# Resistance to PARP inhibition by DNA damage response alterations in BRCA1/2-deficient tumors

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# CHAPTER 8

## General Conclusions and Discussion

Resistance to PARP inhibitors (PARPi) in cancers lacking BRCA1/2 activity is the major focus of research described in this thesis. Heterogenous responses to PARPi were observed in patients carrying *BRCA1/2* mutations already in phase 1 clinical trials more than a decade ago[1]. Nowadays, despite the significant progress in clinical application of PARPi and the regulatory approval of 4 different PARPi compounds[2], drug resistance still presents a major limitation of this therapeutic approach. It is therefore crucial to understand the molecular mechanisms underlying PARPi therapy escape in order to reduce the cancer mortality of patients with tumors in which BRCA1/2 is dysfunctional.

The use of genetically engineered mouse models (GEMMs) of BRCA1/2-associated breast cancer in this work has allowed us to model PARPi resistance *in vivo* in well-defined genetic contexts. By combining high-throughput genetic screens, multiple omics analyses and functional assays, we identified several factors of PARPi resistance and explained their role in therapy failure. Moreover, we established a new tumor-derived organoid system that enables robust *in vivo* validation of putative drug resistance factors. Finally, work described in this thesis has advanced our understanding of basic biological processes involved in DNA damage signaling and repair.
In this final chapter, I wish to briefly discuss what we learnt about PARPi resistance and highlight some of the key outstanding questions that should be addressed in future studies.

A first major conclusion of this work is that tumors possess a substantial capacity to develop PARPi resistance. In fact, all of the *Brca1*$^{-/-}$;*Trp53*$^{-/-}$ and *Brca2*$^{-/-}$;*Trp53*$^{-/-}$ tumors generated in our studies eventually became resistant, despite the initial hypersensitivity to PARPi[3–5] (**Chapter 7, Figure 2c**). Having these two GEM models was critical for this work, as it allowed us to identify distinct mechanisms of resistance, operating on two opposite arms of the synthetic lethal interaction between (1) PARP inhibition and (2) deficiency in homologous recombination (HR).
Our systematic analysis of RAD51 irradiation-induced foci (IRIF) as a surrogate for HR activity revealed a high prevalence of HR recovery in PARPi-resistant *Brca1*$^{-/-}$;*Trp53*$^{-/-}$ tumors (**Chapter 5, Figure 1b**). HR restoration was largely associated with loss of 53BP1 expression (30% of all resistant cases and 47% of all HR-positive tumors, **Chapter 5, Figure 2a and 4d-e**), consistent with the antagonistic roles of BRCA1 and 53BP1 in the regulation of DNA end resection, and consequently, in DNA double-strand break (DSB) repair pathway choice[6,7]. Depletion of 53BP1 was not the only mechanism of HR activation in our model as roughly half of the HR positive tumors remained capable of forming 53BP1 IRIF. Indeed, work from our laboratory[8] and others[9] has shown that DSB resection can be achieved in BRCA1-deficient cells through the loss of REV7 protein, that acts downstream of 53BP1 and its interacting partner, RIF1[10–14] (**Chapter 4**). Follow-up studies demonstrated that

REV7 forms a stable complex called shieldin with three hitherto uncharacterized proteins: C20orf196 (SHLD1), FAM35A (SHLD2), and CT-534A2.2 (SHLD3)[15–18]. The discovery of the 53BP1-RIF1-shieldin pathway finally provided a plausible mechanistic explanation of how broken DNA ends are protected from the nucleolytic degradation: 53BP1 accumulates at the damaged sites and recruits RIF1 in an ATM-dependent manner, which localizes the shieldin complex to the DSBs. SHLD3 binds to RIF1 and REV7, which in turn interacts with SHLD2. Owing to the presence of three tandem OB-folds, SHLD2 possess single-stranded DNA (ssDNA)-binding activity and together with SHLD1 provides a ssDNA-binding module within the complex[19] (**Chapter 2, Figure 2**). Consistent with this model, loss of any of the 53BP1-RIF1-shieldin components in BRCA1-deficient cells rescues the resection of DNA overhangs and promotes homology-directed repair. Importantly, protein expression data and multi-omics analysis of PARPi-resistant *Brca1*$^{-/-}$*;p53*$^{-/-}$ tumors yielded several factors of the 53BP1-RIF1-shieldin pathway, including RIF1, REV7 and SHLD2, as putative mediators of PARPi resistance in our model (**Chapter 4** and **5**).

Despite the emerging view of the shieldin complex as an ultimate effector of 53BP1-mediated end protection, there are several questions that still need to be answered.

Firstly, in the proposed model SHLD2, a main scaffold for the complex, directly associates with ssDNA, suggesting that the pathway acts after the initiation of DNA resection. This is somewhat counterintuitive given the role of the complex in blocking DNA processing. We have recently reported that loss of the members of the CTC1-STN1-TEN1 (CST) complex partially restores DNA resection and renders BRCA1-deficient cells resistant to PARPi[20]. It has also been shown that during telomere replication CST interacts with the telomere end-protecting complex shelterin and antagonizes extensive DNA resection via polymerase alpha (POLA)-dependent fill-in DNA synthesis[21]. Whether CST collaborates with the shieldin complex in buffering end resection at non-telomeric DSBs in an analogous manner remains to be determined.

Secondly, several lines of research suggest that *Trp53bp1*, *Rif1* and the shieldin genes are not entirely epistatic with each other. Using our organoid model of BRCA1-deficient cancer we have demonstrated that genetic ablation of any of these factors confers PARPi resistance *in vivo*. However, PARPi resistance induced by the inactivation of 53BP1 was much more robust than upon depletion of *Shld1* or *Shld2* (PARPi survival: <20 days and >60 days of animals bearing *Brca1*$^{-/-}$*Tpr53*$^{-/-}$ tumors with genetic deletion of *Trp53bp1* and *Shld1/2*, respectively; **Chapter 3, Figure 4b** and **Figure 2f in**[17]), suggesting shieldin-independent contributions of 53BP1 to the PARPi response. Furthermore, not all of the anti-resection activities of 53BP1 engage the RIF1-shieldin axis. While the RIF1-shieldin pathway is required for the fusion of deprotected telomeres in cells deficient for the shelterin subunit TRF2[9,16,18,22], and class-switch recombination (CSR) in B cells[8,15], it does not participate in the 53BP1-mediated V(D)J recombination[15]. It is therefore possible, that RIF1-shieldin-independent 53BP1 function

in V(D)J could depend on its interaction with PTIP, a binding protein that contributes to the repair of DSBs formed in this process[23]. Like RIF1, depletion of PTIP rescued DNA end resection and suppressed the sensitivity of BRCA1-deficient cells to PARPi[24]. Because RIF1 and PTIP act independently of each other[24], PTIP could potentially explain the residual activity of 53BP1 observed in *Shld1/2*-depleted BRCA1-deficient tumors. What dictates the 53BP1-dependent end protection to be channeled into PTIP- or RIF1-mediated pathways and what impact these two branches have on the repair of pathological DSBs induced by PARPi therapy warrant further investigation.

Systematic analysis of RAD51 IRIF formation in our tumor models strongly suggest that HR restoration cannot be achieved in the absence of BRCA2 (**Chapter 5, Figure 1b**), indicating that BRCA2 – unlike BRCA1 – is indispensable for the HR-mediated repair. Thus, BRCA2-deficient tumors – unlike BRCA1-deficient tumors – cannot escape PARPi toxicity through the efficient repair of DSBs. Instead, we find protection of stalled replication forks (RFs) (**Chapter 6**) and partial restoration of PARP1 signaling via the loss of PARG (**Chapter 7**) as underlying causes of the PARPi resistant phenotype in BRCA2-deficient cells. These observations point at the mechanistic basis of cytotoxic effects of PARPi and reflect our incomplete understanding of what the primary PARPi lesions are and how they are resolved by the DNA damage machinery.
Since the publication of work from Ives Pommier's laboratory[25,26], the mechanism of action of PARPi has been reconsidered, and the toxicity of PARPi is now believed to be primarily associated with their ability to trap rather than inhibit PARP1/2. This hypothesis stems from the observation that deletion of *Parp1* in avian B lymphoblast DT40 cells, which naturally lack *Parp2*, completely abolishes PARPi sensitivity[25,26]. PARP trapping also explains why PARPi with equivalent abilities to catalytically inhibit PARP1/2 exhibit widely different cytotoxicities and why the application of these therapeutics extends beyond BRCA-mutated cancers. Finally, the dual mode of action of PARPi accounts for the synergistic effects of PARPi with other DNA damaging agents. This is best exemplified by the synergy with alkylating agents, which is driven by both, catalytic inhibition and PARP trapping, and with topoisomerase 1 (TOP1) inhibitors with which the synergy solely depends on the trapping potency[2,27]. While it is now generally accepted that PARPi act as DNA poisons, it is important to stress that *PARP1* depletion is not tolerated in BRCA1/2-deficient cells[28–30], hence the synthetic lethal interaction between *PARP1* and *BRCA1/2* occurs already at the genetic level, independently of PARP trapping. In line with this, our genetic screens aimed to identify PARPi-resistance factors yielded *Parp1* as a top hit specifically in BRCA-proficient *Trp53*$^{-/-}$ cancer cells but not in the *Brca2*$^{-/-}$;*Trp53*$^{-/-}$ model (**Chapter 7, Figure 1c-e**). Moreover, it has been shown that BRCA1/2-deficient cells are sensitive to the PARPi veliparib, which has very limited trapping activity[25]. Taken altogether, these results suggest that at least in the context of BRCA1/2-

deficiency PARP trapping is a relevant but not the only source of PARPi-mediated toxicity. The fact that we found PARG loss as a main mechanism of PARPi resistance in BRCA2-deficient tumors suggests that catalytic activity of PARP1 is important for the PARPi response. PARG is a major eraser of the poly(ADP-ribose) (PAR) polymers synthesized by PARP enzymes[31]. As we demonstrated in **Chapter 7**, inhibition or silencing of *Parg* expression leads to the restoration of PARylation despite the inhibitory effect of PARPi. These results implicate that PARPi are not fully penetrant and allow residual PARP1/2 activity. Our initial hypothesis was that upon PARPi treatment restored autoPARylation on chromatin-bound PARP1 overcomes PARP1 trapping due to the charge repulsion between PAR and DNA[31]. However, our analysis of PARP1 kinetics at damaged chromatin using laser micro-irradiation and PARP1 trapping assays suggested that rescued PARylation is insufficient to facilitate PARP1 release from the DNA in the presence of PARPi (**Chapter 7, Figure 4c-e**). Instead, we found that *Parg* depletion results in a significant accumulation of PARylated DNA-free PARP1, which has low affinity to chromatin, and overall lower levels of entrapped protein (**Chapter 7, Figure 4b**). Therefore, PARG depletion does not overcome PARP1 trapping *per se*, but limits the amount of PARP1-DNA complexes by retaining substantial population of PARP1 in a PARylated state. The mechanistical link between PARP1 trapping and its catalytic activity is further reinforced by a striking observation that PARGi alone did not enhance PARP1 dissociation from chromatin, but in fact resulted in its prolonged retention on DNA, to a greater extent than treatment with the PARPi olaparib (**Chapter 7, Figure 4e**). These results, consistent with other reports[32], challenge the dogma that the negative charge of PAR branches forces PARP1 off the chromatin[31]. Instead, it suggests that both PARylation as well as dePARylation are critical for the regulation of PARP1 association with DNA. Future studies should provide more detailed insights into this process, which likely involves other proteins[33,34]. This should further inform us about how PARP1-DNA lesions are processed in the presence of PARPi. At the moment little is known about what the primary sites of PARP1 trapping are, how much of the protein is trapped and how much is actually needed for the cytotoxic effects of PARPi. Detection of endogenous PARP-DNA lesions induced by PARPi is challenging, due to their low abundance and lack of sensitive probes. Trapping assays require the induction of acute base damage which is far from the physiological conditions. Importantly, a recent study suggested that, in addition to stochastic single-strand breaks (SSBs), unligated Okazaki fragments are likely DNA intermediates on which PARP1 gets trapped[35]. While the search for different DNA structures that contribute to PARP1 trapping continues, it would also be interesting to consider how PARP1-DNA complexes are sensed and processed. DNA poisoning by PARPi is reminiscent of the mode of action of TOP1 and TOP2 inhibitors, which damage cellular DNA by stabilizing toxic topoisomerase-DNA complexes[36,37]. Due to the high frequency of TOP1/TOP2 trapping by DNA distortions, cells have evolved specific protein-DNA crosslink (DPC) repair pathways to resolve such lesions.

TOP1/2 enzymes form covalent reaction intermediates with DNA, which can be targeted by specialized tyrosyl-DNA phosphodiesterases (TDP), TDP1 or TDP2, respectively[37]. DPCs can also arise spontaneously, as a result of cellular metabolism, and potentially encompass any nuclear protein, various DNA structures and crosslinking molecules. Because of their vast diversity, DPCs require less specific nuclease- and protease-based repair pathways that target DNA or protein component of the crosslink[38,39]. Although PARP1 does not form a covalent bond with DNA, trapping of PARP1 results in a DPC-like lesion, which owing to its bulky structure could interfere with nearly all chromatin transactions. It is therefore plausible that persistent PARP1-DNA complexes are substrates for DPC repair, but experimental data in support of this notion is lacking.

Apart from its impact on PARP1 trapping, loss of PARG resulted in partial restoration of PARP1 signaling. Rescued PARylation was sufficient to recruit the SSB repair scaffold protein, XRCC1, and resulted in alleviated levels of PARPi-induced DNA damage (**Chapter 7, Figure 5d-g**). However, PARP1 is not simply a SSB repair protein and in fact plays multifaceted roles in DNA damage response and chromatin remodeling. PARP1 activity is required to limit the rate of RF progression by modulating fork reversal and preventing premature restart of reversed RFs, which can lead to RF run-off and generation of DSB[40–43]. Inhibition of PARG completely restored controlled RF progression in PARPi-treated cells and prevented DSB formation (**Chapter 7, Figure 5a-c**). The importance of RF speed for PARPi response, first proposed by the Lopes laboratory[42], was further reinforced by a recent study[44]. On the other hand, our work[45] (described in **Chapter 6**) as well as work from other laboratories[46,47] suggested that PARPi treatment leads to the accumulation of stalled RFs that eventually collapse. It has also been shown that stalled RFs persist until mitosis causing mitotic DNA damage and apoptosis[47]. Up until now, several factors that protect stalled RFs from nucleolytic degradation have been implicated in PARPi resistance[46]. The opposite effects that PARPi seem to have on RF regulation, i.e. aberrant acceleration of RF speed and increased RFs stalling require further clarification. The interplay between PARP1 trapping and inhibition of catalytic activity, as well as the genetic context, fork structures, and the source of replication stress are all factors that should be taken into consideration. Additionally, PARP1 has been recently described to sense and mediate the repair of a fraction of unligated Okazaki fragments during replication[35]. It has been proposed that SSB repair proteins, including XRCC1 and LIG3 are recruited in a PAR-dependent manner and facilitate the repair of Okazaki fragments that escaped processing by the canonical FEN1 and LIG1-mediated pathway. It would be interesting to test the effect of PARG depletion on this process and its potential impact on PARPi treatment. So far it is difficult to assess the contribution of restored PARP1 signaling to PARPi response in the absence of PARG, mainly due to the fact that PARylation is intrinsically linked to PARP trapping. Rescued PARP1-mediated repair should prevent excessive accumulation of PARPi-induced DNA damage

and could potentially explain the trapping-independent hypersensitivity of BRCA-deficient cells to PARPi. One way to dissect the distinct PARPi mechanisms would be to compare the resistance profiles of tumors or cells treated with PARPi that exhibit markedly different trapping potencies but similar inhibitory abilities, such as veliparib and talazoparib. Such knowledge could provide rationale for novel drug combinations that would exploit PARP1 trapping or inhibition in specific genetic contexts.
Of note, PARG inhibition has been recently suggested to be synthetic lethal with the inhibition of DNA replication factors in selected ovarian cancer lines that are BRCA1/2-proficient[48]. This observation provides a potential strategy to target PARG-deficient PARPi-resistant tumours, which should acquire collateral sensitivity to CHEK1 inhibitors. The exact mechanism of this synthetic lethal interaction remains elusive, however, and the utility of CHEK1 inhibition in counteracting resistant disease  requires validation.

**Outlook**

Work presented in this thesis exploits, but does not exhaust the utility of our GEM models for BRCA1/2-associated cancer to study therapy resistance. Although we identified several novel mechanisms of PARPi resistance, there is a significant number of cases we cannot explain yet. For instance, loss of PARG was found in about 30% of PARPi-refractory *Brca2$^{-/-}$;Trp53$^{-/-}$* tumors (**Chapter 7, Figure 2f**), suggesting that other HR-independent mechanisms of resistance are operative in our model. The organoid protocol presented in **Chapter 3** should now enable the systematic analysis of RF protection and progression, as well as functional DNA damage assays in samples derived from the resistant tumors. Additionally, recent advances in screening technologies, including gain-of-function approaches should complement the existing toolbox and facilitate the identification of potential determinants of PARPi response.
As mentioned before, all of the tumors generated in this study eventually developed PARPi resistance. This is in stark contrast to the repeated sensitivity that these tumors show in response to the maximal tolerable dose (MTD) of various DNA crosslinking agents, including platinum-based drugs, melphalan, thiotepa and 4-OH-cyclophosphamide[49]. A likely reason for this difference is the fact that the PARPi we used (olaparib and AZD2461) do not induce as much DNA damage as the MTD of DNA crosslinkers[3]. In fact, we were already at the limit of avoiding drug precipitation with the formulation (100 mg/kg) that we used. Obviously, the advantage of these PARPi is the mild toxicity towards normal cells of the body, which is consistent with the concept of synthetic lethality in cancer therapy. It would be interesting to investigate the response to the PARPi talazoparib in these models, which has far greater potency to trap PARP onto the DNA[26]. Maybe more durable responses can be seen. However, substantial side effects of talazoparib are observed[50], which do not really fulfill the ambitious synthetic lethality concept. Similarly, it would be interesting to test

whether lower doses of platinum-based drugs would result in therapy resistance. Cisplatin is the most commonly used therapeutic agent for treating BRCA1/2-mutated cancers. Our data suggest that at least some of the PARPi resistant tumors show decreased sensitivity to cisplatin (**Chapter 6, Figure 4g** and **Chapter 3, Figure 3d,f**). Understanding resistance pathways that are common for PARPi and cisplatin should provide better guidance for patient selection and ultimately improve therapy outcome.

Finally, our organoid models could be utilized to develop approaches that would target resistant disease. We have generated a unique collection of tumors that acquired PARPi resistance *in vivo*, via distinct mechanisms in well-defined genetic contexts. In contrast to our experience with 2D cell lines, organoids can be easily derived from fresh or frozen cancer tissues and preserve the drug response profile of the original tumor (**Chapter 3, Figures 1a and 2**) . As demonstrated in **Chapter 3 and 7**, organoids can be easily genetically modified and used as a model for genetic screens. It should be feasible to optimize conditions for drop-out screens or drug screens, preferably *in vivo*, to identify new synthetic lethal dependencies that often arise at the cost of PARPi resistance[6].

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