The role of DNA polymerase eta in determining cellular responses to chemo-radiation treatment

Submitted by Nils Henrik Nicolay, St Cross College

for the degree of D.Phil in Radiobiology

during Trinity term, 2012

Co-supervised by Dr Ricky Sharma and Professor Thomas Helleday,

Gray Institute for Radiation Oncology & Biology, University of Oxford

ACKNOWLEDGEMENTS

It would not have been possible to write this doctoral thesis without the help and support of the people around me, to only some of whom it is possible to give particular mention here. I would like to thank my primary supervisor, Dr Ricky Sharma for his patience, his good advice and his regular support whenever I needed it during the course of my studies. I would also like to thank my co-supervisor, Professor Thomas Helleday whose insightful comments and ideas helped me a lot to overcome obstacles that I encountered during my research.

I am very grateful for the help and support of my colleagues, above all Dr Rebecca Carter who helped me with the BL-2 cell experiments of my thesis, and Dr Stephanie Hatch who was still approachable for help when I had already left the lab.

I am also grateful beyond words for the continuous emotional and generous financial support of my parents that enabled me to have made it this far.

Finally, I would like to thank my beautiful wife, Julia, for her immense support and understanding during my three years in Oxford. Her patience and her personal sacrifices allowed me to focus on my work and kept my morale up during these years abroad.

CONTENTS

LIST OF TABLES

LIST OF FIGURES

ABBREVIATIONS

°C	degrees Celsius
5-FU	5-fluorouracil
A	adenine
ATM	ataxia teleangiectasia-mutated kinase
ATR	ataxia teleangiectasia mutated-Rad3-related kinase
BER	base excision repair
C	cytosine
c-ABL	Abelson murine leukemia viral oncogene homolog 1
cDNA	complementary deoxyribonucleic acid
Chk	checkpoint kinase
CI	confidence interval
CO_2	carbon dioxide
CPD	cyclobutane pyrimidine dimer
CSA	Cockayne syndrome protein A
CSB	Cockayne syndrome protein B
Ctr	copper transporter
DACH	(trans-R,R)1,2-diaminocyclohexane
DAPI	4',6-diamidino-2-phenylindole
DDR	DNA damage signalling
DFS	disease-free survival
DMEM	Dulbecco’s Modified Eagle Medium
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
DSB	double-strand break

EDTA	ethylenediaminetetraacetic acid
ERCC1	DNA excision repair cross-complementation protein 1
G	guanine
G	tumour grade
GG-NER	global genomic nucleotide excision repair
Gy	Gray
H_2O	water
HIF-1α	hypoxia-inducible factor 1α
HR	homologous recombination
HR	Hazard Ratio
Hsp90	heat shock protein 90
ICL	interstrand crosslink
IR	ionising radiation
MAPK	mitogen-activated protein kinase
mRNA	messenger ribonucleic acid
MT	metallothionein
NER	nucleotide excision repair
NHEJ	non-homologous end joining
nM	nanomolar
OCT	organic cation transporter
OS	overall survival
PARP	Poly ADP ribose polymerase
PBS	phosphate-buffered saline
PCNA	proliferating cell nuclear antigen
PCR	polymerase chain reaction

PIP	PCNA-interacting peptide
pol	polymerase
PVDF	polyvinylidene fluoride
qRT-PCR	quantitative real-time polymerase chain reaction
RNA	ribonucleic acid
RNase	ribonuclease
RPMI	Roswell Park Memorial Institute medium
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
siRNA	small interfering RNA
SSB	single-strand break
T	Thymine
TC-NER	transcription-coupled nucleotide excision repair
TLS	translesion synthesis
U	Uracil
UBZ	ubiquitin-binding zinc finger
UV	ultraviolet
w/v	weight per volume
XP	xeroderma pigmentosum
XPA – XPG	xeroderma pigmentosum complementation groups A – G
XPV	variant form of xeroderma pigmentosum
Δ	Difference

ABSTRACT

DNA polymerase η (pol η), a crucial component of the cellular translesion synthesis pathway, allows cells to bypass and thereby temporarily tolerate DNA damage. Inherited deficiency of pol η, as reported in the variant form of xeroderma pigmentosum, predisposes to UV light-induced skin cancers. To date, pol η is the only DNA polymerase shown to exhibit a causal link to the formation of cancers in humans. However, the role of pol η in the cellular response to forms of DNA damage other than UV-induced lesions is largely unknown.

In the first part of this thesis, it is shown that cells deficient in pol η are resistant to ionising radiation. Deficiency in the polymerase was associated with accumulation of cells in S phase of the cell cycle. Cells deficient in pol η demonstrated increased homologous recombination-directed repair of DNA double-strand breaks created by ionising radiation, and depletion of the homologous recombination protein X-ray repair cross-complementing protein 3 (XRCC3), abrogated the radioresistance observed in pol η-deficient cells compared to pol η-complemented cells. These findings suggest that homologous recombination mediates S phase-dependent radioresistance associated with pol η-deficiency.

In the second part of this thesis, it is shown that pol η-deficient cells have increased sensitivity to the chemotherapeutic compound, oxaliplatin, compared to pol η-deficient expressing cells, but not to the drug 5-fluorouracil that is usually administered in combination with oxaliplatin in the clinical setting. Despite the importance of pol η for cellular survival following exposure to oxaliplatin, the drug did not upregulate the

enzyme after either short-term or long-term exposure. Inhibition of pol η activity by siRNA-mediated knockdown of the protein sensitised cells to oxaliplatin treatment, and partially reversed acquired resistance in oxaliplatin-resistant tumour cell lines. These data suggest that pol η is an interesting target whose function can potentially be interfered with to optimise oxaliplatin-based chemotherapy.

In the third part of this thesis, clinical samples obtained from oesophageal cancer patients before and after treatment with oxaliplatin-containing chemotherapy were analysed for *POLH* mRNA levels encoding pol η protein. Malignant tissue specimens obtained before treatment demonstrated a significantly higher level of *POLH* mRNA than matched normal oesophageal tissue samples. Contrary to the preclinical data, high *POLH* mRNA expression before therapy was shown to correlate with increased overall and disease-free survival of the patient cohort in the clinical trial. Additionally, patients with high *POLH* mRNA-expressing cancers had better therapeutic responses (measured by PET-CT) to oxaliplatin-based treatment than those with low levels. These data suggest that *POLH* mRNA expression should be tested as a biomarker to predict survival and therapeutic responses in oesophageal cancer patients treated with oxaliplatin-containing chemotherapy.

INTRODUCTION

1.1 THE TRANSLESION SYNTHESIS DNA POLYMERASE H

1.1.1 DNA translesion synthesis polymerases

The cellular genome is continuously exposed to endogenous and exogenous DNA damage, and each human cell incurs more than 50 000 damaging events every 24 hours (Lindahl and Wood 1999). Therefore, an elaborate set of different DNA repair mechanisms has evolved to maintain the integrity of the cellular genome and to prevent genetic instability and carcinogenesis. Cellular repair pathways are capable of directly reversing DNA damage or repairing single or double-strand breaks, but the majority of DNA lesions require different forms of repair, such as base excision repair, nucleotide excision repair or mismatch repair (Helleday, Petermann et al. 2008). Since not all lesions can be repaired immediately and may persist to the S phase of cell cycle, they have the potential to impede the replication machinery by stalling or collapsing replication forks. In this way, lesions in the DNA strands may affect the viability of dividing cells (Michel, Grompone et al. 2004; Andreassen, Ho et al. 2006). Cells have developed ways to temporarily tolerate DNA damage without initiating immediate repair of the lesion site. These pathways require specialised enzymes capable of bypassing those forms of damage, therefore allowing cells to continue replication and making lesions amenable to post-replicative repair (Lee and Myung 2008). Human cells contain an array of specialized DNA polymerases that are capable of replicating past various forms of DNA damage, termed DNA translesion (TLS) polymerases (Guo, Kosarek-Stancel et al. 2009; Waters, Minesinger et al. 2009).

Many DNA lesions within the template strand have been characterised that cannot be used as templates by the replicative DNA polymerases to incorporate the correct nucleotides into the newly synthesized strand, and therefore replicative polymerases have been shown to stall at lesion sites (Brieba 2008). In contrast, specialized TLS polymerases are not impeded by conformational changes of lesion sites and can use various modified nucleotides as templates to continue strand synthesis (Goodman and Tippin 2000; Goodman 2002). The TLS machinery is recruited to sites of stalled replication forks or may contribute to the gap filling of single strands created during DNA replication (Watanabe, Tateishi et al. 2004; Fu, Zhu et al. 2008; Waters, Minesinger et al. 2009). Recruitment of the TLS polymerases is believed to result in a polymerase switch, removing the replicative DNA polymerase from the primer terminus and allowing bypass of the distorted lesion site by the TLS enzyme before a second switch leads to the replicative machinery being loaded onto the nascent strand again (**FIGURE 1.1**). This way, lesion bypass allows cells to prevent replication fork stalling and its severe consequences. Following TLS, lesion sites are amenable to repair by the various DNA repair pathways. Certain forms of damage have been described that may even require a subsequent action of two or more TLS polymerases to extend from the lesion site. In those cases, the insertion opposite the lesion site requires the activity of a specific TLS polymerase while the extension from the inserted nucleotide is believed to be performed by a second enzyme (Washington, Johnson et al. 2002; Acharya, Johnson et al. 2006; Zhong, Garg et al. 2006; Lone, Townson et al. 2007).

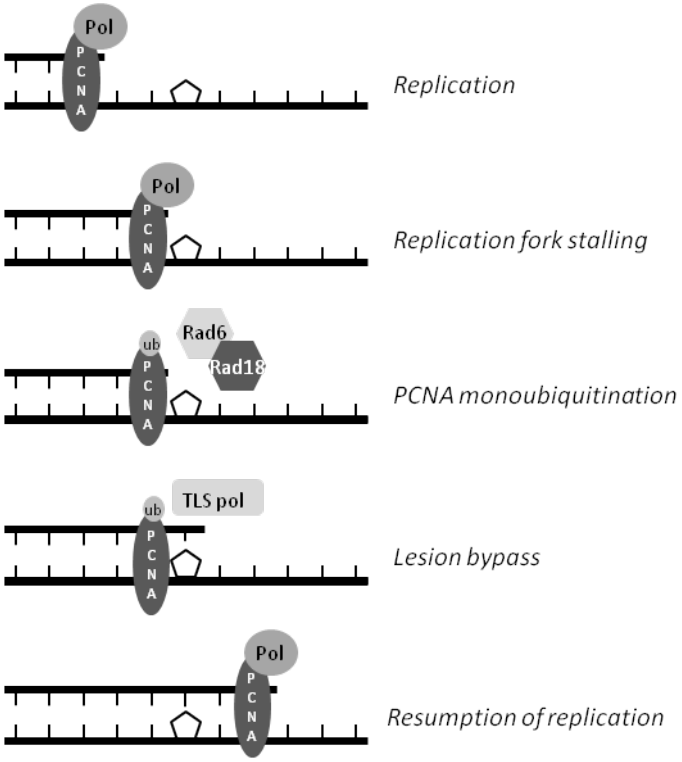


Figure 1.1. Schematic depiction of the TLS pathway

The Y family polymerases, DNA polymerases (pols) η (eta), ι, κ and Rev1, are among the best-characterized TLS enzymes, but other polymerases such as pol ζ and pol β have also been found to exhibit translesional activity. Y family TLS polymerases are widely found throughout prokaryotic and eukaryotic cells. *Sulfolobus solfataricus* DNA polymerase IV (Dpo-4) is an archaeic thermostable enzyme belonging to the Y family of polymerases and is used as a model to investigate the mechanistics underlying TLS (Ling, Boudsocq et al. 2003). In E. coli, Y family polymerases like pol IV (DinB) and the bacterial pol η homologue, pol V, catalyze bacterial DNA lesions (Delmas and Matic 2006; Lee, Chandani et al. 2006). The presence of DNA polymerase η(pol η) has

been shown in various eukaryotic cells including yeast and human while yeast pol η has a slightly higher processivity with a higher number of nucleotides incorporated per association with the DNA template and as several-fold higher fidelity than the human homologue (McCulloch, Wood et al. 2007). To date, pol η has been the most widely studied human TLS polymerase.

1.1.2 DNA polymerase η deficiency as the cause for the variant form of xeroderma pigmentosum

Pol η was first discovered as the enzyme missing in the variant form of xeroderma pigmentosum (XPV) (Johnson, Kondratick et al. 1999; Masutani, Kusumoto et al. 1999). While the seven other subtypes of xeroderma pigmentosum (XP) are characterised by defects in different components of the nucleotide excision repair (NER) pathway and therefore lack the ability to repair damage caused by various DNA-damaging agents, patients suffering from XPV lack the ability to temporarily tolerate these forms of damage. Clinically, XPV patients exhibit a considerably increased sensitivity to sunlight and are prone to develop different forms of UV-induced skin cancers, often on multiple body sites. To date, pol η is the only DNA polymerase that has causally been linked to the development of cancer (Broughton, Cordonnier et al. 2002). Several different mutations in the *POLH* gene encoding pol η protein have been identified in tissues sampled from XPV patients (Broughton, Cordonnier et al. 2002; Tanioka, Masaki et al. 2007).

In vitro experiments have shown that fibroblasts from XPV donors are highly susceptible to the mutagenic effects of UV irradiation, although they are only moderately sensitive to the lethal effects of UV light compared to NER-deficient XP cells that are highly sensitive to UV killing effects. In patients suffering from XPV, UV-induced DNA lesions are 25-fold higher than in healthy individuals and show a unique spectrum of mutations, mainly C-A transversions on the transcribed and T-A transversions on the non-transcribed strand (Wang, Woodgate et al. 2007).

1.1.3 Structural aspects of DNA polymerase η

Human pol η contains 713 amino acids and has a calculated molecular weight of 78 kDa (Zhang, Yuan et al. 2000). The enzyme's N-terminal sequence forms the active site and is highly conserved throughout the Y polymerase family, whereas the C terminal part has been shown to be responsible for targeting and accumulation at DNA damage sites after UV irradiation (Kannouche, Broughton et al. 2001; Broughton, Cordonnier et al. 2002). Pol η's C terminus exhibits a zinc finger structure able to bind to ubiquitin residues on associated proteins like Proliferating Cell Nuclear Antigen (PCNA) and two peptide sequences that allow pol η to interact with PCNA directly (Bomar, Pai et al. 2007; Acharya, Yoon et al. 2008).

Both yeast and human pol η have been co-crystallised with DNA (Alt, Lammens et al. 2007; Biertumpfel, Zhao et al. 2010; Silverstein, Johnson et al. 2010). As for most other TLS polymerases, pol η shares very little sequence homology with the well-defined replicative DNA polymerases; however, it has some basic structural

features in common, like the classic "right-hand" fold of DNA polymerases, consisting of four defined domains: palm, finger, thumb, little finger (Prakash, Johnson et al. 2005; Biertumpfel, Zhao et al. 2010). The central "palm" domain contains the glutamate and aspartate residues coordinating the divalent cations Mg^{2+} or Mn^{2+} that are required to stabilise the incoming deoxynucleotide triphosphates (Baker and Bell 1998). The "finger domain" of human pol η is closed in contrast to its yeast counterpart and makes contact with the template strand and the replicating base pair (Biertumpfel, Zhao et al. 2010). Additionally, the polymerase is connected to the primer by the "thumb" domain (Prakash, Johnson et al. 2005; Biertumpfel, Zhao et al. 2010). Pol η's active site is considerably bigger than that of other crystallised polymerases and able to accommodate two bases of the template strand; the larger active site and its more open confirmation are believed to be able to accommodate bulky DNA adducts such as thymidine-thymidine dimers caused by UV irradiation (Alt, Lammens et al. 2007; Biertumpfel, Zhao et al. 2010; Silverstein, Johnson et al. 2010).

Like other TLS polymerases, pol η lacks an intrinsic 3'-5' exonuclease proofreading activity (Matsuda, Bebenek et al. 2000; Washington, Johnson et al. 2001). Pol η has been reported to make fewer contacts to the newly synthesised base pair and lacks the O helix of replicative polymerases that is involved in checking the steric conformation of forming base pairs (Yang 2003). Selection of the Watson-Crick geometry by DNA-protein interactions is of less importance for TLS polymerases compared to replicative enzymes. In contrast, the dNTP selection of TLS polymerases is largely regulated by interstrand hydrogen bond formation (Hwang and Taylor 2005; DeCarlo, Gowda et al. 2008).

Due to these unique features, pol η is considerably error-prone when replicating past undamaged DNA, and strict regulation has to be in place to limit pol η activity to certain lesion sites and thereby ensure high fidelity of DNA synthesis in the absence of lesions (McCulloch and Kunkel 2008; Rey, Sidorova et al. 2009).

1.1.4 Translesional activity of DNA polymerase η

The bypass mechanism by which pol η acts has been subject to extensive studies, but has not been fully elucidated. Pol η has been discovered in the context of a UV sensitivity disease, and it has been shown that pol η is capable of bypassing UV-induced intrastrand crosslinking DNA adducts, such as thymidine-thymidine dimers (CPDs) or pyrimidine-6/4-pyrimidone adducts in a relatively error-free manner (Johnson, Washington et al. 2000; Yoon, Prakash et al. 2010). Additionally, pol η can also bypass a variety of other DNA lesions *in vitro*, such as 7,8-dihydro-8-oxoguanine, O6-methylguanine, benzo[a]pyrene-N2-deoxyguanine DNA adducts as well as adducts created by the anticancer drugs cisplatin and oxaliplatin (Vaisman, Masutani et al. 2000; Rechkoblit, Zhang et al. 2002; Albertella, Green et al. 2005; Choi, Chowdhury et al. 2006; McCulloch, Kokoska et al. 2009).

After recruitment to the damaged DNA strand, pol η is believed to replace the stalled replicative polymerases and continue replication by inserting nucleotides opposite the lesion. The enzyme can also extend the nascent strand by a few nucleotides beyond the damaged site before the polymerase-primer terminus is destabilized, and a polymerase switch at the lesion site enables replicative DNA polymerases α or δ to

continue high-fidelity strand elongation (McCulloch, Kokoska et al. 2004). Although pol η lacks any proofreading activity, after the incorporation of incorrect nucleotides opposite the lesion, pol η cannot continue chain elongation and dissociates from the damage site (Masutani, Kusumoto et al. 2000; Washington, Johnson et al. 2001). This phenomenon has been attributed to the conformation of certain hydrogen bonds between the enzyme's catalytic domain, the DNA strand and the incoming nucleotides (Alt, Lammens et al. 2007). Therefore, the fidelity of TLS by pol η relies both on the ability of the enzyme to insert the correct nucleotide opposite a DNA lesion and to only continue elongating DNA strands that have a correctly paired primer terminus. Whereas processivity of pol η is low during the replication of undamaged DNA, the enzyme was found to copy CPDs and flanking nucleotides with significantly higher processivity (McCulloch, Kokoska et al. 2004). Unlike replicative DNA polymerases, pol η is able to align distorted DNA strands by forcing it into the normal B form; it does so through strong interactions between its positively charged binding surface and up to four bases upstream of the lesion site as well as through hydrogen bond formation between pol η's "little finger" domain and the phosphate residues on the DNA (Biertumpfel, Zhao et al. 2010). In this respect, pol η is believed to act as a molecular "splint" even for heavily distorted DNA strands. *In vitro* experiments have revealed higher binding affinity of pol η to DNA containing a CPD than to undamaged DNA (Washington, Johnson et al. 2000), and it has been suggested that pol η is restricted from accessing undamaged DNA based on the finding that cellular overexpression of the polymerase does not increase mutagenesis despite its low-fidelity polymerisation activity (King, Nikolaishvili-Feinberg et al. 2005). Pol η's enzymatic activity on damaged and

especially on undamaged DNA needs to be tightly coordinated and regulated due to the polymerase's mutagenic nature.

1.1.5 Recruitment and regulation of activity of DNA polymerase η

Since pol η is considerably error-prone when replicating past undamaged DNA, its recruitment to lesions sites and its activity on DNA has to be tightly regulated. The mechanism is set into action when a replication fork encounters a blocking lesion and stalls, thereby recruiting Rad6 and the E3 ubiquitin ligase Rad18 to the DNA damage site (**FIGURE 1.1**). The heterodimeric Rad6-Rad18 complex leads to the monoubiquitination of the DNA clamping protein Proliferating Cell Nuclear Antigen (PCNA) (Miyase, Tateishi et al. 2005; Hibbert, Huang et al. 2011). The role of PCNA monoubiquitination is not fully understood, but it may play a role in TLS signalling, in strengthening the binding between the sliding clamp and the polymerase or in the switch between the stalled replicative polymerase and the incoming TLS polymerase (Haracska, Unk et al. 2006; Freudenthal, Gakhar et al. 2010). However, in Rad18-deficient cells, pol η was still able to bypass certain DNA lesions that require translesional activity (Schmutz, Janel-Bintz et al. 2010). Accordingly, pol η binds and interacts with both the monoubiquitinated as well as the unmodified form of the DNA processivity clamp PCNA. The pol η protein sequence exhibits two PCNA-interacting peptide (PIP) sequences and a ubiquitin-binding zinc finger (UBZ) motif (Acharya, Yoon et al. 2008). However, it has been a subject of discussion if PCNA monoubiquitination is required for the translesional activity of pol η, and while its PIP domains are indispensable for its ability to perform TLS, binding to monoubiquitinated

PCNA through its UBZ domain does not seem to be required (Acharya, Yoon et al. 2008; Nikolaishvili-Feinberg, Jenkins et al. 2008; Acharya, Yoon et al. 2010).

Pol η itself can be monoubiquitinated, and monoubiquitination inhibits the translesional function of the protein (Bienko, Green et al. 2010). Additionally, phosphorylation of the serine 601 residue of pol η has been reported to be necessary for efficient translesional activity past UV-induced damage, and the phosphorylation links DNA damage signalling by ataxia teleangiectasia mutated-Rad3-related (ATR) kinase and TLS (Gohler, Sabbioneda et al. 2011). The heat shock protein Hsp90 has been identified to regulate pol η function by increasing its folding into the active form, and Hsp90 inhibition was found to lead to a pol η-dependent increase in mutagenesis and cytotoxicity caused by UV irradiation (Sekimoto, Oda et al. 2010). Removal of pol η from the DNA strand is not yet properly understood, but deubiquitination of the PCNA sliding clamp may be involved, allowing a switch back to the replicative DNA polymerase and enabling the replication fork to move along the DNA strand again (Zhuang, Johnson et al. 2008). Additional regulatory mechanisms may also be involved in the polymerase switch during TLS, and alternative protein complexes such as the alternative processivity complex 9-1-1 have been shown to be loaded onto damaged DNA and interact with TLS polymerases in yeast models (Garg, Stith et al. 2005; Fu, Zhu et al. 2008). Additionally, pol η has been shown to be able to interact with the TLS polymerase, REV1 (Acharya, Haracska et al. 2007).

1.2 REPAIR OF IONISING-RADIATION-INDUCED DNA DAMAGE

The human body is constantly exposed to low radiation doses from natural sources and may also be subject to higher doses if exposed to sources used in diagnostic radiology and therapeutic radiation therapy. IR can damage the DNA in different ways, either by depositing its energy directly to the strands, or by ionising cellular water molecules which leads to the creation of hydroxyl radicals that can then cause DNA damage (Mahaney, Meek et al. 2009). Various forms of DNA damage have been described as a consequence of IR treatment, such as damage to DNA bases or the sugar backbone. Dependent on the energy transfer, IR has the potential to induce complex or clustered strand breaks that are characterised by different forms of DNA damage within one region of the DNA (Goodhead 1994). If those lesions remain unrepaired, they may lead to cell death, and their misrepair may result in mutations and genomic instability.

Although base damage and DNA single-strand breaks (SSBs) are much more frequent than double-strand breaks (DSBs), SSBs can be rapidly and accurately repaired using the intact complementary DNA strand. Therefore, DSBs are considered the main toxic lesion by which IR kills cells, and are produced either directly, or as the result of two SSBs in close proximity on opposite strands. As a result, many IR-induced DSBs exhibit overhangs of the 3’ or 5’ strands, as well as 3’-phosphate groups that need processing before the breaks can be ligated (Mimitou and Symington 2011).

1.2.1 Repair of ionising radiation-induced DNA double-strand breaks

Since IR-induced DSBs carry a high risk of losing genetic information and may therefore be potentially lethal to cells, their immediate and efficient repair is crucial for genomic integrity and cell survival. Two major pathways have been described to repair DNA DSBs in mammalian cells, non-homologous end joining (NHEJ) and homologous recombination (HR) (Helleday, Lo et al. 2007; Helleday, Petermann et al. 2008). Since HR uses the sister chromatid or the homologous chromosome as a template for repair of the DSB, it is more accurate than NHEJ. However, HR can only take place after DNA replication, so the majority of IR-induced DSBs are repaired by NHEJ (Branzei and Foiani 2008). "Clean" DSBs with no overhangs or modified strand ends are in most of the cases repaired accurately by NHEJ (Helleday, Lo et al. 2007). However, since IR mostly leads to complex breaks comprising strand ends with non-ligatable groups, NHEJ requires removal of those nucleotides from both sides of the strand break before successful realignment and ligation can take place. Processing of the strand end may lead to the loss of chromosomal material and is therefore potentially error-prone.

1.2.1.1 Non-homologous end joining

The NHEJ pathway employs a variety of different proteins that participate in recognition of DSBs, processing of the strand ends and ligation (**FIGURE 1.2**). Initially, a DSB is recognised by Ku, a heterodimeric protein consisting of Ku70 and Ku80 (Chen, Peterson et al. 1996; Jin and Weaver 1997). As the major DSB-sensing protein involved in NHEJ, Ku localises to sites of DSBs independently of other factors, but is itself required for the recruitment of other repair proteins to DSB sites. *In vivo* data have shown that the C terminal domain of Ku80 is able to interact with the DNA-

dependent protein kinase catalytic subunit (DNA-PKcs), and recruits the kinase to lesion sites which is a key step in NHEJ (Schild-Poulter, Pope et al. 2001). DNA-PKcs has weak enzymatic activity and is able to autophosphorylate and also phosphorylate various other proteins, an essential step in NHEJ (Hartley, Gell et al. 1995; Ding, Reddy et al. 2003). The role of DNA-PKcs autophosphorylation has only been elucidated *in vitro*, but studies suggest that upon phosphorylation, the DNA-PK complex disassembles and opens up the DSB ends to other proteins (Iijima, Ohara et al. 2008; Kusumoto, Dawut et al. 2008).

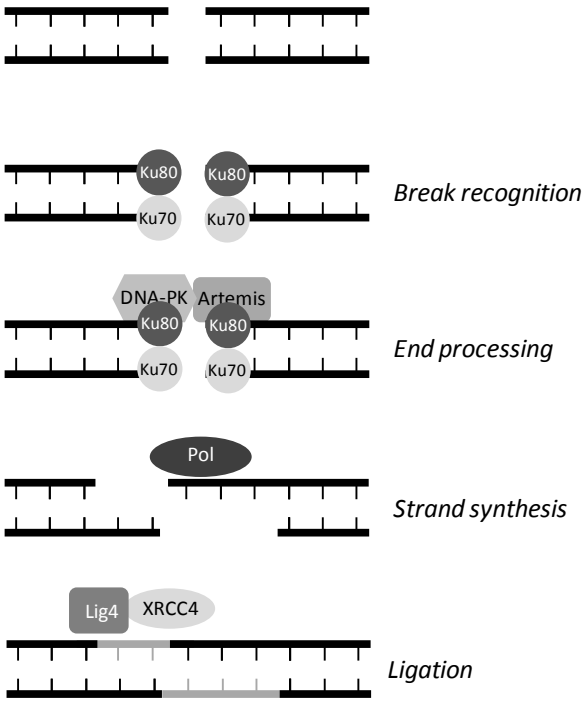


Figure 1.2. Schematic depiction of the NHEJ pathway

Once DSBs have been detected and tethered, successful religation requires processing of the DNA termini to remove other DNA lesions and non-ligatable groups. Since DNA DSBs are highly variable in nature and are usually part of complex lesions, several different enzymes or enzyme combinations are required for processing strand termini. Some proteins that have been linked to end processing include Artemis, poly-nucleotide kinase (PNK), and DNA polymerases λ and μ. Artemis is the main nuclease enzyme for the preparation of strand ends before ligation, it possesses both 5'-3' exonuclease and, interacting with DNA-PK, endonuclease activity (Pannicke, Ma et al. 2004; Coleman and Greenberg 2011). Additional factors have been attributed to end processing during NHEJ. PNK exhibits both 3'-DNA phosphatase and 5'-DNA kinase activity which both may play a role in end processing, and studies have shown PNK involvement in NHEJ (Liu, Doty et al. 2010). In *in vitro* studies, it has been shown that, if IR-induced damage contains gaps that need to be filled prior to ligation, X-family DNA polymerases μ and λ are recruited to lesion sites by Ku and the X4L4 complex, consisting of DNA ligase IV and XRCC4 (McIlwraith, Van Dyck et al. 2000). Eventually, if strand ends are suitable for ligation, the protein complex X4L4, containing DNA ligase IV and XRCC4, carries out the last step of NHEJ with the support of additional proteins like Cernunnos (Callebaut, Malivert et al. 2006).

1.2.1.2 Homologous recombination

In contrast to NHEJ, HR is a pathway for the conservative repair of DSBs; however it requires the presence of a homologous sequence to carry out high-fidelity repair (**FIGURE 1.3**). Analogous to NHEJ, the first step of HR repair requires recognition and marking of the lesion site. The damage recognition proteins in this

pathway are Mre11, Rad50 and Nbs1, which interact and form a functional unit termed the MRN complex, found to be crucial for DSB recognition and early response (Zhang, Fan et al. 2009). MRN complexes form on both ends of the strand break and are believed to interact by homodimerisation of the Rad50 components, thereby aligning the strands. Additionally, interaction of Nbs1 and the phosphoinositol-3 like kinase ATM leads to the recruitment of ATM and the subsequent phosphorylation of multiple proteins involved in damage response (Iijima, Ohara et al. 2008). Strand ends created by IR are processed by various proteins like Exo1, Bloom's syndrome protein (BLM), BRCA1 and C terminus-binding protein of adenovirus E1A-interacting protein (CtIP) (Kusumoto, Dawut et al. 2008; Eid, Steger et al. 2010; Coleman and Greenberg 2011). During end processing, single-strand overhangs are created, that are bound by replication protein A (RPA). Single-strand binding by RPA prevents the formation of DNA secondary structures, allowing RAD51 multimers to assemble and form a nucleoprotein filament along the strand (McIlwraith, Van Dyck et al. 2000). BRCA1 and BRCA2 have been attributed to facilitate RAD51 binding, and both proteins have been shown to be required for IR-induced RAD51 foci formation (Zhang, Fan et al. 2009; Liu, Doty et al. 2010). Since a key feature of HR is the use of a homologous DNA sequence as a template for the broken strand to allow for conservative repair, pairing of homologous DNA segments is required. This mechanism has not been fully elucidated yet, but studies suggest that the RAD51-DNA filament collides randomly with duplex DNA until regions of homology are detected (Bianco, Tracy et al. 1998).

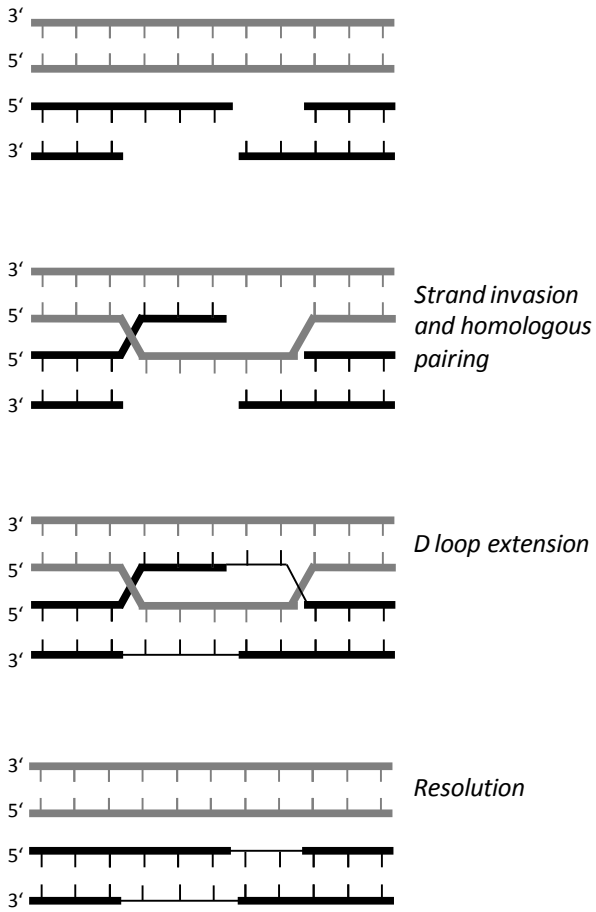


Figure 1.3. Schematic depiction of the HR pathway

Strand invasion by the RAD51-coated single-strand leads to the formation of a temporary D loop, and the 3' end initiates strand elongation using the complementary double-stranded DNA as a template. It is not yet clear which DNA polymerase is required for this process, but both DNA polymerase η and polymerase δ have been shown *in vitro* to be able to perform the D loop extension (McIlwraith, Vaisman et al. 2005; Sebesta, Burkovics et al. 2011). Upon strand invasion and D loop extension of the 3' end, several models have been developed to explain ways of repairing and annealing

the broken strands. The most commonly applied model proposes that, following extension of the invading strand, the strand may be displaced from the duplex DNA, and be re-annealed with the processed second strand to form again an intact double-stranded DNA molecule (Hartlerode and Scully 2009). However, if the second strand end is also captured by the D loop, a so-called double Holliday junction (HJ) is formed. Once a HJ has been formed, helicases like Werner's syndrome protein (WRN), BLM and RecQ5β, enlarge the created "bubble" structure in a mechanism termed "branch migration" (Bachrati and Hickson 2006). Eventually, HJs are resolved in order to untie the two DNA duplex structures, and proteins like Mus81, Eme1, SLX1, SLX4 or Gen1 are believed to participate in this step (Boddy, Gaillard et al. 2001; Bachrati and Hickson 2009; Rass, Compton et al. 2010). The final ligation step is likely carried out by ligase I, and deficiency in ligase I resulted in impaired HR activity (Goetz, Motycka et al. 2005).

1.2.2 Repair of other radiation-induced DNA damage

Although less toxic than DNA strand breaks, the majority of DNA lesions induced by IR are damaged bases, DNA adducts and intra- and inter-strand crosslinks (Cadet, Bellon et al. 2004; Dextraze, Gantchev et al. 2010). These lesions are mostly repaired by two pathways, mismatch repair (MMR) and base excision repair (BER).

BER is a DNA repair pathway that is utilised to remove the majority of DNA adducts induced by both endogenous and exogenous substances. Upon recognition of a base adduct, an adduct-specific DNA glycosylase cleaves the N-glycosidic bond of the damaged base and creates an apurinic/apyrimidinic (AP) site (Friedman and Stivers

2010). The AP site then undergoes processing by AP endonuclease 1 that creates a strand gap that is flanked by a 3'-hydroxyl group on one side, and a 5'-deoxyribose residue on the other (Hegde, Hazra et al. 2008). The subsequent steps of BER either use the short-patch repair way for single nucleotide gaps or long-patch repair for gaps spanning between 2 and 15 nucleotides. Both steps are initiated by DNA polymerase ß that is only able to insert a single nucleotide so that the long-patch pathway requires additional nucleotide synthesis by pol δ or ε. In long-patch BER, a 5' nucleotide flap is created that is subsequently removed by flap endonuclease 1 (FEN1) before final ligation can take place. Ligation in the short-patch pathway is carried out by DNA ligase III using XRCC1 and poly ADP ribose polymerase (PARP) as cofactors, whereas in long-patch BER, DNA ligase I reseals the nick (Tomkinson, Chen et al. 2001). Despite the fact that the BER pathway mostly repairs minor forms of IR-induced damage, changes in the expression of certain BER proteins have been shown to alter cellular responses to IR. Overexpression of the key BER polymerase, pol β, increases radiosensitivity by an increase in IR-induced apoptosis (Frechet, Canitrot et al. 2002). In contrast, extracts from cells either deficient in pol β or expressing a dominant-negative mutant exhibit a lower level of repair of radiation-induced SSBs, albeit with only marginal effects on cellular survival, likely due to the less toxic nature of these IR-induced lesions (Vens, Hofland et al. 2007). Additionally, truncated and non-functional forms of pol β were found to make cells dependent on the HR pathway for the repair of DNA damage caused by IR (Neijenhuis, Verwijs-Janssen et al. 2010). Furthermore, radiosensitisation caused by the expression of a dominant-negative form of pol β has been linked to an inhibition of cellular XRCC1 activity (Neijenhuis, Begg et al. 2005).

MMR repairs base-base mismatches that arise during DNA replication and may otherwise cause point mutations and frameshift mutations. MMR pathway initiation requires the recognition of base mismatches through the MutSα or ß protein dimers, before mismatch excision can be carried out by the exonuclease EXO1. EXO1 is recruited to lesion sites through the binding of MutLα or ß to MutSα, and removes several nucleotides beyond the nucleotide mismatch thus creating a SSB. DNA polymerase δ then re-synthesises the missing strand segment in a process involving the DNA binding proteins PCNA and RPA, before a DNA ligase eventually seals the nick (Li 2008). The role of MMR in the response to IR is largely due to an increase in free 8-oxo-7,8-dihydrodeoxyguanine triphosphate (8-oxodGTP) nucleotides owing to oxidation after IR. These 8-oxodGTPs are incorporated instead of dGTP and are recognised by MMR, leading to G2 arrest by signalling the cell cycle regulation cdc2 pathway. 8-oxoG in DNA is efficiently removed by the human 8-hydroxyguanine glycosylase 1 (hOGG1) via the BER pathway (Martin, Marples et al. 2010). The MMR protein MSH6 was implicated to interact with Ku70 during NHEJ, and MSH6-deficient cells were found to be hypersensitive to IR-induced cell death (Shahi, Lee et al. 2011).

1.2.3 Cellular resistance to ionising radiation

Resistance to IR is a common problem in the clinical setting that may limit the potential of radiotherapy for cancer treatment. Recurrence of tumour diseases is often associated with an acquired increase in radioresistance, and initially sensitive tumours are therefore rendered unsuitable for further radiation treatment (Dutreix, Cosset et al. 2010). The sensitivity of tumours to IR is largely determined by five factors: the

intrinsic radiosensitivity of each cell and tissue, the ability to repair DNA damage induced by radiation, the re-population of the cells not killed by IR, the re-oxygenation of hypoxic tumour cells through the shrinking of the tumour bulk during radiotherapy, and the redistribution of cells that were synchronised in radioresistant phases of the cell cycle to more sensitive phases (Trott 1982; Pajonk, Vlashi et al. 2010).

As DSBs are the main form of DNA damage by which IR kills cells, an increase in DSB repair has been linked to a radioresistant phenotype in different tumour cell models (Negroni, Stronati et al. 2008; Facchino, Abdouh et al. 2010; Lynam-Lennon, Reynolds et al. 2010). Additionally, increased activities in other pathways such as the BER pathway have also been shown to lead to a rise in radioresistance in cell culture (Naidu, Mason et al. 2010; Vens and Begg 2010; Kim, Lee et al. 2011). Therefore, it has been suggested that proteins involved in DSB and other DNA damage repair pathways may be able to predict the radiation response of tumour cells (Welsh, Ellsworth et al. 2009; Moeller, Yordy et al. 2011), and deficiency in certain DNA repair proteins may alter radioresistance and sensitise cells to IR treatment (Vens, Dahmen-Mooren et al. 2002; Zhuang, Li et al. 2011).

Another mechanism leading to an increased radioresistance is termed “re-population”: Tumour cells survive sublethal doses of fractionated radiotherapy and regrow after treatment or in between treatment fractions, thereby re-populating the tumour tissue and selecting for cells with a radioresistant phenotype (Marks and Dewhirst 1991). This problem has come into focus over the recent years with an increased understanding of so-called cancer stem cells that are inherently radioresistant

and have the potential to replace radiosensitive tumour cells killed by IR treatment and make the tumour as a whole more resistant to radiotherapy (Baumann, Krause et al. 2008; Pajonk, Vlashi et al. 2010). Clinically, re-population of irradiated tumours has a major impact on radiotherapy fractionation schemes and overall treatment times for different tumour types.

The importance of oxygen as one of the most potent modifier of cellular radiation responses was first described over a century ago (Schwarz 1909; Gray, Conger et al. 1953; Gray 1957). This phenomenon is of particular relevance for the clinical setting where there usually is an imbalance between oxygen demand and oxygen delivery of tumours rendering cancer sites partially hypoxic. It has been shown both by direct measurement of O_2 partial pressure and using hypoxia-inducible factor 1α (HIF-1α) or pimonidazole as surrogate markers for hypoxia that hypoxia correlates with reduced tumour response to IR and poor outcome (Kaanders, Wijffels et al. 2002; Nordsmark, Bentzen et al. 2005; Silva, Slevin et al. 2008). Hypoxic tumour tissue not only lacks the O_2 required for the formation of reactive oxygen species that mediate IR-induced DNA damage, it also shows altered signalling pathways that modulate transcription and anti-apoptotic cascades and eventually result in a radioresistant phenotype (Cuisnier, Serduc et al. 2003; Voss, Niggemann et al. 2010).

Lastly, the sensitivity of cells depends on the phase of the cell cycle during which radiation treatment is applied. While G1 and G2/M phases have been described to be largely radiosensitive, cells in S phase are relatively radioresistant (Sinclair and Morton 1966; Cheong, Wang et al. 1994). This effect has been attributed to an increase

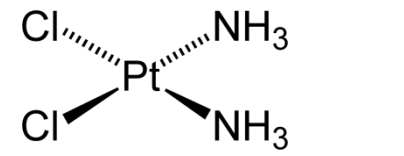
in homology-directed repair, and cells deficient in HR proteins such as RAD51 lose their increased radioresistance during S phase (Tamulevicius, Wang et al. 2007). A consecutive reduction of the HR activity has been reported as cell progress from late S to G2 phase of the cell cycle, which explains an increased radiosensitivity of G2 despite the cellular ability for HR repair (Wilson, Hinz et al. 2010). Accordingly, cells with altered cell cycle distribution profiles may experience changes in resistance to IR treatment, and it has been described that cancer stem cells exhibit an increased S phase population that may help to explain their increased radioresistance (Al-Assar, Mantoni et al. 2011).

1.3 REPAIR AND BYPASS OF PLATINUM-INDUCED DNA ADDUCTS

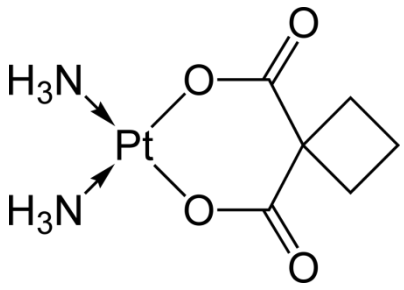
1.3.1 The platinum chemotherapeutic agents

Platinum-based anticancer agents are among the most commonly used drugs to treat patients with solid malignancies (**FIGURE 1.4**). The first compound discovered within this group was cis-diamminedichloroplatinum(II) (cisplatin); its chemical structure and synthesis was described in 1844 (Peyrone 1844). The potential use of cisplatin as a cytotoxic agent was discovered in the 1960s, and it was approved for clinical use by the United States Food and Drug Administration in 1978 (Pasetto, D'Andrea et al. 2006). Although cisplatin has achieved wide approval, its toxicity profile and wide-ranging side effects have led to the development of thousands of similar platinum compounds, of which less than 30 have been tested in clinical trials (Lebwohl and Canetta 1998). To date, only carboplatin and oxaliplatin have been successfully introduced into standard clinical regimens for cancer treatment, and

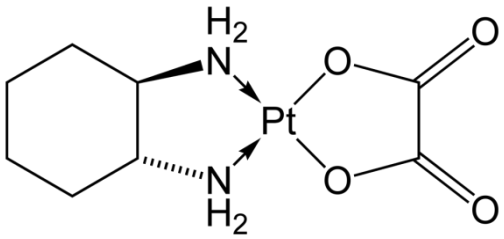
satraplatin and picoplatin are currently undergoing clinical studies for prostate cancer and non-small cell lung cancer respectively (Shah and Dizon 2009).



Cisplatin



Carboplatin



Oxaliplatin

Figure 1.4. Chemical structures of platinum compounds currently in routine clinical use

Cisplatin has been widely used to successfully treat various tumour types, among them epithelial ovarian, testicular, lung, bladder, breast, head and neck cancers, sarcomas and lymphomas (Boulikas and Vougiouka 2004; Pasetto, D'Andrea et al. 2006). However, it has an unfavourable toxicity profile with a marked toxicity to the kidneys, the nervous system, the inner ear; it also causes myelosuppression, electrolyte disturbances and nausea and vomiting (Barabas, Milner et al. 2008). Nephrotoxicity can also prove dose-limiting (Pabla and Dong 2008).

In view of the patients' quality of life, carboplatin treatment has replaced cisplatin as the drug of choice for many cancers (Moncharmont, Auberdiac et al. 2011). Carboplatin was first introduced into the clinic in 1985 and has been used to replace cisplatin for different cancer types (Pasetto, D'Andrea et al. 2006). The compound is filtered through the renal glomeruli but does not get reabsorbed or excreted in the kidneys which reduces its nephrotoxic potential (McKeage 1995). In contrast, the myelosuppressive potential of carboplatin, especially its ability to cause severe thrombocytopenia, usually limits the maximum tolerable dose (van der Vijgh 1991). Despite its favourable toxicity profile, carboplatin has less efficacy than cisplatin in the treatment of germ cell tumours, oesophageal cancer and head and neck cancer, but similar efficacy in lung and ovarian cancer, although definitive studies are lacking (Go and Adjei 1999; Pasetto, D'Andrea et al. 2006).

(Trans-R,R)1,2-diaminocyclohexaneoxalatoplatinum(II) (oxaliplatin) is the only second-generation platinum-based chemotherapeutic drug that has obtained approval for routine cancer treatment. It is widely used as a first-line agent for the therapy of

metastatic colorectal cancer in combination with 5-fluorouracil (5-FU) and folinic acid (de Gramont, Figer et al. 2000; Giacchetti, Perpoint et al. 2000). Oxaliplatin has a distinct toxicity profile from cisplatin and carboplatin; importantly, it does not exhibit nephrotoxicity, and shows delayed-onset reversible neurotoxicity which limits the maximum treatment dose (Schiff, Wen et al. 2009). Oxaliplatin binds to erythrocytic globin proteins, and it has been reported that after a 2-hour infusion of oxaliplatin, up to 40% of the blood oxaliplatin accumulates within erythrocytes (Levi, Metzger et al. 2000). This phenomenon limits the drug's bioavailability and increases the half-life of erythrocyte-bound oxaliplatin to up to 50 days, the elimination half-life of erythrocytes (Levi, Metzger et al. 2000). The drug's mean terminal half-life is about 26 hours, and oxaliplatin is removed from the plasma mainly by renal ultrafiltration and possibly tubular secretion (Kern, Braess et al. 1999).

1.3.2 Cellular effects of platinum agents

Despite being used for patient treatment for over 30 years, the exact mechanism by which platinum-based chemotherapeutic agents exert their cytotoxic effects is still poorly understood. It has been shown that cisplatin, carboplatin and oxaliplatin can bind to DNA strands thereby forming different forms of DNA damage, including monofunctional adducts, intrastrand crosslinks and interstrand crosslinks (Woynarowski, Faivre et al. 2000). Cisplatin and carboplatin exhibit the same cis-diammine carrier ligand; they form the same forms of platinum DNA adducts. The second generation platinum compound oxaliplatin uses the bulkier (trans-R,R)1,2-diaminocyclohexane (DACH) carrier ligand to form DNA adducts (Chaney, Campbell

et al. 2005; Ahmad 2010). Despite differences in the carrier ligand structures, cisplatin, carboplatin and oxaliplatin lead to the same types of platinum adducts at the same DNA sites: 60 – 65 % of platinum DNA lesions are adducts between two adjacent guanine residues (1,2-GG adducts) and a further 25 – 30 % adducts between adjacent adenine and guanine residues (1,2-AG and 1,2-GA adducts) within one DNA strand. About 10 % of platinum adducts are found to span a nucleotide to form 1,3 DNA adducts, and 1 – 3 % lead to adducts between guanine residues on opposite DNA strands (GG-interstrand adducts) (Jennerwein, Eastman et al. 1989; Page, Husain et al. 1990; Woynarowski, Chapman et al. 1998). Additionally, platinum-based drugs have been shown to bind to sulfur-containing amino acids on proteins, especially different methionine residues on histones, which may serve as "platinum reservoirs" from which bound platinum residues are transferred onto DNA molecules (Barnham, Djuran et al. 1996; Wu, Droge et al. 2008).

The *in vitro* activities of oxaliplatin and cisplatin are very similar with oxaliplatin being at least as potent as cisplatin in most of the tumour cells investigated (Rixe, Ortuzar et al. 1996). However, it has been reported that oxaliplatin treatment leads to fewer DNA lesions compared to equimolar doses of cisplatin (Saris, van de Vaart et al. 1996). These discrepancies can be explained by the structural differences between the smaller cisplatin adducts and the bulkier lesions formed by oxaliplatin's DACH ligand that distort the DNA strands to different degrees: oxaliplatin-induced DNA adducts have been suggested to lead to considerably smaller strand distortions (Wu, Pradhan et al. 2004; Wu, Bhattacharyya et al. 2007). The DACH ligand also reaches into the minor groove of the DNA double helix and may therefore have

additional effects on DNA protein interactions during transcription and replication (Scheeff, Briggs et al. 1999).

In addition to the effects caused by binding to the DNA, platinum-based cancer drugs have been shown to interact with various other cellular structures, among them membrane phospholipids, RNA and intracellular proteins (Timerbaev, Hartinger et al. 2006; Jensen, Bjerring et al. 2010), and it has been suggested that those effects may also contribute to the cytotoxic effects exerted by platinum-based cancer drugs (Wang, Reed et al. 2004).

DNA adducts caused by cisplatin, carboplatin or oxaliplatin not only distort the DNA strands, but they also prevent efficient transcription and DNA replication and eventually lead to the collapse of replication forks (Ciccarelli, Solomon et al. 1985; Al-Minawi, Lee et al. 2009; Ahmad 2010). DNA damage signaling (DDR) of platinum-induced lesions involves a variety of cellular signaling cascades (Wang and Lippard 2005). As a result, cells either experience cell cycle arrest and consecutive repair of DNA damage or die by apoptosis or necrosis (Cepeda, Fuertes et al. 2007).

1.3.3 Recognition and repair of DNA damage caused by cisplatin and oxaliplatin

As DNA adducts caused by cisplatin, carboplatin or oxaliplatin can severely affect cellular functions and can result in cell death, cells require mechanisms to cope with those lesions. Platinum-based chemotherapeutic drugs can create different types of adducts, and hence different cellular repair pathways are employed to cope with those

lesions (Ho and Scharer 2010). Intrastrand crosslinks make up the majority of platinum-induced DNA lesions and therefore leave the opposite strand as a template for resynthesis, which makes repair of those lesions relatively straight forward. Distorted platinum-induced DNA lesions are recognized by various cellular proteins like high-mobility group (HMG) proteins, transcription factors, repair proteins and structural proteins like histone H1 (Jamieson and Lippard 1999; Kartalou and Essigmann 2001). Upon recognition of platinum DNA adducts, signals are then transduced by various signaling cascades including the anti-apoptotic c-ABL and AKT pathways, the mitogen-activated protein kinase (MAPK) pathway, p53, the checkpoint kinases Chk1 and Chk2 (Wang and Lippard 2005).

1.3.3.1 Nucleotide excision repair

To remove platinum lesions from the DNA, the role of NER is well established, and the majority of platinum adducts are thought to be removed by this repair pathway (Moggs, Szymkowski et al. 1997). NER employs two distinct sub-pathways: While global genomic NER (GG-NER) is continuously active and deals with DNA lesions throughout the cell cycle phases, damaged sequences actively transcribed by RNA polymerase II undergo focused repair by transcription-coupled NER (TC-NER) (Furuta, Ueda et al. 2002; Tornaletti 2009). Platinum lesions are mostly repaired by TC-NER. After detection of the lesion by the Cockayne's syndrome proteins CSA and CSB, the xeroderma pigmentosum complementation group A (XPA) protein and RPA are recruited that facilitate DNA damage recognition before the helicases XPB and XPD are able to unwind the DNA around the damage site (Sugasawa, Ng et al. 1998; Fousteri, Vermeulen et al. 2006; Krasikova, Rechkunova et al. 2010; Oksenych and Coin 2010).

The strand bearing the platinum adduct is then incised by two endonucleases: on the 5' site by the XPF/DNA excision repair protein 1 (ERCC1) complex and on the 3' site by XPG (Matsunaga, Park et al. 1996). The missing strand segment generated by the excision is then resynthesized by DNA polymerases δ or ε, and DNA ligase III reseals the remaining nick (Moser, Kool et al. 2007; Ogi, Limsirichaikul et al. 2010).

1.3.3.2 Mismatch repair

MMR as another DNA repair pathway has been linked to the cellular repair of DNA lesions caused by first-generation platinum compounds cisplatin and carboplatin, but not the second-generation drug, oxaliplatin. Beyond its repair capacity, the MMR pathway has also been shown to activate cell cycle-regulating proteins like p53, p73 or c-ABL that mediate cell cycle effects or apoptosis (Nehme, Baskaran et al. 1999). Apoptosis after cisplatin treatment depends on functional MMR and p73 activation, and cells deficient in the MMR protein MLH1 or c-ABL fail to activate p73 and show reduced sensitivity to cisplatin (Gong, Costanzo et al. 1999; Nehme, Baskaran et al. 1999). In addition to the direct signalling of apoptosis by different MMR proteins, it has been suggested that the MMR pathway leads to futile repair cycles (Vaisman, Varchenko et al. 1998). Upon recognising a cisplatin-G–C mismatch, the MMR complex removes and refills the corresponding C from the intact strand thereby leaving the cisplatin-bound G residue in place; this futile repair activity eventually leads to the activation of ATM/ATR and consecutive cell cycle arrest or apoptosis (Martin, Hamilton et al. 2008; Pabla, Ma et al. 2011). Whereas the MMR complex responds to damage caused by cisplatin and carboplatin, it fails to recognise oxaliplatin DNA adducts (Vaisman, Varchenko et al. 1998).

1.3.3.3 Translesion synthesis

Damage induced by platinum-based drugs during S phase may not be amenable to immediate repair, and cells therefore depend on a temporary DNA damage tolerance mechanism in order to prevent replication fork stalling and continue DNA replication. In this respect, cells may cope with these forms of damage in a two-step process, first by allowing translesional bypass without repair and consecutively by repairing these lesions by the above-mentioned pathways after replication has taken place. Pol η has been shown to be able to bypass DNA lesions induced by platinum compounds *in vitro* with higher efficiency past oxaliplatin adducts compared to cisplatin adducts (Vaisman, Masutani et al. 2000). Therefore, it has been suggested that this difference in TLS efficiency may contribute to the lower mutagenic potential of oxaliplatin compared to cisplatin (Chaney, Campbell et al. 2005). Pol η preferentially incorporates dCMP opposite platinum 1,2-GG adducts, and upon incorporation of incorrect nucleotides cannot continue chain elongation (Masutani, Kusumoto et al. 2000); on oligonucleotide constructs, correct insertion and extension by pol η across cisplatin and oxaliplatin 1,2-GG adducts is 1000-2000 times more likely than incorrect insertion and extension (Bassett, Vaisman et al. 2003). In contrast, cisplatin-induced 1,3 DNA adducts could not be bypassed by pol η *in vitro* (Chijiwa, Masutani et al. 2010). The ability of pol η to bypass lesions caused by platinum-based agents *in vitro* is reflected in survival differences according to cellular pol η status, and cells deficient in pol η were found to be more sensitive to cisplatin treatment than cells expressing pol η as assessed by clonogenic assay (Albertella, Green et al. 2005). In cells treated with cisplatin, there is a significant increase of intranuclear PCNA and pol η foci, similar to the effects of UV irradiation on nuclei (Albertella, Green et al. 2005).

Recently, another TLS polymerase, pol κ has been suggested as a participant in cellular responses to platinum-induced DNA lesions. Upon recruitment by phosphorylated PCNA and XRCC1, pol κ is thought to form a complex with pol δ and then carries out the re-synthesis of the excised nucleotide fragment thereby filling the remaining gap during the last step of NER (Ogi, Limsirichaikul et al. 2010).

1.3.3.4 Interstrand crosslink repair

While intrastrand crosslinks induced by platinum-based compounds only affect one DNA strand and are easily repaired by NER, interstrand crosslinks (ICLs) disrupt genetic information on both DNA strands and therefore require the interplay of different pathways for their repair. Most of the information about pathways involved in ICL repair has been gathered from studying repair-deficient cell lines (Hinz 2010). In general, ICLs are repaired either in a replication-dependent or a replication-independent manner, and the choice of pathway largely depends on the involvement of a replication fork (Hlavin, Smeaton et al. 2010). In phases of the cell cycle other than replication, ICLs as other forms of platinum-induced DNA adducts are believed to be recognised due to their ability to distort the DNA helix (Clement, Camenisch et al. 2010).

As for NER, the distortion of the double helix is recognised by the XPC-RAD23B complex, and RPA then aids the NER proteins XPB and XPD to unwind the strands, before incisions are made on one of the strands by the ERCC1-XPF complex on the 5’ side and likely by XPG on the 3’ side of the lesion (Staresincic, Fagbemi et al. 2009; Zhao, Jain et al. 2009; Rahn, Adair et al. 2010; Wood 2010). This so-called

"unhooking" leaves an excised nucleotide fragment that is still attached to the remaining strand via the ICL. Unhooked ICL fragments are suitable substrates for TLS polymerases that are able to synthesize the excised strand past the ICL lesion and to restore one strand that can then be used as a template to repair the remaining strand by NER (Ho and Scharer 2010). There is evidence for the involvement of different polymerases to mediate this bypass step: pol ζ, Rev1, pol η, pol κ, pol ι and pol ν have all been linked to ICL bypass either *in vitro* or *in vivo* (Nojima, Hochegger et al. 2005; Sarkar, Davies et al. 2006; Shen, Jun et al. 2006; Minko, Harbut et al. 2008; Zietlow, Smith et al. 2009; Ho and Scharer 2010).

In contrast, replication-dependent repair requires a different set of repair pathways to resolve ICLs that have the potential to collapse replication forks. When a replication fork approaches an ICL, it is believed to stall, and a TLS polymerase extends the nascent leading strand up to the ICL, before a replication-associated, one-ended DSB is formed either mediated by replication past a SSB created by endonucleolytic incision or as a consequence of replication fork collapse (Raschle, Knipscheer et al. 2008; Hinz 2010; Wang, Sengerova et al. 2011). The Mus81-Eme1 complex is believed to initiate the removal of the ICL by incising one of the strands on the 3' side of the lesion, followed by a second incision on the 5' side by the ERCC1-XPF complex (Niedernhofer, Odijk et al. 2004; Hanada, Budzowska et al. 2006). Upon unhooking of the ICL, a nucleotide is inserted opposite the unhooked lesion in a TLS-dependent manner, and pol ζ extends from the nascent strand (Raschle, Knipscheer et al. 2008). It is believed that the unhooked ICL on the leading strand gets removed by NER, and the replicated leading strand then serves as a template to repair the remaining DSB in the

lagging strand in an HR-dependent fashion (Hinz 2010). ICL repair has been established as a prime example for the interplay between TLS, NER and HR.

1.3.4 Clinical relevance of treatment with platinum compounds and resistance formation

Platinum resistance is a major therapeutic problem, and many tumours are either intrinsically resistant or develop resistance during the course of treatment. To date, no treatment option exists in the clinical setting to circumvent platinum resistance. Similar problems exist for other chemotherapeutic agents that lead to increased tumour resistance during the course of treatment. It is believed that the development of resistance to platinum-based compounds is a multifactorial process involving different mechanisms and cellular pathways (Koberle, Tomicic et al. 2010).

1.3.4.1 Cellular drug uptake

It has been reported that cells with an acquired resistance to cisplatin exhibit a decreased intracellular accumulation of the drug (Loh, Mistry et al. 1992). The uptake of cisplatin was shown to be mediated by the copper transporter 1 (Ctr1), and loss of Ctr1 rendered cells resistant to high concentrations of cisplatin and oxaliplatin (Larson, Blair et al. 2009). Upon uptake, cisplatin has been shown to be able to downregulate Ctr1 within minutes of exposure to the drug (Holzer, Katano et al. 2004). In human ovarian cancer samples, low levels of CTR1 mRNA were found to be correlated with poor tumour response to cisplatin (Ishida, McCormick et al. 2010). Other copper transporters such as Ctr2 or the efflux pumps ATM7A and ATM7B have been shown to

influence efflux of platinum-based chemotherapeutic drugs from the cytosol, and high Ctr2 and ATM7A levels increased cellular resistance to those drugs (Katano, Safaei et al. 2003; Samimi, Safaei et al. 2004; Blair, Larson et al. 2009). Another carrier, the organic cation transporter 2 (OCT2) has been linked to cellular uptake of oxaliplatin, and cells that constitutively expressed OCT2 had a marked accumulation of the drug (Burger, Zoumaro-Djayoon et al. 2010)

1.3.4.2 Cytosolic inactivation

Upon uptake, platinum drugs can be inactivated by binding to intracellular thiol-containing molecules like glutathione or metallothionein before reaching the nucleus and damaging the DNA. The enzyme, glutathione-S transferase (GST) is able to catalyse the conjugation of cisplatin to glutathione, and increased levels of glutathione or GST have been shown to correlate with increased resistance to cisplatin in various tumour cell lines (Meijer, Mulder et al. 1992; Mellish, Kelland et al. 1993; Hour, Chen et al. 2000; Jansen, Brouwer et al. 2002). However, studies analysing a possible correlation between the glutathione/GST system and cisplatin resistance in clinical samples have shown conflicting results (van der Zee, Hollema et al. 1995; Bai, Nakanishi et al. 1996; Konishi, Nanbu et al. 1998; Cullen, Newkirk et al. 2003). Similarly with regards to oxaliplatin-based chemotherapy, conflicting findings have been reported that failed to clearly link GST expression and response or survival data (Kim, Kwon et al. 2009; Funke, Timofeeva et al. 2010).

Similar results have been obtained for metallothioneins (MTs), a group of low molecular weight proteins responsible for intracellular detoxification processes. Cell

culture-based experiments have suggested a link between upregulation of MTs and cisplatin resistance (Kennette, Collins et al. 2005), and in specimens from certain tumour types such as colorectal, ovarian and bladder cancer, high MT were found to be associated with resistance to cisplatin treatment and poor outcome (Hishikawa, Kohno et al. 2001; Surowiak, Materna et al. 2007; Wulfing, van Ahlen et al. 2007).

1.3.4.3 DNA repair

In addition to mechanisms preventing platinum compounds from reaching their target structures, increased removal of platinum adducts from DNA strands has been postulated as a means of cells developing resistance to platinum-containing drugs. The NER pathway removes the majority of platinum DNA adducts, and several research teams have reported alterations of expression or function of different NER components leading to a more proficient removal of the drug and hence a more resistant cellular phenotype. Ovarian cancer cell lines with an acquired cisplatin resistance were shown to have increased mRNA levels of ERCC1 (Ferry, Hamilton et al. 2000), and in a different publication, oxaliplatin-resistant HT29 colorectal cancer cells revealed upregulation of the NER gene, XPD (Plasencia, Martinez-Balibrea et al. 2006). On a functional level, enhanced repair of oxaliplatin and cisplatin-induced DNA damage was demonstrated in gastric adenocarcinoma cell lines with increased oxaliplatin resistance using host reactivation assays (Chen, Chen et al. 2007). Similarly, increases in the activity of the NER pathway were found in a cisplatin-resistant cervical cancer cell line (Mukai, Kanzaki et al. 2002). Clinically, high ERCC1 mRNA levels in tumour tissue correlated with decreased response to platinum-based chemotherapy and patient survival in several

published analyses (Dabholkar, Bostick-Bruton et al. 1992; Metzger, Leichman et al. 1998; Shirota, Stoehlmacher et al. 2001; Lord, Brabender et al. 2002).

Apart from the NER pathway, the MMR pathway has been linked to platinum resistance, and mutations leading to MMR deficiency have been shown to result in resistance to cisplatin and carboplatin, but they did not have an effect on oxaliplatin resistance (Fink, Nebel et al. 1996; Fink, Zheng et al. 1997; Lin and Howell 2006). As discussed above, this effect can be explained by a sequence of "futile" repair cycles eventually resulting in irreparable DNA damage and consecutive cell death (Vaisman, Varchenko et al. 1998).

1.3.4.4 DNA damage tolerance

There is increasing evidence that formation of resistance to platinum compounds may be mediated by an increase in tolerance to drug-induced DNA adducts, and several DNA polymerases may have a role in mediating damage tolerance in the context of cancer. *In vitro* assays have shown that pol β, pol ι, pol κ, pol ζ and pol η can bypass DNA lesions created by cisplatin and oxaliplatin (Bassett, Vaisman et al. 2003; Ho, Guainazzi et al. 2011). Knockdown of pol ζ by RNA interference has been shown to increase cellular sensitivity to cisplatin treatment in mismatch repair-deficient colorectal cancer cell lines that are intrinsically resistant to this drug (Lin, Trang et al. 2006). Additionally, pol ζ and Rev1 have been attributed to mediate resistance to cisplatin and oxaliplatin as they participate in both DNA damage tolerance and DNA repair via the HR pathway (Sharma, Hicks et al. 2011).

Overexpression of pol β as reported in a variety of cancer tissues was published to lead to a mutator phenotype in cell culture and a decreased sensitivity to platinum-containing drugs (Canitrot, Cazaux et al. 1998; Bergoglio, Canitrot et al. 2001). Similarly, pol η activity was linked to increased cellular tolerance to cisplatin-induced DNA damage (Albertella, Green et al. 2005). In a different publication, inhibition of the TLS scaffold protein, Rev1 was shown to inhibit cisplatin-induced mutagenesis and thus to prevent the emergence of resistance to the compound (Xie, Doles et al. 2010). Despite some published data derived from *in vitro* assays and cell culture experiments, the relevance of the TLS pathway in respect to treatment with platinum compounds remains largely unknown, and especially its relevance in cancer tissue and patients remains to be elucidated.

1.4 CLINICAL RELEVANCE OF Y FAMILY POLYMERASES IN HUMAN CANCERS

1.4.1 Y family polymerase expression in cancer tissues

Y family polymerases have been implicated in mutagenesis and tumorigenesis, and it has been suggested that their expression and activity has to be tightly regulated for this reason (Waters, Minesinger et al. 2009). To date, pol η is the only DNA polymerase exhibiting a causal connection with the development of cancers (Broughton, Cordonnier et al. 2002). Various severely truncating mutations in the *POLH* gene leading to a deficiency of functional pol η protein have been described in XPV patients from Europe, Asia and North America (Tanioka, Masaki et al. 2007; Inui, Oh et al. 2008). Additional variants of the *POLH* gene have been suggested as low penetrance

alleles predisposing for malignant melanoma (Di Lucca, Guedj et al. 2009). In contrast, no mutations or variations in the *POLH* gene were found in a panel of basal and squamous skin cancers (Glick, White et al. 2006; Flanagan, Rafferty et al. 2007).

Expression patterns of Y family TLS polymerases have been studied in a range of tumour samples, albeit with conflicting results. Albertella et al. reported an upregulation of pol η and ι in a panel of tumour specimens, while pol κ generally showed lower levels compared to corresponding normal tissues (Albertella, Lau et al. 2005). In this study, pol ι was increased more than two-fold in almost 30 % of all analysed samples, while a downregulation was observed in about one quarter of the specimens. In a panel of breast cancer biopsies, pol ι was found generally overexpressed, and it has been suggested that deregulation of the polymerase led to a decrease in fidelity and therefore contributed to increased genomic instability in those tumours (Yang, Chen et al. 2004). In glioma samples, pol ι upregulation was observed in 27 % of all examined cases (Wang, Wu et al. 2010). It has also been shown that hypoxia was able to upregulate pol ι expression in human tumour cell lines (Ito, Koshikawa et al. 2006). A study examining mRNA levels in oesophageal cancer samples reported a general upregulation of pol ι and κ (Zhou, Zhang et al. 2012). In contrast, Pan et al. report a general downregulation of the Y family polymerases, pol ι, η and κ in tumour specimens derived from lung, gastric and colorectal cancers (Pan, Fang et al. 2005).Two further studies reported no alteration in pol η expression in lung and gastric cancer specimens compared to normal tissue samples (Ceppi, Novello et al. 2009; Teng, Qiu et al. 2010). Another study assessed gene expression profiles in non-small cell lung cancer biopsies and reported an upregulation of pol η (Valk, Vooder et

al. 2010). In a small cohort of skin cancer patients, pol η was found downregulated in 4 of the 10 examined basal cell carcinoma biopsies and upregulated in 3 of 10 specimens, while only 1 out of 7 squamous cell cancer samples exhibited a significant pol η increase (Flanagan, Rafferty et al. 2007). In a panel of lung cancer samples, increased pol κ levels were found in 21 of 29 examined specimens (O-Wang, Kawamura et al. 2001), and pol κ upregulation was linked to genetic alterations and tumorigenesis in a mouse model (Bavoux, Leopoldino et al. 2005).

1.4.2 Links of Y family polymerase levels with treatment response and survival in cancer

To date, only few studies have examined the potential link between a deregulation of Y family polymerases and its impact on response to anti-cancer treatment and patient outcome using clinical samples. In a clinical study evaluating pol η expression in patients with non-small cell lung cancer, high *POLH* mRNA levels were found to correlate with reduced therapeutic response to chemotherapeutic treatment regimens containing cisplatin (Ceppi, Novello et al. 2009). Similarly, high expression levels of pol η protein measured by IHC were found to correlate with a decreased response to oxaliplatin-based cancer treatment and reduced overall patient survival (Teng, Qiu et al. 2010), leading to the implication that pol η may have the potential to serve as a biomarker for tumours treated with platinum-based chemotherapy. In tumour samples from glioma patients, expression of pol κ protein as measured by IHC correlated with a higher tumour grade and advanced degree of the disease and hence a

poorer patient prognosis (Wang, Wu et al. 2010). Similarly, high pol ι expression was also found to correlate with a reduced overall survival in this study.

1.5 AIMS

1.5.1 Scope of the research project

The remit of this project is to investigate the TLS polymerase, pol η in the context of cellular response to ionising radiation and platinum-based chemotherapy. Although pol η's role in bypassing platinum DNA adducts has been elucidated at a molecular level, its implications for the clinical setting are currently unclear. Less is known about the involvement of the TLS pathway and its polymerases in response to ionising radiation. Pol η has been causally linked to the development of early-onset cancer in humans. At a molecular level, it has been implicated to participate not only in the TLS cascade, but also in other cellular DNA repair pathways. A more complete understanding of the polymerase's role in DNA repair and temporary tolerance of DNA damage will help to determine if pol η qualifies as a promising target for improving current cancer treatments. Moreover, based on published *in vitro* data showing pol η's link to cellular platinum tolerance, the polymerase may be a useful biomarker to select cancer patients for certain treatment regimens.

1.5.2 Aims of the research project

The overall aim of the research project was to determine if pol η affects cellular responses to treatment with radiotherapy or chemotherapy.

The first specific aim of the project was to investigate the involvement of DNA polymerase η in cellular responses to ionising radiation. As IR induces a broad spectrum of different DNA lesions, various different repair pathways may have an involvement in removing the damage and restoring genomic integrity. The role of the TLS pathway and the possible role of the TLS polymerase, pol η in cellular responses to IR are largely unknown. In Chapter 3.1, I discuss the role of pol η in mediating cellular survival after exposure to ionizing radiation and I study biological mechanisms by which pol η may affect the radioresistance of cells

The second specific aim of the project was to elucidate the role of pol η in mediating cellular sensitivity to the anti-cancer drug, oxaliplatin. Although in-vitro assays have shown the ability of pol η to bypass platinum-induced DNA lesions, little is known about the biological relevance of the polymerase in determining oxaliplatin sensitivity in cancer cells. This part of the project aimed to test if inhibition of pol η potentially sensitised tumour cells to oxaliplatin-based chemotherapeutic treatment and re-sensitised oxaliplatin-resistant cancer cells.

The final specific aim of this project was to translate the preclinical findings described in chapter 3.2 into the clinical setting by studying pol η expression levels in a panel of tumour and normal tissue specimens derived from oesophageal cancer patients. This part of the project aimed to test if pol η levels may serve as a biomarker to predict therapeutic response to platinum-based cancer treatment.

MATERIALS AND METHODS

2.1 GENERAL LABORATORY SUPPLIES

2.1.1 Chemical reagents, kits and equipment

2.1.1.1 Chemical reagents

Chemicals for general laboratory use were obtained of analytical grade; they were purchased from Sigma-Aldrich (Poole, UK), Invitrogen (Paisley, UK) and Fisher Scientific (Loughborough, UK). Bovine serum albumin was purchased from Sigma-Aldrich.

2.1.1.2 Kits

Several kits were used for the research presented here. RNAlater clinical sample storage kit, DNA-RNA extraction kits, reverse transcription and Quantitect Probe PCR kits were purchased from Qiagen (Crawley, UK). Precast Tris-Acetate gels for Western blotting were purchased from Invitrogen. Polyvinylidene fluoride (PVDF) membranes were obtained from Millipore (Watford, UK).

2.1.1.3 Equipment

Irradiation treatment was performed using a 137-Cs irradiator (dose rate of 1.81 Gy/min) at room temperature. DNA damage and repair foci were visualised on an InCell analyser (GE Healthcare, Little Chalfont, UK). Measurements of protein and RNA content were performed using a NanoDrop ND-1000 (Thermo Scientific, Loughborough, UK).Western blots were visualised on a LI-COR Odyssey system (LI-COR Biosciences, Cambridge, UK). Colonies in clonogenic survival experiments were

counted either manually using a pressure-sensitive count system (Stuart, Stone, UK) or using an automated colony counter (Oxford Optronics, Milton Park, UK). A TissueRuptor homogeniser for clinical samples was purchased from Qiagen. Quantitative PCR experiments were performed using an Applied Biosystems 7500 Fast System. Cell viability assays were measured on an Envision 2103 plate reader (Perkin Elmer, Cambridge, UK). FACS analyses were performed on a FACSCalibur system (Becton Dickinson, Oxford, UK).

2.1.2 Buffers and solutions

Buffers used for RNA extraction and purification as well as quantitative real-time PCR were bought from Qiagen. Phosphate-buffered saline tablets were purchased from Oxoid (Basingstoke, UK). Running buffers and transfer buffers for Western blots were obtained from Invitrogen. Blocking buffer for Western blot membranes was bought from Li-Cor (Cambridge, UK). All buffers that were not commercially available were prepared as described in **Table 2.1**. Buffers were made up in purified H_2O which was obtained by reverse osmosis in a Millipore system. Where buffers were used for cell culture or other procedures that required sterility, they were autoclaved at 120°C at 15 pounds/inch^2 for 15 min.

Table 2.1. Buffers and solutions

Buffer/Solution	Constituents
Phosphate buffered saline (PBS)	1 PBS tablet per 100 mL purified H_2O
Fixation and staining solution for clonogenic survival assays	0.4% methylene blue (w/v) in 100% methanol
Protein extraction buffer	Phosphate buffer (10 mMNaH_2PO_4, 10 mMNa_2HPO_4), 500 mMNaCl, 1 mM EDTA, 0.75% w/w Triton-X 100, 10% w/w Glycerol, 5 mM $MgCl_2$
SDS-PAGE loading buffer (5X)	25 mMTris (pH 6.8), 2.5% (v/v) mercaptoethanol, 1% SDS, 5% glycerol, 0.05 mg/mL bromophenol blue, 1 mM EDTA
Running buffer	5 ml NuPage running buffer stock solution per 100 mL purified H_2O
Transfer buffer	5 ml NuPage transfer buffer stock solution per 100 mL purified H_2O, 10% methanol
Washing buffer	PBS, 0.1% Tween 20
4% paraformaldehyde (PFA) solution	4g PFA powder in 100 mL PBS
Permeabilisation buffer for foci staining	PBS, 0.3% Triton-X 100
Blocking buffer for foci staining	PBS, 3% bovine serum albumin (BSA)
DAPI solution	PBS, 5 μg/mL 4',6-diamidino-2-phenylindole (DAPI) powder
Stripping solution for Western blotting	Purified H_2O, 25 mM glycine-HCl, 1% (w/v) sodium dodecyl sulphate (SDS)
Propidium iodide (PI) solution	1 mg PI in 1 mL purified H_2O
Reverse transcription (RT) buffer	4 μL RT buffer, 1 μL reverse transcriptase, 1 μL primer mix (total of 6 μL) per RNA sample
PCR reaction mix	600 μL PCR master mix, 60 μL forward primer (8 μM), 60 μL reverse primer (8 μM), 60 μL probe (2 μM), 360 μL purified H_2O

2.2 MEDIA AND CELL CULTURE SUPPLIES

2.2.1 Media

Dulbecco's Modified Eagle Medium (DMEM), OptiMEM, foetal calf serum, penicillin/streptomycin and trypsin EDTA stock solution were purchased from Invitrogen; Roswell Park Memorial Institute (RPMI) 1640 medium was purchased from Sigma-Aldrich. Dharmafect I transfection reagent was obtained from Thermo Fisher Scientific (Epsom, UK). See **Table 2.2** for a list of media used for cell culture work.

Table 2.2. Cell culture media

Media/Solution	Constituents
Complete DMEM medium	DMEM, 10% foetal calf serum, 100 units penicillin, 100 μg/mL streptomycin
Complete RPMI 1640 medium	RPMI 1640, 10 % foetal calf serum, 100 units penicillin, 100 μg/mL streptomycin
Freezing media	Complete medium (DMEM or RPMI 1640 depending on cell line), 10% dimethyl sulfoxide
Antibiotic-free medium	DMEM, 10% foetal calf serum
Transfection medium	OptiMEM, 0.5% Dharmafect I

2.2.2 Cell culture supplies

25 cm^2, 75 cm^2 and 175 cm^2 cell culture flasks, 10 cm dishes, 6-well plates and 96-well plates and disposable pipettes were ordered from Corning (Birmingham, UK). Disposable pipette tips and filtered pipette tips for RNA work were ordered from VWR (Lutterworth, UK).

2.3 CELLS

2.3.1 Non-tumour cell lines

XP30RO and XP30RO/pol η cells were obtained from Alan Lehmann (University of Sussex, Brighton, UK). MRC5 cells were purchased from the American Type Culture Collection (Teddington, UK).

2.3.2 Tumour cell lines

HCT116 cells were obtained from Bert Vogelstein (Johns Hopkins University, Baltimore, USA). SQ20B cells were received from Gillies McKenna (University of Oxford, UK). P53-deficient HCT116 cells were received from Patrick Johnston (Queens University, Belfast, UK). BL-2 and BL-2 *POLH*-/- cells were donated from Claude-Agnès Reynaud (Université René Descartes, Paris, France).

2.3.3 Drug-resistant cell lines

Oxaliplatin-resistant HCT116 cells were gifted from Patrick Johnston. Oxaliplatin-resistant HCT116 and HT29 cells were obtained from Vanessa Almendro (Institut d'Investigacions Biomèdiques, Barcelona, Spain). Oxaliplatin-resistant and parental KM12-L4 cells were received from Lee Ellis (M.D. Anderson Cancer Center, Houston, USA). Oxaliplatin-resistant and parental AGS cells, MKN45 cells and ST16 cells were obtained from Srinavasan Madhusudan (University of Nottingham, UK).

Table 2.3. Cell lines

Name	Cell type	Medium
XP30RO	Skin fibroblasts	Complete DMEM medium
XP30RO/pol η	Skin fibroblasts	Complete DMEM medium, 100 µg/mL zeocin
MRC5	Lung fibroblasts	Complete DMEM medium
SQ20B	Laryngeal carcinoma cells	Complete DMEM medium
HCT116	Colorectal carcinoma cells	Complete DMEM medium
HCT116 oxaliplatin-resistant	Colorectal carcinoma cells	Complete DMEM medium
HT29	Colorectal carcinoma cells	Complete DMEM medium
HT29 oxaliplatin-resistant	Colorectal carcinoma cells	Complete DMEM medium
KM12-L4	Colorectal carcinoma cells	Complete DMEM medium
KM12-L4 oxaliplatin-resistant	Colorectal carcinoma cells	Complete DMEM medium
AGS	Gastric carcinoma cells	Complete RPMI 1640 medium
AGS oxaliplatin-resistant	Gastric carcinoma cells	Complete RPMI 1640 medium
MKN45	Gastric carcinoma cells	Complete RPMI 1640 medium
MKN45 oxaliplatin-resistant	Gastric carcinoma cells	Complete RPMI 1640 medium
ST16	Gastric carcinoma cells	Complete RPMI 1640 medium
ST16 oxaliplatin-resistant	Gastric carcinoma cells	Complete RPMI 1640 medium
BL-2	Burkitt’s lymphoma	Complete RPMI 1640 medium
BL-2 POLH-/-	Burkitt’s lymphoma	Complete RMPI 1640 medium

2.4 ANTIBODIES AND POLYNUCLEOTIDES

2.4.1 Antibodies

Antibodies were purchased from Atlas Antibodies (Stockholm, Sweden), Abcam (Cambridge, UK), R&D Systems (Abingdon, UK), Bethyl Laboratories (Cambridge, UK), Cell Signalling Technology (Hitchin, UK), Upstate Biotechnology (Watford, UK), LI-COR Biosciences (Cambridge, UK), Calbiochem (Nottingham, UK), Santa Cruz Biotechnology (Heidelberg, Germany), Novus Biologicals (Cambridge, UK) and Invitrogen. The p53 antibody was a gift from Ester Hammond (University of Oxford, UK).

Table 2.4. Antibodies for Western blotting

Epitope	Concentration	Species	Manufacturer
DNA polymerase η	1:1000	Rabbit	Atlas Antibodies (Stockholm, Sweden)
β-actin	1:20000	Mouse	Abcam
p53	1:50	Mouse	Gift from Dr. Ester Hammond
XRCC3	1:5000	Rabbit	Novus Biologicals
anti-rabbit 680 nm	1:10000	Goat	LI-COR Odyssey
anti-rabbit 800 nm	1:10000	Goat	LI-COR Odyssey
anti-mouse 680 nm	1:10000	Goat	LI-COR Odyssey
anti-mouse 800 nm	1:10000	Goat	LI-COR Odyssey

Table 2.5. Antibodies for foci staining

Epitope	Concentration	Species	Manufacturer
γH2AX	1:1000	rabbit	Calbiochem
53BP1	1:500	rabbit	Bethyl
RAD51	1:1000	rabbit	Santa Cruz
anti-rabbit, Alexa 488	1:500	Goat	Invitrogen
anti-mouse Alexa 488	1:500	Goat	Invitrogen

Table 2.6. siRNA sequences for knockdown experiments

Target	Sequence
***POLH* siRNA 1 (sense)**	5'-AAACUGGCCUGUGGACUAA-3'
***POLH* siRNA 1 (antisense)**	5'-UUA GUC CAC AGG CCA GUU U-3'
***POLH* siRNA 2 (sense)**	5'-GAA GUU AUG UCC AGA UCU U-3'
***POLH* siRNA 2 (antisense)**	5'-AAG AUC UGG ACA UAA CUU C-3'
***POLH* siRNA 3 (sense)**	5'-GCA CUU ACA UUG AAG GGU U-3'
***POLH* siRNA 3 (antisense)**	5'-AAC CCU UCA AUG UAA GUG C-3'
***POLH* siRNA 4 (sense)**	5'-GCA AUU AGC CCA GGA ACU A-3'
***POLH* siRNA 4 (antisense)**	5'-UAG UUC CUG GGC UAA UUG C-3'
XRCC3 siRNA (sense)	5'-GGA CCU GAA UCC CAG AAU U-3'
XRCC3 siRNA (antisense)	5'-AAU UCU GGG AUU CAG GUC C-3'
Non-targeting siRNA (sense)	All Star Negative Control

2.4.2 siRNA

siRNA was bought from Eurogentec (Fawley, UK), Dharmacon (Epsom, UK) and Qiagen (Crawley, UK). A list of used sequences can be found in **Table 2.6**.

2.4.3 Primers and probes

Primers and probes were designed as described in the Experimental Procedures section and were ordered custom-made from Sigma-Aldrich. A list of the sequences used for the experiments described here can be found in **Table 2.7**.

Table 2.7. Primers and probes for qRT-PCR

Target	Sequence
***POLH* forward primer**	5'-GCTGGTTGTGAGCATTCGTG
***POLH* reverse primer**	5'-TGCTCAAGAAGCTGGTGATGTC
***POLH* probe**	5'-(FAM)-AAACGCCTCAGCAGCCTGCGC-(TAMRA)
***GAPDH* forward primer**	5'-GAAGATGGTGATGGGATTTC
***GAPDH* reverse primer**	5'-GAAGGTGAAGGTCGGAGTC
***GAPDH* probe**	5'-(FAM)-CAAGCTTCCCGTTCTCAGCC-(TAMRA)

2.5 DRUGS

Oxaliplatin powder, cisplatin powder, 5-fluorouracil powder, resazurin powder and zeocin solution were purchased from Sigma-Aldrich.

2.6 EXPERIMENTAL PROCEDURES

2.6.1 Cell culture

2.6.1.1 Growth of cells

Cells were resuscitated from frozen stocks by rapid thawing at 37°C and addition of complete medium (see **Table 2.2**). DMSO was removed by centrifugation of the cells and re-suspension in fresh medium. Cells were then seeded in 75 cm^2 culture flasks, and viability was checked every 24 hours. When cells reached about 80% confluence, medium was removed and cells were washed with PBS twice before being trypsinised and diluted in fresh medium. Cells were kept in the incubator at 37°C and 5% CO_2. 100 μg/mL of zeocin was added to the medium for XP30RO/pol η cells containing a vector, and cells were continuously exposed to this drug during proliferation.

2.6.1.2 Growth of oxaliplatin-resistant cell lines

Oxaliplatin-resistant AGS, ST16, HT29, MKN45 and KM12-L4 cell lines were resuscitated and seeded using complete RPMI 1640 medium. After the first passage, a maintenance dose of oxaliplatin (5 μM oxaliplatin for AGS and ST16, 3.3 μM for MKN45, 2 μM for HT29 and KM12-L4) was added to the media and cells were cultured with oxaliplatin permanently present in culture. Oxaliplatin-resistant HCT116 cells were cultured without continuous oxaliplatin exposure, but were treated with 8 μM oxaliplatin for 72 once a month to select for resistance.

2.6.1.3 Storage of cells

Permanent stocks of cells were prepared from cultures grown to about 70% confluence, which were trypsinised and resuspended in complete DMEM media (see **Table 2.2**) containing 10% DMSO. Cells were then transferred to Nunc cryo-tubes (Paisley, UK) and stored at -80°C for 24 hours before permanent storage in liquid nitrogen.

2.6.2 siRNA transfection

siRNA stock solution was diluted in OptiMEM to a final concentration of 10 nM, and Dharmafect I transfection reagent was added to a final dilution of 0.5%, and the solution was incubated at room temperature for 20 min. After trypsinisation, cells were re-suspended in antibiotic-free medium (see **Table 2.2**), and 500 000 cells were plated to each well of a 6-well plate. The siRNA-containing solution was added, and cells were incubated with the solution at 37°C. 24 hours after transfection, medium was removed and replaced with complete medium before cells were further grown at 37°C. Knockdown efficiency was checked by Western blotting at 48 and 72 hours after transfection. Sequences for siRNA are shown in **Table 2.6**.

2.6.3 Preparation of drugs

Oxaliplatin powder and 5-fluorouracil powder were diluted in dimethyl sulfoxide (DMSO) to a final concentration of 20 mM, and aliquots were stored at -20°C. Cisplatin powder was diluted in purified H_2O to a final concentration of 5 mM, and

aliquots were stored at -20°C. Drugs were stored to a maximum of 30 days, before fresh solution was prepared from powder.

2.6.4 Clonogenic survival assays

2.6.4.1 Clonogenic survival assays using ionising radiation

Clonogenic survival assays were performed as published by Franken et al. (Franken, Rodermond et al. 2006). After trypsinising, cells were re-suspended in complete medium and counted. A defined number of cells were plated in 10 cm dishes in triplicate and allowed to attach. After 6 hours, dishes were treated with ionising gamma radiation as described above. After treatment, dishes were transferred to an incubator at 37°C and allowed to grow for another 10 to 14 days in order to form colonies. After incubation, growth medium was removed and colonies were fixed and stained using 0.4% methylene blue (w/v) in 100% methanol for 1 hour. Dishes were then rinsed and left to dry overnight before colonies containing more than 50 cells were counted, either manually or using an automated counter. Plating efficiency was calculated as the ratio of colonies counted in the untreated control dishes and the number of cells plated in those dishes:

$$\text{Plating efficiency} = \frac{\text{number of colonies formed}}{\text{number of cells seeded}} \text{ x } 100\%$$

Relative survival for each treatment condition was calculated as the ratio of the surviving colonies per dish and the seeded cell number and normalised to the plating efficiency:

$$\text{Surviving fraction} = \frac{\text{number of colonies formed}}{\text{number of cells plated x plating efficiency}}$$

2.6.4.2 Clonogenic survival assays using drug treatment

For drug treatment, clonogenic survival assays were set up as described above. A defined amount of cells was plated to 10 cm dishes and allowed to attach before treatment was initiated. Cells were treated with increasing doses of drug for a defined period of time (24 hours unless stated otherwise in the results section), after which the drug-containing medium was removed and fresh complete medium was added. Control dishes were only treated with the solvent used to prepare the drug solution (DMSO for oxaliplatin and 5-fluorouracil, purified water for cisplatin). Cells were then allowed to grow for a further 10 to 14 days before being fixed and stained with 0.4% methylene blue (w/v) in 100% methanol. Colonies were counted as described above and the surviving fraction was calculated as described in the previous section.

2.6.4.3 Clonogenic survival assays after siRNA knockdown

siRNA knockdown was performed as described above. In short, transfection solution was prepared to a final siRNA concentration of 10 nM (see **Table 2.2**). Cells were counted and 500 000 cells were transferred to each well of a 6-well plate. Controls

were included in the setup: Cells were mock-transfected with a transfection solution containing only transfection reagent and no siRNA, and a second control was included using non-targeting siRNA in the transfection solution. After 24 hours, the transfection medium was removed, and fresh complete medium was added to each well. After 42 hours, cells were trypsinised and replated to 10 cm dishes and allowed to attach for another 6 hours, so that treatment could be performed at 48 hours after transfection. Knockdown efficiency was checked at 48 and 72 hours to confirm protein depletion for the complete treatment time. At 48 hours after transfection, experiments were performed either using ionising gamma radiation or drug treatment, after which medium was exchanged after another 24 hours. As described above, cells were allowed to grow for a further 10 to 14 days before fixation and staining. Colonies were analysed as described in the previous section.

2.6.5 Cell viability assays

2.6.5.1 Cell viability assays using ionising radiation

The BL-2 cell line and its pol η-deficient clones (BL-2 *POLH*-/- cl82 and cl123) were plated in serial dilutions on 96-well plates (40 000 to 312 cells per well) and treated with ionising radiation at room temperature. Cells were cultured for 5 days following treatment; then media was supplemented by adding resazurin to a concentration of 10 μg/mL. Resazurin reduction to resorufin after 3 hours was measured colorimetrically on an Envision 2103 plate reader (560 – 590 nm). To obtain a measure for relative proliferation, fluorescence data from each treatment dose was plotted against cell number using a minimum of 4 samples per data point. The cell number plated to

obtain 50 % resazurin conversion was interpolated from the graphs. The ratio of untreated cells plated to treated cells plated at 50 % resazurin conversion was plotted on a logarithmic scale to produce relative proliferation curves for each cell line. Experiments were repeated five times, and curves represent five independent experiments.

2.6.5.2 Cell viability assays using drug treatment

For drug treatment experiments, cell viability assays were prepared as described in the previous section. Cells were plated in serial dilutions in 96-well plates, and the drug was added for 5 days, during which cells were cultured in an incubator. For the read-out, resazurin was added to a concentration of 10 μg/mL, and reduction was measured colorimetrically after 3 hours. Relative proliferation was assessed as outlined above: Resazurin conversion rates were plotted against plated cell numbers, and the ratio between the number of untreated cells and cell numbers plated to achieve 50% resazurin conversion were plotted on a logarithmic scale. Graphs represent five independent experiments.

2.6.6 Western blotting

2.6.6.1 Extraction of protein

Cells were grown and treated as described in the individual results chapters. To harvest cells for protein extraction, cells were trypsinised and re-suspended in complete medium, before washed with PBS once and centrifuged to form a cell pellet. Supernatant was removed from the cell pellets, and cell pellets were frozen at -80°C. Protein extraction buffer was prepared as described in **Table 2.1**, and protease and

phosphatase inhibitors were added right before extraction. Cell pellets were re-suspended in extraction buffer and incubated on ice for 10 min before centrifugation to separate the liquid whole-cell extract. The protein-containing supernatant was harvested, and diluted with purified H_2O before protein concentration was determined spectrophotometrically using a NanoDrop ND-1000 machine.

2.6.6.2 Gel electrophoresis and blotting

Samples for Western blotting were diluted in purified H_2O to reach an amount of 40 µg protein. 5x SDS-PAGE loading buffer was added to each sample to a final 1x concentration (see **Table 2.1**). Precast 3-8% Tris-Acetate gels were placed in an electrophoresis tank and submerged in running buffer. Protein samples were loaded into the relevant wells of the gel. Pre-stained protein standard samples with known molecular weights were also loaded to act as size controls.

The protein samples were separated for 80 min using a voltage of 150 V. After separation, the gel was removed from the tank and mounted against a PVDF membrane in a blotting chamber for the transfer. The chamber was submerged in transfer buffer (see **Table 2.1**) and the transfer was performed for 2 hours at a current of 250 mA. The membrane was then removed from the chamber and incubated with LI-COR blocking buffer for 1 hour at room temperature to prevent unspecific binding of the primary antibody against the protein of interest.

The primary antibody was diluted in blocking buffer containing 0.2 % Tween-20, and the membrane was incubated with the solution overnight at 4°C on a shaker (see

Table 2.4). The primary antibody was then removed and the membrane washed with washing buffer (see **Table 2.1**) three times for 5 min, before the secondary antibody containing a fluorescent dye was added in blocking buffer containing 0.2 % Tween-20 for 1 hour at room temperature on a shaker. After incubation with the secondary antibody, the membrane was again washed in washing buffer twice for 5 min and once in PBS, and the binding of the fluorescent secondary antibody was then visualised on a LI-COR Odyssey scanner. Equal loading of protein samples in each gel was confirmed by reprobing the membranes with an antibody against β-actin for one hour at room temperature, followed by three washing steps and incubation with secondary antibody. Actin staining was again visualised using an Odyssey scanner. The amount of target protein and β-actin was measured by the Odyssey software using regions of interest for the target bands and measuring relative fluorescence, and all proteins of interest were normalised to the loading control.

2.6.6.3 Stripping and reprobing of membranes

If membranes were reprobed with another primary antibody, binding of the previous antibody was removed by stripping the membrane. The membrane was washed in PBS and then placed in a shallow tray. Stripping solution (see **Table 2.1**) was added, and the membrane was placed on a shaker for 30 min. The stripping solution was then removed and the membrane was washed with PBS twice for 10 min each on a shaker. PBS was then removed and the membrane incubated with LI-COR blocking buffer for 1 hour at room temperature to prevent unspecific binding of the primary antibody against the protein of interest. Incubation with a new primary antibody was then performed as described in the previous section.

2.6.7 Foci staining

2.6.7.1 Treatment and fixation

After trypsinisation, 16000 cells were plated in each well of a 96-well plate, and 200 µL of complete DMEM medium were added. Cells were incubated at 37°C overnight to allow attachment to the bottom of the wells. Plates were then either irradiated with 5 Gy or mock-irradiated at room temperature. At designated timepoints described in the Results chapter, medium was removed and cells were washed with PBS once before each well was fixed with 4 % PFA solution (see **Table 2.1**) for 15 min. Wells were then washed with PBS and 200 µL of PBS were added to each well before storage at 4°C.

2.6.7.2 Staining

For the staining, PBS was removed and cells were permeabilised with a permeabilisation buffer containing 0.3 % Triton X-100 (see **Table 2.1**) for 10 min. Cells were then blocked for unspecific antibody binding by incubating each well with blocking buffer (see **Table 2.1**) for 1 hour at room temperature. After removal of the blocking buffer, the primary antibody was diluted in fresh blocking buffer and 50 µL of the antibody solution were added to each well. Incubation with the primary antibody was performed overnight at 4°C. For antibody specifications and dilutions see **Table 2.5**. After removal of the primary antibody, each well was washed with PBS three times for 10 min each. The secondary antibody was again diluted in blocking buffer, and 35 µL were added to each well and incubated for 1 hour at room temperature. The wells were then washed with PBS once, and 50 µL of 4',6-diamidino-2-phenylindole (DAPI) solution were added for a nuclear staining. DAPI solution was removed after 5 min and

wells were washed again three times for 10 min, and each well was filled with 100 µL PBS to prepare for visualisation.

2.6.7.3 Foci visualisation and analysis

Foci visualisation was performed using an InCell analyser. 16 wells were imaged for each treatment condition and time point, and 10 fields were taken from different parts of each well. Each plate was individually checked for focus, and focus was adjusted if necessary before the automated imaging programme was run. The images were then used for analysis performed by the InCell analysing software measuring the number and fluorescence intensity of the foci of interest for each individual cell.

2.6.8 FACS analysis for cell cycle profiles

For cell cycle analysis, cells were treated as described in the respective chapter of the Results section and harvested by trypsinisation at defined timepoints after treatment. Cells were then re-suspended in complete medium and centrifuged to form a pellet before the medium was removed and cells were vortexed briefly. 70% ice-cold ethanol was then added drop-wise for fixation, and cells were re-suspended in ethanol and incubated on ice for at least 30 min. After fixation, cells were centrifuged and the ethanol supernatant was removed, before cells were re-suspended in 1 mL PBS. For DNA staining, 20 µL of propidium iodide solution (see **Table 2.1**) were added for every 10^6 cells, and cells were incubated for 30 min at 37°C. A FACSCalibur system was used for the analysis and 10000 events were recorded for each run. Cell cycle profiles were

analysed and modelled using the Modfit LT software. Experiments were repeated at least 3 times, and statistical significance of independent experiments was examined by the Student's t-test.

2.6.9 Extraction of RNA from clinical samples

2.6.9.1 Harvesting, preparation and storage of tissue specimens

Samples were harvested from patients suffering from oesophageal cancer and scheduled for neoadjuvant treatment with 5-FU/oxaliplatin. Informed consent had been obtained from each patient prior to the analysis and ethical approval for the study had been given by the institutional review board of the hospital; all samples were anonymised. Pre-treatment samples of tumour and corresponding normal oesophageal tissue were collected endoscopically, and post-treatment samples were taken from the resected tumours. Pre- and post-therapeutic samples from a total of 49 patients were used for the analysis described in this thesis. Tissue specimens were collected in RNAlater and snap-frozen in liquid nitrogen on site before storage at -80°C until RNA extraction was performed.

2.6.9.2 Tissue preparation

Each clinical sample stored in RNAlater was thawed on ice and carefully removed from the stabilisation reagent, and a small aliquot of each tissue of about 2-3 mm^3 (~3 μg) was separated with a scalpel. It was placed in a 1.5 ml round-bottom tube on ice, and 350 μL of lysis buffer with the addition of 10 μL /mL β-mercaptoethanol were added. The tip of a disposable TissueRuptor probe was placed in the tube, and the

sample was disrupted for 45 s at full speed. The homogenised sample was then kept on ice for about 5 min until foam subsided. The lysate was then centrifuged for 3 min, and the supernatant was harvested and transferred to a spin column and again centrifuged for 30 s after which the flow through was used for RNA purification.

2.6.9.3 Purification of RNA

For each sample, 350 μL of the solution prepared as described in the previous paragraph were mixed with 350 μL of 70% ethanol. The sample was then transferred to an RNeasy MinElute spin column in a collection tube and centrifuged for 15 s at 8000g. The flowthrough was discarded, and washing buffer was added to the spin column and the column centrifuged again. This step was repeated twice. The spin column was then washed with a second washing buffer and the column spun down. A final washing step with 80% ethanol was carried out and the column was centrifuged for 2 min and again for 5 min at 8000g. The spin column membrane was then left to dry. 14 μL RNase-free purified water was then added to the spin column to elute the RNA and the column was centrifuged for 1 min at 8000g. The flow-through containing the purified RNA was then used for further experiments.

2.6.9.4 Quantification and storage of RNA

RNA content of each sample was measured at a wavelength of 260 nm (A_{260}) using a NanoDrop spectrophotometer. RNA purity was checked using the 260 nm to 280 nm ratio (A_{260}/A_{280}), and samples showing A_{260}/A_{280} between 1.9 and 2.1 were considered sufficiently pure RNA. If RNA content and purity were confirmed to be

sufficient for further experiments, samples were frozen in RNase-free purified water and stored at -80°C.

2.6.10 qRT-PCR

2.6.10.1 Generation of cDNA

RNA samples and reagents required for reverse transcription were thawed on ice. A first reaction was carried out to remove all remaining cellular DNA. An aliquot of each sample was incubated with DNA Wipeout buffer diluted in purified water for 3 min at 42°C and then quickly cooled down by placing each tube on ice. The reverse transcription master mix was then prepared from the QuantiTect Qiagen kit: Reverse transcriptase, buffer and primer mix were thawed and mixed and added to the RNA sample. The reverse transcription reaction for each sample was performed in a waterbath at 42°C for 15 min. The reaction was stopped by heating up the samples to 95°C for 3 min to inactivate the polymerase, and samples were consecutively stored on ice. cDNA content of each sample was measured using a NanoDrop spectrophotometer, and a cDNA concentration between 1500 and 2000 ng/µL was achieved for all samples after successful reverse transcription. cDNA purity was confirmed by the 260 nm to 280 nm ratio (A_{260}/A_{280}), and A_{260}/A_{280} around 1.8 was obtained for all samples. cDNA samples were then stored at -20°C until qRT-PCR reactions were performed.

2.6.10.2 Primer design and optimisation

POLH primers were designed to be intron-spanning in order to prevent any residual genomic DNA from being proliferated together with the cDNA samples.

Glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*) was chosen as a housekeeping gene to be used as a control to which the *POLH* cDNA content would be normalised. Sequence-specific fluorescence-coupled probes were used to quantify polymerised *POLH* cDNA. The sequence for primers and probes can be found in **Table 2.7**. Primers were diluted in purified water to a working solution of 8 μM, probes were diluted to 2 μM.

Reaction efficiencies for *POLH* and *GAPDH* were compared by the comparative threshold (ΔC_t) method: A normal oesophageal tissue sample was serially diluted over several orders of magnitudes ($1:10^0$ to $1:10^{-5}$), and qRT-PCR was performed as described in the following section. The amount of fluorescent signal at the threshold cycle (C_t) was determined for both *POLH* and *GAPDH*, and the ratio (ΔC_t) was formed between *POLH* C_t and *GAPDH* C_t for each dilution.

2.6.10.3 Polymerase chain reaction

The components of the master mix for the polymerase chain reaction (PCR) were thawed on ice, and primers and probes for either *POLH* or *GAPDH* were added as described in **Table 2.1**. cDNA stocks were thawed and diluted in purified water, and approximately 400 ng of cDNA were used for the PCR reaction of each sample. PCR reaction mix was added to a total volume of 25 μL per well. Samples were run in triplicate for both *POLH* and *GAPDH*, and each plate contained control wells either lacking cDNA or polymerase activity. Plates were centrifuged and then placed in the cycler. The cycler settings were chosen as outlined in **Table 2.8**, and 40 polymerisation cycles were performed so that all samples could reach the detection threshold.

Table 2.8. Settings for real-time cycler

Step		Temperature	Time	Explanation
Carryover prevention		50°C	2 min	Elimination of dUMP-containing PCR products
Activation step		95°C	15 min	Heating step to activate Taq DNA polymerase
40 cycles	**Denaturation**	94°C	15 s	Melting of double-stranded DNA for polymerase access
	Combined annealing and extension	60°C	60 s	Collection of fluorescence data for quantification

2.6.10.4 Quantification and statistical analysis

The fluorescence signal of the *POLH* and *GAPDH* probes was detected automatically by the PCR cycler. The measured *POLH* mRNA level for each sample was then normalised to the corresponding *GAPDH* mRNA level (ΔC_t value). Each normalised sample was then compared to the normalised *POLH* mRNA content of a normal oesophageal tissue sample (baseline *POLH* sample) that had been acquired independently of the examined samples and was reverse-transcribed and polymerised as described above ($\Delta\Delta C_t$ value). An up-regulation of *POLH* mRNA for an individual sample was defined as a more than two-fold higher mRNA level as measured in the baseline sample ($\Delta\Delta C_t > 2.0$). Correspondingly, a down-regulation for an individual sample was defined as a normalised mRNA level less than half the level of the baseline sample ($\Delta\Delta C_t < 0.5$).

To compare pooled *POLH* mRNA levels in tumour and corresponding normal tissue samples before and after treatment, Wilcoxon signed-rank tests were performed and statistical significance was assumed if the *P* value was below 5 % ($P < 0.05$). Statistical tests were calculated and graphs were plotted using either Microsoft Excel 2007 or PASW 18.0.

2.6.11 Analysis of clinical samples

Statistical dependence between patient and tumour characteristics, i.e. patient age, patient gender, tumour grade and tumour stage, and *POLH* mRNA levels was assessed using Spearman's rank correlation coefficient. Influence of cancer stage, cancer grade and cancer histology on overall and disease-free survival was measured by Logrank test. Correlation between *POLH* mRNA levels and overall and disease-free survival was tested using the Cox proportional hazards model. Survival graphs are shown as Kaplan-Meier curves. A statistical relationship between therapeutic response and *POLH* expression levels was assessed by logistic regression analysis. For all statistical tests, statistical significance was assumed when *P* values were below the 5% significance level ($P < 0.05$).

RESULTS 1: THE INFLUENCE OF POL ETA STATUS ON CELLULAR RESPONSES TO IONISING RADIATION

3.1 INTRODUCTION

As discussed in detail in Chapter 1, pol η enables cells to bypass a variety of DNA lesions which would otherwise stall DNA replication and may therefore threaten cellular viability. The effects of pol η have been mostly studied in the context of DNA damage caused by UV irradiation owing to the enzyme's link with the variant form of xeroderma pigmentosum. It has been shown that the enzyme is able to perform near-error-free bypass past UV-induced CPDs and it also has a role in replicating past other DNA photoproducts arising after UV irradiation (Washington, Johnson et al. 2000; Abdulovic and Jinks-Robertson 2006; Yamamoto, Loakes et al. 2008). In comparison, very little is known about the involvement of pol η in the cellular response to ionising radiation as another form of DNA-damaging radiation. While UV radiation produces a well-defined spectrum of specific lesions, mostly purine nucleotide dimers, IR causes a wide variety of DNA damage including oxidative lesions and strand breaks.

As the spectrum of IR-induced DNA lesions is vast, various different DNA repair pathways may be involved in removing the damage and restoring genomic integrity. To date, the involvement of the TLS pathway and the possible role of the TLS polymerase, pol η, in the cellular response to IR is relatively unknown; no data are currently available on the radiation sensitivity of pol η-deficient cells compared to isogenic pol η-expressing cells.

In this chapter, the role of pol η in mediating cellular survival after exposure to ionizing radiation is examined. Furthermore, possible mechanisms are investigated by which pol η may affect the radioresistance of cells.

3.2 INCREASED RADIATION RESISTANCE IN POL H KNOCKOUT CELL LINES

To investigate a potential influence of the cellular pol η status on cellular survival after exposure to ionising radiation, isogenic cell lines were used that only differed in expression levels of functional pol η protein.

XP30RO cells are derived from a patient with the variant form of xeroderma pigmentosum. The cells contain a homozygous deletion in the *POLH* gene leading to the expression of a non-functional 42-amino acid peptide. They have previously been shown to exhibit an increased sensitivity to cisplatin and UV irradiation that is reversed in cells containing a pol η-expressing vector (Stary, Kannouche et al. 2003). The absence of pol η in XP30RO cells and the expression of the protein in the same cell line complemented with the expression vector (XP30RO/pol η cells) were shown by Western blotting (**FIGURE 3.1 B**). The response of XP30RO and XP30RO/pol η cells to ionising radiation was examined using clonogenic survival assays as described in the "Materials and Methods" section. The XP30RO cells showed significantly increased clonogenic survival compared to the pol η-expressing counterparts ($P < 0.01$, paired Student's t-test) (**FIGURE 3.1 A**).

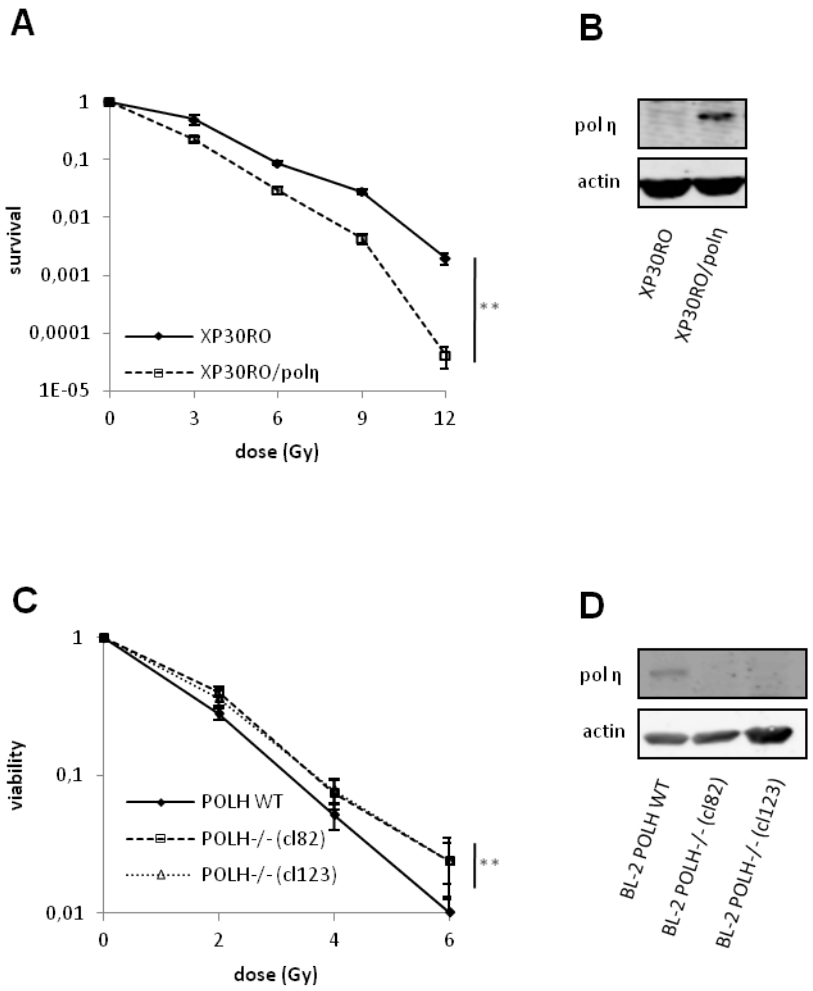


Figure 3.1. Pol η deficiency increases radioresistance in POLH knockout cell lines.

A. Clonogenic survival assay showing increased survival of XP30RO compared to XP30RO/pol η cells after treatment with ionizing radiation. Each data point represents three independent experiments, and error bars show standard error of the mean. **B**. Western blot showing levels of pol η in XP30RO cell lines. **C**. Viability assay in pol η-proficient and pol η-knockout BL-2 cell lines after ionizing radiation. Each data point represents five independent experiments, and error bars show standard error of the mean. **D**. Western blot for pol η levels in *POLH* wild-type and two *POLH* knockout BL-2 cell clones. Statistical analysis performed by paired Student's t-test. ** $P < 0.01$.

As pol η expression in XP30RO/pol η cells was dependent on the presence of a zeocin-selected expression vector, a different system of isogenic cell lines was tested in order to rule out a cell line-specific phenomenon or an indirect effect caused by the POLH vector. Wild-type BL-2 Burkitt's lymphoma cell lines expressed pol η protein, while two BL-2 pol η-/- clones harboured a pol η deletion caused by HR-mediated artificial knockout and did not express functional pol η (Gueranger, Stary et al. 2008). Pol η levels were again confirmed by Western blot (**FIGURE 3.1 D**). Since Burkitt's lymphoma cells do not attach to cell culture dishes, clonogenic survival assays could not be carried out; instead, resazurin-based viability assays were performed in pol η wild-type BL-2 cells and two pol η-knockout clones (cl-82 and cl-123). The resazurin-based assay allowed reliable analysis of survival data obtained with lower irradiation doses of up to 6 Gy. Both pol η-deficient BL-2 clones exhibited a significantly higher viability, as measured at 5 days after treatment ($P < 0.01$, paired Student's t-test) (**FIGURE 3.1 C**).

3.3 INCREASED RADIATION RESISTANCE THROUGH KNOCKDOWN OF POL H

To further investigate the effects of pol η deficiency on cellular survival after exposure to ionizing radiation, siRNA against pol η was used to knock down protein expression in two cell lines. HCT116 colorectal and SQ20B laryngeal cancer cell lines were reverse-transfected with siRNA against pol η or non-targeting siRNA, and knockdown efficiency was tested by Western blotting analysis. Knockdown of more than 75 % was achieved in both cell lines after 48 hours and pol η levels remained

suppressed for the following 24 hours (**FIGURE 3.2 B and D**). SiRNA treatment had no significant effect on the plating efficiencies of both cell lines.

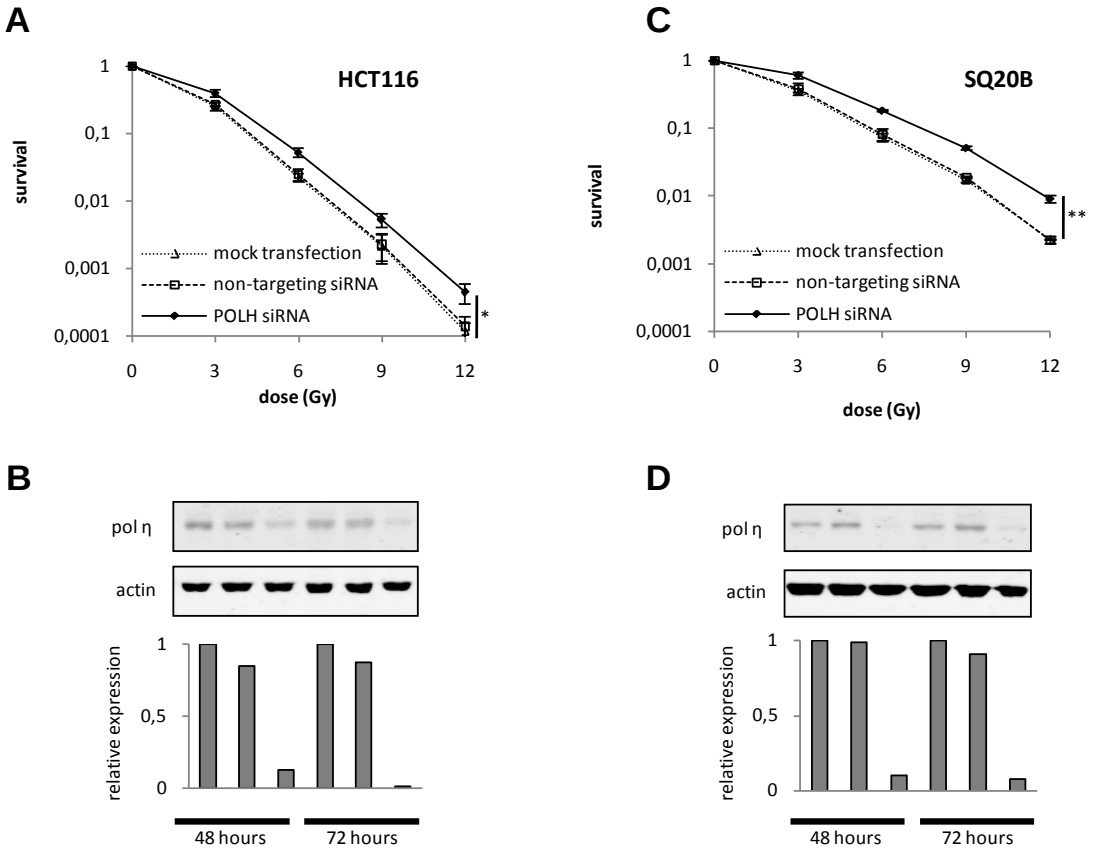


Figure 3.2. Pol η knockdown leads to an increase in radioresistance.

Clonogenic survival in HCT116 (**A**) and SQ20B (**C**) cells after pol η knockdown. Cells were either transfected with non-targeting siRNA or siRNA against pol η for 48 hours before irradiation. Each datapoint represents three independent experiments, and error bars show the standard error of the mean. Statistical analysis performed by Student's t-test. * $P< 0.05$, ** $P< 0.01$. **B**, **D**. Western blot analyses showing efficiency of pol η knockdown after 48 and 72 hours. Knockdown efficiency was above 75 % for both HCT116 and SQ20B cells (bar diagrams).

For survival experiments, cells were irradiated at 48 hours after transfection, and knockdown of pol η in both cell lines led to a significant increase in clonogenic survival ($P< 0.05$ for HCT116 cells, $P< 0.01$ for SQ20B cells, statistical analysis performed by paired Student's t-test) (**FIGURE 3.2 A and C**). The increase in radioresistance using siRNA against pol η was less pronounced than observed for pol η-deficient XP30RO cells, which may have been caused by a remaining low level of pol η expression. Taken together, these experiments in 2 different preclinical model systems clearly show that the differences in survival after ionizing radiation were caused by differences in the expression of pol η protein.

3.4 INCREASED S PHASE POPULATION IN POL H-DEFICIENT CELLS

Although there have been suggestions that cells can carry out translesion synthesis independent of replication (Soria, Belluscio et al. 2009; Daigaku, Davies et al. 2010), the main role of pol η appears to be bypass of lesions to prevent stalling of replication forks in S phase. As described in the Introduction chapter, cell cycle phases influence cellular responses to ionizing radiation considerably, with cells in S phase exhibiting a relative increase in radioresistance compared to cells in other phases of the cell cycle (Sinclair and Morton 1966; Cheong, Wang et al. 1994).

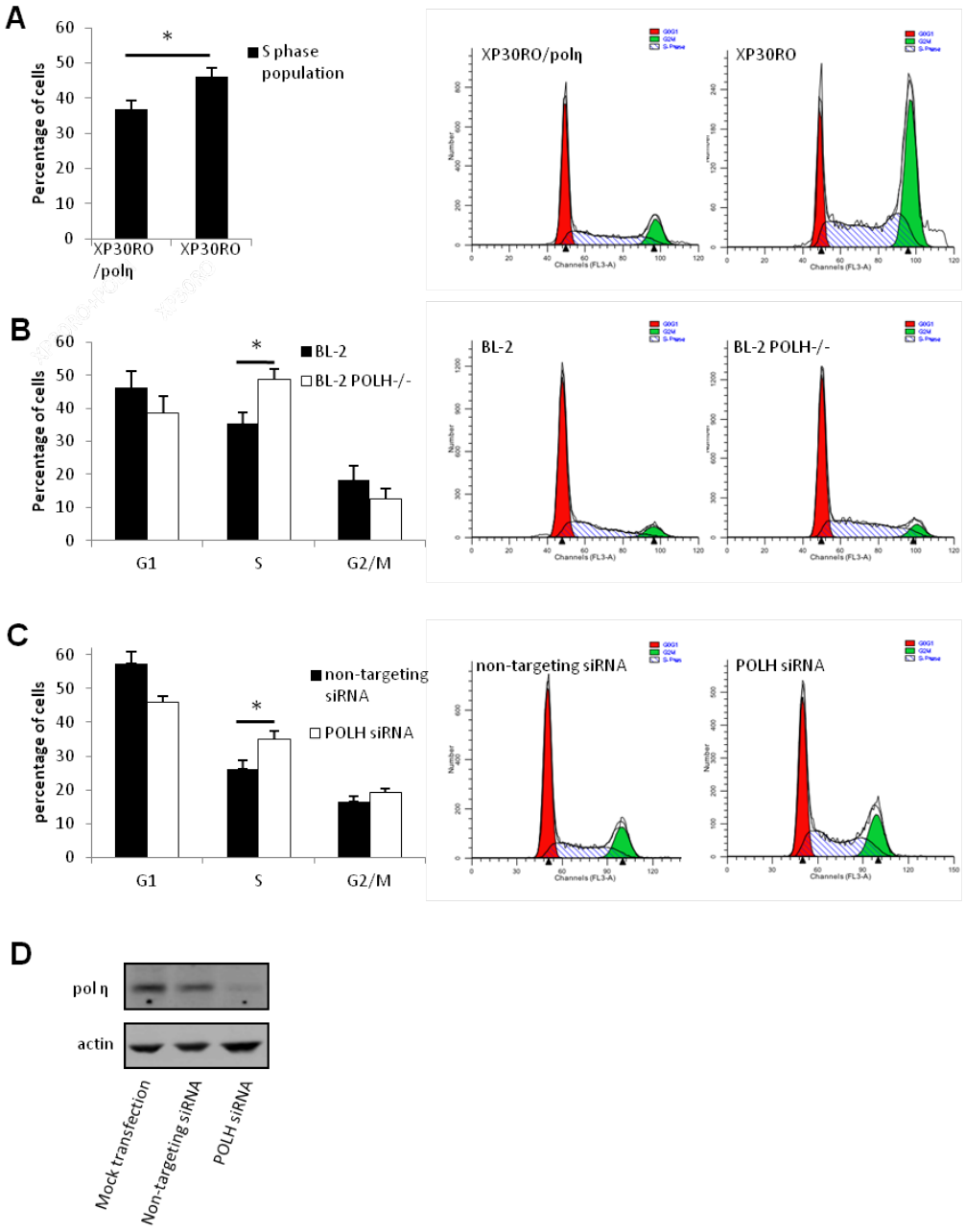


Figure 3.3. Pol η deficiency increases S phase population.

A. FACS analysis showing S phase population in untreated XP30RO and XP30RO/pol η cells. **B**. Cell cycle profile of *POLH* wild-type and *POLH*-/- BL-2 cell lines. **C**, **D**. Cell cycle profile of HCT116 either transfected with 10 nM non-targeting siRNA or siRNA against pol η. Knockdown efficiency was confirmed by Western blot analysis. Data from the original cell cycle histogram data were pooled and depicted as bar diagrams. Error bars show the standard of the mean of at least 5 independent experiments. Statistical analysis was performed by Student's t-test. * $P < 0.05$.

Cell cycle profiles of untreated non-confluent XP30RO and XP30RO/pol η cell lines were analysed, and the percentages of unsynchronised cells in S phase were compared. FACS analysis for cell cycle distribution showed 45.9 % of untreated XP30RO cells in S phase, compared to 36.7 % in XP30RO/pol η cells (**FIGURE 3.3 A**). Since the FACS signals of the diploid G2 population and the tetraploid G1 population were overlapping, no quantitative analysis could be performed for the other cell cycle phases.

In order to confirm these findings in a different pol η-deficient cell system, FACS-based cell cycle profiles were assessed in BL-2 cells. While only 35.4 % of unsynchronised BL-2 *POLH* WT cells were detected in S phase, untreated BL-2 *POLH*-/- cells showed an S phase population of 48.8 % (**FIGURE 3.3 B**). In both knock-out model systems, the difference in the S-phase proportions between corresponding pol η-deficient and pol η-expressing cell lines demonstrated significance ($P < 0.05$, Student's t-test).

To further corroborate these findings, cell cycle profile analysis was performed after knockdown of pol η by siRNA in HCT116 cells. Again, a significant difference in S phase cells was found by comparing cells treated with siRNA against pol η (34.9 % of cells) to cells treated with non-targeting siRNA (26.1% of cells) (**FIGURE 3.3 C and D**).

In summary, in these 3 preclinical models systems, a significant increase of cells in the replication phase of the cell cycle was observed when functional pol η protein was deficient or suppressed.

3.5 DIFFERENCES IN ΓH2AX FOCI BUT NOT 53BP1 FOCI BETWEEN POL H-DEFICIENT AND POL H-EXPRESSING CELLS AFTER IONISING RADIATION

DNA damage induced by ionising radiation leads to phosphorylation of histone 2AX (to γH2AX) and accumulation of p53 binding protein 1 (53BP1) on damaged chromatin; these proteins can be quantified as foci as a means of measuring cellular damage (Costes, Chiolo et al. 2010; Noon and Goodarzi 2011).

To assess possible alterations in the DNA damage repair kinetics in the absence of pol η, immunocytochemical staining for γH2AX and 53BP1 foci was performed in XP30RO and XP30RO/pol η cells after exposure to ionising radiation. Automated foci counting was performed, and adjustments were made for diffuse pannuclear γH2AX staining as observed during S phase of the cell cycle; cells with more than 9 foci were counted as positive as at that threshold, baseline foci levels were found to be not higher than 5%. Without treatment, the XP30RO cell line had about two-fold higher baseline proportions of cells positive for γH2AX foci compared to the proportion for the XP30RO/pol η cell line (5.9 % vs. 3.2 %; $P< 0.001$, Student't t-test). This finding would suggest that those cells are more severely affected by endogenous damage and have a stronger DNA damage response to those lesions.

Exposure to 5 Gy led to an increase in γH2AX-positive cells for both the XP30RO and XP30RO/pol η cell line at 2 and 4 hours post-irradiation. XP30RO/pol η cells showed a higher level of positive cells from 6 hours onwards and had a significant relative delay in the resolution of those DNA damage foci. This again suggested that XP30RO/pol η cells had a higher amount of residual, unrepaired DNA damage created by ionising radiation as determined by residual γH2AX foci after 24 hours ($P< 0.01$, Student's t-test) (**FIGURE 3.4 A and B**). At 24 hours after irradiation, XP30RO/pol η cells still had about 50 % higher percentage of cells positive for γH2AX foci compared to their pol η-deficient counterparts (39.9 % vs 26.9 % of cells; $P< 0.05$, Student's t-test). Therefore, the relative radioresistance of pol η-deficient cells was accompanied by a relatively decreased level of unrepaired DNA damage after IR as highlighted by residual γH2AX foci.

In addition to the analysis of γH2AX staining, 53BP1 foci were also analysed following irradiation of cells. Again, cells with more than 9 foci were counted as positive, as only about 5 % of all untreated cells had more than that number of foci. Although a trend was observed in baseline levels of 53BP1-positive cells being higher in XP30RO cells compared to XP30RO/pol η cells (5.3 % vs. 1.9 %), the difference did not reach statistical significance ($P = 0.12$, Student's t-test). Exposure to a dose of 5 Gy resulted in an increase in 53BP1 foci in both cell lines with no significant differences in foci formation for any of the time points tested ($P = 0.32$, Student's t-test) (**FIGURE 3.5**).

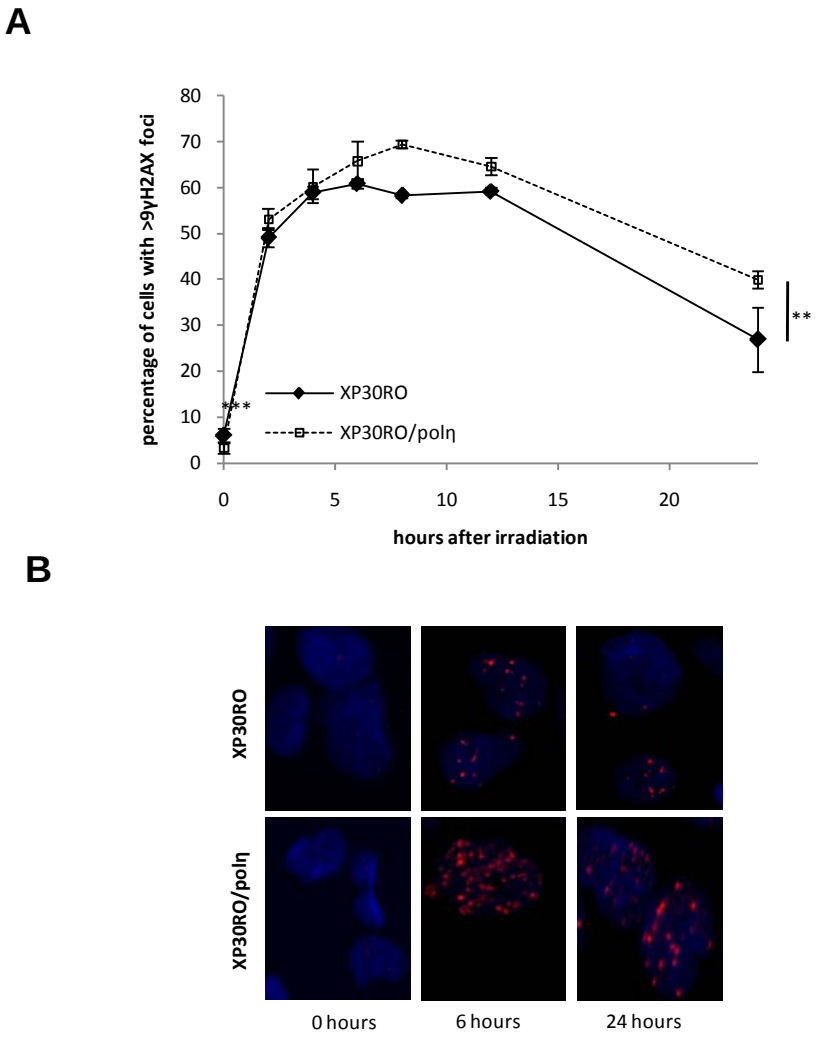


Figure 3.4. Decreased γH2AX foci after irradiation in pol η-deficient cells.

A. Delayed repair in pol η-supplemented cells compared to isogenic pol η-deficient XP30RO cells. Pol η-null cells had higher baseline γH2AX levels in line with an increased level of damage without treatment. Cells were irradiated with 5 Gy and fixed at the indicated time points. γH2AX foci were visualized by immunostaining (**B**), and analysis was performed using the automated InCellAnalyzer system. Cells with more than 9 γH2AX foci were counted as positive, and the percentage of positive cells is plotted against the time after irradiation. Curves represent data from three independent experiments. Statistical analysis was performed by paired Student's t-test. ** $P< 0.01$, *** $P< 0.001$.

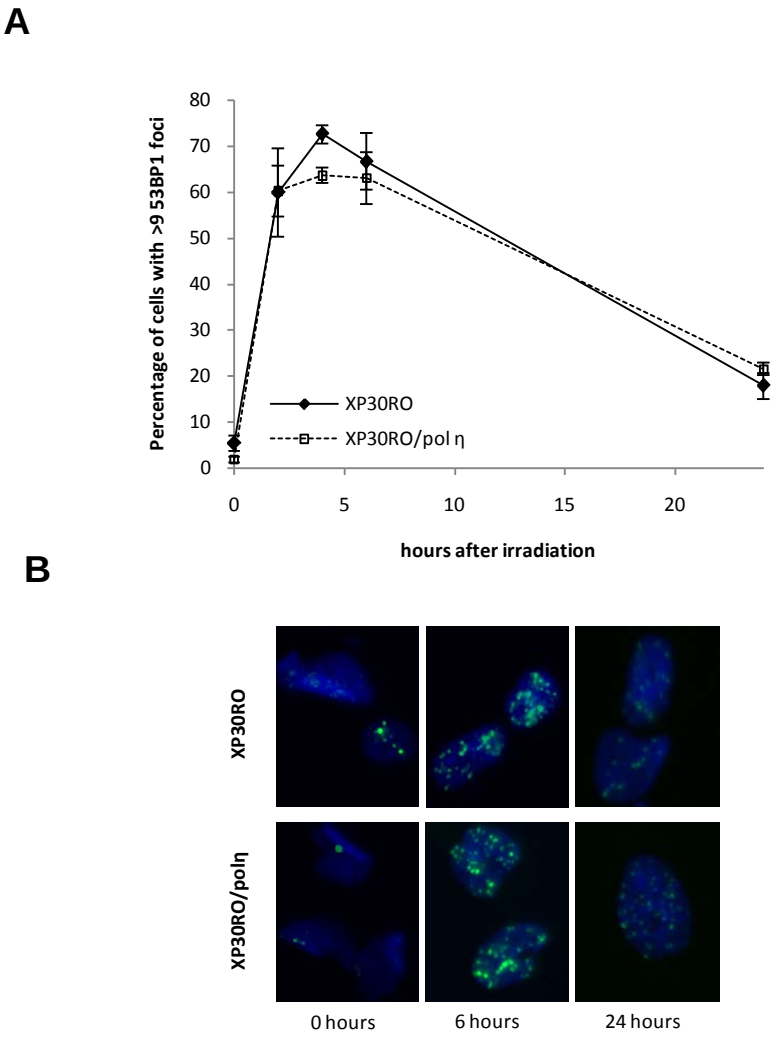


Figure 3.5. No effect of pol η status on 53BP1 foci after irradiation.

A. No difference in the level of 53BP1 foci between pol η-deficient and pol η-supplemented XP30RO cells. Cells were exposed to 5 Gy IR and fixed at the indicated time points. 53BP1 foci were assessed by immunofluorescence staining (**B**) and automated counting. Cells with more than 9 foci were counted as positive, and the percentage of positive cells is plotted against the time after irradiation. Curves represent data from three independent experiments.

3.6 INCREASES OF RAD51 FOCI IN POL H-DEFICIENT CELLS AFTER IONISING RADIATION

Increased radiation resistance during the S phase of the cell cycle has been described previously and it has been attributed to an increase in homology-directed repair (Tamulevicius, Wang et al. 2007; Wilson, Hinz et al. 2010). In order to test if the increased survival observed for pol η-deficient cells after ionising radiation treatment described above was associated with increased homologous recombination repair, analysis of RAD51 foci was performed in the same model system. Automated foci counting was done as described in the Materials and Methods section; cells with more than 5 RAD51 foci were counted as positive, as less than 10 % of all untreated XPV cells exceeded that number of foci (Ying, Myers et al. 2009). After irradiation with 5 Gy, a significantly higher level of RAD51-positive cells was detected in the XP30RO cell line compared with the corresponding pol η-rescued cell line ($P< 0.05$ at 4 hours; $P< 0.001$ at 6 hours; statistical analysis performed by Student's t-test) (**FIGURE 3.6**). The increase in RAD51 foci at 6 hours post-irradiation in the pol η-deficient cell line was consistent with the lower level of γH2AX foci in those cells from 6 hours post-irradiation, and the findings suggest that the increase in clonogenic survival of pol η-deficient cell lines was mediated by an increased homology-directed DNA damage repair in those cells.

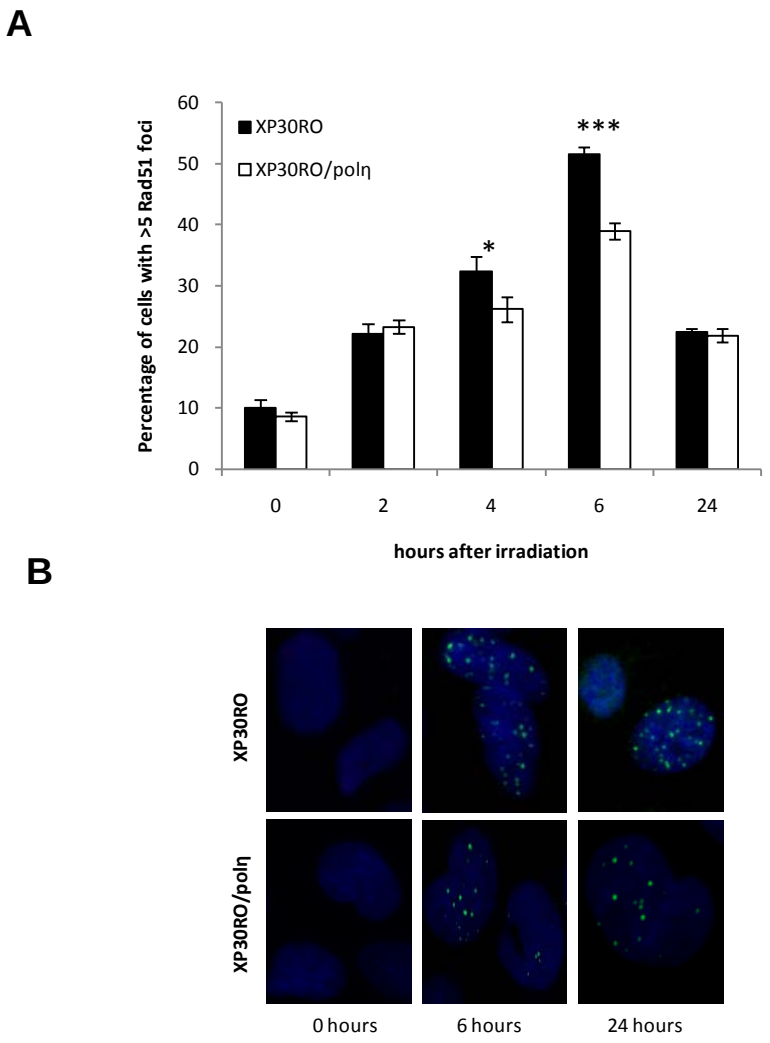


Figure 3.6. Increased RAD51 foci levels in pol η-deficient cells after irradiation

A. Higher levels of RAD51 foci in pol η-deficient cells at 4 and 6 hours after irradiation compared to isogenic pol η-expressing cells. For RAD51 analysis, cells were treated with 5 Gy irradiation dose and fixed and stained at the time points shown. Cells with more than 5 RAD51 foci were counted as positive, and bar graphs represent three independent experiments. Statistical analysis was performed by two-sided Student's t-test. * $P< 0.05$, *** $P< 0.001$. **B**. Sample pictures were prepared from the measured raw data using the ImageJ software.

3.7 ABROGATION OF THE RADIATION SURVIVAL BENEFIT OF POL H-DEFICIENT CELLS BY XRCC3 KNOCKDOWN

Although the analysis of RAD51 foci is often used as a marker of cellular homologous recombination activity, it does not inform regarding the functional aspects of homologous recombination repair. In order to find evidence in favour of the hypothesis that the increase in RAD51 foci formation in the XP30RO cells was associated with increased homologous recombination activity and to further elucidate the role of this DNA repair pathway in the mediation of survival of pol η-deficient cells, siRNA-based knockdown of XRCC3 was performed in XP30RO and XP30RO/pol η cells, and its influence on cellular survival after exposure to ionising radiation was measured. XRCC3 has been established as a protein active in later stages of HR, and it has been suggested to work downstream of Rad51 activity (Brenneman, Wagener et al. 2002).

Knockdown efficiency as assessed by Western blotting was above 70 % in both cell lines (**FIGURE 3.7 B**). Clonogenic survival assays were performed after reverse transfection with XRCC3 siRNA for 48 hours as described in the Materials and Methods chapter. Mock transfection and transfection with non-targeting siRNA resulted in survival levels comparable to untreated XP30RO and XP30RO/pol η cell lines (**FIGURE 3.7 A**). XRCC3 knockdown reduced clonogenic survival in XP30RO cells to the levels of XP30RO/polη cells, suggesting that the increased radioresistance in XP30RO cells was mediated by a homologous recombination-dependent mechanism (**FIGURE 3.7 A**).

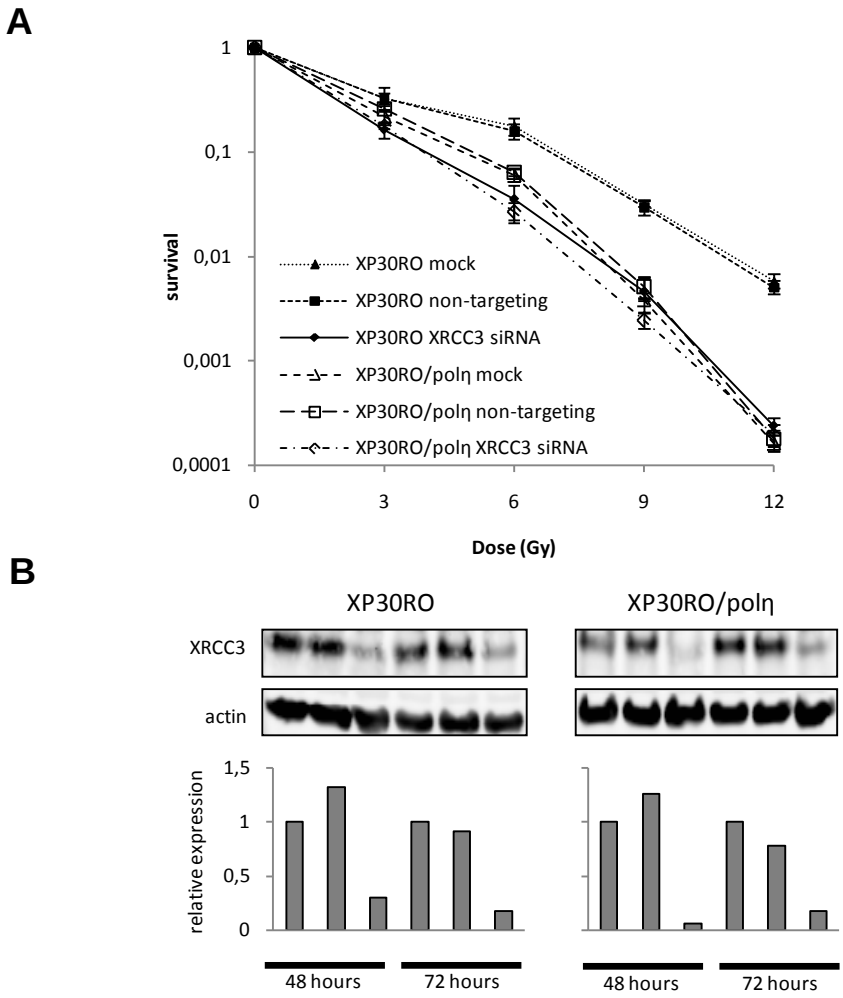


Figure 3.7. Abrogation of radioresistant phenotype in pol η-deficient cells after XRCC3 knockdown.

A. Clonogenic survival assays were performed in XP30RO and XP30RO/pol η cells after XRCC3 knockdown for 48 hours and irradiation. Controls were included using mock transfection and non-targeting siRNA. Each data point represents three independent experiments, and error bars show the standard error of the mean. Statistical analysis performed using Student's t-test. ** $P< 0.01$. **B**. Western blot analysis showing knockdown efficiency of XRCC3 siRNA after 48 and 72 hours. Quantification shows more than 70 % knockdown efficiency for the two cell lines after both indicated timepoints.

Furthermore, compared to control transfections, depletion of XRCC3 decreased clonogenic survival after exposure to ionising radiation significantly in XP30RO cells ($P< 0.01$, Student's paired t-test), but not significantly in XP30RO/pol η cells. This result suggests that XP30RO/pol η cells are less dependent on homologous recombination to repair ionising radiation-induced DNA damage than the pol η-deficient cells.

3.8 SUMMARY

In this study, it has been shown for the first time that pol η status influences cellular survival after exposure to ionising radiation, and that pol η deficiency results in a more radioresistant phenotype. The results show that pol η deficiency alters cell cycle profiles, leading to an increased S phase proportion due to a loss of TLS activity during replication. Higher baseline levels of γH2AX and 53BP1 foci suggest the prolonged presence of unrepaired DNA lesions due to endogenous damage in untreated pol η-deficient compared to pol η-rescued cells. After exposure to IR, pol η-deficient XP30RO cells were found to have a smaller increase in the numbers of γH2AX foci than the increase in levels observed in pol η-expressing cells. A 50 % higher level of residual γH2AX foci was found at 24 hours post-IR in pol η-expressing cells compared to their pol η knockout counterparts. These findings suggest that pol η-deficient cells have a higher proficiency in repairing ionising radiation-induced damage or that pol η expression may be associated with the creation of DNA double-strand breaks or delay the kinetics of their repair.

The increase in RAD51 foci observed in pol η-deficient cells after ionising radiation (**FIGURE 3.6**) compared to pol η-proficient cells, and the abrogation of the survival benefit of pol η-deficient cells after XRCC3 knockdown (**FIGURE 3.7**) compared to pol η-expressing cells suggest that pol η-deficient cells rely on homologous recombination repair more than cells that express pol η and that this discrepancy between the cell lines accounts for differences in cellular fate following ionising radiation. This suggestion is supported by the finding that sensitisation to ionising radiation by XRCC3 knockdown was considerably more pronounced in pol η-deficient cells compared to the corresponding pol η-expressing cells. The higher S phase proportion of pol η-deficient cells, considered in combination with these findings, suggests that increased HR activity is associated with the absence of pol η.

RESULTS 2: THE INFLUENCE OF POL ETA STATUS ON CELLULAR RESPONSES TO PLATINUM-BASED CHEMOTHERAPY

4.1 INTRODUCTION

Platinum-based anticancer drugs are among the most commonly used agents to treat patients with solid malignancies. Cisplatin was the first platinum compound used in the clinical management of cancers, and has been widely tested pre-clinically and has shown efficiency against various cancers in the clinical setting (Pasetto, D'Andrea et al. 2006). However, cisplatin has an unfavourable toxicity profile on many organs, and its nephrotoxic side effects can be dose-limiting. Since cisplatin's introduction into routine clinical use in 1978, multiple second-generation platinum compounds have been developed. Of these, only oxaliplatin has evolved as a standard drug used routinely in the clinic; it is currently a first-line drug used in combination with the antimetabolite drug, 5-FU, to treat colorectal cancers (de Gramont, Figer et al. 2000; Giacchetti, Perpoint et al. 2000).

Like cisplatin, oxaliplatin exerts its effect by binding to DNA and results in different forms of DNA damage including monofunctional adducts, intrastrand and interstrand crosslinks (Woynarowski, Faivre et al. 2000). However, oxaliplatin-induced lesions are much bulkier than cisplatin adducts and distort the DNA strands to a much higher degree (Chaney, Campbell et al. 2005; Wu, Bhattacharyya et al. 2007). The in-vitro activities of cisplatin and oxaliplatin are very similar with oxaliplatin being at least as potent as cisplatin in most of the tumour cells investigated (Rixe, Ortuzar et al. 1996). Oxaliplatin DNA adducts block DNA polymerases from

carrying out replication and transcription, and, if unrepaired, those lesions result in cell cycle arrest and cell death (William-Faltaos, Rouillard et al. 2006). While the repair of oxaliplatin-induced DNA lesions is mainly carried out by the NER pathway, *in vitro* data have shown that the TLS polymerase, pol η is able to bypass oxaliplatin DNA adducts on oligonucleotide strands and may therefore influence cellular responses to the drug.

Pol η has been linked to cellular survival after cisplatin exposure (Albertella, Green et al. 2005), but its role in determining cellular responses to oxaliplatin has not been clearly investigated. This is an interesting research question since oxaliplatin treatment leads to much bulkier and more distorting DNA adducts and has been shown to be active in tumour cells that are resistant to cisplatin (Stordal, Pavlakis et al. 2007; Tozawa, Oshima et al. 2008). This study aimed to elucidate the involvement of pol η in cellular responses to oxaliplatin treatment based on the hypothesis that pol η inhibition may be a strategy to improve oxaliplatin-based cancer treatment.

4.2 INCREASED CELLULAR SENSITIVITY OF XP30RO CELLS TO CISPLATIN AND OXALIPLATIN

To establish the role of pol η in cellular responses to cisplatin in a cell culture-based assay, a pair of isogenic skin fibroblast cell lines derived from an XPV patient were used that only differed in their expression of functional pol η, as described in the previous chapter.

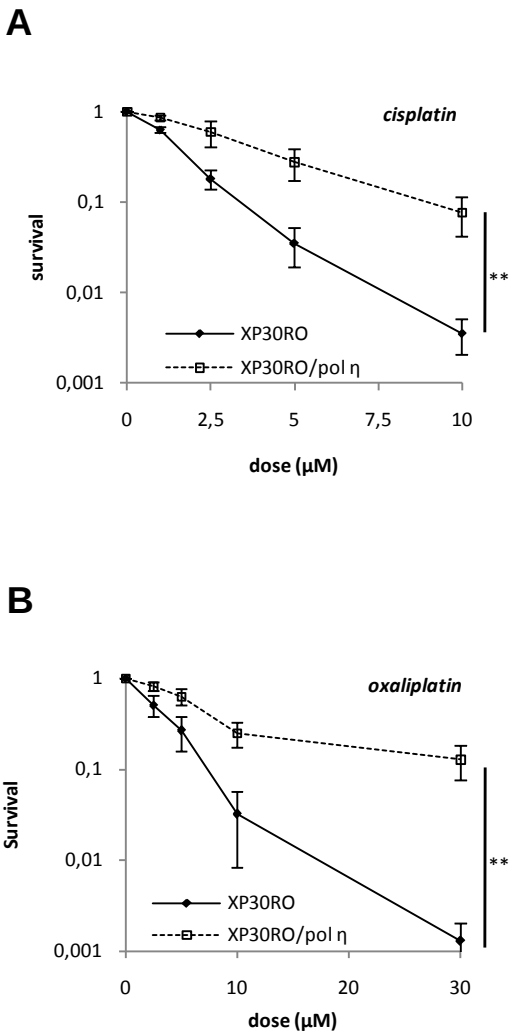


Figure 4.1. Increased sensitivity of pol η-knockout cells to platinum agents.

A. Clonogenic survival assay showing increased sensitivity of XP30RO compared to XP30RO/pol η cells after treatment with increasing concentrations of cisplatin. **B**. Clonogenic assay showing decreased survival of pol η-deficient cells compared to isogenic pol η-expressing cells after exposure to different concentrations of oxaliplatin. Each data point represents three independent experiments, and error bars show standard error of the mean. Statistical analysis performed by paired Student's t-test. ** $P< 0.01$.

Clonogenic survival assays were performed in XP30RO and XP30RO/pol η cells performing 24-hour treatment with cisplatin or oxaliplatin and analysed after 10 days. Pol η-deficient XP30RO cells were found to be significantly more sensitive to cisplatin than their isogenic counterparts supplemented with the pol η-expressing vector (IC_{50} 1.4 μM for XP30RO cells; IC_{50} 3.2 μM for XP30RO/pol η cells for 24-hour treatment) ($P< 0.01$, Student's t-test) (**FIGURE 4.1 A**). This finding is consistent with published data (Albertella, Green et al. 2005).

Sensitivity to oxaliplatin was tested under the same conditions. Treatment with the drug for 24 hours resulted in significantly lower clonogenic survival at 10 days in pol η-deficient XP30RO cells than the corresponding clonogenic survival of pol η-proficient cells (IC_{50} 2.6 μM for XP30RO cells; IC_{50} 6.7 μM for XP30RO/pol η cells with 24-hour treatment) ($P< 0.01$, Student's t-test) (**FIGURE 4.1 B**). Higher micromolar doses of oxaliplatin compared to cisplatin doses were required to inhibit the same percentage of pol η-expressing cells, which is in line with previous *in vitro* data suggesting that pol η is more proficient in bypassing oxaliplatin DNA adducts than cisplatin-induced lesions (Vaisman, Masutani et al. 2000).

4.3 INCREASED SENSITIVITY OF BL-2 POL H KNOCKOUT CELL LINES TO OXALIPLATIN

As XP30RO cells are derived from pol η-deficient patient tissue and can be compared to an isogenic cell line that is supplemented with a pol η-expressing vector, a second preclinical model system was used to rule out a cell line-specific effect or an

indirect effect mediated by the vector. Wild-type BL-2 cells and two pol η-knockout BL-2 clones (cl-82 and cl-123) were treated with different concentrations of oxaliplatin and cellular viability was measured using resazurin assays. Both pol η-deficient clones had a significantly lower viability level compared to the pol η-WT cell line as measured at 5 days after treatment ($P< 0.05$ for both pol η-deficient clones, Student's t-test) (**FIGURE 4.2 A**). These data confirm the findings obtained in XP30RO cells that pol η deficiency renders cells more sensitive to oxaliplatin.

4.4 INCREASED CELLULAR SENSITIVITY TO OXALIPLATIN BY POL H KNOCKDOWN

As shown above, pol η deficiency results in increased sensitivity to oxaliplatin in knockdown cell lines. To test if inhibition of pol η in pol η wild-type cells would also increase sensitivity to this anticancer drug, a siRNA-based approach was used to knock down pol η protein in HCT116 colorectal cancer cells. Experiments were performed 48 hours after knockdown with 10 nM siRNA against pol η protein, and oxaliplatin treatment was carried out for 24 hours. Pol η knockdown after both 48 hours and 72 hours was above 80 % when quantified by Western blotting (**FIGURE 4.2 C**). Knockdown of pol η resulted in decreased clonogenic survival and significantly increased sensitivity to oxaliplatin compared to mock-transfected cells and cells transfected with non-targeting siRNA ($P< 0.05$, Student's t-test) (**FIGURE 4.2 B**). These data show that siRNA-based depletion of pol η had similar effects on oxaliplatin sensitivity as those seen in the 2 pol η knockout cell lines tested.

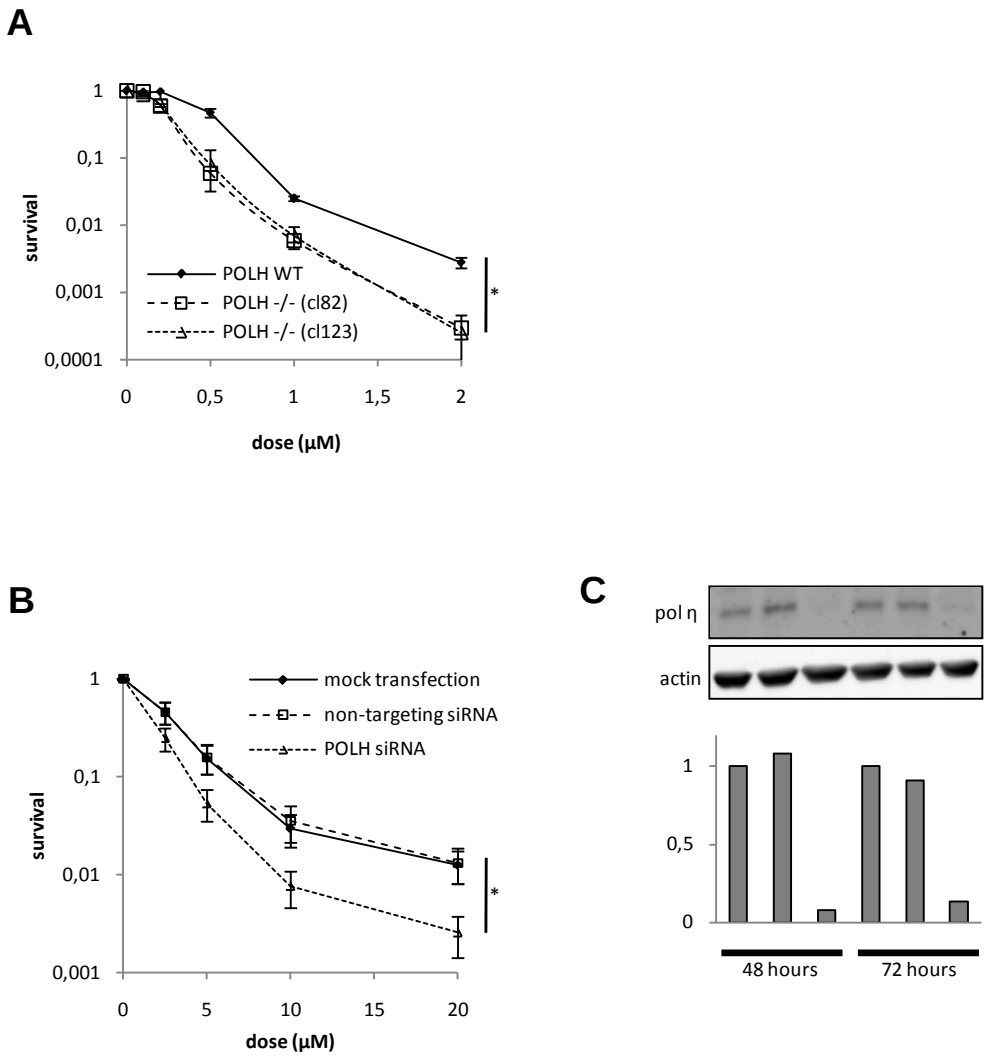


Figure 4.2. Increased sensitivity of pol η-deficient cells to oxaliplatin.

A. Viability assay in pol η-proficient BL-2 cells and two pol η-knockout BL-2 cell clones after continuous exposure to oxaliplatin. **B**. Clonogenic survival in HCT116 cells after pol η knockdown. Cells were either transfected with non-targeting siRNA or siRNA against pol η for 48 hours before treatment with oxaliplatin for 24 hours. Each datapoint represents three independent experiments, and error bars show the standard error of the mean. Statistical analysis performed by Student's t-test. * $P< 0.05$. **C**. Western blot analysis showing efficiency of pol η knockdown after 48 and 72 hours. Knockdown efficiency was above 75 % (bar diagram).

4.5 NO EFFECT OF OXALIPLATIN TREATMENT ON THE EXPRESSION OF POL H PROTEIN

Although pol η protein has been shown to be important for cellular survival after exposure to platinum-containing compounds, the effects of those drugs on the protein expression of the polymerase have not been reported previously. Continuous cellular treatment was performed with different concentrations of oxaliplatin for 24 or 48 hours, and pol η protein levels were assessed by Western blotting. Western blots were repeated three times, and Student's t-tests were used to assess any statistically significant up- or down-regulation. Neither HCT116 colorectal cancer cells nor MRC5 normal lung fibroblasts showed any alteration in pol η expression after short-term treatment with 10 μM oxaliplatin. Nor did a higher dose of 20 μM have any significant effect on pol η expression in either cell line (**FIGURE 4.3**).

Long-term effects of oxaliplatin were tested in a panel of oxaliplatin-resistant cell lines and their isogenic parental counterparts derived from gastric or colorectal cancers. Oxaliplatin resistance had been acquired in all cell lines tested by continuous treatment with the drug for prolonged times up to 6 months and was maintained either by adding oxaliplatin to the culturing media continuously or by spiking the cell lines monthly with the compound (Boyer, McLean et al. 2004; Ametller, Garcia-Recio et al. 2011; Bose, Zimmerman et al. 2011). Pol η protein expression in 7 paired cell lines were measured by Western blotting. Pol η levels varied up to 5-fold between the different colorectal and gastric cancer cell lines (**FIGURE 4.4**). There was no significant difference in pol η protein levels between oxaliplatin-resistant cells and oxaliplatin-sensitive parental cells for the 7 cell lines studied (Student's t-test). Taken

together, these data show that pol η protein expression is stable and cannot be altered by short-term or long-term exposure to oxaliplatin.

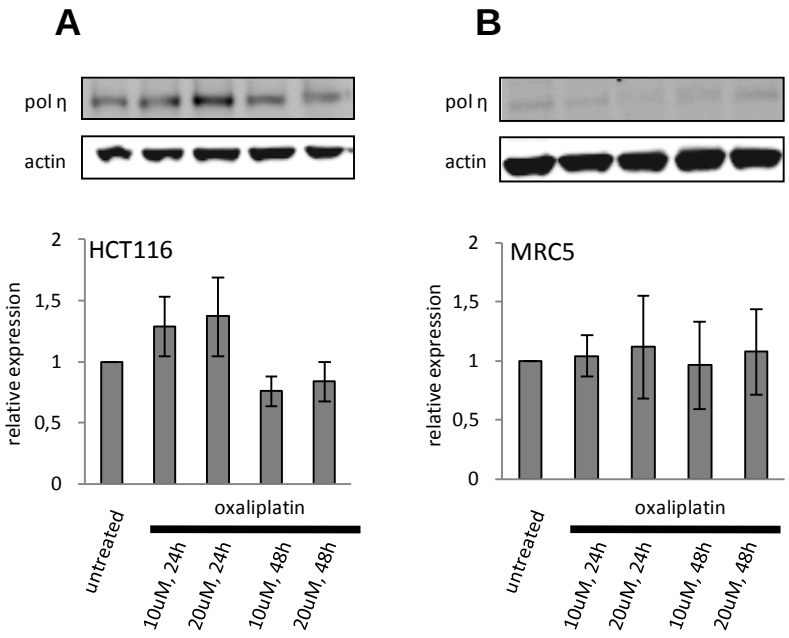


Figure 4.3. Stable expression of pol η protein after short-term exposure to oxaliplatin.

A. Whole cell lysates were harvested from HCT116 and MRC5 cells after treatment with oxaliplatin, and pol η protein levels were assessed by Western blotting. **B**. Bar diagram showing quantification of pol η protein content after treatment with different concentrations of oxaliplatin for 24 or 48 hours. Each bar represents three independent experiments, and error bars show the standard error of the mean.

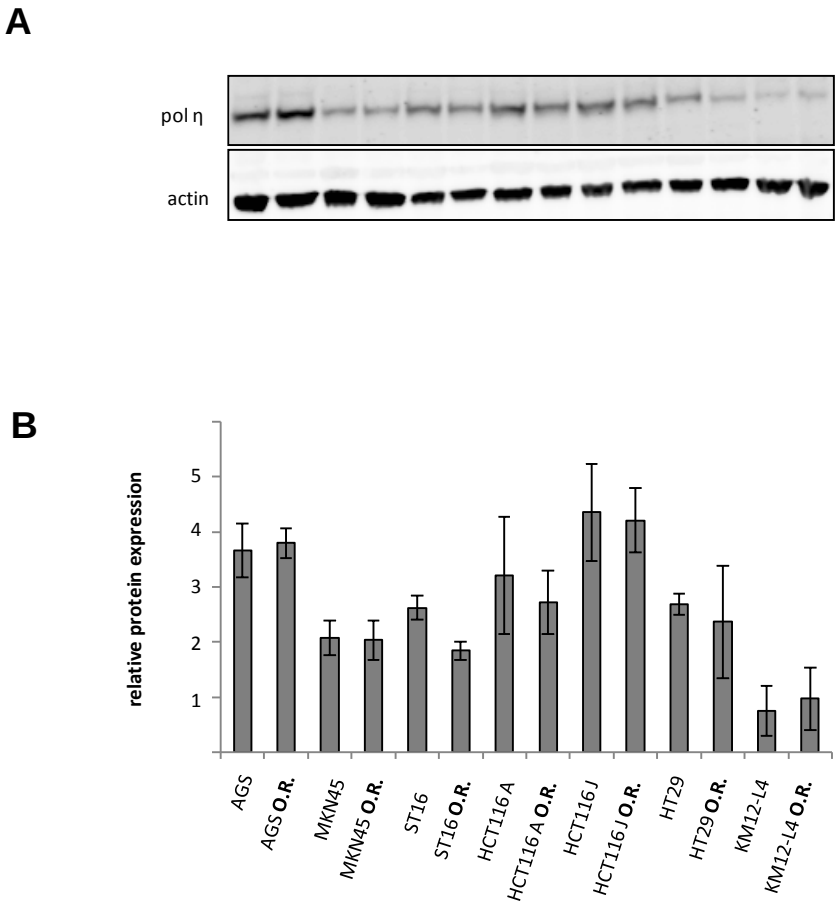


Figure 4.4. Stable expression of pol η protein after long-term exposure to oxaliplatin.

A. Whole cell lysates were harvested from 7 paired gastric and colorectal cancer cell lines with an acquired oxaliplatin resistance, and pol η protein levels were assessed by Western blotting. **B**. Bar diagram showing quantification of pol η protein content in oxaliplatin-resistant (O.R.) and parental wild-type cells. Each bar represents three independent experiments, and error bars show the standard error of the mean.

4.6 ACCUMULATION OF CELLS IN G2 PHASE AFTER OXALIPLATIN TREATMENT

As oxaliplatin may exert its cytotoxic effects through the formation of DNA adducts that can block replication during S phase of the cell cycle, it may therefore affect cell cycle distribution of treated cell lines.

To examine the effects of oxaliplatin treatment on cell cycle distribution, HCT116 colorectal cancer cells and MRC5 normal lung fibroblasts were treated with the drug for 24 or 48 hours, and fluorescence-activated cell sorting was used to measure the different cell cycle phases in those cell lines. Untreated HCT116 cells had a relatively high S phase population (46.9 %) that was completely abrogated after treatment with either 10 or 20 μM oxaliplatin ($P< 0.01$, Student's t-test). An increase in G2 phase cells was measured for both concentrations of oxaliplatin after 24 and 48 hours (**FIGURE 4.5 A and B**). Similarly, the S phase measured in untreated MRC5 cells (21.7 %) was removed by oxaliplatin treatment, and exposure to the drug shifted cells towards G2 phase of the cell cycle ($P< 0.01$, Student's t-test) (**FIGURE 4.5 C and D**). These data suggest that, in this preclinical model system, in the presence of oxaliplatin, cells can bypass platinum-induced damage to carry out replication, but that they stall in G2 phase probably so that the DNA damage can be repaired before entry into mitosis.

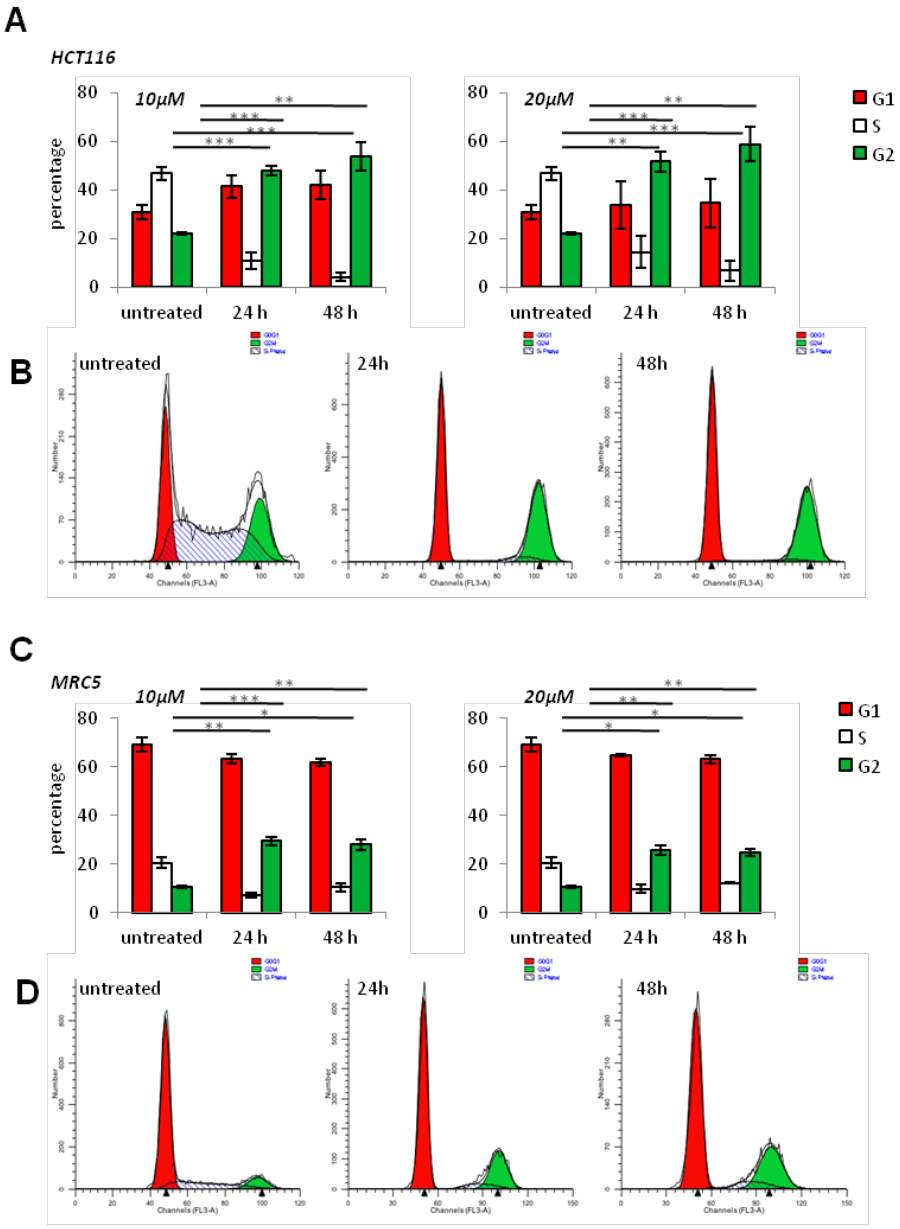


Figure 4.5. Oxaliplatin treatment results in abrogation of S phase and G2 block.

A. Cell cycle distribution of HCT116 cells without treatment and after 24 and 48 hours of exposure to 10 and 20 μM of oxaliplatin. **B**. Example histograms showing cell cycle effects in HCT116 cells with 10 μM oxaliplatin. **C**. Cell cycle distribution of MRC5 lung fibroblast cells without treatment and after 24 and 48 hours of exposure to 10 and 20 μM of oxaliplatin. **D**. Example histograms showing cell cycle effects in MRC5 cells with 10 μM oxaliplatin. Each bar represents three independent experiments, and error bars show the standard error of the mean. Statistical analysis performed by Student's t-test. * $P< 0.05$; ** $P< 0.01$; *** $P< 0.001$.

4.7 NO EFFECT OF POL H STATUS ON CELLULAR SENSITIVITY TO 5-FLUOROURACIL

In the clinic, oxaliplatin is always used in combination regimes together with the antimetabolite drug, 5-FU, as chemotherapy (de Gramont, Figer et al. 2000). 5-FU is an inhibitor of the enzyme, thymidylate synthase and is also incorporated into the nascent DNA strand to lead to disruption of replication. The role of pol η in the repair of 5-FU-induced DNA lesions has not been elucidated.

To test if the DNA polymerase is involved in the bypass of damage caused by 5-FU, clonogenic survival assays were performed using pol η-deficient XP30RO cells and pol η-complemented XP30RO/pol η cells. Cells were treated with increasing concentrations of 5-FU for 24 hours before colony formation was assessed. There was no significant difference in survival between XP30RO and XP30RO/pol η cells (statistical analysis performed by paired Student's t-test), suggesting that pol η does not determine survival after 5-FU treatment (**FIGURE 4.6**).

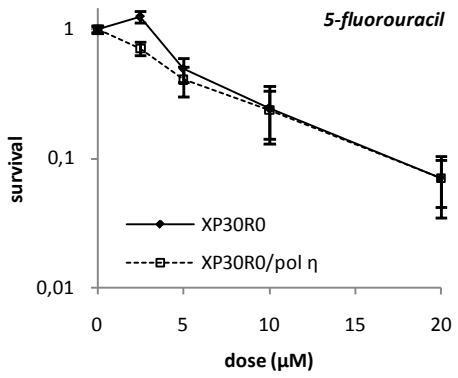


Figure 4.6. No effect of pol η status on cellular sensitivity to 5-fluorouracil.

Clonogenic assay showing no difference in survival of pol η-deficient cells compared to isogenic pol η-expressing XP30RO cells after exposure to different concentrations of 5-fluorouracil. Each data point represents three independent experiments, and error bars show standard error of the mean.

4.8 RESENSITISATION OF OXALIPLATIN-RESISTANT CANCER CELLS TO OXALIPLATIN BY POL H KNOCKDOWN

Data presented above show that pol η status determines cellular response to treatment with oxaliplatin, and pol η can be targeted by siRNA to increase sensitivity of cells to the drug. We postulated that targeting pol η in cells with an acquired resistance to oxaliplatin can attenuate oxaliplatin resistance.

Pol η knockdown was performed in oxaliplatin-resistant HCT116 cells, and efficiency was evaluated by Western blotting. Protein knockdown was above 80 % at

48 hours, and pol η remained suppressed for the following 24 hours (**FIGURE 4.7 B**). For assessment of survival, the polymerase was knocked down for 48 hours prior to treatment with oxaliplatin, and cells were treated with increasing concentrations of the drug for 24 hours before the drug was removed and clonogenic survival was assessed. Compared to the oxaliplatin-sensitive parental HCT116 cell line, resistance was almost 10-fold increased in oxaliplatin-resistant HCT116 cells. Silencing RNA-based knockdown of pol η protein led to a small but statistically significant reduction in clonogenic survival of the resistant cell line after oxaliplatin treatment ($P< 0.001$, paired Student's t-test) (**FIGURE 4.7 A**).

To corroborate these results, the same experimental design was applied to oxaliplatin-resistant and parental AGS gastric adenocarcinoma cells. Pol η knockdown was performed for 48 hours and remained suppressed up to 72 hours (**FIGURE 4.7 D**). Knockdown of the polymerase prior to treatment with oxaliplatin resulted in a significant decrease in clonogenic survival of the resistant AGS cell line after oxaliplatin treatment ($P< 0.05$, paired Student's t-test).

These data, obtained in two oxaliplatin-resistant tumour cell lines of different histological origins, suggest that targeting pol η may re-sensitise oxaliplatin-resistant cells to oxaliplatin chemotherapy.

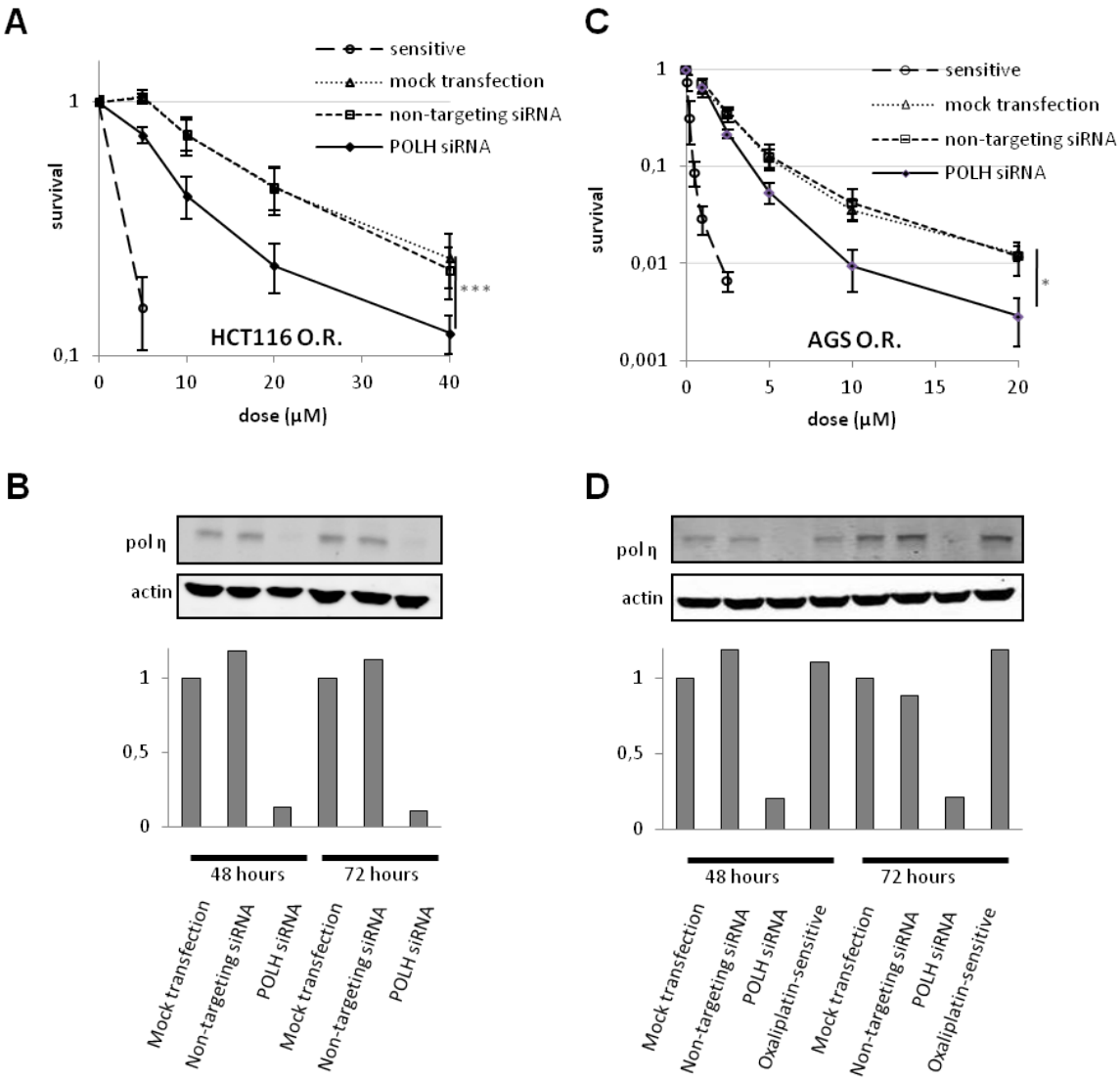


Figure 4.7. Re-sensitisation of oxaliplatin-resistant tumour cell lines to oxaliplatin by pol η knockdown.

A, C. Clonogenic survival in oxaliplatin-resistant HCT116 (**A**) or AGS (**C**) cells after pol η knockdown. Cells were either transfected with non-targeting siRNA or siRNA against pol η for 48 hours before treatment with oxaliplatin for 24 hours. Each datapoint represents three independent experiments, and error bars show the standard error of the mean. Statistical analysis performed by Student's t-test. * $P< 0.05$, *** $P< 0.001$. **B**, **D**. Western blot analyses showing efficiency of pol η knockdown after 48 and 72 hours. Knockdown efficiency was above 75 % (bar diagrams).

4.9 SUMMARY

The cytotoxic drug, oxaliplatin, is widely used to target tumour diseases and has been established as a first-line therapy in combination with 5-FU for colorectal cancer (de Gramont, Figer et al. 2000). Oxaliplatin forms DNA adducts within one strand or between the strands of DNA that block high-fidelity DNA polymerases and therefore block replication and transcription. In order to protect cellular viability, platinum-induced lesions either require immediate repair, or they can be temporarily bypassed by TLS polymerases during S phase and may then be amenable to post-replication repair (Reardon, Vaisman et al. 1999; Chaney, Campbell et al. 2005).

The data presented in this chapter show a role for DNA polymerase η in cellular responses to the platinum compound, oxaliplatin. Pol η-deficient XP30RO and BL-2 cell lines showed increased sensitivity to oxaliplatin compared to their pol η-expressing counterparts. As shown by a siRNA-based approach, pol η can be targeted to re-sensitise oxaliplatin-resistant cells to oxaliplatin. Pol η did not affect sensitivity to the cytotoxic drug, 5-FU, that is often used in combination regimes together with oxaliplatin. Treatment with oxaliplatin did not alter expression of the polymerase in either tumour or non-malignant cell lines. Cell lines with acquired cellular resistance to oxaliplatin did not demonstrate alteration of expression of pol η, suggesting that changes in pol η expression are not involved in the development of acquired resistance to oxaliplatin.

On exposure to oxaliplatin, Pol η wild-type cell lines accumulated in the G2 phase of the cell cycle. This finding probably reflects the fact that cells employ two

distinct pathways to deal with platinum-induced DNA adducts. Cells already carrying out DNA synthesis can employ the TLS pathway to finish replication before stalling in G2 phase where the platinum-induced adducts can be removed by the NER system, whereas cells that are still in G1 phase do not enter S phase with unrepaired platinum lesions.

RESULTS 3: *POLH* MRNA EXPRESSION AS A POTENTIAL BIOMARKER IN OESOPHAGEAL CANCER PATIENTS TREATED WITH OXALIPLATIN

5.1 INTRODUCTION

Oesophageal cancer is the ninth most common cancer in the United Kingdom. In 2007, almost 8000 patients were diagnosed with the disease in the United Kingdom (Office for National Statistics 2010). Despite advances in treatment over the last 2 decades, oesophageal cancer still has a very poor prognosis, and the 5-year survival rate remains below 20 % (Jemal, Siegel et al. 2009). Surgical approaches are the main treatment option for oesophageal carcinoma, but due to the late presentation of symptoms, about half of all oesophageal cancer patients are unresectable at the time of diagnosis because of the development of systemic disease (Shahbaz Sarwar, Luketich et al. 2010).

Platinum-based chemotherapy is now the standard treatment for systemic oesophageal cancer, and cisplatin has been established as a first-line drug in this setting despite considerable side effects that may require termination of further treatment (Go and Adjei 1999; Conroy, Yataghene et al. 2010). Oxaliplatin is the only second-generation platinum-based chemotherapeutic drug approved for cancer therapy, and it is widely used for first-line treatment of metastatic colorectal cancer in combination with 5-fluorouracil and folinic acid (de Gramont, Figer et al. 2000; Giacchetti, Perpoint et al. 2000). In recent years, oxaliplatin has come into focus as an alternative treatment for oesophageal carcinomas. Clinical studies have shown promising efficacy and a

favourable toxicity profile against this type of cancer (Mauer, Kraut et al. 2005; Al-Batran, Hartmann et al. 2008; Cunningham, Starling et al. 2008).

DNA damage caused by oxaliplatin is mainly subject to repair by the nucleotide excision repair pathway, which excised and replaces oligonucleotide sequences within the damaged strand carrying an oxaliplatin adduct (Wang and Lippard 2005). Recently, translesion synthesis has been shown to enable cells to cope with platinum DNA damage independently of excision repair. Of the specialised DNA polymerases involved in this pathway, pol η has been most clearly linked to the bypass of lesions caused by cisplatin and oxaliplatin (Vaisman, Masutani et al. 2000; Albertella, Green et al. 2005), and the enzyme is able to modulate cellular sensitivity to platinum-based agents in cell culture (Chen, Cleaver et al. 2006), although its role in mediating tumour response to oxaliplatin chemotherapy is largely unknown.

The aim of this study was to investigate the role of pol η expression in oesophageal cancers with a view to developing it as a predictive biomarker for oxaliplatin-based cancer treatment.

5.2 PATIENT CHARACTERISTICS

Tissue samples from 49 patients with oesophageal carcinoma were used for this clinical study. Patients had undergone neoadjuvant treatment with two cycles of chemotherapy with oxaliplatin and 5-FU. Pre-treatment samples of tumour and corresponding normal oesophageal tissue were taken from biopsies collected during

initial endoscopic examinations, and post-treatment samples were harvested from the resected tumours. As described in the Materials and Methods chapter, tissue specimens were collected in RNAlater, and RNA was extracted.

Patient characteristics are shown in **TABLE 5.1**. Of the 49 patients from whom pre-treatment biopsies had been taken, 44 underwent surgical resection after two cycles of neoadjuvant treatment. There were 37 male and 12 female patients, and the median age for all patients was 67 years and the mean age 65 years (ranging from 29 to 78 years). Staging and grading was performed by an independent pathologist. At diagnosis, 9 patients had stage I disease, 14 had stage II disease, 18 had stage III disease and 2 had stage IV disease. For analysis purposes, samples from lower stage tumours (stages I and II) and samples from advanced stages (stage III and IV) were grouped together. Three tumours were grade 1, 15 tumours were grade 2 and 25 tumours were grade 3 cancers. 6 patients could not be assessed for tumour stage and grade. On histology, 39 tumours were adenocarcinomas, 8 tumours were squamous cell carcinomas and 2 tumours were adenosquamous and undifferentiated carcinomas, respectively.

Table 5.1. Patient characteristics

49 patients	**N (%)**
Sex	
Male	37 (75.51)
Female	12 (24.49)
Tumour Stage	
I/II	24 (54.55)
III/IV	20 (45.45)
Tumour Grade	
G1	3 (6.98)
G2	15 (34.88)
G3	25 (58.14)
Histological Type	
Adeno	39 (82.98)
Other	8 (17.02)

5.3 OPTIMISATION OF QRT-PCR

qRT-PCR experiments were carried out as described in the Materials and Methods chapter. All primers used for this study were intron-spanning to avoid genomic contamination. The efficiency of primers and probe for *POLH* and *GAPDH* was determined by multiple polymerisation reactions using 1 in 10 serial dilutions of cDNA, and a ratio between threshold values of *POLH* and *GAPDH* polymerisation

(ΔCt values) was calculated for each concentration. As shown in **FIGURE 5.1**, the ΔCt values for each cDNA dilution were comparable and the slope of the line fitted over the individual data points was below 0.1 suggesting a comparable reaction efficiency of both primer pairs.

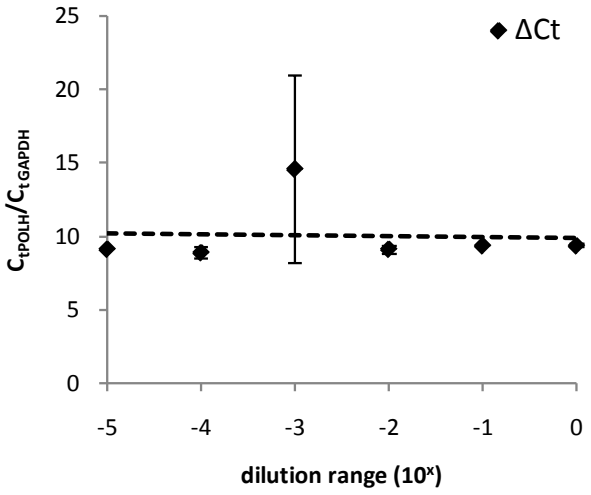


Figure 5.1. Comparable reaction efficiencies of *POLH* and *GAPDH* probes and primers.

cDNA was reverse transcribed from normal oesophageal tissue, and 1 in 10 dilutions were performed over several magnitudes. Ct values were obtained for both sets of primers and probes, and the ratio was plotted against each dilution. The slope of the extrapolated line is 0.07 indicating comparable reaction efficiencies.

5.4 EXPRESSION OF *POLH* MRNA IN TUMOUR AND MATCHING NORMAL TISSUE SAMPLES BEFORE AND AFTER PLATINUM-BASED TREATMENT

As described above, *POLH* mRNA expression was analysed in tumour tissue and corresponding normal tissue before and after treatment with oxaliplatin-based chemotherapy. In pre-treatment biopsies, *POLH* levels were found to be significantly higher than in matching normal tissue samples (median tumour/normal tissue ratio = 1.65 from 0.24 to 7.24; $P< 0.001$, Wilcoxon's sign rank test; **FIGURE 5.3**); 16 tumours had more than two-fold higher *POLH* mRNA levels than the corresponding normal tissue, whereas only one tumour had a more than two-fold lower level (**FIGURE 5.2 A**). After two cycles of oxaliplatin-based chemotherapy, there was no significant difference between *POLH* levels in tumour and matched normal tissue ($P = 0.42$). However, *POLH* levels in the normal tissue samples were found to increase significantly after treatment (median post-treatment/pre-treatment ratio = 1.70 from 0.47 to 10.71; $P< 0.001$, Wilcoxon's sign rank test, **FIGURE 5.3**). This increase in mRNA levels was not observed in the tumour samples ($P = 0.47$).

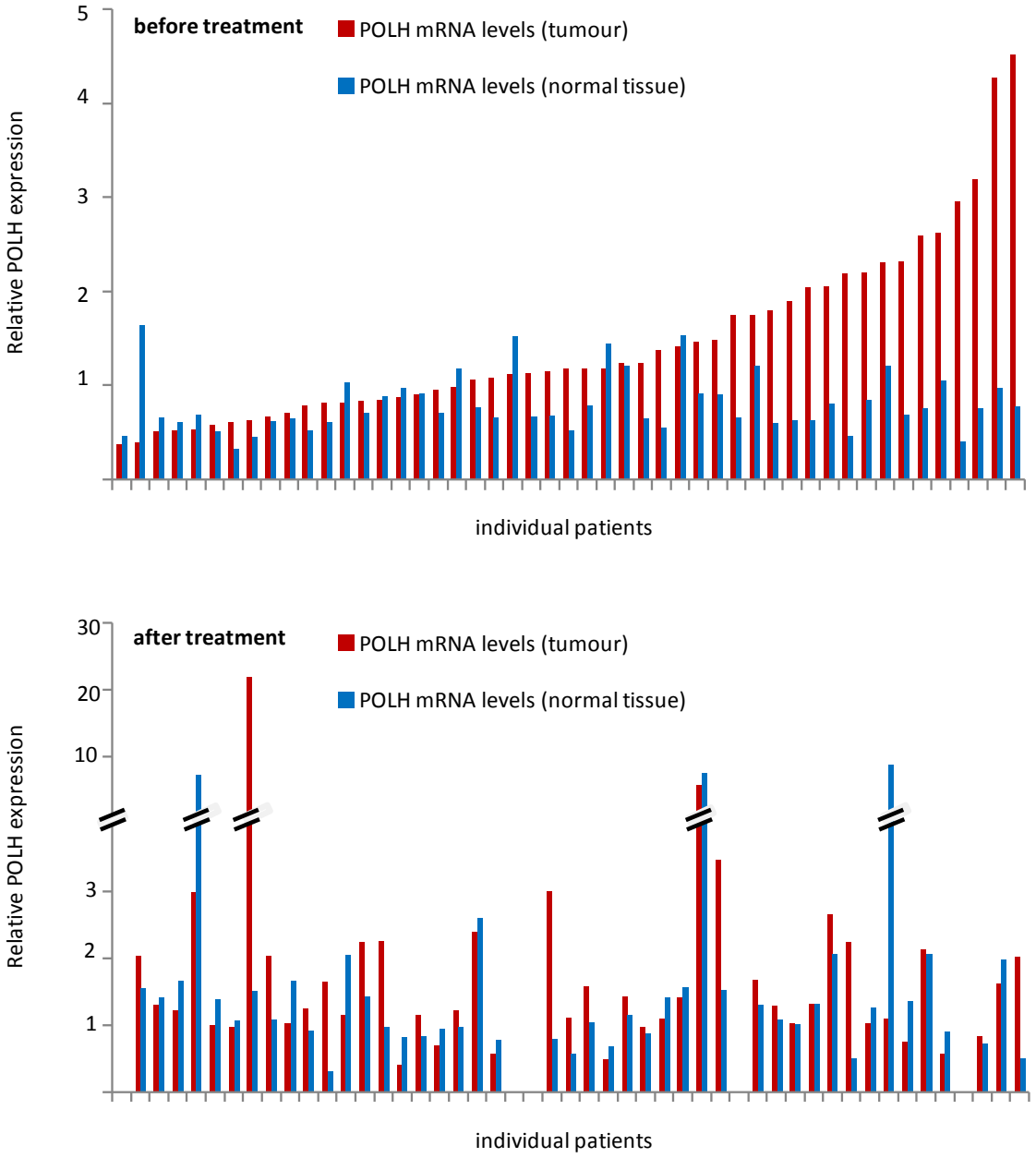


Figure 5.2. Individual *POLH* mRNA levels before and after oxaliplatin-based treatment

Bars represent relative *POLH* mRNA expression in tumour (red) and matched normal tissue samples (blue) before and after two courses of oxaliplatin-based chemotherapy individually for all 49 patients.

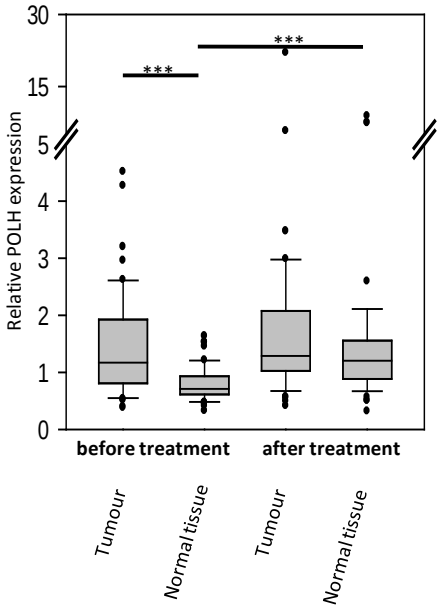


Figure 5.3. *POLH* mRNA levels in tumour and normal tissue

Box plots showing relative *POLH* mRNA levels in oesophageal cancer and matched normal oesophageal tissue before and after oxaliplatin-based treatment. Statistical analysis performed by Wilcoxon's sign rank test. *** $P< 0.001$

No correlation was observed between *POLH* expression in pre-treatment tumour samples and age (P = 0.82, Spearman's rank correlation), gender (P = 0.90), tumour stage (P= 0.23) and tumour grade (P = 0.31, **FIGURE 5.4**). Similarly, there was no significant correlation between *POLH* mRNA levels in pre-treatment normal tissue samples and age (P = 0.81, Spearman's rank correlation) or gender (P = 0.42). Histological tumour type and *POLH* expression did demonstrate a significant effect:

adenocarcinoma samples (n=39) showed a significantly higher *POLH* level than tumours of other histologies (n=10) ($P< 0.001$, Spearman's rank correlation).

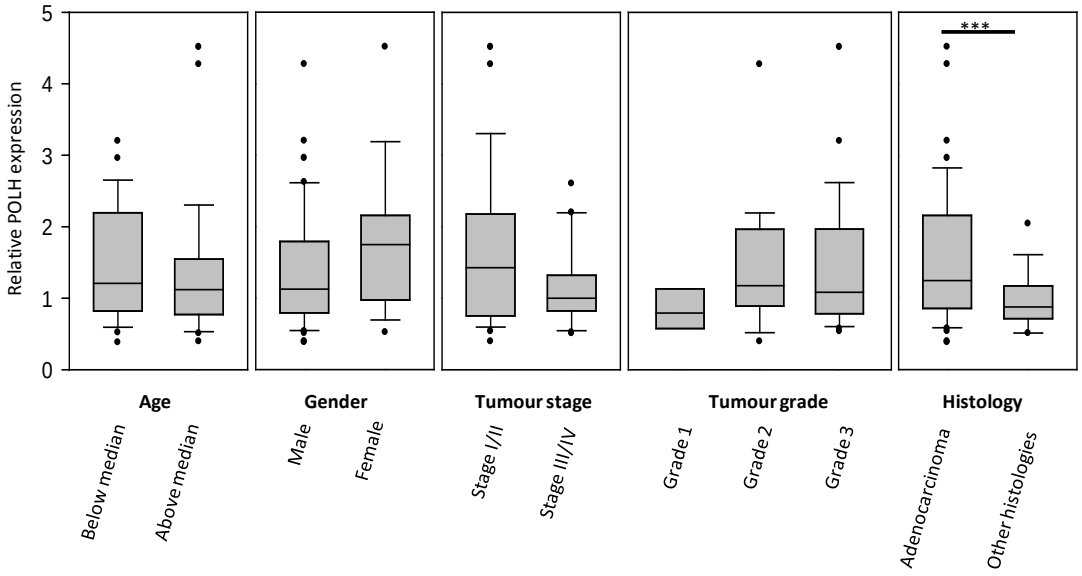


Figure 5.4. Effects of different variables on *POLH* mRNA levels in cancer samples

Box plots showing no influence of age, gender, tumour stage and tumour grade on relative *POLH* mRNA levels in oesophageal cancer samples before treatment. Relative *POLH* mRNA expression was significantly higher in adenocarcinoma samples compared to tumours of other histological types. Statistical analysis performed by Spearman's rank correlation. *** $P< 0.001$

5.5 DETERMINATION OF INDEPENDENT VARIABLES FOR RESPONSE AND SURVIVAL

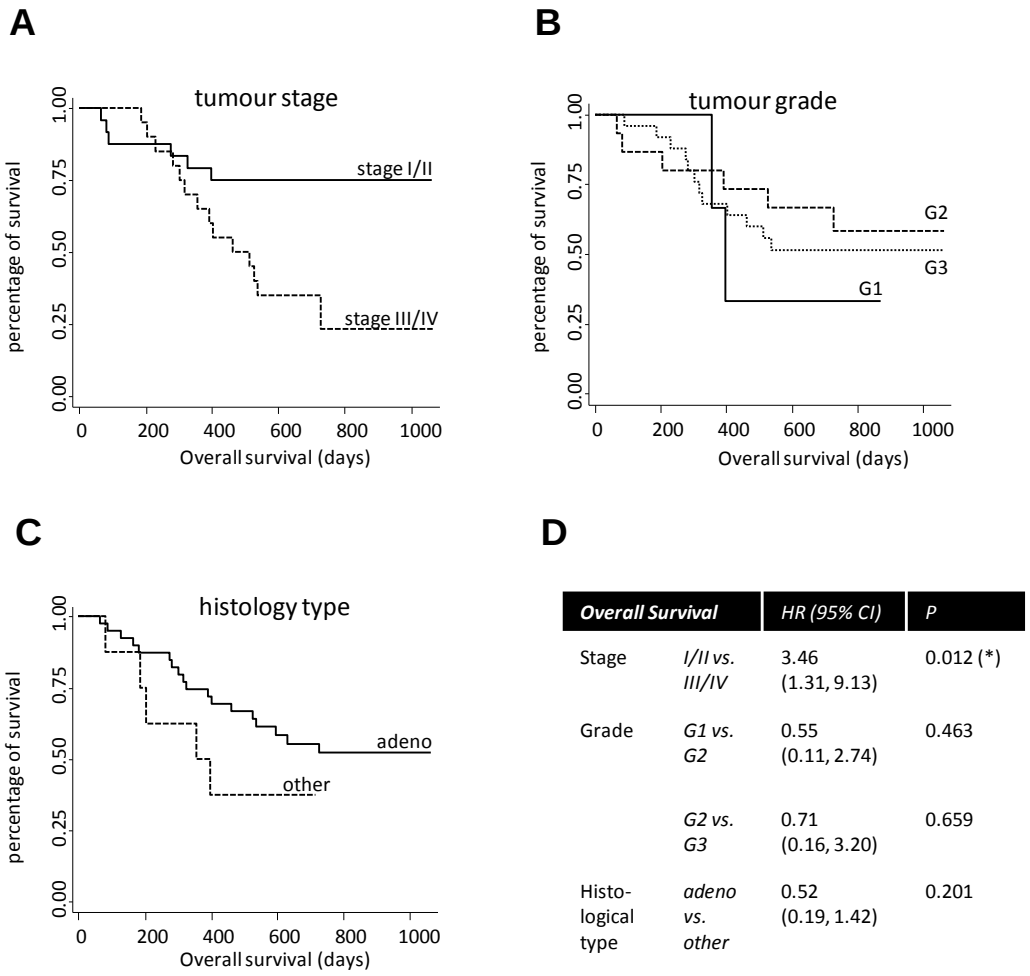
As different characteristics of tumours have a prognostic or predictive relevance and influence patient response and survival, those independent variables

were included so that the analysis could be adjusted to take those effects into consideration.

In this cohort of patients, tumour stage had a significant influence on overall survival (OS), as patients with stage I or II tumours survived longer times than patients with stage III and IV oesophageal cancers (Hazard Ratio 3.46, $P = 0.012$, Log-Rank test). In a similar way, disease-free survival (DFS) was significantly influenced by tumour stage (HR 5.22, $P = 0.005$, Log-Rank test) (**FIGURES 5.5 A and 5.6 A**).

In contrast, tumour grade had no influence on survival. Patients with grade 2 or grade 3 tumours did not have significantly different OS (HR 0.55 for G2, $P = 0.46$; Hazard Ratio 0.71 for G3, $P = 0.66$, Log-Rank test) or DFS (HR 0.33 for G2, $P = 0.21$; HR 0.65 for G3, $P = 0.58$) compared to patients with grade 1 tumours (**FIGURES 5.5 B and 5.6 B**).

Similarly, histological tumour type again had no impact on patient survival in this dataset. Patients with oesophageal adenocarcinoma did not have significantly different OS (HR 0.52, $P = 0.20$) or DFS (Hazard Ratio 0.84, $P = 0.79$) compared to patients with tumours of other histologies (**FIGURES 5.5 C and 5.6 C**). For further correlations between patient survival/therapeutic response and *POLH* expression levels, adjustments were performed for tumour stage to correct for significant effects of those parameters on survival and response alone.

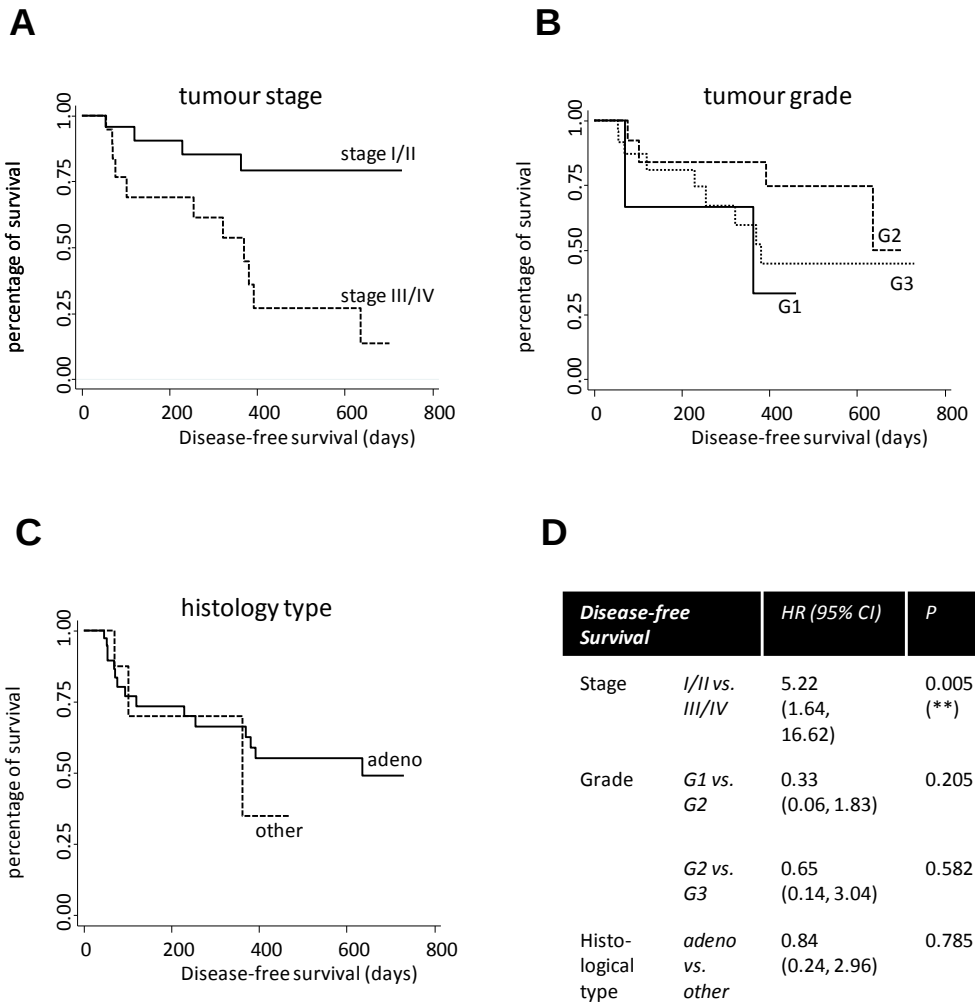


D

Overall Survival		*HR (95% CI)*	*P*
Stage	*I/II vs. III/IV*	3.46 (1.31, 9.13)	0.012 (*)
Grade	*G1 vs. G2*	0.55 (0.11, 2.74)	0.463
	G2 vs. G3	0.71 (0.16, 3.20)	0.659
Histo-logical type	*adeno vs. other*	0.52 (0.19, 1.42)	0.201

Figure 5.5. Variables influencing overall survival

Kaplan-Meier curves showing overall survival split by tumour stages I/II vs. III/IV (**A**), tumour grades (**B**) or histology (**C**). Statistical analysis performed by Logrank test. * $P< 0.05$



Disease-free Survival		*HR (95% CI)*	*P*
Stage	*I/II vs. III/IV*	5.22 (1.64, 16.62)	0.005 (**)
Grade	*G1 vs. G2*	0.33 (0.06, 1.83)	0.205
	G2 vs. G3	0.65 (0.14, 3.04)	0.582
Histo-logical type	*adeno vs. other*	0.84 (0.24, 2.96)	0.785

Figure 5.6. Variables influencing disease-free survival

Kaplan-Meier curves showing disease-free survival split by tumour stages I/II vs. III/IV (**A**), tumour grades (**B**) or histology (**C**). Statistical analysis performed by Logrank test. ** $P< 0.01$

5.6 CORRELATION BETWEEN *POLH* MESSENGER RNA LEVELS AND PATIENT SURVIVAL

To assess a potential influence of *POLH* expression on patient survival, Cox analyses were performed, and results were adjusted to the independent clinical variables as discussed above. Median follow-up was 892 days (range from 892 to 1099 days). At the time of analysis, 25 of 49 patients had died.

Cox analysis revealed that *POLH* levels of oesophageal cancer specimens significantly correlated with patient survival. OS was significantly higher for tumours with high *POLH* expression before treatment, and patients with high *POLH* expression were 75 % less likely to die within the time of follow-up than patients with low *POLH* levels (Hazard Ratio 0.25; 95% CI 0.10, 0.64; $P< 0.005$). After adjustment for tumour stage, patients in the high *POLH* group had a significantly better overall survival (Hazard Ratio 0.29; 95 % CI 0.10, 0.82; $P< 0.05$). Similar results were observed for DFS. Tumours with high pre-treatment *POLH* expression were 63 % less likely to progress, and patients suffering from oesophageal tumours with high *POLH* expression had significantly longer times to progression than patients with low *POLH* tumours (Hazard Ratio 0.37; 95 % CI 0.14, 0.97; $P< 0.05$). However, after adjusting for tumour stage, *POLH* mRNA levels were not significantly related to disease-free survival (Hazard Ratio 0.38; 95 % CI 0.12, 1.23; $P = 0.11$) (**FIGURE 5.7**).

In summary, these data show that high *POLH* expression in oesophageal tumours before oxaliplatin-based chemotherapy correlated with better overall survival.

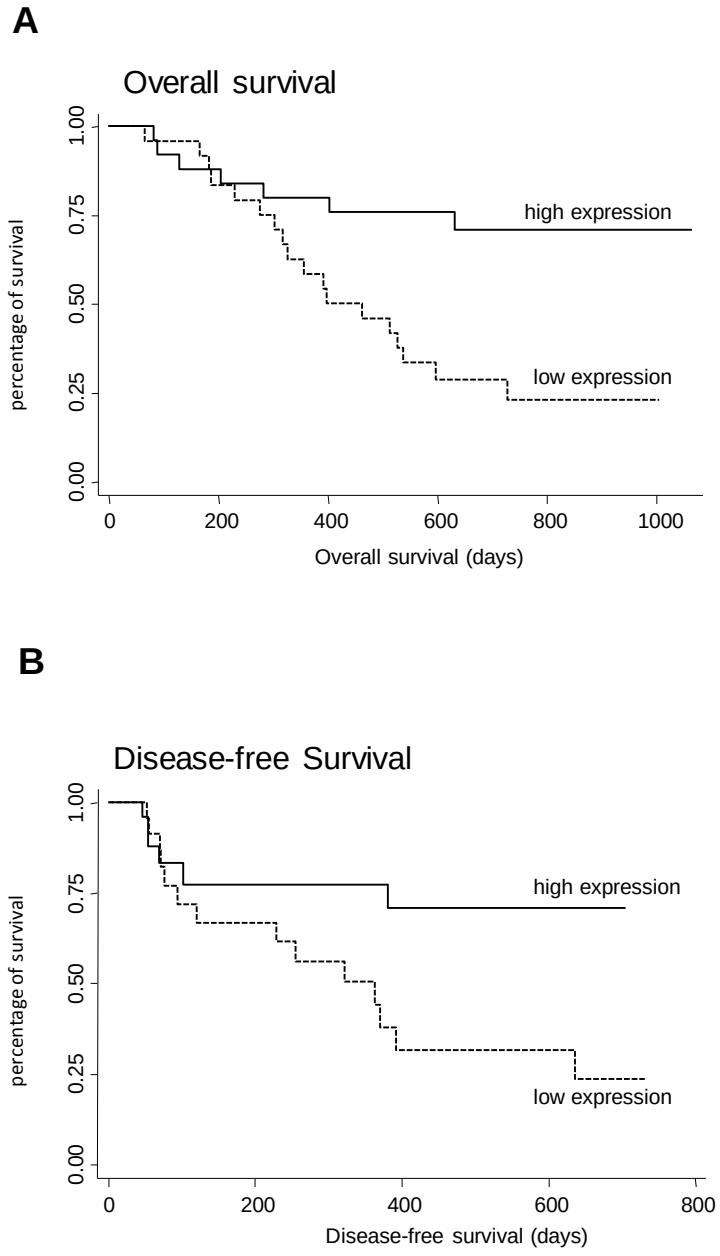


Figure 5.7. High *POLH* levels correlate with improved overall and disease-free survival

Kaplan-Meier curves showing overall survival (**A**) and disease-free survival (**B**) in patients with high *POLH* vs. low *POLH*-expressing tumours as split by the median.

5.7 CORRELATION BETWEEN *POLH* MESSENGER RNA LEVELS AND THERAPEUTIC RESPONSE

For this study, patients' responses to therapy were assessed by two means. Pathological response was investigated in resected tumour specimens using the Mandard score, and metabolic response was assessed using PET-CT data obtained before and after chemotherapeutic treatment (Gillies, Middleton et al. 2012).

Pathological response was assessed by the Mandard score measuring response to neoadjuvant treatment in pathological samples. This score was originally introduced into clinical practice by Anne-Marie Mandard et al. and is based on a grading system for tumour regression measuring post-therapeutic fibrosis and remaining tumour tissue (Mandard, Dalibard et al. 1994). Today, the Mandard scoring system is widely used to assess therapeutic response in oesophageal cancers (Hermann, Horstmann et al. 2006).

Samples from 25 patients (51.0 %) had Mandard scores between 1 and 3 (fibrosis predominant) and were counted as responders, and tumour specimens from 24 patients (49.0 %) had Mandard scores between 4 and 5 (remaining tumour tissue predominant) and were counted as non-responders. By logistic regression analysis, *POLH* mRNA levels did not correlate with pathological response (Odds Ratio 1.53, $P = 0.471$, logistic regression) (**TABLE 5.2**).

Metabolic response was measured by comparing PET-CT data before and after neo-adjuvant treatment with oxaliplatin/5-FU: According to the PERCIST criteria, maximum standardised uptake values (SUV_{max}) before and after two cycles of chemotherapy were compared, and response was defined as more than 30% reduction of the original value of the primary tumour site and no new sites of disease on CT (Wahl, Jacene et al. 2009). 22 patients (44.9 %) had a complete or partial response and were counted as responders, while 27 patients (55.1 %) had stable or progressive disease and were counted as non-responders. By logistic regression analysis, *POLH* levels correlated with metabolic response. 16 patients with high *POLH* levels and only 6 patients with low *POLH* tumour levels had complete or partial response, while tumours from 18 patients with low *POLH* levels, but only from 9 patients with high *POLH* levels did not respond to therapy. Taken together, patients with high *POLH*-expressing tumours had a significantly better response to treatment than patients with low *POLH*-expressing cancers (Odds Ratio 0.19, $P< 0.01$, logistic regression) (**TABLE 5.2**). These results were consistent with the survival data presented in section 5.6 above

Table 5.2. Correlation between high *POLH* levels and pathological (Mandard score) and metabolic (PET-CT) response

Pathological response	**Odds Ratio (95% CI)**	***P* value**
No adjustment	1.53 (0.48, 4.83)	0.471
Adjustment for tumour stage	1.47 (0.41, 5.30)	0.559

Metabolic response	**Odds Ratio (95% CI)**	***P* value**
No adjustment	0.19 (0.05, 0.64)	0.008 (**)
Adjustment for tumour stage	0.21 (0.06, 0.81)	0.023 (*)

5.8 SUMMARY

Previous publications have shown that the translesion DNA polymerase, pol η can bypass oxaliplatin-induced DNA adducts on oligonucleotide constructs (Vaisman, Masutani et al. 2000; Bassett, Vaisman et al. 2003; Chaney, Campbell et al. 2005), and preclinical data presented in chapter 4 have shown that pol η influenced cellular survival after oxaliplatin treatment, and cells deficient in the polymerase were more sensitive to the platinum compound.

The role of the TLS pathway and pol η in mediating cellular tolerance of oxaliplatin-induced damage has not previously been studied in human cancer. In this chapter, mRNA was harvested from tumour and corresponding normal tissue specimens from 49 patients with oesophageal cancer prior to oxaliplatin-based chemotherapy, and *POLH* mRNA levels were analysed. Tumour samples before treatment were found to show significantly higher *POLH* levels than matching normal tissue specimens. Treatment did not alter mRNA expression in tumours, whereas normal tissue after two courses of chemotherapy revealed significantly increased levels of mRNA for *POLH*. Patient characteristics like age and gender as well as tumour stage and tumour grade did not correlate with *POLH* levels, but the histological tumour type was significantly correlated with adenocarcinoma samples having significantly higher *POLH* mRNA expression.

Patients enrolled in this study were followed up for up to 1147 days after inclusion in the trial, and survival and response data were obtained. In apparent contrast to the preclinical data presented in the previous chapter, high *POLH* levels correlated with increased overall and progression-free survival, and patients with high *POLH*-expressing tumours had a significantly better prognosis. Clinical data were also analysed for clinical response, pathological response and radiological response. Clinical and pathological response data did not correlate with *POLH* levels in tumours. Metabolic response data as assessed by PET-CT showed a significant correlation, with high *POLH*-expressing cancers having a higher level of complete or partial response to treatment.

These results show for the first time that *POLH* mRNA levels correlate with patient survival and response to oxaliplatin-based chemotherapy. The data presented above suggest that *POLH* should be developed as a potential biomarker to predict choice of therapy in patients eligible for oxaliplatin-based chemotherapy.

DISCUSSION

6.1 SUMMARY OF FINDINGS

This project focussed on the involvement of the translesion DNA polymerase, pol η in modulating cellular survival after exposure to ionising radiation or oxaliplatin-based chemotherapy. Pol η is a DNA polymerase enabling cells to bypass and temporarily tolerate DNA lesions caused by UV radiation during DNA replication. Its absence results in a rare, autosomal-recessive UV hypersensitivity syndrome, the variant form of xeroderma pigmentosum, resulting in early-onset skin cancers. Although the enzyme is well understood with regard to the repair of UV-induced DNA damage, its role in regulating cellular responses to cancer therapies is less well understood.

The data presented here show that deficiency or knockdown of pol η resulted in increased cellular resistance to ionising radiation. Deficiency of the polymerase led to an accumulation of cells in S phase. In line with the radioresistant phenotype observed in pol η-deficient cells, those cells exhibited faster resolution of γH2AX foci and hence less residual DNA damage after IR treatment. Since IR exerts its main toxic effect by the creation of DNA double-strand breaks, DSB repair activity was examined by RAD51foci staining and XRCC3 knockdown. Pol η-deficient cells were shown to exhibit more efficient DSB repair by the HR pathway during S phase.

The second part of this research project examined the effects of pol η on cellular responses to the anticancer drug, oxaliplatin. Cells lacking the polymerase exhibited increased sensitivity to treatment with oxaliplatin, but not 5-FU, a drug usually

administered together with oxaliplatin in the clinical setting. Cells with wild-type pol η expression were able to cope with oxaliplatin DNA adducts, probably by translesional bypass of oxaliplatin DNA adducts. These cells progressed through the S phase of the cell cycle and accumulated in G2 phase. Despite the apparent importance of pol η in determining cellular survival following treatment with oxaliplatin, neither short-term exposure nor long-term treatment with the drug was found to lead to an upregulation of the enzyme in cell culture. As pol η influences cellular tolerance to platinum compounds, it may be an interesting enzyme to be targeted in combination with platinum-based chemotherapy to increase the efficacy of the cytotoxic chemotherapy. In cell culture, targeting pol η function by siRNA knockdown of the enzyme was found to partially reverse acquired resistance in 2 oxaliplatin-resistant cancer cell lines.

In the third part of this research project, *POLH* mRNA levels were analysed in clinical samples of oesophageal cancer and matching normal oesophageal tissue before and after treatment with oxaliplatin-based chemotherapy. Pre-treatment tumour biopsies exhibited significantly higher levels of *POLH* mRNA than matched normal tissue samples. High *POLH* mRNA levels in tumours before therapy correlated with better overall survival of patients. Importantly, patients with high *POLH* mRNA-expressing cancers had a better therapeutic response than those with low *POLH* tumours as assessed by PET-CT. Although these clinical findings conflict with the results observed in the cell culture experiments, *POLH* expression may hold potential as a predictive biomarker for therapeutic response in patients treated with oxaliplatin-based chemotherapy.

6.2 THE TRANSLESION SYNTHESIS PATHWAY IN CELLULAR RESPONSES TO IONISING RADIATION

Until now, no data have been reported on the involvement of pol η in determining cellular responses to ionising radiation. Unlike UV irradiation that leads to well-defined DNA damage amenable to translesional synthesis by pol η during S phase of the cell cycle, ionising radiation has been shown to induce various different forms of DNA lesions, including base damage, single-strand and DNA double-strand breaks (Helleday, Petermann et al. 2008). Several studies have examined the involvement of the TLS pathway in the bypass of specific IR-induced DNA damage *in vitro*.

Oxidative DNA lesions form the majority of radiation-induced DNA damage, and several publications have examined translesional activity of pol η past specific oxidative DNA lesions. 8-Oxo-7,8-dihydroguanine (8-oxo-G) is a major DNA lesion induced by IR treatment and has a high miscoding potential (Beard, Batra et al. 2010; van Loon, Markkanen et al. 2010). Data from several groups has shown that pol η can bypass 8-oxo-G residues on oligonucleotide constructs with a high efficiency and in a near error-free manner in the presence of auxiliary proteins, PCNA and RP-A (Avkin and Livneh 2002; Maga, Villani et al. 2007; Lee and Pfeifer 2008; McCulloch, Kokoska et al. 2009). Other major forms of oxidative damage observed after IR treatment are crosslinks between guanine and pyrimidine residues, distorting one DNA strand and resulting in mutagenic potential (Jiang, Hong et al. 2007). Using data obtained from oligonucleotide assays, two publications have suggested a role for pol η in the bypass of those IR-induced intrastrand crosslinks (Gu and Wang 2004; Colis, Raychaudhury et al. 2008). Although *in vivo* data investigating the role of pol η in determining cellular

responses to oxidative DNA lesions after IR treatment are lacking, it has been suggested that cells may employ the polymerase to replicate past minor oxidative damage caused by IR.

As TLS permits temporary DNA damage tolerance during genomic replication, one could argue that cellular TLS activity after exposure to IR is mostly confined to the S phase of the cell cycle. However, there is evidence that translesional bypass may also occur independently of cellular replication activity outside of S phase, and, in the yeast model, it has been shown that cells can bypass and tolerate intrastrand crosslinks in G2 phase as well (Daigaku, Davies et al. 2010). As indicated in multiple publications previously, cellular radiosensitivity varies depending on cell cycle phases with S phase cells being relatively radioresistant compared to cells in other phases of the cell cycle (Sinclair and Morton 1966; Iliakis and Okayasu 1990; Cheong, Wang et al. 1994; Biade, Stobbe et al. 1997). Therefore, one would expect that cell lines with higher S phase fractions may exhibit increased radioresistance; indeed, recently published data support this notion (Al-Assar, Mantoni et al. 2011). Previous publications have demonstrated prolonged S phase arrest in pol η-deficient cells upon exogenous DNA damage like UV irradiation (Thakur, Wernick et al. 2001; Cleaver, Bartholomew et al. 2002), and it possible that, in the absence of pol η, continuous endogenous DNA damage also results in S phase accumulation of cells.

In our dataset, the effect of endogenous damage on cells lacking pol η is illustrated by an increase in baseline γH2AX foci arising without cytotoxic treatment. Continuously higher γH2AX foci in those cells suggest an inability to temporarily

tolerate endogenous damage leading to the necessity to repair those lesions before replication can resume. In line with those findings, the results presented in this thesis show that cells that lack pol η accumulate in S phase, and pol η-deficient cell lines have a higher percentage of S phase cells at any given time point compared to cells supplemented with functional pol η. The idea of an S phase-dependent increase in radioresistance due to the absence of pol η would have been further strengthened by survival experiments utilising pol η-null and pol η-supplemented cells after synchronization in G1 phase. Attempts to use XPV or BL-2 cells in G1 phase were undertaken both by serum starvation, by growing cells to confluence and by FACS-based sorting of cells in G1 phase. However, all efforts to synchronise XPV cells in G1 phase were unsuccessful, most likely due to SV40-based immortalization of the cell line, for which problems with synchronization have been reported before (Mitchell and Tupper 1977; Liliensiek, Schell et al. 2006). Similarly, BL-2 cells terminally arrested in G1 phase of the cell cycle upon serum starvation or reaching confluence, and further viability assays could not be performed as those cells did not resume growth after release from the arresting condition.

6.3 RADIOPROTECTION THROUGH INCREASED HOMOLOGOUS RECOMBINATION ACTIVITY

IR exerts its main toxic effect through formation of DNA DSBs, which are primarily repaired by two major pathways as outlined in the introductory chapter of this thesis: Non-homologous end joining and homologous recombination (Helleday, Lo et al. 2007). Deficiencies in both NHEJ and HR have been shown to render cells

hypersensitive to IR treatment (Lobrich and Jeggo 2005; Jeggo and Lavin 2009). In contrast to the continuously active NHEJ pathway, HR only takes place in the presence of a sister chromatid, i.e. during S and G2 phases of the cell cycle, as it requires a DNA strand template for homology-directed DNA repair. Several papers have highlighted the importance of the HR pathway for the cell cycle-dependent changes in cellular radiosensitivity, and it has been suggested that increased radioresistance in S phase is due to an increased HR activity (Cheong, Wang et al. 1994; Tamulevicius, Wang et al. 2007; Frankenberg-Schwager, Gebauer et al. 2009). In contrast, a reduction of HR activity has been implied after IR treatment as cells progress from late S to G2 phase, explaining an increase in radiosensitivity in G2 phase compared to the S phase of the cell cycle (Wilson, Hinz et al. 2010).

In vitro assays have implied direct involvement of pol η in the HR pathway (Kawamoto, Araki et al. 2005), and two publications have described pol η as the only polymerase able to perform D loop extension step on oligonucleotide constructs (McIlwraith, Vaisman et al. 2005; McIlwraith and West 2008), leading to the notion that the enzyme may play a crucial role for cellular DSB repair. These data would suggest increased sensitivity of pol η-deficient cells to IR and an abrogation of the S phase-specific radioresistance formation in the absence of the polymerase. However, a study investigating cellular radiation responses in a panel of XP and XPV cell lines did not report an overall increased sensitivity of pol η-deficient cell lines to IR treatment (Arlett, Green et al. 2008). Indeed, the data presented in this thesis show an increase in radioresistance both in pol η-knockout cell lines as well as cells with a transient pol η knockdown.

More recent *in vitro* data show that other DNA polymerases are also capable of D loop extension, and although pol η can perform this step during HR, there is likely to be considerable redundancy regarding this biological function (Sebesta, Burkovics et al. 2011). Compared to these *in vitro* findings, cell culture data show that cells depleted of pol η were able to perform functional HR repair after exposure to IR as measured by a DR-GFP reporter assay; two other TLS polymerases, pol ζ and Rev1 were shown to facilitate HR (Sharma, Hicks et al. 2011); in another publication by Thakur et al., Mre11-dependent recombination was found as a backup pathway in cells severely depleted of pol η (Thakur, Wernick et al. 2001). In the study presented in this thesis, we found an increased level of RAD51 foci in pol η-deficient cells after IR treatment, which we measured as a marker of early homologous recombination (van Veelen, Cervelli et al. 2005). Additionally, inhibition of HR activity through XRCC3 knockdown rendered pol η-deficient cells more sensitive to IR than their pol η-expressing counterparts, suggesting that the observed increase in radioresistance was probably due to differences in homology-directed DNA repair.

Taken together, these findings suggest that deficiency of cells in the TLS polymerase, pol η results in an accumulation of cells in the radioresistant S phase, leading to an increase in homology-mediated repair of radiation damage while pol η-proficient cells quickly pass through S phase due to their ability to perform TLS. Another explanation for the findings presented here would be the creation of DNA replication lesions by pol η in the wild-type but not the pol η-deficient cells while attempting or completing TLS after IR-induced DNA damage. This would be reflected

in the increased levels of γH2AX but not 53BP1 foci observed in those cells. This "secondary" lesion may not be a substrate for HR, while in the absence of pol η, the lesion is not subject to translesional bypass, so that HR is able to accurately repair the original IR-induced DNA damage (**FIGURE 6.1**).

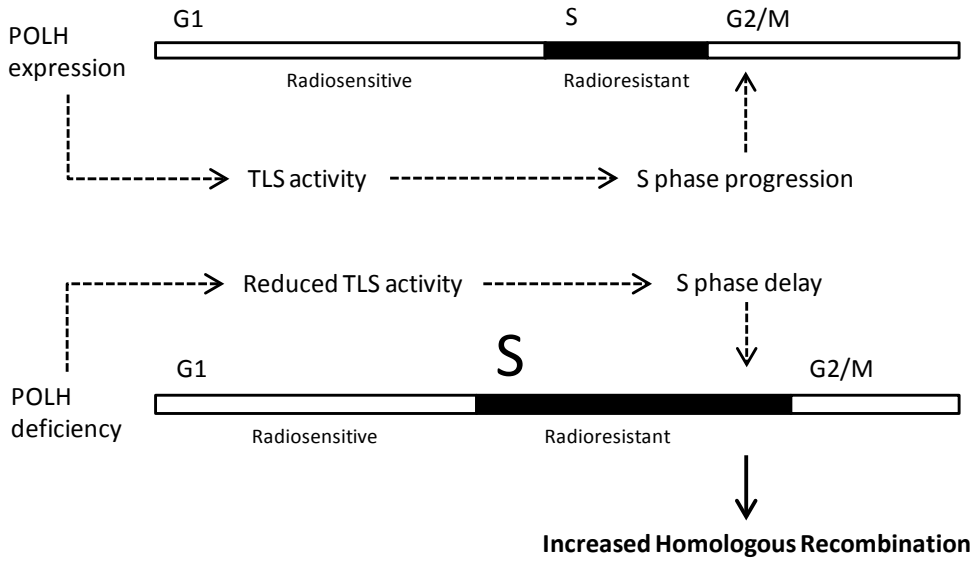


Figure 6.1. Model for the increase in radioresistance in pol η-deficient cells.

Cells proficient in pol η-mediated translesion synthesis progress through S phase and have a higher percentage of cells in radiosensitive phases of the cell cycle. In contrast, cells lacking the ability of translesional bypass by pol η are delayed in S phase since they need to employ other DNA repair mechanisms to deal with DNA damage. Redistribution of pol η-deficient cells towards radioresistant S phase therefore increases the radioresistance, likely through a homologous recombination-dependent mechanism.

6.4 TRANSLESION SYNTHESIS PAST OXALIPLATIN-INDUCED DNA DAMAGE

First-generation platinum compounds, cisplatin and carboplatin, and second-generation platinum drug, oxaliplatin are in wide clinical use for a variety of cancers. As discussed in detail in the introduction, platinum-based cytotoxic drugs exert their effects by forming DNA adducts, mostly between adjacent purine nucleotides. If not repaired quickly by the NER pathway, those lesions have the potential to efficiently block DNA replication and eventually threaten cellular viability. Quantification of the types of different DNA lesions induced by cisplatin and oxaliplatin reveal comparable levels of monofunctional, intrastrand and interstrand adducts, and published crystal structures for cisplatin and oxaliplatin DNA adducts are very similar (Scheeff, Briggs et al. 1999; Spingler, Whittington et al. 2001). However, both cell culture experiments and clinical evidence suggests a fundamental difference, as cells resistant against the first-generation compound cisplatin are sensitive to oxaliplatin treatment (Fink, Nebel et al. 1996; Fink, Zheng et al. 1997). Several authors have attributed this effect to differences in cellular recognition of the adducts. Although cisplatin and oxaliplatin have very similar crystal structures, their solution structures have been published to be considerably different with oxaliplatin distorting the DNA to a much smaller degree and narrowing the minor groove much more than cisplatin (Wu, Pradhan et al. 2004).

Apart from differences in the recognition of cisplatin and oxaliplatin lesions by different components of the MMR pathway that have been widely thought responsible for the differential cellular sensitivities to both drugs (Vaisman, Varchenko et al. 1998; Zdraveski, Mello et al. 2002), there is emerging evidence for differential recognition by

the TLS polymerase, pol η as well. Publications investigating the fidelity of pol η to synthesise past platinum lesions show inconsistent results. Bassett et al. report an about 1000 to 2000-fold higher probability of correct insertion and extension of nucleotides opposite platinum damage by pol η (Bassett, Vaisman et al. 2003). Comparing pol η activity past cisplatin and oxaliplatin adducts *in vitro*, it was shown that the polymerase had a higher fidelity past oxaliplatin lesions than cisplatin lesions, suggesting an explanation for the lower mutagenic potential of oxaliplatin compared to cisplatin (Vaisman, Masutani et al. 2000; Chaney, Campbell et al. 2005). However, in a separate publication, pol η bypass products for both cisplatin and oxaliplatin-damaged oligonucleotides were reported to contain high levels of frameshifts and misinsertions and often multiple replication errors at once (Bassett, Vaisman et al. 2002).

In contrast to the published *in vitro* data, few papers have examined the relevance of pol η levels to cellular responses to platinum compounds. Albertella et al. demonstrated an increased sensitivity of pol η-deficient cells to cisplatin treatment that is very similar to the cisplatin survival data presented in this thesis (Albertella, Green et al. 2005). The survival curves presented here compared cellular responses to cisplatin and oxaliplatin and showed that a higher dosage of oxaliplatin was required to inhibit the same percentage of a fibroblast cell line compared to cisplatin, an observation backed up with published data from oligonucleotide assays showing that pol η can synthesize past oxaliplatin adducts with much higher efficiency compared to cisplatin adducts (Vaisman, Masutani et al. 2000). The relevance of pol η levels in determining cellular responses to oxaliplatin treatment was further underpinned in the dataset presented here by the fact that cells were found capable of progressing through S phase

in the presence of oxaliplatin-induced DNA damage. Both HCT116 and MRC5 cell lines did not have a significant G1 or S phase arrest after oxaliplatin treatment and could progress through replication before stalling in G2 phase, suggesting that their TLS activity allowed temporary oxaliplatin damage tolerance and an opportunity for postreplicative repair before mitosis. Although no published data are available for oxaliplatin in respect to cellular pol η status, experiments performed with cisplatin or carboplatin in pol η-deficient XPV fibroblasts showed a prolonged S phase arrest in the absence of the polymerase (Cruet-Hennequart, Villalan et al. 2009).

6.5 *POLH* EXPRESSION IN NORMAL AND CANCER TISSUES

Genetic analyses have found homologues of the human TLS polymerase, pol η throughout all eukaryotic species (McDonald, Rapic-Otrin et al. 1999). Although comprehensive studies examining pol η expression in normal human tissues are lacking and most available data stem from analyses done in human cell lines, the expression of pol η has been reported for most human tissues with the exception of peripheral lymphocytes, foetal liver and adult muscle (Thakur, Wernick et al. 2001).

Few papers have analysed expression patterns of pol η in human tumours. To date, non-small cell lung cancer has been analysed most thoroughly regarding pol η expression, but the published studies report vastly inconsistent data. In samples harvested from 81 non-small cell lung cancer (NSCLC) patients, Välk et al. reported a significant upregulation of *POLH* mRNA compared to matched normal tissue (Valk, Vooder et al. 2010). In contrast, Ceppi et al. found no significant difference in *POLH*

mRNA between tissue specimens obtained from a group of 72 NSCLC patients compared to matched normal lung samples (Ceppi, Novello et al. 2009). In a third published analysis, *POLH* mRNA levels were found to be downregulated in lung cancer biopsies compared to matched normal tissue, and downregulation was also reported for stomach cancer. The same analysis did not display any significant up- or downregulation of *POLH* mRNA levels in colorectal cancers (Pan, Fang et al. 2005).

Using an immunohistochemical approach to stain for pol η protein, Teng et al. analysed gastric cancer tissue samples from 80 patients and reported very low levels of detectable protein; they quantified all specimens with more than 5 % positive nuclear staining as pol η-positive, which they reported for only 23 of the 80 samples analysed (Teng, Qiu et al. 2010), suggesting that their IHC approach may not be sensitive enough to perform pol η expression analyses in clinical practice. In a different analysis examining TLS polymerase expression in normal and malignant brain tissue, the authors illustrated no difference in *POLH* mRNA between normal neuronal tissue and glioma specimens (Wang, Wu et al. 2010). In this analysis, qRT-PCR data and IHC data were shown in parallel, and, despite the fact that *POLH* mRNA levels were considerably higher than any other TLS polymerase analysed in this study, the study did not find any significant protein levels either by Western blotting or by IHC on tissue slices. It is not currently clear from these 2 studies that pol η immunostaining is sufficiently specific or sensitive using commercial antibodies currently available.

In the dataset presented here, significantly higher *POLH* mRNA levels were found in specimens from oesophageal cancer patients compared to normal oesophageal

tissue from the same patients. To date, no other published analysis has reported *POLH* expression in oesophageal tissue. *POLH* expression was found to differ depending on the histological tumour type, with adenocarcinoma tumours having significantly higher *POLH* levels than tumours of other histologies. Similarly, in a group of NSCLC patients, Ceppi et al. reported higher levels of *POLH* mRNA in lung adenocarcinoma than in NSCLC tumours of other histologies, although in their analysis, the difference did not reach statistical significance (Ceppi, Novello et al. 2009). Regarding the histological analysis performed for this thesis, there may be two factors that have the potential to skew the statistical analysis, namely the relatively small sample size and the uneven distribution between adenocarcinoma tumours and cancers of other histologies. In a separate study, the TLS family polymerase, pol κ, in glioma samples were not found to be upregulated as tumours increased in their grade (Wang, Wu et al. 2010). This finding is consistent with our analysis, in which none of the examined clinical parameters demonstrated significant correlation with *POLH* expression.

6.6 REGULATION OF *POLH* EXPRESSION

As pol η has low fidelity when replicating past undamaged DNA, its expression requires tight regulation (Matsuda, Bebenek et al. 2001). In Saccharomyces cerevisiae, the pol η homologue, Rad30, could be overexpressed by a galactose-inducible vector system (Pavlov, Nguyen et al. 2001). In contrast, human cells with an episomal multicopy pol η vector proved non-viable, and forced pol η overexpression was reported to require both the integration of linear *POLH* copies into the genome and a strong promoter, suggesting that pol η overexpression was toxic (Thakur, Wernick et al. 2001).

Overexpression of Rad30 in yeast cells resulted in a mutagenic phenotype and reduced replication fidelity about ten-fold (Pavlov, Nguyen et al. 2001). In a different analysis, overexpression of pol η in human XPV fibroblasts led to similar mutation rates as in fibroblasts with wild-type levels of the polymerase while at the same time increasing cellular tolerance to UV irradiation. The authors concluded that cells employ mechanisms that prevent unwarranted DNA replication by pol η while allowing access to the damaged strands if needed (King, Nikolaishvili-Feinberg et al. 2005). Therefore, pol η seems to be regulated on different levels, both transcriptionally as well as functionally.

In comparison to artificial systems enabling pol η upregulation, little is known about factors that may alter the cellular expression of chromosomally encoded pol η protein, and the available data are highly inconsistent. There are published data reporting changes in cellular pol η expression in the context of immunological effects as this TLS polymerase has been linked to antibody class switch recombination and immunoglobulin gene somatic hypermutation (Faili, Aoufouchi et al. 2004; Delbos, De Smet et al. 2005). An analysis studying expression patterns in a mouse model of systemic lupus erythematosus reported increased somatic hypermutation activity in those animals, associated with an increased expression of TLS polymerases, pols η, ζ and θ (Zan, Zhang et al. 2009). In adult and SHM-deficient infant B lymphocytes, upregulation of pol η was reported to be induced by stimulation through the B cell receptor (Bowen, Tian et al. 2006). Similarly, in the BL-2 Burkitt's lymphoma cell line, pol η protein expression could be upregulated by co-culturing BL-2 cells with T cells or surface IgM crosslinking (Poltoratsky, Woo et al. 2001).

In comparison to immunoglobulin hypermutation, less is known regarding the impact of DNA damaging agents on the induction of pol η protein. Liu and Chen showed an upregulation of pol η protein by DNA strand breaks caused by exposure to ionising radiation or treatment with the topoisomerase I inhibitor, camptothecin (Liu and Chen 2006). This publication indicated a link between p53 and pol η with the polymerase being a transcriptional target of p53. However comparing p53-/- and p53 WT mice, Ratnam et al. reported virtually identical levels of *POLH* mRNA in both sets of animals, which would argue against an induction of pol η by p53, but potentially indicate a cell-cycle-dependent parallel induction of both p53 and pol η during early S phase (Cleaver, Bartholomew et al. 2002; Ratnam, Bozek et al. 2010). One paper has shown upregulation of the polymerase protein upon UV irradiation in a yeast cell model (Skoneczna, McIntyre et al. 2007). Using human cells, UV-induced lesions were shown to have no significant impact on pol η expression, neither at the mRNA nor at the protein level (Kannouche, Broughton et al. 2001; Chen, Cleaver et al. 2008).

In the data presented for this thesis, it was found that cellular exposure to the platinum compound, oxaliplatin did not lead to any significant alteration in pol η protein levels. Even long-term exposure to the drug that had the potential to engender oxaliplatin resistance did not result in any alteration of pol η expression. In contrast, a publication examining *POLH* mRNA upregulation in lung cancer cell lines found an upregulation of the mRNA transcript in three of five analysed cell lines with one cell line having a more than two-fold increase; an expression analysis on the protein level was not performed (Ceppi, Novello et al. 2009). As only one out of five cell lines in this

study showed a clear two-fold induction of the polymerase upon treatment with cisplatin, the effect may be cell line-specific and therefore may not be relevant to all cancers. Additionally, post-transcriptional regulation need to be taken into consideration, and mRNA induction may not necessarily result in upregulation of stable pol η protein.

In clinical samples, the analysis of *POLH* mRNA expression before and after two cycles of oxaliplatin-based chemotherapy revealed a significant increase in *POLH* levels after treatment in oesophageal normal tissue but not cancer tissue samples. Considering the contradictory results regarding pol η expression in response to treatment with platinum compounds, this finding could potentially be explained by a selection of normal oesophageal mucosal cells with high constitutive expression of pol η. As the oesophageal lining has a high cellular turnover and regenerates during chemotherapy, platinum-based cancer treatment may preferentially kill oesophageal mucosal cells with low pol η expression; cells expressing high constitutive pol η levels may be more resistant to the treatment and therefore survive and repopulate the oesophagus leading to higher overall pol η levels after platinum chemotherapy (Urita, Komuro et al. 2007; Honda, Nakamura et al. 2010).

6.7 INFLUENCE OF *POLH* ON SURVIVAL AND RESPONSE TO PLATINUM-BASED CHEMOTHERAPY

From published preclinical data, a link between the TLS polymerase, pol η and cellular sensitivity to cytotoxic platinum compounds has been suggested. Virtually all

these findings were either obtained in cell-free extracts or comparing pol η-deficient and pol η WT cell lines; no data are available on the effects of different constitutive pol η expression levels on cellular cisplatin sensitivity. To date, two publications have examined the potential association between pol η expression and patient survival or response to therapy. Ceppi et al. studied *POLH* mRNA levels in patients with NSCLC and showed a correlation between polymerase expression and patient survival after treatment with a cisplatin or carboplatin-containing chemotherapy regimen (Ceppi, Novello et al. 2009). High *POLH* mRNA content was reported to be associated with lower overall survival in both univariate and multivariate analysis, and there were significantly less long-term survivors in the high-*POLH* group. However, when dividing the patients by the median *POLH* level, no difference was found between low and high *POLH*-expressing cancers on overall survival. Another problem in the study performed by Ceppi et al. was the fact that more than half of the analysed patients received cisplatin in combination with gemcitabine; it has been shown that pol η levels affect cellular responses to gemcitabine, and gemcitabine vastly augments the sensitivity of pol η-deficient cells to cisplatin (Chen, Cleaver et al. 2006). Therefore the described survival effect in this study cannot clearly be attributed to treatment with cisplatin or carboplatin.

Another research group published an analysis of pol η protein levels in samples derived from gastric adenocarcinoma treated with oxaliplatin-based chemotherapy (Teng, Qiu et al. 2010). Teng et al. reported a significant correlation between tumours with pol η-negative staining and response to oxaliplatin-based chemotherapy as well as increased overall survival. However, very low levels of pol η were detected in their

samples, so the researchers adjusted the cut-off for pol η positivity according to the negative and positive predictive value to predict response to oxaliplatin treatment. Moreover, there was no antibody validation in an independent cohort. As mentioned in the previous section, the antibody used by Teng et al. hardly detected any pol η protein in most of their samples, and as reported similarly for pol η staining in other tissues, a positive staining of > 5% of all nuclei was counted as positive. Because of the very low number of positive cells required for pol η positivity, there is the possibility of increased errors of manual counting and observer bias. Additionally, in combination with previously reported inconsistencies between mRNA and protein quantification of pol η (Wang, Wu et al. 2010), there may be technical problems with antigen retrieval or antibody sensitivity and specificity.

In contrast to the correlations published by Ceppi et al. and Teng et al., we found that high *POLH* mRNA levels in oesophageal cancer samples before treatment with an oxaliplatin-based chemotherapy correlated with an increase in overall and progression-free survival as well as therapeutic response to treatment. There are a number of possible explanations for this discrepancy observed between preclinical models and the effects seen in oesophageal tumours. As pol η appears to be cell cycle-specific and has been implicated in the S phase checkpoint (Thakur, Wernick et al. 2001), low levels of the polymerase may impede cellular progression through S phase and therefore slow down replication phase and subsequently cell doubling times. This may indirectly lead to an increased ability of tumour cells to repair oxaliplatin-induced DNA damage by the NER pathway before cells can resume replication. These tumours may therefore exhibit increased oxaliplatin resistance through increased damage repair.

So far, the role of pol η in the cellular response to platinum compounds has only been established *in vitro* and in cell culture experiments comparing pol η-deficient and pol η-rescued or wild-type cells. In contrast to the polymerase-deficient cell lines employed *in vitro*, tumour samples used in this analysis had detectable *POLH* mRNA levels and were therefore not deficient in pol η-mediated translesional activity. Hence, as their lower pol η expression may still be sufficient to allow for a fully functional TLS pathway, the differential effect seen in those tumours after treatment with oxaliplatin-based chemotherapy may have been due to an indirect effect of pol η on cell survival or proliferation. Additionally, the complex anatomy of tumour tissues with many different cell types, oxygenation levels and connections to blood supply can only to a small degree be predicted by cell culture-based assays, and tumours may behave completely differently from cultured tumour cell lines. To investigate the role of differential pol η expression levels on cellular proliferation and S phase progression, immunohistochemical staining for established proliferation markers such as Ki-67 or S phase-specific proteins like cyclin A in tumour sections may be helpful (Yu, Woods et al. 1992; Erlandsson, Linnman et al. 2000).

Another possible explanation for the observed effect of pol η on patient survival may be due to the mutagenic effect of pol η and especially high pol η expression (Pavlov, Nguyen et al. 2001). As a high percentage of oesophageal cancers was found to over-express pol η, those tumours may acquire mutations during treatment that are not repaired but passed onto subsequent tumour cells and may eventually increase tumour cell death, resulting in improved patient outcomes.

Finally, as POLH mRNA was not assessed for mutations or polymorphisms, the possibility of oesophageal tumours expressing non-functional pol η protein cannot be ruled out as published for another DNA polymerase, pol ε, in colorectal cancers (Cancer Genome Atlas Network 2012).

6.8 VALIDATION OF *POLH* AS A POSSIBLE BIOMARKER TO PREDICT THERAPEUTIC RESPONSE AND SURVIVAL

The findings presented for this thesis comprise the second published dataset analysing *POLH* mRNA levels in the context of patient survival and response to cancer therapy and the third study evaluating pol η as a potential biomarker. Unfortunately, as discussed above, all three analyses have their limitations, especially due to the comparatively small sample size. Therefore, as seen for the other analyses published by Ceppi et al. and Teng et al. (Ceppi, Novello et al. 2009; Teng, Qiu et al. 2010), the relatively small group of patients analysed for this thesis may limit the informative value of the statistical analysis.

In our case, a small number of patients included in the analyses resulted in an uneven distribution in respect to different parameters, especially histological tumour type, as more than two thirds of the oesophageal cancer patients from whom samples were taken, had adenocarcinoma histology. Similarly, in the NSCLC study published by Ceppi et al., about two thirds of samples were lung adenocarcinoma specimens compared to tumours of all other histologies (Ceppi, Novello et al. 2009). As with all

small sample sizes, there is a risk for spurious findings attributable to random variation, so analyses using much bigger collections of tissue specimens would be warranted to make conclusions about the effects of pol η on patient survival and response more statistically robust (Pepe, Etzioni et al. 2001). However, access to a tissue bank with sufficient choice of patient-derived material is a limiting factor for the size of the study. In our case, a significant correlation between tumour stage and patient survival provided an indirect internal validation of this statistical analysis; as those data matched previous findings that had been validated in bigger independent analyses before, it can be assumed that the patient cohort used for the analysis presented here provides a reasonable representation of the oesophageal cancer population in northern European countries (Holscher, Bollschweiler et al. 1995; Rouvelas, Zeng et al. 2005).

Early biomarker development is a stepwise process that starts with preclinical investigations, either hypothesis-driven or screening-based (Pepe, Etzioni et al. 2001; Srivastava 2007; Marchio, Dowsett et al. 2011). In the case of pol η, preclinical results have revealed an involvement of this protein in determining sensitivity to cisplatin and oxaliplatin (Vaisman, Masutani et al. 2000; Albertella, Green et al. 2005), and we have shown that pol η levels vary considerably between cell lines from different tissues and cancers. Oesophageal cancer was chosen as a cancer site that is routinely treated with platinum-based chemotherapy in the neoadjuvant setting and that can be easily accessed during upper GI endoscopies. Variability of pol η at the mRNA level was confirmed by qRT-PCR, both in oesophageal cancer and in normal oesophageal tissue.

As a next step in the development of a predictive biomarker, a correlation between the measured parameter and the clinical features needs to be established. In our dataset, *POLH* mRNA expression significantly correlated with overall and disease-free survival as well as response to therapy. Future steps in evaluating *POLH* levels as a potential biomarker to predict survival and response to platinum-based therapy would comprise analyses of independent collections of oesophageal cancer tissue and other tumours that are routinely treated with cisplatin or oxaliplatin in order to achieve a high degree of reproducibility in independent experiments (Freeman, Bixler et al. 2010). Only if measurements can be reliably reproduced, prospective studies can be rationally set up in order to test whether the potential biomarker will turn out as a feasible tool for clinical routine practice. As in this dataset, *POLH* levels corresponded to both prognosis and therapeutic response, this approach will also be needed to determine if *POLH* expression can be used as a predictive biomarker to foretell therapeutic response to platinum-based cancer therapy or if it a prognostic biomarker only reflecting patient survival. Since the published data analysing pol η as a possible biomarker are inconsistent and rely on relatively small datasets, larger retrospective analyses using samples from different tumour sites are needed before pol η can be tested as a marker for patient survival and response to platinum-based drugs in the clinical setting.

6.9 *POLH* AS A POTENTIAL TARGET TO OPTIMISE PLATINUM-BASED CHEMOTHERAPY

As the deficiency of pol η has been reported to increase cellular sensitivity to the chemotherapeutic agents, cisplatin and oxaliplatin, the polymerase may be an interesting

protein target in order to increase the cytotoxic activity of platinum-based drugs. To date, platinum sensitivity of pol η-deficient cells has been only established by comparing pol η-null XPV cells with pol η wild-type fibroblasts (Albertella, Green et al. 2005; Chen, Cleaver et al. 2006). Here we report an increased sensitivity to oxaliplatin not only for XPV fibroblasts but also for *POLH*-knockout BL-2 cells and wild-type cells after knocking down the polymerase using siRNA. These findings suggest that pol η can be targeted to increase the cytotoxic activity of oxaliplatin, although more in-depth analyses are warranted to establish the polymerase as a useful target, especially with regard to the seemingly conflicting finding presented here showing that high POLH levels were associated with higher survival rates.

It has been reported previously that siRNA-based pol η knockdown recapitulates most of the phenotypic features seen in pol η-deficient XPV cells (Laposa, Feeney et al. 2003), and likely inhibition of the polymerase activity by small-molecule inhibitors will produce a similar effect. Several publications have characterised pol η inhibitors *in vitro*, but most of the discovered substances block other DNA polymerases as well. Gallotannin penta-O-galloyl-beta-D-glucose was reported to exert its inhibitory effect in the nanomolar range but also efficiently blocked the B family replicative enzymes (Mizushina, Zhang et al. 2010). Mizushina et al. found that 3-O-methylfunicone was a selective inhibitor of the Y family polymerases with only minimal activity against any other tested polymerase (Mizushina, Motoshima et al. 2009). Similarly, penicilliols A and B were characterised to specifically block Y family polymerases using micromolar concentrations with no measurable effect on other polymerase families (Kimura, Takeuchi et al. 2009). So far, the published inhibitors were only tested in cell-free

enzyme assays, and only 3-O-methylfunicone was shown to sensitise HeLa cells to UV irradiation in cell culture (Mizushina, Motoshima et al. 2009). Pol η-targeting drugs have not been tested in combination with platinum compounds.

Data presented in this thesis show that targeting pol η by an siRNA-based knockdown approach can increase sensitivity to oxaliplatin not only in wild-type cancer cell lines, but also in cell lines with acquired oxaliplatin resistance. This is potentially an important finding as cancers often develop resistance to chemotherapeutic agents during the course of treatment that requires termination of this treatment and changes of the treatment plan to a second-line anticancer regime which usually has lower clinical response rates. Although resistance to platinum compounds is a multifactorial process involving a variety of mechanisms (Koberle, Tomicic et al. 2010), inhibition of pol η activity warrants closer examination as a possible way of re-sensitising resistant tumour cells to oxaliplatin.

6.10 FUTURE DIRECTIONS

The findings presented here, showing that pol η may have the potential to mediate cellular effects to cisplatin and oxaliplatin, warrant further analyses. As discussed above, inhibiting pol η activity may eventually prove a feasible method to increase the efficiency of platinum-based cancer treatment. Although different inhibitors have been reported in *in vitro* assays, it is not known if they can be used on living cells. Cell-based survival assays combining platinum compounds with a pol η inhibitor will give an idea if a combination treatment has the potential to increase the sensitivity of

tumour cells to cisplatin or oxaliplatin. The few compounds published to inhibit pol η *in vitro* are fairly unselective and inhibit various other polymerases as well, so further studies into combining platinum drugs with TLS inhibition would require highly specific inhibitors with minimised off-target effects on other cellular proteins. Considering the reported over-expression of pol η in a variety of cancer tissues, a potential pol η inhibitor may help sensitising or even re-sensitising those tumours to platinum-based treatment.

As in this thesis, high *POLH* mRNA levels were shown to correlate with improved therapeutic response and longer patient survival. As discussed above, pol η may mediate the cell cycle in tumour cells and have an impact on cellular turnover and cancer proliferation, immunohistochemical analyses may shed light on cell cycle distribution and proliferation rate depending on *POLH* levels. Ki-67 is a well-established marker that can be reliably detected by IHC and has been shown to strongly correlate with the growth fraction of a given tissue (Scholzen and Gerdes 2000). The proliferation index for all oesophageal cancer specimens will be measured by Ki-67 staining using tissue sections prepared from the same material from which mRNA extraction was performed. Similarly, cell cycle distribution in oesophageal tissue specimens before and after treatment with oxaliplatin-based chemotherapy will be established using IHC-based markers for different cell cycle phases. Especially differences in S phase population as measured by cyclin A staining have been measured in oesophageal tissue specimens before and may reveal an indirect effect of pol η on cell cycle distribution in the tumour samples used in this thesis (Huang, Xiao et al. 2010; Huang, Chen et al. 2012). If *POLH* expression holds potential as a biomarker to predict

therapeutic response in oesophageal cancer patients by the criteria discussed above, a clinical trial will be warranted to select patients for chemotherapeutic treatment according to their *POLH* status. Although platinum-based therapies are most commonly used to treat oesophageal cancer, there is still a large percentage of patients who do not respond satisfactorily to this treatment and may benefit more from alternative compounds such as irinotecan or cetuximab (Ajani, Correa et al. 2010; Moehler, Mueller et al. 2011; Schonnemann, Yilmaz et al. 2012). Depending on those further data, *POLH* may potentially prove a viable marker to select different treatment regimens for oesophageal cancer patients and may eventually help to offer personalised therapy for oesophageal cancer.

REFERENCES

Abdulovic, A. L. and S. Jinks-Robertson (2006). "The in vivo characterization of translesion synthesis across UV-induced lesions in Saccharomyces cerevisiae: insights into Pol zeta- and Pol eta-dependent frameshift mutagenesis." Genetics **172**(3): 1487-98.

Acharya, N., L. Haracska, et al. (2007). "Complex formation of yeast Rev1 with DNA polymerase eta." Mol Cell Biol **27**(23): 8401-8.

Acharya, N., R. E. Johnson, et al. (2006). "Complex formation with Rev1 enhances the proficiency of Saccharomyces cerevisiae DNA polymerase zeta for mismatch extension and for extension opposite from DNA lesions." Mol Cell Biol **26**(24): 9555-63.

Acharya, N., J. H. Yoon, et al. (2008). "Roles of PCNA-binding and ubiquitin-binding domains in human DNA polymerase eta in translesion DNA synthesis." Proc Natl Acad Sci U S A **105**(46): 17724-9.

Acharya, N., J. H. Yoon, et al. (2010). "DNA polymerase eta lacking the ubiquitin-binding domain promotes replicative lesion bypass in humans cells." Proc Natl Acad Sci U S A **107**(23): 10401-5.

Ahmad, S. (2010). "Platinum-DNA interactions and subsequent cellular processes controlling sensitivity to anticancer platinum complexes." Chem Biodivers **7**(3): 543-66.

Ajani, J. A., A. M. Correa, et al. (2010). "Trimodality therapy without a platinum compound for localized carcinoma of the esophagus and gastroesophageal junction." Cancer **116**(7): 1656-63.

Al-Assar, O., T. Mantoni, et al. (2011). "Breast cancer stem-like cells show dominant homologous recombination due to a larger S- G2 fraction." Cancer Biol Ther **11**(12).

Al-Batran, S. E., J. T. Hartmann, et al. (2008). "Phase III trial in metastatic gastroesophageal adenocarcinoma with fluorouracil, leucovorin plus either oxaliplatin or cisplatin: a study of the Arbeitsgemeinschaft Internistische Onkologie." J Clin Oncol **26**(9): 1435-42.

Al-Minawi, A. Z., Y. F. Lee, et al. (2009). "The ERCC1/XPF endonuclease is required for completion of homologous recombination at DNA replication forks stalled by inter-strand cross-links." Nucleic Acids Res **37**(19): 6400-13.

Albertella, M. R., C. M. Green, et al. (2005). "A role for polymerase eta in the cellular tolerance to cisplatin-induced damage." Cancer Res **65**(21): 9799-806.

Albertella, M. R., A. Lau, et al. (2005). "The overexpression of specialized DNA polymerases in cancer." DNA Repair (Amst) **4**(5): 583-93.

Alt, A., K. Lammens, et al. (2007). "Bypass of DNA lesions generated during anticancer treatment with cisplatin by DNA polymerase eta." Science **318**(5852): 967-70.

Ametller, E., S. Garcia-Recio, et al. (2011). "Differential regulation of MMP7 in colon cancer cells resistant and sensitive to oxaliplatin-induced cell death." Cancer Biol Ther **11**(1): 4-13.

Andreassen, P. R., G. P. Ho, et al. (2006). "DNA damage responses and their many interactions with the replication fork." Carcinogenesis **27**(5): 883-92.

Arlett, C. F., M. H. Green, et al. (2008). "Minimal ionizing radiation sensitivity in a large cohort of xeroderma pigmentosum fibroblasts." Br J Radiol **81**(961): 51-8.

Avkin, S. and Z. Livneh (2002). "Efficiency, specificity and DNA polymerase-dependence of translesion replication across the oxidative DNA lesion 8-oxoguanine in human cells." Mutat Res **510**(1-2): 81-90.

Bachrati, C. Z. and I. D. Hickson (2006). "Analysis of the DNA unwinding activity of RecQ family helicases." Methods Enzymol **409**: 86-100.

Bachrati, C. Z. and I. D. Hickson (2009). "Dissolution of double Holliday junctions by the concerted action of BLM and topoisomerase IIIalpha." Methods Mol Biol **582**: 91-102.

Bai, F., Y. Nakanishi, et al. (1996). "Immunohistochemical expression of glutathione S-transferase-Pi can predict chemotherapy response in patients with nonsmall cell lung carcinoma." Cancer **78**(3): 416-21.

Baker, T. A. and S. P. Bell (1998). "Polymerases and the replisome: machines within machines." Cell **92**(3): 295-305.

Barabas, K., R. Milner, et al. (2008). "Cisplatin: a review of toxicities and therapeutic applications." Vet Comp Oncol **6**(1): 1-18.

Barnham, K. J., M. I. Djuran, et al. (1996). "Ring-Opened Adducts of the Anticancer Drug Carboplatin with Sulfur Amino Acids." Inorg Chem **35**(4): 1065-1072.

Bassett, E., A. Vaisman, et al. (2003). "Efficiency of extension of mismatched primer termini across from cisplatin and oxaliplatin adducts by human DNA polymerases beta and eta in vitro." Biochemistry **42**(48): 14197-206.

Bassett, E., A. Vaisman, et al. (2002). "Frameshifts and deletions during in vitro translesion synthesis past Pt-DNA adducts by DNA polymerases beta and eta." DNA Repair (Amst) **1**(12): 1003-16.

Baumann, M., M. Krause, et al. (2008). "Exploring the role of cancer stem cells in radioresistance." Nat Rev Cancer **8**(7): 545-54.

Bavoux, C., A. M. Leopoldino, et al. (2005). "Up-regulation of the error-prone DNA polymerase {kappa} promotes pleiotropic genetic alterations and tumorigenesis." Cancer Res **65**(1): 325-30.

Beard, W. A., V. K. Batra, et al. (2010). "DNA polymerase structure-based insight on the mutagenic properties of 8-oxoguanine." Mutat Res **703**(1): 18-23.

Bergoglio, V., Y. Canitrot, et al. (2001). "Enhanced expression and activity of DNA polymerase beta in human ovarian tumor cells: impact on sensitivity towards antitumor agents." Oncogene **20**(43): 6181-7.

Biade, S., C. C. Stobbe, et al. (1997). "The intrinsic radiosensitivity of some human tumor cells throughout their cell cycles." Radiat Res **147**(4): 416-21.

Bianco, P. R., R. B. Tracy, et al. (1998). "DNA strand exchange proteins: a biochemical and physical comparison." Front Biosci **3**: D570-603.

Bienko, M., C. M. Green, et al. (2010). "Regulation of translesion synthesis DNA polymerase eta by monoubiquitination." Mol Cell **37**(3): 396-407.

Biertumpfel, C., Y. Zhao, et al. (2010). "Structure and mechanism of human DNA polymerase eta." Nature **465**(7301): 1044-8.

Blair, B. G., C. A. Larson, et al. (2009). "Copper transporter 2 regulates the cellular accumulation and cytotoxicity of Cisplatin and Carboplatin." Clin Cancer Res **15**(13): 4312-21.

Boddy, M. N., P. H. Gaillard, et al. (2001). "Mus81-Eme1 are essential components of a Holliday junction resolvase." Cell **107**(4): 537-48.

Bomar, M. G., M. T. Pai, et al. (2007). "Structure of the ubiquitin-binding zinc finger domain of human DNA Y-polymerase eta." EMBO Rep **8**(3): 247-51.

Bose, D., L. J. Zimmerman, et al. (2011). "Chemoresistant colorectal cancer cells and cancer stem cells mediate growth and survival of bystander cells." Br J Cancer **105**(11): 1759-67.

Boulikas, T. and M. Vougiouka (2004). "Recent clinical trials using cisplatin, carboplatin and their combination chemotherapy drugs (review)." Oncol Rep **11**(3): 559-95.

Bowen, A. L., C. Tian, et al. (2006). "Transcriptional control of activation-induced cytidine deaminase and error-prone DNA polymerases is functionally mature in the B cells of infants at birth." Hum Immunol **67**(1-2): 43-6.

Boyer, J., E. G. McLean, et al. (2004). "Characterization of p53 wild-type and null isogenic colorectal cancer cell lines resistant to 5-fluorouracil, oxaliplatin, and irinotecan." Clin Cancer Res **10**(6): 2158-67.

Branzei, D. and M. Foiani (2008). "Regulation of DNA repair throughout the cell cycle." Nat Rev Mol Cell Biol **9**(4): 297-308.

Brenneman, M. A., B. M. Wagener, et al. (2002). "XRCC3 controls the fidelity of homologous recombination: roles for XRCC3 in late stages of recombination." Mol Cell **10**(2): 387-95.

Brieba, L. G. (2008). "Template dependent human DNA polymerases." Curr Top Med Chem **8**(15): 1312-26.

Broughton, B. C., A. Cordonnier, et al. (2002). "Molecular analysis of mutations in DNA polymerase eta in xeroderma pigmentosum-variant patients." Proc Natl Acad Sci U S A **99**(2): 815-20.

Burger, H., A. Zoumaro-Djayoon, et al. (2010). "Differential transport of platinum compounds by the human organic cation transporter hOCT2 (hSLC22A2)." Br J Pharmacol **159**(4): 898-908.

Cadet, J., S. Bellon, et al. (2004). "Radiation-induced DNA damage: formation, measurement, and biochemical features." J Environ Pathol Toxicol Oncol **23**(1): 33-43.

Callebaut, I., L. Malivert, et al. (2006). "Cernunnos interacts with the XRCC4 x DNA-ligase IV complex and is homologous to the yeast nonhomologous end-joining factor Nej1." J Biol Chem **281**(20): 13857-60.

Cancer Genome Atlas Network (2012). "Comprehensive molecular characterization of human colon and rectal cancer." Nature **487**(7407): 330-7.

Canitrot, Y., C. Cazaux, et al. (1998). "Overexpression of DNA polymerase beta in cell results in a mutator phenotype and a decreased sensitivity to anticancer drugs." Proc Natl Acad Sci U S A **95**(21): 12586-90.

Cepeda, V., M. A. Fuertes, et al. (2007). "Biochemical mechanisms of cisplatin cytotoxicity." Anticancer Agents Med Chem **7**(1): 3-18.

Ceppi, P., S. Novello, et al. (2009). "Polymerase eta mRNA expression predicts survival of non-small cell lung cancer patients treated with platinum-based chemotherapy." Clin Cancer Res **15**(3): 1039-45.

Chaney, S. G., S. L. Campbell, et al. (2005). "Recognition and processing of cisplatin- and oxaliplatin-DNA adducts." Crit Rev Oncol Hematol **53**(1): 3-11.

Chen, C. C., L. T. Chen, et al. (2007). "Combined modalities of resistance in an oxaliplatin-resistant human gastric cancer cell line with enhanced sensitivity to 5-fluorouracil." Br J Cancer **97**(3): 334-44.

Chen, F., S. R. Peterson, et al. (1996). "Disruption of DNA-PK in Ku80 mutant xrs-6 and the implications in DNA double-strand break repair." Mutat Res **362**(1): 9-19.

Chen, Y. W., J. E. Cleaver, et al. (2006). "A novel role of DNA polymerase eta in modulating cellular sensitivity to chemotherapeutic agents." Mol Cancer Res **4**(4): 257-65.

Chen, Y. W., J. E. Cleaver, et al. (2008). "Human DNA polymerase eta activity and translocation is regulated by phosphorylation." Proc Natl Acad Sci U S A **105**(43): 16578-83.

Cheong, N., X. Wang, et al. (1994). "Loss of S-phase-dependent radioresistance in irs-1 cells exposed to X-rays." Mutat Res **314**(1): 77-85.

Chijiwa, S., C. Masutani, et al. (2010). "Polymerization by DNA polymerase eta is blocked by cis-diamminedichloroplatinum(II) 1,3-d(GpTpG) cross-link: implications for cytotoxic effects in nucleotide excision repair-negative tumor cells." Carcinogenesis **31**(3): 388-93.

Choi, J. Y., G. Chowdhury, et al. (2006). "Translesion synthesis across O6-alkylguanine DNA adducts by recombinant human DNA polymerases." J Biol Chem **281**(50): 38244-56.

Ciccarelli, R. B., M. J. Solomon, et al. (1985). "In vivo effects of cis- and trans-diamminedichloroplatinum(II) on SV40 chromosomes: differential repair, DNA-protein cross-linking, and inhibition of replication." Biochemistry **24**(26): 7533-40.

Cleaver, J. E., J. Bartholomew, et al. (2002). "Polymerase eta and p53 jointly regulate cell survival, apoptosis and Mre11 recombination during S phase checkpoint arrest after UV irradiation." DNA Repair (Amst) **1**(1): 41-57.

Clement, F. C., U. Camenisch, et al. (2010). "Dynamic two-stage mechanism of versatile DNA damage recognition by xeroderma pigmentosum group C protein." Mutat Res **685**(1-2): 21-8.

Coleman, K. A. and R. A. Greenberg (2011). "The BRCA1-RAP80 Complex Regulates DNA Repair Mechanism Utilization by Restricting End Resection." J Biol Chem **286**(15): 13669-80.

Colis, L. C., P. Raychaudhury, et al. (2008). "Mutational specificity of gamma-radiation-induced guanine-thymine and thymine-guanine intrastrand cross-links in mammalian cells and translesion synthesis past the guanine-thymine lesion by human DNA polymerase eta." Biochemistry **47**(31): 8070-9.

Conroy, T., Y. Yataghene, et al. (2010). "Phase II randomised trial of chemoradiotherapy with FOLFOX4 or cisplatin plus fluorouracil in oesophageal cancer." Br J Cancer **103**(9): 1349-55.

Costes, S. V., I. Chiolo, et al. (2010). "Spatiotemporal characterization of ionizing radiation induced DNA damage foci and their relation to chromatin organization." Mutat Res **704**(1-3): 78-87.

Cruet-Hennequart, S., S. Villalan, et al. (2009). "Characterization of the effects of cisplatin and carboplatin on cell cycle progression and DNA damage response activation in DNA polymerase eta-deficient human cells." Cell Cycle **8**(18): 3039-50.

Cuisnier, O., R. Serduc, et al. (2003). "Chronic hypoxia protects against gamma-irradiation-induced apoptosis by inducing bcl-2 up-regulation and inhibiting mitochondrial translocation and conformational change of bax protein." Int J Oncol **23**(4): 1033-41.

Cullen, K. J., K. A. Newkirk, et al. (2003). "Glutathione S-transferase pi amplification is associated with cisplatin resistance in head and neck squamous cell carcinoma cell lines and primary tumors." Cancer Res **63**(23): 8097-102.

Cunningham, D., N. Starling, et al. (2008). "Capecitabine and oxaliplatin for advanced esophagogastric cancer." N Engl J Med **358**(1): 36-46.

Dabholkar, M., F. Bostick-Bruton, et al. (1992). "ERCC1 and ERCC2 expression in malignant tissues from ovarian cancer patients." J Natl Cancer Inst **84**(19): 1512-7.

Daigaku, Y., A. A. Davies, et al. (2010). "Ubiquitin-dependent DNA damage bypass is separable from genome replication." Nature **465**(7300): 951-5.

de Gramont, A., A. Figer, et al. (2000). "Leucovorin and fluorouracil with or without oxaliplatin as first-line treatment in advanced colorectal cancer." J Clin Oncol **18**(16): 2938-47.

DeCarlo, L., A. S. Gowda, et al. (2008). "Formation of purine-purine mispairs by Sulfolobus solfataricus DNA polymerase IV." Biochemistry **47**(31): 8157-64.

Delbos, F., A. De Smet, et al. (2005). "Contribution of DNA polymerase eta to immunoglobulin gene hypermutation in the mouse." J Exp Med **201**(8): 1191-6.

Delmas, S. and I. Matic (2006). "Interplay between replication and recombination in Escherichia coli: impact of the alternative DNA polymerases." Proc Natl Acad Sci U S A **103**(12): 4564-9.

Dextraze, M. E., T. Gantchev, et al. (2010). "DNA interstrand cross-links induced by ionizing radiation: an unsung lesion." Mutat Res **704**(1-3): 101-7.

Di Lucca, J., M. Guedj, et al. (2009). "Variants of the xeroderma pigmentosum variant gene (POLH) are associated with melanoma risk." Eur J Cancer **45**(18): 3228-36.

Ding, Q., Y. V. Reddy, et al. (2003). "Autophosphorylation of the catalytic subunit of the DNA-dependent protein kinase is required for efficient end processing during DNA double-strand break repair." Mol Cell Biol **23**(16): 5836-48.

Dutreix, M., J. M. Cosset, et al. (2010). "Molecular therapy in support to radiotherapy." Mutat Res **704**(1-3): 182-9.

Eid, W., M. Steger, et al. (2010). "DNA end resection by CtIP and exonuclease 1 prevents genomic instability." EMBO Rep **11**(12): 962-8.

Erlandsson, F., C. Linnman, et al. (2000). "A detailed analysis of cyclin A accumulation at the G(1)/S border in normal and transformed cells." Exp Cell Res **259**(1): 86-95.

Facchino, S., M. Abdouh, et al. (2010). "BMI1 confers radioresistance to normal and cancerous neural stem cells through recruitment of the DNA damage response machinery." J Neurosci **30**(30): 10096-111.

Faili, A., S. Aoufouchi, et al. (2004). "DNA polymerase eta is involved in hypermutation occurring during immunoglobulin class switch recombination." J Exp Med **199**(2): 265-70.

Ferry, K. V., T. C. Hamilton, et al. (2000). "Increased nucleotide excision repair in cisplatin-resistant ovarian cancer cells: role of ERCC1-XPF." Biochem Pharmacol **60**(9): 1305-13.

Fink, D., S. Nebel, et al. (1996). "The role of DNA mismatch repair in platinum drug resistance." Cancer Res **56**(21): 4881-6.

Fink, D., H. Zheng, et al. (1997). "In vitro and in vivo resistance to cisplatin in cells that have lost DNA mismatch repair." Cancer Res **57**(10): 1841-5.

Flanagan, A. M., G. Rafferty, et al. (2007). "The human POLH gene is not mutated, and is expressed in a cohort of patients with basal or squamous cell carcinoma of the skin." Int J Mol Med **19**(4): 589-96.

Fousteri, M., W. Vermeulen, et al. (2006). "Cockayne syndrome A and B proteins differentially regulate recruitment of chromatin remodeling and repair factors to stalled RNA polymerase II in vivo." Mol Cell **23**(4): 471-82.

Franken, N. A., H. M. Rodermond, et al. (2006). "Clonogenic assay of cells in vitro." Nat Protoc **1**(5): 2315-9.

Frankenberg-Schwager, M., A. Gebauer, et al. (2009). "Single-strand annealing, conservative homologous recombination, nonhomologous DNA end joining, and the cell cycle-dependent repair of DNA double-strand breaks induced by sparsely or densely ionizing radiation." Radiat Res **171**(3): 265-73.

Frechet, M., Y. Canitrot, et al. (2002). "Deregulated DNA polymerase beta strengthens ionizing radiation-induced nucleotidic and chromosomal instabilities." Oncogene **21**(15): 2320-7.

Freeman, W. M., G. V. Bixler, et al. (2010). "A multistep validation process of biomarkers for preclinical drug development." Pharmacogenomics J **10**(5): 385-95.

Freudenthal, B. D., L. Gakhar, et al. (2010). "Structure of monoubiquitinated PCNA and implications for translesion synthesis and DNA polymerase exchange." Nat Struct Mol Biol **17**(4): 479-84.

Friedman, J. I. and J. T. Stivers (2010). "Detection of damaged DNA bases by DNA glycosylase enzymes." Biochemistry **49**(24): 4957-67.

Fu, Y., Y. Zhu, et al. (2008). "Rad6-Rad18 mediates a eukaryotic SOS response by ubiquitinating the 9-1-1 checkpoint clamp." Cell **133**(4): 601-11.

Funke, S., M. Timofeeva, et al. (2010). "Genetic polymorphisms in GST genes and survival of colorectal cancer patients treated with chemotherapy." Pharmacogenomics **11**(1): 33-41.

Furuta, T., T. Ueda, et al. (2002). "Transcription-coupled nucleotide excision repair as a determinant of cisplatin sensitivity of human cells." Cancer Res **62**(17): 4899-902.

Garg, P., C. M. Stith, et al. (2005). "Proliferating cell nuclear antigen promotes translesion synthesis by DNA polymerase zeta." J Biol Chem **280**(25): 23446-50.

Giacchetti, S., B. Perpoint, et al. (2000). "Phase III multicenter randomized trial of oxaliplatin added to chronomodulated fluorouracil-leucovorin as first-line treatment of metastatic colorectal cancer." J Clin Oncol **18**(1): 136-47.

Gillies, R. S., M. R. Middleton, et al. (2012). "Metabolic response at repeat PET/CT predicts pathological response to neoadjuvant chemotherapy in oesophageal cancer." Eur Radiol **22**(9): 2035-43.

Glick, E., L. M. White, et al. (2006). "Mutations in DNA polymerase eta are not detected in squamous cell carcinoma of the skin." Int J Cancer **119**(9): 2225-7.

Go, R. S. and A. A. Adjei (1999). "Review of the comparative pharmacology and clinical activity of cisplatin and carboplatin." J Clin Oncol **17**(1): 409-22.

Goetz, J. D., T. A. Motycka, et al. (2005). "Reduced repair of DNA double-strand breaks by homologous recombination in a DNA ligase I-deficient human cell line." DNA Repair (Amst) **4**(6): 649-54.

Gohler, T., S. Sabbioneda, et al. (2011). "ATR-mediated phosphorylation of DNA polymerase eta is needed for efficient recovery from UV damage." J Cell Biol **192**(2): 219-27.

Gong, J. G., A. Costanzo, et al. (1999). "The tyrosine kinase c-Abl regulates p73 in apoptotic response to cisplatin-induced DNA damage." Nature **399**(6738): 806-9.

Goodhead, D. T. (1994). "Initial events in the cellular effects of ionizing radiations: clustered damage in DNA." Int J Radiat Biol **65**(1): 7-17.

Goodman, M. F. (2002). "Error-prone repair DNA polymerases in prokaryotes and eukaryotes." Annu Rev Biochem **71**: 17-50.

Goodman, M. F. and B. Tippin (2000). "The expanding polymerase universe." Nat Rev Mol Cell Biol **1**(2): 101-9.

Gray, L. H. (1957). "Oxygenation in radiotherapy. I. Radiobiological considerations." Br J Radiol **30**(356): 403-6.

Gray, L. H., A. D. Conger, et al. (1953). "The concentration of oxygen dissolved in tissues at the time of irradiation as a factor in radiotherapy." Br J Radiol **26**(312): 638-48.

Gu, C. and Y. Wang (2004). "LC-MS/MS identification and yeast polymerase eta bypass of a novel gamma-irradiation-induced intrastrand cross-link lesion G[8-5]C." Biochemistry **43**(21): 6745-50.

Gueranger, Q., A. Stary, et al. (2008). "Role of DNA polymerases eta, iota and zeta in UV resistance and UV-induced mutagenesis in a human cell line." DNA Repair (Amst) **7**(9): 1551-62.

Guo, C., J. N. Kosarek-Stancel, et al. (2009). "Y-family DNA polymerases in mammalian cells." Cell Mol Life Sci **66**(14): 2363-81.

Hanada, K., M. Budzowska, et al. (2006). "The structure-specific endonuclease Mus81-Eme1 promotes conversion of interstrand DNA crosslinks into double-strands breaks." EMBO J **25**(20): 4921-32.

Haracska, L., I. Unk, et al. (2006). "Ubiquitylation of yeast proliferating cell nuclear antigen and its implications for translesion DNA synthesis." Proc Natl Acad Sci U S A **103**(17): 6477-82.

Hartlerode, A. J. and R. Scully (2009). "Mechanisms of double-strand break repair in somatic mammalian cells." Biochem J **423**(2): 157-68.

Hartley, K. O., D. Gell, et al. (1995). "DNA-dependent protein kinase catalytic subunit: a relative of phosphatidylinositol 3-kinase and the ataxia telangiectasia gene product." Cell **82**(5): 849-56.

Hegde, M. L., T. K. Hazra, et al. (2008). "Early steps in the DNA base excision/single-strand interruption repair pathway in mammalian cells." Cell Res **18**(1): 27-47.

Helleday, T., J. Lo, et al. (2007). "DNA double-strand break repair: from mechanistic understanding to cancer treatment." DNA Repair (Amst) **6**(7): 923-35.

Helleday, T., E. Petermann, et al. (2008). "DNA repair pathways as targets for cancer therapy." Nat Rev Cancer **8**(3): 193-204.

Hermann, R. M., O. Horstmann, et al. (2006). "Histomorphological tumor regression grading of esophageal carcinoma after neoadjuvant radiochemotherapy: which score to use?" Dis Esophagus **19**(5): 329-34.

Hibbert, R. G., A. Huang, et al. (2011). "E3 ligase Rad18 promotes monoubiquitination rather than ubiquitin chain formation by E2 enzyme Rad6." Proc Natl Acad Sci U S A.

Hinz, J. M. (2010). "Role of homologous recombination in DNA interstrand crosslink repair." Environ Mol Mutagen **51**(6): 582-603.

Hishikawa, Y., H. Kohno, et al. (2001). "Expression of metallothionein in colorectal cancers and synchronous liver metastases." Oncology **61**(2): 162-7.

Hlavin, E. M., M. B. Smeaton, et al. (2010). "Initiation of DNA interstrand cross-link repair in mammalian cells." Environ Mol Mutagen **51**(6): 604-24.

Ho, T. V., A. Guainazzi, et al. (2011). "Structure-dependent bypass of DNA interstrand crosslinks by translesion synthesis polymerases." Nucleic Acids Res **39**(17): 7455-64.

Ho, T. V. and O. D. Scharer (2010). "Translesion DNA synthesis polymerases in DNA interstrand crosslink repair." Environ Mol Mutagen **51**(6): 552-66.

Holscher, A. H., E. Bollschweiler, et al. (1995). "Prognosis of early esophageal cancer. Comparison between adeno- and squamous cell carcinoma." Cancer **76**(2): 178-86.

Holzer, A. K., K. Katano, et al. (2004). "Cisplatin rapidly down-regulates its own influx transporter hCTR1 in cultured human ovarian carcinoma cells." Clin Cancer Res **10**(19): 6744-9.

Honda, M., T. Nakamura, et al. (2010). "Process of healing of mucosal defects in the esophagus after endoscopic mucosal resection: histological evaluation in a dog model." Endoscopy **42**(12): 1092-5.

Hour, T. C., J. Chen, et al. (2000). "Characterization of chemoresistance mechanisms in a series of cisplatin-resistant transitional carcinoma cell lines." Anticancer Res **20**(5A): 3221-5.

Huang, J. X., W. C. Chen, et al. (2012). "Clinicopathological significance of cyclooxygenase-2 and cell cycle-regulatory proteins expression in patients with esophageal squamous cell carcinoma." Dis Esophagus **25**(2): 121-9.

Huang, J. X., W. Xiao, et al. (2010). "Relationship between COX-2 and cell cycle-regulatory proteins in patients with esophageal squamous cell carcinoma." World J Gastroenterol **16**(47): 5975-81.

Hwang, H. and J. S. Taylor (2005). "Evidence for Watson-Crick and not Hoogsteen or wobble base pairing in the selection of nucleotides for insertion opposite pyrimidines and a thymine dimer by yeast DNA pol eta." Biochemistry **44**(12): 4850-60.

Iijima, K., M. Ohara, et al. (2008). "Dancing on damaged chromatin: functions of ATM and the RAD50/MRE11/NBS1 complex in cellular responses to DNA damage." J Radiat Res (Tokyo) **49**(5): 451-64.

Iliakis, G. E. and R. Okayasu (1990). "Radiosensitivity throughout the cell cycle and repair of potentially lethal damage and DNA double-strand breaks in an X-ray-sensitive CHO mutant." Int J Radiat Biol **57**(6): 1195-211.

Inui, H., K. S. Oh, et al. (2008). "Xeroderma pigmentosum-variant patients from America, Europe, and Asia." J Invest Dermatol **128**(8): 2055-68.

Ishida, S., F. McCormick, et al. (2010). "Enhancing tumor-specific uptake of the anticancer drug cisplatin with a copper chelator." Cancer Cell **17**(6): 574-83.

Ito, A., N. Koshikawa, et al. (2006). "Hypoxia-inducible factor-1 mediates the expression of DNA polymerase iota in human tumor cells." Biochem Biophys Res Commun **351**(1): 306-11.

Jamieson, E. R. and S. J. Lippard (1999). "Structure, Recognition, and Processing of Cisplatin-DNA Adducts." Chem Rev **99**(9): 2467-98.

Jansen, B. A., J. Brouwer, et al. (2002). "Glutathione induces cellular resistance against cationic dinuclear platinum anticancer drugs." J Inorg Biochem **89**(3-4): 197-202.

Jeggo, P. and M. F. Lavin (2009). "Cellular radiosensitivity: how much better do we understand it?" Int J Radiat Biol **85**(12): 1061-81.

Jemal, A., R. Siegel, et al. (2009). "Cancer statistics, 2009." CA Cancer J Clin **59**(4): 225-49.

Jennerwein, M. M., A. Eastman, et al. (1989). "Characterization of adducts produced in DNA by isomeric 1,2-diaminocyclohexaneplatinum(II) complexes." Chem Biol Interact **70**(1-2): 39-49.

Jensen, M., M. Bjerring, et al. (2010). "Cisplatin interaction with phosphatidylserine bilayer studied by solid-state NMR spectroscopy." J Biol Inorg Chem **15**(2): 213-23.

Jiang, Y., H. Hong, et al. (2007). "In vivo formation and in vitro replication of a guanine-thymine intrastrand cross-link lesion." Biochemistry **46**(44): 12757-63.

Jin, S. and D. T. Weaver (1997). "Double-strand break repair by Ku70 requires heterodimerization with Ku80 and DNA binding functions." Embo J **16**(22): 6874-85.

Johnson, R. E., C. M. Kondratick, et al. (1999). "hRAD30 mutations in the variant form of xeroderma pigmentosum." Science **285**(5425): 263-5.

Johnson, R. E., M. T. Washington, et al. (2000). "Fidelity of human DNA polymerase eta." J Biol Chem **275**(11): 7447-50.

Kaanders, J. H., K. I. Wijffels, et al. (2002). "Pimonidazole binding and tumor vascularity predict for treatment outcome in head and neck cancer." Cancer Res **62**(23): 7066-74.

Kannouche, P., B. C. Broughton, et al. (2001). "Domain structure, localization, and function of DNA polymerase eta, defective in xeroderma pigmentosum variant cells." Genes Dev **15**(2): 158-72.

Kartalou, M. and J. M. Essigmann (2001). "Recognition of cisplatin adducts by cellular proteins." Mutat Res **478**(1-2): 1-21.

Katano, K., R. Safaei, et al. (2003). "The copper export pump ATP7B modulates the cellular pharmacology of carboplatin in ovarian carcinoma cells." Mol Pharmacol **64**(2): 466-73.

Kawamoto, T., K. Araki, et al. (2005). "Dual roles for DNA polymerase eta in homologous DNA recombination and translesion DNA synthesis." Mol Cell **20**(5): 793-9.

Kennette, W., O. M. Collins, et al. (2005). "Basal and zinc-induced metallothionein in resistance to cadmium, cisplatin, zinc, and tertbutyl hydroperoxide: studies using MT knockout and antisense-downregulated MT in mammalian cells." Toxicol Sci **88**(2): 602-13.

Kern, W., J. Braess, et al. (1999). "Oxaliplatin pharmacokinetics during a four-hour infusion." Clin Cancer Res **5**(4): 761-5.

Kim, S. H., H. C. Kwon, et al. (2009). "Prognostic value of ERCC1, thymidylate synthase, and glutathione S-transferase pi for 5-FU/oxaliplatin chemotherapy in advanced colorectal cancer." Am J Clin Oncol **32**(1): 38-43.

Kim, Y. M., Y. M. Lee, et al. (2011). "Ataxia telangiectasia and Rad3-related overexpressing cancer cells induce prolonged G arrest and develop resistance to ionizing radiation." DNA Cell Biol **30**(4): 219-27.

Kimura, T., T. Takeuchi, et al. (2009). "Penicilliols A and B, novel inhibitors specific to mammalian Y-family DNA polymerases." Bioorg Med Chem **17**(5): 1811-6.

King, N. M., N. Nikolaishvili-Feinberg, et al. (2005). "Overproduction of DNA polymerase eta does not raise the spontaneous mutation rate in diploid human fibroblasts." DNA Repair (Amst) **4**(6): 714-24.

Koberle, B., M. T. Tomicic, et al. (2010). "Cisplatin resistance: preclinical findings and clinical implications." Biochim Biophys Acta **1806**(2): 172-82.

Konishi, I., K. Nanbu, et al. (1998). "Tumor response to neoadjuvant chemotherapy correlates with the expression of P-glycoprotein and PCNA but not GST-pi in the tumor cells of cervical carcinoma." Gynecol Oncol **70**(3): 365-71.

Krasikova, Y. S., N. I. Rechkunova, et al. (2010). "Localization of xeroderma pigmentosum group A protein and replication protein A on damaged DNA in nucleotide excision repair." Nucleic Acids Res **38**(22): 8083-94.

Kusumoto, R., L. Dawut, et al. (2008). "Werner protein cooperates with the XRCC4-DNA ligase IV complex in end-processing." Biochemistry **47**(28): 7548-56.

Laposa, R. R., L. Feeney, et al. (2003). "Recapitulation of the cellular xeroderma pigmentosum-variant phenotypes using short interfering RNA for DNA polymerase H." Cancer Res **63**(14): 3909-12.

Larson, C. A., B. G. Blair, et al. (2009). "The role of the mammalian copper transporter 1 in the cellular accumulation of platinum-based drugs." Mol Pharmacol **75**(2): 324-30.

Lebwohl, D. and R. Canetta (1998). "Clinical development of platinum complexes in cancer therapy: an historical perspective and an update." Eur J Cancer **34**(10): 1522-34.

Lee, C. H., S. Chandani, et al. (2006). "Homology modeling of four Y-family, lesion-bypass DNA polymerases: the case that E. coli Pol IV and human Pol kappa are orthologs, and E. coli Pol V and human Pol eta are orthologs." J Mol Graph Model **25**(1): 87-102.

Lee, D. H. and G. P. Pfeifer (2008). "Translesion synthesis of 7,8-dihydro-8-oxo-2'-deoxyguanosine by DNA polymerase eta in vivo." Mutat Res **641**(1-2): 19-26.

Lee, K. Y. and K. Myung (2008). "PCNA modifications for regulation of post-replication repair pathways." Mol Cells **26**(1): 5-11.

Levi, F., G. Metzger, et al. (2000). "Oxaliplatin: pharmacokinetics and chronopharmacological aspects." Clin Pharmacokinet **38**(1): 1-21.

Li, G. M. (2008). "Mechanisms and functions of DNA mismatch repair." Cell Res **18**(1): 85-98.

Liliensiek, S. J., K. Schell, et al. (2006). "Cell sorting but not serum starvation is effective for SV40 human corneal epithelial cell cycle synchronization." Exp Eye Res **83**(1): 61-8.

Lin, X. and S. B. Howell (2006). "DNA mismatch repair and p53 function are major determinants of the rate of development of cisplatin resistance." Mol Cancer Ther **5**(5): 1239-47.

Lin, X., J. Trang, et al. (2006). "DNA polymerase zeta accounts for the reduced cytotoxicity and enhanced mutagenicity of cisplatin in human colon carcinoma cells that have lost DNA mismatch repair." Clin Cancer Res **12**(2): 563-8.

Lindahl, T. and R. D. Wood (1999). "Quality control by DNA repair." Science **286**(5446): 1897-905.

Ling, H., F. Boudsocq, et al. (2003). "Replication of a cis-syn thymine dimer at atomic resolution." Nature **424**(6952): 1083-7.

Liu, G. and X. Chen (2006). "DNA polymerase eta, the product of the xeroderma pigmentosum variant gene and a target of p53, modulates the DNA damage checkpoint and p53 activation." Mol Cell Biol **26**(4): 1398-413.

Liu, J., T. Doty, et al. (2010). "Human BRCA2 protein promotes RAD51 filament formation on RPA-covered single-stranded DNA." Nat Struct Mol Biol **17**(10): 1260-2.

Lobrich, M. and P. A. Jeggo (2005). "Harmonising the response to DSBs: a new string in the ATM bow." DNA Repair (Amst) **4**(7): 749-59.

Loh, S. Y., P. Mistry, et al. (1992). "Reduced drug accumulation as a major mechanism of acquired resistance to cisplatin in a human ovarian carcinoma cell line: circumvention studies using novel platinum (II) and (IV) ammine/amine complexes." Br J Cancer **66**(6): 1109-15.

Lone, S., S. A. Townson, et al. (2007). "Human DNA polymerase kappa encircles DNA: implications for mismatch extension and lesion bypass." Mol Cell **25**(4): 601-14.

Lord, R. V., J. Brabender, et al. (2002). "Low ERCC1 expression correlates with prolonged survival after cisplatin plus gemcitabine chemotherapy in non-small cell lung cancer." Clin Cancer Res **8**(7): 2286-91.

Lynam-Lennon, N., J. V. Reynolds, et al. (2010). "Alterations in DNA repair efficiency are involved in the radioresistance of esophageal adenocarcinoma." Radiat Res **174**(6): 703-11.

Maga, G., G. Villani, et al. (2007). "8-oxo-guanine bypass by human DNA polymerases in the presence of auxiliary proteins." Nature **447**(7144): 606-8.

Mahaney, B. L., K. Meek, et al. (2009). "Repair of ionizing radiation-induced DNA double-strand breaks by non-homologous end-joining." Biochem J **417**(3): 639-50.

Mandard, A. M., F. Dalibard, et al. (1994). "Pathologic assessment of tumor regression after preoperative chemoradiotherapy of esophageal carcinoma. Clinicopathologic correlations." Cancer **73**(11): 2680-6.

Marchio, C., M. Dowsett, et al. (2011). "Revisiting the technical validation of tumour biomarker assays: how to open a Pandora's box." BMC Med **9**: 41.

Marks, L. B. and M. Dewhirst (1991). "Accelerated repopulation: friend or foe? Exploiting changes in tumor growth characteristics to improve the "efficiency" of radiotherapy." Int J Radiat Oncol Biol Phys **21**(5): 1377-83.

Martin, L. M., B. Marples, et al. (2010). "DNA mismatch repair and the DNA damage response to ionizing radiation: making sense of apparently conflicting data." Cancer Treat Rev **36**(7): 518-27.

Martin, L. P., T. C. Hamilton, et al. (2008). "Platinum resistance: the role of DNA repair pathways." Clin Cancer Res **14**(5): 1291-5.

Masutani, C., R. Kusumoto, et al. (2000). "Mechanisms of accurate translesion synthesis by human DNA polymerase eta." EMBO J **19**(12): 3100-9.

Masutani, C., R. Kusumoto, et al. (1999). "The XPV (xeroderma pigmentosum variant) gene encodes human DNA polymerase eta." Nature **399**(6737): 700-4.

Matsuda, T., K. Bebenek, et al. (2000). "Low fidelity DNA synthesis by human DNA polymerase-eta." Nature **404**(6781): 1011-3.

Matsuda, T., K. Bebenek, et al. (2001). "Error rate and specificity of human and murine DNA polymerase eta." J Mol Biol **312**(2): 335-46.

Matsunaga, T., C. H. Park, et al. (1996). "Replication protein A confers structure-specific endonuclease activities to the XPF-ERCC1 and XPG subunits of human DNA repair excision nuclease." J Biol Chem **271**(19): 11047-50.

Mauer, A. M., E. H. Kraut, et al. (2005). "Phase II trial of oxaliplatin, leucovorin and fluorouracil in patients with advanced carcinoma of the esophagus." Ann Oncol **16**(8): 1320-5.

McCulloch, S. D., R. J. Kokoska, et al. (2004). "Enzymatic switching for efficient and accurate translesion DNA replication." Nucleic Acids Res **32**(15): 4665-75.

McCulloch, S. D., R. J. Kokoska, et al. (2009). "The efficiency and fidelity of 8-oxo-guanine bypass by DNA polymerases delta and eta." Nucleic Acids Res **37**(9): 2830-40.

McCulloch, S. D., R. J. Kokoska, et al. (2004). "Preferential cis-syn thymine dimer bypass by DNA polymerase eta occurs with biased fidelity." Nature **428**(6978): 97-100.

McCulloch, S. D. and T. A. Kunkel (2008). "The fidelity of DNA synthesis by eukaryotic replicative and translesion synthesis polymerases." Cell Res **18**(1): 148-61.

McCulloch, S. D., A. Wood, et al. (2007). "Effects of accessory proteins on the bypass of a cis-syn thymine-thymine dimer by Saccharomyces cerevisiae DNA polymerase eta." Biochemistry **46**(30): 8888-96.

McDonald, J. P., V. Rapic-Otrin, et al. (1999). "Novel human and mouse homologs of Saccharomyces cerevisiae DNA polymerase eta." Genomics **60**(1): 20-30.

McIlwraith, M. J., A. Vaisman, et al. (2005). "Human DNA polymerase eta promotes DNA synthesis from strand invasion intermediates of homologous recombination." Mol Cell **20**(5): 783-92.

McIlwraith, M. J., E. Van Dyck, et al. (2000). "Reconstitution of the strand invasion step of double-strand break repair using human Rad51 Rad52 and RPA proteins." J Mol Biol **304**(2): 151-64.

McIlwraith, M. J. and S. C. West (2008). "DNA repair synthesis facilitates RAD52-mediated second-end capture during DSB repair." Mol Cell **29**(4): 510-6.

McKeage, M. J. (1995). "Comparative adverse effect profiles of platinum drugs." Drug Saf **13**(4): 228-44.

Meijer, C., N. H. Mulder, et al. (1992). "Relationship of cellular glutathione to the cytotoxicity and resistance of seven platinum compounds." Cancer Res **52**(24): 6885-9.

Mellish, K. J., L. R. Kelland, et al. (1993). "In vitro platinum drug chemosensitivity of human cervical squamous cell carcinoma cell lines with intrinsic and acquired resistance to cisplatin." Br J Cancer **68**(2): 240-50.

Metzger, R., C. G. Leichman, et al. (1998). "ERCC1 mRNA levels complement thymidylate synthase mRNA levels in predicting response and survival for gastric cancer patients receiving combination cisplatin and fluorouracil chemotherapy." J Clin Oncol **16**(1): 309-16.

Michel, B., G. Grompone, et al. (2004). "Multiple pathways process stalled replication forks." Proc Natl Acad Sci U S A **101**(35): 12783-8.

Mimitou, E. P. and L. S. Symington (2011). "DNA end resection-Unraveling the tail." DNA Repair (Amst) **10**(3): 344-8.

Minko, I. G., M. B. Harbut, et al. (2008). "Role for DNA polymerase kappa in the processing of N2-N2-guanine interstrand cross-links." J Biol Chem **283**(25): 17075-82.

Mitchell, B. F. and J. T. Tupper (1977). "Synchronization of mouse 3T3 and SV40 3T3 cells by way of centrifugal elutriation." Exp Cell Res **106**(2): 351-5.

Miyase, S., S. Tateishi, et al. (2005). "Differential regulation of Rad18 through Rad6-dependent mono- and polyubiquitination." J Biol Chem **280**(1): 515-24.

Mizushina, Y., H. Motoshima, et al. (2009). "3-O-methylfunicone, a selective inhibitor of mammalian Y-family DNA polymerases from an Australian sea salt fungal strain." Mar Drugs **7**(4): 624-39.

Mizushina, Y., J. Zhang, et al. (2010). "Anti-cancer gallotannin penta-O-galloyl-beta-D-glucose is a nanomolar inhibitor of select mammalian DNA polymerases." Biochem Pharmacol **80**(8): 1125-32.

Moehler, M., A. Mueller, et al. (2011). "Cetuximab with irinotecan, folinic acid and 5-fluorouracil as first-line treatment in advanced gastroesophageal cancer: a prospective multi-center biomarker-oriented phase II study." Ann Oncol **22**(6): 1358-66.

Moeller, B. J., J. S. Yordy, et al. (2011). "DNA repair biomarker profiling of head and neck cancer: Ku80 expression predicts locoregional failure and death following radiotherapy." Clin Cancer Res **17**(7): 2035-43.

Moggs, J. G., D. E. Szymkowski, et al. (1997). "Differential human nucleotide excision repair of paired and mispaired cisplatin-DNA adducts." Nucleic Acids Res **25**(3): 480-91.

Moncharmont, C., P. Auberdiac, et al. (2011). "Cisplatin or carboplatin, that is the question." Bull Cancer **98**(2): 164-175.

Moser, J., H. Kool, et al. (2007). "Sealing of chromosomal DNA nicks during nucleotide excision repair requires XRCC1 and DNA ligase III alpha in a cell-cycle-specific manner." Mol Cell **27**(2): 311-23.

Mukai, M., A. Kanzaki, et al. (2002). "Enhanced nucleotide excision repair in cisplatin resistant human KB carcinoma cells." Oncol Rep **9**(4): 839-44.

Naidu, M. D., J. M. Mason, et al. (2010). "Radiation resistance in glioma cells determined by DNA damage repair activity of Ape1/Ref-1." J Radiat Res (Tokyo) **51**(4): 393-404.

Negroni, A., L. Stronati, et al. (2008). "Radioresistance in a tumour cell line correlates with radiation inducible Ku 70/80 end-binding activity." Int J Radiat Biol **84**(4): 265-76.

Nehme, A., R. Baskaran, et al. (1999). "Induction of JNK and c-Abl signalling by cisplatin and oxaliplatin in mismatch repair-proficient and -deficient cells." Br J Cancer **79**(7-8): 1104-10.

Neijenhuis, S., A. C. Begg, et al. (2005). "Radiosensitization by a dominant negative to DNA polymerase beta is DNA polymerase beta-independent and XRCC1-dependent." Radiother Oncol **76**(2): 123-8.

Neijenhuis, S., M. Verwijs-Janssen, et al. (2010). "Targeted radiosensitization of cells expressing truncated DNA polymerase {beta}." Cancer Res **70**(21): 8706-14.

Niedernhofer, L. J., H. Odijk, et al. (2004). "The structure-specific endonuclease Ercc1-Xpf is required to resolve DNA interstrand cross-link-induced double-strand breaks." Mol Cell Biol **24**(13): 5776-87.

Nikolaishvili-Feinberg, N., G. S. Jenkins, et al. (2008). "Ubiquitylation of proliferating cell nuclear antigen and recruitment of human DNA polymerase eta." Biochemistry **47**(13): 4141-50.

Nojima, K., H. Hochegger, et al. (2005). "Multiple repair pathways mediate tolerance to chemotherapeutic cross-linking agents in vertebrate cells." Cancer Res **65**(24): 11704-11.

Noon, A. T. and A. A. Goodarzi (2011). "53BP1-mediated DNA double strand break repair: Insert bad pun here." DNA Repair (Amst).

Nordsmark, M., S. M. Bentzen, et al. (2005). "Prognostic value of tumor oxygenation in 397 head and neck tumors after primary radiation therapy. An international multi-center study." Radiother Oncol **77**(1): 18-24.

O-Wang, J., K. Kawamura, et al. (2001). "DNA polymerase kappa, implicated in spontaneous and DNA damage-induced mutagenesis, is overexpressed in lung cancer." Cancer Res **61**(14): 5366-9.

Office for National Statistics (2010). "Cancer Statistics Registration: Registration of cancer diagnosed in 2007, England " **Series MB1 no. 38**.

Ogi, T., S. Limsirichaikul, et al. (2010). "Three DNA polymerases, recruited by different mechanisms, carry out NER repair synthesis in human cells." Mol Cell **37**(5): 714-27.

Oksenych, V. and F. Coin (2010). "The long unwinding road: XPB and XPD helicases in damaged DNA opening." Cell Cycle **9**(1): 90-6.

Pabla, N. and Z. Dong (2008). "Cisplatin nephrotoxicity: mechanisms and renoprotective strategies." Kidney Int **73**(9): 994-1007.

Pabla, N., Z. Ma, et al. (2011). "hMSH2 recruits ATR to DNA damage sites for activation during DNA damage-induced apoptosis." J Biol Chem **286**(12): 10411-8.

Page, J. D., I. Husain, et al. (1990). "Effect of the diaminocyclohexane carrier ligand on platinum adduct formation, repair, and lethality." Biochemistry **29**(4): 1016-24.

Pajonk, F., E. Vlashi, et al. (2010). "Radiation resistance of cancer stem cells: the 4 R's of radiobiology revisited." Stem Cells **28**(4): 639-48.

Pan, Q., Y. Fang, et al. (2005). "Down-regulation of DNA polymerases kappa, eta, iota, and zeta in human lung, stomach, and colorectal cancers." Cancer Lett **217**(2): 139-47.

Pannicke, U., Y. Ma, et al. (2004). "Functional and biochemical dissection of the structure-specific nuclease ARTEMIS." Embo J **23**(9): 1987-97.

Pasetto, L. M., M. R. D'Andrea, et al. (2006). "The development of platinum compounds and their possible combination." Crit Rev Oncol Hematol **60**(1): 59-75.

Pavlov, Y. I., D. Nguyen, et al. (2001). "Mutator effects of overproducing DNA polymerase eta (Rad30) and its catalytically inactive variant in yeast." Mutat Res **478**(1-2): 129-39.

Pepe, M. S., R. Etzioni, et al. (2001). "Phases of biomarker development for early detection of cancer." J Natl Cancer Inst **93**(14): 1054-61.

Peyrone, M. (1844). "Ueber die Einwirkung des Ammoniaks auf Platinchlorür." Ann Chem Pharm **51**(1): 1-29.

Plasencia, C., E. Martinez-Balibrea, et al. (2006). "Expression analysis of genes involved in oxaliplatin response and development of oxaliplatin-resistant HT29 colon cancer cells." Int J Oncol **29**(1): 225-35.

Poltoratsky, V., C. J. Woo, et al. (2001). "Expression of error-prone polymerases in BL2 cells activated for Ig somatic hypermutation." Proc Natl Acad Sci U S A **98**(14): 7976-81.

Prakash, S., R. E. Johnson, et al. (2005). "Eukaryotic translesion synthesis DNA polymerases: specificity of structure and function." Annu Rev Biochem **74**: 317-53.

Rahn, J. J., G. M. Adair, et al. (2010). "Multiple roles of ERCC1-XPF in mammalian interstrand crosslink repair." Environ Mol Mutagen **51**(6): 567-81.

Raschle, M., P. Knipscheer, et al. (2008). "Mechanism of replication-coupled DNA interstrand crosslink repair." Cell **134**(6): 969-80.

Rass, U., S. A. Compton, et al. (2010). "Mechanism of Holliday junction resolution by the human GEN1 protein." Genes Dev **24**(14): 1559-69.

Ratnam, S., G. Bozek, et al. (2010). "The pattern of somatic hypermutation of Ig genes is altered when p53 is inactivated." Mol Immunol **47**(16): 2611-8.

Reardon, J. T., A. Vaisman, et al. (1999). "Efficient nucleotide excision repair of cisplatin, oxaliplatin, and Bis-aceto-ammine-dichloro-cyclohexylamine-platinum(IV) (JM216) platinum intrastrand DNA diadducts." Cancer Res **59**(16): 3968-71.

Rechkoblit, O., Y. Zhang, et al. (2002). "trans-Lesion synthesis past bulky benzo[a]pyrene diol epoxide N2-dG and N6-dA lesions catalyzed by DNA bypass polymerases." J Biol Chem **277**(34): 30488-94.

Rey, L., J. M. Sidorova, et al. (2009). "Human DNA polymerase eta is required for common fragile site stability during unperturbed DNA replication." Mol Cell Biol **29**(12): 3344-54.

Rixe, O., W. Ortuzar, et al. (1996). "Oxaliplatin, tetraplatin, cisplatin, and carboplatin: spectrum of activity in drug-resistant cell lines and in the cell lines of the National Cancer Institute's Anticancer Drug Screen panel." Biochem Pharmacol **52**(12): 1855-65.

Rouvelas, I., W. Zeng, et al. (2005). "Survival after surgery for oesophageal cancer: a population-based study." Lancet Oncol **6**(11): 864-70.

Samimi, G., R. Safaei, et al. (2004). "Increased expression of the copper efflux transporter ATP7A mediates resistance to cisplatin, carboplatin, and oxaliplatin in ovarian cancer cells." Clin Cancer Res **10**(14): 4661-9.

Saris, C. P., P. J. van de Vaart, et al. (1996). "In vitro formation of DNA adducts by cisplatin, lobaplatin and oxaliplatin in calf thymus DNA in solution and in cultured human cells." Carcinogenesis **17**(12): 2763-9.

Sarkar, S., A. A. Davies, et al. (2006). "DNA interstrand crosslink repair during G1 involves nucleotide excision repair and DNA polymerase zeta." EMBO J **25**(6): 1285-94.

Scheeff, E. D., J. M. Briggs, et al. (1999). "Molecular modeling of the intrastrand guanine-guanine DNA adducts produced by cisplatin and oxaliplatin." Mol Pharmacol **56**(3): 633-43.

Schiff, D., P. Y. Wen, et al. (2009). "Neurological adverse effects caused by cytotoxic and targeted therapies." Nat Rev Clin Oncol **6**(10): 596-603.

Schild-Poulter, C., L. Pope, et al. (2001). "The binding of Ku antigen to homeodomain proteins promotes their phosphorylation by DNA-dependent protein kinase." J Biol Chem **276**(20): 16848-56.

Schmutz, V., R. Janel-Bintz, et al. (2010). "Role of the ubiquitin-binding domain of Poleta in Rad18-independent translesion DNA synthesis in human cell extracts." Nucleic Acids Res **38**(19): 6456-65.

Scholzen, T. and J. Gerdes (2000). "The Ki-67 protein: from the known and the unknown." J Cell Physiol **182**(3): 311-22.

Schonnemann, K. R., M. Yilmaz, et al. (2012). "Phase II study of biweekly cetuximab in combination with irinotecan as second-line treatment in patients with platinum-resistant gastro-oesophageal cancer." Eur J Cancer **48**(4): 510-7.

Schwarz, G. (1909). "Ueber Desensibilisierung gegen Roentgen und Radium Strahlen." Muenchner Medizinische Wochenschrift **24**: 1-2.

Sebesta, M., P. Burkovics, et al. (2011). "Reconstitution of DNA repair synthesis in vitro and the role of polymerase and helicase activities." DNA Repair (Amst).

Sekimoto, T., T. Oda, et al. (2010). "The molecular chaperone Hsp90 regulates accumulation of DNA polymerase eta at replication stalling sites in UV-irradiated cells." Mol Cell **37**(1): 79-89.

Shah, N. and D. S. Dizon (2009). "New-generation platinum agents for solid tumors." Future Oncol **5**(1): 33-42.

Shahbaz Sarwar, C. M., J. D. Luketich, et al. (2010). "Esophageal cancer: an update." Int J Surg **8**(6): 417-22.

Shahi, A., J. H. Lee, et al. (2011). "Mismatch-repair protein MSH6 is associated with Ku70 and regulates DNA double-strand break repair." Nucleic Acids Res **39**(6): 2130-43.

Sharma, S., J. K. Hicks, et al. (2011). "REV1 and polymerase {zeta} facilitate homologous recombination repair." Nucleic Acids Res.

Shen, X., S. Jun, et al. (2006). "REV3 and REV1 play major roles in recombination-independent repair of DNA interstrand cross-links mediated by monoubiquitinated proliferating cell nuclear antigen (PCNA)." J Biol Chem **281**(20): 13869-72.

Shirota, Y., J. Stoehlmacher, et al. (2001). "ERCC1 and thymidylate synthase mRNA levels predict survival for colorectal cancer patients receiving combination oxaliplatin and fluorouracil chemotherapy." J Clin Oncol **19**(23): 4298-304.

Silva, P., N. J. Slevin, et al. (2008). "Prognostic significance of tumor hypoxia inducible factor-1alpha expression for outcome after radiotherapy in oropharyngeal cancer." Int J Radiat Oncol Biol Phys **72**(5): 1551-9.

Silverstein, T. D., R. E. Johnson, et al. (2010). "Structural basis for the suppression of skin cancers by DNA polymerase eta." Nature **465**(7301): 1039-43.

Sinclair, W. K. and R. A. Morton (1966). "X-ray sensitivity during the cell generation cycle of cultured Chinese hamster cells." Radiat Res **29**(3): 450-74.

Skoneczna, A., J. McIntyre, et al. (2007). "Polymerase eta is a short-lived, proteasomally degraded protein that is temporarily stabilized following UV irradiation in Saccharomyces cerevisiae." J Mol Biol **366**(4): 1074-86.

Soria, G., L. Belluscio, et al. (2009). "DNA damage induced Pol eta recruitment takes place independently of the cell cycle phase." Cell Cycle **8**(20): 3340-8.

Spingler, B., D. A. Whittington, et al. (2001). "2.4 A crystal structure of an oxaliplatin 1,2-d(GpG) intrastrand cross-link in a DNA dodecamer duplex." Inorg Chem **40**(22): 5596-602.

Srivastava, S. (2007). "Cancer biomarker discovery and development in gastrointestinal cancers: early detection research network-a collaborative approach." Gastrointest Cancer Res **1**(4 Suppl 2): S60-3.

Staresincic, L., A. F. Fagbemi, et al. (2009). "Coordination of dual incision and repair synthesis in human nucleotide excision repair." EMBO J **28**(8): 1111-20.

Stary, A., P. Kannouche, et al. (2003). "Role of DNA polymerase eta in the UV mutation spectrum in human cells." J Biol Chem **278**(21): 18767-75.

Stordal, B., N. Pavlakis, et al. (2007). "Oxaliplatin for the treatment of cisplatin-resistant cancer: a systematic review." Cancer Treat Rev **33**(4): 347-57.

Sugasawa, K., J. M. Ng, et al. (1998). "Xeroderma pigmentosum group C protein complex is the initiator of global genome nucleotide excision repair." Mol Cell **2**(2): 223-32.

Surowiak, P., V. Materna, et al. (2007). "Nuclear metallothionein expression correlates with cisplatin resistance of ovarian cancer cells and poor clinical outcome." Virchows Arch **450**(3): 279-85.

Tamulevicius, P., M. Wang, et al. (2007). "Homology-directed repair is required for the development of radioresistance during S phase: interplay between double-strand break repair and checkpoint response." Radiat Res **167**(1): 1-11.

Tanioka, M., T. Masaki, et al. (2007). "Molecular analysis of DNA polymerase eta gene in Japanese patients diagnosed as xeroderma pigmentosum variant type." J Invest Dermatol **127**(7): 1745-51.

Teng, K. Y., M. Z. Qiu, et al. (2010). "DNA polymerase eta protein expression predicts treatment response and survival of metastatic gastric adenocarcinoma patients treated with oxaliplatin-based chemotherapy." J Transl Med **8**: 126.

Thakur, M., M. Wernick, et al. (2001). "DNA polymerase eta undergoes alternative splicing, protects against UV sensitivity and apoptosis, and suppresses Mre11-dependent recombination." Genes Chromosomes Cancer **32**(3): 222-35.

Timerbaev, A. R., C. G. Hartinger, et al. (2006). "Interactions of antitumor metallodrugs with serum proteins: advances in characterization using modern analytical methodology." Chem Rev **106**(6): 2224-48.

Tomkinson, A. E., L. Chen, et al. (2001). "Completion of base excision repair by mammalian DNA ligases." Prog Nucleic Acid Res Mol Biol **68**: 151-64.

Tornaletti, S. (2009). "DNA repair in mammalian cells: Transcription-coupled DNA repair: directing your effort where it's most needed." Cell Mol Life Sci **66**(6): 1010-20.

Tozawa, K., T. Oshima, et al. (2008). "Oxaliplatin in treatment of the cisplatin-resistant MKN45 cell line of gastric cancer." Anticancer Res **28**(4B): 2087-92.

Trott, K. R. (1982). "Experimental results and clinical implications of the four R's in fractionated radiotherapy." Radiat Environ Biophys **20**(3): 159-70.

Urita, Y., H. Komuro, et al. (2007). "Regeneration of the esophagus using gastric acellular matrix: an experimental study in a rat model." Pediatr Surg Int **23**(1): 21-6.

Vaisman, A., C. Masutani, et al. (2000). "Efficient translesion replication past oxaliplatin and cisplatin GpG adducts by human DNA polymerase eta." Biochemistry **39**(16): 4575-80.

Vaisman, A., M. Varchenko, et al. (1998). "The role of hMLH1, hMSH3, and hMSH6 defects in cisplatin and oxaliplatin resistance: correlation with replicative bypass of platinum-DNA adducts." Cancer Res **58**(16): 3579-85.

Valk, K., T. Vooder, et al. (2010). "Gene expression profiles of non-small cell lung cancer: survival prediction and new biomarkers." Oncology **79**(3-4): 283-92.

van der Vijgh, W. J. (1991). "Clinical pharmacokinetics of carboplatin." Clin Pharmacokinet **21**(4): 242-61.

van der Zee, A. G., H. Hollema, et al. (1995). "Value of P-glycoprotein, glutathione S-transferase pi, c-erbB-2, and p53 as prognostic factors in ovarian carcinomas." J Clin Oncol **13**(1): 70-8.

van Loon, B., E. Markkanen, et al. (2010). "Oxygen as a friend and enemy: How to combat the mutational potential of 8-oxo-guanine." DNA Repair (Amst) **9**(6): 604-16.

van Veelen, L. R., T. Cervelli, et al. (2005). "Analysis of ionizing radiation-induced foci of DNA damage repair proteins." Mutat Res **574**(1-2): 22-33.

Vens, C. and A. C. Begg (2010). "Targeting base excision repair as a sensitization strategy in radiotherapy." Semin Radiat Oncol **20**(4): 241-9.

Vens, C., E. Dahmen-Mooren, et al. (2002). "The role of DNA polymerase beta in determining sensitivity to ionizing radiation in human tumor cells." Nucleic Acids Res **30**(13): 2995-3004.

Vens, C., I. Hofland, et al. (2007). "Involvement of DNA polymerase beta in repair of ionizing radiation damage as measured by in vitro plasmid assays." Radiat Res **168**(3): 281-91.

Voss, M. J., B. Niggemann, et al. (2010). "Tumour reactions to hypoxia." Curr Mol Med **10**(4): 381-6.

Wahl, R. L., H. Jacene, et al. (2009). "From RECIST to PERCIST: Evolving Considerations for PET response criteria in solid tumors." J Nucl Med **50 Suppl 1**: 122S-50S.

Wang, A. T., B. Sengerova, et al. (2011). "Human SNM1A and XPF-ERCC1 collaborate to initiate DNA interstrand cross-link repair." Genes Dev **25**(17): 1859-70.

Wang, D. and S. J. Lippard (2005). "Cellular processing of platinum anticancer drugs." Nat Rev Drug Discov **4**(4): 307-20.

Wang, G., E. Reed, et al. (2004). "Molecular basis of cellular response to cisplatin chemotherapy in non-small cell lung cancer (Review)." Oncol Rep **12**(5): 955-65.

Wang, H., W. Wu, et al. (2010). "Analysis of specialized DNA polymerases expression in human gliomas: association with prognostic significance." Neuro Oncol.

Wang, Y., R. Woodgate, et al. (2007). "Evidence that in xeroderma pigmentosum variant cells, which lack DNA polymerase eta, DNA polymerase iota causes the very high frequency and unique spectrum of UV-induced mutations." Cancer Res **67**(7): 3018-26.

Washington, M. T., R. E. Johnson, et al. (2001). "Accuracy of lesion bypass by yeast and human DNA polymerase eta." Proc Natl Acad Sci U S A **98**(15): 8355-60.

Washington, M. T., R. E. Johnson, et al. (2002). "Human DINB1-encoded DNA polymerase kappa is a promiscuous extender of mispaired primer termini." Proc Natl Acad Sci U S A **99**(4): 1910-4.

Washington, M. T., R. E. Johnson, et al. (2000). "Accuracy of thymine-thymine dimer bypass by Saccharomyces cerevisiae DNA polymerase eta." Proc Natl Acad Sci U S A **97**(7): 3094-9.

Watanabe, K., S. Tateishi, et al. (2004). "Rad18 guides poleta to replication stalling sites through physical interaction and PCNA monoubiquitination." EMBO J **23**(19): 3886-96.

Waters, L. S., B. K. Minesinger, et al. (2009). "Eukaryotic translesion polymerases and their roles and regulation in DNA damage tolerance." Microbiol Mol Biol Rev **73**(1): 134-54.

Welsh, J. W., R. K. Ellsworth, et al. (2009). "Rad51 protein expression and survival in patients with glioblastoma multiforme." Int J Radiat Oncol Biol Phys **74**(4): 1251-5.

William-Faltaos, S., D. Rouillard, et al. (2006). "Cell cycle arrest and apoptosis induced by oxaliplatin (L-OHP) on four human cancer cell lines." Anticancer Res **26**(3A): 2093-9.

Wilson, P. F., J. M. Hinz, et al. (2010). "Influence of homologous recombinational repair on cell survival and chromosomal aberration induction during the cell cycle in gamma-irradiated CHO cells." DNA Repair (Amst) **9**(7): 737-44.

Wood, R. D. (2010). "Mammalian nucleotide excision repair proteins and interstrand crosslink repair." Environ Mol Mutagen **51**(6): 520-6.

Woynarowski, J. M., W. G. Chapman, et al. (1998). "Sequence- and region-specificity of oxaliplatin adducts in naked and cellular DNA." Mol Pharmacol **54**(5): 770-7.

Woynarowski, J. M., S. Faivre, et al. (2000). "Oxaliplatin-induced damage of cellular DNA." Mol Pharmacol **58**(5): 920-7.

Wu, B., P. Droge, et al. (2008). "Site selectivity of platinum anticancer therapeutics." Nat Chem Biol **4**(2): 110-2.

Wu, Y., D. Bhattacharyya, et al. (2007). "Solution structures of a DNA dodecamer duplex with and without a cisplatin 1,2-d(GG) intrastrand cross-link: comparison with the same DNA duplex containing an oxaliplatin 1,2-d(GG) intrastrand cross-link." Biochemistry **46**(22): 6477-87.

Wu, Y., P. Pradhan, et al. (2004). "NMR solution structure of an oxaliplatin 1,2-d(GG) intrastrand cross-link in a DNA dodecamer duplex." J Mol Biol **341**(5): 1251-69.

Wulfing, C., H. van Ahlen, et al. (2007). "Metallothionein in bladder cancer: correlation of overexpression with poor outcome after chemotherapy." World J Urol **25**(2): 199-205.

Xie, K., J. Doles, et al. (2010). "Error-prone translesion synthesis mediates acquired chemoresistance." Proc Natl Acad Sci U S A **107**(48): 20792-7.

Yamamoto, J., D. Loakes, et al. (2008). "Translesion synthesis across the (6-4) photoproduct and its Dewar valence isomer by the Y-family and engineered DNA polymerases." Nucleic Acids Symp Ser (Oxf)(52): 339-40.

Yang, J., Z. Chen, et al. (2004). "Altered DNA polymerase iota expression in breast cancer cells leads to a reduction in DNA replication fidelity and a higher rate of mutagenesis." Cancer Res **64**(16): 5597-607.

Yang, W. (2003). "Damage repair DNA polymerases Y." Curr Opin Struct Biol **13**(1): 23-30.

Ying, S., K. Myers, et al. (2009). "BRCA2-dependent homologous recombination is required for repair of Arsenite-induced replication lesions in mammalian cells." Nucleic Acids Res **37**(15): 5105-13.

Yoon, J. H., L. Prakash, et al. (2010). "Error-free replicative bypass of (6-4) photoproducts by DNA polymerase zeta in mouse and human cells." Genes Dev **24**(2): 123-8.

Yu, C. C., A. L. Woods, et al. (1992). "The assessment of cellular proliferation by immunohistochemistry: a review of currently available methods and their applications." Histochem J **24**(3): 121-31.

Zan, H., J. Zhang, et al. (2009). "Lupus-prone MRL/faslpr/lpr mice display increased AID expression and extensive DNA lesions, comprising deletions and insertions, in the immunoglobulin locus: concurrent upregulation of somatic hypermutation and class switch DNA recombination." Autoimmunity **42**(2): 89-103.

Zdraveski, Z. Z., J. A. Mello, et al. (2002). "MutS preferentially recognizes cisplatin- over oxaliplatin-modified DNA." J Biol Chem **277**(2): 1255-60.

Zhang, F., Q. Fan, et al. (2009). "PALB2 functionally connects the breast cancer susceptibility proteins BRCA1 and BRCA2." Mol Cancer Res **7**(7): 1110-8.

Zhang, Y., F. Yuan, et al. (2000). "Error-prone lesion bypass by human DNA polymerase eta." Nucleic Acids Res **28**(23): 4717-24.

Zhao, J., A. Jain, et al. (2009). "Mismatch repair and nucleotide excision repair proteins cooperate in the recognition of DNA interstrand crosslinks." Nucleic Acids Res **37**(13): 4420-9.

Zhong, X., P. Garg, et al. (2006). "The fidelity of DNA synthesis by yeast DNA polymerase zeta alone and with accessory proteins." Nucleic Acids Res **34**(17): 4731-42.

Zhou, J., S. Zhang, et al. (2012). "Over-expression of DNA Polymerase iota (Pol iota) in Esophageal Squamous Cell Carcinoma." Cancer Sci.

Zhuang, W., B. Li, et al. (2011). "Knockdown of the DNA-dependent protein kinase catalytic subunit radiosensitizes glioma-initiating cells by inducing autophagy." Brain Res **1371**: 7-15.

Zhuang, Z., R. E. Johnson, et al. (2008). "Regulation of polymerase exchange between Poleta and Poldelta by monoubiquitination of PCNA and the movement of DNA polymerase holoenzyme." Proc Natl Acad Sci U S A **105**(14): 5361-6.

Zietlow, L., L. A. Smith, et al. (2009). "Evidence for the involvement of human DNA polymerase N in the repair of DNA interstrand cross-links." Biochemistry **48**(49): 11817-24.

APPENDICES

Nicolay, N. H., Berry, D. P., Sharma, R. A. **Liver metastases from colorectal cancer: radioembolization with systemic therapy**. Nat Rev Clin Oncol. 2009 Dec;6(12):687-97.

Yang, J., Parsons, J., Nicolay, N. H., Caporali, S., Harrington, C. F., Singh, R., Finch, D., D'Atri, S., Farmer, P. B., Johnston, P. G., McKenna, W. G., Dianov, G., Sharma, R. A. **Cells deficient in the base excision repair protein, DNA polymerase beta, are hypersensitive to oxaliplatin chemotherapy**. Oncogene. 2010 Jan 21;29(3):463-8.

Nicolay, N. H., Helleday, T., Sharma, R. A. **Biological relevance of DNA polymerase β and translesion synthesis polymerases to cancer and its treatment**. Curr Mol Pharmacol. 2012 Jan;5(1):54-67.

Hill, E. J., Nicolay, N. H., Middleton, M. R., Sharma, R. A. **Oxaliplatin as a radiosensitiser for upper and lower gastrointestinal tract malignancies: What have we learned from a decade of translational research**? Crit Rev Oncol Hematol. 2012 Sep;83(3):353-87.

Parsons, J. L., Nicolay, N. H., Sharma, R. A. **Biological and therapeutic relevance of non-replicative DNA polymerases to cancer**. Antioxid Redox Signal. 2012 Sep 5. [Epub ahead of print]

Nicolay, N. H., Carter, R., Hatch, S. B., Schultz, N., Prevo, R., McKenna, W. G., Helleday, T., Sharma, R. A. **Homologous recombination mediates S-phase-dependent radioresistance in cells deficient in DNA polymerase eta**. Carcinogenesis. 2012 Nov;33(11):2026-34.