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# Cancer chess: molecular insights into PARP inhibitor resistance

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*“Logic will get you from A to B,*
*imagination will take you everywhere“*

(Albert Einstein)

# Cancer Chess: Molecular Insights Into PARPi Resistance

## General Discussion and Future Perspectives

## Chapter 8

## General Discussion and Future Perspectives

Personalized medicine is aimed at improving cancer treatment by defining the genetic background of a tumor and tailoring the treatment to exploit tumor-specific characteristics. However, the complexity of cancer genomes poses researchers and clinicians with the challenge to extract the information that is of clinical relevance. In addition to direct targeting of cancer cell proliferation and survival, tumors may also be targeted by exploiting the concept of synthetic lethality (SL). A synthetic lethal (SL) interaction is an interaction between two genes in which inactivation of either of the single gene has no or little effect on cell survival, whereas simultaneous inactivation of both genes is highly lethal [1-3]. While the concept of SL was first described almost a century ago, its clinical utility for cancer treatment has been long-awaited. Indeed, the initial discovery that chemical inhibition of poly(ADP-ribose) polymerase (PARP)1/2 (PARPi) is selectively toxic to cells that are defective for the double-strand break (DSB) repair mechanism homologous recombination (HR) was received with great enthusiasm as it provided a strong rationale for the targeted treatment of BRCA1/2 deficient tumors [4, 5]. Mechanistically, PARPi treatment is believed to induce one-sided DSBs upon replication fork collapse which in the absence of HR become substrates for toxic canonical non-homologous end joining (c-NHEJ), driving radial chromosome formation and ultimately cell death [4] – a model which still stands today (Fig. 1). However, despite encouraging clinical responses initially, the majority of patients are eventually faced with a recurrent tumor that has become refractory to PARPi treatment [6]. Thus, it is evident that (acquired) resistance remains a major hurdle which must be overcome to achieve long-lasting responses in more patients, even when exploiting SL genetic interactions. This unsatisfactory outcome drives the interest to map molecular mechanisms that may explain PARPi resistance with the ultimate goal to translate this knowledge into strategies that may increase PARPi efficacy in more patients. This thesis contributes to these efforts by describing the identification of several new factors that modulate PARPi sensitivity in BRCA1 deficient cells, namely DYNLL1 (Chapter 4), the Shieldin (SHLD) complex (Chapter 5) and the CTC1-STN1-TEN1 (CST) complex (Chapter 6). This thesis also describes that 53BP1 pathway inactivation creates a new treatment vulnerability in the form of radiotherapy (Chapter 7). At the same time, the study of PARPi resistance mechanisms is providing unprecedented insights in the DNA damage response.

DYNLL1, SHLD and CST were found to be linked to the 53BP1-RIF1-MAD2L2/REV7 pathway in DSB repair [7-12], a pathway that is presumed to promote repair via c-NHEJ by protecting DSB ends from resection and is normally counteracted by BRCA1 (reviewed in [13, 14]). It is well-described that inactivation of the 53BP1 pathway in BRCA1 deficient cells partially restores end resection and HR activity [15-24], providing an explanation for PARPi resistance. However, since neither 53BP1 nor RIF1 nor MAD2l2/REV7 is known to possess direct DNA binding properties, it has remained elusive how end protection may be achieved by this pathway. The identification of two novel single-stranded DNA

(ssDNA) binding complexes that function downstream in the 53BP1 pathway, SHLD and CST, provides valuable mechanistic insights: active recruitment of ssDNA binding complexes may 'shield' the DSB end from further resection. At the same time, the involvement of ssDNA binding complexes in the 53BP1 pathway raises new questions about the nature of the DSB end on which this pathway is engaged. Indeed, it hints to the generation of DSB intermediates that comprise a 3' ssDNA tail. At first sight, this seems at odds with the hitherto presumed role of 53BP1 in DSB end protection. However, this seeming paradox could also imply that 53BP1 mediated end protection is a more finely regulated process.

## 53BP1 Mediated DSB End Protection Revisited

The notion that the 53BP1 pathway facilitates c-NHEJ is supported by a number of assays that serve as a proxy for c-NHEJ activity, including IR sensitivity assays [25], plasmid

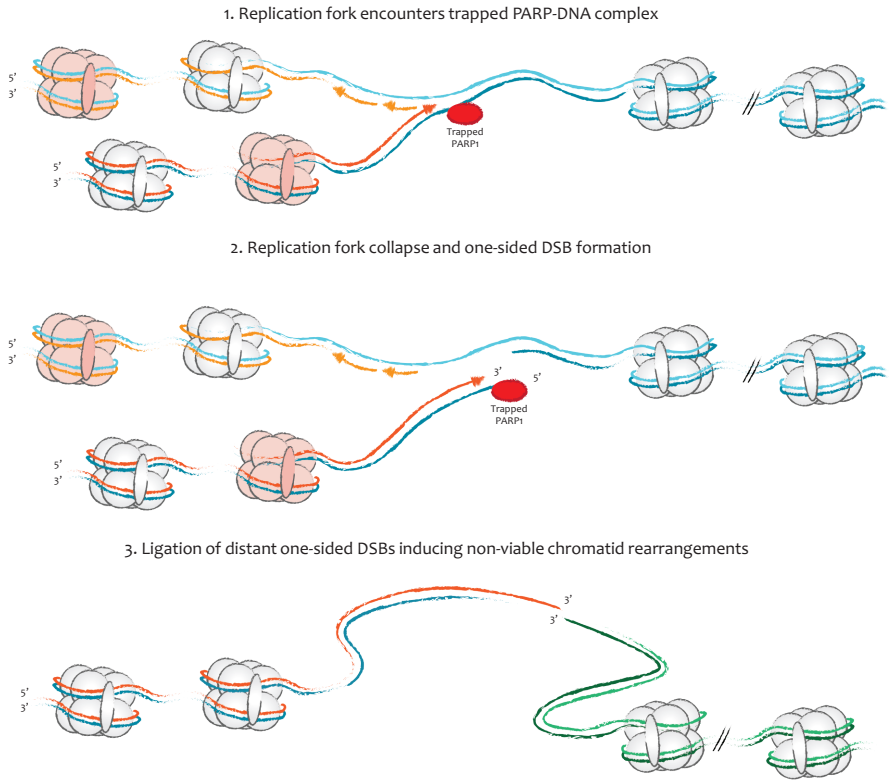


**FIGURE 1 |** Simplified model for the generation of non-viable chromatid rearrangements upon PARPi treatment in BRCA1 deficient cells. When a trapped PARP-DNA complex is encountered by a replication fork, a one-sided DSB is formed, which in the absence of BRCA1 is prone to 53BP1 pathway mediated ligation with distant one-sides DSBs.

integration assays [17], CSR assays [26, 27], and the fusion of uncapped telomeres [28]. These assays strongly rely on KU70/80, XRCC4 or LIG4 activity and 53BP1 acts epistatic with these factors. Moreover, cells in which the 53BP1 pathway is suboptimal become increasingly dependent on alternative end joining (a-EJ) pathways such as alternative non-homologous end joining (a-NHEJ) and polymerase-theta mediated end joining (TMEJ) to repair DSBs, which demonstrates that the 53BP1 pathway does not function in a-NHEJ or TMEJ [29, 30]. The observation that DSB end resection is enhanced upon 53BP1 depletion [15, 16] provides a plausible explanation for why repair may be shuttled towards these alternative repair pathways, which require ssDNA overhangs, and corroborates the hypothesis that the 53BP1 pathway facilitates c-NHEJ by inhibiting end resection.

However, several peculiarities emphasize that the 53BP1 pathway is not essential for c-NHEJ. For example, the IR sensitivity that is observed upon depletion of 53BP1 pathway factors is relatively mild compared to core c-NHEJ factors, suggesting that not all IR induced DSBs require the 53BP1 pathway for its repair ([25], this thesis). Moreover, the role of the 53BP1 pathway in other c-NHEJ driven processes such as V(D)J recombination is limited [31]. Together, these findings argue that the 53BP1 pathway acts as a positive regulator of c-NHEJ in a subset rather than in all DSB contexts, although it is unclear how this substrate is defined.

It has been shown that when DSB ends are directly compatible for end joining, c-NHEJ can be executed efficiently and proceeds primarily by the activities of KU70/80, XRCC4 and LIG4 [32]. The role of the 53BP1 pathway in the repair of blunt DSB ends or DSB ends with short overhangs (<4 nts) may thus be limited, although 53BP1 induced changes in the chromatin environment and enhancement of DSB end mobility may enhance the efficacy of the repair process [28, 33, 34]. It also seems unlikely that the ssDNA binding complexes SHLD and CST would be required for the repair of 'clean' breaks due to the limited length or absence of ssDNA overhangs. However, more complex breaks that are not amenable for direct ligation and/or contain (protein) blocks on DSB ends may require additional processing and in this context one can speculate about a role for the 53BP1 pathway and ssDNA binding complexes.

*The 53BP1 pathway at DSB ends - protection from or by resection?*

The findings described in this thesis may be incorporated in the accepted model, which proposes that an important function of the 53BP1 pathway is to regulate DSB repair pathway choice by controlling end resection. As outlined in more detail in the introduction (Chapter 1), the widespread abundance of the H4K20me2 chromatin mark primes the chromatin for rapid recruitment of 53BP1 to DSBs following ATM/MRN activation and seems consistent with such a coordinating role [35-38]. Once 53BP1 is engaged, however, rather than inducing a block to resection the role of the 53BP1 pathway may be more versatile.

It is conceptually tempting to speculate that the 53BP1 pathway may allow limited resection to proceed in a resection dependent c-NHEJ sub-pathway. This sub-pathway might resemble the slow component of c-NHEJ [39], which is LIG4 dependent and thus differs from a-NHEJ. In contrast to resection in S/G2, which requires the endonuclease activity of MRN, resection dependent c-NHEJ was previously shown to depend on the exonuclease activity of MRN and EXO1 [40]. However, it is possible that distinct DSB lesions might be subjected to endonucleolytic rather than exonucleolytic resection by MRN in G1, and a protein block (i.e. TOP2 or KU) at the DSB end has been suggested as a candidate [41-43]. Here, MRE11 initiated endonucleolytic cleavage followed by 3' – 5' resection towards the DSB end resembles MRN activity in G2 and provides a means to remove the protein block, leaving behind a free 3' ssDNA overhang in the process (Fig. 2). This scenario is analogous to the removal of covalently bound SPO11 during meiotic recombination, which also requires MRE11 nuclease activity [44, 45]. Subsequent binding of SHLD or CST could be an effective way to occlude the ssDNA tail from 5' – 3' nuclease access and/or to shorten the resected overhang by a POLA mediated fill-in reaction (discussed below). Future studies are required to unravel the precise PARPi induced DSB substrate and how it is generated. Possibly, the trapping of PARP1 on the DNA by PARPi is also processed as a protein block by MRN. The high sensitivity of BRCA1 deficient cells to both PARPi and topoisomerase inhibitors, together with the finding that 53BP1 pathway inactivation induces cross-resistance to both treatments corroborates this possibility [this thesis, [23, 46, 47]]. However, it cannot be excluded that the 3' ssDNA tail results from incomplete DNA synthesis at a collapsed replication fork or upon fork reversal [48], in which case MRN mediated resection would not be required for the loading of SHLD-CST on PARPi induced DSBs.



*The 53BP1 pathway at DSB ends – competition for the overhang?*

Although the identification of SHLD and CST may be incorporated in the accepted model of end protection by the 53BP1 pathway, a number of implications on DSB end metabolism remain obscure. Both SHLD and CST are RPA like complexes that bind ssDNA through their OB-fold domains [8, 49]. However, whilst RPA binding to ssDNA is an intermediate step in HR, SHLD-CST binding facilitates repair via c-NHEJ. How the biochemical properties of SHLD-CST binding to ssDNA compare to RPA remains to be investigated. The affinity of the heterotrimeric RPA complex for ssDNA has been shown to depend on its conformation and on the OB-fold domains that make contact with ssDNA (reviewed in [50], [51]). In its low-affinity mode, RPA binds 8-10 nucleotides of ssDNA, while 30-nts are bound per complex in its high-affinity mode. As such, RPA may progressively 'unroll' on a short ssDNA overhang. This bimodal distribution of RPA is believed to be important for the regulation of its interacting proteins (reviewed in [52]). While it is currently unknown if SHLD also adopts different conformations, this has been suggested in the literature for CST [53]. Furthermore, it has been shown that 18-nt of

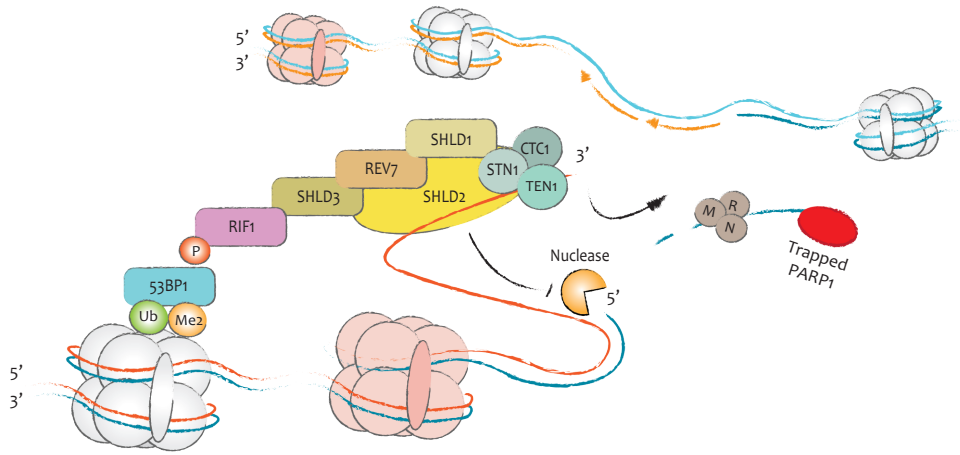


**FIGURE 2 |** A speculative model in which the MRN-mediated exonycleolytic activity followed by 3' to 5' resection towards the DSB end removes a trapped PARP-DNA complex. SHLD-CST is subsequently loaded on the 3' ssDNA tail to occlude nuclease access and thereby protect the DSB end from extensive resection.

G-rich ssDNA is sufficient for CST binding, although this sequence specificity may be diminished for longer stretches of ssDNA [49, 53, 54]. It has also been shown that CST is stabilized at the ssDNA-dsDNA junction and that a 10-nt overhang is sufficient for binding [55]. Notably, these ssDNA lengths are in the same ballpark as the ~20-35nt ssDNA overhang that is initially generated by MRE11 mediated endonucleolytic resection on blocked dsDNA ends [43, 56]. Thus, although limited, the insights into the biochemical properties of SHLD-CST binding to ssDNA may be consistent with a proposed role of the 53BP1 pathway downstream of MRN. It would be useful to learn more about the characteristics of the overhangs that engage SHLD and CST, which may help understand how the overhang is generated. Such data may also provide insights into whether SHLD is exchanged for CST, or if both complexes coexist on ssDNA overhangs.

Regardless of its generation, it seems evident that the ssDNA overhang can in principle be bound by both RPA and SHLD-CST, implying a direct competition between these protein complexes for ssDNA binding. The relatively low abundance of SHLD and CST suggests that these complexes require the 53BP1 pathway for efficient loading on resected DNA. Thereby, active recruitment further distinguishes SHLD-CST from RPA. Upon DNA binding, it is possible that the subsequent antagonizing effect of SHLD-CST on end resection is explained solely by steric hindrance of resection nucleases. However, additional consequences on DNA repair cannot be excluded as ssDNA-RPA has been shown to activate ATR signaling [57, 58]; terminate EXO1 mediated resection [59]; and is exchanged for RAD51 during HR [60, 61]. Future work is required to investigate if these processes are affected by SHLD-CST coated ssDNA.

*The 53BP1 pathway at DSB ends – is POLA involved?*

It is noteworthy that while SHLD and CST could be expected to act redundantly in the processes described above, depletion of either SHLD or CST alone is sufficient to inactivate the 53BP1 pathway [7-12]. This dual requirement for the ssDNA binding complexes SHLD and CST in modulating end protection is enigmatic. The finding that CST is recruited to DSB ends by SHLD [12] suggests that CST might perform a function that cannot be executed by SHLD alone. The previously described activities of CST in other DNA contexts provide food for thought to speculate on such function. The preference of CST for binding G-rich DNA and its demonstrated ability to unfold G-quadruplexes [55, 62] raise the possibility that CST aids SHLD specifically in the protection of G-rich ssDNA. This would imply that the requirement for CST is sequence dependent, providing a testable hypothesis. Alternatively, the recognition of the ssDNA-dsDNA junction by CST [55] may aid in coordinating end capture or in providing a tighter restriction against resection nucleases. However, a more exciting hypothesis stems from the described role of CST at resected telomeres, where it recruits POLA to mediate a fill-in reaction as a way to buffer resection length and thereby limit telomere erosion [63]. This would extend the analogy between end protection at telomeres by shelterin and at DSB ends by shieldin [8]. Interestingly, resection at telomeres is mediated by EXO1 [63], demonstrating that POLA has the capacity to counteract substantially resected telomeric ends. Although the evidence that CST mediates a similar reaction at resected DSB ends is limited [12], it may provide the most appealing explanation for the requirement of CST beyond SHLD (Fig. 3). Indeed, the ability of CST to bind ssDNA substrates dynamically and to recognize the ssDNA-dsDNA junction seems most appropriate for mediating a fill-in reaction via sequential extension [55].

The prospect of active fill-in at resected DSB ends certainly warrants further investigation as it could invoke a paradigm shift. Depending on the processivity – i.e. whether fill-in is limited to the terminal nucleotides or if it may proceed over longer distances – initiation of long-range end resection might be a reversible process and commitment to HR repair might be revoked by active fill-in. Similarly, it raises the possibility that the length of the 3' overhang may be buffered by POLA mediated fill-in synthesis during end resection. Capturing this process *in vivo*, however, is challenging due to the error-free nature of fill-in. Hence, differentiation between a sequence that has never been resected versus a sequence that has been resected and subsequently filled-in is problematic, at least on the sequence level. The essential role of POLA in lagging-strand replication further complicates experiments directed at demonstrating a role of POLA at DSB ends. Fiddling with POLA activity may not be tolerated by cells, and care must be taken to separate fill-in synthesis during DSB repair from fill-in during replication. Conducting experiments in arrested cells, or using mutants that impair POLA binding to CST [64] may provide a solution to circumvent these issues.

If fill-in of resected DSB ends can be unequivocally demonstrated, the next question that arises is how these filled-in ends are subsequently processed. It seems unlikely that the RNA primer from which a fill-in reaction is initiated invariably binds the terminus of the ssDNA overhang; hence the fill-in reaction possibly leaves behind an ssDNA overhang that is too short to initiate a new round of fill-in. Furthermore, the faith of the RNA primer requires further study. Since the majority of 53BP1 driven DSB substrates require LIG4 for their repair, it is tempting to believe that LIG4 is ultimately responsible for the ligation of filled-in ends. This is compatible with the observation that LIG4 retains activity on DSB ends with short overhangs [32]. A possible physiological role for 53BP1-RIF1-SHLD-CST-POLA could thus be to allow the removal of end blocking lesions through MRN mediated resection, whilst protecting these ends from engaging long-range resection and simultaneously mediating fill-in synthesis to channel repair through c-NHEJ. Future studies are required to determine if fill-in indeed occurs at DSBs, which DSB substrate is amenable for fill-in, and how a filled-in DSB end is subsequently processed.

*The 53BP1 pathway at DSB ends – convergence with other DSB repair pathways on ssDNA?*

The finding that the 53BP1 pathway acts on DSB substrates that entail an ssDNA tail also raises new questions for the regulation of DSB repair pathway choice. DSB repair pathway choice is commonly referred to as the choice between NHEJ and HR, depending on whether end resection is initiated or not. The finding that a short ssDNA tail may be the actual substrate that is protected from extensive resection by the 53BP1 pathway raises the possibility that this pathway competes with polymerase theta (POLQ).

POLQ was recently described to join 3' ssDNA overhangs that share ≥2 bp homology at or near the break end, by using these overhangs as a template for DNA synthesis (extensively reviewed in [65]). This activity is prone to generate deletions with microhomology and templated insertions, making TMEJ an intrinsically mutagenic repair pathway. The possibility that 53BP1 and POLQ act on the same DSB substrate is supported by the observation that 53BP1 and POLQ are synthetic lethal [30]. Moreover, c-NHEJ and TMEJ function synergistically in response to IR [66]. Thus, c-NHEJ and TMEJ are each exclusively involved in the repair of a subset of DSBs, while acting redundantly on others. This may be interpreted such that POLQ is able to take over the repair of at least a subset of DSB ends that would otherwise have been repaired via the 53BP1 pathway. Since repair via the 53BP1 pathway appears to ultimately require LIG4 rather than POLQ for completion, this may implicate that the 53BP1 pathway somehow obstructs the engagement of POLQ. How this may be achieved, however, is puzzling, since the helicase domain of POLQ has been shown to facilitate the removal of RPA from ssDNA overhangs [67] and thus may similarly be able to strip off SHLD-CST. Perhaps the anchor to 53BP1-RIF1 sufficiently enforces SHLD-CST protection of ssDNA such that POLQ is disfavored in the presence of 53BP1.

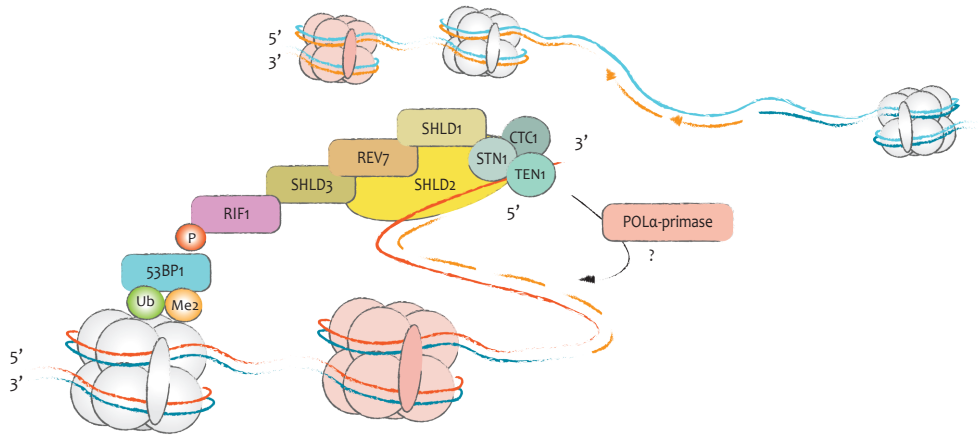


**Figure 3 |** POLA mediated fill-in synthesis may further reduce the length of the 3' ssDNA tail to allow ligation via LIG4.

Interestingly, TMEJ has also been shown to be essential for the survival of cells in which HR is compromised [68, 69]. The finding that BRCA1 deficient tumors are characterized by genetic scars that are typical for TMEJ activity provides a solid case for its significance [70]. It is hypothesized that TMEJ is required to rescue cells from toxic DSB repair intermediates that are generated when HR is initiated but cannot be completed [65, 71]. Similar to the 53BP1 pathway, this implies that the HR pathway may somehow preclude POLQ from acting on its substrate.

Together, these studies hint towards a potential competition between TMEJ, c-NHEJ and HR. How the engagement of these pathways is coordinated remains to be investigated.

*The 53BP1 pathway at DSB ends – an inconvenient observation*

Throughout this thesis, 3D organoid technology was used to evaluate the effect of genetic depletion of the 53BP1 pathway on PARPi response in BRCA1 deficient mouse mammary tumors. Hereby, previous data on 53BP1 and REV7/MAD2L2 were confirmed, and it was demonstrated for the first time that loss of RIF1, DYNLL1, SHLD or CST drives PARPi resistance *in vivo* (this thesis). The tumor data for 53BP1, RIF1 and REV7/MAD2L2 were generated in the same experiment and therefore resemble a direct isogenic comparison (Chapter 3 - addendum). Interestingly, genetic depletion of different 53BP1 pathway members showed different potencies in driving PARPi resistance. Although a technical explanation cannot be excluded, it is peculiar that the time before resistant tumors emerged matched the hierarchy of the 53BP1 pathway. Indeed, PARPi treated mice bearing 53BP1 depleted tumors had a median survival of only 8 days, which did not differ significantly from tumor-bearing mice that were left untreated. PARPi treated mice bearing RIF1 depleted tumors had a significantly longer median survival of

25 days, whereas PARPi treated mice with REV7/MAD2l2 depleted tumors had an even longer median survival of approximately 41 days. Although it remains unclear how these differences should be interpreted, it could be that loss of these downstream factors is not fully epistatic with loss of 53BP1. Perhaps the physical presence of 53BP1 also interferes with HR directly, even when the downstream pathway members are depleted. Alternatively, 53BP1 might perform RIF1 independent functions that influence PARPi toxicity. This is not unlikely, since 53BP1 has been described to enhance chromatin mobility [33, 34] and additional effector proteins have been identified, including PTIP [18, 72].

PTIP forms the other known branch of the 53BP1 pathway [18, 72]. Even though PTIP has been implicated in the 53BP1 pathway for several years and is also thought to stimulate DSB end protection, its precise role is still poorly understood. Moreover, besides its role in DSB end protection, PTIP has been shown to regulate replication fork (RF) stability in an MLL3/4-CHD4-MRE11 dependent manner [73]. In the model proposed by Chaudhuri et al., the collision of a replication fork with trapped PARP-DNA causes fork stalling. Stalled forks are normally protected from degradation by BRCA1/2 [74, 75], but become substrates for MRE11 mediated degradation of the nascent strands in the absence of BRCA1/2. Loss of PTIP was shown to rescue RFs from MRE11 mediated degradation by preventing MRE11 recruitment to stalled RFs [73]. RF protection by PTIP loss was sufficient to induce resistance to therapeutics that cause RF stalling (such as PARPi) in BRCA1 /2 deficient cells, without restoring HR. In light of the observation that 53BP1 depletion may be more potent *in vivo* than depletion of the downstream factors of the RIF1 branch, it would be interesting to test if dual depletion of the RIF1- and PTIP axis recapitulates the full extent of 53BP1 depletion on PARPi resistance *in vivo*. Admittedly, the plethora of genome-wide CRISPR screens for PARPi resistance adequately picked up the RNF8-RNF168-53BP1-RIF1-REV7-SHLD-CST pathway [8, 9, 11], while the PTIP axis was conspicuous by its absence. Direct testing of two PTIP targeting sgRNAs in KB1P and KB2P mammary tumor cell lines also did not show a notable effect on PARPi sensitivity *in vitro*, although it must be noted that these sgRNAs were not validated for protein knockout (data not shown). Certainly, the extent to which PTIP loss and/or replication fork protection contributes to PARPi resistance requires further study.

Whilst the components of the 53BP1 pathway seem set with the identification of the SHLD and CST complexes, it is clear that several questions regarding its spatiotemporal regulation and its precise substrate remain to be addressed.

## On the Origin of PARPi Induced DSBs

The prevailing model for PARPi induced toxicity holds that DSBs are formed when replication forks collide with trapped PARP1-DNA complexes, resulting in replication fork stalling and collapse. However, the model of fork stalling and fork collapse has been challenged by Maya-Mendoza et al., who rather proposed that PARPi accelerates

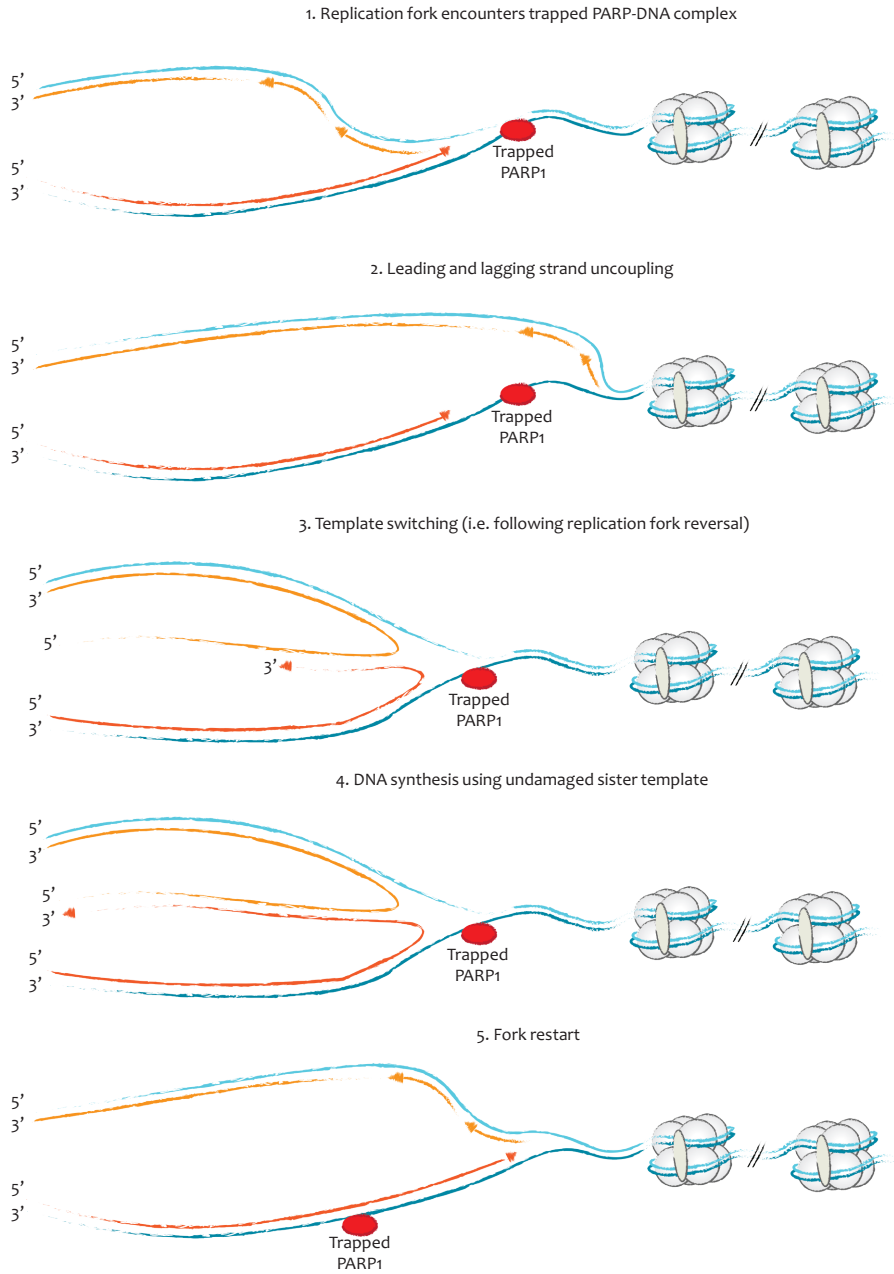


**FIGURE 4 |** a hypothetical model for template switching to continue replication upon collision of a replication fork with a PARP-DNA complex. Uncoupling of the leading and the lagging strand followed by fork stabilization and template switching might allow the use of the nascent strand as a template for replication.

replication fork progression and that cells accumulate ssDNA gaps as a result [76]. Unfortunately, the authors have not attempted to unify their model with existing data on the 53BP1-BRCA1 interaction. Indeed, the strong influence of the 53BP1 pathway on the PARPi response in BRCA1 deficient cells is adequately explained by the formation of PARPi induced DSBs, presumably resulting from fork collapse. Moreover, PARPi resistance in BRCA1/2 deficient cells due to restoration of replication fork stability was explained by the generation of a reversed fork intermediate upon PARPi treatment [73]. If PARPi treatment does not lead to fork reversal and/or collapse [76], alternative explanations for the 53BP1-BRCA1 interaction on PARPi sensitivity must be found. Moreover, the possibility that ssDNA gaps induce fork collapse in the next round of replication has not been investigated, which would only be a minor deviation from the accepted model. The study is also subject to certain limitations, as the experiments were performed under high concentrations of PARPi (10 µM), while PARPi toxicity in BRCA1/2 deficient cells is already observed in the nM range – a dose at which Maya-Mendoza et al. describe no effect on fork speed [76]. Moreover, a 10 µM concentration of the PARPi olaparib was previously shown to release the PARP mediated break on RECQ1 mediated fork restart, providing an alternative explanation for the finding that PARPi accelerates fork progression [48, 77]. Lastly, Maya-Mandoza et al. describe that accelerated elongation was dependent on the p21-p53 axis, which is known to be impaired in the majority of BRCA1/2 deficient tumors. Therefore, the relevance of these findings for the clinical utility of PARPi requires further study.

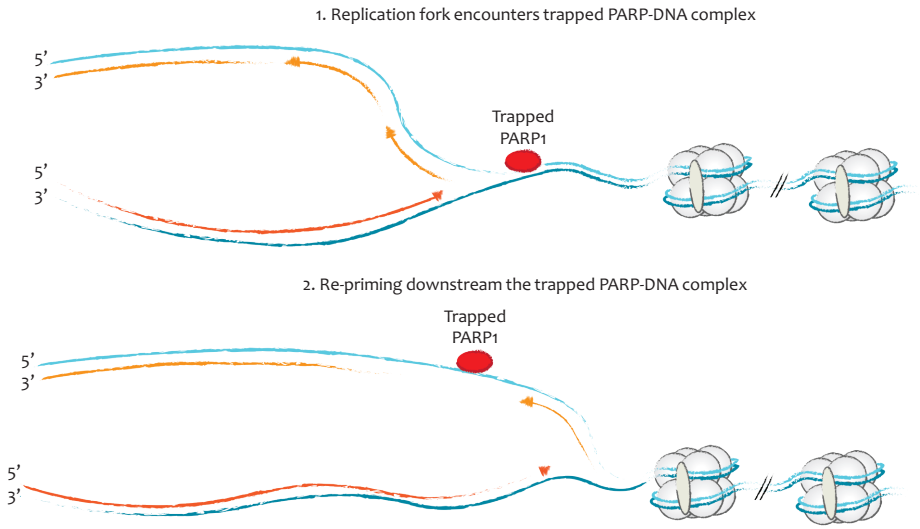


**FIGURE 5 |** a hypothetical model for re-priming downstream a trapped PARP-DNA complex as a way to avoid replication fork collapse.

The previous section illustrates the poor understanding of the cellular response that is initiated when a replication fork encounters a trapped PARP1-DNA complex. Besides the possibility of fork reversal and/or collapse, it is unclear if the replication machinery may be able to replicate past PARP1-DNA complexes by the use of template switching (Fig. 4). Collision of an ongoing replication fork with a PARP1-DNA complex might result in leading-lagging strand uncoupling. Thereby, replication might continue on the undamaged strand. Although not absolutely required for the model, the exposure of ssDNA on the damaged strand could trigger fork reversal to stabilize the replication fork. DNA synthesis might subsequently continue using the nascent strand as a template, after which the fork could be restarted to proceed with replication. This process might also explain how increased fork stabilization leads to chemoresistance in HR deficient cells. Similarly, it would be interesting to investigate if re-priming downstream of PARP1-DNA complexes occurs as it would allow replication to proceed at the expense of ssDNA gaps (Fig. 5). Interestingly, replication fork uncoupling and re-priming would both lead to increased levels of ssDNA gaps [76]. At the same time, it is questionable if such strategies are viable in the long-term since the PARP1-DNA complex is not removed and thus continues to pose a barrier for transcription and replication.

## Checking in on PARPi

In the last decade, a number of mechanisms have been described that may affect the responsiveness of tumors to PARPi treatment. Functional restoration of the genetic alteration underlying the HR defect and PARPi sensitivity is a major resistance mechanism and remains the only clinically validated mechanism to date (reviewed in [6]). A surprising number of ways by which this is achieved in tumor cells have been described (reviewed in Chapter 2 [78]).

In case functional restoration of BRCA1 is not possible (e.g. due to large, irreversible deletions as is the case in the BRCA1 deficient mouse tumor model that was used throughout this thesis), disruption of the 53BP1 pathway appears to be a dominant route to PARPi resistance. The absence of clinical data has thus far precluded full validation of the mechanisms described in this thesis, although the existence of disruptive mutations in 53BP1 and REV7/MAD2L2 has been identified in breast explants [79]. These explants were derived from BRCA1 germline mutation carriers and were refractory to PARPi treatment when transplanted in mice; however, a causal relation cannot be confirmed.

Restoration of HR via BRCA1/2 re-expression or disruption of the 53BP1 pathway is not the only route to resistance that has been described. PARPi resistance may also occur independently of HR restoration, for example due to replication fork protection [73]. Additionally, certain PARP inhibitors, such as olaparib (Lynparza) were found to be a substrate for the drug efflux-transporter MDR1 [80]. Overexpression of MDR1 reduces intracellular levels of these drugs, thereby alleviating toxicity. Although this was found to be a prominent resistance mechanism in mouse models, clinical

indications of this mechanism occurring in patients remain limited. The most compelling evidence comes from the finding that approximately 8% of chemoresistant high-grade serous carcinoma had upregulated MDR1 [81, 82]. However, it is not known if this was causal for the lack of response, nor that MDR1 was specifically upregulated by PARPi treatment since these tumors were heavily pre-treated. Finally, the drug target of PARPi may also be modulated. Indeed, it was shown that PARP1 depletion alleviates PARPi toxicity in wildtype and BRCA1 deficient cells [83, 84]. PARPi cytotoxicity can also be relieved by depleting PAR glycohydrolase (PARG) [85], the enzyme responsible for degrading PAR chains. Hereby, the residual catalytic activity of PARP1 is thought to be sufficient to mediate the recruitment of downstream DDR factors and to mediate its release from the DNA in the presence of PARPi. Collectively, these and future studies will provide a 'roadmap' of routes that tumors follow to alleviate PARPi toxicity. The next step will be to capitalize on this knowledge by identifying new treatment options for resistant tumors.

*From bench to bedside*

Since resistance mechanisms evolve to provide a survival advantage in the presence of therapy pressure, this may be accompanied by a fitness cost in other settings [86, 87]. The existence of such an acquired vulnerability or Achilles' heel in PARPi resistant clones that have inactivated the 53BP1 pathway is described in Chapter 7 [9, 46]. Resistance to PARPi came at the cost of an enhanced sensitivity to IR, which could be exploited to deplete these PARPi resistant cells from mouse tumors *in vivo* (Fig. 6) [46]. Thus, radiotherapy may be effective in such cases. Similar studies need to be employed for each known mechanism of resistance in order to provide actionable solutions to combat resistant tumors.

The identification of new vulnerabilities in resistant tumors also advocates that the molecular cause of resistance must be evaluated in the clinic. Hereto, carefully designed studies are required that directly compare each resistant tumor to its matched treatment-naïve counterpart. Ideally, these matched tumors are compared on the genetic level to pinpoint the alteration(s) that may be causal for resistance. Hopefully, these and equivalent studies will provide sufficient incentive to address the lack of clinical validation in the future.

*Hypothesis-driven questions for the clinic*

Several propositions follow from the resistance mechanisms that have been described. Certain HR deficient/PARPi sensitive genetic backgrounds might be more prone to developing resistance than others. This can be deduced from the number of resistance mechanisms that accompany each gene defect. For example, loss of the 53BP1 pathway renders BRCA1 deficient cells resistant to PARPi, thus providing a number of genes that

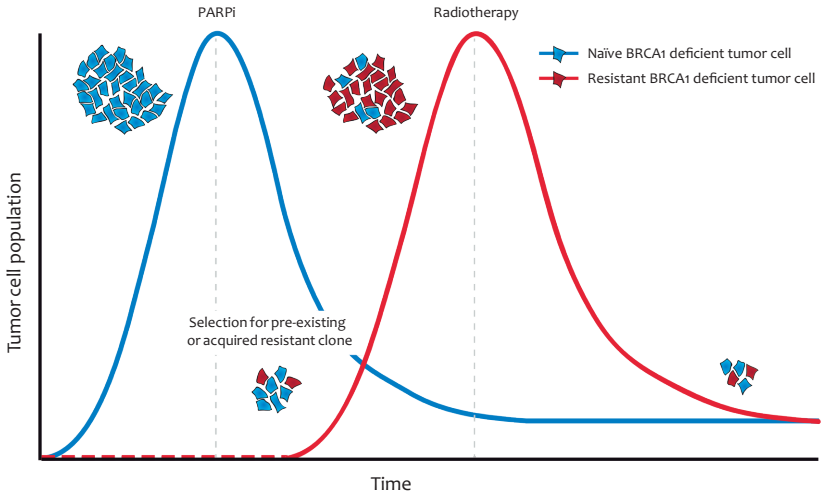


**FIGURE 6 |** Certain resistance mechanisms might expose a new treatment vulnerability which can be therapeutically exploited. For example, loss of the 53BP1 pathway renders BRCA1 deficient cells resistant to PARPi leading to their enrichment. These cells may subsequently be depleted from the population by radiotherapy.

may induce synthetic viability when inactivated. However, these same genes have no effect on BRCA2 deficient cells. Therefore, BRCA1 deficient tumors might have more options to acquire resistance and therefore might show a less durable response to PARPi. Similarly, ATM mutations induce PARPi sensitivity, but this is alleviated when members of the c-NHEJ pathway or the BRCA1-A complex are inactivated [88]. This shows that resistance mechanisms may vary per genetic background. Therefore, the ongoing efforts to evaluate PARPi efficacy in other HR deficient backgrounds should be met with, ideally saturating, screens to pinpoint the genetic contexts that rescue the synthetic lethal interaction with PARPi. This becomes more complex if an HR defect can be implied on the basis of a BRCAness "scar", while no causal gene disruption can be identified [70]. Thus, besides testing whether such tumors are responsive to PARPi treatment, it will be pivotal to eventually pinpoint the underlying cause of the HR defect and to investigate which mechanisms may rescue its SL interaction with PARPi.

Not only alterations in different HR genes might affect PARPi efficacy; different mutations in the same HR gene may also affect the outcome. Mutations in essential domains could be expected to carry a lower potential for functional restoration by secondary mutations, since large in-frame deletions would render the protein non-functional and thereby preclude these events from driving resistance. Although it remains speculative if specific mutations indeed influence clinical outcome, it has been described that BRCA1null alleles cause embryonic lethality at E7.5-9.5, whilst C-terminal truncating BRCA1 mutations show delayed lethality at E9.5-E10.5 [89]. Similarly, it has been shown that a subset of inactivating mutations in BRCA1 or BRCA2 can be

by-passed with splice-variants that possess hypomorphic activity [90-93]. Inactivating events that cause synthetic viability may also depend on the expression of such allelic variants. This is most evident from PARP1 inactivation as a means to alleviate the toxicity by PARP trapping, which is tolerated if cells possess residual BRCA1 activity [84]. A dependency on BRCA1-Δ11q expression was recently also described for 53BP1 pathway inactivation [94]. However, this finding warrants further study as 53BP1 pathway inactivation potently induced resistance in the KB1P mouse model, which develops mammary tumors that lack Brca1 exons 5-13 and do not express BRCA1-Δ11q [8, 11, 22, 23, 47]. Regardless, the existence of allelic variants must be considered if we are to fully understand the genetic network that determines PARPi sensitivity.

*An alternative strategy to enhance PARPi efficacy*

An interesting new angle to PARPi was provided by the recent finding that BRCA1/2 deficient tumors are prone to activating the cGAS-STING pathway [95, 96]. This pathway normally functions as an antiviral defense mechanism by activating the immune system in response to cytosolic dsDNA of viral origin [97, 98]. It was found that this pathway is also triggered by cytoplasmic dsDNA arising from faulty mitosis, which may be enhanced in HR deficient tumors due to their genomic instability [95]. The use of PD-L1 and CTLA4 immune-checkpoint inhibitors (ICI) might work synergistically in this context by unleashing cytotoxic T-cells to boost immune cell activity against tumor cells. Not mutually exclusive, tumors with unstable genomes might be more likely to create neo-antigens, which could subsequently elicit an immune response. This may be boosted further by treatment, and PARPi seem ideal for this task as they have few side effects and are likely to be more tumor-specific than conventional chemotherapy [99-101]. This concept is supported by the observation that PD-1/PD-L1 activation limits the activity of PARPi in HR deficient mouse tumors, which can be diminished by co-administration of a PD-L1 inhibitor with PARPi [100]. Furthermore, preclinical work using the KB1P transplantation model has shown that PARPi treatment enhances the activation of the cGAS/STING pathway, triggering cytotoxic CD8+ T-cell recruitment and an antitumor immune response [101]. It was further demonstrated that this response was more pronounced in BRCA deficient cells compared to BRCA proficient cells, which is significant from a translational perspective. These prospects have drawn PARPi therapy closer to the field of immunotherapy, which has already achieved promising clinical responses for several tumor types [102]. Indeed, the clinical utility of combinations of PARPi with ICI is currently being investigated in several clinical trials [103]. The notion that the immune system may eradicate genomically unstable tumor cells also predicts that immune-modulation may play a role in PARPi resistance and this certainly calls for further study. Here, genetically engineered mouse models may provide a shortcut to test the role of the immune system in modulating PARPi response. In this respect, it is

intriguing that a matched treatment-naïve and treatment-resistant KB1P tumor-derived organoid model did not recapitulate PARPi resistance *in vitro* but maintained resistance *in vivo*, indicating that PARPi resistance in this tumor may be driven by a cancer cell-extrinsic mechanism (Chapter 3). Although many explanations for this discrepancy could be pursued, an obvious difference between *in vitro* and *in vivo* culture systems is the lack of an immune system. Perhaps it is not that the resistant organoid model does not recapitulate its resistance *in vitro*, but that the naïve organoid model does not recapitulate its sensitivity *in vitro*. Perhaps *in vivo* the naïve tumor cells are efficiently eradicated by the immune system upon PARPi treatment. A straightforward experiment would be to test the response of these matched models in NMRI/Nude mice, which lack a cytotoxic T-cell response. If this hypothesis were true one would predict to observe no differences in PARPi response between this naïve and resistance organoid model in athymic NMRI/Nude mice. In line with this thought, Pantelidou et al. indeed observed an enhanced antitumor response upon PARPi treatment when KB1P tumor pieces were engrafted in syngeneic FVB mice compared to immunodeficient SCID mice [101].

***Although it is clear that PARPi may not immediately put the tumor in checkmate, the game of chess is far from over.***

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