Cancer chess: molecular insights into PARP inhibitor resistance

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Preface

Do you believe in coincidence? My instinct says 'no', and my scientific background strengthens me not to take coincidence for an answer. So why is it that in a room full of patients diagnosed with the same subtype of cancer, due to a defect in the same gene, and treated in the same way, some patients are faced with a relapsing tumor soon after treatment is initiated, while others live disease-free for many years? This intriguing question drives the motivation to the work presented in this thesis, which is focused on the subgroup of breast tumors that are hallmarked by a defect in BRCA1. Although this defect can be therapeutically targeted using PARP1 inhibitors, resistance to this treatment may develop. As a strong advocate of genetics, this work centers on identifying genetic interactions that may be able to explain why some of these tumors respond to PARP1 inhibition whereas others do not. At the same time, these studies have provided valuable insights in the regulation of DNA double-strand break repair. Insights which may interest not only the fundamental biologist, but may also serve a purpose in gene targeting and, hopefully, may be translated to improve cancer treatment. Indeed, the genetic alterations that were identified in this thesis not only render BRCA1-deficient tumor cells unresponsive to PARP1 inhibition, but also expose a new vulnerability that can be exploited.

Cancer Chess:

General Introduction

Molecular Insights

Into PARPi Resistance

Chapter 1

The Cancer Chess Analogy

From a more distant perspective, cancer treatment resembles a game of chess where the outcome is influenced by the choices that are made. At any given time, a number of chess pieces can be moved making it challenging to identify the right move for each situation. In response to each move, the opponent will set a tailored counter-move to prevent being driven into checkmate. While this might indeed neutralize the original strategy, it might at the same time open up a back door that can be taken advantage of. Moreover, since the number of possible moves is not endless it is theoretically possible to predict and have an adequate response for each move that can be made by the opponent. Thereby, with the right strategy one can stay in the driver's seat and force the opponent into a dead alley. In a similar fashion, tumors may be cornered by exploiting vulnerabilities that they acquire upon developing resistance to initial treatment.

This introduction elaborates on the rationale behind specific treatments for specific tumors, ultimately closing in on the function of and interaction between Breast Cancer 1 (BRCA1) and poly(ADP-ribose) polymerase (PARP)1. In addition, it describes the molecular mechanism thought to be at the basis of the successful clinical exploitation of the BRAC1-PARP1 interaction. The abundant role of DNA double-strand break (DSB) repair regulation is highlighted, as well as the unresolved questions that form the starting point for this thesis.

Personalized medicine: a shift in focus

The central dogma to cancer treatment is, and has been for many decades, to disentangle the heterogeneity in cancer and to find the optimal therapeutic strategy for each different type of cancer. While treatment choice has traditionally been guided by classifications based on the organ-of-origin and (histo)pathological characteristics (Fig. 1), the introduction of high-throughput sequencing (HTS) technology and collected efforts such as The Human Genome Project have made it possible to determine the genetic make-up and gene-expression profile for each tumor [1-3]. Therefore, the current interpretation of personalized medicine is aimed at improving cancer treatment by defining the genetic makeup of a tumor and tailoring the treatment to exploit tumor-specific vulnerabilities (Fig. 1). However, HTS has unequivocally shown that cancer genomes can be highly complex: tumor cells contain many genetic changes compared to normal cells, comprising single base changes, insertions, deletions, copy number changes and chromosome rearrangements [4-6]. Hence, it is evident that no tumor between two patients is completely identical, known as inter-tumor heterogeneity. This is further complicated by the realization that even within an individual tumor not all tumor cells are identical, known as intra-tumor heterogeneity [7]. Thus, while HTS theoretically allows sub-classifications to be made in unprecedented detail, it is not straightforward to use this information to guide treatment.

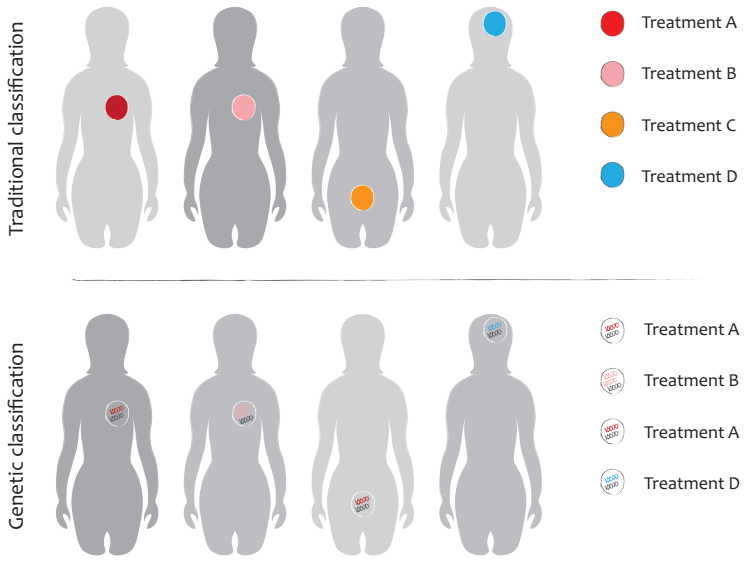


FIGURE 1 | Traditional classifications to guide treatment choice have been based on the organ-of-origin and (histo)pathological characteristics of the tumor. The development of NGS in principle allows this to be further refined with genetic classifications, using defined genetic alterations that may be targeted by a specific treatment irrespective of organ-of-origin.

Lessons learned from targeting driver signaling pathways

One approach in personalized medicine is to target the key signaling pathways that fuel tumor cell growth. For example, a subset of melanomas is driven by the BRAF V600E mutation, which leads to the constitutive activation of the MAPK signaling pathway [8]. BRAF V600E can be potently inhibited by the small molecule vemurafenib, which improved the survival of patients with this subset of melanoma while it had little effect in melanomas that are wild type for BRAF [9, 10]. This has provided a proof-of-principle for the concept of tailoring treatment to tumor-specific alterations. However, a subset of colon cancers that carried the same BRAF V600E mutation were not responsive to vemurafenib treatment [11]. This discordance was explained by the finding that colon cancers circumvent BRAF inhibition by feedback activation of EGFR. Strong synergistic effects were observed by the dual inhibition of BRAF (V600E) and EGFR. This was not required in melanoma cells since these cells naturally express low levels of EGFR. At the same time, it shows that pathway reactivation mitigates the inhibitory effect of BRAF inhibition and indeed, multiple mechanisms have been described to reactivate MAPK signaling and drive resistance to this targeted treatment [12]. Importantly, while EGFR feedback activation increased fitness in the presence of BRAF (V600E) inhibition, it was shown to be disadvantageous in the absence of the inhibitor [13]. Therefore, some relief was

found by withdrawing treatment in resistant cells, known as a "drug holiday". A drug holiday restored the resistant cancer cells to their original state, by which they regained sensitivity to V600E inhibition. While this strategy provided a temporary relief, it did not result in durable responses.

More recently, it was shown that MAPK reactivation increases the cellular levels of reactive oxygen species (ROS). This provides a back door that can be exploited by subsequent treatment with the histone deacetylase inhibitor vorinostat, which further boosts ROS levels to a lethal point selectively in drug-resistant cells [14]. The concept that drug-resistance might coincide with a fitness cost was first described in bacteria and is known as collateral sensitivity [15, 16]. Hereby, collateral sensitivity provides an opportunity to improve the outcome of targeted treatment in one-two punch treatment approaches that may drive resistant cancer cells into checkmate. At the same time, this exemplifies the importance to identify the molecular cause of resistance and the new vulnerability that it might expose.

Exploiting synthetic lethality for cancer treatment

While therapeutic targeting of key signaling pathways can be successful, there are also limitations to this approach. As illustrated in the previous section, reactivation of the targeted pathway is frequent and many different mechanisms have been described. Moreover, some tumor driver mutations are deemed undruggable, meaning that it is difficult to develop specific inhibitors for certain proteins. Examples include RAS mutations and MYC amplification, which are frequently found across cancer types [17,18]. In addition, cancer driver pathways are often important for the normal physiology of somatic cells, and therefore the therapeutic window may not be as large as one would hope.

Another promising approach in personalized medicine is to exploit the concept of synthetic lethality. A synthetic lethal (SL) interaction is an interaction between two genes which have no or little effect on cell viability when inactivated individually, while combined inactivation of these two genes is highly toxic to the cell (Fig. 2) [19]. This can be used for cancer treatment by identifying a tumor-specific disruption in a gene that takes part in a SL interaction, "Gene A". Disruption of another component, "Gene B", will be toxic selectively to tumor cells, since somatic cells have no defect in "Gene A". This can lead to a large therapeutic window and relatively few side effects. Hence, the challenge lies in identifying tumor-specific SL interactions that can be targeted by a drug. A famous success story is the acquired sensitivity of cells with a defect in BRCA1 or BRCA2 to chemical inhibition of PARP1/2 using PARP inhibitors (PARPi) such as olaparib [20].

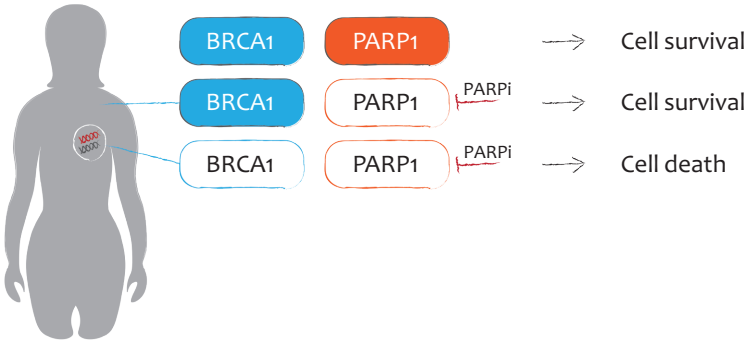


FIGURE 2 | Example of a synthetic lethal interaction exploited in patients. Inhibition of PARP1/2 is tolerated in somatic cells, but highly toxic to tumor cells in which BRCA1 is inactivated.

The BRCA1/PARP Interaction

The clinical side of BRCA1 and PARPi

BRCA1 is a tumor suppressor protein which functions in the pathway that governs DNA double-strand break (DSB) repair via homologous recombination (HR) [21, 22]. Many pathogenic single-nucleotide polymorphisms (SNPs) in BRCA1 have been identified, and these were shown to disrupt the function of BRCA1 in HR [23]. Carriers of gene disrupting mutations in BRCA1 have an increased susceptibility for the development of cancer, primarily breast- or ovarian cancer [24, 25]. Breast tumors that arise in BRCA1 mutation carriers often show loss-of-heterozygosity (LOH) of the wild type allele resulting in a full BRCA1 defect [26, 27]. These tumors are associated with the basal-like and hormone-receptor negative breast cancer subtype [28]. On a genetic level, these tumors are characterized by a high extent of DNA copy-number alterations indicating that these tumors have unstable genomes [29]. The high level of genome instability is most likely tolerated due to the inactivation of the tumor suppressor protein p53, which is frequent in BRCA1 deficient tumors [30]. Besides familial breast- and ovarian cancer, inactivation of BRCA1, for example through promoter hyper-methylation and subsequent LOH, is also observed in sporadic breast- and ovarian cancer patients [31, 32]. Finally, defects in BRCA1 are not exclusive to breast- and ovarian cancer as HTS studies have reported on BRCA1 inactivation in prostate and pancreatic cancer [33, 34]. This potentially enlarges the population of patients that may benefit from PARPi treatment.

The use of PARPi for patients with BRCA1/2 deficient tumors is a promising strategy, because the defect is tumor-specific: somatic cells still carry at least one functional allele in both hereditary and sporadic cancer patients. Indeed, large therapeutic windows have been reported between BRCA1/2 proficient cells and BRCA1/2 deficient cells, which can be up to 1,000 fold [35, 36]. Moreover, the clinical

application of PARPi, such as olaparib, has relatively few side effects and the majority of germline BRCA1 mutant patients show clinical benefit from this treatment [37]. Since PARPi is well-tolerated, it can be administered as maintenance therapy in which patients receive daily treatment [38]. PARPi treatment reduced the risk of disease progression by 70% in patients that were newly diagnosed with advanced ovarian cancer carrying a BRCA1/2 mutation [39]. Moreover, a durable response was achieved in a fraction of patients demonstrating that PARPi treatment has curative potential. These clinical data confirm the synthetic lethality concept in cancer treatment. However, the majority of patients are eventually faced with a recurrent tumor that has become refractory to PARPi treatment (Fig. 3). Hence, resistance remains a major hurdle that must be overcome to achieve long-lasting responses in more patients, even when exploiting SL interactions. A more complete molecular understanding of PARPi's mechanism-of-action and resistance mechanisms may aid the design of rational treatment strategies to combat PARPi resistant tumors.

Physiological functions of PARP1

The PARP family of proteins catalyze the post-translational modification of target proteins by synthesizing branched homopolymer poly(ADP)-ribose (PAR) chains of up to 200-300 units, and are countered by the action of the PAR glycohydrolase (PARG) enzyme which degrades PAR. The best understood functions of ADP-ribosylation occur in response to stress, such as DNA damage, in which PARP1 and PARP2 play a major role (reviewed in [40]). Indeed, PARP1 and PARP2 have partially overlapping functions as their combined deletion is required to induce embryonic lethality in mice [41]. PARP1 is a highly conserved enzyme that contains N-terminal zinc finger motifs to detect damaged DNA, including single-strand DNA (ssDNA) nicks and ssDNA breaks (SSBs), bulky DNA lesions and DSBs [42, 43]. PARP1 binding to damaged DNA activates the C-terminal catalytic domain, which uses NAD+ as a substrate to impose negatively

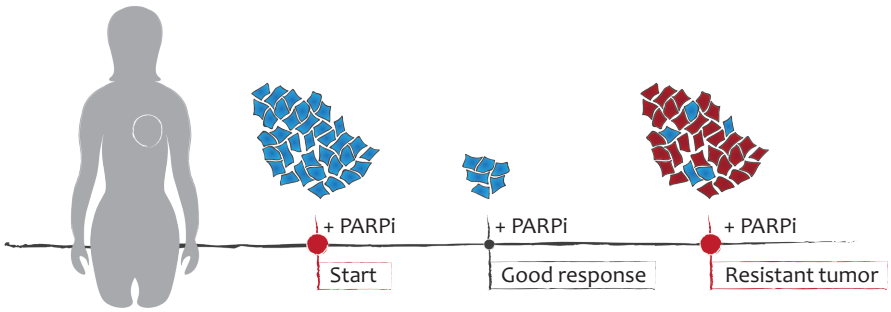


FIGURE 3 | Although the majority of BRCA1 mutant patients show clinical benefit from PARPi, eventually intrinsic or acquired resistance leads to tumor recurrence.

charged PAR chains (PARylation) onto target proteins. These target proteins include chromatin proteins to promote the recruitment of DNA repair effector proteins such as XRCC1 [44-46]. In addition, PARP1 also PARylates itself to mediate its release from the DNA after repair has been completed [47]. The PARylation of histone tails relaxes the chromatin environment by facilitating nucleosome eviction, which enhances the accessibility of damaged DNA [48, 49]. However, a major purpose of PARP1 (auto)-PARylation is to promote the recruitment of DNA damage response (DDR) proteins – which bind PAR non-covalently using PAR-binding motifs – in the presence of damaged DNA or when cells encounter replication stress.

The recognition of SSBs by PARP1 and subsequent PARP1/2 dependent recruitment of XRCC1 is well-described [40, 44, 45]. XRCC1 acts as a molecular scaffold for DNA polymerase β, bifunctional polynucleotide kinase 3′-phosphatase (PNKP) and DNA ligase 3 (LIG3) to efficiently repair SSBs. Additionally, PARP1 functions in the removal of covalent topoisomerase 1 (TOP1)-DNA cleavage complexes (TOP1cc) by tyrosyl-DNA phosphodiesterase 1 (TDP1) [50, 51]. TOP1cc is an intermediate that is formed to relax supercoiling ahead of the replication fork by making a ssDNA incision, but can also arise from endogenous or exogenous DNA lesions (reviewed in [52]). However, inadequate removal of TOP1cc induces DSB formation either directly by a subsequent cleavage event on the opposite strand, or indirectly when an incoming replication fork collides with TOP1cc. TDP1 hydrolyses the phosphodiester bond between the TOP1 tyrosyl moiety and the DNA 3'-end, but requires stabilization via PARP1 mediated PARylation for its activity. This subsequently exposes a nick that is a substrate for SSB repair. Indeed, recruitment of XRCC1 to TOP1cc is promoted by PARP1-TDP1 [50].

PARP1 has also been implicated in the response to replicative stress by stabilizing replication forks via a process termed replication fork reversal [53, 54]. Conceptually, this allows the sensing and repair of replication obstacles prior to their collision with incoming forks. Thereby, DSB formation as a consequence of replication fork collapse or run-off could be prevented. Mechanistically, fork reversal comprises the conversion of replication forks into so-called chicken-foot structures effectuated by annealing of the two nascent strands. While it remains unclear if and how this structure is formed under physiological conditions, a role for RECQ1 has been described in reversing four-way chicken-foot structures back into three-way structures using its helicase activity to mediate fork restart. The helicase activity of RECQ1 is inhibited by its interaction with PARylated PARP1 [55], whereby untimely restart is prevented until the lesion is removed. However, fork reversal inherently creates a one-sided DSB at the replication fork and thus requires protection from canonical DSB processing.

While it is well established that PARP1/2 orchestrates the maintenance of genome stability, its role herein is multifaceted and still not fully understood. The interest in PARP1/2, however, gained a significant boost when it was identified as a potent target for the treatment of tumors that are defective for HR due to a genetic defect in BRCA1 or BRCA2 [35, 36].

Mechanism of action of PARPi

The mechanism underlying the SL interaction between PARP1 and BRCA1/2 was initially explained by the effect of PARPi on inhibiting SSB repair [35, 36]. The inhibition of PARP1 mediated XRCC1 recruitment and its retention on SSBs impairs the repair of SSB intermediates formed during base excision repair (BER). These persistent SSBs subsequently drive the formation of DSBs during DNA replication, which require HR for accurate repair. In the absence of HR, these DSBs may be processed by error-prone mechanisms such as non-homologous end-joining (NHEJ). Since PARPi induced DSBs are one-sided, these repair activities drive the formation of non-viable chromatid rearrangements such as triradial and quadriradial chromosomes, ultimately resulting in cell death during mitosis [36, 56]. However, this model does not capture all available data, as it was found that depletion of XRCC1, a core factor in BER, did not induce equal synthetic lethality [57], suggesting that PARPi has additional effects that contribute to its toxicity in HR deficient cells. One of these was found to be the "trapping" of PARP1 on the DNA [58], a concept that is supported by several observations.

First, a number of PARP inhibitors have been developed, all of which inhibit the catalytic activity of PARP1 and PARP2 but with a different ability to trap PARP1 on the DNA. This trapping ability is correlated with its cytotoxicity: the most potent "trapper" has the strongest cytotoxic effect [59]. More direct evidence for the trapping concept comes from the finding that PARP1 retention at damage sites is extended by PARPi treatment [60]. Furthermore, PARP1 wildtype cells are more responsive to PARPi than to RNAi mediated depletion or genetic inactivation of PARP1 [61], and BRCA1 mutated cells were shown to become resistant to PARPi treatment by depleting PARP1 [62]. These studies show that the absence of PARP1 is less cytotoxic than the sole inhibition of its catalytic activity. Finally, PARPi cytotoxicity can be relieved by depleting PAR glycohydrolase (PARG) [63], the enzyme responsible for degrading PAR chains. Hereby, the residual catalytic activity of PARP1 - despite the presence of PARPi - is sufficient for the recruitment of downstream DDR factors and for its release from the DNA. These findings can be unified in a model in which PARP inhibition not only impairs SSB repair, but also invokes PARP1-DNA complexes [58]. Importantly, this implies that PARPi acts synergistically with processes that increase the load of DNA lesions on which PARP enzymes can become trapped. Indeed, additional DNA structures – besides the SSB intermediate formed during BER – that engage PARP1 have recently been identified [64, 65]. However, how these trapped complexes subsequently drive toxicity in HR deficient cells remains ambiguous. The accepted model is that ssDNA breaks are converted to one-sided DSBs during replication. Additionally, trapped PARP1-DNA complexes might induce replication fork stalling, ultimately leading to replication fork collapse and the formation of one-sided DSBs (Fig. 4) [20]. These replication-associated DSBs can be accurately repaired via HR in wild type cells, explaining why PARPi treatment is well-tolerated in patients. However, these DSBs become highly toxic to cells that have

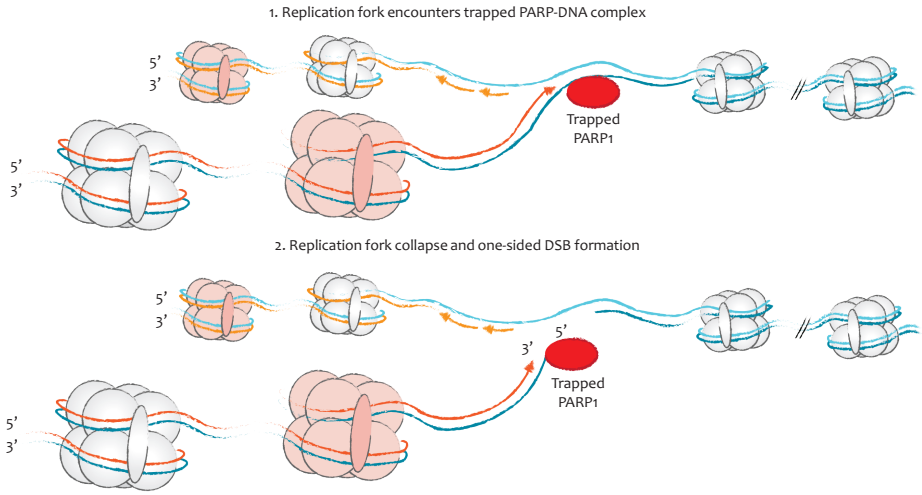


FIGURE 4 | The accepted model through which PARPi exerts its cytotoxic effects. During replication, unrepaired ssDNA breaks may be converted to one-sided DSBs, or trapped PARP1-DNA complexes might lead to replication fork stalling and subsequent replication fork collapse.

a defect in the HR pathway, such as cells that have lost BRCA1 or BRCA2 expression [35, 36]. It is this interaction between the formation of replication-associated DSBs by PARP inhibitors and the lack of an adequate DSB repair mechanism that is believed to be the mechanistic basis for the observed synthetic lethality in BRCA deficient cells. While it is still debated how trapped PARP1-DNA complexes induce toxicity (further discussed in Chapter 8), the notion that the HR pathway is paramount for its removal is supported by the findings that defects in other HR genes, such as PALB2, BRCA2, RAD51C or RAD51D also lead to PARPi sensitivity [58, 66, 67]. Tumors that are defective in one of these genes can acquire PARPi resistance by restoring protein function, for instance via reversion mutations [68-73]. Moreover, *in vitro* and *in vivo* model systems indicate that mechanisms that restore HR activity independent of BRCA1 restoration are prominent drivers of PARPi resistance in BRCA1 deficient cells. In the years hereafter, restoration of HR activity and resistance to PARPi in BRCA1 deficient cells have been powerful readouts to significantly advance the understanding of DSB repair regulation.

Bringing Double-Strand Breaks to a Close

Two pathways for DSB repair

A single persistent DSB is thought to be sufficient to drive a cell to lethality. DSB repair mechanisms have evolved to resolve DSBs that occur in a variety of cellular contexts to preserve genome integrity. The two most studied and fundamentally different

pathways are non-homologous end joining (NHEJ) and HR, which are highlighted below. Additionally, several backup mechanisms have been described, presumably to complete repair if NHEJ or HR fail. These mechanisms include theta-mediated end-joining (TMEJ) [74-76] and single-strand annealing (SSA) [77].

Simplistically, NHEJ is the sequence-independent fusion of two DSB ends. In reality, this apparently simple mechanism requires the action of a complex signaling cascade to recognize DSBs, make the chromatin accessible, and modify the DSB ends prior to their ligation (reviewed in [78]). Depending on the complexity of the DSB end, these steps may comprise the limited resection of DNA ends, fill-in of DNA gaps, or the removal of blocking end groups. Thus, NHEJ can resolve a large variety of DSB substrates. This capacity is further enhanced by LIG4, which retains DNA ligase activity on incompatible ends [79]. As a consequence, NHEJ is active throughout the cell cycle [80], but comes with an increased risk of introducing genome alterations in the form of small deletions, insertions or even translocations. The latter may be formed when two distant DSB ends are ligated together.

In contrast to NHEJ, HR uses a homologous DNA sequence as a template for DSB repair, making this the most accurate DSB repair mechanism (reviewed in [81]). The repair template is preferentially provided by the sister chromatid on replicated regions. Hence, HR is primarily active during S and G2 phases of the cell cycle [80]. A critical step in HR is the initiation of extensive 5' to 3' end resection on the DSB end to generate a 3' single-stranded DNA (ssDNA) overhang. Resection occurs in a two-step model, in which the endonuclease and 3' to 5' exonuclease activity of the Mre11, Rad50, Nbs1 (MRN) complex generates short stretches of ssDNA and removes DNA-bound proteins from the DSB termini, most notably Ku (reviewed in [82]). The second step involves more extensive end resection, which requires the action of 5' to 3' exonucleases, such as EXO1 or DNA2 [83], and is promoted by CtIP [84]. Following resection, the ssDNA overhang is protected from the formation of secondary structures by the binding of replication protein A (RPA). The subsequent replacement of RPA with RAD51 requires the action of mediators, such as BRCA2 [85]. The resulting RAD51-ssDNA nucleofilament searches for and pairs with homologous sequences [86-88]. Upon pairing, the homologous sequence serves as a template for DNA synthesis, by which the invading strand is extended across the original break site. This process of strand invasion produces a displacement D-loop, which is resolved in the final step of HR.

While repair of DSBs via the HR pathway is generally preferred due to its higher fidelity, a regulatory mechanism is required to restrict its activity to DSBs that arise during or post-replication, while at the same time allowing NHEJ activity on the remaining DSBs. The differential requisite to inhibit or initiate end resection commits DSB repair to the NHEJ or HR pathway, respectively, and it is this step that is tightly controlled by several mechanisms – often referred to as 'pathway choice'. Critical in this process are the opposing roles of 53BP1 and BRCA1, which favor repair via NHEJ or HR, respectively (discussed below).

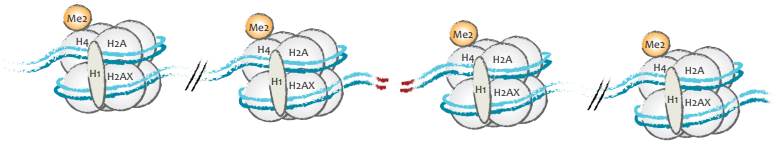
Regulating the End Problem: 53BP1

Upon recognition of a DSB, a signaling cascade is initiated which changes the chromatin environment around the break site (reviewed in [89-91]) (Fig. 5). One of these changes is the phosphorylation of histone H2AX (γ-H2AX) by ATM, which serves as a molecular signal to ensure local recruitment of DNA response factors, including MDC1. Subsequently, MDC1 recruits the E3 ubiquitin ligases RNF8 and RNF168, which together with the E2 ubiquitin-conjugating enzyme 13 (UBC13) catalyze the formation of Lys63-linked ubiquitin chains on H2AK13 and H2AK15 [92]. This local ubiquitylation is required for the retention of 53BP1 and BRCA1 [93]. The accumulation of 53BP1 is further dependent on mono- and di-methylated H4K20 [94]. Di-methylated H4K20 (H4K20me2) is a widespread mark that "primes" the chromatin for 53BP1 binding. The specificity towards DSBs is thought to arise by masking H4K20me2 from 53BP1 binding in undamaged DNA; the exposure of which is coordinated by RNF8 and RNF168 [95]. While the methylation state is rather stable, the dynamic acetylation of H4K16 negatively influences the binding of 53BP1 to H4K20me2 as another regulatory mechanism to fine-tune DSB repair pathway choice [96, 97]. Ultimately, these marks are integrated to locally accumulate 53BP1 near the break site where the 53BP1 pathway protects DSB ends from resection and facilitates repair via NHEJ.

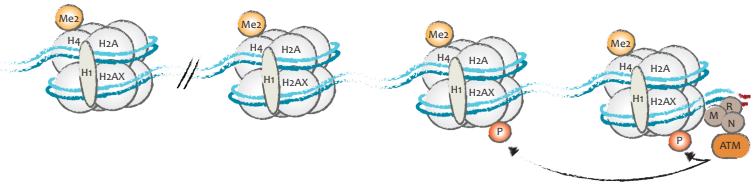
The role of 53BP1 in end protection and facilitating the NHEJ pathway has been demonstrated in a number of cellular contexts. One compelling example occurs in activated mature B-cells, which are restricted to the production of antibodies (immunoglobulins) of the IgM or IgD isotype. The controlled induction of a DSB at conserved motifs – switch regions - in the heavy chain locus (IgH) is followed by a recombination event in the heavy chain exon clusters, Cμ, Cγ, Cα and Cε. This process of class switch recombination (CSR) is required for the production of antibodies of a different isotype, such as IgG, IgA or IgE [98]. CSR is stimulated by the activity of activation-induced (cytidine) deaminase (AID), which produces deoxyuracils by deaminating deoxycytosines [98-100]. Due to the high density and staggered position of deoxyuracils in switch regions, the repair process leads to the formation of DSBs and subsequent repair by NHEJ activity. DSB induction in switch regions is not affected by the absence of 53BP1; rather, 53BP1 plays a major role to ensure correct ligation. Indeed, the knockout of 53BP1 impairs isotype switching and results in a severe CSR defect [101, 102]. The role of 53BP1 is less pronounced in V(D)J recombination, which occurs during the maturation of B-cells. V(D)J recombination is the driving force to generate diversity in the antigen binding domain of immunoglobulins and T cell receptors. The assembly of exons from the variable (V), diversity (D) and joining (J) gene segments follows an ordered pattern of DSB induction by the RAG1/2

FIGURE 5 | A (simplified) schematic overview of the signaling cascade leading to DSB repair via the 53BP1 pathway. Key activities are exerted by MRN, ATM, RNF8, RNF168, 53BP1, RIF1 and REV7, which are highlighted. The connections that are still unresolved are depicted by a dashed line.

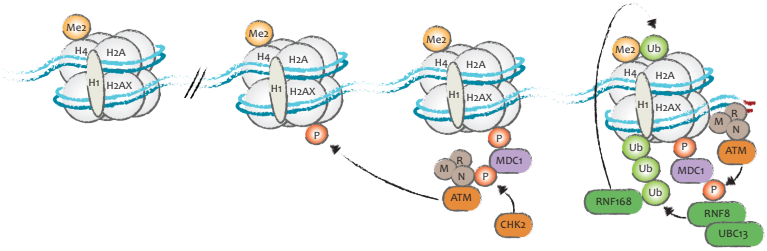
1. DSB formation



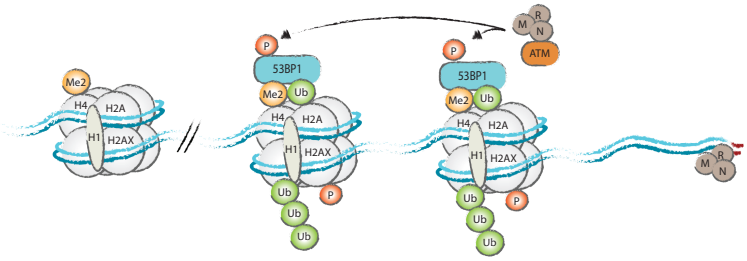
2. Break recognition and ATM mediated phosphorylation of H2AX



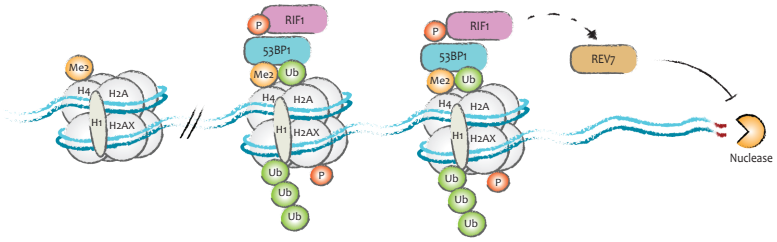
3. Recruitment of MDC1 and RNF8/168 ultimately lead to exposure of H4K20Me2



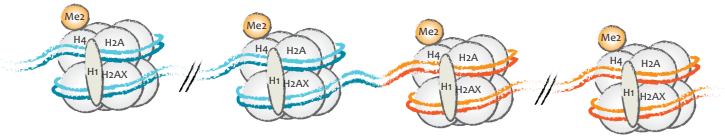
4. Recruitment of 53BP1, which also gets phosphorylated by ATM



5. Nuclease activity inhibition by 53BP1 through RIF1 and REV7, favoring NHEJ



6. Potential for toxic repair by joining distant DSBs



nuclease and is followed by repair via NHEJ (reviewed in [103]). Interestingly, while inactivation of core NHEJ factors such as LIG4 severely impairs V(D)J recombination [104, 105], 53BP1 knockout exclusively impairs distal V(D)J joining [106]. The function of 53BP1 is also demonstrated in the context of dysfunctional telomeres. The telomeric ends of chromosomes resemble DSBs and are sheltered from invoking a DDR by a process known as telomere capping (reviewed in [107]). Mechanistically, the repetitive nature of telomeric sequences (TTAGGG in vertebrates) allows the formation of a T-loop, a loop structure by which a telomeric 3' overhang folds back into the DNA [108, 109]. The protein complex that mediates the formation of T-loops is the Shelterin complex, in which TRF2 is an essential component. The absence of TRF2 leads to telomere uncapping and triggers DSB repair signaling [110]. Since these misrecognized DSBs are one-sided, the activity of NHEJ induces chromosomal fusions. Indeed, this phenotype is driven by NHEJ as it was shown to be rescued by the depletion of core NHEJ components such as LIG4, as well as by the depletion of 53BP1 [111, 112]. A role for 53BP1 in the repair of DSBs arising from exogenous sources has also been demonstrated. Knockout of 53BP1 in DT40 cells induced an intermediate sensitivity to ionizing radiation (IR) compared to knockout of LIG4 or KU70 [113, 114]. Moreover, the double knockout did not further enhance IR sensitivity, suggesting that 53BP1 acts on a subset of the DSB substrates that are repaired by NHEJ. Together, these studies are consistent with a role of 53BP1 in promoting DSB repair by NHEJ, rather than being an essential component of the NHEJ pathway itself or playing a role in an alternative repair pathway.

Soon after the identification of 53BP1 it was shown that RIF1 and PTIP are two downstream proteins in the 53BP1 pathway [114-119] (Fig. 5). The interaction with 53BP1 depends on specific phosphoresidues on the N-terminus of 53BP1, which are phosphorylated by ATM. Both RIF1 and PTIP phenocopy 53BP1 as the loss of either protein was shown to facilitate repair via NHEJ of dysfunctional telomeres and cause radiosensitivity. However, while RIF1 plays an essential role in CSR, the role of PTIP is dispensable in this context suggesting the existence of additional layers of regulation depending on the DSB substrate. The 53BP1 pathway was further extended by the identification of REV7/MAD2L2, which function downstream of RIF1 [120, 121]. Importantly, all of these factors are present in the nucleus throughout the cell cycle. Thus, a mechanism needs to be in place to inhibit the 53BP1 pathway in S-phase to allow repair via the HR pathway.

Regulating the end problem: BRCA1

The notion that BRCA1 functions in HR originates from the finding that BRCA1 colocalizes with RAD51 [22]. The BRCA1 gene contains an N-terminal RING domain, two nuclear localization signals (NLS), a coiled-coil (CC) motif and a C-terminal BRCT domain. The RING domain interacts with a similar domain in BARD1, and this interaction is required for the ubiquitin E3 ligase activity of BRCA1 [122-124]. BRCA1

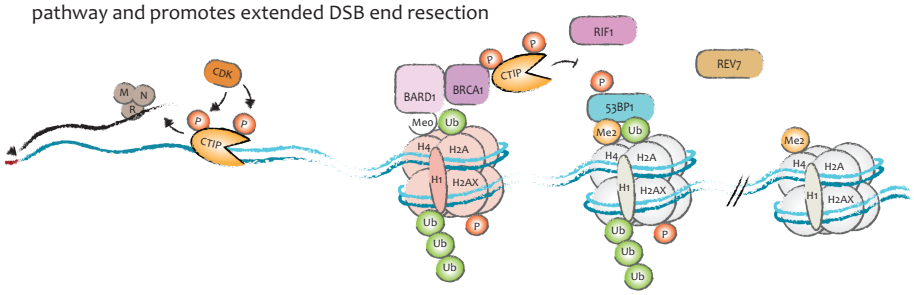
directly partners with PALB2 through its CC motif to facilitate the accumulation of BRCA2, thereby linking BRCA1 to downstream proteins in the HR pathway [125, 126]. The BRCT domain allows phosphorylation-dependent binding to a number of proteins, including Abraxas, BACH1/BRIP1 and CtIP (forming the BRCA1-A, BRCA1-B and BRCA1-C complexes, respectively) [127-131]. Thereby, BRCA1 can take part in a number of protein complexes, each with different functions in DNA repair. The BRCA1-C complex, together with MRE11-RAD50-NBS (MRN), facilitates 5' end resection to promote homologous recombination [84, 129, 132, 133].

Importantly, the RING, CC and BRCT domains are all important for the physiological function of BRCA1 and mutations in these regions are often deleterious. Indeed, cancer-predisposing pathogenic mutations tend to cluster in these domains and impair the function of BRCA1 in HR [23]. BRCA1 deficient cells show a defect in the localization of PALB2, BRCA2 and RAD51 into nuclear foci at DSBs, which is most likely caused by the impaired 5' end resection of DSBs in the absence of BRCA1, as evidenced by decreased loading of pRPA [133].

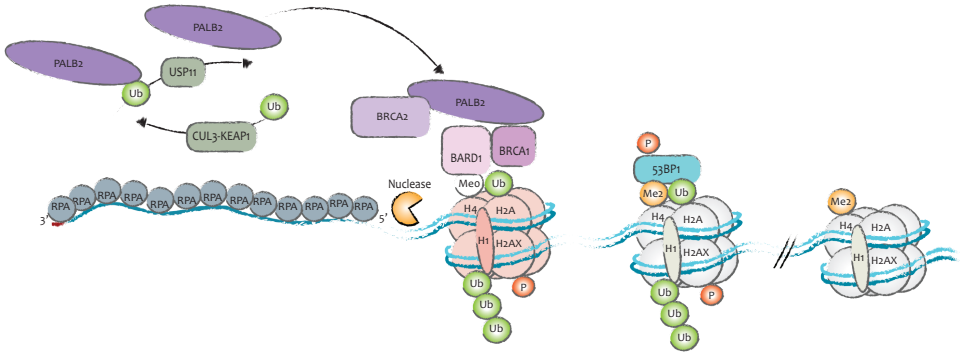
Releasing the break on DSB resection

The outcome of DSB repair pathway choice is influenced by the cell cycle phase (Fig. 6). Paradoxically, 53BP1 is recruited to DSBs irrespective of the cell cycle phase, demonstrating that DSBs are initially primed for repair via NHEJ. Rather, 53BP1 is actively occluded from the core of the break site and gets redistributed to the periphery during S phase [134]. The repositioning of 53BP1 coincides with the recruitment of BRCA1 and is diminished in the absence of BRCA1. BRCA1 has a more striking effect on RIF1 recruitment: while RIF1 recruitment is largely restricted to G1 cells, its localization in S/G2 cells can be restored by depletion of BRCA1 [114]. This unequivocally shows the antagonistic relation between the 53BP1 pathway and BRCA1. Like 53BP1, recruitment of BRCA1 to DSBs depends on a chromatin environment marked by γ-H2AX and the ubiquitin cascade imposed by RNF8 and RNF168 activity. Integration with the cell cycle comes from the BRCT dependent interaction between BRCA1 and CtIP. Both this interaction and the activity of CtIP to promote end resection are mediated by CDK dependent phosphorylation of specific residues on CtIP, S327 and T847 respectively [129, 132, 135]. These phosphorylation events were shown to be required to antagonize RIF1 localization to DSBs in S/G2 [114]. Moreover, the localization of RIF1 to DSBs in G1 cells can be reduced by the expression of a phosphomimicking mutant of CtIP that is constitutively active (CtIP-T847E). Thus, CDK activity regulates a 'switch' to inhibit the 53BP1 pathway specifically in S phase. Together, these data demonstrate a regulatory circuit of 53BP1-RIF1 and BRCA1-CTIP to control DSB pathway choice. Another cell cycle regulatory step is the interaction between BRCA1 and PALB2. It was shown that this interaction is inhibited by the ubiquitylation of Lys20 on PALB2 by the CUL3-KEAP1 E3 ligase [136]. The PALB2Lys20 mark requires removal by the deubiquitylase USP11, which

1. BRCA1/BARD1 are recruited to post-replicative DSBs through recognition of the H4oK20meo chromatin mark. CDK mediated phosphorylation on BRCA1 and CTIP antagonizes the 53BP1 pathway and promotes extended DSB end resection



2. ssDNA exposed by end resection is bound by RPA.
Formation of the BRCA1-PALB2-BRCA2 complex is under cell cycle control through USP11.



3. BRCA2 replaces RPA with RAD51 and repair proceeds via HR

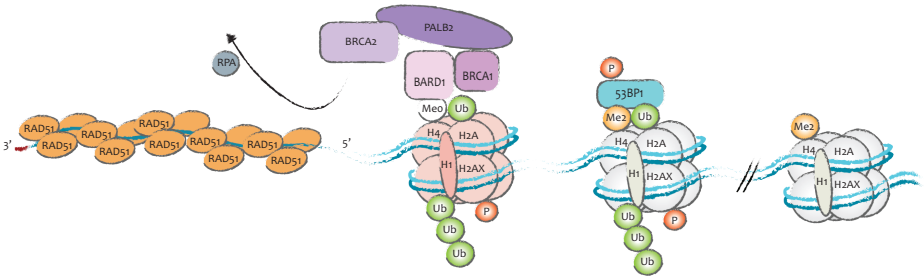


FIGURE 6 | A schematic overview of the first steps of the HR pathway, in which BRCA1 and BRCA2 play a pivotal role. Several cell cycle regulatory mechanisms that antagonize the 53BP1 pathway and restrict HR to post-replicative regions are depicted.

is controlled by a CRL4 complex that targets USP11 for degradation upon DNA damage in G1. Loss of 53BP1, artificial stimulation of CtIP by expression of CtIP-T847E, plus depletion of KEAP1 lead to (partial) HR activation even in G1 phase cells demonstrating that these factors normally function to restrict HR to S phase cells.

The regulation is likely more complex as in early S phase not all the DNA has been replicated and therefore not all DSBs that occur in S phase cells are suitable for repair via HR. Recent insights to this regulatory requirement suggest a role for the H4K20me2 mark, which is required for the recruitment of 53BP1. The histones that are newly incorporated during replication do not yet carry this mark, which dilutes the concentration of H4K20me2 on post-replicative regions [137, 138]. It is conceivable that the lower concentration of H4K20me2 or potentially other chromatin marks facilitates the redistribution of 53BP1 specifically on DSBs for which a sister-chromatid is available. In conjunction, the recently shown recognition of H4K20me0 by BARD1 provides an elegant mechanism by which BRCA1 may be specifically recruited to post-replicative regions [139].

The Interplay of 53BP1 and BRCA1 on PARPi Sensitivity

The antagonistic relation between 53BP1 and BRCA1 is especially evident from the phenotypes in BRCA1 deficient cells that are rescued by the depletion of 53BP1. Loss of BRCA1 in otherwise wild type cells induces cell cycle arrest and subsequent apoptosis, presumably due to the genomic instability that arises in these cells. This cellular response can be overcome by the depletion of P53 to allow cell survival in *Brca1*$^{\Delta 11/\Delta 11}$ cells [140]. However, this did not rescue the underlying BRCA1 defect as *Brca1*$^{\Delta 11/\Delta 11}$*;p53*$^{-/-}$ mice are hallmarked by a high degree of genomic instability and are prone to tumor development and premature aging. In contrast, mice in which *Trp53bp1* was inactivated in the *Brca1*$^{\Delta 11/\Delta 11}$ background had a low incidence of tumor development and showed a near normal lifespan [141]. Two landmark studies independently demonstrated that the concurrent loss of 53BP1 in *Brca1*$^{\Delta 11/\Delta 11}$ or BRCA1 null cells coincides with a (partial) restoration of homologous recombination activity [142, 143]. While inhibition of 53BP1 had previously been shown to enhance HR [144], these studies demonstrated that BRCA1 function is not essential for HR, as HR is reactivated in the absence of 53BP1. Strikingly, the loss of 53BP1 was shown to drive PARPi resistance in BRCA1 deficient cells, presumably due to the restoration of HR activity. Indeed, HR activity and PARPi resistance could be reversed by inhibition of ATM signaling supporting the notion that the HR defect is the primary source driving PARPi sensitivity [142]. Similar phenotypes have been described upon depletion of the downstream factors PTIP, RIF1 or REV7/MAD2L2 [115-117, 119-121]. While the significance of 53BP1 pathway inactivation as a driver of PARPi resistance in BRCA1 deficient cancer patients requires further study, the occurrence of mutations in TRP53BP1 and REV7/MAD2L2 has been described [145]. These data have put the 53BP1 pathway at the center of attention due to its potential clinical implications for successful PARPi treatment.

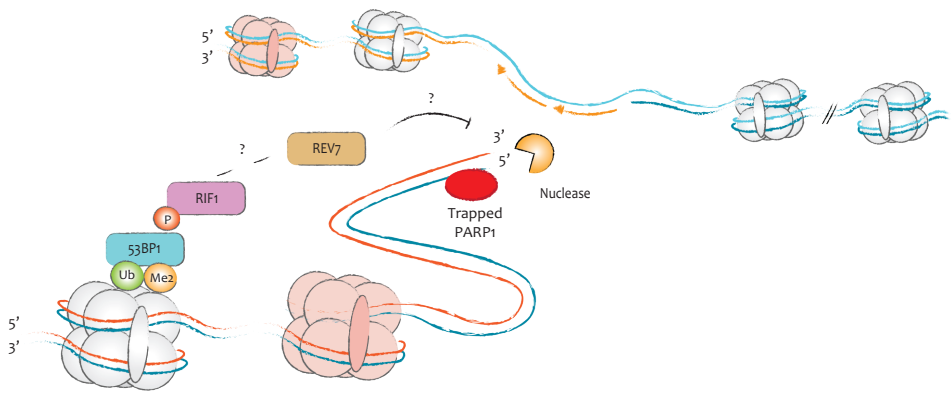


FIGURE 7 | The 53BP1 pathway is believed to protect PARPi induced one-sided DSBs from resection by nucleases. Steps that are incompletely understood are depicted with a '?'.

Scope of This Thesis

The BRCA1/PARP1 paradigm exploits the HR defect of BRCA1 deficient cells and has great potential for cancer treatment. However, durable responses are infrequent as most tumors eventually become resistant to PARPi treatment. Thus, resistance is a hurdle that must be overcome in order to maximally exploit this synthetic lethal interaction in the clinic. In reference to the cancer chess analogy, we are now beginning to grasp the counter-moves that may be played by resistant tumors as the molecular mechanisms that underlie PARPi resistance are being mapped at an increasing pace. Reactivation of HR activity through restoration of functional BRCA1 expression is one class of such mechanisms; however, it is clear that resistance may also develop while BRCA1 remains inactivated. A prominent route towards BRCA1 independent PARPi resistance is the reactivation of HR activity owing to the inactivation of the 53BP1 pathway. The 53BP1 pathway normally antagonizes resection at DSB ends and it has been shown that the removal of this "brake" allows DSB ends to be resected with sufficient efficiency to restore HR. However, several aspects of this class of resistance mechanisms and its implications for cancer treatment remain unexplained, which is the focus of this thesis.

Mechanistically, there is an incomplete understanding of how the 53BP1 pathway protects DSB ends from resection, since neither 53BP1, RIF1 nor REV7/MAD2L2 has direct DNA binding capabilities (Fig. 7). Two models exist to explain this discrepancy: (i) the 53BP1 pathway changes the chromatin environment to block nuclease access, or (ii) the 53BP1 pathway recruits yet unidentified factors with DNA binding capacity. The second model implies the existence of additional proteins that may be inactivated in cancer cells to withstand treatment, thus suggesting that our view on the BRCA1/PARP1 chessboard is still incomplete. With this second model in mind, several

CRISPR/Cas9 based high-throughput loss-of-function screens were performed to identify such factors. Chapter 3 describes an advance in technology to accelerate the *in vivo* validation of putative resistance factors by combining CRISPR/Cas9 technology and 3D tumor organoid culture protocols. The identification and validation of multiple factors of the 53BP1 pathway are described in Chapters 4-6, thus providing support for the second model.

From a translational perspective, the question is whether inactivation of the 53BP1 pathway puts the patient and clinician checkmate, or if a new synthetic lethal interaction is acquired upon the loss of the 53BP1 pathway, thus allowing for one-two punch approaches. The 53BP1 pathway has a physiological function in DSB repair and it is therefore conceivable that its inactivation is disadvantageous in specific contexts. One such context was identified and exploited as described in Chapter 7 of this thesis.

Together, this thesis aims to further the molecular understanding of the 53BP1 pathway and its role in driving PARP inhibitor resistance in the absence of BRCA1; and to identify new vulnerabilities of these resistant cells that can be exploited.

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