Characterization of genetic modulators of PARP inhibitor sensitivity: molecular mechanisms and therapeutic implications

Ph.D. Thesis

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Introduction

Targeted cancer therapy aims to exploit the very same feature that gives the cancer cell an edge over its neighboring normal ones and turns that feature into Achilles' heel by designing a drug that would eliminate only those cells where this feature is present, thus, achieving precision therapy. This is reflected by a phenomenon called synthetic lethality, where two mutations with a viable phenotype for either of them alone lead to a lethal phenotype if they are present together. An ideal example of a synthetic lethal interaction exploited for personalized cancer therapy is the Poly ADP-ribose Polymerase (PARP) inhibitors (PARPi) and their synthetic lethality with Breast Cancer Susceptibility protein (BRCA).

BRCA1 and BRCA2 are tumor suppressors playing crucial roles in maintaining genomic integrity through their functions within the double-strand breaks (DSB) repair pathway homologous recombination (HR). Loss of either of them predisposes to cancer and impairs the HR pathway, making the cells reliant on the non-homologous

end joining (NHEJ) pathway for repairing DSB, which results in genomic instability.

PARPi refers to small molecule inhibitors that target mainly PARP1 as the catalyzer of the posttranslational modification ADP-ribosylation. Active PARP1 is fundamental to the DNA damage response and the repair of single-stranded DNA (ssDNA) breaks. When such lesions occur, PARP1 is recruited to the damage site, where it ADP-ribosylates itself and neighboring proteins, allowing for the release of PARP1 from the damage site and signaling to chromatin remodelers and repair factors to be recruited and execute faithful repair of the lesion.

PARPi not only block ADP-ribosylation, but also prolong the retention of PARP1 on the damage site causing a so-called “PARP trapping” phenomenon. Trapped PARP1 on the chromatin, when unresolved, renders the cell prone to DSB formation. In a BRCA-proficient cell, DSB will be repaired by HR preserving genomic integrity, while in a BRCA-deficient cell, DSB either accumulate or are repaired through NHEJ causing genomic instability and

eventually cell death. This mechanism underlies the synthetic lethality between PARPi and BRCA deficiency.

PARPi have been approved in the clinics for treatment of breast, ovarian, prostate and pancreatic cancers, with HR deficiency, accumulation of ssDNA breaks and prolonged PARP1 retention being the main hallmarks of PARPi sensitivity. Despite the promising outcomes of PARPi usage at the bedside, cases of innate or acquired resistance started to emerge. Several molecular mechanisms have been identified to underlie PARPi resistance, however, only the mechanisms arising from reactivation of HR either through reversion mutations, BRCA1 hypomorphs or rewiring of HR pathway due to loss of NHEJ factors have been observed clinically or in patient-derived xenograft (PDX) models, making them the subject of extensive research aiming to deepen our understanding of these mechanisms and how to overcome it.

Advances in genetic engineering and next-generation sequencing made it possible to conduct high throughput genetic screens with PARPi, which aimed to identify genetic alterations that regulate PARPi sensitivity. In a

genome wide CRISPR knockout screen, we identified the loss of the PAR-binding chromatin remodeler ALC1/CHD1L and the accessory subunits of DNA polymerase epsilon POLE3 and POLE4 to underlie sensitivity to the PARPi, Olaparib.

ALC1 is a chromatin remodeler activated by binding Poly(ADP-ribose) chains via its C-terminal Macro domain and catalyzes chromatin remodeling by nucleosome sliding through its ATPase domain. Clinically, ALC1 is an oncogene associated with poor prognosis and amplified in different solid tumors including hepatocellular carcinoma,

POLE3 and POLE4 are the accessory subunits of DNA polymerase epsilon (POLε), a protein complex responsible for executing replication of DNA leading strand. The importance of these accessory subunits in stabilizing the POLε complex is highlighted by the sensitivity to replication stress inducers upon their loss. POLE3 and POLE4 are crucial for ensuring symmetrical histone segregation upon DNA replication through their H3-H4 histone chaperon activity have a role in regulating heterochromatin silencing.

Aims and methods

By employing CRISPR gene editing technique, along with molecular, cellular, biochemical and immune-based assays performed in well-characterized cancer cell lines, this work aimed to advance our understanding of the molecular mechanisms underlying PARPi sensitivity and resistance from a fundamental science perspective, with a potential clinical impact in the context of diagnosis and/or drug target identification.

Specifically, we worked towards:

- Validating three of the top candidates identified in our CRISPR screen (ALC1, POLE3, POLE4) in the context of PARPi sensitivity.

- Characterizing the molecular mechanism by which PARPi sensitivity is induced when ALC1 and POLE4 are deleted.

- Investigating whether targeting these genes (ALC1 and POLE4) enhances the sensitivity of BRCA-deficient cells to PARPi and overcomes acquired resistance due to loss of 53BP1.

Results and discussion

To validate the results of the screen, we tested the sensitivity of ALC1 KO to PARPi. Indeed, ALC1 KO showed hypersensitivity to treatment with different PARPi, namely, Olaparib, Veliparib and Niraparib. This sensitivity was dependent on the presence of PARP1 as the main target of PARPi, as the sensitivity of ALC1 KO to Olaparib was reversed in ALC1/PARP1 double knockouts. Importantly, reintroducing wild-type ALC1 in ALC1 KO by ectopic overexpression rescued PARPi sensitivity. This was not the case when introducing mutant ALC1 that was unable to recruit to the damage site or and ATPase-dead ALC1 that cannot remodel the chromatin, indicating that fully functional ALC1 is the driver of PARPi resistance.

The role of ALC1 in the early steps of the DNA damage response prompted us to assess the levels of DSB in ALC1 KO cells upon PARPi treatment. Olaparib induced elevated levels of DSB in ALC1 KO compared to their parental wild-type reflected by stronger staining of the phosphorylated histone variant H2AX (γH2AX) as an indicator of DSB, as well as longer tail moment in an

alkaline comet assay. Mechanistically, this accumulation of DSB in ALC1 KO upon PARPi treatment is due to failure of these cells mobilize PARP1 from the sites of DNA damage. This enhanced levels of trapped PARP1 hinders the repair process leading to genomic instability and cell death in ALC1 KO cells.

Importantly, our data demonstrated that ALC1 is upstream of the repair pathway choice between HR and NHEJ, as loss of BRCA1, a key player in HR, or loss of 53BP1, a crucial factor in NHEJ, did not enhance ALC1 KO sensitivity to Olaparib. Crucially, concomitant loss of both BRCA1 and 53BP1, a common resistance mechanism to PARPi, did not rescue ALC1 KO sensitivity to Olaparib, indicating that ALC1 loss could bypass this acquired resistance.

Clinically, we showed that overexpression of ALC1, a common feature of certain solid tumors, induced PARPi resistance even in BRCA deficient background. An observation that holds significance for directing therapeutic intervention to such tumors.

Similarly, loss of the accessory subunits of POLε resulted in PARPi sensitivity, as both POLE3 KO and POLE4 KO were sensitive to different PARPi. Considering that POLE3 serves as a shared subunit between the POLε holoenzyme and the CHRAC chromatin remodeling complex, where it forms a heterodimer with POLE4 and CHRAC15, respectively. Knocking out POLE3 led to loss of both POLE4 and CHRAC15 proteins. To avoid potential confounding phenotypes stemming from the absence of both POLE3-POLE4 and POLE3-CHRAC15 heterodimers, we opted to further investigate the effects of PARPi treatment specifically in POLE4 KO cells.

Our experiments revealed that sensitivity of POLE4 KO to Olaparib is reversed upon loss of PARP1 and not PARP2. Nevertheless, live-cell imaging assay showed that PARP1 dynamics at the sites of laser-induced damage was not affected by loss of POLE4, indicating that the sensitivity of POLE4 KO to PARPi, although dependent on PARP1 presence, is not due to dysregulated PARP1 kinetics at DNA damage sites.

Since PARPi has been shown to increase replication fork speed, we assessed this speed in POLE4 KO with and without PARPi. DNA fiber assay illustrated mild decrease in replication fork speed in POLE4 KO which was further potentiated by PARPi treatment. suggesting accumulation of replicative defects in POLE4 KO. Indeed, native-BrdU staining assay to uncover ssDNA gaps demonstrated elevation of these lesions in POLE4 KO upon Olaparib treatment. Mechanistically, our data revealed that POLE4 played a role in ensuring seamless execution of post-replicative repair (PRR), as BrdU comet assay showed that in POLE4 KO cells, the levels of post-replicative gaps after hydroxyurea (HU) treatment were higher than in wild-type cells. This phenotype persisted even after 3h recovery time, due to impaired PRR in POLE4 KO.

These ssDNA gaps led to a replication stress phenotype in POLE4 KO as indicated by upregulation of the activity of the Ataxia-telangiectasia mutated and RAD3-related (ATR) kinase, and the phosphorylation of replication protein A (RPA) on serine 33 (S33) even without PARPi treatment. Phenotypes that got exacerbated by Olaparib.

Failure to mitigate replication stress in POLE4 KO upon PARPi caused a striking increase in the replication stress phenotype indicated by phosphorylation of RPA on Threonine 21 (T21), a signal of sever replication stress. This sever replication stress phenotype in POLE4 KO was rescued by reintroducing wild-type POLE4 by ectopic overexpression.

Considering that ATR is a member of the phosphatidylinositol 3-kinase-related kinase (PIKK) family, we assessed the activity of the other two kinases, DNA-PK and Ataxia-telangiectasia mutated (ATM). Our data showed overactivation of these kinases in POLE4 KO compared to wild-type upon Olaparib or ATR inhibitor (ATRi) treatment. This activity is reflected in their regulation of pRPA (T21) signal in POLE4 KO, as inhibiting ATM potentiated the signal while inhibiting DNA-PK suppressed it. Moreover, we demonstrated that PARPi treatment arrested POLE4 KO in G2 phase of the cell cycle due to ATR activity, and when ATR was inhibited along with Olaparib treatment, this led to

premature mitotic entry of POLE4 KO and replication catastrophe phenotype indicated by accumulation of DSB.

Importantly, we illustrated that sensitivity of POLE4 KO to PARPi is not due to HR deficiency, as POLE4 KO were able mount Rad51 foci upon PARPi treatment unlike BRCA1-depelted cells. Moreover, loss of both POLE4 and BRCA1 enhanced PARPi sensitivity compared to losing either of them. Crucially, simultaneous depletion of BRCA1 and 53BP1 did not rescue PARPi sensitivity in POLE4 KO cells as it did in wild-type cells, demonstrating that targeting POLE4 hold the potential of exacerbating PARPi-induced synthetic lethality in BRCA1-depleted cells and circumventing the acquired resistance mechanism triggered by HR rewiring upon loss of 53BP1 in BRCA1-compromised cells.

Finally, this work represents an effort to expand our understanding of targeted cancer therapy with PARPi by exploring how sensitivity to these drugs can be induced by targeting different genes. While both ALC1 KO and POLE4 KO demonstrate HR-independent PARPi sensitivity that could be helpful to evade acquired

resistance due to HR restoration, the underlying molecular mechanisms for this sensitivity are different. This offers clear insight into the intertwined nature of the mechanisms by which PARPi induce cytotoxicity. Yet, it projects the promising possibilities to expand the applicability of these inhibitors to uncharted territories in the context of cancer treatment.

List of Publications (MTMT Number: 10076533)

- Hasan Mamar*, Roberta Fajka-Boja*, Mónika Mórocz, Eva Pinto Jurado, Siham Zentout, Alexandra Mihuț, Anna Georgina Kopasz, Mihály Mérey, Rebecca Smith, Abhishek Bharadwaj Sharma, Nicholas D. Lakin, Andrew James Bowman, Lajos Haracska, Sébastien Huet and Gyula Timinszky. **The loss of DNA polymerase epsilon accessory subunits POLE3-POLE4 leads to BRCA1-independent PARP inhibitor sensitivity.** *Nucleic Acids Research* (2024). doi: https://doi.org/10.1093/nar/gkae439. (* Shared first authors). **Impact factor: 14.9**

- Szilvia Juhász*, Rebecca Smith*, Tamás Schauer, Dóra Spekhardt, Hasan Mamar, Siham Zentout,

Catherine Chapuis, Sébastien Huet, And Gyula Timinszky. **The chromatin remodeler ALC1 underlies resistance to PARP inhibitor treatment.** *Science Advances.* (2020). doi: https://doi.org/10.1126/sciadv.abb8626. (* Shared first authors). **Impact factor: 15.4**

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- EMBO short-term fellowship at University of Warwick, UK. (2023)

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- Poster Presentation at **Characterization of posttranslational modifications in cellular signaling,** Odense, Denmark. (2023)
- Selected Talk at **FEBS Advances Lectures Course PARP 2023**, Hvar, Croatia. (2023)
- Selected Talk at **Straub days,** Szeged, Hungary. (2023)
- Selected Talk at **The Biochemistry Global Summit,** Lisbon, Portugal. (2022)
- Poster Presentation at **Young Scientist Forum**, Vimeiro, Portugal. (2022)
- Poster Presentation at **Hungarian Molecular Life Sciences,** Eger, Hungary. (2021)

I declare as the supervisor and the corresponding author of the below listed publications, that the contribution of Hasan Mamar was significant in these publications and that the doctoral process is based on these publications. The results reported in the PhD dissertation were not used to acquire any PhD degree in the past and will not be used in the future either.

- Hasan Mamar*, Roberta Fajka-Boja*, Mónika Mórocz, Eva Pinto Jurado, Siham Zentout, Alexandra Mihuț, Anna Georgina Kopasz, Mihály Mérey, Rebecca Smith, Abhishek Bharadwaj Sharma, Nicholas D. Lakin, Andrew James Bowman, Lajos Haracska, Sébastien Huet and Gyula Timinszky. **The loss of DNA polymerase epsilon accessory subunits POLE3-POLE4 leads to BRCA1-independent PARP inhibitor sensitivity.** *Nucleic Acids Research* (2024). doi: https://doi.org/10.1093/nar/gkae439. (* Shared first authors). **Impact factor: 14.9**

- Szilvia Juhász*, Rebecca Smith*, Tamás Schauer, Dóra Spekhardt, Hasan Mamar, Siham Zentout, Catherine Chapuis, Sébastien Huet, and Gyula Timinszky. **The chromatin remodeler ALC1 underlies resistance to PARP inhibitor treatment.** *Science Advances.* (2020). DOI: https://doi.org/10.1126/sciadv.abb8626. (* Shared first authors). **Impact factor: 15.4**

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