

Identifying mechanisms involved in the resistance of homologous recombination defective *Dictyostelium* cells to ART inhibitors

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Identifying mechanisms involved in the resistance of homologous recombination defective *Dictyostelium* cells to ART inhibitors

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Poly(ADP-ribose) polymerase (PARP) inhibitors (PARPi) are toxic to cells with defects in homologous recombination (HR), which is exploited in the clinic as therapy of *BRCA1/2*-mutated tumours. Although the concept of synthetic lethality between PARPi and HR deficiency is well-known, the exact mechanisms resulting in cell death are still unclear. Here, we establish *Dictyostelium* as model to investigate the molecular basis of synthetic lethality of HR dysfunction with PARPi. We show that disrupting the HR-critical factor *exonuclease I (exo1)* sensitises *Dictyostelium* cells to PARPi. We further identify that HR inhibition is toxic for cells disrupted in the single-strand break responding PARP Adprt2, whereas an additional loss of the non-homologous end-joining (NHEJ)-promoting PARP Adprt1a renders cells resistant to the treatment. Accordingly, a defect in NHEJ suppresses PARPi toxicity to HR-defective *exo1*⁻ strains which is not dependent on the alternative NHEJ (alt-NHEJ) factors Lig3 and PolQ but instead due to re-activation of HR. Furthermore, inhibiting Mre11 re-sensitises *dnapkcs*⁻*exo1*⁻ cells to PARPi, indicating that redundancy between nucleases induce PARPi resistance. To identify further mechanisms that suppress PARPi toxicity to HR-defective cells, we perform a screen to select for PARPi-resistant *exo1*⁻ cells. Using whole genome sequencing we detect mutations that might be accountable for the tolerance of HR-compromised cells to PARPi. Taken together, our data indicate that NHEJ drives the toxicity of PARPi in the absence of HR

and that its disruption results in PARPi resistance due to nuclease redundancy, which restores HR.

Author's Publications

Kolb, A.-L., Gunn, A. R. & Lakin, N. D. Redundancy between nucleases required for homologous recombination promotes PARP inhibitor resistance in the eukaryotic model organism *Dictyostelium*. *Nucleic Acids Res.* 1–12 (2017). doi:10.1093/nar/gkx639

Kolb A.-L., Hsu, D. W., Wallis A. B. A., Ura, S., Rakhimova, A., Pears, C. J., Lakin N. D., *Dictyostelium* as a model to assess site-specific ADP-ribosylation events (chapter for Methods in Molecular Biology volume focused on Mono ADP-ribosylation(Springer), in press)

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Abbreviations

5'-dRP	5' deoxyribose phosphate
Adprt	ADP-ribosyl-transferase
Alt-NHEJ	Alternative non-homologous end-joining
APE	AP-endonuclease
APL	Aprataxin-like protein
APLF	Aprataxin and PNKP-like factor
AP-site	Apurinic/Apyrimidinic site
ART	ADP-ribosyltransferase
ATM	ataxia-telangiectasia mutated
ATR	Ataxia telangiectasia and Rad3 related
ATP	Adenosine triphosphate
BER	Base excision repair
BIR	Break-induced replication
BRCA1	Breast cancer-associated protein 1
BRCA2	Breast cancer-associated protein 2
<i>Bsr</i>	Blasticidin resistance
cAMP	Cyclic adenosine monophosphate
CtiP	CtBP-interacting protein
DDR	DNA damage response
D-loop	Displacement loop
DNA	Deoxyribonucleic acid
DNA-PKcs	DNA-dependent protein kinase catalytic subunit
dNTP	deoxynucleoside triphosphate
DSB	Double-strand break
DSBR	Double-strand break repair

DTT	Dithiothreitol
EDTA	Ethylenediaminetetraacetic acid
Fen1	Flap Endonuclease 1
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HR	Homologous recombination
ICL	Inter-strand cross-link
IR	Ionising radiation
KA	<i>Klebsiella aerogenes</i>
M	Molar
MAR	Mono(ADP-ribose)
MART	Mono-ADP-ribosyl-transferase
MARylation	Mono(ADP-ribosyl)ation
MEF	Mouse embryonic fibroblast
MMS	Methyl methanesulphonate
MRN	Mre11/Rad50/Nbs1
MRX	Mre11/Rad50/Xrs2
NAD ⁺	Nicotinamide adenosine dinucleotide
NER	Nucleotide excision repair
NHEJ	Non-homologous end-joining
PAR	Poly(ADP-ribose)
PARG	Poly(ADP-ribose) glycohydrolase
PARP	Poly(ADP-ribose) polymerase
PARylation	Poly(ADP-ribosyl)ation
PCNA	Proliferating cellular nuclear antigen
PCR	Polymerase chain reaction
Pipes	Piperazine-N,N'-bis(2-ethanesulfonic acid)
PNKP	Polynucleotide kinase phosphatase
Pol A	DNA polymerase A

Pol β	DNA polymerase β
Pol δ/ϵ	DNA polymerase δ/ϵ
PolQ	DNA polymerase Θ
Pol X	DNA polymerase X
REMI	Restriction enzyme-mediated integration
ROS	Reactive oxygen species
RPA	Replication protein A
RT	Reverse Transcriptase
SDSA	Synthesis-directed strand annealing
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
SSB	Single-strand break
SSBR	Single-strand break repair
TDP1	Tyrosyl-DNA phosphodiesterase 1
TOP I	DNA topoisomerases I
TOP II	DNA topoisomerases II
UV	Ultraviolet
XRCC1	X-ray cross-complementing protein 1
XRCC4	X-ray cross-complementing protein 4

Table of contents

1. Introduction.....	1
1.1 Introduction of DNA damage response.....	1
1.2 ADP-ribosylation	2
1.3 ADP-ribosylation in DNA single-strand break repair	6
1.3.1 Sources of single-strand breaks.....	6
1.3.2 Base excision repair	7
1.3.3 Single-strand break repair.....	7
1.3.4 PARPs in single-strand break repair	10
1.4 ADP-ribosylation in DNA double-strand break repair	12
1.4.1 Sources of DNA double-strand breaks.....	12
1.4.2 DSB repair pathways	13
1.4.2.1 Homologous recombination	13
1.4.2.2 Non-homologous end joining (NHEJ).....	16
1.4.2.3 Alternative non-homologous end joining (alt-NHEJ).....	19
1.4.3 PARPs in DSB repair	22
1.5 PARP inhibition in the clinic.....	24
1.5.1 HR deficiency is associated with a high risk of developing cancer	24
1.5.2 Synthetic lethality of HR deficiency and PARP inhibition.....	25
1.5.3 Development of PARPi.....	27
1.5.4 Mechanism of PARP inhibition.....	28
1.5.5 Resistance of HR-defective tumours to PARPi in the clinic	30
1.6 Model organisms to study ADP-ribosylation	33
1.7 <i>Dictyostelium</i> as model organism for DNA repair	35
1.7.1 The genetic model organism <i>Dictyostelium</i>	35
1.7.2 <i>Dictyostelium</i> as model organism to study DDR	36
1.7.3 PARPs involved in DDR in <i>Dictyostelium</i>	38

1.8 Aims of this thesis	40
2. Materials and Methods	43
2.1 Materials	43
2.2 Methods	47
2.2.1 <i>Dictyostelium</i> cell culture and genetic manipulation	47
2.2.1.1 Cell culture.....	47
2.2.1.2 Transformation.....	48
2.2.1.3 Generating of disruption strains	48
2.2.1.4 PCR screening of disruption strains	49
2.2.1.5 Cre-loxing	49
2.2.1.6 Storage of strains.....	49
2.2.2 Molecular biology.....	50
2.2.2.1 Phenol-chloroform DNA extraction.....	50
2.2.2.2 Southern blotting.....	51
2.2.2.3 RNA Extraction and cDNA synthesis	52
2.2.2.4 DNA manipulations techniques	52
2.2.3 Phenotypic analysis	53
2.2.3.1 Sensitivity assay	53
2.2.3.2 Clonogenic assay	53
2.2.3.3 Luminescent cell viability assay	53
2.2.3.4 Chromatin extraction.....	54
2.2.3.5 HR assay	55
2.2.4 Protein techniques	55
2.2.4.1 Protein Purification.....	55
2.2.4.2 Poly-acrylamide gel electrophoresis and Western blotting	56
2.2.4.3 Auto-PARylation assay	57
2.2.5 Sample preparation for whole genome sequencing	58
2.2.5.1 Screen to obtain PARPi-resistant HR deficient cells.....	58

2.2.5.2	DNA Preparation.....	58
2.2.5.3	Library generation using Nextera	58
2.2.5.4	Library quantification and quality control using qPCR	60
2.2.6	Bioinformatics	61
2.2.6.1	Sequence alignments	61
2.2.6.2	Genome sequencing.....	61
2.2.6.3	Analysis of sequencing data	61
3.	Synthetic lethality of HR-deficient strains with PARPi is conserved in	
	<i>Dictyostelium</i>	63
3.1	Introduction	63
3.1.1	PARPi as cancer therapy.....	63
3.1.2	ADP-ribosylation and DDR in <i>Dictyostelium</i>	64
3.1.3	Aims	64
3.2	Results	65
3.2.1	Efficacy of different PARPi on inhibiting auto-PARylation in <i>Dictyostelium</i> ..	65
3.2.2	Generation of HR-deficient <i>Dictyostelium</i> strains	70
3.2.3	PARPi are toxic to cells disrupted in the <i>exo1</i> gene	76
3.2.4	Loss of Adprt2 sensitises cells to HR inhibition	82
3.3	Discussion.....	87
4.	Disrupting NHEJ suppresses PARPi toxicity to HR-deficient strains	91
4.1	Introduction	91
4.1.1	Synthetic lethality of HR dysfunction with PARPi is conserved in	
	<i>Dictyostelium</i>	91
4.1.2	Aims	92
4.2	Results	92
4.2.1	Identification of a putative PolQ orthologue.....	92
4.2.2	Disrupting <i>polq</i> sensitises cells to DNA DSBs.....	96

4.2.3	Disruption of NHEJ or PolQ-dependent repair increases the sensitivity of <i>exo1⁻</i> strains to DNA DSBs	101
4.2.4	Disrupting <i>dnapkcs</i> but not <i>polq</i> suppresses PARPi toxicity to <i>exo1⁻</i> cells	108
4.3	Discussion.....	117
5.	Resistance to PARPi of <i>exo1⁻</i> cells is due to restoration of HR	125
5.1	Introduction	125
5.1.1	Potential pathways rendering HR defective cells resistant to PARPi.....	125
5.1.2	Aims	126
5.2	Results	127
5.2.1	PARPi tolerance of <i>dnapkcs⁻exo1⁻</i> cells is not dependent on Lig3 and PolQ..	127
5.2.2	The <i>exo1⁻dnapkcs⁻</i> strains restore HR to suppress PARPi toxicity	133
5.3	Discussion.....	139
6.	Identification of mutations that suppress PARPi toxicity to <i>exo1⁻</i> cells	144
6.1	Introduction	144
6.1.1	<i>Dictyostelium</i> as model to identify mutations involved in PARPi resistance of HR-defective cells.....	144
6.1.2	Aims	145
6.2	Results	146
6.2.1	Generation of PARPi-resistant HR-deficient strains	146
6.2.2	Quality control of whole genome sequencing data	153
6.2.3	Analysis of whole genome sequencing data	158
6.3	Discussion.....	164
7.	General Discussion and Conclusion.....	173
7.1	Synthetic lethality of HR dysfunction with PARPi is conserved in <i>Dictyostelium</i> .	173
7.2	Loss of NHEJ renders <i>exo1⁻</i> cells tolerant to PARPi.....	174
7.3	Survival of <i>dnapkcs⁻exo1⁻</i> cells to PARPi is independent of PolQ and Lig3	175

7.4 Nuclease redundancy as resistance mechanism of <i>dnapkcs⁻exo1⁻</i> cells to PARPi ...	178
7.5 Novel mechanisms involved in the tolerance of HR-defective cells to PARPi	180
7.6 Conclusion	183
8. References	184
9. Appendix	211
9.1 Primer Sequences.....	211

1. Introduction

1.1 Introduction of DNA damage response

Our genome is under continuous assault from genotoxic endogenous and exogenous agents, inducing different kinds of DNA damages. Sources of exogenous factors include UV-light, ionising radiation (IR) and various chemical compounds. Endogenous mutagenic agents are formed during spontaneous enzymatic reactions, as free radical species or alkylating agents. Another source for endogenous damage is base mismatches occurring during DNA replication. All of these mutagenic sources cause different kinds of DNA damage, including single strand breaks (SSB), Base damage, inter- and intra-strand crosslinks (ICL) and double-strand breaks (DSB). If left unrepaired, DNA damage accumulates resulting in genomic instability, causing apoptosis or senescence. Furthermore, loss of genomic integrity predisposes the cells to different clinical disorders, such as neurodegenerative diseases and cancer¹⁻³. Therefore, cells have evolved a network of repair mechanisms to signal and correct DNA damage, which is collectively known as DNA damage response (DDR).

Execution of DDR is highly conserved and relies on various protein modifications to induce a signal transduction cascade. Detection of DNA lesions occurs by specialised sensor proteins, which in turn activate mediator proteins. The mediator or adapter proteins recruit additional substrates and act as scaffolds for downstream effector proteins that resolve the DNA damage, regulate the cell cycle and potentially induce apoptosis⁴. Signalling of DNA damage is mediated by various protein post-translational modifications, such as phosphorylation, methylation and acetylation⁵. For example, phosphorylation of the histone H2AX upon DNA DSB detection, mediated by kinases such as ATM, ATR or DNA PKcs, generates a scaffold for several effector proteins necessary for DNA repair⁶⁻⁸. Furthermore, regulation of chromatin reorganization during DNA repair is dependent on

the acetylation of several lysine residues of histones H3 and H4⁵. Methylation of histone H4 is also critical for the recruitment of 53BP1 thereby initiating one of the DSB repair pathways⁹. This is only a small subset of protein-post translational modifications involved in DDR. Further modifications, such as ubiquitination, SUMOylation and ADP-ribosylation are involved in signal transduction of DDR¹⁰. The role of ADP-ribosylation during DNA repair will be discussed in detail and is the major topic of this thesis.

1.2 ADP-ribosylation

ADP-ribosylation is a reversible post translational protein modification that regulates a variety of cellular processes, including cell cycle, inter- and intracellular signalling, transcription, apoptosis and DNA repair pathways¹¹. The modification is catalysed by an enzyme family with 17 members, called ADP-ribosyltransferases (ARTs). The enzymes transfer ADP-ribose from nicotinamide adenine dinucleotide (NAD⁺) to an acceptor protein. Typically the amino acids glutamate, aspartate, arginine, serine and lysine are ADP-ribosylated¹². The substrates are either modified with a single ADP-ribose unit (mono ADP-ribosylation) or with multiple units, forming linear or branched poly ADP-ribose (PAR) chains¹³. ARTs that add a mono ADP-ribose are referred to as mono-ADP-ribosyltransferases (MARTs), whereas enzymes catalysing the generation of PAR chains are called poly (ADP-ribose) polymerases (PARPs)¹⁴.

PARPs can produce chains with up to 200 ADP-ribose units, joining them by unique O-glycosidic ribose-ribose bonds, thereby adding a bulk and highly negatively charged modification to the substrates^{13,15}. Besides substrate modification, most of the ARTs are also competent in auto-ADP-ribosylation. ADP-ribosylation is a reversible protein modification and exhibits a high turnover rate. Degradation of PAR chains is mediated by ADP-ribose hydrolases, such as PAR glycohydrolase (PARG), leaving mono-ADP-ribose

at the substrate^{14,16}. Enzymes such as MacroD1 and MacroD2 are competent in removing a single ADP-ribose unit, thereby reversing the substrate back to the unmodified state¹⁷.

Whether the ART generates MAR or PAR can be determined by comparing different characteristics. The most important factor is the sequence motif in the NAD⁺ binding core, which is conserved from the organism in which ADP-ribosylation was first discovered *Corynebacterium diphtheria* to mammalian cells¹⁸. Three specific amino acid residues, that mediate the binding to NAD⁺ and catalyse the transfer reaction, form an evolutionary conserved characteristic triad^{12,19}. ARTs containing a His-Tyr-Glu (H-Y-E) are predicted to generate PAR-chains, whereas enzymes containing leucine, isoleucine or valine instead of the glutamate are predicted to have MAR activity^{12,19}. The amino acid residues histidine and tyrosine mediate NAD⁺ binding and coordination, whereas glutamate is crucial for catalysing the elongation of PAR polymers^{20–24}. ARTs lacking the histidine, such as PARP9 and PARP13, are predicted to be enzymatically inactive. The role of the three amino acids was investigated by structural studies and auto-modification activities of the ART enzymes^{20,21,24}. The best studied members of the ART family, PARP1, PARP2, PARP5a and PARP5b contain the H-Y-E motif and generate PAR chains, supporting the prediction¹⁹. However, experimental evidence suggest that PARP3 and PARP4, although possessing the H-Y-E triad, exhibit MAR instead of PAR activity^{12,25–28}. Recent experimental evidence suggest, that in addition to the NAD⁺ binding core secondary structural features, such as the donor loop, shape the substrate binding pocket, which influences the catalytic activity of the enzymes. Changes in the amino acid sequence of the donor loop effects the length of the PAR chain, whereas the acceptor loop (A-loop) is potentially important for the enzymatic activity of the ARTs in general^{27,29}.

DNA dependent PARylation was first discovered by Chambon *et al.* in 1963 in rat liver nuclear extracts^{30,31}. Just a few years later, the first ART enzyme with its DNA dependent ADP-ribosylation activity was described³². For the next 30 years only one enzyme was

ADP-ribosyltransferase	Activity	Triad motif	Automodification
ARTD1 (PARP1)	PAR	H-Y-E	+
ARTD2 (PARP2)	PAR	H-Y-E	+
ARTD3 (PARP3)	MAR	H-Y-E	+
ARTD4 (PARP4)	MAR	H-Y-E	+
ARTD5 (PARP5a)	PAR	H-Y-E	+
ARTD6 (PARP5b)	PAR	H-Y-E	+
ARTD7 (PARP15)	MAR	H-Y-L	+
ARTD8 (PARP14)	MAR	H-Y-L	+
ARTD9 (PARP9)	inactive	Q-Y-T	ND
ARTD10 (PARP10)	MAR	H-Y-I	+
ARTD11 (PARP11)	ND	H-Y-I	ND
ARTD12 (PARP12)	MAR	H-Y-I	ND
ARTD13 (PARP13)	inactive	Y-Y-V	ND
ARTD14 (PARP7)	MAR	H-Y-I	+
ARTD15 (PARP16)	MAR	H-Y-Y	ND
ARTD16 (PARP8)	ND	H-Y-I	ND
ARTD17 (PARP6)	ND	H-Y-I	ND

Table 1.1: Human ADP-ribosyltransferases. The first column shows the recently proposed nomenclature of the 17 human ADP-ribosyltransferases sharing homology with bacterial diphtheria toxin (ARTDs) as well as the old names of the enzymes. The catalytic activity of each ART, either poly (ADP-ribosylation) (PAR) or mono (ADP-ribosylation) (MAR) is shown in the second column. The triad motif, which represents the amino acid sequence in the NAD⁺ binding core, is exhibit in the third column. This motif predicts the catalytic activity, with only ARTs, containing H-Y-E able to generate PAR chains, whereas enzymes lacking the Glutamate possess MAR activity. However, some exceptions of this rule have already been identified. The last column depicts the ability to auto-ADP-ribosylation. ND: not determined, H: Histidine, Y-Tyrosine, E: Glutamate, L: Leucine, I: Isoleucine, Q: Glutamine, T: Threonine, V: Valine Adapted from Hottinger *et al.* (2010) and Vyas *et al* (2014).

thought to be responsible for ADP-ribosylation and has mostly been studied with regards to its activity in DDR³³. Then, within a few years several enzymes with ADP-ribosylation activity were discovered, resulting in renaming the first identified enzyme of this family into PARP1. A two-hybrid screen in human cell lines identified the tankyrase PARP5a, which possesses a highly similar catalytic domain to PARP1. PARP5a localises to DNA telomeres, maintaining telomere length and synthesises PAR-chains independently of the presence of DNA^{34–36}. Another two ARTs, PARP2 and PARP3, were identified in human tissues³⁷. Both ARTs respond to DNA damage in the nucleus, whereas PARP3 was also identified to interfere with the G1-S cell cycle transition in HeLa cells^{38–41}. At the same time, PARP4 was identified in a two-hybrid screen as member of a cytoplasmic ribonucleoprotein complex⁴². PARP4 also localises to nuclear pores and interacts with the mitotic spindle during mitosis⁴². Hence, within a short time several enzymes containing ADP-ribosylation activity were identified, which was thought to be restricted to one enzyme for about 30 years. Further enzymes with an ART catalytic domain were discovered by human genome sequencing⁴³. To date, the ART family consists of 17 members and not all of their functions have been determined yet. More recently PARP10 and PARP14 were identified to play a role in the protection of replication associated stress^{44,45}. Additionally, PARP10 is involved in cell signalling and proliferation, whereas PARP14 also acts as transcription factor^{46,47}.

Since all members of the family were previously named PARPs despite their different enzymatic activities, a new nomenclature was recently suggested, in which all ART family members that share homology with bacterial diphtheria toxin are referred to as ARTDs (Table 1.1)¹². However, it is still being discussed in the scientific community whether the ADP-ribosylation enzymes are called “ARTs” or “PARPs”. Hereafter the enzymes will be referred to with the more common name “PARPs”.

The best defined role of PARPs is in DNA damage signalling. This was suggested by various studies showing that intracellular NAD and ATP levels decrease rapidly upon DNA damage^{48–50}. Furthermore, PARP inhibition and NAD depletion renders mouse leukaemia cells sensitive to DNA alkylating agents, indicating that ADP-ribosylation is important for DNA damage repair⁵¹. ADP-ribosylation is hypothesised to possess two distinct roles in DNA repair. The negative charge of the post-translational protein modification relaxes the chromatin structure, making the DNA lesion accessible for repair proteins^{52,53}. Furthermore, PARylation is required for the recruitment of downstream repair factors, which contain specific PAR-binding domains⁵⁴. The specific role of ADP-ribosylation in DNA repair is discussed in the next section of this thesis.

1.3 ADP-ribosylation in DNA single-strand break repair

1.3.1 Sources of single-strand breaks

Single-strand breaks (SSBs) are gaps in one of the DNA double helix strands and thousands of these breaks occur in every cell each day. If unrepaired, SSBs can be converted into the more deleterious DNA double-strand breaks (DSBs) during DNA replication. The most common source of DNA SSBs is an oxidative attack by reactive oxygen species (ROS), which are either generated endogenously as natural by-product during cell respiration or exogenously by different environmental factors, such as ionising radiation or UV light⁵⁵. The oxidative attack either directly induces DNA SSBs or forms abnormal DNA bases, such as thymine glycol or 8-oxo-guanin. Other Base lesions might result from deamination or methylation⁵⁶. The damaged bases are subsequently removed by base excision repair (BER), generating a SSB^{57–61}. SSBs can also occur as a result of defects in cellular enzymes, such as DNA topoisomerases I (TOP I). Those enzymes introduce transient SSBs to relax DNA during replication and transcription that are normally resealed by TOP I⁶².

1.3.2 Base excision repair

Abnormal base modifications are repaired via base excision repair (BER), generating SSBs during the process. BER is initiated with the excision of the damaged base by DNA glycosylases, which cut the N-glycosidic bond between the base and the DNA backbone, leaving an abasic site (AP-site) that lacks the purine or pyrimidine base. Mammalian cells possess several different DNA glycosylases that can be divided into two subgroups, mono- and bi-functional enzymes⁵⁶. An AP-site, generated by a mono-functional DNA glycosylase needs to be further processed into a SSB using different enzymes^{56,63}. Therefore, an AP lyase or AP endonuclease, such as APE1, incises the DNA backbone directly 5' of the AP-site to generate a strand break^{60,64}. Bi-functional DNA glycosylases not only generate the AP-site, but also exhibit AP lyase activity to catalyse the incision into the DNA backbone to form a SSB⁵⁶. The SSB is subsequently repaired via SSB repair (SSBR), which is explained in the next section. A model of BER and SSBR is presented in Figure 1.1.

1.3.3 Single-strand break repair

Repair of DNA SSBs can be divided into four steps. First, the break is detected followed by DNA end processing. Afterwards the DNA gap is filled and the ends are subsequently ligated together⁶⁵. PARP1 binds to and is activated by single-stranded DNA (ssDNA) ends, making this enzyme responsible for the first step, detection of the SSB. This induces PARP1 auto-modification, as well as ADP-ribosylation of target proteins. Subsequently, the scaffolding protein X-ray repair cross-complementing protein 1 (XRCC1) is recruited to the damaged DNA by interaction with the PAR chain^{54,66,67}. Although no enzymatic activity of XRCC1 has been detected so far, it is an essential protein for SSB repair due to its role in recruiting various repair factors. For example, XRCC1 interacts with and stimulates Polynucleotide kinase 3' phosphate (PNKP), which

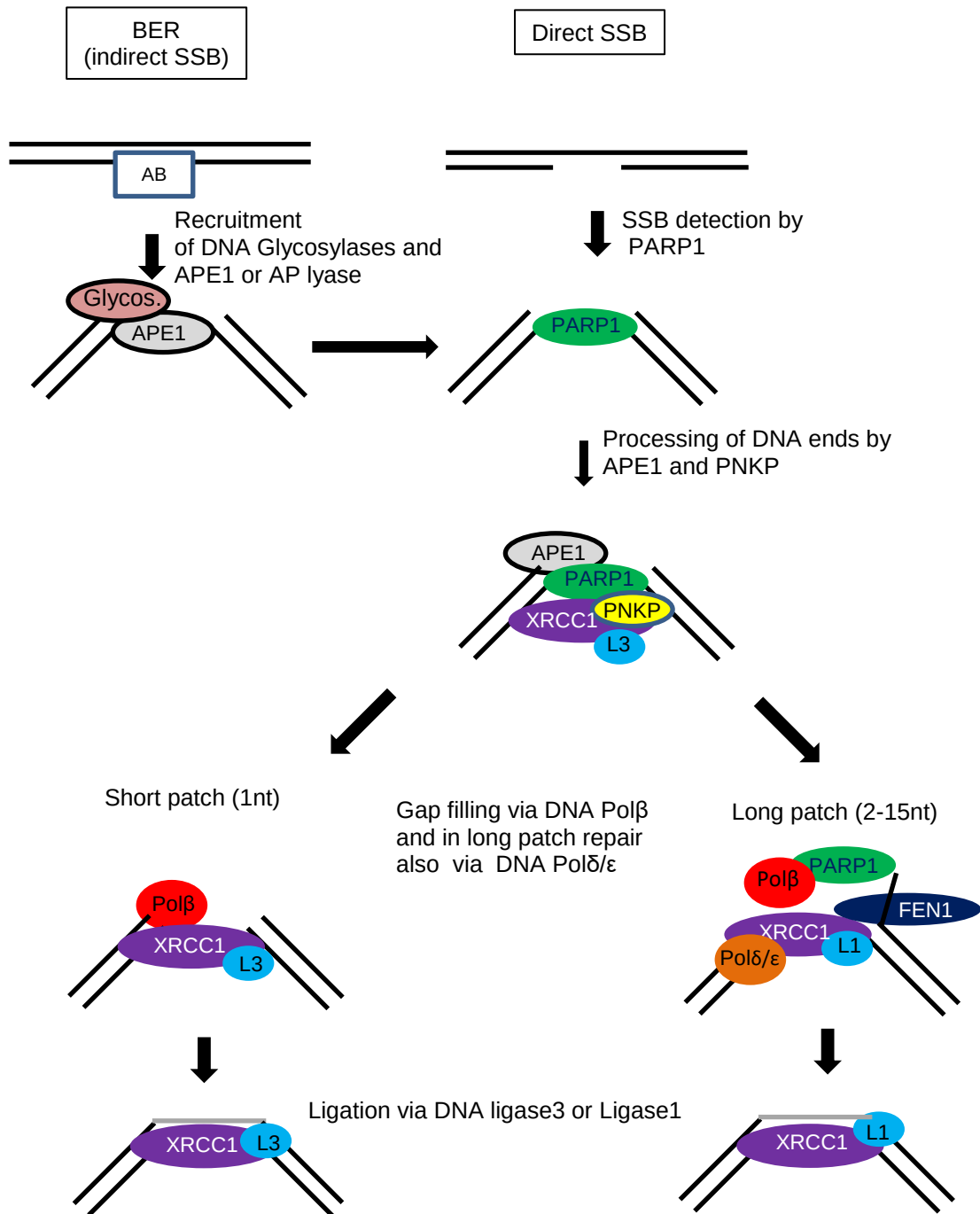


Figure 1.1: Model for Base excision repair (BER) and repair of single strand breaks (SSB). Abnormal bases (AB) are detected and excised by DNA glycosylases (Glycos.), and further processed into a SSB by an AP-Lyase or AP endonuclease such as APE1. The SSB is repaired via the same way as direct SSB. PARP1 binds to the SSB, inducing auto-ADP-ribosylation and substrate modification. XRCC1 binds to the ADP-ribosyl chain of PARP1 and recruits downstream repair factors, such as the Polynucleotide kinase 3'phosphate (PNKP) which mediates DNA end processing. Another enzyme involved in DNA end processing is APE1. XRCC1 also recruits the DNA ligase (L3). Following DNA processing, the missing nucleotides are inserted using the intact strand as template. If only 1 nucleotide is missing short patch repair is performed using the DNA polymerase β (Pol β). If 2-15 nucleotides are missing long path repair is performed. Thereby, nucleotide synthesis is potentially mediated by Pol β and DNA polymerase δ and Pol ϵ (Pol δ/ϵ). The flap endonuclease (Fen1) removes the displaced 5'DNA end, creating a ligatable DNA substrate. Ligation of the ends is mediated by DNA Ligase 1 (L1) or DNA Ligase 3 (L3). Adapted from Caldecott, 2008.

possesses 3'-DNA phosphatase and 5'-DNA kinase activity and is involved in DNA end processing^{68,69}. Depending on the type of damage and the modification at the DNA ends, different enzymes are required for the processing step to render the DNA ends compatible for re-ligation. For example, APE1 and tyrosyl-DNA phosphodiesterase 1 (TDP1) catalyse the removal of 3'-phosphoglycolate groups. Thereby, TDP1 generates 3'-phosphate ends that need to be further processed by PNKP⁵⁵. Another enzyme involved in end processing is aprataxin, which removes 5'AMP that occur by abortive DNA ligation^{61,70-73}.

Following DNA end processing, the missing nucleotides in the DNA gap are replaced using the intact complementary DNA strand as template. In most instances only a single nucleotide needs to be inserted, which is known as short patch repair. Sometimes, however, the gap consists of 2-15 absent nucleotides, requiring long-patch repair^{69,74}. DNA polymerase β (Pol β), recruited to the break by direct interaction with XRCC1, plays a role in DNA synthesis in both pathways⁷⁵⁻⁷⁷. Pol β inserts single DNA nucleotides in short patch repair and also possesses β -lyase activity to remove the 5'-deoxyribose phosphate group, which is required for ligation of the two ends^{78,79}. It also initiates DNA synthesis during long-patch repair, stimulated by PARP1, in combination with two other DNA polymerases, Pol δ/ϵ ^{64,76,80}. The repair mechanism is proposed to be performed by displacing the 5'DNA end, forming a flap intermediate, which is then removed by flap endonuclease (FEN1) to create a ligatable DNA substrate^{76,81}.

Once the DNA gap is filled, the final step during SSB repair is sealing the DNA ends. A number of studies have implicated DNA ligase III (LIG3), which interacts with XRCC1, and DNA ligase I (LIG1) in the repair of DNA SSBs⁸²⁻⁸⁴. Both enzymes catalyse the formation of a covalent phosphodiester bond between the 3'-OH of the upstream nucleotide and the 5'-PO₄ of the downstream DNA end in an ATP-dependent way^{64,69}. It is proposed that following the degradation of the PAR chain, ADP-ribose even serves as energy source for

the ligation step. In the presence of pyrophosphate, the pyrophosphatase NUDIX5 catalyses the conversion of ADP-ribose to nuclear ATP and ribose-5-phosphate, which implies that PARPs are not only important for the detection of SSBs⁸⁵⁻⁸⁷.

1.3.4 PARPs in single-strand break repair

The importance of PARPs in SSBR was identified over 30 years ago when Park *et al* showed that treatment with PARP inhibitors (PARPi) delays the repair of SSBs in HeLa cells⁸⁸. More than 10 year later, PARP1 was identified to play an important role in DNA damage repair *in vivo* using knockout mice. Exposure of *Parp1*^{-/-} mice to DNA damage significantly decreases the survival rate compared to PARP1-proficient mice^{89,90}. Furthermore, cells derived from *Parp1*^{-/-} mice are highly sensitive to alkylating agents, indicating the importance of this enzyme in DNA damage response, especially in DNA SSBR⁹¹. PARP1 possesses three zinc-fingers, two of them promote the interaction with DNA, one with DSBs the other with SSBs, and the third one stimulates its DNA-dependent catalytic activity⁹²⁻⁹⁵. DNA binding induces auto-ADP-ribosylation of PARP1, which facilitates the interaction with SSBR factors such as XRCC1 and Lig3^{54,66,77,96,97}. Whilst ADP-ribosylation is required for the recruitment of XRCC1 to sites of SSBs, interaction of Polβ to PARP1 is only DNA dependent, indicating that XRCC1 and Polβ bind to different domains of PARP1^{54,67,98}. Furthermore, *in vitro* data show that PARP1 promotes strand displacement synthesis of Polβ on long patch repair, but only in the presence of FEN-1^{75,80}. Moreover, depletion of PARP1 in HeLa cells severely decreases XRCC1 recruitment to DNA breaks and delays SSBR in G1 phase⁹⁹. However, the repair of DNA SSBs is potentially carried out via two different sub-pathways of BER, dependent on the type of lesion. XRCC1 and PARP1 are both involved in SSBR and repair of damaged purine bases, whereas damages in pyrimidine bases are resolved in a PARP1-independent XRCC1-dependent pathway¹⁰⁰. Interestingly, PARP1-depleted A549 cells exhibit a similar amount of SSBs as wild type cells following exposure to alkylating

agents, indicating that PARP1 either plays a minor in repair of this specific lesion, or there is a redundancy with other PARP enzymes, potentially PARP2¹⁰¹. Auto-modification of PARP1 decreases its binding affinity to DNA and leads to its dissociation, making the damaged DNA accessible for repair⁴⁰. The PAR-chain is then rapidly degraded by poly ADP- ribose glycohydrolase (PARG), restoring the PARP enzyme into its unmodified state, active for further rounds of SSB detection¹⁰².

PARP2 is also proposed to be involved in DDR, has auto-ADP-ribosylation activity and is potentially responsible for PARylation in PARP1-depleted cells following SSBs^{38,103}. PARP2 structure is similar to PARP1, although it contains a shorter N-terminal region the function of which is poorly understood⁹²⁻⁹⁴. Similarly to *Parp1*^{-/-} mice, disruption of the *Parp2* gene renders mice sensitive to ionizing radiation, supporting a role of PARP2 in DNA damage repair. Moreover, *Parp1*^{-/-}*Parp2*^{-/-} mice are embryonically lethal, indicating that both enzymes are important for early development and potentially exhibit overlapping functions¹⁰⁴. *In vitro* data show that PARP1 and PARP2 form homo- and heterodimers and furthermore, can ADP-ribosylate each other, indicating that both enzymes can act together. Furthermore, similar to PARP1, PARP2 interacts with XRCC1, Pol β and LIG3¹⁰⁵. Using human A549 cells it was shown that depletion of PARP2 only has a minor effect on SSB kinetics, whereas PARP1 knockdown significantly decreases the rate of SSBR. Even simultaneous depletion of PARP1 and PARP2 does not further reduce the repair rate, indicating that PARP1 is the primary PARP in SSBR¹⁰⁶. Interestingly, PARP1 and PARP2 are both recruited to DNA SSBs but with different kinetics. PARP1 rapidly, but transiently accumulates at damaged DNA sites, whereas PARP2 recruitment was slower but more persistent. Besides, PARP2 recruitment to DNA lesions is impaired in *PARP1*^{-/-} cells indicating that PARP2 potentially plays a role downstream of PARP1. Although PARP2 is competent in binding to XRCC1, recruitment of this protein to SSBs is solely dependent on PARP1¹⁰⁷. Overall, these data suggest that PARP1 is the main PARP involved in SSBR and that PARP2 plays a minor but unique role which has not been determined yet.

It was proposed that ADP-ribosylation provides the energy source for sealing the DNA ends in the final step of SSB repair. Nucleotide insertion into the DNA gap catalysed by Pol β generates pyrophosphate from dNTPs, which forms ATP in conjunction with the degradation product of the PAR chain^{65,85}. The generated ATP is subsequently used to ligate the DNA ends by LIG3 or LIG1, completing SSB repair^{85,86}. Hence, PARP enzymes are important during SSBR as sensor of SSBs as well as provider of the energy source with NAD as substrate. Interestingly, a recent publication implicated PARP3 in chromatin modification upon SSBs. Histone H2B was identified as the target of PARP3-mediated ADP-ribosylation at nicked DNA *in vitro* implicating a role of this enzyme in SSBR¹⁰⁸.

1.4 ADP-ribosylation in DNA double-strand break repair

1.4.1 Sources of DNA double-strand breaks

DNA double-strand breaks, in which the DNA backbone of two complementary DNA strands is simultaneously broken, are considered to be the most toxic form of DNA lesion. This damage can be induced by endogenous and exogenous sources. Examples of exogenous sources are ionising radiation (IR), UV-light or anticancer chemotherapeutic drugs, such as DNA crosslinking agents, phleomycin or topoisomerase inhibitors¹⁰⁹. Endogenous sources for DNA DSBs include ROS, generated during cellular metabolism, errors in DNA replication or chromosome miss-segregation during mitosis. Some DNA DSBs are intentionally generated during biological processes, such as meiotic recombination or V(D)J recombination, which is important for the maturation of B- and T-cells^{110–114}. Furthermore, Topoisomerase II (Top2) catalyses the transient formation of DNA DSBs to manage DNA supercoils. Defects in this enzyme might prevent the re-ligation of the DNA ends, causing DNA DSBs^{62,115}.

1.4.2 DSB repair pathways

Classically, DNA DSBs are repaired via two different pathways, either homologous recombination (HR) or non-homologous end joining (NHEJ). Recently, a third DNA repair pathway, alternative non-homologous end-joining (alt-NHEJ) has been described to resolve DSB^{116,117}. One of the main differences between the three pathways is the accuracy of DNA repair. HR requires the presence of the homologous sister chromatid to serve as template for accurate DNA synthesis. Therefore, this repair pathway is restricted to S- and G2 phases of the cell cycle after DNA replication took place^{118,119}. On the contrary, the error-prone DSB repair pathways NHEJ and alt-NHEJ can be performed throughout the cell cycle. NHEJ re-ligates the DNA ends after a short processing step of the DSB without nucleotide insertion. Hence, the repaired strand contains deletions flanking the original break, explaining the error-prone nature of this pathway¹¹¹. On the other hand, some types of alt-NHEJ require the alignment of a short microhomology region of 2-25 bp before ligating the broken ends, which usually results in nucleotide deletions surrounding the DSB¹²⁰. All three pathways share some of the repair proteins, especially those involved in processing DNA ends.

1.4.2.1 Homologous recombination

The HR pathway is activated upon DNA DSB recognition in S- and G2 phases of the cell cycle. This repair pathway uses the homologous sister chromatid as template to synthesise and insert missing nucleotides in an error free manner. The mechanism of this pathway is shown in Figure 1.2. Sensing of the DNA DSB and activation of HR is performed in a complex mechanism, requiring the interplay of different proteins and kinases. The MRN complex, composed of a MRE11 dimer, RAD50 dimer and NBS1 rapidly recognises and binds to the DNA DSB^{121–123}. During S and G2 phase of the cell cycle, cyclin dependent kinase 1 (Cdk1) phosphorylates CtIP, resulting in the interaction of CtIP with the MRN complex^{124–127}. Furthermore, CtIP also interacts with the breast

cancer gene 1 (BRCA1), controlling the G2/M DNA damage checkpoint^{124,128}. The MRN complex in association with CtIP recruits the ATM kinase as an inactive dimer and promotes dimer dissociation, which results in auto-phosphorylation and activation of the ATM kinase. All three MRN complex members are in turn phosphorylated and activate a variety of events important for DSB repair^{4,129}. Other substrates of the ATM kinase are BRCA1 and the Histone H2AX, which serves as marker for DNA DSBs^{8,130}. BRCA1 is involved in the pathway choice between NHEJ and HR, antagonizing 53BP1 and promoting HR^{131–134}. Mice deficient in BRCA1 are embryonically lethal, underlining the importance of this protein and indicating that HR dysfunction is deleterious for the cells^{135,136}. Phosphorylation of the endo/exonuclease MRE11 by ATM controls the extent of DNA end resection flanking the DSB. Primary 3′-5′ DNA end processing is mediated by MRE11 and the endonuclease CtIP, promoted by the ATPase activity of Rad50. Thereby, ATP binding of RAD50 is associated with tight interaction of the MRN complex to the DNA, whereas ATP hydrolysis induces DNA end processing^{129,130,137–141}.

DNA end resection is performed in a two-step model. Primary DNA end processing is followed by long range 5′-3′ DNA resection performed either by the exonuclease 1 (EXO1) or in a backup pathway, using a protein complex consisting of the Bloom syndrome helicase (BLM), Werner syndrome helicase (WRN) and the Dna2 nuclease^{142,143}. Phosphorylation of EXO1 during S/G2 phase of the cell cycle by CDK1/2 and the ATM kinase is essential for its activity. Phosphorylated EXO1 interacts with BRCA1, initiating localization of EXO1 via an unknown mechanism to sites of DNA DSBs^{143–146}. EXO1 or Dna2 resect the DNA DSB, thereby generating a 3′DNA overhang of single-stranded DNA, which is coated by RPA to prevent degradation and unwanted secondary structure formation^{147–150}. RPA is subsequently replaced by RAD51, which is loaded onto the ssDNA by direct interaction with BRCA2^{151–153}. Germline mutations in BRCA2, resulting in the loss of function of this protein, is associated with high risks of developing breast and ovarian cancer^{154,155}. The ssDNA-RAD51 nucleofilament along with

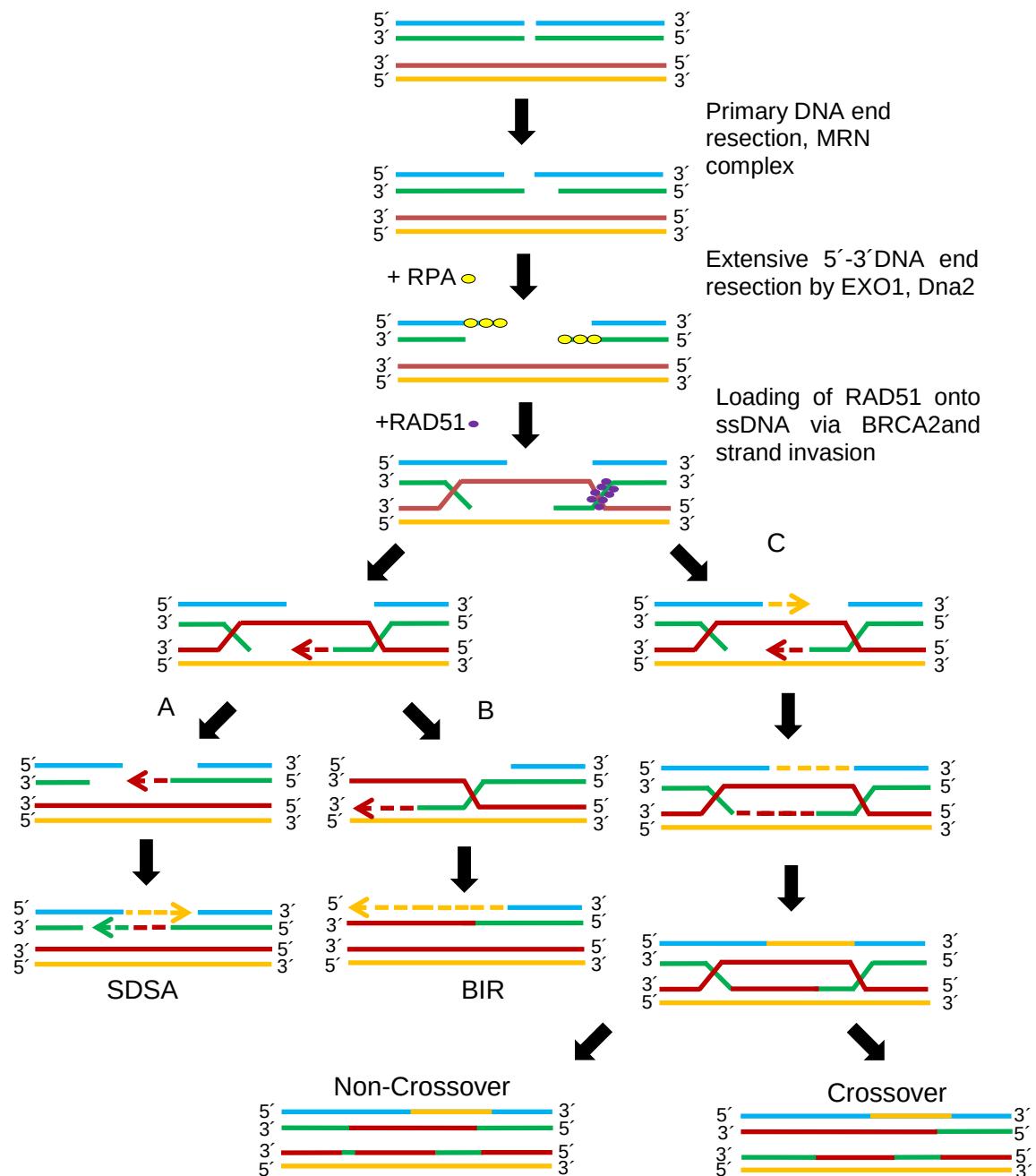


Figure 1.2: Model for DNA DSB repair by homologous recombination (HR). Once a DNA DSB is encountered and the HR pathway is activated, primary 3'-5' DNA end resection is mediated by the MRN complex (MRE11, NBS1 and RAD50). Extensive resection is performed by exonucleases such as EXO1 or Dna2. RPA binds the ssDNA preventing it from forming secondary structures. RAD51 replaces RPA and forms nucleofilaments that mediate the homology search and strand invasion, generating the Holliday junction. The DNA DSB can be resolved via different ways depending on the type of break. HR via synthesis dependent strand annealing (SDSA) (**A**) results in a non-crossover product. Thereby, the invading 3' strand is extended along the template DNA but released as the Holliday junction would slide along the DNA. The newly synthesised 3' strand anneals with the other 3' overhang of the damaged DNA strand by complementary base pairing and the remaining gap is filled and ligated. **B**: Break-induced replication (BIR) is performed if DSBs are encountered at replication forks. The D-loop is converted into a replication fork and DNA synthesis proceeds until the end of the sister chromatid. **C**: Both 3' DNA overhangs are extended using the sister chromatid as template. Resolution of the Holliday junction can result in two products, non-crossover or crossover. Adopted from Li and Heyer, 2008.

RAD54 invades the sister chromatid and performs homology search, thereby forming a D-loop intermediate DNA structure that leads to Holliday junction formation^{4,156}. Then, DNA synthesis occurs on the invading DNA strand, which is followed by LIG1-dependent sealing of the DNA ends¹⁵⁷. Resolving the DNA crossover (Holliday junctions) by cleavage and ligation to obtain two intact DNA molecules is the final step of HR^{158,159}.

HR is generally an error-free DSB repair pathway. However, one variant of this pathway, single-strand annealing (SSA), repairs the DNA in an error-prone way. This variant is independent of the homologous sister chromatid, but requires the presence of two direct repeats with more than 30 base pairs to flank the DSB. The direct repeats are aligned and annealed, resulting in the restoration of the DNA duplex strand. Thereby, the nucleotides located in between the direct repeats are deleted, resulting in the loss of genetic material^{160,161}. Two more HR variants exist that repair the DNA in an error free manner: synthesis dependent strand annealing (SDSA) and break-induced replication (BIR). Both variants result in a non-crossover product. In SDSA the invading 3' DNA is extended by DNA polymerases in the D-loop until the break is passed. Then, the newly synthesised DNA is released and joined with the 3' end of the previously damaged strand, whereas the template is sealed back with its original strand^{131,158}. BIR requires long range DNA synthesis and uses the entire sister chromatid downstream of the break as template for repair, resulting in the loss of heterozygosity between the sister chromatids^{158,162}.

1.4.2.2 Non-homologous end joining (NHEJ)

The majority of DNA DSBs that are induced by ionizing radiation, generating chemically heterogeneous ends, as well as breaks produced during immune cell maturation, are repaired via NHEJ. This repair pathway is active throughout the cell cycle and proceeds in three steps: break recognition, DNA end processing and ligation of the ends. This error-

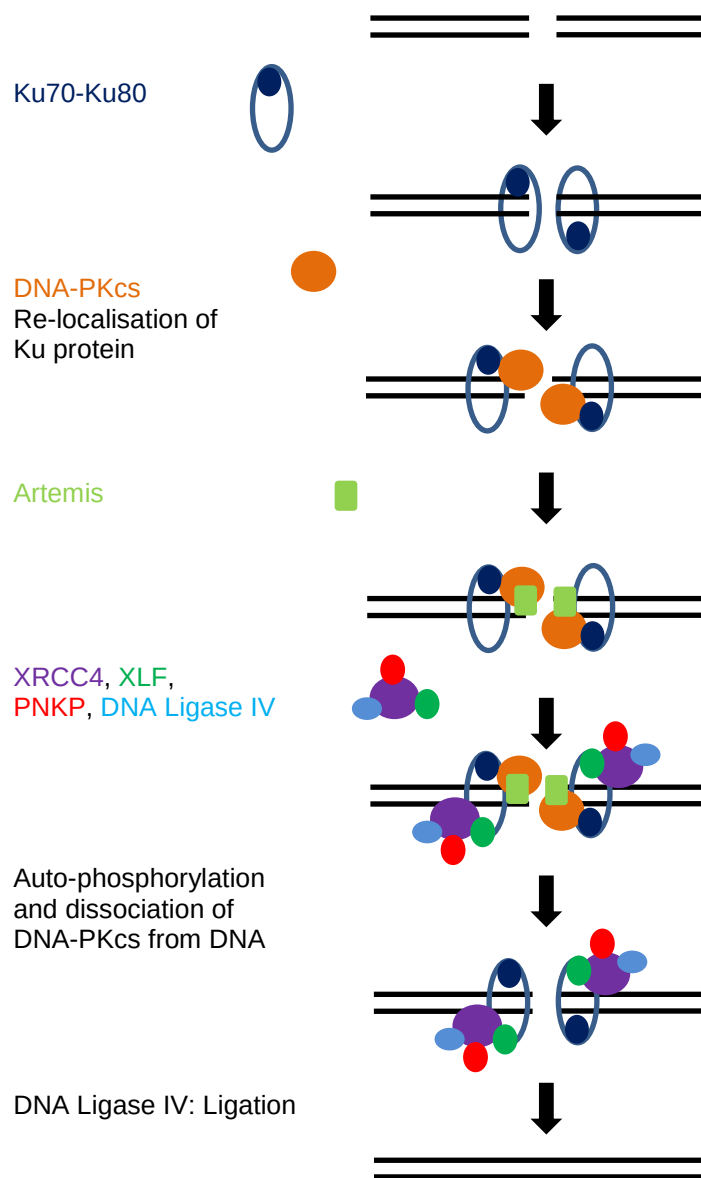


Figure 1.3: Model for DNA DSB repair via non-homologues end joining (NHEJ). The DNA DSB is encountered by the Ku70/Ku80 heterodimer, which forms a complex with the DNA dependent protein kinase DNA-PKcs. This results in the activation of the kinase activity and re-localisation of the Ku proteins. DNA-PKcs recruits and activates the nuclease Artemis, which mediates DNA end processing if necessary. The scaffold protein XRCC1 binds to the DNA break and mediates the recruitment of various downstream repair factors such as XLF, PNKP, and DNA Ligase IV. PNKP further processes DNA to create a ligatable DNA substrate. Auto-phosphorylation of DNA-PKcs results in its dissociation from the break, making the DNA accessible for ligation, which is mediated by the DNA Ligase IV. Adopted from Collis *et al.* 2005.

prone DSB repair pathway does not necessarily require resection and results in deletions of less than 10 base pairs^{163–166}. The DSB is recognised independent of the DNA structure and mostly independent of the sequence by the Ku heterodimer, consisting of Ku70 and Ku80 proteins. *In vitro* data show that once bound, the Ku heterodimer can slide along the DNA enabling the binding of further Ku proteins to the DSB^{167–169}. Ku binding protects the DNA ends from degradation and therefore inhibits the HR pathway. The Ku heterodimer also serves as a scaffold and directly interacts with downstream repair factors^{170,171}. As such, Ku recruits the DNA-dependent protein kinase catalytic subunit (DNA-PKcs), resulting in translocation of Ku and the positioning of DNA-PKcs at DNA termini^{163,172–174}. DNA interaction subsequently activates the kinase activity of DNA-PKcs, which is essential for NHEJ. DNA-PKcs phosphorylates several proteins involved in this pathway such as Ku70/80, XRCC4, Artemis, DNA Ligase IV *in vitro*.^{111,165,175,176} However, the significance of DNA-PKcs dependent phosphorylation events *in vivo* have yet to be determined¹¹¹.

In vitro data illustrate that Artemis, which is involved in DNA processing, is recruited to DSBs via interaction with DNA-PKcs¹⁷⁷. Artemis possesses 5′ to 3′ DNA single-strand exonuclease activity. Upon phosphorylation and interaction with DNA-PKcs, Artemis acquires an endonucleolytic activity, which enables it to process 5′ and 3′ DNA overhangs, and an activity critical for the hairpin-opening during V(D)J recombination *in vitro*^{175,177}. The scaffolding protein XRCC4 is recruited to DSBs by interaction with the Ku heterodimer^{172,178}. XRCC4 tightly binds to XRCC4-like factor (XLF), DNA Ligase IV and the polynucleotide kinase/phosphatase (PNKP), thereby recruiting essential factors for the last steps of NHEJ^{178–181}. PNKP removes 3′ phosphate groups prior to ligation. The MRN complex is another processing factor that can mediate DNA end resection to generate ligatable DNA ends^{163,182,183}.

Following end processing, the DNA strands are re-joined by Ligase IV, whose activity is stimulated by XRCC4^{178,184}. If the DNA ends are not compatible for ligation, the X family DNA polymerases, such as Pol λ or Pol μ , fill the DNA gap^{185–187}. Prior to the DNA ligation, DNA-PKcs auto-phosphorylates, resulting in the dissociation of the kinase from the nucleic acid, liberating the DNA ends and enabling the ligation^{163,188,189}. Figure 1.3 shows a simplified model of the NHEJ pathway.

1.4.2.3 Alternative non-homologous end joining (alt-NHEJ)

The first evidence of alt-NHEJ was collected by Albertini *et al.*, who showed that spontaneous nucleotide deletions of various length preferable occur in close proximity to short microhomologies using bacteria¹⁹⁰. The existence of a DSB repair pathway that is dependent on two or more bases of microhomologies for DNA ligation was demonstrated a few years later¹⁹¹. Two different studies illustrated that a low amount of DSBs in Ku80-deficient yeast and mice is repaired via an error-prone repair pathway that requires microhomologies to flank the DSB, resulting in nucleotide deletions^{116,117}. Alt-NHEJ shows slower kinetics than NHEJ, and mice models lacking an essential NHEJ protein are sensitive to ionizing radiation, indicating that alt-NHEJ is not sufficient to ensure viability^{192,193}. Hence, alt-NHEJ was considered to be active only when NHEJ is compromised and was originally classified as a backup DSB repair pathway. However, 10-20% of DSBs that are thought to be resolved via HR are actually channelled through alt-NHEJ in mammalian cells¹⁹⁴. This verified that alt-NHEJ is active in cells that are capable of HR and NHEJ. Furthermore, combined deletion of HR and alt-NHEJ is embryonically lethal for mice, and knockdown of the alt-NHEJ essential factor *POLQ* increases the cell death of HR-defective ovarian cancer cells¹⁹⁵. This indicates that alt-NHEJ is an important DSB repair pathway to confer viability of HR-dysfunctional cells.

Alt-NHEJ is employed throughout the cell cycle, but is functionally enhanced during S-phase and especially active in response to replicative stress^{196–198}. Furthermore, this pathway is engaged following excessive resection, when HR is aborted or if the nucleic acid serves as poor NHEJ substrate¹⁹⁹. Besides, extensive sequencing of the DNA flanking the repaired DSB revealed that microhomologies are not required for alt-NHEJ. However a subset of this pathway depends on a 2-25bp microhomology region to flank the DSB. Thus alt-NHEJ is also referred to as microhomology-mediated end-joining (MMEJ)^{120,200,201}.

The exact mechanism and the functions of the proteins involved in this pathway are still to be defined in detail. A proposed model suggests four steps of this repair pathway: DNA resection, microhomology annealing, nucleotide insertion and ligation²⁰². Audebert *et al.* first suggested that PARP1 is involved in an alternative pathway to re-ligate DNA DSBs²⁰³. Further studies verified that recognition of DSBs is mediated by PARP1, which directly competes with Ku for binding to DNA ends^{203–207}. Studies in mouse embryonic fibroblast and human cell lines showed that MRE11 and CtIP recognise and interact with the DSB, initiating DNA end processing, which is required for the progression of alt-NHEJ^{208,209}. As such, alt-NHEJ and HR use the same initial DNA end resection machinery, however, alt-NHEJ, as opposed to HR, only requires limited DNA end processing¹⁹⁴.

Following DNA end processing, the DNA polymerase θ (POLQ), containing a helicase and polymerase domain, plays an essential role in alt-NHEJ. It is proposed that POLQ binds as dimer to the resected DNA strands, stabilises the DNA synapse and facilitates the interaction of both DNA overhangs. POLQ also mediates microhomology annealing, catalyses dNTP incorporation and overhang extension^{198,210,211}. The importance of POLQ is confirmed by various studies, showing that POLQ-dependent alt-NHEJ is required for the survival of cells deficient in HR^{195,212}. Before POLQ can extend the overhang the

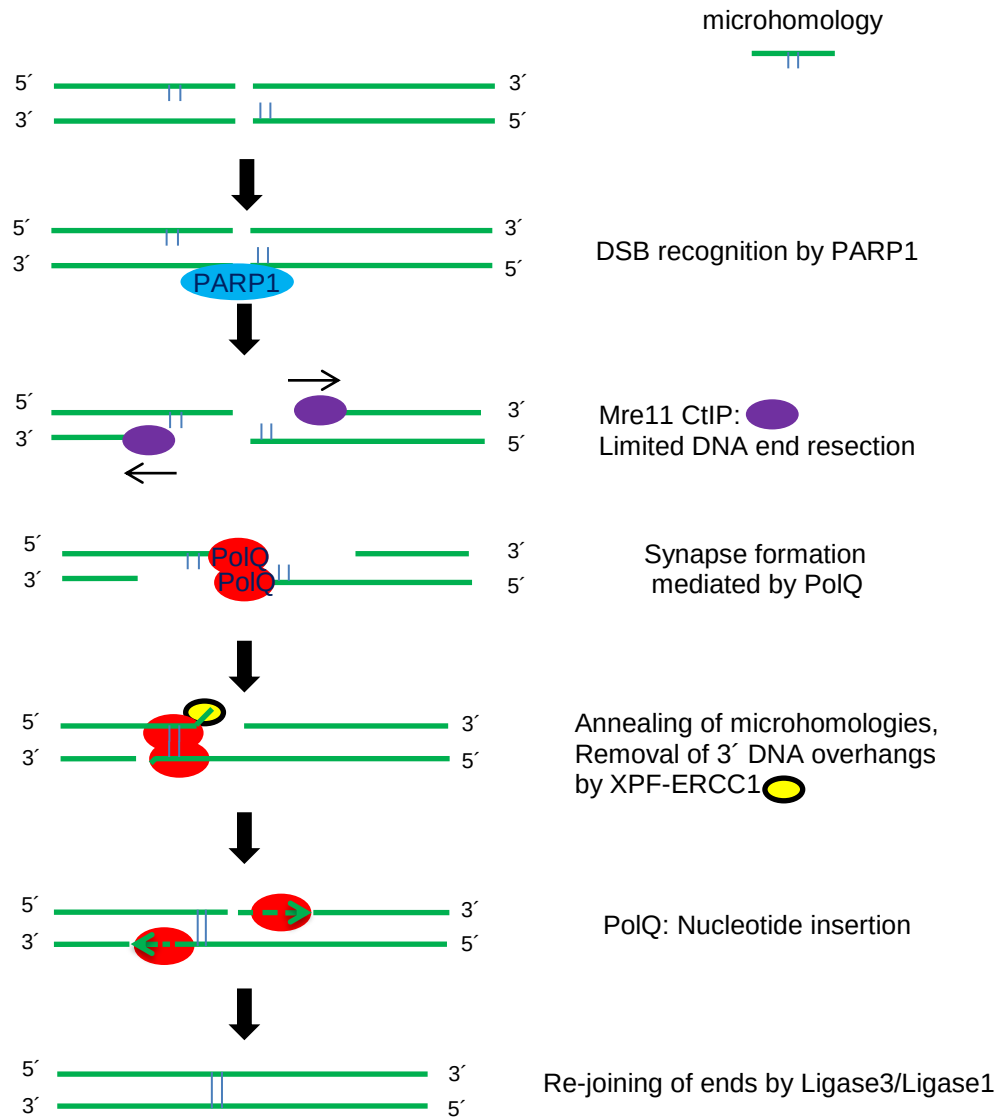


Figure 1.4: Model for DNA DSB repair via alternative non-homologues end joining using a 2 nucleotide microhomology (alt-NHEJ). The DNA DSB is detected by PARP1. Then, the Mre11 and/or CtIP mediate DNA end resection. PolQ forms a dimer interacting with both DNA ends and thereby promotes microhomologies annealing. The non-homologous 3' DNA overhangs are removed by endonucleases such as XPF-ERCC1. Then, PolQ fills in the missing nucleotides and Lig3 or Lig1 ligates the DNA ends together. Adopted from Wang and Xu, 2017.

3'flaps, that are generated during microhomology annealing, need to be removed by the structure-specific endonuclease XPF-ERCC1^{202,213–216}. Several studies show that rejoining of the DNA ends is catalysed by the DNA Ligase III (LIG3), with its redundant factor DNA Ligase I (LIG1) as backup ligase^{203,217–219}. Figure 1.4 shows a simplified model of the alt-NHEJ pathway.

1.4.3 PARPs in DSB repair

When a DNA DSB is encountered, the chromatin surrounding the lesion is ADP-ribosylated, resulting in the recruitment of remodelling factors. This in turn restructures and modifies the chromatin, rendering the DNA DSB accessible for downstream repair factors, such as BRCA1 and BRCA2^{220–225}. Hence, besides its direct involvement in DDR, PARP enzymes initiate chromatin modification to induce DNA repair. So far, PARP1-3, PARP10 and PARP14, are known to be involved in the DDR in mammalian cells, with all of them being part of the DSB repair machinery. PARP1 was proposed to bind to stalled replication forks, as it mediates replication fork restart *in vitro* and is enriched on chromatin following DNA damage in S-phase *in vivo*^{226–228}. Furthermore, PARPi-treated mouse embryonic fibroblasts (MEFs) and *Parp1*^{-/-} MEFs are both sensitive to agents that induce replication stress²²⁷. Moreover, *PARP1*-depleted HeLa cells are hypersensitive to γ-irradiation in S-phase⁹⁹. Another study showed that *PARP1* knockout decreases the viability of embryonic stem cells in response to replication fork damage, indicating that PARP1 is necessary for the restart of stalled replication forks²²⁶. Interestingly, according to Bryant *et al.* depletion of either *PARP1* or *PARP2* reduces Rad51 foci formation and sensitises cells to replication stress, indicating that both enzymes are involved in restarting stalled replication forks²²⁷. Furthermore, different studies illustrate that PARP1 and PARP2 promote Mre11-dependent DNA end resection, thereby inducing HR^{227,229}. However, other data reveal that PARPi increase Mre11-dependent DNA resection of stalled replication forks in BRCA2-defective VC-8 cells, indicating that PARPs protect the

ends of stalled replication forks from degradation in the absence of BRCA2²³⁰. Overall, PARP1 and PARP2 play a role in HR-induction upon replication stress, but the exact function has not been determined yet.

PARP3 is recruited to DNA DSBs, interacts with several proteins involved in NHEJ, such as DNA-PKcs, Ku70, Ku80 and DNA Ligase IV, thereby promoting this DSB repair pathway. Depletion of *PARP3* reduces the recruitment of Ku80 to laser induced DNA damage sites, which impairs NHEJ and potentially promotes recombination²³¹. Furthermore, the aprataxin and PNKP like factor (APLF) binds to ADP-ribosylated PARP3, resulting in its recruitment to DNA DSBs.^{84,232} APLF is associated with the retention of the NHEJ factors XRCC4/DNA Ligase IV to chromatin, thereby promoting re-joining of the DNA ends.^{84,233} PARP3 has also been implicated in ADP-ribosylation of histone H1 and H2B following DSBs *in vitro*^{108,234}. Rulten *et al* verified that histone H1.2 is ADP-ribosylated *in vitro*, which is potentially involved in the recruitment of APLF to the break⁸⁴. The role of PARP2 is less studied than the one of PARP1 or PARP3. However, PARP2 is implicated to play a minor role in NHEJ, especially during V(D)J recombination as its deficiency combined with a p53-defect results in the accumulation of unrepaired DSBs in developing T-cells, thereby enhancing T-cell lymphoma development^{235,236}.

PARP1 is also implicated in DNA DSB repair by alt-NHEJ²⁰³. PARPi treatment of NHEJ-defective cells increases the sensitivity to ionizing radiation, indicating that PARPs are involved in a repair mechanism other than NHEJ²⁰⁴. Accordingly, *Parp1*^{-/-} cells exhibit impaired DSB re-joining with only 50% re-ligated DNA ends within 60 minutes after ionizing radiation, underlining the importance of PARP1 in DSB repair²³⁷. PARP1 binds to DNA DSBs via its first zinc finger domain and directly interacts with LIG3 promoting alt-NHEJ^{203,204,206,218,219}. However, PARP1 directly competes with the Ku heterodimer for binding to the DNA break *in vivo*. As PARP1 and Ku70/80 possess similar expression levels in human tissues, whereas Ku70/80 demonstrates a higher affinity to DNA ends

than PARP1, this limits the rate of DSB repair via alt-NHEJ^{204,206,238}. Furthermore, PARP1 is implicated to ADP-ribosylate histone H1, which potentially activates LIG3 and therefore enhances DNA ligation in alt-NHEJ. PARP1 itself is also associated with the enrichment of LIG3 at damaged chromatin, further promoting the re-joining of DNA ends²⁰⁶. Localisation of PolQ at DSBs is reduced in U2OS cells depleted of *PARP1* by siRNA or treated with PARPi, providing further evidence for the activity of PARP1 in alt-NHEJ²¹².

It was recently discovered that PARP10 interacts with PCNA, which regulates DNA replication and S-phase coupled repair, in HeLa cells. Furthermore, PARP10 deficiency results in genomic instability and it was proposed that this enzyme is involved in the restart of stalled replication forks^{44,45}. Nicolae *et al.* also discovered the importance of PARP14 for HR. RAD51 is proposed to be MARYlated by PARP14 in response to DNA DSBs, which induces strand invasion of RAD51 nucleofilaments⁴⁴. However, further studies are necessary to verify the role of PARP10 and PARP14 in DNA DSB repair.

1.5 PARP inhibition in the clinic

1.5.1 HR deficiency is associated with a high risk of developing cancer

Since their discovery in the early 1990 by analysing the DNA of families with an autosomal dominant occurrence of breast and ovarian cancer, the tumour suppressor proteins BRCA1 and BRCA2 have been studied extensively^{239–242}. Both proteins are required for accurate DSB repair via HR and loss of either is associated with chromosomal aberrations, increased radio sensitivity and a high risk of developing cancer^{136,243–245}. Homozygous inactivation of *BRCA1* or *BRCA2* is usually embryonically lethal, however, a heterozygous defect of one of the factors renders cells viable, but predisposed for cancer, such as breast and ovarian cancer^{240,242,246}. Before tumour

development, the cells lose their intact *BRCA1/2* allele, leaving them with the inactive protein, indicating that certain cancers mediate cell proliferation in the absence of HR¹¹⁸. Strikingly, carriers of *BRCA1* and *BRCA2* mutations have a lifetime risk of approximately 80% and 40%, respectively, to develop breast and ovarian cancer^{154,247–249}.

As cells, that are deficient in HR essential factors, such as RAD51, *BRCA1*, *BRCA2* or EXO1, are hypersensitive to γ -irradiation, this verifies that DNA damage is lethal for HR-compromised cells^{145,244,245,250}. This further indicates that besides prone to cancer, HR dysfunction sensitises cells to DNA damaging agents, such as chemo- and radio-therapy, which is exploited in the clinic to target *BRCA1/2*-mutated tumours.²⁵¹ Interestingly, patients with a germline mutation in *BRCA1* and *BRCA2* have a more favourable clinical course and are associated with longer survival rates than patients with cancer that is not associated with a HR-deficiency^{252–254}. Furthermore, patients with germline mutation in *BRCA1/2* respond better to platinum and non-platinum based chemotherapeutic treatments, such as cisplatin and carboplatin^{255,256}. In 2005, two independent studies illustrated that various cell lines defective in *BRCA1* or *BRCA2* are hypersensitive to PARP inhibition resulting in chromosomal instability and apoptosis^{257,258}. Hence, these data provide the platform for a more specific treatment of *BRCA1/2*-associated breast and ovarian tumours. Several PARPi have been developed in the last years and are currently being tested in clinical trials to treat *BRCA1/2*-defective breast and ovarian cancer patients. Two of them, Lynparza (olaparib) and rucaparib are already applied as therapy of patients with *BRCA*-mutated advanced ovarian cancer.

1.5.2 Synthetic lethality of HR deficiency and PARP inhibition

Synthetic lethality of two proteins arises when the deficiency of either alone is viable, but the loss of both leads to cell death²⁵⁹. This effect is described for cells that are defective in proteins essential for correct HR, such as *BRCA1* or *BRCA2*, and PARP inhibition *in vitro*

and *in vivo*^{257,258}. Initial studies classified *Parp1* knockout mice as viable and healthy²⁶⁰. However, DNA repair defects in *Parp1* deficient mice were discovered a few years later, making it an important protein for genomic integrity⁹⁰. Loss of PARP1 renders cells impaired in SSB repair, resulting in chromosomal instability and an increased rate of apoptosis^{91,98,261}. Accordingly, *Parp1*^{-/-} mice are sensitive to alkylating agents and γ -irradiation but are not prone to developing cancer^{89,260,262}. Hence, although more susceptible for DNA damage, cells and mice deficient in PARP1 are viable. As explained previously, HR defective cells are viable, but prone to cancer and hypersensitive to DNA damage^{145,244,245,250}. Thus, a deficiency of HR or PARP alone is viable, but a defect in both is synthetically lethal^{257,258}.

PARP inhibition induces RAD51 foci formation and H2AX phosphorylation, indicating that this drug generates DNA damage that is channelled through HR in wild type cells²⁵⁷. Furthermore, PARPi exposure of cells defective in BRCA1 or BRCA2 results in chromosomal instability and cell cycle arrest, leading to apoptosis^{257,258}. Interestingly, PARPi treatment of mice transplanted with BRCA2-deficient cancer cells inhibited tumour formation, whereas tumour growth in animals that were injected with wild-type cells was not affected by the treatment²⁵⁸. This indicates that whilst cancer cells that are deficient in HR are killed when exposed to PARPi, surrounding cells competent in this DSB repair pathway survive the treatment²⁶³. Hence, this synthetic lethal interaction is used in the clinic to specifically target tumour cells defective in the HR pathway.

Interestingly, PARP1 is hyper-activated in replicating HR-defective cells indicating that replication associated damage is repaired in a PARP1-dependent way if HR is absent²⁶⁴. Furthermore, PARPi that are more specific for PARP1/2 significantly decrease the cell viability of BRCA1- and BRCA2-defective cells, whereas unspecific compounds had no effect²⁶³. This indicates that the main PARPs conferring synthetic lethality are PARP1/2.

Whilst PARP1 is involved in different damage repair pathways including SSBR, alt-NHEJ and possibly for the restart of stalled replication forks, PARP2 plays a role in SSBR. Hence, the inhibitors developed to treat HR-defective cancer cells are supposed to specifically target PARP1/2. However, when comparing the IC₅₀ values of the agents used in the clinic, it became apparent that the drugs not only inhibit PARP1/2 but due to the high similarity in structure and catalytic activity of the enzyme family also target other PARPs, especially PARP3^{234,265–267}. Due to the lack of specific inhibitors, it is unclear whether the inhibition of other PARPs positively or negatively affects synthetic lethality of HR-defective cells.

1.5.3 Development of PARPi

Once activated PARPs transfer an ADP-ribose moiety from NAD⁺ onto a substrate or auto-PARYlate. Thereby, the enzymes produce nicotinamide, which in turn weakly inhibits the catalytic activity of the enzymes. This feedback inhibition is important for the control of the NAD⁺ levels in the cell²⁶⁸. Due to this identified inhibitory effect several of the developed PARPi are nicotinamide analogues. The first inhibitors, which were 90% more effective in inhibiting PARPs than nicotinamide, were generated by replacing the heterocyclic nitrogen at the 3 position with a carbon, to form benzamide analogues. These drugs also had the advantage of not being metabolised by NAD biosynthetic enzymes^{269,270}. However, some of the first developed PARPi were highly insoluble and had to be applied in high concentrations, leaving them unsuitable for the use in animals. Besides, some of the drugs were quite unspecific and target other enzymes than PARPs, such as the NAD-dependent lysine deacylase activity of sirtuins, which is important for various cellular processes^{271,272}. Nevertheless, benzamide/nicotinamide analogues provided a good platform for the development of more specialised drugs with increased potency. The effective inhibitor PJ-34 was developed based on the second generation of agents, quinazoline analogues^{270,273,274}.

As an alternative approach, chemical drug synthesis, based on the analysis of the structure and activity relationship (SAR), was used for the generation of the highly potent 8-Hydroxy-2-methylquinazolin-4[3H]-one (Nu1025) and other effective compounds^{275,276}. Further structural studies determined that a carboxamide group in the second aromatic ring increases specificity and activity of the PARPi^{275,276}. Olaparib (lynparza), the first approved PARPi that is applied as monotherapy for *BRCA*-mutated advanced ovarian cancer, was developed as part of the third generation of PARPi^{277–282}. Furthermore, rucaparib has recently been approved (December 2016) by the FDA as therapy of patients with *BRCA*-mutated advanced ovarian cancer who have previously been treated with two or more chemotherapies.

1.5.4 Mechanism of PARP inhibition

Even a PARPi exposure of less than 24 hours kills HR-deficient cells. However, the exact inhibitory mechanism triggering the death of HR-dysfunctional cells remains an open question²⁶⁵. The first proposed model suggests that PARPi treatment leads to the accumulation of unrepaired SSBs. This was first described by Boulton *et al.* showing that SSB and DSB levels are increased in Chinese hamster ovary cells following PARPi exposure and γ -irradiation²⁸³. Further studies identified that PARPi treatment induces γ H2AX foci formation in *BRCA2*-deficient and -proficient cell lines, supporting the model that PARPi increase DSB levels^{257,258}. Once an unrepaired SSB is encountered during replication it leads to the collapse of the replication fork, forming toxic one ended DSBs. Those lesions are substrates for HR, as evidenced by an increase in RAD51 foci formation in *BRCA2*-proficient cells following PARPi treatment, and result in cell death of HR-deficient cells^{228,257,258}. However, *BRCA2*-defective cells do not exhibit increased SSBs following PARPi treatment²⁶⁴. Consistent with this, SSBs do not accumulate in *PARP1* depleted A549 and *PARP1*^{-/-} DT40 cells^{101,106}. As siRNA knockdown of *PARP1*

but not of *XRCC1* decreases the survival of BRCA2-deficient cells, this verifies that the role of PARP1 in SSB repair is not important for synthetic lethality of HR dysfunctional cells with PARPi²⁸⁴.

PARP inhibition delays SSB repair to a greater extent than *PARP1*-depletion, indicating that PARPi not only impede the activity of the enzymes^{101,106}. Hence, a PARP1 trapping model has been proposed based on the idea that PARPi promote tight interaction of PARP1 to DNA, preventing the release of the enzyme from damaged lesions^{101,265}. *In vivo* crosslinking and ChIP experiments showed, that the amount of DNA interacting with PARP1 was greater in samples treated with DNA damaging agents and PARPi than in cells exposed to only one of the agents²⁸⁵. Furthermore, in the presence of MMS, olaparib exposure increases the interaction of PARP1 and PARP2 with chromatin in several human cancer cell lines, which can be reversed upon drug removal, supporting the PARP1-trapping model²⁸⁶. Once PARP1 encounters a DNA damage it binds to the damaged DNA, resulting in the activation of its enzymatic activity, including auto-modification. PARP1 auto-ADP-ribosylation is necessary for the dissociation from DNA, making the damaged site accessible to facilitate DNA repair progression⁴⁰. By binding to the NAD⁺ binding site, PARPi impair the PARylation activity of the enzymes and thereby inhibit the dissociation of PARP from the damaged site²⁸⁷. It is further suggested that PARPi induce an allosteric conformational change of the enzyme, thereby stabilising the DNA-PARP complex^{287,288}. This traps PARPs onto the DNA, forming a barrier for replication fork progression and impeding DNA repair. It has not been directly studied yet whether and how trapping PARP onto the DNA stalls the progression of the replication fork. However, it is known that tight DNA-Protein complexes can cause replication fork pausing, which may require recombination for its restart^{289,290}. Another possibility is that following PARPi treatment DNA damage persists due to the inaccessibility of the DNA lesion. During S-phase the replication fork stalls once it encounters the trapped PARP and the unrepaired DNA damage²⁹⁰. HR is essential for the viability of PARPi treated cells

as it is required to bypass the trapped PARP and repair the DNA damage to facilitate the restart of the replication fork^{101,265,286}. Downregulation of PARP1 expression levels but not of PARP2 reduces the sensitivity of HR-defective cells to olaparib, indicating that PARP1 is the major mediator of PARPi-induced synthetic lethality of HR dysfunctional cells²⁸⁷.

A third model, explaining the lethal effect of PARPi on HR-compromised cells, is based on data showing that during S-Phase PARP1 is hyper-activated in BRCA2-defective cells²⁶⁴. Previously, it was illustrated that *PARP1*^{-/-} cells exhibit an increase in RAD51 foci formation after stalling replication forks due to hydroxyurea (HU) treatment. This indicates an elevated HR activity in PARP1-deficient strains and a potential role of PARP1 in the restart of stalled replication forks²²⁶. Besides, DNA end resection in *Parp1*^{-/-} mouse embryonic fibroblasts is impaired following HU treatment, suggesting that PARP1 is involved in resolving replication stress²²⁷. This indicates that restart of stalled replication forks can potentially be mediated by two pathways, either via HR or dependent on PARP1^{264,265}. Whilst the deficiency of one of the two is viable, lacking both mechanisms is lethal for the cells.

1.5.5 Resistance of HR-defective tumours to PARPi in the clinic

Due to synthetic lethality of PARP inhibition with HR dysfunction, several PARPi are being tested in clinical trials as therapy for different types of tumours that are associated with a HR deficiency but especially for *BRCA*-mutated breast and ovarian cancers. Two of these drugs are already approved as monotherapy by the FDA for the treatment of advanced ovarian cancer that is associated with mutations in one of the *BRCA* genes. However, despite the promising results in the clinic, some tumours develop a resistance to this treatment^{251,282,291}. Studies in genetically engineered mouse models for *BRCA1*-associated breast cancer showed that some mice upregulated the expression of genes encoding for drug efflux pumps following long term treatment with PARPi, resulting in drug

resistance. This can be circumvented by the combined treatment of PARPi with either an inhibitor targeting the drug efflux pumps, or by chemotherapeutic agents, such as cisplatin or carboplatin, indicating that inducing DNA damage potentiates the effect of PARPi^{292,293}. However, attempts in reversing the drug resistance by targeting the efflux pumps failed in clinical trials, indicating that a reduction in the drug concentration due to the pumps is not the main resistance mechanism^{293,294}.

Several studies illustrate that restoration of HR renders BRCA1-defective cells refractory to PARPi. 53BP1 antagonizes BRCA1 in the pathway choice between HR and NHEJ, promoting the latter^{133,295,296}. Interestingly, *Brca1*^{Δ11/Δ11}*53BP1*^{-/-} mice, lacking full length BRCA1 as well as 53BP1, are genomically unstable, yet not prone to tumour formation in the first 20 months of their life²⁹⁷. Furthermore, cells deficient in BRCA1 and 53BP1 form RAD51 foci following γ-irradiation similar to wildtype cells, whereas 53BP1-proficient and BRCA1-deficient cells only exhibit a low number of RAD51 foci¹³³. Moreover, mice transplanted with cells that are defective in BRCA1 and depleted of *53BP1* grew tumours that were non-responsive to PARPi, whereas control tumours expressing 53BP1 were sensitive²⁹⁸. This suggests that loss of 53BP1 results in restoration of HR in BRCA1-defective cells, which renders the cells resistant to PARPi. Furthermore, knockdown of NHEJ factors such as *KU80* and *ARTEMIS* diminishes the PARPi toxicity to BRCA2-deficient ovarian cancer cell lines^{284,291}. A similar loss of sensitivity to PARPi was detected when ovarian cancer cell lines were exposed to DNA-PKcs inhibitors²⁸⁴. This indicates that downregulation of essential NHEJ components renders BRCA2-deficient cells refractory to PARPi.

Similarly, depletion of the DNA Helicase B (*HELB*) leads to the resistance of BRCA1-deficient cells to PARPi. HELB directly binds to ssDNA and controls DNA processing dependent on the stage of the cell cycle^{299,300}. *In vitro* data show that HELB inhibits DNA

resection by MRN, BLM-Dna2 and EXO1 and therefore, antagonizes HR. During M and G1 phase HELB is present in the nucleus. As cells enter S-phase CDK phosphorylates HELB, resulting in the export of the helicase from the nucleus, allowing repair via HR³⁰¹. Interestingly, *Helb* depletion in *Brca1*^{-/-}*p53*^{-/-} mouse mammary tumour cell lines restores RAD51 foci formation and renders the cells resistant to PARPi. This indicates that loss of HELB reactivates HR potentially by the loss of nuclease inhibition, which suppresses PARPi toxicity³⁰⁰. Xu *et al.* discovered that depletion of the NHEJ promoting factor *Rev7* similarly results in the tolerance of *Brca1*^{-/-}*p53*^{-/-} mouse mammary tumour cells to PARPi³⁰². Furthermore, *Rev7* knockdown restores DNA end resection and RAD51 foci formation in *Brca1*^{-/-}*p53*^{-/-} cells, indicating that loss of REV7 re-activates HR, resulting in the resistance to PARPi³⁰². ATM inhibitor treatment of *Brca1*^{-/-}*p53*^{-/-} mouse cells depleted of *Helb* or *Rev7* abolishes RAD51 foci formation and furthermore re-sensitises cells to PARPi^{300,302}. This indicates that reactivation of HR and resistance to PARPi is dependent on the ATM kinase activity in these cells.

Studying breast cancer stem cells (CSC), Liu *et al.* identified that RAD51 expression levels can determine the sensitivity of BRCA1-deficient cells to PARPi. Overexpression of RAD51 compensates for the absence of BRCA1 and restores HR, resulting in the resistance to PARPi, whereas targeted knockdown of *RAD51* by shRNA sensitises the cells to PARPi³⁰³.

BRCA1- and *BRCA2*-mutated tumours can acquire resistance to chemotherapeutic agents as well as PARPi through secondary mutations after an initial clinical response³⁰⁴⁻³⁰⁸. For example, the pancreatic cancer cell line Capan-1 exhibits a frameshift mutation in *BRCA2*, resulting in the expression of a truncated *BRCA2*, which is defective in promoting damage induced RAD51 foci formation^{307,309}. This cell line is highly sensitive to PARPi but can acquire resistance to the treatment due to a mutation in the *BRCA2* gene, generating

a new isoform that restores the open reading frame, resulting in the expressing of an almost full length BRCA2³⁰⁷. Another resistance mechanism was identified using the PARPi sensitive breast cancer cell line SUM1315MO2, which harbours a hemizygous *BRCA1* mutation, producing a non-functional and shortened protein. Some of these breast cancer cells survive PARPi exposure due to expressing a truncated BRCA1 by employing a translational start site downstream of the mutation. This truncated BRCA1 version facilitates RAD51 foci formation, which re-activates HR and therefore renders the cells tolerant to PARPi³⁰⁶.

Previous data illustrate that breast cancer cells exhibit several alterations in DNA sequences and copy numbers³¹⁰. Furthermore, an abnormal number of chromosomes, which is often detected in cancer cells is associated with a significantly increased mutation rate compared to diploid cells³¹¹. Hence, due to the high mutagenic rate of cancer cells, further yet undiscovered alteration are probably involved in resistance mechanisms of HR defective cells to PARPi³¹². However, despite the observed resistance, the positive effect of PARPi in cancer therapy is still obvious, making them an effective agent for specific types of tumours. Besides the application as monotherapy, PARPi are being tested in combination with radio- and chemo-therapy to enhance their lethal effect on HR-defective tumour cells³¹³.

1.6 Model organisms to study ADP-ribosylation

All species require repairing DNA damage, which occurs throughout their life, to maintain genome integrity. One of the most convenient model organisms to study DNA repair mechanisms are bacteria, which exhibit all major repair pathways. ADP-ribosylation was initially identified as a pathogenic mechanism of bacterial toxins, such as pertussis and cholera toxin^{14,314,315}. However, in those prokaryotic cells only MARTs have been identified that are not involved in the DNA repair machinery. Furthermore, due to the very simple

form of DNA repair in bacteria the molecular function and interaction between the proteins might be different compared to eukaryotic cells. As such, bacteria are an unsuitable model organism to study complex DNA repair mechanisms. Another single cell model organism used to study DNA repair is the budding yeast, *S.cerevisiae*. Several proteins important for the HR pathway such as RAD51, RAD50, MRE11 have been identified by a genetic screen using this model^{316,317}. Hence, the budding yeast is an ideal model organism for studying DNA repair processes, due to the conservation of various proteins involved in recombination from yeast to human as well as its genetic tractability^{318,319}. However, as *S.cerevisiae* lack proteins containing an ART catalytic domain as opposed to 17 PARP family members in mammalian cells, it is an unsuitable organism to study ADP-ribosylation events important for DDR³²⁰.

Hence, other model organisms are required to investigate DNA damage responsive ADP-ribosylation. For example, the nematode worm *C.elegans* has proved to be a useful organism to elucidate the activity of PARPs during DNA repair. Orthologues of PARP1 and PARP2 have been identified in *C.elegans*, which are activated following DNA damage, with one of them even being involved in alt-NHEJ^{198,321–323}. Similarly, the fruit fly *Drosophila melanogaster* contains genes for two PARP enzymes^{35,320,324}. Interestingly, PolQ and its implication in alt-NHEJ was first discovered in the fruit fly and *C.elegans*, making both organisms suitable models to study DDR³²⁵. However, since only two PARPs were identified in *C.elegans* and *Drosophila melanogaster* so far, studying the activity of ADP-ribosylation is difficult in these organisms. Besides model organisms, specific cell lines might be employed to study the effect of ADP-ribosylation. For example, the chicken B-lymphocyte cell line DT40 naturally lack PARP2, making it a useful tool to identify the activity of PARP1 without the presence of its partially redundant PARP2^{326–328}. But due to the missing PARP2, the findings of the effect of ADP-ribosylation mediated by PARP1 cannot be directly transferred to humans. Surprisingly, *in silico* analysis revealed that the social amoeba *Dictyostelium discoideum* expresses 15 proteins containing ART catalytic

domains. Several of the enzymes show conservation with the PARPs involved in DDR in mammalian cells, making it a suitable model to study the effect of ADP-ribosylation in DNA repair^{329–331}.

1.7 *Dictyostelium* as model organism for DNA repair

1.7.1 The genetic model organism *Dictyostelium*

Dictyostelium discoideum is a eukaryotic social amoeba that is found in the soil and is feeding on bacteria in the wild. If food is available it grows as an individual cell. However, exhaustion of the food supply leads to its aggregation into a multicellular organism. Thereby, the cells differentiate either into stalk or spore cells, which is induced by extracellular cAMP signalling and communication between the cells. The *Dictyostelium* cells are forming a slug, which can either directly be transformed into a fruiting body or can move as motile slug to search for a more suitable environment. The spore cells form the tip of the fruiting body and are released to find a better feeding ground for germination, whereas the stalk cells die after differentiation^{332–334}. Whilst wild type *Dictyostelium* only feed on bacteria, cells that are used in the lab have been modified to grow axenically in shaking suspension, containing glucose^{335,336}. These axenic strains, referred to as Ax2 or Ax4 cells, are cultivated at 22°C with a doubling time of about 8 hours.

The *Dictyostelium* genome is divided into 6 haploid chromosomes, which contain around 12 200 protein coding genes on a total of 34Mb. The genome was recently fully sequenced despite the vast amount of complex repeats and the overall A-T rich genome (78%) complicating the process³³⁷. Interestingly, the chromatin structure with its histones seems to be well conserved with vertebrate organisms³³⁸. Disruption strains are generated by homologous recombination using a construct, containing an antibiotic

resistance gene flanked by sequences that are homologues to the targeted genomic locus. Recycling of the resistance gene is performed using the Cre-LoxP system, which is useful when double or triple knockout strains are prepared³³⁹. However, due to its haploidy, genes essential for living cannot be disrupted in *Dictyostelium* cells.

Dictyostelium have a simple lifecycle and reproduce generally asexual, although the cells are capable of a sexual cycle under certain conditions. Therefore, two cells fuse that contain different mating types out of the three potential ones^{337,340}. The cell cycle of vegetative axenic cells has been studied by various techniques. *Dictyostelium* cells exhibit a short mitotic phase, lasting approximately 10-20 minutes. As there is no detectable G1 period, the cells directly progress into S-Phase, which is no longer than 30 minutes³³⁴. The G2-phase with a length of 4-8 hours is by far the longest period of the *Dictyostelium* cell cycle³³⁴. However, the length of each phase is highly variable³⁴¹. Unlike other eukaryotic cell *Dictyostelium* can only be synchronised into G2, but not into G1-phase^{334,342}. Several studies suggest that differentiation into spore or stalk cells depends on the phase of the cell cycle at the time of starvation. Cells that are in the middle or in late G2-phase preferentially differentiate into spore cells, whereas cells that are in the other stages of the cell cycle develop into stalk cells³⁴³⁻³⁴⁶. However, due to the lack of markers to distinguish the cell cycle phases of living *Dictyostelium*, the correlation between the stage of the cell cycle and development into spore or stalk cells is still under debate.

1.7.2 *Dictyostelium* as model organism to study DDR

An interesting feature of *Dictyostelium* is the unusual conservation of DNA repair pathways and proteins with humans, making it an attractive model to study DDR. This organism shows conservation of several factors important for BER, HR and NHEJ³⁴⁷⁻³⁵⁰. The Ku proteins, identified a few years ago, show a very similar overall domain structure

compared to the human proteins. The C-terminus of *Dictyostelium* Ku80, containing the DNA-PKcs binding site, which is necessary for NHEJ in vertebrate cells, is highly similar to its human counterpart^{351,352}. Our laboratory and others identified Artemis and the protein kinase DNA-PKcs in *Dictyostelium*, with the latter being the first orthologue found in a single celled eukaryote, verifying further conservation of the NHEJ pathway in *Dictyostelium*^{349,353,354}. Besides, AP-endonucleases and an uracil DNA glycosylase, both involved in BER, have been identified in *Dictyostelium* cells, indicating that BER is also conserved in this model^{348,355,356}. Another DNA repair pathway, which seems to be conserved is nucleotide excision repair (NER) since several proteins involved in his pathway are expressed in *Dictyostelium*^{357–359}. Other DNA repair factors such as components required for the Fanconi anaemia pathway, are also present in *Dicytoystelium*³⁶⁰. Unsurprisingly, several proteins necessary for HR, which is conserved throughout all kingdoms of life, are expressed in this model organism³⁶¹. This includes the ATR kinase, promoting HR following DSB recognition, as well as Exo1, Rad51 and Mre11³⁵⁰. With the exception of few components necessary for human HR, such as Nbs1 which is part of the MRN complex with Mre11, Rad50, and the endonuclease CtIP, most proteins required for this pathway have been identified in *Dictyostelium*.

Although resumption of cell growth following DNA DSBs is impaired in NHEJ mutants, DNA damaging agents are not toxic for these strains, indicating that NHEJ is active but not essential for the survival of vegetative *Dictyostelium* cells. Interestingly, *Dictyostelium* spore cells lacking one of the Ku proteins or DNA-PKcs are defective in DNA DSB repair^{353,354}. It is proposed that spore cells are arrested in G1-phase and due to the absence of a sister chromatid required for HR rely on NHEJ to repair DNA damage^{353,362}. However, other data reveal that spore cells mostly reside in G2-phase, in which the cells are proficient in both DSB repair pathways^{341,354}.

Interestingly, *Dictyostelium* are very resistant to all sorts of DNA damage, including UV-light, X-rays or DNA damage inducing drugs and furthermore exhibit the lowest spontaneous mutation rate reported for any eukaryote^{363–365}. One potential reason for the resistance might be that *Dictyostelium* repair DNA DSBs mostly using highly accurate HR. This might be due to the fact that *Dictyostelium* are lacking the G1-phase of the cell cycle but exhibit a prolonged G2-phase, in which the homologous sister chromatids required for HR are present in the cell. However, HR is considered to be the main DNA repair pathway in most of the lower eukaryotes including yeast, which lack this resistance to genotoxic stress⁴. Hence, further investigations of the reduced sensitivity of *Dictyostelium* to genotoxic stress might give insights in resistance mechanism to DNA damaging agents of other organisms.

1.7.3 PARPs involved in DDR in *Dictyostelium*

The model organism *Dictyostelium* contains 15 genes with a putative ART catalytic domain with three of them potentially being involved in DDR: Adprt1a, Adprt1b and Adprt2^{320,329,331,366}. Similar to vertebrate cells, ADP-ribosylation occurs in *Dictyostelium* following DNA SSBs, indicating that this protein modification is important for repair. Disruption of *adprt2* renders the cells sensitive to base damage and DNA SSBs throughout the cell cycle³⁶⁷. Similarly, *adprt1b*⁻ cells are sensitive to DNA SSBs, suggesting that *adprt2* and *adprt1b* are necessary for repairing DNA SSBs, leading to the conclusion that the role of PARylation is largely conserved in *Dictyostelium*³⁶⁷. Surprisingly, ADP-ribosylation is still intact in *adprt2*⁻ and *adprt1b*⁻ cells. This indicates a redundancy in DNA SSB signalling between the enzymes, which is similar to vertebrate cells where PARP2 has been implicated to catalyse ADP-ribosylation following DNA SSBs in *Parp1*^{-/-} cells^{38,367,368}. However, transient depletion of PARP1 and PARP2 individually and in combination showed that PARP1 is the major enzyme mediating ADP-ribosylation following SSB in mammalian cells, whereas PARP2 only plays a minor role¹⁰⁶.

Knocking out *PARP1* and *PARP2* in combination proves to be difficult in mammalian cells, making it hard to investigate the potential redundancy in detail¹⁰⁴. Similarly, as combined disruption of *adprt1b* and *adprt2* was unsuccessful so far, it is unknown if both enzymes act together to signal SSBs³⁶⁸.

The *adprt2*⁻ cells exhibit reduced ADP-ribosylation following phleomycin treatment, indicating that this PARP might play a role in signalling DSB repair. However, disrupting *adprt1a* further decreases PARylation upon DSBs compared to *adprt2*⁻ cells, suggesting that Adprt1a is the main PARP involved in DSB repair. Furthermore, *adprt1a*⁻ cells are defective in the recruitment of Ku80 onto the chromatin following DNA damage. Hence, similar to PARP3, Adprt1a is promoting NHEJ to repair DNA DSBs^{367,368}. Although Adprt1a is dispensable for SSB signalling, combined disruption of *adprt1a* with *adprt2* further sensitises cells to agents that induce SSBs³⁶⁷. This either indicates that in the absence of Adprt2, Adprt1a takes over the role in signalling SSBs to maintain cell viability, or that SSBs are converted to DSBs, which are channelled through Adprt1a-dependent NHEJ^{367–369}. Interestingly, loss of *dnapkcs* does not sensitise cells to alkylating agents, but its disruption in *adprt2*⁻ cells further increases their sensitivity to SSBs. Besides, *adprt2*⁻ cells show an upregulation of Ku80 recruitment onto the chromatin following SSBs, supporting the hypothesis that unrepaired SSBs are converted to DSBs which are repaired via NHEJ in *Dictyostelium*³⁶⁸.

ADP-ribosylation is induced when cells are incubated with cisplatin, which introduces intra- and inter-strand crosslinks. Adprt2 has been identified to be the main PARP responsible for PARylation in response to this kind of damage in *Dictyostelium*. The conserved NHEJ factor Apl (Aprataxin/APPLf-and PNKP-Like protein) is capable of binding to Adprt2 mediated ADP-ribosylation following cisplatin exposure and is therefore potentially involved in resolving the damage³⁶⁹. Interestingly, in the absence of Adprt2,

Adprt1a responds to cisplatin treatment, further indicating a redundancy between the two enzymes.

Taken together, DNA DSB repair pathways and ADP-ribosylation in response to DNA strand damages are unusually conserved in *Dictyostelium*. This makes this model organism, in conjunction with its simple genetics and the high resistance to genotoxic stress, a useful tool to study DNA repair pathways and ADP-ribosylation following DNA damage.

1.8 Aims of this thesis

Although the concept of synthetic lethality between PARP inhibition and HR deficiency in human cells is well-known, the exact mechanisms resulting in cell death are still unclear. When HR is dysfunctional DNA DSBs are probably resolved via one of the other DSB repair pathways, which either induces genomic instability, or allows cells to tolerate PARPi to some degree^{195,212,284}. Whilst knockdown of the alt-NHEJ component *POLQ* enhances the lethality of HR compromised cells, NHEJ is proposed to be stimulated following PARPi treatment in HR-defective cells. This indicates that both pathways are possibly employed when HR is dysfunctional^{195,212,284}. Identifying which repair pathway is engaged in HR-deficient cells will help elucidate the molecular basis of synthetic lethality with PARPi, with the potential of giving information to improve the treatment of BRCA1/2-associated tumours. Due to the similarity of *Dictyostelium* DNA DSB repair pathways to human cells and the conservation of DNA-damage responsive ADP-ribosylation, the major aim of this thesis was to develop this system as a model to study the events following PARPi treatment in HR-defective cells. A further goal was to exploit the genetic tractability of *Dictyostelium* to disrupt DSB repair pathways in combination and assess the effect of DSBs and PARPi on HR-compromised cells.

It is proposed that inhibiting PARP1/PARP2 is lethal for HR-defective cells, whereas incubation with unspecific inhibitors has no effect²⁶³. Accordingly, PARPi such as olaparib that target the SSB-responsive enzymes PARP1/2 are the most promising and attractive compounds for the therapy of *BRCA*-mutated tumours due to their selectivity and lack of toxicity for normal cells³⁷⁰. However, because of structural similarity and similar mode of action other family members, especially PARP3, are targeted by these compounds, which might influence their lethal effect on HR-defective cells^{234,263,265,266}. Given the lack of an inhibitor solely targeting PARP1/2 it is still unclear whether synthetic lethality of HR-compromised cells is caused by inhibition of PARP1/2, or by a combinatory disruption of several PARPs that are involved in DDR. Therefore, I also wanted to assess the impact of HR dysfunction on cell viability in different PARP-defective backgrounds. Thereby, I sought to identify the most appropriate PARP to target, to kill HR-defective cells with a view of improving the treatment of HR-compromised tumours.

After an initial clinical response some HR-associated tumours develop a resistance to PARPi. A few factors involved in rendering HR-defective cells refractory to the treatment are already known, such as secondary mutations in HR genes or loss of the NHEJ-promoting factor 53BP1^{308,371}. However, as *BRCA1/BRCA2*-defective cancer cells are impaired in accurate HR and therefore highly mutagenic, it is most likely that several other gene alterations contribute to the resistance to PARPi³⁷². Since *Dictyostelium* possess one of the lowest mutation rates of nuclear DNA reported for any eukaryote, indicating a very low level of spontaneous variants, this model is ideal to identify mutations involved in PARPi tolerance of HR-dysfunctional cells³⁶⁵. Given our overall aim was to identify mechanisms resulting in the loss of PARPi toxicity, we first wanted to select PARPi-resistant HR-defective *Dictyostelium* cells using a genetic screen. Our goal was to identify novel unique mutations in protein coding genes that are involved in rendering *exo1⁻* cells resistant to PARPi.

Taken together, we wanted to investigate the mechanisms resulting in the death of HR-defective cells to PARPi using the genetically tractable model *Dictyostelium*. Furthermore, we sought to identify mechanisms rendering HR-dysfunctional cells resistant to PARPi as well as elucidate which PARP is the most appropriate to target to kill HR-defective cells. This might help to improve the treatment of patients with BRCA-mutated tumours.

2. Materials and Methods

2.1 Materials

Church Buffer

0.5 M NaHPO₄ (pH 7.2)

1% (w/v) bovine serum albumin fraction V

1 mM EDTA (pH 8)

7% (w/v) SDS

[Filter sterilised]

DNA loading dye

0.25% bromophenol blue

40% sucrose

Freezing media

47.5% HL5

47.5% fetal bovine serum (FBS)

5% DMSO

[from sterile stocks]

H50 Buffer

50 mM KCl

20 mM HEPES

10 mM NaCl

5 mM NaHCO₃

1 mM NaH₂PO₄·H₂O

1 mM $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$

[pH 7.0; filter sterilised]

HL5

53 mM maltose

1.4% bacteriological peptone

0.7% yeast extract

4 mM Na_2HPO_4

3.5 mM KH_2PO_4

340 μM dihydrostreptomycin sulphate

74 μM vitamin B12 (cyanocobalamin)

82 μM biotin

532 μM riboflavin

[pH 6.5; autoclaved; 25 ml 20% glucose added to 475 ml HL5 immediately before use]

IMAC buffer

50mM HEPES (pH 8.0)

150mM NaCl

10% Glycerol

1mM Imidazol

1mM DTT

KK2

19 mM KH_2PO_4

3.6 mM K_2HPO_4

[Autoclaved]

LB Agar

1% bactotryptone

0.5% yeast extract

85 mM NaCl

1.5% agar

[Autoclaved]

LB Broth

1% bactotryptone

0.5% yeast extract

85 mM NaCl

[Autoclaved]

MHK solution

40mM MgOAc

20mM KCl

50mM HEPES

NEB buffer

10mM Pipes (pH 6.8)

300mM Sucrose

3mM MgCl₂

20mM NaCl

0.5% Triton

NLB buffer

10mM MgOAc

10mM NaCl

30mM HEPES

10% Sucrose

2% NP-40

SDS-PAGE Running Buffer

25 mM Tris

192 mM glycine

0.1% SDS

SDS-PAGE Transfer Buffer

25 mM Tris

192 mM glycine

20% methanol

SDS Loading Buffer (2×)

50 mM Tris (pH 6.8)

20% glycerol

4% SDS

0.2% bromophenol blue

100 mM DTT

SM Agar

1% peptone

56 mM glucose

0.1% yeast extract

16 mM KH_2PO_4

5.5 mM $\text{K}_2\text{HPO}_4 \cdot 3\text{H}_2\text{O}$

4 mM MgSO_4

1.7% agar

[Autoclaved]

SSC (20×)

3 M NaCl

300 mM sodium citrate tribasic ($\text{C}_6\text{H}_5\text{Na}_3\text{O}_7 \cdot 2\text{H}_2\text{O}$)

[Autoclaved]

TAE (50×)

2 M Tris Base

50 mM EDTA

5.71% glacial acetic acid

[pH 8.2]

Tris-buffered saline supplemented with Tween-20 (TBS-T)

2.48 mM Tris

137 mM NaCl

[pH 7.4; autoclaved]

2.2 Methods

2.2.1 *Dictyostelium* cell culture and genetic manipulation

2.2.1.1 Cell culture

Dictyostelium cells were grown on SM agar plates, in association with *Klebsiella aerogenes* (KA) at 22°C. For axenic growth, *Dictyostelium* cells were grown in HL5 media, at 22°C in the dark. Therefore, 90 cm dishes, 6-, 24-, 96- well plates were used when appropriate. For growth in shaking suspension, cells were put in flasks and rotated at

220 rpm. Cells were kept between 5×10^5 and 6×10^6 cells/ml and experiments were performed in the exponential growth range ($2\text{--}6 \times 10^6$ cells/ml). Cell density was calculated by counting cells using a haemocytometer and a Motic AE21 trinocular microscope. Antibiotic selection, blasticidin (Enzo) or G418 (Sigma) at 20-10 $\mu\text{g/ml}$, was added when appropriate.

2.2.1.2 Transformation

5×10^7 vegetative cells were collected by centrifugation (3000 rpm for 3 minutes). Cells were washed twice in ice cold H50 buffer, and resuspended in H50 buffer to a density of 5×10^7 cells/ml. 5×10^6 cells were incubated with 3-8 μg of plasmid DNA on ice for 5 minutes. 1 μg of pREP plasmid was simultaneously added for transfections with PDXA-3C vectors. The mixture was transferred into a 1 mm gap electroporation cuvette (Bio-Rad) and pulsed twice (0.65 kV and 25 μF , separated by 5 seconds) with the Gene Pulser Xcell (Bio-Rad). After a 5 minute recovery on ice, cells were transferred into HL5. For transfections with extrachromosomal vectors, cells were transferred in 90 cm dishes with 10 ml HL5. Antibiotic selection, 20 $\mu\text{g/ml}$ G418, was added 24 hours after transfection. For transfection with a disruption construct, cells were transferred in 50 ml HL5 and subsequently diluted 1:10 and 1:100 in HL5. Diluted cells were transferred in 96-well plates and antibiotic selection, 10 $\mu\text{g/ml}$ blasticidin was added 24 hours later. The plates were incubated in the dark at 22 °C.

2.2.1.3 Generating of disruption strains

800-1000bp long DNA sequences, homologous for each gene that is targeted for disruption were amplified using PCR and ligated into the pLPBLP vector, flanking a blasticidin resistance cassette (*bsr* cassette). After transformation into XL1-Blue *E. coli* cells, the pLPBLP disruption vector was obtained by Maxi-preparation (Macherey-Nagel). The disruption construct was excised using suitable restriction enzymes, purified using

the PCR purification kit (Qiagen) and transformed into *Dictyostelium* cells as described in 2.2.1.2. Cells growing after 14 days in 96-well plates indicate potential gene disruption, which were collected and screened by PCR. Cells that were positive in the screen were spread on SM agar in association with KA, for clonal selection. Clones were screened by PCR, as before.

2.2.1.4 PCR screening of disruption strains

Genomic DNA was obtained from potential disruption strains using the Wizard® genomic DNA purification kit (Promega). Amplification of DNA fragments was performed by PCR using 10 µM synthesised primers (Sigma, sequences in the Appendix), 100 µg DNA and PCR BIO HiFi Polymerase (PCR Biosystems), using the manufacturer's protocol.

2.2.1.5 Cre-loxing

Transformation of *Dictyostelium* cells was performed as described in 2.2.1.2, with the pDXA-NLS-Cre plasmid encoding Cre recombinase. After transformation, the cells were transferred in a 90 cm dish containing 10 ml HL5, 20 µg/ml G418 (Sigma) was added 24 hours later. The cells were incubated in the dark at 22 °C. After 5 days the medium was replaced with HL5 containing no selection. After another 4-5 days, the cells were spread on SM agar plates in association with KA. Clones were selected and replica-plated into HL5, containing 20 µg/ml G418 or 10 µg/ml blasticidin, and those killed by both antibiotics were identified as successful strains.

2.2.1.6 Storage of strains

Permanent stocks of *Dictyostelium* strains were generated by removing HL5 from exponential growing cells and resuspending them to 5×10^7 cells/ml in freezing media. Cells were transferred into cryotubes (Thermo Fisher Scientific) or put as 100 µl aliquots

in 0.5 ml Eppendorf tubes. The tubes were placed on ice for 5 minutes, followed by 1-2 hours at -20 °C and finally at -80 °C for long-term storage. For recovery of cells from long-term storage, aliquots were thawed and transferred into 10 ml of HL5 medium in a 90 mm dish. After 30 minutes, which allows the cells to adhere to the dish, the media was replaced with fresh HL5.

2.2.2 Molecular biology

2.2.2.1 Phenol-chloroform DNA extraction

$1-4 \times 10^8$ exponentially growing cells were collected, removed from HL5 by centrifugation (3000 rpm, 3 min) and washed twice with KK2. The cell pellets were resuspended in nuclear lysis buffer (10 mM MgOAc, 10 mM NaCl, 30 mM HEPES pH 7.5, 10% sucrose, 2% NP-40) to a density of 1×10^8 cells/ml and incubated on ice for 10 minutes, followed by centrifugation (9000xg, 10 minutes, 4°C) to collect the nuclei. The cell pellets were resuspended in twice the volume of MHK (40 mM MgOAc, 20 mM KCl, 50 mM HEPES pH 7.5) than used NLB buffer. After adding NaCl to 200 mM and SDS to 1% final concentration, phenol-chloroform-isoamyl alcohol (Sigma) was added in a 1:1 ratio, followed by gentle inversion and centrifugation (20,000xg, 5 minutes). The upper aqueous layer containing the DNA was transferred to a fresh tube and if it was not clear, the phenol-chloroform extraction was repeated. If the aqueous layer was clear, chloroform was added in a 1:1 ratio followed by centrifugation (20,000 xg, 5 minutes). The upper aqueous layer was transferred to a fresh tube containing two volumes of 100% ethanol and 100 mM sodium acetate. DNA was precipitated at -80 °C for 20 minutes and pelleted by centrifugation at 20,000 xg for 5 minutes at 4 °C. The DNA pellet was washed with 70% ethanol followed by a repetition of the centrifugation step. The ethanol was removed and the pellet was air-dried, prior to resuspension of the DNA in a minimal volume ddH₂O. The DNA concentration was quantified using the NanoDrop ND-1000 spectrophotometer.

2.2.2.2 Southern blotting

Genomic DNA was purified and quantified as described in 2.2.2.1. 50 µg DNA was digested overnight with 500 units of the desired restriction enzyme. The DNA-enzyme mix was purified using PCR purification kit (Qiagen) and eluted in 50 µl ddH₂O. The DNA was loaded into a 0.7% TAE-agarose/ethidium bromide gel and separated by gel electrophoresis. The gel was washed in 0.2 N HCl for 10 minutes, rinsed in dH₂O, followed by a washing step with 1.5 M NaCl and 0.5 M NaOH for 45 minutes, before rinsing with dH₂O. The gel was further washed in 1 M Tris (pH 7.4) for 30 minutes, rinsed in dH₂O and washed again in 1 M Tris (pH 7.4) for 15 minutes. The DNA was transferred overnight by capillary action onto a nylon Hybond-N⁺ membrane (Amersham) using 10× SSC, subsequently washed with 5×SSC and air-dried. The DNA covalently cross-linked to the membrane by UV irradiation using a UV Stratalinker (Stratagene).

The probe for the Southern blot was amplified from genomic Ax2 DNA by PCR, and radioactively-labelled with [α^{32} P]-dATP (Hartmann Analytic) using the DecaprimeTM II DNA Labelling Kit (Thermo Fisher Scientific). Unincorporated [α^{32} P]-dATP was removed using SigmaSpin Post-reaction Clean-up columns (Sigma), according to the provided instructions. The membrane was rotated in Church buffer for 1 hour at 65 °C. The probe was denatured by boiling at 100°C for 5 minutes, before adding it to the Church buffer. Hybridisation occurred overnight at 65°C, while rotating. The membrane was washed twice in 2×SSC with 0.01% SDS, followed by washing twice with 0.2×SSC with 0.1% SDS at 65°C, while rotating. Detection of radioactively-labelled bands was performed using autoradiographic film (Hyperfilm, GE Healthcare). Blots were stripped by incubating the membrane three times with boiling 0.1% SDS, before re-probing using the same protocol as above.

2.2.2.3 RNA Extraction and cDNA synthesis

1×10^7 *Dictyostelium* cells were isolated by centrifugation (3000 rpm, 3 minutes) and lysed in 1 ml TRI reagent (Sigma) by repeated pipetting, followed by incubation at room temperature for 5 minutes. The samples were shaken vigorously after adding 0.2 ml chloroform, before incubating the mixture for a further 2-15 minutes at room temperature. After centrifugation (12,000×g, 15 minutes, 4 °C), the colourless upper phase containing the RNA was transferred to a fresh tube containing 0.5 ml isopropanol. The samples were allowed to stand for 5-10 minutes at 4°C, following centrifugation at 12,000×g for 20 minutes. The resulting pellet was washed with 70% ethanol and centrifuged at 7,500×g for 5 minutes at 4°C. The RNA pellet was air-dried for 5-10 minutes before resuspension in ddH₂O by repeated pipetting at 55-60°C for 10-15 minutes. RNA concentration was quantified using the NanoDrop ND-1000 spectrophotometer and stored at -80°C.

The cDNA synthesis from 5 µg RNA was performed using the SUPERScript II Reverse Transcriptase (Invitrogen), according to the provided protocol. The final product was used in RT-PCR reactions.

2.2.2.4 DNA manipulations techniques

Standard techniques of DNA manipulations such as transformation, amplification via PCR, restriction digestion, ligation, isopropanol and ethanol precipitation as well as TAE gel electrophoresis were performed using well-established protocols. Gel extraction and PCR purification (Qiagen) were performed according to the manufacturer's instructions. Plasmids were transformed into XL1-Blue competent *E. coli*, plasmid DNA purification was performed using either a Miniprep Kit (Qiagen) or a Maxi-preparation kit (Macherey Nagel). Sequencing of DNA fragments occurred by Sanger sequencing, performed locally by Source Bioscience, using synthesised primers (Sigma).

2.2.3 Phenotypic analysis

2.2.3.1 Sensitivity assay

Dictyostelium cells were collected, diluted to 1×10^6 cells/ml in HL5, and separated into 1 ml aliquots. These cells were exposed to the indicated concentrations of DNA damaging agents such as MMS (Sigma), phleomycin (Sigma) or mock-treated. Phleomycin was prepared by resuspending it in ddH₂O to a stock concentration of 20 mg/ml. Cells were incubated at 100 rpm for 1 hour while shaking. Afterwards, cells were diluted to 1×10^4 cells/ml in KK2. Then, 250 cells were mixed with 350 μ l *K. aerogenes* and spread onto 140 mm SM agar plates, which was performed twice for each condition. The plates were incubated in the dark and survival was assessed by observing *Dictyostelium* plaque formation after 3- 7 days.

2.2.3.2 Clonogenic assay

Exponentially growing *Dictyostelium* cells were diluted to 2.5×10^3 cells/ml in HL5 and 100 μ l were transferred into a 14 cm dish. The relevant PARPi concentration was added and the plates were incubated for 9-14 days at 22°C. When using Benzamide the media was refreshed every second day to ensure PARPi activity. When colonies were visible, the plates were washed twice with ice cold PBS followed by fixation with ice-cold Methanol for 10 minutes. Colonies were stained using 0.5% Crystal Violet solution. After 10 minutes the plates were carefully washed with ddH₂O and dried at room temperature. Survival was assessed by counting the stained *Dictyostelium* colonies.

2.2.3.3 Luminescent cell viability assay

Exponentially growing *Dictyostelium* cells were diluted to 2×10^3 cells/ml in HL5 and 50 μ l, containing 100 cells were transferred as replicates in 96-well plates. The cells were exposed to the indicated PARPi concentrations such as Benzamide, Nu1025, olaparib

and rucaparib or treated with the respective carrier (100% Ethanol for benzamide; DMSO for the other three inhibitors). Light sensitive Nu1025 was resuspended in DMSO to a concentration of 142 mM, whereas Benzamide was solved in 100% Ethanol to a concentration of 1 mM, olaparib and rucaparib were both resuspended in DMSO to a concentration of 46 mM. Cells were incubated for 5 days in the dark. Afterwards, the media was replaced by a 1:1 dilution of CellTiter-Glo® reagent (Promega) in HL5 and incubated for 30 minutes whilst shaking. The CellTiter-Glo® reagent causes cell lysis and mediates a luminescent signal proportional to the amount of ATP present. The ATP level in turn is directly proportional to the number of living cells in culture. Luminescence was measured using a platereader (Pherastar). After averaging the luminescence signal of the two replicates, the log2 fold change was computed by subtracting the base 2 logarithm of the carrier treated sample from the base 2 logarithm of the drug treated sample.

2.2.3.4 Chromatin extraction

Exponentially growing *Dictyostelium* cells were diluted to 1×10^6 cells/ml in HL5 if treated with Nu1025, and to 5×10^6 cells/ml in HL5 when treated with Phleomycin or MMS. For Nu1025 (Enzo Life sciences) incubation time was 2.5 h. When treated with MMS (Sigma), cells were incubated for 1 h, for phleomycin (Sigma) incubation was for 1.5 hours. All incubation steps occurred on a rotating wheel. Following the treatment, cells were washed with ice cold KK2 and resuspended in nuclear extraction buffer (NEB) with 0.1% Triton X-100 to a density of 1×10^7 cells/ml. After incubating on ice for 15 minutes, the samples were spun down at $14000 \times g$ for 3 minutes at 4°C . The pellet was resuspended in the same volume of NEB with 0.1% Triton X-100, and left on ice for a further 15 minutes, before the centrifugation step was repeated. Then, the pellet was resuspended in NEB with 200 $\mu\text{g/ml}$ RNase A, and rotated for 30 minutes at room temperature, following another centrifugation step ($14000 \times g$, 3 minutes). The final pellet was resuspended in $2 \times$ SDS loading buffer and boiled at 100°C for 5 minutes. Chromatin extracts were analysed

by SDS-PAGE and Western blotting (Sections 2.2.4.2). Besides Chromatin extracts, whole-cell extracts were also prepared following the treatment and washing in ice-cold KK2. Samples were resuspended in 2×SDS loading buffer, followed by boiling at 100°C for 5 minutes.

2.2.3.5 HR assay

The *cdk8* knockout plasmid was digested with KpnI and NotI to obtain DNA fragments homologous to the *Dictyostelium cdk8* genomic locus, which flank the blasticidin resistance cassette.^{373,374} The fragments were purified using the PCR purification kit (Qiagen) and transformed into *Dictyostelium* cells as described in 2.2.1.2. Cells were grown for 14 days in 96-well plates with blasticidin as selection. Clonal suspensions of blasticidin resistant transformants were spotted onto SM agar containing a lawn of KA. Plaques were grown for 5-7 days until they were large enough for phenotypic analysis. Targeted disruption of the *cdk8* genomic locus is indicated by an aggregation- deficient phenotype. Some aggregation-proficient and -deficient colonies were selected, and the genomic DNA was analysed using PCR to confirm targeted or random integration.

2.2.4 Protein techniques

2.2.4.1 Protein Purification

Transformation of *E.coli* M15 bacteria with pQE30 plasmids containing either His6-Adprt1a or His6-Adprt2 was performed using standard protocols. A single colony was transferred in 10 ml LB with Ampicillin and incubated overnight at 37°C. The next morning, this pre-culture was transferred into 400 ml fresh LB with Ampicillin and cultivated at 37°C to an OD of 0.6-0.8. Protein expression was induced with 1 mM IPTG and after shaking at 30°C for 4 h, cells were harvested by centrifugation (4700 rpm, 10 minutes, 4°C) and cell pellets were frozen at -20°C. Cell pellets were resuspended in

20 ml lysis buffer (IMAC buffer, 1:1000 PMSF (100xstock), 1 protein inhibitor tablet per 20 ml IMAC buffer). Bacteria cells were disrupted using Sonication (80 A for 10 s, 5x). The lysates were spun down (15000 rpm, 10 minutes, 4°C), the supernatant was collected and incubated with Ni-NTA on a spin column for 1 h at 4°C. The column was washed three times (wash buffer1: IMAC buffer, 1 mM PMSF, 10 mM Imidazol; wash buffer2: IMAC buffer, 1 mM PMSF, 20 mM Imidazol; wash buffer3: IMAC buffer, 1 mM PMSF, 30 mM Imidazol) before eluting the protein (elution buffer: IMAC buffer, 1 mM PMSF, 250 mM Imidazol). The elution was transferred into a dialysis bag and stirred in dialysis buffer (50 mM Tris pH8, 10 % Glycerol) for 2 h at 4°C, after 1 h the dialysis buffer was replaced with fresh buffer. The samples aliquoted in 0.5 ml tubes, shock frozen in liquid nitrogen and stored at -80°C.

2.2.4.2 Poly-acrylamide gel electrophoresis and Western blotting

Protein samples were loaded onto 8-15% sodium dodecyl sulphate polyacrylamide gels and separated at 100 V along a pre-stained protein marker (PageRuler Plus, Fermentas). The protein samples were transferred onto a PVDF membrane (Millipore, 0.45 µm pore size) at 100 V for 1 hour. The membrane was blocked in 5% milk in TBS-T for 1 h. Then, the membrane was incubated with the primary antibody, which was diluted in 5% milk in TBS-T. Dilutions of the primary antibodies are given in Table 2.1. The membrane was washed 3x with TBS-T before it was incubated with the respective horseradish peroxidase (HRP) conjugated secondary antibody, diluted 1:5000 in 5% milk in TBS-T. The membrane was washed three more times and detected with ECL (Millipore), following the manufacturer's specifications.

Antibody	company	Dilution	Secondary antibody
Anti-Actin	Sigma-Aldrich	1:1000	Anti-goat
Anti-H3	abcam	1:2000	Anti-goat
Anti-Flag	Sigma-Aldrich	1:5000	Anti-mouse
Anti-Myc	Sigma-Aldrich	1:1000	Anti-mouse
Anti- γ H2AX	abcam	1:1000	Anti-rabbit

Table 2.1: Primary Antibodies used for Western Blotting

2.2.4.3 Auto-PARylation assay

Expression and purification of recombinant His-tagged Adprt1a or Adprt2 is described in 2.2.4.1. The reaction mixture, as shown in Table 2.2, was prepared with the relevant PARPi concentration. 1 μ M of His-tagged Adprt1a or Adprt2 were added to start the reaction. The mixtures was incubated for 30 minutes room temperature and terminated by addition of 1xSDS buffer and 1 mM DTT and boiling for 10 minutes. The reaction mixture was subjected to 8% SDS-PAGE, vacuum-dried on whatman paper and auto-PARylation signal was detected using the Phosphorimager.

Reagent	Concentration
10xreaction buffer(500mM Tris buffer pH 8.0, 20mM MgCl ₂)	1x
³² P-NAD ⁺	100nM
NAD ⁺	75 μ M
DNA	5 μ g/ml
PARPi (benzamide, olaparib, rucaparib, Nu1025)	
Adprt	1 μ M

Table 2.2: Reaction Mixture for the auto-PARylation assay

2.2.5 Sample preparation for whole genome sequencing

2.2.5.1 Screen to obtain PARPi-resistant HR deficient cells

Exponentially growing *Dictyostelium* cells were diluted to 40 and 200 cells/ml in HL5 and 100 µl were transferred into each well of a 96-well plate. 5 mM benzamide or ethanol as mock treatment was added and the plates were incubated at 22°C. The media was refreshed every second day to ensure PARPi activity. When colonies were visible (after 2-3 weeks), the cells were collected and spread clonally on a KA lawn on SM agar. The clonal cells were collected and frozen down. To test if the cells are permanently resistant to PARPi a luminescent cell viability assay was performed.

2.2.5.2 DNA Preparation

DNA preparation was performed according to the specifications of the kit Quick-gDNA MidiPrep (Zymo Research). The obtained DNA was quantified using AccuBlue NextGen dsDNA Quantitation Kit (Biotium) according to the manufacturer's specifications.

2.2.5.3 Library generation using Nextera

Library generation was performed in collaboration with Rob Kay's lab (MRC, Laboratory of Molecular Biology, Cambridge). Nextera DNA library generation kit and the Nextera Index kit were used for the library generation. The Library preparation was performed according to the manufacturer's instructions. After encountering various problems with the library during sequencing on a MiSeq, the protocol was adjusted accordingly.

Tagmentation of genomic DNA: 20 µl of 7.5 ng/µl genomic DNA was combined with 25 µl of Tagmentation buffer and 2.5 µl Tagmentation enzyme. After mixing and centrifugation at 280xg at room temperature for 1 minute the mixture was subjected to the

following program in a thermal cycler with a preheated lid set to 55°C: 55°C for 5 minutes, holding at 10°C.

Clean up of tagmented DNA: Clean up and concentration of the tagmented DNA was performed using DNA Clean and Concentrator-5 Capped (Zymo Research) kit according to the manufacturer's specifications. Elution of the DNA was performed using 25 µl resuspension buffer (RSB) provided in the Nextera DNA library generation kit.

Amplify Tagmented DNA: The DNA samples were amplified and adapters were added using Q5 Hot Start High-Fidelity DNA Polymerase (NEB) instead of the DNA polymerase supplied with the Nextera kit. Table 2.3 shows the sample set up for amplification and adapter attachment to the tagmented DNA. The used PCR program is shown in Table 2.4.

Solution/buffer/enzyme added	Amount [µl]
Tagmented and cleaned up genomic DNA	20
Index adapter 1	5
Index Adapter 2	5
Q5 DNA polymerase	1
dNTP (10mM)	1
Q5 buffer (5x buffer)	10
PPC (primer mix)	5
MQ H ₂ O	3

Table 2.3: Sample set up for amplification and addition of the adapters to tagmented DNA

step	Temperature	time
1	72°C	3 minutes
2	98°C	30 sec
3	98°C	10 sec
4	63°C	30 sec
5	62°C	5 minutes
6	Go to step 3, 5 cycles	

Table 2.4: PCR program used for amplification

Clean up libraries: 25 µl of magnetic AMPure XP beads (Beckman Coulter) were thoroughly mixed with each sample, followed by incubation for 5 minutes at room temperature. The samples were placed on a magnetic stand. Once the liquid is clear (about 2 minutes) the supernatant is removed and discarded. The beads were washed two times with 200 µl 80% Ethanol, while still on the magnetic stand. Residual Ethanol was removed and the beads were air dried for about 15 minutes. Afterwards, the samples were removed from the magnetic stand and thoroughly mixed with 32.5 µl resuspension buffer. The samples were incubate for 2 minutes at room temperature before placing them on the magnetic stand and incubating for another 2 minutes until the liquid is clear. Then, 30 µl were transferred into a fresh tube which contain 300 bp long DNA fragments.

2.2.5.4 Library quantification and quality control using qPCR

KAPA Library Quantification kit (Complete Kit Rox Low, Kapa Biosystems) was used to accurately quantify the amount of adapter-ligated-DNA molecules in each individual library through qPCR. This is necessary to ensure equal representation of each individual library in the pool that was prepared for whole genome sequencing. To verify the accuracy of the qPCR, three different dilutions of each library in water (1:1 000, 1:10 000, 1:100 000) were analysed in duplicates. Therefore, 4 µl of each dilution was mixed with 12 µl 2x Kapa SYBR FAST qPCR Master Mix + 10x Primer Premix, containing the

polymerase and the oligonucleotides and 0.4 µl ROX Low and subjected to the qPCR program shown in Table 2.5.

step	Temperature	time
1	95°C	5 minutes
2	95°C	30 sec
3	60°C	45 sec
4	Go to step 2, 35 cycles	

Table 2.5: qPCR program

2.2.6 Bioinformatics

2.2.6.1 Sequence alignments

Protein amino acid and DNA sequences were obtained from UniProt or dictyBase^{375,376}. Protein and DNA sequences were aligned using BLAST³⁷⁷. Protein domains, structure and functional sites were detected using InterProScan^{378,379}.

2.2.6.2 Genome sequencing

Whole genome sequencing was performed by the Oxford Genomic Centre based at the Wellcome Trust Centre for Human Genetics, Oxford, on an Illumina HiSeq2500 system producing 150bp paired-end reads. The 31 barcoded samples were pooled and run altogether on a single lane of a flowcell.

2.2.6.3 Analysis of sequencing data

The FASTQ files were aligned to the *Dictyostelium* reference genome (dicty_2.7) obtained from Ensemble Protists using Burrows-Wheeler alignment tool (BWA, version 0.7.5a)^{380,381}. The aligned files were processed (merged, indexed, removal of PCR duplicates) using SAMtools (version 0.1.19)^{382,383}. Specific site were identified as variants

using freebayes (version 1.0.2) with a threshold of at least 90% agreement among the sequenced reads mapped to the reference genome³⁸⁴. The mutations were further filtered using BEDtools (version 2.17.0) to identify unique mutations in the single strains³⁸⁵. To exclude false positive variants the mutations were checked using SAMtools and Integrative genomics viewer (IGV, version 2.3.92)^{382,383,386,387}. The effect of the mutation on the gene was determined using the online tool Variant effect predictor³⁸⁸. Various quality control parameters of the sequencing data were calculated using R (version 3.0.2)³⁸⁹.

3. Synthetic lethality of HR-deficient strains with PARPi is conserved in *Dictyostelium*

3.1 Introduction

3.1.1 PARPi as cancer therapy

Mutations in the HR essential proteins BRCA1 and BRCA2 result in an increased lifetime risk to develop breast and ovarian cancer of 80% and 40%, respectively^{154,247–249}. As HR dysfunction is associated with misrepair of DNA DSBs, cells defective in BRCA1 or BRCA2 accumulate somatic mutations, such as genetic deletions or -rearrangements^{390–392}. Accordingly, mice lacking important HR factors are sensitive to γ -irradiation, indicating that a deficiency in this repair pathway increases the lethality to DNA damaging agents^{244,245,250,251}. This is exploited in the clinic as chemo- and radio-therapy to target HR-defective tumours^{250,251}. Recently, a novel therapeutic strategy was developed, which is based on the concept of synthetic lethality. Two genes are defined as synthetically lethal when the deficiency of one alone is viable, but deletion of both results in cell death. For example, cells defective in BRCA1, BRCA2 or other HR essential proteins undergo synthetic lethality with PARP inhibition^{257,258}. Although the exact mechanism resulting in cell death is not entirely understood, the concept of synthetic lethality with PARP inhibition is being applied as treatment for HR-defective tumours.

PARPi that specifically inhibit the SSB-responsive PARP1 and PARP2 reduce the viability of BRCA1- and BRCA2-deficient cells, whereas compounds that target PARP1/2 less effectively have no effect²⁶³. However, because of the high similarity between the PARP enzymes, most compounds used in the clinic not only inhibit PARP1/2 but also other PARPs, especially PARP3. Hence, due to the lack of specific PARPi, the exact effect of inhibiting different PARPs on HR-defective cells remains unclear. Identifying if inhibiting

PARP1/2 alone or other members of this enzyme family is lethal for HR-compromised cells or whether targeting PARPs besides PARP1/2 is counteracting the lethal effect of PARPi on HR-dysfunctional cells will help understand the molecular mechanisms underlying this pathway.

3.1.2 ADP-ribosylation and DDR in *Dictyostelium*

In contrast to other commonly used systems such as yeast, *Dictyostelium* express 15 proteins containing ART catalytic domains, of which several of the enzymes show conservation with the mammalian orthologues involved in DDR^{320,329–331,366}. Similar to mammalian PARP3, Adprt1a is recruited to DNA DSBs in *Dictyostelium* where it promotes NHEJ³⁶⁷. Cells defective in *adprt2* or *adprt1b* are sensitive to MMS, indicating that these PARPs play a role in tolerance to DNA SSBs and are therefore possibly the *Dictyostelium* orthologues of mammalian PARP1 and PARP2^{329,367}. Hence, the three *Dictyostelium* PARPs exhibit similar functions in the DDR to mammalian PARP1-3. Additionally, the DSB repair pathways HR and NHEJ are equally conserved in this genetically tractable model organism. This suggests that *Dictyostelium* is a useful tool to study the molecular basis of synthetic lethality between PARP inhibition and HR dysfunction^{341,353}.

3.1.3 Aims

Given the conservation of DNA DSB repair pathways and DNA damage responsive PARPs *Dictyostelium* is a suitable model to study the molecular basis of synthetic lethality of HR deficiency with PARP inhibition. However, as it is unclear if PARPi are killing HR-defective *Dictyostelium* cells, our first aim was to assess the efficacy of clinically relevant PARPi on inhibiting ADP-ribosylation in this organism. Secondly, we exploited the genetic tractability of *Dictyostelium* to identify whether PARP inhibition is lethal for HR-compromised cells. We also aimed to define which combination of PARP disruption is

synthetically lethal with HR dysfunction. This will indicate the appropriate PARP to be targeted in synthetic lethal strategies to kill HR-deficient cells and might help improve the treatment of HR-defective cancer patients.

3.2 Results

3.2.1 Efficacy of different PARPi on inhibiting auto-PARylation in *Dictyostelium*

My overall aim was to exploit *Dictyostelium* to investigate the molecular mechanisms involved in synthetic lethality of HR deficiency with PARP inhibition. Therefore, I first assessed whether currently available PARPi reduce DNA damage responsive ADP-ribosylation in *Dictyostelium*, with a view to employ the most appropriate compounds in synthetic lethality studies. *Dictyostelium* cells possess two PARPs that confer resistance to SSBs, Adprt2 and Adprt1b, and one, Adprt1a, that is required to promote NHEJ^{367,368}. It is proposed that synthetic lethality occurs in mammalian cells when HR-defective cells are exposed to agents that preferably inhibit PARP1 and PARP2, which are involved in single strand break repair²⁶³. Thus, we not only sought to assess if PARPi target the *Dictyostelium* orthologues *in vitro*, but also whether these agents show any specificity for one of the enzymes. Therefore, recombinant His6-tagged Adprt1a and His6-tagged Adprt2 were expressed and purified from the bacterial expression vector pQE30 in M15 *E.coli* cells (Figure 3.1A-B). The protein concentration of His-tagged Adprt1a (109 kDa) and Adprt2 (80 kDa) in the various elutions was quantified by comparing the signal intensities of the purified enzymes with a BSA standard containing a known protein quantity (Figure 3.1C). The protein concentration of His6-Adprt1a was 25 ng/μl for elution E1 and 15 ng/μl for E2. The quantified concentration of purified His6-Adprt2 was 100 ng/μl for elution E1 and 80 ng/μl for E2.

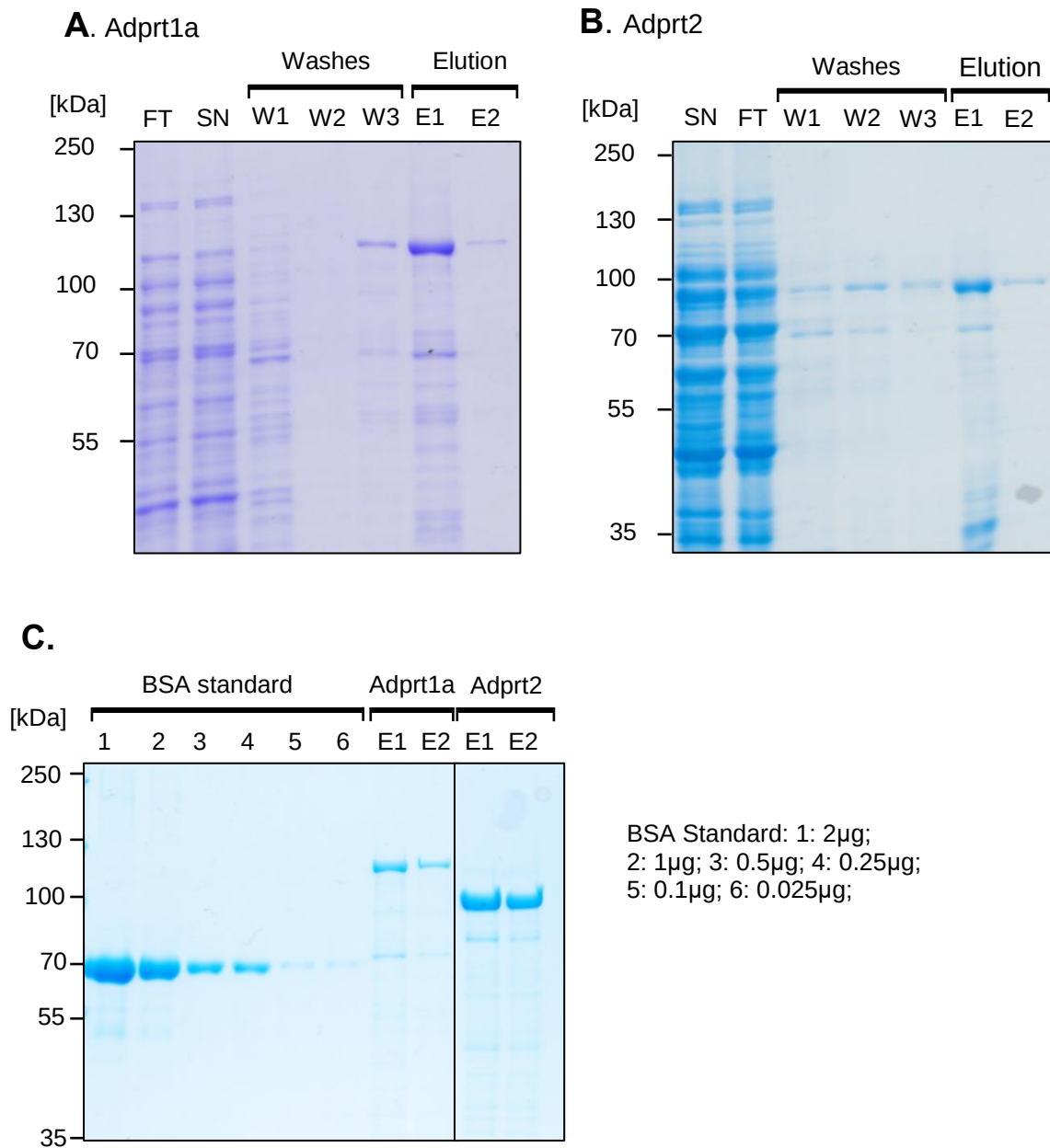


Figure 3.1: Protein purification and quantification of His-tagged Adprt1a and Adprt2. **A.** Purification of His6-Adprt1a SDS-PAGE from *E.coli* M15 bacteria cells. Samples were separated by SDS-PAGE and detected by Coomassie staining. Abbreviations: supernatant (SN), flow-through (FT). **B.** Purification of His6-Adprt2 SDS-PAGE from *E.coli* M15 bacteria cells. Samples were separated by SDS-PAGE and detected by Coomassie staining. Abbreviations: supernatant (SN), flow-through (FT). **C.** Quantification of the eluted His6-tagged Adprt1a and Adprt2 (10 µl per lane) by comparison with BSA standards. The indicated elutions and amounts of BSA were separated by SDS-PAGE and detected by Coomassie staining.

Similar to Human PARP1-3, the *Dictyostelium* PARPs exhibit auto-ADP-ribosylation activity when incubated with sheared salmon sperm DNA *in vitro*^{393–397}. Therefore, purified His6-tagged Adprt1a and Adprt2 were used in an *in vitro* assay to investigate the efficacy of different PARPi to inhibit their auto-ADP-ribosylation activity. As we wanted to analyse a broad spectrum of inhibitors, we applied four different compounds: benzamide, Nu1025, olaparib and rucaparib. Whilst benzamide is a member of the first generation of PARPi, Nu1025 represents the second generation^{269,275}. Olaparib and rucaparib were developed as part of the third generation and are already approved as therapy for ovarian cancer patients^{277–282}. For the *in vitro* assay, different concentrations of each PARPi or the solvent were incubated with ³²P-labelled NAD⁺. Sheared salmon sperm DNA was added to activate auto-ADP-ribosylation of the enzymes, which were added at last. The ADP-ribosylation level of the enzymes was analysed by separating proteins using SDS PAGE and detecting the radioactive signal, emitted from ³²P-labelled NAD⁺ incorporated into the ADP-ribose chain. We observed a radioactively labelled protein at the molecular weight of recombinant Adprt1a (Figure 3.2) or Adprt2 (Figure 3.3), which are approximately 108 kDa and 80 kDa, respectively. This indicates that *Dictyostelium* PARPs auto-ADP-ribosylate *in vitro*. The four PARPi decreased the auto-ADP-ribosylation level of Adprt1a and Adprt2 (Figure 3.2-3.3) with increasing concentrations, confirming their inhibitory effect on the enzymatic activity of *Dictyostelium* PARPs *in vitro*. When comparing the compound quantity that is required to reduce ADP-ribosylation by approximately 50%, it became apparent that the PARPi have different efficacies in targeting Adprt1a and Adprt2. Olaparib and rucaparib were more effective in inhibiting auto-ADP-ribosylation of Adprt1a, whereas Nu1025 was the most effective to decrease auto-modification of Adprt2. On the contrary, Benzamide reduced auto-ADP-ribosylation of both enzymes similarly. As Adprt2 is responding to SSBs, whereas Adprt1a promotes NHEJ, the PARPi that is most specifically targeting Adprt2, Nu1025, is the most appropriate inhibitor to study synthetic lethality of HR deficiency with PARPi in *Dictyostelium*.

Adprt1a

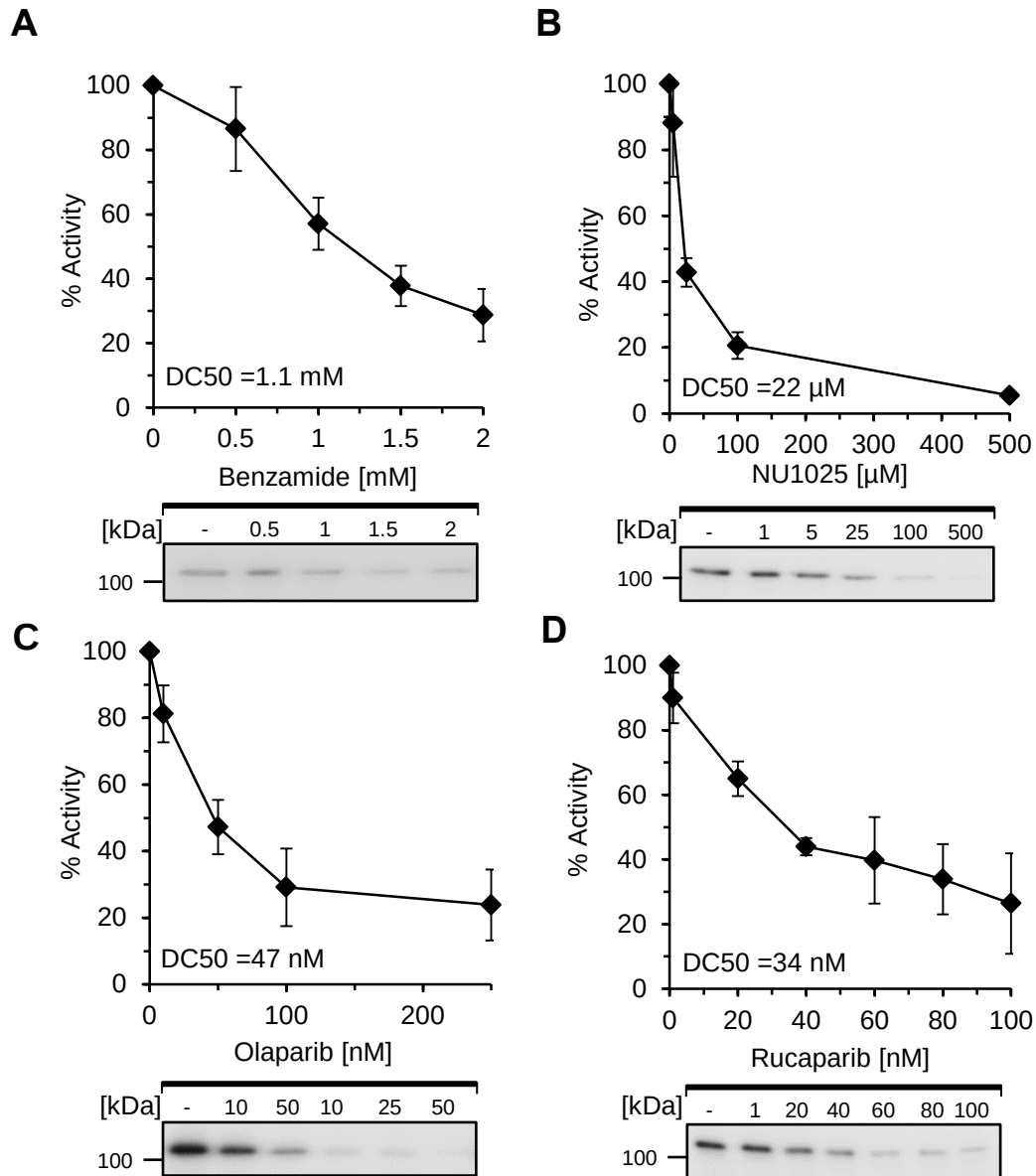


Figure 3.2: Differential inhibition of Adprt1a auto-ADP ribosylation by PARP inhibitors *in vitro*. A-D. Recombinant His- tagged Adprt1a was employed in ADP-ribosylation assays using ^{32} P-labelled NAD $^{+}$ in the absence or presence of increasing concentrations of PARPi: benzamide (A), Nu1025 (B), olaparib (C), rucaparib (D). Following SDS-PAGE, ADP-ribosylated proteins were detected and quantified by phosphor-imager analysis. Error Bars represent the standard error of the mean (SEM) from three independent experiments. DC50: Drug concentration required to decrease ADP-ribosylation by approximately 50%.

Adprt2

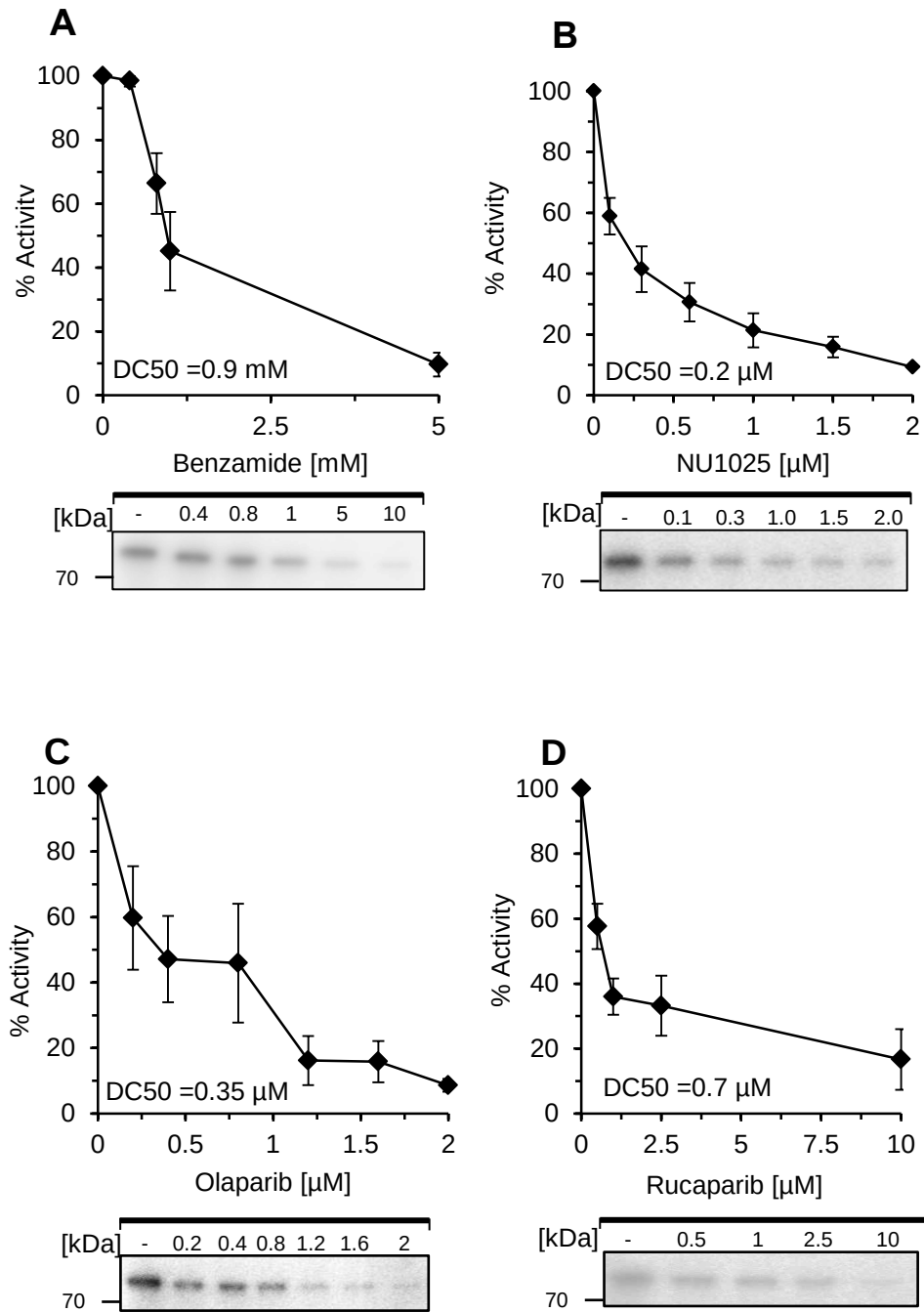


Figure 3.3: Differential inhibition of Adprt2 auto-ADP ribosylation by PARP inhibitors *in vitro*. A-D. Recombinant His-tagged Adprt1a was employed in ADP-ribosylation assays using ^{32} P-labelled NAD $^{+}$ in the absence or presence of increasing concentrations of PARP inhibitors: benzamide (A), Nu1025 (B), olaparib (C), rucaparib (D). Following SDS-PAGE, ADP-ribosylated proteins were detected and quantified by phosphor-imager analysis. Error bars represent the standard error of the mean (SEM) from three independent experiments. DC50: Drug concentration required to decrease ADP-ribosylation by approximately 50%.

3.2.2 Generation of HR-deficient *Dictyostelium* strains

Following the verification that PARPi impair the enzymatic activity of *Dictyostelium* PARPs, we subsequently wished to explore if synthetic lethality of HR-deficient strains to PARPi is conserved in *Dictyostelium*. Studying recombination in *Dictyostelium* proves to be difficult as most of the HR knock-out mutations are lethal. However, cells disrupted in the HR factor *exo1*, which mediates extensive 5'-3' DNA resection during the processing step, are viable. Although it is unclear why this is the case, it is likely due to redundancy with other nucleases such as Dna2 and Mre11^{354,398}. We therefore designed a strategy to disrupt the *exo1* genomic locus by targeted homologous recombination starting 47bp upstream of the start codon and leaving 950bp of the last exon in the genome (Figure 3.4). This strategy removes the nuclease domain and also prevents the expression of the entire gene, generating Exo1-deficient *Dictyostelium* cells. The homologous arms were amplified by PCR and ligated into the pLPBLP vector, using the indicated restriction enzyme sites. The pLPBLP vector contains a blasticidin resistance (BSR) cassette encoding a gene for resistance to an antibiotic, blasticidin, which is toxic to Ax2 cells. The homologous arms were ligated into the vector on either side of the BSR cassette, generating the *exo1* disruption vector which was subsequently verified by restriction digestion (Figure 3.4C). The disruption construct, containing the left and right winged arm as well as the BSR cassette, was excised from the *exo1* disruption vector using KpnI/PmlI and PCR purified (Figure 3.4D).

Ax2 cells were transfected with the disruption construct using standard protocols and selected in blasticidin for approximately 2 weeks until cell growth was observed. Blasticidin resistant cells were spread onto a KA lawn on SM agar to generate single clones. To identify *exo1*⁻ cells and exclude random integration of the disruption construct we devised different screening strategies. First, we generated genomic DNA from potential mutants and the parental Ax2 cells and employed PCR amplification. Primers

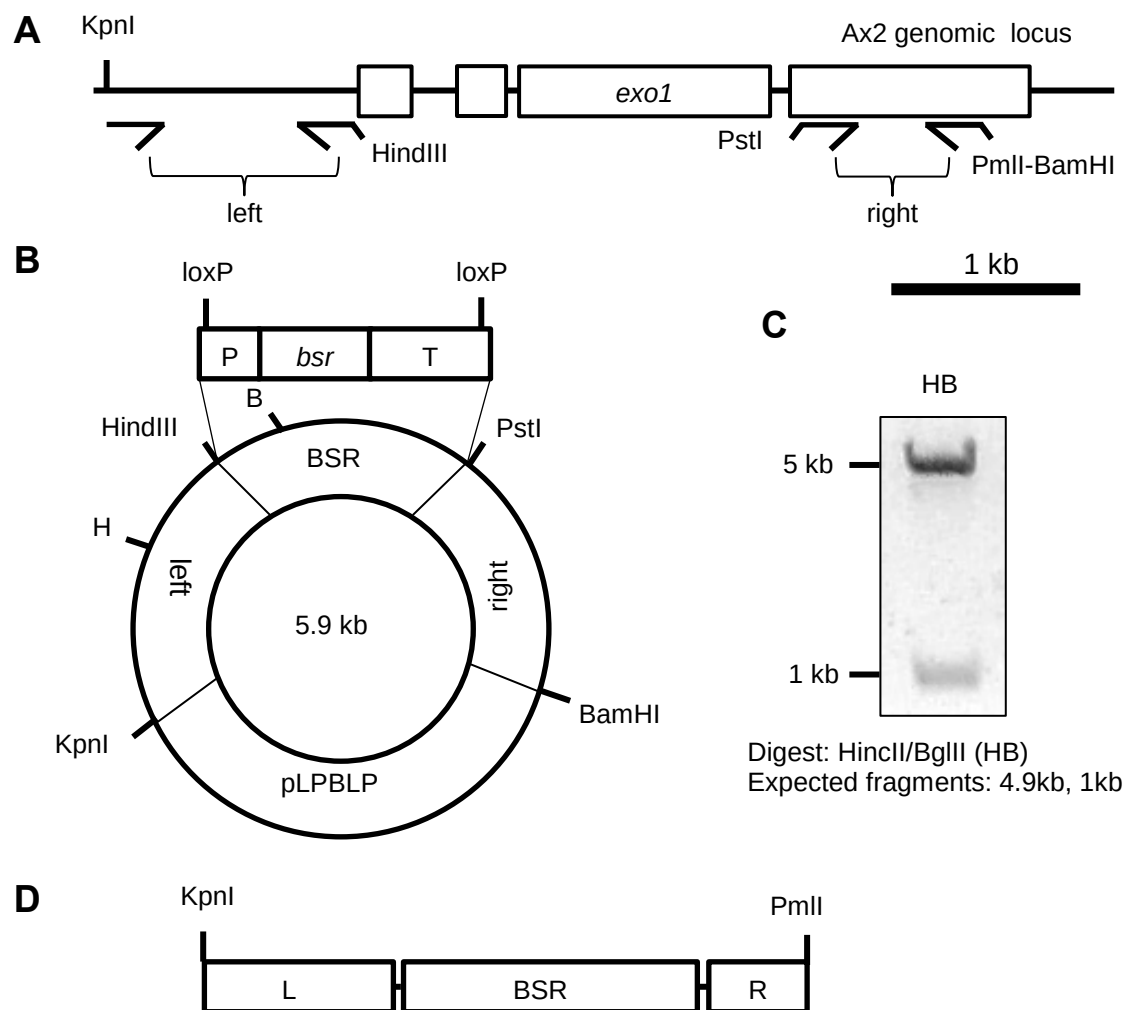


Figure 3.4: Strategy for the disruption of the *exo1* gene by targeted homologous recombination. **A.** Representation of the *exo1* genomic locus in *Dictyostelium*. The location of the homologous arms used for the disruption of the *exo1* gene by targeted homologous recombination are depicted as "left" and "right". Primers used to amplify the homologous arms are shown with attached restriction enzyme sites. Endogenous KpnI restriction site marks the end of one of the homologous arms. **B.** Map of the pLPBLP *exo1* disruption vector. The homologous arms are ligated on either side of the cassette coding for blasticidin resistance (BSR) using the indicated restriction enzymes (KpnI and HindIII for the left arm, PstI and BamHI for the right). Blasticidin is a toxic antibiotic for the parental Ax2 strain. The structure of the BSR cassette is shown in the upper panel containing the *bsr* gene, the actin6 promoter (P) and the terminator (T). The location of the LoxP sites are also indicated which can be used to excise the BSR cassette using the *Cre* recombinase. The restriction enzyme sites used for the verification of the plasmid, HincII (H) and BglIII (B) are located on the map. **C.** Verification of the pLPBLP *exo1* disruption vector by a test digest using HincII (H) and BglIII (B) as restriction enzymes. Sizes of the expected fragments are shown in the table. **D.** Representation of the final fragment that was used for the disruption of the *exo1* genomic locus in Ax2 cells. This fragment was obtained through digestion with the restriction enzymes KpnI and PmlI.

spanning from the BSR cassette to a region external to the disruption construct were used to verify targeted integration of the homologous arms and insertion of the *bsr* gene. In addition, oligonucleotides that amplify an internal region of *exo1*, which is deleted in the knockout strains, were employed to confirm the disruption of *exo1* (Figure 3.5A-B). The generation of *exo1*⁻ strains was further assessed with a Southern blot using two different probes (Figure 3.5D). One of them binds to the BSR cassette, verifying a single integration event (probe2), whereas the other probe (probe1) interacts with a region external to the disruption construct. Restriction digestion with the enzyme HaeIII produced different fragment sizes of the parental Ax2 strain (7kb) and the *exo1*⁻ strains (6.5kb) that interacted with the probes. These results confirm that we successfully generated three independent *exo1*⁻ strains.

To further validate the *exo1*⁻ strains, we first assessed whether disrupting *exo1* results in a deficiency of the HR pathway. Therefore, we employed the HR critical factor Rad51, which is recruited to DNA DSBs through interaction with Brca2 and replaces Rpa on ssDNA during HR^{151–153}. Once loaded, Rad51 searches for homologous DNA on the sister chromatid to serve as template for the repair of DNA double-strand breaks^{399,400}. Given that this protein is pivotal for HR, failure in Rad51 recruitment onto DNA indicates a defect in this DSB repair pathway. Hence, we compared the enrichment of Rad51 on chromatin following DNA damage in the *exo1*⁻ cells to the parental Ax2 strain. Therefore, both cell lines were transfected with the extrachromosomal pDXA-3C vector carrying Flag-Rad51 and an empty pDXA-3C vector as control using standard procedures. The pDXA-3C vector contains the neomycin resistance cassette and the *cis*-acting element Ddp2 origin of replication (ORI). Co-transfection with the helper plasmid pREP, containing the Ddp2 ORI *trans*-acting element, is required to confer expression of the pDXA-3C vector in *Dictyostelium*^{401,402}. Following whole cell extract preparation, Flag-Rad51 expression levels were analysed using Western blotting (Figure 3.6A). AX2 and *exo1*⁻ strains, transfected with pDXA-3C-Flag-Rad51, showed a signal at the size of Flag-Rad51

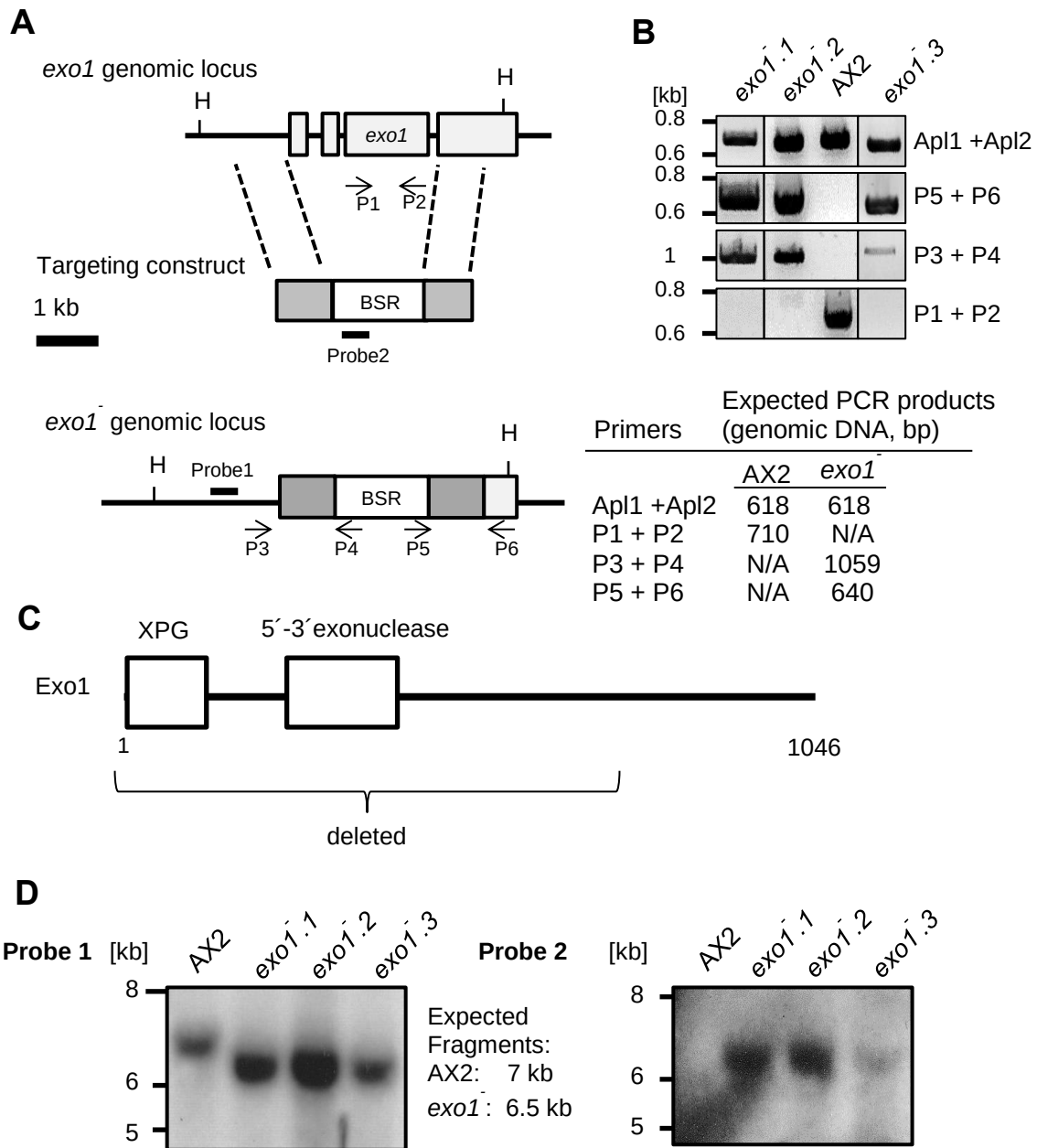


Figure 3.5: Generation and verification of *exo1⁻* *Dictyostelium* strains. **A.** Representation of the desired recombination event of the *exo1* genomic locus (top) with the *exo1* targeting construct (middle). A blasticidin resistance (BSR) cassette flanked by the homology regions (grey boxes) was inserted into the *exo1* genomic locus via targeted homologous recombination, generating an *exo1⁻* strain (bottom). The light grey box in the bottom figure represents the remaining 950bp in the final *exo1* exon. P indicates the primers used for screening by PCR, H indicates the restriction enzyme site of *Hae*III used for Southern blotting, Probe 1 and Probe 2 indicate the location of the probes used for Southern blotting. **B.** PCR verification of the *exo1⁻* strains using the primers at locations indicated in (A). The expected fragment sizes for Ax2 and *exo1⁻* strains are shown in the table. **C.** The domain map of Exo1 indicates the deleted regions in the *exo1⁻* strains. This strategy disrupted the entire exonuclease activity of *exo1* including the start codon preventing gene expression. **D.** Southern Blot verification of the *exo1⁻* strains. Genomic DNA of Ax2 and *exo1⁻* strains were digested with *Hae*III (H) and incubated with the probes, indicated in (A), resulting in detection of DNA fragments shown in the table. Probe 1 confirmed disruption of the *exo1* genomic locus, whereas blotting with probe 2 which interacted with blasticidin resistance cassette (BSR) confirmed a single integration event had occurred in the disruption strains.

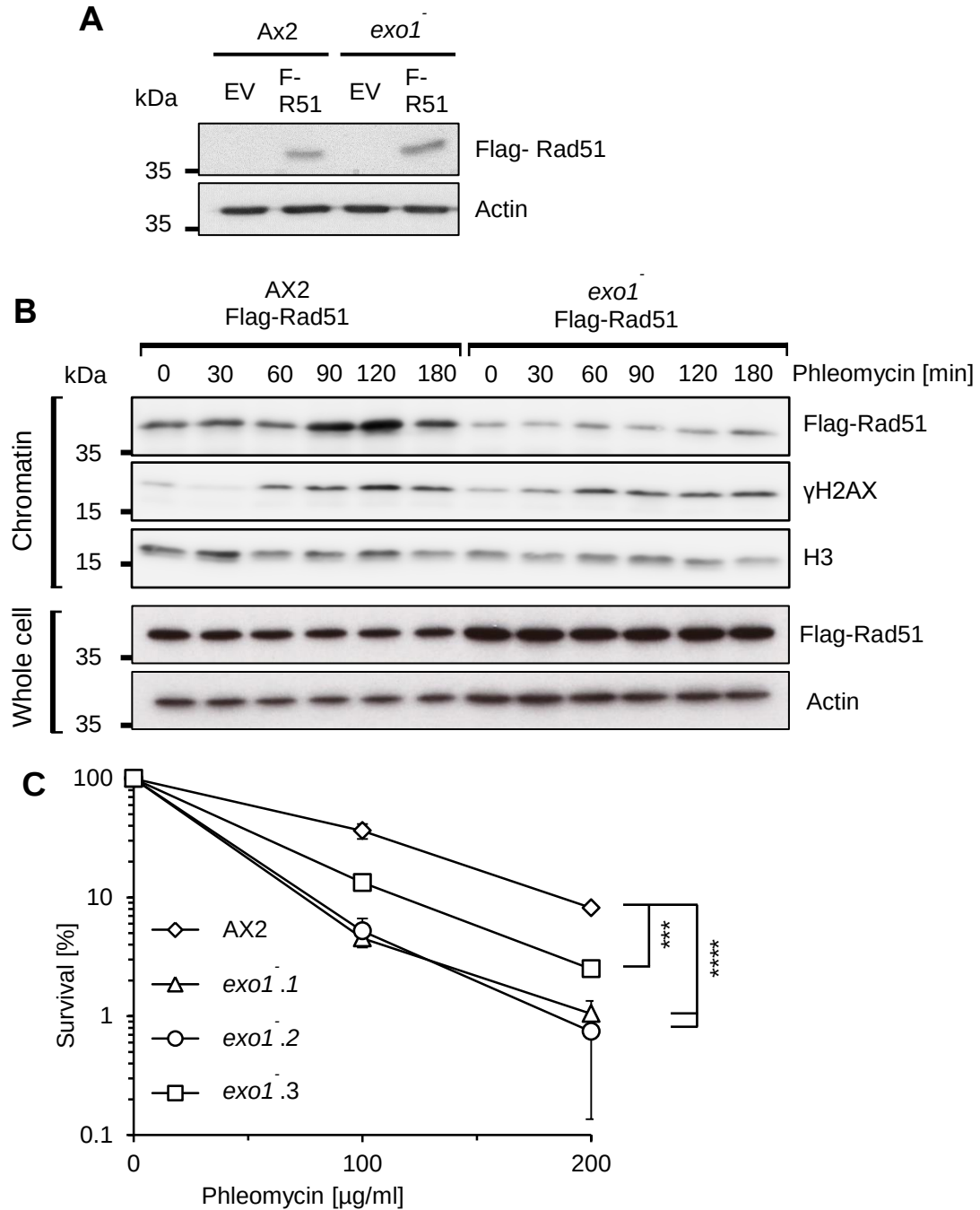


Figure 3.6: The *exo1*⁻ strains are defective in Rad51 recruitment following DNA DSBs and are sensitive to the DNA damaging agent phleomycin. **A.** Ax2 and *exo1*⁻ cells were transfected with an empty pDXA-3C vector (EV) or with pDXA-3C-Flag-Rad51 (F-R51) using standard procedures. Whole cell extracts were prepared and Flag-Rad51 expression was detected by Western blotting using the indicated antibodies. **B.** Ax2 and *exo1*⁻ cells expressing Flag-Rad51 were left untreated or exposed to 300 μg/ml phleomycin. Chromatin and whole cell extracts were prepared after the indicated times and Western blotting was performed with the indicated antibodies. **C.** Ax2 and three independent *exo1*⁻ strains were treated with the indicated phleomycin concentrations for 1 hour before plating on SM agar. After 3-7 days survival was assessed by plaque formation relative to a mock-treated control. Error bars represent the SEM from three independent experiments. Statistical significance was determined with respect to the parental Ax2 strain by two-way ANOVA, * $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$, **** $P \leq 0.0001$.

(40 kDa) when incubated with the Flag antibody, which is absent in the strains transfected with the empty vector.

Ax2 and *exo1⁻* strains expressing Flag-Rad51 were treated with the DSB-inducing agent phleomycin for up to three hours, before extracting the chromatin of these cells. The level of Flag-Rad51 enriched with chromatin was determined by SDS-PAGE and Western blotting. The γ H2AX levels, which serve as marker for DSBs, increased on chromatin with the length of the phleomycin treatment in both cell lines, verifying that DNA damage is induced in Ax2 and *exo1⁻* strains⁸. Although Flag-Rad51 expression is slightly increased in *exo1⁻* cells compared to the parental Ax2 strain, its enrichment onto chromatin is severely reduced in *exo1⁻* cells following phleomycin treatment compared to Ax2 (Figure 3.6B). Given the defect in DNA end-resection, it is possible that there is a positive selection on *exo1⁻* strains to over-express other components of the HR pathway, such as Rad51. Overall, *exo1⁻* cells are defective in recruiting the HR essential protein Rad51 onto damaged chromatin, indicating a defect in this DSB repair pathway.

It is well established that HR deficiency is associated with an increased lethality to DNA damaging agents due to inefficient DNA DSB repair^{136,243–245}. We assessed whether the *exo1⁻* mutants are sensitive to DSBs, which would further indicate a defect in HR. Therefore, Ax2 and three independent *exo1⁻* strains were incubated with increasing concentrations of the DNA DSB inducing agent phleomycin for one hour. Following the treatment, cells were plated onto SM agar and their survival was assessed by analysing plaque formation (Figure 3.6C). Survival to phleomycin of all three independent *exo1⁻* strains is significantly decreased compared to Ax2 cells, indicating that disruption of *exo1* sensitizes the cells to DSBs. Consistent with this, *exo1⁻ Dictyostelium* strains are unable to perform targeted HR at the *cdk8* locus in a HR assay, further supporting the hypothesis that *exo1⁻* strains are deficient in HR³⁵⁴. Taken together, the data indicate that disrupting *exo1* renders the cells more susceptible to DSBs due to a defect in HR.

3.2.3 PARPi are toxic to cells disrupted in the *exo1* gene

Having established a HR-defect in the *exo1*⁻ strains, I next sought to investigate whether *Dictyostelium* is a suitable model to study synthetic lethality between HR deficiency and PARPi. Although it is well-known that HR-defective mammalian cells are synthetically lethal with PARPi, it is unclear whether this is conserved in *Dictyostelium*. The current dogma describes that inhibiting the SSB-responsive enzymes using PARPi results in DNA damage that is resolved through HR. We identified Nu1025 as the most effective PARPi that is targeting the *Dictyostelium* SSB-responsive Adprt2. Thus, we initially explored if this compound induces DNA damage that is channelled through HR for repair in the parental Ax2 cells. Therefore, Flag-Rad51 expressing Ax2 cells were exposed to 1 mM Nu1025 for 2.5 hours, before chromatin extracts were prepared. Exposure to Nu1025 induced Flag-Rad51 enrichment onto the chromatin and H2AX phosphorylation (Figure 3.7A), suggesting that this PARPi causes DNA damage which is channelled through HR for repair. The experiment was repeated but including *exo1*⁻ strains expressing Flag-Rad51 to elucidate if the observed effect of Nu1025 is dependent on Exo1. As described previously, Flag-Rad51 was enriched onto chromatin and γH2AX levels were increased following Nu1025 exposure in parental Ax2 cells (Figure 3.7). The basal level of γH2AX in *exo1*⁻ cells, as shown in the mock-treated sample (Figure 3.7B), was upregulated compared to Ax2 cells. This suggests that the *exo1* disruption strain exhibits an elevated endogenous DNA damage level due to defects in DNA repair. The level of γH2AX is further enhanced when exposed to Nu1025, indicating that PARPi treatment is associated with an increase in DNA damage. Whilst Flag-Rad51 is enriched on chromatin in response to Nu1025 in parental Ax2 cells this is reduced in *exo1*⁻ cells (Figure 3.7B). Together, these data indicate that Nu1025 induces DNA damage that is channelled through Exo1-dependent HR.

Subsequently, we sought to investigate if the defect in repairing Nu1025 induced DNA damage through HR is reflected in the sensitivity of *exo1⁻* strains to this compound and whether this extends to other PARPi. As shown in Figure 3.2 and Figure 3.3, whilst Nu1025 is more specific in targeting the SSB-responsive Adprt2, olaparib and rucaparib are more effective at inhibiting the DSB-responsive Adprt1a, whereas benzamide similarly inhibits both Adprt1a and Adprt2 to a certain degree *in vitro*. Given that the four PARPi have different specificities for *Dictyostelium* PARPs, we sought to assess whether this is reflected in their toxicity to *exo1⁻* cells. Ax2 and *exo1⁻* strains were incubated with various concentrations of the PARPi, or with the carrier for 5 days before cell viability was detected using the CellTiter-Glo® Luminescent Cell Viability Assay (Promega). With this assay we quantified the ATP level in the cells, which is directly proportional to the number of living cells in culture. The log₂ fold change was computed by subtracting the base 2 logarithm of the carrier-treated sample from the base 2 logarithm of the drug-treated sample. A log₂ fold change of below zero means a decreased survival compared to the mock-treated sample.

The log₂ surviving fraction of *exo1⁻* strains exposed to olaparib and rucaparib (Figure 3.8A-B) was not significantly different from the Ax2 strain. This indicates that olaparib and rucaparib are not decreasing the cell viability of *exo1⁻* strains and are therefore not toxic for HR-defective *Dictyostelium* cells. Interestingly, treatment with benzamide or Nu1025 significantly reduced the log₂ surviving fraction of both independent *exo1⁻* strains compared to the Ax2 strain (Figure 3.9A-B). As the log₂ surviving fraction of *exo1⁻* strains decreased with increasing concentrations of benzamide or Nu1025, both PARPi significantly reduced the viability of *exo1⁻* cells in a dose-dependent manner but are not affecting the parental Ax2 strain. Hence, this suggests that in contrast to olaparib and rucaparib, benzamide and Nu1025 are toxic for *exo1⁻* *Dictyostelium* strains, whereas the parental cells survive these compounds.

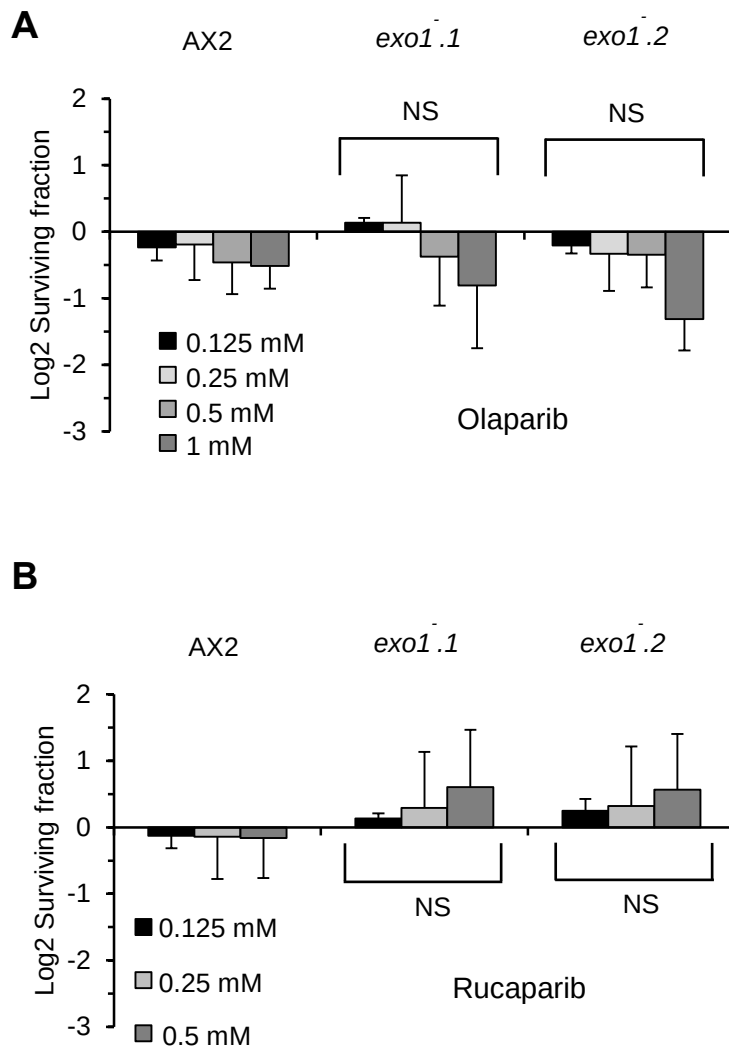


Figure 3.8: Olaparib and rucaparib do not affect cell viability of *exo1*⁻ strains. A-B. Ax2 cells and two independent *exo1*⁻ strains were incubated with the indicated concentrations of olaparib (**A**) or rucaparib (**B**) for 5 days. Cell survival was assessed using CellTiter-Glo® (Promega). Cell viability is represented as log2 fold changes between untreated cells and those exposed to the indicated PARPi. Error bars represent the standard error of the mean (SEM) from three independent experiments. The p-values are calculated with respect to the parental Ax2 strain using two-sided student's t-test, NS $P > 0.05$, * $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$.

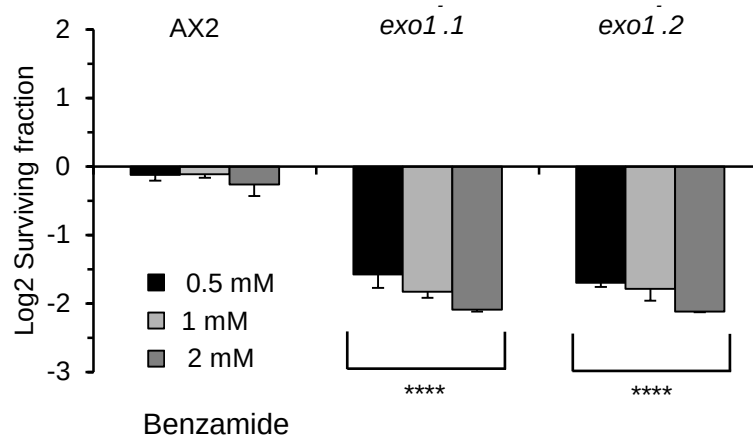
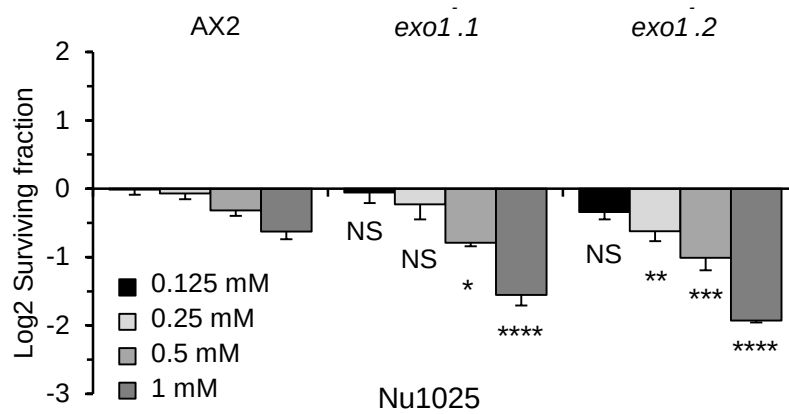
A**B**

Figure 3.9: Benzamide and Nu1025 decrease the cell viability of *exo1*⁻ strains. A-B. Ax2 cells and two independent *exo1*⁻ strains were incubated with the indicated concentrations of benzamide (A) or Nu1025 (B) for 5 days. Cell survival was assessed using CellTiter-Glo® (Promega). Cell viability is represented as log2 fold changes between untreated cells and those exposed to the indicated PARPi. Error bars represent the standard error of the mean (SEM) from three independent experiments. The p-values are calculated with respect to the parental Ax2 strain using two-sided student's t-test, NS P>0.05, * P≤0.05, ** P ≤ 0.01, *** P ≤ 0.001 **** P ≤ 0.0001

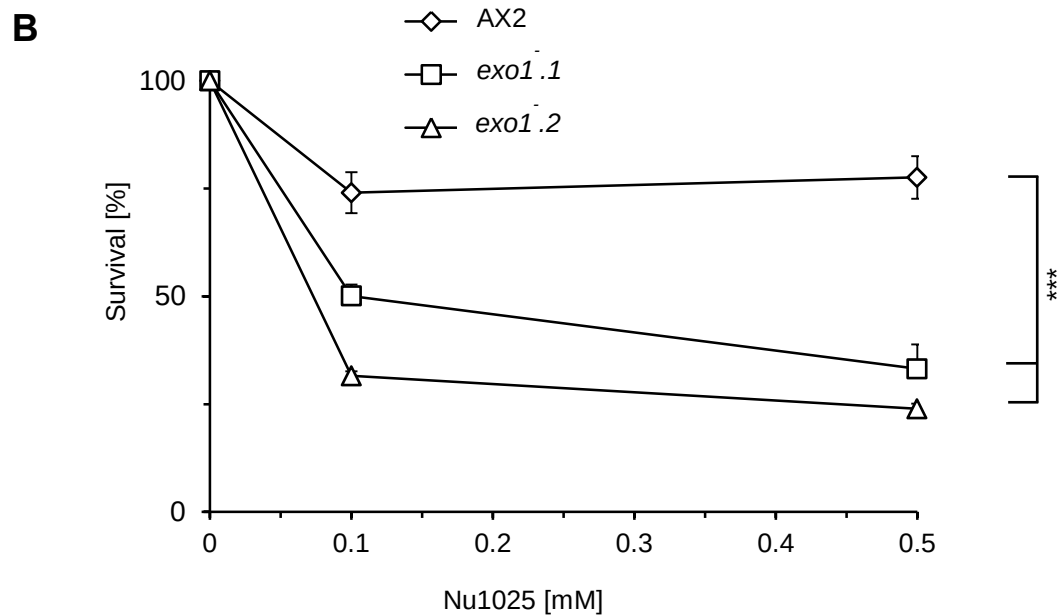
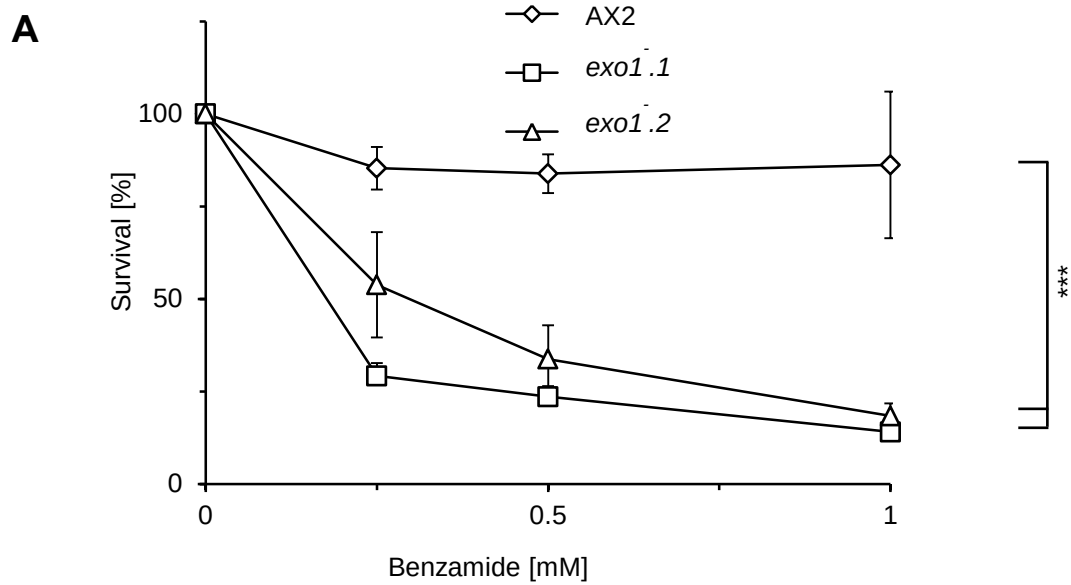


Figure 3.10: Benzamide and Nu1025 are synthetically lethal with *exo1*⁻ strains. A-B. Ax2 cells and two independent *exo1*⁻ strains were incubated with the indicated concentrations of benzamide (A) or Nu1025 (B) in 14 cm dishes in HL5. Cell survival was assessed after 9-12 days by plaque formation relative to the mock-treated sample. Data represent mean $\pm$ SEM from three independent experiments. The p-values are calculated with respect to the parental Ax2 strain by two-way ANOVA, NS $P > 0.05$, * $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$.

As the CellTiter-Glo® Luminescent Cell Viability Assay detects the amount of living cells it is possible, that benzamide and Nu1025 only decrease the cell growth of *exo1⁻* strains compared to the mock-treated samples, instead of being lethal for the cells. Additionally, given that PARPs consume ATP during auto-ADP-ribosylation, which is impaired by PARPi, the activity of benzamide and Nu1025 might affect the outcome of this ATP-based assay^{403,404}. Hence, to confirm that benzamide and Nu1025 are toxic for HR-defective cells, we cultivated *exo1⁻* and parental Ax2 cells under constant PARPi exposure until cell growth was visible by plaque formation after 9-14 days. As before, both *exo1⁻* strains were significantly more sensitive to benzamide and Nu1025 as the parental Ax2 strain (Figure 3.10), confirming that both PARPi are lethal for *exo1⁻* strains but not for the Ax2 strain.

Taken together, olaparib and rucaparib, which are more specific in targeting the DSB-responsive PARP Adprt1a *in vitro*, are not affecting the survival of HR-defective strains. On the contrary, Nu1025 which targets the SSB-involved PARP Adprt2 *in vitro*, and benzamide, which similarly inhibits Adprt1a and Adprt2 *in vitro*, decrease the cell viability of *exo1⁻* strains. This confirms that synthetic lethality of HR-deficient cells with PARPi is conserved in *Dictyostelium*.

3.2.4 Loss of Adprt2 sensitises cells to HR inhibition

Having confirmed that PARPi are synthetically lethal with HR dysfunction in *Dictyostelium*, we next wanted to identify which PARP is the most appropriate to target in HR-defective cells to induce cell death. The current dogma proposes that inhibiting PARP1 and PARP2 confers synthetic lethality with HR deficiency⁴⁰⁵. This is supported by our data, illustrating that PARPi which effectively target the SSB-responsive Adprt2 are toxic for HR-compromised *Dictyostelium* cells. However, PARPi that are used in the clinic as therapy for HR-defective ovarian tumours not only inhibit PARP1/2 but also target other PARP family members, especially PARP3. Hence, it is unclear whether the observed lethality of

HR-defective cells with PARPi is due to inhibiting a specific PARP and whether targeting other family members is beneficial or counteractive for cell survival. Previously, we generated a library of *Dictyostelium* strains that are disrupted in the different PARPs. The *adprt2*⁻ cells are lacking the enzyme involved in DNA SSB repair. Whilst *adprt1a*⁻ cells are deficient in the PARP that mediates NHEJ, *adprt1a*⁻*adprt2*⁻ mutants are disrupted in both enzymes^{354,368}. We sought to assess whether inhibiting HR is toxic for these strains. Thereby, we wanted to identify the PARP that has to be disrupted to kill HR-dysfunctional cells.

For this analysis we first had to identify an agent that inhibits HR in *Dictyostelium*. There are several compounds on the market that target proteins involved in different steps of this DSB repair pathway. Following DNA DSB recognition, DNA resection into single-stranded DNA is critical for HR, which can be impaired by targeting DNA nucleases, such as Mre11, Exo1 or Dna2. While the DNA DSB is being processed, RPA interacts with the ssDNA intermediates, protecting it from forming secondary structures. Several compounds are developed to inhibit RPA, however, in addition to its function in HR, RPA is involved in various cellular processes, such as DNA replication, cell cycle and DNA damage checkpoints⁴⁰⁶. As we sought to identify an inhibitor that only impairs HR we did not assess the efficacy of RPA inhibitors on the *Dictyostelium* orthologue^{407,408}. During HR, Rad51 replaces RPA on the ssDNA forming a nucleofilament, which invades the sister chromatid to perform homology search^{399,400}. As Rad51 activity is crucial to complete HR, this protein is a potential target for compounds aimed to compromise HR. For example, RI-1 and its analogue RI-2 covalently bind to the amino acid cysteine 319 on the surface of Rad51, thereby impairing the oligomerization of Rad51 monomers on DNA and inhibiting HR^{409,410}. As the cysteine 319 residue is not conserved in *Dictyostelium* Rad51, these inhibitors are not applicable to diminish HR in this model organism. The activity of another Rad51 inhibitor, B-02, was assessed by analysing the survival of Ax2 cells to B-02 upon DSB induction^{411,412}. As *Dictyostelium* preferably repair

DNA DSBs using HR, inhibition of this pathway should sensitise the cells to DNA damage. However, B-02 is not sensitising the Ax2 cells to DSBs (data not shown), indicating that this compound is not effectively inhibiting HR in *Dictyostelium*.

We then focused on compounds that impair DNA end resection following DSB recognition to identify an inhibitor that effectively impairs HR in *Dictyostelium*. As DNA processing is pivotal for channelling the DSB through HR instead of NHEJ, inhibiting DNA nucleases negatively affects HR. Besides Exo1, Mre11 is required for DNA end resection during HR, and inhibiting this protein using a drug called mirin disrupts homology-dependent repair in mammalian cells⁴¹³. We assessed whether mirin has a similar inhibitory effect on HR activity in *Dictyostelium*. Since Mre11 is potentially partially redundant with other nucleases, we included the *exo1*⁻ strain in the sensitivity assay to avoid missing the inhibitory effect of mirin due to this redundancy⁴¹⁴. Ax2 and *exo1*⁻ cells were pre-incubated with 0.5 mM mirin for 30 minutes before phleomycin was added to induce DNA DSBs. While exposure to mirin did not sensitise Ax2 cells to phleomycin (Figure 3.11), mirin treatment significantly increased the sensitivity of *exo1*⁻ cells to DSBs. This indicates that inhibiting Mre11 using mirin in Ax2 cells has no or only a marginal effect on the sensitivity to DNA DSBs and is thereby not impairing HR activity due to a redundancy with Exo1. The increased sensitivity of *exo1*⁻ strains to DNA DSBs when exposed to mirin confirms a redundancy of Exo1 and Mre11. This further indicates that in the absence of Exo1, DNA DSBs are potentially repaired using Mre11-dependent HR, explaining why exposure to mirin increases the sensitivity to DNA damage.

Having established that mirin is an effective inhibitor of HR in *Dictyostelium*, we wanted to assess its effect on the cell viability of different PARP-defective strains. If inhibiting Adprt1a and/or Adprt2 is synthetically lethal with HR deficiency, the respective disruption strain should be sensitive to mirin. To test this, the cell viability of Ax2, *adprt2*⁻, *adprt1a*⁻ and *adprt1a*⁻*adprt2*⁻ strains was assessed using the CellTiter-Glo® Luminescent Cell

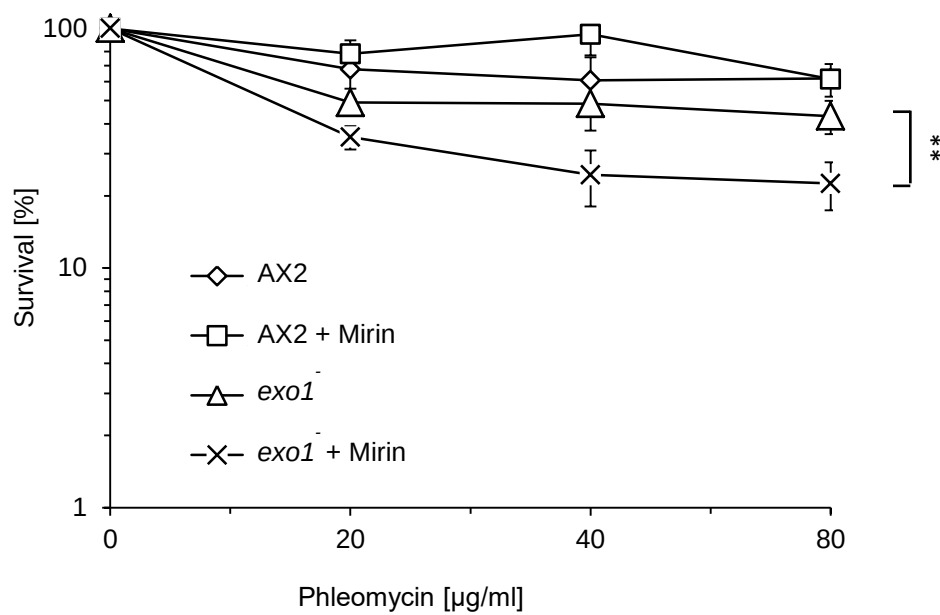


Figure 3.11: Mirin enhances the sensitivity of *exo1*⁻ strains to phleomycin. Ax2 and *exo1*⁻ strains were exposed to 0.5 mM mirin or mock-treated for 30 minutes, before the strains were treated with various concentrations of phleomycin (0, 20, 40, 80 µg/ml) for 1 hour. The cells were plated on SM agar. After 3-7 days survival was assessed by plaque formation relative to a mock-treated control. Error bars represent the SEM from three independent experiments. Statistical significance was determined for the *exo1*⁻ strain exposed to mirin compared to the mock-treated *exo1*⁻ strain by two-way ANOVA, * P≤0.05, ** P≤0.01.

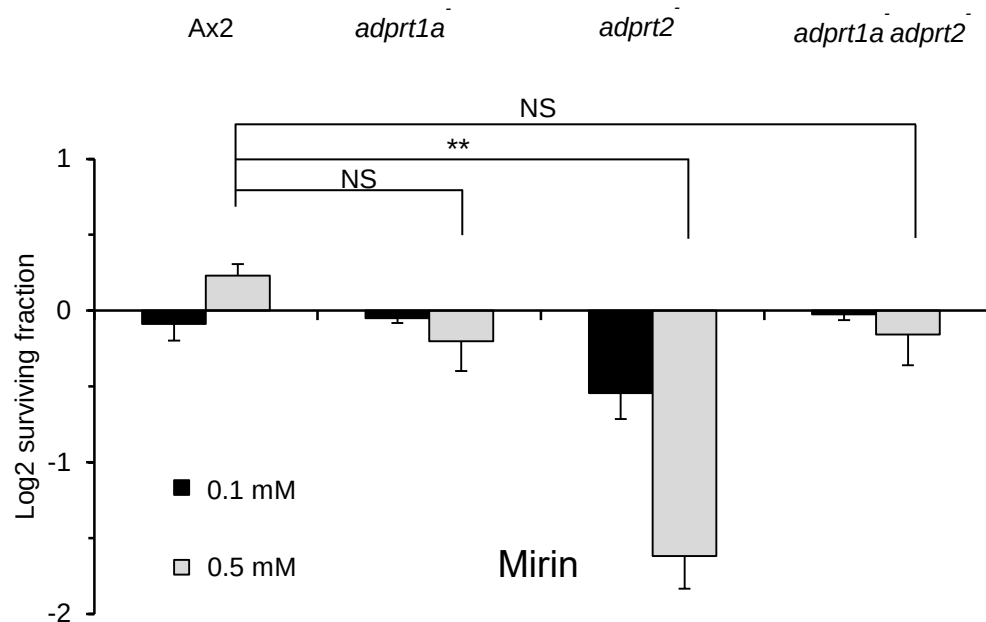


Figure 3.12: Exposure to mirin decreases the cell viability of *adprt2*⁻ strains. *Ax2*, *adprt1a*⁻, *adprt2*⁻ and *adprt1a*⁻*adprt2*⁻ strains were treated with the indicated mirin concentration. Cell viability was assessed after 5 days using CellTiter-Glo® (Promega). Cell viability is represented as log2 fold changes between untreated cells and those exposed to PARPi. Error bars represent the SEM from three independent experiments. Statistical significance was calculated for the disruption strains compared to the *Ax2* strain exposed to the same mirin concentration using two-sided student's t-test, NS $P > 0.05$, * $P \leq 0.05$, ** $P \leq 0.01$

Viability Assay after cultivating the cells with mirin for 5 days. As expected, exposure to mirin did not affect parental Ax2 cells (Figure 3.12). Interestingly, the viability of *adprt2*⁻ cells was significantly decreased when exposed to mirin, whereas *adprt1a*⁻ cells were not affected by the treatment. Surprisingly, mirin was not toxic for the *adprt1a*⁻*adprt2*⁻ strain. Overall, we identified that cells solely deficient in *adprt2* are synthetically lethal with the HR inhibitor, whereas loss of *adprt1a* renders the cells refractory to the treatment. This indicates that Adprt2 has to be targeted by PARPi to induce synthetic lethality of HR-defective *Dictyostelium* strains and that disruption of *adprt1a* suppresses the sensitivity to the compound.

3.3 Discussion

Previously, our group and others discovered that the role of PARPs in DNA damage repair is conserved in *Dictyostelium*^{329–331,367}. Adprt1a binds to DNA DSBs where it promotes Ku70 loading onto damaged DNA through its interaction with the PAR binding domain of Ku70. In contrast, Adprt2 is involved in the repair of SSBs^{367,368}. In mammalian cells, PARP3 is recruited to DNA DSBs and interacts with several proteins involved in the NHEJ pathway. Depletion of PARP3 impairs the activation of NHEJ and potentially promotes DNA resection, thereby possibly inducing HR²³¹. On the contrary, PARP1 is recruited to DNA SSBs, which induces auto-ADP-ribosylation and promotes the interaction with SSBR factors such as XRCC1 and Lig3^{54,66,77,96,97}. Due to a similar function in promoting NHEJ, we propose Adprt1a as the orthologue of PARP3, whereas Adprt2 shares the activity of PARP1 in SSBR^{97,231,367,368}. Here, we further exploit ADP-ribosylation in *Dictyostelium* and illustrate that synthetic lethality between PARP inhibition and HR-deficiency is similarly conserved in this organism.

We generated HR-defective *Dictyostelium* strains to investigate whether synthetic lethality between PARP inhibition and HR deficiency is conserved in this model organism.

Therefore, we disrupted the *exo1* genomic locus to develop strains that are impaired in DNA end resection, resulting in failure of HR following DNA DSBs. The *exo1*⁻ strains are defective in the enrichment of Flag-Rad51 onto chromatin following DNA DSB induction (Figure 3.6A-B). Moreover, the strains are sensitive to DNA DSBs (Figure 3.6C) further verifying that *exo1*⁻ strains display aberrant DSB repair due to disruption of the HR pathway. Additionally, *Dictyostelium* lacking *exo1* are unable to perform targeted HR at the *cdk8* locus due to defective HR (Figure 5.6A)³⁵⁴. Overall, we generated HR-defective *Dictyostelium* strains by disrupting the *exo1* genomic locus. Interestingly, whilst *Dictyostelium* disrupted in *exo1* remain viable, loss of other HR important factors is lethal for this model organism. We proposed that the survival of *exo1*⁻ strains is due to redundancy with other nuclease, such as Dna2 and Mre11^{354,398}. We tested this hypothesis by analysing whether the Mre11 inhibitor mirin further sensitises *exo1*⁻ strains to DNA DSBs. Whilst mirin did not decrease the survival of Ax2 cells to phleomycin, it further decreased the viability of *exo1*⁻ strains to DNA DSBs (Figure 3.11). Although Mre11 is active in the primary DNA end processing step upon DSB recognition, whereas Exo1 mediates extensive DNA resection in mammalian cells, Mre11 and Exo1 have redundant roles in yeast and humans^{414–417}. As mirin is only sensitising *exo1*⁻ cells to DSBs, Exo1 and Mre11 are equally redundant in *Dictyostelium*, explaining the viability of *exo1*-deficient strains. This further indicates that mirin is a potent inhibitor of the HR factor Mre11 and impairs HR in *Dictyostelium*.

Given our goal to establish *Dictyostelium* as model to investigate the mechanisms underlying synthetic lethality, we assessed whether PARPi target and inhibit *Dictyostelium* DNA damage-responsive PARPs. We therefore exploited the auto-ADP-ribosylation activity of Adprt1a and Adprt2 following activation with single-stranded DNA and incubation with the substrate ³²P-NAD⁺ in an *in vitro* assay (Figure 3.2-3.3). Incubation with the inhibitors (Nu1025, benzamide, olaparib and rucaparib) impairs auto-ADP-ribosylation of recombinant His-tagged Adprt1a and Adprt2 with increasing concentration

of the compounds. Hence, all four agents inhibit the enzymatic activity of *Dictyostelium* PARPs *in vitro*. Furthermore, our data indicate that the PARPi exhibit differential specificity for Adprt1a and Adprt2. Whilst benzamide similarly inhibits the auto-catalytic activity of both enzymes *in vitro*, Nu1025 is more specific in targeting the SSB-responsive Adprt2. On the contrary, olaparib and rucaparib preferentially inhibit the DSB-responsive Adprt1a. This is unexpected as olaparib and rucaparib, which are already used as therapy for patients with BRCA1/2-associated ovarian cancer, are supposed to target the SSB-responsive PARP²⁸¹. However, two published studies suggest that Lynparza/olaparib preferable inhibits PARP3 (IC₅₀=4 nM) instead of PARP1 (IC₅₀=5-6 nM) supporting our data showing that this compound preferentially targets the DSB-responsive PARP^{281,418}.

The best supported model to explain PARPi toxicity to HR-deficient cells is referred to as “trapping-model”. PARPi treatment disrupts PARP1- and PARP2-dependent SSB repair by trapping these enzymes at DNA breaks. When PARP bound DNA lesions are encountered by a replication fork this leads to replication stress and collapse of the replication fork, which needs to be resolved by HR^{419,420}. Consistent with this model, exposure to Nu1025 induces the enrichment of the HR essential protein Rad51 onto chromatin as well as H2AX phosphorylation in Ax2 cells (Figure 3.7). As Rad51 is not enriched on chromatin following Nu1025 treatment in the *exo1*⁻ strain, this indicates that this PARPi induces DNA damage in *Dictyostelium* that is resolved through Exo1-dependent HR. Interestingly, exposure to Nu1025 and benzamide is lethal for HR-defective *exo1*⁻ cells (Figure 3.9 - 3.10), whereas olaparib and rucaparib are not toxic for these cells (Figure 3.8). Although several factors might influence the efficacy of the agents, such as insufficient absorption or upregulation of drug export using efflux pumps or even compound stability, this might indicate that inhibition of Adprt2, but not Adprt1a is toxic for HR-deficient cells. In further support of this hypothesis, inhibiting HR by exposure to mirin is not toxic for *adprt1a*⁻ cells (Figure 3.12), whereas mirin treatment is lethal for

adprt2⁻ cells. Taken together, whilst inhibition of the SSB-responsive Adprt2 is lethal for HR-deficient cells, targeting Adprt1a, which is promoting NHEJ, is not toxic for HR-defective strains. This confirms that synthetic lethality of PARPi and HR deficiency is conserved in *Dictyostelium*.

Since Adprt2 is the potential orthologue of PARP1 and the main PARP involved in SSB repair this indicates that in mammalian cells PARP1 should be targeted to induce synthetic lethality of HR-defective cells. Similar to PARP3, Adprt1a is required to promote the error-prone NHEJ pathway, explaining why *adprt1a⁻* strains exhibit a reduced NHEJ activity³⁶⁷. Interestingly, disrupting *adprt1a* in *adprt2⁻* strains suppresses the toxicity of mirin, rendering the cells viable when exposed to the HR inhibitor. This indicates that impairing the PARP involved in NHEJ is counteracting the lethal effect of the HR inhibitor on cells that are defective in the SSB-responsive PARP. Accordingly, aberrant NHEJ suppresses the sensitivity of HR-deficient strains to PARPi, suggesting that NHEJ is toxic for the cells²⁸⁴. Hence, *adprt2⁻* strains exposed to mirin repair endogenous DNA damage using error-prone NHEJ, which decreases the viability, possibly due to increasing genomic instability. Disruption of the error-prone pathway suppresses the sensitivity to HR inhibitors and renders the cells viable despite mirin treatment. As the PARPi currently used in the clinic not only inhibit PARP1/2, but also target other PARPs, novel PARPi with no inhibitory effect on the NHEJ mediating PARP should be developed to treat HR-deficient cancer patients^{234,265–267}.

4. Disrupting NHEJ suppresses PARPi toxicity to HR-deficient strains

4.1 Introduction

4.1.1 Synthetic lethality of HR dysfunction with PARPi is conserved in *Dictyostelium*

In the previous chapter we identified that synthetic lethality of PARPi with HR dysfunction is conserved in *Dictyostelium*, making it a suitable model to investigate the molecular basis behind this concept. Given that *exo1⁻* *Dictyostelium* cells exhibit an upregulation of endogenous DNA lesions, other repair pathways might be activated to resolve the damages. Interestingly, DSB-inducing agents such as phleomycin or replication stress through PARPi treatment further increase the amount of DNA breaks, suggesting that those damages are channelled through a DSB repair mechanism other than HR. This compensatory repair pathway can either be beneficial and mediate the survival of a subset of HR-defective cells, or be responsible for the increased lethality of HR-compromised cells to DNA DSBs and PARPi. In mammalian cells, two DSB repair mechanisms are active besides HR: alt-NHEJ and NHEJ. The alt-NHEJ pathway is employed following excessive end resection, when HR is aborted or if the DNA serves as poor NHEJ substrate. As such, alt-NHEJ is engaged when either canonical repair pathway fails, making it the backup pathway for HR and NHEJ¹⁹⁹. Given alt-NHEJ has been suggested to compete with HR in the repair of replication stress, such as PARPi treatment, this makes alt-NHEJ a likely candidate to repair PARPi-mediated damages in HR-compromised cells^{421,422}. Accordingly, depletion of the alt-NHEJ essential factor PolQ reduces the survival of HR-deficient cells, supporting the activity of this repair pathway when HR is dysfunctional^{195,212}. However, the classical NHEJ pathway is proposed to be

engaged in HR-deficient cells that are exposed to PARPi²⁸⁴. Hence, alt-NHEJ and NHEJ are both attractive alternatives to repair DSBs and PARPi-induced DNA damage in HR-compromised cells.

4.1.2 Aims

Whilst NHEJ is conserved in *Dictyostelium*, alt-NHEJ is less well-characterised in this model organism. We identified a putative orthologue of the alt-NHEJ essential protein PolQ and sought to assess the role of this protein in DDR in *Dictyostelium*, which might indicate whether this organism is possibly able to employ this DSB repair pathway.

Whilst the concept of synthetic lethality of PARPi with HR-defective cells is known, the mechanisms resulting in cell death remain unclear. It is suggested that treatment with PARPi promotes NHEJ when HR is compromised, indicating that DNA repair using this error-prone pathway is lethal for the cells²⁸⁴. On the contrary, HR-defective cells are hypersensitive to PolQ knockdown, indicating that alt-NHEJ is engaged when HR is absent^{195,212}. As it is still under debate which DSB repair mechanism is engaged in HR-deficient cells, we sought to exploit the genetic tractability of *Dictyostelium* to define whether NHEJ or alt-NHEJ resolve DNA DSBs in HR-deficient cells. As both end joining pathways are error-prone, we also assessed whether either of the pathways directs synthetic lethality following PARPi treatment of HR-defective cells.

4.2 Results

4.2.1 Identification of a putative PolQ orthologue

Whilst it is known that the NHEJ pathway is conserved in *Dictyostelium*, it is unclear whether this model organism is equally competent in performing alt-NHEJ^{347–350}.

Interestingly, this organism possesses most proteins required for alt-NHEJ, such as DNA Lig1, Lig3 and Mre11^{203–209}. However, the alt-NHEJ essential factor DNA polymerase Θ (POLQ), which is conserved from flies to worms to humans, has not been identified in *Dictyostelium* to date^{211,323,423}. During alt-NHEJ, the resected DNA strands are recognised by a POLQ dimer, which mediates the interaction of both DNA overhangs and stabilises the DNA synapse¹⁹⁸. Furthermore, POLQ facilitates microhomology annealing and DNA synthesis^{198,210,211}. Human POLQ contains an ATPase-helicase domain in the N-terminal region of the protein, and a DNA polymerase domain at the C-terminal end, which are separated by a large central domain^{424,425}. Interestingly, solely POLQ polymerase activity but not the helicase domain is essential for resistance to DNA DSBs^{195,426}. However, only the helicase activity of the alt-NHEJ enzyme PolQ is annotated in the *Dictyostelium* genome, whereas the essential polymerase domain has not been located so far. Given the helicase and the polymerase domain are separated by a large central domain in mammalian cells, it is possible that in *Dictyostelium* the domains are rearranged in the genome and have evolved into two separated proteins⁴²⁷.

To locate the PolQ polymerase domain, we used BLAST to align human POLQ to the *Dictyostelium* proteome, thereby searching for local sequence homologies with the human enzyme³⁷⁷. The output of the BLAST search is illustrated in Figure 4.1A alongside the detected expectation score (E-values). The E-value describes the probability that the match occurred randomly, taking into account the length of the query sequence, as well as the database size. The closer the E-value is to zero, the more significant the match is to the input sequence. As we applied a stringent threshold of less than e^{-25} , we only detected three matches (Figure 4.1A) with the best score for the already known PolQ-like helicase domain. Another annotated protein, ascc3l, also aligned to the helicase domain but with a higher E-value compared to the PolQ-like helicase. Ascc3l has not been studied so far, but it contains a DEAD box helicase domain, thereby explaining the sequence similarity with the helicase domain of PolQ. Only the unknown protein

DDB_G0277749 aligns to the PolQ polymerase domain with an E-value of $9e^{-56}$. Hence, this BLAST search suggests that *Dictyostelium* PolQ is spread over at least two proteins, one containing the helicase domain and the other the polymerase domain. The sequence alignment of human POLQ polymerase domain to the potential *Dictyostelium* PolQ is shown in Figure 4.1B. Identical amino acids are marked as yellow, whereas amino acids of a similar type are coloured in turquoise. To verify that the identified gene is coding for the PolQ-polymerase domain, we used BLAST to align the protein sequence of DDB_G0277749 to the human proteome. As expected, the first hit was human POLQ, which aligned with 26.5% to the *Dictyostelium* PolQ-like polymerase indicating that we identified the *Dictyostelium* orthologue of the human POLQ polymerase domain. Hence, from now on DDB_G0277749 will be referred to as PolQ. The essential amino acids required for the catalytic activity of PolQ are conserved in *Dictyostelium* PolQ and are encircled in red in Figure 4.1B^{428–431}. Two positively charged residues, R2202 and R2254 (marked with an asterisk in Figure 4.1B), that are only present in PolQ but no other polymerases are partially conserved in *Dictyostelium*. Structural studies in humans suggest that both residues interact with the phosphate backbone of the primer^{431,432}. Other important residues that are also conserved in *Dictyostelium* are encircled in black, although their functions have yet to be determined^{431,432}. The human POLQ contains three sequence insertions, which are only partially conserved in the *Dictyostelium* PolQ-like polymerase (underlined in Figure 4.1B)^{431,433}. The first insertion is potentially involved in the processivity of POLQ, whereas the second and third insertion promote the polymerase's ability to bypass abasic sites^{425,431,434}. However, although the *Drosophila* orthologue of PolQ is lacking all three insertions, the enzyme is still essential for the alt-NHEJ pathway in this model, indicating that the insertions might not be important for the role of PolQ in alt-NHEJ²¹¹.

Using InterProScan, the protein sequence of *Dictyostelium* PolQ was compared to the predicted domains, structures and function sites of human POLQ^{378,379}. These predicted

protein domains are displayed in Figure 4.1C. Similar to human POLQ, the *Dictyostelium* counterpart belongs to the DNA polymerase A family. Besides the DNA-directed DNA polymerase domain, *Dictyostelium* PolQ contains a ribonuclease H like domain as well as a WD40/YVTN repeat-like-containing domain. The latter describes a repeated motif consisting of around 40 residues that are involved in protein binding^{435,436}. Strikingly, the WD40/YVTN domain is missing in human POLQ, indicating that this region is a unique feature of *Dictyostelium* PolQ and possibly required for the interaction with the PolQ-like helicase domain. The ribonuclease H like (RNaseH) domain is also present in mammalian and *Drosophila* PolQ and hydrolyses RNA-DNA intermediates and binds to nucleic acids⁴³⁷.

4.2.2 Disrupting *polq* sensitises cells to DNA DSBs

Having identified a potential gene encoding the PolQ polymerase domain, we sought to assess its effect on DNA damage repair in *Dictyostelium*. Hence, we generated a *polq* disruption strain using the strategy of targeted homologous recombination, thereby removing the *polq* genomic locus starting 3bp upstream of the start codon and leaving 800bp of the last exon in the genome (Figure 4.2A). Both homologous arms were amplified by PCR and ligated into the pLPBLP vector on either side of the BSR cassette, using the indicated restriction enzyme sites, generating the *polq* disruption vector (Figure 4.2B). The homologous arms were checked for mutations using Sanger sequencing. The targeting construct, containing the homologous arms flanking the BSR cassette, was excised from the vector using KpnI/SacII and PCR purified.

Dictyostelium Ax2 cells were transfected with the targeting construct using standard protocols and selection in blasticidin occurred until cell growth was observed. To obtain clonal cells, the blasticidin resistant cells were spread onto a KA lawn on SM agar. Targeted integration into the *polq* genomic locus was confirmed by PCR (Figure 4.3A).

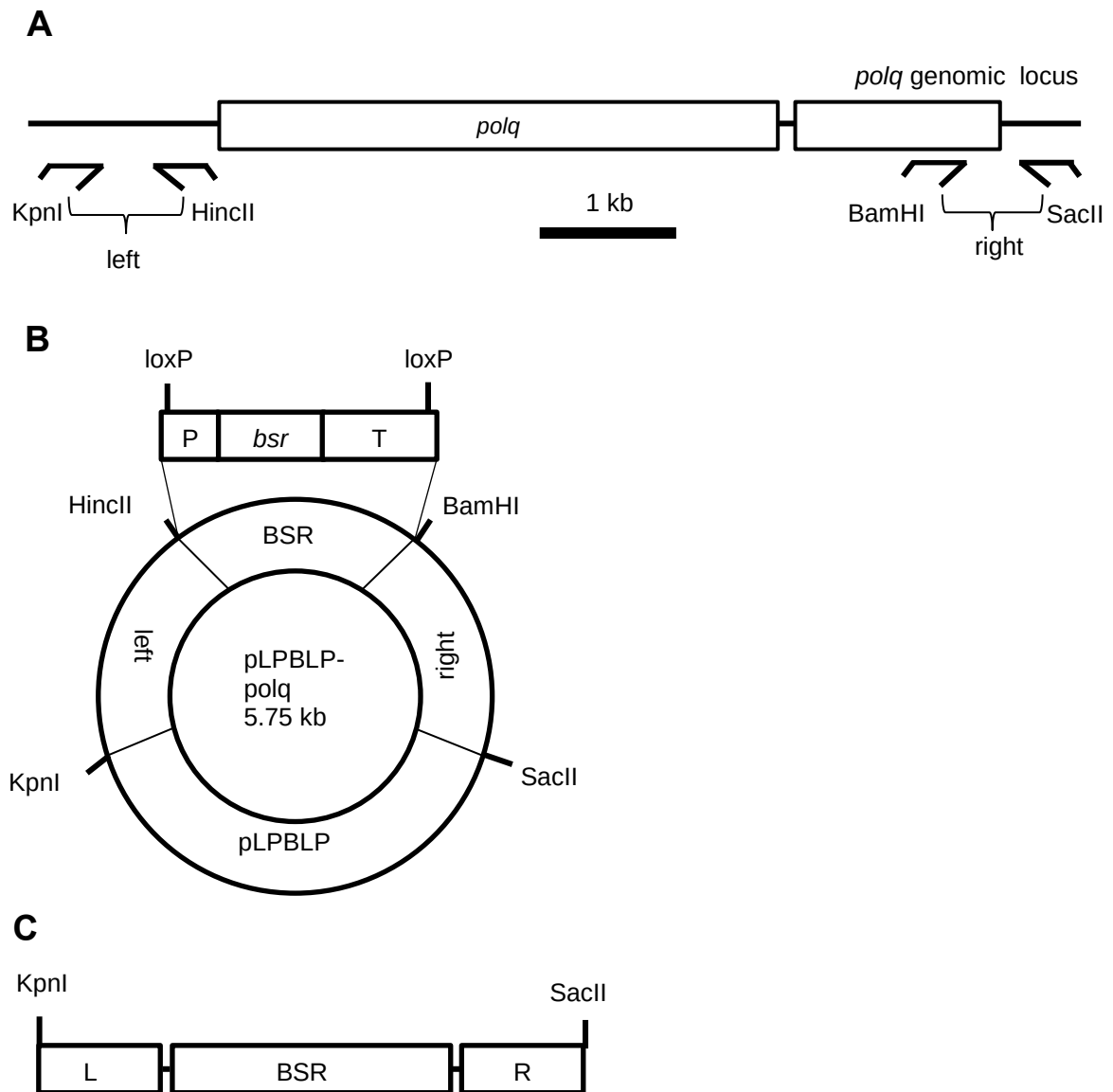


Figure 4.2: Strategy for the disruption of the potential *polq* gene coding for the polymerase domain by targeted homologous recombination. **A.** Representation of the *polq* genomic locus in *Dictyostelium*. The locations of the homologous arms, used for the disruption of the *polq* gene by targeted homologous recombination, are depicted as “left” (L) and “right” (R). Primers used to amplify the homologous arms are shown with attached restriction enzyme sites. **B.** Map of the pLPBLP *polq* disruption vector. The homologous arms are ligated on either side of the cassette coding for blasticidin resistance (BSR), using the indicated restriction enzymes (KpnI and HincII for the left arm, BamHI and SacII for the right). Blasticidin is a toxic antibiotic for the parental Ax2 strain. The structure of the BSR cassette is shown in the upper panel containing the *bsr* gene, the actin6 promoter (P) and the terminator (T). The location of the LoxP sites are also indicated which can be used to excise the BSR cassette using Cre recombinase. **C.** Representation of the final fragment that is used for the disruption of the *polq* genomic locus in Ax2 cells, which is obtained through digestion with the restriction enzymes KpnI and SacII.

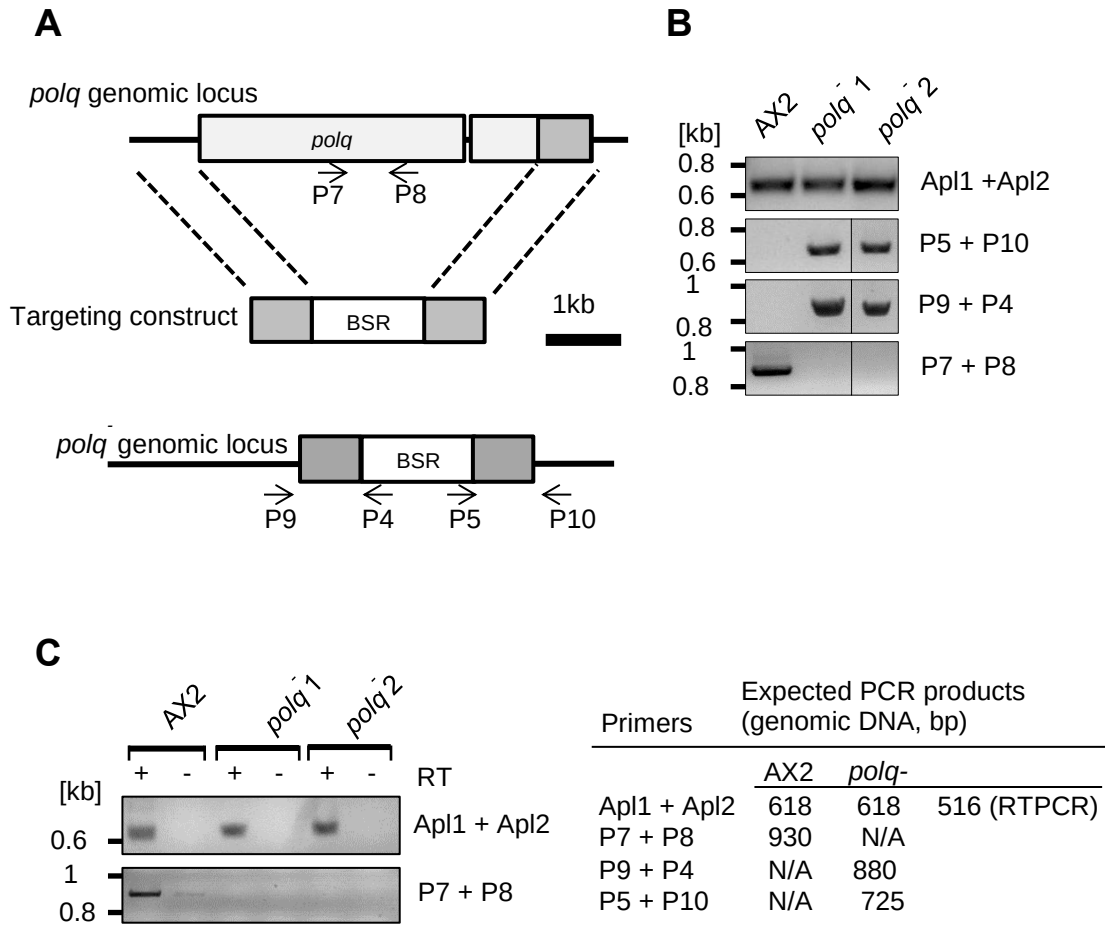


Figure 4.3: Generation and verification of *polq*⁻ *Dictyostelium* strains. **A.** Representation of the desired recombination event of the potential *polq* genomic locus (top) with the *polq* targeting construct (middle). A blasticidin resistance (BSR) cassette flanked by homology regions, depicted as grey boxes, is inserted via targeted homologous recombination, generating a *polq*⁻ strain (bottom). Thereby, the potential *polq* genomic locus is disrupted 3 bp upstream of the ATG start codon. The light grey box on the right side in the bottom figure represents the remaining 800bp in the final *polq* exon in the disruption strain. P indicates the primers used for screening by PCR. **B.** PCR verification of the *polq*⁻ strains using the primers at locations indicated in (A). The expected fragment sizes for Ax2 and *polq*⁻ strains are shown in the table. As a control, oligonucleotides were used that amplify a region of the *apl* genomic locus. **C.** RT-PCR verification of *polq*⁻ strain using the primers at locations indicated in (A). As control, oligonucleotides were used that amplify a region of the *apl* genomic locus. RT: Reverse Transcriptase

Genomic DNA of potential *polq*⁻ cells and parental Ax2 strains was amplified using oligonucleotides spanning from the BSR cassette to a region external to the disruption construct. Thereby, targeted integration of the homologous arms and insertion of the BSR cassette was confirmed (Figure 4.3B). Furthermore, primers binding to a region in the first exon of *polq*, which is deleted in the disruption strain, were used to verify the generation of *polq*⁻ strains (Figure 4.3B). Additionally, cDNA of potential *polq*⁻ strains was prepared using standard protocols and subsequently used in a RT-PCR to test PolQ expression levels (Figure 4.3C). In contrast to Ax2 strains, the two *polq*⁻ strains were lacking PolQ expression confirming the successfully generation of two independent *polq*⁻ strains.

As PolQ has been implicated to play a role in base excision repair and DSB repair in different eukaryotes, we studied the effect of various DNA damaging agents on the survival of *polq*⁻ strains⁴³⁸. Thereby, we wanted to explore the role of the PolQ polymerase domain in DDR and identify if *Dictyostelium* PolQ is the functional orthologue of the human protein. Therefore, two independent *polq*⁻ strains and Ax2 cells were exposed to different concentrations of the indicated DNA damage inducing agents and their survival was assessed as previously described. We included the *exo1*⁻ strain in the experiments as control for the activity of the DNA damaging agents (Figure 4.4). The *exo1*⁻ cells were slightly, but significantly sensitive to the oxidative DNA damage inducing compound H₂O₂ compared to AX2 cells (Figure 4.4B). Exposure to the DSB inducing agent phleomycin and to the alkylating agent MMS significantly reduced the survival of the HR-deficient *exo1*⁻ cells, verifying the activity of both agents (Figure 4.4A+C). Exposure to MMS did not affect cell survival of the *polq*⁻ strains, indicating that the PolQ-like polymerase is not involved in resolving DNA damage induced by this agent (Figure 4.4A). Treatment with H₂O₂ significantly decreased the survival of one of the *polq*⁻ strains compared to the Ax2 strain. However, the calculated p-values did not severely differ between the two *polq*⁻ strains, 0.0230 for *polq*⁻.2 being significantly different from Ax2 and 0.0526 for *polq*⁻.1 marginally above the 0.05 threshold (Figure 4.4B). Hence, PolQ is possibly playing a

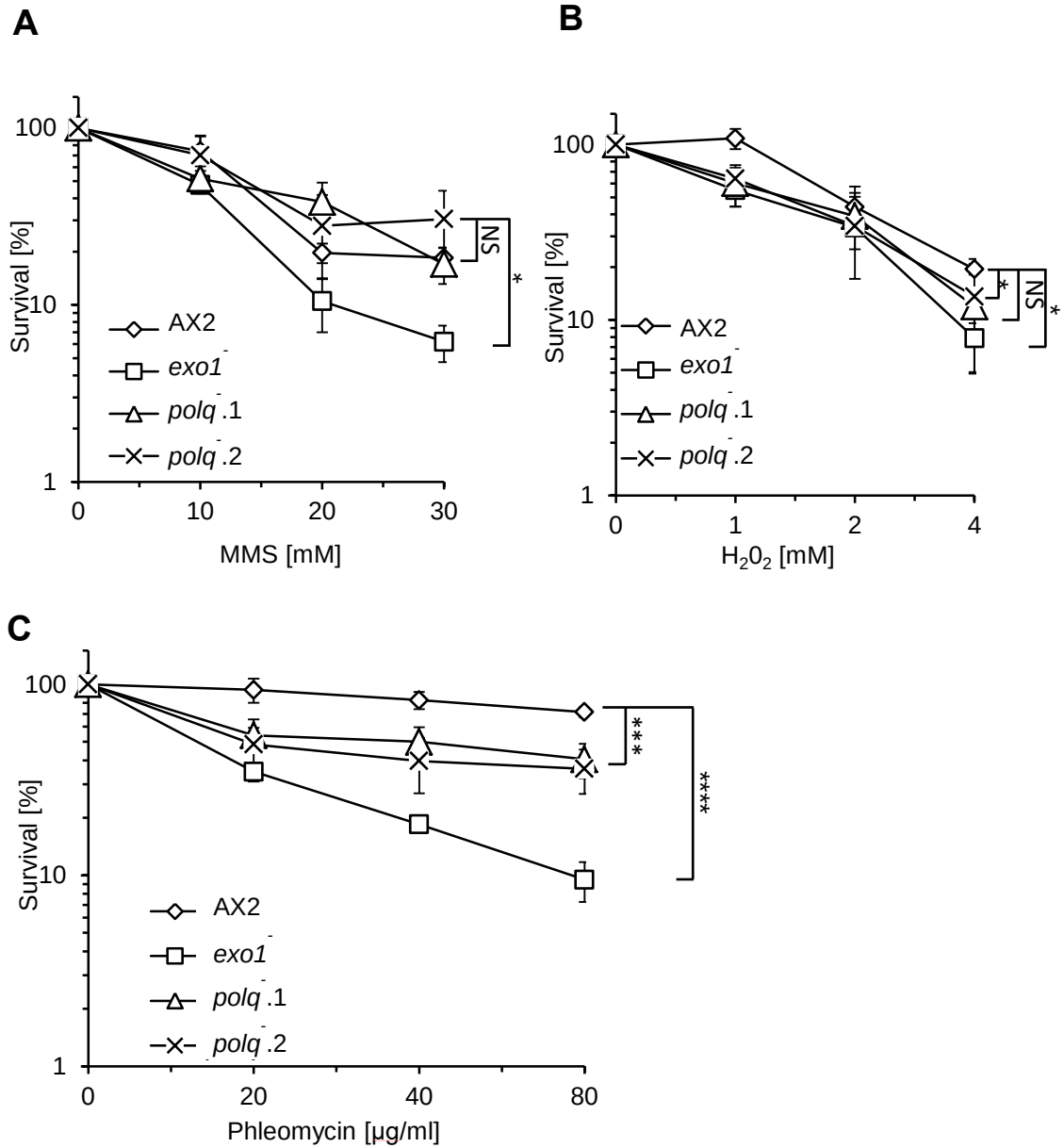


Figure 4.4: The *polq*⁻ strains are sensitive to DNA DSB. A-C. Ax2, *exo1*⁻ and two independent *polq*⁻ strains were treated with the indicated DNA damaging agent, MMS (A), H₂O₂ (B), phleomycin (C) or left untreated for 1 hour before plating onto a KA lawn on SM agar plates. After 3-7 days survival was assessed by plaque formation relative to an untreated control. Error bars represent the SEM from three independent experiments. Statistical significance was determined by two-way ANOVA compared to the AX2 strain, NS P>0.05, * P≤0.05, ** P≤0.01, *** P≤0.001, **** P≤0.0001.

minor role in the repair of oxidative DNA damage in *Dictyostelium*. Exposure to phleomycin significantly decreased the survival of both *polq*⁻ strains compared to Ax2 cells (Figure 4.4C). This indicates that the PolQ-like polymerase is involved in DNA DSB repair in *Dictyostelium*. Since the *exo1*⁻ strain was more sensitive to phleomycin as the *polq*⁻ cells, this suggests that the function of PolQ in DNA DSB repair is less important than the role of Exo1. Given that *Dictyostelium* PolQ is similar to its human orthologue involved in DSB repair, this indicates that it is possibly required for alt-NHEJ. However, as we did not formally test whether *Dictyostelium* are competent in alt-NHEJ other roles of PolQ in DNA repair cannot be excluded at this stage. Hence, further investigations are required to elucidate the exact function of PolQ.

4.2.3 Disruption of NHEJ or PolQ-dependent repair increases the sensitivity of *exo1*⁻ strains to DNA DSBs

Next, we sought to investigate the hierarchy of the different DNA DSB repair mechanisms and whether a redundancy between these pathways allows cells to tolerate DNA DSBs. Since HR is the main repair pathway in *Dictyostelium*, we considered whether PolQ-dependent repair or classical NHEJ is engaged to resolve DSBs in HR-deficient strains. This may indicate which DSB repair pathway is employed in HR-compromised cells and could further address the long-term aim of identifying the molecular basis behind synthetic lethality of HR-defective cells with PARPi. Whilst PolQ-dependent repair is proposed to compete with HR in resolving replication stress, NHEJ is suggested to repair PARPi-induced damage if HR is compromised, making both pathways likely candidates to repair DSBs in HR-deficient cells^{284,421,422}. To identify which pathway is engaged when HR is defective, we exploited the genetic tractability of *Dictyostelium* to disrupt two DSB repair pathways in combination by targeting genes important for the respective repair pathways. We employed the already established *dnapkcs*⁻ strain as NHEJ-deficient *Dictyostelium*

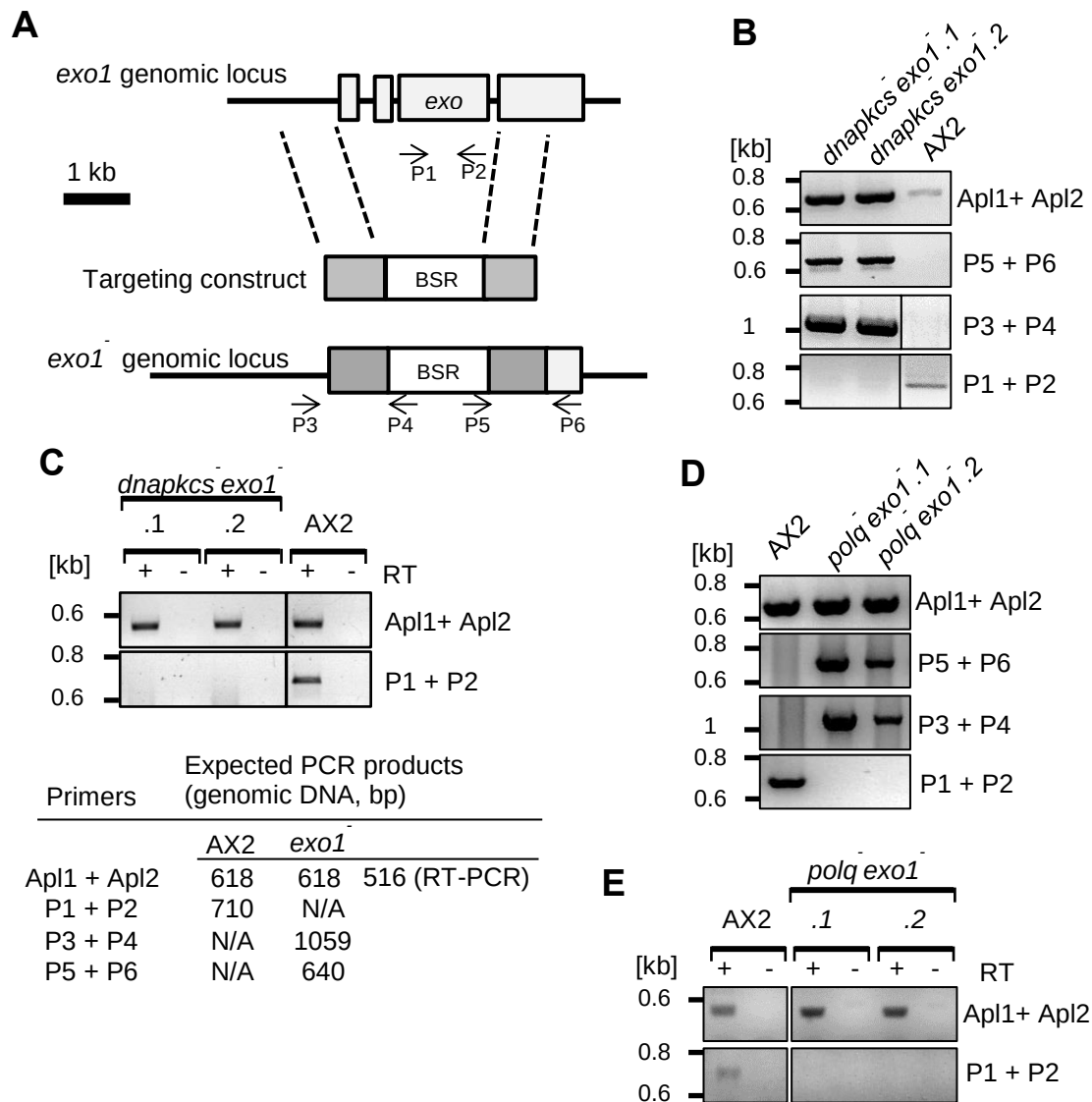


Figure 4.5: Generation and verification of *dnapkcs*⁻*exo1*⁻ and *polq*⁻*exo1*⁻ strains. **A.** Representation of the desired recombination event of the *exo1* genomic locus (top) with the *exo1* targeting construct (middle). A blasticidin resistance (BSR) cassette flanked by the homology regions (grey boxes) is inserted into the *exo1* genomic locus via targeted homologous recombination, generating an *exo1*⁻ strain (bottom). The light grey box in the bottom figure represents the remaining 950bp in the final *exo1* exon. P indicates the primers used for screening by PCR. **B.** PCR verification of the *dnapkcs*⁻*exo1*⁻ strains using the primers at locations indicated in (A). As control, oligonucleotides were used that amplify a region of the *apl* genomic locus. The expected fragment sizes for Ax2 and disrupted *exo1* in the *dnapkcs*⁻ and *polq*⁻ background strains are shown in the table. **C.** RT-PCR verification of *dnapkcs*⁻*exo1*⁻ strains using the primers at locations indicated in (A). **D.** PCR verification of the *polq*⁻*exo1*⁻ strains using the primers at locations indicated in (A). The expected fragment sizes for Ax2 and disrupted *exo1* in the *dnapkcs*⁻ and *polq*⁻ background strains are shown in the table. **E.** RT-PCR verification of *polq*⁻*exo1*⁻ strains using the primers at locations indicated in (A).

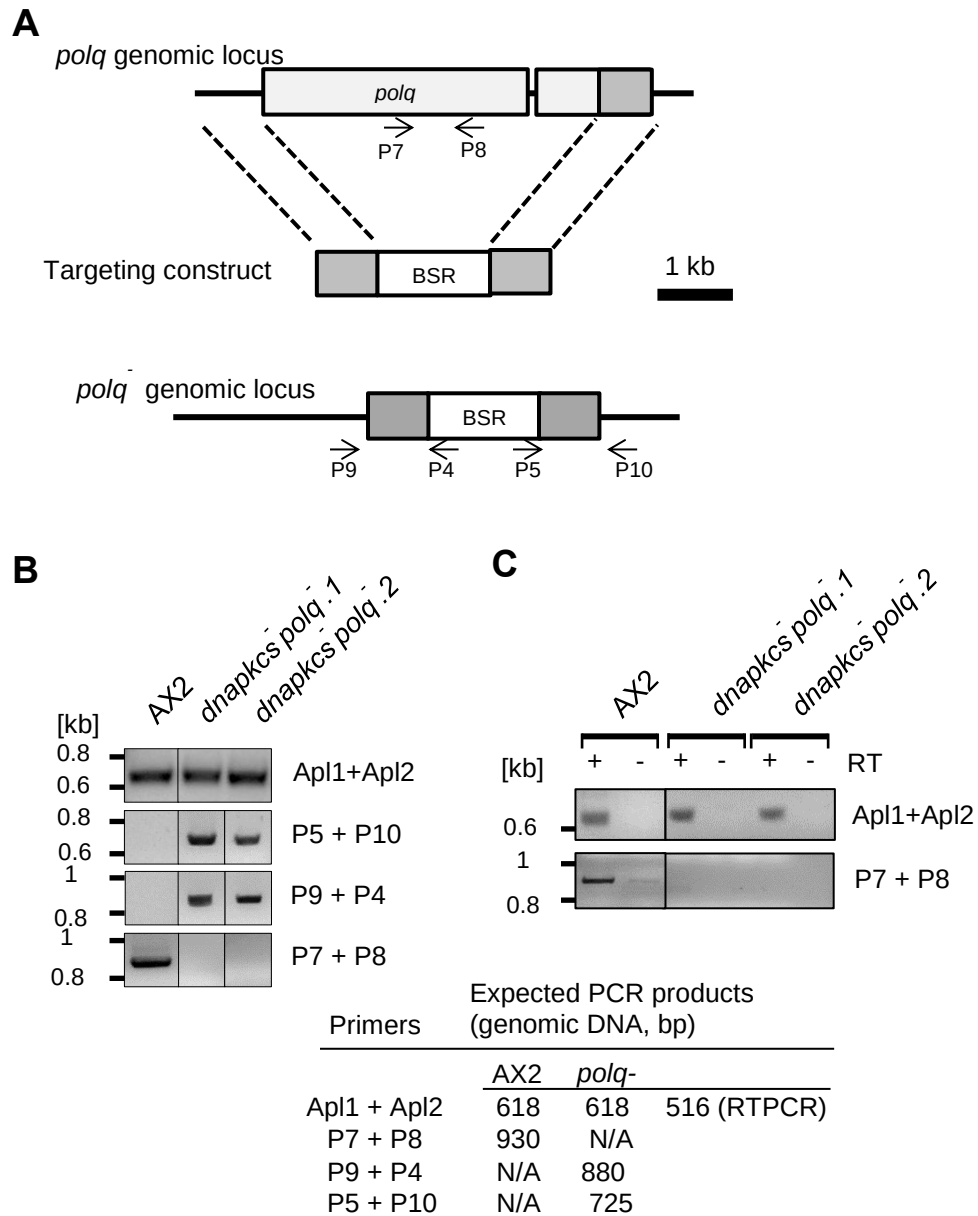


Figure 4.6: Generation and verification of *dnapkcs*⁻*polq*⁻ strains. **A.** Representation of the desired recombination event of the potential *polq* genomic locus (top) with the *polq* targeting construct (middle). A blasticidin resistance (BSR) cassette flanked by homology regions, depicted as grey boxes, is inserted via targeted homologous recombination, generating a *polq*⁻ strain (bottom). Thereby, the *polq* genomic locus is disrupted 3 bp upstream of the ATG start codon. The light grey box on the right side in the bottom figure represents the remaining 800bp in the final *polq* exon in the disruption strain. P indicates the primers used for screening by PCR. **B.** PCR verification of the *dnapkcs*⁻*polq*⁻ strains using the primers at locations indicated in (A). Oligonucleotides that amplify a region of the *apl* genomic locus were used as a control. The expected fragment sizes for Ax2 and disrupted *polq* in the *dnapkcs*⁻ are shown in the table **C.** RT-PCR verification of *dnapkcs*⁻*polq*⁻ strains using the primers at locations indicated in (A).

strain³⁵³. Disruption of the *exo1* genomic locus in the *dnapkcs*⁻ background strain was performed using the same strategy as described previously (Figure 4.5A), thereby generating *Dictyostelium* cells that are defective in HR and NHEJ. Verification of the *dnapkcs*⁻*exo1*⁻ strains was performed as described previously but instead of Southern blotting, RT-PCR using oligonucleotides amplifying a region deleted in the *exo1* disruption strain was performed to confirm the lack of *exo1* expression (Figure 4.5B-C). For the generation of strains defective in HR and PolQ-dependent repair, we first recycled the floxed BSR cassette in the *polq*.2 strain by excising the loxP sites flanking the BSR cassette using *Cre*-recombinase following standard procedures. We disrupted the *exo1* genomic locus in the *polq*.2 strain using the previously described strategy (Figure 4.5A). Verification of *exo1* disruption occurred as described previously using PCR and RT-PCR (Figure 4.5D-F), confirming the generation of two independent *polq*⁻*exo1*⁻ strains.

For complete analysis of the interplay between the different DSB repair pathways, and to further investigate the role of PolQ, we generated *dnapkcs*⁻*polq*⁻ strains, which are deficient in NHEJ and PolQ-dependent repair. Therefore, the *polq* genomic locus was deleted in the *dnapkcs*⁻ strain, using the previously described strategy (Figure 4.6A) Strain verification was performed by PCR and RT-PCR (Figure 4.6). In summary, we verified the generation of the following *Dictyostelium* strains: *dnapkcs*⁻*exo1*⁻, *polq*⁻*exo1*⁻ and *dnapkcs*⁻*polq*⁻.

Whilst NHEJ is the main DNA DSB repair pathway in human somatic cells, it plays a minor role in HR-proficient *Dictyostelium*^{353,439}. However, NHEJ-deficient mutants are defective in restriction enzyme mediated integration (REMI) of DNA into the *Dictyostelium* genome and exhibit delayed cell growth following DNA DSBs, indicating that NHEJ is employed in vegetative cells^{341,354}. As HR-defective ovarian cancer cell lines are dependent on DNA damage repair using the polymerase domain of POLQ, and therefore rely on DSB repair via alt-NHEJ, we wanted to identify whether NHEJ or PolQ-dependent

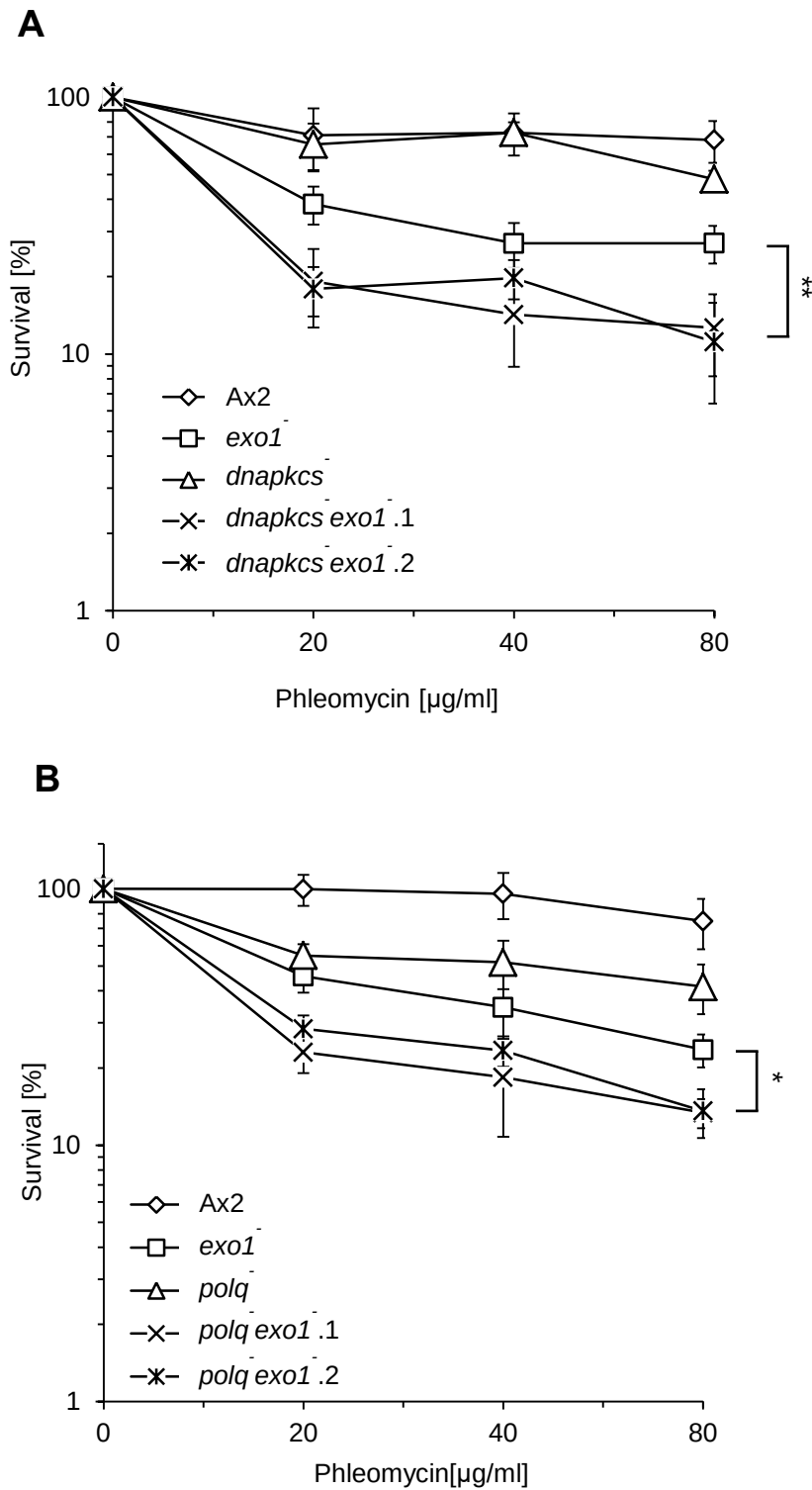


Figure 4.7: Disruption of *dnapkcs* and *polq* further sensitises *exo1*⁻ cells to phleomycin. A. Ax2, *exo1*⁻, *dnapkcs*⁻ and two independent *dnapkcs*⁻*exo1*⁻ strains were treated with the indicated phleomycin concentrations for one hour before plating on SM agar. After 3-7 days survival was assessed by plaque formation relative to a mock-treated control. Error bars represent the SEM from three independent experiments. Statistical significance was determined with respect to the *exo1*⁻ strain by two-way ANOVA, NS $P > 0.05$, * $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$. **B.** Ax2, *exo1*⁻, *polq*⁻ and two independent *polq*⁻*exo1*⁻ strains were treated with the indicated phleomycin concentrations for one hour and analysed as described in (A). Error bars represent the SEM from three independent experiments. Statistical significance was determined with respect to the *exo1*⁻ strain by two-way ANOVA, NS $P > 0.05$, * $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$.

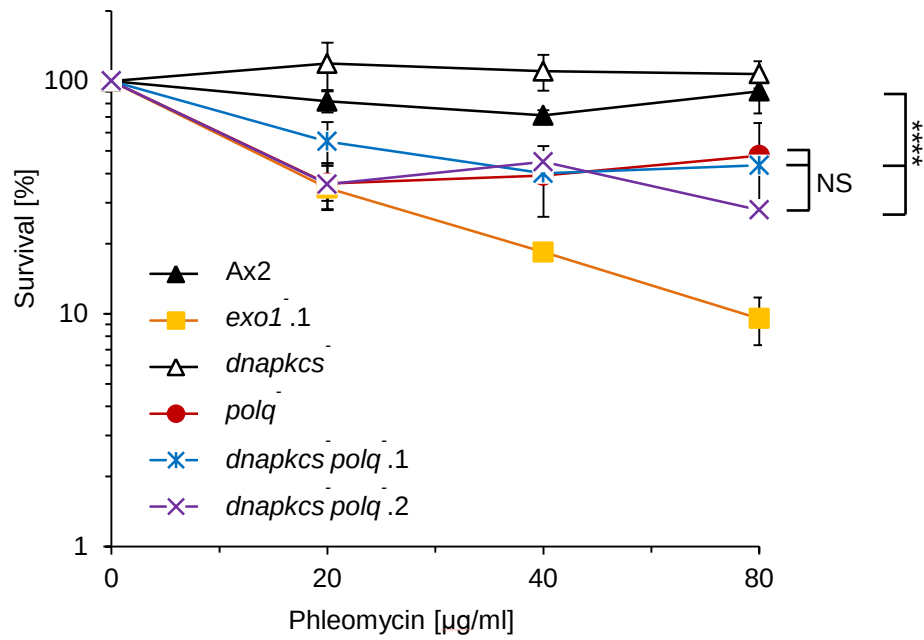


Figure 4.8: Disruption *dnapkcs* of does not further sensitise *polq* cells to phleomycin. Ax2, *exo1*, *polq*, *dnapkcs* and two independent *dnapkcs polq* strains were treated with the indicated phleomycin concentrations for one hour before plating on SM agar. After 3-7 days survival was assessed by plaque formation relative to a mock-treated control. Error bars represent the SEM from three independent experiments. Statistical significance was determined with respect to the Ax2 strain, *polq* and the *dnapkcs* strain by two-way ANOVA, NS $P > 0.05$, * $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$, **** $P \leq 0.0001$.

repair is engaged to resolve DSBs in HR-deficient *Dictyostelium* cells¹⁹⁵. We assessed the sensitivity of *dnapkcs⁻exo1⁻* and *polq⁻exo1⁻* strains to phleomycin and quantified the survival relative to the untreated control. The survival of *dnapkcs⁻exo1⁻* strains was significantly reduced with respect to the *exo1⁻* strain, indicating that disrupting NHEJ further increases the sensitivity of HR-defective cells to DNA DSBs (Figure 4.7A). A similar result was observed when *polq⁻exo1⁻* strains were exposed to phleomycin (Figure 4.7B). Disruption of *polq* significantly further sensitised the *exo1⁻* strain to DSBs, suggesting that PolQ plays a role in DNA DSB repair other than HR. Concluding, disrupting *polq* or *dnapkcs* reduces the survival of *exo1⁻* cells to DSBs, indicating that NHEJ and PolQ-dependent repair are involved in DNA DSB repair when HR is compromised.

The *polq⁻* mutants are sensitive to DNA DSBs (Figure 4.4C), whereas disrupting *dnapkcs* does not affect the viability of *Dictyostelium* cells to bleomycin³⁵³. This indicates that in HR-proficient *Dictyostelium* PolQ-dependent repair is more frequently employed than NHEJ. As both pathways, PolQ-dependent repair and NHEJ, are resolving DNA lesions in HR-compromised cells, we sought to identify whether NHEJ equally compensates for the deficiency of PolQ. These data either assign NHEJ an important function in resolving lesions if other pathways fail, or support HR as the main DSB repair pathway in *Dictyostelium*. The cell survival of *dnapkcs⁻polq⁻* strains was significantly reduced when treated with phleomycin compared to *dnapkcs⁻* cells (Figure 4.8). However, since the double knockout strains were similarly sensitive to DSBs as the *polq⁻* mutant, this indicates that disrupting NHEJ is not enhancing the sensitivity of *polq⁻* cells to DNA DSBs (Figure 4.8). This suggests that cells defective in PolQ, but competent in HR, are not relying on NHEJ to repair DNA lesions. Taken together, NHEJ and PolQ-dependent repair are both employed when HR is dysfunctional. Interestingly, *Dictyostelium* preferable resolve DSBs through HR and maybe to a lesser extent via PolQ-dependent repair instead of NHEJ.

4.2.4 Disrupting *dnapkcs* but not *polq* suppresses PARPi toxicity to *exo1⁻* cells

In addition to the role of *dnapkcs* and *polq* in DSB repair, we wanted to investigate their function in synthetic lethality of PARP inhibition with HR deficiency. We previously established that Rad51 is enriched on chromatin following PARPi treatment, which is defective in *exo1⁻* cells (Chapter 3). This is in line with the current dogma explaining that PARPi impair SSB repair, resulting in increased DNA damage, which is channelled through HR. Given the HR defect of *exo1⁻* strains, another DSB repair pathway possibly repairs PARPi-induced DNA lesions, either resulting in cell death or rendering a subset of cells viable despite the PARPi treatment. Enrichment of the NHEJ-essential protein Ku70 onto chromatin was used to assess whether this DSB repair pathway was employed in *exo1⁻* cells. Given that PolQ depletion similarly reduces the survival of *Dictyostelium* and mammalian HR-deficient cells to DSBs, *Dictyostelium* PolQ might be equally important for alt-NHEJ^{195,212}. As we also considered whether *Dictyostelium* are able to employ this DSB repair pathway, we investigated the chromatin recruitment of another factor important for mammalian alt-NHEJ, DNA Ligase III (Lig3), following PARPi treatment.

Myc-Ku70 and Myc-Lig3, respectively, were expressed in Ax2 and *exo1⁻* cells using the extrachromosomal pDXA-3C vector. When incubated with the Myc antibody, whole-cell extracts from Ax2 and *exo1⁻* cells transfected with pDXA-3C-Myc-Ku70 (Figure 4.9A) showed a signal at the size of 104 kDa, which is absent in the cells expressing the empty pDXA-3C vector. The same result was observed when using the pDXA-3C-Myc-Lig3 vector (Figure 4.9B). Only the cells transfected with the vector containing Myc-Lig3 but not the empty vector exhibited a signal at the size of Lig3 (133 kDa). Hence, we generated Ax2 and *exo1⁻* cell lines that express Myc-Ku70 and Myc-Lig3 using an extrachromosomal vector system.

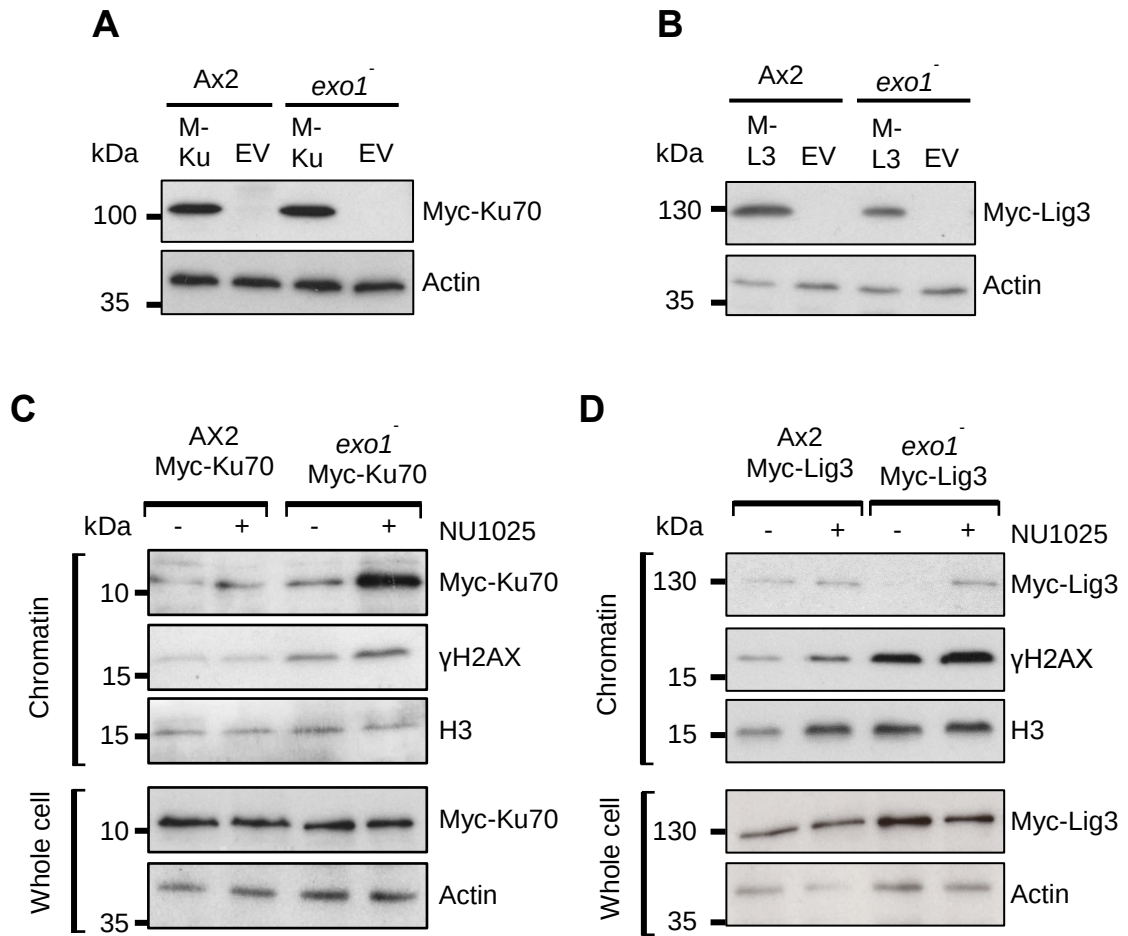


Figure 4.9: Myc-Ku70 is enriched on Chromatin in *exo1*⁻ strain following PARPi treatment. **A-B.** Ax2 and *exo1*⁻ cells were transfected with an empty pDXA-3C vector (EV) or with pDXA-3C-Myc-Ku70 (M-Ku) (**A**) or pDXA-3C-Myc-Lig3 (M-L3) (**B**) using standard procedures. Whole cell extracts were prepared and expression of the Myc-tagged proteins was detected by Western blotting using the indicated antibodies. **C-D.** Ax2 cells and *exo1*⁻ cells expressing Myc-Ku70 (**C**) or Myc-Lig3 (**D**) were left untreated or exposed to 1mM Nu1025 for 2.5 hours. Chromatin and whole cell extracts were prepared and Western blotting was performed with the indicated antibodies.

As we wanted to assess whether NHEJ or Lig3-dependent repair is employed to resolve PARPi-induced damage in HR-defective *Dictyostelium* strains, we analysed the enrichment of Myc-Ku70 and Myc-Lig3 on chromatin following PARP inhibition. Therefore, Ax2 and *exo1*⁻ cells expressing Myc-Ku70 or Myc-Lig3, respectively, were exposed to Nu1025, followed by chromatin extraction and analysis of the Myc-tagged protein levels on chromatin by Western blotting. Compared to the loading control H3, γ H2AX levels were increased in *exo1*⁻ cells following Nu1025 treatment and to a lesser extent in Ax2 cells, confirming that Nu1025 induces DNA damage in *Dictyostelium* cells (Figure 4.9C-D). Myc-Ku70 was slightly enriched on chromatin in the Ax2 cells treated with Nu1025, indicating that a small amount of PARPi-induced DNA damage is potentially repaired by NHEJ. Strikingly, Myc-Ku70 was highly enriched on chromatin in Nu1025 exposed *exo1*⁻ cells, indicating that in contrast to the parental strain, PARPi-mediated damage is channelled through NHEJ if HR is dysfunctional. Whilst Nu1025 did not induce Myc-Lig3 enrichment on chromatin in the parental strain, Myc-Lig3 was slightly enriched in *exo1*⁻ cells following PARPi treatment. It is possible that a small amount of PARPi-induced DNA damage is resolved through a Lig3-dependent pathway. In contrast to Myc-Lig3, Myc-Ku70 was highly enriched on chromatin in *exo1*⁻ cells following PARPi exposure, suggesting that NHEJ is engaged to repair PARPi-mediated DNA damage in HR mutants.

Lig3 is involved in mammalian alt-NHEJ and furthermore, A. Gunn illustrated that *Dictyostelium* Lig3 is enriched on chromatin following MMS treatment, indicating that this protein is involved in DNA repair in *Dictyostelium* (unpublished data). Hence, besides *polq* and *dnapkcs*, we wanted to assess whether disrupting *lig3* is affecting synthetic lethality of *exo1*⁻ cells to PARPi. Thereby, we were hoping to further explore the role of Lig3 in resolving PARPi-induced DNA damage. We disrupted the *exo1* genomic locus in the *lig3*⁻ strain (kindly provided by A. Gunn) and verified *exo1* disruption as described previously using PCR, confirming the generation of two independent *lig3*⁻*exo1*⁻ strains (Figure 4.10A-B). For complete analysis of the interplay between the different DSB repair

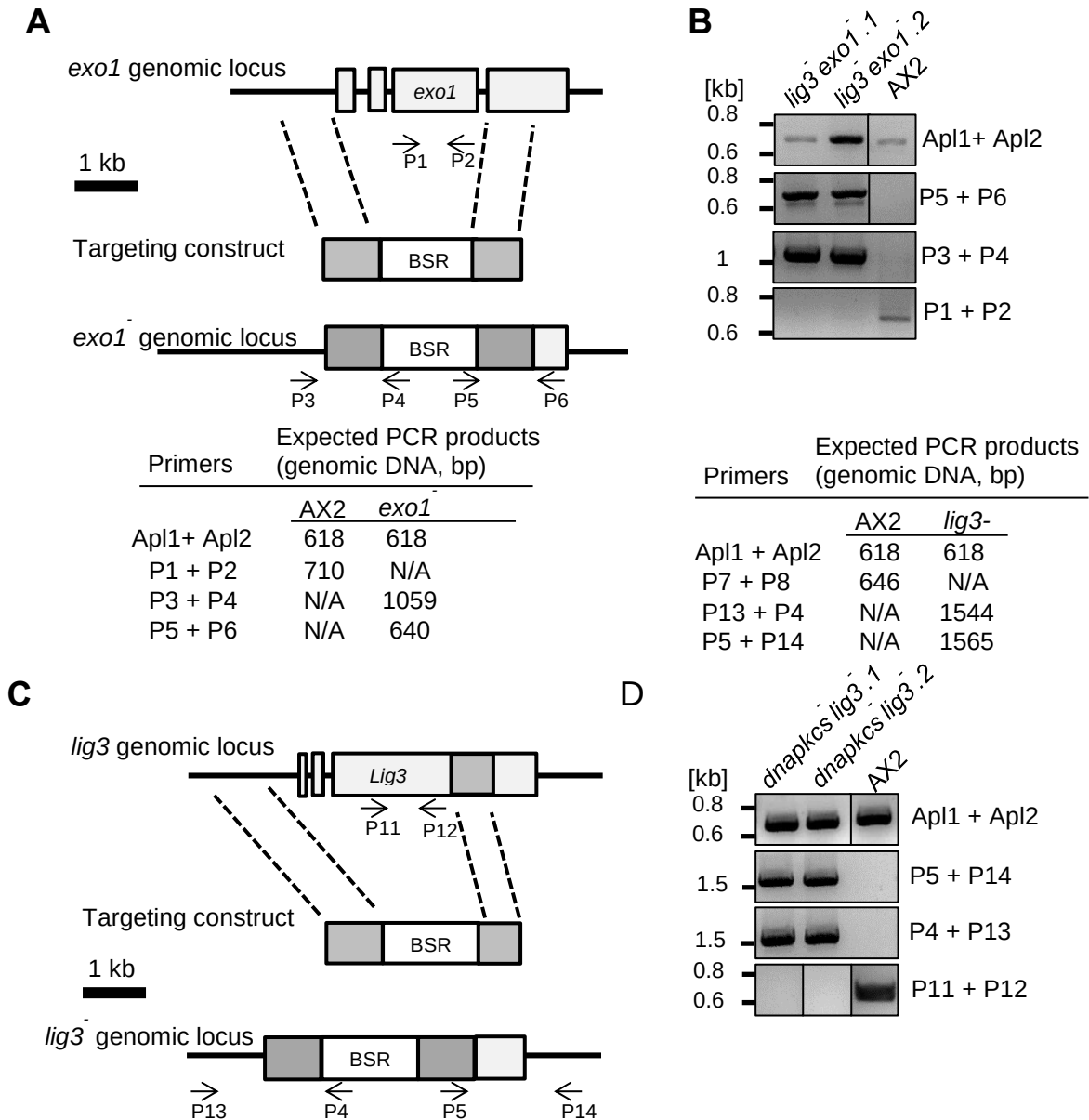


Figure 4.10: Generation and verification of *lig3*⁻*exo1*⁻ and *dnapkcs*⁻*lig3*⁻ strains. The *lig3*⁻ disruption strategy and the *lig3*⁻ strain was provided by Alasdair Gunn. **A.** Representation of the desired recombination event of the *exo1* genomic locus (top) with the *exo1* targeting construct (middle). A blasticidin resistance (BSR) cassette flanked by the homology regions (grey boxes) is inserted into the *exo1* genomic locus via targeted homologous recombination, generating an *exo1*⁻ strain (bottom). The light grey box in the bottom figure represents the remaining 950bp in the final *exo1* exon. P indicates the primers used for screening by PCR. **B.** PCR verification of the *lig3*⁻*exo1*⁻ strains using the primers at locations indicated in (A). The expected fragment sizes for Ax2 and disrupted *exo1* in the *lig3*⁻ background strain are shown in the table. **C.** Representation of the desired recombination event of the *lig3* genomic locus (top) with the *lig3* targeting construct (middle). A blasticidin resistance (BSR) cassette flanked by homology regions, shown as grey boxes, is inserted via targeted homologous recombination generating *lig3*⁻ strain (bottom). This strategy disrupts most of the *lig3* genomic locus, with only the last 600bp remaining of the final exon, depicted in grey, downstream of the BSR cassette. P indicates the primers used for screening by PCR. **D.** PCR verification of the *dnapkcs*⁻*lig3*⁻ strains occurred using the primers at locations indicated in (C). Oligonucleotides that amplify a region of the *apl* genomic locus serve as control. The expected fragment sizes for Ax2 and disrupted *lig3* in the *dnapkcs*⁻ background strain are shown in the table.

pathways and to further investigate the role of Lig3, we also generated *dnapkcs⁻lig3⁻* strains. The *lig3* targeting construct, kindly provided by A. Gunn (A), was used to disrupt the *lig3* genomic locus in the *dnapkcs⁻* strain (Figure 4.10C). Verification of the *dnapkcs⁻lig3⁻* strain was performed by PCR using oligonucleotides spanning from the BSR cassette to the external region of the homologous arms, thereby confirming targeted integration of the BSR cassette (Figure 4.10D). Further verification occurred with a PCR using primers amplifying a region deleted in the *lig3⁻* strain, thereby confirming the generation of two independent *dnapkcs⁻lig3⁻* strains.

Subsequently, we wanted to investigate if synthetic lethality of HR deficiency with PARPi is affected by disrupting *dnapkcs*, *lig3* or *polq* in combination with *exo1*. We hypothesised that if an error prone repair pathway is engaged to repair PARPi-induced DNA damage, disrupting this pathway might increase the cell viability. Another possibility is that deleting another repair pathway further decreases the viability when treated with PARPi. To test this, we incubated the *dnapkcs⁻exo1⁻*, *lig3⁻exo1⁻*, *polq⁻exo1⁻*, including the respective control strains, with different concentrations of Benzamide and Nu1025, and quantified the cell viability using CellTiter-Glo® Luminescent Cell Viability Assay (Promega).

As expected, the cell viability of the *exo1⁻* strain was severely decreased when treated with Nu1025 or benzamide (Figure 4.11-4.14). Whilst the viability of the *lig3⁻* strain was not affected by both compounds (Figure 4.11), *lig3⁻exo1⁻* cells showed a similar viability as the *exo1⁻* strain when treated with PARPi. This indicates that disruption of *lig3* is not affecting the synthetic lethality of the *exo1⁻* strain to PARPi. A similar result was observed when using the *polq⁻exo1⁻* strains (Figure 4.12). Whilst one of the *polq⁻* strains showed a significant lower viability as the Ax2 strain when treated with 1mM Nu1025, the other *polq⁻* mutant possessed a similar viability as the Ax2 cells to PARPi. Accordingly, the viability of both *polq⁻exo1⁻* strains was slightly decreased compared to the *exo1⁻* strain when exposed to Nu1025. This indicates that PolQ-dependent repair might be beneficial for the

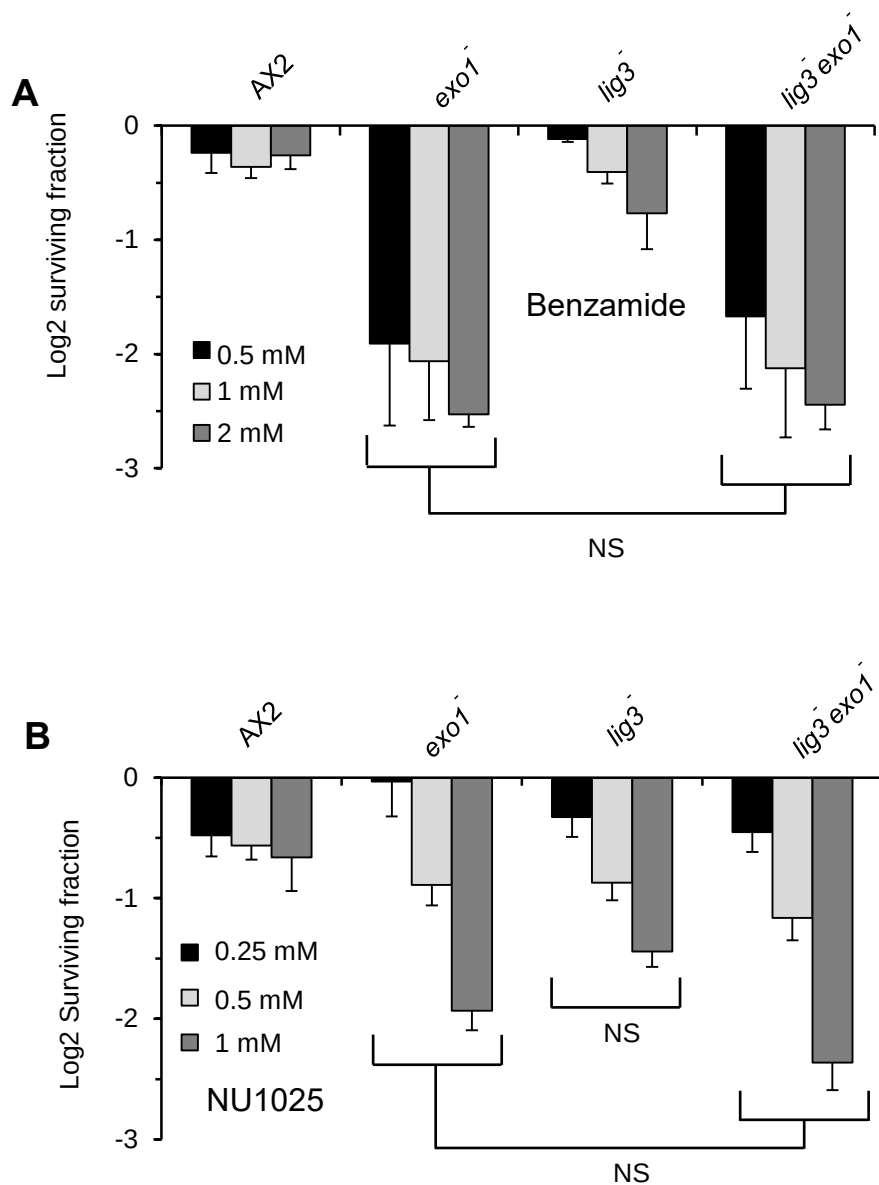


Figure 4.11: Disruption of *lig3* is not affecting cell viability of *exo1*⁻ cells when exposed to PARPi. A-B. *Ax2*, *exo1*⁻, *lig3*⁻ and *lig3*⁻*exo1*⁻ strains were treated with the indicated benzamide (A) and Nu1025 (B) concentrations. Cell viability was assessed after 5 days using CellTiter-Glo® (Promega). Cell viability is represented as log2 fold changes between untreated cells and those exposed to PARPi. Error Bars represent the SEM from three independent experiments. Statistical significance was calculated for the single disruption strains compared to the *Ax2* strain and for the double knockout strain compared to the *exo1*⁻ strain exposed to the same PARPi concentration using two-sided student's t.test, NS $P > 0.05$, * $P \leq 0.05$, ** $P \leq 0.01$

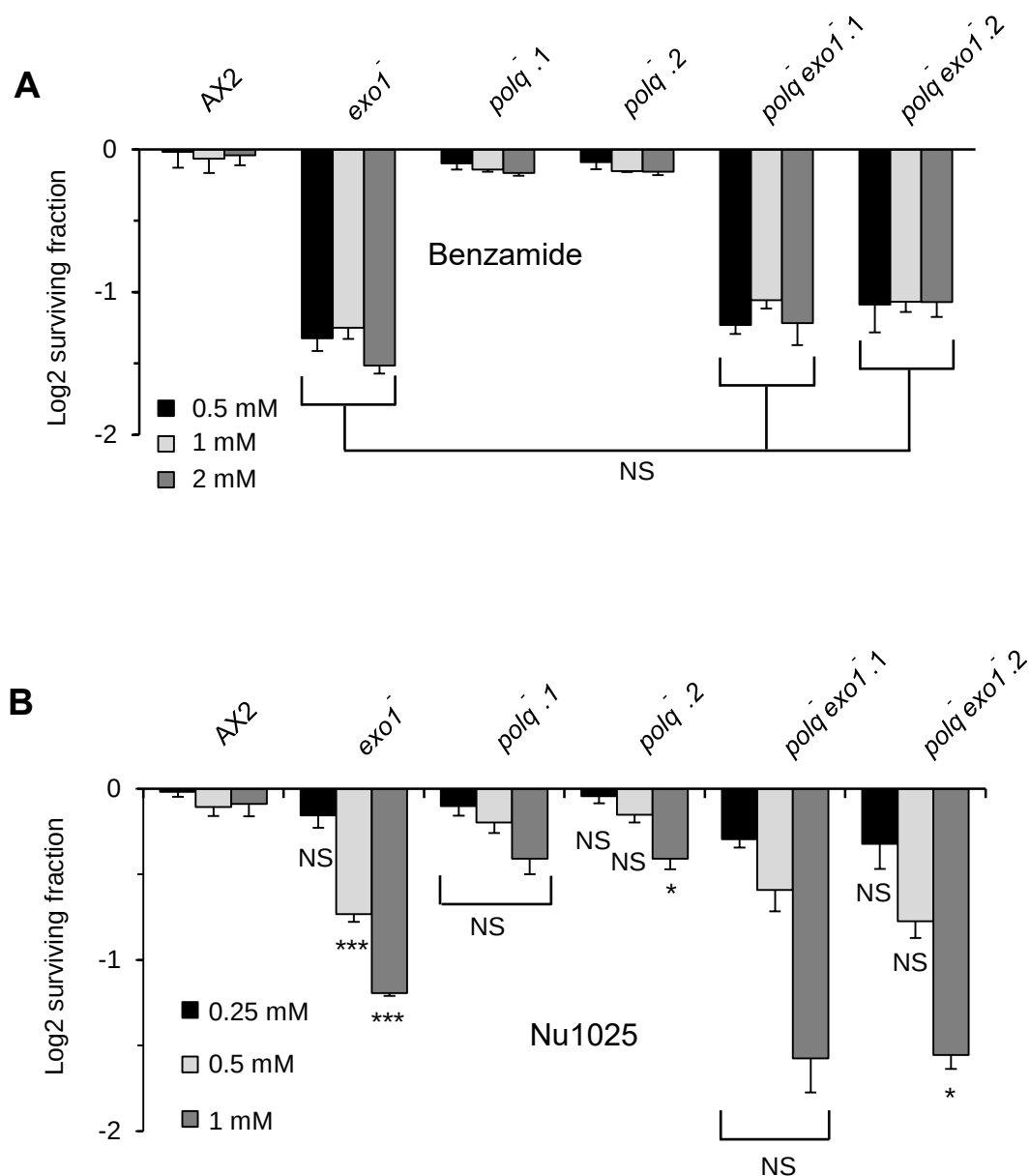


Figure 4.12: Disruption of *polq* is not affecting the cell viability of *exo1⁻* cells to PARPi. A-B. Ax2, *exo1⁻*, *polq⁻* and *polq⁻exo1⁻* strains were treated with the indicated benzamide (A) and Nu1025 (B) concentrations. Cell viability was assessed after 5 days using CellTiter-Glo® (Promega). Cell viability is represented as log2 fold changes between untreated cells and those exposed to PARPi. Error Bars represent the SEM from three independent experiments. Statistical significance was calculated for the single disruption strains compared to the Ax2 strain and for the double knockout strain compared to the *exo1⁻* strain exposed to the same PARPi concentration using two-sided student's t-test, NS $P > 0.05$, * $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$

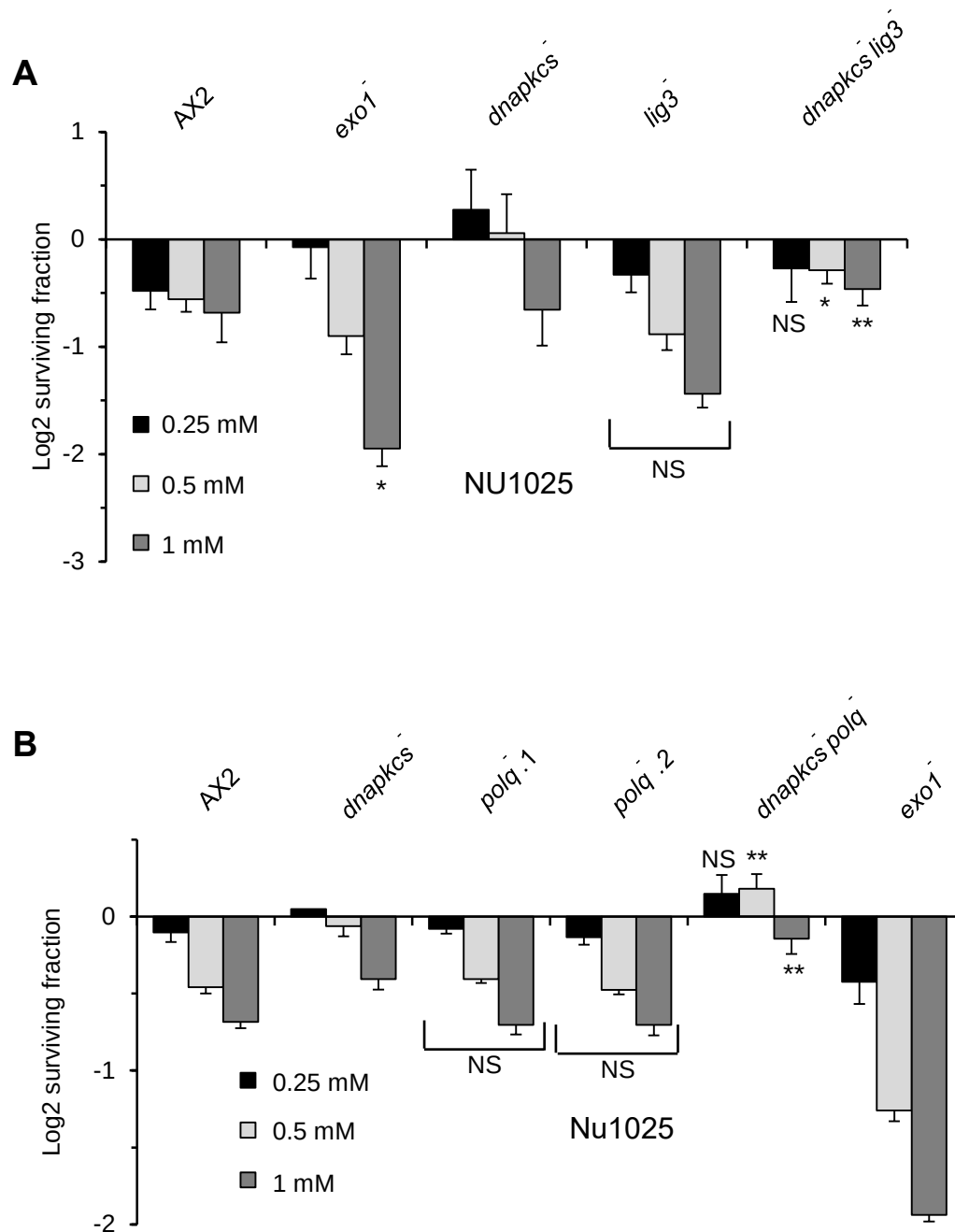


Figure 4.13: Combined disruption of *dnapkcs* and *polq/lig3* does not sensitise the cells to PARPi. A-B. The indicated strains were treated with the indicated Nu1025 concentrations. Cell viability was assessed after 5 days using CellTiterGlo® (Promega). Cell viability is represented as log2 fold changes between untreated cells and those exposed to PARPi. Error bars represent the SEM from three independent experiments. Statistical significance was calculated for the single disruption strains compared to the Ax2 strain exposed to the same PARPi concentration and for the double disruption strains compared to *lig3* (A) and *polq*.1 (B) using two-sided student's t-test, NS $P > 0.05$, * $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$

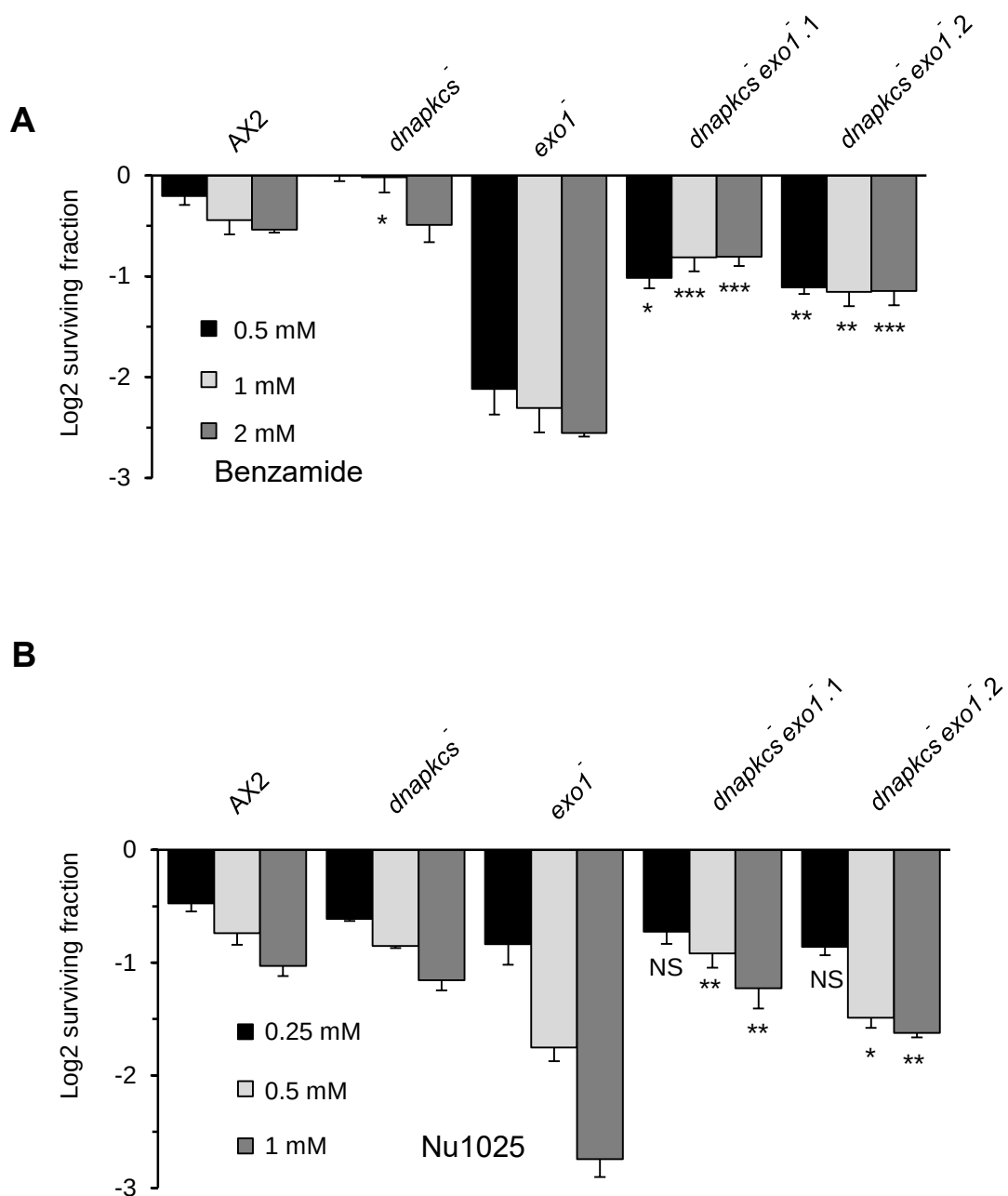


Figure 4.14: Disruption of *dnapkcs* de-sensitises the *exo1*⁻ cells to PARPi. A-B. Ax2, *exo1*⁻, *dnapkcs*⁻ and *dnapkcs*⁻*exo1*⁻ strains were treated with the indicated benzamide (A) and Nu1025 (B) concentration. Cell viability was assessed after 5 days using CellTiter-Glo® (Promega). Cell viability is represented as log2 fold changes between untreated cells and those exposed to PARPi. Error bars represent the SEM from three independent experiments. Statistical significance was calculated for the single disruption strains compared to the Ax2 strain and for the double knockout strain compared to the *exo1*⁻ strain exposed to the same PARPi concentration using two-sided student's t-test, NS P>0.05, * P≤0.05, ** P≤0.01, *** P≤0.001

survival of *exo1*⁻ cells to Nu1025 and its disruption increases the lethality of the HR-deficient strain. However, this effect was only visible with one *polq*⁻*exo1*⁻ strain and is not apparent when using benzamide as both *polq*⁻*exo1*⁻ strains exhibit a similar viability as the *exo1*⁻ cells when exposed to this PARPi. Furthermore, the slight sensitivity of *polq*⁻ strains to Nu1025 compared to Ax2 cells is not apparent in Figure 4.13B, indicating that the effect is either marginal or an artefact of this experiment.

As expected, *dnapks*⁻*polq*⁻ and *dnapks*⁻*lig3*⁻ strains were not sensitive to Nu1025 (Figure 4.13) as the strains are competent in HR to resolve PARPi-mediated damage. Interestingly, disruption of *dnapks* increased the viability of *lig3*⁻ and *polq*⁻ strains, indicating that disruption of NHEJ is beneficial for the cell survival when exposed to PARPi. Accordingly, *dnapks*⁻ cells were more viable compared to the Ax2 strain (Figure 4.14A) when exposed to benzamide, whereas Nu1025 similarly affected the viability of *dnapks*⁻ and Ax2 cells. Strikingly, disrupting *dnapks* significantly increased the viability of the *exo1*⁻ cells to PARPi (Figure 4.14). Hence, impairing NHEJ by disrupting *dnapks* suppresses the synthetic lethality of HR-defective strains to PARPi. This indicates that in the absence of HR, PARPi-mediated DNA damage is resolved using error-prone NHEJ, resulting in cell death possibly by inducing genomic instability. This suggests that NHEJ might be the driving force of synthetic lethality of HR deficiency with PARP inhibition, and impairing this pathway renders the cell refractory to PARPi treatment.

4.3 Discussion

Whilst it is known that PARPi are toxic for HR-dysfunctional cells, the mechanism resulting in cell death is unclear. Given PARPi-induced DNA damage is channelled through HR for repair, cells compromised in recombination possibly resolve the lesions using another DSB repair pathway which either promotes cells death, or is responsible for the survival of a subset of HR-defective cells. As the activity of POLQ is essential for the

survival of HR-compromised mammalian cells, one possibility is that PARPi-mediated damage is repaired through alt-NHEJ²¹⁰. Accordingly, inactivation of POLQ in HR-deficient mice is embryonic lethal, supporting the role of POLQ in cell survival when HR is absent¹⁹⁵. Additionally, PolQ is implicated to promote end-joining of replication-associated DSBs in *Caenorhabditis elegans* and *Drosophila*, indicating its conservation in several species^{211,323}. Although *Dictyostelium* possesses most of the required proteins for alt-NHEJ, including the PolQ-like helicase domain, located on a protein lacking the polymerase domain, it is unclear whether this organism is competent in this pathway. As the POLQ helicase and polymerase domain are separated by a large central domain in mammalian cells, it is possible that PolQ is spread on two proteins in *Dictyostelium*. As 20% of all predicted *Dictyostelium* proteins are developed by gene duplication, the helicase and polymerase domain might have been split due to gene duplication and evolved on different proteins³³⁷. Another possibility is that PolQ polymerase and helicase domain were separated onto different DNA locations by inter-chromosomal segment exchange³³⁷. PolQ evolved into an enzyme complex in a similar manner to other polymerases, which consist of several proteins each performing an important function of the polymerase complex. Using BLAST, we identified an unknown protein with high similarity to the human POLQ polymerase domain in the *Dictyostelium* genome, which we referred to as *polq*. This protein possesses high sequence and structural similarity to the polymerase domain of POLQ, with an e-value of $9e^{-56}$ (Figure 4.1). *Dictyostelium* PolQ aligned with a similarity of 26.5% to the human counterpart, confirming that we identified the potential *Dictyostelium* orthologue of human POLQ polymerase domain. This indicates that *Dictyostelium* PolQ is spread on at least two proteins, one contains the helicase domain the other the polymerase domain, supporting our hypothesis that PolQ evolved into two proteins in *Dictyostelium*.

The essential amino acids required for the catalytic activity of PolQ are conserved in *Dictyostelium* PolQ (Figure 4.1B, encircled in red)^{428–431}. For example, these amino acids

coordinate the association of a Ca^{2+} ion with the phosphate tail of the nucleotide, which is important for polymerase activity. Two positively charged residues, R2202 and R2254 (Figure 4.1B, marked with an asterisk), that are only present in PolQ but no other polymerases are partially conserved in *Dictyostelium*. Several other conserved residues that have an important but unknown function in PolQ are also identified (Figure 4.1B, encircled in black)^{431,432}. The human POLQ contains three sequence insertions that are only partially conserved in the *Dictyostelium* PolQ-like polymerase (underlined in Figure 4.1B)^{431,433}. Whilst the first insertion possibly mediates the processivity of POLQ, the other two insertions are involved in bypassing abasic sites^{425,431,434}. The first and second insertion are partially conserved in *Dictyostelium*, whereas the third is missing in this organism (Figure 4.1B). Although none of the insertions are conserved in the *Drosophila* orthologue of PolQ, the enzyme is still essential for the alt-NHEJ pathway in this organism, indicating that the insertions might not be important for the role of PolQ in alt-NHEJ²¹¹. However, as some of the essential residues are only partially conserved in *Dictyostelium*, we cannot exclude the possibility that the protein containing the identified PolQ-like polymerase domain is not the functional orthologue of mammalian POLQ, but another polymerase.

Applying InterProScan, we identified further domains and potential functions of *Dictyostelium* PolQ (Figure 4.1C). Similar to human POLQ, the *Dictyostelium* protein belongs to the DNA Polymerase A family based on its primary amino acid sequence^{424,440}. Interestingly, in contrast to the human version, *Dictyostelium* PolQ contains a WD40/YVTN domain at its N-terminal end. This domain is characterised by at least 4 copies of the WD-repeat, which consists of a cluster of approximately 40–60 amino acids, with each repeat comprising a four-stranded antiparallel β -sheet^{435,441}. Together, these repeats are thought to form a circularised propeller structure of β -sheets⁴⁴². The WD-repeat is conserved in all eukaryotes and the domain is implicated in a vast variety of functions, including protein-protein interaction^{435,442}. Interestingly, this domain is absent in

human, *Drosophila* and *C.elegans* PolQ. As the PolQ -helicase and polymerase domains are separated only in *Dictyostelium* this indicates that the WD40/YVTN domain is a unique feature of *Dictyostelium* PolQ and possibly required for the interaction with the PolQ-like helicase.

Similar to mammalian and *Drosophila* PolQ, a ribonuclease H-like (RNaseH) domain is located on the *Dictyostelium* orthologue. RNaseH binds to nucleic acids, hydrolyses RNA-DNA intermediates and possibly degrades RNA Primers that might be used during DNA replication or repair^{437,443}. Due to the lack of proofreading activity of PolQ, the RNaseH domain might be necessary to remove mistakenly incorporated ribo- instead of deoxyribonucleotides⁴⁴³. However, the exact function of this domain in PolQ has not been studied yet. Taken together, we identified the potential orthologue of PolQ in *Dictyostelium* which contains the essential motifs, except for the helicase domain, which was identified on a separate protein.

POLQ knockdown decreases alt-NHEJ efficiency in U2OS cells, which can be rescued by expression of full-length *POLQ*, but not with the protein lacking the polymerase domain, suggesting that this domain is required for end-joining¹⁹⁵. Furthermore, disrupting the polymerase domain of *POLQ* enhances the cell death of HR-defective ovarian cancer cells, indicating their dependence on alt-NHEJ¹⁹⁵. To test if *Dictyostelium* PolQ is equally important for DNA damage repair, we disrupted *polq* to generate two independent *polq*⁻ strains (Figure 4.2-4.3) and assessed their sensitivity to various DNA damaging agents. DNA damage caused by the alkylating agent MMS is predominantly repaired via base excision repair (BER) and only transferred into a DSB when the damage is encountered by a replication fork^{444,445}. The effect of MMS treatment on *polq* disruption cells is not consistent between different organisms and cell lines. Whereas disrupting *polq* is not sensitising *Drosophila* to MMS treatment, *polq-1*⁻ *C.elegans* show a defect in BER, indicating that PolQ is involved in this pathway⁴⁴⁶⁻⁴⁴⁸. Interestingly, disruption of *POLQ* in

DT40 cells that are deficient in the BER polymerase Pol β further sensitises the cells to MMS, indicating that POLQ might serve as a backup polymerase for BER⁴⁴⁹. However, no sensitivity to MMS was observed in *Dictyostelium polq*⁻ cells, suggesting that the PolQ polymerase domain is not required for BER in this organism (Figure 4.4A).

Exposure to H₂O₂ induces oxidative damage which is repaired by SSB repair and BER⁴⁵⁰. Interestingly, one of the *polq*⁻ strains is slightly sensitive to H₂O₂, indicating that PolQ might potentially play a role in SSB repair (Figure 4.4B). However, as this sensitivity is only significant for one of the *polq*⁻ strains this suggests that the effect is only an artefact and PolQ is probably not involved in SSB repair. Interestingly, exposure to the DSB-inducing agent phleomycin significantly decreases the viability of the *polq*⁻ strains compared to the parental Ax2 strain (Figure 4.4C). However, the sensitivity to phleomycin is not as severe as in the *exo1*⁻ strain, suggesting that PolQ is involved in DNA DSB repair, but to a lesser extent than Exo1. Based on our results and previously published data we believe that PolQ is required for DSB repair other than NHEJ, potentially for alt-NHEJ³⁵⁴. However, to date alt-NHEJ and the role of PolQ in this pathway have not been investigated in *Dictyostelium*. For example, the activity of alt-NHEJ in *Dictyostelium* can be assessed using an *in vitro* assay described by Sharma *et al*⁴⁵¹. In this assay, two double stranded oligonucleotides containing microhomologies and overhangs on both ends are incubated with cell-free protein extracts from *Dictyostelium*. End-joining of the two oligonucleotides using alt-NHEJ usually results in the annealing of microhomologies and the deletion of nucleotides which can be detected by sequencing of the substrate⁴⁵¹. For an *in vivo* test, the Ej2-GFP vector, developed by Bennardo *et al* can be employed⁴⁵². This vector contains a NLS-tag fused to a GFP coding sequence, which is disrupted by an I-SceI restriction enzyme that is flanked by 8nt microhomologies. If the cut I-SceI restriction enzyme site is repaired via alt-NHEJ, the microhomology region is annealed resulting in the deletion of several nucleotides and expression of GFP⁴⁵².

We wanted to identify the importance of the different DSB repair pathways and elucidate which repair pathway is engaged should another one fail. Therefore, we analysed the sensitivity of *Dictyostelium* strains that are defective in two of the three possible DSB repair pathways to phleomycin. Disrupting *dnapkcs* or *polq* in combination with *exo1* further sensitises the single disruption strains to phleomycin (Figure 4.7). This significant decrease in the survival indicates that in HR-defective *exo1*⁻ strains DSBs are resolved via both pathways, NHEJ and PolQ-dependent repair. Interestingly, *dnapkcs*⁻*polq*⁻ strains are similarly sensitive to phleomycin as *polq*⁻ cells (Figure 4.8), indicating that NHEJ plays a minor role in repairing DSBs in HR-competent but *polq*-defective cells. This is supported by the fact that DSBs are primarily repaired via HR in vegetative *Dictyostelium* cells. However, in contrast to mammalian cells, *Dictyostelium* preferably repair DSBs using HR, suggesting that the effect of combined disruption of NHEJ and alt-NHEJ on the sensitivity to DSBs might be different in mammalian cells. Overall, DSBs are channelled through NHEJ and PolQ dependent repair in HR-defective cells.

Although it is known that HR-deficient cells are hypersensitive to PARPi, the exact mechanism resulting in cell death is still unclear. In the previous chapter we established that Ax2 cells, in contrast to *exo1*⁻ cells, recruit Rad51 onto the chromatin following PARPi treatment, thereby verifying the activation of HR upon PARP inhibition. Here, we show that Lig3 is not enriched on chromatin in Ax2 or *exo1*⁻ cells following PARPi treatment, indicating that Lig3-dependent repair is not involved in resolving PARPi-mediated damage (Figure 4.9B, D). Accordingly, disruption of *lig3* does not affect the viability of *exo1*⁻ cells to PARPi (Figure 4.11), indicating that Lig3 is not required for repairing PARPi-mediated damage in HR-deficient cells.

Interestingly, the viability of *polq*⁻*exo1*⁻ cells is slightly decreased compared to the *exo1*⁻ cells when treated with Nu1025 (Figure 4.12B), which might be due to the fact that HR-deficient mammalian cells rely on PolQ-dependent repair for survival^{195,212}. Furthermore,

polq⁻ strains exhibit a slightly reduced viability following Nu1025 incubation compared to the Ax2 cells, indicating that disruption of PolQ mildly sensitises the cells to PARPi. However, this effect is only apparent when using Nu1025, which preferably targets the PARP1 orthologue Adprt2 *in vitro*, but not with benzamide (Figure 4.12). Besides, only one of the *polq*⁻ and *polq*⁻*exo1*⁻ strains display a significant decrease in viability to Nu1025 compared to Ax2 and *exo1*⁻ strains, respectively, indicating that the observed effect is only an artefact. Furthermore, as PARP1 is promoting PolQ-dependent alt-NHEJ, PARPi are probably not affecting the viability of PolQ-depleted cells⁴⁵³. However, it is possible that on some occasions PolQ-dependent alt-NHEJ is activated independently of PARP1. For example, *exo1*⁻ cells are potentially competent in initial DNA end resection using Mre11, but might abort HR due to the lack of Exo1, resulting in the activation of alt-NHEJ. Combined disruption of HR and the backup pathway alt-NHEJ possibly further decreases the viability of *Dictyostelium* cells to PARPi.

Surprisingly, disruption of *dnapkcs* in *polq*⁻ or *lig3*⁻ cells increases the viability of the cells to PARPi (Figure 4.13). This indicates that disruption of the error-prone NHEJ pathway is beneficial for the survival of the cells to PARPi. Interestingly, Myc-Ku70 is highly enriched on chromatin in the *exo1*⁻ cells when exposed to Nu1025 (Figure 4.9A, C), indicating that PARPi induce DNA damage that is channelled through error-prone NHEJ when HR is compromised. Strikingly, PARPi treatment is not lethal for *dnapkcs*⁻*exo1*⁻ cells, indicating that knockdown of NHEJ factors renders the cells refractory to the compounds (Figure 4.14). This supports previous data suggesting that disruption of NHEJ reduces the toxicity of PARPi to HR-deficient cells^{133,298}. Absence of the NHEJ promoting factor 53BP1 results in restoration of HR and therefore induces resistance to PARPi treatment in cancer cells that are deficient in BRCA1. Accordingly, knockdown of NHEJ essential factors diminish the PARPi toxicity to BRCA2-deficient ovarian cancer cell lines²⁸⁴. We identified a similar loss of sensitivity to PARPi by combined disruption of HR and NHEJ in *Dictyostelium*. Hence, synthetic lethality of *exo1*⁻ cells with PARPi is due to hyper-activation of the error-

prone NHEJ pathway, leading to cell death possibly due to increased genomic instability. Disruption of NHEJ renders HR-defective cells unresponsive to PARPi, supporting the deleterious effect of NHEJ on the survival of the cells²⁸⁴.

5. Resistance to PARPi of *exo1*⁻ cells is due to restoration of HR

5.1 Introduction

5.1.1 Potential pathways rendering HR defective cells resistant to PARPi

PARP inhibition is toxic to cells with defects in HR, providing the platform for a therapeutic application using PARPi in a synthetic lethal strategy to specifically target HR-deficient tumours^{257,258,419,454}. Several PARPi are currently being tested in clinical trials with two of them already approved as monotherapy for HR-deficient ovarian cancer patients. The best supported model explaining the inhibitory mechanism of PARPi is the trapping model. This model suggests that PARPi promote the tight interaction of PARPs with DNA lesions, forming a barrier that needs to be bypassed by HR for replication fork restart^{419,420,101,265,286}. When HR is dysfunctional, DNA damage is channelled through mutagenic pathways, resulting in genome instability and cell death^{419,454}. Accordingly, in our experimental system the NHEJ protein Ku70 is enriched on chromatin in HR-deficient cells following PARPi treatment, indicating that this DSB repair pathway is employed when HR is compromised. Furthermore, disruption of NHEJ renders HR-deficient *exo1*⁻ strains refractory to PARPi treatment, suggesting that the toxicity of PARPi to HR-dysfunctional cells is due to increased error-prone repair by NHEJ. In line with this, we discovered that loss of the SSB-responsive enzyme Adprt2 is synthetically lethal with HR inhibition, whereas an additional lack of Adprt1a, which promotes NHEJ, rescues the lethality of *Dictyostelium* cells. Given that disrupting NHEJ leads to loss of PARPi toxicity in HR-dysfunctional cells, this suggests that *dnapks*⁻*exo1*⁻ cells employ another repair pathway to survive the PARPi treatment. In mammalian cells, alt-NHEJ, which is

dependent on Mre11, PolQ and Lig3, is a third possible repair pathway to resolve DNA DSBs. Loss of the putative *Dictyostelium* orthologue of PolQ sensitises the cells to DNA DSBs (Chapter 4). Additionally, disruption of *polq* further decreases the viability of *exo1*⁻ cells to DSBs, indicating that PolQ-dependent repair is engaged in HR-deficient *Dictyostelium* cells. As *dnapkcs*⁻*exo1*⁻ cells are defective in HR and NHEJ these cells might rely on PolQ-dependent repair to resolve PARPi-induced DNA lesions. However, another possible explanation for the survival of *dnapkcs*⁻*exo1*⁻ cells to PARPi is based on our finding that Exo1 and Mre11 share redundant activities. Thus, cells defective in NHEJ and *exo1* might engage another nuclease to resect DNA ends and therefore re-activate HR to survive the PARPi treatment.

5.1.2 Aims

Whilst we showed that disrupting NHEJ renders HR-defective cells refractory to PARPi, the mechanism resulting in the resistance of *dnapkcs*⁻*exo1*⁻ strains to PARPi is unclear. Hence, we sought to assess whether cells disrupted in NHEJ and HR engage PolQ-dependent repair, or if another pathway allows these cells to tolerate PARPi. Our lab previously found that the alt-NHEJ factor Lig3 is enriched on chromatin upon MMS treatment, indicating that this protein is involved in DNA repair in *Dictyostelium*²¹². Hence, we wanted to test if Lig3 and/or PolQ play a role in the survival of *dnapkcs*⁻*exo1*⁻ strains to PARPi. However, we also demonstrated that Exo1 and Mre11 are redundant in *Dictyostelium*, suggesting that *dnapkcs*⁻*exo1*⁻ strains might survive PARPi treatment due to the re-activation of HR using Mre11 for DNA resection. Therefore, we also assessed whether the resistance of NHEJ and HR defective strains to PARPi is due to restoration of HR through Mre11-dependent mechanisms.

5.2 Results

5.2.1 PARPi tolerance of *dnapkcs⁻exo1⁻* cells is not dependent on Lig3 and PolQ

Initially, we wanted to assess whether the resistance of *dnapkcs⁻exo1⁻* strains to PARPi is due to PolQ- and/or Lig3-dependent DNA repair. Therefore, we first recycled the floxed BSR cassette in the *dnapkcs⁻polq⁻* and *dnapkcs⁻lig3⁻* strains by excising the loxP sites flanking the BSR cassette using the Cre-recombinase following standard procedures. We subsequently generate two different triple knockout strains, *dnapkcs⁻polq⁻exo1⁻* and *dnapkcs⁻lig3⁻exo1⁻*, by disrupting the *exo1* genomic locus in these cells. The strategy of *exo1* disruption was described previously and is shown again in Figure 5.1A. The generation of two independent *dnapkcs⁻lig3⁻exo1⁻* strains was confirmed by PCR (Figure 5.1B). Verification of two independent *dnapkcs⁻polq⁻exo1⁻* strains (Figure 5.1C-D) was performed by PCR and RT-PCR using the same primers as previously described. Concluding, we generated *dnapkcs⁻polq⁻exo1⁻* and *dnapkcs⁻lig3⁻exo1⁻* strains by disrupting *exo1* in the respective background strain.

Previously, we identified that disrupting *polq* further sensitises *exo1⁻* strains to DSBs, indicating that DNA damage is not only resolved through NHEJ, but also via a PolQ-dependent repair mechanism when HR is compromised. This suggests that DSBs might be channelled through PolQ-dependent repair in *dnapkcs⁻exo1⁻* strains. To test whether cells that are defective in HR and NHEJ rely on PolQ-dependent repair, we assessed the sensitivity of *dnapkcs⁻polq⁻exo1⁻* cells to DNA DSBs. Therefore, two independent *dnapkcs⁻polq⁻exo1⁻* strains and the respective control strains were exposed to various concentrations of the DNA DSB-inducing agent phleomycin and their survival was assessed as described previously. As expected, *exo1⁻* cells were highly sensitive to DNA DSBs, and this is further increased when *polq* or *dnapkcs* are disrupted (Figure 5.2).

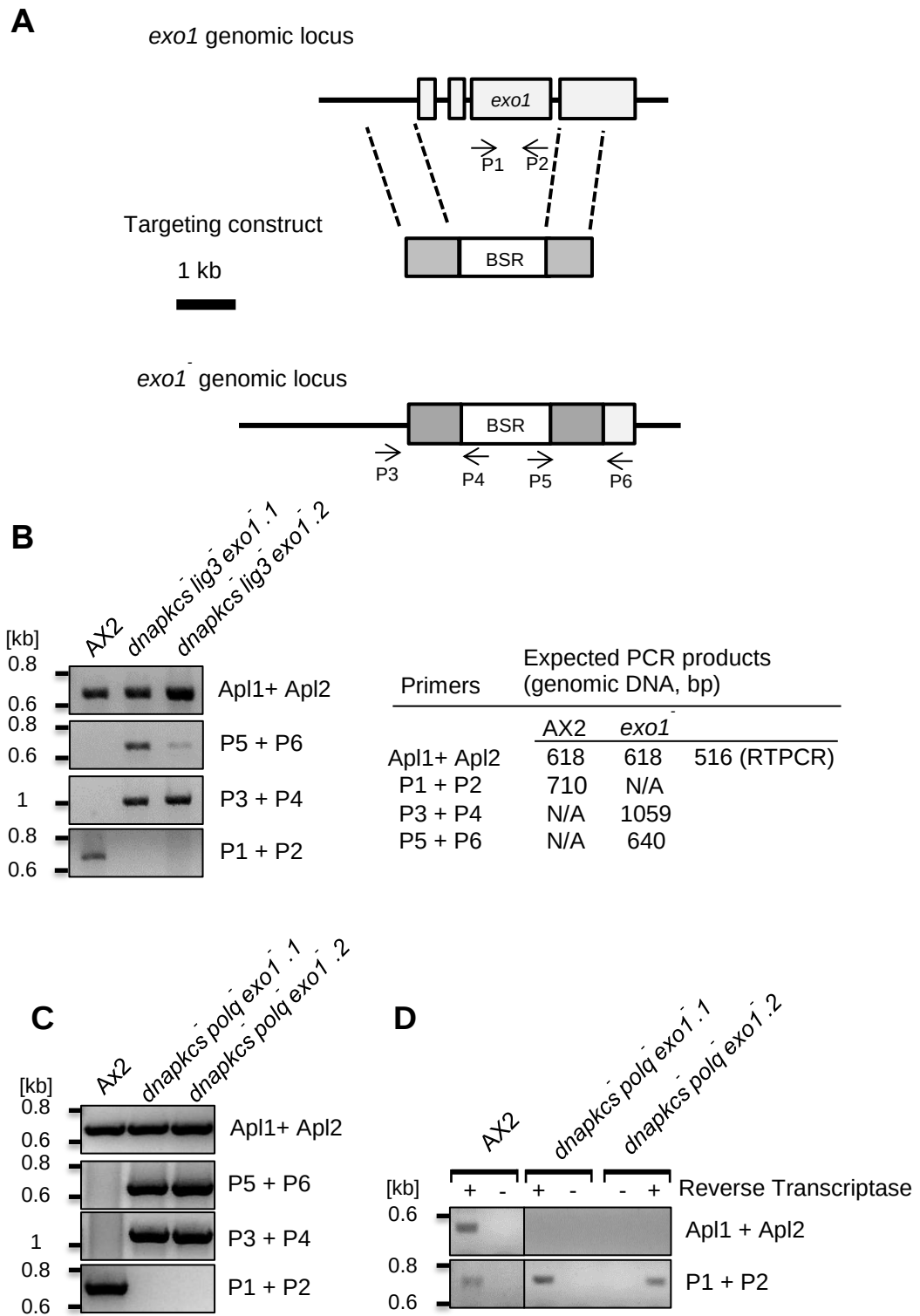
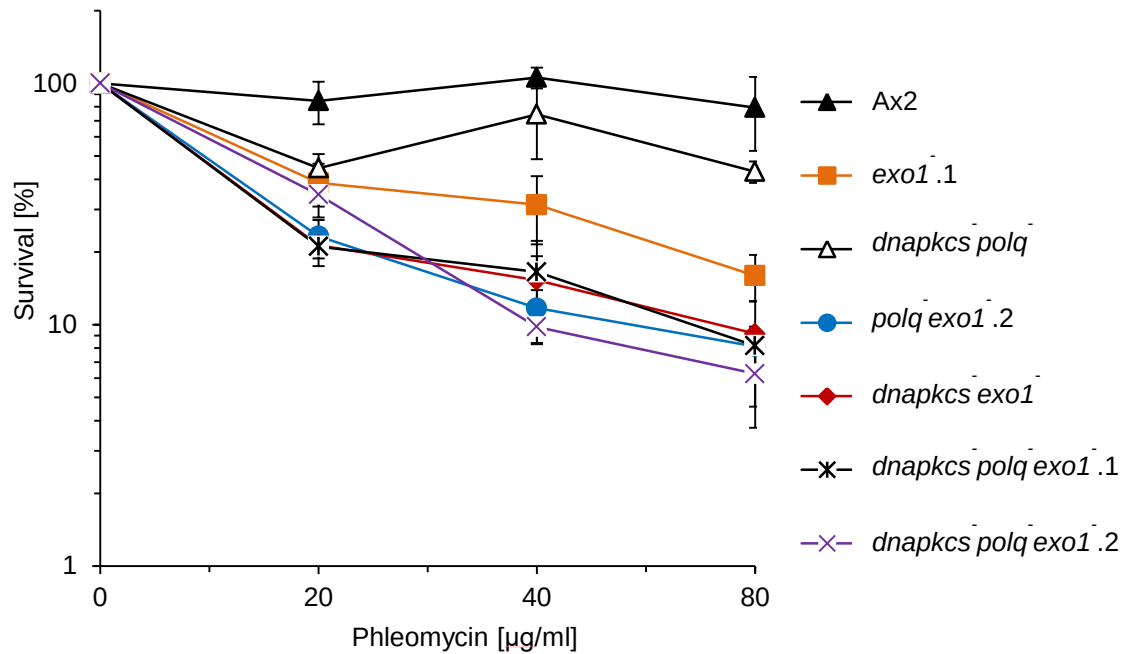


Figure 5.1: Verification of *dnapks lig3 exo1*⁻ and *dnapks polq exo1*⁻ Dictyostelium strain generation . **A.** Representation of the desired recombination event of the *exo1* genomic locus (top) with the *exo1* targeting construct (middle). The light grey box in the bottom figure represents the remaining 950bp in the final *exo1* exon. P indicates the primers used for screening by PCR. **B.** PCR verification of disrupting *exo1* in *dnapks lig3* strains using the primers at locations indicated in (A). The expected fragment sizes for Ax2 and *exo1* disruption strains are shown in the table. Oligonucleotides that amplify a region of the *apl* genomic locus were used as control. **C.** PCR verification of disrupting *exo1* in *dnapks polq* strains using the primers at locations indicated in (A). The expected fragment sizes for Ax2 and *exo1* disruption strains are shown in the table. **D.** RT-PCR verification of *dnapks polq exo1*⁻ strains using the primers at locations indicated in (A).



Strain	P value (Ax2)	P value (exo1)	P value (dnapkcs exo1)	P value (polq exo1)
Ax2	N/A	N/A	N/A	N/A
exo1 .1	0.0001 (***)	N/A	N/A	N/A
dnapkcs polq	0.0236 (*)	0.0223 (*)	0.0008 (***)	0.0006 (***)
polq exo1 .2	<0.0001 (****)	0.0101 (*)	0.7886 (NS)	N/A
dnapkcs exo1	<0.0001 (****)	0.0168 (*)	N/A	N/A
dnapkcs polq exo1 .1	<0.0001 (****)	0.0108 (*)	0.9999 (NS)	0.7447 (NS)
dnapkcs polq exo1 .2	<0.0001 (****)	0.0296 (*)	0.6454 (NS)	0.4488 (NS)

Figure 5.2 The *dnapkcs polq exo1* strains are similarly sensitive to DNA DSBs than the double disruption strains. Ax2, *exo1*, *dnapkcs polq*, *polq exo1*, *dnapkcs exo1*, and two independent *dnapkcs polq exo1* strains were treated with the indicated phleomycin concentrations for one hour before plating onto a KA lawn on SM agar. After 3-7 days, survival was assessed by plaque formation relative to a mock-treated control. Error bars represent the SEM from three independent experiments. Statistical significance was determined with respect to the Ax2 strain, *exo1*, *dnapkcs exo1* and *polq exo1* strain by two-way ANOVA, NS $P > 0.05$, * $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$, **** $P \leq 0.0001$.

However, the triple knockout strains showed a similar sensitivity to phleomycin as the *polq⁻exo1⁻* and *dnapkcs⁻exo1⁻* strains, indicating that disrupting *dnapkcs* or *polq*, respectively, does not further sensitise the double knockout cells to DNA DSBs. This indicates that in the absence of HR, *polq* and *dnapkcs* possibly act in the same DNA repair pathway. Another possibility is that *dnapkcs⁻polq⁻exo1⁻* strains can re-engage recombination, resulting in no further sensitivity to DSBs when *dnapkcs* or *polq* are disrupted.

In the previous chapter, we illustrated that disrupting *dnapkcs* and thereby impairing NHEJ in a HR-deficient strain suppresses synthetic lethality with PARPi. As HR and NHEJ are both disrupted in such a strain, these cells could potentially engage PolQ-dependent repair to resolve PARPi-induced damage, thereby rendering the cells viable to PARPi. Besides PolQ, Lig3 is also important for mammalian alt-NHEJ. Given that *Dictyostelium* Lig3 also functions in DDR, it is possible that both proteins are similarly required for alt-NHEJ in this model organism. We therefore tested whether Lig3- and/or PolQ-dependent repair are responsible for the survival of *dnapkcs⁻exo1⁻* strains in the presence of PARPi. We hypothesised that if Lig3 and/or PolQ are required to tolerate PARPi, disrupting either of them should re-sensitise *dnapkcs⁻exo1⁻* cells to these compounds. This will not only shed light into the repair pathway mediating the survival of HR/NHEJ compromised cells to PARPi, but also indicate whether PolQ and Lig3 are involved in the same mechanism. Hence, we investigated the effect of disrupting *lig3* and *polq* on the viability of *dnapkcs⁻exo1⁻* cells to PARPi. Therefore, triple knockout strains and their respective control strains were exposed to different concentrations of Nu1025 and benzamide. The viability of each strain was assessed using the CellTiter-Glo® Luminescent Cell Viability Assay. As before, the *exo1⁻* strain was synthetically lethal with both compounds, which was suppressed in the *dnapkcs⁻exo1⁻* strain (Figure 5.3-5.4). Neither *dnapkcs⁻lig3⁻exo1⁻* (Figure 5.3) or *dnapkcs⁻polq⁻exo1⁻* (Figure 5.4) showed a decrease in cell viability when exposed to PARPi compared to the *dnapkcs⁻exo1⁻* strains.

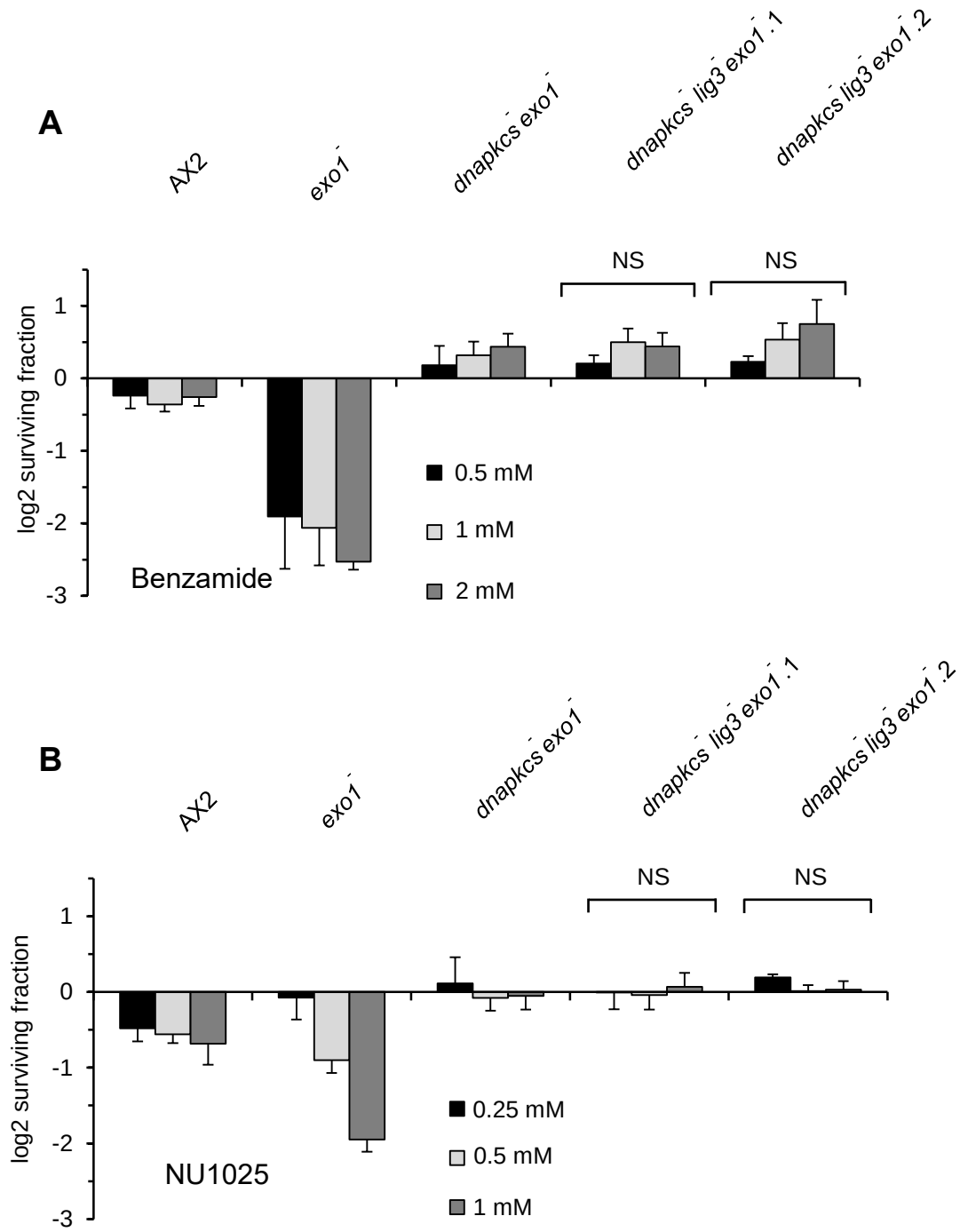


Figure 5.3: Disruption of *lig3* does not affect the tolerance of *dnapkc*⁻*exo1*⁻ cells to PARPi. **A-B.** Ax2, *exo1*⁻, *dnapkc*⁻*exo1*⁻, and *dnapkc*⁻*lig3*⁻*exo1*⁻ strains were treated with the indicated PARPi concentration, benzamide (**A**) and NU1025 (**B**). Cell viability was assessed after 5 days using CellTiter-Glo® (Promega). Cell viability is represented as log₂ fold change between untreated cells and those exposed to PARPi. Error bars represent the SEM from three independent experiments. Statistical significance was calculated for the *dnapkc*⁻*lig3*⁻*exo1*⁻ strains compared to the *dnapkc*⁻*exo1*⁻ strain exposed to the same PARPi concentration using two-sided student's t-test, NS P>0.05, * P≤0.05, ** P ≤ 0.01

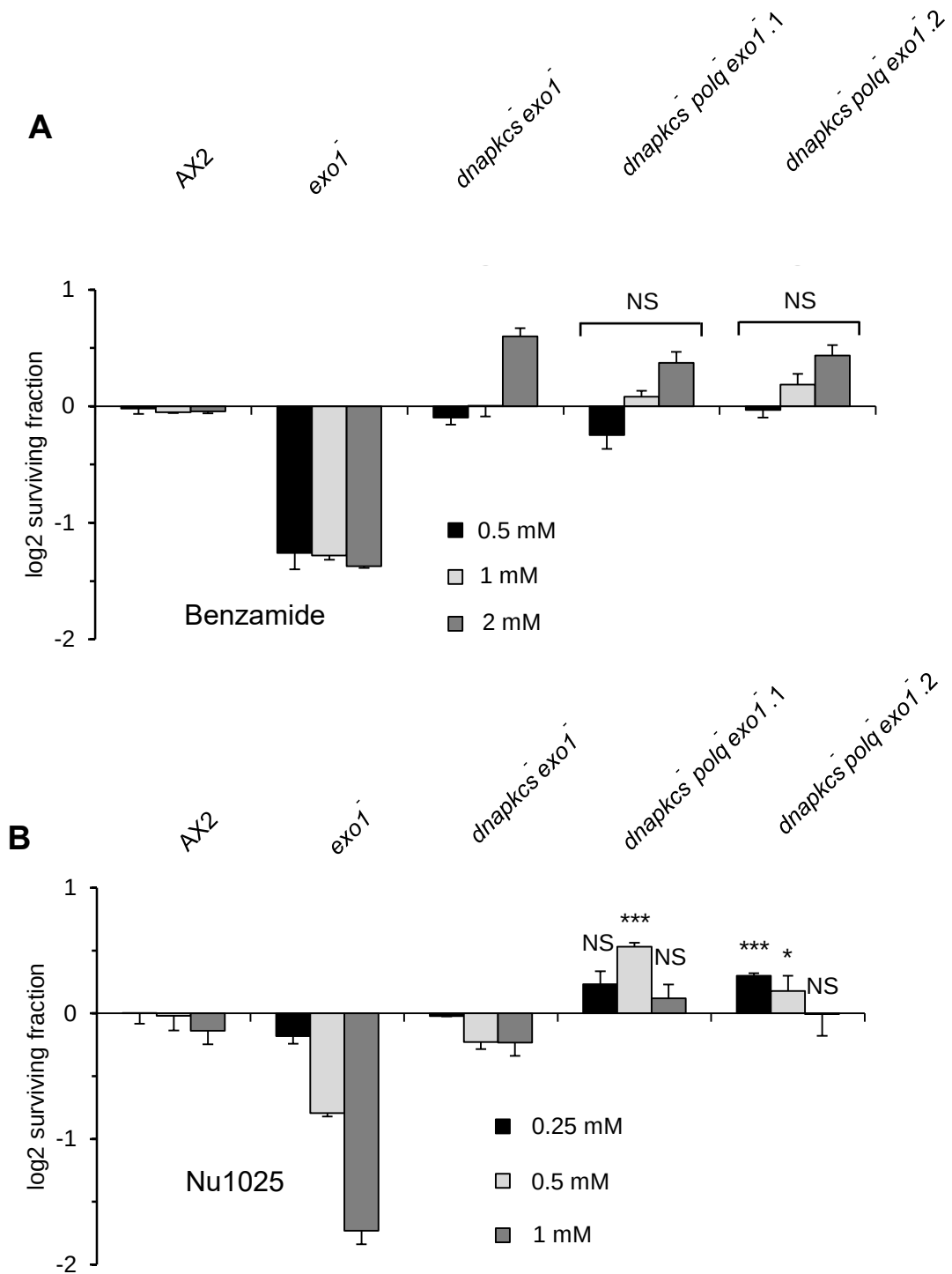


Figure 5.4: Disruption of *polq* does not affect the loss of PARPi toxicity to *dnapKcs*⁻*exo1*⁻ cells. A-B. Ax2, *exo1*⁻, *dnapKcs*⁻*exo1*⁻, and *dnapKcs*⁻*polq*⁻*exo1*⁻ strains were treated with the indicated PARPi concentrations: benzamide (A) and Nu1025 (B). Cell viability was assessed after 5 days using CellTiter-Glo® (Promega). Cell viability is represented as log2 fold change between untreated cells and those exposed to PARPi. Error bars represent the SEM from three independent experiments. Statistical significance was calculated for the *dnapKcs*⁻*polq*⁻*exo1*⁻ strains compared to the *dnapKcs*⁻*exo1*⁻ strain exposed to the same PARPi concentration using two-sided student's t-test, NS P>0.05, * P≤0.05, ** P≤0.01, *** P≤0.001

Interestingly, disruption of *polq* increases the viability of *dnapks⁻exo1⁻* cells to Nu1025 (Figure 5.4), suggesting that loss of PolQ possibly further suppresses the toxicity of Nu1025 to HR/NHEJ-deficient cells. However, as this effect is not apparent with benzamide, increased tolerance to Nu1025 due to PolQ disruption is probably not transferable to other PARPi in general. Taken together, this suggests that neither Lig3- or PolQ-dependent repair is responsible for the survival of the *dnapks⁻exo1⁻* strain to PARPi.

5.2.2 The *exo1⁻dnapks⁻* strains restore HR to suppress PARPi toxicity

As disrupting NHEJ increases the viability of *exo1⁻* cells to PARPi independent of PolQ and Lig3, this indicates that Lig3- and PolQ-dependent repair is not employed to resolve PARPi induced DNA damage. Given our previous finding that the nucleases Exo1 and Mre11 are partially redundant in response to DSBs, we hypothesised that the *dnapks⁻exo1⁻* strain can restore HR using Mre11, and thus suppress PARPi toxicity. To test this we first assessed whether HR is re-activated in *dnapks⁻exo1⁻* cells compared to the *exo1⁻* strain by analysing the enrichment of the HR factor Rad51 onto chromatin following PARPi exposure. Therefore, *exo1⁻*, *dnapks⁻* and *dnapks⁻exo1⁻* strains were transfected with the pDXA-3C-Flag-Rad51 vector or the empty pDXA-3C vector as control. As expected, only cells transfected with pDXA-3C-Flag-Rad51, and not those containing the empty plasmid, expressed Flag-Rad51 (Figure 5.5A).

Next, we tested whether Rad51 is enriched on chromatin following PARPi treatment in order to establish whether DNA lesions induced by this agent are repaired using HR in *dnapks⁻exo1⁻* cells. Hence, we exposed the *exo1⁻*, *dnapks⁻* and *dnapks⁻exo1⁻* strains expressing Flag-Rad51 to Nu1025, extracted chromatin from these cells, and analysed the samples using Western blotting with the indicated antibodies. As observed previously,

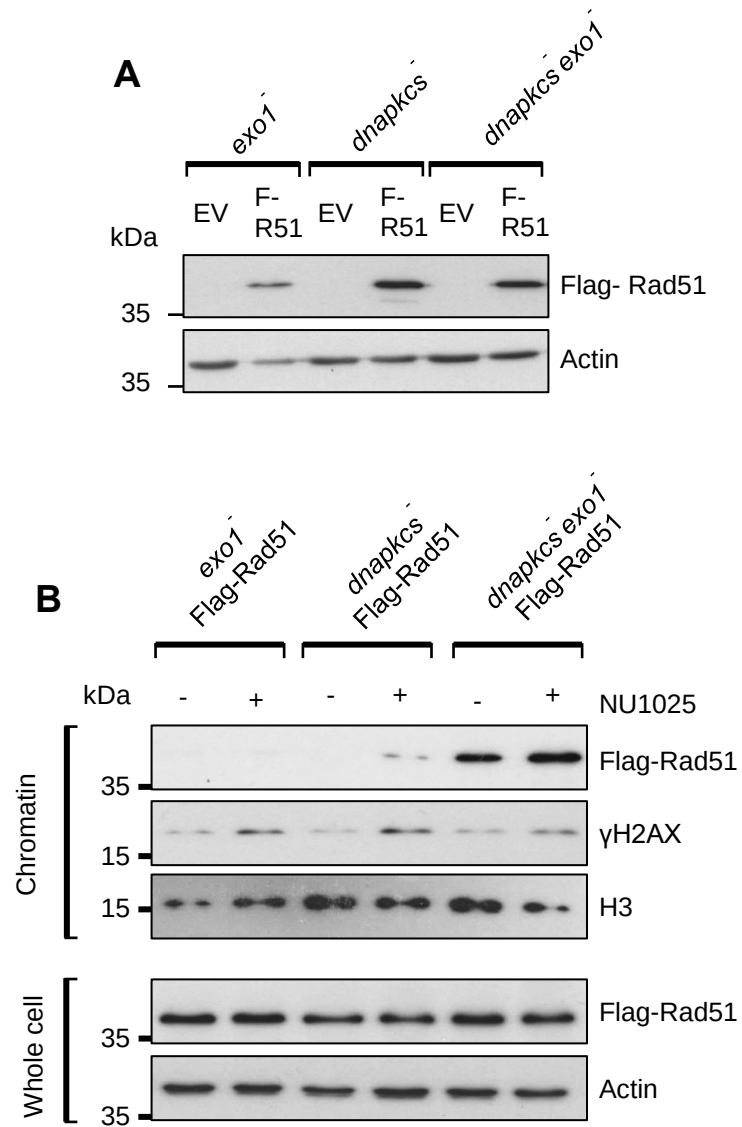


Figure 5.5: The *dnapkcs exo1* strain recruits Flag-Rad51 onto chromatin when exposed to Nu1025. **A.** The *exo1*⁻, *dnapkcs*⁻, *dnapkcs exo1*⁻ cells were transfected with an empty pDXA-3C vector (EV) or with pDXA-3C-Flag-Rad51 (F-R51) using standard protocols. Whole cell extracts were prepared and expression of the Flag-tagged protein was detected by Western blotting using the indicated antibodies. **B.** The *exo1*⁻, *dnapkcs*⁻, *dnapkcs exo1*⁻ cells expressing Flag-Rad51 were left untreated or exposed to 1mM Nu1025 for 2.5 hours. Chromatin and whole cell extracts were prepared and Western blotting was performed with the indicated antibodies.

Flag-Rad51 was not enriched on chromatin in *exo1⁻* cells following PARPi treatment, indicating that the cells are deficient in HR (Figure 5.5B). As expected, Flag-Rad51 was enriched on chromatin in the *dnapkcs⁻* cells upon Nu1025 treatment, indicating that repair of PARPi induced damage is channelled through Rad51 dependent repair. Furthermore, the mock-treated *dnapkcs⁻exo1⁻* strain exhibited high levels of Flag-Rad51 on chromatin, which was further increased when exposed to Nu1025 (Figure 5.5B). This indicates that *dnapkcs⁻exo1⁻* cells employ a Rad51-dependent pathway to repair endogenous DNA lesions as well as DNA damage that occurs through PARPi treatment. Given that HR is the only known repair pathway to recruit Rad51 onto the chromatin during the repair process, we conclude that *dnapkcs⁻exo1⁻* strains can restore recombination, and this potentially results in the resistance to PARPi⁴⁵⁵.

Subsequently, we wanted to assess whether the enhanced Rad51 enrichment onto chromatin in *dnapkcs⁻exo1⁻* cells is reflected in increased HR activity compared to the *exo1⁻* strain. Hence, we performed a HR assay with Ax2, *exo1⁻*, *dnapkcs⁻* and *dnapkcs⁻exo1⁻* strains to quantify the level of recombination of the different *Dictyostelium* strains. For this assay we first recycled the floxed BSR cassette in the different disruption strains by Cre- mediated recombination of the loxP sites flanking the blasticidin resistance gene, using standard procedures. Then, the Ax2, *exo1⁻*, *dnapkcs⁻* and *dnapkcs⁻exo1⁻* strains were transfected with the *cdk8* disruption construct, obtained from the *cdk8*KO-pLPBLP vector, which carries the blasticidin resistance gene flanked by sequences homologous to the *cdk8* genomic locus. When the cells are competent in homologous recombination, the *cdk8* disruption construct is inserted in place of the *cdk8* genomic locus by targeted integration, replacing the *cdk8* gene with the BSR cassette³⁷⁴. This results in *cdk8⁻* cells that have lost the ability to develop aggregates (*agg⁻* cells) and are impaired in spore formation³⁷³. Following transfection, the aggregation efficiency of the blasticidin resistant cells was assessed by plating the cells on a KA lawn and observing their development. The HR efficiency of each strain was calculated as the amount of *agg⁻* colonies compared

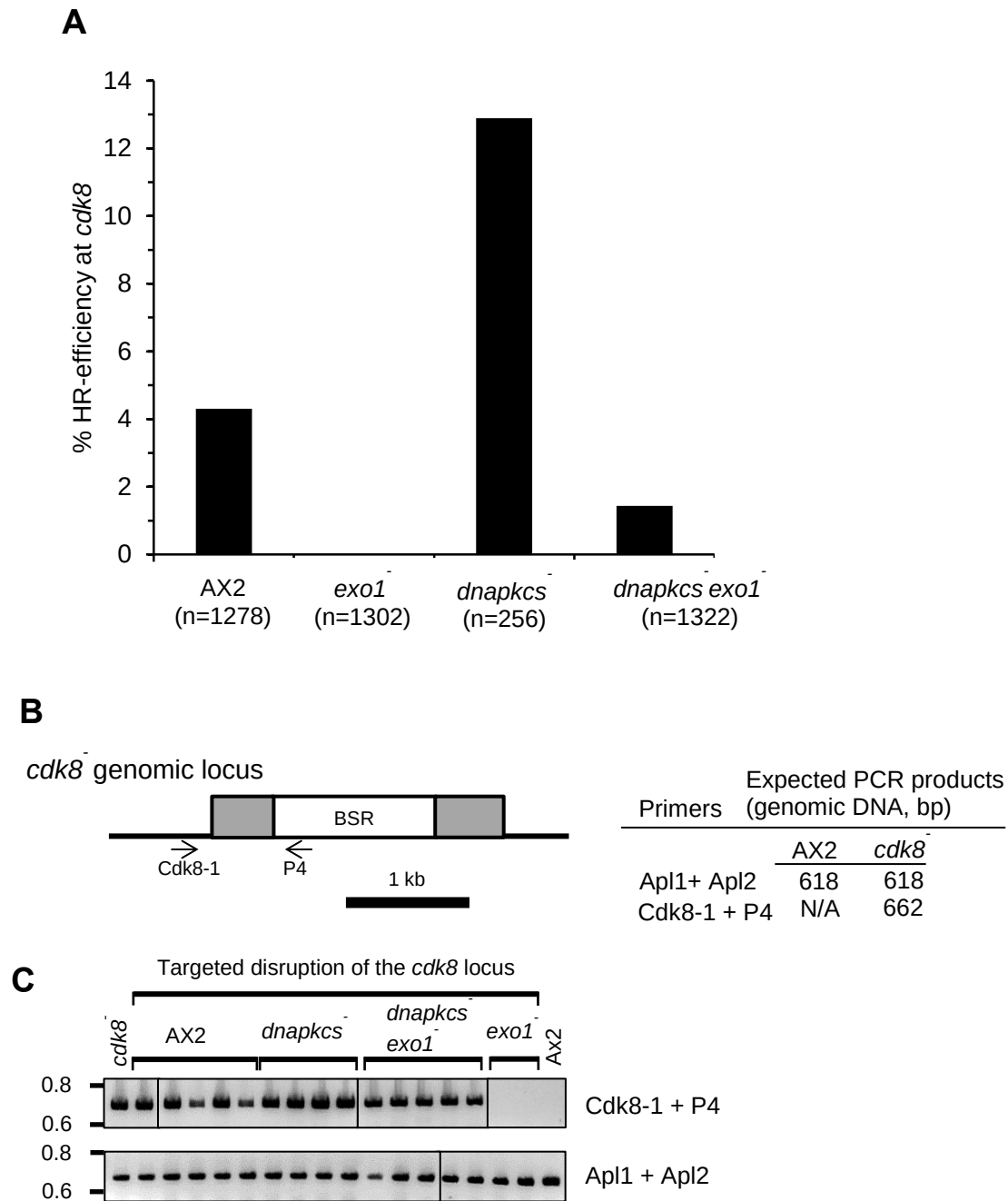


Figure 5.6: The *dnapkcs*⁻*exo1*⁻ strain can reengage HR **A.** *Ax2*, *exo1*⁻, *dnapkcs*⁻ and *dnapkcs*⁻ *exo1*⁻ cells were assessed for HR efficiency by measuring targeted integration of the blasticidin cassette at the *cdk8* locus. Multiple transfections were performed and drug-resistant clones analysed for targeting *cdk8* by PCR analysis (**C**). The percentage of targeted HR efficiency at the *cdk8* locus is calculated as the proportion of aggregate-null colonies against the total number of colonies analysed. The *n* number represents the total number of clones analysed. **B.** Representation of the *cdk8*⁻ genomic locus with the blasticidin resistance cassette in place of *cdk8*. Cdk8 and P4 are the primers used for verification of targeted insertion. **C.** PCR verification of a selection of cells with targeted integration of the BSR cassette at the *cdk8* genomic locus using the primers at locations indicated in (**B**). The expected fragment sizes are shown in the table.

to the total number of plaques. For verification, targeted insertion of the BSR cassette in the *cdk8* locus was confirmed by PCR (Figure 5.6B-C). Whilst 1300 colonies were screened resulting in a 4.3% HR efficiency of the Ax2 strain (Figure 5.6A), no aggregation null colony was detected in the *exo1*⁻ strain, confirming that this strain is unable to perform HR. Interestingly, HR efficiency of *dnapkcs*⁻ cells was highly enhanced (12.9%) compared to the Ax2 strain, suggesting that disruption of NHEJ increases the HR activity. This is in line with previous results illustrating that disrupting the DSB-responsive PARP Adprt1a impairs NHEJ but promotes HR³⁶⁷. Moreover, an increase in HR efficiency was observed in the *dnapkcs*⁻*exo1*⁻ cells compared to the *exo1*⁻ strain. As the double knockout strains possessed a HR efficiency of 1.4% compared to 0% in the *exo1*⁻ strain, this suggests that disrupting a component of the NHEJ pathway renders the cells more effective in targeted HR. This confirms that the *dnapkcs*⁻*exo1*⁻ strain can compensate the absence of *exo1* and restore HR.

Given that redundancy between Exo1 and Mre11 allows cells to tolerate DNA DSBs, we hypothesised this redundancy might also suppress PARPi toxicity to *exo1*⁻ cells when NHEJ is dysfunctional. Hence, we assessed whether *dnapkcs*⁻*exo1*⁻ cells survive PARPi through Mre11-dependent HR, and whether we can re-sensitise these cells to PARPi by impairing Mre11. Therefore, the cell viability of Ax2, *exo1*⁻, *dnapkcs*⁻ and *dnapkcs*⁻*exo1*⁻ strains was assessed following exposure to PARPi in combination with the Mre11 inhibitor mirin. Whilst mirin significantly decreased the viability of the Ax2 strain to benzamide (Figure 5.7A) it did not affect the viability of these cells to Nu1025 (Figure 5.7B). This indicates that inhibition of Mre11 is not inducing synthetic lethality with PARPi in general. Treatment of *exo1*⁻ cells with PARPi and mirin in combination further decreased the cell viability, supporting the hypothesis that Mre11-dependent HR is engaged to repair PARPi-induced DNA damage in *exo1*⁻ strains (Figure 5.7 5.7). However, mirin only significantly reduced the viability to benzamide but not to Nu1025, indicating that probably only a subset of PARPi-induced damages is channelled through Mre11-dependent HR in the

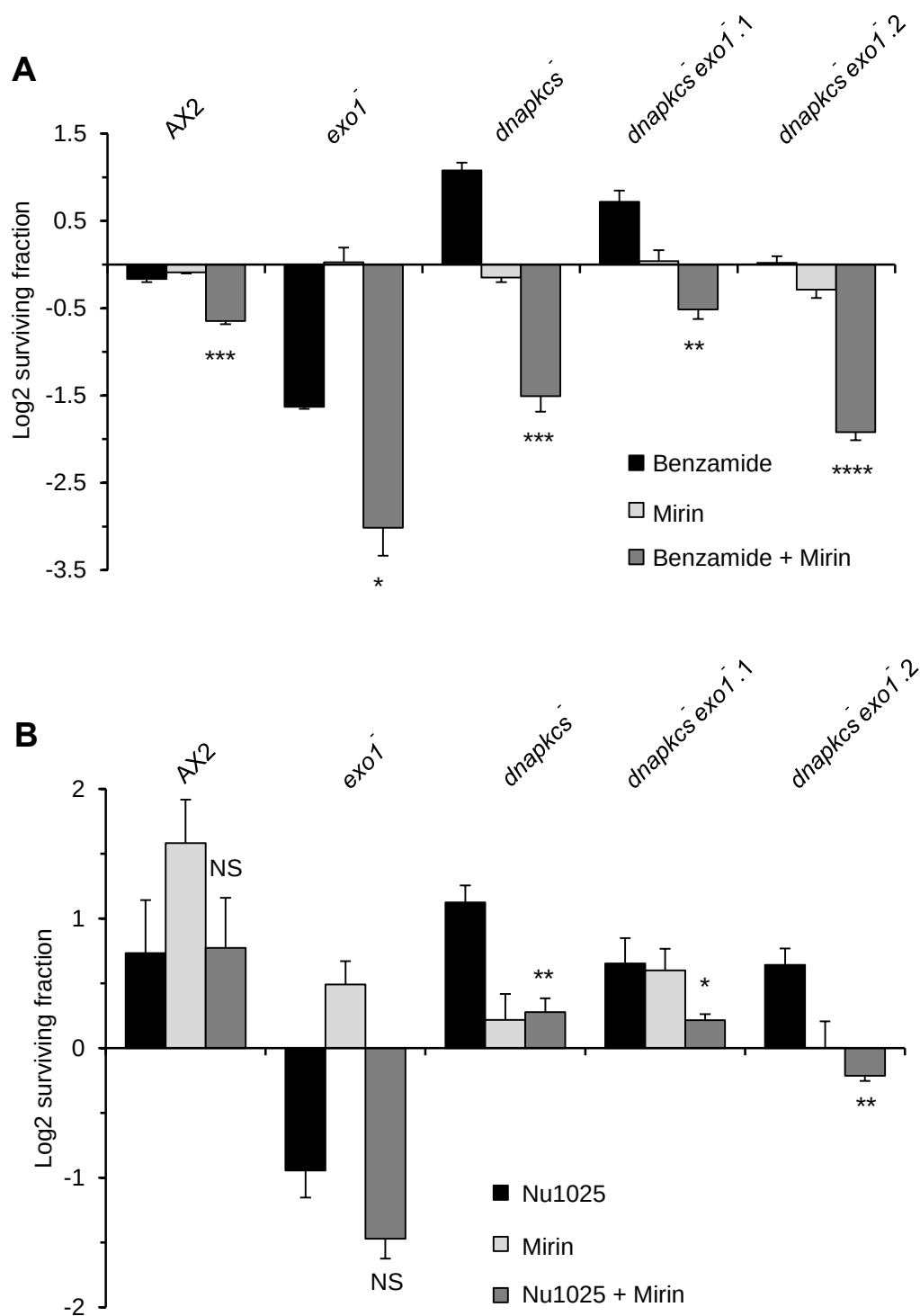


Figure 5.7: Mirin treatment re-sensitises *dnapkcs*⁻*exo1*⁻ cells to PARPi. A-B. *Ax2*, *exo1*⁻, *dnapkcs*⁻ and *dnapkcs exo1*^{-1,2} strains were treated with 2 mM benzamide (A) and 1 mM Nu1025 (B), respectively, and/or 0.5 mM mirin. Cell viability was assessed after 5 days using CellTiter-Glo® (Promega). Cell viability is represented as log₂ fold change between untreated cells and those exposed to the compounds. Error Bars represent the SEM from three independent experiments. Statistical significance was calculated for the samples treated with PARPi and mirin in combination compared to the PARPi-treated one using two-sided student's t-test, NS $P > 0.05$, * $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$, **** $P \leq 0.0001$;

exo1⁻ strains. Surprisingly, exposure to mirin and PARPi significantly reduced the viability of *dnapkcs*⁻ strains compared to only PARPi-treated cells. This might indicate that inhibiting Mre11 negatively affects HR activity in *dnapkcs*⁻ cells, which renders the cells overtly sensitive to PARPi. Interestingly, mirin significantly decreased the viability of *dnapkcs*⁻*exo1*⁻ strains to PARPi. Hence, inhibiting Mre11 re-sensitises HR/NHEJ-deficient cells to PARPi, indicating that those strains engage Mre11-dependent HR to repair PARPi-induced damage resulting in tolerance to these compounds. This confirms that *dnapkcs*⁻*exo1*⁻ strains survive PARPi treatment due to re-activation of HR and that inhibiting Mre11 re-sensitises the cells to these agents.

5.3 Discussion

In this chapter we identified the mechanism that results in the survival of *dnapkcs*⁻*exo1*⁻ cells to PARPi. The POLQ-dependent pathway has been suggested to compete with HR, especially in resolving replication stress^{195,212}. As such, knockdown of *POLQ* in HR-deficient human cells promotes cell death, indicating that when HR is defective DNA DSBs are preferably repaired via this repair pathway¹⁹⁵. Consistent with this, disruption of *polq* further sensitises *exo1*⁻ *Dictyostelium* cells to DNA DSBs. Furthermore, in NHEJ-deficient mammalian cells, POLQ-dependent repair is engaged with increased kinetics, indicating that this mechanism is required for the survival when HR and/or NHEJ is compromised¹⁹⁹. Hence, PolQ-dependent repair is an attractive alternative to mediate the restart of stalled replication forks induced by PARPi when HR is absent. To test whether PolQ-dependent repair is employed in *dnapkcs*⁻*exo1*⁻ cells, we disrupted *polq* in combination with *exo1* and *dnapkcs* (Figure 5.1) and first assessed the sensitivity of these strains to DNA DSBs (Figure 5.2). Survival of *dnapkcs*⁻*polq*⁻*exo1*⁻ strains to phleomycin is similar compared to *dnapkcs*⁻*exo1*⁻ and *polq*⁻*exo1*⁻ cells, indicating that disrupting *polq* or *dnapkcs* does not further sensitise the respective double knockout strain. This suggests that DNA PKcs and PolQ might act in the same DNA repair pathway, thereby resulting in

no further sensitisation to DSBs when both are disrupted in the same strain. Another possible explanation for the similar DSB sensitivity of the triple knockout cells to the *dnapks⁻exo1⁻* and *polq⁻exo1⁻* cells is that these cells can restore recombination. Interestingly, we previously illustrated (Chapter 3) that, similar to yeast and humans, Mre11 and Exo1 share a redundant function in DNA end resection in *Dictyostelium*^{414–416}. Hence, cells deficient in Exo1 and NHEJ and/or PolQ dependent repair might be able to restore HR via DNA end resection using Mre11, resulting in viable cells when exposed to phleomycin or PARPi.

Given the conservation of factors that are required for mammalian alt-NHEJ besides PolQ, such as Lig1 and Lig3, we wanted to test if disrupting *lig3* induces a similar phenotype as *polq* to PARPi treatment. This might indicate whether PolQ and Lig3 are similarly required for DNA repair and possibly shed light on whether *Dictyostelium* are able to employ alt-NHEJ. Hence, we generated *dnapks⁻lig3⁻exo1⁻* strains by disrupting *exo1* in *dnapks⁻lig3⁻* strains (Figure 5.1). We assessed the viability of these triple knockout strains to PARPi to test whether *dnapks⁻exo1⁻* strains remain viable despite PARPi treatment due to Lig3- and/or PolQ-dependent repair. The triple knockout strains are not sensitive to PARPi (Figure 5.3-5.4), indicating that neither PolQ nor Lig3 is involved in rendering *dnapks⁻exo1⁻* cells tolerant to these compounds. Given PARP1 was suggested to regulate the alt-NHEJ pathway in mammalian cells, it is not surprising that PolQ- and Lig3-dependent repair are not mediating the survival of *dnapks⁻exo1⁻* cells to PARPi. However, this indicates that PolQ and Lig3 are possibly similarly involved in alt-NHEJ in *Dictyostelium* and that PARPs are potentially similarly promoting this repair pathway^{203,207,456}. As we did not formally test whether alt-NHEJ is conserved in *Dictyostelium*, no further conclusions can be drawn. Given that disruption of *polq* or *lig3* might not fully impair the alt-NHEJ pathway in *Dictyostelium*, we cannot entirely exclude the possibility of this mechanism being involved in the resistance of HR/NHEJ-deficient cells to PARPi.

As resistance of *dnapkcs⁻exo1⁻* cells to PARPi is independent of Lig3- and PolQ-dependent repair, we sought to identify whether these cells restore HR to render themselves tolerant to PARPi. We previously detected the redundancy of Exo1 with the nuclease Mre11, suggesting that the latter can be employed to mediate DNA end resection following DSB recognition in the *exo1⁻* strain. Surprisingly, inhibition of Mre11 using mirin decreases the viability of Ax2 cells to benzamide (Figure 5.7). Since mirin impairs HR, this reduced viability of Ax2 cells might reflect synthetic lethality of HR dysfunction with PARP inhibition. However, we have shown previously that whilst Mre11 inhibition is not sensitising Ax2 cells to DSBs, mirin further sensitises *exo1⁻* strains to phleomycin. This indicates that a defect in HR following mirin treatment is only apparent when cells are lacking Exo1. Given that mirin decreases the viability of Ax2 cells to benzamide but not to the more specific PARPi Nu1025, the observed effect is probably an artefact.

As mirin further decreases the viability of *exo1⁻* cells to PARPi, this indicates that PARPi-induced damage is possibly channelled through Mre11-dependent repair in the absence of Exo1. However, mirin only significantly reduces cell viability to benzamide but not to Nu1025. This suggests that probably only a small amount of PARPi-mediated DNA lesions are repaired in a manner that is dependent on Mre11, whereas the majority of DNA damage is resolved through NHEJ in *exo1⁻* cells. As end-resection is critical for regulating whether repair of DSBs is channelled through HR or NHEJ, loss of HR-associated DNA nucleases, such as Exo1, possibly promotes the latter, supporting previous findings that *exo1⁻* cells employ NHEJ to resolve PARPi-induced damage.

Surprisingly, inhibition of Mre11 decreases the viability of *dnapkcs⁻* cells to PARPi (Figure 5.7). However, incubation with PARPi alone increases the viability of *dnapkcs⁻* strains, which might be due to the absence of the error prone NHEJ pathway and hyper-activation of HR in these cells (Figure 5.6A). Interestingly, combined treatment of Nu1025 and mirin

is similarly affecting the viability of *dnapkcs*⁻ cells as exposure to mirin alone, suggesting that this combined treatment is not lethal for *dnapkcs*⁻ cells due to the ability to employ Exo1-dependent HR. As combined benzamide and mirin treatment significantly reduces the viability of *dnapkcs*⁻ cells, this indicates that benzamide might inhibit other targets that potentiate its negative effect on cell viability.

The first evidence supporting the hypothesis that *dnapkcs*⁻*exo1*⁻ cells can restore HR were collected by detecting enrichment of the HR-essential factor Rad51 onto chromatin following Nu1025 treatment in *dnapkcs*⁻*exo1*⁻ cells, which is absent in *exo1*⁻ cells (Figure 5.5). Furthermore, *dnapkcs*⁻*exo1*⁻ cells exhibit increased HR activity compared to *exo1*⁻ cells, confirming that disrupting *dnapkcs* and thereby impairing NHEJ can restore HR in *exo1*-deficient cells (Figure 5.6). This indicates that, although lacking Exo1, *dnapkcs*⁻*exo1*⁻ cells can resect DNA, resulting in the enrichment of Rad51 on chromatin and reactivation of HR in response to PARPi. Following DSB recognition, DNA end resection is a critical point in the pathway choice between HR and NHEJ⁴⁵⁷. The NHEJ factor 53BP1, in interaction with Rif1 and Rev7, protects DNA ends from resection, whereas BRCA1 promotes DNA end resection, thereby activating HR^{458,459}. Accordingly, cells lacking 53BP1 or its binding partners restore HR in BRCA1-defective cells, resulting in tolerance to PARPi^{133,302,460}. Our data indicate that disruption of *dnapkcs* similarly inhibits NHEJ and reactivates HR by promoting DNA end resection, restoring Rad51 enrichment on chromatin and rendering the cells refractory to PARPi treatment. Published data supporting our hypothesis illustrate that depletion of the DNA Helicase B (HELB), which antagonises HR during M and G1 phase of the cell-cycle, rescues the toxicity of PARPi in BRCA1-deficient cells^{299–301}. Furthermore, HELB-deficiency restores RAD51 foci formation, indicating that in the absence of HELB, HR is potentially reactivated due to the loss of nuclease inhibition, supporting our findings that restoration of HR can be due to effective DNA end resection³⁰⁰. However, as generation of HR-deficient *Dictyostelium* cells by disrupting *exo1* allows for the restoration of HR following NHEJ inhibition, through

activation of Mre11, this might not be possible if *brca1* or *brca2* are deleted. Hence, it is unknown whether reactivation of HR due to the redundancy between nucleases, and therefore activation of DNA resection, is transferable to human cancer cells lines that are mutated in *BRCA1* or *BRCA2*.

Importantly, we also illustrate that the redundancy of Exo1 and Mre11 is essential for the survival of *dnapks⁻exo1⁻* cells to PARPi (Figure 5.7). Our data show that inhibition of Mre11, using mirin restores the toxicity of PARPi to *dnapks⁻exo1⁻* cells. This suggests that the redundancy between the nucleases is a crucial factor for the resistance of *exo1⁻* cells to PARP inhibition. Interestingly, Mre11 has been implicated in the mammalian alt-NHEJ pathway. Therefore, mirin treatment might not only impair DNA resection required for HR, but also for alt-NHEJ, suggesting a possible role for alt-NHEJ in the tolerance of *dnapks⁻exo1⁻* cells to PARPi¹²⁰. However, we showed that disrupting the putative alt-NHEJ factors *polq* and *lig3* in *dnapks⁻exo1⁻* cells does not re-sensitise them to PARPi (Figure 5.3-5.4). Furthermore, as PARP1 promotes alt-NHEJ, exposure to PARPi is possibly inhibiting this DSB repair pathway, making it unlikely that this mechanism is responsible for the resistance to PARPi. Taken together, *exo1⁻* cells resolve PARPi induced DNA lesions using NHEJ, resulting in cell death. Disruption of NHEJ promotes end resection via the redundant nuclease Mre11, restores HR and therefore leads to the resistance to PARPi.

6. Identification of mutations that suppress PARPi toxicity to *exo1*⁻ cells

6.1 Introduction

6.1.1 *Dictyostelium* as model to identify mutations involved in PARPi resistance of HR-defective cells

Whilst HR is the predominant pathway to repair canonical DSBs and PARPi-induced damage in *Dictyostelium*, these types of DNA lesions are resolved by NHEJ in *exo1*⁻ cells. As disrupting NHEJ renders *exo1*⁻ cells refractory to PARPi, this indicates that synthetic lethality of HR-compromised cells to PARPi is due to error-prone NHEJ, supporting the deleterious effect of this DSB repair pathway on cell survival. Further data indicate that in the absence of NHEJ and Exo1, cells engage the nuclease Mre11, resulting in re-activation of HR and tolerance to PARPi. Hence, we identified a novel mechanism leading to PARPi resistance by restoring HR through the redundancy between nucleases.

Synthetic lethality of HR dysfunction with PARP inhibition is already exploited in the clinic to treat tumours that are defective in HR proteins, such as BRCA1 or BRCA2. However, several HR-defective cancer cells develop a resistance to this therapy. Some of the mechanisms rendering HR-defective cells tolerant to PARPi are already known, such as re-activation of HR due to loss of 53BP1²⁸⁴. As these tumour cells exhibit a significantly increased mutation rate compared to healthy diploid cells due to aberrant HR, several yet undiscovered gene alterations may contribute to tolerating PARPi^{310–312}. For example, BRCA1-defective mice can develop a resistance to PARPi through upregulation of drug efflux pumps, indicating that further mechanisms apart from re-activation of recombination are possible to suppress PARPi toxicity in HR-dysfunctional cells^{292,293}. Due to the high

mutation rate, identifying mutations responsible for PARPi tolerance proves to be difficult in human cancer cells. Interestingly, *Dictyostelium* possess one of the lowest spontaneous DNA mutation rates reported for eukaryotes and furthermore are highly resistant to DNA damage^{363–365}. In contrast to human cells with a haploid genome size of 3,300 Mb, *Dictyostelium* have a relatively small genome of 34 Mb, which is divided into 6 haploid chromosomes, containing approximately 12,200 protein coding genes. The genetic tractability and reduced spontaneous mutation rate of *Dictyostelium*, in addition to its small genome makes this model an ideal organism to perform whole genome sequencing to explore genetic alterations³⁶⁵. Moreover, by means of illustrating that HR-defective *Dictyostelium* cells are able to obtain a tolerance to PARPi, we established this organism as a suitable model to identify novel genes, that when mutated, suppress PARPi toxicity.

6.1.2 Aims

Given the characteristics of its genome, *Dictyostelium* proves to be a useful organism to investigate resistance mechanisms of HR-compromised cells to PARPi using whole genome sequencing. Our aim in this chapter was to identify novel mutations in protein coding genes that are involved in the resistance of HR-defective cells to PARPi. To this end, we performed a screen to obtain PARPi-resistant *exo1⁻* *Dictyostelium* cells, and then employed whole genome sequencing to identify unique mutations that suppress PARPi toxicity to HR-deficient cells.

6.2 Results

6.2.1 Generation of PARPi-resistant HR-deficient strains

Previously, we established that exposure to Nu1025 or benzamide is toxic to *exo1⁻* cells (Chapter 3). In this chapter, we sought to perform a genetic screen to identify mutations that render HR-compromised cells refractory to PARPi. Therefore, we first assessed the conditions in which HR-defective *exo1⁻* cells are killed due to benzamide exposure, whereas parental Ax2 cells remain viable, with a view to use these conditions to select for PARPi-resistant *exo1⁻* cells. We hypothesised that HR-dysfunctional *Dictyostelium* cells that survive benzamide exposure, and exhibit a persistent resistance to further PARPi, will have obtained genetic mutations that decrease the toxicity of PARPi, leading to the survival of the cells. To identify the required screening conditions, Ax2 and two independent *exo1⁻* cells were incubated with various benzamide concentrations in a 14cm dish until cell growth was visible. As expected, benzamide was similarly toxic to two independent *exo1⁻* strains, whereas Ax2 cells were significantly more viable (Figure 6.1A). Whilst only 5% of Ax2 cells survived 7.5 mM benzamide, 50% of these cells remained viable when exposed to 5 mM benzamide. Interestingly, 7.5 mM benzamide was lethal for all *exo1⁻* cells, whereas 12% and 0.2%, respectively, of the *exo1⁻* strains survived 5 mM benzamide. As we wanted to verify the difference in cell survival between *exo1⁻* and Ax2 strains, the 5 mM benzamide treatment was repeated with a greater number of cells. As before, whilst 50% Ax2 cells survived, 0.1% *exo1⁻* cells remained viable when exposed to benzamide (Figure 6.1). Thereby, we identified that 5 mM benzamide is the required concentration for the screen to kill most of the *exo1⁻* cells, whereas HR-competent cells survive. Hence, we hypothesised that *exo1⁻* cells that survive 5 mM benzamide will have acquired a mutation that suppresses PARPi toxicity.

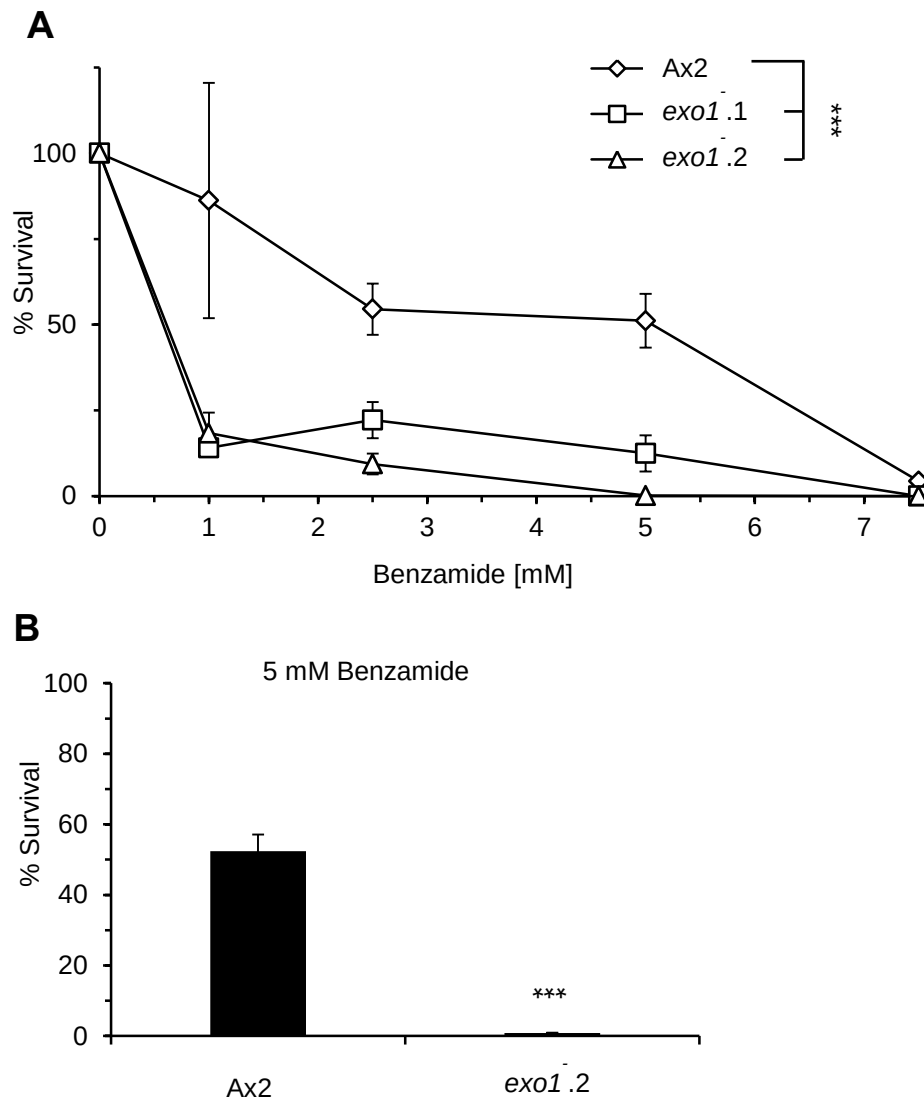


Figure 6.1: 5 mM benzamide is the required concentration for the screen to select for PARPi-resistant *exo1*⁻ cells. **A** Ax2 cells and two independent *exo1*⁻ strains were incubated with the indicated concentrations of benzamide in 14cm dishes in HL5. Cell survival was assessed after 9-17 days by plaque formation relative to the mock-treated sample. Data represent mean $\pm$ SEM from three independent experiments. The p-values are calculated with respect to the parental Ax2 strain by two-way ANOVA, NS $P > 0.05$, * $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$. **B**. 670 cells of Ax2 and *exo1*⁻ strains were incubated with 5 mM benzamide or left untreated. Cell survival was assessed after 9-17 days by plaque formation relative to the mock-treated sample. Data represent mean $\pm$ SEM from three independent experiments. The p-values are calculated with respect to the parental Ax2 strain by two-sided student's t-test NS $P > 0.05$, * $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$.

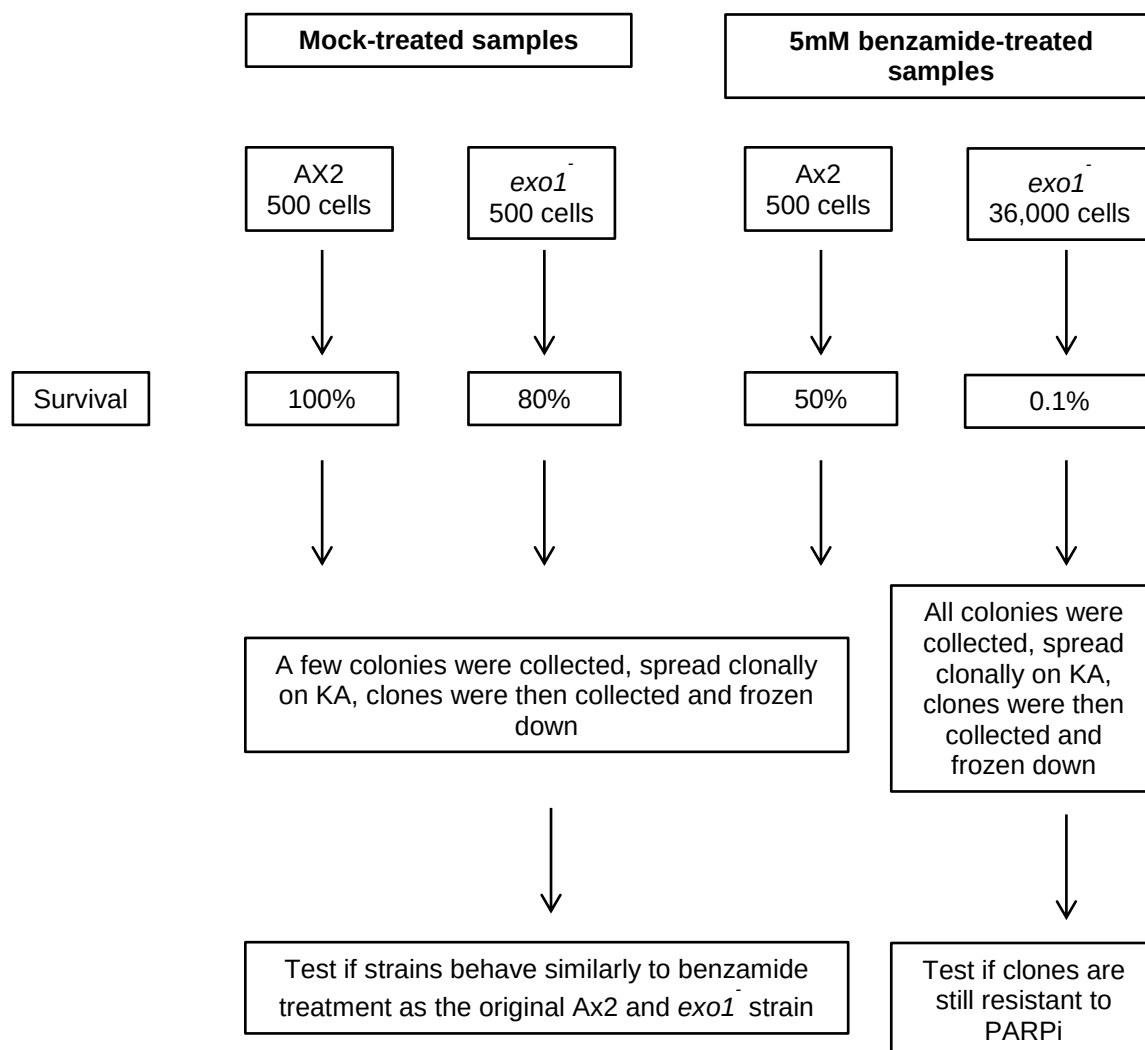


Figure 6.2: Workflow of the screen. For the screen, 36 000 *exo1*⁻ cells were spread in 96-well plates: 40 plates with 4 cells/well and 30 plates with 20 cells/well, and exposed to 5 mM benzamide. As control, 500 cells Ax2 cells were exposed to 5 mM benzamide and the same number of Ax2 and *exo1*⁻ cells were mock-treated. 0.1% of the *exo1*⁻ cells survived the benzamide treatment, whereas 80% of the *exo1*⁻ cells were viable following mock exposure. 100% Ax2 cells survived the mock treatment, whereas 50% were killed by 5 mM benzamide. The *exo1*⁻ cells that survived the benzamide treatment were spread clonally onto a KA lawn, selected and frozen down. 10 cells of each control sample were collected, spread clonally and frozen down. The collected cells were subsequently tested in a cell viability assay to assess their survival to further PARPi.

Next, we performed the genetic screen using the established benzamide concentration, with a view to induce selection pressure on *exo1*⁻ cells to generate mutations that lead to PARPi resistance. As *exo1*⁻ cells are dysfunctional in HR, which possibly results in genomic instability, these cells might have a higher spontaneous mutation rate compared to the parental Ax2 strain. By including mock-treated *exo1*⁻ and parental Ax2 cells in the screen we were able to compare the basal mutation rates of both strains and therefore identify variants that promote a survival advantage of the *exo1*⁻ cells to PARPi.

Additionally, Ax2 cells that underwent benzamide selection pressure were also added in the screen to help exclude spontaneous variants that are promoted by PARPi exposure, as opposed to being involved in suppressing PARPi toxicity in HR-compromised cells. To perform the screen, 4-20 Ax2 or *exo1*⁻ cells were plated per well and cultivated in 96-well plates, with either ethanol as mock treatment or 5 mM benzamide (Figure 6.2). In total, 36,000 *exo1*⁻ cells were exposed to benzamide, whereas 500 cells of the control strains (Ax2 or *exo1*⁻ cells) were incubated with ethanol or benzamide (Figure 6.2). After 2 weeks, Ax2 cells exposed to benzamide or ethanol were collected. As the *exo1*⁻ cells possess a growth defect, colony formation of the mock-treated control was visible after 17 days, whereas *exo1*⁻ cells exposed to benzamide were collected after 19 days. Overall, *exo1*⁻ cells remained viable in 36 wells despite benzamide treatment, resulting in a survival fraction of 0.1%. As before, approximately 50% of Ax2 cells survived PARPi treatment. To obtain clonal cells, viable colonies were spread onto a KA lawn on SM agar, which were then subsequently collected and frozen down. We obtained 36 clonal *exo1*⁻ cells that survived benzamide treatment, possibly due to a mutation that is providing an advantage to the cells to survive PARPi exposure. In addition, 10 clones of each control, Ax2 and *exo1*⁻ cells that underwent the ethanol treatment, and Ax2 cells that were selected with benzamide, were also collected.

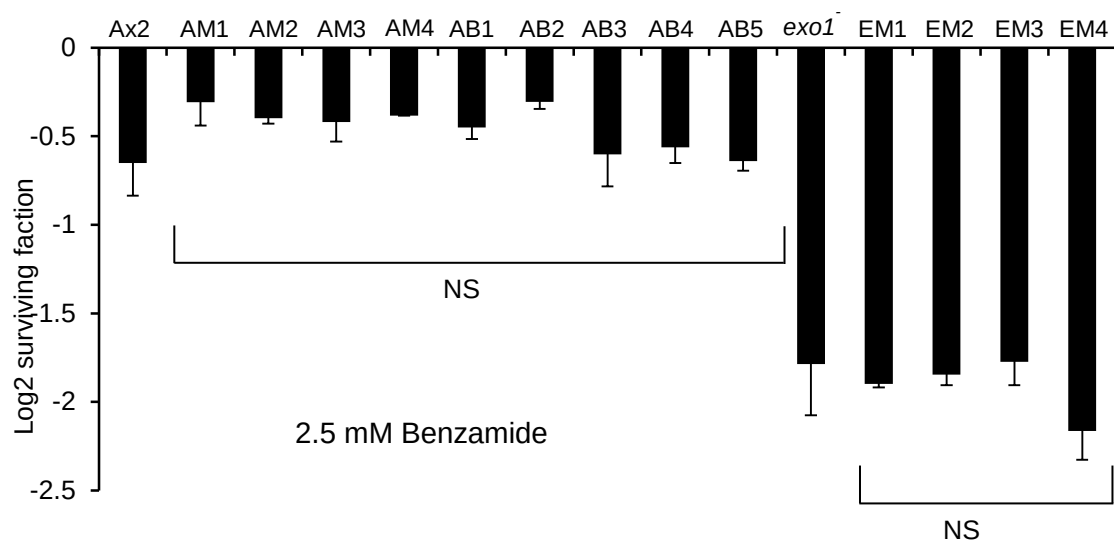


Figure 6.3: Long-term benzamide and/or mock treatment does not affect cell sensitivity to PARPi. Ax2 and *exo1⁻* strains that were selected using ethanol and Ax2 cells that were exposed to benzamide during the screen, were incubated with the indicated benzamide concentration for 5 days. Cell survival was assessed using CellTiter-Glo® (Promega). Cell viability is represented as log2 fold changes between untreated cells and those exposed to the indicated PARPi. Error bars represent the standard error of the mean (SEM) from three independent experiments. The p-values, provided in the table, are calculated with respect to the parental Ax2 strain or *exo1⁻* strains that did not go through the screen, using two-sided student's t-test, NS $P > 0.05$, * $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$. AB: Ax2 that were exposed to benzamide in the screen. AM: Ax2 cells that were mock-treated in the screen. EM: *exo1⁻* cells that were mock-treated in the screen

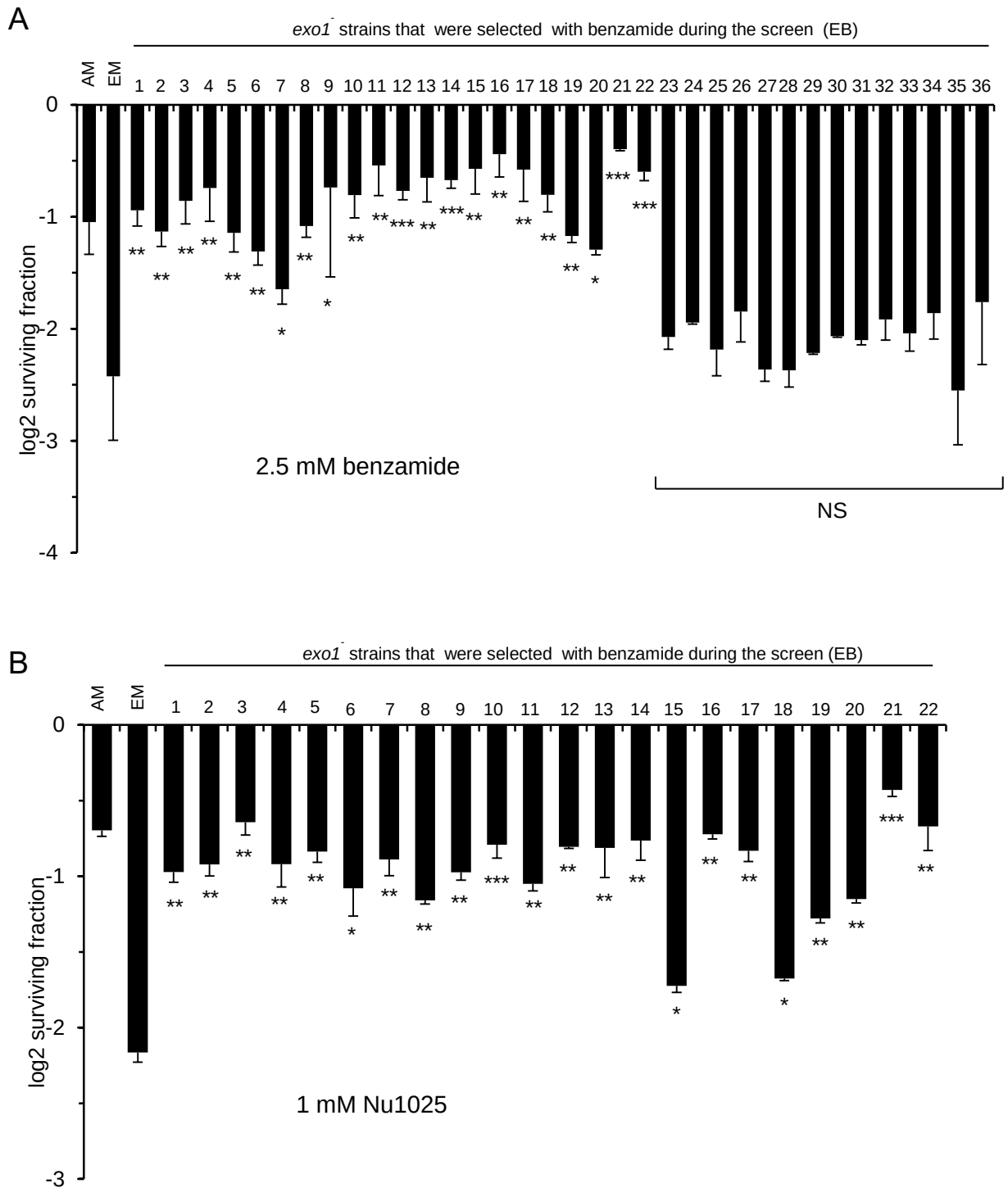


Figure 6.4: 22 *exo1*⁻ cells that were selected using benzamide are resistant to PARPi in a reproducible manner. A-B. The *exo1*⁻ strains that were selected using benzamide in the screen were incubated with the indicated concentrations of benzamide (A) or Nu1025 (B). Ax2 (AM) and *exo1*⁻ cells (EM) that were mock-treated in the screen serve as a control. After 5 days, cell survival was assessed using CellTiter-Glo® (Promega). Cell viability is represented as log₂ fold changes between untreated cells and those exposed to the indicated PARPi. Error bars represent the standard error of the mean (SEM) from three independent experiments. The p-values, provided in the table, are calculated with respect to the *exo1*⁻ strain that was mock-treated in the screen, using two-sided student's t-test, NS $P > 0.05$, * $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$ **** $P \leq 0.0001$

We then tested whether the long-term exposure to benzamide or ethanol, as conducted in the screen, changes the viability of *Dictyostelium* to PARPi. The cell viability of mock- (AM) and benzamide-treated Ax2 cells (AB), along with the mock exposed *exo1*⁻ cells (EM), was compared to Ax2 and *exo1*⁻ strains, respectively, that did not undergo the screening procedure. Ax2 and *exo1*⁻ strains that went through the screening procedure were similarly sensitive to benzamide as the respective parental cells that were not selected with the screen (Figure 6.3). We thereby established that exposure to ethanol and/or benzamide for 2-3 weeks does not affect the viability of the control strains to PARPi.

Next, we sought to assess whether the *exo1*⁻ cells that were selected using benzamide possess a reproducible resistance to benzamide and other PARPi, such as Nu1025. Firstly, we tested if the 36 *exo1*⁻ cells that survived the benzamide treatment (EB strains) are still resistant to this PARPi by assessing their viability to benzamide using the CellTiter-Glo® Luminescent Cell Viability Assay (Promega). Ax2 (AM strains) and *exo1*⁻ cells (EM strains) that were mock-treated throughout the screen, serve as control (Figure 6.4A). Since only 22 out of the 36 EB cells were significantly more viable to benzamide as the EM strain, this indicates that 22 *exo1*⁻ cells possess a reproducible resistance to benzamide. To detect whether these 22 *exo1*⁻ cells tolerate other PARPi or only suppress benzamide toxicity, we performed a secondary screen with Nu1025 (Figure 6.4B). All of the tested *exo1*⁻ cells were significantly more viable to Nu1025 compared to the *exo1*⁻ strain that was mock-treated throughout the screen. Hence, we collected 22 *exo1*⁻ cells that possess a reproducible resistance to different PARPi. We therefore hypothesised that these strains gained a mutation that is beneficial for the survival of HR-defective cells under benzamide selection, resulting in the suppression of PARPi toxicity.

6.2.2 Quality control of whole genome sequencing data

To identify mutations that might suppress PARPi toxicity in HR-compromised cells, we sequenced the genomes of the 22 *exo1*⁻ cells that show a reproducible resistance to benzamide and Nu1025. The genomes of three Ax2 cells, either exposed to benzamide (AB) or ethanol (AM), as well as three *exo1*⁻ clones that were mock-treated (EM) were sequenced as controls. Library generation was optimised and performed as described in the Materials and Methods section with kind help of Gareth Bloomfield in Rob Kay's lab (MRC, Laboratory of Molecular Biology, Cambridge). Sequencing of the genomes was performed on an Illumina HiSeq2500 system.

The workflow of sample preparation and data analysis is illustrated in Figure 6.5. After genome sequencing, the reads were aligned to the *Dictyostelium* reference genome obtained from Ensemble Protists using the Burrows-Wheeler alignment tool (BWA) and further processed using SAMtools^{380–383}. As a PCR was performed to amplify the adapter-ligated DNA molecules during library preparation, PCR duplicates were generated. Following genome sequencing, those duplicates have either identical reads or align to the exact same start position and are therefore a possible source of bias for the analysis^{461,462}. Hence, the read pair with the highest mapping quality was kept, whilst PCR duplicates were removed using SAMtools. For variant calling, we set the threshold to 90% agreement among the sequenced reads that are mapped to the reference genome, before identifying a specific site as a mutation using freebayes³⁸⁴. To detect mutations that are unique to benzamide resistant *exo1*⁻ cells we excluded gene alterations that occur in mock- and benzamide-treated Ax2 cells and mock exposed *exo1*⁻ cells using BEDtools³⁸⁵. As we were interested in mutations in protein-coding regions that result in the suppression of PARPi toxicity to HR-defective cells, we focused on variants in the exon sequences. Furthermore, we required a depth of at least four reads to identify the obtained variant as a true mutation, which was assessed using SAMtools and integrative

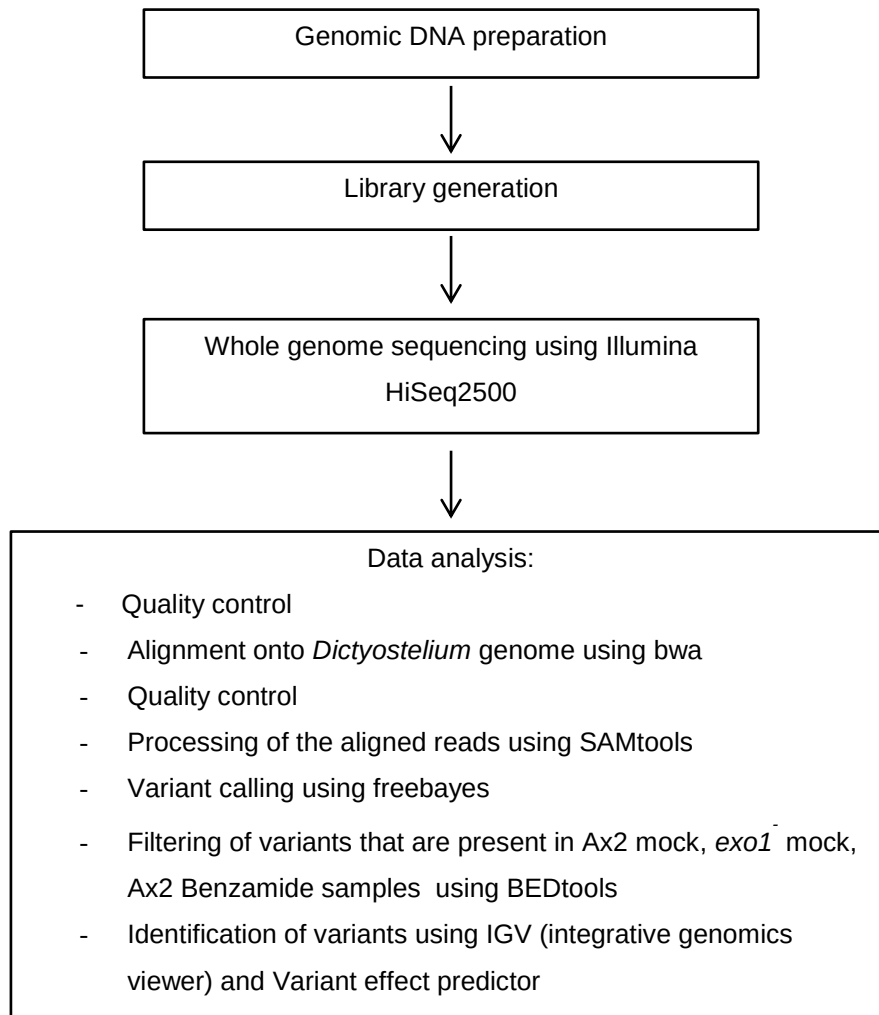


Figure 6.5: Workflow of the whole genome sequencing analysis. Genomic DNA was prepared from 22 *exo1*⁻ strains that are resistant to benzamide and 3 *exo1*⁻ strains that were mock-treated. Additionally, DNA was prepared from Ax2 cells that were either mock- or benzamide-treated. Following library generation, the whole genome was sequenced using an Illumina HiSeq2500. Quality control was performed at two stages: of the raw data and after alignment to the reference *Dictyostelium* genome obtained from Ensemble Protists using the Burrows-Wheeler alignment tool (BWA)^{380,381}. The aligned reads were processed using SAMtools^{382,383}. Freebayes was used for the identification of variants³⁸⁴. We filtered the mutations present in the Ax2 samples and the mock-treated *exo1*⁻ strains to obtain variants that are unique for the benzamide resistant *exo1*⁻ strains using BEDtools³⁸⁵. Further filtering was performed using IGV, Variant effect predictor and SAMtools^{382,383,386–388}.

genomics viewer (IGV)^{382,383,386,387}. The effect of the identified mutation on the protein coding region was investigated using Variant effect predictor³⁸⁸.

Once we aligned the sequenced reads to the reference genome, we first wanted to confirm that the 22 PARPi-resistant *exo1*⁻ strains are lacking the *exo1* genomic locus. Surprisingly, 11 out of the 22 EB strains possessed the endogenous *exo1* gene and are therefore probably competent in expressing this nuclease, rendering the cells proficient in HR. As we were interested in identifying mutations that suppress PARPi toxicity to HR-compromised cells, we excluded those 11 strains from the analysis (EB12-22) and focused on the remaining PARPi-resistant *exo1*⁻ strains (EB1-11).

The genome sequencing data were subjected to two rounds of quality control: of the raw data upon data acquisition and then again once the reads were aligned to the reference genome. The quality of the raw data was monitored by the sequencing service, providing the parameters for the base quality (Phred Quality score), the GC content of the sequenced data, and the duplication rate (Table 6.1). The Phred quality score (Q score) indicates the accuracy of identifying each nucleobase of the sequencing reads^{463,464}. The Q scores are logarithmically linked to base-calling error probabilities and are defined by $Q = -10\log_{10}(\text{Error Rate})$, with higher values corresponding to higher quality of the sequencing reads. As Illumina systems usually obtain a Q score between 30 and 40, the sequenced reads are within this range, with Q scores varying from 34.3 to 35.2, resulting in 99.62 - 99.70% accuracy per nucleobase call^{465,466}. The GC content of the sequenced data varies with an error rate of 9-10%, from 27.2 to 29.8%, which is slightly higher than the GC concentration of the *Dictyostelium* nuclear genome (22.4%). This might be due to problems sequencing highly AT-rich repetitive sequences³³⁷. However, as it is still in range of the *Dictyostelium* nuclear genome composition, this indicates that the sequenced DNA belongs to this model organism. Library complexity is indicated by the amount of duplicate reads, which are defined as sequencing reads that are either exactly identical or

	GC concentration [%]	Average Q score	duplication rate of paired reads [%]
AM1	28.9 ± 9.8	35.0	4.3
AM2	29.0 ± 9.5	35.1	5.3
AE3	28.9 ± 9.5	35.1	3.4
AB1	28.0 ± 9.8	35.1	3.9
AB2	27.8 ± 9.6	35.0	4.1
AB3	29.0 ± 9.5	35.2	4.3
EM1	28.0 ± 9.7	34.6	2.7
EM2	28.6 ± 9.9	34.9	2.8
EM3	27.8 ± 9.8	34.6	4.1
EB1	28.5 ± 9.9	35.0	3.9
EB2	27.6 ± 10.0	34.4	2.4
EB3	27.2 ± 9.6	34.6	4.5
EB4	29.1 ± 9.5	35.0	3.1
EB5	29.8 ± 10.1	35.2	2.6
EB6	29.5 ± 9.6	35.0	3.0
EB7	27.4 ± 9.8	35.0	2.7
EB8	29.2 ± 9.7	34.9	3.9
EB9	27.3 ± 9.6	34.3	3.1
EB10	27.6 ± 9.4	34.3	2.5
EB11	29.0 ± 9.8	34.9	2.0

Table 6.1: Quality of raw whole genome sequencing data. Whole genome sequencing was performed by the Oxford Genomic Centre based at the Wellcome Trust Centre for Human Genetics, Oxford, using an Illumina HiSeq2500 system. The quality of the raw data was monitored by the sequencing service, providing the GC concentration with the error rate of each sample, the Phred score, and the PCR duplication rate. The Phred quality score (Average Q score) is a parameter for the quality of the identification of each nucleobase by DNA sequencing^{463,464}. The duplication rate of paired reads is defined as paired sequencing reads that are either exactly identical or align to the exact same start position^{461,462}. AM: Ax2 mock-treated samples, AB: Ax2 benzamide-treated samples. EM: *exo1*⁻ mock-treated samples, EB: benzamide-resistant *exo1*⁻ cells.

	reads in total [million]	reads mapped to the Genome [%]	median depth	bases covered with >5 reads [%]	bases covered with >20 reads [%]
AM1	18.3	99.0	54	96.9	83.5
AM2	26.9	98.5	43	93.1	73.1
AE3	13.5	98.8	42	92.6	72.0
AB1	22	98.8	76	97.6	87.9
AB2	23.2	98.7	84	97.9	89.6
AB3	16.6	99.1	52	98.0	77.3
EM1	14.6	98.7	48	96.6	80.1
EM2	14.4	98.9	44	95.6	76.4
EM3	14.5	99.2	46	96.4	78.8
EB1	22.8	98.8	74	97.8	88.4
EB2	14.2	98.8	46	96.9	81.0
EB3	18.1	99.1	63	97.7	87.2
EB4	17.2	98.2	54	96.3	80.4
EB5	12.4	98.2	34	92.7	68.6
EB6	13.3	98.6	39	93.2	71.5
EB7	16.3	99.1	54	96.9	83.5
EB8	19.1	98.8	58	97.0	82.8
EB9	15.5	99.1	53	97.3	83.3
EB10	10.2	99.2	33	92.8	68.0
EB11	7.7	99.2	22	86.4	52.7

Table 6.2: Quality of aligned whole genome sequencing data. The raw data were aligned to the *Dictyostelium* reference genome obtained from Ensemble Protists using the Burrows-Wheeler alignment tool (BWA)^{380,381}. The data presented in this table were calculated using R³⁸⁹. The median depth describes the number of reads that are likely to be aligned at a given base. Data analysis was performed with the help of Gareth Bloomfield. AM: Ax2 mock-treated samples, AB: Ax2 benzamide-treated samples. EM: *exo1*⁻ mock-treated samples, EB: benzamide-resistant *exo1*⁻ cells.

align to the exact same start position^{461,462}. These duplicates arise due to the PCR amplification step in the library generation. Higher duplication rates can indicate a low amount of starting DNA, or a high variance in fragment size in the library, as smaller fragments are more efficiently amplified by PCR. Thus, a low duplication rate indicates a higher complexity of the sequencing reads. Table 6.1 shows the duplication rate of paired reads, which is below 5% for all samples except for one, indicating a reasonable complexity of the sequencing data. Taken together, we obtained high quality sequencing data of the Ax2 and the *exo1⁻* cells.

Different quality parameters were monitored after aligning the sequenced reads to the reference genome using R³⁸⁹. Approximately 98.2- 99.2% of the reads mapped onto the *Dictyostelium* genome, indicating that no substantial contamination of the sequences occurred (Table 6.2). The median depth of each base, describing the median number of reads that are likely to be aligned at a given base, was calculated for each sample after removing PCR duplicates. The calculated metrics for the median depth indicates that a nucleotide in EB11 is sequenced on average 22 times, whereas AB2 possess the highest median depth score with 84. This variation might be due to differences in the amount of sequenced DNA between the samples. Whilst 86.4- 98% of the bases are covered with at least 5 reads in the samples, 52.7- 89.6% of the nucleotides are sequenced with a depth of more than 20. Overall, the read depth of the samples is higher than in a previous publication about *Dictyostelium* genome sequencing, indicating that the obtained sequencing reads have a suitable coverage to identify mutations³⁶⁵.

6.2.3 Analysis of whole genome sequencing data

After verifying the quality of the data, we analysed the sequencing reads regarding DNA alterations compared to the reference genome, with kind help from Gareth Bloomfield. We sought to identify unique mutations in protein-coding regions that are responsible for the

	amount of variants in total	variants in exons
AM1	18866	2501
AM2	18041	2514
AM3	40861	3330
AB1	18134	2488
AB2	17797	2496
AB3	19050	2544
EM1	18773	2476
EM2	18773	2476
EM3	19455	2512
EB1	18097	2503
EB2	18901	2486
EB3	18547	2527
EB4	18762	2511
EB5	19968	2482
EB6	19556	2448
EB7	18130	2491
EB8	18281	2505
EB9	18381	2492
EB10	21206	2632
EB11	20851	2503

Table 6.3: Amount of detected variants in the sequenced samples. The reads were aligned to the *Dictyostelium* reference genome obtained from Ensemble Protists using the Burrows-Wheeler alignment tool (BWA)^{380,381}. The amount of total variants was calculated using freebayes³⁸⁴. The amount of variants present in exons were identified using BEDtools³⁸⁵. AM: Ax2 mock-treated samples, AB: Ax2 benzamide-treated samples. EM: *exo1*⁻ mock-treated samples, EB: benzamide-resistant *exo1*⁻ cells.

	Variants in exons that are not present in the mock-treated Ax2 and <i>exo1</i> ⁻ cells or Ax2 benzamide-treated samples	Variants present only in PARPi-resistant <i>exo1</i> ⁻ cells that induce a change in the protein sequence
EB1	40	2
EB2	70	2
EB3	35	1
EB4	126	3
EB5	71	4
EB6	48	3
EB7	52	1
EB8	55	2
EB9	40	2
EB10	184	14
EB11	111	12

Table 6.4: Amount of unique variants in the PARPi-resistant *exo1*⁻ strains. We filtered the mutations present in Ax2 samples and mock-treated *exo1*⁻ strains to obtain variants that are unique to the benzamide-resistant *exo1*⁻ strains using BEDtools³⁸⁵. Further filtering was performed using IGV and SAMtools to identify mutations that induce a change in the protein sequence^{382,383,386–388}. EB: benzamide-resistant *exo1*⁻ cells.

resistance of *exo1*⁻ cells to PARPi. Several mutations were discovered in transposons, which contain highly repetitive sequences and are usually not coding for a protein, except for the enzyme transposase^{467,468}. Hence, variants in transposon elements were excluded from the analysis as mutations are difficult to detect and are probably not in protein-coding regions. The amount of total variants is similar in all sequenced strains and varies from 18,000 to 21,000 (Table 6.3). Although AM3 contains more than double the amount of total mutations than the other AM samples, only 3,300 of these mutations reside in protein-coding regions. This indicates that the majority of the variants are located in non-coding regions, possibly in complex AT-rich sequences. On average, each PARPi-resistant *exo1*⁻ cell contains a similar amount of mutations in the protein-coding region as the *exo1*⁻ mock-treated samples and the Ax2 benzamide-treated samples (Table 6.3). This indicates that long-term benzamide treatment is not promoting gene mutations and that, although deficient in HR, *exo1*⁻ cells do not exhibit a higher mutation rate than the Ax2 cells.

Approximately 3% of the variants in protein-coding regions that were detected in the PARPi-resistant *exo1*⁻ cells are not present in any of the mock-treated samples or benzamide-treated Ax2 cells (Table 6.4). EB10 possesses the highest number of variants in exons (184 mutations), whereas EB1 has the least with 35. However, most of these variants are silent mutations and therefore do not induce changes in protein sequences, indicating that they are not involved in acquiring resistance to PARPi. In total, we detected 46 unique mutations that affect the protein sequences in the PARPi-resistant *exo1*⁻ cells. The genes containing these mutations are illustrated in Table 6.5. Only variants with coverage of at least four reads were considered a true mutation. The read depth of each variant is shown in the second column of Table 6.5, with alterations that were classified as true mutations marked in blue. As three of the EB strains (EB1, EB5 and EB8) do not possess a true mutation one of the identified variants with coverage of less than four reads might be responsible for the survival of these cells. Another possibility is that these

	mutation	amount of reads	Gene	gene domains
EB1	missense	2	set1	histone methyltransferase
	missense	3	DDB_G0273497	alkaline phosphatase like,
EB2	missense	69	DDB_G0271238	non cytoplasmic protein
	amino acid change in splice site	2	DDB_G0285187	/
EB3	missense	45	DDB_G0270330	Pesticidal crystal protein
EB4	missense	2	DDB_G0277057	/
	missense	78	actin7	encodes one of the actin proteins
	missense	5	DDB_G0286361	Zinc finger
EB5	missense	2	DDB_G0268072	/
	missense	2	DDB_G0276145	/
	missense	3	DDB_G0283833	/
	missense	3	DDB_G0290435	Leucine-rich repeat domain,
EB6	premature stop codon	4	DDB_G0285767	contains a ubiquitin domain, protein binding
	missense	2	DDB_G0285981	type A von Willebrand factor (VWFA) domain
	missense	3	DDB_G0290815	Immunoglobulin like transcription factor, mannose6-phosphate receptor binding
EB7	deletions of 10 nucleotides	40	DDB_G0270056	CheY-like superfamily, receiver domain of phosphorylation signal
EB8	missense	3	DDB_G0274677	WD40/YVTN repeat-like-containing domain
	premature stop codon	2	DDB_G0277721	fszA mitochondrial cell division protein, tubulin and GTPase domain
EB9	missense	4	DDB_G0275965	transmembrane protein with cytoplasmic and non-cytoplasmic domain
	missense	58	DDB_G0287869	R3H domain, Transcription factor NFX1 family
EB10	premature stop codon	2	DDB_G0268838	/
	missense	3	DDB_G0279539	RNA-binding domain
	premature stop codon	2	DDB_G0288859	zinc finger, jmjC domain
	missense	3	DDB_G0289119	serine/threonine kinase
	7 missense mutations	4-15	pks30	polyketide synthase, beta-ketoacetyl synthase family protein
	3 missense mutations	3-5	pks32	polyketide synthase, beta-ketoacetyl synthase family protein
EB11	premature stop codon	2	fnkB	c-terminal E3 ligase domain, n-terminal serin/threonin protein kinase
	lost stop codon	2	alrC	Aldehyde Reductase
	2 missense mutations	2	tsin	membrane associate ATPases
	missense	2	DDB_G0270522	WD40/YVTN repeat-like-containing domain
	lost stop codon	2	DDB_G0269860	nuclear import protein
	missense	2	DDB_G0275797	/
	missense	47	DDB_G0278431	transmembrane protein with unknown function
	missense	2	DDB_G0288601	/
	missense	2	valS2	valyl tRNA synthetase
	Missense, splice region variant	4	DDB_G0289609	putative acetoin dehydrogenase
	missense	2	DDB_G0293782	/

Table 6.5: List of detected variants in the PARPi-resistant *exo1* strains. The genes that contain the mutations in the PARPi-resistant *exo1* cells and the read depth per mutations was identified using IGV, SAMtools and Variant effect predictor^{382,383,386-388}. The type of each mutation was detected using Variant effect predictor. The gene domains and possible gene function were assessed using InterProScan^{378,379}. EB: benzamide-resistant *exo1* cells. Variants with a read depth of at least four were marked in blue, whereas interesting gene alterations were marked in purple.

strains contain changes in non-coding DNA possibly controlling transcription, resulting in resistance to PARPi. However, as we did not have the time to investigate mutations in non-coding DNA sequences, we cannot confirm these assumptions.

Most of the mutated genes encode for unknown proteins, with unpredicted functions, except for some proteins such as *actin7*, *pks30* and *pks32* (Table 6.5). Actin7 is one of the *Dictyostelium* Actin proteins, whereas *pks30* and *pks32* encode polyketide synthases that produce the secondary metabolite polyketide. The protein domains, and thereby the potential protein function encoded by the mutated unknown genes, were determined using InterproScan and are illustrated in the last column in Table 6.5^{378,379}. Interestingly, EB10 exhibits 8 unique variants, with 7 being in the same protein-coding gene, *pks30* (polyketide synthase 30). Most of the other sequenced PARPi-resistant *exo1*⁻ strains exhibit 1-2 unique mutations in protein-coding regions with a read depth of at least four. Hence, the amount of mutations only present in the *exo1*⁻ strains that are resistant to benzamide is very low, supporting published data of a low mutation rate of *Dictyostelium*³⁶⁵.

We detected 12 different protein-coding genes that were mutated with a read depth of at least four in the PARPi-resistant *exo1*⁻ strains. Hence, we were focusing on these alterations and their potential contribution to the tolerance of PARPi. The protein coding sequence DDB_G0286361, containing a missense mutation in EB4, possesses a Zinc finger at its N-terminus and can interact with nucleic acid. However, the function of this protein, and therefore its role in the resistance of *exo1*⁻ cells to PARPi, is unclear. With the exception of three mutations, most of the detected variants are point mutations, resulting in an amino acid change in the respective protein. The mutation of DDB_G0285767 in EB6 results in a premature stop codon, while DDB_G0270056 in EB7 lacks 10 nucleotides, and a mutation in EB11 leads to an amino acid change in the splice site of DDB_G0289609. The latter protein contains two cystathionine-beta-synthase (CBS)

domains and is a putative acetoin dehydrogenase. The gene with the premature stop codon, DDB_G0285767 is coding for a polypeptide containing an ubiquitin domain, and possibly possesses a molecular function in protein binding. The gene DDB_G0270056, which lacks 10 nucleotides in EB7 codes for a protein containing a domain that receives a phosphorylation signal as part of the response regulator family CheY⁴⁶⁹. Some of the genes we identified, such as Set1, contain known mutations that change their cellular activity⁴⁷⁰. Although the mutations are in different domains possible correlating effects of the variants on cell viability will be discussed in the next section. As most of the mutated genes encode for unknown proteins, we could not detect a direct correlation of a variant with the survival of HR-defective strains to PARPi.

6.3 Discussion

The overall aim of this thesis was to exploit *Dictyostelium* as model to identify mechanisms that suppress PARPi toxicity to HR-deficient cells. We therefore selected for benzamide-resistant HR-defective *exo1*⁻ cells under conditions which kill HR-dysfunctional cells, but not Ax2 cells. By assessing the required conditions for the genetic screen, we detected that 7.5 mM benzamide is lethal for all *Dictyostelium* cells, independent of HR efficiency. However, whilst 50% Ax2 cells survive 5 mM benzamide, only 0.1% *exo1*⁻ cells survive this treatment (Figure 6.1). Hence, we selected *exo1*⁻ cells under constant 5 mM benzamide exposure and hypothesised that only HR-compromised cells survive the treatment if they have gained a mutation resulting in resistance to PARPi. By performing genome sequencing, we hoped to identify mutations that contribute to PARPi tolerance of HR-compromised cells. Given that *exo1*⁻ cells are possibly genetically unstable, they might possess a high number of spontaneous variants, making it difficult to identify mutations responsible for suppressing PARPi toxicity. Hence, we included Ax2 cells in the screen to assess differences in the mutation rate of both cell lines. Furthermore, *exo1*⁻ cells that underwent the genetic screen using ethanol as mock-treatment will help to

identify and exclude spontaneous mutations. We selected 36 *exo1*⁻ cells that survived the benzamide treatment and 10 clones of each control: Ax2 and *exo1*⁻ cells that were mock-treated, and Ax2 cells that were selected with benzamide.

Following the screen (Figure 6.2), we assessed whether *exo1*⁻ cells that were selected under benzamide pressure possess a reproducible resistance to PARPi, and whether the control strains exhibit a similar viability to benzamide as the respective cells that did not undergo the screening procedure. The controls that were selected through the screen did exhibit a similar sensitivity to benzamide as the respective Ax2 and *exo1*⁻ cells that did not undergo the screening procedure (Figure 6.3). Hence, cultivation for 2-3 weeks in media supplemented with ethanol, or in the case of Ax2 with benzamide, does not affect the behaviour of these cells to benzamide. 22 out of the 36 *exo1*⁻ cells that survived the benzamide treatment possess a reproducible resistance to this PARPi (Figure 6.4A). Hence, 14 of the selected *exo1*⁻ cells are not persistently resistant to benzamide and were excluded from the following analysis. These 14 clones might have survived the benzamide selection due to transient adjustments to the PARPi treatment, such as changes in protein expression. We confirmed that the 22 *exo1*⁻ cells suppress PARPi toxicity in general, instead of only being resistant to benzamide by employing a secondary screen using Nu1025 (Figure 6.4B). Given our hypothesis, that these 22 *exo1*⁻ cells tolerate PARPi due to gene alterations, we employed whole genome sequencing to identify unique mutations that are responsible for the survival of these HR-defective cells to PARPi.

After library preparation and DNA quantification, the barcoded 22 PARPi-resistant *exo1*⁻ cells and 9 control strains were pooled for whole genome sequencing on an Illumina HiSeq2500 system. The sequencing service provided several parameters to control the quality of the raw sequencing reads. The obtained Phred score (Q-score) of the raw data indicates that each nucleobase in the sequenced samples is identified with an accuracy of

99.62 - 99.70% (Table 6.1), which is within the suggested range^{465,466}. The GC-content distribution represents another quality parameter, as the concentration of the four nucleotides varies across different species (Table 6.1). Hence, an abnormal GC-percentage compared to the sequenced organism indicates possible DNA contamination. However, as PCR amplification was performed during library generation, which is a possible source of nucleotide bias, differences in GC-AT distributions of the sequenced reads to the *Dictyostelium* genome might not only be due to DNA contamination⁴⁷¹. The detected GC-content distribution varies from 27.2 to 29.8% with an error rate of 9-10%, indicating that the sequenced reads represent the *Dictyostelium* genome. Moreover, the amount of PCR duplicates, which is defined as sequencing reads that are either exactly identical or align to the exact same start position, indicate the library complexity^{461,462}. As a high duplication rate might be due to a low amount of input DNA or variances in fragment sizes, a low duplication rate indicates a higher complexity of the sequencing reads. The highest detected duplication rate of paired reads in the samples is 5.3%, suggesting a reasonable complexity of the sequencing data.

After aligning the sequenced reads to the *Dictyostelium* reference genome obtained from Ensemble Protists, we controlled the data quality for a second time^{380,381}. We first tested whether the 22 PARPi-resistant *exo1*⁻ strains are disrupted in the *exo1* genomic locus. Surprisingly, 11 out of the 22 EB strains possess the endogenous *exo1* gene, which renders them competent in HR and therefore tolerant to PARPi. These strains might have re-gained the ability to express Exo1, rendering the cells viable to PARPi. However, as almost the entire gene was deleted while generating the *exo1*⁻ strain, a gene-transfer of a whole gene must have occurred. As this is unlikely, it is more plausible that some wells containing *exo1*⁻ strains were contaminated with Ax2 cells during the screening procedure, suggesting that these 11 strains might be Ax2 cells. Since we were interested in identifying novel mutations that suppress PARPi toxicity to HR-compromised cells, we

excluded those 11 strains from the analysis (EB12-22) and focused on the remaining PARPi-resistant *exo1⁻* strains (EB1-11).

The amount of reads of the remaining 20 sequenced samples varied between 7.7- 26.9 million reads, which is probably due to differences in the DNA amount that was pooled for sequencing (Table 6.2). However, 98.2-99.2% of those reads aligned to the genome, verifying that we sequenced the *Dictyostelium* genome. Another quality parameter is the median depth of each base, which describes the median number of reads that align to a given base. The calculated median depth indicates that a nucleotide in EB11 is sequenced on average 22 times, whereas AB2 possess the highest median depth with 84. Since EB11 also possess the least amount of sequenced reads, this low median depth is probably due to a low DNA input. Given our threshold of four reads per base to define an identified variant as a true mutation, the calculated median depth of the samples should be sufficient. This assumption is supported by the percentage of bases covered with at least 5 reads, which varies between 86.4 and 98%. Besides, excluding EB11, 68-89.6% of the nucleobases are sequenced at least 20 times, further indicating that the read depth is adequate to identify unique mutations in the individual samples.

The sequencing reads of the 20 samples were aligned to the *Dictyostelium* reference genome obtained from Ensemble Protists, which originated from Ax4, whereas our *Dictyostelium* samples evolved from the Ax2 strain^{380,381}. Ax2 and Ax4 strains share a common ancestor, but originated independently and therefore differ in some genetic loci⁴⁷². For example, in contrast to Ax2, Ax4 strains carry a large chromosome 2 duplication, which was removed from the reference genome before aligning our sequenced samples. Further genomic differences in Ax2 and Ax4 possibly exist, which increases the amount of mutations of the sequenced samples. However, due to the lack of a fully sequenced and annotated Ax2 genome, we employed the sequenced Ax4 strain as our reference.

As we were interested in identifying genes that, when mutated confer resistance of HR-defective cells to PARPi, we focused on variants in the protein-coding regions of the *Dictyostelium* genome. The amount of mutations in exons varies from 2,400 to 3,300 (Table 6.3), which is surprising as *Dictyostelium* is considered to possess one of the lowest mutation rates³⁶⁵. However, the amount of mutations is possibly due to employing the Ax4 strain as reference genome instead of Ax2. Interestingly, the mock-treated Ax2 and *exo1*⁻ cells possess a similar number of mutations, indicating that the mutation rate of the HR-defective cells is similar to that of parental Ax2. Furthermore, Ax2 cells that were exposed to benzamide for 2 weeks contain a similar amount of variants as the mock-treated samples, suggesting that benzamide treatment is not promoting gene mutations. Overall, the sequencing data of the PARPi-resistant *exo1*⁻ cells are suitable to identify unique mutations which might be responsible for their survival to PARPi.

Our aim was to identify mutations in protein coding genes that are responsible for the resistance of *exo1*⁻ strains to PARPi. Hence, we excluded mutations in the PARPi-resistant *exo1*⁻ cells that are present in the Ax2 mock- and benzamide-treated samples, as well as variants that appear in the mock-treated *exo1*⁻ cells (Table 6.4). Thereby, we decreased the number of false positive mutations by excluding variants that are possibly induced by benzamide treatment without conferring a resistance to PARPi, as well as spontaneous mutations that appear due to HR dysfunction. After further excluding silent mutations that do not alter protein sequences, we identified 46 unique mutations that might be involved in suppressing PARPi toxicity in HR-defective cells (Table 6.4). Most of these mutations possess a read depth of less than four, and were filtered as we set a minimum depth of four reads per nucleobase for a mutation to be considered a true mutation (Table 6.5, marked in blue). This results in a total of 18 unique variants in the 11 benzamide-resistant *exo1*⁻ cells. Seven of these variants are missense mutations in the *pks30* gene in EB10 (Table 6.4-6.5). Interestingly, the same strain contains further mutations in *pks32*. The proteins that are encoded by *pks30/32* are polyketide synthases,

which produce the secondary metabolite polyketide. Although the effect of these mutations is unknown, they possibly alter the enzymatic activity of the polyketide synthases. As Pks produce secondary metabolites, any changes in the enzymatic activity might have an impact on the production of polyketides. This in turn potentially impairs other cellular activities, resulting in a survival advantage to PARPi and the resistance of HR-defective cells to benzamide^{473,474}. Another possibility is that the mutations in Pks indirectly diminish the number of secondary metabolites that induce DNA lesions, thereby decreasing the amount of endogenous damage, making the cells more resistant to exogenous damaging agents, such as PARPi. Hence, the detected mutations in *pks30/32* might not directly suppress PARPi toxicity but potentially influence other cellular pathways, which play a role in the survival of *exo1*⁻ cells to PARPi.

Another interesting unique and true mutation appears in EB6 (Table 6.5). The gene DDB_G0285767 contains an amino acid change resulting in a premature stop codon, leading to a truncated protein with 174 amino acids instead of 1361. The full length protein contains an ubiquitin-related domain at its N-terminal end, indicating a potential involvement in targeting cellular proteins for degradation. However, the ubiquitin domain is still present in the truncated version. As no further domains or motifs were detected in the full length polypeptide, the exact function of the protein is still unknown. Thus, whether the expression of the truncated protein instead of the full length version is involved in rendering the *exo1*⁻ cells refractory to PARPi remains unclear.

As three of the benzamide resistant *exo1*⁻ cells (EB1, EB5 and EB8) do not exhibit a unique and true mutation in protein coding regions, they potentially contain unique variants in non-coding DNA. Some of these mutations might reside in regulatory elements, such as enhancers or promoters, controlling the expression of a specific gene, which in turn renders the cells refractory to PARPi⁴⁷⁵. However, as time was limited we could not analyse the variants in non-coding regions of benzamide-resistant *exo1*⁻ cells.

Some interesting variants in benzamide-resistant *exo1⁻* cells did not pass the coverage threshold of at least four reads per nucleobase (marked in purple in Table 6.5). For example, the histone methyltransferase *set1* contains a missense mutation in EB1. This variant is interesting as a defect in Histone H3 tri-methylation, mediated by Set1, results in aberrant NHEJ in yeast⁴⁷⁰. As such, this mutation might decrease the methylation activity of Set1, leading to a reduced NHEJ activity, which in turn promotes the survival of HR-defective cells to PARPi. The observed mutation is not in the well-conserved Set domain but in a yet unknown protein domain, which might regulate the methylation activity. However, as the variant in *set1* only possesses a read depth of two, the mutation needs to be confirmed by conventional Sanger sequencing. Interestingly, we detected another mutation in a protein that is involved in chromatin modification. EB10 contains a unique variant resulting in a premature stop codon in *jcdJ*, leading to the expression of a severely truncated protein (Table 6.5). This gene codes for a protein containing a zinc finger, mediating nucleic acid binding, and a jumonji C domain, which is present in several transcription factors, but also functions in histone demethylation mechanisms^{476,477}. As chromatin structure regulates the access of DNA repair proteins to damaged sites, expression of a truncated JcdJ might reduce its histone demethylation activity, thereby influencing DNA damage repair⁴⁷⁸. As this variant only has a read depth of two further studies are required to verify this as a reliable mutation. However, we found two mutations in proteins that are involved in histone methylation processes in two independent PARPi-resistant *exo1⁻* strains, indicating that changes in proteins responsible for histone modification might affect the tolerance of HR-defective cells to PARPi.

The PARPi-resistant *exo1⁻* strain EB6 contains another interesting mutation with a read depth of two. The protein coding gene DDB_G0285981 possesses a variant resulting in a missense mutation. The function of the protein encoded by this gene is unknown, but it includes a von Willebrand A (VWF-A) domain, which is found in several extracellular proteins, but also in proteins that are involved in transcription, membrane transport and

DNA repair⁴⁷⁹. Interestingly, mammalian and *Dictyostelium* Ku proteins contain a VWF-A domain, which alongside mediating protein binding is necessary for progression of NHEJ, possibly by DNA end processing⁴⁸⁰. Thus, this unknown protein might be involved in DNA repair via its VWF-A domain. However, further studies are required to confirm this mutation and analyse its function.

Another interesting mutation with a read depth of less than four was detected in EB11 (Table 6.5). DDB_G0267934, which is coding for a Serine-Threonine protein kinase, contains a premature stop codon, resulting in a shortened protein with 60 instead of 530 amino acids. As protein kinases are often required for signal transduction and control different complex pathways, expression of this truncated Serine-Threonine protein kinase might negatively affect its activity. However, given the exact role of DDB_G0267934 is unknown the effect of this mutation on the viability of *exo1*⁻ cells to PARPi is disputable.

Overall, although whole genome sequencing of PARPi-resistant *exo1*⁻ cells resulted in the detection of interesting unique variants, none of the identified mutations are directly involved in the survival of *exo1*⁻ cells to PARPi. However, some of the mutations might change protein function, which potentially influences other cellular activities, resulting in the resistance of HR-defective cells to PARPi. Moreover, several mutations might be present in non-coding regions, controlling gene expression and leading to the survival of HR-dysfunctional cells to PARPi. Further analysis is required to identify gene alterations in non-coding regions and fully investigate the detected individual mutations.

Given the sequenced *Dictyostelium* strains originated from single clones, it is possible that the single clones acquired mutations independently of the PARPi treatment, which were passed on to their daughter cells. Since these mutations would be unique for the individual strains, and differ to the reference genome, we might identify these false-positive mutations as unique variants that suppress PARPi toxicity. However, as

Dictyostelium possess a very low mutation rate, we assume that these gene alterations are minimal. One first experiment to verify the identified mutations would be to PCR amplify the region containing the variants in the respective strains and sequence the fragments using a conventional Sanger sequencing service. Once the mutation is confirmed, the same variant would be introduced in a HR-deficient *Dictyostelium* strain to assess its effect on the cell viability to PARPi. Thereby, we would investigate whether the mutation is rendering the cells refractory to PARPi, or if it's just a random variant that occurred in the single clone and is therefore not involved in PARPi resistance.

7. General Discussion and Conclusion

7.1 Synthetic lethality of HR dysfunction with PARPi is conserved in *Dictyostelium*

In contrast to other single celled eukaryotes, *Dictyostelium* exhibit unusual conservation of certain DDR proteins required for NHEJ and HR, as well as DNA damage-responsive ADP-ribosylation^{320,329,331,347–350,366}. Together this makes this organism a suitable model to study synthetic lethality of PARPi with HR dysfunction. Therefore, we first assessed whether synthetic lethality of PARPi and HR deficiency is conserved in *Dictyostelium*. Thus we exploited the auto-ADP-ribosylation activity of *Dictyostelium* PARPs in an *in vitro* screen to elucidate the efficacy and specificity of four PARPi on inhibiting Adprt2 and Adprt1a. Interestingly, the clinically relevant PARPi rucaparib and olaparib are more specific for inhibiting the DSB-responsive PARP Adprt1a than in targeting the SSB-responsive Adprt2 *in vitro*. As both of these PARPi are not synthetically lethal with HR-deficient *exo1⁻* *Dictyostelium* strains, this might suggest that inhibiting Adprt1a is not inducing cell death of HR-compromised cells. In support of this hypothesis, the viability of *adprt1a⁻* cells is not affected when incubated with the HR inhibitor mirin, indicating that impairing the DSB-responsive PARP is not lethal for HR-defective cells. On the contrary, Nu1025 is more specific in inhibiting the SSB-responsive PARP Adprt2, whereas benzamide is targeting both PARPs similarly *in vitro*. Interestingly, exposure to Nu1025 and benzamide is lethal for *exo1⁻* cells. This is in line with previous assumptions that PARPi that are more specific for PARP1/2 decrease the viability of BRCA-defective cells, whereas unspecific PARPi do not²⁶³. Interestingly, in contrast to the *adprt1a⁻* strain, HR inhibition kills cells that are disrupted in the SSB-responsive PARP Adprt2. Hence, inhibition of Adprt2 is lethal for HR-defective cells, whereas inhibiting the NHEJ-promoting PARP Adprt1a has no effect. Given Adprt2 is the potential orthologue of PARP1, this

suggests that in order to induce cell death of HR-dysfunctional tumours solely PARP1, but not the DSB-responsive PARP3, has to be targeted. As the clinically relevant PARPi olaparib and rucaparib seem to target the DSB-responsive PARP in *Dictyostelium* and mammalian cells more effective PARPi that only inhibit PARP1/2 with no effect on PARP3 should be developed and applied as treatment of HR-defective tumours^{281,418}. Taken together, HR defective *Dictyostelium* cells are synthetically lethal with PARPi that target the SSB-responsive PARP Adprt2.

7.2 Loss of NHEJ renders *exo1⁻* cells tolerant to PARPi

Although the concept of synthetic lethality between PARPi and HR deficiency has been known for several years, the exact mechanism resulting in cell death is still unclear. The current dogma dictates that PARPi impair PARP1- and PARP2-dependent SSB repair by trapping the enzymes to DNA breaks, forming an obstacle for replication fork progression. Once the PARP bound DNA lesions are encountered by a replication fork, this results in replication stress and replication fork collapse, which is resolved by HR^{419,420}. Accordingly, Nu1025 induces Rad51 enrichment on chromatin as well as H2AX phosphorylation, indicating that PARPi cause a similar type of DNA damage in *Dictyostelium*, which is channelled through HR for repair. Whilst Rad51 is not enriched on chromatin in the *exo1⁻* strains following Nu1025 treatment, γ H2AX generation is upregulated. Thus, repair of PARPi induced DNA damage through HR is dependent on Exo1. Interestingly, the *exo1⁻* strains exhibit highly enriched Ku70 on chromatin when exposed to Nu1025, suggesting that if HR is defective PARPi-mediated DNA damage is channelled through NHEJ. Loss of Exo1 leads to a defect in DNA end resection following DNA DSB recognition, which is critical for the pathway choice between HR and NHEJ, supporting our data that end-joining is upregulated in the *exo1⁻* strains⁴⁵⁷. Consistent with this, disrupting the NHEJ factor *dnapkcs* further sensitises *exo1⁻* cells to DNA DSBs, verifying that repair of DNA lesions is dependent on NHEJ when HR is dysfunctional.

In contrast to *exo1⁻* strains, benzamide and Nu1025 treatment is not lethal for *dnapkcs⁻exo1⁻* cells, indicating that suppressing NHEJ renders *exo1⁻* cells refractory to PARPi. Accordingly, disrupting the NHEJ-promoting PARP *adprt1a* rescues the lethality of *adprt2⁻* cells to HR inhibitors. This is in line with other data, suggesting that loss of core NHEJ factors reduces the toxicity of PARPi to HR-deficient cells^{133,298}. The NHEJ essential protein 53BP1 together with its interaction partners RIF1 and REV7 competes with BRCA1 for binding to DSBs, protects the DNA ends from resection and activates NHEJ⁴⁵⁸. Depleting *53BP1* in BRCA1-defective cancer cells renders the cells resistant to PARPi²⁸⁴. We identified a similar loss of PARPi toxicity by combined disruption of HR and NHEJ in *Dictyostelium*. However, so far it was unclear if impairing the DSB-responsive PARP affects the lethality of HR-dysfunctional cells to PARPi. Our data show that a defect in the NHEJ-promoting PARP suppresses PARPi toxicity to HR-defective cells. As the PARPi on the market also target the DSB-responding PARP, we stress for the development of compounds that only inhibit the SSB-responding PARPs with no inhibitory effect on PARP3. Applying PARPi that solely target PARP1/2 will more effectively kill HR-defective tumour cells as the compounds that are currently used in the clinic. Overall, our data suggests that if HR is compromised PARPi induced DNA damage is channelled through the error-prone NHEJ pathway resulting in apoptosis possibly due to increased genomic instability. Disruption of NHEJ renders HR-defective *exo1⁻* cells unresponsive to PARPi, supporting the deleterious effect of NHEJ on the survival of the cells²⁸⁴.

7.3 Survival of *dnapkcs⁻exo1⁻* cells to PARPi is independent of PolQ and Lig3

Although disrupted in two DSB repair mechanisms, the *dnapkcs⁻exo1⁻* *Dictyostelium* strain survives PARPi treatment, indicating that the cells can engage a third DSB repair mechanism to restart collapsed replication forks and bypass trapped PARPs. In addition

to HR and NHEJ, mammalian cells possess the alt-NHEJ pathway. Given alt-NHEJ is activated following excessive end resection, when HR is aborted or if the DNA serves as poor NHEJ substrate, this mechanism is a potential alternative to resolve PARPi-induced damage¹⁹⁹. Although most of the proteins required for alt-NHEJ are conserved in *Dictyostelium*, it is unclear whether this organism is competent in executing this DSB repair pathway. We detected the putative orthologue of the alt-NHEJ essential PolQ polymerase domain using BLAST. However, the helicase domain of PolQ is annotated on a different protein coding region in the *Dictyostelium* genome, indicating that the two domains of PolQ are expressed on different proteins³³⁷. It is possible that PolQ evolved similar to other eukaryotic polymerases into an enzyme complex, consisting of several polypeptides with each of them being important for another function of the polymerase. Another possibility is that the PolQ polymerase and helicase domain were separated onto different DNA locations by inter-chromosomal segment exchange³³⁷. As 20% of all predicted *Dictyostelium* proteins developed by gene duplication, it is possible that the PolQ domains have arisen on two proteins³³⁷. The identified PolQ-like polymerase domain, referred to as PolQ, aligned with an e-value of $9e^{-56}$ and a similarity of 26.5% to the human POLQ counterpart. As explained in the fourth chapter, the PolQ-like polymerase domain possesses the essential amino acids required for the catalytic activity of PolQ. Several other crucial residues are partially conserved, indicating that we might have identified the *Dictyostelium* orthologue of the human PolQ polymerase domain. However, as not all residues and motifs important for PolQ polymerase activity are conserved, it is possible that the identified *Dictyostelium* protein is not the functional orthologue of mammalian POLQ.

Depletion of POLQ reduces alt-NHEJ efficiency in mammalian cells, which can only be rescued by full-length PolQ but not with the protein lacking the polymerase domain, suggesting that the polymerase activity is essential for end-joining¹⁹⁵. Accordingly, disruption of *polq* significantly sensitises the *Dictyostelium* cells to phleomycin, indicating

that PolQ plays a role in DNA DSB repair in this organism. Given *polq*⁻ cells are not as sensitive to phleomycin as *exo1*⁻ cells this indicates that Exo1 is more important for DSB repair than PolQ. Whilst vegetative *Dictyostelium* do not require NHEJ to survive phleomycin treatment, DNA DSBs are toxic for HR-defective cells, suggesting that PolQ is involved in a DSB repair pathway other than NHEJ and HR³⁵⁴. Furthermore, PolQ-dependent repair is possibly more important in resolving DSBs in vegetative *Dictyostelium* cells than NHEJ. Since disruption of *polq* significantly decreases the survival of *exo1*⁻ cells to DSBs, this suggests that HR-dysfunctional cells rely on PolQ-dependent repair. This is in line with previous data illustrating that DSBs are channelled through POLQ-dependent alt-NHEJ in HR-defective human cancer cells, supporting the possibility that *Dictyostelium* employ alt-NHEJ^{195,212}. Disrupting NHEJ similarly decreases the viability of *exo1*⁻ cells to DSBs as deleting *polq*, indicating that survival of HR-dysfunctional *Dictyostelium* similarly depends on NHEJ and PolQ-dependent repair. Taken together, we identified the protein carrying the PolQ polymerase domain in *Dictyostelium*, which is involved in DSB repair and potentially required for alt-NHEJ. However, to assess the exact function of PolQ and the conservation of alt-NHEJ in *Dictyostelium* further investigations are required.

Alt-NHEJ has been suggested to compete with HR in resolving replication stress in mammalian cells. Given PARPi induce this type of damage, it is possible that the DNA lesions obtained by PARP inhibition are resolved through PolQ-dependent repair when HR and NHEJ are compromised^{421,422}. As HR- and NHEJ-defective cells possibly rely on PolQ-dependent repair for suppressing PARPi toxicity, disrupting *polq* might re-sensitise the cells to these compounds. We tested this hypothesis and included a strain that is disrupted in another alt-NHEJ factor, Lig3 which mediates re-joining of DNA ends. Thereby, we sought to support the results obtained with the *polq*⁻ strains and strengthen the indications that alt-NHEJ is conserved in this organism. Since disrupting *polq* or *lig3* does not re-sensitise the *dnapkcs*⁻*exo1*⁻ strains to PARPi, this indicates that neither PolQ nor Lig3 are responsible for PARPi tolerance of HR- and NHEJ-deficient *Dictyostelium*

cells. Interestingly, as loss of PolQ further increases the viability of *dnapkcs⁻exo1⁻* strains to Nu1025, PolQ-dependent repair might even be deleterious for HR- and NHEJ-dysfunctional cells. Taken together, survival of *dnapkcs⁻exo1⁻* cells to PARPi is independent of Lig3 and PolQ. This is not surprising as PARP1 was proposed to mediate POLQ localization to DNA DSBs, thereby promoting alt-NHEJ in mammalian cells^{203,212}. As we expose the cells to compounds that inhibit the potential orthologue of PARP1 we possibly similarly impair PolQ-dependent repair in *Dictyostelium*.

7.4 Nuclease redundancy as resistance mechanism of *dnapkcs⁻exo1⁻* cells to PARPi

Although Mre11 mediates primary DNA end processing of DNA DSBs, whilst Exo1 extensively resects the DNA 5'-3' during HR, the nucleases Mre11 and Exo1 share redundant roles in yeast and human cells⁴¹⁴⁻⁴¹⁷. Consistent with this, the Mre11 inhibitor mirin only sensitises *exo1⁻* *Dictyostelium* cells but not the parental Ax2 strain to DSBs, indicating that Exo1 and Mre11 are equally redundant in this model organism⁴⁵⁵. Interestingly, mirin further sensitises the *exo1⁻* cells to benzamide, suggesting that some PARPi-induced DNA lesions are channelled through Mre11-dependent HR in these cells. However, as the *exo1⁻* cells exhibit highly enriched Ku70 on chromatin following Nu1025 treatment the majority of PARPi-mediated DNA damages are resolved using NHEJ⁴⁸¹. Given DNA DSB resection is critical for the pathway choice between NHEJ and HR, a defect in the DNA nuclease Exo1 promotes NHEJ to repair PARPi-mediated DNA damage possibly before Mre11 is engaged to mediate extensive DNA end resection⁴⁵⁷.

As *dnapkcs⁻exo1⁻* strains survive PARPi treatment this illustrates that resolving PARPi induced DNA damage through NHEJ is lethal for the cells. In contrast to *exo1⁻* cells, Rad51 is enriched on chromatin in the *dnapkcs⁻exo1⁻* strain following PARPi treatment,

indicating that disruption of NHEJ rescues the defect of recruiting the HR factor onto damaged chromatin. During HR, Rad51 displaces RPA on resected ssDNA forming a nucleofilament and catalysing strand invasion^{399,400}. Thus, although defective in Exo1, *dnapkcs⁻exo1⁻* strains are competent in DNA end processing, which is possibly catalysed by the nucleases Mre11. Moreover, the *dnapkcs⁻exo1⁻* strain exhibit an enhanced HR activity compared to the *exo1⁻* cells, further supporting our hypothesis that loss of *dnapkcs* restores recombination in the *exo1⁻* cells. Interestingly, *dnapkcs⁻* strains exhibit a highly upregulated HR activity compared to the Ax2 cells. This indicates that although HR is the predominant pathway to repair DSBs in vegetative cells, NHEJ is possibly engaged to resolve some DNA lesions. Disruption of NHEJ upregulates the HR activity in Ax2 and *exo1⁻* cells, indicating that loss of NHEJ renders the cells more efficient in HR. As *exo1⁻* cells lack an important protein required for extensive DNA end resection, restoration of HR is dependent on DNA processing possibly through Mre11, a nuclease which is redundant with Exo1. Consistent with this, exposure to the Mre11 inhibitor mirin re-sensitises the *dnapkcs⁻exo1⁻* strains to Nu1025 and benzamides. This confirms that PARPi-induced DNA damage is channelled through Mre11-dependent HR, rendering *dnapkcs⁻exo1⁻* cells viable to PARPi. Exposure to mirin impairs Mre11 activity, resulting in dysfunctional HR, which restores the synthetic lethality of *dnapkcs⁻exo1⁻* cells with PARPi.

Hence, we identified that redundancy between nucleases that catalyse end resection is another mechanism resulting in the resistance of HR-defective cells to PARPi. However, most of the HR-compromised tumours that are treated with PARPi are dysfunctional in BRCA1 or BRCA2 and therefore either deficient in promoting HR over NHEJ (BRCA1) or defective in Rad51 loading onto damaged DNA (BRCA2)^{151–155,482}. As such, it is unknown whether this nuclease redundancy is a determining factor in tolerance to PARPi of human HR-defective breast and ovarian cancer cells. Interestingly, BRCA1 interacts with the nuclease CtIP in mammalian cells thereby accelerating DNA end resection, indicating a role of BRCA1 in the DNA end processing step⁴⁸³. Cells lacking BRCA1 are not only

defective in promoting HR but also exhibit reduced kinetics of CtIP-dependent DNA resection⁴⁸³. As loss of core NHEJ factors result in PARPi tolerance of *BRCA1* mutated cells, this might be due to redundancy of nucleases catalysing DNA resection, restoring DNA processing kinetics and re-activating HR. As such, a similar mechanism as identified in *Dictyostelium dnapkcs⁻exo1⁻* cells resulting in the resistance to PARPi is possible in human cells lacking *BRCA1* or *BRCA2*. It is important to more formally test whether nuclease redundancies render *BRCA1/BRCA2*- mutated human tumour cells tolerant to PARPi, before suggesting adjustments to the therapy of HR-defective cancer cells with PARPi.

7.5 Novel mechanisms involved in the tolerance of HR-defective cells to PARPi

Our long-term aim was to identify genes that when mutated result in the resistance of HR-defective cells to PARPi. To this end, we selected *exo1⁻* cells that seem resistant to benzamide. After whole genome sequencing of *exo1⁻* cells with a reproducible PARPi resistance, we filtered mutations that appeared in Ax2 and *exo1⁻* cells that were mock-treated during the screen and variants that are present in benzamide selected Ax2 cells. We detected that, although Exo1-defective cells are deficient in HR, they do not exhibit an increased mutation rate compared to parental Ax2 cells. Hence, due to the potential low spontaneous mutation rate, *exo1⁻* cells are suitable to identify gene alterations responsible for the resistance to PARPi. We identified 46 unique mutations in protein coding genes in PARPi-resistant *exo1⁻* cells, 18 of them were considered true mutations due to a read depth of at least four. Three out of the eleven sequenced *exo1⁻* strains do not contain unique variants in protein coding genes with a depth of at least four. This indicates that either a mutation with a lower read depth is responsible for tolerating PARPi, or mutations in non-coding regions, regulation gene expression, result in the loss

of PARPi toxicity. However, as we were focusing on protein coding genes, we did not scan the non-coding sequences for mutations.

Only three of the identified genes containing true mutations are annotated. EB4 obtained a missense mutation in *act7*, which is coding for one of the Actin proteins. However, as *Dictyostelium* possess 17 distinct genes expressing identical conventional Actin, a mutation in one of these genes is most probably not affecting the molecular function of the protein group⁴⁸⁴. The other two annotated genes that contain mutations are coding for polyketide synthases (*pks*). Both genes are altered in the same PARPi-resistant *exo1*⁻ strain, with *pks30* possessing seven missense mutations, whereas *pks32* only obtained three point mutations, resulting in amino acid changes. The detected gene alterations in the *pks* enzymes, which produce secondary metabolites called polyketides, might not directly induce the resistance to PARPi. However, changes in the production of the secondary metabolites might influence downstream cellular pathways that possibly play a role in the survival of *exo1*⁻ cells to PARPi^{473,474}.

Interesting mutations affecting proteins involved in epigenetic mechanisms were detected in two independent PARPi-resistant *exo1*⁻ cells. EB1 contains a missense mutation in the histone methyltransferase *set1*, which is interesting as a defect in Set1-dependent Histone H3 tri-methylation results in dysfunctional NHEJ in yeast⁴⁷⁰. As explained previously, PARPi-mediated damage is channelled through error-prone NHEJ when HR is absent, resulting in cell death. Disruption of NHEJ renders *exo1*⁻ cells refractory to the compounds. The amino acid change in Set1 might possibly affect its enzymatic activity and similar to yeast de-regulate NHEJ, leading to an increased survival of HR-deficient cells to PARPi. On the other hand, EB10 obtained a variant leading to a premature stop codon, resulting in the expression of a severely truncated protein called JcdJ. This protein contains a jumonji C domain, which is present in several transcription factors but also functions in histone demethylation mechanisms^{476,477}. As histone demethylases are linked

to drug resistance in cancer cells, it is possible that loss of JcdJ activity downregulates the methylation status of target proteins. This might affect the gene expression, or alter the access of repair proteins to damaged DNA due to changes in the chromatin structure^{478,485}. This in turn possibly renders *exo1⁻* cells resistant to PARPi. Previous publications contradict our theory, as drug tolerant cancer cells exhibit an upregulation of histone demethylases, such as Jarid1A, instead of a downregulation^{485,486}. Given JcdJ is not the functional orthologue of Jarid1A, expression of truncated JcdJ in HR-defective cells might induce a novel mechanism in suppressing PARPi toxicity. However, as the mutations detected in *set1* and *jcdJ* only possess a read depth of two, further tests are required to verify that these gene alterations are true mutations and result in the loss of PARPi toxicity to *exo1⁻* cells.

Nine unique variants with a read depth of at least four that were detected in the PARPi-resistant *exo1⁻* cells are located in unknown proteins. Although we detected some of the domains present in the mature polypeptides using InterProScan, we could not conclude any direct correlation of changing the protein activity with the survival of HR-defective strains to PARPi. However, we only performed a primary screen to identify gene alterations in PARPi-resistant *exo1⁻* cells compared to parental Ax2 and mock exposed *exo1⁻* cells. Thus, further experiments are required to verify that these mutations are involved in acquiring resistance to PARPi. Besides, the molecular functions of the proteins containing the variants need to be analysed to understand whether and how the mutation is affecting the toxicity of PARPi to HR-defective cells. Once the mutations have been validated in *Dictyostelium* we will test whether these gene alteration are suppressing PARPi toxicity to HR-deficient human cell lines. Hence, we performed the initial experiments to identify novel resistance mechanisms of HR-dysfunctional cells to PARPi using the model organism *Dictyostelium*, which might help improve the treatment of *BRCA1/2*-mutated tumours.

7.6 Conclusion

Taken together, we identified that synthetic lethality of HR deficiency with PARPi is conserved in *Dictyostelium*, and thereby established this organism as useful tool to study the molecular basis of this concept. Whilst lack of the SSB-responsive PARP combined with HR inhibition is lethal for the cells, an additional loss of the NHEJ-promoting PARP renders the cells refractory to HR inhibitors. Accordingly, disrupting error-prone NHEJ rescues the survival defect of *exo1⁻* cells to PARPi. As the current PARPi used in the clinic to target HR-defective tumours inhibit SSB- and DSB-responding PARPs they possibly promote PARPi resistance. According to our data, PARPi that only inhibit SSB-responsive PARPs more effectively kill HR-defective tumour cells. We therefore stress for the development of PARPi that solely inhibit the SSB-responsive PARPs with no inhibitory effect on the NHEJ-promoting PARP. The mechanism responsible for the survival of *dnapks⁻exo1⁻* cells to PARPi depends on the redundancy between nucleases mediating end resection, leading to the restoration of HR. Consistent with this, inhibiting Mre11 re-sensitises *dnapks⁻exo1⁻* cells to PARPi, confirming that we identified a novel resistance mechanism to these compounds, which relies on the redundancy of nucleases⁴⁵⁵. So far, it is unclear whether nuclease redundancy is a determining factor of acquiring PARPi tolerance in human HR-compromised tumours. Thus, we first need to confirm these data using human cell lines before suggesting adjustments of treating HR-defective cancer patients. Using whole genome sequencing we identified further potential mechanisms that are involved in the resistance of *exo1⁻* strains to PARPi. However, further experiments are required to validate the hits.

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9. Appendix

9.1 Primer Sequences

Sequenced of the oligonucleotides containing restriction sites are italicised.

Generation of the *exo1* disruption construct

KpnI-*exo1*: 5´ - AGGTACCTCTA GAAAAGGTAAATTAATCATTG -3´
HindIII-*exo1*: 5` CAAAGCTTCCTCCACTCCTACCTATCTATTCACC-3´
PstI-*exo1*: 5´-AACTGCAGCCCAAGTAGTATCGGTGATGAC-3´
PmlI-BamHI-*exo1*: 5- CCGGATCCCACGTGGTGCACCTTCACTTTTTGGTCC-3´

General screening Primer

P4: 5´ - ATGCTATACGAAGTTATCCGTGG-3´
P5: 5´ - GAAGTTATCATATGCCGCATGG-3´
Apl1: 5´-GGTATTAATCCATCATACTTAAAGAAAGC-3´
Apl2: 5´-GAACTGTATTTTGTACTTTTGAACCTG-3´
Cdk8-1: 5´-TACATACACACACACGCTCTC-3´

Screening of *exo1* disruption

P1: 5´ - GATGGTGATAGTAATGGTGATGG-3´
P2: 5´ - CTTCTTTTTCATCCTCGTAGTCAC-3´
P3: 5´ - GCAAGGTGATATGCGTTACATAG-3´
P6: 5` - TGTTGTTGTTGCTACAGCTATTC-3´

Generation of the *polq* disruption construct

KpnI-*polq*: 5´-GGGGTACCCAGTTCTCAAGTAATTTCAAAGAG-3´
HincII- *polq*: 5´-CCGTCGACCCC CATTTCTTTTTTATCTTTATT-3´

SacII- *polq*: 5'-GGCCGCGGCTAAAGAACCCCAATTAGTG CC-3'
BamHI-*polq*: 5'-GGGGATCCGTATCACTTAGAAATTCTTTTACTTGTGC-3'

Screening of *polq* disruption

P7 5' - AATGTCACCTATAAAATCCAATTG-3'
P8 5' - GACCAACTATTTTCGTTTTAAAGG-3'
P9 5'-TCTCTCCAATCAAAAAGGTAAAGT-3'
P10 5`- GGTTATTTTTTGGTTGTTGAGAATAAGTA-3'

Screening of *lig3* disruption

P11: 5'-TGATAATGATAATGGTGATGGCG-3'
P12: 5'-CACCATTAACAATTG TCACCTCAG-3'
P13: 5'-GGTATTGGTAGAATTGGTATTGTAGGTTG-3'
P14: 5'-AAACTCGAGGTAGAAAAGTTTATAATTTTTTACATCTAAAAGATCACTC-3'