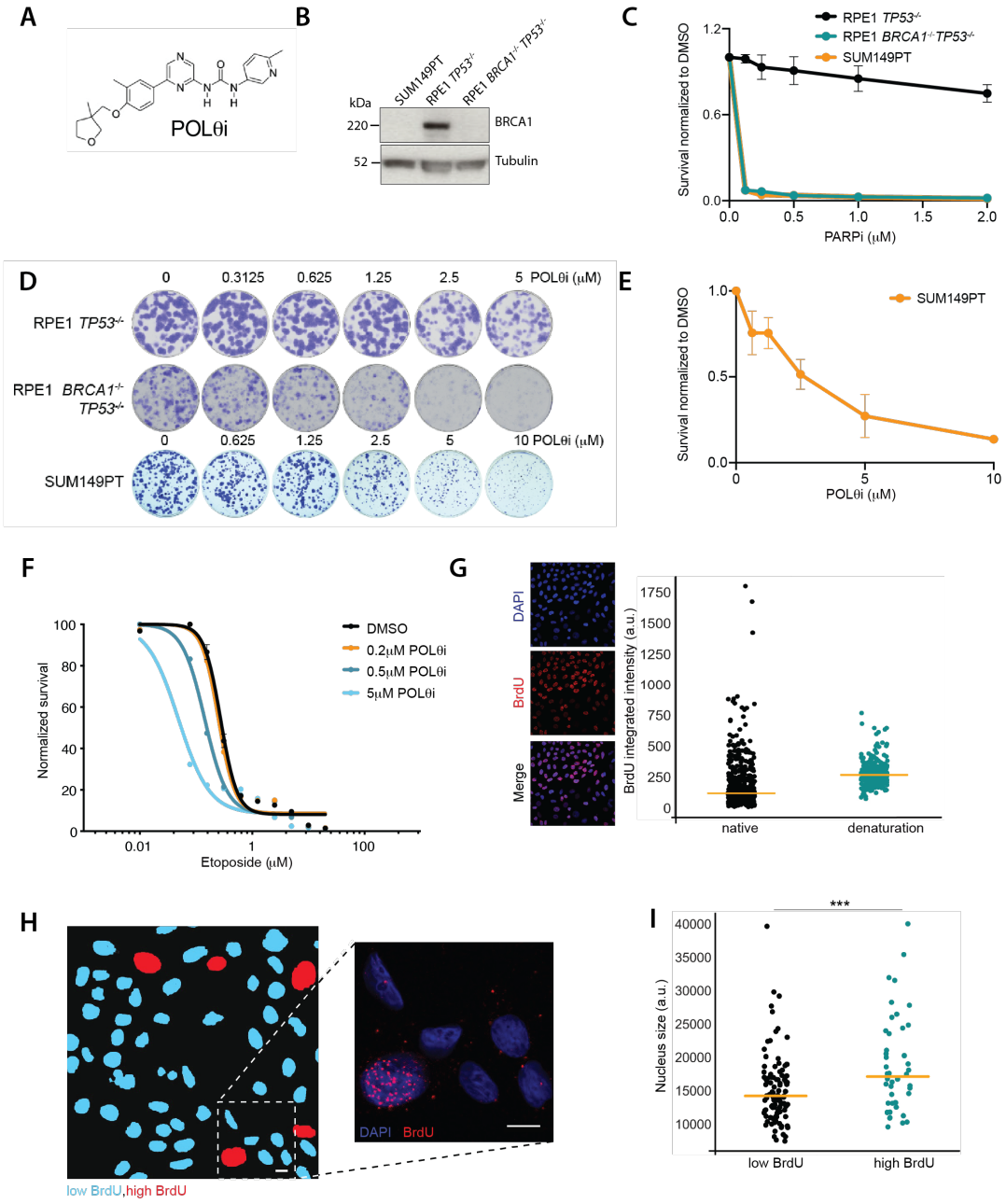
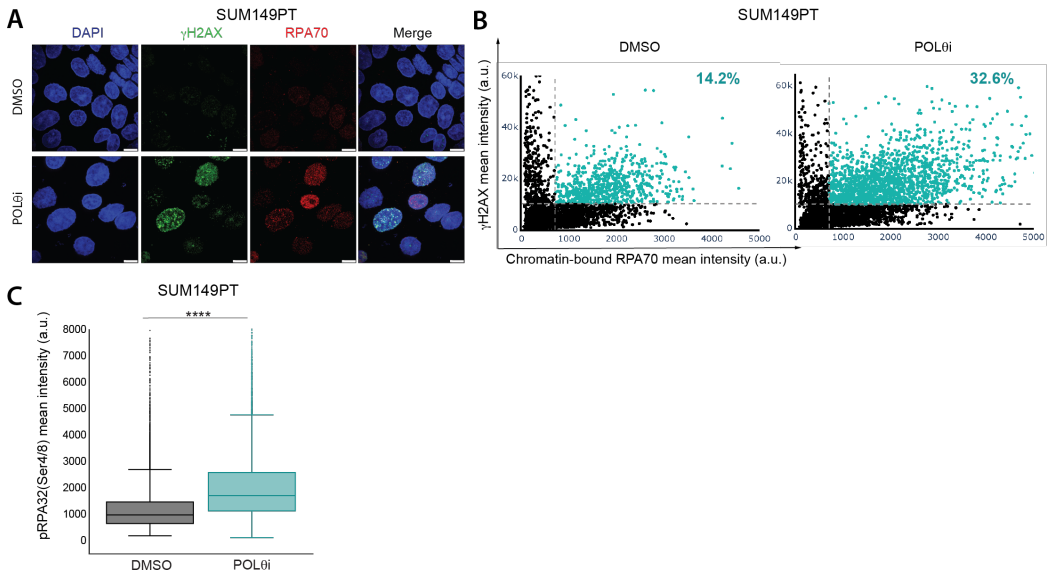


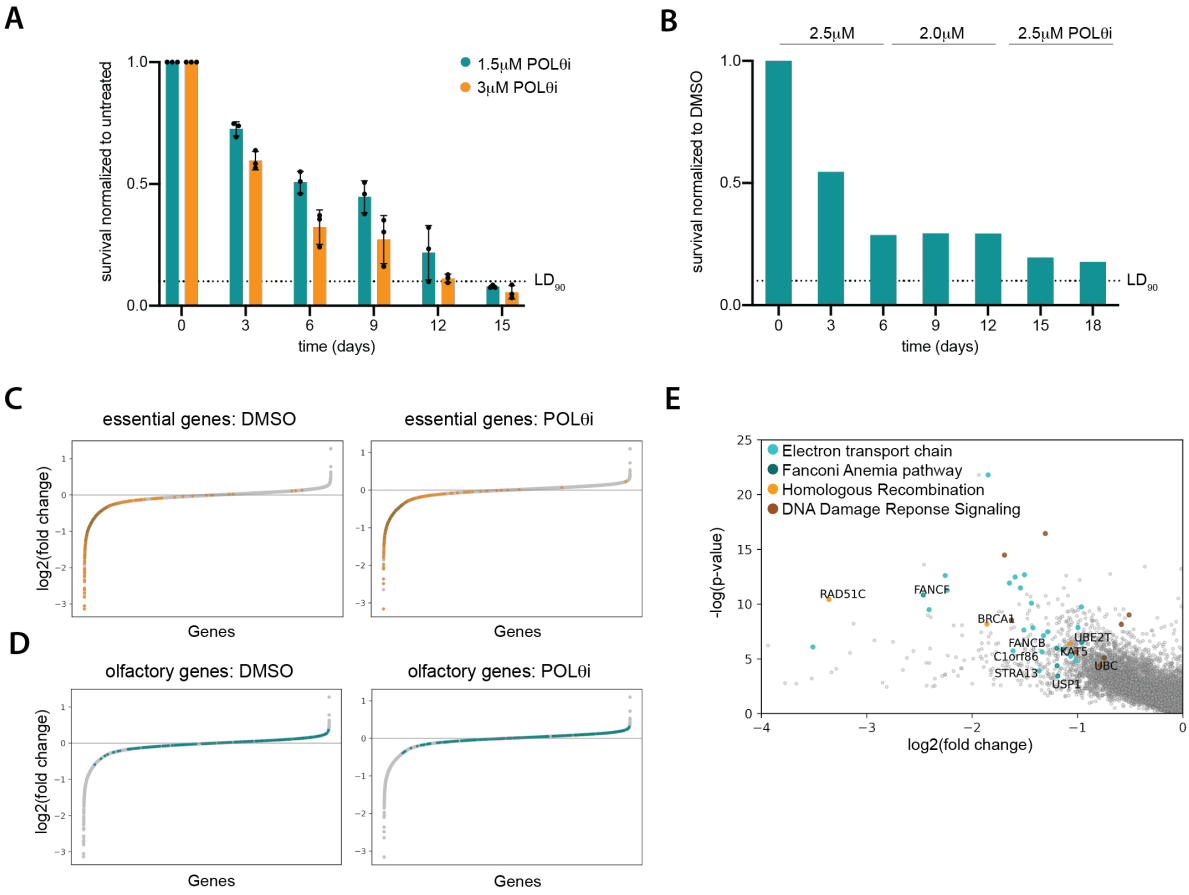
Fig4: Loss of activity of the MRN complex and CDK6 alleviate the functional interaction between BRCA1 and POLθ.



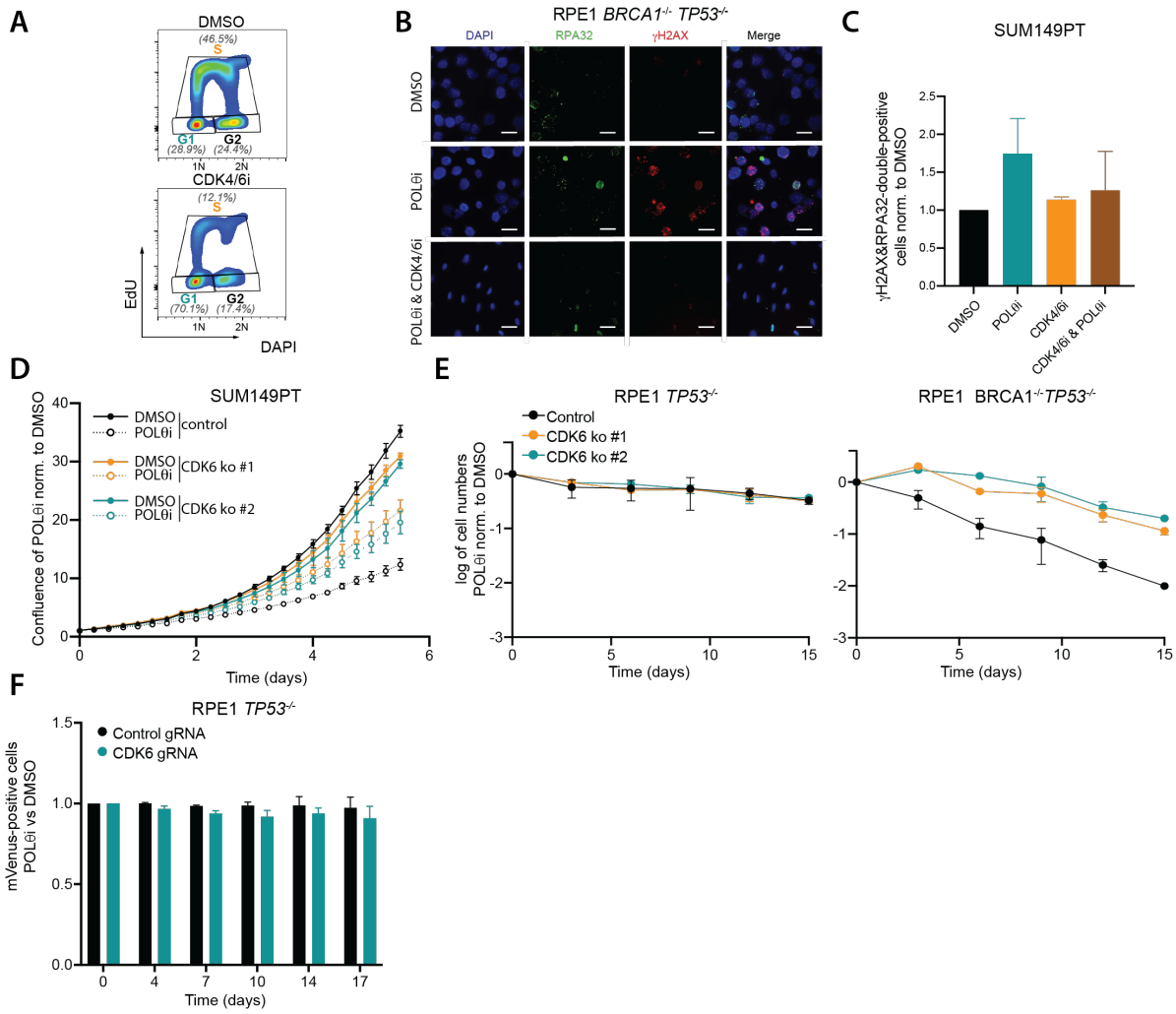
SuppFig1: POLθ processes ssDNA gaps generated in BRCA1 deficient cells.



SuppFig2: POLθ inhibition induced replication stress is exacerbated in BRCA1 deficient cells.



Supp Fig3: A genome-wide CRISPR-Cas9 ko screen identified modulators of the BRCA1-POLθ interaction.



Supp Fig4: Loss of activity of the MRN complex and CDK6 alleviate the functional interaction between BRCA1 and POLθ.

4 Chapter 4: Discussion

4.1 The concept of synthetic lethality revolutionized cancer treatment

Utilizing synthetic lethality for cancer treatment has numerous advantages over traditional chemotherapy. First, inhibition of targets that are essential specifically in cancer cells, opens a therapeutic window that allows specific killing of cancer cells while leaving normal cells unharmed. Second, more than half of all known cancer mutations are loss-of-function mutations in tumor suppressors that are more difficult to target with some even considered as being “undruggable”. In this case, inhibitors aiming at the synthetic lethal partner allows indirect targeting of cancer-specific loss-of-function mutations.

While the identification of genetic interactions used to be time-consuming, the advent of functional genomic screening and next generation sequencing now enables the systemic probing of entire genetic landscapes for interactions that could potentially be exploited in cancer treatment. Recently, CRISPR screening approaches have been developed to not only allow knock-out but also inhibition (CRISPRi) and overexpression (CRISPRa) of genes. The public availability of large amounts of screening data in thousands of samples spanning dozens of cancer types, as provided by projects such as the TCGA, further fuels the understanding of the relevance of genetic interactions in different tissues. In addition, improvements in medicinal chemistry such as high throughput screening of large numbers of candidate compounds allows time- and cost-efficient identification of potent inhibitors that can be directly utilized for target inhibition and potentially further developed for clinical use.

The combined utilization of functional genomic screening, publicly available data and medicinal chemistry can be exemplified by a recently published report aiming at the identification of a novel treatment strategy for cancers with overexpression of cyclin E, which occurs in around 20% of ovarian cancer patients (Gallo *et al*, 2022; Patch *et al*, 2015). Genome-scale knock-out CRISPR screens in cellular models of cyclin E overexpression revealed that mutations in PKYMT confer selective sensitivity in cells with high cyclin E expression compared to cells with normal cyclin E levels. A structure-guided medicinal chemistry approach then facilitated the identification of a small-molecule inhibitor of PKYMT, RP-6306, which was subsequently used to (1) probe the molecular mechanism underlying the synthetic lethal interaction between cyclin E overexpression and PKYMT loss-of-function and (2) validate this interaction in mouse implantation models as well as patient-derived xenografts of cyclin E overexpression. Finally, this compound entered clinical trials as monotherapy and in combination with gemcitabine (ClinicalTrials.gov identifiers NCT04855656 and NCT05147272). The short turnover time between target identification and clinical utilization, as showcased in this example, will dramatically increase the number of exploitable tumor targets in cancer treatment.

4.2 Utilizing synthetic lethal interactions for cancer treatments requires an understanding of their underlying molecular mechanisms

While the above-described pipeline from functional genomic screening to clinical utilization of DDR targets allows rapid identification of numerous targets, understanding the molecular mechanisms underlying the newly discovered synthetic lethal interactions is still a time-consuming process that is difficult to automate. However, acquiring such knowledge is vital for many reasons.

First, a deep molecular understanding of the mechanisms explaining genetic interactions allows the identification of biomarkers that confer sensitivity to a given therapy. This can be exemplified using WRN, a synthetic lethal target in cancers with microsatellite instability, a type of genetic hypermutability that arises as a result of defective mismatch repair (Behan *et al*, 2019; Chan *et al*, 2019; Lieb *et al*, 2019). Mechanistic studies revealed that TA-dinucleotide repeats are expanded in microsatellite unstable cancer cells and eventually form secondary DNA structures that can stall replication forks and cause replication fork collapse, if not resolved by WRN helicase. In the absence of WRN helicase activity, for example as a result of small-molecule inhibition, MUS81 nuclease cleaves stalled replication forks and causes chromosome shattering (van Wietmarschen *et al*, 2020). Extensive DNA resection, RPA exhaustion and chromosome shattering as a result of MUS81 activity are therefore novel biomarkers that underlie the synthetic lethality between microsatellite instability and WRN inhibitor sensitivity.

Second, insights into the biological mechanisms behind synthetic lethal interactions allows predicting treatment combinations. As an example, a recent study showed that cancer cells with amplification of *CCNE1*, leading to overexpression of cyclin E, depend on the negative regulation of CDK1 by the PKMYT1 kinase (Gallo *et al*, 2022). If CDK1 activity is no longer suppressed, for example after inhibition of PKMYT1, cells prematurely enter mitosis before finishing replication, resulting in chromosome pulverization. Based on this observed replication stress phenotype, it was proposed that agents perturbing DNA replication such as hydroxyurea and gemcitabine, might confer additional sensitivity to PKMYT1 inhibition in models of cyclin E overexpression. Indeed, the combination of PKMYT1 inhibition and gemcitabine acts synergistically and is currently explored in human clinical trials (ClinicalTrials.gov identifier NCT05147272).

Third, understanding the underlying molecular connections allows prediction of possible resistance mechanisms and ideally, their collateral vulnerabilities that can be exploited in a secondary treatment strategy. One known example is restoration of HR by loss-of-function mutations in 53BP1 that confers resistance to PARP inhibitor treatment (Jaspers *et al*, 2013). 53BP1 is a key player in DSB repair choice that suppresses end resection and promotes NHEJ (Bunting *et al*, 2010). Loss of 53BP1 function restores end resection and facilitates HR even in the absence of BRCA1. However, cells bearing mutations in 53BP1 become dependent on

POLθ, which can be exploited by targeting this enzyme with small-molecule inhibitors in a second-line treatment (Zatreanu *et al*, 2021; Zhou *et al*, 2021; Wyatt *et al*, 2016).

Fourth, by understanding individual interactions, we gather new insights into the complex interplay between DDR pathways. For instance, studying the hypersensitivity and resistance mechanisms of HR deficient cells to PARP inhibitors, allowed the identification of a missing link in DSB repair pathway choice: the Shieldin complex. Based on the identification of several subunits of the Shieldin complex in independent CRISPR screens aimed at the identification of PARP inhibitor resistance genes, it became clear that this complex promotes NHEJ by shielding DNA ends from resection (Noordermeer *et al*, 2018; Dev *et al*, 2018; Gupta *et al*, 2018).

Summing up, while efficient screening procedures allow the interrogation of the entire genomic landscape for novel anti-cancer targets, the molecular understanding of the identified interactions often lags behind. Obtaining such an understanding, however, is vital not only for learning more about the DDR framework but also for identifying therapy-specific biomarkers, predicting efficient treatment combinations, foreseeing potential origins of resistance as well as determining the respective second-line treatments.

4.3 The accumulation of replication gaps drives cell death in BRCA1 deficient cells

Few synthetic lethal interactions within the DDR have been investigated as intensively as the interaction between BRCA1 and PARP. Interestingly, despite numerous years of research, the exact molecular mechanisms underlying this interaction are still highly debated. Although the DSB was long believed to be the primary lesion responsible for the cellular toxicity of genotoxic chemotherapy, this “DSB model” often fails to align cellular models with their clinical outcomes, for instance in the case of PARP inhibition. As PARP inhibitors are toxic specifically in cells with defects in BRCA or other HR genes that are involved in replication fork protection, PARPi toxicity was believed to stem from DSBs that are generated during fork collapse. This is also consistent with the observation that restoration of HR and fork protection supports chemoresistance (Bouwman *et al*, 2010; Bunting *et al*, 2010; Chaudhuri *et al*, 2016; Sakai *et al*, 2008). In 2018, this model was confronted by the finding that PARP inhibition does not block or slow DNA replication but instead supports an acceleration of fork speed (Maya-Mendoza *et al*, 2018). To accommodate for this, the DSB model was adjusted by proposing that unrestrained replication leads to DSB formation (Maya-Mendoza *et al*, 2018; Quinet. & Vindigni, 2018). A landmark study in 2021 then addressed the causes of PARPi sensitivity in an elegant series of separation-of-function models (Cong *et al*, 2021). First, the authors showed that FANCJ mutant cells, despite having defective HR and fork protection, were not hypersensitive to PARP inhibition, challenging the hypothesis that lack of HR and fork protection are the main drivers of sensitivity. Furthermore, unrestrained replication was also not the origin of PARPi sensitivity as shown by lack of hypersensitivity in p21 deficient cells.

This raised the question whether DSBs generated upon fork collapse or unrestrained replication fork progression are truly the cause of PARPi sensitivity. An alternative explanation could be provided based on a newly discovered role of BRCA1 in the suppression of ssDNA gaps during replication which have been visualized in *Xenopus* egg extracts using electron microscopy (Schlacher *et al*, 2011; Hashimoto *et al*, 2010). Although ssDNA gaps were long neglected as primary toxic lesions based on the assumption that they were easy to repair, ssDNA formation is a common event in chemotherapy. Etoposide and ionizing radiation, for instance, generate a 30- and 100-fold increased load of ssDNA gaps compared to DSBs, respectively (Yue Gao, 2009). In the case of cisplatin, ssDNA levels allow predicting therapy response, suggesting that this lesion might mediate some of the observed toxicity (Chen & Zeller, 1990). Recent studies indicate that multiple doses of cisplatin treatment promote ssDNA gap formation by fork repriming mediated by PRIMPOL (Quinet *et al*, 2020). Considering ssDNA as the primary toxic lesion and taking a newly described role of PARP1 in Okazaki fragment processing into account, a novel model of PARPi functionality that aligns well with the observed clinical outcomes, has been suggested (Cong *et al*, 2021; Hanzlikova *et al*, 2018). Okazaki fragments formed on the lagging strand that escape processing by the canonical Okazaki fragment processing pathway consisting of FEN1 and LIG1, are recognized by PARP1 (Hanzlikova *et al*, 2018; Zheng & Shen, 2011; Levin *et al*, 1997). PARP1 then undergoes poly(ADP-ribosyl)ation (PARylation) to recruit XRCC1, a player in back-up Okazaki fragment processing together with LIG3 (Arakawa & Iliakis, 2015). The role of PARP1 as a sensor of ssDNA gaps from unligated Okazaki fragments is also highlighted by increased PARylation upon inhibition of canonical LIG1 and FEN1 (Hanzlikova *et al*, 2018). In BRCA1 deficient cells, back-up Okazaki fragment processing fails to engage as aberrantly high 53BP1 levels prevent PARP1 from recruiting XRCC1 (Cong *et al*, 2021). Several lines of evidence support this novel ssDNA gap model. (1) PARP inhibition is synthetic lethal with loss of function of genes that have no described role in HR or fork protection, but instead function in Okazaki fragment processing, such as FEN1, XRCC1, PCNA and LIG1 (Thakar *et al*, 2020; Ström *et al*, 2011). (2) Several HR proteins including BRCA1, BRCA2 and RAD51 have roles in replication gap suppression, as shown by abnormally high ssDNA levels in cells harboring mutations in these genes (Cong *et al*, 2021; Taglialatela *et al*, 2021; Kolinjivadi *et al*, 2017; Hashimoto *et al*, 2010). (3) HR proficiency fails to predict PARPi response in the clinics (Ledermann and Pujade-Lauraine 2019). (4) PARPi resistance through loss of 53BP1 can be explained by restoration of back-up Okazaki fragment processing, as 53BP1 no longer prevents XRCC1 recruitment through PARP1. This is supported by restoration of PARP1-dependent PARylation to recruit LIG3-XRCC1 to chromatin upon 53BP1 loss (Cong *et al*, 2021). (5) PARPi re-sensitization of BRCA1-53BP1 deficient cells through loss of LIG3 can be

explained by loss of back-up Okazaki fragment processing that depends on XRCC1 and LIG3 (Dias 2021).

While overall gap levels are increased upon inhibition of PARP, it remains partially unclear why the resulting toxicity is more profound in BRCA-deficient cells. Several explanations were put forward. (1) When the replication machinery faces an obstacle, PrimPol allows repriming of DNA synthesis downstream of the lesion, which leads to generation of single-stranded gaps. BRCA2 was recently shown to restrain DNA replication fork progression after DNA damage by counteracting PrimPol activity (Kang *et al*, 2021). In the absence of BRCA2 function, increased PrimPol activity could generate more ssDNA gaps. (2) BRCA proteins inhibit the extension of ssDNA gaps by nucleases such as MRE11 (Tirman *et al*, 2021; Quinet *et al*, 2020). Therefore, the lack of BRCA activity in combination with PARP inhibition increases the overall ssDNA gap burden. (3) BRCA2 was shown to promote the interaction between RAD51 and Polymerase α, potentially supporting efficient lagging strand synthesis (Kolinjivadi *et al*, 2017).

While this model is now slowly being accepted for contributing to PARPi toxicity, we are the first to propose that ssDNA levels also determine the outcome of POLθ inhibitor therapy in BRCA1 mutant cells. An increase of ssDNA levels upon POLθ inhibition in mouse embryonic fibroblasts lacking both BRCA1 and 53BP1, was shown previously (Zatreanu *et al*, 2021). However, this was attributed to increased resection of DNA DSBs. Using S1 nuclease assays and detection of intracellular PAR-levels, we show that increased ssDNA levels upon POLθ inhibition stem from faulty processing of gaps on the lagging strand of replication. This pinpoints to a novel role of POLθ in replication gap suppression and is in line with ssDNA being the toxic lesion in BRCA1 mutant cells.

4.4 POLθ is synthetic lethal with BRCA1 by suppressing replication gap formation

For many years, POLθ has been perceived merely as a back-up enzyme for DNA DSB repair when both NHEJ and HR fail. Only when it became clear that POLθ overexpression is a frequent event in cancer and that HR deficiency confers a dependence on POLθ for DNA repair, did this enzyme received more attention. While first inhibitors are now already entering the clinics, the molecular mechanisms underlying this observed synthetic lethal interaction is still not entirely clear. We have shown that in addition to its role in DSB repair, POLθ also functions in unperturbed replication by suppressing the formation of single-stranded gaps on the lagging strand that form during Okazaki fragment generation. This novel role of POLθ becomes limiting in cells bearing mutations in BRCA1 that have decreased replication fork fidelity and single-stranded gap formation. If POLθ is absent in this setting, single-stranded gaps accumulate that eventually exhaust the nuclear levels of RPA, a common hallmark of replication stress. This leaves single-stranded regions unprotected and accessible to

nucleolytic degradation, for example by the MRN-complex, forming DSBs that can be visualized with γH2AX. Therefore, POLθ is required for maintaining genome stability by suppressing replication gap formation on the lagging strand of replication, a role which becomes limiting in BRCA1 deficient cells and might partially explain their dependence on POLθ function **(Figure 12).**

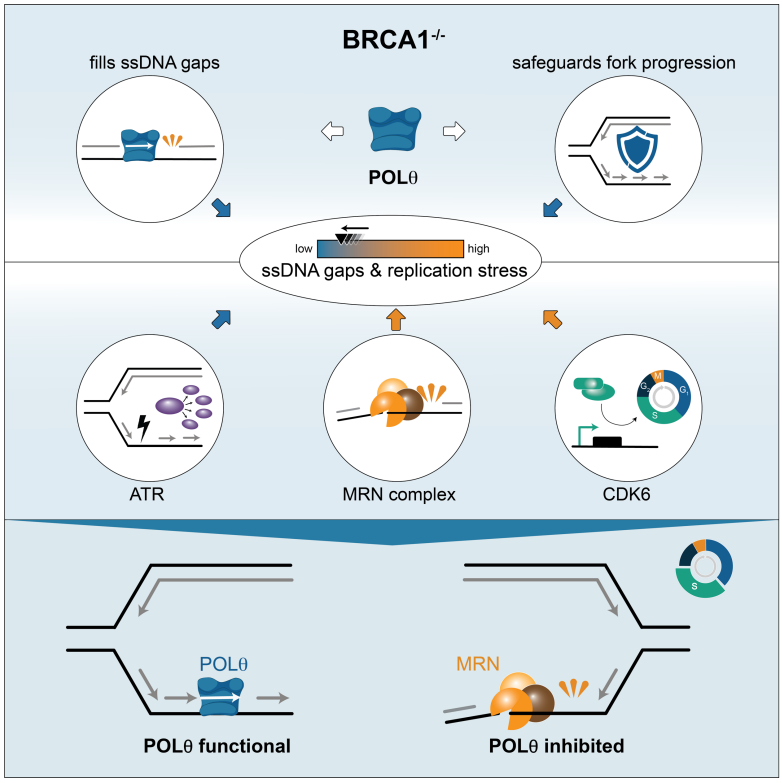


Figure 12: Graphical model of the role of POLθ in replication gap suppression.

4.5 The unique combination of helicase and polymerase domain make POLθ ideal for replication gap suppression

POLθ is a multifunctional enzyme that contains both an N-terminal helicase domain and a C-terminal polymerase domain, which makes it the only eukaryotic polymerase known to date that possesses helicase activity (Black *et al*, 2016; Ozdemir *et al*, 2018; Wood & Doublié, 2016). While the functions of each individual domain were actively studied, it remains largely unclear why both polymerase and helicase activity are required in one polypeptide. The helicase domain has been shown to unwind DNA and DNA-RNA hybrids and similar to the related HELQ helicase, was shown to preferentially act on DNA substrates resembling the lagging strand of replication (Ozdemir *et al*, 2018). This is in line with our findings implicating POLθ in the processing of lagging strand replication. The C-terminal polymerase domain, on the other hand, is a “Swiss Army knife” for DNA repair: it can support terminal transferase activity as well as templated extension *in cis* and *in trans* (Kent *et al*, 2016). Moreover, it catalyzes translesion synthesis, opposite different types of DNA lesions such as abasic sites or thymine glycols (Hogg *et al*, 2011, 2004; Seki *et al*, 2003; Kusumoto *et al*, 2002; Takata *et*

al, 2006; Yoon *et al*, 2019). Only recently, the polymerase domain was found to be capable of accommodating RNA-DNA hybrids to promote RNA-templated DNA repair (Chandramouly *et al*, 2021). Given our findings of POLθ in lagging strand processing, one can speculate that POLθ might require this activity to initiate repair from RNA primers of Okazaki fragments. Elegant *in vitro* studies in the manuscript accompanying our study, led by the Costanzo lab, have shed light on the interplay between the two domains (Mann et al, under review, 2022). In specific, POLθ depleted *Xenopus* egg extracts were reconstituted with either full-length POLθ or one of the two domains, followed by electron microscopy of replication intermediates. While reconstitution of full-length POLθ efficiently decreased the observed ssDNA load at replication intermediates compared to the non-reconstituted control, overexpression of the polymerase domain resulted in replication intermediates with small flaps. Reconstitution of the helicase domain alone, on the other hand, failed to decrease gap levels on replication intermediates. Based on these observations, the following model is proposed. The POLθ polymerase domain fills the inter-Okazaki fragment gaps while the helicase domain removes stalled Okazaki fragments along the way, providing an explanation for the unique combination of both helicase and polymerase activities within one polypeptide. While translesion synthesis polymerases are also capable of filling gaps but lack a helicase domain to remove any obstacles along the way, one could speculate that translesion synthesis polymerases are responsible for gap filling on the leading strand of replication while POLθ is responsible for lagging strand gap filling.

PARP1 has been shown to recruit POLθ to initiate TMEJ (Mateos-Gomez *et al*, 2015). Considering that PARP1 acts a sensor of unligated Okazaki fragments, this route of recruitment would also fit with a role of POLθ in ssDNA gap processing (Hanzlikova *et al*, 2018). Alternatively, translesion synthesis signaling could recruit POLθ, as supported by the finding that POLθ binds to ubiquitinated PCNA in UV-irradiated human fibroblasts (Yoon *et al*, 2019). Furthermore, POLθ-dependent displacement of RAD51, which was originally thought to determine pathway choice between HR and TMEJ on ssDNA overhangs of DSBs, would also fit with a role of POLθ in the processing of ssDNA regions that are pre-coated with RAD51 (Ceccaldi *et al*, 2015). Similarly, POLθ helicase domain was shown to counteract RPA to promote annealing to initiate end joining wile suppressing recombination of ssDNA (Mateos-Gomez *et al*, 2017). This antagonistic function could also be involved in lagging strand synthesis as RPA continues to coat single-stranded regions, which is no longer required upon POLθ binding.

Summarizing, the unique domain architecture composed of both a helicase domain, capable of unwinding the lagging strand of replication and displacing stalled Okazaki fragments, as well as a polymerase domain, which promotes translesion synthesis, make POLθ an ideal candidate for lagging strand processing.

4.6 POLθ maintains replication fork stability in BRCA1 deficient cells

In our study, we have discovered a novel function of the DSB repair enzyme POLθ in gap suppression during lagging strand replication. Given this finding, it remains to be addressed to which extent POLθ affects the overall fidelity of replication. In our study, we have demonstrated that POLθ inhibition decreases fork speed in BRCA1 mutant cells and their parental cell line, indicating a role of POLθ in unperturbed replication. Furthermore, this is outlined by a decrease in fork symmetry in BRCA1 mutant cells upon POLθ inhibition, indicating problems with fork progression that result in fork collapse. This ties well with other studies, showing decreased fork speed in the absence of exogenous DNA damaging agents as well as increased fork collapse or stalling with hydroxyurea, upon loss of POLθ activity (Ceccaldi *et al*, 2015). A certain expression level seems to be required for intact replication dynamics as POLθ overexpression has also been shown to reduce fork speed (Lemée *et al*, 2010). The involvement of POLθ in maintaing replication fork dynamics is also highlighted by hypersensitivity of POLθ knock down or knock out cells to replication stress inducing agents such as hydroxyurea (Wang *et al*, 2019). Based on their culminating roles in replication stress, POLθ knock out cells were also shown to be hypersensitive to inactivation of ATR (Wang *et al*, 2019). This is consistent with our finding that ATR and POLθ inhibition act synergistically in BRCA1 deficient cells, which endure elevated replication stress levels.

Another link between replication and POLθ is provided by the finding that POLθ determines the timing of replication in human cells (Fernandez-Vidal *et al*, 2014). In specific, this is enabled by POLθ binding to the Orc2 and Orc4 subunits of the Origin recognition complex, bringing POLθ in close physical proximity of the replication fork. The role of POLθ in replication timing is demostrated by delayed replication in cells overexpressing POLθ.

Overall, apart from its well described role in DSB repair, POLθ appears to be involved in maintaining replication fork stability, a function which becomes limiting under challenged conditions, such as in a BRCA1/2 deficient background.

4.7 Modulators of POLθ inhibitor (POLθi) response and their implications for the clinical use of POLθ inhibitors

Using a genome-wide CRISPR-Cas9 knock out screen, we have demonstrated that several processes, such as cell cycle regulation and ssDNA processing, modulate the effect of POLθ inhibition in BRCA1 deficient cells. In specific, we have identified CDK6 as a suppressor of the genetic interaction between POLθ and BRCA1, highlighted by an alleviated POLθi response upon loss of CDK6 activity. We could pinpoint this to a decreased percentage of S phase cells, which are most vulnerable to concomitant loss of POLθ and BRCA1 activity. This is in line with previous reports, demonstrating that CDK4/6 inhibition protects from the cytotoxic effects of

chemotherapy, such as treatment with anthracyclines, as cells are blocked from S phase entry (McClendon *et al*, 2012). This protective effect has been shown to strongly depend on the timing of treatment. The sequential administration of CDK4/6 inhibitors after taxane treatment, for example, has been shown to potentiate the chemotherapeutic effect, based on the repressive activity of CDK4/6 inhibitors on HR proteins (Salvador-Barbero *et al*, 2020). Based on these studies and our findings, there should be caution when considering combination treatments of POLθ with CDK4/6 inhibitors.

Moreover, our CRISPR-Cas9 knock out screen led to the identification of NBN, a member of the MRN-complex. We could further show that inhibition of the activity of MRE11, another member of this complex, suppresses ssDNA load, supporting a model in which MRE11 processes ssDNA gaps exposed in the absence of POLθ activity. *In vitro* assays have shown that the MRE11 endonuclease activity favors ssDNA regions adjacent to dsDNA with a protein bound to the 5' or 3' end, a structure closely resembling a replication gap (=ssDNA) neighbouring an Okazaki fragment (=dsDNA), possibly bound by a stalled polymerase (Paull, 2018; Williams *et al*, 2008). This finding suggests that POLθ inhibitors will be less potent and should therefore be used with caution in patients with mutations in the MRN-complex that are frequent in human cancers (Park *et al*, 2011).

In contrast to MRE11 and CDK6 loss, we have shown that co-treatment with ATR inhibitors acts synergistically with POLθ inhibition. Based on the culminating roles of ATR and POLθ in the maintaing replication fork stability, we propose a model in which combined loss of ATR and POLθ surpasses the tolerable threshold of replication stress. This has also been reported in the context of PARP inhibition. ATR and PARP inhibitors were shown to act synergistically in PARPi resistant patient-derived xenograft models as well as in ATM-deficient cells (Kim *et al*, 2020; Lloyd *et al*, 2020). Considering that several ATR inhibitors are currently in clinical trials, combination therapy of ATRi and POLθi might potentiate tumor response.

In addition to proposing individual genetic interactions of POLθ, our study also supports a rethinking of the DSB dogma in the therapy of BRCA mutant tumors. Instead of increased DSB levels by defective HR, "BRCAness" could describe impaired lagging strand processing that renders BRCA deficient cells sensitive to treatments that support the toxic accumulation of replication gaps. This is strongly supported by the dependence of BRCA mutant cells on translesion synthesis for gap filling, highlighted by the resistance observed in BRCA deficient cells with upregulated translesion synthesis (Chen *et al*, 2022; Nayak *et al*, 2020; Cuella-Martin *et al*, 2021; Wojtaszek *et al*, 2019). Therefore, revisiting our understanding of BRCAness might allow the accurate prediction of biomarkers and efficient combination therapies based on gap vulnerability.

4.8 Limitations of this study

In this study, we have identified an unanticipated role of POLθ in replication gap suppression. However, our study has certain limitations. First, our findings are limited to one POLθ inhibitor targeting the polymerase domain. Future studies utilizing different POLθ inhibitors or genetic mutants of the enzymatic domains, will allow studying the functions of individual domains. Second, the generation of BRCA1 and POLθ double knock out cell lines is technically challenging due to the strong lethal interaction shared by these two factors. To allow the functional interrogation of POLθ in a genetic model of concomitant POLθ and BRCA1 loss, the use of technologies allowing a partial knock down such as CRISPRi will be helpful. This might also enable electron microscopy of replication intermediates, which is technically not feasible in knockout cell lines due to insufficient biological material. Third, although we have demonstrated that POLθ functions in lagging strand processing, we cannot exclude a contribution of impaired DSB repair upon POLθ inhibition in BRCA1 deficient cells. Several observations suggest that the role of POLθ in DSB repair becomes limiting under challenged conditions. First, the TMEJ mutational signature, characterized by microhomology flanked deletions, is increased in tumor carrying mutations in *BRCA1/2*. Second, *C. elegans* with mutations in the FANCJ homolog DOG-1, depends on POLθ for the suppression of big deletions at sites with G4 quadruplex forming potential (G4 sites), highlighted by an increased TMEJ mutational signature at such loci in the absence of POLθ activity. However, the roles of POLθ in ssDNA and DSB processing are not mutually exclusive. We propose a model in which POLθ is both responsible for maintaining lagging strand processing as well as recapturing broken ends generated upon fork collapse for TMEJ.

4.9 Conclusions and Future Directions

Within the last decades, cancer therapy has transformed from using “hammers” such as chemotherapeutic compounds to “scalpels” targeting selected nodes in cancer signaling pathways with high specificity. While initially, such nodes were identified by low-throughput hypothesis-driven approaches, genome-wide perturbation approaches such as CRISPR screens combined with low sequencing costs now enable systematic screening of the entire genome for synthetic lethal interactions that are exploitable for cancer treatment. PARP inhibitors, as the first DDR-targeted anti-cancer therapy, have proven to be highly effective in BRCA mutant tumors. The PARPi induced toxicity is thought to stem from the inability of BRCA mutant cells to prevent and repair DSBs that are generated upon PARP trapping. However, the observation that PARPi response can be uncoupled from the role of BRCA in HR or fork protection has challenged this DSB model. Unlike DSBs, levels of replication-associated

ssDNA gaps allow predicting therapy response, inspiring a revised model of PARPi action in BRCA mutant cells.

Despite the initial success of PARP inhibitors, resistance is common, launching a search for new targets that could be utilized in PARPi resistant cancers. In this context, the DNA repair polymerase POLθ was suggested as its inhibition not only confers cellular lethality in BRCA mutant cells but in addition, shares a synthetic lethality with 53BP1, a common resistance gene for PARPi. Based on these encouraging findings, POLθ inhibitors are under active investigation and have already entered clinical trials. The synthetic lethality between POLθ and BRCA is thought to stem from their roles in DSB repair. In the absence of BRCA proteins, HR is no longer functional, making the cells dependent on TMEJ for repair of resected DNA ends. However, POLθ has also been reported to function in replication by affecting replication timing and becoming limiting for replication integrity under challenged conditions. Having a role of BRCA in the maintenance of replication fidelity in mind, we asked whether the BRCA1- POLθ synthetic lethality could also stem for their culminating roles in replication.

Indeed, we identified a role of POLθ at the replication fork. Specifically, we show that POLθ is required for the suppression of replication-associated gaps on the lagging strand of BRCA1 mutant cells. Increased gap formation in the absence of POLθ drives decreased fork stability, as shown by decreased fork speed and symmetry. This induces a cellular replication stress phenotype as manifested by increased RPA-chromatinization and γH2AX formation, ultimately impacting on cell cycle progression and hypersensitivity to ATR inhibition. Furthermore, using an unbiased genome-wide approach, we identified two cellular processes, ssDNA processing and cell cycle regulation, that modulate POLθ inhibitor response in BRCA1 mutant cells. Overall, our findings question the DSB dogma of therapy response in BRCA mutant cells and support the relevance of ssDNA gaps, as already suggested for PARPi.

While this provides a starting point for the investigation of replication fork stability in cells lacking POLθ and BRCA activity, further studies are required to dissect the molecular mechanisms of POLθ in more detail. In specific, the contributions of DSB and ssDNA to the phenotype of POLθi in BRCA mutant cells remain largely unclear. The finding that ssDNA levels drive POLθi response also have implications for the clinical use of these inhibitors: Which biomarkers, apart from BRCA mutations, predispose cells to ssDNA gap formation and therefore cause hypersensitivity to POLθ loss? Which treatment combinations synergize in ssDNA gap formation and would therefore have increased anti-tumor potency? How do cancer cells manage to circumvent excessive gap formation to develop resistance?

Unlike classical chemotherapeutic agents, targeted anti-cancer therapy depends on an understanding of the molecular events responsible for the development of cancer. Despite the cutting-edge, high-throughput methodologies recently developed to identify novel synthetic lethal interactions, our understanding of the mechanisms underlying cancer treatments used

in the clinics is lagging. We are still at the drawing board to define the mechanism of PARP inhibitors, after their clinical use for more than ten years. This highlights the importance of basic research to refine our understanding of DNA repair mechanisms to ultimately instruct the use of DDR-targeted therapies in the clinics.

References

Alexandrov LB, Ju YS, Haase K, Van Loo P, Martincorena I, Nik-Zainal S, Totoki Y, Fujimoto A, Nakagawa H, Shibata T, *et al* (2016) Mutational signatures associated with tobacco smoking in human cancer. *Science (80-)* 354: 618–622

Alexandrov LB, Nik-Zainal S, Wedge DC, Aparicio SAJR, Behjati S, Biankin A V., Bignell GR, Bolli N, Borg A, Børresen-Dale AL, *et al* (2013) Signatures of mutational processes in human cancer. *Nature* 500: 415–421

Ammazzalorso F, Pirzio LM, Bignami M, Franchitto A & Pichierri P (2010) ATR and ATM differently regulate WRN to prevent DSBs at stalled replication forks and promote replication fork recovery. *EMBO J* 29: 3156–3169

Arakawa H & Iliakis G (2015) Alternative okazaki fragment ligation pathway by dna ligase III. *Genes (Basel)* 6: 385–398

Bansbach CE, Bétous R, Lovejoy CA, Glick GG & Cortez D (2009) The annealing helicase SMARCAL1 maintains genome integrity at stalled replication forks. *Genes Dev* 23: 2405–2414

Bartkova J, Hor Z, Koed K, Kra A, Tort1 F, Zieger K, Guldberg1 P, Sehested M, Nesland JM, Lukas C, *et al* (2006a) DNA damage response: An important anticancer barrier in early human tumorigenesis. *Nature* 434: 864–870

Bartkova J, Rezaei N, Liontos M, Karakaidos P, Kletsas D, Issaeva N, Vassiliou LVF, Kolettas E, Niforou K, Zoumpourlis VC, *et al* (2006b) Oncogene-induced senescence is part of the tumorigenesis barrier imposed by DNA damage checkpoints. *Nature* 444: 633–637

Beà S, Valdés-Mas R, Navarro A, Salaverria I, Martín-Garcia D, Jares P, Giné E, Pinyol M, Royo C, Nadeu F, *et al* (2013) Landscape of somatic mutations and clonal evolution in mantle cell lymphoma. *Proc Natl Acad Sci U S A* 110: 18250–18255

Beck H, Nähse V, Larsen MSY, Groth P, Clancy T, Lees M, Jørgensen M, Helleday T, Syljuåsen RG & Sørensen CS (2010) Regulators of cyclin-dependent kinases are crucial for maintaining genome integrity in S phase. *J Cell Biol* 188: 629–638

Bednarski JJ & Sleckman BP (2019) At the intersection of DNA damage and immune responses. *Nat Rev Immunol* 19: 231–242

Behan FM, Iorio F, Picco G, Gonçalves E, Beaver CM, Migliardi G, Santos R, Rao Y, Sassi F, Pinnelli M, *et al* (2019) Prioritization of cancer therapeutic targets using CRISPR–Cas9 screens. *Nature* 568: 511–516

Belousova EA, Maga G, Fan Y, Kubareva EA, Romanova EA, Lebedeva NA, Oretskaya TS & Lavrik OI (2010) DNA Polymerases β and λ Bypass Thymine Glycol in Gapped DNA Structures. *Biochemistry* 49: 4695–4704

Bétous R, Couch FB, Mason AC, Eichman BF, Manosas M & Cortez D (2013) Substrate-Selective Repair and Restart of Replication Forks by DNA Translocases. *Cell Rep* 3: 1958–1969

Bhat KP, Bétous R & Cortez D (2015) High-affinity DNA-binding domains of replication protein A (RPA) direct SMARCAL1-dependent replication fork remodeling. *J Biol Chem* 290: 4110–4117

Bhat KP & Cortez D (2018) RPA and RAD51: Fork reversal, fork protection, and genome stability. *Nat Struct Mol Biol* 25: 446–453

Bindea G, Mlecnik B, Fridman WH, Pagès F & Galon J (2010) Natural immunity to cancer in humans. *Curr Opin Immunol* 22: 215–222

Black SJ, Kashkina E, Kent T & Pomerantz RT (2016) DNA polymerase θ: A unique multifunctional end-joining machine. *Genes (Basel)* 7

Blackburn EH (2001) Switching and signaling at the telomere. *Cell* 106: 661–673

Blasco MA (2005) Telomeres and human disease: Ageing, cancer and beyond. *Nat Rev Genet* 6: 611–622

Bock FJ & Chang P (2016) New directions in poly(ADP-ribose) polymerase biology. *FEBS J* 283: 4017–4031

Bouwman P, Aly A, Escandell JM, Pieterse M, Bartkova J, Van Der Gulden H, Hiddingh S, Thanasoula M, Kulkarni A, Yang Q, *et al* (2010) 53BP1 loss rescues BRCA1 deficiency and is associated with triple-negative and BRCA-mutated breast cancers. *Nat Struct Mol Biol* 17: 688–695

Branzei D & Foiani M (2007) Template Switching: From Replication Fork Repair to Genome Rearrangements. *Cell* 131: 1228–1230

Brookes P & Lawley P (1961) The reaction of mono- and di-functional alkylating agents with nucleic acids. *Biochem J* 80: 496–503

Budman J, Kim SA & Chu G (2007) Processing of DNA for nonhomologous end-joining is controlled by kinase activity and XRCC4/ligase IV. *J Biol Chem* 282: 11950–11959

Buisson R, Niraj J, Rodrigue A, Ho CK, Kreuzer J, Foo TK, Hardy EJL, Dellaire G, Haas W, Xia B, *et al* (2017) Coupling of Homologous Recombination and the Checkpoint by ATR. *Mol Cell* 65: 336–346

Bunting SF, Callén E, Wong N, Chen HT, Polato F, Gunn A, Bothmer A, Feldhahn N, Fernandez-Capetillo O, Cao L, *et al* (2010) 53BP1 inhibits homologous recombination in brca1-deficient cells by blocking resection of DNA breaks. *Cell* 141: 243–254

Byun TS, Pacek M, Yee MC, Walter JC & Cimprich KA (2005) Functional uncoupling of MCM helicase and DNA polymerase activities activates the ATR-dependent checkpoint. *Genes Dev* 19: 1040–1052

Caldecott KW (2014) DNA single-strand break repair. *Exp Cell Res* 329: 2–8

Campbell PJ, Getz G, Korbel JO, Stuart JM, Jennings JL, Stein LD, Perry MD, Nahal-Bose HK, Ouellette BFF, Li CH, *et al* (2020) Pan-cancer analysis of whole genomes. *Nature* 578: 82–93

Cao X, Lu Y, Liu Y, Zhou Y, Song H, Zhang W, Davis D, Cui J, Hao S, Jung J, *et al* (2019) Combination of PARP inhibitor and temozolomide to suppress chordoma progression. *J*

Mol Med 97: 1183–1193

Ceccaldi R, Liu JC, Amunugama R, Hajdu I, Primack B, Petalcorin MIR, O'Connor KW, Konstantinopoulos PA, Elledge SJ, Boulton SJ, *et al* (2015) Homologous-recombination-deficient tumours are dependent on Polθ -mediated repair. *Nature* 518: 258–262

Chan EM, Shibue T, McFarland JM, Gaeta B, Ghandi M, Dumont N, Gonzalez A, McPartlan JS, Li T, Zhang Y, *et al* (2019) WRN helicase is a synthetic lethal target in microsatellite unstable cancers. *Nature* 568: 551–556

Chandramouly G, Zhao J, McDevitt S, Rusanov T, Hoang T, Borisonnik N, Treddinick T, Lopezcolorado FW, Kent T, Siddique LA, *et al* (2021) Polθ reverse transcribes RNA and promotes RNA-templated DNA repair. *Sci Adv* 7: 1–12

Chang HHY, Pannunzio NR, Adachi N & Lieber MR (2017) Non-homologous DNA end joining and alternative pathways to double-strand break repair. *Nat Rev Mol Cell Biol* 18: 495–506

Chaudhuri AR, Callen E, Ding X, Gogola E, Duarte AA, Lee JE, Wong N, Lafarga V, Calvo JA, Panzarino NJ, *et al* (2016) Replication fork stability confers chemoresistance in BRCA-deficient cells. *Nature* 535: 382–387

Chen D, Gervai JZ, Póti Á, Németh E, Szeltner Z, Szikriszt B, Gyüre Z, Zámborszky J, Ceccon M, Szallasi Z, *et al* (2022) BRCA1 deficiency specific base substitution mutagenesis is dependent on translesion synthesis and regulated by 53BP1. *Nat Commun*

Chen G & Zeller WJ (1990) In vitro investigations on induction and reversal of cisplatin resistance in a rat ovarian tumor cell line. *J Cancer Res Clin Oncol* 116: 443–447

Chen YH, Jones MJK, Yin Y, Crist SB, Colnaghi L, Sims RJ, Rothenberg E, Jallepalli P V. & Huang TT (2015) ATR-Mediated Phosphorylation of FANCI Regulates Dormant Origin Firing in Response to Replication Stress. *Mol Cell* 58: 323–338

Cheng K, Samimi R, Xie G, Shant J, Drachenberg C, Wade M, Davis RJ, Nomikos G & Raufman JP (2008) Acetylcholine release by human colon cancer cells mediates autocrine stimulation of cell proliferation. *Am J Physiol - Gastrointest Liver Physiol* 295: 591–597

Ciccia A, Nimonkar A V., Hu Y, Hajdu I, Achar YJ, Izhar L, Petit SA, Adamson B, Yoon JC, Kowalczykowski SC, *et al* (2012) Polyubiquitinated PCNA Recruits the ZRANB3 Translocase to Maintain Genomic Integrity after Replication Stress. *Mol Cell* 47: 396–409

Clauson C, Schärer OD & Niedernhofer L (2013) Advances in understanding the complex mechanisms of DNA inter strand cross-link repair. *Cold Spring Harb Perspect Med* 3

Collins NB, Wilson JB, Bush T, Thomashevski A, Roberts KJ, Jones NJ & Kupfer GM (2009) ATR-dependent phosphorylation of FANCA on serine 1449 after DNA damage is important for FA pathway function. *Blood* 113: 2181–2190

Cong K, Peng M, Kousholt AN, Lee WTC, Lee S, Nayak S, Krais J, VanderVere-Carozza PS, Pawelczak KS, Calvo J, *et al* (2021) Replication gaps are a key determinant of PARP inhibitor synthetic lethality with BRCA deficiency. *Mol Cell* 81: 3128-3144.e7

Cortez D, Glick G & Elledge SJ (2004) Minichromosome maintenance proteins are direct targets of the ATM and ATR checkpoint kinases. *Proc Natl Acad Sci U S A* 101: 10078–10083

Couch FB, Bansbach CE, Driscoll R, Luzwick JW, Glick GG, Bétous R, Carroll CM, Jung SY, Qin J, Cimprich KA, *et al* (2013) ATR phosphorylates SMARCAL1 to prevent replication fork collapse. *Genes Dev* 27: 1610–1623

Cuella-Martin R, Hayward SB, Fan X, Chen X, Huang JW, Taglialatela A, Leuzzi G, Zhao J, Rabadan R, Lu C, *et al* (2021) Functional interrogation of DNA damage response variants with base editing screens. *Cell* 184: 1081-1097.e19

Davies SL, North PS, Dart A, Lakin ND & Hickson ID (2004) Phosphorylation of the Bloom's Syndrome Helicase and Its Role in Recovery from S-Phase Arrest. *Mol Cell Biol* 24: 1279–1291

DeBerardinis RJ & Chandel NS (2020) We need to talk about the Warburg effect. *Nat Metab* 2: 127–129

Dev H, Chiang TWW, Lescale C, de Krijger I, Martin AG, Pilger D, Coates J, Sczaniecka-Clift M, Wei W, Ostermaier M, *et al* (2018) Shieldin complex promotes DNA end-joining and counters homologous recombination in BRCA1-null cells. *Nat Cell Biol* 20: 954–965

Dianov GL & Hübscher U (2013) Mammalian base excision repair: The forgotten archangel. *Nucleic Acids Res* 41: 3483–3490

Difilippantonio MJ, Zhu J, Chen HT, Meffre E, Nussenzweig MC, Max EE, Ried T & Nussenzweig A (2000) Dna repair protein Ku80 suppresses chromosomal aberrations and malignant transformation. *Nature* 404: 510–514

Dobzhansky T (1946) Genetics of natural populations; recombination and variability in populations of Drosophila pseudoobscura. *Genetics* 31: 269–290

Du X, Li J, Luo X, Li R, Li F, Zhang Y, Shi J & He J (2020) Structure-activity relationships of Wee1 inhibitors: A review. *Eur J Med Chem* 203: 112524

Van Egeren D, Escabi J, Nguyen M, Liu S, Reilly CR, Patel S, Kamaz B, Kalyva M, DeAngelo DJ, Galinsky I, *et al* (2021) Reconstructing the Lineage Histories and Differentiation Trajectories of Individual Cancer Cells in Myeloproliferative Neoplasms. *Cell Stem Cell* 28: 514-523.e9

El-shemerly M, Hess D, Pyakurel AK, Moselhy S & Ferrari S (2008) ATR-dependent pathways control hEXO1 stability in response to stalled forks. *Nucleic Acids Res* 36: 511–519

Elbæk CR, Petrosius V, Benada J, Erichsen L, Damgaard RB & Sørensen CS (2022) WEE1 kinase protects the stability of stalled DNA replication forks by limiting CDK2 activity. *Cell Rep* 38: 110261

Esposito F, Giuffrida R, Raciti G, Puglisi C & Forte S (2021) Wee1 kinase: A potential target to overcome tumor resistance to therapy. *Int J Mol Sci* 22: 1–23

Falck J, Mailand N, Syljuåsen RG, Bartek J & Lukas J (2001) The ATM-Chk2-Cdc25A checkpoint pathway guards against radioresistant DNA synthesis. *Nature* 410: 842–847

Fernandez-Vidal A, Guitton-Sert L, Cadoret JC, Drac M, Schwob E, Baldacci G, Cazaux C & Hoffmann JS (2014) A role for DNA polymerase Q in the timing of DNA replication. *Nat Commun* 5

Ferrara N (2009) Vascular endothelial growth factor. *Arterioscler Thromb Vasc Biol* 29: 789–791

Fishel R, Lescoe MK, Rao MRS, Copeland NG, Jenkins NA, Garber J, Kane M & Kolodner R (1993) The human mutator gene homolog MSH2 and its association with hereditary nonpolyposis colon cancer. *Cell* 75: 1027–1038

Fynan TM & Reiss M (1993) Resistance to inhibition of cell growth by transforming growth factor-beta and its role in oncogenesis. *Crit Rev Oncog* 4: 493–540

Gaillard H, García-Muse T & Aguilera A (2015) Replication stress and cancer. *Nat Rev Cancer* 15: 276–280

Gallo D, Young JTF, Fourtounis J, Martino G, Álvarez-Quilón A, Bernier C, Duffy NM, Papp R, Roulston A, Stocco R, *et al* (2022) CCNE1 amplification is synthetic lethal with PKMYT1 kinase inhibition. *Nature* 604

Gao Y, Mutter-Rottmayer E, Zlatanou A, Vaziri C & Yang Y (2017) Mechanisms of post-replication DNA repair. *Genes (Basel)* 8

García-Gómez S, Reyes A, Martínez-Jiménez MI, Chocrón ES, Mourón S, Terrados G, Powell C, Salido E, Méndez J, Holt IJ, *et al* (2013) PrimPol, an Archaic Primase/Polymerase Operating in Human Cells. *Mol Cell* 52: 541–553

Giannattasio M, Zwicky K, Follonier C, Foiani M, Lopes M & Branzei D (2014) Visualization of recombination-mediated damage bypass by template switching. *Nat Struct Mol Biol* 21: 884–892

Gill SJ, Travers J, Pshenichnaya I, Kogera FA, Barthorpe S, Mironenko T, Richardson L, Benes CH, Stratton MR, McDermott U, *et al* (2015) Combinations of PARP inhibitors with temozolomide drive PARP1 trapping and apoptosis in Ewing's sarcoma. *PLoS One* 10: 1–17

Goldberg FW, Finlay MR V., Ting AKT, Beattie D, Lamont GM, Fallan C, Wrigley GL, Schimpl M, Howard MR, Williamson B, *et al* (2020) The Discovery of 7-Methyl-2-[(7-methyl[1,2,4]triazolo[1,5- a]pyridin-6-yl)amino]-9-(tetrahydro-2 H-pyran-4-yl)-7,9-dihydro-8 H-purin-8-one (AZD7648), a Potent and Selective DNA-Dependent Protein Kinase (DNA-PK) Inhibitor. *J Med Chem* 63: 3461–3471

Goodwin S, McPherson JD & McCombie WR (2016) Coming of age: Ten years of next-generation sequencing technologies. *Nat Rev Genet* 17: 333–351

Gorgoulis VG, Vassiliou LVF, Karakaidos P, Zacharatos P, Kotsinas A, Liloglou T, Venere M, DiTullio RA, Kastrinakis NG, Levy B, *et al* (2005) Activation of the DNA damage checkpoint and genomic instability in human precancerous lesions. *Nature* 434: 907–913

Greaves M & Maley CC (2012) Clonal evolution in cancer. *Nature* 481: 306–313

Green DR & Reed JC (1998) Mitochondria and apoptosis. *Science (80-)* 281

Grivennikov SI, Greten FR & Karin M (2010) Immunity, Inflammation, and Cancer. *Cell* 140: 883–899

Gupta R, Somyajit K, Narita T, Maskey E, Stanlie A, Kremer M, Typas D, Lammers M, Mailand N, Nussenzweig A, *et al* (2018) DNA Repair Network Analysis Reveals Shieldin as a Key Regulator of NHEJ and PARP Inhibitor Sensitivity. *Cell* 173: 972-988.e23

Hae YY, Shevchenko A, Shevchenko A & Dunphy WG (2004) Mcm2 is a direct substrate of ATM and ATR during DNA damage and DNA replication checkpoint responses. *J Biol Chem* 279: 53353–53364

Hanahan D & Folkman J (1996) Patterns and emerging mechanisms of the angiogenic switch during tumorigenesis. *Cell* 86: 353–364

Hanahan D & Weinberg RA (2000) The Hallmarks of Cancer. *Cell* 100: 57–70

Hanahan D & Weinberg RA (2011) Hallmarks of cancer: The next generation. *Cell* 144: 646–674

Hanzlikova H, Kalasova I, Demin AA, Pennicott LE, Cihlarova Z & Caldecott KW (2018) The Importance of Poly(ADP-Ribose) Polymerase as a Sensor of Unligated Okazaki Fragments during DNA Replication. *Mol Cell* 71: 319-331.e3

Hashimoto Y, Chaudhuri AR, Lopes M & Costanzo V (2010) Rad51 protects nascent DNA from Mre11-dependent degradation and promotes continuous DNA synthesis. *Nat Struct Mol Biol* 17: 1305–1311

Helleday T (2011) The underlying mechanism for the PARP and BRCA synthetic lethality: Clearing up the misunderstandings. *Mol Oncol* 5: 387–393

Hoege C, Pfander B, Moldovan GL, Pyrowolakis G & Jentsch S (2002) RAD6-dependent DNA repair is linked to modification of PCNA by ubiquitin and SUMO. *Nature* 419: 135–141

Hogg M, Seki M, Wood RD, Doublié S & Wallace SS (2011) Lesion bypass activity of DNA polymerase θ (POLQ) is an intrinsic property of the pol domain and depends on unique sequence inserts. *J Mol Biol* 405: 642–652

Hogg M, Wallace SS & Doublié S (2004) Crystallographic snapshots of a replicative DNA polymerase encountering an abasic site. *EMBO J* 23: 1483–1493

Howlader N, Noone A, Krapcho M, Miller D, Brest A, Yu M, Ruhl J, Tatalovich Z, Mariotto A & Lewis D (2019) Cancer Statistics Review 1975-2009. *Cancer Stat Rev - SEER Stat*

Hu J, Sun L, Shen F, Chen Y, Hua Y, Liu Y, Zhang M, Hu Y, Wang Q, Xu W, *et al* (2012) The intra-S phase checkpoint targets Dna2 to prevent stalled replication forks from reversing. *Cell* 149: 1221–1232

Hudson TJ, Anderson W, Aretz A, Barker AD, Bell C, Bernabé RR, Bhan MK, Calvo F, Eerola I, Gerhard DS, *et al* (2010) International network of cancer genome projects. *Nature* 464: 993–998

Ibarra A, Schwob E & Méndez J (2008) Excess MCM proteins protect human cells from replicative stress by licensing backup origins of replication. *Proc Natl Acad Sci U S A* 105: 8956–8961

Jaspers JE, Kersbergen A, Boon U, Sol W, Deemter L Van, Zander SA, Drost R, Wientjens E, Ji J, Aly A, *et al* (2013) Loss of 53BP1 Causes PARP Inhibitor Resistance in Brca1 - Mutated Mouse Mammary Tumors. *Cancer Discov* 3: 68–81

Junttila MR & Evan GI (2009) P53 a Jack of all trades but master of none. *Nat Rev Cancer* 9: 821–829

Kaina B, Christmann M, Naumann S & Roos WP (2007) MGMT: Key node in the battle against genotoxicity, carcinogenicity and apoptosis induced by alkylating agents. *DNA Repair (Amst)* 6: 1079–1099

van de Kamp G, Heemskerk T, Kanaar R & Essers J (2021) DNA Double Strand Break Repair Pathways in Response to Different Types of Ionizing Radiation. *Front Genet* 12

Kang Z, Fu P, Alcivar AL, Fu H, Redon C, Foo TK, Zuo Y, Ye C, Baxley R, Madireddy A, *et al* (2021) BRCA2 associates with MCM10 to suppress PRIMPOL-mediated repriming and single-stranded gap formation after DNA damage. *Nat Commun* 12

Kannouche PL & Lehmann AR (2004) Ubiquitination of PCNA and the polymerase switch in human cells. *Cell Cycle* 3: 1009–1011

Kent T, Mateos-Gomez PA, Sfeir A & Pomerantz RT (2016) Polymerase θ is a robust terminal transferase that oscillates between three different mechanisms during end-joining. *Elife* 5: 1–25

Kile AC, Chavez DA, Bacal J, Eldirany S, Korzhnev DM, Bezsonova I, Eichman BF & Cimprich KA (2015) HLTF's Ancient HIRAN Domain Binds 3' DNA Ends to Drive Replication Fork Reversal. *Mol Cell* 58: 1090–1100

Kim C, Paulus BF & Wold MS (1994) Interactions of Human Replication Protein A with Oligonucleotides. *Biochemistry* 33: 14197–14206

Kim H, Xu H, George E, Hallberg D, Kumar S, Jagannathan V, Medvedev S, Kinose Y, Devins K, Verma P, *et al* (2020) Combining PARP with ATR inhibition overcomes PARP inhibitor and platinum resistance in ovarian cancer models. *Nat Commun* 11

Kinzler KW & Vogelstein B (1996) Lessons from Hereditary Colorectal Cancer. *Cell* 87: 159–170

Klimovskaia IM, Young C, Strømme CB, Menard P, Jasencakova Z, Mejlvang J, Ask K, Ploug M, Nielsen ML, Jensen ON, *et al* (2014) Tousled-like kinases phosphorylate Asf1 to promote histone supply during DNA replication. *Nat Commun* 5

Knudson AG (1971) Mutation and cancer: statistical study of retinoblastoma. *Proc Natl Acad Sci U S A* 68: 820–823

Koh G, Degasperi A, Zou X, Momen S & Nik-Zainal S (2021) Mutational signatures: emerging concepts, caveats and clinical applications. *Nat Rev Cancer* 21: 619–637

Kok YP, Guerrero Llobet S, Schoonen PM, Everts M, Bhattacharya A, Fehrmann RSN, van den Tempel N & van Vugt MATM (2020) Overexpression of Cyclin E1 or Cdc25A leads to replication stress, mitotic aberrancies, and increased sensitivity to replication checkpoint inhibitors. *Oncogenesis* 9

Kolinjivadi AM, Sannino V, De Antoni A, Zadorozhny K, Kilkenny M, Técher H, Baldi G, Shen

R, Ciccia A, Pellegrini L, *et al* (2017) Smarcal1-Mediated Fork Reversal Triggers Mre11-Dependent Degradation of Nascent DNA in the Absence of Brca2 and Stable Rad51 Nucleofilaments. *Mol Cell* 67: 867-881.e7

Kotsantis P, Petermann E & Boulton SJ (2018) Mechanisms of oncogene-induced replication stress: Jigsaw falling into place. *Cancer Discov* 8: 537–555

Kottemann MC & Smogorzewska A (2013) Fanconi anaemia and the repair of Watson and Crick DNA crosslinks. *Nature* 493: 356–363

Krishnakumar R & Kraus WL (2010) The PARP Side of the Nucleus: Molecular Actions, Physiological Outcomes, and Clinical Targets. *Mol Cell* 39: 8–24

Kusumoto R, Masutani C, Iwai S & Hanaoka F (2002) Translesion synthesis by human DNA polymerase η across thymine glycol lesions. *Biochemistry* 41: 6090–6099

Landau DA & Wu CJ (2013) Chronic lymphocytic leukemia: Molecular heterogeneity revealed by high-throughput genomics. *Genome Med* 5: 1–13

Langevin F, Crossan GP, Rosado I V., Arends MJ & Patel KJ (2011) Fancd2 counteracts the toxic effects of naturally produced aldehydes in mice. *Nature* 475: 53–59

Lavin MF (2008) Ataxia-telangiectasia: From a rare disorder to a paradigm for cell signalling and cancer. *Nat Rev Mol Cell Biol* 9: 759–769

Lemaçon D, Jackson J, Quinet A, Brickner JR, Li S, Yazinski S, You Z, Ira G, Zou L, Mosammaparast N, *et al* (2017) MRE11 and EXO1 nucleases degrade reversed forks and elicit MUS81-dependent fork rescue in BRCA2-deficient cells. *Nat Commun* 8

Lemée F, Bergoglio V, Fernandez-vidal A, Machado-silva A & Pillaire M (2010) DNA polymerase θ up-regulation is associated with poor survival in breast cancer , perturbs DNA replication , and promotes genetic instability. *PNAS* 107

Levin DS, Bai W, Yao N, O'Donnell M & Tomkinson AE (1997) An interaction between DNA ligase I and proliferating cell nuclear antigen: Implications for Okazaki fragment synthesis and joining. *Proc Natl Acad Sci U S A* 94: 12863–12868

Li GM (2008) Mechanisms and functions of DNA mismatch repair. *Cell Res* 18: 85–98

Lieb S, Blaha-Ostermann S, Kamper E, Rippka J, Schwarz C, Ehrenhöfer-Wölfer K, Schlattl A, Wernitznig A, Lipp JJ, Nagasaka K, *et al* (2019) Werner syndrome helicase is a selective vulnerability of microsatellite instability- high tumor cells. *Elife* 8: 1–22

Llobet SG, Vegt B Van Der, Jongeneel E, Bense RD, Zwager MC & Schröder CP (2020) Cyclin E expression is associated with high levels of replication stress in triple-negative breast cancer. *npj Breast Cancer*: 1–13

Lloyd RL, Wijnhoven PWG, Zena AR, Giuditta W, Falenta K, Jones GN, James N, Chabbert CD, Stott J, Dean E, *et al* (2020) Combined PARP and ATR inhibition potentiates genome instability and cell death in ATM-de fi cient cancer cells. *Oncogene*: 4869–4883

Lord CJ & Ashworth A (2017) PARP inhibitors: Synthetic lethality in the clinic. *Science (80-)* 355: 62–75

Mailand N, Falck J, Lukas C, Syljuåsen RG, Welcker M, Bartek J & Lukas J (2000) Rapid

destruction of human Cdc25A in response to DNA damage. *Science (80-)* 288: 1425–1429

Martínez-Jiménez F, Muiños F, Sentís I, Deu-Pons J, Reyes-Salazar I, Arnedo-Pac C, Mularoni L, Pich O, Bonet J, Kranas H, *et al* (2020) A compendium of mutational cancer driver genes. *Nat Rev Cancer* 20: 555–572

Mateos-Gomez PA, Gong F, Nair N, Miller KM, Lazzerini-Denchi E & Sfeir A (2015) Mammalian polymerase θ promotes alternative NHEJ and suppresses recombination. *Nature* 518: 254–257

Mateos-Gomez PA, Kent T, Deng SK, Mcdevitt S, Kashkina E, Hoang TM, Pomerantz RT & Sfeir A (2017) The helicase domain of Polθ counteracts RPA to promote alt-NHEJ. *Nat Struct Mol Biol* 24: 1116–1123

Maya-Mendoza A, Moudry P, Merchut-Maya JM, Lee M, Strauss R & Bartek J (2018) High speed of fork progression induces DNA replication stress and genomic instability. *Nature* 559: 279–284

McClendon AK, Dean JL, Rivadeneira DB, Yu JE, Reed CA, Gao E, Farber JL, Force T, Koch WJ & Knudsen ES (2012) CDK4/6 inhibition antagonizes the cytotoxic response to anthracycline therapy. *Cell Cycle* 11: 2747–2755

McLendon R, Friedman A, Bigner D, Van Meir EG, Brat DJ, Mastrogianakis GM, Olson JJ, Mikkelsen T, Lehman N, Aldape K, *et al* (2008) Comprehensive genomic characterization defines human glioblastoma genes and core pathways. *Nature* 455: 1061–1068

McNeely S, Beckmann R & Bence Lin AK (2014) CHEK again: Revisiting the development of CHK1 inhibitors for cancer therapy. *Pharmacol Ther* 142: 1–10

Méchali M (2010) Eukaryotic DNA replication origins: Many choices for appropriate answers. *Nat Rev Mol Cell Biol* 11: 728–738

Mejlvang J, Feng Y, Alabert C, Neelsen KJ, Jasencakova Z, Zhao X, Lees M, Sandelin A, Pasero P, Lopes M, *et al* (2014) New histone supply regulates replication fork speed and PCNA unloading. *J Cell Biol* 204: 29–43

Mendez-Bermudez A, Lototska L, Bauwens S, Giraud-Panis MJ, Croce O, Jamet K, Irizar A, Mowinckel M, Koundrioukoff S, Nottet N, *et al* (2018) Genome-wide Control of Heterochromatin Replication by the Telomere Capping Protein TRF2. *Mol Cell* 70: 449-461.e5

Murai J, Huang SYN, Das BB, Renaud A, Zhang Y, Doroshow JH, Ji J, Takeda S & Pommier Y (2012) Trapping of PARP1 and PARP2 by clinical PARP inhibitors. *Cancer Res* 72: 5588–5599

Murga M, Campaner S, Lopez-Contreras AJ, Toledo LI, Soria R, Montaña MF, D'Artista L, Schleker T, Guerra C, Garcia E, *et al* (2011) Exploiting oncogene-induced replicative stress for the selective killing of Myc-driven tumors. *Nat Struct Mol Biol* 18: 1331–1335

Nayak S, Calvo JA, Cong K, Peng M, Berthiaume E, Jackson J, Zaino AM, Vindigni A, Hadden MK & Cantor SB (2020) Inhibition of the translesion synthesis polymerase REV1 exploits replication gaps as a cancer vulnerability. *Sci Adv* 6

Neelsen KJ & Lopes M (2015) Replication fork reversal in eukaryotes: From dead end to dynamic response. *Nat Rev Mol Cell Biol* 16: 207–220

Negrini S, Gorgoulis VG & Halazonetis TD (2010) Genomic instability an evolving hallmark of cancer. *Nat Rev Mol Cell Biol* 11: 220–228

Nih C, Paolo P, Fiore D, Pierce JH, Fleming TF, Hazan R, Ullrich A, King CR, Schlessinger J & Aaronson SA (1987) Overexpression of the Human EGF Receptor Confers an EGF-Dependent Transformed Phenotype to. *Cell* 51: 1063–1070

Nik-Zainal S, Alexandrov LB, Wedge DC, Van Loo P, Greenman CD, Raine K, Jones D, Hinton J, Marshall J, Stebbings LA, *et al* (2012) Mutational processes molding the genomes of 21 breast cancers. *Cell* 149: 979–993

Noordermeer SM, Adam S, Setiaputra D, Barazas M, Pettitt SJ, Ling AK, Olivieri M, Álvarez-Quilón A, Moatti N, Zimmermann M, *et al* (2018) The shieldin complex mediates 53BP1-dependent DNA repair. *Nature* 560: 117–121

Nouspikel T (2009) Nucleotide excision repair: Variations on versatility. *Cell Mol Life Sci* 66: 994–1009

Ozdemir AY, Rusanov T, Kent T, Siddique LA & Pomerantz RT (2018) Polymerase θ-helicase efficiently unwinds DNA and RNA-DNA hybrids. *J Biol Chem* 293: 5259–5269

Palovcak A, Liu W, Yuan F & Zhang Y (2017) Maintenance of genome stability by Fanconi anemia proteins. *Cell Biosci* 7: 1–18

Panzarino NJ, Krais JJ, Cong K, Peng M, Mosqueda M, Nayak SU, Bond SM, Calvo JA, Doshi MB, Bere M, *et al* (2021) Replication gaps underlie BRCA deficiency and therapy response. *Cancer Res* 81: 1388–1397

Park YB, Chae J, Kim YC & Cho Y (2011) Crystal structure of human Mre11: Understanding tumorigenic mutations. *Structure* 19: 1591–1602

Patch AM, Christie EL, Etemadmoghadam D, Garsed DW, George J, Fereday S, Nones K, Cowin P, Alsop K, Bailey PJ, *et al* (2015) Whole-genome characterization of chemoresistant ovarian cancer. *Nature* 521: 489–494

Paull TT (2018) 20 Years of Mre11 Biology: No End in Sight. *Mol Cell* 71: 419–427

Pfeiffer P, Goedecke W & Obe G (2000) Mechanisms of DNA double-strand break repair and their potential to induce chromosomal aberrations. *Mutagenesis* 15: 289–302

Polak P, Kim J, Braunstein LZ, Karlic R, Haradhavala NJ, Tiao G, Rosebrock D, Livitz D, Kübler K, Mouw KW, *et al* (2017) A mutational signature reveals alterations underlying deficient homologous recombination repair in breast cancer. *Nat Genet* 49: 1476–1486

Poli J, Tsaponina O, Crabbé L, Keszthelyi A, Pantesco V, Chabes A, Lengronne A & Pasero P (2012) dNTP pools determine fork progression and origin usage under replication stress. *EMBO J* 31: 883–894

Quinet. A & Vindigni A (2018) Breaking the replication speed limit. *Nature*: 1–2

Quinet A, Lemaçon D & Vindigni A (2017) Replication Fork Reversal: Players and Guardians. *Mol Cell* 68: 830–833

Quinet A, Tirman S, Jackson J, Šviković S, Lemaçon D, Carvajal-Maldonado D, González-Acosta D, Vessoni AT, Cybulla E, Wood M, *et al* (2020) PRIMPOL-Mediated Adaptive Response Suppresses Replication Fork Reversal in BRCA-Deficient Cells. *Mol Cell* 77: 461-474.e9

Rahman N (2014) Realizing the promise of cancer predisposition genes. *Nature* 505: 302–308

Ramsden DA, Carvajal-Garcia J & Gupta GP (2021) Mechanism, cellular functions and cancer roles of polymerase-theta-mediated DNA end joining. *Nat Rev Mol Cell Biol* 0123456789

Reddy EP, Reynolds RK, Santos E & Barbacid M (1982) A point mutation is responsible for the acquisition of transforming properties by the T24 human bladder carcinoma oncogene. *Nature* 300: 149–152

Ren CH, Yan Q & Zhang ZY (2014) Minimum length of direct repeat sequences required for efficient homologous recombination induced by zinc finger nuclease in yeast. *Mol Biol Rep* 41: 6939–6948

Rosado I V., Langevin F, Crossan GP, Takata M & Patel KJ (2011) Formaldehyde catabolism is essential in cells deficient for the Fanconi anemia DNA-repair pathway. *Nat Struct Mol Biol* 18: 1432–1434

Sakai W, Swisher EM, Karlan BY, Agarwal MK, Higgins J, Friedman C, Villegas E, Jacquemont C, Farrugia DJ, Couch FJ, *et al* (2008) Secondary mutations as a mechanism of cisplatin resistance in BRCA2-mutated cancers. *Nature* 451: 1116–1120

Salvador-Barbero B, Álvarez-Fernández M, Zapatero-Solana E, El Bakkali A, Menéndez M del C, López-Casas PP, Di Domenico T, Xie T, VanArsdale T, Shields DJ, *et al* (2020) CDK4/6 Inhibitors Impair Recovery from Cytotoxic Chemotherapy in Pancreatic Adenocarcinoma. *Cancer Cell* 37: 340-353.e6

San Filippo J, Sung P & Klein H (2008) Mechanism of Eukaryotic Homologous Recombination. *Annu Rev Biochem* 77: 229–257

Schlacher K, Christ N, Siaud N, Egashira A, Wu H & Jasin M (2011) Double-strand break repair-independent role for BRCA2 in blocking stalled replication fork degradation by MRE11. *Cell* 145: 529–542

Sedgwick B (2004) Repairing DNA-methylation damage. *Nat Rev Mol Cell Biol* 5: 148–157

Seki M, Marini F & Wood RD (2003) POLQ (Pol θ), a DNA polymerase and DNA-dependent ATPase in human cells. *Nucleic Acids Res* 31: 6117–6126

Seki M, Masutani C, Yang LW, Schuffert A, Iwai S, Bahar I & Wood RD (2004) High-efficiency bypass of DNA damage by human DNA polymerase Q. *EMBO J* 23: 4484–4494

Sharma RA & Dianov GL (2007) Targeting base excision repair to improve cancer therapies. *Mol Aspects Med* 28: 345–374

Sobeck A, Stone S, Landais I, de Graaf B & Hoatlin ME (2009) The Fanconi anemia protein FANCM is controlled by FANCD2 and the ATR/ATM pathways. *J Biol Chem* 284:

25560–25568

Sogo JM, Lopes M & Foiani M (2002) Fork reversal and ssDNA accumulation at stalled replication forks owing to checkpoint defects. *Science (80-)* 297: 599–602

Solimini NL, Luo J & Elledge SJ (2007) Non-Oncogene Addiction and the Stress Phenotype of Cancer Cells. *Cell* 130: 986–988

Ström CE, Johansson F, Uhlén M, Szigyarto CAK, Erixon K & Helleday T (2011) Poly (ADP-ribose) polymerase (PARP) is not involved in base excision repair but PARP inhibition traps a single-strand intermediate. *Nucleic Acids Res* 39: 3166–3175

Sturzenegger A, Burdova K, Kanagaraj R, Levikova M, Pinto C, Cejka P & Janscak P (2014) DNA2 cooperates with the WRN and BLM RecQ helicases to mediate long-range DNA end resection in human cells. *J Biol Chem* 289: 27314–27326

Sugasawa K, Okamoto T, Shimizu Y, Masutani C, Iwai S & Hanaoka F (2001) A multistep damage recognition mechanism for global genomic nucleotide excision repair. *Genes Dev* 15: 507–521

Sung P & Klein H (2006) Mechanism of homologous recombination: Mediators and helicases take on regulatory functions. *Nat Rev Mol Cell Biol* 7: 739–750

Symington LS & Gautier J (2011) Double-strand break end resection and repair pathway choice. *Annu Rev Genet* 45: 247–271

Taglialatela A, Alvarez S, Leuzzi G, Sannino V, Ranjha L, Huang JW, Madubata C, Anand R, Levy B, Rabadan R, *et al* (2017) Restoration of Replication Fork Stability in BRCA1- and BRCA2-Deficient Cells by Inactivation of SNF2-Family Fork Remodelers. *Mol Cell* 68: 414-430.e8

Taglialatela A, Leuzzi G, Sannino V, Cuella-Martin R, Huang J-W, Wu-Baer F, Baer R, Costanzo V & Ciccia A (2021) REV1-Polζ maintains the viability of homologous recombination-deficient cancer cells through mutagenic repair of PRIMPOL-dependent ssDNA gaps. *Mol Cell*: 1–18

Takata KI, Shimizu T, Iwai S & Wood RD (2006) Human DNA polymerase N (POLN) is a low fidelity enzyme capable of error-free bypass of 5S-thymine glycol. *J Biol Chem* 281: 23445–23455

Talmadge JE & Fidler IJ (2010) AACR centennial series: The biology of cancer metastasis: Historical perspective. *Cancer Res* 70: 5649–5669

Taylor AM., Harnden D., Arlett CF, Harcourt SA, Lehmann AR, Stevens S & Bridges B. (1975) Ataxia telengiectasia: a human mutation in with abnormal radiation sensitivity. *Nature* 258: 427–429

Técher H & Pasero P (2021) The Replication Stress Response on a Narrow Path Between Genomic Instability and Inflammation. *Front Cell Dev Biol* 9

Thakar T, Leung W, Nicolae CM, Clements KE, Shen B, Bielinsky AK & Moldovan GL (2020) Ubiquitinated-PCNA protects replication forks from DNA2-mediated degradation by regulating Okazaki fragment maturation and chromatin assembly. *Nat Commun* 11: 1–14

Tibbetts RS, Cortez D, Brumbaugh KM, Scully R, Livingston D, Elledge SJ & Abraham RT (2000) Functional interactions between BRCA1 and the checkpoint kinase ATR during genotoxic stress. *Genes Dev* 14: 2989–3002

Timme CR, Rath BH, O'Neill JW, Camphausen K & Tofilon PJ (2018) The DNA-PK inhibitor VX-984 enhances the radiosensitivity of glioblastoma cells grown in vitro and as orthotopic xenografts. *Mol Cancer Ther* 17: 1207–1216

Tirman S, Quinet A, Wood M, Meroni A, Cybulla E, Jackson J, Pegoraro S, Simoneau A, Zou L & Vindigni A (2021) Temporally distinct post-replicative repair mechanisms fill PRIMPOL-dependent ssDNA gaps in human cells. *Mol Cell* 81: 4026-4040.e8

Tiwari M (2012) Antimetabolites: Established cancer therapy. *J Cancer Res Ther* 8: 510–519

Toledo L, Neelsen KJ & Lukas J (2017) Replication Catastrophe: When a Checkpoint Fails because of Exhaustion. *Mol Cell* 66: 735–749

Tomimatsu N, Mukherjee B, Catherine Hardebeck M, Ilcheva M, Vanessa Camacho C, Louise Harris J, Porteus M, Llorente B, Khanna KK u. & Burma S (2014) Phosphorylation of EXO1 by CDKs 1 and 2 regulates DNA end resection and repair pathway choice. *Nat Commun* 5: 3561

Truong LN, Li Y, Shi LZ, Hwang PYH, He J, Wang H, Razavian N, Berns MW & Wu X (2013) Microhomology-mediated End Joining and Homologous Recombination share the initial end resection step to repair DNA double-strand breaks in mammalian cells. *Proc Natl Acad Sci U S A* 110: 7720–7725

Tuduri S, Tourrière H & Pasero P (2010) Defining replication origin efficiency using DNA fiber assays. *Chromosom Res* 18: 91–102

Vaitsiankova A, Burdova K, Sobol M, Gautam A, Benada O, Hanzlikova H & Caldecott KW (2022) PARP inhibition impedes the maturation of nascent DNA strands during DNA replication. *Nat Struct Mol Biol*

Vogelstein B, Papadopoulos N, Velculescu VE, Zhou S, Diaz LA & Kinzler KW (2013) Cancer genome landscapes. *Science (80-)* 340: 1546–1558

Vousden KH & Lane DP (2007) P53 in Health and Disease. *Nat Rev Mol Cell Biol* 8: 275–283

Wan L, Lou J, Xia Y, Su B, Liu T, Cui J, Sun Y, Lou H & Huang J (2013) HPrimpol1/CCDC111 is a human DNA primase-polymerase required for the maintenance of genome integrity. *EMBO Rep* 14: 1104–1112

Wang JC (2002) Cellular roles of DNA topoisomerases: A molecular perspective. *Nat Rev Mol Cell Biol* 3: 430–440

Wang Z, Song Y, Li S, Kurian S, Xiang R, Chiba T & Wu X (2019) DNA polymerase (POLQ) is important for repair of DNA double-strand breaks caused by fork collapse. *J Biol Chem* 294: 3909–3919

Ward IM & Chen J (2001) Histone H2AX Is Phosphorylated in an ATR-dependent Manner in Response to Replicational Stress. *J Biol Chem* 276: 47759–47762

Weaver AN & Yang ES (2013) Beyond DNA repair: Additional functions of PARP-1 in cancer.

Front Oncol 3 NOV: 1–11

Weinstein IB (2002) Cancer: Addiction to oncogenes - The Achilles heal of cancer. *Science (80-)* 297: 63–64

van Wietmarschen N, Sridharan S, Nathan WJ, Tubbs A, Chan EM, Callen E, Wu W, Belinky F, Tripathi V, Wong N, *et al* (2020) Repeat expansions confer WRN dependence in microsatellite-unstable cancers. *Nature* 586: 292–298

Williams N, Lee J, Mitchell E, Moore L, Baxter EJ, Hewinson J, Dawson KJ, Menzies A, Godfrey AL, Green AR, *et al* (2022) Life histories of myeloproliferative neoplasms inferred from phylogenies. *Nature* 602: 162–168

Williams RS, Moncalian G, Williams JS, Yamada Y, Limbo O, Shin DS, Groocock LM, Cahill D, Hitomi C, Guenther G, *et al* (2008) Mre11 Dimers Coordinate DNA End Bridging and Nuclease Processing in Double-Strand-Break Repair. *Cell* 135: 97–109

Wojtaszek JL, Chatterjee N, Najeeb J, Ramos A, Lee M, Bian K, Xue JY, Fenton BA, Park H, Li D, *et al* (2019) A Small Molecule Targeting Mutagenic Translesion Synthesis Improves Chemotherapy. *Cell* 178: 152-159.e11

Wong RP, Petriukov K & Ulrich HD (2021) Daughter-strand gaps in DNA replication – substrates of lesion processing and initiators of distress signalling. *DNA Repair (Amst)* 105: 103163

Wood RD & Doublié S (2016) DNA polymerase θ (POLQ), double-strand break repair, and cancer. *DNA Repair (Amst)* 44: 22–32

Wyatt DW, Feng W, Conlin MP, Yousefzadeh MJ, Roberts SA, Mieczkowski P, Wood RD, Gupta GP & Ramsden DA (2016) Essential Roles for Polymerase θ-Mediated End Joining in the Repair of Chromosome Breaks. *Mol Cell* 63: 662–673

Yang L, Pang Y & Moses HL (2010) TGF-β and immune cells: an important regulatory axis in the tumor microenvironment and progression. *Trends Immunol*

Ying S, Hamdy FC & Helleday T (2012) Mre11-dependent degradation of stalled DNA replication forks is prevented by BRCA2 and PARP1. *Cancer Res* 72: 2814–2821

Yoon JH, Bhatia G, Prakash S & Prakash L (2010) Error-free replicative bypass of thymine glycol by the combined action of DNA polymerases κ and ζ in human cells. *Proc Natl Acad Sci U S A* 107: 14116–14121

Yoon JH, McArthur MJ, Park J, Basu D, Wakamiya M, Prakash L & Prakash S (2019) Error-Prone Replication through UV Lesions by DNA Polymerase θ Protects against Skin Cancers. *Cell* 176: 1295-1309.e15

Yue Gao OHAMSN (2009) Numerical Analysis of Etoposide Induced DNA Breaks. *PLoS One* 4

Yun MH & Hiom K (2009) CtIP-BRCA1 modulates the choice of DNA double-strand-break repair pathway throughout the cell cycle. *Nature* 459: 460–463

Zatreanu D, Robinson HMR, Alkhatib O, Boursier M, Finch H, Geo L, Grande D, Grinkevich V, Heald RA, Langdon S, *et al* (2021) Polθ inhibitors elicit BRCA-gene synthetic lethality and target PARP inhibitor resistance. *Nat Commun*: 1–15

Zellweger R, Dalcher D, Mutreja K, Berti M, Schmid JA, Herrador R, Vindigni A & Lopes M (2015) Rad51-mediated replication fork reversal is a global response to genotoxic treatments in human cells. *J Cell Biol* 208: 563–579

Zenke FT, Zimmermann A, Sirrenberg C, Dahmen H, Kirkin V, Pehl U, Grombacher T, Wilm C, Fuchss T, Amendt C, *et al* (2020) Pharmacologic inhibitor of DNA-PK, M3814, potentiates radiotherapy and regresses human tumors in mouse models. *Mol Cancer Ther* 19: 1091–1101

Zhao H, Watkins JL & Piwnica-Worms H (2002) Disruption of the checkpoint kinase 1 / cell division cycle 25A pathway abrogates ionizing radiation-induced S and G2 checkpoints. *Proc Natl Acad Sci U S A* 99: 14795–14800

Zhao Y, Thomas HD, Batey MA, Cowell IG, Richardson CJ, Griffin RJ, Calvert AH, Newell DR, Smith GCM & Curtin NJ (2006) Preclinical evaluation of a potent novel DNA-dependent protein kinase inhibitor NU7441. *Cancer Res* 66: 5354–5362

Zheng L & Shen B (2011) Okazaki fragment maturation: Nucleases take centre stage. *J Mol Cell Biol* 3: 23–30

Zhou BS & Elledge SJ (2000) Checkpoints in Perspective. *Nature* 408: 433–439

Zhou J, Gelot C, Pantelidou C, Li A, Yücel H, Davis RE, Färkkilä A, Kochupurakkal B, Syed A, Shapiro GI, *et al* (2021) A first-in-class polymerase theta inhibitor selectively targets homologous-recombination deficient tumors. *Nat Cancer*

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Personal information

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Personal profile

I am a highly motivated PhD student, currently finishing my degree in Molecular Biology. My strive for excellence has earned me five performance scholarships of the University of Vienna as well as a three-year Individual DOC Fellowship of the Austrian Academy of Sciences. I am highly organized, enjoy overcoming challenges and have proven to be able to produce innovative research under pressure. While spending more than five years in various collaborative research environments, I particularly enjoyed presenting my scientific work as well as the coordination and planning of tasks within my project team.

Education

09/2018 - now	**PhD Program** at the **Research Center for Molecular Medicine (CeMM),** Vienna
03/2016 – 07/2018:	**Master studies** in **Molecular Biology** at the **University of Vienna,** graduated with honors, Vienna
10/2012 – 02/2016:	**Bachelor studies** in **Microbiology and Genetics** at the **University of Vienna**, graduated with honors, Vienna
2004 – 2012:	**Bundesrealgymnasium Anton-Bruckner-Straße** with focus on scientific subjects, graduated with honors, Wels

Research experience

09/2018 - now	**PhD Thesis** in **Joanna Loizou's group** and **Georg Winter's group** at the **Research Center for Molecular Medicine (CeMM)**, Vienna ("Dissecting the role of Polymerase θ in BRCA mutant cancers")
09/2017 – 07/2018	**Master Thesis** in **Luisa Cochella's group** at the **Research Institute of Molecular Pathology (IMP)**, Vienna ("Regulatory role of *miR-1* in the pharyngeal development of *C. elegans*")
04/2017 – 05/2017	**Internship** in **Dea Slade's group** at the **Max Perutz Laboratories (MPL),** Vienna ("Protein purification of PHD and SPOC domains of the SPEN protein family")
02/2017 – 04/2017	**Internship** in **Luisa Cochella's group** at the **Research Institute of Molecular Pathology (IMP), V**ienna ("Regulatory role of *miR-1* in the pharyngeal development of C. *elegans*")
08/2016– 10/2016:	**Internship** in **Sandra Siegert's group** at the **Institute of Science and Technology Austria** (IST), Klosterneuburg ("Analysis of microglial gene expression in different mouse lines")
09/2015-10/2015:	**Bachelor Thesis** in **Tim Skern's group** at the **Max Perutz Laboratories (MPL),** Vienna („Mutational analysis to investigate the binding of 2A proteinase to eIF4E")

Scholarships and fellowships

10/2012 – 09/2013	Performance scholarship of the University of Vienna
10/2013 – 09/2014	Performance scholarship of the University of Vienna
10/2014 – 09/2015	Performance scholarship of the University of Vienna
10/2016 – 09/2017	Performance scholarship of the University of Vienna
10/2017 – 09/2018	Performance scholarship of the University of Vienna
12/2019 - 12/2022	Individual DOC Fellowship of the Austrian Academy of Sciences covering 38 000€ annually over 3 years

Publications

Schrempf A., Bernardo S., Arasa-Verge E.A., Ramirez-Otero M.A., Wilson J., Kirchhofer D., Timethaler G., Ambros A.M., Kaya A., Wieder M., Ecker G.F., Winter G.E., Costanzo V., Loizou J.I. POLθ processes ssDNA gaps and promotes replication fork progression in BRCA1 deficient cells. Under review.

Schrempf, A., Slyskova, J., Loizou, J.I. (2021). Targeting the DNA Repair Enzyme Polymerase q in Cancer Therapy. Trends in Cancer, 7(2),98-111, https://doi.org/10.1016/j.trecan.2020.09.007

Amayr, M., **Schrempf, A.**, Üzülmez, Ö., Olek, K. M., & Skern, T. (2017). Interaction of 2A proteinase of human rhinovirus genetic group A with eIF4E is required for eIF4G cleavage during infection. Virology, 511(July), 123–134. https://doi.org/10.1016/j.virol.2017.08.020

Serwas, N. K., Hoeger, B., Ardy, R. C., Stulz, S. V., Sui, Z., Memaran, N., Meeths, Marie, Krolo, Anna, Petronczki, Ö., Pfajfer, L., Hou, T., Halliday, N., Santos-Valente, E., Kalinchenko, A., Kennedy, A., Mace, E., Mukherjee, M., Tesi, B., **Schrempf, A.**, Pickl, W., Loizou, J., Kain, R, Bidmon-Fliegenschnee, B., Schickel J., Glauzy, S., Huemer, J., Garncarz, W., Salzer, E., Pierides, I., Bilic, i., Thiel, J., Priftakis, P., Banerjee, P., Förster-Waldl, E., Medgyesi, D., Huber, W., Orange, J., Meffre, E., Sansom, DM., Bryeceson, Y., Altman, A., Boztug, K. (2019). Human DEF6 deficiency underlies an immunodeficiency syndrome with systemic autoimmunity and aberrant CTLA-4 homeostasis. Nature Communications, 10(1), 3106. https://doi.org/10.1038/s41467-019-10812-x

Personal skills

Languages:
- German (mother tongue)
- English (B2)
- Italian (A2)

Computer skills:
- MS Office
- Molecular Biology Software: ApE, Fiji, Prism, NemAcquire, NemAnalysis, PyMOL, FlowJo, SnapGene, scanR acquisition, scanR analysis
- Python

Driving licence:
- Class B

Personal training:
- Career Coaching with Gabriela Abado, LBG Career Center, 05/2022
- Skills Training "Time Management", LBG Career Center,11/2021
- European Business Competence License (EBCL) for Accounting, Investment Calculation, Commercial Law and Finance, 01/2021
- 3-month Management Skills and Leadership programme "NaturTalente", University of Vienna in collaboration with six industry partners, 03/2020-06/2020
- Workshop „Co-Create Career Circle", LBG Career Center, 05/2019
- Workshop "Presentation Skills", Medical University of Vienna, 04/2019
- Workshop "Die Kunst des freien Sprechens", WIFI Wien, 06/2017
- Workshop "Effektiv präsentieren. Effizient kommunizieren", University of Vienna, 06/2017
- Workshop "Rhetorik und Verhalten 1", WIFI OÖ, 08/2017

Personal interests:
- Music: active member of the choir "Cantus Novus Wien" since 2012
- Sports: hiking, skiing, ski touring, biking, jogging, climbing

Appendix

Conferences & Collaborations

03/2020 - now	Member of **EU-funded DDREAM Synergy Project,** a collaboration between three labs (Cambridge, Zürich and Vienna)
11/2019	**Signalling and Gene Regulation in Health and Disease Conference**, Poster presentation of my scientific work, Babramham Institute, Cambridge
10/2019	**Genome Engineering: Frontiers of CRISPR/Cas Conference**, Poster presentation of my scientific work, Cold Spring Harbor Laboratories (CSHL), New York
09/2019	**Collaborative research stay** in the **Corn Lab,** ETH Zürich
05/2019	**Ubiquitin & Friends Symposium,** Poster presentation of my scientific work, Vienna
02/2019	**CeMM Scientific Recess**, Poster presentation of my scientific work, Waidhofen
05/2018	**RNA-spring Meeting**, Poster presentation of my scientific work, Retz