THE ROLE OF PARPS AND APLF IN DNA INTERSTRAND CROSSLINK REPAIR

A thesis submitted to the Board of the faculty of Biological Sciences, University of Oxford, in partial fulfilment of the requirements for the degree of Master of Science by Research

FLORIAN FRANZ HENNING ZOBEL

Word count: 26,140

St Catherine's College Trinity Term, 2017

“*Ja schreib er es nieder*”

Günther Zobel

The role of PARPs and APLF in DNA interstrand crosslink repair

Florian Franz Henning Zobel, St Catherine's College

Submitted for the degree of Master of Science by Research, Trinity Term 2017

ABSTRACT

Over the duration of every organism's life its cells continuously face insults to their DNA. The accumulation of lesions results in cell death, inflammation and, following the activation of oncogenes or inactivating mutations of DNA repair factors, cancer formation. One particularly deleterious form of DNA damage are DNA interstrand crosslinks (ICLs), which prevent DNA strand seperation, therefore interfering with replication and transcription. Here we uncover a novel function for PARPs and the DNA repair protein APLF in the tolerance of human cells to DNA ICLs. Consistent with a role of PARPs in ICL repair, we observe ADP-ribosylation in response to the ICL inducing agent cis-platin. Furthermore, the inhibition of PARPs by Olaparib sensitizes human cells to ICLs. We identify the importance of PARP1 and PARP2 in these events and uncover that they are epistatic. We propose that PARP1 and PARP2 participate in ICL removal and that this is independent of the Fanconi Anemia pathway. The NHEJ repair factor APLF is recruited to chromatin in response to DNA ICLs and localizes to sites of laser induced damage in combination with the photreactive ICL inducing drug 4,5',8-trimethylpsoralen. Disruption of APLF sensitizes cells to ICLs. Additionally, the ADP-ribose binding tandem PBZ domain of APLF is essential for the restoration of MMC tolerance in *aplf*Δ cells. We suggest that APLF is an early responder to ICL formation. Together these data identify a novel role for PARP1, PARP2 and APLF in the tolerance of human cells to ICLs.

ACKNOWLEDGEMENTS

I would like to thank my supervisor Associate Professor Dr Nicholas Lakin for his support throughout my degree, for challenging my understanding of the topic, helping me see the bigger picture of my work and everything else he has taught me. Many thanks also to George Ronson, who equipped me with the practical skills and in many ways was the Obi-Wan Kenobi to my Anakin Skywalker. I am grateful to the members of my lab Alisdair Gunn, Seiji Ura and Anna-Lena Kolb for their input. Eric Liang, David Lopez Martinez, Juan Bada Juarez, Steve Lavington, Javier Vinals Camallonga, Anna Motnenko thank you for all the great times and for making the lab such an enjoyable and unique place to work in.

My thanks to Professor Keith Caldecott and Dr Ivan Ahel for providing vital materials for my experiments.

My gratitude also goes to my College friends, especially Roxanna Abhari, Alexander Bucknell and Mikesh Udani. I shall not attempt naming everyone that comes to mind, but they will know that I mean them.

Finally, and most importantly, I would like to to thank my family, without the financial, moral, intellectual and mental support of which I would not have been able to pursue this degree. My sisters Dr Isabelle and Theresa Zobel, whom I am immensely proud of, my grandparents Dr Franz and Elfriede Weber and my dear parents, Günther and Dr Michaela Zobel, to whom I dedicate this work to.

ABBREVIATIONS

Adprt	ADP-ribosyl transferase
alt-NHEJ	alternative nonhomologous end-joining
APE1	apurinic-apyrimidinic (AP) endonuclease 1
APL	aprataxin/APLF-and-PNKP-like protein
APLF	aprataxin and PNK like factor
ATM	ATM serine/threonine kinase
ATR	serine/threonine-protein kinase
Ax2	Dictyostelium discoideum cells
BER	base excision repair
BRCA1	breast cancer 1
BRCA2	breast cancer 2
Cas9	CRISPR associated protein 9
Chk1	checkpoint kinase 1
CPD	cylcobutane pyrimidine dimers
CRISPR	Clustered Regularly Interspaced Short Palindromic Repeats
DDR	DNA damage response
DNA-PK	DNA protein kinase
DNA-PKcs	DNA protein kinase catalytic subunit
DSB	double strand break
FA	Fanconi Anemia
FANCA - M	Fanconi Anemia complementation group A to M
FEN1	Flap endonuclease 1
FHA	forkhead associated

GG-NER	global genome nucleotide excision repair
ɣH2AX	phosphorylated H2AX
HR	homologous recombination
ICL	DNA interstrand crosslink
IR	ionizing radiation
LP-BER	long patch BER
MMC	mitomycin C
MMS	methyl methanesulfonate
MRN	Mre11/Rad50/Nbs1 complex
NER	nucleotide excision repair
NHEJ	classical nonhomologous end-joining
PAR	poly-(ADP) ribose
PARP	poly-(ADP)ribose polymerase
PBZ	PAR-binding zinc finger
PNKP	Bifunctional polynucleotide phosphatase/kinase
RPE-1	hTERT immortalized retinal pigmented epithelial cells
ROS	reactive oxygen species
RPA	replication protein A
SP-BER	short patch BER
SSB	single strand break
SSBR	single strand break repair
TC-NER	transcription coupled nucleotide excision repair
TLS	translesion synthesis
TMP	4,5',8-Trimethylpsoralen
U2OS	human bone osteosarcoma epithelial cells

UV	ultraviolet
XLF	XRCC4 like factor
XPA	Xeroderma pigmentosum complementation group A
XPC	Xeroderma pigmentosum complementation group C
XRCC4	X-ray repair cross-complementing protein 4
ZnF	zinc finger motif

TABLE OF CONTENTS

1. INTRODUCTION

1.1. THE HUMAN DNA DAMAGE RESPONSE

The genetic content in every single one of our cells is constantly exposed to a great number of threats to its integrity. These threats can be the result of external sources, metabolic products, error-prone DNA replication or even the instability of the DNA itself (Lindahl et al. 1993, Wogan et al. 2004, Brooks et al. 2005, Tubbs and Nussenzweig 2017). Causes of endogenous DNA damage can be deamination and reactive oxygen species (ROS) generated in human metabolism, or the exposure to ionizing radiation or H_2O_2 (Helleday et al. 2014). Environmental mutagens on the other hand can be physical, such as UV irradiation, or chemical. Examples of naturally occurring chemical are aflatoxins, which are formed by molds such as *Aspergillus flavus*, ptaquilosides and pyrrolizidine alkaloids, herbal ingredients. Dioxins, furans, aliphatic hydrocarbons and polycyclic aromatic hydrocarbons such as vinyl chloride, used in plastic polymers, however are considered artificial mutagens.

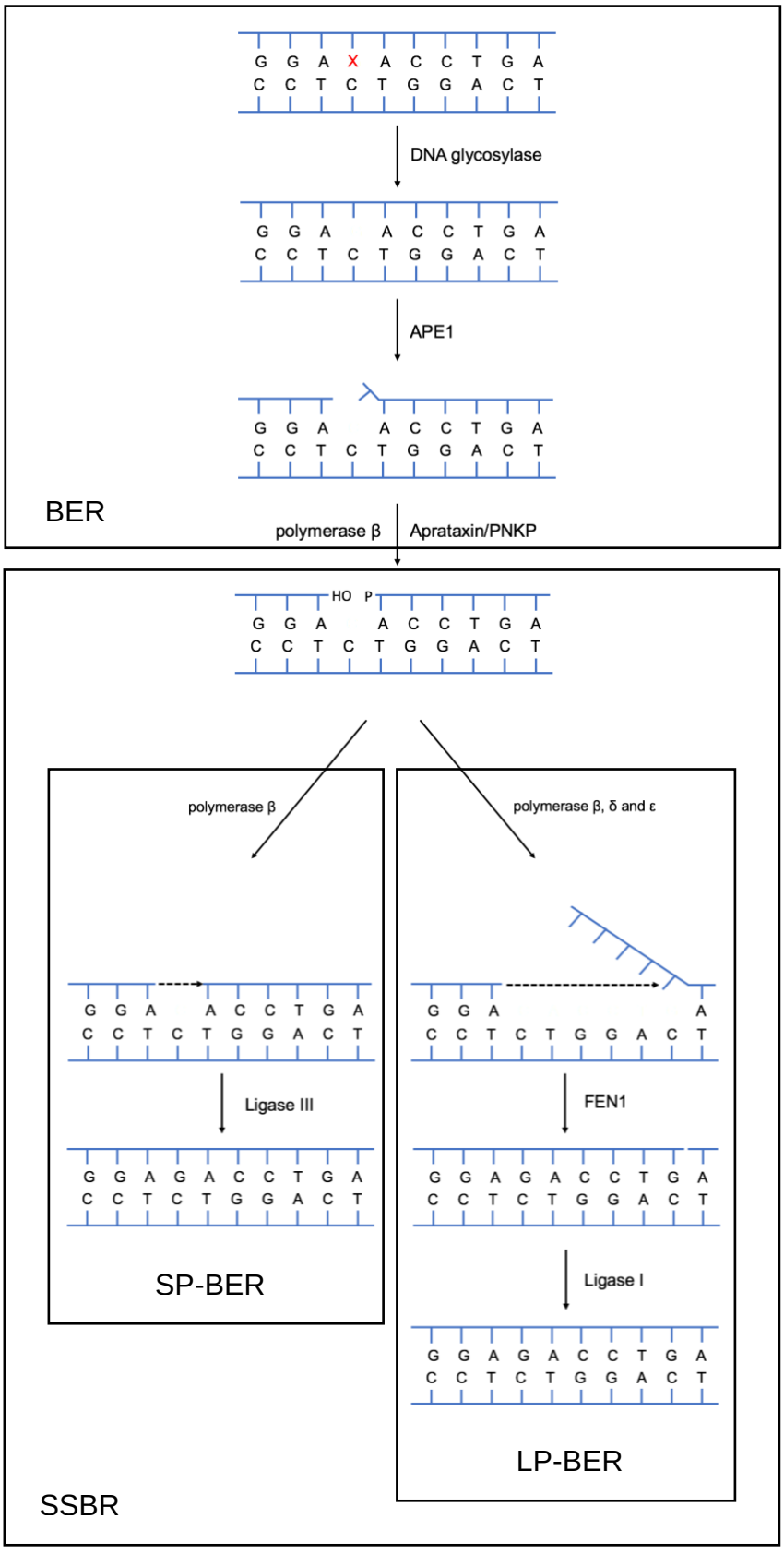
Intake of other substances can produce carcinogens – compounds supporting the cancer formation by causing DNA damage – in the process of the human metabolism. One example is benzapyrene, a by-product of combustion processes, that is oxidized by cytochrome P 450 in Phase I of the metabolism, yielding epoxides. Some epoxides cannot be metabolised in Phase II, releasing them to react with guanine. Another such substance, the industrially produced and rarely food-based N-nitrosodimethylamine, is partly catabolized into formaldehyde, which reacts with the phosphate of guanine

and also other bases to form DNA adducts.

The result is the formation of a variety of DNA damage types, namely DNA adducts, depurination, depyrimidination, DNA-protein crosslinks, DNA intra- and inter-strand crosslinks, base mismatches and thymine-dimers. The progression of the cell cycle to replication or the activation of DNA repair pathways confers many of these types of damage into single strand breaks (SSBs) or double strand breaks (DSBs). Other DNA damage sources such as ionizing radiation (IR) or the antibiotic phleomycin directly cause SSBs and DSBs formation.

Depending on the type of damage encountered and the stage of the cell cycle, the human body possesses equally diverse ways to protects its genetic material and ensure its faithful repair. Mismatch repair (MMR) recognizes and repairs base mismatches, whilst base excision repair (BER) takes place following bulky DNA adduct formation (Jackson and Bartek 2009). Lyase activity of DNA glycosylase catalyzes the removal of a damaged base, leaving an abasic (AB) site. These sites as well as direct single strand breaks induced by reactive oxygen species or others, or by topoisomerase1-DNA structures are processed by the SSB repair (SSBR) machinery (Figure 1.1.). The DNA ends are prepared for gap filling by polynucleotide kinase 3'-phosphatase (PNKP) and by apurinic-apyrimidinic (AP) endonuclease (APE1). Gap filling can occur through the short patch (SP-BER) or the long patch (LP-BER) sub-pathway (Caldecott 2008). In the presence of an AB site 5'-dRP, which can be removed by polymerase β, the polymerization and ligation step proceeds via SP-BER featuring polymerase β and Ligase III. (Klungland and Lindahl 1997). If DNA end processing by polymerase β is unsuccessful, polymerase β together with polymerase δ and polymerase ε synthesizes

Figure 1.1. Schematic description of the base excision repair pathway (BER). DNA bulky adducts such as 8-oxo-guanine, formed by the reaction of guanine with hydroxyl free radicals, topoisomerase I conjugates, but also single strand breaks induced by IR or endonucleases are channelled through this pathway. Adducts and other lesions are converted into SSBs by the combined efforts of DNA glycosylase and APE1. Thus, the lower part of the pathway can also be referred to as SSBR. The DNA ends are then prepared for gap filling by polymerase β, PNKP and aprataxin. Depending on this event, long patch or short patch BER subsequently occurs through different polymerases. DNA flaps in long patch repair are removed by FEN1 and the remaining nick sealed by Ligase III or Ligase I activity.

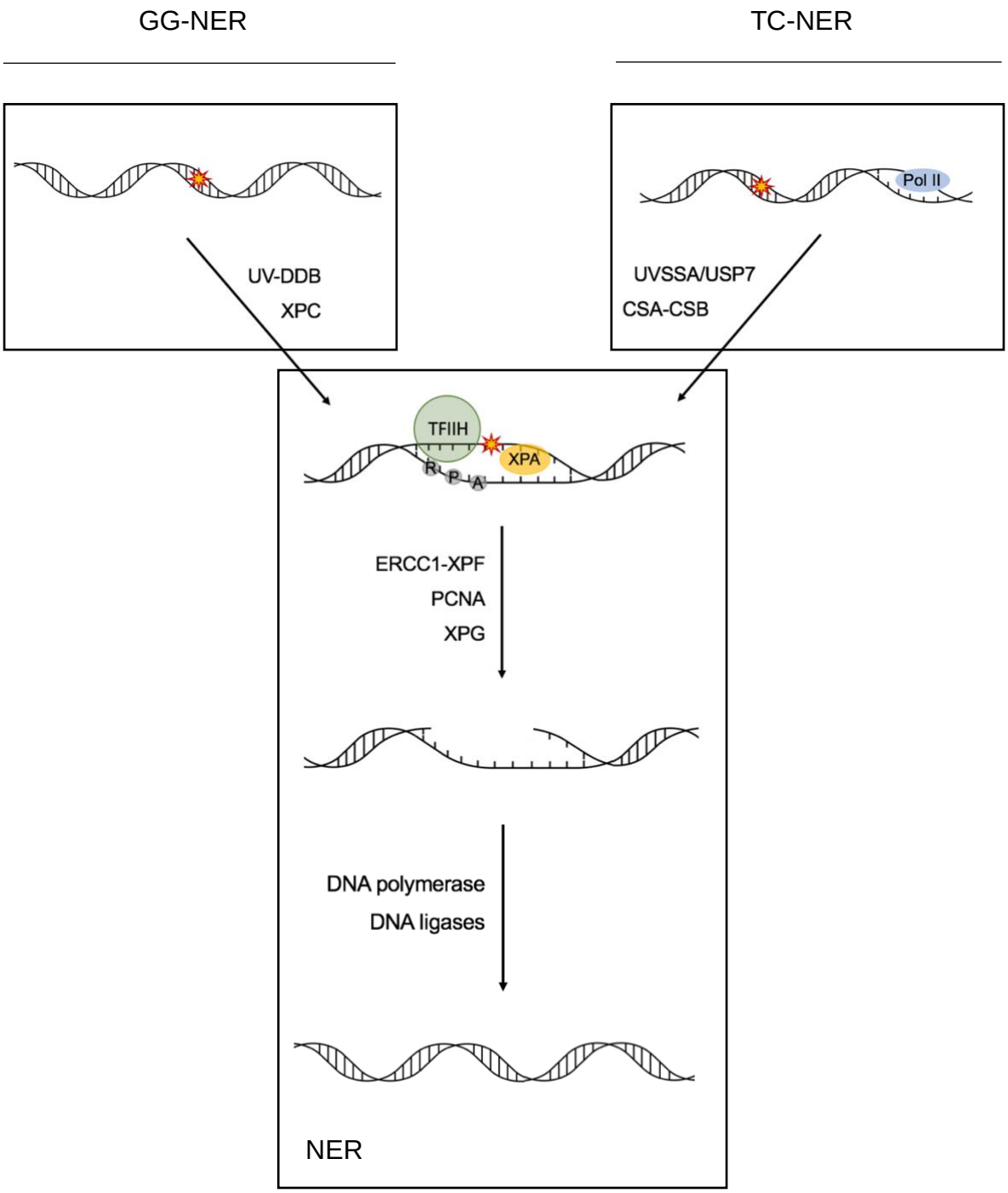

G G A X A C C T G A
C C T C T G G A C T
DNA glycosylase
G G A A C C T G A
C C T C T G G A C T
APE1
G G A A C C T G A
C C T C T G G A C T
BER
polymerase β
Aprataxin/PNKP
HO P
G G A A C C T G A
C C T C T G G A C T
polymerase β
polymerase β, δ and ε
G G A A C C T G A
C C T C T G G A C T
Ligase III
FEN1
G G A G A C C T G A
C C T C T G G A C T
SP-BER
Ligase I
LP-BER
SSBR

a longer patch and displaces the DNA 3' of the SSB. The displaced DNA is cut by FEN1 and LP-BER completed by Ligase I activity (Robertson et al. 2008). Recently, a new mode of gap filling was proposed, which might present the dominant sub-pathway in BER. (Woodrick et al. 2017).

Nucleotide excision repair (NER) responds to a scale of unrelated DNA helix lesions ranging from UV induced thymidine dimers to cyclopurines and interstrand crosslinks. It is divided into transcription-coupled and whole-genome nucleotide excision repair (TC-NER and GG-NER, respectively) (Hoeijmakers 2001). XPC, supported by DDB2 of the UV-DDB complex and possibly PARP1, recognizes DNA lesions in GG-NER, whereas TC-NER is initiated following stalling of RNA polymerase II (Figure 1.2.). Access to the lesion is provided by a group of proteins including Cockayne syndrome B (CSB), which move with RNA polymerase II during transcription elongation (Marteijn et al. 2014, Robu et al. 2017). After the recognition step, TC-NER and GG-NER converge into one pathway (Figure 1.2.). Subsequent repair includes DNA unwinding, lesion verification and RPA recruitment by the TFIIH complex. DNA incision 5' of the lesion site by the endonuclease dimer XPF-ERCC1 is directed by XPA. 3' incision is carried out by XPG and gap filling by polymerase δ, ε or κ is signalled by PCNA. DNA sealing by DNA ligase I or III competes the repair.

DSBs are conventionally repaired by either homologous recombination (HR) or non-homologous end-joining (NHEJ). HR is only exercised, if an identical template DNA is available, whereas NHEJ acts throughout the cell cycle and in competition with HR (Hoeijmakers 2001). In HR, a complex of the exo- and endonuclease Mre11, BRCA1 and DNA binding Nbs1 and Rad50 possibly responsible for homology search and DNA end binding (MRN complex) recognizes the lesion and performs DNA end resection.

Figure 1.2. Simplified schematic of nucleotide excision repair (NER) (inspired by Marteijn et al. 2014). The pathway is split in global genome NER (GG-NER) and transcription coupled NER (TC-NER), which differ in the nature of lesion recognition. Later stages of the two sub-pathways use the same repair proteins. The TFIIH unwinds the DNA around the lesion site and loads RPA on single stranded DNA. XPA, another repair factor recruited following lesion recognition, directs 5' incision by XPF-ERCC1 and possibly interacts with XPG, which cuts the DNA 5' of the lesion. PCNA loads the repair polymerases δ, ε or κ and the nick is sealed by DNA ligase I or DNA ligase III.


GG-NER
TC-NER
UV-DDB
XPC
Pol II
UVSSA/USP7
CSA-CSB
TFIIH
XPA
R
P
A
ERCC1-XPF
PCNA
XPG
DNA polymerase
DNA ligases
NER

This initial resection step is further extended through three partially redundant long-range resection reactions catalysed by EXO1 and/or by DNA2 and supported by the helicase BLM (Karanja et al. 2012). BRCA2 together with PALB2 then localizes Rad51, which assembles to form the presynaptic filament on a ssDNA stretch 3' of the break, replacing RPA (Li and Heyer 2008, Lord and Ashworth 2016). The Rad51-covered ssDNA invades the double-stranded sister chromosome to search for and bind its homologous DNA sequence. This structure is called the synaptic complex (Filippo et al. 2008). Polymerases extend the DNA of both strand ends of the damaged duplex using their homologues as template. Ligation ensues and generates a double Holliday that is resolved by endonucleases leaving crossover or non-crossover DNA.

NHEJ does not require sequence homology and is more likely to occur in the wake of radiation induced DSBs, but is an error prone pathway (Friedberg et al. 2003, Dueva and Iliakis 2013). Damage recognition is conducted by the Ku70/Ku80 heterodimer, which interacts with the catalytic subunit DNA-PKcs to form protein kinase DNA-PK (Davis et al. 2013). DNA-PKcs interacts with Artemis, the exonucleolytic activity of which produces ssDNA overhangs to reveal microhomologies between the broken DNA ends. APLF binds the XRCC4-Ligase IV complex and links it with DNA-PK, thus connecting the different stages of NHEJ. XRCC4 is a scaffold protein without catalytic activity and together with XLF bridges the DNA ends. XLF also enhances the ligation activity of Ligase IV (Yano et al. 2008). Finally, DSBs can be repaired by alternative NHEJ (alt-NHEJ), a potential back-up pathway of classical NHEJ (c-NHEJ). It repairs DSBs in the absence of Ku70/Ku80 and Ligase IV, although it has also been shown to act in the presence of function NHEJ.

It has emerged that the different DNA repair pathways do not act isolated in their DNA damage response, but are interconnected (Mouw et al. 2014, Haynes et al. 2015). With a number of DNA damage types, more than one repair pathway is required and/or present for their successful removal. In the case of DNA interstrand crosslinks (ICLs), a particularly deleterious type of DNA damage, several repair pathways are required for detection, conversion to DSBs and repair.

1.2. DNA INTERSTRAND CROSSLINKS

DNA interstrand crosslinks are a form of DNA damage in which the two opposing strands of the DNA are covalently connected with each other. If left unrepaired, ICLs can effectively block DNA replication and transcription (Grillari et al. 2007). This makes them a particularly toxic and carcinogenic type of DNA damage. Of the worldwide 20 most genotoxic recorded agents, 12 are bifunctional alkylating agents, i.e. substances inducing DNA intra- and inter-strand crosslinks. Their rodent TD_{50}, a carcinogen's required life-time dose to increase the risk of tumour formation by 50%, is 10-1000 fold higher than monoalkylating agents (Noll et al. 2006). As few as 55 interstrand crosslinks are required to kill human Xeroderma pigmentosum (XP) fibroblasts, cells deficient in nucleotide excision repair (Lawley and Phillips 1996). This supports the notion that ICLs are a much more deleterious form of DNA damage than either single or double strand breaks.

ICLs can arise via exogenous or endogenous sources. The unsaturated aldehydes crotonaldehyde and acrolein are two of the most prominent ICL inducing carcinogenics and are by-products of metabolic as well as external processes. They derive from the oxidisation of unsaturated lipids increased in high-fat diets and cyclic N-nitrosamines found in tobacco smoke (Kozekov et al. 2002, Stone et al. 2007, Folmer et al. 2003, Deans and West 2011). Furthermore, they are by-products of combustion processes. Acrolein also emerges when glycerol in burning fat is broken down at a temperature of >280°C (Wilson et al. 1991). Acetaldehyde and formaldehyde, contained in coffee and bread, are also members of this group. Elevated levels of acetaldehyde in the body can result from partial oxidation of alcohol (Brooks et al. 2005). Formaldehyde arises

following metabolization of dimethylnitrosamine present in certain foods or methane oxidisation (Huang & Hopkins 1993).

The potency of interstrand crosslinking agents has led to their introduction to cancer treatment as chemotherapeutic drugs. Naturally occurring examples are mitomycin C (MMC), a product of the mold *Streptomyces caespitosis*, and psoralens, a class of furocoumarins produced by leafy vegetables and other plants as a type of defense mechanism against predators. Cis-platin and nitrogen mustard on the other hand are artificially produced, with the latter being a form of cytotoxic chemotherapeutic agent including nitrogen gas used as a chemical weapon in WWI. Although only 5% - 10% of the DNA crosslinks induced by MMC and 10% of cis-platin induced crosslinks are interstrand crosslinks, they are thought to be the predominant cause of toxicity of these drugs. (Deans and West 2011, Williams et al. 2013). Whereas MMC, cis-platin and psoralens all induce ICLs, their distorting effects on the DNA vary (Williams et al. 2013). In more recent years it has been suggested, that this might influence the cellular response to their removal (Smeaton et al. 2008).

Chemotherapeutic treatment with ICL forming drugs has been shown to be particularly successful in combination with PARP inhibitors for BRCA1 and BRCA2 mutated breast and ovarian cancer (Lheureux 2017, Song et al. 2017). This highlights the importance of a better insight of how interstrand crosslinks are repaired in human cells and how this could positively impact cancer treatment. Below, I provide a short review of our current understanding of ICL repair.

1.3. DNA INTERSTRAND CROSSLINK REPAIR

ICL repair requires multiple DNA repair pathways including the Fanconi Anemia (FA) pathway, translesion synthesis (TLS), homologous recombination, nucleotide excision repair and checkpoint signaling. The nature of ICL repair is dependent on the stage of the cell cycle. Whereas in G0/G1 phase cells may be able to repair ICLs via NER, the majority of ICLs are encountered by two converging or a single replication fork in S phase cells, the collapse of which triggers a response by the FA pathway, followed by ICL unhooking, TLS, HR and NER (Deans and West 2011). The different possibilities of ICL repair are illustrated in Figure 1.3.

1.3.1. ICL repair in S/G2 phase

ICL repaired in replicating cells is channeled through the FA pathway. Defects in this pathway result in the human chromosome instability syndrome Fanconi Anemia that is characterized by cancer predisposition, bone marrow failure and development defects (Schwab et al. 2015, Liang et al. 2016). To date, at least 19 FA genes have been identified, mutation of which causes the disease phenotype (Martinez et al. 2016). The first two FA proteins localized to sites of ICLs are FANCM and FAAP24, although other sensor proteins such as UHRF1 and MHF1 also exist (Ciccia et al. 2007, Williams et al. 2013, Tian et al. 2015). The FANCM/FAAP24 heterodimer recruits an oligomer of FANCA, B, C, E, F, G, and L that together forms the FA core complex. The core complex substrate FANCL possesses E3 ubiquitin ligase activity and monoubiquitylates a dimer of two other proteins, FANCD2 and FANCI. (Li and Heyer 2008). (Kim and D'Andrea

2012, Ishiai et al. 2017). Independently of this function, FANCM/FAAP24 also remodel DNA of stalled replication forks with their translocase activity and could act together with the helicase FANCJ and BRCA2, which function downstream of FANCD2/FANCI ubiquitylation (Li and Heyer 2008). The translocase activity may allow binding of RPA to ssDNA, which recruits the cell cycle checkpoint proteins ATR/Chk1 (Kim and D'Andrea 2012). They in turn phosphorylate members of the FA core complex and FANCI to trigger monoubiquitylation of FANCD2/FANCI (D2/I) (Ishiai et al. 2017). This explains the dependence of ATR/Chk1 mediated checkpoint signaling on FANCM and on FAAP24 (Collis et al. 2008).

D2/I ubiquitylation is required for the recruitment of nucleases to incise the DNA 3' and 5' of the interstrand crosslink. It attracts both FAN1, which excises the 5' cut, and the scaffold protein SLX4, which localizes MUS81 and XPF to the DNA. Subsequently, a complex with the MUS81-EME1 endonuclease heterodimer, which incises the DNA 3' of the lesion site, assembles. Together with the SLX1-SLX4 and the ERCC1-XPF heterodimer, which also possesses endonuclease activity and might be responsible for 3' incision at helix distorting lesions, they form an unhooking complex. (Hanada et al. 2006, Deans and West 2011, MacKay et al. 2010, Yamamoto 2011). These proteins achieve the unhooking of the interstrand crosslinks, allowing TLS by polymerase ζ, η or Rev1 to take place (Haynes et al. 2015). How exactly the FA pathway directs TLS is not clear. It has been suggested that the monoubiquitylated D2/I complex replaces DNA polymerase δ and loads either Rev1/Rev3 or polymerase η onto the DNA (Wang et al. 2007, Haynes et al. 2015).

Two circumstances have been proposed, under which FA pathway directed repair can

occur: a single or two converging replication forks (Figure 1.3.). Whereas in the vicinity of a single stalled replication fork TLS is followed by the removal of the unhooked ICL via NER, it leaves a double strand break when two replication forks converge, which requires repair by HR. (Deans and West 2011). Similar to TLS, FANCD2 contributes to the recruitment of HR repair factors through interacting with BRCA1, BRCA2 and Nbs1 (Michl et al. 2011). Furthermore, FA proteins D1/J/N/O acting downstream of the D2/I complex facilitate Rad51 loading and the resolution of repair intermediates (Kim and D'Andrea 2012). Ultimately, the unhooked ICL is repaired by components of the NER pathway, most prominently the endonuclease XPF/ERCC4 identified as an FA gene, FANCQ (Mouw and D'Andrea 2014). This signifies the crosstalk between FA pathway directed ICL repair in replicating cells and NER directed interstrand crosslink removal in G0/G1 cells, which is exemplified below.

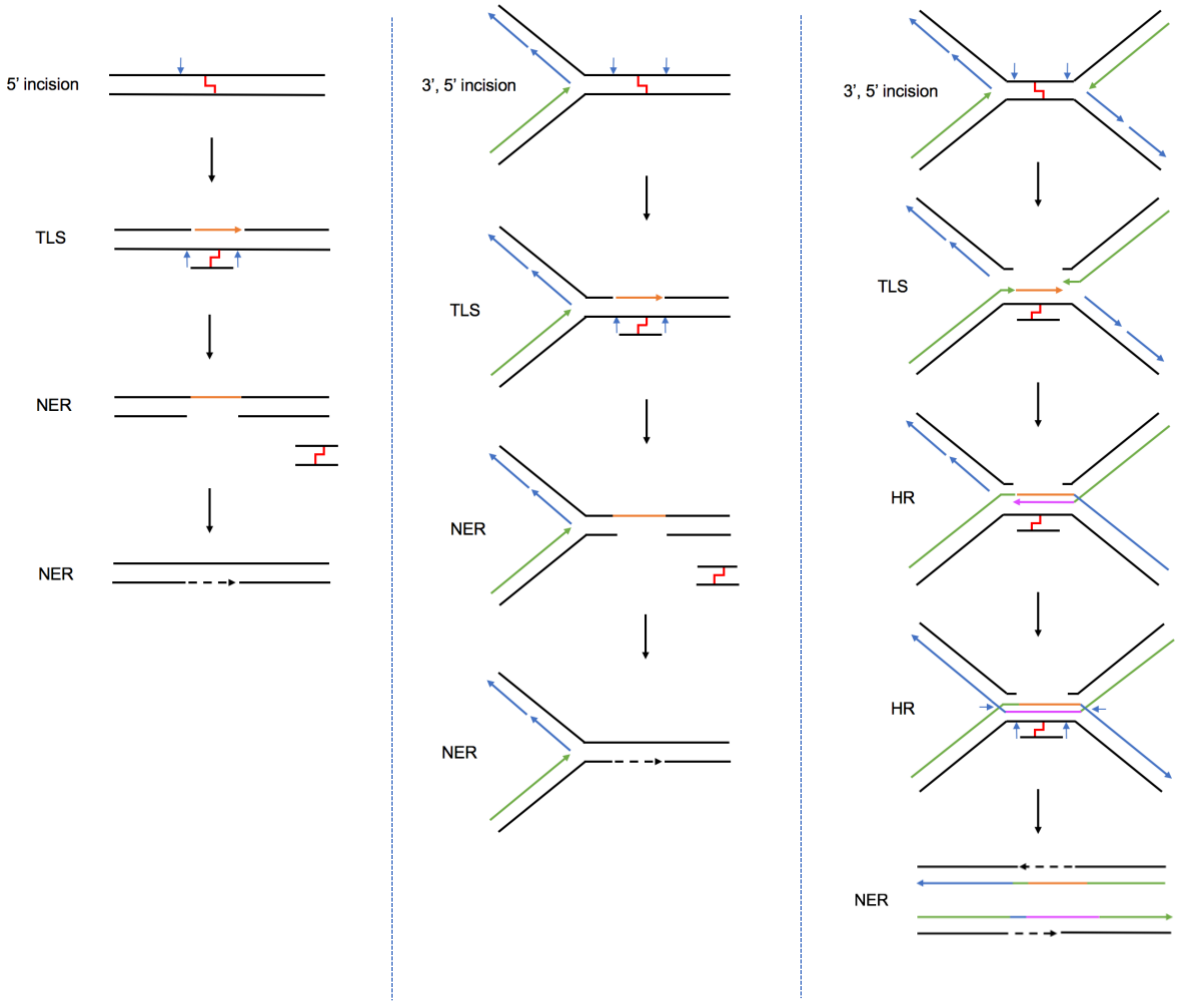
1.3.2. ICL repair in G0/G1 cells

The removal of DNA interstrand crosslinks outside of replication is dominated by the lesion unhooking via NER, followed by translesion synthesis and a second round of NER (Figure 1.3.). The existence of a Fanconi Anemia independent ICL repair pathway has been confirmed in ERCC6 depleted human fibroblasts that are more sensitive to cis-platin than WT. ERCC6 is a protein involved in global genome and transcription coupled NER that recruit repair factors to the stalled RNA polymerase II (Zhang et al. 2012). Knock-down of FANCD2 further sensitized ERCC6$^{-/-}$ cells to cis-platin. At the same time, repair of cis-platin produced DNA intrastrand crosslinks, which make up 95% of the DNA damage caused by the drug, does not require ERCC6

Figure 1.3. The different known repair mechanisms of DNA interstrand crosslinks. NER is the simplest form of ICL repair and can act in G0/G1 cells and possibly throught the cell cycle, whereas Fanconi Anemia (FA) directed repair only takes place during replication. Depending on whether one replication fork encounters the lesion (more likely the case in early S phase cells), or two converging replication forks stall (more likely in late S phase cells), the repair can include a homologous recombination step. NER directed ICL repair includes an incision step followed by TLS, and finally ICL removal and gap filling through the NER pathway. The majority of ICLs is thought to be channeled through the FA pathway, which acts in replicating cells. Following its activation by monoubiquitylation, the FANCD2/FANCI heterodimer recruit nucleases, which incise the DNA 3' and 5' of the lesion. This is followed by TLS and NER. In the case of two converging replication forks, strand incision and subsequent TLS prduces a double strand break that is repaired by HR. Repair is completed by NER.

FA directed repair

NER | One replication fork | Two replication forks



and other NER proteins, supporting the notion that these proteins are involved in ICL repair distinct from the replication-dependent FA pathway (Enoiu et al. 2012). Furthermore, removal of a single psoralen ICL by replication-independent repair (RIR) in human fibrosarcoma (HC-1080) and Chinese hamster (AA8) cells deficient in NER was impaired in contrast to HR deficient mutants, underlining the importance of NER for ICL repair as well as its independence of replication (Wang et al. 2001).

In RIR, DNA strand incision 3' and 5' of the lesion is first carried out by NER (Figure 1.3.). This is indicated by the MMC sensitivity of HT-1080 cells lacking NER nucleases XPF and of AA8 cells void of the 3' nuclease ERCC5 (Zheng et al. 2003). Reduced cis-platin repair efficiency is also evident in XPF and XPG depleted U2OS cells (Graf et al. 2011). Unhooking of the lesion is followed by futile replicative polymerase δ activity and polymerase ζ or η directed TLS (Sarkar et al. 2006). Activation of the translesion polymerases is dependent on Rad6/Rad18 complex induced monoubiquitylation of PCNA (McHugh and Sarkar 2006, Williams et al. 2012, Haynes et al. 2015). In a third and final step, the unhooked interstrand crosslink is removed by a second round of NER (Figure 1.3.) (Zheng et al. 2001).

1.3.3. Posttranslational modifications in ICL repair

Multiple post-translational modifications (PTMs) have been implicated in the repair of interstrand crosslinks, including ubiquitylation, SUMOylation and phosphorylation. In recent years, our research group was interested in exploring whether another PTM, ADP-ribosylation, might be involved in the detection or removal of ICLs. Many cellular

functions are regulated by ADP-ribosylation, including the activity and recruitment of target proteins (Corda et al. 2003, Kalisch et al. 2012). The enzymes catalyzing the covalent addition of ADP-ribose units onto themselves and onto other substrates, poly-ADPribose-polymerases (PARPs), are involved in several DNA repair pathways including BER, NER, MMR, HR, c-NHEJ and alt-NHEJ, some of which feature in ICL repair. To understand how PARPs might exert a contributory function in ICL repair, it is worth examining their known DNA repair roles.

1.4. POLY-ADP-RIBOSE POLYMERASES

PARPs are a group of 18 proteins that carry sequence homology with the catalytic domain of its founding member, PARP1. The PARP signature motif is located at the C-terminus for all PARP members except PARP4 (Schreiber et al. 2006). The PARP domain of the majority of PARP proteins possesses catalytic activity for mono- or poly-ADP-ribosylation, (Vyas and Chang 2014). The addition of either single ADP-ribose units or form long branched ADP-ribose chains on themselves or target proteins using NAD^+ as a substrate. The first ADP-ribose molecule is attached to a lysine, glutamate or aspartate residue of the target protein through O-glycosidic binding (Gibson and Kraus 2012). Recently serine was also identified as an acceptor amino acid for ADP-ribose (Leidecker et al. 2016). In PARylation this initial step is followed by the linking of ADP ribose units via ribose-ribose 2' – 1'' bonds up to PAR chains of 200 moieties. Branching of the PAR polymer occurs on average every 20 ADP ribose units through a 2'' – 1''' glycosidic bond (d'Amours et al. 1999).

The function of target proteins can be changed by covalent attachment of PAR chains. Equally, a number of proteins carry PAR recognition motifs, allowing them to recognize and bind PAR modified proteins. There are four groups of ADP ribose binding domains: the PAR binding motif (PBM), poly(ADP-ribose)-binding zinc finger (PBZ), WWE domain and macrodomain, that recognize different regions of a poly(ADP-ribose) chain (Zaja et al. 2013). Conversely, poly-(ADP-ribose) chains are removed by the combined activities of Poly-(ADP-ribose) glycohydrolase (PARG) ADP-ribosylhydrolase 3 (ARH3), which reduced PAR to MAR, that can subsequently be removed by MacroD1, MacroD2 and TARG1 (Perina et al. 2014).

PARPs are involved in many physiological processes ranging from mitosis, cell division and cell death to inflammation, transcription regulation and DNA repair. For example, PARP7 is involved in regulating metabolizing enzymes such as cytochrome P450, PARP10 interacts with c-Myc and PARP3 and PARP14 influence the expression levels of inflammatory genes. (Bai et al. 2015).

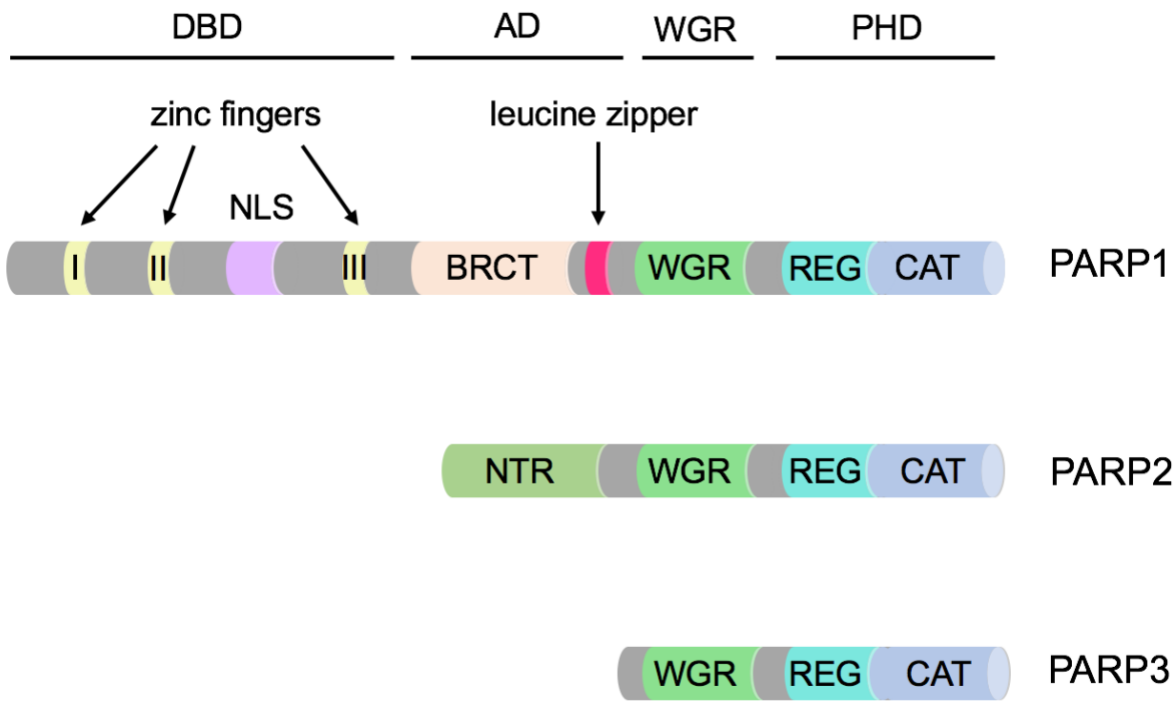
1.5. PARPS AND DNA REPAIR

According to their functions PARPs are grouped into six clades (Perina et al 2014). The most prominent is clade 1, which includes PARP1, PARP2 and PARP3. These proteins have been implicated in DNA repair and transcriptional regulation. (Tallis et al. 2014). PARP1 possesses four main domains. Amino-terminal is a DNA binding domain (DBD) consisting of three zinc-finger motifs and a nuclear localisation signal (NLS). It neighbours an auto-modification domain (AD) including a BRCT motif and a leucine-zipper motif potential responsible for dimerization, followed by a WGR domain that might contribute to DNA binding. Carboxyl-terminal is a PARP homology domain (PHD) that contains a regulatory and a catalytic domain (Altmeyer et al 2009). The domain architecture of PARP2 is simpler, consisting of an N-terminal region (NTR) and – shared with PARP1 – a central WGR domain and C-terminal PHD (Riccio et al. 2015). PARP3 is even smaller, consisting only of a WGR domain and PHD. The WGR domain in all three PARPs has been shown to be essential for their DNA dependent catalytic activation (Langelier et al. 2014). PARP1, 2 and 3 are able to bind damaged DNA and participate in multiple DDR pathways (Tallis et al. 2014). Their domain architecture is shown in Figure 1.4.

1.5.1. PARPs in SSBR

SSBs arise as a result of strand incision in BER following the detection of a DNA adduct, strand break formation through IR or topoisomerase II, or DNA helix distorting or transcription blocking lesions. Formation of poly-(ADP-ribose) chains in mammalian

Figure 1.4. Domain architecture of PARP1, PARP2 and PARP3. PARP1 has a DNA binding domain (DBD) and is continuously bound to DNA. PARP2 and PARP3 lack such a region, but are also located to sites of DNA damage. All three PARPs share a PARP homology domain (PHD), which consists of a catalytic (CAT) and regulatory (REG) region.


DBD
AD
WGR
PHD
zinc fingers
leucine zipper
NLS
I
II
III
BRCT
WGR
REG
CAT
PARP1
NTR
WGR
REG
CAT
PARP2
WGR
REG
CAT
PARP3

cells is dependent on the presence of a single strand break (Benjamin and Gill 1980). PARP1 and, to a lesser extent PARP2, are responsible for the formation of the DNA damage dependent PAR chains and play a role in SSBR.

Base excision repair (BER)

In BER, PARP1 and XRCC1 are at the forefront of the damage recognition. PARP1 localizes XRCC1, which interacts with the subsequent repair factors APE1, PNK, DNA ligase III and DNA polymerase β (Caldecott 2003). An alkylated base is removed by an N-glycosylase, followed by strand incision through the AP endonuclease (APE1). 3' OH and 5' phosphate of the DNA strand ends around the SSB are recovered by the catalytic activities of aprataxin and PNKP (Ahel et al. 2006, Weinfeld et al. 2011). DNA synthesis is carried out by polymerase β and ligation by ligase III (Vodenicharov et al. 2000). Previous studies presented conflicting results on the importance of PARP1 and PARP2 in BER. PARP1 and subsequently XRCC1 are recruited to UV + UV damage enonuclease SSBs in dependence of DNA strand incision and PARP1 PARylation activity following H_2O_2 induced damage is dependent on the oxoguanine glycosylase (OGG1) (Okano et al. 2003, Hooten et al. 2011). SSB formation via BER activates PARP1 auto-PARylation and histone PARylation, giving rise to a more open chromatin structure as well as recruiting PAR-binding DNA repair factors (Pachkowski et al. 2009). In line with this, PARP1 and PARP2 can form complexes with XRCC1, polymerase β, ligase III APE1 and Tdp1 (Schreiber et al. 2002, Moor et al. 2015, Prasad et al. 2015). PARP1 and PARP2 also recognize and bind apurinic/apyrimidinic (AP) sites (Khodyreva et al. 2010, Kutuzov et al. 2015). Furthermore, PARP1 and PARP2 human single knock-out cells present impaired SSB, 8-oxoG and abasic site repair kinetics (Dantzer et al. 2000, Schreiber et al. 2002, Le Page et al. 2003, Fisher

et al. 2007). However, other studies did not yield less proficient timely repair of MNNG induced damage in PARP1$^{-/-}$ MEFs and of SSBs in PARP1^{KD} A549 compared to the parental cell lines (Vodenicharov et al. 2000, Ström et al. 2011). PARP inhibition, which stabilizes PARP binding to DNA, is not equivalent to the knock-out of PARPs, potentially explaining observed differences in importance of PARP1 and PARP2 for BER (Horton et al. 2014). Recently, a new RECQ1 sub-pathway of BER was proposed that features un-PARylated PARP1. This is reminiscent of earlier findings that highlight the importance of un-PARylated PARP1 for long-patch BER (Prasad et al. 2001, Woodrick et al. 2017). Overall, whereas PARP1 and PARP2 have attributed roles in BER, their exact contribution and the importance of PARylation in it require further investigation. Both PARPs promote BER, but are not indispensable in this repair pathway (De Vos et al. 2012).

Nucleotide excision repair (NER)

NER is divided into two sub-pathways, global genome NER (GG-NER) and transcription coupled NER (TC-NER), which respond to different DNA lesions. GG-NER repairs DNA helix distortions, while TC-NER takes place upon polymerase II stalling (Mitchell et al. 2003, Marteijn et al. 2014).

PARP1 contributes to GG-NER by retaining and preventing degradation of the repair factor DDB2 at UV DNA damage sites and recruiting the chromatin remodeller ALC1 through PARylation (Pines et al. 2012, Robu et al. 2013). Independently of DDB2, PARP1 binds the GG-NER protein XPC through direct protein-protein interaction and promotes its recruitment to DNA through its catalytic activity (Robu et al. 2017). Furthermore, PARP1 poly-ADP-ribosylation is dependent on DNA strand incision and

the presence of the DDB2 binding scaffold protein XPA, which non-covalently interacts with PAR (Okano et al. 2000, Okano et al. 2003, Fischer et al. 2014). PARP1 inhibition impairs the repair of UV induced damage, namely CPDs and 6-4 photoproducts (Flohr et al. 2003, Fischer et al. 2014). Although both GG-NER and TC-NER funnel into one pathway, which requires XPA, so far no TC-NER specific function of PARP1 has been described (Pines et al. 2013).

Mismatch repair (MMR)

MMR is responsible for the repair of base-base mismatches and indel loops (IDLs). Depending on the nature of the damage, it is either recognized by a MutLα or a MutLβ heterodimer, which forms a tetramer with MutLα, another heterodimer, and undergoes a conformational change. The resulting sliding clamp can move either towards the 3' or the 5' end of the DNA strand, removing PCNA and replication factor C (RFC). In the case of 5' to 3' repair, the RFC is replaced by the exonuclease EXO1. Following single strand stabilization by RPA, EXO1 deactivation and DNA synthesis by a polymerase β/PCNA complex, the final nick is sealed by ligase I. If the clamp moves towards the 3' terminus, PCNA is detached from RFC, which remains at its position on the DNA, preventing 5' to 3' strand removal. The DNA containing the mismatch is removed by EXO1 loading and several 5' to 3' cutting events, the homologous strand RPA coated, PCNA moved past the mismatch and polymerase β directed DNA synthesis and ligation carried out (Jiricny 2006).

PARP1 has been suggested to participate in 5' to 3' directed MMR due to its ability to co-localize with MutSα. It is able to form protein-protein interactions with EXO1, RPA, PCNA and RFC (Liu et al. 2011).

1.5.2. PARPs in DSBR

Double strand breaks are a more severe lesion than single strand breaks. They can be the cause of genome rearrangements, including chromosome translocations, and their unfaithful repair by NHEJ leads to loss of sequence information and potentially gene function (Richardson and Jasin 2000). Many DNA damage forms ultimately result in DSB formation, such as SSBs encountered by a replication fork, which triggers HR directed repair (Jeggo and Löbrich 2007). PARP1, PARP2 and PARP3 have all been implicated in three DSB repair pathways, namely HR, NHEJ and alt-NHEJ. An overview of these is given in Figure 1.5.

Homologous recombination (HR)

Homologous recombination is one of the main repair pathways by which the repair of double strand breaks is governed. However, it is largely involved in the repair of not only DSBs, but also stalled replication forks and therefore is mostly active in S and G2 phase. Other than for meiotic recombination and for synthesis-dependent strand annealing (SDSA) repair, HR mediated DSB repair can result in the formation of both non-crossovers or crossover (Sung and Klein 2006). In SDSA, the sister chromosome strand invasion and DNA synthesis follows strand displacement, before reannealing of the strand with its 3' sequence.

In the first step of HR, 3' overhangs are generated by DNA end resection through the Mre11/Rad50/Nbs1 (MRN) and CtIP complex as well as EXO1 and DNA2. (Symington and Gautier 2011, Karanja et al. 2012). CtIP activates ATR and Mre11 recruits ATM to DSBs, where it is acetylated and sets into motion cell cycle arrest by phosphorylating

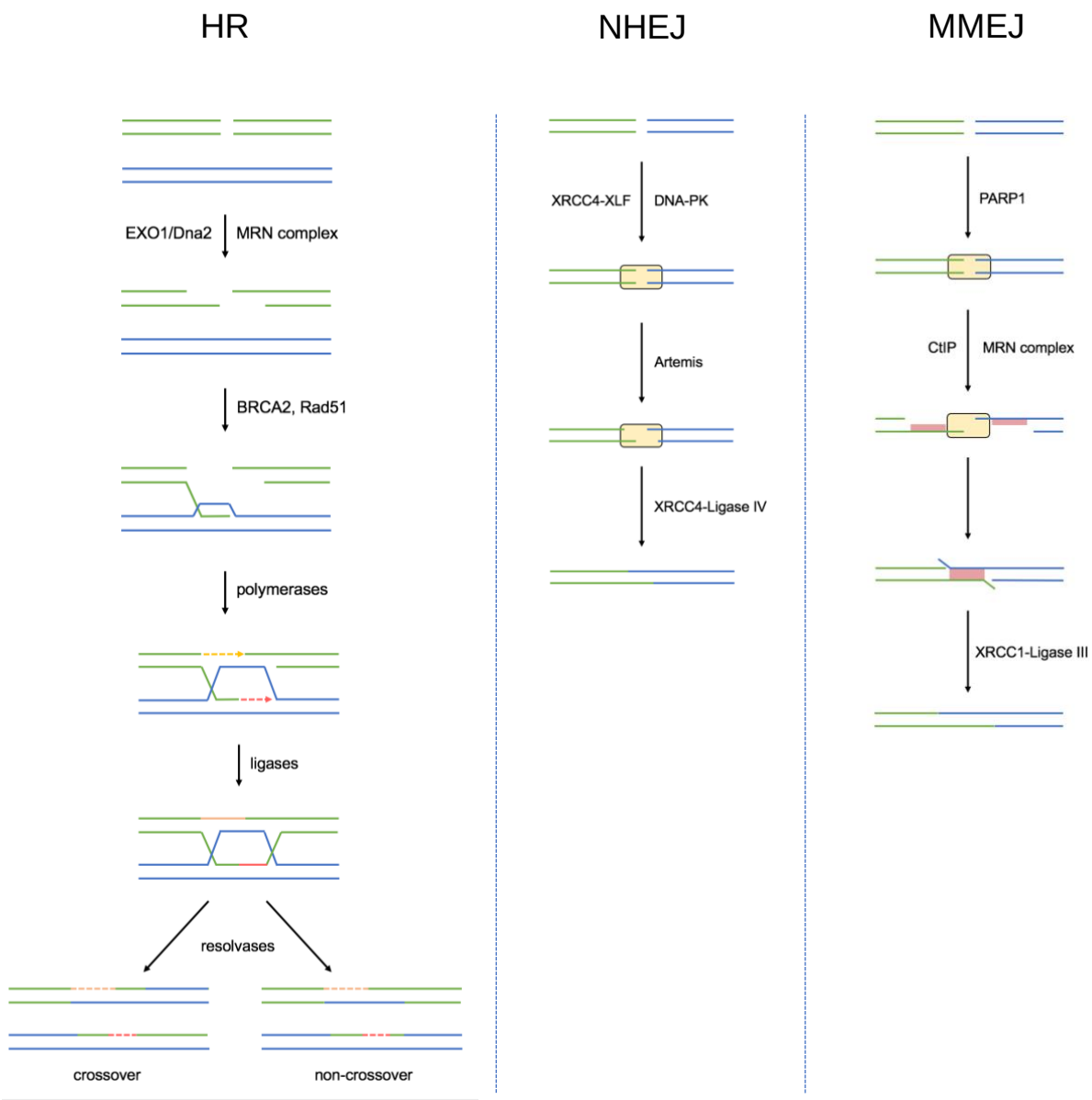
the checkpoint kinase Chk2 and histone H2AX (Sartori et al. 2007). BRCA2 recruited Rad51 then guides strand invasion and homology search followed by DNA synthesis, strand incision and ligation (Li and Heyer 2008, Lord and Ashworth 2016).

Similar to BER, partially conflicting hypotheses have been established over the last 17 years concerning the role of PARPs in HR. Whereas earlier findings assign the effect of PARPs inhibition or deletion on HR to their functions in BER and replication fork restart, more recent findings propose direct involvement of PARP1 and potentially PARP2 in the repair kinetics of this pathway. Given its role in BER, it was suggested that PARP1 is not actively involved in DSBR by HR, but that its knock-out rather exacerbates the DNA damage load in a cell due to the progression of unrepaired SSBs into S-phase, where they encounter replication forks and form DSBs (De Murcia et al. 2000, McCabe et al. 2006, Bryant et al. 2006). This is supported by the observation of increased Rad51 foci formation in PARP1 depleted cells (Schultz et al. 2003, Yang et al. 2004). PARP1 has also been attributed an essential role in activating replication fork restart, delaying fork restart and decelerating fork progression in damaged cells, rather than acting in DSB repair itself (Yang et al. 2004, Sugimura et al. 2008, Bryant el al. 2009, Wei and Yu 2016). However, there is evidence for a more direct role of PARPs in HR. PARP1 has been shown to be critical for the balance between HR and NHEJ directed DSBR by competing with Ku70/Ku80 and with XRCC4/Ligase IV for DSB ends (Wang et al. 2006, Beck et al. 2014). Knocking-out Ku70 in PARP1$^{-/-}$ DT40 cells even rescues IR sensitivity of these cells (Hochegger et al. 2006). Furthermore, many more recent sources record protein-protein interactions of PARP1 and several DNA repair proteins of the homologous recombination pathway. PARP1 promotes recruitment to DNA breaks of the MRN complex and Rad51 and co-localizes with

ɣH2AX and ATM, but not Rad51 following microirradiation (Schultz et al. 2003, Haince et al. 2008, Beck et al. 2014, Tallis et al. 2014, Wei and Yu 2016). Direct or PAR chain mediated interactions with PARP1 was shown in the case of Mre11 and suggested for Nbs1, ATM and BRCA1 (Boulton et al. 2002, Gagne et al. 2008, Haince et al. 2008). Whether this is enough for PARP1 knock-out to compromise HR-directed repair is still unclear (de Murcia et al. 2000, Hochegger et al. 2006, Wei and Yu 2016). Double knock-outs of PARP1 or PARP2 with H2AX, Ku80 and ATM are embryonically lethal or, in the case of DNA-PKcs, lead to severe growth retardation in mice, further sparking the decade old debate on the extent of PARP contribution to HR in the context with other DSB response proteins (de Murcia et al. 2000, Gosh et al. 2016). Finally, immediate PAR chain formation by PARP1 is evidenced in response to micro-irradiation and H_2O_2 treatment (Hochegger et al. 2006, Haince et al. 2008). PARP2 participation in HR is still a topic of controversy, although similar to PARP1, it might interact with and promote the MRN complex (Yelamos et al. 2008, Wei and Yu 2016).

Altogether, the interconnection of multiple DNA repair pathways, not least through the different stages of the cell cycle, complicates the task of shining light onto the precise functions of PARPs in HR. In particular the interpretation of phenotypes in knock-out cell lines leaves room for interpretation, while the confirmation of PARP interactions with HR proteins is not necessarily an indication of the importance of PARPs for the functionality of HR. A more stringent seperation of the SSB and DSB repair functions of PARPs is therefore required to draw a more holistic picture of their contribution to homologous recombination.

Figure 1.5. Homologous recombination vs. non-homologous end-joining vs. microhomology mediated end-joining of alt-NHEJ in the repair of double strand breaks. HR acts in S phase, where replication fork collapse leads to DSB formation, or G2 phase. It uses the sister chromosome as a template for faithful repair of the broken strand, producing a double Holliday junction. Resolution of the junction can lead to crossover and non-crossover products. NHEJ in contrast acts throughout the cell cycle and re-joins broken DNA ends after tethering them. This leads to error-prone repair and can cause gene deletions. MMEJ is dependent on long terminal stretches of microhomology (red), that require end resection and lead to substantial deletions or insertions


HR
NHEJ
MMEJ
EXO1/Dna2
MRN complex
BRCA2, Rad51
polymerases
ligases
resolvases
crossover
non-crossover
XRCC4-XLF
DNA-PK
Artemis
XRCC4-Ligase IV
PARP1
CtIP
MRN complex
XRCC1-Ligase III

.

Nonhomologous end-joining

NHEJ is another principal pathway for the repair of DSBs. Whereas it can function at any stage of the cell cycle and stands in completion with HR, it mostly acts in G0 and in G1 phase (Ceccaldi et al. 2016). NHEJ is initiated by the binding of the Ku70/Ku80 heterodimer (Ku) to DNA ends.

Ku interacts with the DNA protein kinase catalytic subunit (DNA-PKcs) to for the DNA protein kinase (DNA-PK). Direct interaction of Ku70 with XRCC4 and binding of APLF to XRCC4-Ligase IV and DNA-PK, links DNA-PK with the later steps in NHEJ directed DSB repair, namely DNA end bridging, microhomology search and ligation (Hammel et al. 2016). This aggregation of NHEJ factors, in particular Ku and potentially an XRCC4-XLF filament, holds the broken DNA strands together in what has been termed a synaptic complex (Davis and Chen 2013). Prior to DNA ligation by ligase IV, the two broken DNA ends require re-alignment. In order to achieve this, DNA-PKcs interacts with a protein called Artemis, that expresses 3’ as well as 5’ exonucleolytic activity in combination with DNA-PKcs. Artemis trims the DNA ends to reveal ssDNA until a region of microhomology (1 - 4 homologous bases) is found that allows complementary strands of the two DNA duplexes to bind (Lieber et al. 2008).

To facilitate NHEJ recognition and repair, opening of the chromatin structure around the DSB could occur through PARP3 PARylation of histone H1, causing its repulsion from DNA (Chiruvella et al. 2017). PARP3 catalytic activity is required for targeting APLF to DNA damage sites. PARP3 PARylated H1 might recruit APLF, which in turn is phosphorylated by ATM and accelerates NHEJ through its interaction with the DNA end ligation performing XRCC4-Ligase IV complex (Rulten et al. 2011, Boehler and

Dantzer 2011, Fenton et al. 2013). PARP3 has been associated with further functions beyond targeting APLF. It significantly affects DSBR, since PARP3 depleted cells are sensitive to DSB inducing agents and show more persistent H2AX formation (Boehler et al. 2011, Beck et al. 2014). Additionally, direct interactions of PARP3 with Ku70, Ku80, DNA-PKcs, DNA ligase IV, DNA ligase III and PARP1 have been reported in the past (Rouleau et al. 2007, Boehler and Dantzer 2011, Beck et al. 2014). Interaction of Ku with PARP3, mediated by DNA, is particularly well studied (Rouleau et al. 2007). PARP3 is able to PARylate Ku70 *in vitro* and recruits both Ku70 and Ku80 to sites of DNA damage (Beck et al. 2014). Finally, PARP3 prevents increased end resection through alternative NHEJ, thus promoting classical NHEJ. This becomes apparent in PARP3 knock-down cells, which show increased 53BP1 foci formation in response to etoposide, and is consistent with the observation that 53BP1 promotes a subtype of alternative non-homologous end-joining (Beck et al. 2014, Xiong et al 2015).

It has been suggested that PARP1 and PARP3 act synergistically in NHEJ (Boehler et al. 2011). However, although PARP1 was shown to directly interact with DNA-PK, this binding event appears to take place outside of an NHEJ context, but rather pertain to replication fork restart (Li et al. 2004, Spagnolo et al. 2012, Ying et al. 2015).

Alternative nonhomologous end-joining (alt-NHEJ)

Alt-NHEJ emerged as a pathway, when in the absence of classical Ku and Ligase IV dependent non-homologous end-joining (NHEJ, c-NHEJ) residual end-joining activity was observed in cells (Guirouilh-Barbat et al. 2007, Boboila et al. 2010, Fattah et al. 2010). Since alt-NHEJ in XRCC4-ligase IV deficient cells shares its DNA repair proteins with c-NHEJ except for the ligation step, its existence as an independent DSB

repair pathway remains the subject of debate (Nussenzweig and Nussenzweig 2007, Lieber 2010, Boboila et al. 2012, Chang et al. 2017). Characteristically, alt-NHEJ is more reliant on microhomology mediated repair and also longer microhomologies than c-NHEJ, which more frequently leads to deletions or insertions (Chiruvella et al. 2017, Deriano and Roth 2013). However, cells deficient in XRCC4 are capable of direct end joining, throwing open the question of whether alt-NHEJ can be separated into two sub-pathways, microhomology mediated end-joining (MMEJ) and direct alternative end-joining (direct a-EJ) (Boboila et al. 2012). Supporting this hypothesis, in end-joining directed class switch recombination (CSR) $Ku70^{-/-}$ cells and $Ku70^{-/-}$ $LigaseIV^{-/-}$ cells revealed more direct and less microhomology mediated CSR junctions than $XRCC4^{-/-}$ $LigaseIV^{-/-}$ cells (Boboila et al. 2010). Surprisingly, V(D)J recombination intermediates are to a small extent repaired by alt-NHEJ even in c-NHEJ proficient cells, which changed the perception of alt-NHEJ from a back-up to a competitor to c-NHEJ (Corneo et al. 2007).

In general, alt-NHEJ is independent of Ku and Ligase IV activity and can be separated into a synapsis, processing and ligation step. It shows more extensive end resection than c-NHEJ, possibly mediated by the MRN complex and CtIP to reveal longer stretches of terminal microhomology than in c-NHEJ. The DNA ends are ligated by either ligase I or ligase III in complex with XRCC1 (Lieber 2010, Della-Maria et al. 2011, Bunting and Nussenzweig 2013).

In comparison to other DNA repair pathways, the role of PARP1 in alt-NHEJ is well defined. At the initial stage, it is responsible for synapse formation independent of the presence of microhomology (Audebert et al. 2004, Audebert et al. 2008). However,

the knock-out of PARP1 in XRCC4 or ligase IV deficient cells induces a decrease in microhomology, which indicates that PARP1 promotes MMEJ (Robert et al. 2010).

PARP1 is particularly vital for alt-NHEJ in Ku70/Ku80 depleted cells, which rely on it for successful end-joining and are sensitized to IR, when it is inhibited (Wang et al. 2006, Mansour et al. 2010, Cheng et al. 2011). Therefore, alt-NHEJ directed DNA repair in Ku depleted results in enhanced PARP1 chromatin recruitment and PAR synthesis (Audebert et al. 2004, Cheng et al. 2011). Additionally, PARP1 suppresses XRCC4 and DNA-PKcs phosphorylation in Ku deficient cells, thus favouring alt-NHEJ (Audebert et al. 2004, Cheng et al. 2011).

Finally, PARP1 also contributes to the ligation step of alt-NHEJ. It interacts with XRCC1, which forms a complex with ligase III, and is necessary for its recruitment to chromatin (Audebert et al. 2004).

1.5.3. PARPs and APLF

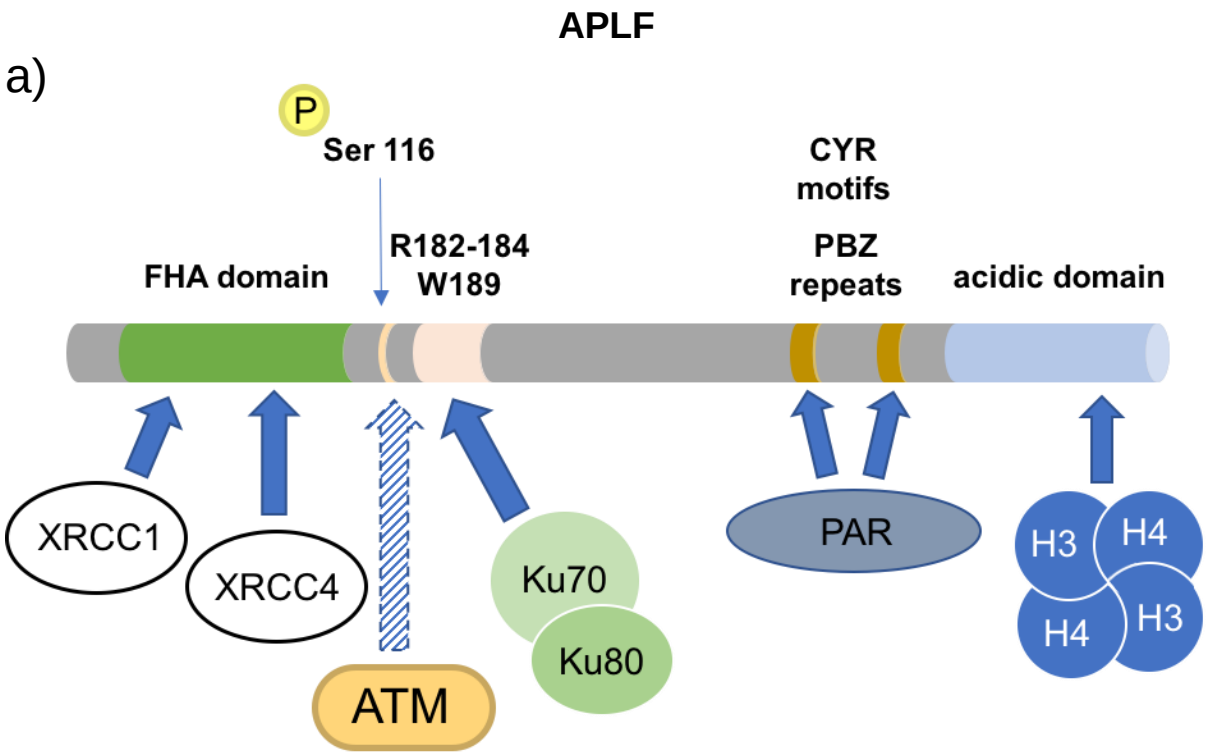
PARPs share their participation in two unrelated DNA repair pathways, BER and NHEJ, with the PBZ domain containing protein APLF. XRCC1, which also contains a PAR recognition motif and co-localizes to site of SSBs together with PARP1, binds to APLF (Okano et al. 2000, Bekker-Jensen et al. 2007). Furthermore, a link between PARP3 catalytic activity and APLF recruitment to DNA damage sites has been established in NHEJ (Rulten et al. 2011, Fenton et al. 2013) Finally, members of our group recently showed in the model organism *Dictyostelium discoideum* that localization of APLF to

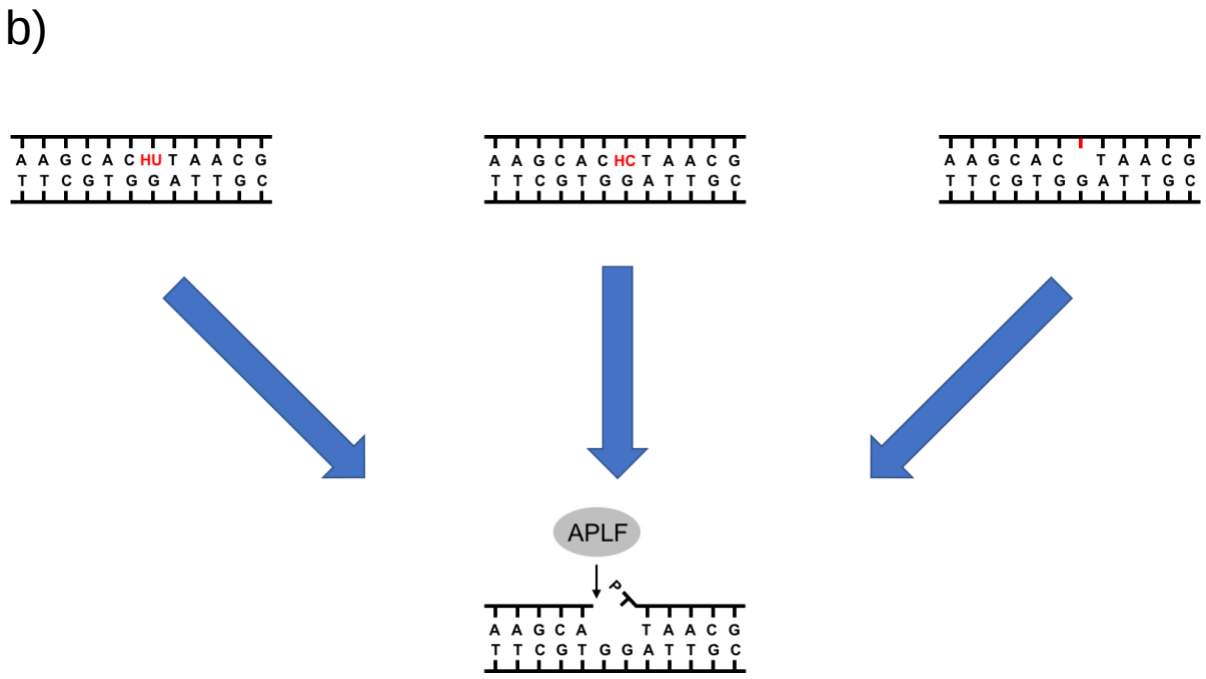
sites of cis-platin induced DNA damage was dependent on Adprt2, the Dictyostelium orthologue of PARP1 (Gunn et al. 2016). APLF and PARPs might therefore be closely linked in the repair of multiple types of DNA damage, including the removal of DNA interstrand crosslinks.

1.6. APLF

APLF, first described by Iles et al. 2007, is a protein named after its similarity in domain architecture to aprataxin and PNK (**a**prataxin- and **P**NK-**l**ike **f**actor). The third and most recent member of the family of FHA domain carrying proteins, it was shown to interact with the CK2-phosphorylated form of the single strand break repair protein XRCC1 as well as XRCC4 and XRCC5, both of which take part in NHEJ (Bekker-Jensen et al. 2007). This observation was connected with the speculation that APLF – again not unlike aprataxin and PNK – might harbour a DNA end processing capacity at sites of single- strand breaks. Evidence of a nucleolytic activity confirmed 3' nicking activity of APLF against AP sites, hydroxyuracil and hydroxycytosine and 3'-5' exonuclease activity (Figure 1.6. b) (Kanno et al. 2007). The former observed function was ascribed to a cysteine-tyrosine-arginine (CYR) sequence in tandem zinc-finger like motifs. Since hydroxyuracil, AP sites and hydroxycytosine are repaired by BER, a function of APLF in that pathway was proposed. However, any attempts to connect APLF deficiency with an increased sensitivity to single- and double strand break inducing agents yielded poor results. In later studies the previously discovered zinc-finger motifs (ZnF) in APLF with the sequence $CX_5CX_6HX_5H$ were identified to be poly-ADP-ribose (PAR) binding zinc fingers (PBZ) (Ahel et al. 2008, Rulten et al. 2008). *In vitro* and *in vivo* data revealed PAR binding - most importantly of poly-ADP-ribosylated (PARylated) PARP1, supporting an APLF function in base excision repair. Furthermore, the PBZ repeats showed ADP-ribosylation themselves, but only if both motifs were unmodified. Further insights into the residues responsible for the interaction with ADP-ribose pinpointed it on the previously mentioned conserved CYR and C(M/P)Y motifs in the PBZ repeats (Li et al. 2010). The notion of APLF as a DSBR protein was further

Figure 1.6. APLF domain architecture and potential role in BER **a)** The interaction domains of APLF. The dotted line signifies that ATM is phosphorylating Ser116 of APLF, rather than permanently binding the protein. APLF is able to bind XRCC1, XRCC4, the Ku70/Ku80 dimer, poly-ADP-ribose and histone tetramers **b)** Possible APLF mode of action in BER. APLF has shown 3' endo- and also exonucleolytic activity against double stranded DNA containing hydroxyuracil (HU), hydroxycytosin (HC) and AP sites, all of which are being repaired in base excision repair (BER).


APLF
a)
P
Ser 116
R182-184
W189
FHA domain
CYR
motifs
PBZ
repeats
acidic domain
XRCC1
XRCC4
ATM
Ku70
Ku80
PAR
H3
H4
H4
H3

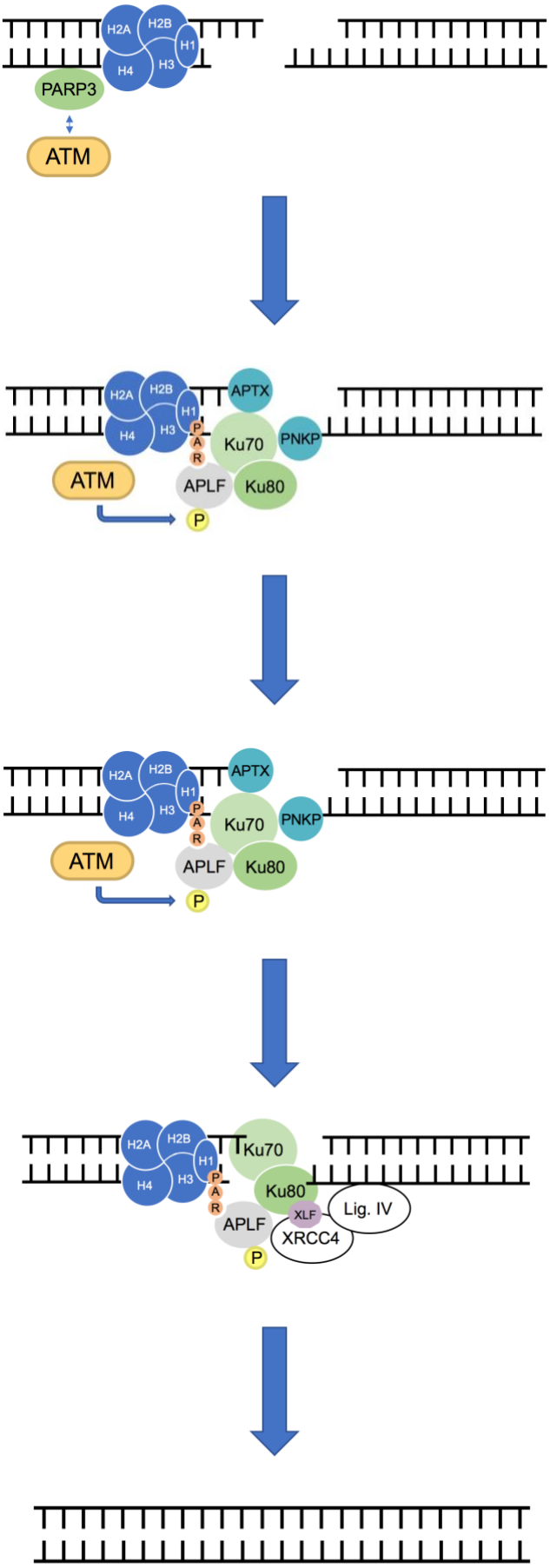

b)
APLF

corroborated by findings on the existence (Kanno et al. 2007, Macrae et al. 2008) and the amino acid specification of (Shirodkar et al. 2013, Grundy et al. 2013) Ku70/Ku80 binding residues in APLF.

Figure 1.6.a provides an overview of the existing body of literature on the structural characteristics and functions of APLF. A recent addition to this model has been the observation that the tandem PBZ repeats of APLF are capable of not only recognizing auto-poly-ADP-ribosylated PARP1 and trans-poly-ADP-ribosylated histone H1, but are also recruited to DNA damage sites in dependence of PARylation in NHEJ, possibly catalyzed by PARP3. It was postulated that upon localization to sites of DSB by PARylated H1, APLF in turn retains the XRCC4-LigaseIV complex to promote NHEJ (Rulten et al. 2011). Before that, however, APLF interacts with the Ku heterodimer, which is crucial for its retention in the nucleus and therefore its interaction with XRCC4-LigaseIV (Shirodkar et al. 2013). APLF is therefore thought to accelerate end ligation in NHEJ by promoting the stability, recruitment and/or retention of XRCC4-Ligase IV by linking it with Ku (Grundy et al. 2013, Hammel et al. 2016). This is reflected in the sensitivity to ionizing radiation of DT40 cells void of APLF (Grundy et al. 2013). Taken together, these findings present a strong case for the importance of APLF in the resolution of not only single-strand breaks, but also double-strand breaks.

In Figure 1.7. a model of the mode of action of APLF is proposed on the basis of the publications reviewed above. By judgement of these, it can be safely assumed that APLF has over the past decade been established as a contributor to single-strand break repair (SSBR) by BER and to double-strand break repair (DSBR) as a key player in non-homologous end-joining.

Figure 1.7. Proposed mode of action of APLF in NHEJ. PARP3, upon interacting with ATM at sites of double-strand breaks, poly-ADP-ribosylates H1 and recruits APLF via latter's PBZ repeats. PARP3 and APLF also interact in a PBZ dependent manner .ATM dependent APLF phosphorylation requires PARP3 and is essential for function double strand break repair by NHEJ. Following its chromatin recruitment, APLF binds the Ku heterodimer through Ku80 and accelerates its presence at the DSB. Simultaneously, processing of the DNA ends is carried out by aprataxin (APTX) and polynucleotide kinase 3' phosphatase (PNKP). APLF also binds XRCC4, promoting the recruitment and retention of the dimer XRCC4-LigaseIV. A stable complex of Ku70/80 and XRCC4-LigaseIV linked by APLF is formed Since this complex is involved in DNA end ligation, APLF links two separate steps in the NHEJ pathway. Another repair protein, non-homologous end-joining factor 1 (XLF), is also thought to bind both Ku and XRCC4.


H2A
H2B
H1
H4
H3
PARP3
ATM
APTX
P
A
R
Ku70
PNKP
APLF
Ku80
XLF
Lig. IV
XRCC4

What remains widely unanswered is whether APLF is also capable of assisting in the resolution of DNA damage forms other than SSBs and DSBs and whether this would be dependent on already established functions of APLF in BER and NHEJ or pertain to a novel feature of the protein.

More recently, findings from our group were published that connect the *Dictyostelium discoideum* protein APL (aprataxin/APLF-and-PNKP-like protein) with Adprt2, the human orthologue of which is PARP1, in DNA interstrand crosslink repair (Gunn et al. 2016). APL, bearing resemblance to human APLF, contains an FHA-like domain and a PAR binding PBZ domain. In addition, it also harbours a macrodomain, another type of poly-ADP-ribose interaction domain. Adprt2 expresses ADP-ribosylation activity in response to cis-platin and this leads to APLF recruitment to chromatin. Interestingly, although knocking-out Adprt2 abolishes APL recruitment, it does not fully disrupt ADP-ribosylation in response to that type of damage, suggesting redundancy between Adprt2 and other ARTs (ADP-ribosyl-transferases). APL recruitment was found to be dependent on its macrodomain. It remains unresolved whether the absence of APL sensitizes *D. discoideum* to ICLs. This data encouraged us to explore whether human APLF might be involved in the repair of DNA interstrand crosslinks and whether this involvement might be dependent on PARPs.

1.7. AIMS

PARPs have been shown to be crucial components in the repair of an array of highly diverse DNA insults. Particularly the contribution of the DNA binding PARPs PARP1, PARP2 and PARP3 has become evident in the reduced viability of knock-out cells towards various unrelated DNA damage forms. Many DNA damage types can be repaired by more than just one DNA repair pathway. This means that DNA damage response PARPs may be involved in multiple pathways channelling the same sort of DNA damage or may act in competing pathways. Redundancy between pathways, however, might mask their impact on the repair of a particular type of damage. At the same time, mechanisms are in place to favour one pathway over another depending on certain circumstances as well as the stage of the cell cycle. This can sometimes lead to the underappreciation of specific pathway and proteins, the importance of which on the overall repair of a form of damage is neglected. In several instances, PARPs have been attributed auxiliary or accelerating functions rather than essential ones, impeding the identification of the mechanistic background of their function. Furthermore, the way PARPs contribute to the DDR is highly versatile through sensing sites of DNA damage, PARylating target proteins, recruiting repair factors to DNA, catalysing chromatin decondensation and influencing transcription regulation. These circumstances make it equally exciting and difficult to explore the extent of PARP contribution on a specific DNA damage source or a pathway. Many questions remain to try and explain the extensive impact of PARPs and ADP-ribosylation on DNA repair.

A body of literature has implicated PARPs in multiple DNA repair pathways resolving SSBs and DSBs including BER, NER, HR, NHEJ and alt-NHEJ. Whether PARPs also

contribute to the resolution of interstrand crosslinks, resolved through the FA pathway as well as NER, remains unknown. Studies in our group using the model organism *Dictyostelium discoideum* revealed that the proteins Adprt2 and Adprt1a, the human orthologues of which are PARP1 and PARP3, are responsible for the resistance of cells against DNA interstrand crosslinks. Adprt1a and Adprt2 are recruited to chromatin following treatment with the ICL inducing drug cis-platin. Furthermore, mutants of any of the two proteins showed increased sensitivity towards cis-platin compared to the parental Ax2 cells (Gunn et al. 2016). Recruitment of the *D. discoideum* protein APL in response to cis-platin induced ICL formation is also dependent on the presence of Adprt2. On the APL side, its localisation to Adprt2 is dependent on its PAR-binding macrodomain. PARP catalytic activity in *D. discoideum* is therefore directly linked to cis-platin induced damage. Given the complexity of interstrand crosslink repair, these findings left us with many questions: Does the inhibition of PARPs impair the tolerance to ICL inducing agents in human cells? What is the nature of PARP contribution to ICL repair? Which pathways are involved? Is APL required for faithful ICL repair? How does APL recruitment to chromatin after cis-platin treatment play into ICL repair? Is there a human orthologue to *D. discoideum* APL?

We hypothesised, that PARPs might be involved in ICL repair/removal in human cells. Therefore, our aim was to investigate whether the absence of PARPs has an impact on the cell viability of human cells following treatment with the ICL inducing drug MMC and if so, what the molecular implications of this absence are. Structural and functional similarities suggest that APL is the *D. discoideum* orthologue to the human APLF, a protein involved in NHEJ of mammalian cells (1.5.2.). This encouraged us to explore a potential involvement of APLF in ICL removal.

2. MATERIALS & METHODS

2.1. MATERIALS

Squish Buffer

10 mM Tris-HCl

1 mM EDTA

25 mM NaCl

4x SDS loading buffer

200 mM Tris-HCl (pH 6.8)

8% SDS

0.4% bromphenol blue

40% glycerol

1x SDS loading buffer

25% 4x SDS loading buffer

100 mM DTT

10x BNS Buffer

480 mM Tris-HCl

390 mM glycine

1x BNS Transfer Buffer

10% 10x BNS Buffer

0.0375% SDS

20% methanol

1x PBS

137 mM NaCl

2.7 mM KCl

10 mM Na_2HPO_4

1.8 mM KH_2PO_4

1x TBS-T

50 mM Tris-HCl, pH 7.5

150 mM NaCl

0.2% Tween 20

1x Running buffer

25 mM Tris-HCl

192 mM glycine

0.1% SDS

1x Transfer buffer

50 mM Tris-HCl

384 mM glycine

20% methanol

Extraction buffer 1

10 mM Tris-HCl pH 7.5

150 mM NaCl

1.5 mM $MgCl_2$

0.34 mM sucrose

10% glycerol

1 mM DTT

1 protease inhibitor cocktail tablet (Sigma, 11836170001) per 10 mL extraction buffer

10 mM sodium fluoride

1 mM sodium orthovanadate

Extraction buffer 2

3 mM EDTA

0.2 mM EGTA

1 mM DTT

1 protease inhibitor cocktail tablet (Sigma, 11836170001) per 10 mL extraction buffer

NETN150 buffer

20 mM Tris-HCl

150 mM NaCl

1 mM EDTA

Nonident P-40

Figure 2.1. List of primary and secondary antibodies used in Western Blotting and plasmids. The antibody from the Caldecott group (University of Sussex) was raised in rabbits

Primary antibodies for Western Blotting

APLF	1:500	1% milk in TBS-T	Caldecott lab, rabbit AB
myc	1:1000	5% milk in TBS-T	Santa Cruz, 9E10
FANCD2	1:1000	5% milk in TBS-T	Santa Cruz, FI17
Actin	1:5000	5% milk in TBS-T	Santa Cruz, C-11
H3	1:1000	5% milk in TBS-T	Abcam, ab12079
pan-ADP-ribose	1:10000	5% milk in TBS-T	Millipore, MABE1016

Secondary antibodies for Western Blotting

goat	1:10 000	5% milk in TBS-T	Dako, P0449
mouse	1:10 000	5% milk in TBS-T	Dako, P0260
rabbit	1:10 000	5% milk in TBS-T	Dako, P0448

Plasmids

myc-APLF WT	pCI-puro (1x myc tag)	N-terminal	Caldecott lab
myc-APLF R27A	pCI-puro (1x myc tag)	N-terminal	Caldecott lab
myc-APLF C379/385A	pCI-puro (1x myc tag)	N-terminal	Caldecott lab
YFP-APLF	pcDNA6.2/N-YFP-DEST	N-terminal	Ivan Ahel lab

2.2. METHODS

2.2.1. Cell biology and gene manipulation

Cell growth

Human retinal pigmented epithelial cells (RPE-1) and bone osteosarcoma (U2OS) cells were grown in the dark at 37°C, 5% CO_2. Cells were maintained in 75mm cell culture flasks. Cells attached to the bottom of the flask were culture in DMEM with 20mM L-glutamine, 100 units/mL Penicillin, 100 µg/mL Streptomycin and 10% fetal bovine serum (FBS), here referred to as DMEM comp. + P/S. Cells were allowed to grow to 80% confluency. At that stage, the growth media was removed, cells washed in PBS and trypsin-EDTA added to detach the cells from the bottom of the flask. Following detachment, cells were resuspended in growth media, the cellular concentration counted with a haemocytometer and 1x 10^5 cells left in the flask. All the flasks contained 15 mL growth media at any time. For several experimental set-ups, cells were seeded in 96, 24, and 6-well plates. Several plasmid transfected strains were grown in the presence of Puromycin.

CRISPR Cas9 double nickase strategy

The *aplf*Δ strains of RPE-1 cells were generated using the CRISPR Cas9 double nickase method. Two gRNAs guide a mutated Cas9, which can only introduce single strand breaks, to proximal sites on opposing DN strands in exon1 of the APLF gene, generating a DSB. The target sequence of the gRNAs used is as follows:

gRNA1: 5’ ACCCGGGGACCGCCGTCCCGCGG 3’

gRNA2: 5’ GCGCCCGGGGAGACGGTGATCGG 3’

CRISPR Cas9 transformation into RPE-1

RPE-1 cells were seeded at a concentration of 3 x 10^5 cells in wells of a 6-well plate and left to attach overnight. On the following day, the cells were transfected with two plasmids each carrying a distinct sequence for a guide RNA (gRNA) and the cDNA for the CRISPR Cas9 nickase. For one well, 2 µg of each plasmid were added to 400 µL serum-free DMEM (DMEM containing L-glutamine, Penicillin and Streptomycin, but not FBS). As a negative control, the two plasmid backbones without the sequences for the gRNAs were added to serum-free DMEM in a separate transfection mixture. Turbofect Transfection Reagent was thoroughly vortexed and 6 µL added to complete the transfection mixture. The solution was homogenized by pipetting and incubated for 15-20 minutes at room temperature. Finally, it was added in a dropwise manner to 3.6 mL DMEM comp. + P/S for each well to make up 4 mL. For even distribution, the 6-well plate was gently rocked before incubation at 37°C, 5% CO_2.

After no more than 24 hours following the transfection, the growth media was removed and 2 mL DMEM comp. + P/S placed back into each well. Transfection with the two plasmid containing the sequences for the gRNAs and transfection with the control plasmids were each carried out for 3 different wells and each of those wells was exposed to a different concentration of Puromycin (3, 4 and 5 µg/mL) to select for successfully transfected cells.

24hr following Puromycin treatment, DMEM comp. was taken off of all wells, cells were washed twice with PBS and trypsonized adding 200 µL trypsin-EDTA solution. Cells were incubated for 5-10 minutes at 37°C, 5% CO_2 to detach from the bottom of the wells and were then resuspended in 800 µL DMEM comp. + P/S. Of each transfection

5, 20, 50 and 200 µL were placed into 10 mL dishes containing 10 mL DMEM comp. + P/S. The dishes are kept at 37°C, 5% CO2 for 10 days.

By that time, successfully transfected cells and also others that survived Puromycin selection had grown into colonies that were selected and harvested by trypsonisation in dedicated harvesting cylinders. Colonies were selected independent of their size, but based on the presence of other colonies and single cells in close proximity. Each selected colony was detached in 30 µL Trypsin-EDTA and grown in a well of a 96-well plate in 200 µL media.

CRISPR Cas9 verification by PCR and sequencing

The growth of the colonies was continued from 96 into 24 and 6-well plates, at which stage DNA extracts were prepared from the cells. 50% of confluent cells in a well of a 24-well plate were spun down for 5' at 200g and resuspended in 100 µL Squish buffer. Samples were frozen down to -20°C to pre-lyse cells. Afterwards, proteinase K was added to a final concentration of 200 µg/mL and samples first incubated at 65°C for 30' and then at 95°C for 2' to inactivate proteinase K.

Taq polymerase PCR was run of each DNA sample with the below primers to amplify exon 1 of the APLF gene, in the centre of which a double strand break is induced via the CRISPR cas9 double nickase method (see 3.2.2.). Agarose gel electrophoresis of the PCR products was carried out with 2% agarose gels. The undisrupted sequence is 315 bp long, whereas CRISPR Cas9 double nickase disrupted, PCR amplified DNA stretches carry deletions or insertions and therefore ran faster or slower, respectively, in the agarose gel.

PCR primer sequences:

Forward: TGTTTTTTCCCAGGGCGTGG

Reverse: TTCTAAAATCCGGACCGGCG

PCR reaction:

Denaturing:	95°C	1 minute	⎫
	95°C	15 seconds	⎬ Repeated 34x
Annealing:	68°C	15 seconds	⎭
Elongation:	72°C	30 seconds	
	72°C	10 minutes	
Finish:	4°C	∞	

Colonies producing one or more shifted band(s), but no wildtype band, were selected, the bands cut out, the DNA extracted using QIAquick gel extraction kit, cloned using the QIAGEN PCR cloning kit and transformed into E.coli. Single E.coli colonies were picked, grown in suspension and minipreps performed with QIAprep miniprep kit. The identity of the cloned sequence was confirmed by restriction digest and agarose gel electrophoresis, before sending the sample for sequencing.

siRNA transfection of U2OS/RPE-1 cells

0.6 x 10^5 cells were seeded in wells of a 24-well plate and incubated at 37°C, 5% CO_2 overnight. On the next morning, the transfection mixture was prepared. 50 µL of 1 µM siRNA solution in DMEM was prepared. For each 50 µL of siRNA solution, 50 µL of 1.25 µL DharmaFECT transfection reagent in DMEM were prepared and both mixtures left for 5 minutes at room temperature. They were mixed and left at room temperature

for 20 minutes. For each 100 µL siRNA/DharmaFECT transfection mixture 400 µL DMEM + FBS were added. The growth media was taken off the wells and 500 µL transfection solution placed in each well.

For each cell line a transfection reaction with control siRNA and an siRNA pool against the target gene were prepared. After adding the transfection mix, cells were incubated for 24 hours at 37°C, 5% CO_2. After 24 hours, the transfection solution was replaced with a newly prepared one and the cells were again incubated for 24 hours at 37°C, 5% CO_2. The cells were then rescued from the siRNA transfection, trypsonized and redistributed into 6-well plates. On the following day, clonogenic survival assays were prepared from the transfected cells.

Clonogenic survival assay

Cells were seeded at 400 or 600 cells/well in 2 mL DMEM comp. + P/S in the wells of 6-well plates and incubated at 37°C, 5% CO_2 overnight. On the next morning they were treated with the appropriate concentrations of MMC or, as a negative control, the carrier solution (50% ethanol, 50% Tris-HCl pH 8.0). After 24 hours the wells were washed twice with PBS, 4 mL DMEM comp. + P/S was placed back into each wells and the cells were allowed to recover for 7 days at 37°C, 5% CO_2.

To test the sensitivity of cells to MMC after PARP inhibition, 10 µM Olaparib was added 1 hour prior to MMC treatment and DMSO used as a negative control. After the end of the MMC treatment, 2 mL DMEM comp. + P/S were placed back into the wells and Olaparib was added to the wells for another 24 hours. Finally, cells were washed twice with PBS, 4 mL DMEM comp. + P/S was placed back into the wells and the cells were

allowed to recover for 6 days at 37°C, 5% CO_2.

At the end of the recovery period, the 6-well plates were taken out of the incubator and the wells washed with PBS. The cells were fixed with 100% methanol at -20°C for 20 minutes, the methanol taken off and the cells stained at room temperature with crystal violet for 20 minutes. The plates were then washed and dried. Colonies were counted by eye.

Laser stripes

3 x 10^5 U2OS cells were seeded in 2 mL media in 35 mm glass bottom dishes and let grown for 24 hours at 37°C and 5% CO_2. Similar to the CRISPR Cas9 transformation 4 µg YFP-APLF and 6 µL pre-vortexed Turbofect transfection reagent were mixed with 400 µL DMEM for every dish, left for 15-20 minutes at room temperature and the media in the dishes replaced with the transfection mixture + 3.6 mL. 24 hours later the media was taken off and 2 mL pre-calibrated (37°C, 5% CO_2) microscope media (phenol-red free DMEM + 10% FBS + 2 mM L-glutamine) added. Dishes are either left untreated or treated for 30 minutes with 20 µg/mL TMP before laser stripe treatment.

For microirradiation, a 405 nm Solid State light source was used. Cells were irradiated over a period of 5 cycles at 21 milliseconds each with a laser intensity of 13%. Recruitment of YFP-APLF to the laser stripe was observed through a GFP filter, since the pass wavelength of the filter is within in range of the wavelengths of the YFP emitted light. To monitor and record YFP-APLF laser stripe formation, we used a Spinning-disk confocal microscope (PerkinElmer Ultra-VIEW Vox on an Olympus IX81 microscope with a Hamamatsu Photonics ImagEM camera). The objective used was

a 40x oil UPlan FL with an NA of 1.3. In order to avoid sample bleaching, photos were taken ever 20 seconds for the first minute and, if the signal persisted, every minute after that.

2.2.2. Biochemistry

Whole cell and nuclear extracts

Whole cell extracts were prepared by washing cells in 1x PBS and then scraping them off the bottom of the wells with 100 – 200 µL 1x SDS loading buffer boiled at 100°C or by dissolving a centrifuged cell pellet of a suspension of measured cell concentration in the appropriate amount of 1x SDS loading buffer boiled at 100°C. The samples were boiled at 100°C for 5 minutes, quick-spun and frozen at -20°C.

For nuclear extracts, 5 x 10^6 cells were spun at 2000g for 5 minutes, washed with PBS, spun again and resuspended in 200 µL sucrose buffer supplemented with 20 µL 0.5% Triton-X. The solution was vortexed briefly, kept on ice for 5 minutes and then spun with 1300g for 5 minutes at 4°C. The supernatant was discarded and the pellet washed again in 200 µL sucrose buffer and spun down with 1300g for 5 minutes at 4°C. The pelleted nuclei were resuspended in 50 µL NETN150 buffer supplemented with 5 µM MgCl2 and 1:1000 benzonase nuclease. The extract was incubated on ice for 30 minutes and afterwards frozen down for future Bradford assay. For western blotting, aliquots of the extracts equivalent of a specific protein content were diluted in SDS loading buffer to make up 1x SDS loading buffer.

Cellular fractionation of chromatin

1x 10^5 RPE-1 cells were seeded in wells of 6-well plates and on the following day transfected with myc-APLF plasmid DNA. 24hr after transfection, cells were treated with MMC 1.65 µM MMC for 1 hour and biochemical fractionation performed 0, 1 and 3 hours post MMC treatment.

The cells were scraped off the bottom the wells, washed with ice-cold PBS and spun for 5 minutes at 3000 rpm. The pellets were resuspended in 65 µL extraction buffer 1 supplemented with 0.1% TritonX-100 (cell concentration must remain below 1 x 10^7 cells/mL). The solution was incubated on ice for 10 minutes, centrifuged at maximum speed for 5 minutes at 4°C and the supernatant (cytosolic fraction) separated from the pellet. The pellet was resuspended in 65 µL extraction buffer 2.1 and the integrity of the nuclei confirmed with a phase contrast microscope. The solution was incubated on ice for 30 minutes and after that centrifuged with 1700g for 4 minutes at 4°C. Again, the supernatant (soluble nuclear fraction) was separated from the pellet. The pellet was resuspended in 50 µL at 100°C pre-heated 1x SDS loading buffer and 4x SDS loading buffer pre-heated at 100°C added to the cytosolic and soluble nuclear fraction to yield samples of 1x SDS loading buffer concentration. All samples were heated at 100°C for 5 minutes, quick-spun and frozen at -20°C.

In the same way that cells were collected for biochemical fractionation, whole cell extracts were prepared of all time points post MMC treatment to ensure that myc-APLF expression levels were equal between them.

Western blotting

A range of polyacrylamide gels from 6% to 15% were prepared (see 2.1. Materials) and used for the optimal resolution of the desired protein(s). Chromatin, nuclear or whole cell extracts in 1x SDS loading buffer were loaded to a maximum of 20 µL per well. The samples were boiled for 1-5 minutes at 100°C and vortexed before application to the gel. Empty wells were filled with the appropriate amount of 1x SDS loading buffer. Gels were run in 1x Running Buffer at 150 Volts for 1 hour. The gels were then placed into 1x BNS transfer buffer. Semi-dry transfer was carried out onto 0.45 µm methanol activated Immobilon PVDF membranes with the BioRad Trans-Blot® Turbo™ transfer System at 1.0 A and 25 Volts for 30 minutes. Alternatively, 2 hours wet transfer at 250 mA was performed at room temperature using 1x transfer buffer.

Membranes were blocked for 30 minutes at room temperature with 5% milk in TBS-T. Primary antibody in 5% milk in TBS-T solutions were distributed onto the membranes and left at 4°C overnight. On the next morning, the membranes were washed 3x for 10 minutes with PBS and covered in secondary antibody in 5% milk in TBS-T solutions at room temperature for 1 hour. Following 3 more PBS washing steps for 10 minutes each, the membranes were treated with Immobilon Western Chemiluminsecent HRP Substrate for 5 minutes and the signal then developed onto Amersham Hyperfilm MP films or recorded with LI-COR Odyssey Fc Imager and the images analysed for signal quantification with ImageStudioLite. For the concentrations and company names of the primary and secondary antibodies used, see Figure 2.1.

3. PARPS IN INTERSTRAND CROSSLINK REPAIR

3.1. INTRODUCTION

Poly-ADPribose-polymerases (PARPs), a group of 17 proteins with the founding father PARP1, catalyse the transfer of one or more ADP-ribose moieties onto target proteins or themselves – called mono- and poly(ADP-ribosylation), respectively – using NAD^+ as substrate (D'Amours et al. 1999, Amè et al. 2004, Perina et al. 2014). They exercise a broad range of functions in DNA repair, cell division, transcription regulation, RNA interference, chromatin regulation, apoptosis, inflammation, mitochondrial function as well as cell cycle checkpoints in eukaryotic cells (Schreiber et al. 2006, Haince et al. 2007, Wang et al 2011, Gibson & Kraus 2012, Tallis et al. 2014). One sub-group of PARPs, namely PARP1, PARP2 and PARP3, and possibly tankyrase 1 and tankyrase 2, bind DNA and have been implicated in the DNA damage response (DDR) (Pleschke et al. 2000, Schreiber et al. 2006, De Vos et al. 2012, Tallis et al. 2014, Wei et al. 2016).

PARP1, being the most well-studied of the PARPs, has been attributed several roles in single- and double-strand break repair (SSBR and DSBR respectively). It rapidly recognizes and binds SSBs, triggering auto-ADP-ribosylation and trans-PARylation of other proteins that promotes recruitment of the SSB repair factors XRCC1-Ligase3 to DNA lesions (Caldecott 2003, Caldecott 2008, Fisher et al. 2007). It also acts upon SSBs formed in base excision repair (BER) (Dantzer et al 2000), promoting APE1 strand incision (Harris et al. 2009, Prasad et al. 2015) and co-functioning with pol β in long-patch BER (Prasad et al. 2001, Le Page 2003). In DSBR, PARP1 contributes to

homologous recombination (HR) by promoting replication fork restart and competing with Ku70/Ku80 and XRCC4/Ligase 4 of NHEJ for DNA ends. (Hochegger et al. 2006, Beck et al. 2014) and recruits Mre11 to stalled replication forks (Bryant et al. 2009, Beck et al 2014). Finally, it competes with NHEJ by participating in alternative NHEJ (a-NHEJ) (Wang et al. 2006).

Similarly, to PARP1, PARP2 also acts in BER, cooperating with FEN1 to determine the size of the patch generated (Huber et al. 2004) and being an additional member of the PARP1-XRCC1-Ligase3 complex (Schreiber et al. 2002). A role for PARP2 in HR is still being debated. PARP3, on the other hand, has been mostly implicated in DSB associated repair (Boehler et al. 2011). It facilitates NHEJ by recruiting APLF to DNA damage sites (Rulten et al. 2011) and aids Ku80 in limiting end resection through HR to promote classical NHEJ (Beck et al. 2014).

ADP-ribosylation is conserved across many domains of life and homologues of PARPs have been found in representatives of all major eukaryotic supergroups, including the model organism *Dictyostelium discoideum* (Perina et al. 2014). *Dictyostelium* is a model organism particularly suited for experimental work, since it is a simple multicellular eukaryote, the physiology of which is applicable for studying many problems of development and cell signalling (*The Development of Dictyostelium discoideum* by W. Loomis et al. 1982, Raper et al. 1935). The extensive research on this slime mold, which is often used in its vegetative stage, has allowed it to become a successful system for exploring drugs, molecular functions of human diseases and more (Gaudet et al. 2008, Eichinger et al. 2005).

Similar to human cells, *Dictyostelium* harbours multiple PARPs. Data published by Gunn et al. 2016 suggests that the PARPs Adprt2 and Adprt1a, the *Dictyostelium* orthologues of human PARP1 and PARP3, are important factors for the repair of DNA interstrand crosslinks (ICLs). ICLs are a particularly deleterious form of DNA damage, the removal of which is cell cycle dependent and requires multiple layers of at least partly interwoven repair pathways. An *adprt2* knock-out cell line shows increased sensitivity to the ICL inducing drug cis-platin in comparison to Ax2 wildtype cells. This phenotype was further enhanced by simultaneous knock-out of Adprt1a, indicating at least partial redundancy between these two proteins. Adprt1a knock-out cells alone did not harbour a sensitivity to cis-platin, suggesting that only in the absence of Adprt2, ICL repair is mediated by a secondary pathway, NHEJ, and ADP-ribosylation activity of Adprt1a. Taken together, this supports a role for Adprt2 and ADP-ribosylation in the immediate repair of ICLs.

Given these observations, in addition to the findings that DNA repair pathways are generally well conserved between *Dictyostelium* and humans, we speculated that PARPs might also be involved in the repair of ICLs in human cells. To this end, one of the aims of this chapter was to inquire whether PARP catalytic activity could be observed in response to ICLs and whether the absence of PARP catalytic activity had an impact on the survival of cells to ICL inducing agents. We were also interested to find which, if any, PARPs specifically contribute to the resistance of human cells to DNA interstrand crosslinks.

Finally, if PARPs are indeed shown to support the survival of cells to ICLs, an exciting prospect is to explore the nature of that positive influence and the implications of it on

our current understanding of the functions of PARPs in DNA repair. ICLs are repaired through multiple pathways involving numerous repair steps and – in S-phase – the stalling of replication forks and cell cycle arrest. These include the FA pathway, TLS, HR and NER and are partly interconnected through the cell cycle. Given the complexity and versatility of the human DNA damage response to ICLs, many possibilities for a PARP contribution to ICL repair can be imagined. We therefore wanted to investigate, if the inhibition of PARPs sensitizes cells to MMC am how this could fit into our existing knowledge of PARPs and the framework of ICL repair pathways.

3.2. RESULTS

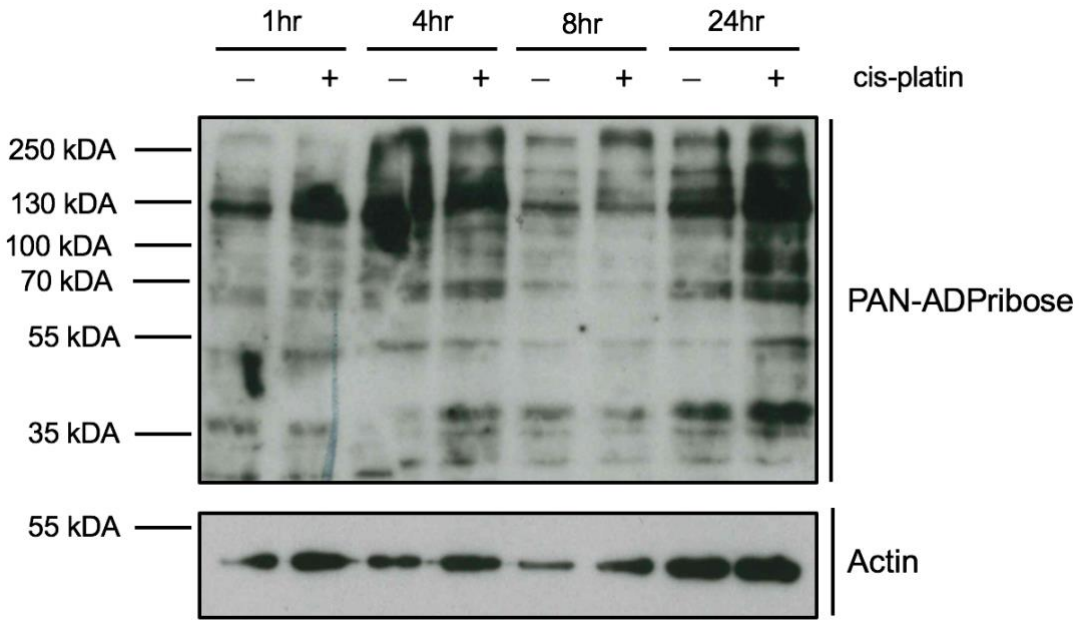
3.2.1. Cis-platin treatment in U2OS cells upregulates ADP-ribose levels

Between them, PARP1 and PARP2 contribute to BER, c-NHEJ, alt-NHEJ and HR and exercise multiple DNA repair functions within these pathways. Although both are capable of directly interacting with proteins involved in the DDR, their main contribution to DNA repair comes from ADP-ribosylation of themselves or target proteins at sites of DNA damage (Liu et al. 2011, Tallis et al. 2014). Therefore, as a first step to look into a possible role of PARPs in ICL repair, we considered if cells formed ADP-ribose chains in response to DNA interstrand crosslinks.

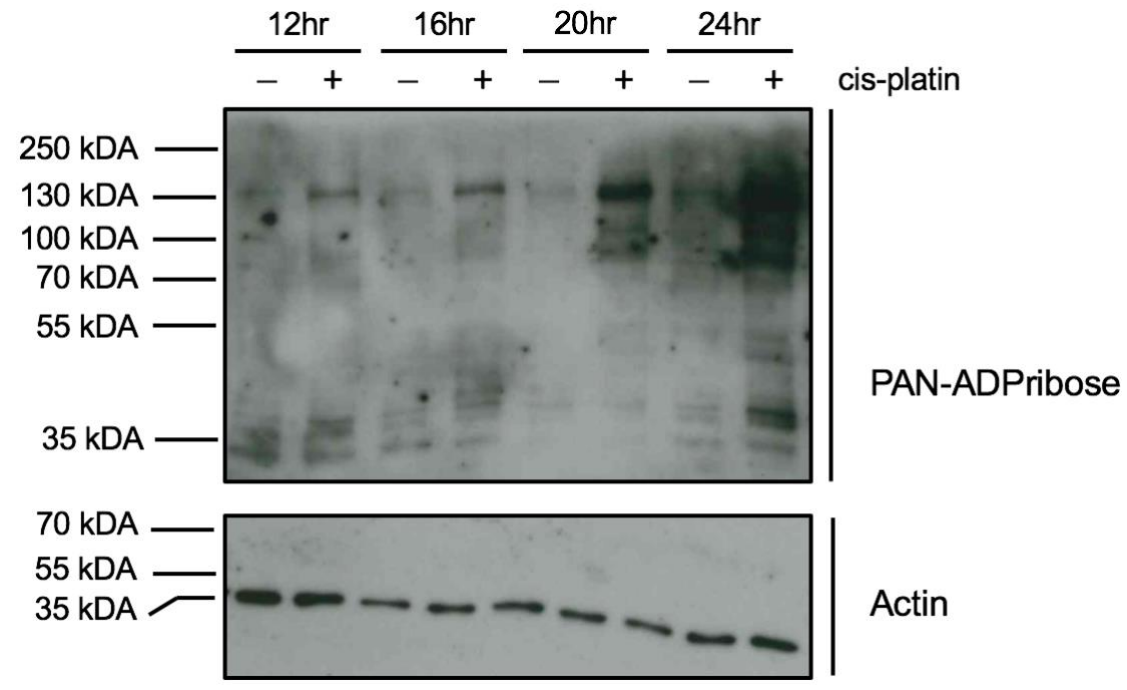
ADP-ribose levels in bone osteosarcoma epithelial (U2OS) cells were investigated in response to a continuous treatment with the ICL inducing drug cis-platin. Whole cell extracts were prepared 1, 4, 8, 12, 16, 20 and 24 hours after the start of the continuous exposure of cells to cis-platin. All forms of mono- and poly-ADP-ribosylation were determined using immunoblotting. Seperation over a brad size range of proteins was achvied including higher molecular PAR substrates such as PARP1, but low molecular weight PAR-modified histones could not be resolved. Between 1 and 8 hours after the start of cis-platin treatment, no difference in ADP-ribosylation levels between treated and untreated U2OS cells was observed (Figure 3.1. a). However, between 12 and 24 hours ADP-ribose levels continually increased in cis-platin treated samples against the untreated samples. ADP-ribose levels rose most prominently at close to 130 kDA (Figure 3.1. b). Elevated ADP-ribose levels also appeared above the 70 kDA band in treated samples from 20 hours and 24 hours. Although the identity of

Figure 3.1. ADP-ribose formation is elevated in U2OS cells treated with cis-platin. **a)** U2OS cells were treated with cis-platin and whole cell extracts prepared at 1, 4, 8 and 24 hours of treatment. The extracts were loaded onto a polyacrylamide gel, Western Blotting carried out and ADP-ribose levels investigated with a PAN-APD-ribose antibody. **b)** Similar to a), but this time whole cell extracts were prepared 12, 16, 20 and 24 hours after the start of the cis-platin treatment.

a)


1hr
4hr
8hr
24hr
– + – + – + – +
cis-platin
250 kDA
130 kDA
100 kDA
70 kDA
55 kDA
35 kDA
PAN-ADPribose
55 kDA
Actin

b)


12hr
16hr
20hr
24hr
– + – + – + – +
cis-platin
250 kDA
130 kDA
100 kDA
70 kDA
55 kDA
35 kDA
PAN-ADPribose
70 kDA
55 kDA
35 kDA
Actin

the bands is unclear, these findings suggest that ADP-ribosyation can be observed in human cells treated with ICL inducing agents. PARP1 has a molecular weight of 116kDA, PARP2 of 66 kDA. Since PARP1 and PARP2 account for the majority of poly-ADPribosylation in cells and strongly auto-modify themselves in response to DNA damage, it is possible that the bands represent auto-PARylated versions of these proteins.

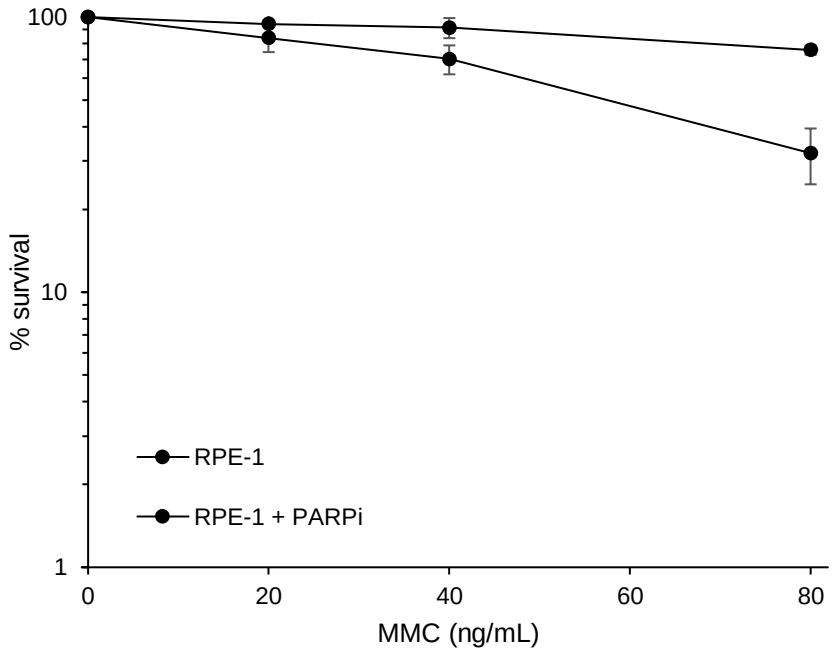
3.2.2. The PARP inhibitor Olaparib sensitizes cells to mitomycin C

Our group previously found that the absence of the *Dictyostelium* proteins Adprt2 but not Adprt1a, the human orthologues of which are PARP1 and PARP3 respectively, produces sensitivity to the DNA ICL inducing drug mitomycin C (MMC). In light of these findings, we were interested to investigate whether human PARPs were involved in the resolution of ICLs.

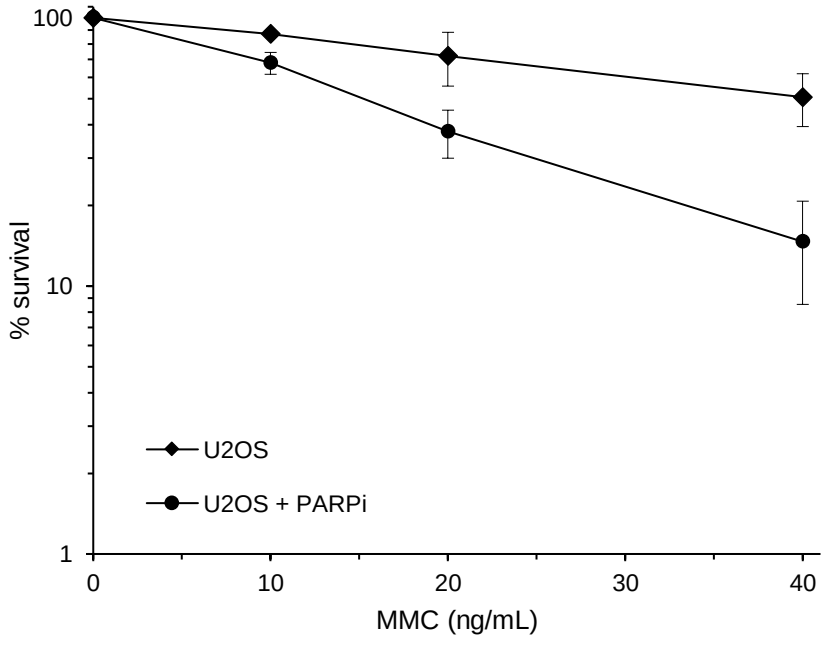
By means of clonogenic survival assay, we examined whether the PARP inhibitor (PARPi) Olaparib sensitizes cells to MMC. In this type of assay, cells were exposed to increasing concentrations of MMC and survival was measured by normalizing the number of the colonies in the treated against the untreated population. Olaparib is a clinically tested PARP inhibitor (Brown et al. 2016), that has recently been approved by the US Food and Drug Administration (FDA) and the European Commission (EC) for the treatment of advanced ovarian cancer. Maintenance treatment with the drug during and following platinum-based chemotherapy improved the progression-free survival in patients, especially those harbouring BRCA1/2 mutations (Lheureux et al.

Figure 3.2. The PARP inhibitor Olaparib sensitizes cells to mitomycin C. **a)** RPE-1 cells were seeded in a clonogenic survival assay and their sensitivity to MMC assessed in the absence or presence of Olaparib treatment The error bars represent the standard error of the mean of three independent experiments. **b)** Similarly to a) the sensitivity of U2OS cells to MMC +/- Olaparib was detected in a clonogenic survival assay. The error bars represent the standard error of the mean of three independent experiments

a)


100
10
1
% survival
RPE-1
RPE-1 + PARPi
0
20
40
60
80
MMC (ng/mL)

b)


100
10
1
% survival
U2OS
U2OS + PARPi
0
10
20
30
40
MMC (ng/mL)

2017). While PARP inhibitors such as Olaparib act as competitive inhibitors against NAD^+ for the catalytic pocket of PARPs, their ability to trap PARPs through allosteric changes at the sites of DNA damage is another important feature that contributes towards their potency (Ekblad et al. 2013, Shen et al. 2015). This is particularly true for PARP1 and PARP2 and to a lesser degree PARP3 (Murai et al. 2012), although other studies also include other PARPs in the list of PARPs inhibited by Olaparib (Wahlberg et al. 2012).

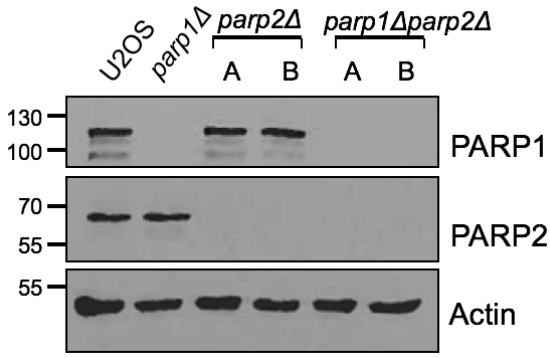
The sensitivity to MMC in the presence or absence of Olaparib was evaluated in two independent human cell lines, U2OS (Figure 3.2. a) and RPE-1 (Figure 3.2. a). RPE-1 is an hTERT immortalized human retinal pigmented epithelium cell line. Olaparib significantly sensitizes RPE-1 as well as U2OS cells to ICL induction by MMC (Figure 3.2.). In the case of RPE-1 cells, Olaparib was left for 48 hours following the end of the MMC treatment (Figure 3.2. a). Olaparib treatment in the U2OS cell line was continued for 72 hours post MMC treatment (Figure 3.2. b). At the highest mitomycin C concentration shown, the p-value for Olaparib against control treated RPE-1 cells was 0.0115. For Olaparib vs. control treated U2OS cells, the p-value at 40 ng/mL MMC is 0.0486. From these data we conclude, that PARP inhibition by Olaparib causes significant mitomycin C sensitivity in human cells, indicating a potential role of these proteins in ICL repair.

3.2.3. U2OS *parp1*Δ, *parp2*Δ and *parp1/2*Δ cells are sensitive to mitomycin C

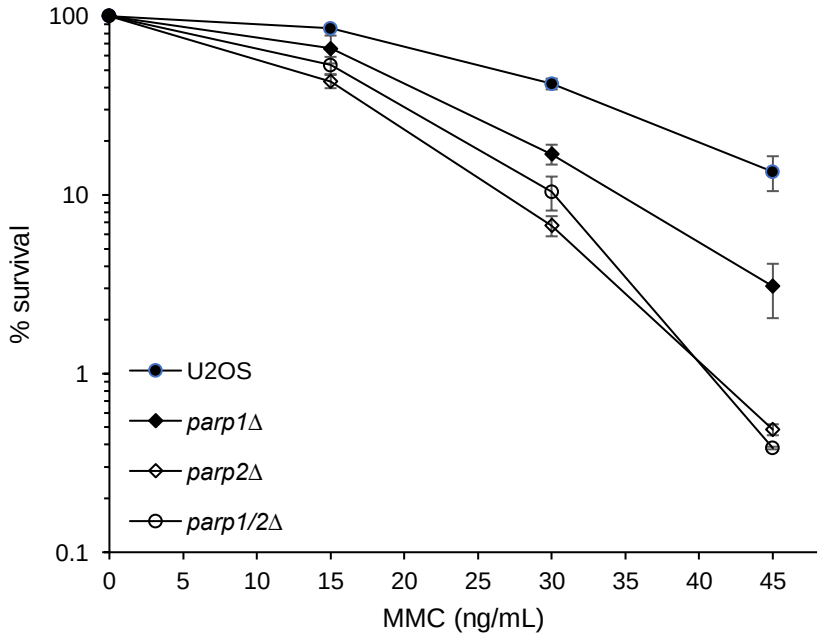
Although PARP inhibition through Olaparib sensitizes cells to MMC, this agent targets

Figure 3.3. MMC sensitivity of U2OS prenatal and *parp1*Δ*, parp2*Δ and *parp1/2*Δ KO cell lines **a)** The PARP1 and PARP2 expression levels of *parp1*Δ, *parp2*Δ and *parp1/2*Δ were tested by Western Blotting (data of George Ronson) **b)** The MMC sensitivity of U2OS in comparison to *parp1*Δ, *parp2*Δ and *parp1/2*Δ were evaluated by clonogenic survival assay. The error bars represent the standard error of the mean of three independent experiments **c)** p-values were calculated of the different knock-out cell lines in comparison with the parental cell line at all MMC concentrations.

a)



b)



c)

against RPE-1	15 ng/mL MMC	30 ng/mL MMC	45 ng/mL MMC
p-value(*parp1*Δ)	0.1766	0.0024	0.0301
p-value(*parp2*Δ)	0.0003	0.0003	0.0491
p-value(*parp1/2*Δ)	0.0056	0.0010	0.0483

multiple PARPs and possesses inhibitory features beyond PARP1, 2 and 3. Therefore, we wanted to independently confirm our findings and determine the PARP responsible for preventing sensitivity to MMC. To achieve this, we exploited cell lines disrupted in the PARP1 and PARP2 gene alone or in combination to establish their sensitivity to ICLs induced by MMC. U2OS PARP1, PARP2 and PARP1/2 KO lines were generated and validated by George Ronson using the CRISPR Cas9 double nickase strategy and confirmed by sequencing and western blotting. Expression levels of the proteins in the knock-out strains against the parental U2OS strain are shown in Figure 3.3. a.

*parp1*Δ is significantly more sensitive to MMC than U2OS parental cells at an MMC concentration of 30 and 45 ng/mL (Figure 3.3. c). *parp2*Δ and *parp1/2*Δ are more sensitive to MMC than U2OS parental cells at all MMC concentrations (15, 30 and 45 ng/mL). Interestingly, *parp2*Δ and parp1/2Δ are also more sensitive than *parp1*Δ at the two highest MMC concentrations, but *parp1/2*Δ is not more sensitive than *parp2*Δ. This indicates, that knocking-out PARP2 has a greater impact on the survival of cells to MMC than PARP1. Furthermore, from the fact that the *parp1/2*Δ is not more sensitive than *parp2*Δ we conclude that PARP1 and PARP2 are epistatic and function in the same pathway.

3.2.4. Olaparib sensitizes U2OS, *parp1*Δ and *parp2*Δ, but not *parp1/2*Δ to MMC

Having confirmed PARP1 and PARP2-dependent sensitization of human cells to MMC by two independent means, the question remained whether PARP1 and PARP2 are the sole players among PARPs in the removal of ICLs. We therefore addressed the

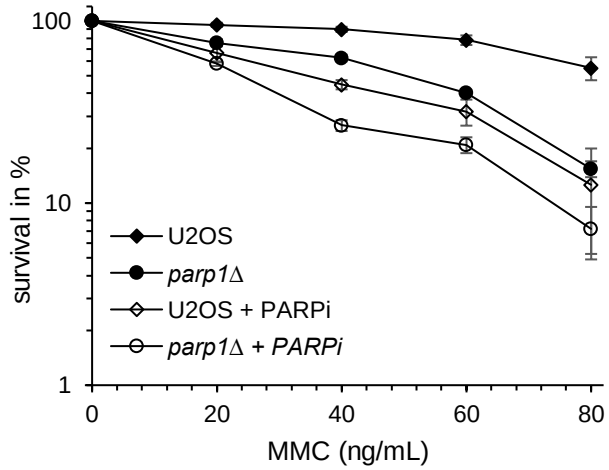
question of the potential involvement of other PARPs in the tolerance of human cells to ICLs. To test this, we established whether Olaparib could further sensitize *parp1/2*Δ cells to MMC. Beyond PARP1 and PARP2, Olaparib is documented to inhibit PARP3 and also exert minor inhibitory properties towards other PARPs, including PARP4, PARP12, PARP15 and PARP16 (Wahlberg et al. 2012).

*parp1*Δ and *parp2*Δ were less sensitive than PARPi treated U2OS parental cells and were significantly further sensitized to MMC by addition of Olaparib (Figure 3.4.). In contrast, Olaparib treated parental cells were similarly sensitive to MMC as *parp1/2*Δ and Olaparib treatment did not induce a further increase in MMC sensitivity in *parp1/2*Δ cells (Figure 3.4. c and d). This indicates that PARP1 and PARP2 are the only two PARPs that are involved in the tolerance of cells to ICLs. The further sensitisation of *parp1*Δ and *parp2*Δ cells to MMC in combination with Olaparib stands in contrast to our hypothesis that PARP1 and PARP2 are epistatic and act in the same pathway.

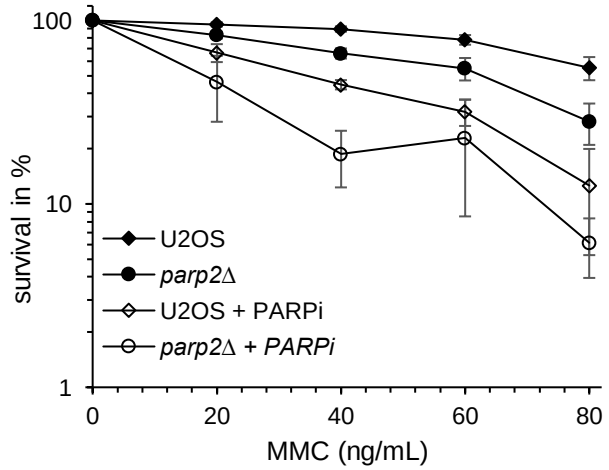
If PARP1 and PARP2 impair the same pathway to an equal extent, knock-out of either should not further sensitize cells to ICLs. However, the PARP inhibitor Olaparib does not only enzymatically suppress PARPs, but also traps PARP1 and PARP2 at sites of DNA damage leading to the formation of DSBs that are repaired by HR (Murai et al. 2012, Brown et al. 2016). Since PARP1 or PARP2 knock-out cells already harbour a DNA repair defect to DSBs by HR, Olaparib and other PARP inhibitors additionally sensitize *parp1*Δ and *parp2*Δ cells to MMC due to trapping of the remaining PARP. Olaparib therefore exhibits additional toxicity that does not stem from its suppression of PARP catalytic activity, but its trapping of PARP1 or PARP2. This is in line with the finding that U2OS *parp1/2*Δ are not further sensitized to MMC by Olaparib. In this cell

Figure 3.4. Olaparib sensitizes *parp1*Δ and *parp2*Δ cells, but not *parp1/2*Δ cells to MMC. **a)** Clonogenic survival assay to test the MMC sensitivity of U2OS and *parp1*Δ cells +/- Olaparib. The error bars represent the standard error of the mean of three independent experiments **b)** Clonogenic survival assay to test the MMC sensitivity of U2OS and *parp1/2*Δ cells +/- Olaparib. The error bars represent the standard error of the mean of three independent experiments. **c)** Clonogenic survival assay to test the MMC sensitivity of U2OS and *parp1/2*Δ cells +/- Olaparib. The error bars represent the standard error of the mean of three independent experiments **d)** p-values showing the difference in sensitivity of Olaparib against DMSO treated cells for *parp1*Δ, *parp2*Δ and *parp1/2*Δ cells.

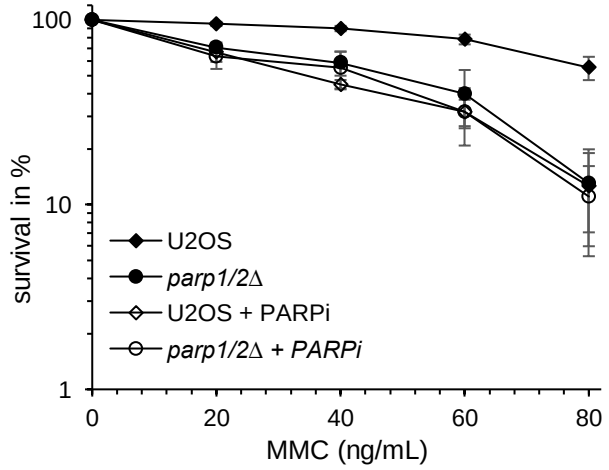
a)



b)



c)



d)

Olaparib vs. DMSO	**20 ng/mL**	**40 ng/mL**	**60 ng/mL**	**80 ng/mL**
p-value (*parp1*Δ)	0.0088	0.0003	0.0017	0.0599
p-value (parp2Δ)	0.1148	0.0084	0.1431	0.0422
p-value (parp1/2Δ)	0.5231	0.8270	0.6757	0.8128

line both PARP1 and PAR2 are absent, leaving none of the two DNA binding PARPs to be trapped. PARP1 and PARP2 are therefore the only PARPs contributing to the tolerance of human cells to ICL inducing agents.

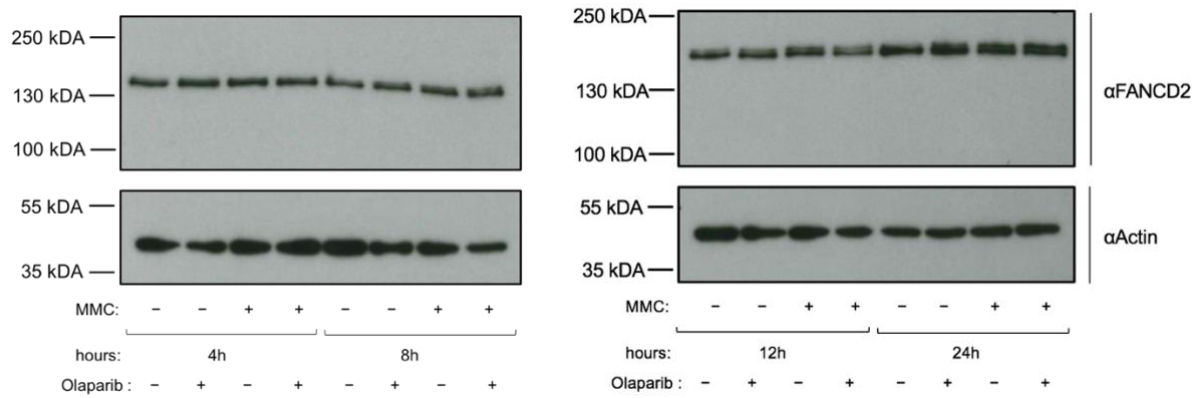
3.2.5. FANCD2 monoubiquitylation is independent of PARP inhibition

Following our findings that the presence of PARPs protects cells from sensitisation to ICL inducing agents, we inquired which ICL repair pathway PARPs might contribute towards. Since the Fanconi Anemia pathway is the main ICL repair pathway, our aim was to establish at which stage, if any, PARPs might function in the resolution of DNA interstrand crosslinks through FA-dependent mechanisms (Moldovan and D'Andrea 2009). FANCD2 monoubiquitylation is a hallmark of this S-phase dependent ICL repair pathway, preceding ICL incision and unhooking. We therefore used immunoblotting to detect FANCD2 monoubiquitylation levels in MMC treated cells in the presence or absence of PARP inhibition.

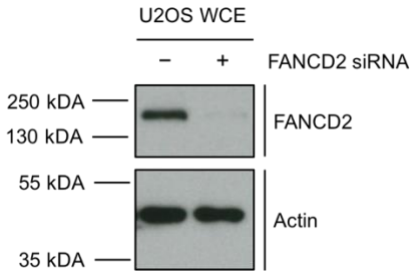
Cells were either MMC treated for 1 hour or left untreated and whole cell extracts prepared 4, 8, 12 or 24 hr after the end of the treatment. The PARP inhibitor Olaparib or the carrier solution (DMSO) were added one hour prior to MMC treatment. At each time point, whole cell extracts were prepared and monoubiquitylated against unmodified FANCD2 levels were examined using αFANCD2 antibody (Figure 3.5.) Whereas FANCD2 ubiquitylation levels steadily increased over time in MMC treated samples, this effect remained unaltered by the addition of Olaparib. We concluded that the inhibition of PARPs does not have an influence on the ubiquitylation of FANCD2

Figure 3.5. FANCD2 and PARPs act independent of each other in preventing sensitivity of cells to MMC. **a)** Cells were treated first with Olaparib or the negative control and then with MMC for 1 hour or left untreated and whole cell extracts prepared 4, 8, 12 or 24 hours following MMC treatment. FANCD2 monoubiquitylation levels of the extracts were tested through western blotting **b)** Western blotting of the successful FANCD2 knock-down in FANCD2 siRNA vs. control siRNA treated cells **c)** Clonogenic survival assay of U2OS to MMC after control siRNA vs. FANCD2 siRNA treatment +/- Olaparib. The error bars represent the standard error of the mean of three independent experiments

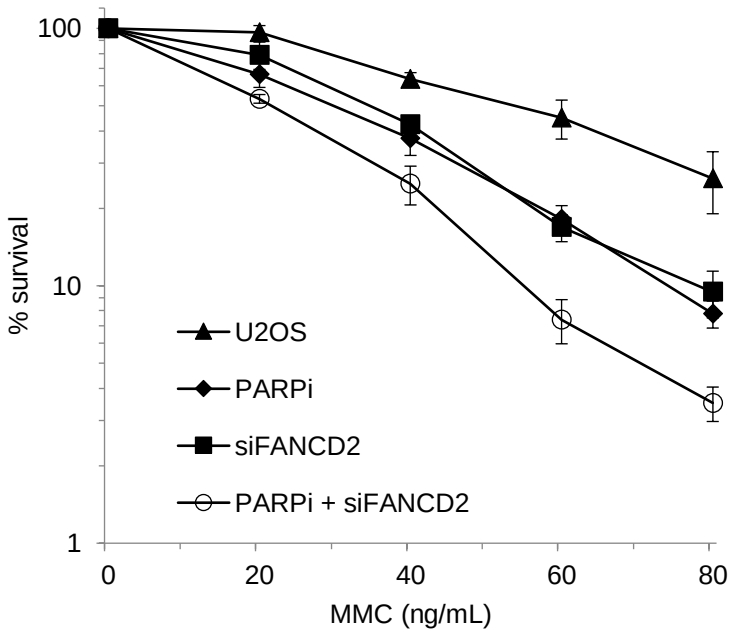
a)


250 kDA
130 kDA
100 kDA
55 kDA
35 kDA
MMC: – – + + – – + +
hours: 4h 8h
Olaparib : – + – + – + – +
250 kDA
130 kDA
100 kDA
55 kDA
35 kDA
αFANCD2
αActin
MMC: – – + + – – + +
hours: 12h 24h
Olaparib : – + – + – + – +

b)


U2OS WCE
– + FANCD2 siRNA
250 kDA
130 kDA
FANCD2
55 kDA
Actin
35 kDA

c)


100
10
1
% survival
0
20
40
60
80
MMC (ng/mL)
U2OS
PARPi
siFANCD2
PARPi + siFANCD2

in response to ICLs. PARPs thus act downstream of FANCD2 ubiquitylation or outside of the FA pathway. These results are in agreement with our previous findings, that the increase in ADP-ribosylation in cis-platin treated samples occurs significantly later than the rise of FANCD2 ubiquitylation levels (Bhagwat et al. 2009).

3.2.6. PARPs and FANCD2 do not act in the same ICL repair pathway

Given that PARP inhibition does not influence the monoubiquitylation of FANCD2, we investigated whether PARPs potentially act further downstream in the FA pathway. We therefore performed an epistasis analysis by means of clonogenic survival assay using FANCD2 siRNA and Olaparib.

U2OS cells were transfected with FANCD2 siRNA or control siRNA. One hour prior to MMC treatment, Olaparib or empty carrier solution (DMSO) were added to FANCD2 siRNA and control siRNA transfected cells. Cells treated with either only Olaparib or FANCD2 were both similarly more sensitive to MMC than control siRNA treated cells (Figure 3.5.), pointing towards a similar importance of PARPs and FANCD2 for the tolerance of human cells to interstrand crosslinks. FANCD2 knock-down cells treated with Olaparib were further sensitized to MMC against Olaparib treated or FANCD2 knock-down cells. This indicates that PARPs and FANCD2 act in separate pathways to prevent sensitivity of cells to ICLs. Since Olaparib treatment further exacerbates the phenotype against MMC observed in FANCD2 knock-down cells and this phenotype is more severe than the one in solely Olaparib treated cells, this phenotype can only in part be explained by an ICL repair defect resulting from the absence of FANCD2.

We therefore conclude that the contribution of PARPs to MMC tolerance might be the results of an ICL repair role of PARPs independent from the Fanconi Anemia pathway. However, using this approach we cannot exclude the possibility that knock-down of FANCD2 also inhibits any potential role of PARPs in ICL repair and that the observed increased sensitivity to MMC is owed to the toxicity of PARP trapping though Olaparib.

3.3. DISCUSSION

Poly-ADP-ribose-polymerases are involved in many cellular processes from mitosis and inflammation to cell death. PARPs contribute to the DDR through regulation of transcription and active roles in multiple DNA damage response pathways (De Vos et al. 2012). PARP1, PARP2 and PARP3 in particular exert functions in BER, HR, c-NHEJ and alt-NHEJ. In chemotherapy, PARP inhibition sensitizes BRCA-mutated ovarian, breast and pancreatic cancer cells to interstrand crosslink inducing agents such as cis-platin, MMC and psoralens. (Yarchoan et al. 2016, Liu et al. 2017, Song et al. 2017). A recent study in our lab in the model organism *Dictyostelium discoideum* has provided evidence that Adprt2, the *Dictyostelium* orthologue of PARP1, is crucial for maintaining wildtype sensitivity levels of cells to cis-platin (Gunn et al. 2016). We therefore considered whether PARPs in human cells also contributed to the resolution of interstrand crosslinks.

ADP-ribose levels in U2OS cells are elevated in response to cis-platin

ADP-ribose levels in U2OS cells continuously treated with cis-platin, a DNA interstrand crosslink inducing agent, were elevated from 12 hours after the start of the treatment. Interestingly, ADP-ribosylation appeared most prominently at 130 kDA from 12 hours ICL treatment and at 70 kDA from 20 hours. PARP1 has a molecular weight of 116 kDA. One ADP ribose unit weighs approximately 0.5 kDA (Leung 2014). Although we cannot formally attribute these bands to specific proteins, the observed 130 kDA band might correspond to poly-ADP-ribosylated PARP1 and the 70 kDA band to PARylated PARP2, which has a molecular weight of 66 kDA. We hypothesize that ICL induction might trigger PARP1 and PARP2 poly-ADP-ribosylation.

The increase in ADP-ribosylation in response to cis-platin treatment in human cells was apparent from 12 hours and 20 hours after the start of the treatment and increased further at 24 hours. We speculated how the ADP-ribose formation dynamics compared with other events in the cell cycle arrest and repair of ICLs, such as in the Fanconi Anemia pathway. Nuclear foci of the endonuclease FAN1, which is speculated to participate in DNA interstrand crosslink unhooking, appear after 16 hours in response to MMC treatment, while the appearance of γH2AX, a hallmark of double-strand break formation, was recorded by immunoblotting 24hr post MMC treatment (MacKay et al. 2010, Wang et al. 2011). The endonuclease dimer Mus81-Eme1, which incises DNA 5' of the ICL in the unhooking step of the FA pathway, induces DSBs after 24hr of MMC treatment (Hanada et al. 2006). These time frames coincide with the observed increase in ADP-ribosylation levels in response to cis-platin and might be connected to PARP catalytic activity. Other events within the Fanconi Anemia pathway, such as FANCD2 mono-ubiquitination and FANCM/FAAP24 dependent RPA formation occur significantly earlier, suggesting they might take place up-stream of ADP-ribosylation (Bhagwat et al. 2009, Huang et al. 2010)

Since we investigated an asynchronous cell population, we were unable to determine whether elevated ADP-ribosylation levels derived from a certain proportion of cells going through a specific part of the cell cycle. ADP-ribosylation in response to ICLs might arise independent of replication. ICLs are also repaired by nucleotide excision repair and this pathway is particularly important for cells in G0/G1 phase, where the FA pathway is absent, although its importance to reduce the genotoxic load of ICLs is disputed (Bergstralh and Sekelsky 2008). In NER, other repair dynamics than in the FA pathway apply (McHugh and Sarkan 2006, Wood 2010, Hashimoto et al. 2016)

Whereas many participants of ICL dependent NER and their order of function have been identified, the time frames of their appearance remain elusive (Zheng et al. 2003, Fousteri and Mullenders 2008, Enoiu et al. 2012, Williams et al. 2012). Given the delayed formation of ADP-ribose to MMC treatment, an initial role of PARPs in ICL damage recognition and DNA repair factor recruitment for replication independent ICL repair is unlikely. Examining poly(ADP)ribose levels in G1 arrested cells after MMC treatment could help determine the importance of PARylation for an NER mediated response to interstrand crosslinks.

PARP1 and PARP2 produce the overwhelming majority of PAR chains, making them strong candidates for causing the observed ADP-ribosylation. However, one caveat of our experiments is that most ICL inducing agents, including MMC and cis-platin, induce not only DNA interstrand crosslinks and that PARP1 and PARP2 are versatilely engaged in multiple DNA repair pathways. Only 5-14% of the lesions induced by MMC and 1-5% of lesions induced by cis-platin are ICLs, whereas a large proportion of the remainder consists of DNA intrastrand crosslinks in the case of cis-platin. MMC also produces other adducts and ROS (Wang et al. 2010). PARP1 facilitates the repair of UV induced damage, namely CPDs and 6-4 photoproducts through NER (Flohr et al. 2003, Fischer et al. 2014). Indeed, PARP catalytic response might thus be the result of cis-platin produced DNA intrastrand crosslinks, since both global genome and transcription coupled NER respond to these types of lesions (Jung and Lippard 2006). Nevertheless, a more recent publication points towards an ICL specific NER pathway, which the repair of cis-platin based intrastrand crosslinks does not depend on (Enoiu et al. 2012).

PARP inhibition or knock-out sensitizes U2OS and RPE-1 cells to MMC

We treated two independent human cell lines with the PARP inhibitor Olaparib, which sensitized them to MMC. Olaparib strongly inhibits PARP1 and PARP2 and to a lesser extent PARP3, all of which are involved in the DNA damage response. Although we cannot disregard the potential inhibitory activity of Olaparib against other PARPs, we hypothesized that PARP1 and PARP2 are responsible for the sensitivity of human knock-out cells against mitomycin C. To this end, we tested U2OS *parp1*Δ, *parp2*Δ and *parp1/2*Δ cells for their sensitivity against MMC in the presence or absence of Olaparib in clonogenic survival assays. Our results showed both *parp1*Δ and *parp2*Δ cells to be sensitive to MMC. Interestingly, *parp1/2*Δ cells were not more sensitive than *parp2*Δ cells, suggesting that PARP1 and PARP2 are epistatic in their ability to confer tolerance against ICLs in human cells. Both single KO cell lines, but not the double KO cell line were further sensitized by Olaparib. We therefore conclude, that PARP1 and PARP2 are the only PARPs contributing to ICL resistance and that the increased sensitivity to MMC in PARPi treated *parp1*Δ and *parp2*Δ cells is the result of a cytotoxic effect resulting from PARP1 or PARP2 trapping.

Our data points towards a potential role of PARP1 and PARP2 in the repair of ICLs through their catalytic activities. However, PARP1 and PARP2 domain architecture is more complicated, containing DNA and DDR protein binding activities. It would be intriguing to gain further insights into the context of PARP participation in ICL repair by investigating which functional domains of PARP1 and PARP2 are essential to prevent MMC sensitivity in U2OS cells. Both the DNA binding and the catalytic activity of PARP1 are vital for its functions in multiple DNA repair pathways. Nevertheless, PARP1 can also contribute to DNA damage repair through protein-protein interaction.

Future work could include introducing truncated PARP1 or PARP2 into PARP1 and PARP2 knock-out cells to determine the minimal sequence required of both PARPs to exercise their function in preventing sensitivity to ICL inducing agents. To identify this sequence would allow more precise speculations towards the role of PARPs in the resolution of DNA interstrand crosslinks. Additionally, immunoprecipitation and mass spectrometry experiments could be utilized to identify any novel interaction partners of PARP1 in the presence of ICLs and the interaction's dependence on the domain architecture of PARP1. Finally, *in vitro* assays could be employed to examine the DNA binding properties of PARP1 in the presence of ICLs and whether initiation of PARP1 and PARP2 catalytic activities requires the presence of specific DNA repair proteins in addition to ICL damaged DNA. Exemplary of this is a set-up recently used to study the role of PARP1 in delivering XPC to damaged DNA and promoting the initiation of NER (Robu et al. 2017).

PARP contribution to ICL repair is independent of the FA pathway

In order to determine a molecular function of PARPs in ICL repair, we were interested to see whether the monoubiquitination of FANCD2 in response to continuous MMC treatment is affected by the inhibition of PARPs through Olaparib. PARP inhibition did not suppress FANCD2 monoubiquitination, indicating that PARPs, if they are involved in the FA pathway, act downstream of FANCD2. Epistatic analysis revealed that cells sensitized to MMC by knock-down of FANCD2 were even further sensitized by addition of Olaparib. This points towards a role of PARPs in ICL repair outside of the Fanconi Anemia pathway. However, it was impossible to exclude the possibility that further sensitization of FANCD2 knock-down cells derived from the toxic effect of the trapping of PARPs at DNA by Olaparib. It therefore remains possible that PARPs

contribute to the tolerance of cells to ICL inducing agents through an active role in the Fanconi Anemia pathway. Unaltered FANCD2 ubiquitination levels in combination with the previously observed comparatively late appearance of ADP-ribosylation could be interpreted insofar that PARPs act downstream of the ubiquitination of FANCD2 at the unhooking step or in HR directed DSB repair or NER. From previously described functions of PARP1 and PARP2 and from the reported time frames for chromatin recruitment of nucleases involved in the FA pathway, one interesting line of inquiry would be to examine whether the recruitment to DNA damage sites of endonucleases involved in ICL unhooking is dependent on PARP catalytic activity. Impairment of HR and NER following ICL recognition and unhooking in PARP1 and PARP2 deficient cells might result in increased DSB formation or other DNA repair intermediate that can be quantified. Since ICL sensitivity results from both repair and checkpoint defects, the formation of ADP-ribose chains could also be important to promote cell cycle arrest (Ben-Yehoyada 2009). It would also be interesting to determine whether cells depleted of specific PARPs show a different cell cycle distribution in comparison to their parental cells in response to ICLs.

Alternatively, future work could include the inhibition of FANCD2 expression and other FA pathway proteins by siRNA in *parp1*Δ and *parp2*Δ cells lines. In addition, *parp1*Δ and *parp2*Δ sensitivity to MMC could also be tested in combination with the siRNA depletion of NER factors that were shown to produce milder sensitivities in response to ICL inducing drugs (De Silva et al. 2000). Whereas these factors might not be involved in ICL repair in S-phase cells, the resistance of cells to ICLs in their presence might be derived from their ICL repair capabilities in G0/G1 cells (Mouw et al. 2014). The effect of a combined absence of PARP1 or PARP2 and a specific NER factor on

cell survival against MMC would therefore be indicative of whether PARPs contribute to NER directed ICL repair.

The involvement of PARPs – especially PARP1 and PARP2 – in multiple DNA repair pathways of partial redundancy remains to constitute a substantial obstacle in the separation of PARP functions. Many suggestions for modes of action of PARPs in ICL repair in this discussion thus remain speculative and the particular contribution of PARPs to the tolerance to ICLs elusive.

Much work remains to identify the role of PARP1 and PARP2 in interstrand crosslink repair. The molecular basis of this can be established through multiple approaches, one being alkaline and neutral comet assays. These could resolve whether PARP1 and PARP2 contribute to the formation of single- or double-strand breaks following MMC treatment. Formation of single- but not double-strand breaks in cells depleted of PARP1 and PARP2 would indicate a role for the two proteins in translesion synthesis, whereas the absence of both single- and double-strand breaks would indicate a participation of PARP1 and PARP2 in the 5' and the 3' incision around the ICL site. If sensitivity of U2OS *parp1*Δ and *parp2*Δ cells to MMC was the results of a homologous recombination replication fork restart defect instead, an increase in DSBs, but not in SSBs formation could be expected.

Of course these are only a few of the many approaches that can be taken to unravel the nature of the implication of PARP1 and PARP2 in ICL repair. It is worth considering our observations of PARP catalytic activity and phenotypical studies in the light of the importance of PARPs for many physiological aspects of a cell, including chromatin

remodelling and transcription regulation. This gains particular significance considering an indication that levels of FANCG, a member of the FA core complex, are regulated by PARP1 acting as a transcription factor (Ko et al. 2012). Keeping an open mind therefore has to stand at the heart of expanding the importance of PARPs for the DDR into the realm of interstrand crosslink repair.

4. APLF AS A SENSOR IN ICL REPAIR

4.1. INTRODUCTION

APLF (aprataxin- and PNK-like factor) is a protein of the open reading frame C2orf13 of the human genome and a member of the FHA domain family. It possesses only one translated isoform and has homologues in many vertebrates including *mus musculus* and *gallus gallus* (Iles et al. 2007).

Over the years, a role for APLF in DNA single- and double- strand break repair (SSBR and DSBR respectively) has been revealed. The protein shares a forkhead-associated (FHA) domain with aprataxin and PNK – both proteins involved at different stages of the base excision repair (BER) pathway (Rass et al. 2007, Weinfeld et al. 2011). Via this domain, APLF binds the SSBR protein XRCC1 and the NHEJ protein XRCC4 (Becker-Jensen et al. 2007). Beyond that, APLF harbours a Ku70/80 interaction motif. (Kanno et al. 2007, Macrae et al. 2008, Shirodkar et al. 2013, Grundy et al. 2013). In addition, tandem poly(ADP)-ribose (PAR) binding zinc finger (PBZ) motifs are located towards the C-terminus of the protein. Catalytic activities of PARP1 and PARP3 are responsible for APLF recruitment to chromatin in SSBR and NHEJ, respectively. These events are dependent on the presence and functionality of the tandem PBZ repeats (Ahel et al. 2008, Rulten et al. 2008, Li et al. 2010). Furthermore, the C-terminus of APLF possesses an acidic H3/H4 tetramer binding motif that may contribute to its retention at chromatin (Mehrotra et al. 2011). Finally, PBZ-dependent phosphorylation of APLF through ATM serine/threonine kinase at Ser116 is crucial for its auxiliary function in NHEJ. (Macrae et al. 2008).

Until recently, no other DNA repair functions have been uncovered or suggested for APLF. Nevertheless, the diversity of its multiple interaction domains leaves room for speculation on undiscovered functions of APLF in other DNA damage response (DDR) pathways.

Our research group undertook work in the model organism *Dictyostelium discoideum* to investigate the repair of DNA interstrand crosslinks. Following a search for proteins with ADP-ribose binding domains in *Dictyostelium,* a novel protein, named APL, was discovered (aprataxin/APLF-and-PNKP-like protein). Similar to APLF, PNK and aprataxin, APL carries a N-terminal FHA domain in addition to a central PBZ domain and C-terminal macrodomain, another PAR-binding module (Gunn et al. 2016). The PBZ domain and the macrodomain both exhibited PAR-binding activities. Whilst no enrichment of APL in chromatin is detected after MMS induced DNA base damage (methyl-methane sulfonate) or bulky adducts induced by 4-nitroquinoline-1-oxide (4NQO), it is recruited to chromatin in response to the DNA interstrand crosslink inducing agent cis-platin. This recruitment is dependent on the presence of the PAR binding macrodomain and on Adprt2, indicating a role for ADP-ribosylation in recruiting and/or retaining APL at sites of DNA ICLs. APL also carries a PBZ domain, although its contribution to chromatin binding in response to DNA damage has not been tested. Due to the structural similarities of *Dictyostelium* APL and human APLF, including the presence of an FHA domain and DNA binding motif, we speculated that APLF might be the human orthologue of APL. APLF carries a tandem PBZ repeat and binds to chromatin in SSBR and NHEJ dependent on PARP1 and PARP3 catalytic activity, respectively. APLF might therefore have a hitherto unrecognized role in the detection and/or the repair of DNA ICLs (Iles et al. 2007).

The experiments described in this chapter explored this hypothesis by determining whether, similar to *Dictyostelium* APL, human APLF participates in the resolution of ICLs. Additionally, we were also interested to determine the domains in APLF that contribute towards this function.

4.2. RESULTS

4.2.1. APLF is recruited to chromatin in response to interstrand crosslinks

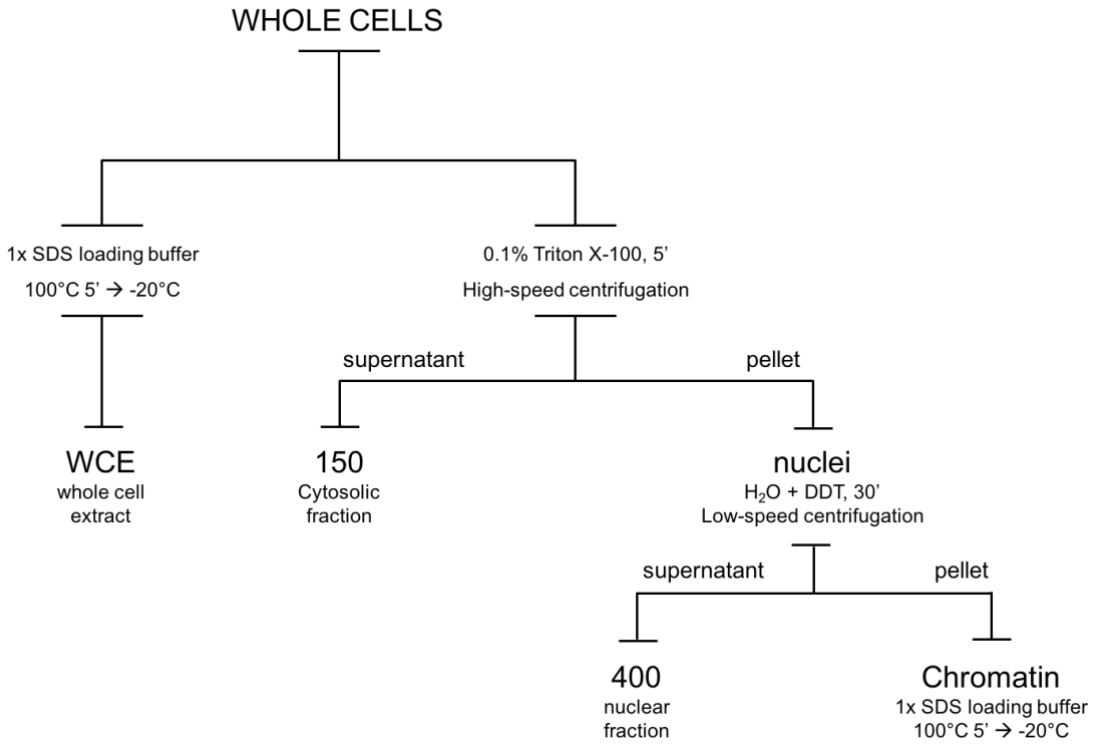
Dictyostelium discoideum APL is recruited to chromatin following treatment with the interstrand crosslink inducing drug cis-platin. Given our speculation that APLF is the human orthologue of *Dictyostelium* APL, we first investigated if APLF is subsequently recruited to sites of DNA interstrand crosslinks.

MMC is a DNA interstrand crosslinking inducing agent used in anti-cancer therapy treatment (Blasiak et al. 2017). We assessed whether APLF is enriched in chromatin following exposure of cells to this DNA damaging drug. RPE-1 cells were transfected with a plasmid expressing myc-tagged APLF. Following a transient exposure to 1.65 µM MMC for 1 hour, cells were harvested at multiple time points after the end of the treatment and whole cell extracts and chromatin fractions prepared (Figure 4.1. a). APLF levels at the different time points after MMC treatment were assessed by immunoblotting with myc antibody.

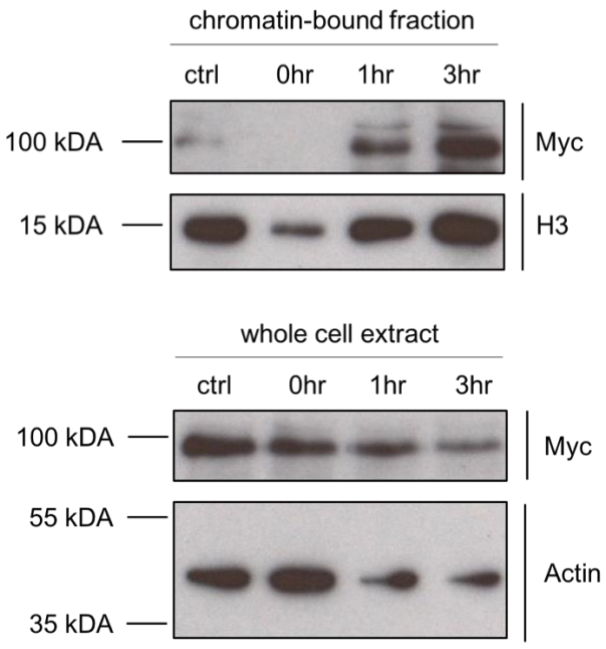
APLF levels in chromatin fractions 1 and 3 hours after MMC treatment were highly elevated against the control sample indicating rapid recruitment of APLF to chromatin and possibly ICLs. APLF was completely absent from the 0 hour sample, likely due to a strongly diminished sample concentration indicated by the H3 loading control. The loading controls for the other samples varied less strongly, but might have passed the linear range. APLF levels in the whole cell extracts relative to the overall sample concentrations remained mostly unchanged throughout, even though uneven loading

Figure 4.1. APLF is recruited to chromatin in response to MMC induced ICLs. **a)** Scheme of chromatin fractionation and whole cell extract preparation. **b)** RPE-1 cells were treated with 1.65 µM MMC for 1 hour. Whole cell extracts and chromatin-bound fractions were prepared at the indicated time points after the end of the MMC treatment and APLF levels recorded by Western blotting. Actin and H3 were used as loading controls for the whole cell extracts and chromatin-bound fraction, respectively.

a)


WHOLE CELLS
1x SDS loading buffer
100°C 5' → -20°C
0.1% Triton X-100, 5'
High-speed centrifugation
supernatant
pellet
WCE
whole cell extract
150
Cytosolic fraction
nuclei
H2O + DDT, 30'
Low-speed centrifugation
supernatant
pellet
400
nuclear fraction
Chromatin
1x SDS loading buffer
100°C 5' → -20°C

b)


chromatin-bound fraction
ctrl
0hr
1hr
3hr
100 kDA
Myc
15 kDA
H3
whole cell extract
ctrl
0hr
1hr
3hr
100 kDA
Myc
55 kDA
Actin
35 kDA

complicates the interpretation of these results as well. In spite of the fact that irregular sample concentration does not allow for a definitive judgement, elevated APLF levels in the 1 dn 3 hour chromatin fractions appear not to be the result of differences in the APLF expression levels between the samples at the different time point. Instead, the amount of APLF binding to chromatin increases sharply after MMC treatment due to an active APLF recruitment.

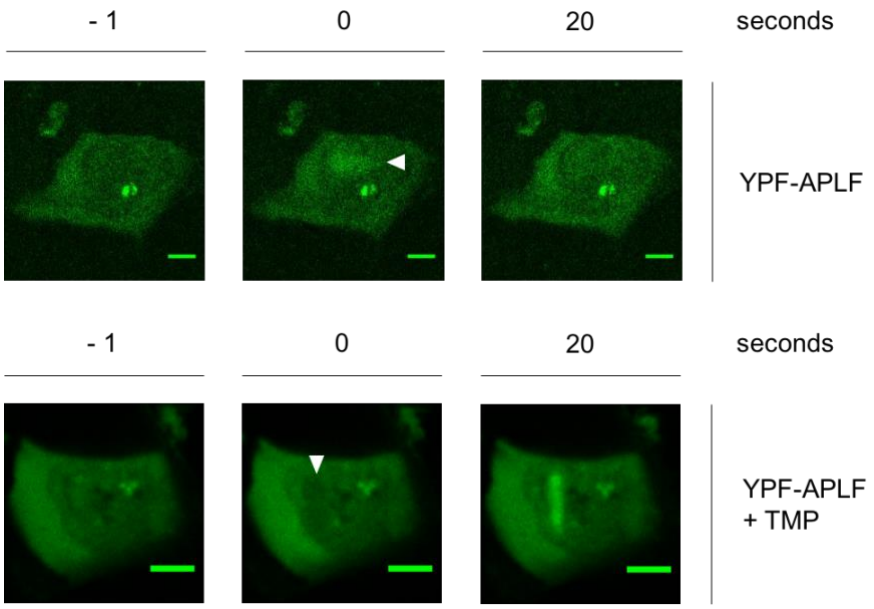
4.2.2. APLF is recruited to TMP induced interstrand crosslinks

Although mitomycin C is widely used as a DNA interstrand crosslink inducing agent, only 4-15% of the induced lesion are ICLs. Other adducts, including DNA intrastrand crosslinks and ROS also arise from MMC treatment (Wang et al. 2010). To verify that the localization of APLF to chromatin is dependent on ICL formation, we used a more quantitative, DNA ICL damage specific method – laser microirradiation in combination with the psoralen 4,5',8-Trimethylpsoralen (TMP).

TMP is a furocoumarin that intercalates into the DNA. When absorbing light in the near UV range, it photoreacts with pyrimidines to form DNA interstrand crosslinks (Ross and Yu 1988). Through live cell imaging, we assessed whether APLF was recruited to laser stripes of 405 nm wavelength in combination with TMP. RPE-1 cells were transfected with a plasmid carrying YFP-APLF, generously provided by Dr Ivan Ahel at the Sir William Dunn School of Pathology, University of Oxford. In previous studies, immunofluoresence set-ups detected nuclear as well as cytosolic APLF staining in stably expressing and transiently transfected, untreated cells (Iles et al. 2007, Ahel et

Figure 4.2. Establishing conditions to detect an ICL specific localization of APLF to DNA damage sites. The green line in the images indicates a lenth of 10 µm, the white arrows indicate the location of the laser stripe induction. **a)** RPE-1 cells transiently expressing YFP-APLF were microirradiated with a 405 nm laser at 13% laser intensity, with 5 cycles at 21 ms exposure time each in the presence or absence of TMP **b)** Transiently YFP-APLF expressing RPE-1 cells were microirradiated with a 405 nm laser at 6,5% intensity, with 1 cycle at 1 ms exposure time in the presence or absence of TMP.

a)


- 1
0
20
seconds
YPF-APLF
- 1
0
20
seconds
YPF-APLF
+ TMP

b)

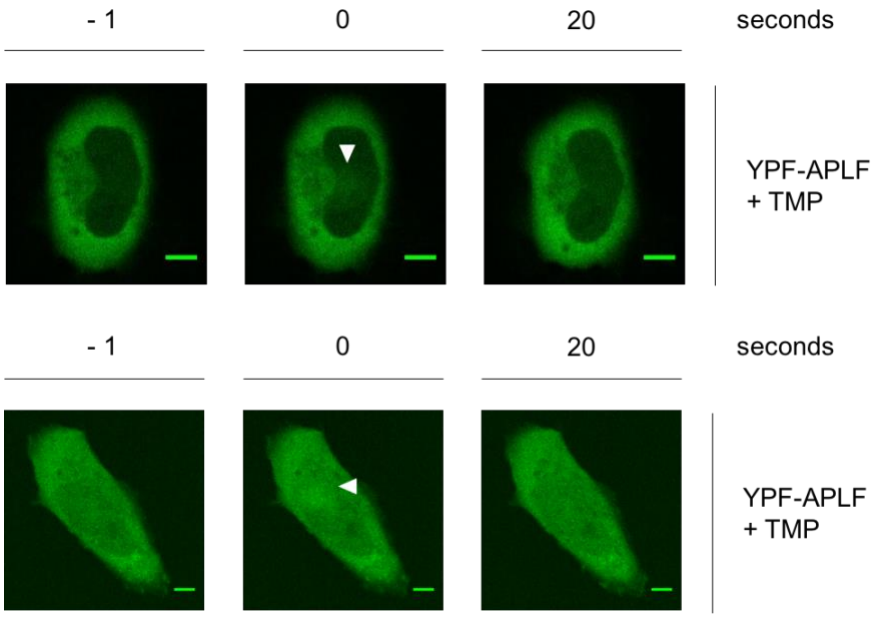
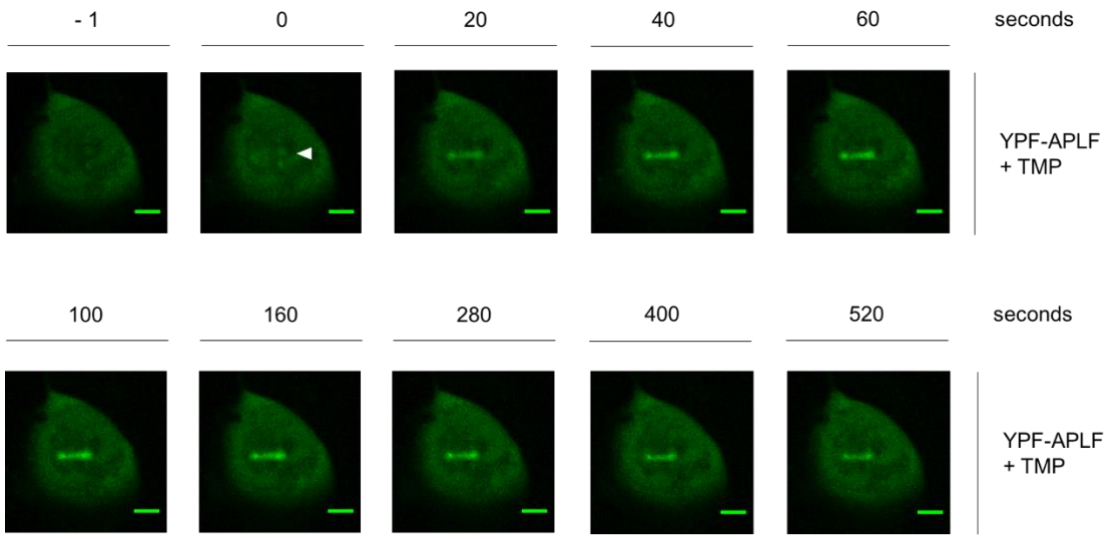
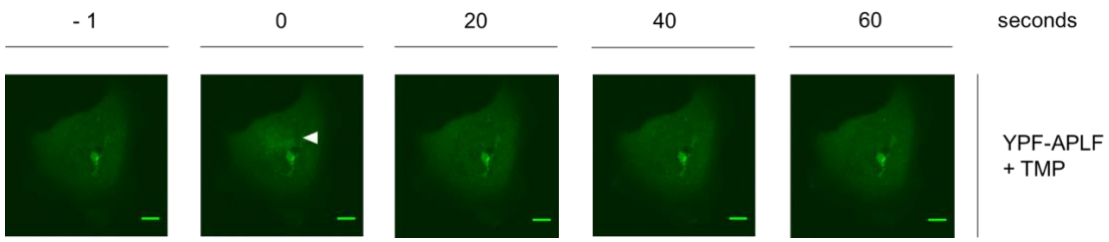

- 1
0
20
seconds
YPF-APLF
+ TMP
- 1
0
20
seconds
YPF-APLF
+ TMP

Figure 4.3. APLF localization to microirradiation induced damage. The green line in the images indicates a lenth of 10 µm, the white arrows indicate the location of the laser stripe induction. **a)** RPE-1 cells transfected with YFP-APLF were treated with TMP for 30 minutes before microirradiation with a 405 nm laser at 13% laser intensity, for 5 cycles at 21 ms exposure time each. YFP-APLF recruitment to the laser stripe was recorded at the indicated time points. **b)** RPE-1 cells were transfected with YFP-APLF, TMP added and the celles exposed to a 405 nm laser at 13% laser intensity, for 5 cycles at 21 ms exposure time each**.** YFP-APLF recruitment to the laser stripe was recorded at the indicated time points. **c)** The strength of the YFP-APLF stripe normalized against the background signal of the cell was quantified and the intensity normalized against the YFP-APLF signal in the cell before microirradiation. The values for TMP treatment in combination with microirradiation represent the mean of 5 independent measurements, the values for microirradiated cells are represented by the mean of 3 independent experiments.

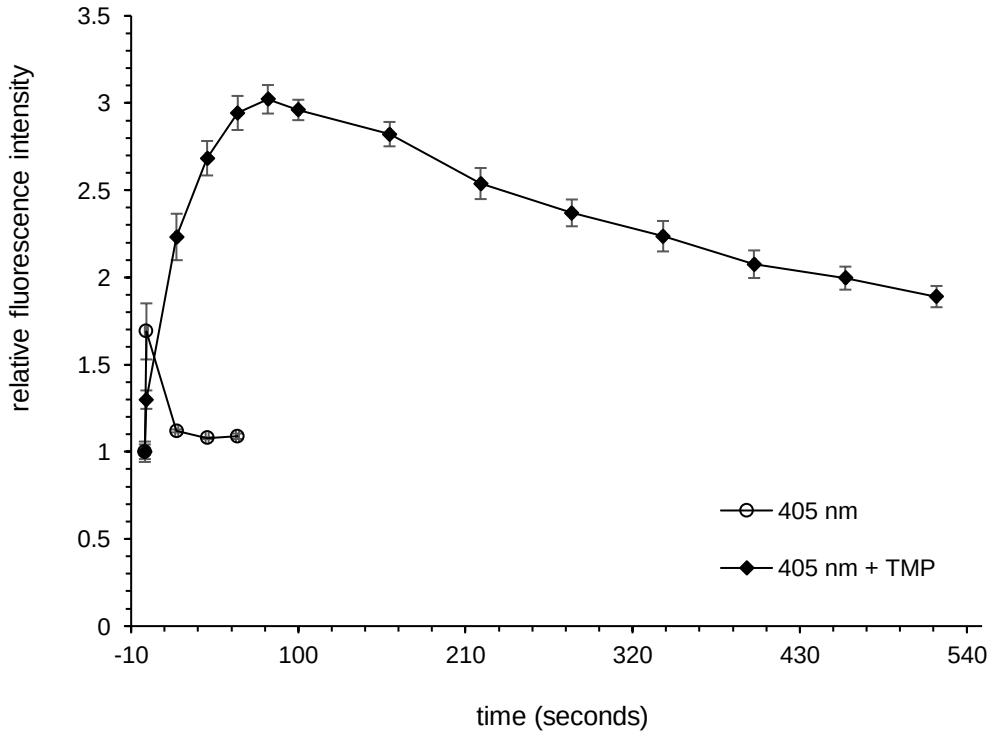
a)


- 1
0
20
40
60
seconds
YPF-APLF
+ TMP
100
160
280
400
520
seconds
YPF-APLF
+ TMP

b)


- 1
0
20
40
60
seconds
YPF-APLF
+ TMP

c)


relative fluorescence intensity
3.5
3
2.5
2
1.5
1
0.5
0
-10
100
210
320
430
540
time (seconds)
405 nm
405 nm + TMP

al. 2008, Macrae et al. 2008) Rulten et al. 2008, Rulten et al. 2011, Shirodkar et al. 2013). Stable expression of GFP tagged APLF in cells resulted in its nuclear presence in live cell laser stripe experiments (Bekker-Jensen et al. 2007, Li et al. 2010, Fenton et al. 2013) However, live cell laser stripe experiments featuring transient transfection of human cells with YFP-APLF resulted in whole-cellular staining (Mehrotra et al. 2011, Grundy et al. 2013). The fluoresesence signal in these findings was stronger in the cytosol than in the nucleus, which is in line with our obersvations.

TMP was added 30 minutes before the start of the experiment to be allowed to intercalate into DNA before laser stripe induction. However, the laser itself also induces single and double strand breaks and APLF has previously been shown to act in NHEJ. We therefore had to establish conditions, under which the intensity, duration and number of irradiation cycles would not trigger strong APLF recruitment in the absence of TMP. We defined conditions, under which only residual, diffused APLF recruitment could be observed immediately after microirradiation, but a clear, prolonged localization of YFP-APLF to the laser stripes was visible after microirradiation in combination with TMP (Figure 4.2.). Irradiation was carried out at 21 ms for 5 cycles with a laser intensity of 13%. Further reduction in the settings to 1 ms,1 cycle and 6.5% laser intensity still left a blurred localization signal immediately after damage induction in the absence of TMP, but also resulted in the disappearance of YFP-APLF stripes 20 second after irradiation together with TMP. We had thus created conditions under which we were able to observe the response of APLF specifically to DNA interstrand crosslinks.

We recorded the dynamics of YFP-APLF recruitment to laser + TMP induced damage

and observed a peak in the intensity of the fluorescence signal at 80 seconds following the end of microirradiation, after which the signal gradually decreases (Figure 4.3. a and c). The recruitment dynamics of YFP-APLF after 405 nm + TMP are represented by 5 independent time course measurements. In comparison, irradiated cells in the absence of TMP showed a significantly smaller local concentration of APLF at the irradiated area, which was only visible immediately after microirradiation. At 20 seconds, fluorescence had decreased almost to background levels (Figure 4.3. b and c). For this negative control time course experiment, the mean value of three independent measurements was blotted in Figure 4.3. c.

We concluded, that APLF is rapidly recruited to sites of interstrand crosslinks, appearing first at 20 seconds after irradiation, peaking at 80 seconds and then gradually declining. We were therefore able to confirm the results observed in 4.2.1., that APLF is rapidly recruited to chromatin and that this recruitment is dependent on the presence of ICLs. The gradual reduction of the GFP signal furthermore indicates that APLF persists at sites of DNA interstrand crosslinks.

4.2.3. RPE-1 cells treated with siAPLF show increased sensitivity to MMC

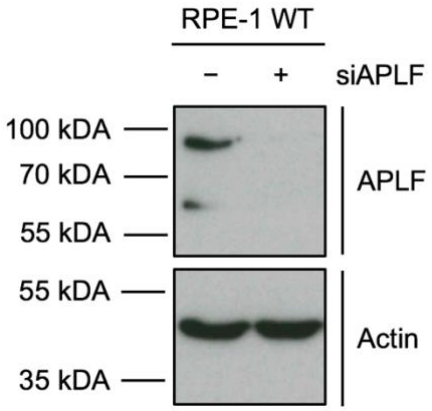
Having established that human APLF is enriched in chromatin following MMC induced damage as well as laser + TMP induced ICLs, we next wanted to address whether the absence of APLF had phenotypical consequences on cells treated with mitomycin C. To test this, we knocked down APLF by siRNA in RPE-1 cells and compared their survival rate to increasing concentrations of MMC in a clonogenic survival assay with

control siRNA treated cells. Successful knock-down of APLF was verified through immunoblotting of whole cell extracts from APLF siRNA and control siRNA transfected cells harvested at the start of the MMC treatment (Figure 4.4.).

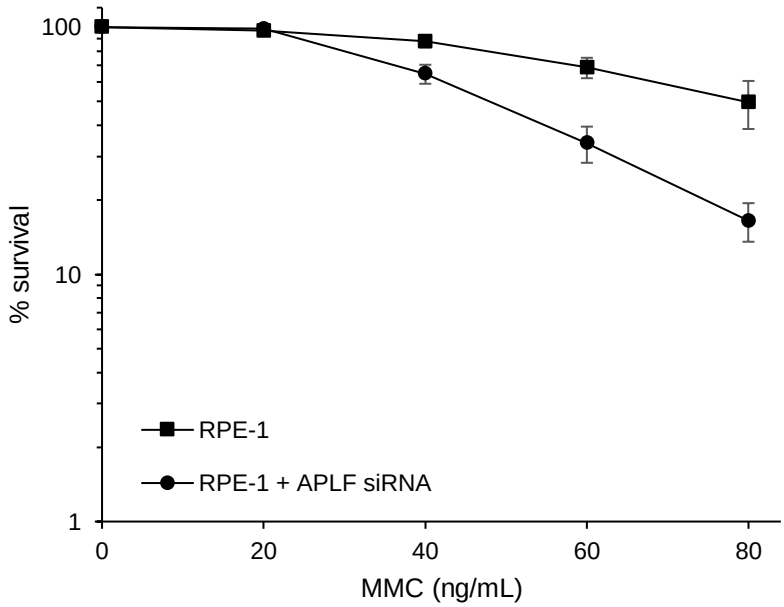
APLF siRNA treated RPE-1 cells were significantly more sensitive to MMC than their control siRNA treated counterpart at the three highest MMC concentrations (Figure 4.4. b). p-values for all concentrations at a significance level of 5% are given in Figure 4.4. c. We concluded that knocking-out APLF in RPE-1 cells leads to decreased survivability of human cells to MMC.

Figure 4.4. RPE-1 cells are sensitized to MMC following APLF siRNA treatment. **a)** RPE-1 cells were transfected with control siRNA or APLF siRNA and expression levels of APLF detected through Western Blotting **b)** control siRNA and APLF siRNA transfected cells were seeded in a clonogenic survival assay and survival to MMC tested. The values and error bars represent the mean and standard error of the mean of three independent experiments. **c)** p-values were calculated for the sensitivity of RPE-1 control siRNA vs. APLF siRNA transfected cells to MMC from the values of three independent experiments.

a)



b)



c)

MMC (ng/mL)	20 ng/mL	40 ng/mL	60 ng/mL	80 ng/mL
p-value	0,74	0,037	0,016	0,043

4.3. DISCUSSION

The *Dictyostelium* protein APL possesses a similar domain architecture to human APLF. It associates with chromatin in cells after treatment with cis-platin. This is dependent on Adprt2, the *Dictyostelium* orthologue to human PARP1. APL holds a PAR binding PBZ motif and macro domain (Gunn et al. 2016.). Its Adprt2 dependent chromatin recruitment might therefore be dependent on the catalytic activity of Adprt2 and its ability to bind PAR chains. On the basis of this information, we were encouraged to look for a role of APLF, the potential human orthologue of APL, in the repair of ICLs, a particularly deleterious DNA damage that prevents the seperation of complementary DNA strands and causes replication fork stalling and collapsing.

APLF is recruited to chromatin following ICL formation

We showed that APLF was rapidly recruited to chromatin in response to MMC induced damage and to ICLs formed by TMP in combination with microirradiation. This suggests an early role for APLF in sensing or removing ICLs or in the recruitment of other repair factors. APLF possesses a 3' exonucleolytic activity towards double-stranded DNA and endonucleolytic activity to single-stranded DNA (Kanno et al. 2007, Li et al. 2011, Pannunzio et al. 2014, Menon et al. 2016). This may point towards an early role of APLF in ICL unhooking, especially considering that the identity of the endonucleases cutting 3' and 5' of the interstrand crosslink have been a recent topic of controversy. FAN1, XPF, SLX4 and the dimer MUS81-EME1 have all been shown to be necessary to prevent the sensitivity of cells to MMC and cis-platin (Hanada et al. 2006, Svendsen et al. 2009). In FA pathway initiated ICL repair of two converging, stalled replication forks, strand incision followed by TLS generates a DSB. The time frames reported for

DSB formation, however, strongly differ from the rapid recruitment of APLF observed in our experiments. A role of APLF in the initial ICL recognition step of the Fanconi Anemia pathway might therefore be more likely. Besides FANCM/FAAP24, MHF1, MHF2 and UHRF1 have been reported as ICL sensors (Williams et al. 2013, Liang et al. 2015, Lopez-Martinez 2016). APLF might belong to this group of proteins. It would therefore be interesting to see whether it influences the recruitment of FANCD2 to ICLs or its monoubiquitylation by the FA core complex.

Alternatively, APLF might participate in NER, which has been implicated in ICL repair of G0/G1 phase cells (McHugh and Sarkan 2006, Wood 2010, Dean and West 2011, Hashimoto et al. 2016). It lacks the initial step of FA core complex formation. Strand incision might therefore arise comparatively quickly in NER directed ICL repair. Given the nucleolytic activity of APLF and its rapid recruitment to chromatin, it might be involved in ICL unhooking or in flap removal in NER mediated ICL repair. Evidence has been provided for the repair of ICLs by a TC-NER pathway that does not resolve intrastrand crosslinks such as a 1,3-GTG cis-platin intrastrand crosslink (Enoiu et al. 2012). Cells deficient in repair factors involved in TC-NER such as XPA and XPB are sensitive to cisplatin, while absence of the GG-NER factor XPC did not influence cisplatin sensitivity. APLF could therefore act in ICL specific TC-NER to prevent the sensitization of cells to ICL inducing drugs.

APLF localization to laser stripes in combination with TMP or in response to MMC induced damage indicates an active role APLF in ICL removal. To determine at which stage of the cell cycle and in what pathway APLF participates in ICL repair presents an important future goal. There are multiple ways to follow up this question. One way

to investigate APLF participation in ICL unhooking and flap removal would be by exploring whether γH2AX levels in MMC treated cells decline in the absence of APLF. If DNA interstrand crosslinks are not unhooked, translesion synthesis (TLS) is impaired and DSB formation prevented. APLF might also effect the recruitment or catalytic activity of TLS and NER factors including polymerase ζ, polymerase ν and others, the localization of which to DNA damage sites can be detected by immunoblotting or cell imaging. In continuation of our microirradiation experiment, YFP-tagged truncated forms of APLF could be introduced into cells, so determine, which domain(s) of the protein are required for its DNA damage localization and retention at sites of DNA interstrand crosslinks.

The difference in timing of ADP-ribosylation and APLF recruitment to chromatin in response to cis-platin and MMC treatment, respectively, suggests that APLF might act independently of PARP catalytic activity. Whether PARPs and APLF act at different stages of the same pathway or in different pathways is unclear. However, retention of APLF at laser stripe + TMP induced DNA damage sites could point towards prolonged presence of APLF at DNA interstrand crosslinks. In NHEJ, APLF was shown to link multiple stages of the repair pathway through its interaction domain. The same might be true for APLF in ICL repair. Early APLF recruitment could therefore still be linked to the observed ADP-ribosylation events. APLF recruitment experiments featuring later time points could provide insights into the dissociation times of APLF.

APLF siRNA sensitizes RPE-1 cells to MMC

The importance of APLF for ICL repair is visualized in the sensitivity of cells to ICLs in its absence. Whereas the loss of non-essential DNA repair facilitating or accelerating

domains or proteins does often not have an effect on the sensitivity of cells to repair a particular type of DNA damage, the absence of essential repair factors in a specific repair pathway can trigger sensitivity of cells to one sort of DNA damage or another. APLF might therefore exhibit not just an auxiliary, but vital role in the successful repair of ICLs through either the FA pathway or NER. Considering our observations of an active role of APLF in ICL repair, epistatic analysis through clonogenic survival assays could help answer the question of which pathway is the source of the ability of APLF to protect cells from ICL sensitivity. If the knock-out of APLF in addition to the removal of well-studied ICL repair proteins such as FANCD2, does not further sensitize cells to ICL inducing drugs, this would support a role of APLF in the pathway of the removed protein. Further sensitization in cells following the combined absence of both proteins would indicate that they function in separate pathways. Sensitivity of APLF depleted cells could additionally be studied in a cell cycle context, given that the Fanconi Anemia pathway acts specifically in replicating cells, whereas NER directed ICL repair could be found in G0/G1 cells.

In conclusion, the ability of APLF to prevent sensitivity of cells to MMC might be linked to its recruitment to interstrand crosslinks. To determine which APLF domain is vital for the survival of cells against MMC will provide new insights into its function in ICL repair and the nature of its recruitment to ICLs. APLF harbors several protein binding domains and a PBZ domain, which could be essential for cell survival against MMC as well as chromatin localization. This way, a connection could be formed between our recruitment and phenotypical studies.

5. THE ROLE OF APLF IN ICL REPAIR

5.1. INTRODUCTION

APLF has previously been identified as a repair protein that acts in NHEJ and BER. It's contribution to these pathways relies on its ability to bind other DNA repair proteins. In BER, APLF interacts with XRCC1 through its fork head associated (FHA) domain and its recruitment to SSBs is dependent on PARP1 (Iles et al. 2007, Bekker-Jensen et al. 2007, Kanno et al. 2007). Conversely, the tandem PAR binding PBZ repeats of APLF are required for its recruitment to sites of SSBs and DSBs (Li et al. 2010). Through FHA domain dependent interaction with XRCC4-Ligase IV, it promotes the heterodimer's retention at sites of DNA damage (Grundy et al. 2013). Furthermore, it interacts with the Ku heterodimer and thus serves as a scaffold protein for a bigger complex, bringing proteins of different stages of the NHEJ pathway together (Shirodkar et al. 2103, Grundy et al. 2013, Hammel et al. 2016).

The absence of APLF sensitizes cells to DNA single and double strand breaks and it is also required for the resistance of cells to ɣ-irradiation and DNA adducts induced by MMS (Kanno et al. 2007, Bekker-Jensen et al. 2007, Tong et al. 2016). A direct correlation between specific APLF domains and the survival of cells to different types of DNA damage was provided in the case of cells carrying APLF mutated at its phosphorylation that displayed increased radiosensitivity (Fenton et al. 2013). Deletion of the APLF C-terminal acidic domain, reported to be responsible for histone binding, produces a reduced survival rate of cells against Phleomycin (Mehrotra et al. 2011). In short, the absence of specific APLF domains can have phenotypic consequences.

The nature of the reported domains crucial for cell survival in both studies referred to above is that of a protein binding motif, highlighting the importance of protein-protein interactions for the function of APLF in DNA repair.

Given the sensitivity of APLF siRNA knock-down cells to MMC, we were interested to see whether knock-out of APLF through CRISPR Cas9 also sensitized cells to MMC. Furthermore, we wanted to assess the domain responsible for this sensitivity and if it was different from the APLF domains required for the tolerance against other types of DNA damage mentioned above, implicating it in novel regulatory mechanisms. To this end we reintroduced truncated or mutated forms of APLF into *aplf*Δ cells. This would allow us new insights into which function APLF could potentially play in the removal of DNA interstrand crosslinks.

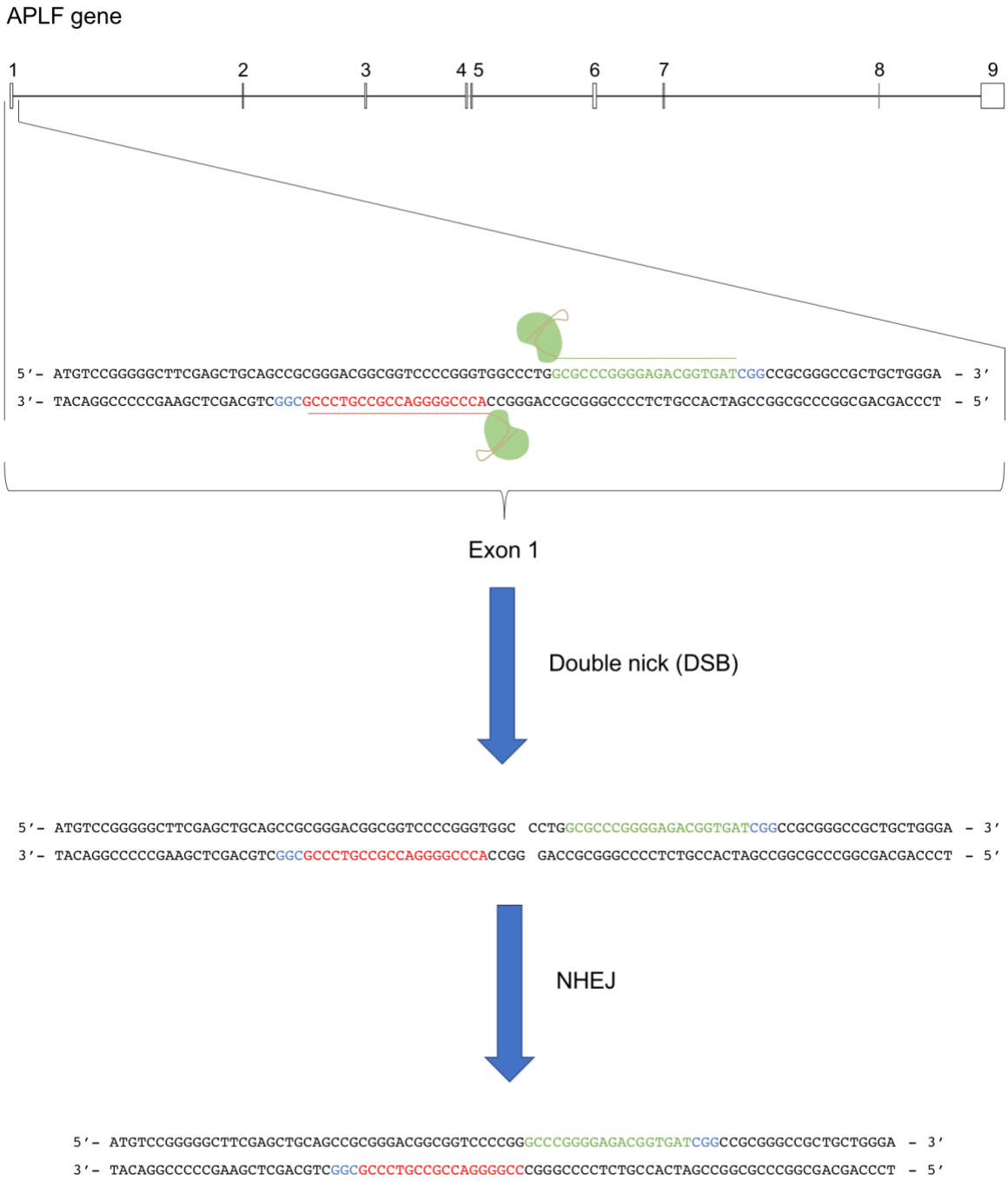
5.2. RESULTS

5.2.1. Generation of RPE-1 *aplf*Δ knock-out cell lines

Having established in the previous chapter that knocking down APLF through siRNA sensitizes cells to MMC induced damage, we wanted to confirm these findings with a more rigorous method and examine whether the same phenotypical observation is true for cells disrupted in the APLF gene. To address this, we generated *aplf*Δ knock-out cell lines using CRISPR Cas9 genome editing in RPE-1 cells and assessed the impact of the loss of function of the gene on ICL repair.

We decided to employ the CRISPR Cas9n double nickase method to disrupt the APLF gene in RPE-1 cells (CRISPR 101 2nd Edition, Trevino & Zhang 2014). Whilst the initial CRISPR Cas9 knock-out method introduces a DSB at the target sequence of the guide RNA (gRNA), the double nickase strategy introduces two SSBs in close proximity. Therefore, the enzyme requires two gRNAs to cut both DNA strands. Two physically close SSBs on opposing DNA strands produce a DSB. This greatly reduces off-target effects, since the probability for the two gRNAs to bind in close proximity at a different location in the genome is significantly reduced. APLF possesses only one isoform that includes exon1. Since we wanted to interrupt translation of the mRNA as early as possible, gRNAs where designed with target sites in that region of the gene. Unfaithful DNA repair by NHEJ can produce deletions of varying length. These could potentially remove the start codon of the translated region of APLF, or stretch past exon 1 into the following intron. Two target sequences were found in the centre of exon 1, which both harbour a gRNA target site and a PAM motive (5' NGG 3'), allowing the initial

Figure 5.1. The CRISPR Cas9 double nickase strategy for the APLF gene. Two gRNAs bind target sequences (coloured in red and in green) on the two homologous strands of exon 1 of the APLF gene. Cas9n introduces two close, single-strand breaks on the opposing strands, leading to double strand break formation. The double strand break is unfaithfully repaired by non-homologous end-joining, which produces insertions or deletions in exon1 of the gene.


APLF gene
1
2
3
4 5
6
7
8
9
5′- ATGTCCGGGGGCTTCGAGCTGCAGCCGCGGGACGGCGGTCCCCGGGTGGCCCTGGCGCCCGGGGAGACGGTGATCGGCCGCGGGCCGCTGCTGGGA - 3′
3′- TACAGGCCCCCGAAGCTCGACGTCGGCGCCCTGCCGCCAGGGGCCCACCGGGACCGCGGGCCCCTCTGCCACTAGCCGGCGCCCGGCGACGACCCT - 5′
Exon 1
Double nick (DSB)
5′- ATGTCCGGGGGCTTCGAGCTGCAGCCGCGGGACGGCGGTCCCCGGGTGGC CCTGGCGCCCGGGGAGACGGTGATCGGCCGCGGGCCGCTGCTGGGA - 3′
3′- TACAGGCCCCCGAAGCTCGACGTCGGCGCCCTGCCGCCAGGGGCCCACCGG GACCGCGGGCCCCTCTGCCACTAGCCGGCGCCCGGCGACGACCCT - 5′
NHEJ
5′- ATGTCCGGGGGCTTCGAGCTGCAGCCGCGGGACGGCGGTCCCCGGGCCCGGGGAGACGGTGATCGGCCGCGGGCCGCTGCTGGGA - 3′
3′- TACAGGCCCCCGAAGCTCGACGTCGGCGCCCTGCCGCCAGGGGCCCGGGCCCCTCTGCCACTAGCCGGCGCCCGGCGACGACCCT - 5′

binding of the gRNAs (Figure 5.1.). The target first site stretches from 25 bp – 48 bp and the second site from 55 bp – 78 bp. Given exon 1 of the APLF gene is 96 bp long, this places the target sites in its centre, as required.

The work flow for generating *aplf*Δ cells is illustrated in Figure 5.2. We transfected RPE-1 cells with two plasmids, one carrying the cDNA for one gRNA and Cas9, the other carrying the cDNA for the other gRNA and Cas9. Afterwards, cells were selected in Puromycin. After 10 days, clones were picked and screened by PCR and agarose gel electrophoresis for the introduction of indels in exon 1 of APLF. Chromatin was prepared from each clone and a sequence spanning the CRISPR Cas9 cut site amplified by PCR. In the RPE-1 parental strain this sequence is 319 bp long (Figure 5.3.). Agarose-gel electrophoresis was used to compare the length of the amplified sequences of the different clones with the parental sequence length. Of the 117 clones screened, 10 were homozygous for indels in the amplified sequence, whilst 7 clones were heterozygous mutants, i.e. harbouring a WT band (Figure 5.3 b, c and d) 5 of the homozygous clones carried only a single shifted band (Figure 5.3 b, c and e). Only clones carrying no WT APLF bands were selected for further investigation. The DNA bands were extracted from the gel, cloned into transformation vectors and grown in *E. coli*. After purification by mini-prep the plasmids were sent for DNA sequencing. Using this strategy, we generated 3 independent RPE-1 *aplf*Δ cell lines, the sequencing data of which is shown in Figure 5.4 b. RPE-1 *aplf*Δ*1* carries a 26 bp deletion, *aplf*Δ*2* a 53 bp deletion and *aplf*Δ*3* a 13 bp deletion.

To further validate these cell lines, APLF expression levels were tested in these three clones. Due to its highly acidic C-terminal domain, APLF does not run at its predicted

Figure 5.2. Schematic description of the generation and verification of *aplf*Δ knock-out cell lines. Cells were transfected with two vectors carrying the gRNAs and Cas9. Selection by Puromycin revealed successfully transfected clones. Resistant cells were harvested and APLF knock-out verified by PCR amplification of the CRISPR Cas9 cut site and sequencing. Furthremore, APLF expression levels were tested by immnoblotting.

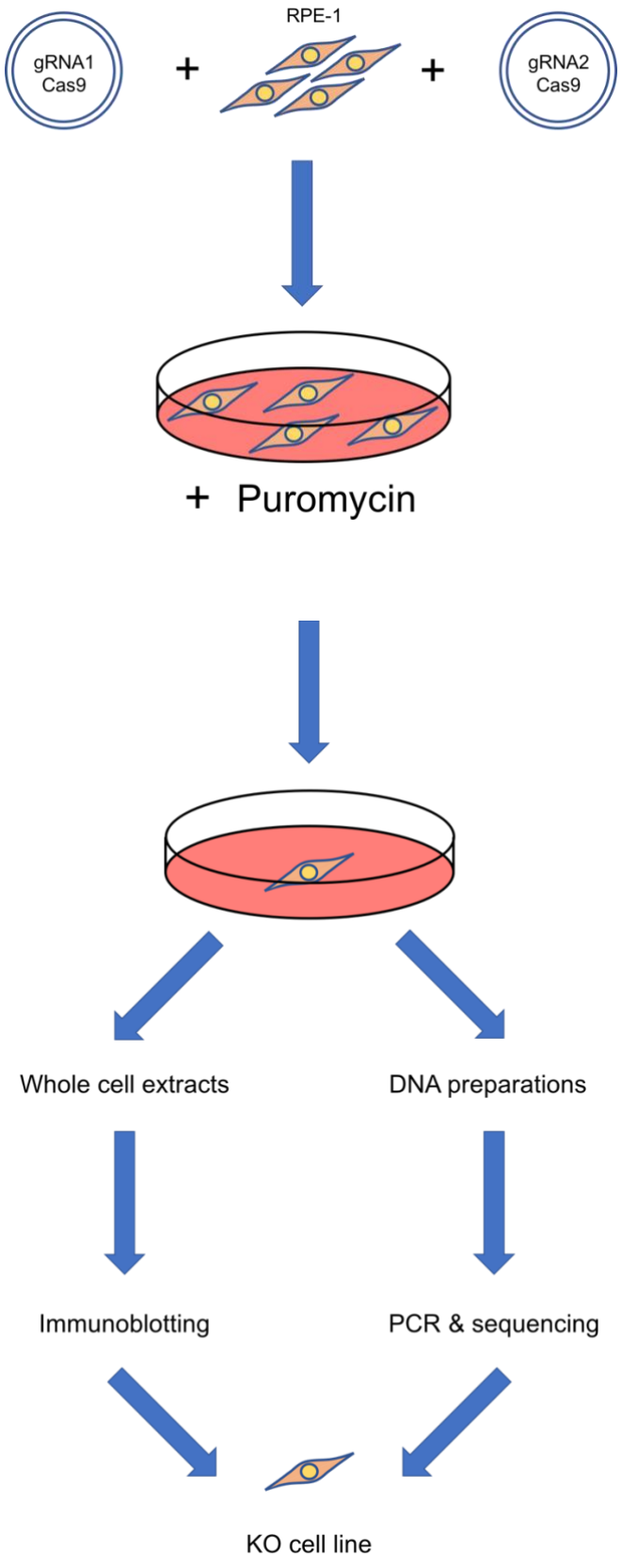
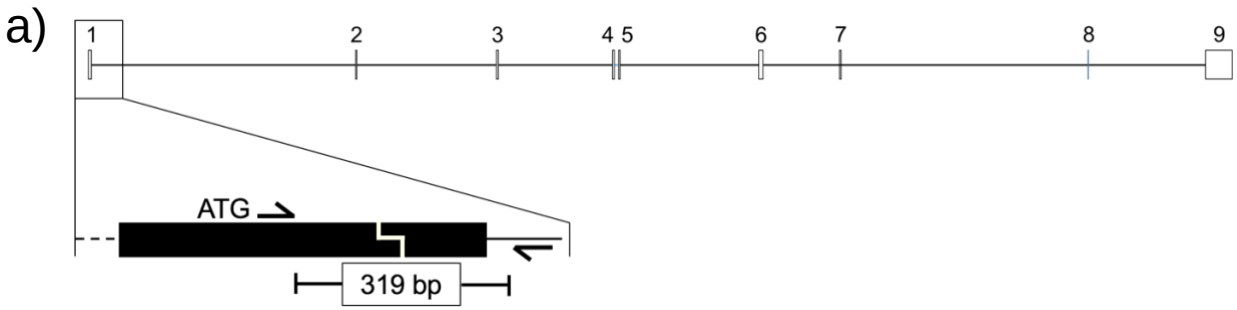
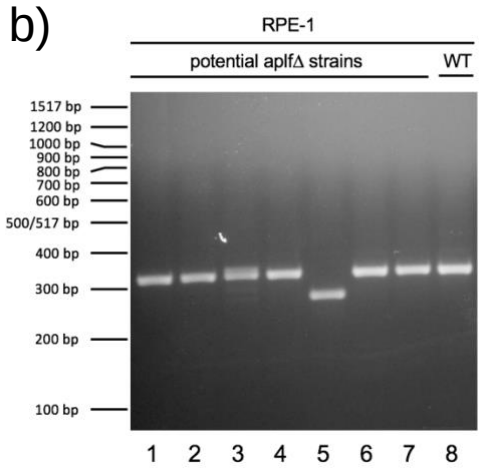
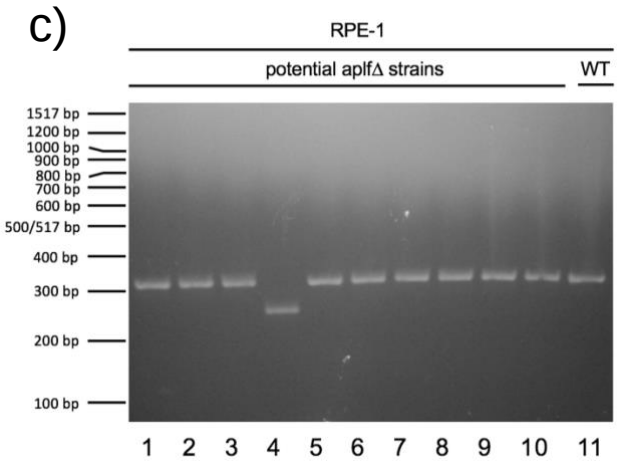
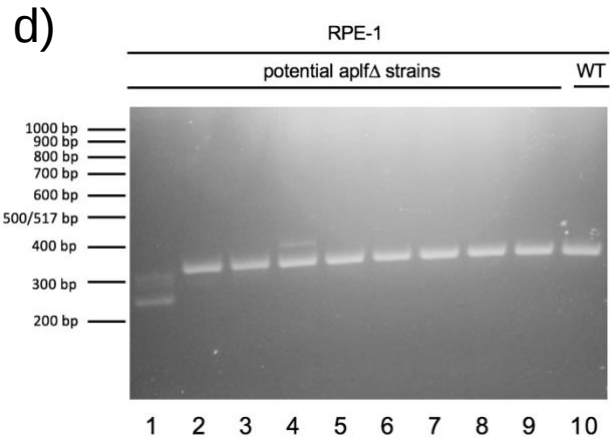

gRNA1
Cas9
+
RPE-1
+
gRNA2
Cas9
+ Puromycin
Whole cell extracts
DNA preparations
Immunoblotting
PCR & sequencing
KO cell line

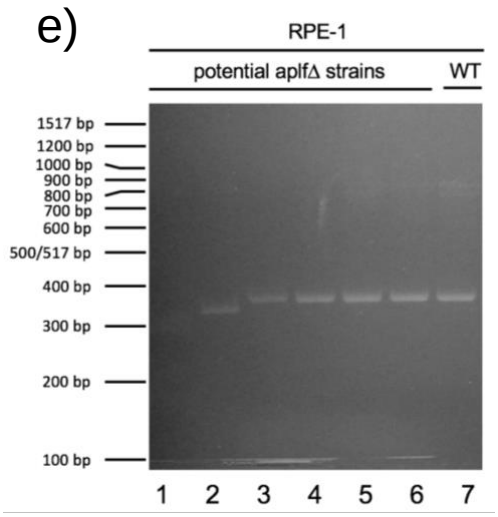
Figure 5.3. Representative screens looking for RPE-1 clones harbouring insertions or deletions in the CRISPR Cas9 cut site in exon 1 of the APLF gene. **a)** Outline of the 319 bp PCR sequence around the CRISPR Cas9 cut site within exon1 of the APLF gene. **b)**, **c)**, **d)** and **e)** RPE-1 cells were transfected with plasmids for the gRNAs and Cas9 and successfully transfected cells selected through Puromycin selection. Colonies of single resistant cells were grown, DNA extracts prepared from the cells and the sequence outlined in a) PCR amplified. Agarose gel electrophoresis was perforedm on the PCR products of the various colonies.


a)
1
2
3
4 5
6
7
8
9
ATG
319 bp


b)
RPE-1
potential aplfΔ strains
WT
1517 bp
1200 bp
1000 bp
900 bp
800 bp
700 bp
600 bp
500/517 bp
400 bp
300 bp
200 bp
100 bp
1 2 3 4 5 6 7 8


c)
RPE-1
potential aplfΔ strains
WT
1517 bp
1200 bp
1000 bp
900 bp
800 bp
700 bp
600 bp
500/517 bp
400 bp
300 bp
200 bp
100 bp
1 2 3 4 5 6 7 8 9 10 11


d)
RPE-1
potential aplfΔ strains
WT
1000 bp
900 bp
800 bp
700 bp
600 bp
500/517 bp
400 bp
300 bp
200 bp
1 2 3 4 5 6 7 8 9 10


e)
RPE-1
potential aplfΔ strains
WT
1517 bp
1200 bp
1000 bp
900 bp
800 bp
700 bp
600 bp
500/517 bp
400 bp
300 bp
200 bp
100 bp
1 2 3 4 5 6 7

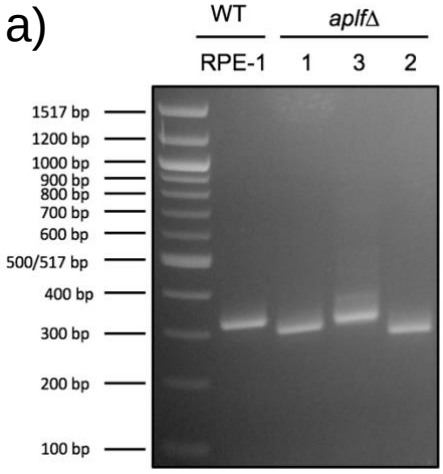
molecular weight of 57 kDA, but instead migrates close to the 100 kDA mark between 75 kDa and 90 kDa in a poly-acrylamide gel (Iles et al. 2007, Kanno et al. 2007). With the generous donation of APLF antibody raised in rabbits by Keith Caldecott (University of Sussex) we were able to confirm these findings with RPE-1 parental cells. Subsequently, the three RPE-1 *aplf*Δ clones were screened for their APLF expression levels (Figure 5.4. c). Consistent with the disruption the gene, APLF is absent in nuclear extracts of all three cell lines. Due to the existence of only one APLF isoform and frameshift deletions in all three cell lines causing premature stop codons at the start of exon 2, we were able to exclude the existence of functional, truncated APLF form(s) in these three cell lines.

5.2.2. Sensitivity of RPE-1 *aplf*Δ cell lines to different DNA damaging agents

Having successfully generated RPE-1 APLF knock-out cell lines, we next decided to test whether they were sensitive to any specific variety of DNA damaging agents. Several agents were used in these experiments, including methyl-methane sulfonate (MMS), that induces bulky DNA adducts, Phleomycin, that causes DSBs, and MMC that produced DNA interstrand crosslinks. DNA adducts are removed by BER, leaving an abasic site that is repaired by single strand break repair (SSBR). DSBs induced by Phleomycin are repaired by homologous recombination (HR) in S phase cells, where the sister chromosome is used as a template faithful repair, or by non-homologous end-joining (NHEJ), which seals broken DNA ends in an error-prone manner. Similar to DSBs, ICLs can be repaired by multiple pathways, the presence of which depends on the cell cycle. In replicating cells, ICL repair is directed by the Fanconi Anemia (FA)

Figure 5.4. Confirmation by sequencing and immunoblotting of three independent RPE-1 *aplf*Δ cell lines. **a)** DNA extracts of the three independent *aplf*Δ cell lines were prepared, the sequence of the APLF gene around the CRISPR Cas9 cut site PCR amplified and gel electrophoresis performed of the samples. **b)** The DNA bands from the agarose gel in a) were extracted, cloned into a transformation vector and the vectors amplified in E.coli. Plasmids extracted by Mini-Prep were sent for DNA sequencing and the sequences compared against the APLF wildtype sequence using SnapGene **c)** Nuclear extracts were prepared of *aplf*Δ*1* and *aplf*Δ*2* and the APLF expression levels analysed by Western Blotting. **d)** Whole cell extracts were prepared of *aplf*Δ*1* and *aplf*Δ*3* and the APLF expression levels analysed by Western Blotting.

a)



b)

RPE-1 *aplfΔ1* APLF exon 1

GTGAAGCTGCGCTGGGGCCGGGCTCTGAGAGGACCGG
CGCAGCCGCGGGGAGCCTTTGAGGCCCTCCCTCGGTG
TTTTTTCCCAGGGCGTGGGCTTGCCCCGCGCGTGTCT
GTGGAGGGCGGAAACAGCGGAGGGGCCAGTCTCCTGG
CGAAGGGGCCTAATCCTTGCCCGCCATGTCCGGGGGC
TTCGAGCTGCAGCCGCGGGACG----------
----------GGGGAGACGGTGATCGGCCGCGGGCC
GCTGCTGGGA

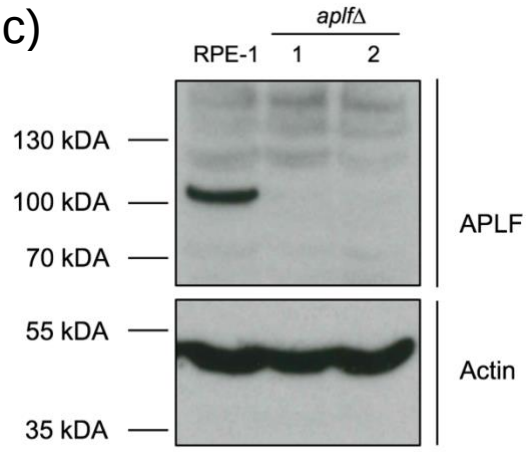
RPE-1 *aplfΔ2* APLF exon 1

GTGAAGCTGCGCTGGGGCCGGGCTCTGAGAGGACCGG
CGCAGCCGCGGGGAGCCTTTGAGGCCCTCCCTCGGTG
TTTTTTCCCAGGGCGTGGGCTTGCCCCGCGCGTGTCT
GTGGAGGGCGGAAACAGCGGAGGGGCCAGTCTCCTGG
CGAAGGGGCCTAATCCTTGCCCGCCATGTCCGGGGGC
TTCGAGCTG----------
----------CGGCCGCGGGCC
GCTGCTGGGA

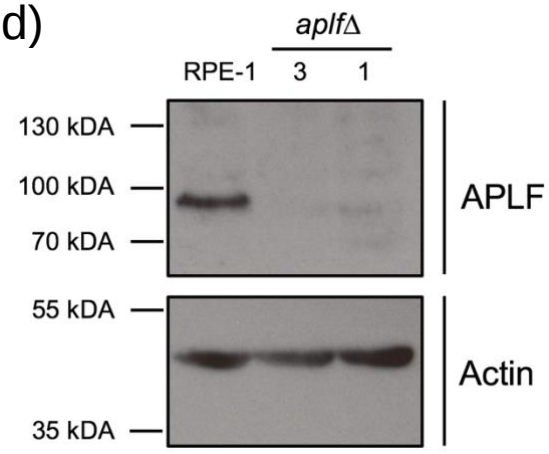
RPE-1 *aplfΔ3* APLF exon 1

GTGAAGCTGCGCTGGGGCCGGGCTCTGAGAGGACCGG
CGCAGCCGCGGGGAGCCTTTGAGGCCCTCCCTCGGTG
TTTTTTCCCAGGGCGTGGGCTTGCCCCGCGCGTGTCT
GTGGAGGGCGGAAACAGCGGAGGGGCCAGTCTCCTGG
CGAAGGGGCCTAATCCTTGCCCGCCATGTCCGGGGGC
TTCGAGCTGCAGCCG----------CCCGGGTGG
CCCTGGCGCCCGGGGAGACGGTGATCGGCCGCGGGCC
GCTGCTGGGA

c)



d)



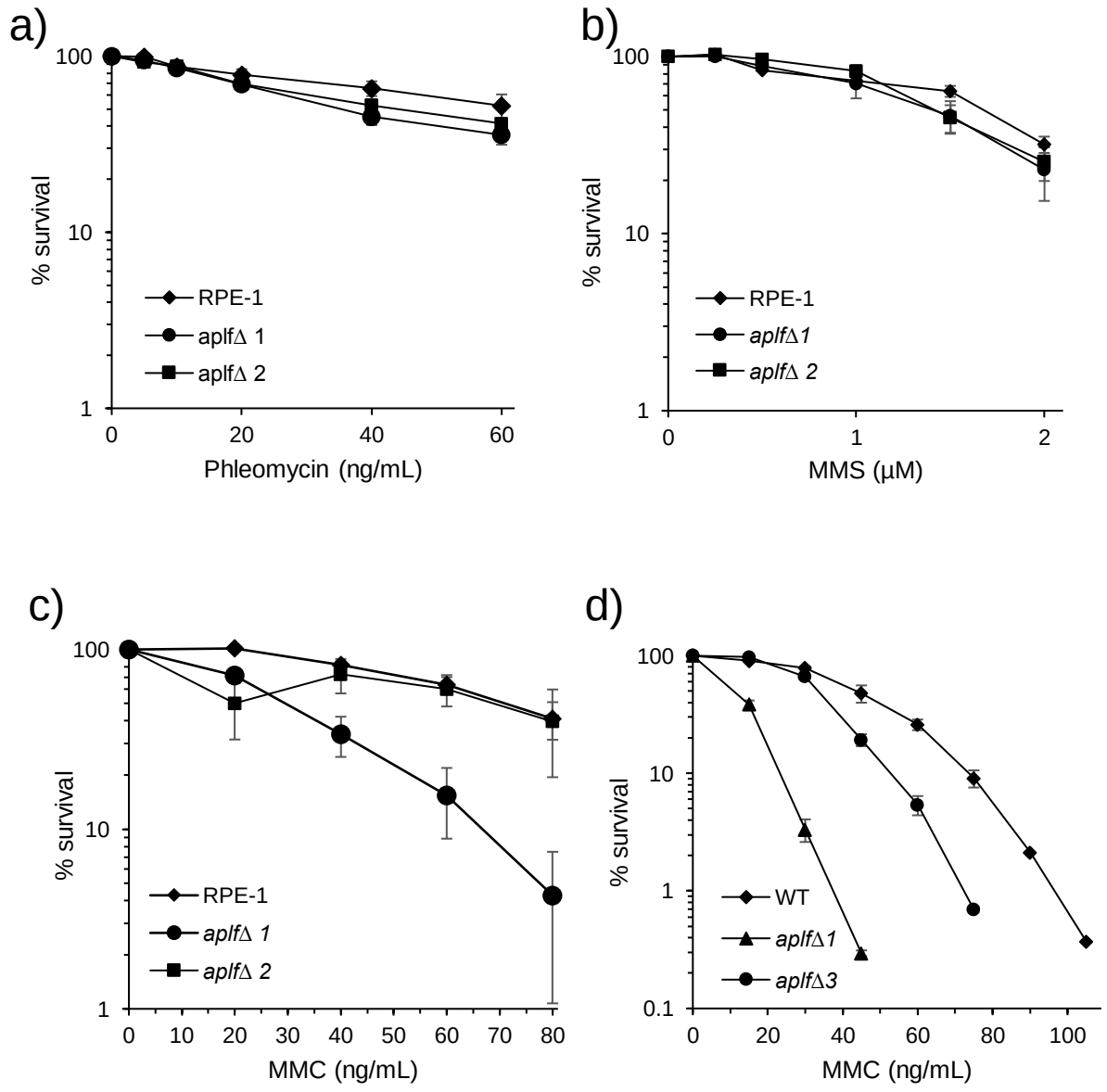
pathway, followed by translesion synthesis (TLS), HR and nucleotide excision repair (NER) (Deans and West 2011, Lopez-Martinez 2016). In addition to this, ICLs can be repaired by FA independent 3' and 5' DNA strand incision, TLS and consecutive NER (Hashimoto et al. 2016, Deans and West 2011). Sensitivity to the above DNA damaging agents was tested by means of clonogenic survival assays. (Figure 5.5.).

Both *aplf*Δ*1* and *aplf*Δ*2* showed mild, but insignificant sensitivity to MMS or Phleomycin compared to the parental cells. In contrast, *aplf*Δ*1* exhibited strong sensitivity towards MMC compared to parental cells and *aplf*Δ*2*. Given the inconclusive results on the sensitivity of APLF knock-out cells to MMC, we decided to test a third cell lines, *aplf*Δ*3*, for its sensitivity to MMC (Figure 3.6. d). RPE-*1 aplf*Δ*3* was significantly sensitive to MMC, albeit not as drastic as RPE-1 *aplf*Δ*1*. We concluded, that the knock-out of APLF in human cells generally confers sensitivity to ICLs induced by MMC, although with phenotypic variations.

5.2.3. Complementing RPE-1 *aplf*Δ cells with myc-APLF

Since *aplf*Δ cells were sensitized to ICLs, we were interested to determine whether the reintroduction of APLF wildtype into the genomic DNA would reverse the observed phenotype. Additionally, we wanted to identify which domains of APLF are required for the tolerance to ICLs. To achieve this, we complemented RPE-1 *aplf*Δ cells with wildtype APLF and APLF mutated in the FHA domain or the first of the tandem PBZ repeats (APLF FHA* and APLF PBZ, respectively). Subsequently, we assessed whether they were able to complement the observed phenotypes.

Figure 5.5. Sensitivity of RPE-1 parental strain and at least two independent RPE-1 *aplf*Δ cell lines to three different DNA damaging agents. **a)** RPE-1, *aplf*Δ*1* and *aplf*Δ*2* cells were seeded in a clonogenic survival assay and their sensitivities to Phleomycin compared. The surival curves represent the mean and standard error of the mean of three independent experiments **b)** RPE-1, *aplf*Δ*1* and *aplf*Δ*2* cells were seeded in a clonogenic survival assay and their survival rate in the presence of increasing MMC concentrations quantified. The surival curves represent the mean and standard error of the mean of three independent experiments **c)** and **d)** RPE-1 and three confirmed, independent *aplf*Δ cell lines – *aplf*Δ*1*, *aplf*Δ*2* and *aplf*Δ*3* – were seeded in clonogenic survival assays and their sensitivities to MMC tested. The surival curves represent the mean and standard error of the mean of three independent experiments

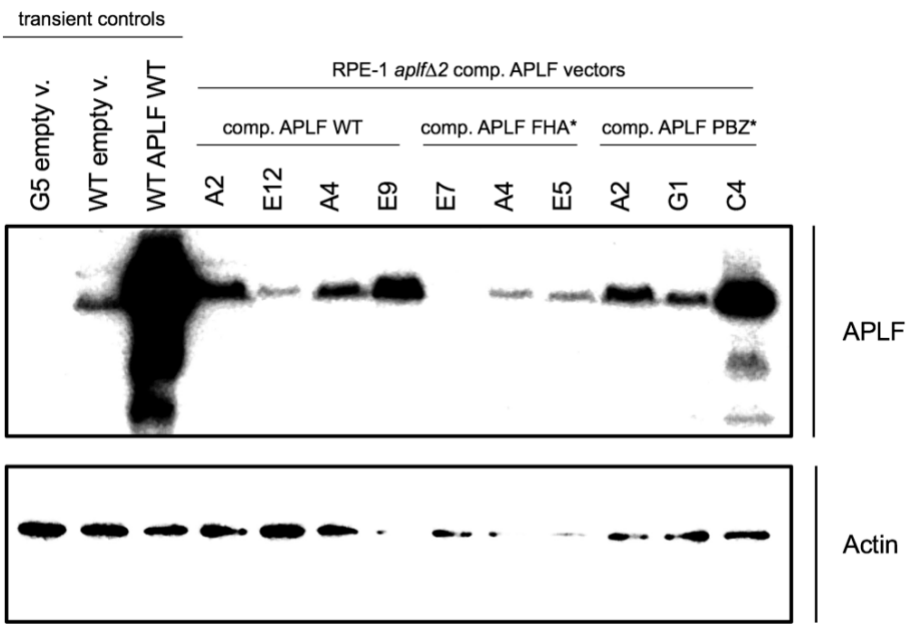

a)
100
10
1
% survival
RPE-1
aplfΔ 1
aplfΔ 2
0
20
40
60
Phleomycin (ng/mL)
b)
100
10
1
% survival
RPE-1
aplfΔ1
aplfΔ 2
0
1
2
MMS (μM)
c)
100
10
1
% survival
RPE-1
aplfΔ 1
aplfΔ 2
0
10
20
30
40
50
60
70
80
MMC (ng/mL)
d)
100
10
1
0.1
% survival
WT
aplfΔ1
aplfΔ3
0
20
40
60
80
100
MMC (ng/mL)

RPE-1 *aplf*Δ cell lines were transfected with plasmids carrying myc-tagged WT APLF, APLF FHA* or APLF PBZ*, kindly provided by Keith Caldecott (University of Sussex). APLF FHA* carries an inactivating R27A mutation, which abrogates its ability to bind phosphothreonine (Macrae et al. 2008). APLF PBZ* carries a C379A and a C385A mutation of the first PBZ repeat. Both of these cysteines are zinc-binding amino acids of the PBZ domain (Eustermann et al. 2010). The APLF constructs were a generous donation by Keith Caldecott (University of Sussex) and the constructs confirmed by our own independent DNA sequencing. All plasmids carry a Puromycin resistance cassette. Therefore, after transfection and subsequent growth in Puromycin, only cells that had integrated the transfected APLF plasmid into their genome were able to form colonies. These clones were screened for the expression of APLF through immunoblotting. Successful insertion of the APLF transgene into the genome yielded varying expression levels (Figure 5.6.a). Therefore, APLF expression levels in all clones were quantified and cells with expression levels resembling endogenous APLF in the RPE-1 parental strain were taken forward. We were unable to reintroduce APLF into *aplf*Δ*1* cells, but generated *aplf*Δ*2* cells stably expressing APLF WT, APLF FHA*, and APLF PBZ* (Figure 5.6. b).

The phenotypic consequences of APLF WT, FHA* and PBZ* reintroduction into *aplf*Δ*2* cells were investigated by clonogenic survival assay following MMC treatment. RPE-1 *aplf*Δ*2* did not initially show a significant MMC sensitivity (Figure 5.5.), and *aplf*Δ*2* complemented with the empty vector control was slightly, but not significantly sensitive to MMC in comparison to the parental RPE-1 cell line (Figure 5.7. a). When complemented with WT APLF, cells were able to tolerate MMC to a similar degree to parental cells. Whilst APLF FHA* was able to complement the slight MMC sensitivity of *aplf*Δ*2* cells, this was not the case for APLF PBZ* (Figure 5.7. b).

Figure 5.6. Quantitative immunoblots measuring APLF expression levels in RPE-1 *aplf*Δ*2* cells complemented with APLF WT, APLF FHA* and APLF PBZ*. **a)** *aplf*Δ cells were transfected with myc-APLF WT, FHA* or PBZ* and successfully transfected cells selected with Puromycin. The selection was continued in resistant cells, to ensure integration of the APLF gene into the genomic DNA. Resistant colonies from single cells were grown and whole cell extracts of each colony prepared. APLF expression levels of the cell lines were tested by immunoblotting and recorded with the LI-COR Odyssey Fc. APLF expression levels were quantified and normalized against the parental cells using ImageStudioLite **b)** Continuation of a) showing the quantified APLF expression levels of various complemented cell lines normalized against the parental control. Marked in red and underlines are the APLF expression levels of one APLF WT, one APLF PBZ* and one APLF FHA* complemented *aplf*Δ*2* cell line normalized against the APLF expression levels of parental cells.

a)


transient controls
G5 empty v.
WT empty v.
WT APLF WT
RPE-1 aplfΔ2 comp. APLF vectors
comp. APLF WT
comp. APLF FHA*
comp. APLF PBZ*
A2
E12
A4
E9
E7
A4
E5
A2
G1
C4
APLF
Actin

b)

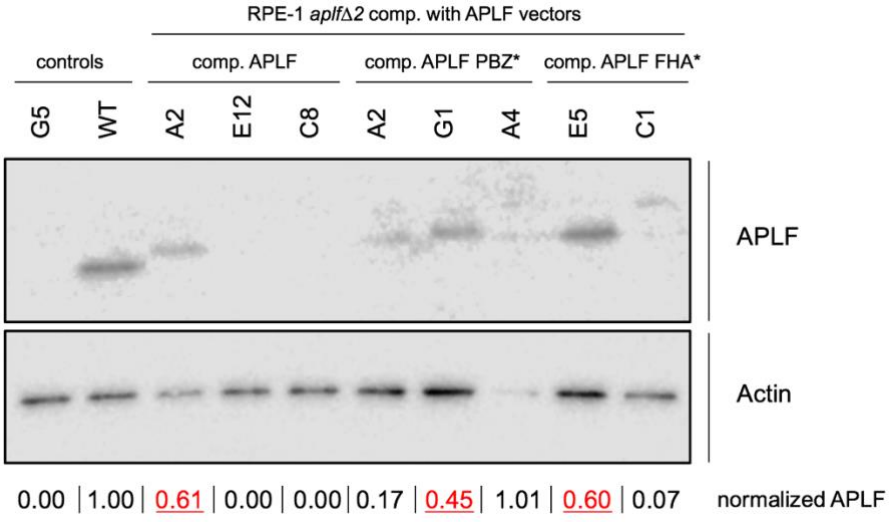
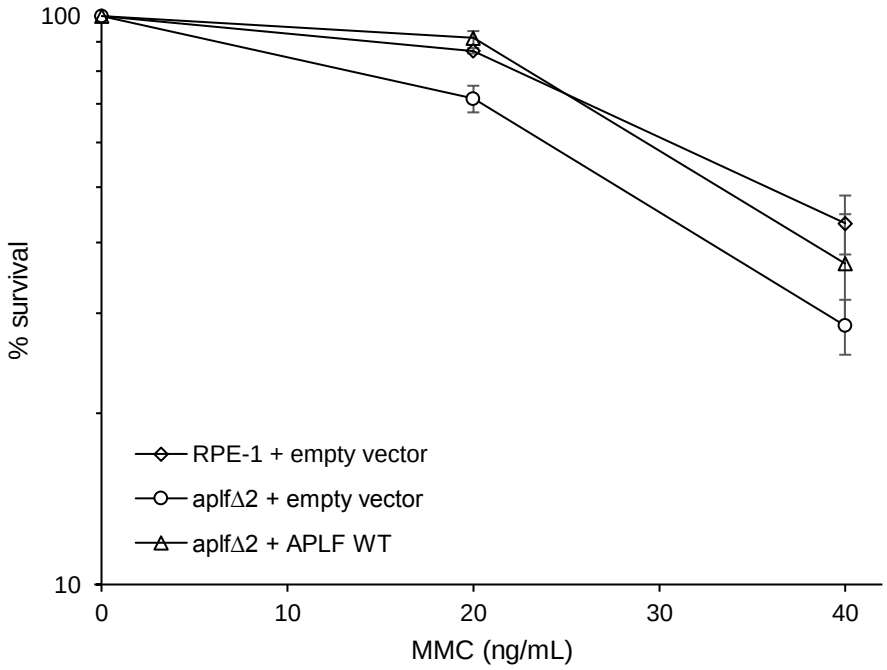
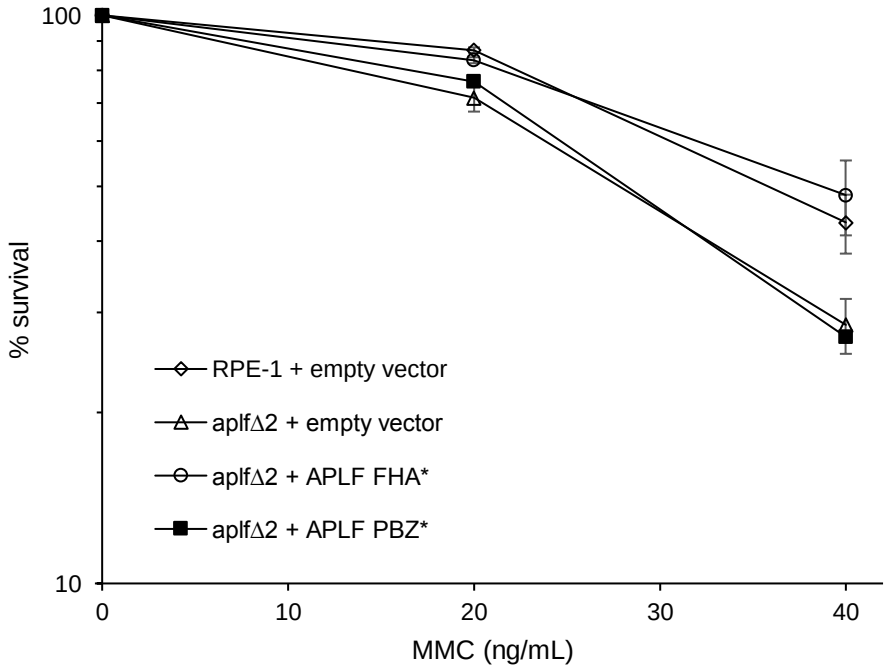

RPE-1 aplfΔ2 comp. with APLF vectors
controls
comp. APLF
comp. APLF PBZ*
comp. APLF FHA*
G5
WT
A2
E12
C8
A2
G1
A4
E5
C1
APLF
Actin
0.00 | 1.00 | 0.61 | 0.00 | 0.00 | 0.17 | 0.45 | 1.01 | 0.60 | 0.07
normalized APLF

Figure 5.7. Clonogenic survival assay of APLF WT, APLF PBZ*, APLF FHA* or empty vector complemented RPE-1 *aplf*Δ*2* against RPE-1 WT **a)** RPE-1 complemented with empty vector, *aplf*Δ*2* complemented with empty vector and *aplf*Δ*2* complemented with APLF WT were seeded in a clonogenic survival assay and their sensitivity to MMC tested. The surival curves represent the mean and standard error of the mean of three independent experiments **b)** Within the same clonogenic survival assay as a) *aplf*Δ*2* complemented with APLF FHA* and aplfΔ2 complemented with APLF PBZ* were seeded and equally their sensitivity to MMC evaluated. The surival curves represent the mean and standard error of the mean of three independent experiments

a)


100
10
% survival
0
10
20
30
40
MMC (ng/mL)
RPE-1 + empty vector
aplfΔ2 + empty vector
aplfΔ2 + APLF WT

b)


100
10
% survival
0
10
20
30
40
MMC (ng/mL)
RPE-1 + empty vector
aplfΔ2 + empty vector
aplfΔ2 + APLF FHA*
aplfΔ2 + APLF PBZ*

Based on these data, we tentatively hypothesize that the reintroduction of APLF into human *aplf*Δ strains can complement the MMC sensitivity observed in *aplf*Δ cells. This provides evidence that APLF is indeed required for the tolerance of cell to ICL inducing agents. Furthermore, the restoration of MMC sensitivity levels comparable with the parental cell line is dependent on the functionality of the tandem PBZ motifs, but does not require the presence of a functional FHA domain.

5.3. DISCUSSION

The absence of APLF renders human cells sensitive to ICLs

CRISPR Cas9 knock-out of APLF in RPE-1 cells revealed a sensitivity to MMC, thus confirming our findings from APLF siRNA knock-down experiments. However, *aplf*Δ cells were not more sensitive to MMS or Phleomycin than parental cells. The lack of sensitivity to DSBs induced by Phleomycin and bulky adducts induced by MMS is in line with previous observations, reporting that neither MMS nor UV produces a phenotype in DT40 cells depleted of APLF (Iles et al. 2007). However, knock-down of APLF in HeLa cells produced sensitivity to MMS (Kanno et al. 2007). Another study identified a phenotype in APLF$^{-/-}$ DT40 cells to IR, itself a source of single strand breaks (Grundy et al. 2007). DNA damage by MMS is repaired predominantly by BER and UV damage is channeled through NER whereas phleomycin induced DSBs are repaired by HR or NHEJ. APLF possesses abasic site endonuclease activity and 3' exonuclease activity, implicating it in BER (Kanno et al. 2007, Li et al. 2011). It also acts in NHEJ, where it links the Ku70/80 heterodimer with the XRCC4/LigaseIV complex (Grundy et al. 2013, Hammel et al. 2016).

Our data indicates, that the repair defects in BER and NHEJ induced by the absence of APLF are not severe enough to result in increased sensitivity of *aplf*Δ cells to MMS or Phleomycin. Our data on the absence of sensitivity to Phleomycin in *aplf*Δ cells disagrees with the clonogenic survival assay of a previous study on APLF (Mehrotra et al. 2011). However, in this study only a slight sensitivity of the *aplf*Δ cell line was observed against the parental U2OS cell line, when the survival rate was blotted on a linear graph. No error bars were provided, making it impossible to determine if the

displayed sensitivity is significant. A survival curve of our parental cell line with similar similar numbers to the graph in question did not lead to a sensitivity of *aplf*Δ cells to Phleomycin in three independent experiments.

In contrast, however, we detected a significant sensitivity to ICLs in the absence of APLF. The repair of ICLs requires many steps and multiple interconnected pathways. The nature of the repair also varies between the different stages of the cell cycle. In the S-phase dependent FA pathway, ICLs are recognized by the Fanconi Anemia core complex and following the 3' and 5' unhooking of the damage site and translesion synthesis, a double strand break forms in the case of two replication forks converging on the ICL. These DSBs are repaired by HR. Therefore, cells lacking NHEJ repair factors including Ku, DNA-PKcs, DNA Ligase IV and XRCC4 are not sensitive to MMC. On the contrary, inhibition of the NHEJ pathway in patients with Fanconi anemia reduces the observed sensitivity to interstrand crosslink inducing agents (Deans & West 2011, Kim & D'Andrea 2012). We conclude that the NHEJ repair properties of APLF cannot be the cause for its ability to prevent increased ICL sensitivity in human RPE-1 cells. Equally, there is no indication that BER is involved in ICL repair. We hypothesized that we uncovered the phenotypic manifestations of a novel role of APLF in the DNA damage response.

The importance of APLF for ICL repair is visualized in the sensitivity of cells to ICLs in its absence. APLF recruitment in response to MMC induced DNA damage as well as to laser stripes + TMP induced ICLs indicates an active role of APLF in ICL removal. To determine at which stage of the cell cycle and in what pathway APLF participates in ICL repair presents an important future goal. There are multiple ways to follow up

on this question. One way to investigate APLF participation in ICL unhooking and flap removal would be by exploring whether γH2AX levels in MMC treated cells decline in the absence of APLF. If the DNA interstrand crosslinks are not unhooked, translesion synthesis (TLS) is impaired and the formation of DSBs prevented. APLF might also effect the recruitment or catalytic activity of TLS and NER factors including polymerase ζ, polymerase ν and others, the localization of which to damage sites can be detected by immunoblotting or cell imaging.

WT, FHA, but not PBZ* APLF rescue slight ICL sensitivity*

APLF can bind ADP-ribose via its tandem PBZ repeats and harbours multiple protein interaction domains, with which it binds the Ku heterodimer, XRCC4 and XRCC1 (Kanno et al. 2007, Macrae et al. 2008, Ahel et al. 2008, Li et al. 2010, Eustermann et al. 2010, Shirodkar et al. 2013, Grundy et al. 2013, Hammel et al. 2016). We therefore considered whether the ability of APLF to prevent sensitivity to MMC is dependent on one or more of its interaction motifs, in particular the XRCC1/XRCC4 interacting FHA domain and the poly-ADP-ribose binding tandem PBZ repeats. Our results revealed that the first of the tandem PBZ repeats, but not the FHA domain, is critical for restoring wildtype sensitivity levels to MMC in *aplf*Δ cells. These findings are consistent with our previous speculation, that the contribution of APLF to ICL repair does not originate from its role in NHEJ. APLF is recruited to chromatin through the tandem PBZ repeats in dependence of PARP1 catalytic activity in BER and PARP3 catalytic activity in NHEJ. The dispensability of the FHA domain for the tolerance of cells to ICL repair reveals a seperation of function between this region and the tandem PBZ repeats. Since the FHA domain is crucial for NHEJ, PARP3 catalytic activity dependent APLF recruitment to chromatin mediated by the PBZ repeats in NHEJ might therefore also

be expendable. Instead, the ability of APLF to prevent sensitivity to ICL inducing drugs might depend on the catalytic activity of PARP1 outside of its described cooperation with APLF in base excision repair.

Supporting our hypothesis of a potential role of APLF in NER mediated ICL repair and dependence of that role on PARP1 catalytic activity, the latter participates in NER. Although it is involved in GG-NER mediated UV repair, which brings into question our hypothesis of APLF and PARP1 in TC-NER, it also interacts with XPA (Pines et al. 2012, King et al. 2012, Fischer et al. 2014). Similar to the rapid APLF chromatin recruitment levels detected by us, chromatin-bound PARP1 association with XPA was visible within several minutes following UV irradiation (King et al. 2012). In light of the requirement of the PBZ repeats of APLF to prevent sensitivity of cells to ICLs, we hypothesized that APLF might act in TC-NER mediated ICL repair and that it may be recruited to chromatin by PARP catalytic activity.

Altogether, we conclude that our observations highlight the importance of APLF for the tolerance of cells to interstrand crosslinks and the requirement for the APLF tandem PBZ repeats for this tolerance. Together with our observations on APLF chromatin recruitment dynamics, this allows room for discussed speculations on the molecular basis of APLF involvement in ICL repair.

6. GENERAL DISCUSSION

PARPs are a group of proteins that catalyze ADP-ribosylation, a post-translational modification required in many cellular functions. The DNA binding PARPs PARP1, PARP2 and PARP3 catalyze poly-ADPribosylation in response to various insults to the DNA or the hindrance of its faithful replication and transcription including DNA bulky adducts, SSBs, DSBs, DNA intrastrand crosslinks and replication fork stalling. However, the evidence on the mode of action underlying their necessity, expressed through their disruption resulting in sensitization of cells to different DNA damaging agents or the failure of repair proteins to localize to DNA damage sites in their absence, is often conflicting and incomplete. One such case is BER, where PARP1 and PARP2 form a complex with polymerase β, XRCC1, ligase III, APE1 and Tdp1. The absence of PARP1 sensitizes cells to MNU and H_2O_2 and DNA strand break resealing is considerably slower in $PARP2^{-/-}$ than in $PARP2^{+/+}$ MEFs (Dantzer et al. 2000, Schreiber et al. 2002). Nevertheless, the amount of SSBs and AB sites in response to the methylating agent MNNG is unchanged between PARP1 proficient and deficient cells, indicating that the sensitivity of cells to alkylating and SSB inducing agents in the absence of PARP1 is not the result of impaired BER (Vodenicharov et al. 2000). Similarly, in HR interactions of PARP1 with repair proteins were reported, although the requirement for PARP1 might stem from its initiation of replication fork restart and its competition with Ku70 and Ligase IV of NHEJ (Hochegger et al. 2006, Haince et al. 2008, Sugimura et al. 2008).

On the basis of experiments undertaken in the course of this thesis, we propose that PARPs promote the repair of DNA interstrand crosslinks. Firstly, ADP-ribosylation is

elevated in cis-platin treated cells (Figure 3.1.) Secondly, we present evidence that PARPs are vital for the survival of human cells in the face of ICL inducing agents (Figure 3.2.). Furthermore, PARP1 and PARP2 both contribute to the tolerance of cells to ICLs (Figure 3.3. b). Epistasis analysis revealed that Olaparib treatment does not further sensitize *parp1/2*Δ cells to mitomycin C and that PARP1 and PARP2 exert their roles through the same mechanism or pathway (Figure 3.4.) Finally, PARP inhibition does not influence FANCD2 monoubiquitylation, an early event in the Fanconi Anemia pathway, and sensitizes FANCD2 knock-down cells further to MMC, suggesting a role outside of the FA pathway (Figure 3.5.).

We have identified PARP1 and PARP2 as contributors to the tolerance of human cells to the DNA interstrand crosslink inducing agent MMC. Although we are not able to exclude the potential involvement of any other mono- or poly-ADPribosylating PARPs implicated in the DDR (Nicolae et al. 2014, Nicolae et al. 2015), the fact that *parp1/2*Δ cells were not further sensitized to MMC by Olaparib gave us confidence that other Olaparib sensitive PARPs do not influence the survival of cells to ICLs (Wahlberg et al. 2012). We therefore conclude that PARP1 and PARP2 are the main PARPs that sensitize cells to DNA ICLs.

Experiments to place PARP1 and PARP2 within an ICL repair pathway point towards a function either downstream of FANCD2 monoubiquitylation within the FA pathway, or a role of PARPs outside the FA pathway. The majority of DNA interstrand crosslinks are considered to be ultimately repaired by the FA pathway, making it the most obvious pathway for PARPs to act in (Moldovan and D'Andrea 2009). Following ICL unhooking and TLS, PARP1 and PARP2 might load members of the MRN complex onto broken

DNA ends, as it does in HR mediated DSB repair (Haince et al. 2008). Nevertheless, this might not be the cause of sensitivity to MMC in *parp1*Δ and *parp2*Δ cells, since the absence of PARP1 does not compromise DSBR in itself (Yang et al. 2003). A more plausible explanation is that replication fork restart is compromised in *parp1*Δ cells (Bryant et al. 2009, Sugimura et al. 2008). This could be tested by observing Rad51 chromatin recruitment or foci formation following MMC treatment.

Contrary to NHEJ, NER is not an error-prone pathway and defects in several NER proteins significantly reduce the ability of cells to remove ICLs (Zheng et al. 2003, Enoiu et al. 2012). It therefore remains difficult to attribute MMC sensitivity of *parp1*Δ and *parp2*Δ cells to one pathway or another. PARP1 interacts with several NER factors, including XPA (King et al. 2012, Fischer et al. 2014). XPA was implicated in a replication-independent ICL repair pathway involving NER and TLS (Enoiu et al. 2012). Testing the presence of PARP dependent NER factors in *parp1*Δ and *parp2*Δ cells against parental U2OS cells after ICL induction could shed light on the question whether PARPs might function in replication independent ICL repair.

One aspect of DNA interstrand crosslinks that has been given more consideration in recent years is the extent to which DNA is distorted. MMC induced ICLs produce only a minor distortion to the DNA, whereas ICLs resulting from cis-platin yield a strong DNA helix distortion (Lopez-Martinez et al. 2016). Structural specificities of ICLs lead to preferential bypass by different translesion synthesis polymerases (Ho et al. 2011). If and to what degree DNA helix distortion influences repair through one pathway or the other is still unclear (Smeaton et al. 2008). This leaves the question whether ADP-ribosylation in response to cis-platin treatment might represent a different PARP

dependent repair event than underlying MMC sensitivity of *parp1*Δ and *parp2*Δ cells. One way of resolving the persisting uncertainty in pathway placement of PARP1 and PARP2 in ICL repair would be to investigate the cell cycle distribution in the *parp1*Δ and *parp2*Δ cells against the parental cell line. ICL repair capabilities could also be explored in cell extracts of non-replicating and replicating cells of all three cell lines. Finally, cells could be arrested at a specific stage of the cell cycle and the impact of PARPs on ICL repair at each stage examined.

In contrast to PARPs, APLF has been established as a key factor in classical NHEJ, where it acts as a scaffold for factors in the DNA end bridging and ligation step of NHEJ and is recruited to chromatin in dependence of ADP-ribosylation events. The function of its protein interactions in other DNA repair pathways, namely BER, remains elusive, since APLF disrupted cells present no impairment in their tolerance to MMS or Phleomycin (Figure 5.5. b).

Similar to PARP1 and PARP2, we propose that APLF positively influences the survival of cells to MMC (Figure 4.4.). Additionally, APLF is recruited to sites of laser stripe and TMP induced ICLs (Figure 4.3.) and to chromatin following DNA damage through MMC (Figure 4.1.). In both cases a rapid localization is observed. The importance of APLF for the survivability of cells to ICL inducing agents was confirmed in knock-out cell lines. The reintroduction of mutated forms of APLF into *aplf*Δ cells tentatively points towards a requirement of the PAR binding PBZ domain of APLF for the tolerance of cells to ICL inducing agents (Figure 5.7.).

Our data suggests that APLF is an early responder to DNA interstrand crosslinks and that this constitutes a hitherto unrecognized function of the protein. We observe similar localization kinetics of APLF to laser stripes in combination with TMP as in previous studies on roles of APLF in NHEJ (Rulten et al. 2011, Grundy et al. 2013). However, the appearance of YFP-APLF stripes following microirradiation was strictly dependent on the presence of TMP, a drug which forms ICLs in combination with light in the near UV range. Experiments using chromatin fractions confirmed APLF recruitment in MMC treated cells. Thus, APLF might be a novel ICL sensor of the FA pathway, although it could also contribute to replication independent ICL repair. One possibility is a distinct NER pathway, which specifically repairs this type of lesion (Enoiu et al. 2012). The repair of other NER channeled lesions such as cis-platin intrastrand crosslinks and possibly UV induced damage does not rely on this pathway, which could explain the tolerance of *aplf*Δ cells to UV induced damage (Grundy et al. 2013). The impact of the absence of APLF on specific ICL repair pathways could be evaluated by investigating the arrival and disappearance of repair intermediates such as DBSs for the Fanconi Anemia pathway, or gaps in the case of NER directed ICL repair. Furthermore, the recruitment of known ICL repair factors to laser stripes + TMP could be recorded in the presence or absence of APLF.

A link exists between APLF and ADP-ribosylation through its tandem PAR binding zinc finger (PBZ) domain (Eustermann et al. 2010). Recently, a connection between the *Dictyostelium* orthologue of PARP1, Adprt2, and a potential orthologue of human APLF, APL, was drawn by studies in our group (Gunn et al. 2016). Adprt2 is recruited to the chromatin fraction in response to cis-platin induced DNA interstrand crosslinks. On a phenotypical level, *adprt2*Δ cells suffer cis-platin sensitivity in comparison to parental

Ax2 cells. APLF chromatin localization was also observed and found to be dependent on Adprt2.

Interestingly, some of our data tentatively suggests that the sensitivity of human RPE-1 *aplf*Δ cells to DNA interstrand crosslinks could be dependent on the functionality of its tandem PBZ domain. However, the dynamics of APLF chromatin recruitment and appearance of ADP-ribosylation in response to ICL induction differ significantly. One interpretation is that APLF might recognize DNA interstrand crosslinks in a manner that does not require PAR chain formation. The absence of the PBZ domain, however, could prevent APLF from binding trans-PARylated proteins or auto-PARylated PARP1 or PARP2 at a later stage of the repair process. Alternatively, our ADP-ribosylation data presents an unrelated event in ICL repair and we were not able to detect the ADP-ribose signal responsible for APLF chromatin recruitment. Unfortunately, at this stage we are not able to confirm whether the presence of APLF at chromatin coincides with ADP-ribosylation at any stage after treatment of cells with an ICL inducing drug.

The fact that PARP3 does not influence the survival of cells to ICLs supports a potential novel connection of PARP1 and PARP2 catalytic activity and APLF, which differs from the described dependence of APLF on PARP3 in NHEJ (Rulten et al. 2011). Given the discrepancies in the timely arrival of ADP-ribosylation and APLF chromatin localization, one possible interpretation of the above data would be that PARP1 and PARP2 as well as APLF act in the same pathway, albeit at different stages. Although further sensitization of FANCD2 knock-down cells to MMC after Olaparib treatment points to FA pathway independent ICL removal, we can also not

exclude the possibility that PARPs might be acting in the FA pathway downstream of FANCD2 monoubiquitylation. PARP1 might be required to compete with Ku70 and Ligase IV, both APLF interaction partners, to favour the channeling of FA pathway produced DSBs through HR rather than NHEJ. Since the inhibition of NHEJ in FA patients reduced the sensitivity of cells to ICL inducing agents, epistasis analysis experiments of *parp1*Δ and *parp2*Δ cells in combination with APLF knock-out would be an interesting prospect (Deans & West 2011, Kim & D'Andrea 2012). The involvement of APLF in NHEJ could mean that APLF knock-out in FA pathway impaired cells produces a different effect on the sensitivity of those cells to ICLs than in cells with a functional FA pathway. Therefore, APLF knock-out in *parp1*Δ and *parp2*Δ cells might result in varying additional sensitivities to MMC and other drugs depending on whether FA pathway directed or FA independent repair is impaired.

Taken together, we might have discovered a novel role for APLF, PARP1 and PARP2 in DNA repair. We conclude, that APLF is a potential novel sensor protein in ICL repair and that APLF, PARP1 and PARP2 all effect the survivability of cells to ICLs. We also present tentative evidence that these effects are connected, since epistasis analysis revealed that PARP1 and PARP2 act in the same pathway and the PAR binding PBZ domain of APLF is required for cells to tolerate ICLs. Many questions remain about the nature of these roles and how the identified contributions of PARP1, PARP2 and APLF to the survival of cells to ICLs are connected.

REFERENCES

Ahel, I, Rass, U, El-Khamisy, SF, Katyal, S, Clements, P, McKinnon, P, Caldecott, K, West, S (2006). The neurodegenerative disease protein aprataxin resolves abortive DNA ligation intermediates. Nature *443*, 713–716.

Ahel, A, Ahel, D, Matsusaka, T, Clark, A, Pines, J, Boulton, S, West, S (2008). Poly(ADP-ribose)-binding zinc finger motifs in DNA repair/checkpoint proteins. Nature *451*, 81–5.

Altmeyer, M, Messner, S, Hassa, P, Fey, M,Hottiger, M (2009). Molecular mechanism of poly(ADP-ribosyl)ation by PARP1 and identification of lysine residues as ADP-ribose acceptor sites. Nucleic Acids Research *37*, 3723–38.

Amé, GC, Spenlehauer, C, Murcia, G (2004). The PARP superfamily. BioEssays : News and Reviews in Molecular, Cellular and Developmental Biology *26*, 882–93.

Audebert, M, Salles, B, Calsou, P (2004). Involvement of Poly(ADP-ribose) Polymerase-1 and XRCC1/DNA Ligase III in an Alternative Route for DNA Double-strand Breaks Rejoining. Journal of Biological Chemistry *279*, 55117–55126.

Audebert, M, Salles, B, Calsou, P (2008). Effect of double-strand break DNA sequence on the PARP-1 NHEJ pathway. Biochemical and Biophysical Research Communications *9*;369(3):982-8

Bai, P (2015). Biology of Poly(ADP-Ribose) Polymerases: The Factotums of Cell Maintenance. Molecular Cell *58*, 947–58.

Beck, C, Robert, I, Reina-San-Martin, B, Schreiber, V, Dantzer F, (2014). Poly(ADP-ribose) polymerases in double-strand break repair: focus on PARP1, PARP2 and PARP3. Experimental Cell Research *329*, 18–25.

Beck, C, Boehler, C, Barbat, J, Bonnet, ME, Illuzzi, G, Ronde, P, Gauthier, LR, Magroun, N, Rajendran, A, Lopez, BS, Scully R, Boussin FD, Schreiber V, Dantzer, F (2014). PARP3 affects the relative contribution of homologous recombination and nonhomologous end-joining pathways. Nucleic Acids Research *42*, 5616–32.

Bekker-Jensen S, Fugger K, Danielsen JR, Gromova I, Sehested M, Celis J, Bartek J, Lukas J, Mailand N. (2007). Human Xip1 (C2orf13) is a novel regulator of cellular responses to DNA strand breaks. The Journal of Biological Chemistry *282*, 19638–43.

Benjamin RC, Gill DM (1980). ADP-ribosylation in mammalian cell ghosts. Dependence of poly(ADP-ribose) synthesis on strand breakage in DNA. The Journal of Biological Chemistry *255*, 10493–501.

Ben-Yehoyada M, Wang LC, Kozekov ID, Rizzo CJ, Gottesman ME, Gautier J (2009). Checkpoint signaling from a single DNA interstrand crosslink. Molecular Cell *35*, 704–15.

Bergstralh DT, Sekelsky J (2008). Interstrand crosslink repair: can XPF-ERCC1 be

let off the hook? Trends in Genetics : TIG *24*, 70–6.
Bhagwat N, Olsen AL, Wang AT, Hanada K, Stuckert P, Kanaar R, D'Andrea A, Niedernhofer LJ, McHugh PJ. (2009). XPF-ERCC1 participates in the Fanconi anemia pathway of cross-link repair. Molecular and Cellular Biology *29*, 6427–37.
Blasiak J 2017. DNA-damaging anticancer drugs – a perspective for DNA repair-oriented therapy. Current Medicinal Chemistry *24*(15):1488-1503
Boboila C, Yan C, Wesemann DR, Jankovic M, Wang JH, Manis J, Nussenzweig A, Nussenzweig M, Alt FW (2010). Alternative end-joining catalyzes class switch recombination in the absence of both Ku70 and DNA ligase 4. The Journal of Experimental Medicine *207*, 417–427.
Boboila, C, Alt, FW, Schwer, B (2012). Advances in Immunology. Advances in Immunology *116*, 1–49.
Boehler C, Dantzer F (2011). PARP-3, a DNA-dependent PARP with emerging roles in double-strand break repair and mitotic progression. Cell Cycle (Georgetown, Tex.) *10*, 1023–4.
Boehler C, Gauthier LR, Mortusewicz O, Biard DS, Saliou JM, Bresson A, Sanglier-Cianferani S, Smith S, Schreiber V, Boussin F, Dantzer F (2011). Poly(ADP-ribose) polymerase 3 (PARP3), a newcomer in cellular response to DNA damage and mitotic progression. Proceedings of the National Academy of Sciences of the United States of America *108*, 2783–8.
Boulton SJ, Gartner A, Reboul J, Vaglio P, Dyson N, Hill DE, Vidal M (2002). Combined functional genomic maps of the C. elegans DNA damage response. Science (New York, N.Y.) *295*, 127–31.
Brooks PJ, Theruvathu JA (2005). DNA adducts from acetaldehyde: implications for alcohol-related carcinogenesis. Alcohol (Fayetteville, N.Y.) *35*, 187–93.
Brown JS, Kaye SB, Yap TA (2016). PARP inhibitors: the race is on. British Journal of Cancer *114*, 713–715.
Bryant, HE, and Helleday, T (2006). Inhibition of poly (ADP-ribose) polymerase activates ATM which is required for subsequent homologous recombination repair. Nucleic Acids Research *34*, 1685–91.
Bryant HE, Petermann E, Schultz N, Jemth AS, Loseva O, Issaeva N, Johansson F, Fernandez S, McGlynn P, Helleday T (2009). PARP is activated at stalled forks to mediate Mre11-dependent replication restart and recombination. The EMBO Journal *28*, 2601–15.
Bunting, SF, Nussenzweig, A (2013). End-joining, translocations and cancer. Nature Reviews Cancer *13*, 443–454.
Caldecott, K. (2003). XRCC1 and DNA strand break repair. DNA Repair *2*, 955–69.
Caldecott, K. (2008). Single-strand break repair and genetic disease. Nature Reviews. Genetics *9*, 619–31.
Ceccaldi, R, Rondinelli, B, D'Andrea, AD (2016). Repair Pathway Choices and Consequences at the Double-Strand Break. Trends in Cell Biology *26*, 52–64.
Chang, HH, Pannunzio, NR, Adachi, N, Lieber, MR (2017). Non-homologous DNA end joining and alternative pathways to double-strand break repair. Nature

Reviews Molecular Cell Biology *18*, 495–506.
Cheng, Q, Barboule, N, Frit, P, Gomez, D (2011). Ku counteracts mobilization of PARP1 and MRN in chromatin damaged with DNA double-strand breaks. *39*(22):9605-19
Chiruvella, K, Liang, Z (2013). Repair of double-strand breaks by end joining. *1*;5(5):a012757
Ciccia A, Ling C, Coulthard R, Yan Z, Xue Y, Meetei AR, Laghmani el H, Joenje H, McDonald N, de Winter JP, Wang W, West SC (2007). Identification of FAAP24, a Fanconi anemia core complex protein that interacts with FANCM. Molecular Cell *25*, 331–43.
Collis SJ, Ciccia A, Deans AJ, Horejsí Z, Martin JS, Maslen SL, Skehel JM, Elledge SJ, West SC, Boulton SJ (2008). FANCM and FAAP24 function in ATR-mediated checkpoint signaling independently of the Fanconi anemia core complex. Molecular Cell *32*, 313–24.
Corda, D, Girolamo, M (2003). Functional aspects of protein mono-ADP-ribosylation. The EMBO Journal *22*, 1953–8.
Corneo B, Wendland RL, Deriano L, Cui X, Klein IA, Wong SY, Arnal S, Holub AJ, Weller GR, Pancake BA, Shah S, Brandt VL, Meek K, Roth DB (2007). Rag mutations reveal robust alternative end joining. Nature *449*, 483–6.
D'Amours, D, Desnoyers, S, D'Silva, I, Poirier, GG (1999). Poly(ADP-ribosyl)ation reactions in the regulation of nuclear functions. The Biochemical Journal *342 (Pt 2)*, 249–68.
Dantzer F, de La Rubia G, Ménissier-De Murcia J, Hostomsky Z, de Murcia G, Schreiber V (2000). Base excision repair is impaired in mammalian cells lacking Poly (ADP-ribose) polymerase-1. Biochemistry *39*(25):7559-69
Davis AJ, Chen DJ (2013). DNA double strand break repair via non-homologous end-joining. Translational Cancer Research 130–143.
Deans, AJ, West, (2011). DNA interstrand crosslink repair and cancer. Nature Reviews. Cancer *11*, 467–80.
Della-Maria, J, Zhou, Y, Tsai, MS, Kuhnlein, J (2011). Human Mre11/human Rad50/Nbs1 and DNA ligase IIIα/XRCC1 protein complexes act together in an alternative nonhomologous end joining pathway. *286*(39):33845-53
Deriano, and Roth (2013). Modernizing the nonhomologous end-joining repertoire: alternative and classical NHEJ share the stage. Annual Review of Genetics *47*:433-55
De Silva IU, McHugh PJ, Clingen PH, Hartley JA (2000). Defining the roles of nucleotide excision repair and recombination in the repair of DNA interstrand cross-links in mammalian cells. Molecular and Cellular Biology *20*, 7980–90.
De Vos M, Schreiber V, Dantzer F. (2012). The diverse roles and clinical relevance of PARPs in DNA damage repair: current state of the art. Biochemical Pharmacology *84*, 137–46.
Dueva, L, Iliakis, D (2013). Alternative pathways of non-homologous end joining (NHEJ) in genomic instability and cancer. Translational Cancer Research

2(3):163-177

Eichinger L, Pachebat JA, Glöckner G, Rajandream MA, Sucgang R, Berriman M, Song J, Olsen R, Szafranski K, Xu Q, Tunggal B, Kummerfeld S, Madera M, Konfortov BA, Rivero F, Bankier AT, Lehmann R, Hamlin N, Davies R, Gaudet P, Fey P, Pilcher K, Chen G, Saunders D, Sodergren E, Davis P, Kerhornou A, Nie X, Hall N, Anjard C, Hemphill L, Bason N, Farbrother P, Desany B, Just E, Morio T, Rost R, Churcher C, Cooper J, Haydock S, van Driessche N, Cronin A, Goodhead I, Muzny D, Mourier T, Pain A, Lu M, Harper D, Lindsay R, Hauser H, James K, Quiles M, Madan Babu M, Saito T, Buchrieser C, Wardroper A, Felder M, Thangavelu M, Johnson D, Knights A, Loulseged H, Mungall K, Oliver K, Price C, Quail MA, Urushihara H, Hernandez J, Rabbinowitsch E, Steffen D, Sanders M, Ma J, Kohara Y, Sharp S, Simmonds M, Spiegler S, Tivey A, Sugano S, White B, Walker D, Woodward J, Winckler T, Tanaka Y, Shaulsky G, Schleicher M, Weinstock G, Rosenthal A, Cox EC, Chisholm RL, Gibbs R, Loomis WF, Platzer M, Kay RR, Williams J, Dear PH, Noegel AA, Barrell B, Kuspa A (2005). The genome of the social amoeba Dictyostelium discoideum. Nature *435*, 43–57.

Ekblad T, Camaioni E, Schüler H, Macchiarulo A (2013). PARP inhibitors: polypharmacology versus selective inhibition. The FEBS Journal *280*, 3563–75.

Enoiu M, Jiricny J, Schärer OD (2012). Repair of cisplatin-induced DNA interstrand crosslinks by a replication-independent pathway involving transcription-coupled repair and translesion synthesis. Nucleic Acids Research *40*, 8953–64.

Eustermann S, Brockmann C, Mehrotra PV, Yang JC, Loakes D, West SC, Ahel I, Neuhaus D (2010). Solution structures of the two PBZ domains from human APLF and their interaction with poly(ADP-ribose). Nature Structural & Molecular Biology *17*, 241–3.

Fattah F, Lee EH, Weisensel N, Wang Y, Lichter N, Hendrickson EA (2010). Ku Regulates the Non-Homologous End Joining Pathway Choice of DNA Double-Strand Break Repair in Human Somatic Cells. PLoS Genetics.

Fenton AL, Shirodkar P, Macrae CJ, Meng L, Koch CA (2013). The PARP3- and ATM-dependent phosphorylation of APLF facilitates DNA double-strand break repair. Nucleic Acids Research *41*, 4080–92.

Fischer JM, Popp O, Gebhard D, Veith S, Fischbach A, Beneke S, Leitenstorfer A, Bergemann J, Scheffner M, Ferrando-May E, Mangerich A, Bürkle A (2014). Poly(ADP-ribose)-mediated interplay of XPA and PARP1 leads to reciprocal regulation of protein function. The FEBS Journal *281*, 3625–41.

Fisher AE, Hochegger H, Takeda S, Caldecott KW (2007). Poly(ADP-ribose) polymerase 1 accelerates single-strand break repair in concert with poly(ADP-ribose) glycohydrolase. Molecular and Cellular Biology *27*, 5597–605.

Flohr C, Bürkle A, Radicella JP, Epe B (2003). Poly (ADP-ribosyl) ation accelerates DNA repair in a pathway dependent on Cockayne syndrome B protein. Nucleic Acids Research *31*(18):5332-7

Folmer V, Soares JC, Gabriel D, Rocha JB (2003). A high fat diet inhibits δ-aminolevulinate dehydratase and increases lipid peroxidation in mice (Mus musculus). Journal of Nutrition *133*(7):2165-70

Fousteri M, Mullenders LH (2008). Transcription-coupled nucleotide excision repair in mammalian cells: molecular mechanisms and biological effects. Cell Research *18*, 73–84.

Friedberg EC (2003). DNA damage and repair. Nature *421*(6921):436-40

Gagné JP, Isabelle M, Lo KS, Bourassa S, Hendzel MJ, Dawson VL, Dawson TM, Poirier GG (2008). Proteome-wide identification of poly(ADP-ribose) binding proteins and poly(ADP-ribose)-associated protein complexes. Nucleic Acids Research *36*, 6959–76.

Gaudet P, Fey P, Chisholm R (2008). Dictyostelium discoideum: The Social Ameba. CSH Protocols *2008*, pdb.emo109.

Ghosh R, Roy S, Kamyab J, Danzter F, Franco S (2016). Common and unique genetic interactions of the poly(ADP-ribose) polymerases PARP1 and PARP2 with DNA double-strand break repair pathways. DNA Repair *45*, 56–62.

Gibson BA, Kraus WL (2012). New insights into the molecular and cellular functions of poly(ADP-ribose) and PARPs. Nature Reviews. Molecular Cell Biology *13*, 411–24.

Graf N, Ang WH, Zhu G, Myint M, Lippard SJ (2011). Role of endonucleases XPF and XPG in nucleotide excision repair of platinated DNA and cisplatin/oxaliplatin cytotoxicity. Chembiochem: A European Journal of Chemical Biology *12*, 1115–23.

Grillari J, Katinger H, Voglauer R (2007). Contributions of DNA interstrand cross-links to aging of cells and organisms. Nucleic Acids Research *35*(22):7566-76

Grundy GJ, Rulten SL, Zeng Z, Arribas-Bosacoma R, Iles N, Manley K, Oliver A, Caldecott KW (2013). APLF promotes the assembly and activity of non-homologous end joining protein complexes. The EMBO Journal *32*, 112–25.

Guirouilh-Barbat J, Rass E, Plo I, Bertrand P, Lopez BS (2007). Defects in XRCC4 and KU80 differentially affect the joining of distal nonhomologous ends. Proceedings of the National Academy of Sciences *104*(52):20902-7

Gunn AR, Banos-Pinero B, Paschke P, Sanchez-Pulido L, Ariza A, Day J, Emrich M, Leys D, Ponting CP, Ahel I, Lakin ND (2016). The role of ADP-ribosylation in regulating DNA interstrand crosslink repair. Journal of Cell Science *129*, 3845–3858.

Haince JF, Kozlov S, Dawson VL, Dawson TM, Hendzel MJ, Lavin MF, Poirier GG (2007). Ataxia telangiectasia mutated (ATM) signaling network is modulated by a novel poly(ADP-ribose)-dependent pathway in the early response to DNA-damaging agents. The Journal of Biological Chemistry *282*, 16441–53.

Haince JF, McDonald D, Rodrigue A, Déry U, Masson JY, Hendzel MJ, Poirier GG (2008). PARP1-dependent kinetics of recruitment of MRE11 and NBS1 proteins to multiple DNA damage sites. Journal of Biological Chemistry *283*, 1197–1208.

Hammel M, Yu Y, Radhakrishnan SK, Chokshi C, Tsai MS, Matsumoto Y, Kuzdovich

M, Remesh SG, Fang 2, Tomkinson AE, Lees-Miller SP, Tainer JA (2016). An Intrinsically Disordered APLF Links Ku, DNA-PKcs, and XRCC4-DNA Ligase IV in an Extended Flexible Non-homologous End Joining Complex. The Journal of Biological Chemistry *291*, 26987–27006.

Hanada K, Budzowska M, Modesti M, Maas A, Wyman C, Essers J, Kanaar R (2006). The structure-specific endonuclease Mus81-Eme1 promotes conversion of interstrand DNA crosslinks into double-strands breaks. The EMBO Journal *25*, 4921–32.

Harris JL, Jakob B, Taucher-Scholz G, Dianov GL, Becherel OJ, Lavin MF (2009). Aprataxin, poly-ADP ribose polymerase 1 (PARP-1) and apurinic endonuclease 1 (APE1) function together to protect the genome against oxidative damage. Human Molecular Genetics *18*, 4102–17.

Hashimoto S, Anai H, Hanada K (2016). Mechanisms of interstrand DNA crosslink repair and human disorders. Genes and Environment : The Official Journal of the Japanese Environmental Mutagen Society *38*, 9.

Haynes B, Saadat N, Myung B, Shekhar MP (2015). Crosstalk between translesion synthesis, Fanconi anemia network, and homologous recombination repair pathways in interstrand DNA crosslink repair and development of chemoresistance. Mutation Research. Reviews in Mutation Research *763*, 258–66.

Helleday T, Eshtad S, Nik-Zainal S (2014). Mechanisms underlying mutational signatures in human cancers. Nature Reviews. Genetics *15*, 585–98.

Ho TV1, Guainazzi A, Derkunt SB, Enoiu M, Schärer OD (2011). Structure-dependent bypass of DNA interstrand crosslinks by translesion synthesis polymerases. Nucleic Acids Research *39*(17):7455-64.

Hochegger H, Dejsuphong D, Fukushima T, Morrison C, Sonoda E, Schreiber V, Zhao GY, Saberi A, Masutani M, Adachi N, Koyama H, de Murcia G, Takeda S (2006). Parp-1 protects homologous recombination from interference by Ku and Ligase IV in vertebrate cells. The EMBO Journal *25*, 1305–1314.

Hoeijmakers JH (2001). Genome maintenance mechanisms for preventing cancer. Nature *411*, 366–74.

Horton JK, Stefanick DF, Prasad R, Gassman NR, Kedar PS, Wilson SH (2014). Base excision repair defects invoke hypersensitivity to PARP inhibition. Molecular Cancer Research : MCR *12*, 1128–39.

Huang H, and Hopkins PB (1993). DNA interstrand cross-linking by formaldehyde: nucleotide sequence preference and covalent structure of the predominant cross-link formed in synthetic oligonucleotides. Journal of the American Chemical Society *115* (21), pp 9402–9408

Huang M, Kim JM, Shiotani B, Yang K, Zou L, D'Andrea AD (2010). The FANCM/FAAP24 complex is required for the DNA interstrand crosslink-induced checkpoint response. Molecular Cell *39*, 259–68.

Huber A, Bai P, de Murcia JM, de Murcia G (2004). PARP-1, PARP-2 and ATM in the DNA damage response: functional synergy in mouse development. DNA Repair *3*, 1103–8.

Iles N, Rulten S, El-Khamisy SF, Caldecott KW (2007). APLF (C2orf13) is a novel human protein involved in the cellular response to chromosomal DNA strand breaks. Molecular and Cellular Biology *27*, 3793–803.
Ishiai M, Sato K, Tomida J, Kitao H, Kurumizaka H, Takata M (2017). Activation of the FA pathway mediated by phosphorylation and ubiquitination. Mutation Research 803-805:89-95
Jackson SP, Bartek J (2009). The DNA-damage response in human biology and disease. Nature *461*, 1071–1078.
Jeggo PA, Löbrich M (2007). DNA double-strand breaks: their cellular and clinical impact? Oncogene *10*;26(56):7717-9.
Jiricny J (2006). The multifaceted mismatch-repair system. Nature Reviews Molecular Cell Biology *7*, 335–346.
Jung Y, Lippard SJ (2007). Direct cellular responses to platinum-induced DNA damage. Chemical Reviews *107*, 1387–407.
Kalisch T, Amé JC, Dantzer F, Schreiber V (2012). New readers and interpretations of poly (ADP-ribosyl)ation.
Kanno S, Kuzuoka H, Sasao S, Hong Z, Lan L, Nakajima S, Yasui A (2007). A novel human AP endonuclease with conserved zinc-finger-like motifs involved in DNA strand break responses. The EMBO Journal *26*, 2094–103.
Karanja KK, Cox SW, Duxin JP, Stewart SA, Campbell JL (2012). DNA2 and EXO1 in replication-coupled, homology-directed repair and in the interplay between HDR and the FA/BRCA network. Cell Cycle *11*, 3983–3996.
Khodyreva SN, Prasad R, Ilina ES, Sukhanova MV, Kutuzov MM, Liu Y, Hou EW, Wilson SH, Lavrik OI (2010). Apurinic/apyrimidinic (AP) site recognition by the 5’-dRP/AP lyase in poly(ADP-ribose) polymerase-1 (PARP-1). Proceedings of the National Academy of Sciences of the United States of America *107*, 22090–5.
Kim H, D'Andrea AD (2012). Regulation of DNA cross-link repair by the Fanconi anemia/BRCA pathway. Genes & Development *26*, 1393–408.
King BS, Cooper KL, Liu KJ, Hudson LG (2012). Poly(ADP-ribose) contributes to an association between poly(ADP-ribose) polymerase-1 and xeroderma pigmentosum complementation group A in nucleotide excision repair. The Journal of Biological Chemistry *287*, 39824–33.
Klungland A, Lindahl T (1997). Second pathway for completion of human DNA base excision-repair: reconstitution with purified proteins and requirement for DNase IV (FEN1). The EMBO Journal *16*, 3341–3348.
Ko HL, Ren EC (2012). Functional Aspects of PARP1 in DNA Repair and Transcription. Biomolecules *2*, 524–48.
Kozekov ID, Nechev LV, Moseley MS, Harris CM, Rizzo CJ, Stone MP, Harris TM (2003). DNA interchain cross-links formed by acrolein and crotonaldehyde. Journal of the American Chemical Society *125*(1):50-61.
Kutuzov MM, Khodyreva SN, Ilina ES, Sukhanova MV, Amé JC, Lavrik OI (2015). Interaction of PARP-2 with AP site containing DNA. Biochimie *112*, 10–9.
Langelier MF, Riccio AA, Pascal JM (2014). PARP-2 and PARP-3 are selectively

activated by 5’ phosphorylated DNA breaks through an allosteric regulatory mechanism shared with PARP-1. Nucleic Acids Research *42*, 7762–75.
Lawley PD, Phillips DH (1996). DNA adducts from chemotherapeutic agents. Mutation Research *355*, 13–40.
Leidecker O, Bonfiglio JJ, Colby T, Zhang Q, Atanassov I, Zaja R, Palazzo L, Stockum A, Ahel I, Matic (2016). Serine is a new target residue for endogenous ADP-ribosylation on histones. Nature Chemical Biology *12*, 998–1000.
Le Page F, Schreiber V, Dherin C, De Murcia G, Boiteux S (2003). Poly(ADP-ribose) polymerase-1 (PARP-1) is required in murine cell lines for base excision repair of oxidative DNA damage in the absence of DNA polymerase beta. The Journal of Biological Chemistry *278*, 18471–7.
Leung AK (2014). Poly (ADP-ribose): an organizer of cellular architecture. J Cell Biol *205*, 613–619.
Lheureux S, Lai Z, Dougherty BA, Runswick S, Hodgson DR, Timms KM, Lanchbury JS, Kaye S, Gourley C, Bowtell D, Kohn EC, Scott C, Matulonis U, Panzarella T, Karakasis K, Burnier JV, Gilks CB, O'Connor MJ, Robertson JD, Ledermann J, Barrett JC, Ho TW, Oza AM (2017). Long-term responders on olaparib maintenance in high-grade serous ovarian cancer: Clinical and molecular characterization. Clinical Cancer Research : An Official Journal of the American Association for Cancer Research. *23*(15):4086-4094
Li X, Heyer WD (2008). Homologous recombination in DNA repair and DNA damage tolerance. Cell Research *18*, 99–113.
Li B, Navarro S, Kasahara N, Comai L (2004). Identification and biochemical characterization of a Werner’s syndrome protein complex with Ku70/80 and poly (ADP-ribose) polymerase-1. Journal of Biological Chemistry *279*, 13659–13667.
Li GY, McCulloch RD, Fenton AL, Cheung M, Meng L, Ikura M, Koch CA (2010). Structure and identification of ADP-ribose recognition motifs of APLF and role in the DNA damage response. Proceedings of the National Academy of Sciences of the United States of America *107*, 9129–34.
Li S, Kanno S, Watanabe R, Ogiwara H, Kohno T, Watanabe G, Yasui A, Lieber MR (2011). Polynucleotide kinase and aprataxin-like forkhead-associated protein (PALF) acts as both a single-stranded DNA endonuclease and a single-stranded DNA 3’ exonuclease and can participate in DNA end joining in a biochemical system. The Journal of Biological Chemistry *286*, 36368–77.
Liang CC, Li Z, Lopez-Martinez D, Nicholson WV, Vénien-Bryan C, Cohn MA (2016). The FANCD2-FANCI complex is recruited to DNA interstrand crosslinks before monoubiquitination of FANCD2. Nature Communications *7*, 12124.
Lieber MR (2010). The mechanism of double-strand DNA break repair by the nonhomologous DNA end-joining pathway. Annual Review of Biochemistry *79*, 181–211.
Lindahl T (1993). Instability and decay of the primary structure of DNA. Nature *362*, 709–15.

Liu Y, Kadyrov FA, Modrich P (2011). PARP-1 enhances the mismatch-dependence of 5'-directed excision in human mismatch repair in vitro. DNA Repair *10*, 1145–53.

Liu Y, Niu Z, Lin X, Tian Y (2017). MiR-216b increases cisplatin sensitivity in ovarian cancer cells by targeting PARP1. Cancer Gene Therapy *24*, 208–214.

Loomis WF (1982). The development of Dictyostelium discoideum. New York: Academic Press. *551 p*. ISBN: 9780124556201

Lopez-Martinez D, Liang CC, Cohn MA (2016). Cellular response to DNA interstrand crosslinks: the Fanconi anemia pathway. Cellular and Molecular Life Sciences : CMLS *73*, 3097–114.

Lord CJ, Ashworth A (2016). BRCAness revisited. Nature Reviews. Cancer *16*, 110–20.

MacKay C, Déclais AC, Lundin C, Agostinho A, Deans AJ, MacArtney TJ, Hofmann K, Gartner A, West SC, Helleday T, Lilley DM, Rouse J (2010). Identification of KIAA1018/FAN1, a DNA repair nuclease recruited to DNA damage by monoubiquitinated FANCD2. Cell *142*, 65–76.

Macrae CJ, McCulloch RD, Ylanko J, Durocher D, Koch CA (2008). APLF (C2orf13) facilitates nonhomologous end-joining and undergoes ATM-dependent hyperphosphorylation following ionizing radiation. DNA Repair *7*, 292–302.

Mansour WY, Rhein T, Dahm-Daphi J (2010). The alternative end-joining pathway for repair of DNA double-strand breaks requires PARP1 but is not dependent upon microhomologies. Nucleic Acids Research *38*, 6065–77.

Marteijn JA, Lans H, Vermeulen W, Hoeijmakers JH (2014). Understanding nucleotide excision repair and its roles in cancer and ageing. Nature Reviews. Molecular Cell Biology *15*, 465–81.

McCabe, N, Turner, NC, Lord, CJ, Kluzek, K, Białkowska, A, Swift, S, Giavara, S, O'Connor, M, Tutt, AN, Zdzienicka, MZ, Smith, GCM (2006). Deficiency in the repair of DNA damage by homologous recombination and sensitivity to poly (ADP-ribose) polymerase inhibition. Cancer Res. *15*;66(16):8109-15

McHugh PJ1, Sarkar S (2006). DNA interstrand cross-link repair in the cell cycle: a critical role for polymerase zeta in G1 phase. Cell Cycle (Georgetown, Tex.) *5*, 1044–7.

Mehrotra PV, Ahel D, Ryan DP, Weston R, Wiechens N, Kraehenbuehl R, Owen-Hughes T, Ahel I (2011). DNA repair factor APLF is a histone chaperone. Molecular Cell *41*, 46–55.

Menon V, Povirk LF (2016). End-processing nucleases and phosphodiesterases: An elite supporting cast for the non-homologous end joining pathway of DNA double-strand break repair. DNA Repair *43*, 57–68.

Michl J1, Zimmer J1, Tarsounas M (2016). Interplay between Fanconi anemia and homologous recombination pathways in genome integrity. The EMBO Journal *35*, 909–23.

Mitchell JR, Hoeijmakers JH, Niedernhofer LJ (2003). Divide and conquer: nucleotide excision repair battles cancer and ageing. Current Opinion in Cell Biology *15*, 232–40.

Moldovan GL, D'Andrea AD (2009). How the Fanconi anemia pathway guards the genome. Annual review of genetics *43*: 223-249.
Moor NA, Vasil'eva IA, Anarbaev RO, Antson AA, Lavrik OI (2015). Quantitative characterization of protein-protein complexes involved in base excision DNA repair. Nucleic Acids Research *43*, 6009–22.
Mouw KW, D'Andrea AD (2014). Crosstalk between the nucleotide excision repair and Fanconi anemia/BRCA pathways. DNA Repair *19*, 130–4.
Murai J, Huang SY, Das BB, Renaud A, Zhang Y, Doroshow JH, Ji J, Takeda S, Pommier Y (2012). Trapping of PARP1 and PARP2 by Clinical PARP Inhibitors. Cancer Research *72*, 5588–99.
Ménisser-de Murcia J, Mark M, Wendling O, Wynshaw-Boris A, de Murcia G (2001). Early embryonic lethality in PARP-1 Atm double-mutant mice suggests a functional synergy in cell proliferation during development. Molecular and Cellular Biology *21*, 1828–32.
Nicolae CM1, Aho ER, Vlahos AH, Choe KN, De S, Karras GI, Moldovan GL (2014). The ADP-ribosyltransferase PARP10/ARTD10 interacts with proliferating cell nuclear antigen and is required for DNA damage tolerance. The Journal of Biological Chemistry *289*(19):13627-37
Nicolae CM, Aho ER, Choe KN, Constantin D, Hu HJ, Lee D, Myung K, Moldovan GL (2015). A novel role for the mono-ADP-ribosyltransferase PARP4/ARTD8 in promoting homologous recombination and protecting against replication stress. Nucleic Acids Research *43*(6):3143-53
Noll DM, Mason TM, Miller PS (2006). Formation and repair of interstrand cross-links in DNA. Chemical Reviews *106*, 277–301.
Noren Hooten N, Kompaniez K, Barnes J, Lohani A, Evans MK (2011). Poly(ADP-ribose) polymerase 1 (PARP-1) binds to 8-oxoguanine-DNA glycosylase (OGG1). The Journal of Biological Chemistry *286*, 44679–90.
Nussenzweig A, Nussenzweig MC (2007). A backup DNA repair pathway moves to the forefront. Cell *131*, 223–5.
Okano S, Kanno S, Nakajima S, Yasui A (2000). Cellular responses and repair of single-strand breaks introduced by UV damage endonuclease in mammalian cells. The Journal of Biological Chemistry *275*, 32635–41.
Okano S, Lan L, Caldecott KW, Mori T, Yasui A (2003). Spatial and temporal cellular responses to single-strand breaks in human cells. Molecular and Cellular Biology *23*, 3974–81.
Pachkowski BF, Tano K, Afonin V, Elder RH, Takeda S, Watanabe M, Swenberg JA, Nakamura J (2009). Cells deficient in PARP-1 show an accelerated accumulation of DNA single strand breaks, but not AP sites, over the PARP-1-proficient cells exposed to MMS. Mutation Research *671*, 93–9.
Pannunzio, Li, Watanabe, and Lieber (2014). Non-homologous end joining often uses microhomology: implications for alternative end joining. DNA Repair (Amst) *17*:74-80
Perina D, Mikoč A, Ahel J, Ćetković H, Žaja R2, Ahel I (2014). Distribution of protein poly(ADP-ribosyl)ation systems across all domains of life. DNA Repair *23*, 4–

16.
Pines, Vrouwe, Marteijn, Typas, Luijsterburg, Cansoy, Hensbergen, Deelder, Groot, Matsumoto, et al. (2012). PARP1 promotes nucleotide excision repair through DDB2 stabilization and recruitment of ALC1. The Journal of Cell Biology *199*, 235–49.
Pines A, Vrouwe MG, Marteijn JA, Typas D, Luijsterburg MS, Cansoy M, Hensbergen P, Deelder A, de Groot A, Matsumoto S, Sugasawa K, Thoma N, Vermeulen W, Vrieling H, Mullenders L (2013). Touching base with PARPs: moonlighting in the repair of UV lesions and double-strand breaks. Trends in Biochemical Sciences *38*, 321–30.
Pleschke JM, Kleczkowska HE, Strohm M, Althaus FR (2000). Poly(ADP-ribose) binds to specific domains in DNA damage checkpoint proteins. The Journal of Biological Chemistry *275*, 40974–80.
Prasad R, Lavrik OI, Kim SJ, Kedar P, Yang XP, Vande Berg BJ, Wilson SH (2001). DNA polymerase beta -mediated long patch base excision repair. Poly(ADP-ribose)polymerase-1 stimulates strand displacement DNA synthesis. The Journal of Biological Chemistry *276*, 32411–4.
Prasad R, Dyrkheeva N, Williams J, Wilson SH (2015). Mammalian Base Excision Repair: Functional Partnership between PARP-1 and APE1 in AP-Site Repair. PloS One *10*, e0124269.
Raper KB (1935). Dictyostelium discoideum, new species of slime mold from decaying forest leaves. Journal of Agricultural Research *50*(2):135-147
Rass U, Ahel I, West SC (2007). Actions of aprataxin in multiple DNA repair pathways. The Journal of Biological Chemistry *282*, 9469–74.
Riccio AA, Cingolani G1, Pascal JM (2016). PARP-2 domain requirements for DNA damage-dependent activation and localization to sites of DNA damage. Nucleic Acids Research *44*, 1691–702.
Richardson C1, Jasin M (2000). Frequent chromosomal translocations induced by DNA double-strand breaks. Nature *405*(6787):697-700
Robert I, Dantzer F, Reina-San-Martin B (2009). Parp1 facilitates alternative NHEJ, whereas Parp2 suppresses IgH/c-myc translocations during immunoglobulin class switch recombination. The Journal of Experimental Medicine *206*(5):1047-56
Robertson AB, Klungland A, Rognes T, Leiros I (2009). DNA Repair in Mammalian Cells. Cellular and Molecular Life Sciences *66*, 981–993.
Robu M, Shah RG, Petitclerc N, Brind'Amour J, Kandan-Kulangara F, Shah GM (2013). Role of poly(ADP-ribose) polymerase-1 in the removal of UV-induced DNA lesions by nucleotide excision repair. Proceedings of the National Academy of Sciences of the United States of America *110*, 1658–63.
Robu M, Shah RG1, Purohit NK, Zhou P2, Naegeli H, Shah GM (2017). Poly(ADP-ribose) polymerase 1 escorts XPC to UV-induced DNA lesions during nucleotide excision repair. Proceedings of the National Academy of Sciences of the United States of America *114*, E6847–E6856.
Ross PM, Yu HS (1988). Interstrand crosslinks due to 4,5',8-trimethylpsoralen and

near ultraviolet light in specific sequences of animal DNA. Effect of constitutive chromatin structure and of induced transcription. Journal of Molecular Biology *201*, 339–51.

Rouleau M, McDonald D, Gagné P, Ouellet ME, Droit A, Hunter JM, Dutertre S, Prigent C, Hendzel MJ, Poirier GG (2007). PARP-3 associates with polycomb group bodies and with components of the DNA damage repair machinery. Journal of Cellular Biochemistry *100*, 385–401.

Rulten SL, Cortes-Ledesma F, Guo L, Iles NJ, Caldecott KW (2008). APLF (C2orf13) is a novel component of poly(ADP-ribose) signaling in mammalian cells. Molecular and Cellular Biology *28*, 4620–8.

Rulten SL, Fisher AE, Robert I, Zuma MC, Rouleau M, Ju L, Poirier G, Reina-San-Martin B, Caldecott KW (2011). PARP-3 and APLF function together to accelerate nonhomologous end-joining. Molecular Cell *41*, 33–45.

San Filippo J, Sung P, Klein H (2008). Mechanism of eukaryotic homologous recombination. Annual Review of Biochemistry *77*, 229–57.

Sarkar S, Davies AA, Ulrich HD, McHugh PJ (2006). DNA interstrand crosslink repair during G1 involves nucleotide excision repair and DNA polymerase zeta. The EMBO Journal *25*, 1285–94.

Sartori AA, Lukas C, Coates J, Mistrik M, Fu S, Bartek J, Baer R, Lukas J, Jackson SP (2007). Human CtIP promotes DNA end resection. Nature *450*, 509–14.

Schreiber V, Amé JC, Dollé P, Schultz I, Rinaldi B, Fraulob V, Ménissier-de Murcia J, de Murcia G (2002). Poly(ADP-ribose) polymerase-2 (PARP-2) is required for efficient base excision DNA repair in association with PARP-1 and XRCC1. The Journal of Biological Chemistry *277*, 23028–36.

Schreiber V, Dantzer F, Ame JC, de Murcia G (2006). Poly(ADP-ribose): novel functions for an old molecule. Nature Reviews Molecular Cell Biology *7*, 517–528.

Schultz N, Lopez E, Saleh-Gohari N, Helleday T (2003). Poly(ADP-ribose) polymerase (PARP-1) has a controlling role in homologous recombination. Nucleic Acids Research *31*, 4959–4964.

Schwab RA, Nieminuszczy J, Shah F, Langton J, Lopez Martinez D, Liang CC, Cohn MA, Gibbons RJ, Deans AJ, Niedzwiedz (2015). The Fanconi Anemia Pathway Maintains Genome Stability by Coordinating Replication and Transcription. Molecular Cell *60*, 351–61.

Shen Y, Aoyagi-Scharber M, Wang B (2015). Trapping Poly(ADP-Ribose) Polymerase. The Journal of Pharmacology and Experimental Therapeutics *353*, 446–57.

Shirodkar P, Fenton AL, Meng L, Koch CA (2013). Identification and functional characterization of a Ku-binding motif in aprataxin polynucleotide kinase/phosphatase-like factor (APLF). The Journal of Biological Chemistry *288*, 19604–13.

Smeaton MB, Hlavin EM, McGregor Mason T, Noronha AM, Wilds CJ, Miller PS (2008). Distortion-dependent unhooking of interstrand cross-links in

mammalian cell extracts. Biochemistry *47*, 9920–30.
Song W, Tang L, Xu Y, Xu J, Zhang W, Xie H, Wang S, Guan X (2017). PARP inhibitor increases chemosensitivity by upregulating miR-664b-5p in BRCA1-mutated triple-negative breast cancer. Scientific Reports *7*, 42319.
Spagnolo L, Barbeau J, Curtin NJ, Morris EP, Pearl LH (2012). Visualization of a DNA-PK/PARP1 complex. Nucleic Acids Research *40*, 4168–77.
Stone MP, Cho YJ, Huang H, Kim HY, Kozekov ID, Kozekova A, Wang H, Minko IG, Lloyd RS, Harris TM, Rizzo CJ (2008). Interstrand DNA cross-links induced by α, β-unsaturated aldehydes derived from lipid peroxidation and environmental sources. Accounts of Chemical Research *41*(7):793-804
Ström CE, Johansson F, Uhlén M, Szigyarto CA, Erixon K, Helleday T (2011). Poly (ADP-ribose) polymerase (PARP) is not involved in base excision repair but PARP inhibition traps a single-strand intermediate. Nucleic Acids Research *39*, 3166–75.
Sugimura K, Takebayashi S, Taguchi H, Takeda S, Okumura K (2008). PARP-1 ensures regulation of replication fork progression by homologous recombination on damaged DNA. The Journal of Cell Biology *183*, 1203–12.
Sung P, Klein H (2006). Mechanism of homologous recombination: mediators and helicases take on regulatory functions. Nature Reviews Molecular Cell Biology. *7*(10):739-50
Svendsen JM, Smogorzewska A, Sowa ME, O'Connell BC, Gygi SP, Elledge SJ, Harper JW (2009). Mammalian BTBD12/SLX4 assembles a Holliday junction resolvase and is required for DNA repair. Cell *138*, 63–77.
Symington LS, Gautier J (2011). Double-strand break end resection and repair pathway choice. Annual Review of Genetics *45*, 247–71.
Tallis M, Morra R, Barkauskaite E, Ahel I (2014). Poly(ADP-ribosyl)ation in regulation of chromatin structure and the DNA damage response. Chromosoma *123*, 79–90.
Tian Y, Paramasivam M, Ghosal G, Chen D, Shen X, Huang Y, Akhter S, Legerski R, Chen J, Seidman MM, Qin J, Li L (2015). UHRF1 contributes to DNA damage repair as a lesion recognition factor and nuclease scaffold. Cell Reports *10*, 1957–66.
Tong KI, Ota K, Komuro A, Ueda T, Ito A, Anne Koch C, Okada H (2016). Attenuated DNA damage repair delays therapy-related myeloid neoplasms in a mouse model. Cell Death & Disease *7*, e2401.
Trevino AE, Zhang F (2014). Genome Editing using CRISPR Cas9. Methods in Enzymology *546*, 162-173
Tubbs A, Nussenzweig A (2017). Endogenous DNA Damage as a Source of Genomic Instability in Cancer. Cell *168*, 644–656.
Vodenicharov MD, Sallmann FR, Satoh MS, Poirier GG (2000). Base excision repair is efficient in cells lacking poly(ADP-ribose) polymerase 1. Nucleic Acids Research *28*, 3887–96.
Vyas S, Chang P (2014). New PARP targets for cancer therapy. Nature Reviews. Cancer *14*, 502–9.

Wahlberg E, Karlberg T, Kouznetsova E, Markova N, Macchiarulo A, Thorsell AG, Pol E, Frostell Å, Ekblad T, Öncü D, Kull B, Robertson GM, Pellicciari R, Schüler H, Weigelt J (2012). Family-wide chemical profiling and structural analysis of PARP and tankyrase inhibitors. Nature Biotechnology *30*, 283–8.

Wang W(2007). Emergence of a DNA-damage response network consisting of Fanconi anaemia and BRCA proteins. Nature Reviews. Genetics *8*, 735–48.

Wang LC1, Gautier J (2010). The Fanconi anemia pathway and ICL repair: implications for cancer therapy. Critical Reviews in Biochemistry and Molecular Biology *45*, 424–439.

Wang X, Peterson CA, Zheng H, Nairn RS, Legerski RJ, Li L (2001). Involvement of nucleotide excision repair in a recombination-independent and error-prone pathway of DNA interstrand cross-link repair. Molecular and Cellular Biology *21*, 713–20.

Wang M, Wu W, Wu W, Rosidi B, Zhang L, Wang H, Iliakis G (2006). PARP-1 and Ku compete for repair of DNA double strand breaks by distinct NHEJ pathways. Nucleic Acids Research *34*, 6170–82.

Wang Y, Kim NS, Haince JF, Kang HC, David KK, Andrabi SA, Poirier GG, Dawson VL, Dawson TM (2011). Poly(ADP-ribose) (PAR) binding to apoptosis-inducing factor is critical for PAR polymerase-1-dependent cell death (parthanatos). Science Signaling *4*, ra20.

Wang AT, Sengerová B, Cattell E, Inagawa T, Hartley JM, Kiakos K, Burgess-Brown NA, Swift LP, Enzlin JH, Schofield CJ, Gileadi O, Hartley JA, McHugh PJ (2011). Human SNM1A and XPF-ERCC1 collaborate to initiate DNA interstrand cross-link repair. Genes & Development *25*, 1859–70.

Wei H, Yu X (2016). Functions of PARylation in DNA Damage Repair Pathways. Genomics, Proteomics & Bioinformatics *14*, 131–9.

Weinfeld M1, Mani RS, Abdou I, Aceytuno RD, Glover JN (2011). Tidying up loose ends: the role of polynucleotide kinase/phosphatase in DNA strand break repair. Trends in Biochemical Sciences *36*, 262–271.

Williams HL, Gottesman ME, Gautier J (2012). Replication-independent repair of DNA interstrand crosslinks. Molecular Cell *47*, 140–7.

Williams HL, Gottesman ME, Gautier J (2013). The differences between ICL repair during and outside of S phase. Trends in Biochemical Sciences *38*, 386–93.

Wilson VL, Foiles PG, Chung FL, Povey AC, Frank AA, Harris CC (1991). Detection of acrolein and crotonaldehyde DNA adducts in cultured human cells and canine peripheral blood lymphocytes by 32 P-postlabeling and nucleotide chromatography. Carcinogenesis *12*, 1483–1490.

Wogan GN, Hecht SS, Felton JS, Conney AH, Loeb LA (2004). Environmental and chemical carcinogenesis. Seminars in Cancer Biology *14*, 473–86.

Wood RD (2010). Mammalian nucleotide excision repair proteins and interstrand crosslink repair. Environmental and Molecular Mutagenesis *51*, 520–6.

Woodrick J, Gupta S, Camacho S, Parvathaneni S, Choudhury S, Cheema A, Bai Y, Khatkar P, Erkizan HV, Sami F, Su Y, Schärer OD, Sharma S, Roy R (2017). A new sub-pathway of long-patch base excision repair involving 5’ gap

formation. The EMBO Journal *36*, 1605–1622.
Xiong X, Du Z, Wang Y, Feng Z, Fan P, Yan C, Willers H, Zhang J (2015). 53BP1 promotes microhomology-mediated end-joining in G1-phase cells. Nucleic Acids Research *43*, 1659–1670.
Yamamoto KN, Kobayashi S, Tsuda M, Kurumizaka H, Takata M, Kono K, Jiricny J, Takeda S, Hirota K (2011). Involvement of SLX4 in interstrand cross-link repair is regulated by the Fanconi anemia pathway. Proceedings of the National Academy of Sciences of the United States of America *108*, 6492–6.
Yang YG, Cortes U, Patnaik S, Jasin M, Wang ZQ (2004). Ablation of PARP-1 does not interfere with the repair of DNA double-strand breaks, but compromises the reactivation of stalled replication forks. Oncogene *23*, 3872–3882.
Yano K, Morotomi-Yano K, Akiyama H (2009). Cernunnos/XLF: a new player in DNA double-strand break repair. The International Journal of Biochemistry & Cell Biology *41*, 1237–40.
Yarchoan M, Myzak MC, Johnson BA 3rd, De Jesus-Acosta A, Le DT, Jaffee EM, Azad NS, Donehower RC, Zheng L, Oberstein PE, Fine RL, Laheru DA, Goggins M (2017). Olaparib in combination with irinotecan, cisplatin, and mitomycin C in patients with advanced pancreatic cancer. Oncotarget *8*(27):44073-44081
Yélamos J, Schreiber V, Dantzer F (2008). Toward specific functions of poly(ADP-ribose) polymerase-2. Trends in Molecular Medicine *14*, 169–78.
Ying S, Chen Z, Medhurst AL, Neal JA, Bao Z, Mortusewicz O, McGouran J, Song X, Shen H, Hamdy FC, Kessler BM, Meek K, Helleday T (2016). DNA-PKcs and PARP1 Bind to Unresected Stalled DNA Replication Forks Where They Recruit XRCC1 to Mediate Repair. Cancer Research *76*, 1078–88.
Zaja R, Mikoč A, Barkauskaite E, Ahel I (2012). Molecular Insights into Poly(ADP-ribose) Recognition and Processing. Biomolecules *3*, 1–17.
Zhang X, Horibata K, Saijo M, Ishigami C, Ukai A, Kanno S, Tahara H, Neilan EG, Honma M, Nohmi T, Yasui A, Tanaka K. (2012). Mutations in UVSSA cause UV-sensitive syndrome and destabilize ERCC6 in transcription-coupled DNA repair. Nature Genetics *44*, 593–7.
Zheng H, Wang X, Warren AJ, Legerski RJ, Nairn RS, Hamilton JW, Li L (2003). Nucleotide excision repair- and polymerase eta-mediated error-prone removal of mitomycin C interstrand cross-links. Molecular and Cellular Biology *23*, 754–61.