Regulation of the Base Excision Repair Pathway

A thesis presented for the degree of Doctor of Philosophy

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Declaration

I declare that this thesis is wholly my own work apart from the following figures:

Experimental data presented in Figure 4.3 and Figure 6.11 was generated in close collaboration with Dr. Mattia Poletto.

The qPCR analysis presented in Figure 6.8 was carried out by Dr. Mattia Poletto using experimental samples generated by me.

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Abstract

Maintenance of genomic stability is paramount for survival of an organism; failure to repair DNA damage ultimately leads to the accumulation of genetically unstable cells and the onset of different human diseases including cancer. DNA single strand breaks and base oxidation/alkylation are among the most frequent types of DNA damage occurring spontaneously in cells. Base excision repair (BER), which copes with the majority of these lesions, is therefore a fundamental DNA repair system. Accordingly, it is important to understand how BER is regulated, and particularly, how and if BER is affected by the cellular load of DNA damage.

Although functions of key BER proteins are well-defined, regulation of their expression is poorly understood. During BER, the protein XRCC1 is particularly important. It functions as a scaffold, stabilising repair complexes at sites of DNA damage thereby promoting efficient DNA repair. As a central coordinator in BER, it is therefore of great interest to understand how expression of XRCC1 is controlled. In this thesis I demonstrate that modulation of XRCC1 expression is mediated by transcription factor Sp1.

Importantly, Sp1 is also affected during the DNA damage response, suggesting an indirect mechanism promoting BER modulation in response to the cellular DNA damage load. In fact, I show that, in response to persistent DNA strand breaks, the key DNA damage signalling factor ATM phosphorylates Sp1. This initiates Sp1 degradation, negatively affecting BER. Therefore, this thesis identifies a mechanism involving signalling from ATM that regulates BER in response to persistent DNA damage, which I link to susceptibility to apoptosis and cell elimination.

I hypothesise that regulation of DNA repair in response to persistent DNA damage constitutes a mechanism to promote the elimination of potentially pre-cancerous cells that accumulate unrepairable levels of DNA lesions.

Table of contents

List of figures

List of tables

Abbreviations

8-oxo-dG	7,8-dihydro-8-oxoguanine
AOA	Ataxia with oculomotor apraxia
AP	Apurinic/apyrimidinic (site)
APE1	Apurinic/apyrimidinic endonuclease 1
APTX	Aprataxin
ARF	p14 alternative reading frame
ATM	Ataxia telangiectasia mutated
ATR	Ataxia telangiectasia and Rad3-related
BER	Base excision repair
BRCT	BRCA1 C Terminus
BSA	Bovine serum albumin
CHIP	Carboxyl terminus of Hsc70 interacting protein
ChIP	Chromatin Immunoprecipitation
Chk1	Checkpoint kinase 1
Chk2	Checkpoint kinase 2
CHO	Chinese hamster ovary
CK2	Casein kinase II
Cmap	Connectivity map
CS	Cockayne syndrome
DDR	DNA damage response
DHFR	Dihydrofolate reductase
DNA-PKcs	DNA-dependent protein kinase catalytic subunit
dRP	Deoxyribosephosphate
DSBs	Double strand breaks
DTT	Dithiothreitol
EDTA	Ethylenediaminetetraacetic acid
EMSA	Electrophoretic Mobility Shift Assay
FA	Fanconi Anaemia
FBS	Foetal Bovine Serum
FEN-1	Flap endonuclease 1
FOXM1	Forkhead box protein M1
FPLC	Fast protein liquid chromatography
GG-NER	Global genome nucleotide excision repair
GSK3β	Glycogen synthase kinase-3 β
H_2O_2	Hydrogen peroxide
HIPK2	Homeodomain-interacting protein kinase 2
HR	Homologous recombination
IPTG	Isopropyl β-D-1-thiogalactopyranoside
IR	Ionising radiation
JNK1	Jun N-terminal kinase 1
KLF	Krüppel-like factor
Lig I	DNA ligase I
Lig III	DNA ligase IIIα
LP	Long patch
MDC1	Mediator of DNA damage checkpoint 1

MEF	Mouse embryonic fibroblast
MGMT	O^6-methylguanine-DNA methyltransferase
MMR	Mismatch repair
MMS	Methyl methanesulfonate
MPG	3-methylpurine-DNA glycosylase
MRN	MRE11-RAD50-NBS1
Mule	Mcl-1 ubiquitin ligase E3
NEM	N-Ethylmaleimide
NER	Nucleotide excision repair
NHEJ	Non-homologous end joining
NK	Natural killer
NLS	Nuclear localisation signal
NTD	N-terminal domain
O^6-meG	O^6-methylguanine
OGG1	8-oxoguanine DNA glycosylase
PAGE	Polyacrylamide gel electrophoresis
PARP1	Poly(ADP-ribose) polymerase
PCNA	Proliferating cell nuclear antigen
PIKK	Phosphatidylinositol 3-kinase-related-kinase
PMSF	Phenylmethylsulfonyl fluoride
PNKP	Polynucleotide kinase 3'-phosphatase
Pol β	DNA polymerase β
PTM	Post translational modification
RFC	Replication factor C
RNF4	Ring finger protein 4
ROS	Reactive oxygen species
RPA	Replication protein A
SCAN-1	Spinocerebellar ataxia with axonal neuropathy
SDS	Sodium dodecylsulfate
SP	Short patch
Sp1	Specificity protein 1
SSB	Single strand break
SSBR	Single strand break repair
TAD	Transactivation domain
TC-NER	Transcription-coupled nucleotide excision repair
TDP-1	Tyrosyl-DNA-phosphodiesterase
TFIIH	Transcription factor II H
TOP1	DNA topoisomerase I
UNG	Uracil DNA glycosylase
UV	Ultraviolet
XP	Xeroderma pigmentosum
XRCC1	X-ray cross complementing 1
β-TRCP	β-transducin repeat-containing protein

1 Introduction

In this thesis the role of transcription factor Sp1 in regulating the base excision repair (BER) pathway will be discussed. For this reason the introduction will give an overview of DNA damage and repair, focussing particularly on BER. As we hypothesise a central role for the ATM kinase in the mechanism that regulates BER, the role of this molecule in the DNA damage response will also be discussed. Furthermore, Sp1 will be examined particularly with respect to its proposed functions in DNA repair.

1.1 DNA damage and repair

The genetic code required for an organism to exist is stored in an intrinsically unstable molecule called DNA. Maintaining the integrity of DNA is paramount for cell survival, however, there exists a constant threat of genome modification from reactive molecules of both endogenous and exogenous origin. By-products of cellular metabolism for example, exposure to ultraviolet light (UV) and the use of chemotherapeutic drugs all expose our genome to potentially mutagenic DNA modifications. If allowed to persist, DNA damage can cause mutagenesis, including base substitutions, insertions, deletions and chromosomal rearrangements; these changes can directly, or progressively, lead to disease, especially cancer, neurodegeneration and premature ageing. Consequently, constant genome maintenance is essential to ensure the continued viability and longevity of organisms. In order to avoid such deleterious outcomes, cells have evolved mechanisms collectively termed the DNA damage response (DDR),

which includes different DNA repair systems, to resolve DNA lesions and restore genome integrity. This section will introduce the concept of DNA damage and briefly review our current knowledge of different DNA repair pathways, discussing the types of lesions that each repair system routinely deals with.

The chemistry of the cellular environment is such that the genomes of all organisms are continuously modified by endogenously produced molecules and by spontaneous chemical reactions resulting from the inherent instability of DNA. In fact, it is estimated that ~10^5 DNA lesions occur in a single mammalian cell per day (Lindahl, 1993) as a result of spontaneous decay, cellular metabolism and replication errors. Of these, reactive oxygen species (ROS) generated as a by-product of aerobic respiration, are considered a major source of endogenous DNA lesions (De Bont and van Larebeke, 2004). Furthermore, everyday exposure to exogenous agents also poses a severe threat to genomic stability; significant environmental DNA-damaging agents include UV light and ionising radiation (IR). In fact, it is reported that a single day in the sun may induce up to 10^5 photoproducts per exposed cell (Hoeijmakers, 2009). The spectrum of DNA damage types that can occur in cells is vast, although the lesions can be broadly divided into (i) single base alterations (e.g. oxidation, alkylation, generation of abasic sites, base mispairing), (ii) breakage in the DNA phosphate backbone (single and double strand breaks), (iii) inter/intra-strand crosslinks and (iv) DNA-protein crosslinks. Figure 1.1 gives an overview of the key DNA repair systems and the lesions that each repairs.

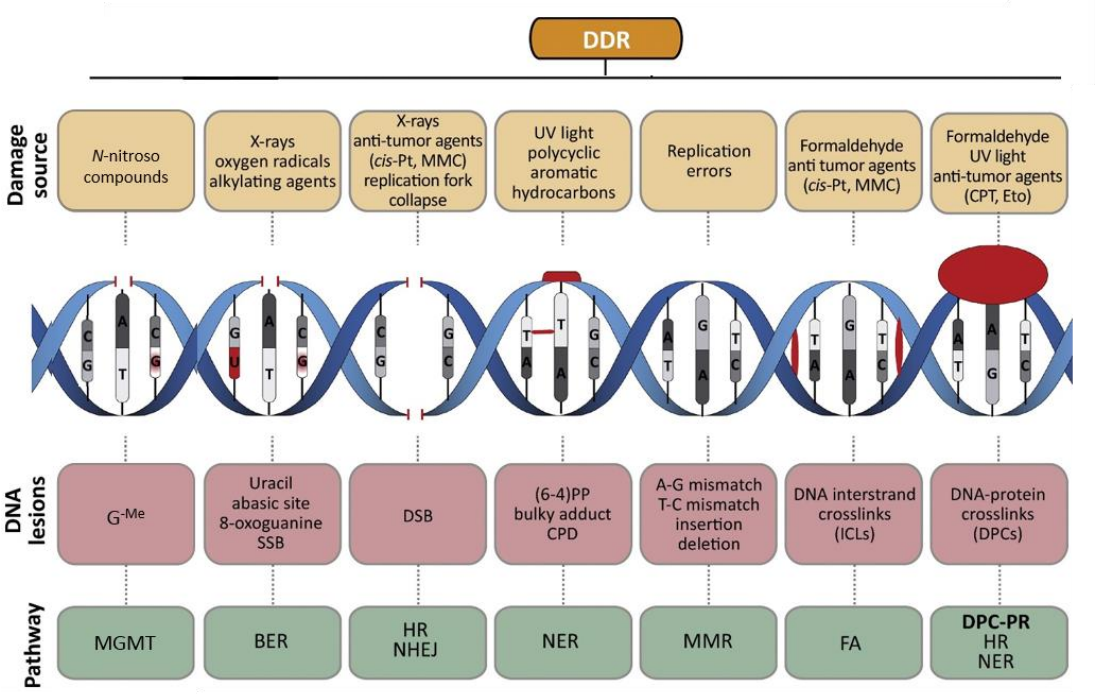

Figure 1.1 Schematic representation of major DNA repair pathways operating in eukaryotic cells.

Specialised DNA repair pathways cope with specific DNA lesions. DDR, DNA damage response; *cis*-Pt, cisplatin; CPD, cyclobutane pyrimidine dimers; CPT, camptothecin; Eto, etoposide; MMC, mitomycin C; DDR, DNA damage response; G^{-Me}, O^6-methylguanine; SSB, single strand break; DSB, double strand break, BER, base excision repair; DPC-PR, DNA-protein crosslink proteolysis repair FA, Fanconi anaemia; HR, Homologous recombination; MGMT, O^6-methylguanine-DNA methyl transferase; MMR, mismatch repair; NER, nucleotide excision repair; NHEJ, non-homologous end joining; Adapted from:(Vaz et al., 2017).

1.1.1 Direct reversal: O^6-methylguanine-DNA methyltransferase (MGMT)

Most of the DNA repair systems operating in mammalian cells are organised into multi-step pathways. The exception to this, however, is the repair of O^6-alkylguanine adducts such as O^6-methylguanine (O^6-meG), which is commonly generated by exposure to *N*-nitroso compounds such as those found in processed meat (Tricker, 1997). O^6-meG is particularly dangerous as it can block DNA replication (Ceccotti et al., 1993), affect cytosine methylation (Hepburn et al., 1991) and mispairs with thymine causing G:C to A:T transitions in DNA (Esteller and Herman, 2004). To defend against these adducts, cells are able to

directly reverse the lesion through the action of the suicide enzyme MGMT, which transfers the aberrant alkyl group to a cysteine residue within its own active site. This restores the guanosine to an undamaged state while inactivating the MGMT protein, which is then degraded (Xu-Welliver and Pegg, 2002). Direct lesion reversal by a single enzyme, however, is a rare mechanism in mammalian cells that is only recognised for a small subset of methylated DNA lesions (Sedgwick et al., 2007). In response to other types of DNA damage, therefore, cells must instead rely on the coordinated action of several enzymes operating within a pathway.

1.1.2 Nucleotide Excision Repair (NER)

Bulky DNA lesions that lead to a distortion in the helical structure of duplex DNA will often impede progression of DNA and RNA polymerases resulting in replication fork collapse or stalled transcription (Gillet and Scharer, 2006). Typically, these lesions are repaired through NER. Broadly, NER is separated into two pathways – global-genome NER (GG-NER), and the more specialised transcription-coupled NER (TC-NER), which deals specifically with lesions blocking RNA polymerase progression. The pathways are thought to differ at the recognition step, utilising the same machinery to carry out the repair steps (Chatterjee and Walker, 2017). DNA adducts commonly repaired by NER include cyclobutane pyrimidine dimers and other complex photo-adducts resulting from exposure to environmental mutagens e.g. UV light or chemotherapeutic compounds such as cisplatin. Equally, helix-distorting lesions can arise from endogenous sources including products of lipid peroxidation (malendialdehyde-related pyrimidopurinone adducts) or ROS, in the case of cyclopurine lesions.

Defects in NER lead to the human diseases Xeroderma Pigmentosum (XP) and Cockayne Syndrome (CS), which are both associated with UV sensitivity (Rapin et al., 2000). Briefly, NER involves four major steps: (i) damage recognition, (ii) incisions flanking the DNA lesion and subsequent removal of an oligonucleotide fragment spanning several nucleotides around the site of damage, (iii) gap filling with undamaged nucleotides, (iv) ligation to seal the remaining nick. During GG-NER, the XPC-RAD23B complex initiates repair after recognition of a helix-distorting lesion by binding to the DNA strand opposite the lesion. XPC-RAD23B then mediates the recruitment of the transcription factor II H (TFIIH) complex. This contains ten subunits, including the two helicases XPB and XPD. Through the activity of these helicase subunits, TFIIH promotes denaturation of the DNA duplex surrounding the lesion, facilitating the recruitment of XPA and replication protein A (RPA). Two endonucleases are then recruited: the XPF-ERCC1 complex through direct interaction with XPA, and XPG by interaction with TFIIH. These endonucleases remove an oligonucleotide fragment containing the DNA lesion by cleaving 5' (XPF-ERCC1) and 3' (XPG) to the site of damage. Gap filling is carried out by DNA polymerases δ,ε or κ in cooperation with Proliferating Cell Nuclear Antigen (PCNA) and Replication Factor C (RFC). Finally, the nick is sealed either by the X-ray cross complementing 1-DNA ligase IIIα (XRCC1-Lig III) complex or a Flap endonuclease 1-DNA ligase I (FEN-1-Lig I) complex (Iyama and Wilson, 2013). TC-NER, on the other hand, is thought to be initiated by stalling of an RNA polymerase on the transcribed strand of an active gene. This serves as a signal for engagement of the CS proteins, CSA and CSB, which facilitate damage removal and the restoration of transcription (Fousteri et al., 2006). After recruitment of the CS proteins, the same protein machinery as

described for GG-NER, starting from recruitment of the TFIIH complex, is believed to execute the lesion excision, gap-filling and nick ligation stages of repair (Iyama and Wilson, 2013).

1.1.3 Mismatch Repair (MMR)

DNA base mismatches and insertion-deletion loops are primarily removed using MMR. These lesions arise mainly from errors during DNA replication or deleterious repair intermediates resulting from homologous recombination (HR) (Jiricny, 2006, Modrich, 2006). In fact, MMR is critical for increasing DNA replication fidelity by preventing base substitutions. To preserve genome integrity, it is imperative that MMR operates only on the newly synthesised strand of DNA, which contains the mispaired base. In eukaryotes, strand discrimination is thought mainly to be achieved through association of the MMR apparatus with the cellular replication machinery (Wagner and Meselson, 1976). MMR involves four major steps (i) damage recognition, (ii) exonuclease resection of the region flanking the mismatch (iii) resynthesis to correct the mismatch (iv) ligation, to seal the remaining nick. Damage recognition is carried out by the MutS complexes MutSα and MutSβ, (Iyama and Wilson, 2013). Following recognition, a MutL complex, either MutLα or MutLγ depending on damage type, is recruited. These complexes carry out the initial incision step on the appropriate strand as well as organising other proteins at the damage site. After incision, PCNA coordinates with exonuclease Exo1 to excise the mismatch, generating a gap. The multi-nucleotide gap is then filled by DNA polymerase δ, and the nick is sealed by Lig I (Larrea et al., 2010).

1.1.4 Base Excision Repair (BER)

The BER pathway copes with small, non-bulky lesions that do not induce major distortion in the structure of the DNA double helix. As DNA bases are highly vulnerable to chemical modification, base lesions occur very frequently in eukaryotic cells. This makes the BER pathway especially important to prevent DNA damage accumulation. Examples of BER substrates include abasic (AP) sites, single strand breaks (SSBs) and base modifications such as oxidative and alkylation damage. Many oxidative lesions, including 7,8-dihydro-8-oxoguanine (8-oxo-dG) result from attack by ROS produced during normal mitochondrial respiration. For example, the superoxide anion radical ($O_2^{\cdot -}$), hydrogen peroxide (H_2O_2) and the hydroxyl radical ($^{\cdot}OH$) are all significant intracellular ROS; side reactions between the electron transport chain and molecular oxygen have been estimated to produce superoxide at a rate of 1-2% of the total oxygen consumption per day (Murphy, 2009, Abbotts and Wilson, 2017).

Broadly, BER can be divided into five main steps: (i) damage recognition and removal of the DNA lesion, (ii) backbone incision, (iii) end processing, (iv) gap filling, (v) ligation of the remaining nick in the DNA backbone. As the BER pathway is central to this thesis, the molecular steps of BER, and its regulation, will be explored in much greater detail in Section 1.4.

1.1.5 Double Strand Break Repair

DNA strand breaks, particularly double strand breaks (DSBs), are among the most deleterious forms of DNA damage that cells experience, as they are both difficult to repair and highly toxic. In fact, it has been calculated that a single DSB is severe enough to induce cell cycle arrest in a eukaryotic cell (Huang et al., 1996). Although a rare event, DSBs can arise endogenously from molecules such as ROS, or clustered SSBs. They can also be caused by a collision between the DNA replication or transcription machinery and unrepaired DNA lesions (Kuzminov, 2001). Exogenous sources of DSBs include IR and some anti-cancer chemotherapeutic agents. In order to cope with danger posed by DSBs, cells have evolved two major mechanisms – non-homologous end joining (NHEJ) and HR. An overview of each pathway is shown in Figure 1.2. Competition occurs between the DSB recognition complexes from NHEJ and HR, with pathway choice mainly dictated by the cell cycle phase, although the fine molecular details are not currently understood. NHEJ can operate during any phase in the cell cycle, whereas HR predominates during S and G_2 phases. Canonical NHEJ is initiated by the Ku70/Ku80 heterodimer binding directly to the broken DNA ends; it serves as a scaffold to engage other NHEJ factors such as DNA-dependent protein kinase catalytic subunit (DNA-PKcs). Binding of DNA-PKcs leads to the recruitment of components such as Artemis and polynucleotide kinase 3'-phosphatase (PNKP), which carry out end processing and resection. Gaps are then filled either by DNA polymerase μ or λ. Finally, DNA ligase IV in complex with XRCC4 carries out the end joining step (Iyama and Wilson, 2013). Due to the end processing step, NHEJ is often error-prone, resulting in some loss of genetic information at the site of the DSB. In contrast, HR is commonly

considered to be error-free as it uses the homologous sister chromatid as the template for repair. It is used to restart stalled replication forks, as well as to repair interstrand crosslinks (ICLs). In general, HR is initiated by the MRN (Mre-11-Rad50-Nbs-1) complex recognising a DSB. Binding of CtIP (C-terminal binding protein interacting protein) then promotes end resection to generate the 3' single stranded DNA overhang required for strand invasion. The 3' overhang is then stabilised by RPA binding, before RPA is subsequently displaced by Rad51. Rad51-coated ssDNA then invades the intact homologous DNA region and a D loop is created. Following D loop formation, the DSB is resolved via one of two distinct mechanisms - formation of a Holliday junction, or through the synthesis-dependent strand annealing pathway (Chatterjee and Walker, 2017).

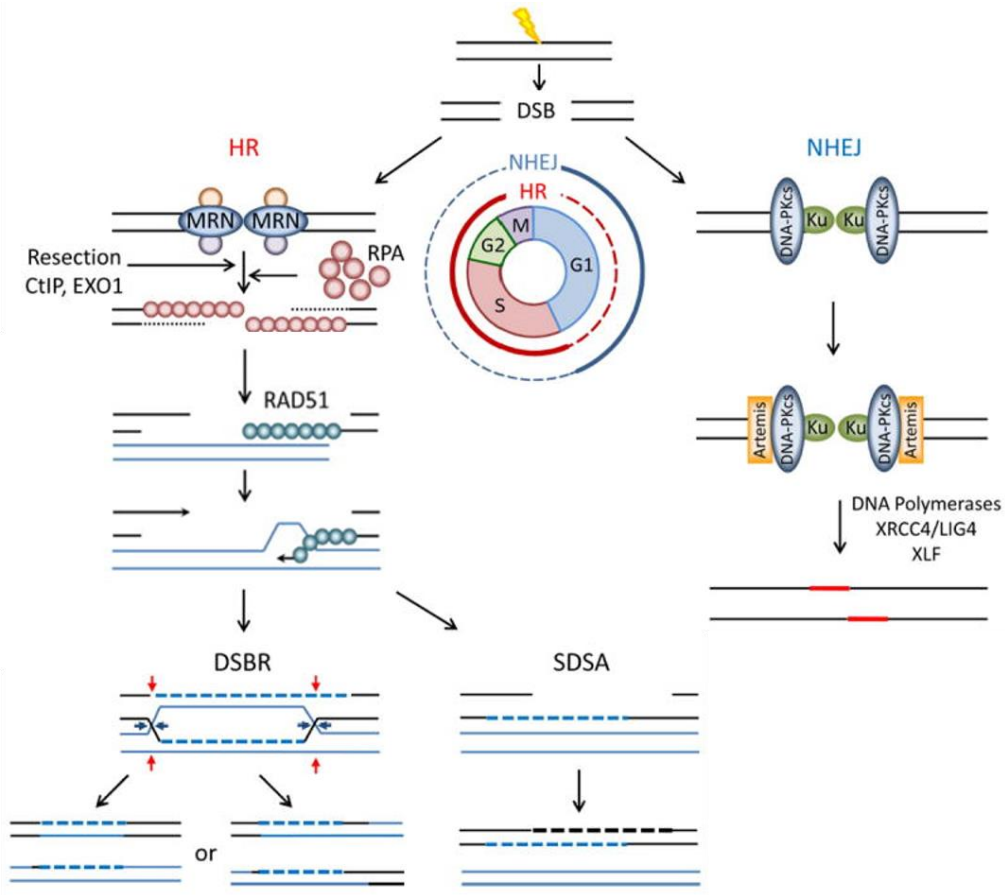

Figure 1.2 Schematic of DSBR mechanisms HR and NHEJ.

DSBR is divided into two major pathways: HR and NHEJ. HR operates during S phase, whereas NHEJ functions in all cell cycle phases. HR is initiated by recognition of a DSB by the MRN complex (Mre11-Rad50-Nbs1). Binding of CtIP initiates 5′–3′ end resection to create a 3′ ssDNA overhang. Further resection is carried out by exonucleases (possibly EXO1), and the resulting ssDNA is stabilised by RPA coating. RPA is subsequently displaced by Rad51 to form Rad51 nucleoprotein filaments, enabling strand invasion of the intact homologous DNA region. In classical DSBR, the second DSB end may be captured by the D-loop resulting in an intermediate with double Holliday junctions. This leads to non-crossover (cleavage at blue arrows) or crossover (cleavage at blue arrows on one side and red arrows on other side) products. In SDSA, the newly synthesised strand is displaced to permit annealing to the other DSB end. This results in a non-crossover product. Canonical NHEJ is initiated by the Ku70/Ku80 heterodimer binding directly to broken DNA ends followed by recruitment of DNA-PKcs. DNA-PKcs activates Artemis, which generates terminal overhangs prior to ligation. Gap-filling is then performed before end joining by XRCC4–LIG4 in collaboration with XLF. CtIP, C-terminal binding protein-interacting protein; DNA-PKcs, DNA-dependent protein kinase catalytic subunit; DSBR, DNA double strand break repair; HR, homologous recombination; NHEJ, nonhomologous end joining; SDSA, synthesis-dependent strand annealing; XRCC4, X-ray repair cross-complementing protein 4. Adapted from: (Iyama and Wilson, 2013).

1.1.6 DNA-Protein Crosslink Repair (DPCs)

Another specific type of DNA lesion is DNA-protein crosslinks (DPCs). If unrepaired, DPCs cause replication fork stalling which ultimately leads to the formation of DSBs. DPCs are caused by the irreversible binding of proteins to DNA. Endogenous sources of DPC induction include aldehydes generated through normal metabolic processes, ROS, and helical alterations in DNA. DPCs are commonly divided into two groups, (i) nonenzymatic DPCs (Type 1) such as histones crosslinked to DNA and (ii) enzymatic DPCs (Type 2) such as Topoisomerase I and II cleavage complexes (Top1-ccs and Top2-ccs) (Vaz et al., 2017).

Despite being an abundant DNA lesion, the mechanism of DPC removal and repair had remained unknown until very recently, although previous studies had suggested a role for both NER and HR (Vaz et al., 2017). In 2014, a protease-based DPC repair mechanism was identified in budding yeast, involving DNA-dependent protease Wss1 (Stingele et al., 2014). This was later followed by the identification of protease SPARTAN (SPRTN), as a critical component of DPC repair in human cells (Lopez-Mosqueda et al., 2016, Stingele et al., 2016, Vaz et al., 2016, Morocz et al., 2017). The repair of Top1-ccs and Top2-ccs, for example, demonstrate the requirement for SPRTN in cells. Until recently, TDP1 and TDP2 respectively, were assumed to be the major enzymes involved in the removal of these lesions. However, both enzymes can only efficiently process peptides approximately 150 amino acids long (Interthal and Champoux, 2011, Vaz et al., 2017), indicating that upstream proteolysis of topoisomerase I and topoisomerase II into smaller peptides must be required for repair. Therefore, it was shown that SPRTN proteolytically digests DNA-crosslinked topoisomerase I

and topoisomerase II, which enables further processing by TDPs (Vaz et al., 2016). The importance of SPRTN function is highlighted by Ruijs-Aalfs syndrome resulting from germline mutations in *SPRTN*, and is characterised by genomic instability, premature ageing and early-onset hepatocellular carcinoma (Lessel et al., 2014).

1.1.7 Fanconi Anaemia Pathway

DNA interstrand cross-links (ICLs) are another highly dangerous DNA lesion. ICLs can inhibit both replication and transcription, causing mutations, chromosome breakage and mititotic catastrophe if left unrepaired (Lopez-Martinez et al., 2016). ICLs can be generated endogenous sources including metabolic by-products such as reactive aldehydes, or have exogenous origin resulting from commonly used chemotherapeutic agents such as cisplatin. To protect against ICLs, cells rely on the action of members of the Fanconi Anaemia (FA) pathway, and the coordination of several different DNA repair pathways including NER, HR and translesion synthesis. Defects in the FA pathway cause the autosomal recessive disease Fanconi Anaemia, which is characterised by developmental abnormalities, progressive bone marrow failure and cancer predisposition (Ceccaldi et al., 2016, Lopez-Martinez et al., 2016). To date, 19 different FA genes have been identified (FANCA-T), which can be divided according to function. FANCA, FANCB, FANCC, FANCE, FANCF, FANCG, FANCL, FANCM, FANCT form part of the FA core complex, FANCD2-FANCI forms a heterodimer that is thought to coordinate the different stages of ICL repair, and finally FANCD1, FANCJ, FANCN, FANCO, FANCP, FANCQ, FANCR and FANCS are effector proteins.

The FA pathway is activated during S phase by ICLs as incoming replication forks are unable to bypass the lesions, causing fork stalling. ICLs are detected by UHRF1 and the FANCM-MHF1-MHF2 complex. UHRF1 recruits the FANCD2-I heterodimer, whereas the FANCM-MHF1-MHF2 complex recruits the FA core complex to chromatin. The FA core complex then monoubiquitinates FANCD2-FANCI through the action of E3 ligase FANCL and E2 enzyme FANCT. Ubiquitylated FANCD2-FANCI coordinates nucleolytic incision by endonucleases MUS81, SLX1, XPF/FANCQ to release the ICL from one of the two parental strands in a process known as unhooking (Ceccaldi et al., 2016, Lopez-Martinez et al., 2016). During unhooking, these endonucleases cleave the DNA strand contiguous to the ICL, generating a DNA adduct and an ICL-derived DSB. Translesion synthesis by the REV1-polζ complex then bypasses the DNA adduct. The DSB created by the incision is then repaired through HR (Ceccaldi et al., 2016). To ensure fidelity during DSB repair, the FA pathway blocks repair through error-prone NHEJ, instead funnelling the ICL-derived DSB into FA-pathway-dependent HR repair (Rodriguez and D'Andrea, 2017).

Although each of the repair pathways here has been described in isolation, it should be noted that this is an increasingly outdated view of the general cellular response to DNA damage. As the pathways have been further characterised, it has become clear that DNA repair should in fact be considered as a group of dynamic systems that cooperate and sometimes even compete during the repair of DNA lesions. This pathway crosstalk ensures that cells are able to adapt accordingly to the nature and load of DNA damage. For example, various BER components are reported to cooperate with proteins from NER, MMR, NHEJ and HR (Limpose et al., 2017), which highlights the plasticity with which the DDR is able to promote DNA repair.

1.2 The DNA Damage Response

The DDR is a collective term that encompasses the signalling events and enzymatic activities related to the detection and reaction to DNA damage. In addition to the canonical DNA repair pathways, the DDR involves mechanisms that ultimately regulate cell fate decisions according to the level and severity of the damage detected. These include halting the cell cycle to allow adequate time to repair damage, modifying transcription in the appropriate repair pathway to cope with the lesion, or triggering cell death or senescence when the level of damage exceeds the repair capacity of the cell.

The signalling network of the DDR is a complex response that works as a classical signal transduction pathway. Signals are passed in a hierarchical manner from “sensors” through “transducers” to the various “effector” molecules, operating across at least five different levels (Figure 1.3) (Zhou and Elledge, 2000). The ataxia telangiectasia mutated (ATM) and ataxia telangiectasia and Rad3-related (ATR) protein kinases are the central coordinators of this far-reaching network, which is primarily driven by phosphorylation events; over 700 substrates have been recognised to have some involvement in the DDR, demonstrating the complexity at which the system operates (Matsuoka et al., 2007). Sensors in the network are the proteins at sites of DNA damage that directly recognise aberrant DNA structures; these include the MRN complex and the Rad9-Rad1-Hus1 “911 complex”, which respond to DSBs or stalled replication forks respectively, and activate ATM or ATR the transducer kinases (Bakkenist and Kastan, 2003, Zou and Elledge, 2003). In addition to the protein kinase cascade, mediator proteins work in cooperation to facilitate

phosphorylation events, by recruiting ATM/ATR substrates to amplify DDR signalling (Zhou and Elledge, 2000). Mediator of DNA damage checkpoint 1 (MDC1) is an example of a mediator; MDC1 forms foci at DSBs to promote the recruitment of DNA repair proteins (Stewart et al., 2003). ATM and ATR phosphorylate and activate checkpoint kinases 1 (Chk1) and 2 (Chk2) which are responsible for regulating the activity of many downstream effectors. Important effectors include the p53 tumour suppressor, stabilisation of which leads to a number of potential outcomes including cell cycle arrest, senescence or apoptosis (Roos et al., 2016). For example, a G1 phase cell cycle arrest can be stimulated through direct phosphorylation of p53 by Chk2 (Hirao et al., 2000). In this case, p53 promotes G1 arrest by stimulating the expression of p21, which blocks the action of the CDK2-cyclin E complex, thereby preventing a G1/S transition until the damage is repaired (Bartek and Lukas, 2003). Alternatively, when the level of DNA damage is too severe, the DDR can promote cell death through one of several mechanisms including apoptosis, regulated necrosis or autophagy. During apoptosis, Ser46 phosphorylation of p53 is a central event, although p53-independent methods of cell death also exist (Roos and Kaina, 2006). Ser46 phosphorylation is suggested to alter p53 promoter selection to favour the induction of pro-apoptotic genes such as PUMA (*BBC3*) (Mayo et al., 2005, Pietsch et al., 2008). Several kinases acting downstream of ATM/ATR are proposed to carry out this modification, including homeodomain-interacting protein kinase 2 (HIPK2) and p38 (Hofmann et al., 2002, Bulavin et al., 1999).

DNA damage triggers wide-ranging cellular consequences, and it is still very unclear how cells switch between survival or death pathways. These outcomes seem to depend on a number of different factors including the amount of DNA

damage, repair capacity of the cell, and the status of key DDR proteins. The current paradigm is that low levels of DNA damage trigger survival mechanisms including DNA repair and cell cycle arrest. High levels of DNA damage, on the other hand, can saturate the DNA repair capacity leading to cell death instead. The precise dynamics that govern these cell fate outcomes, though, are extremely complex and evidently require further research. As a central coordinator in the DDR network, however, ATM is clearly important in the decision-making process.

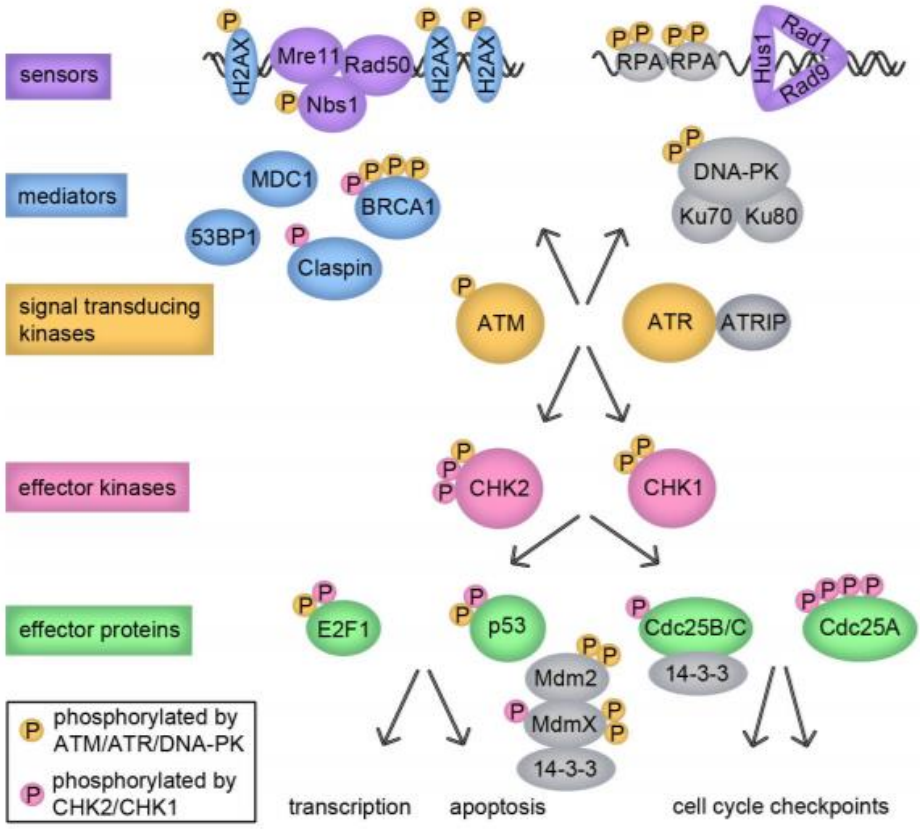

Figure 1.3 Overview of the DDR

DNA damage or replicative stress detected by specific sensors (purple) is signalled to signal transducing kinases (orange). Mediators (blue) facilitate phosphorylation events during DDR activation. The signal is then transduced to the effector kinases (pink) and through to effector proteins (green). Effector proteins govern the final outcome of the response resulting in different cellular outcomes, depending on the severity of damage. Proteins phosphorylated by ATM, ATR and/or DNA-PK are shown with an orange phosphate group. Proteins phosphorylated by Chk1 and/or Chk2 are shown with a pink phosphate group. Adapted from (Freeman and Monteiro, 2010).

1.3 ATM

ATM is a very large serine/threonine kinase that belongs to the phosphatidylinositol 3-kinase-related-kinase (PIKK) family. PIKK family members, which also include the DDR-related proteins ATR and DNA-PKcs, contain a C-terminal kinase domain that exhibits homology to the PI3 family of lipid kinases (Savitsky et al., 1995, Paull, 2015). The rare autosomal recessive disorder ataxia telangiectasia (A-T) is caused by loss of ATM. A-T is a multisystem disorder characterised by progressive loss of cerebellar neuron function, predisposition to cancer - especially lymphomas - and hypersensitivity to ionising radiation (Lavin et al., 2007).

Within the DDR, ATM functions as one of the central coordinators. Canonically, ATM is thought to be recruited to the sites of DNA DSBs by the MRN complex (Lee and Paull, 2005). ATM is initially recruited as an inactive dimer; however, following re-localisation, the kinase domain of each ATM molecule phosphorylates the other at Ser1981 causing dissociation into two active monomers. Upon activation, ATM then phosphorylates a large number of substrates to implement different cellular responses to DNA damage. For example, this includes the phosphorylation of histone H2AX which stimulates the recruitment of proteins involved in DNA repair (Burma et al., 2001). Furthermore, ATM is also able to directly phosphorylate p53 at Ser15 in response to DNA damage (Banin et al., 1998, Canman et al., 1998), which is important for p53 function including inducing cell cycle arrest (Meek and Anderson, 2009).

It is becoming increasingly clear that the role of ATM in the DDR is more wide-ranging than previously thought. As alluded to earlier, given its central role in

orchestrating the DDR it is plausible that ATM plays a role in affecting cell fate outcomes. Aside from signalling to promote cell cycle delay in response to DNA damage, ATM has also been implicated cell fate decisions such as senescence and apoptosis as well as differentiation. For example, senescence induced by telomere shortening was shown to be dependent on signalling through ATM (Herbig et al., 2004). Furthermore Fausti and colleagues demonstrated a role for ATM in promoting senescence in cells lacking Werner (Fausti et al., 2013). There are several lines of evidence linking ATM with the induction of apoptosis. During neurogenesis for example, ATM was shown to initiate an apoptotic response in neurons that had incurred genomic damage (Lee et al., 2001). Furthermore, ATM-dependent apoptosis has been linked to different members of the E2F family of transcription factors (Powers et al., 2004, Paulson et al., 2008).

Although ATM has primarily been considered a DSB-responsive protein, a growing body of evidence has indicated that, at least with respect to modulating DNA repair pathways, its function is far broader. For example, there are increasing indications that ATM is linked to the repair of SSBs in addition to the cellular response to oxidative stress. Its proposed roles also extend to activation in response to different types of DNA damage, as well as modulating the functions of proteins involved in the repair of these lesions. For example, the end-processing enzymes PNKP and TDP-1 can both be phosphorylated by ATM. PNKP phosphorylation was shown to promote the repair of oxidative lesions by preventing its proteasomal degradation (Parsons et al., 2012) whereas TDP-1 phosphorylation promoted its interaction with molecular scaffold XRCC1 to enhance DNA repair (Das et al., 2009). In addition, Agnihotri and colleagues identified 3-methylpurine-DNA glycosylase (MPG) as a novel ATM substrate.

They showed that ATM-mediated phosphorylation of MPG, which removes alkylated DNA bases during the initial step of BER, enhanced its activity and promoted resistance to temozolomide (Agnihotri et al., 2014). As detailed in section 1.5.2, it was also shown that ATM-activated kinase Chk2 phosphorylates XRCC1 which was associated with promoting BER (Chou et al., 2008). Finally, research in our lab has demonstrated impaired BER capacity in cells lacking ATM (Poletto et al., 2017). These examples demonstrate that ATM seems to assist in fine-tuning the cellular response to DNA lesions by modulating the recruitment and activity of DNA repair proteins, and that this is not just limited to DSB repair.

With respect to activation, it has become clear that ATM is also able to respond to a wider range of cellular stresses than previously recognised. Firstly, a study from our lab demonstrated that ATM can be activated by DNA strand breaks. This occurred in response to cell treatment with alkylating agent MMS, and took place in the absence of detectable DSBs, suggesting a potential role for SSBs in ATM activation. Moreover, ATM could be activated by endogenous strand breaks originating under conditions of SSB repair deficiency, which can be generated by siRNA-mediated knock down of XRCC1 (Khoronenkova and Dianov, 2015). Another study in support of this hypothesis indicated a requirement for MPG and APE1-dependent formation of SSBs in MMS-induced ATM activation (Chou et al., 2015). Secondly, ATM response to ROS is well-documented (Shiloh, 2014), with widespread oxidative stress now a recognised feature of A-T (Reichenbach et al., 2002). Elevated levels of ROS have been detected in ATM knockout mice (Kamsler et al., 2001) and studies in our own lab demonstrated that fibroblasts depleted of ATM showed progressive accumulation of ROS (Poletto et al., 2017).

In 2010, Guo and colleagues demonstrated ATM activation in response to oxidative stress, occurring via different mode of activation to that associated with DSBs. In this case, oxidative stress induced by H_2O_2 caused disulphide bond formation between two ATM monomers at C2991 resulting in an active dimer (Guo et al., 2010a, Guo et al., 2010b). Under these circumstances, phosphorylation of p53 and CHK2 was still observed, although H2AX phosphorylation was not, indicating context-specific activation of ATM targets (Guo et al., 2010b). These studies demonstrate that our understanding of ATM function is constantly evolving and it is no longer seen simply as a DSB-responsive protein. In fact, ATM function has much more wide-reaching implications, particularly in relation to BER where increasing evidence points at a role for ATM in the response to DNA lesions that are repaired through the pathway.

1.4 The Base Excision Repair Pathway

As previously described, the BER pathway is a major cellular defence against non-bulky DNA lesions, including oxidative base modifications, deamination products and SSBs. Its importance is signified by the high frequency and variation with which BER substrates occur in cells. It is estimated, for instance, that there are more than 100 types of oxidative base modification (Iyama and Wilson, 2013). The presence of abnormally modified bases can be highly mutagenic; if left unrepaired, certain base modifications can lead to transition or transversion mutations, for example. Alternatively, lesions such as thymine glycol can block DNA polymerase progression which activates cell death responses (Evans et al., 1993, Roos and Kaina, 2013). Therefore, BER is one of the most fundamental systems operating in higher eukaryotes to ensure DNA integrity. Accordingly, knock out of major components of the pathway such as apurinic/apyrimidinic endonuclease 1 (APE1), XRCC1, DNA polymerase β (Pol β), Lig I and Lig III leads to either embryonic or early post-natal lethality (Bentley et al., 1996, Xanthoudakis et al., 1996, Tebbs et al., 1999, Sugo et al., 2000, Puebla-Osorio et al., 2006), indicating that a lack of BER is not compatible with survival and normal development.

The following paragraphs will give an overview of the BER pathway, including types of lesions that are routinely repaired, and the molecular steps involved. There is also particular emphasis on our current understanding of how the pathway is regulated.

Oxidation-induced DNA backbone and base modifications are particularly prevalent forms of DNA damage. In fact, SSBs, which arise mainly through ROS

attack, are estimated to be among the most frequently occurring (Thompson and West, 2000, Abbotts and Wilson, 2017). Oxidative base modifications are also a common form of DNA damage, of which 8-oxo-dG is one of the most abundant. In fact, it has been estimated that 180 guanines are oxidised to 8-oxo-dG per cell per day (Lindahl, 1993), although steady state measurements using mass spectrometry suggest that this number may be higher (Iyama and Wilson, 2013). 8-oxo-dG is potentially mutagenic due to its ability to mispair with adenine which can cause G:C to T:A transversions after replication (Shibutani et al., 1991). Furthermore, DNA containing 8-oxo-dG can lead to the generation of mutant mRNA transcripts (Bregeon and Doetsch, 2011), and correspondingly, mutant proteins (Bregeon et al., 2009).

Spontaneous deamination reactions are also potentially harmful due to the risk of transition mutations. For example, the deamination of cytidine to uracil can result in a C:G to T:A transition. Furthermore, spontaneous deamination of adenine to inosine, which can pair with cytidine, can lead to a A:T to G:C transition. Uracil and inosine residues in nuclear DNA are estimated to be present at a similar level to 8-oxo-dG indicating that deaminated bases are also a significant threat to genome stability (Iyama and Wilson, 2013). Common examples of base lesions are shown in Figure 1.4.

Genomic damage can also result from enzymatic reactions. SSBs for example, are generated as an obligate intermediate of APE1 activity during BER. However, they can also arise as a result of failed enzyme reactions such as abortive DNA topoisomerase I (TOP1) activity (Wang, 2002).

1.4.1 Stages of BER

BER is considered to occur through five major steps: (i) damage recognition and removal of the DNA lesion, (ii) backbone incision at the resulting AP site, (iii) end processing, (iv) gap filling, (v) sealing the remaining nick in the DNA backbone. An overview of the pathway is given in Figure 1.5.

During the first step, the damage is detected by a class of enzymes called DNA glycosylases, which are thought to scan the DNA helix and excise the damage base in a lesion-specific manner. Through cleavage of the N-glycosidic bond, which links the base to the sugar phosphate backbone, the damaged base is removed. DNA glycosylases can be categorised as monofunctional or bifunctional enzymes, depending on their mode of action. Monofunctional DNA glycosylases such as uracil DNA glycosylase (UNG) simply cleave the C1'N-glycosidic bond and generate an AP site. Bifunctional DNA glycosylases, on the other hand, also have an associated β-lyase activity which can generate a SSB with modified DNA ends (Kim and Wilson, 2012). The bifunctional DNA glycosylase 8-oxoguanine DNA glycosylase (OGG1), for example, removes the damaged base and cleaves the DNA backbone leaving a 3'-α,β-unsaturated aldehyde blocking moiety. In contrast, the endonuclease VIII-like proteins (NEIL1-3), carry out a β,δ-elimination reaction creating a nick with a blocked 3'-phosphate end (Jacobs and Schär, 2012). The ends generated by these enzymes must be additionally processed before continuation of "classical" BER. Currently at least 11 known human DNA glycosylases have been described (Table 1.1), which seem to exhibit considerable functional redundancy as to their substrate specificity. However, because many of the studies investigating DNA glycosylase substrate specificity were carried out *in vitro* it is difficult to be certain

of their true specificity *in vivo.* Nonetheless, some degree of functional redundancy between the glycosylases is undeniable as single knock out of any of these enzymes, with the exception of TDG, is not embryonic lethal (Jacobs and Schär, 2012).

Table 1.1 Human DNA glycosylases and primary substrates.

Adapted from (Jacobs and Schär, 2012).

NAME		MONO/ BIFUNCTIONAL	LESION TYPE	PHYSIOLOGICAL SUBSTRATES
UDG	Uracil DNA Glycosylase	Mono	Uracil bases	U, 5-FU
SMUG1	Single-strand-selective monofunctional uracil DNA glycosylase I	Mono		U, 5-hmU, 5-FU
TDG	Thymine DNA Glycosylase	Mono	Modified pyrimidines	T, U, 5-FU,
MBD4	Methyl-CpG binding domain 4	Mono		T,U, 5-FU
MPG	3-methylpurine-DNA glycosylase	Mono	Alkylated bases	3-meA, 7-meG, hypoxanthine
MYH	MutY Homolog	Mono	Oxidised bases	A opposite 8-oxo-dG
OGG1	8-oxo-guanine glycosylase 1	Bi		8-oxo-dG, FaPy
NTH1	Endonuclease III-like 1	Bi	Oxidised, ring-fragmented or saturated pyrimidines	Tg, FaPyG, 5-hC, 5-hU
NEIL 1	Endonuclease VIII-like 1	Bi		Tg, FaPyG, FaPyA, 8-oxoG,
NEIL 2	Endonuclease VIII-like 2	Bi		Tg, 5-hC, 5-hU FaPyG, FaPyA, 8-oxo-dG
NEIL 3	Endonuclease VIII-like 3	Bi		FaPyG, FaPyA

U, uracil; A, adenine; T, thymine; C, cytosine; G, guanine; 5-FU, 5-fluorouracil; 5-hmU, 5-hydroxymethyl U; me, methyl; 8-oxo-dG, 7,8-dihydro-8-oxoguanine; Tg, thymine glycol; FaPy, 2,6-diamino-4-hydroxy-5-N-methylformamidopyrimidine; h, hydroxyl;

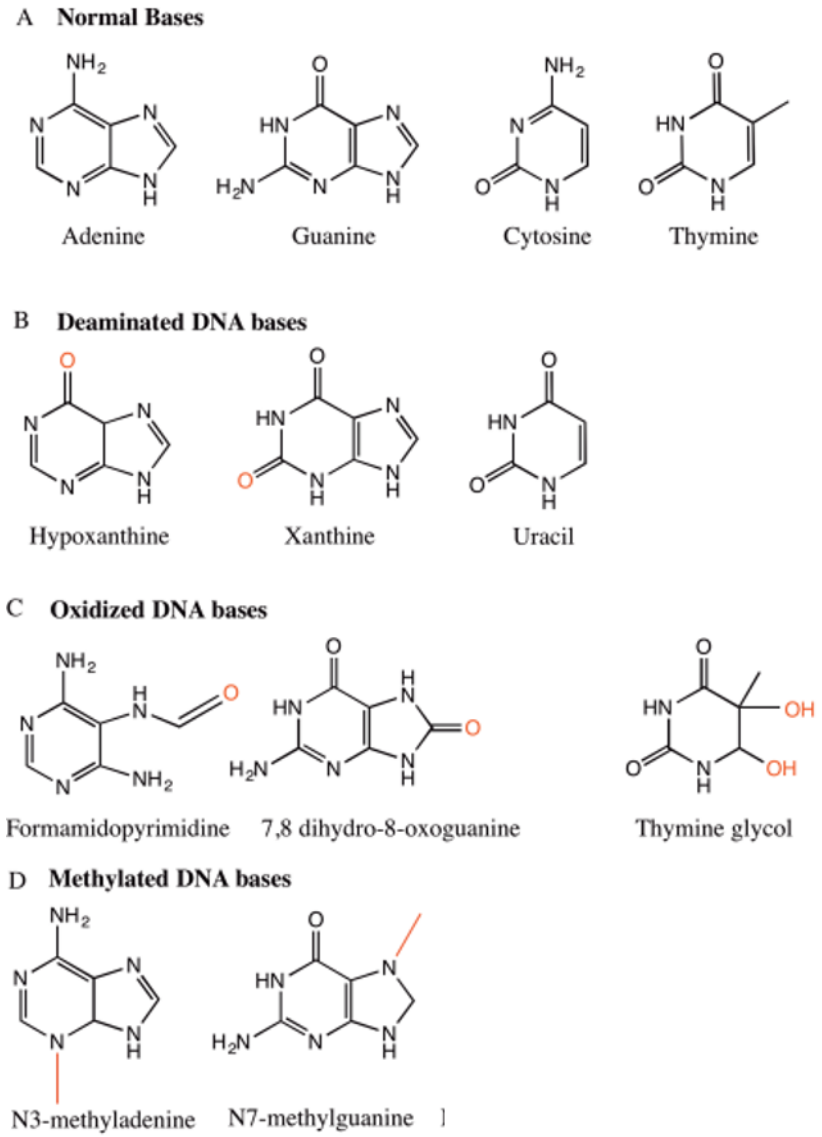

Figure 1.4 Examples of common DNA base lesions

(A) Normal structures of DNA bases. **(B)** Deaminated bases. **(C)** Oxidised DNA bases. **(D)** methylated DNA bases. Adapted from (Chatterjee and Walker, 2017).

The base excision step in the pathway generally leaves behind an AP site which is then recognised and processed by an AP endonuclease. In mammalian cells, this is carried out by APE1, which cleaves the phosphodiester backbone immediately 5' to the AP site, generating a SSB intermediate containing 3'-hydroxyl (3'OH) and 5'-deoxyribosephosphate (dRP) ends (Demple et al., 1991, Wilson and Barsky, 2001).

Following APE1 incision at an AP site, BER most commonly proceeds through removal of the 5'dRP group. Pol β is the main enzyme responsible for removal of the 5'dRP moiety through its dRP lyase activity (Prasad et al., 1998). APE1 incision is usually sufficient to generate termini that can be processed. However, complex repair intermediates or base excision steps carried out by a bifunctional DNA glycosylase require further intervention by end-processing enzymes aprataxin (APTX), PNKP or tyrosyl-DNA phosphodiesterase (TDP-1). These enzymes convert the SSB into a single nucleotide gap with 5'-P and 3'-OH moieties that can then be efficiently filled in and re-ligated. APTX processes 5'-AMP groups resulting from failed DNA ligation reactions (Ahel et al., 2006). TDP1 is involved in the removal of TOP1 cleavage complexes from DNA (Yang et al., 1996), generating a SSB with 3'-P and 5'-OH termini. These termini are processed by PNKP using both its kinase and phosphatase activities to generate the 5-'P and 3'-OH moieties needed for gap filling (Jilani et al., 1999). PNKP also processes 3'-P intermediates generated by bifunctional DNA glycosylases. Besides its AP endonuclease activity, APE1 is also reported to have weak 3'-phosphodiesterase activity which can remove 3'-α,β-unsaturated aldehyde blocking moieties (Wiederhold et al., 2004).

After the end-processing step, BER typically proceeds via the short-patch (SP) pathway, where the stretch of DNA affected by repair is restricted just to the damaged nucleotide. In this case, Pol β is the major enzyme which inserts the missing nucleotide into the gap (Sobol et al., 1996) although a back-up role has also been proposed for DNA polymerase λ, especially during the repair of oxidative lesions (Maga et al., 2007, van Loon and Hübscher, 2009). After gap filling, the XRCC1-Lig III complex then seals the remaining nick (Caldecott et al.,

1994), although this can also be carried out Lig I (Krokan and Bjørås, 2013). However, under some circumstances when repair involves insertion of more than one nucleotide, BER instead continues via a strand displacement-dependent gap filling process termed the long-patch (LP) pathway. This sub-pathway is thought to act when cellular ATP concentrations are low – resulting in low ligation efficiency (Petermann et al., 2006), or during S phase of the cell cycle when replication-associated proteins are more abundant. While the factors that dictate sub-pathway choice are still being defined, it is also thought that the initiating DNA glycosylase, as well as whether the 5'-moiety is a suitable substrate for Pol β both help determine the final molecular steps of BER (Iyama and Wilson, 2013, Abbotts and Wilson, 2017). LP BER relies on replication-associated polymerases δ and ε in cooperation with the sliding clamp PCNA and the clamp loading factor RFC. Pol β can also carry out strand-displacement synthesis but only when at high concentration, or through stimulation by certain protein-protein interactions (Fan and Wilson, 2005). During LP BER, a stretch of 2-12 nucleotides is synthesised, with strand displacement resulting in the formation of a 5' flap. This flap is removed by FEN-1. Given that Lig I is physically associated with PCNA, this ligase appears to be the major nick sealing enzyme involved in this sub-pathway (Levin et al., 1997).

The regulation and functions of scaffold protein XRCC1 will be described in much greater detail in a dedicated section (1.5) as this protein is central to BER and is a main topic of this thesis.

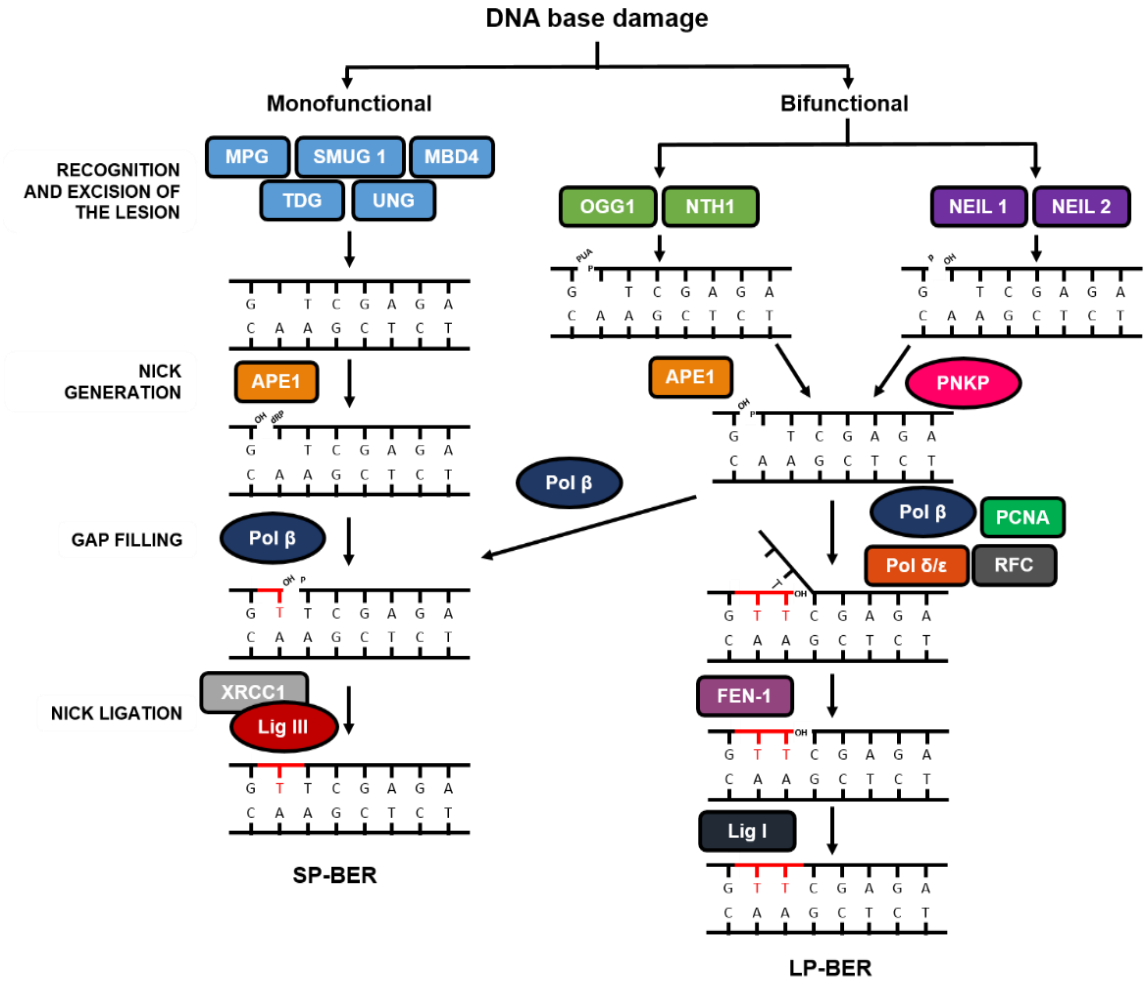

Figure 1.5 Overview of the BER pathway.

BER is initiated through substrate-specific recognition and removal of a damaged base by a DNA glycosylase. Monofunctional DNA glycosylases (light blue) generate an AP site. APE1 then incises the DNA backbone at the AP site generating an SSB with 3'-OH and 5'-dRP ends. Bifunctional DNA glycosylases (light green or purple), which have AP lyase activity, also cleave the AP site producing an SSB with 3'-α,β-unsaturated aldehyde (PUA), 3'-phosphate or 5' phosphate ends. These termini require further processing by APE1 or PNKP. After end-processing, the excised nucleotide is replaced by a DNA polymerase. During short patch (SP) repair, Pol β inserts a single nucleotide before the nick is sealed by the XRCC1-Lig III complex. In the long-patch (LP) pathway, a replicative polymerase (usually Pol δ or Pol ε although Pol β can participate) incorporates 2-12 nucleotides through a strand displacement mechanism in conjunction with loading factor RFC and sliding clamp PCNA. The flap intermediate is removed by FEN-1 before Lig I seals the nick. Adapted from (Kim and Wilson, 2012).

1.4.2 BER Sub-pathways

Canonical BER does not operate in isolation. It involves many different enzymes, and can occur via different sub-pathways that vary in repair patch size and requisite proteins. Two such examples are LP-BER and single strand break repair (SSBR). SSBs can arise from a number of different sources. Some are unavoidable, arising as obligate intermediates during BER. However, SSBs can also be formed by ROS attack, IR, radiomimetic drugs or topoisomerase I inhibitors. As many canonical BER proteins are involved in the repair of direct SSBs, SSBR is tightly connected to BER and often viewed as a specialised BER sub-pathway (Abbotts and Wilson, 2017). The SSBR pathway is initiated through the recruitment of poly(ADP-ribose) polymerase (PARP1) which acts as a molecular nick sensor by recognising exposed SSBs (Okano et al., 2003). PARP1 then modulates repair through ADP-ribosylation of protein substrates, including itself (Hayaishi and Ueda, 1977, Abbotts and Wilson, 2017). Interestingly, many BER proteins including XRCC1, Pol β and Lig III interact with PARP1 (Masson et al., 1998, Dantzer et al., 2000, Leppard et al., 2003). In fact, recruitment of XRCC1 to sites of DNA damage by activated PARP1 has been proposed as PARP1's primary role in coordinating SSBR (Abbotts and Wilson, 2017). However, PARP1 seems to be dispensable for the repair of SSBs initiated by DNA glycosylases, demonstrating that BER and SSBR operate through distinct mechanisms (Vodenicharov et al., 2000, Allinson et al., 2003, Strom et al., 2011a). Once recruited, the same enzymes described in BER (SP or LP) carry out the end-processing, nucleotide insertion and nick ligation steps (Caldecott, 2008).With respect to other sub-pathways, Woodrick and colleagues have recently described a "new" sub-pathway of LP BER involving formation of a

nucleotide gap 5' to the lesion. It remains uncertain, however, the relevance of this mechanism *in vivo* (Woodrick et al., 2017).

1.4.3 Coordination of BER

As described previously, there are five defined steps in BER; how each step is orchestrated to form a coordinated response remains under debate, however. Several different models have been proposed in the literature to explain how the pathway has evolved to maximise repair efficiency.

Back in 2000, Wilson and Kunkel presented the so-called "passing the baton" model, based on transient protein-protein interactions (Wilson and Kunkel, 2000); they proposed that repair intermediates are channelled from a DNA glycosylase through to the DNA ligase in a coordinated manner, without exposing the cellular environment to toxic repair intermediates such as SSBs (Wilson and Kunkel, 2000). Several lines of evidence supported this hypothesis. Firstly, multiple protein-protein interactions exist between components of the pathway, including interactions between Pol β and APE1 bound to an AP site (Bennett et al., 1997). Secondly, co-crystal structures of BER enzymes bound to DNA showed that APE1 favours AP sites produced by the action of upstream DNA glycosylases (Mol et al., 2000). Moreover, Mol and colleagues also calculated that APE1 has high affinity for its cleaved product, all of which are consistent with the hypothesis of enzyme-product handoff (Mol et al., 2000). This model provides a neat mechanism for the coordination of "classic" short-patch BER. However, it does not explain how more complex lesions requiring end-processing are handled.

An alternative model proposes that BER is carried out in specialised multi-protein repair complexes, known in some cases as “BERosomes” (Hegde et al., 2010, Prasad et al., 1996). Historically, several groups published co-immunoprecipitation experiments demonstrating interactions between different BER proteins (Prasad et al., 1996, Akbari et al., 2004, Dianov and Hübscher, 2013). More recently, Prasad and colleagues reported, using a Pol β immunoaffinity-capture technique, a repair complex present in mouse embryonic fibroblasts (MEFs) containing PARP-1, XRCC1, Lig III, PNKP and TDP-1, capable of BER activity (Prasad et al., 2012, Prasad et al., 2015), which supports this hypothesis. However, the physiological relevance of such large complexes remains controversial given the wide spectrum of DNA lesions that BER must handle (Dianov and Hübscher, 2013). Moreover, given that BER is not the only source of AP sites, it seems reasonable to conclude that AP site incision by APE1 can also operate independently to the rest of the pathway. Despite this, given the broad list interaction partners for XRCC1, and the sensitivity to exogenous mutagens loss of XRCC1 confers, however, some degree of multi-protein complex formation is likely necessary for efficient BER. Although these somewhat opposing models have been proposed, it is reasonable that they simply describe slightly different aspects of this highly dynamic process. What is clear, however, is that efficient BER requires a complex network of protein-protein interactions with coordination by the non-enzymatic scaffolds XRCC1 and PCNA. In addition, it has become evident that post translational modifications (PTMs) are necessary for fine-tuning BER. For example, phosphorylation, acetylation, methylation, ubiquitylation and SUMOylation of almost every

component of the pathway has been suggested to play a role in modulation (Fan and Wilson, 2005, Parsons and Dianov, 2013).

In addition to understanding the coordination of different steps in the repair process, it is also important to consider how steady-state modulation of specific pathway components occurs. Of particular interest is whether regulation is dependent on the cellular load of DNA damage, and if modulations operate at both protein and transcriptional levels. Variations in BER gene expression are significant, both between tissues and among individuals, (Srivastava et al., 1999), suggesting that some degree of BER regulation occurs according to the cellular environment. Because BER is continuously required by mammalian cells, it is logical to assume that BER activity is regulated through a steady-state rather than an “on/off” mechanism. Given the highly dynamic cellular environment, it is clear that BER must adjust to the cellular level of DNA damage as fluctuations can arise under conditions such as inflammation (Jaiswal et al., 2000, Meira et al., 2008), during high levels of transcription (Datta and Jinks-Robertson, 1995), or hormonal signalling (Stork et al., 2016). Furthermore, variations in the level of spontaneous endogenous DNA damage experienced by different cell types also exist; highly metabolically active cells such as neurons, for example, are presumed to generate more oxidative lesions (Wilson et al., 2011).

Moreover, it is imperative that BER is tightly controlled as imbalance between the different steps of the pathway can cause genomic instability (Coquerelle et al., 1995, Glassner et al., 1998). During repair, nick ligation of SSBs is slower than the generation of incised DNA by APE1 (Wilson et al., 2011, Khoronenkova and Dianov, 2015). Therefore, cells must balance carrying out repair of endogenous lesions against the generation of unrepaired intermediates to ensure DNA fidelity

and prevent accumulation of SSBs. In line with this, mutations associated with decreased amounts or activities of BER enzymes have been linked to increased genomic instability and decreased viability (Cabelof et al., 2003, Lang et al., 2004, Ochs et al., 1999, Sweasy et al., 2005, Fan et al., 2007, Horton et al., 2008). However, overexpression of BER genes can also have negative consequences in cells (Coquerelle et al., 1995, Chan et al., 2007, Albertella et al., 2005, Frosina, 2000). For example, high levels of APE1 are associated with increased tumour aggressiveness and poorer prognosis in cancer (Li and Wilson, 2014). Moreover, overexpression of Pol β in mammalian cells has been shown to increase genomic instability due to a higher rate of frameshift mutations (Canitrot et al., 1998, Chan et al., 2007). Taken together, this strongly indicates that the levels of BER components must be highly regulated to promote genome stability.

It is assumed that the steady-state level of BER proteins exists as a dynamic equilibrium between protein synthesis and degradation. Although early studies indicated a dose and time-dependent increase in Pol β mRNA levels in mammalian cells treated with DNA damaging agents (Fornace et al., 1989), a later quantitative study was unable to replicate this (Inoue et al., 2004). It is now generally accepted that BER protein levels do not change substantially in response to acute DNA damage induced by exogenous mutagens (Parsons and Dianov, 2013). Instead, it has been postulated that cells can regulate BER steady-state levels using an elegant mechanism to link BER enzyme levels to the load of endogenous DNA damage. This is achieved through stabilisation of the enzymes that are actively engaged in repair, and proteasomal degradation of excessive components that are not directly involved. The realisation that members of the ubiquitin-proteasome system are involved in modulating BER

stability has uncovered a new perspective on the regulation of BER dynamics, demonstrating that the pathway is a damage-responsive system (Dianov and Hübscher, 2013, Parsons and Dianov, 2013).

One example of proteasome-mediated regulation of BER is the case of Pol β, where the balance between the monoubiquitylation, polyubiquitylation and deubiquitylation reactions regulate Pol β turnover. In this mechanism, if the level of Pol β exceeds the requirement for the level of DNA lesions, the majority of newly synthesised enzyme is ubiquitylated and degraded. This occurs through sequential ubiquitylation reactions by the E3 ligases Mcl-1 ubiquitin ligase E3 (Mule) and carboxyl terminus of Hsc70 interacting protein (CHIP). Mule monoubiquitylates Pol β, promoting polyubiquitylation by CHIP and subsequent degradation via the proteasome (Parsons et al., 2008, Parsons et al., 2009) (Figure 1.6). However, Mule activity can be inhibited by binding of p14 alternative reading frame (ARF) (Chen et al., 2005), which accumulates in response to DNA damage (Khan et al., 2004, Orlando et al., 2014). Therefore, when DNA damage accumulates, Mule is inhibited by ARF which decreases the rate of Pol β ubiquitylation. This shifts the equilibrium towards deubiquitlyation by USP47 (Parsons et al., 2011), generating more active Pol β to participate in repair. This steady-state mechanism describes an important system for cells to adapt accordingly to their levels of endogenous DNA damage by directly linking DNA damage sensing to modulation of protein amount.

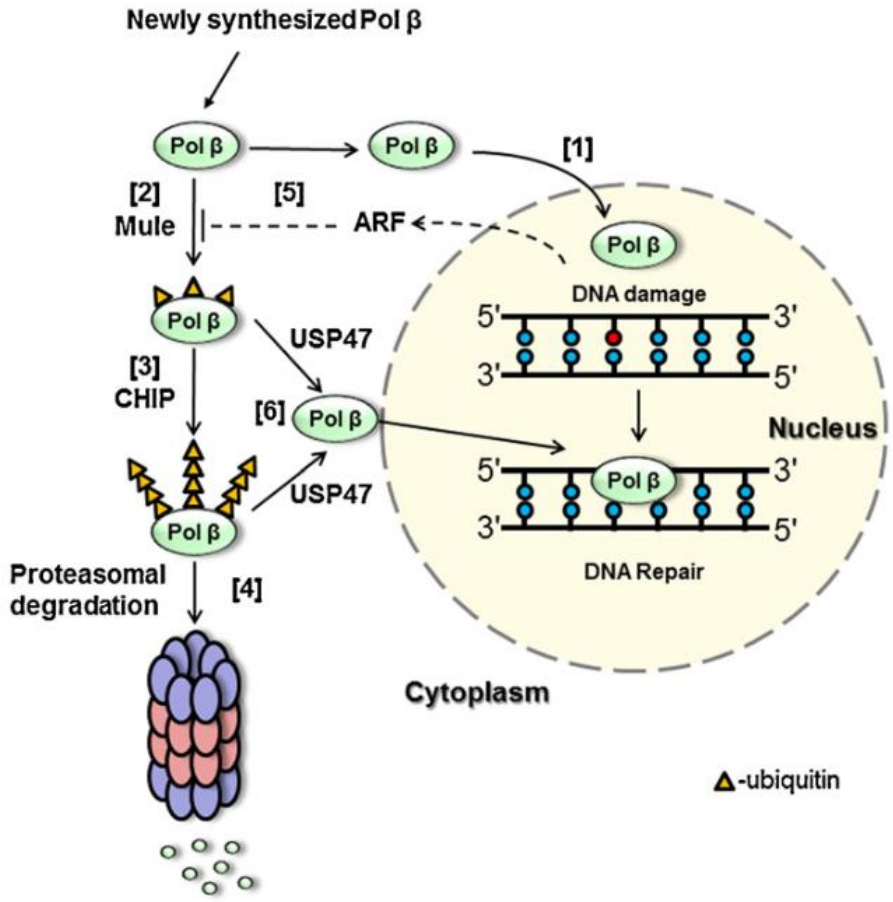

Figure 1.6 Modulation of Pol β protein levels

Newly synthesised Pol β is either transported to the nucleus [1] to take part in DNA repair, or monoubiquitylated by E3 ligase Mule [2]. Monoubiquitylated Pol β is targeted for polyubiquitylation by E3 ligase CHIP leading to degradation via the proteasome [4]. In the presence of DNA damage Mule activity is inhibited by accumulated ARF, decreasing the rate of Pol β ubiquitylation, and shifts the equilibrium towards deubiquitylation by USP47. This results in more active Pol β able to enter the nucleus and take part in DNA repair. Source: (Parsons and Dianov, 2013).

Furthermore, there is some evidence that steady state levels can be modified through transcriptional changes. In a recent publication, we showed that cells can modulate BER coordination by affecting *APEX1* transcription. Specifically, we demonstrated that APE1 could be downregulated in response to the accumulation of unrepaired DNA strand breaks through modulating the stability of transcription factor Sp1 in order to prevent genomic instability. The role of Sp1 in regulating BER is a central topic to this thesis; functions of Sp1 will be

discussed in greater detail in Section 1.6. Furthermore, we demonstrated that impairment of p53 function also led to a failure in this BER coordination mechanism, indicating that BER can easily become dysregulated in cancer (Poletto et al., 2016).

From the proposed mechanisms to coordinate different stages of BER, to the precise systems modulating steady state protein levels, it is clear that BER is a highly regulated process at the molecular level. Increasing lines of evidence suggest that this is also true at the cellular level.

1.4.4 BER regulation during different biological processes

It is generally accepted that DNA repair capacity differs greatly between cell types. In particular, there is now increasing evidence that BER varies between progenitor cells and their differentiated counterparts. For example, it was reported that human embryonic stem cells are able to repair most types of DNA lesion more effectively than differentiated cell types (Maynard et al., 2008). In line with this, Narciso and colleagues noted that terminally differentiated muscle cells are defective in BER. This was ascribed to strong downregulation of XRCC1, subsequent destabilisation of Lig III and a lack of Lig I (Narciso et al., 2007), which resulted in susceptibility to oxygen injury. Furthermore, higher BER capacity has also been recorded in proliferative neural progenitor cells compared to their terminally differentiation progeny (Sykora et al., 2013). Regulation of BER in the immune system also provides strong evidence for BER modulation during normal biological processes. In this case, it was observed that monocytes lack XRCC1 and Lig III, leaving them sensitive death induced by oxidative stress. The

macrophages and dendritic cells derived from them, however, were found to be repair competent (Briegert and Kaina, 2007, Bauer et al., 2011). These findings have recently been reconciled into a mechanism termed “killing in trans” which hypothesises that monocyte susceptibility to ROS-induced DNA damage restricts the levels of monocyte-derived macrophages in tissues. Activated macrophages produce high levels of ROS, so this system is proposed to protect healthy tissue from excessive ROS-induced damage (Ponath and Kaina, 2017). These examples indicate that BER is clearly modulated during different biological processes, and importantly, that this regulation has biological significance. Therefore, as BER is such a highly regulated process, it is unsurprising that its dysregulation is associated with pathological outcomes.

1.4.5 BER and human disease

Despite the obvious importance of this pathway is maintaining genomic integrity, very few inherited disorders are actually associated with genetic defects in BER components. Two exceptions to this are DNA glycosylases *MUTYH* and *UNG* mutations, which are associated with cancer predisposition and immunological defects respectively (Wilson et al., 2011). More recently, a patient carrying *XRCC1* mutations linked to cerebellar ataxia has been identified (Hoch et al., 2017). However, the general lack of human diseases linked to inactivating mutations of core BER factors is likely due to the previously discussed embryonic lethality associated with loss of key BER proteins. The fact that complete loss of BER function is incompatible with life reaffirms the serious threat imposed by endogenous DNA damage. As a result, research has instead been directed towards understanding whether variations in repair capacity among individuals,

can influence disease incidence (Sokhansanj and Wilson, 2006, Wilson et al., 2011).

As discussed previously, high levels of BER proteins can be hazardous for cells. In fact, overexpression of BER proteins is a common feature in many different cancer types, where it is often associated with resistance to therapy. For example, overexpression of APE1 is reported to confer resistance to cisplatin (Wang et al., 2009). Furthermore, Pol β has been shown to be overexpressed in a variety of human tumours (Srivastava et al., 1999). In fact, it has been estimated that approximately 30% of human tumours carry Pol β mutations. In addition to overt changes in expression reported in tumours, more subtle variations, mainly polymorphisms, are also expected to affect predisposition to cancer. In line with this, polymorphic variants of APE1, OGG1, Pol β and XRCC1 have all been shown to affect BER capacity. Some polymorphic variants have also been linked to an increased predisposition to specific cancers although more research is necessary to fully understand the implications (Wallace et al., 2012).

It is clear that BER dysregulation can lead to disease. In addition to promoting tumourigenesis, reduced BER capacity has also been linked to diseases associated with high levels of oxidative stress, including neuropathologies and complications from strokes (Wilson et al., 2011); decline in BER capacity has also been observed during ageing (Xu et al., 2015). The association between BER and neurological diseases likely exists because neurons are post-mitotic, and produce substantial levels of ROS due to their high metabolic turnover. As these terminally differentiated cells cannot use replication-associated DNA repair pathways such as HR, they are more reliant on the remaining systems, which

include BER. This therefore renders these cells more sensitive to modulations in BER efficiency. In line with this, BER impairment caused by limited DNA glycosylase and Pol β activity was identified in brains of Alzheimer's patients, (Weissman et al., 2007). Furthermore, loss of any of the end-processing enzymes APTX, PNKP or TDP-1 is associated with neurological disease. Ataxia with oculomotor apraxia-1 (AOA1) is caused by mutations in *APTX* (Moreira et al., 2001), for example. Several different neuropathologies have been linked to *PNKP* mutations. An unnamed disorder characterised by neurodegeneration and cerebellar ataxia as well as the AOA sub-type, AOA4, are linked to different homozygous mutations in *PNKP* (Poulton et al., 2013, Bras et al., 2015). Additionally, compound heterozygous *PNKP* mutations have been implicated in a separate developmental disorder, characterised by microcephaly, seizures and developmental delay (Shen et al., 2010). Finally, homozygous mutation in TDP-1 leads to spinocerebellar ataxia with axonal neuropathy-1 (SCAN-1) (Takashima et al., 2002). The tissue-specific nature of these disorders clearly highlights the importance of maintaining functional BER, especially in non-dividing cells.

1.5 XRCC1

XRCC1 is central to the coordination of the BER pathway. It operates as a molecular scaffold interacting with enzymatic components from each stage of BER. Through this ability to bind multiple proteins, and also to DNA, it is able to support many of the key protein-protein interactions required for the efficient repair of DNA lesions.

1.5.1 Protein Structure and Function

XRCC1 was first identified from mutant studies using Chinese hamster ovary (CHO) cell lines that showed sensitivity to alkylating agents, defective rejoining of strand breaks after exposure to various mutagens, and elevated rates of sister chromatid exchange (Thompson et al., 1982, Thompson et al., 1990). The *XRCC1* gene, located on chromosome 19q13.2 produces a 69.5 kDa protein composed of 633 amino acids. The basic structure comprises an N-terminal domain (NTD) spanning residues 1-155, and two BRCA1 C Terminus (BRCT) domains, BRCT I (residues 310-405) and BRCT II (residues 529-633), which share sequence homology with the highly conserved BRCT protein interaction domain (Figure 1.7) and (Bork et al., 1997). Described briefly below are some of the key XRCC1 protein-protein interactions important in BER.

Previous studies have shown that the NTD is critical for binding Pol β, both independently and in complex with a DNA substrate containing a single nucleotide gap (Dianova et al., 2004, Marintchev et al., 2003). The NTD may also be able to bind directly to DNA substrates including gapped and nicked

structures (Marintchev et al., 1999). The central BRCT I domain is essential for binding molecular nick sensor PARP1 (Masson et al., 1998) while the C terminal BRCT II domain is critical for interaction with Lig III (Caldecott et al., 1994, Nash et al., 1997). The linker regions between the different domains are also important sites for protein-protein interaction. For example, deletion analyses have indicated that the region between the NTD and BRCT I domains is required for APE1 (Vidal et al., 2001), OGG1 (Marsin et al., 2003) and PCNA (Fan et al., 2004) binding. This region also contains the nuclear localisation signal (NLS) (residues 241-279) (Thompson et al., 1990). In addition, the region between the two BRCT domains is essential for PNKP interaction (Whitehouse et al., 2001, Della-Maria et al., 2012); this region also contains a cluster of casein kinase 2 (CK2) phosphorylation sites immediately downstream of the BRCT I domain (Loizou et al., 2004). In addition to these mapped interactions, XRCC1 binding to a number of DNA glycosylases has also been reported. Pull-down experiments carried out by Campalans and colleagues, for example, described XRCC1 interaction with MPG, NEIL2 and NTH1 (Campalans et al., 2005). Functional interaction has also been reported with UNG (Akbari et al., 2010).

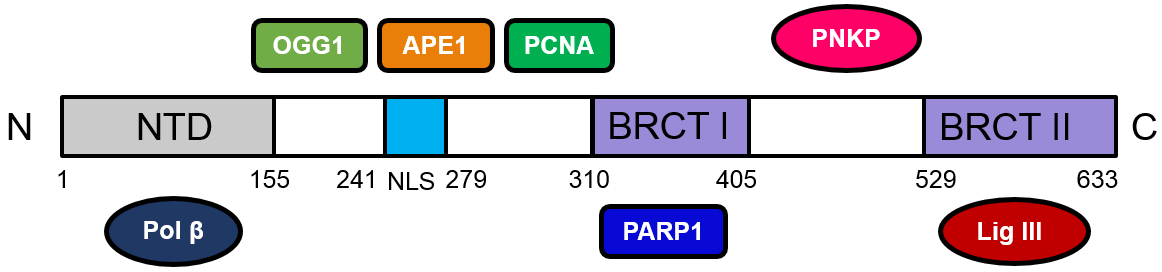

Figure 1.7 XRCC1 domain organisation and overview of major protein-protein interactions.

XRCC1 interacts with a large number of proteins from BER. Pol β interacts with the N terminal domain (NTD). The region between the N terminal domain (NTD) and BRCA 1 C terminus domain I (BRCT I) interacts with OGG1, APE1 and PCNA. PARP1 interacts with the BRCT I domain. The region between the two BRCT domains is important for PNKP interaction. Lig III interacts with the C terminal BRCT II domain. NLS; Nuclear localisation signal.

As is the case for core BER proteins, homozygous *XRCC1* deletion is embryonically lethal in mice indicating an essential role for XRCC1 in embryo development; (Tebbs et al., 1999). However, the presence of XRCC1 even at greatly reduced levels seems to provide sufficient protein to promote survival; complementation experiments with a transgene expressing XRCC1 at less than 10% normal levels rescued the embryonic lethality phenotype for example (Tebbs et al., 2003). Furthermore, these mice showed only reduced body weight compared to wildtype littermates, and no obvious increase in cancer incidence (Ladiges, 2006), suggesting that very low levels of XRCC1 protein expression are sufficient to maintain genomic stability, at least in mice. In support of this hypothesis, *XRCC1* has been shown to have a large number of single-nucleotide polymorphisms (SNPs), some of which are maintained at high frequency in the human population (Ladiges, 2006). In particular, three nonsynonymous SNPs resulting in the amino acid changes R399Q, R280H and R194W, have been widely studied in cancer epidemiological investigations. Although trends with specific cancers have occasionally been reported in a variety of ethnic backgrounds, reports are frequently conflicting making it difficult to draw

substantial conclusions. Furthermore, studies investigating the functional impact of these polymorphic amino acid variants have not produced a clear answer. The population variants R399Q, R280H, and R194W had little or no effect on XRCC1 protein stability or protein-protein interactions with PARP1, Lig III, Pol β or PCNA (Berquist et al., 2010). Another study showed slight variation in the repair profiles of cells expressing either the R280H or R399Q variants, although recruitment of Pol β and PNKP to sites of damage was unaffected (Hanssen-Bauer et al., 2012). As a result, the functional significance of *XRCC1* SNPs remains largely unknown. This suggests that variable XRCC1 expression levels are compatible with life, at least in the absence of genotoxin exposure.

More recently however, evidence is emerging that there is likely to be a cell type-specific dependency on XRCC1 function. This is particularly relevant in the brain. In support of this, increased strand break accumulation and cell death was reported in primary cerebellar granule cells derived from haploinsufficient $XRCC1^{+/-}$ animals following menadione exposure (Kulkarni et al., 2008). Furthermore, $XRCC1^{+/-}$ mice displayed increased brain damage following oxidative stress induced by ischemic stroke (Ghosh et al., 2015). Finally, very recently, a study by Hoch and colleagues described a patient with compound heterozygous mutations in *XRCC1* exhibiting ocular motor apraxia, axonal neuropathy and progressive cerebellar ataxia (Hoch et al., 2017). These are all features also observed in other cerebellar ataxias caused by mutations in proteins involved in the end-processing steps of BER that form complexes with XRCC1, including TDP-1, APTX and PNKP as described previously (Section 1.4.1).This finding therefore highlights the importance of XRCC1-containing repair complexes in the prevention of neurodegeneration in humans.

Dysregulation of XRCC1 levels has also been reported in a wide number of tumour types. In particular, low XRCC1 expression has been reported in breast (Sultana et al., 2013), gastric (Wang et al., 2012) and lung (Blomquist et al., 2009) carcinomas where it is generally associated with poorer outcomes. This includes increased tumour aggressiveness, reduced survival, and unresponsiveness to radiotherapy (Sak et al., 2005, Blomquist et al., 2009, Sultana et al., 2013). In contrast, overexpression of XRCC1 has also been reported to confer resistance to cisplatin in some gastric cancers (Xu et al., 2014). This clearly demonstrates that tight regulation of XRCC1 expression is necessary to ensure genome stability and survival, and it is therefore important to understand how XRCC1 expression is modulated.

1.5.2 XRCC1 Regulation

PTMs are known to be important for regulating XRCC1 function. For example, both DNA-PK and CHK2 have been shown to phosphorylate XRCC1 in response to DNA damage (Levy et al., 2006, Chou et al., 2008). CHK2-mediated phosphorylation of XRCC1 was suggested to promote BER, partially through enhanced XRCC1 interactions with MPG and UNG2 (Chou et al., 2008). Multiple phosphorylation sites for CK2 have also been identified at the XRCC1 C-terminus, located between the two BRCT domains (Loizou et al., 2004), which are postulated to enhance BER via different methods. Two studies independently showed that CK2 phosphorylation was important for XRCC1 protein-protein interactions, demonstrating that mutation of the proposed phosphorylation sites,

or inhibition of CK2 activity decreased the rate of SSB repair (Loizou et al., 2004, Luo et al., 2004). Moreover, Luo and colleagues specifically showed that CK2 phosphorylation at Ser518, Thr519 and Thr523 promoted XRCC1 interaction with PNKP and APTX (Luo et al., 2004). More recently, it has been proposed that CK2 phosphorylation also aids BER by promoting XRCC1 dissociation from DNA to facilitate backbone incision by APE1 and ensure fast repair. In this case, the authors observed decreased SSB formation after alkylating DNA damage in cells expressing XRCC1 where the CK2 phosphorylation sites had been mutated. They hypothesised that the decrease was due to an increased affinity of mutant XRCC1 for DNA, thereby reducing APE1 recruitment and subsequent SSB formation (Strom et al., 2011b). In addition to this, a study from our lab linked CK2 phosphorylation to XRCC1 stability; it was shown that direct phosphorylation by CK2 occurs on newly synthesised XRCC1, and is required for XRCC1-Lig III complex stability (Parsons et al., 2010).

The role of the ubiquitin-dependent proteasome pathway in regulating XRCC1 protein stability has been highlighted by several studies (Parsons et al., 2008, Kang et al., 2011, Xu et al., 2014). In particular, two different E3 ligases have been linked to XRCC1 stability. For example, it was shown that XRCC1 can be ubiquitylated by the E3 ligase CHIP (Parsons et al., 2008), the same enzyme that polyubiquitylates Pol β as discussed previously. In this case, ubiquitylation was mapped to the C terminal BRCT motif, as truncated XRCC1 protein lacking this region showed increased stability compared to the wild-type protein. Furthermore, siRNA-mediated knock down of CHIP also increased the stability of XRCC1, indicating that CHIP-dependent ubiquitylation results in proteasomal-mediated degradation of XRCC1 (Parsons et al., 2008) In addition to this, it was

shown that XRCC1 can be ubiquitylated in a poly(ADP-ribose)-dependent manner by the E3 ligase Iduna, although further study is needed to fully understand the cellular context of this modification (Kang et al., 2011).

With respect to transcriptional regulation, previous studies have identified two different transcription factors involved in modulating XRCC1 transcription. Firstly, two studies have reported that E2F1 is involved in XRCC1 transcriptional regulation. Chen et al. showed that overexpression of wild-type but not mutant E2F1 in Saos2 cells increased XRCC1 mRNA and protein levels (Chen et al., 2008b). A later study also showed that *XRCC1* expression correlated with E2F1 levels after release from HU-induced cell cycle arrest (Jin et al., 2011). Secondly, FOXM1 (Forkhead box protein M1) was reported to affect *XRCC1* transcription, with the authors showing that overexpression of FOXM1 stimulated XRCC1 promoter expression (Tan et al., 2007). Furthermore, with respect to DNA damage, it was shown that XRCC1 mRNA levels were increased in prostate cancer cells in a MAPK-dependent manner after ionising radiation (Yacoub et al., 2001). However, this finding has not been followed up in other cell lines and it is unclear how *XRCC1* transcription is affected by DNA damage. Therefore, it is evident that further study is needed to understand *XRCC1* transcriptional regulation. In particular, it is of interest to identify which transcription factors are involved in regulating basal expression of the *XRCC1* gene, and if XRCC1 levels can be modulated by the cellular DNA damage load.

As discussed previously, there is increasing evidence that ATM and BER are connected; ATM modifies proteins involved in BER (Agnihotri et al., 2014), and can be activated by SSBs, in the absence of detectable DSBs (Khoronenkova and Dianov, 2015). This led us to hypothesise that ATM might function as a

signalling molecule linking the cellular DNA damage load with BER modulation. Moreover, we hypothesised that transcription factor Sp1 might also be involved, acting as a transducer between ATM activation and BER, as Sp1 can be modified by ATM. The following section reviews our current understanding of the functions of transcription factor Sp1 in greater detail.

1.6 Sp1 (Specificity Protein 1)

Sp1 is the founding member of a family of highly related zinc finger proteins. The human Sp1 gene encodes a 785 amino acid, 90-105 kDa protein which is highly conserved in mammalian species; its DNA binding domain is also conserved in fish, reptiles and birds (Beishline and Azizkhan-Clifford, 2015). Sp1 was first identified in the 1980s, as a promoter-specific binding factor that was able to activate the SV40 early promoter (Dynan and Tjian, 1983), before subsequently being shown to recognise GC-rich regions of DNA (Gidoni et al., 1984). As Sp1 was found to be present in all mammalian cell types (Briggs et al., 1986), it was initially thought to function as a constitutive transcriptional activator of housekeeping genes. This was supported by the evidence that knockout of Sp1 causes embryonic lethality in mice. In fact, Sp1-deficient embryos die very early during development (around day 10.5), and display wide-ranging phenotypic abnormalities (Marin et al., 1997) suggesting a general function for Sp1 across many different cell types. However, this view is now somewhat out-dated and it is clear that Sp1 plays a role not just in maintaining basal transcription, but also in the induction/inhibition of transcription for a large number of genes. Estimates suggest that there are at least 12000 Sp1 binding sites in the human genome (Cawley et al., 2004); therefore, it is not surprising that Sp1 has been implicated in the expression of genes involved in many aspects of cellular function including DNA repair, cell cycle, differentiation and apoptosis. Moreover, although Sp1 is widely expressed, there are significant differences among cell types; in mice for example, Sp1 protein levels were shown to be low in Purkinje cells in the cerebellum, whereas high levels were reported in developing haematopoietic

cells, (Saffer et al., 1991). In contrast, low level Sp1 expression was reported during spermatogenesis (Ma et al., 2008). This highlights the wide-reaching roles for Sp1 in modulating different cellular processes but also strongly suggests that Sp1 functions can be highly context-specific. Therefore, to understand Sp1 functions, it is imperative to understand how Sp1 itself can be regulated.

1.6.1 Sp-family members

Sp1 belongs to the Sp/KLF (Krüppel-like factor) family of transcription factors, which has a total of 26 members (van Vliet et al., 2006). The family is characterised by a highly conserved DNA binding domain comprising three adjacent Cys_2His_2-type zinc fingers (Kadonaga et al., 1987), which bind GC or GT boxes. The wider Sp/KLF family is divided into two groups - Sp-like transcription factors, and KLF-like transcription factors. The Sp members are then further sub-divided into Sp1-4 and Sp5-9. In general, Sp1-4 have a similar overall domain structure, although Sp1, Sp3 and Sp4 are more closely related (Figure 1.8). In fact, the DNA binding domains of Sp1 and Sp3 share over 90 percent DNA sequence identity (Kingsley and Winoto, 1992). However, the two factors are believed to perform distinct functions in cells. Unlike Sp1, knockout of Sp3 is not embryonic lethal, for example. Instead, Sp3 mice die postnatally of respiratory failure (Bouwman et al., 2000). In addition, high resolution microscopy has revealed that the two factors reside in discrete regions of the nucleus, and are found in different promoter complexes (He et al., 2005). This further supports the hypothesis that Sp1 and Sp3 are not functionally equivalent.

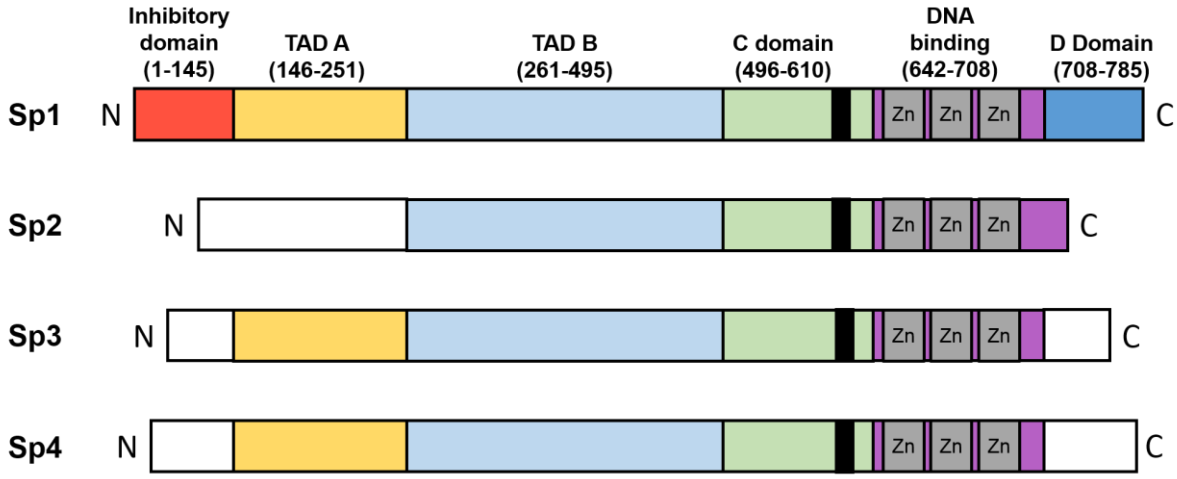

Figure 1.8 Domain organisation of Sp-like transcription factors

Conserved domains of the Sp-like transcription factors are shown. Sp1 contains an N terminal inhibitory domain (red) which is not present in other family members. With the exception of Sp2, the Sp-like family members contain two N-terminal transactivation domains (TAD A (yellow) and TAD B (light blue)). The C domain (green) contains a highly charged region adjacent to the DNA binding domain (purple) comprising three zinc fingers. Sp1 contains a C-terminal D domain (blue) important for multimerisation. Black box indicates highly conserved buttonhead domain.

1.6.2 Sp1 Protein Structure

An overview of Sp1 domain organisation is depicted in Figure 1.8. Sp1 contains two main N-terminal transactivation domains (TAD A and TAD B) which are characterised by glutamine-rich regions. In most cases the glutamine-rich regions are flanked by a serine/threonine-rich region, important for PTMs. In cooperation with the DNA binding domain, these two transactivation domains support the majority of Sp1 transcriptional activity. An inhibitory domain has also been mapped to the N-terminus (Murata et al., 1994). The C domain contains highly charged amino acids, and supports DNA binding and transactivation although it is not essential for either process (Courey and Tjian, 1988). As highlighted in Figure 1.8 Sp1 also contains a small region of homology to the *Drosophila* protein buttonhead (Athanikar et al., 1997). This so-called buttonhead element is thought to be important in synergistic promoter activation, as was

reported for the low density lipoprotein receptor gene (Yieh et al., 1995). The DNA binding domain comprises the three contiguous Cys_2His_2-type zinc fingers, which preferentially bind to the consensus sequence 5'T-GGGCGG-G/A-G/A-C/T-3' (Wierstra, 2008, Briggs et al., 1986); the three zinc fingers show differential sequence preferences (Kriwacki et al., 1992). However, the conservation of amino acids in the linker regions between the fingers suggests that the three zinc fingers function as a single unit (Harrison et al., 2000). Moreover, Sp1 does not contain a consensus NLS and it has been shown that the zinc finger domains actually serve as the NLS (Ito et al., 2009). In contrast to other Sp family members, Sp1 also contains a C-terminal multimerisation domain, termed the D domain, which mediates the super-activation of promoters that contain multiple adjacent Sp sites. Here, both DNA-bound and unbound Sp1 molecules can interact and form tetramers and ultimately larger multimers in order to promote the association of proximal promoter regions with distal enhancers. This is exemplified by regulation of the human DNA topoisomerase II α promoter where activation occurs by bridging Sp1 binding sites at a distal enhancer with those in the proximal promoter (Magan et al., 2003, Williams et al., 2007).

Interestingly, Sp1 is believed to be largely unstructured. The two transactivation domains, in addition to the C and D domains do not contain any known structural elements (Beishline and Azizkhan-Clifford, 2015), although an NMR structure has been elucidated for the DNA binding domain (Oka et al., 2004). It is thought that the disordered structure aids Sp1 interaction with a large number of protein partners to promote its own PTMs and therefore affect its functions (Beishline and Azizkhan-Clifford, 2015).

1.6.3 Sp1 in Human Disease

Given the role of Sp1 in modulating the expression of genes linked to a multitude of cellular processes, it is unsurprising that dysregulation of Sp1 is associated with the onset of disease. The most highly studied is cancer, although Sp1 has also been implicated in a number of neurodegenerative disorders including Huntington's, Alzheimer's disease and multiple sclerosis (O'Connor et al., 2017). With respect to cancer, Sp1 overexpression has been described in breast, colorectal, gastric, lung, pancreatic and thyroid cancers as well as in glioma (Beishline and Azizkhan-Clifford, 2015, Bajpai and Nagaraju, 2017); high levels of Sp1 normally correlate with poorer prognosis. With this in mind, Sp1 is an attractive therapeutic target. However, Sp1 regulated genes include oncogenes and tumour suppressors as well as anti-apoptotic and pro-apoptotic proteins. Therefore, the ability of Sp1 to regulate genes with opposing functions highlights the need to fully understand context-dependent regulation of Sp1 function.

1.6.4 Sp1 Regulation

While Sp1 is active in all cell types, it must also be tightly regulated to ensure appropriate responses to changing cellular conditions and signalling pathways. One major mechanism through which this is achieved is PTMs. Growing evidence indicates that a very large number of PTMs can influence Sp1 function including phosphorylation, acetylation, SUMOylation, ubiquitylation and *O*-linked glycosylation (Tan and Khachigian, 2009). These modifications not only affect Sp1 activity, but also its stability through modulation of proteolytic cleavage and proteasomal degradation. Given that a single molecule of Sp1 has the potential

to be extensively modified, this raises the question as to whether Sp1 proteins in the same cell can be differentially modified depending on their environment. Additionally, several modifications that have been identified either *in vitro* or from screens have yet to be associated with specific cellular functions, including the Ser101 modification which will be discussed in greater detail in Section 1.6.6.

Phosphorylation is the most highly studied Sp1 PTM. This is likely due to the sheer potential for Sp1 to undergo phosphorylation; prediction software suggests that Sp1 has 110 putative phosphorylation sites, with 67 of these being serine, 39 threonine and four tyrosine (from NetPhos 3.1 at www.cbs.dtu.dk/services/NetPhos/). It is already known that Sp1 can be phosphorylated at a number of these sites by the actions of various kinases (see Table 1.2) yet our understanding of the context and influence of these modifications is still in its infancy.

Sp1 phosphorylation is known to have different effects on Sp1 function. For example, studies using rat hepatoma cells showed that phosphorylation by CK2 at Thr579 (corresponding to Thr668 in human Sp1) reduced the DNA binding capacity of Sp1, without affecting protein stability (Armstrong et al., 1997). In contrast, a separate study showed that Ser59 phosphorylation by cyclin A-CDK2 was associated with increased transcription of known Sp1 responsive genes including dihydrofolate reductase (*DHFR*) (Fojas de Borja et al., 2001). ERK1/2-dependent Ser59 phosphorylation was also shown to increase transcription of the *CDKN1A* gene, encoding for the cell cycle regulator p21, thereby promoting senescence (Kim and Lim, 2009). It is clear, therefore, that the phosphorylation state of Sp1 influences its transcriptional activity. Interestingly, Sp1 phosphorylation has also been shown to affect its stability. For example, a study

by Chuang et al. found that Jun N-terminal kinase 1 (JNK1)-mediated Sp1 phosphorylation at Thr278 and Thr739 in HeLa cells protected the protein from ubiquitin-dependent degradation. This was found to increase Sp1 stability during mitosis (Chuang et al., 2008). Furthermore, ubiquitin-mediated degradation of Sp1 may also be modulated through Ser728 and Ser732 phosphorylation by Glycogen synthase kinase-3 beta (GSK3β) and ERK phosphorylation, also at Thr739 (Wei et al., 2009). Despite strong evidence for ubiquitin-mediated Sp1 degradation via the proteasome, to date no residue(s) that are directly ubiquitylated have been identified (Beishline and Azizkhan-Clifford, 2015).

In addition to phosphorylation, other PTMs are also known to modulate Sp1 protein stability. For example, an early study showed that reduced *O*-linked glycosylation under conditions of glucose starvation was associated with increased Sp1 degradation (Han and Kudlow, 1997). Using deletion mutants, it was later shown that the N-terminal 54 amino acids of Sp1 were required for its degradation (Su et al., 1999), and this was proposed to occur via interaction with the proteasome (Su et al., 2000). Sp1 stability is also negatively affected by SUMOylation at Lys16 (Spengler et al., 2008). More recently, Wang and colleagues showed that this SUMOylation event promotes Sp1 interaction with the E3 ligase Ring Finger Protein 4 (RNF4) ultimately leading to its degradation (Wang et al., 2011). Therefore it is clear that PTMs are extremely important for modulating different aspects of Sp1 function.

Table 1.2 Sp1 Post translational Modifications.

Adapted from (Beishline and Azizkhan-Clifford, 2015).

RESIDUE	MODIFICATION	ENZYME	FUNCTION
Ser7	Phosphorylation	Unknown	Stability
Lys16	SUMOylation	RNF4	Stability
Ser56	Phosphorylation	ATM/ATR	Unknown
Ser59	Phosphorylation	CDK2, ERK1/2	Stability/DNA binding
Ser101	Phosphorylation	ATM/ATR	Unknown
Ser111-114	Glycosylation	OGT	Unknown
Ser220	Phosphorylation	DNA-PK	Transcription
Thr278	Phosphorylation	JNK1, ERK1/2	Transcription
Thr453	Phosphorylation	ERK1/2	Transcription
Thr668	Phosphorylation	CK2	DNA binding
Lys703	Acetylation	p300	Unknown
Ser728	Phosphorylation	GSK3β	Degradation
Ser732	Phosphorylation	GSK3β	Degradation
Thr739	Phosphorylation	ERK1/2, JNK1	Transcription/stability

1.6.5 Sp1 is a pleiotropic modulator of cell fate

Sp1 is linked to the regulation of genes involved in a wide number of different cellular processes. In terms of the DNA damage response, Sp1 is recognised to modulate the expression of genes involved in cell cycle arrest, apoptosis as well as in DNA repair. For example, numerous studies have investigated the role of Sp1 in regulating p21 expression. This was first shown in an early study from Biggs and colleagues which identified Sp1 binding sites in the *CDKN1A* promoter important for p21 induction in response to okadaic acid (Biggs et al., 1996). However, it is now clear that there multiple signalling pathways associated with Sp1-dependent modulation of p21 (Beishline and Azizkhan-Clifford, 2015). Sp1-dependent regulation of multiple components of apoptosis, both pro- and anti-apoptotic, has also been described. Grande and colleagues demonstrated that Sp1 modulates expression of the pro-apoptotic protein NOXA. Specifically, they showed in testicular embryonal carcinoma cells that downregulation of Sp1 increased expression of NOXA indicating that Sp1 plays a repressive role in

NOXA regulation (Grande et al., 2012). In contrast, it has been reported that Sp1 is important for expression of the anti-apoptotic factor survivin (Xu et al., 2007). In fact, the overexpression of survivin, which has been reported in many tumour types, was directly linked to Sp1 activity in a study using colon cancer cells (Asanuma et al., 2004). These examples demonstrate that Sp1 clearly plays a role in regulating multiple cell fates.

As well as modulating genes involved in cell fate decisions, it has become increasingly clear that Sp1 functions are linked to the DNA damage response. For example, Sp1 DNA binding in human cell lines exposed to IR was shown to increase in a transient and reversible manner (Meighan-Mantha et al., 1999, Yang et al., 2000). Furthermore, Sp1 DNA binding was shown to be affected by oxidative stress. In a model of cortical neurons, Sp1 binding to a canonical Sp1 DNA sequence increased during homocysteate-induced oxidative stress, with loss of Sp1 under these conditions associated with decreased viability (Ryu et al., 2003). This suggests that Sp1 function can be modulated by DNA damage. Additionally, depletion of Sp1 was shown to inhibit repair of site-specific DNA breaks (Beishline et al., 2012) and was also associated with increased sensitivity to IR (Iwahori et al., 2008). Specifically with respect to transcription, Sp1 has also been shown to regulate the expression of a number of genes involved in DNA repair, including *APEX1* in BER (Poletto et al., 2016). Taken together, these examples suggest that Sp1 does indeed perform important roles in the cellular response to DNA damage.

1.6.6 Ser101 phosphorylation

In further support of a role for Sp1 in the DNA damage response, some serine and threonine residues within the transactivation domains are organised into two SQ/TQ clusters (Olofsson et al., 2007). These clusters are frequently found in ATM substrates. Interestingly two groups reported ATM-dependent phosphorylation of Sp1 in response to DNA damage including IR and H_2O_2 as well as HSV-1 infection (Iwahori et al., 2007, Olofsson et al., 2007), mapped to Ser101. Several studies then attempted to investigate the role of this modification. Ser101 phosphorylation was shown not to affect transcriptional activity at Sp1-responsive promoters, for example (Iwahori et al., 2008). Furthermore, the same study showed co-localisation of Ser101-phosphorylated Sp1 with activated ATM at sites of DNA damage. A non-transcriptional role for Ser101 phosphorylation was also proposed by another study demonstrating that a deletion mutant containing only the N-terminal 182 amino acids could still co-localise with γH2AX at sites of DNA damage. Since this deletion mutant lacked both the DNA binding and multimerisation domains, the study concluded that the role of Sp1 in DSB repair is distinct to its role in transcriptional regulation (Beishline et al., 2012). The absolute role of Ser101 phosphorylation, however, is still unclear.

1.7 Aims of the study

It is clear that much is known about the molecular steps that comprise the BER pathway. Our knowledge regarding how the pathway is regulated, however, is more incomplete. For example, although it is known that variations in the cellular BER capacity occur, how this is mediated is mainly unknown. In particular, it is uncertain how BER responds to fluctuations in the cellular level of DNA damage, especially at the transcriptional level. This understanding is vital for deciphering how BER can adapt in the long term to changes in the cellular DNA damage load. Therefore, this study seeks to address this gap in our knowledge, to understand how variations in the DNA damage load are sensed and communicated to the BER pathway to adjust repair capacity.

The major question that will be addressed by this thesis is to understand how BER is modulated by the cellular DNA damage load at the level of transcription. Specifically, this will be studied with respect to *XRCC1* transcription, due to its central role in BER coordination. Furthermore, as we hypothesise that Sp1 may act as a transducer between ATM and BER, this study will investigate the function of Sp1 Ser101 phosphorylation after DNA damage.

2 Materials and Methods

2.1 Cell culture

TIG-1 normal human fibroblasts were obtained from the Coriell Institute Cell Repository (AG06173). Cells were cultured in DMEM low glucose (Life Technologies) supplemented with 15% FBS at 37°C in a humidified atmosphere with 5% CO_2. The Nishi NK cell line has been previously described (Cerboni et al., 2001). NK cells were grown in IMDM GlutaMAX™ (Life Technologies) supplemented with 10% FBS, 2% heat-inactivated human serum (Sigma-Aldrich), 100 IU/mL penicillin, 100 µg/mL streptomycin and 10 ng/mL recombinant human IL-15 (PeproTech), at 37°C in a humidified atmosphere with 7.5% CO_2. Cells were routinely checked for mycoplasma.

2.2 Cell viability and co-culture assays

Cell viability was assessed using resazurin (Sigma-Aldrich). For co-culture experiments, TIG-1 cells were treated as described before a suspension of NK cells was aliquoted onto adherent fibroblasts at the indicated NK:TIG-1 ratio. Cell cytotoxicity was assessed after co-incubation by washing off NK cells and evaluating the viability of fibroblasts using resazurin.

2.3 Cell treatments

Campothecin, cisplatin, cycloheximide, etoposide, H_2O_2, MMS, theobromine, the APE1 inhibitor (Inhibitor III), the Chk1 inhibitor (UCN-01) and midostaurin were all purchased from Sigma-Aldrich. Mithramycin A and MG132 were from Enzo Life Sciences. Zeocin was obtained from Life Technologies. The Chk2 inhibitor (CCT 241533) was from Tocris. The ATM inhibitors (KU-55933 and KU-60019) were obtained from Abcam and R&D systems, respectively. The DNA-PK inhibitor (Inhibitor III) and staurosporine were obtained from Millipore while Navitoclax (ABT-263) and sulindac were purchased from Cayman Chemicals. The ATR inhibitor (VE-821) was a kind gift from Dr Anderson Ryan (University of Oxford) while irinotecan was a kind gift from Dr Ricky Sharma (University of Oxford).Timings and doses are indicated for each experiment.

2.4 siRNA Transfections

Transient transfections with siRNAs were carried out using the Lipofectamine RNAiMAX reagent (Life Technologies), according to the manufacturer's instructions. Cells were treated with 30 nM siRNA and analysed 72 h after transfection, unless otherwise indicated. Control transfections were carried out using a non-targeting siRNA from Eurogentec (SR-CL000-005), all custom siRNA oligonucleotides were also obtained from Eurogentec. A detailed list of the sequences can be found in Appendix II.

2.5 Whole cell extracts

Whole cell extracts were prepared essentially as described (Tanaka et al., 1992). Buffers were completed with protease inhibitors (aprotinin, chymostatin, leupeptin and pepstatin, all 1 µg/mL, 1 mM PMSF) in addition to 1 mM DTT and 1 mM N-Ethylmaleimide (NEM). For harvesting, cells were washed in ice cold phosphate buffered saline (PBS) and scraped, before centrifugation at 240 g. Cells were then resuspended in one packed cell volume of buffer I (10 mM Tris-HCl (pH7.8), 200 mM KCl). Following this, two packed cell volumes of buffer II (10 mM Tris-HCl pH7.8, 600 mM KCl, 40% glycerol, 2 mM EDTA, 0.2% IGEPAL® CA-630) were added and mixed thoroughly before incubating for 15 min on ice. Cell lysates were then centrifuged at 16100 g for 20 min and the supernatant was collected.

2.6 Subcellular fractionation

To generate cytoplasmic and nuclear fractions, cells were sequentially resuspended in two different buffers, both of which were completed with protease inhibitors (aprotinin, chymostatin, leupeptin and pepstatin, all 1 µg/mL, 1 mM PMSF) in addition to 1 mM DTT and 1 mM NEM. For experiments looking at S101 phosphorylation of Sp1, phosphatase inhibitor cocktail V (Millipore) was also included. Firstly, cells were resuspended in one packed cell volume of buffer I (20 mM Tris-HCl (pH 7.4), 2.5 mM $MgCl_2$, 0.5% (v/v) IGEPAL® CA-630) and vortexed before incubation on ice for 10 min. Nuclei were pelleted by centrifugation at 10600 g for two min. The supernatant, containing the

cytoplasmic fraction, was collected and stored separately on ice. The nuclear pellet was then resuspended in buffer II (20 mM phosphate buffer pH 8.0, 500 mM NaCl, 1 mM EDTA, 0.75% (v/v) Triton X-100, 10% glycerol, 5 mM $MgCl_2$) in the same volume as with buffer I, vortexed, and incubated on ice for 10 min. Insoluble components were removed by centrifugation at 10600 g for two min, and the supernatant, containing the nuclear fraction, was collected.

2.7 Bradford assay

The colourimetric Bradford assay was used to determine protein concentration in cell extracts. The Bio-Rad Protein Assay reagent (Bio-Rad) was used to dilute the sample to be assayed five hundred fold; optical density at 595 nm was then measured using a spectrophotometer. Bovine serum albumin (BSA) was used as protein standard.

2.8 Western blotting

Separation of proteins was performed using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Protein samples were prepared in Laemmli Loading Buffer (25 mM Tris, pH 6.8, 2.5% β-mercaptoethanol, 1% (v/v) SDS, 5% (v/v) glycerol, 1 mM EDTA and 0.05 mg/mL bromophenol blue) before being boiled at 95°C for five min and loaded onto either 8%, 10%, 12% or 4-16% polyacrylamide gels, all made in-house. Electrophoresis was performed in Tris-Glycine SDS running buffer (25 mM Tris-HCl, pH 8.3, 192 mM glycine, 0.1%

(w/v) SDS). Following electrophoresis, separated proteins were transferred onto an Immobilon-FL polyvinylidene difluoride (PVDF) membrane which had been activated in methanol prior to use. Transfer buffer contained 25 mM Tris, pH 8.3, 192 mM glycine, 20% methanol. After transfer, the membrane was blocked for one hour at room temperature in Odyssey blocking buffer (Li-Cor Biosciences) diluted 1:1 in PBS, and incubated overnight at 4°C in primary antibody. Membranes were washed three times for five minutes in PBS supplemented with 0.1% Tween-20 before incubation for 1 h in the appropriate fluorescently-labelled secondary antibody. Membranes were again rinsed three times in PBS with 0.1% Tween-20 and analysed using an Odyssey image analysis system (Li-Cor Biosciences). Quantification of band intensities was also performed using the Odyssey Imager software. A detailed list of the antibodies used in this study is given in Appendix II.

2.9 Flow cytometry

For flow cytometry analyses, cells were harvested by trypsinisation before fixation in ice-cold 70% ethanol. Cells were treated with 100 µg/mL RNase A (Qiagen) before staining with 20 µg/mL propidium iodide (Sigma). After staining, samples were run on a Becton-Dickinson FACScan (BD-Biosciences) and cell cycle distribution was analysed using Modfit LT software (Verity Software House). Apoptosis was measured using the AnnexinV-FITC Apoptosis Detection Kit (Abcam) as per the manufacturer's instructions. Samples were acquired using a Becton-Dickinson FACSCalibur™ instrument and data analysis was carried out using BD CellQuest Pro.

2.10 Quantitative Real-time PCR (qPCR)

Total RNA was extracted using the RNeasy® Mini Kit (Qiagen), according to the manufacturer's instructions. RNA concentrations were measured using a Nanodrop ND-1000 (Thermo Scientific) and quality assessed using A260/A280 and A260/A230 ratios. Generation of cDNA was completed using the Superscript™ First Strand Synthesis System for RT-PCR (Life Technologies), according to the manufacturer's protocol. qPCR was performed using the Fast SYBR® Green Master Mix (Applied Biosystems) as per instructions provided by the manufacturer. Reactions were carried out using a 7500 Fast Real-Time PCR-System (Applied Biosystems). The comparative CT method was applied for quantification of gene expression, using *GAPDH* and *B2M* as endogenous controls, unless otherwise indicated. A list of the primers used can be found in Appendix II.

2.11 Chromatin Immunoprecipitation (ChIP)

After the relevant treatment, cells were cross-linked with 1.5% formaldehyde diluted in culture medium for 15 min at room temperature before quenching with 125 mM glycine. Lysis and dilution buffers were completed with protease inhibitors (aprotinin, chymostatin, leupeptin and pepstatin, all 1 µg/mL, 1 mM PMSF). Cells were scraped, collected by centrifugation and lysed in lysis buffer (50 mM Tris-HCl pH 8.1, 10 mM ethylenediaminetetraacetic acid (EDTA), 1% sodium dodecylsulfate (SDS) for 10 min on ice. Chromatin was sheared to yield average sizes of 200-1000 bp using a Bioruptor® (Diagenode) (Figure 9.5), and

clarified by centrifugation at 16100 *g* for 10 min at 4°C. Supernatant protein concentration was quantified using a Bradford assay (Bio-Rad) and an equal amount of protein between samples was diluted 5-fold in dilution buffer (16.7 mM Tris–HCl pH 8.1, 1.2 mM EDTA, 0.01% SDS,1.1% Triton X-100, 167 mM NaCl). To decrease non-specific binding, chromatin was pre-cleared using protein A/G magnetic beads (New England Biolabs) by end-over-end rotation for 1 h at 4°C. For immunoprecipitation, four µg of Sp1 antibody (Millipore) was added to the pre-cleared supernatants and rotated end-over-end overnight at 4°C. Control reactions were instead incubated with four µg normal rabbit IgG (Santa Cruz Biotechnology). Protein-DNA complexes were pulled down by incubation with protein A/G magnetic beads for 2 h at 4°C with end-over-end rotation. To further decrease non-specific interactions, beads were pre-saturated with 100 ng/µL BSA and 100 ng/µL salmon sperm DNA (Invitrogen) for 30 min at room temperature. After pull-down, protein-DNA complexes were washed consecutively with solutions containing increasing concentrations of salt, each for five min end-over-end at 4°C. Firstly, low salt wash solution (20 mM Tris–HCl pH 8.1, 2 mM EDTA, 0.1% SDS, 1% Triton X-100, 150 mM NaCl), then high salt wash solution (20 mM Tris–HCl pH 8.1, 2 mM EDTA, 0.1% SDS, 1% Triton X-100, 500 mM NaCl), followed by a lithium chloride wash solution (10 mM Tris–HCl pH 8.1, 1 mM EDTA, 1% sodium deoxycholate, 1% IGEPAL® CA-630, 0.25 M LiCl) and two washes in TE buffer (10 mM Tris–HCl pH 8.1, 1 mM EDTA). Protein-DNA complexes were eluted using freshly prepared elution buffer (0.1 M $NaHCO_3$, 1% SDS) by end-over-end rotation at room temperature for 30 min. Proteins were removed by digestion with proteinase K for 3 h at 55°C (40 mM Tris-HCl pH 6.5, 200 mM NaCl, 10 mM EDTA, 40 ng/µL proteinase K (Sigma), 20

ng/μL RNase A). Cross-linking was reversed by overnight heating at 65°C. DNA was then extracted using a QIAquick® PCR purification kit. qPCR analysis was carried out, as detailed in Section 2.10.

2.12 Electrophoretic Mobility Shift Assay (EMSA)

For EMSAs, an *XRCC1* promoter probe (-189 to -165) was obtained by annealing a 5′-IRDyeR800-labelled oligonucleotide (5'-GTGTGGCGGAGGGAGGCGGGGCTGGAGGAAACG-3' (IntegratedDNA Technologies)) with its unlabelled complementary sequence 5'-CGTTTCCTCCAGCCCCGCCTCCCTCCGCCACAC-3' in annealing solution (40 mM Tris-HCl pH 8.0, 50 mM NaCl). An unlabelled Sp1 consensus probe (forward: 5′-ATTCGATCGGGGCGGGGCGAGC-3', reverse: 5'-GCTCGCCCCGCCCCGATCGAAT-3') (Kadonaga et al., 1986) was used as a cold competitor to confirm binding specificity. Nuclear extracts or recombinant Sp1 protein were used in EMSA reactions; both were obtained as described in Sections 2.6 and 9.1 although the nuclear extracts used in EMSA were produced without the addition of DTT or NEM. Binding reactions were set up by incubating either 10 μg nuclear extract or 500 ng recombinant Sp1 in 20 mM Tris-HCl pH 7.5, 100 mM KCl, 10 mM $ZnSO_4$, 0.2% IGEPAL® CA-630, 20% glycerol, 5 mM DTT, supplemented with 1 μg salmon sperm DNA (Invitrogen) and 50 nM double stranded *XRCC1* promoter probe for 15 min at 37°C. For experiments using mithramycin A, the probe was pre-incubated with mithramycin A for five min at 37°C before the addition of the remaining reaction components for a further ten min. Samples were separated on a 6% native PAGE gel at 150 V for 50 min,

which was pre-electrophoresed for 10 min at 150 V. The gel was then analysed on an image analysis system (Li-Cor Biosciences). A reaction scheme is provided in Appendix I (Figure 9.6).

2.13 Luciferase assays

For luciferase assays, the pGL3-XRCC1 reporter plasmid containing the *XRCC1* promoter was used. See section 2.24.1 for cloning details. TIG-1 cells were transfected with 30 nM relevant siRNA using Lipofectamine® RNAiMAX according to the manufacturer's protocol. Twenty four hours after siRNA transfection, cells were transferred into 96-well plates. Once cells had attached, transfection of pGL3-XRCC1 was carried out using the Viromer® YELLOW reagent (Lipocalyx) as per the manufacturer's instructions. Cells were analysed for luciferase activity 48 h post plasmid transfection using a Dual-Glo® Luciferase Assay System (Promega). To correct for bias in transfection efficiency, the pGL3-XRCC1 plasmid, encoding a firefly luciferase (*P. pyralis*)-based reporter, was co-transfected with a pCMV-RL vector. The latter expresses a Renilla luciferase (from *R. reniformis*)-based reporter under the control of a strong Cytomegalovirus (CMV) promoter. The luminescence generated from the first construct, pGL3-XRCC1, was then normalised to signal originating from the second, pCMV-RL, to obtain *XRCC1* promoter activity.

2.14 Apoptosis assays

Apoptosis induction was measured using the Apo-ONE® Homogeneous Caspase-3/7 Assay kit (Promega), according to the manufacturer's protocol. Cells were treated as indicated and fluorescence was assessed using a POLARstar Omega plate reader (BMG Labtech). Measurement of cell viability with resazurin was used to normalise for fluorescence signal.

2.15 Alkaline comet assays

The alkaline comet assay was used to detect alkali-sensitive DNA lesions; these include AP sites, SSBs and DSBs. Briefly, cells were trypsinised, mixed in suspension with 1% low melting point agarose (Bio-Rad) and embedded on a microscope slide. The slides were then placed in freshly prepared lysis buffer (2.5 M NaCl, 100 mM EDTA, 10 mM Tris adjusted to pH 10.5 using NaOH, 1% (v/v) DMSO, 1% (v/v) Triton X-100), for 1 h at 4°C. Slides were then incubated for 30 min in the dark in cold electrophoresis buffer (300 mM NaOH, 1 mM EDTA, 1% (v/v) DMSO, pH 13.0) to allow DNA to unwind, before electrophoresis for 25 min at 25 V. Following electrophoresis, slides were neutralised with 0.5 M Tris-HCl, pH 8.0, and left to dry overnight. Slides were rehydrated in H_2O and stained for 30 min with Sybr Gold (Life Technologies), before imaging on a Nikon Eclipse 90i microscope and analysed using Komet 5.5 image analysis software (Andor Technology). The percentage of DNA present in the tail was analysed for at least 50 cells per slide.

2.16 Neutral comet assays

Neutral comet assays were used to detect DSBs. Cells were trypsinised, mixed in suspension with 1% low melting point agarose (Bio-Rad) and embedded on a microscope slide. The slides were then placed in freshly prepared cold lysis buffer (2.5 M NaCl, 100 mM EDTA, 10 mM Tris adjusted to pH 9.5 using NaOH, 1% (v/v) sodium lauryl sarcosinate, 10% (v/v) DMSO, 0.5% (v/v) Triton X-100) for 2 h at 4°C. Slides were then washed three times for 20 min in cold 1x Tris borate EDTA (TBE) (National Diagnostics) buffer, before electrophoresis for 25 min at 25 V. Following electrophoresis, slides were analysed as described for the alkaline comet assays.

2.17 *In vitro* ligation assays

For nick ligation reactions, 1 µg of nuclear extract was incubated in 50 mM Tris-HCl pH 7.5, 10 mM $MgCl_2$, 10 mM DTT, and 1 mM ATP at 37°C for the indicated time. The oligonucleotide substrate was used at 50 nM and has been previously described (Wilson, 2003, McNeill et al., 2004). Sequences are provided in Table 2.1 and a schematic for the substrate is provided in Figure 2.1. The DNA substrate was 5′-labelled with IRDye®800 (IDT). Reactions were halted with 96% formamide and 10 mM EDTA before analysis by electrophoresis on a 20% denaturing urea-polyacrylamide gel. The percentage of substrate converted to product was determined by using an Odyssey image analysis system (Li-Cor Biosciences).

Table 2.1 Sequences of oligonucleotides used for *in vitro* ligation assays.

Oligonucleotide	Nucleotide sequence (5'-3')
15P	CTGCAGCTGATGCGC
pC19	pCGTACGGATCCCCGGGTAC
34G	GTACCCGGGGATCCGTACGGCGCATCAGCTGCAG

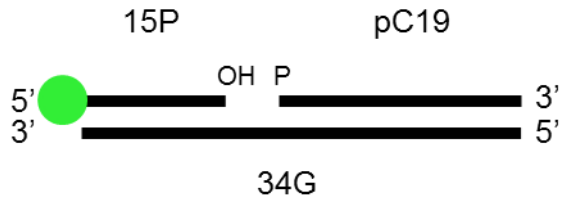

Figure 2.1 Schematic of oligonucleotide substrate used for *in vitro* ligation studies

Green circle represents IRDye®800 tag.

2.18 *In vitro* phosphorylation assays

Phosphorylation reactions were carried out in phosphorylation buffer (50 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), pH 7.5, 50 mM KCl, 10 mM $MgCl_2$, 1 mM ATP, 1 mM DTT and 5% (v/v) glycerol) using 500 ng recombinant Sp1 and 100 ng active recombinant ATM (Millipore). Reactions were incubated for 2 h at 30°C, and stopped with the addition of Laemmli loading buffer.

2.19 Immunofluorescence

For immunofluorescence analysis, TIG-1 cells were seeded onto coverslips and treated as indicated. Immunostaining was carried out on plate using standard procedures. Cells were fixed with paraformaldehyde (4% in PBS) for 15 min at

room temperature, washed three times with PBS, then permeabilised using 0.2% (v/v) Triton-X in PBS for 10 min at 4°C. After fixation, cells were blocked in 5% BSA in PBS for 1 h. Incubation with a primary antibody was carried out in 5% BSA-PBS supplemented with 0.01% Tween-20. An Alexa Fluor 488-conjugated secondary antibody (Life Technologies) was used for indirect detection of the antigens. Hoechst 33342 (Life Technologies) was used to visualise nuclei.

2.20 High-throughput microscopy

For double strand break repair assays, TIG-1 cells were first transfected with 30 nM indicated siRNA using Lipofectamine® RNAiMAX then transferred to 96-well plates 24 h after transfection. Zeocin (50 µg/mL) was added to cells for 1 h, before being washed away, and cells left to repair in fresh medium for times indicated. Immunostaining was carried out as detailed in 2.19. Images were acquired using an IN Cell Analyzer 1000 Imaging System and data were analysed using IN Cell Investigator Software (GE Healthcare Life Sciences). At least 500 cells per condition were analysed. For quantification of γH2AX foci, TIG-1 cells were treated with H_2O_2 for 1 h at doses indicated before release for either 0 or 24 h in fresh medium. Immunostaining and IN Cell analysis were carried out as for 53BP1 foci.

2.21 Reactivation assays

2.21.1 Plasmid Preparation

For use in these assays, a pCMV-RL plasmid was depurinated by incubation at 65°C for 40 min in 0.1 M sodium citrate and 0.5 M NaCl. DNA was recovered by ethanol precipitation. Briefly,1/10th reaction volume 3 M sodium acetate pH5.2 was added to the sample followed by three reaction volumes 100% ethanol before incubation at -80°C for 30 min. Following this, the reaction was centrifuged at 4°C for 40 min at 16100 g and supernatant removed. The pellet was then washed with 70% ethanol before further centrifugation at 4°C for 5 min at 16100 g. The supernatant was removed and the pellet left to air dry for 10 min before resuspension in H_2O. Depurinated plasmid was stored at -80 in single-use aliquots. Success of depurination was checked by APE1 incision activity (Appendix I, Figure 9.7, panel B).

2.21.2 Assay set -up

A reaction scheme is provided in Appendix I. TIG-1 cells were first transfected with 30 nM relevant siRNA using Lipofectamine® RNAiMAX as per the manufacturer's recommendations. Twenty four hours after siRNA transfection, cells were transferred into 96-well plates. A further 24 hours later, cells were transfected with 50 ng of either damaged or undamaged pCMV-RL in combination with 25 ng pEGFP-N1 (encoding for GFP, used to correct for bias in transfection efficiency) using the Viromer® YELLOW reagent (Lipocalyx) as per the manufacturer's instructions. Cells were analysed for luciferase activity either 48 or 72 h post plasmid transfection, using the Renilla-Glo® Luciferase Assay

System (Promega) as per the manufacturer's indications. Repair efficiency was expressed as percentage of luciferase activity normalised to the undamaged plasmid signal.

2.22 Bioinformatics analysis

2.22.1 XRCC1 promoter analysis

Bioinformatics analysis of the *XRCC1* promoter was carried out using the Genomatix Software Suite. Putative transcription factor binding sites in the *XRCC1* promoter were identified using the MatInspector tool. Potential positive sites were identified by a high matrix similarity score, indicating high sequence similarity with the consensus site for the transcription factor of interest. *XRCC1* promoter sequences (1 kb upstream from the transcription start site) from multiple species were downloaded from the Genomatix Software Suite and multiple sequence alignment was completed using ClustalX (Larkin et al., 2007).

2.22.2 Connectivity Map analysis

The Connectivity Map dataset contains genome-wide microarray expression data for 453 different treatment and vehicle control pairs, using 164 distinct small molecules in three different cell lines. The tool builds a hypothetical gene expression signature based on down-regulated and up-regulated genes ("down tag" and "up tag", respectively). In our case, the "down tag" included *XRCC1* and *APEX1* whereas the "up tag" contained *CDKN1A* (encoding for p21), in order to mimic conditions of ATM activation. Enrichment of the induced (p21) and repressed (*XRCC1* and *APEX1*) genes for each treatment was estimated using

the Kolmogorov-Smirnov statistic as described (Lamb et al., 2006), and an overall connectivity score for the signature was generated. A high positive connectivity score indicates that the genes in the signature are behaving as predicted whereas a negative score indicates negative correlation. Drugs with both positive and negative connectivity scores were analysed by Western Blotting for the expression of Sp1, and induction of ATM autophosphorylation. Connectivity Map data are available at *http://www.broad.mit.edu/cmap* and GEO (Gene Expression Omnibus, accession number GSE5258).

2.23 Bacterial transformation

E. coli strains XL1-Blue Competent Cells (Agilent Technologies) and DH5α were used to amplify plasmid DNA for experimental use. XL10-Gold® Ultracompetent Cells (Agilent Technologies) were used for site directed mutagenesis. Rosetta™ *E. coli* cells were used for protein expression. All bacterial strains were cultured in Luria-Bertani (LB) broth, supplemented with the appropriate antibiotic for each plasmid. Heat shock conditions were as per the manufacturer's recommendations for each bacterial strain.

2.24 Cloning

2.24.1 pGL3-XRCC1

For generation of the pGL3-XRCC1 plasmid, containing the *XRCC1* promoter upstream of a luciferase reporter, genomic DNA was first extracted from TIG-1

cells using a GenElute™ Mammalian Genomic DNA miniprep kit (Sigma). The genomic region upstream of the *XRCC1* gene (-3969-+174) was then amplified using primers KpnI_XRCC1_F and NheI_XRCC1_R, and cloned into a pGL3 plasmid (Promega). Primers to amplify the 4 kb region upstream of the *XRCC1* gene were designed against the DNA sequence provided by the Biomart tool at *Ensembl.org*. Empty pGL3 was linearised using KpnI and NheI (both New England Biolabs), and digested plasmid was recovered using a QIAquick® Gel Extraction Kit (Qiagen), after electrophoresis on a 1% agarose gel. After digestion, vector and insert were ligated at a 1:3 ratio (vector:insert) using T4 ligase (New England Biolabs), overnight at 16°C. In every ligation reaction two controls were included, the first containing no DNA ligase and the second with no insert, in order to correct for incomplete plasmid restriction and vector self-circularisation. Ligation products were transformed into XL1-Blue Competent Cells (Agilent Technologies). Colonies were screened by colony PCR, and positive clones were verified by sequencing. A map of pGL3-XRCC1 is given in Figure 9.4.

2.24.2 pET28a-Sp1

Sub-cloning Sp1 cDNA into a bacterial expression vector was carried out by amplification of pN3-Sp1, a gift from Guntram Suske (Addgene plasmid #24543) using the primers EcoRI_Sp1_F and EcoRI_Sp1_R. The empty vector pET28a-Sp1 was linearised using EcoRI (New England Biolabs) and digested plasmid was recovered using a QIAquick® Gel Extraction Kit (Qiagen), after electrophoresis on a 1% agarose gel. Ligation and transformation steps were carried out as for the generation of *pGL3*-XRCC1. Colonies were also screened by colony PCR and positive clones verified by sequencing.

2.25 Site-directed mutagenesis

The Quikchange Lightning Site-Directed Mutagenesis Kit (Agilent Technologies) was used to introduce targeted mutations in different plasmids. Primers were designed to only introduce mismatches in the middle of each oligonucleotide sequence. PCR was carried out as per the manufacturer's recommendations. Amplification products were digested using DpnI to remove methylated and hemi-methylated parental DNA strands, and transformed into XL10-Gold® Ultracompetent Cells. Bacteria were grown for 1 h at 37°C in Super optimal broth with catabolite repression (SOC) medium before being plated onto an LB-agar plate containing the appropriate antibiotic. Positive colonies were verified by sequencing.

Plasmids used in all assays were purified using a Qiafilter™ Plasmid Maxi Kit (Qiagen).

2.26 Statistical analysis

Statistical data are presented as a mean ± standard deviation of at least three independent biological experiments. P values were calculated using the two-tailed Student's t-test using Microsoft Excel or SPSS. A P value of <0.05 was considered significant.

3 Transcription factor Sp1 regulates expression of *XRCC1*

Understanding more about the regulation of BER is imperative. The cellular environment and inherent instability of the DNA molecule means that there is constant endogenous damage that cells must cope with. SSBs and base lesions comprise the majority of this DNA damage, which is mainly repaired through BER. Given that XRCC1 is essential for the coordination of the pathway, it is important to understand how XRCC1 is regulated, and particularly whether it may be modulated according to the DNA damage load within a cell. Studies have already elucidated much about regulation of the XRCC1 protein itself, particularly that post translational modifications are important for regulating its stability. What is mainly unknown, however, is how *XRCC1* is regulated at the transcriptional level. Understanding transcriptional regulation is important for inferring the mechanisms that contribute to the long-term regulation of BER in order to promote adaptation to changes in the cellular environment. We, therefore, sought to address this gap in our knowledge. Importantly, we were interested in understanding the regulation occurring in response to endogenous DNA damage in normal cells. To achieve this, the use of non-transformed cells was, therefore, imperative in this study as cancer cells are genetically unstable. We have previously demonstrated, for example, that impaired p53 function, a feature of many cancer cells, leads to a failure in the coordination of BER (Poletto et al., 2016). Therefore, in an attempt to avoid any bias, all of the experiments in this thesis have been carried out using normal human fibroblasts.

3.1 Bioinformatics analysis of the *XRCC1* promoter highlights several putative transcription factors

The initial aim of my project was to identify transcription factors that might modulate basal transcription of the *XRCC1* gene. By using a combination of bioinformatics and literature searches, I produced a shortlist of candidate transcription factors, which were then validated. *In silico* analysis of promoter sequences using the MatInspector Tool (Cartharius et al., 2005) ranks short DNA sequences/potential transcription factor binding sites by their homology to the consensus sequence for different transcription factors. Examining a 1 kb region of the proximal *XRCC1* promoter (Figure 3.1) produced an extensive list of putative transcription factors including OCT1, E2F family members and Sp1. These findings were then cross-referenced against a literature search for XRCC1 transcriptional regulation to further narrow down potential candidates.

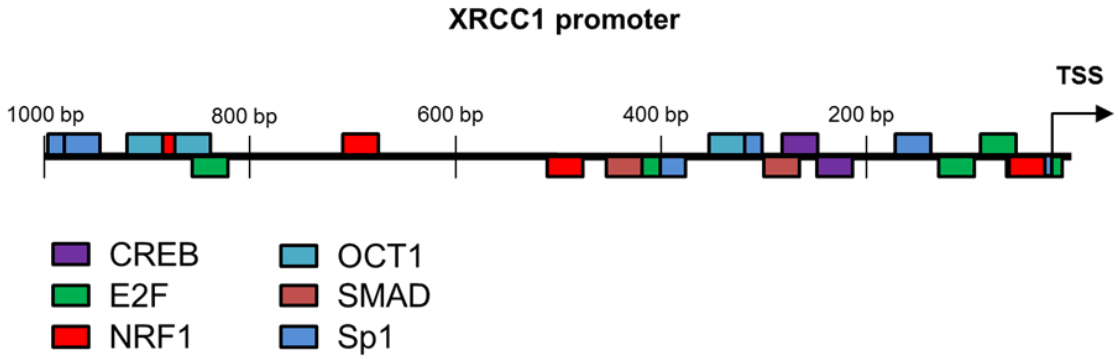

Figure 3.1 Bioinformatics analysis of the *XRCC1* promoter

Analysis of the region of the *XRCC1* promoter 1 kb upstream of the proposed transcriptional start site performed using the MatInspector tool. Colours are used to indicate different putative transcription factor families and their proposed binding sites, which were highlighted by the analysis. CREB; cAMP response element binding protein; E2F; E2F transcription factor; NRF1; nuclear respiratory factor 1; OCT1; Octamer transcription factor; SMAD; SMAD family member; TSS; Transcription start site.

Previous studies have linked two different transcription factors to *XRCC1* transcriptional regulation, namely E2F1 (Chen et al., 2008b) and FOXM1 (Tan et al., 2007). Specifically, Chen and colleagues showed that overexpression of E2F1 led to increased XRCC1 expression whereas Tan et al. demonstrated that Chk2-mediated stabilisation of FOXM1 after irradiation stimulated XRCC1 expression. However, several lines of reasoning indicated that transcription factor Sp1 might be a promising candidate for modulation of *XRCC1* basal transcription. Firstly, data published by our lab described a role for Sp1 in the regulation of APE1, another core BER component (Poletto et al., 2016). Secondly, Sp1 has previously been shown to bind to the MGMT promoter (Bocangel et al., 2009). As mentioned in section 1.1.1, MGMT repairs O^6-meG, the common and highly mutagenic base lesion. Therefore, there are at least two different examples of Sp1-regulated proteins associated with the repair of DNA base lesions, suggesting that this phenomenon might be more widespread. Furthermore, early analysis of the baboon XRCC1 promoter revealed the presence of GC-rich regions, indicative of potential binding sites for Sp1 (Zhou and Walter, 1998). Moreover, Hao and colleagues showed that Sp1 could bind at a polymorphic site within the 5'UTR of the *XRCC1* gene (Hao et al., 2006). This narrowed down our list to the three most promising candidates, Sp1, E2F1 and FOXM1.

3.2 Sp1 is important to maintain XRCC1 levels

In order to assess putative transcriptional regulation, we first looked at the contribution of each candidate transcription factor to basal *XRCC1* expression. To achieve this, we measured *XRCC1* mRNA levels using qPCR after knock

down of each of our putative transcription factors. Surprisingly, in our model of primary human fibroblasts (TIG-1 cells), siRNA-mediated knock down of either FOXM1 or E2F1 did not result in a significant change in *XRCC1* mRNA levels, in contrast to previous findings (Chen et al., 2008b, Tan et al., 2007), (Figure 3.2, panel A). E2F1 knock down was validated by Western blotting (Figure 3.2, panel B) and FOXM1 knock down by qPCR (Figure 3.2, panel C). The discrepancies between our findings and the previous studies may be due to the use of different cell models. Our research uses normal diploid human fibroblasts, whereas the studies described here use a combination of MEFs and cancer cell lines including U2OS and Saos2. Moreover, data from our lab has previously shown that BER becomes dysregulated in transformed cells (Poletto et al., 2016); this may explain the differences between the results. Depletion of Sp1, on the other hand, did produce a significant decrease in *XRCC1* mRNA levels Figure 3.2, panel A). This indicated that Sp1 might be involved in regulating the basal transcription of *XRCC1*. Similarly, Western blot analysis revealed that XRCC1 protein levels were also decreased after depletion of Sp1 (Figure 3.3, panels A and B). Accordingly, protein levels of Lig III, which is stabilised in a complex with XRCC1, were also negatively affected by Sp1 knock down (Figure 3.3, panels A and B). Furthermore, the reduction in XRCC1 protein levels after Sp1 depletion was reproduced using multiple siRNA sequences indicating that this was not due to an off-target effect of the siRNA (Figure 3.3).

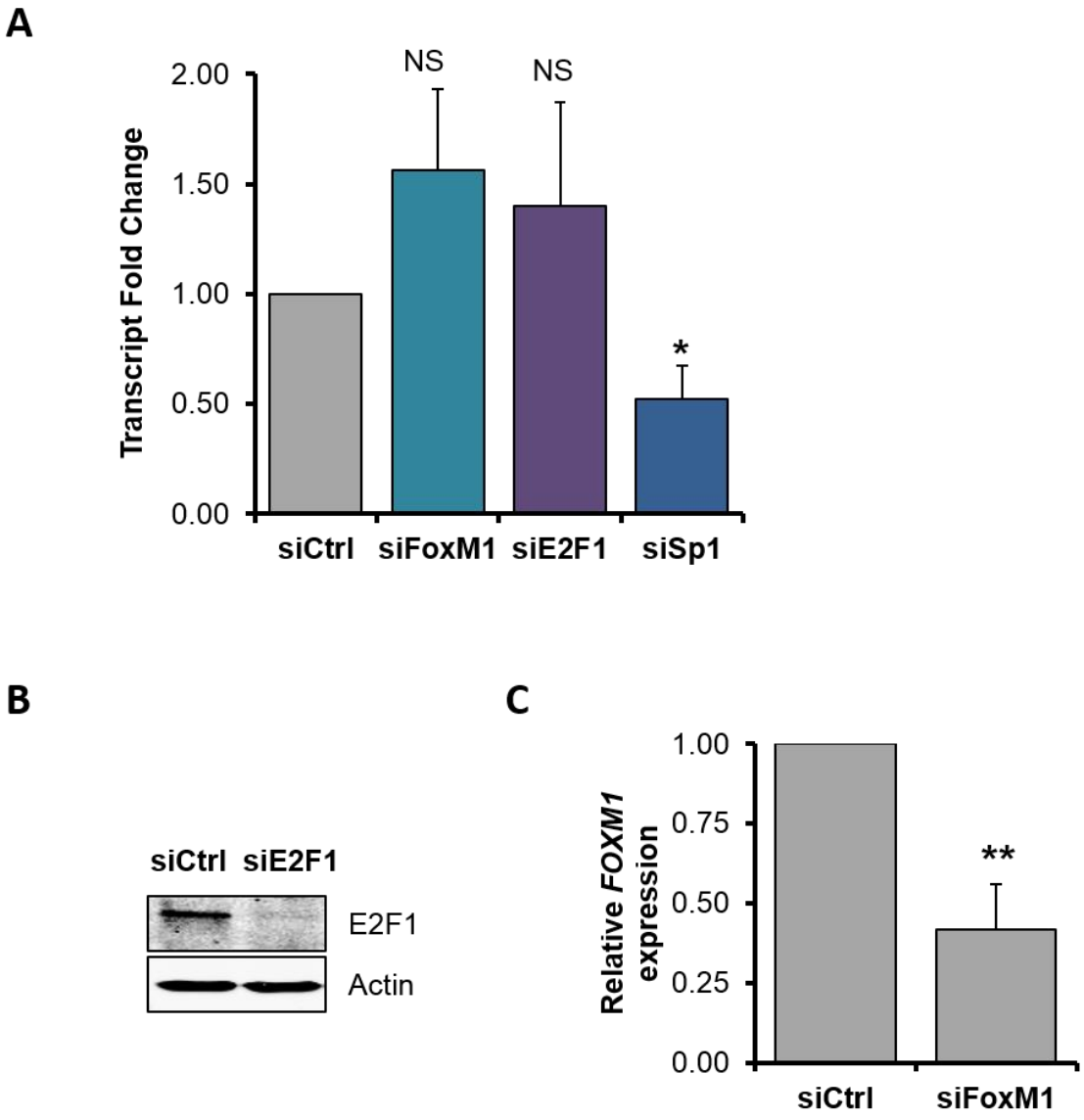

Figure 3.2 XRCC1 mRNA levels after knock down of different candidate transcription factors

(A) Histogram illustrating transcript levels of XRCC1 in TIG-1 cells after depletion of candidate transcription factors FOXM1, E2F1 and Sp1. Loss of Sp1 results in a statistically significant reduction in *XRCC1* mRNA levels whereas depletion of FOXM1 or E2F1 has no negative impact on *XRCC1* transcript levels. Results are expressed as mean ± SD from three independent experiments. SF3B2, GAPDH and B2M were used as endogenous controls. (**B**) Representative Western blotting to validate knock down of E2F1 using siRNA. Actin was used as loading control. (**C**) Histogram showing *FOXM1* mRNA levels after knock down with FOXM1 siRNA (siFOXM1) as a validation for the success of the knock down. Data are presented as mean ± SD from three independent experiments. SF3B2, GAPDH and B2M were used as endogenous controls. siCtrl; control siRNA-treated sample. *:p<0.05; **:p<0.01.

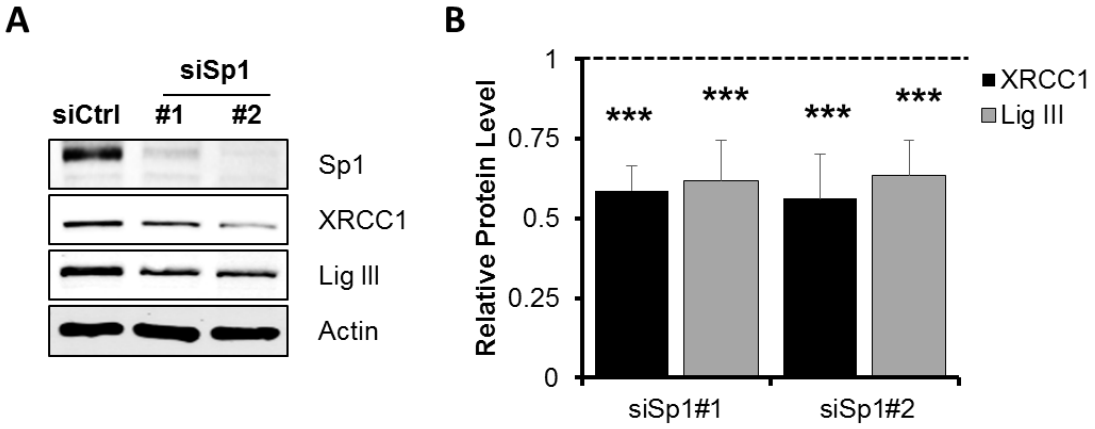

Figure 3.3 Sp1 depletion negatively affects XRCC1 protein levels

(A) Representative Western blotting analysis on TIG-1 cells transfected with either control siRNA (siCtrl) or Sp1 targeting siRNA (siSp1). Loss of Sp1 using different sequences of Sp1 siRNA results in a reduction in XRCC1 protein levels with a corresponding reduction in Lig III protein levels. Actin was used as a loading control. **(B)** Densitometric quantification of data presented in panel A. Data are expressed as mean ± SD from at least three independent experiments and confirm Sp1 depletion results in a reduction to XRCC1 and Lig III protein levels ***:p<0.001.

To confirm that Sp1 contributes to basal transcription of *XRCC1*, we used an approach alternative to siRNA-mediated depletion. We exploited the Sp1 inhibitor mithramycin A (Figure 3.4, panel A) to investigate Sp1 effect on XRCC1 levels. Mithramycin A binds to GC-rich stretches of DNA, thereby preventing the binding of Sp1 to its consensus sequence (Snyder et al., 1991). Treatment of TIG-1 fibroblasts with mithramycin A had a severe impact on XRCC1 mRNA levels. Within eight hours, XRCC1 mRNA levels showed approximately an 80% decrease (Figure 3.4, panel B). Similarly, XRCC1 protein levels were strongly reduced by mithramycin A treatment, which were statistically significant at all time points investigated (Figure 3.4, panels C and D). The apparent discrepancy observed when comparing the kinetics of decrease can likely be ascribed to the very long half-life of the XRCC1 protein (see below) in comparison with its mRNA (approx. 6h, data not shown). Inhibiting induction of a known Sp1 target gene, namely *CDKN1A* which encodes for p21 (Biggs et al., 1996), was used to check

the efficacy of mithramycin A. Importantly, we found that mithramycin A treatment in TIG-1 cells successfully blocked induction of p21 expression after etoposide treatment, indicating that the conditions used in our experiments were sufficient to inhibit Sp1 DNA binding (Figure 3.4, panel E). Taken together, these results suggested that the Sp1 transcription factor is involved in the regulation of XRCC1 basal expression.

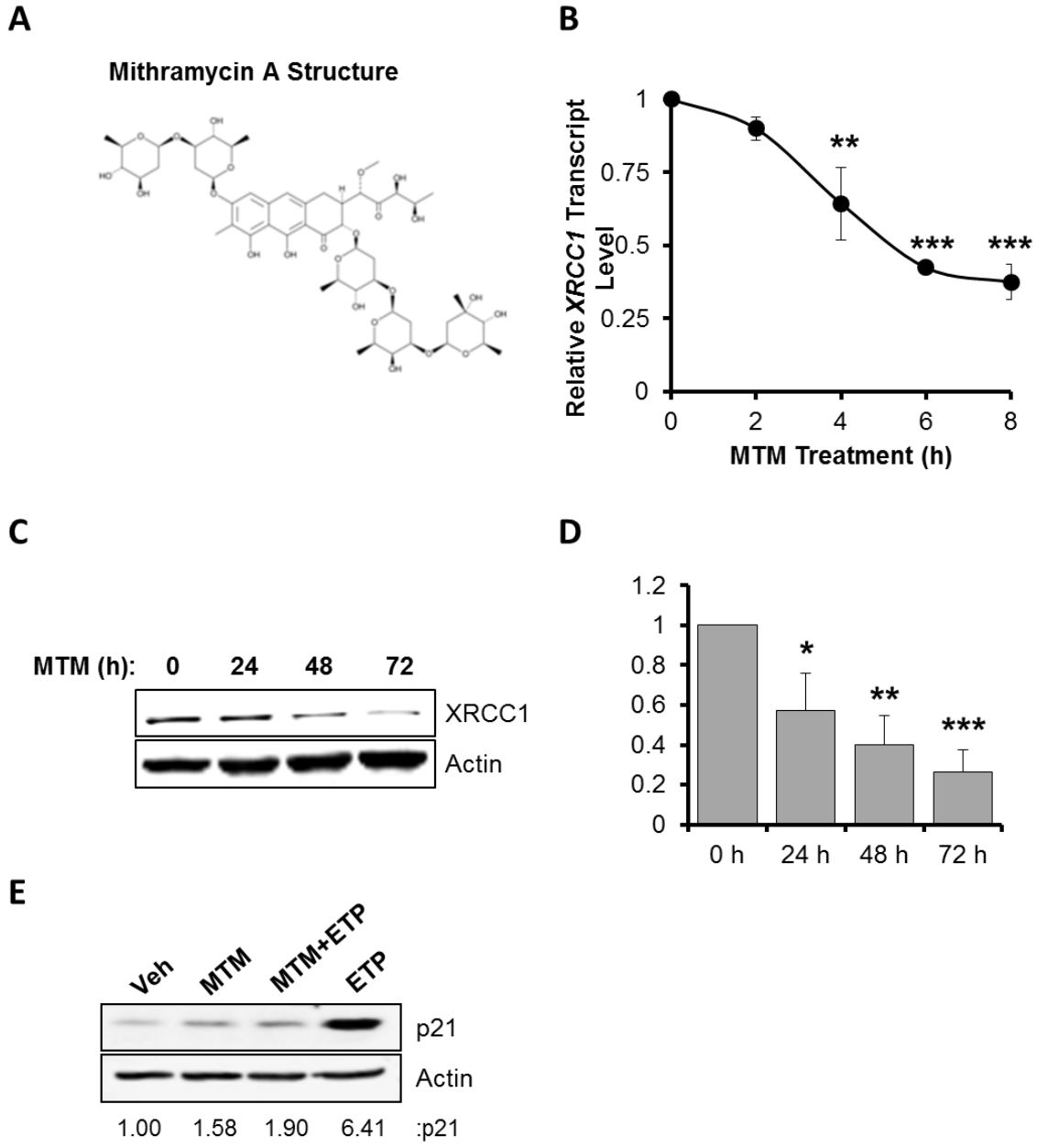

Figure 3.4 Inhibition of Sp1 negatively affects XRCC1

(A) Chemical structure of Sp1 inhibitor mithramycin A. **(B)** Analysis of *XRCC1* transcript levels in a representative Sp1 inhibitor (Mithramycin A) time course in TIG-1 cells. The level of XRCC1 transcript decreases in a time-dependent manner. Results are expressed as mean ± SD from three independent experiments. GAPDH and B2M were used as endogenous controls. **(C)** Western blotting from a representative mithramycin A time course in TIG-1 cells. XRCC1 protein levels reduce in a time-dependent manner. Actin was used as loading control. **(D)** Densitometric quantification of data presented in panel C confirming mithramycin A treatment decreases protein levels of XRCC1 **(E)** Validation of mithramycin A activity. Induction of p21 after treatment of TIG-1 cells with etoposide (10 μM, 6 h) is prevented by the addition of mithramycin A. Actin was used as a loading control. Quantifications are reported at the bottom of the blots. In all experiments mithramycin A was used at 1 μM. *:p<0.05; **:p<0.01; ***:p<0.001. Veh; vehicle, MTM; mithramycin A, ETP; etoposide.

3.3 Sp1 does not affect XRCC1 protein stability

Having established a role for Sp1 in XRCC1 regulation, we wanted to investigate if XRCC1 protein degradation or turnover were also altered after loss of Sp1, or if the effect was purely at the transcriptional level. Using proteasome inhibitor MG132, we did not observe a rescue in XRCC1 protein levels after Sp1 depletion (Figure 3.5, panels A and B). This indicated that Sp1 depletion does not increase proteasomal degradation of XRCC1 protein, suggesting that Sp1 does not regulate XRCC1 protein stability. To reinforce our hypothesis that Sp1 is only involved in transcriptional regulation of XRCC1, we used cycloheximide to assess XRCC1 protein turnover. Cycloheximide inhibits the elongation stage of translation and is routinely used to determine protein half-life. Comparison of XRCC1 degradation kinetics between control and Sp1-depleted fibroblasts during a cycloheximide time course, however, showed no difference in XRCC1 protein turnover, indicating that Sp1 depletion does not affect XRCC1 protein stability (Figure 3.5, panels B and C). A sharp decrease in p53 protein level confirmed the activity of cycloheximide in this assay. Taken together, these results indicated that Sp1 is mainly involved in modulating transcription of the XRCC1 gene rather than involved in the post translational regulation of XRCC1 protein.

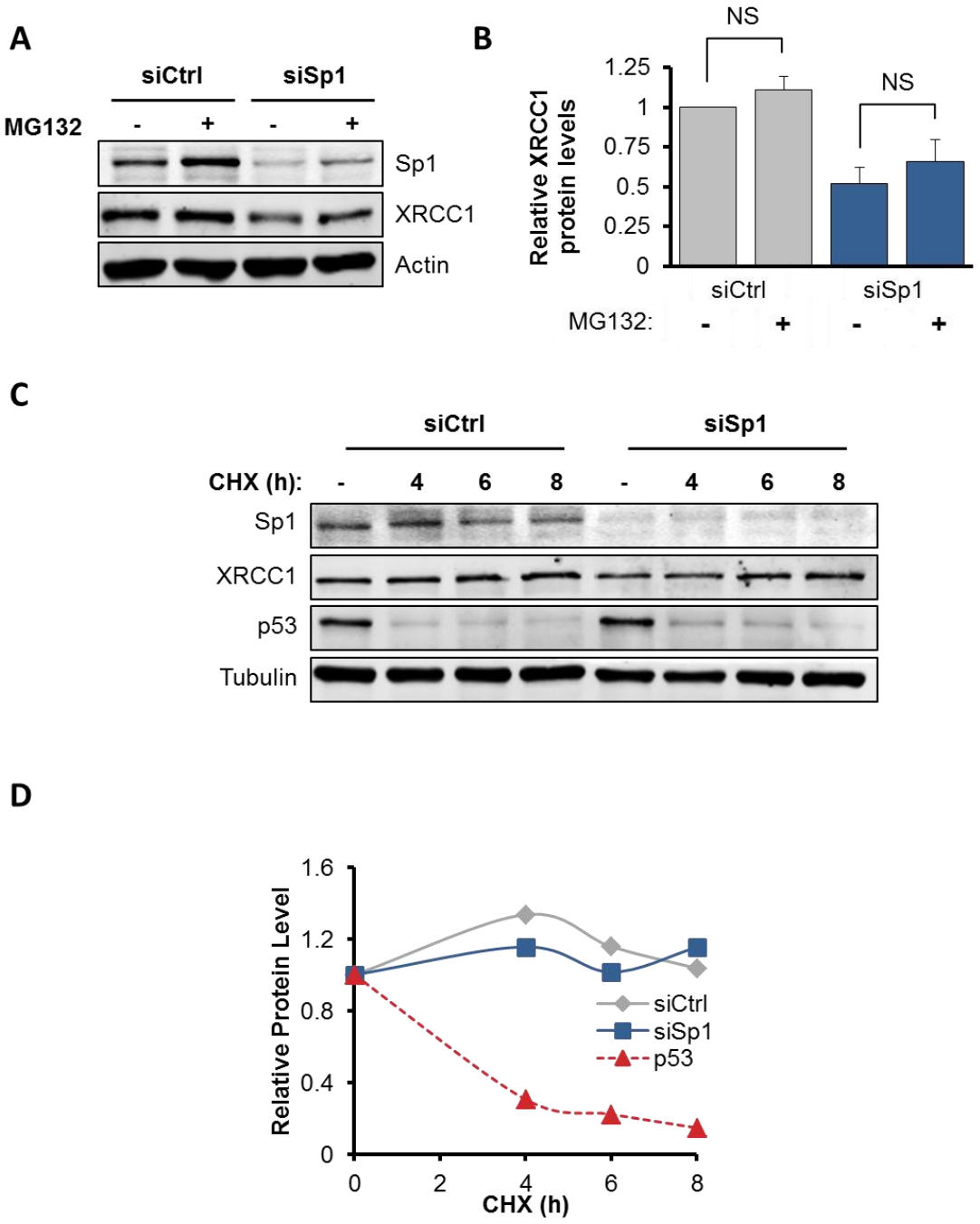

Figure 3.5 Sp1 depletion does not affect XRCC1 protein stability

(A) Representative Western blotting analysis on siCtrl-or siSp1-transfected TIG-1 cells treated with proteasome inhibitor MG132 (10 μM, 6 h). XRCC1 protein levels in Sp1-depleted cells are not rescued to a greater extent by proteasome inhibition compared to control cells. Actin was used as loading control. **(B)** Densitometric quantification of data presented in (A), confirming Sp1 knockdown does not affect XRCC1 protein stability. Data are presented as mean ± SD from at least three independent experiments. **(C)** Representative Western blotting analysis on control or Sp1 knock down TIG-1 cells treated with cycloheximide (CHX). XRCC1 protein levels do not change after the addition of cycloheximide. p53, which has a short half-life, is used as a positive control for cycloheximide efficacy. Tubulin was used as a loading control. **(D)** Densitometric quantification of Western blot presented in (B) showing XRCC1 protein levels in siCtrl and siSp1-treated cells (grey and blue lines, respectively) are unaffected by CHX addition. p53 protein levels (red line), from siCtrl-treated cells, decline during CHX treatment. NS: Not significant.

3.4 Characterisation of Sp1 binding activity to the *XRCC1* promoter

Given the indications that Sp1 is involved in *XRCC1* transcriptional regulation, we next wanted to establish whether *XRCC1* promoter activity is dependent on the presence of Sp1. To this end, we cloned a 4 kb region of the *XRCC1* promoter into a luciferase reporter vector, directly upstream of the luciferase gene. (See section 2.24.1 for cloning details). Luciferase activity was detected when the *XRCC1* promoter plasmid was transfected into siCtrl-treated TIG-1 cells. Depletion of Sp1 in TIG-1 fibroblasts, on the other hand, resulted in almost complete ablation of luciferase activity (Figure 3.6); furthermore, this finding was verified using multiple siRNA sequences, decreasing the possibility that the loss of promoter activity was due to an off-target effect of the siRNA. These data demonstrated that Sp1 is essential for *XRCC1* promoter activity.

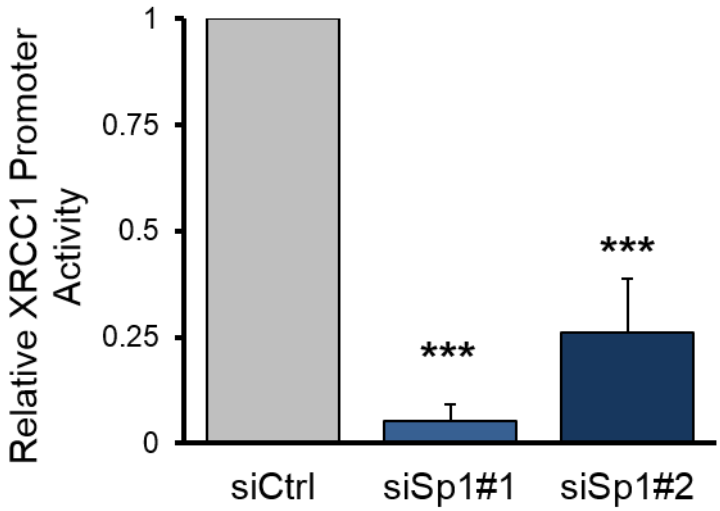

Figure 3.6 Sp1 is important for *XRCC1* promoter activity

Histogram showing reduction in *XRCC1* promoter activity in TIG-1 cells upon Sp1 knock down with two different siRNA sequences as measured by luciferase assay. Results are expressed as mean ± SD from four independent experiments. ***:p<0.001.

Our initial bioinformatics study, in combination with the finding that Sp1 is essential for *XRCC1* promoter activity, led us to further characterise the *XRCC1* promoter. Given that the initial analysis indicated several potential Sp1 binding sites (Figure 3.1), we aimed to identify which of the putative Sp1 sites was most likely to be important in *XRCC1* transcriptional regulation. To this end, we investigated whether any of the most promising putative Sp1 sites were evolutionarily conserved. In fact, alignment of different mammalian *XRCC1* proximal promoters using ClustalX (Larkin et al., 2007) revealed that one of the DNA sequences identified is highly conserved across seven different mammalian species (Figure 3.7). This strongly suggested that this sequence might be important for *XRCC1* transcriptional regulation. Therefore, this spurred us to investigate Sp1 binding at this site in greater detail.

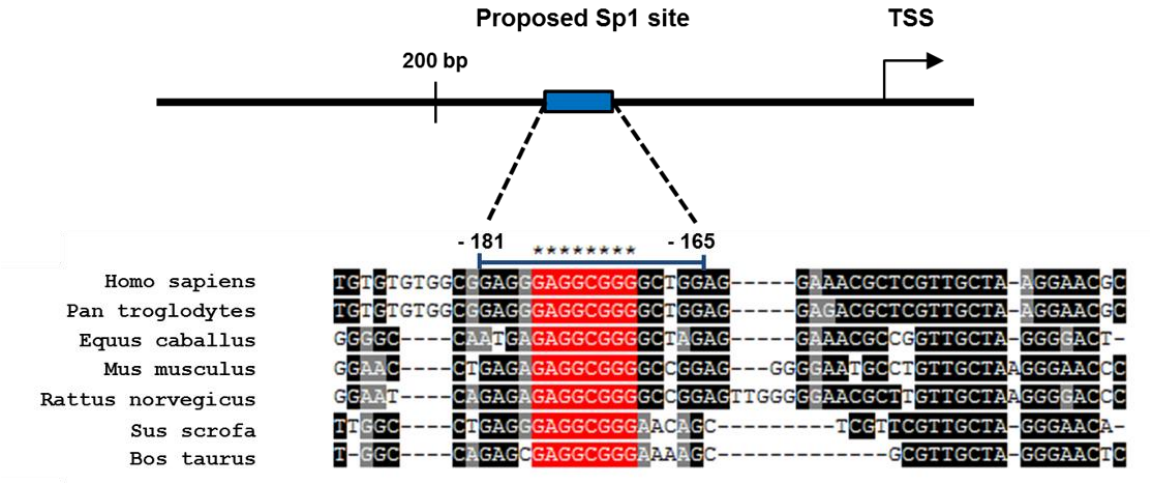

Figure 3.7 Putative Sp1 binding site is highly conserved among mammalian species

Multiple sequence alignment of the *XRCC1* promoter from different mammalian species using ClustalX shows that the Sp1 binding site identified in our analysis is highly conserved across all of the promoters aligned. Alignment was carried out using regions 1 kb upstream of the transcription start site in each species, downloaded from the Genomatix Software Suite. *Homo sapiens*, human; *Pan troglodytes*, chimpanzee; *Equus caballus*, horse; *Mus musculus*, mouse; *Rattus norvegicus*, rat; *Sus scrofa,* pig; *Bos taurus*, cow. Blue line indicates sequence contained in EMSA probe in Figure 1.10. Core binding site is highlighted in red. * denote bases conserved across all species in this analysis. Numbering is relative to the transcription start site (TSS) of human *XRCC1*.

In order to further characterise the role of Sp1 in *XRCC1* transcription, we checked whether Sp1 is present at the *XRCC1* promoter *in vivo* by performing chromatin immunoprecipitation (ChIP) assays on the *XRCC1* proximal promoter region. Using information from the bioinformatics analysis, we designed primers against the region containing the most promising putative binding site (Figure 3.8, panel A). Importantly, this region was amplified after chromatin pull-down with an anti-Sp1 specific antibody, but not with a control IgG from non-immune serum (Figure 3.8, panel B). The primer pair XRCC1_ 2, which amplified a different region of the *XRCC1* promoter not predicted to contain any Sp1 sites, showed no enrichment after chromatin IP, confirming the resolution of the assay (Figure 3.8, panel B).

To further validate that the observed enrichment was indeed due to the presence of Sp1 at the region of interest in the *XRCC1* promoter, we carried out the same analysis in TIG-1 fibroblasts depleted of Sp1. Importantly, the enrichment in the region amplified by primer pair XRCC1_1 was lost upon Sp1 depletion, suggesting that Sp1 is indeed present at the *XRCC1* proximal promoter under basal conditions (Figure 3.8 panel C).

To verify that the ChIP assay was working properly, positive and negative control primer pairs were included in the experiments. A positive control, which amplifies a known Sp1 binding site in the *DHFR* proximal promoter, showed enrichment after pull-down with a specific Sp1 antibody, which was lost upon Sp1 depletion (Figure 3.8, panel D). An additional negative control pair, on the other hand, designed against a non-GC rich region of the *APEX1* proximal promoter not associated with Sp1 binding, did not amplify after pull-down with a specific Sp1 antibody, above the level of the IgG control (Figure 3.8, panel E). These data

indicated that Sp1 is present at the *XRCC1* promoter under basal conditions and confirmed binding of the transcription factor to a region consistent with our bioinformatics predictions.

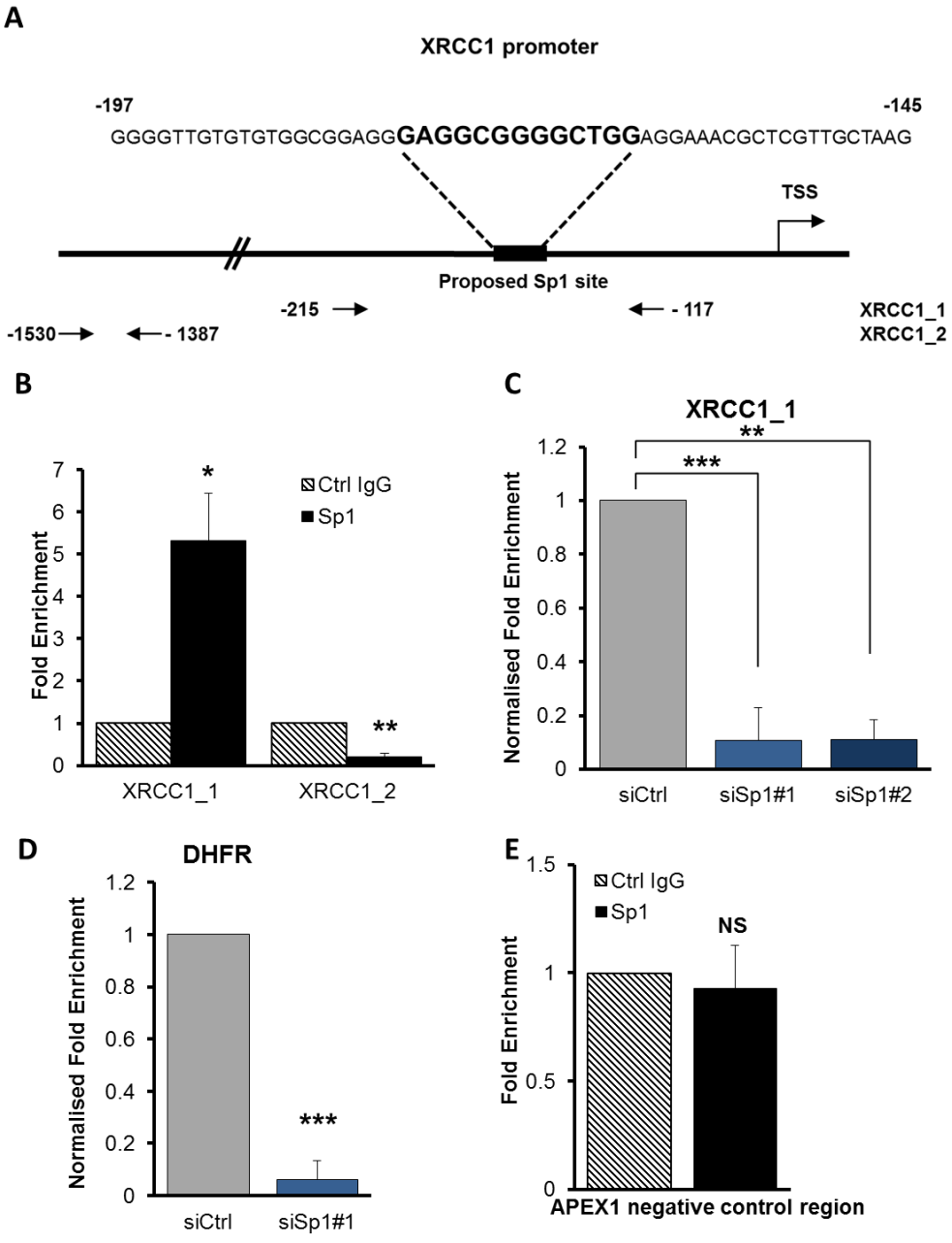

Figure 3.8 Sp1 is present at the *XRCC1* promoter under basal conditions

(A) Schematic of the *XRCC1* promoter indicating locations of primers used in ChIP assays. **(B)** Histogram showing ChIP analysis in siCtrl-treated TIG-1 cells assessing ability of Sp1 to bind to the XRCC1 proximal promoter. Enrichment of Sp1 is seen with primer pair XRCC1_1, covering the proposed Sp1 binding site. No enrichment is seen with XRCC1_2, which amplifies a non-GC rich region of the *XRCC1* promoter and is used as a negative control. Results are expressed as mean fold enrichment ± SD from two independent experiments. **(C)** Histogram showing ChIP analysis in TIG-1 cells using primer pair XRCC1_1. Sp1 is enriched at the XRCC1 promoter under basal conditions, which is lost upon Sp1 depletion using two different siRNA sequences. Results are expressed as the mean fold enrichment of Sp1 binding relative to the unspecific IgG, normalised to the enrichment of Sp1 in siCtrl-treated cells. SD is from three independent experiments, **(D)** Histogram indicating that Sp1 enrichment at the DHFR promoter is lost upon Sp1 depletion. DHFR is a known Sp1 regulated gene and is used as a positive control for Sp1 binding. Results are expressed as mean fold enrichment normalised to the enrichment of Sp1 in siCtrl-treated cells. SD from two independent experiments. **(E)** Histogram showing ChIP analysis in siCtrl-treated TIG cells using a primer pair against a region of the *APEX1* promoter where there are no known Sp1 binding sites to serve as a negative control. Results are expressed as mean fold enrichment ± SD from two independent experiments. **:p<0.01, ***:p<0.001. TSS; Transcription Start Site.

We next wanted to ascertain if Sp1 is able to bind directly to the DNA sequence of the putative Sp1 binding site *in vitro*. It is possible, for example, that Sp1 binds the *XRCC1* promoter as part of a multi-protein complex. Using the information provided by ChIP in addition to the bioinformatics data, we designed a fluorescently-tagged DNA probe containing the putative Sp1 binding site (Figure 3.9, panel A). The ability of Sp1 to bind to this sequence was then assessed using Electrophoretic Mobility Shift Assays (EMSAs). A reaction scheme for EMSAs can be found in Appendix I (Figure 9.6). Nuclear extracts from TIG-1 fibroblasts were incubated with the fluorescently tagged probe (region -165 to -189). A specific DNA/protein complex, detected as a slowly migrating band, was observed in the control extracts (Figure 3.9, panel B). The formation of this complex was greatly reduced in Sp1-depleted nuclear extracts, indicating the likely presence of Sp1 in the protein-DNA complex. This hypothesis was further confirmed by the use of an unlabelled competitor sequence containing a consensus Sp1 binding site (Kadonaga et al., 1986). When the control nuclear extract was incubated with the *XRCC1* promoter probe in the presence of 100 fold excess of the competitor sequence, formation of the protein-DNA complex was also prevented. This indicated that Sp1 can bind to the *XRCC1* promoter probe, but can be sequestered away by the excess of competitor.

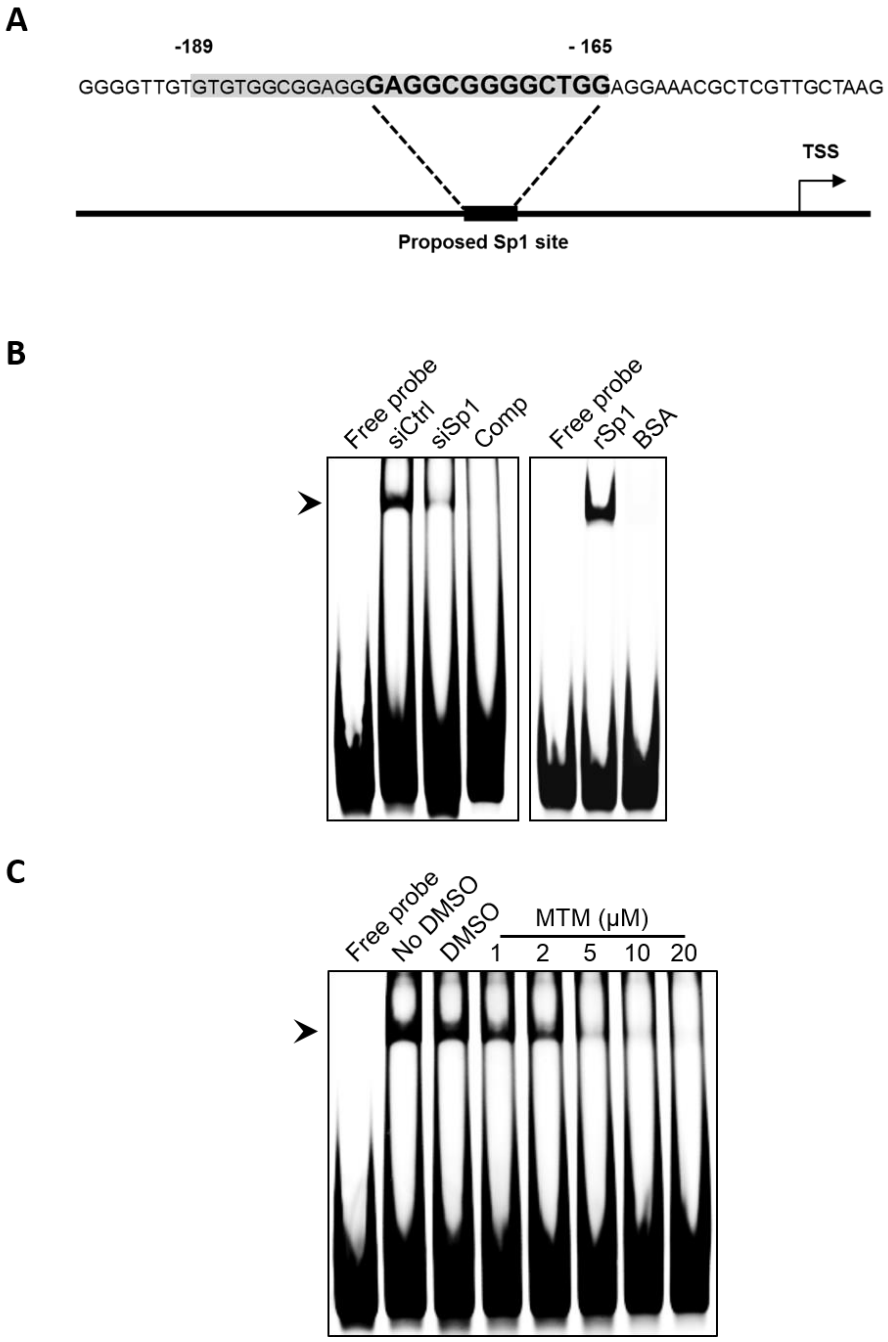

Figure 3.9 Sp1 binds to the *XRCC1* promoter *in vitro*

(A) Schematic representation of the *XRCC1* promoter showing location of the DNA sequence used in the EMSA probe, relative to the putative Sp1 binding site. The full-length probe is highlighted in grey. **(B)** Representative EMSA measuring Sp1 binding activity to the XRCC1 probe. Left: TIG-1 cells were treated with control (siCtrl) or Sp1-targeting (siSp1)siRNA and generated nuclear extracts were used to assess Sp1 binding activity to the XRCC1 promoter probe. Arrow indicates formation of Sp1-containing protein-DNA complex which is lost upon Sp1 depletion. Comp: unlabelled Sp1 competitor sequence, shows specificity of protein-DNA complex formation. Right: The binding activity of recombinant Sp1 protein (rSp1) to the XRCC1 promoter probe was assessed. Formation of protein-DNA complex indicates binding of recombinant Sp1 to the promoter probe. BSA was used as negative control for binding activity. **(C)** Representative EMSA measuring Sp1 binding activity of TIG-1 nuclear extract to the *XRCC1* proximal promoter probe in the presence of Sp1 inhibitor mithramycin A. Increasing concentrations of Sp1 inhibitor prevent formation of the protein-DNA complex. DMSO was used as a control for mithramycin A (MTM) binding. The “No DMSO” control was used to control for any interference in Sp1 binding by DMSO. TSS; Transcription start site.

Additionally, the ability of Sp1 to bind to the *XRCC1* promoter *in vitro* was also assessed using recombinant Sp1 protein (Figure 3.9, panel B right hand side); this assay was carried out using partially purified recombinant Sp1 from *E.coli* (for purification details, see section 9.1). Incubation of recombinant Sp1 with the promoter probe resulted in formation of a protein-DNA complex, further suggesting that Sp1 is able to bind the *XRCC1* promoter independently of other cofactors. In this experiment purified BSA was used as a negative control for DNA binding.

To further investigate Sp1 binding to the *XRCC1* promoter, we carried out EMSAs using the Sp1 inhibitor, mithramycin A. By incubating the DNA probe with mithramycin A prior to the binding reaction, we observed a dose-dependent decrease in complex formation (Figure 3.9, panel C). Taken together this data suggests that Sp1 is able to directly bind to the DNA sequence of the putative binding site *in vitro*.

In summary, this chapter describes the identification of Sp1 as an important transcriptional regulator for *XRCC1* expression. These experiments showed that Sp1 binds both *in vitro* and *in vivo* to the XRCC1 promoter under basal conditions, and identified a putative binding site that is conserved during evolution. The observation that Sp1 depletion modulates the steady-state levels of XRCC1 highlights the role of this transcription factor in the regulation of the basal expression of a core BER protein. Given the essential role for this pathway in the repair of endogenous DNA damage, and the gap in our knowledge regarding modulation of BER in response to the cellular load of DNA damage, this led us to investigate the regulation of Sp1 in response to DNA damage.

4 ATM phosphorylation of Sp1 at Ser101

The major question we want to address is whether BER can be modulated long-term, at the transcriptional level, by the level of endogenous DNA lesions. To investigate this, we sought to understand if *XRCC1* transcription can be affected by the cellular load of DNA damage. Given that Sp1 is important for XRCC1 transcriptional regulation, it was therefore essential, in turn, to understand how Sp1 is modulated. Sp1 is heavily regulated through post translational modifications (Beishline and Azizkhan-Clifford, 2015). As discussed in the introduction, phosphorylation has been identified as an important regulatory feature for different Sp1 activities (Table 1.2). In particular, phosphorylation at Ser101 is of specific interest as a number of different studies have indicated that Ser101 can be targeted for phosphorylation in an ATM-dependent manner in response to DNA damage (Olofsson et al., 2007, Iwahori et al., 2008, Hau et al., 2015). However, the function of this modification is currently unknown. Furthermore, although Ser101 phosphorylation is known to be ATM-dependent, it is uncertain whether it is accomplished directly through ATM itself, another PIKK family member, or by an ATM-activated kinase acting downstream of ATM. Therefore, we investigated Ser101 phosphorylation in greater depth.

4.1 Sp1 is phosphorylated at Ser101 in response to DNA damage

We first assessed whether there is any DNA lesion specificity for Sp1 Ser101 phosphorylation. Treatment of normal human fibroblasts with increasing concentrations of H_2O_2 resulted in a dose-dependent increase in both Ser101 phosphorylation and ATM phosphorylation (Figure 4.1, panel A). Phosphorylation of Sp1 was also induced by treatment with alkylating agent MMS (Figure 4.1, panel B). Furthermore, treatment with either camptothecin or etoposide (Figure 4.1, panels C and D), which are topoisomerase poisons, resulted in both Sp1 and ATM phosphorylation. Similarly, treatment of normal human fibroblasts with cisplatin, a DNA cross-linking agent (Figure 4.1, panel E), or zeocin, a radiomimetic (Figure 4.1, panel F), also showed activation of ATM and phosphorylation of Sp1. These results indicated that while Ser101 phosphorylation occurs in response to DNA damage, this modification does not appear to be DNA lesion-specific.

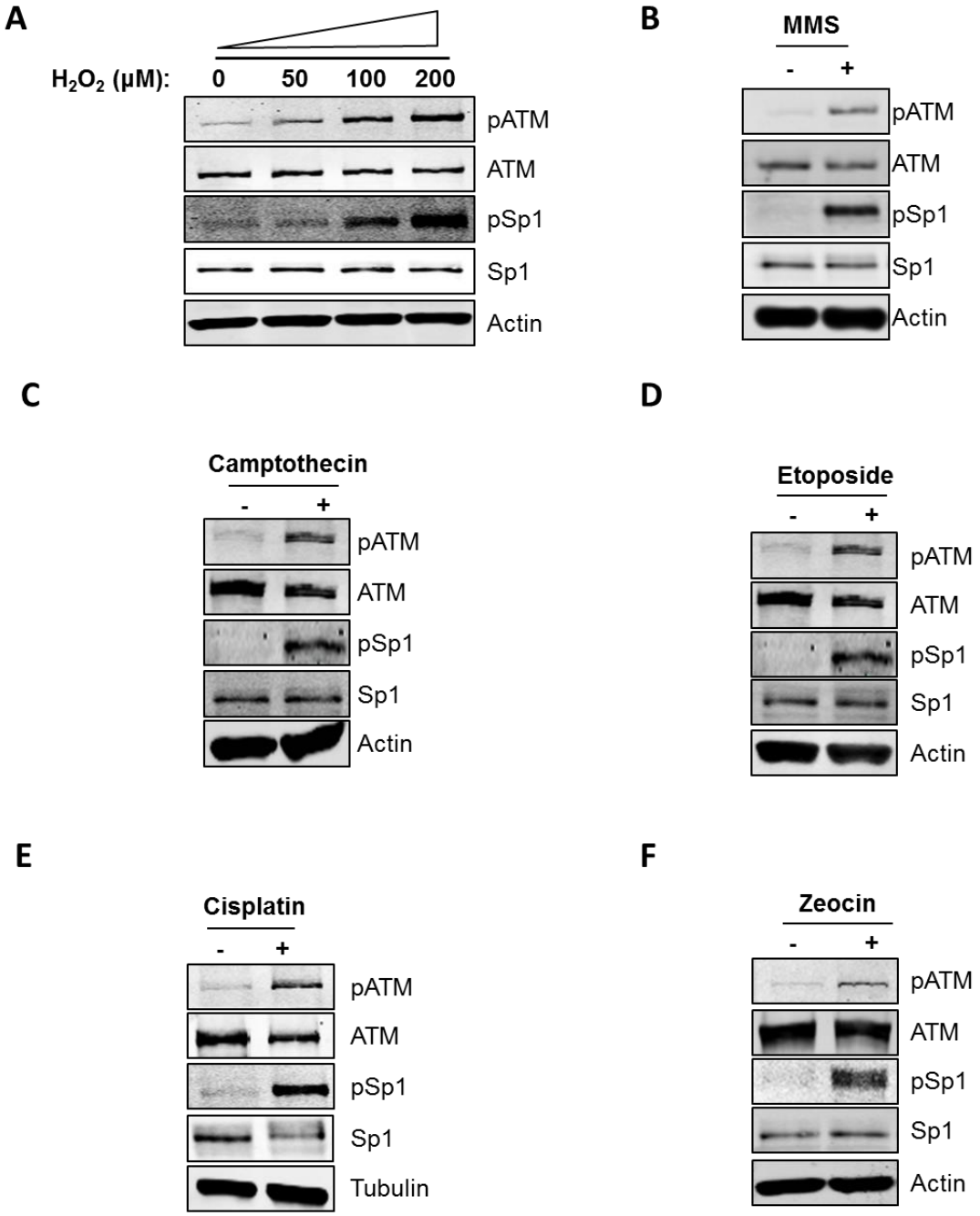

Figure 4.1 Sp1 is phosphorylated at Ser101 in response to DNA damage

(A) Representative Western blot analysis on TIG-1 cells shows dose-dependent induction of ATM phosphorylation at Ser1981 and Sp1 phosphorylation at Ser101 upon treatment with H_2O_2 (50-100-200 μM, 1 h). **(B)** Representative Western blotting analysis on TIG-1 cells showing ATM and Sp1 phosphorylation upon treatment with MMS (1 mM, 3 h). **(C)** and **(D)** Representative Western blotting analysis on TIG-1 cells after treatment with camptothecin (10 μM, 1 h) (C) or etoposide (10 μM, 1 h) (D) showing ATM and Sp1 phosphorylation. **(E)** Representative Western blotting analysis on TIG-1 cells treated with cisplatin (100 μM, 6 h) indicating phosphorylation of both Sp1 and ATM. **(F)** Representative Western blotting analysis on TIG-1 cells showing ATM and Sp1 phosphorylation after treatment with zeocin (50 μg/mL, 1 h). In all Western blots, either actin or tubulin was used as a loading control.

4.2 Sp1 is phosphorylated at Ser101 in an ATM-dependent manner

Next, we investigated whether Ser101 phosphorylation is accomplished by ATM. Consistent with previous findings, (Olofsson et al., 2007, Hau et al., 2015), Ser101 phosphorylation was prevented after H_2O_2 treatment by inhibition of ATM kinase activity (Figure 4.2). This confirmed that Ser101 phosphorylation was either mediated through ATM itself, or by a kinase acting further downstream.

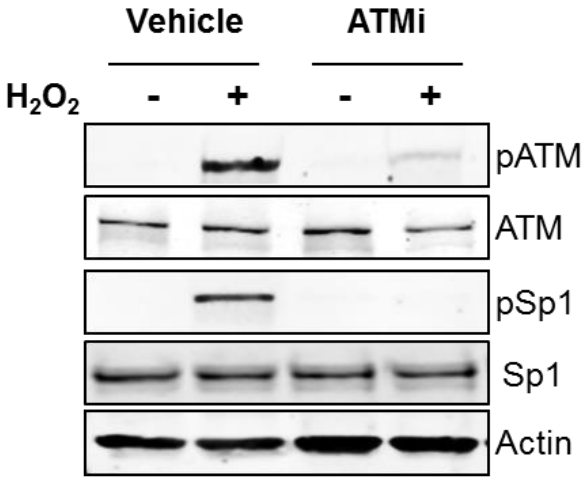

Figure 4.2 Ser101 phosphorylation is dependent on ATM activity

Representative Western blot analysis on TIG-1 cells showing loss of Sp1 phosphorylation in the presence of ATM inhibitor (ATMi - KU55933, 10 μM for 2 h prior to 200 μM H_2O_2 for 1 h). Actin was used as loading control.

To investigate if ATM is directly responsible for Sp1 phosphorylation at Ser101, we used an *in vitro* kinase assay. Co-incubation of active recombinant ATM with recombinant purified Sp1 revealed that Sp1 can be phosphorylated by ATM *in vitro*. Importantly, an Sp1 mutant protein where Ser101 was mutated into an alanine (S101A) could not be phosphorylated by ATM *in vitro* (Figure 4.3, panel A). This confirmed the specificity of the antibody used and further suggested that

ATM can directly phosphorylate Sp1 at Ser101. In addition, when the same kinase reaction was probed using an antibody specific to the phospho-ATM/ATR substrate motif (pSpT QG), wild-type Sp1 was clearly phosphorylated (Figure 4.3, panel B). The S101A mutant, however, did not show any phosphorylation, suggesting that Ser101 is the only residue phosphorylated by ATM under these experimental conditions. Taken together, these data suggested that Ser101 phosphorylation can be accomplished by the ATM kinase itself.

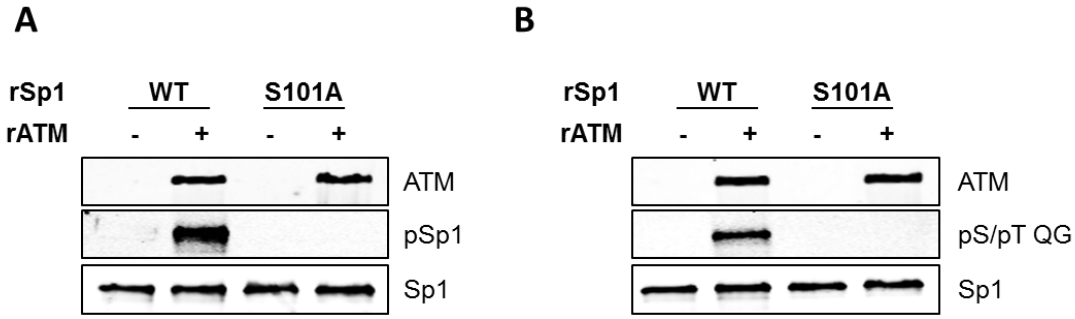

Figure 4.3 ATM directly phosphorylates Sp1 at Ser101 *in vitro*

(A) Representative *in vitro* kinase assay using recombinant ATM (rATM) and wild-type (WT) or Ser101-Ser101Ala mutant (S101A) Sp1 recombinant proteins (rSp1). ATM can phosphorylate wild-type Sp1 but is unable to phosphorylate the S101A mutant. Reactions were probed with the phosphorylated Ser101-specific antibody. **(B)** Representative *in vitro* kinase assay performed as in (A) showing that Ser101 is the major site of ATM-mediated Sp1 phosphorylation. Reactions were probed using an ATM/ATR substrate-specific pS/pT QG antibody.

To further support our hypothesis of direct Sp1 phosphorylation by ATM at Ser101, we next investigated downstream ATM-activated kinases for their involvement. Using specific small molecule inhibitors we demonstrated after DNA damage induced by H_2O_2 that Ser101 phosphorylation is not dependent on Chk1 (Figure 4.4, panel A) or Chk2 activity (Figure 4.4, panel C). Importantly these observations were confirmed by siRNA-mediated knock down of either Chk1 (Figure 4.4, panel B) or Chk2 (Figure 4.4, panel D). This led us to conclude that

neither Chk1 nor Chk2 are involved in Ser101 phosphorylation of Sp1 in response to DNA damage.

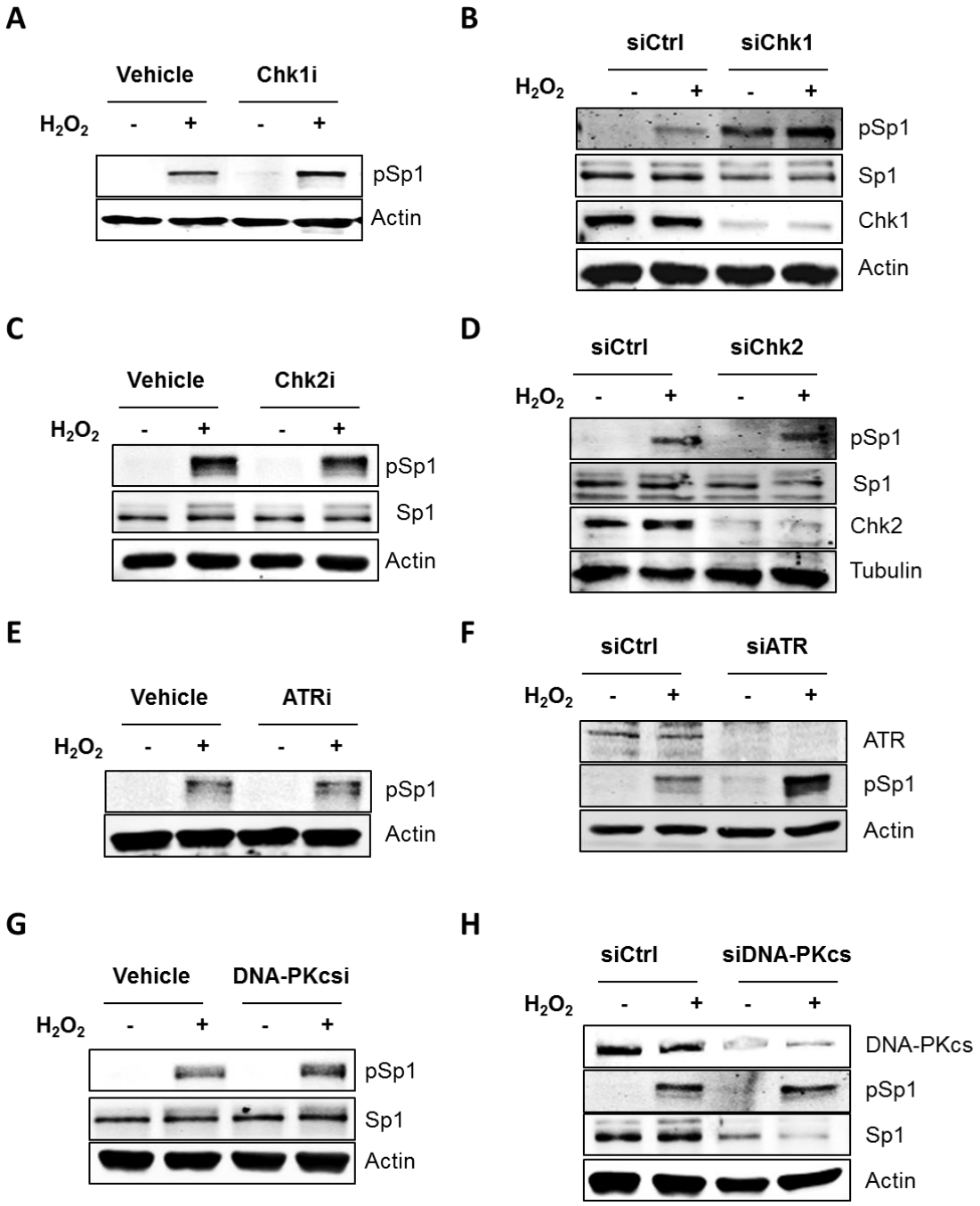

Figure 4.4 ATM is the main kinase responsible for Ser101 phosphorylation after DNA damage

(A) Representative Western blot analysis on TIG-1 cells shows presence of Sp1 phosphorylation after treatment with H_2O_2 (200 μM, 1 h) in the presence of Chk1 inhibitor (UCN-01, 10 μM for 2 h prior to H_2O_2). **(B)** Representative Western blot analysis on TIG-1 cells depleted of Chk1 using siRNA. Phosphorylation of Sp1 is still detected after treatment with H_2O_2 (conditions as in panel A) **(C)** Same as (A) but cells were treated with Chk2 inhibitor (CCT 241533, 3 nM for 2 h prior to H_2O_2). **(D)** Same as (B), but cells were treated with Chk2 siRNA. **(E)** Same as (A), but cells were treated with an ATR inhibitor (VE-821, 1 μM for 2 h prior to H_2O_2). **(F)** Same as (B), but cells were treated with ATR targeting siRNA. **(G)** Same as (A), but cells were treated with a DNA-PKcs inhibitor (DNA-PK Inhibitor III, 10 μM for 2 h prior to H_2O_2). **(H)** Same as (B), but cells were treated with a DNA-PKcs siRNA. Either actin or tubulin was used as a loading control.

Additionally, we checked if other members of the phosphatidylinositol 3-kinase-related kinase (PIKK) family involved in the DDR, namely ATR and DNA-PKcs, were involved in Ser101 phosphorylation. We found that neither ATR inhibition (Figure 4.4, panel E) nor ATR knock down (Figure 4.4, panel F) abrogated Ser101 phosphorylation. Furthermore, the inhibition of DNA-PKcs did not lead to loss of Ser101 phosphorylation after H_2O_2 treatment (Figure 4.4, panel G). Similarly, siRNA-mediated knock down of DNA-PKcs did not block Ser101 phosphorylation (Figure 4.4, panel H). This indicated that Ser101 phosphorylation was not dependent on either ATR or DNA-PKcs.

In summary, these data demonstrate that, in response to DNA damage, ATM is the major kinase responsible for Sp1 phosphorylation at Ser101. As ATM activation has been suggested to occur in response to endogenous DNA damage (Khoronenkova and Dianov, 2015), we hypothesised that XRCC1, and in turn BER, might be modulated by ATM activation through Sp1 phosphorylation. In order to address this hypothesis, we sought to understand the biological consequences of Ser101 phosphorylation of Sp1.

5 Sp1 regulation by DNA damage

The previous chapter in this thesis established that ATM directly phosphorylates Sp1 at Ser101 in response to DNA damage. As the function of this phosphorylation event is currently unknown, we sought to understand the functional consequences of Ser101 phosphorylation. Considering that ATM is activated in response to DNA damage, and that Sp1 is important for the transcriptional regulation of *XRCC1*, we hypothesised that Ser101 phosphorylation might be important in DNA damage-dependent regulation of BER through modulation of Sp1 activity. To this end, we investigated how Sp1 protein function is affected by DNA damage, under conditions promoting Ser101 phosphorylation.

5.1 Sp1 is downregulated by persistent DNA damage

As shown earlier (Figure 4.1), TIG-1 normal human fibroblasts treated with H_2O_2 show activation of ATM in a dose-dependent manner, accompanied by accumulation of Ser101-phosphorylated Sp1. Interestingly, we not only observed a correlation between the level of ATM activation and the Sp1 phosphorylation pattern, but also with the amount of γH2AX foci (Figure 5.1, panel B). After 24 h repair, for example, Sp1 phosphorylation persisted only at the highest dose of H_2O_2 (Figure 5.1, panel A). This was concomitant with persistent γH2AX foci (Figure 5.1, panel B), suggesting the presence of persistent unrepaired DNA damage or stalled replication forks. Surprisingly, this was accompanied by a

reduction in total Sp1 protein levels (Figure 5.1, panels A and B) suggesting that Sp1 phosphorylation at Ser101 might trigger downregulation of the protein when there is persistent unrepaired DNA damage.

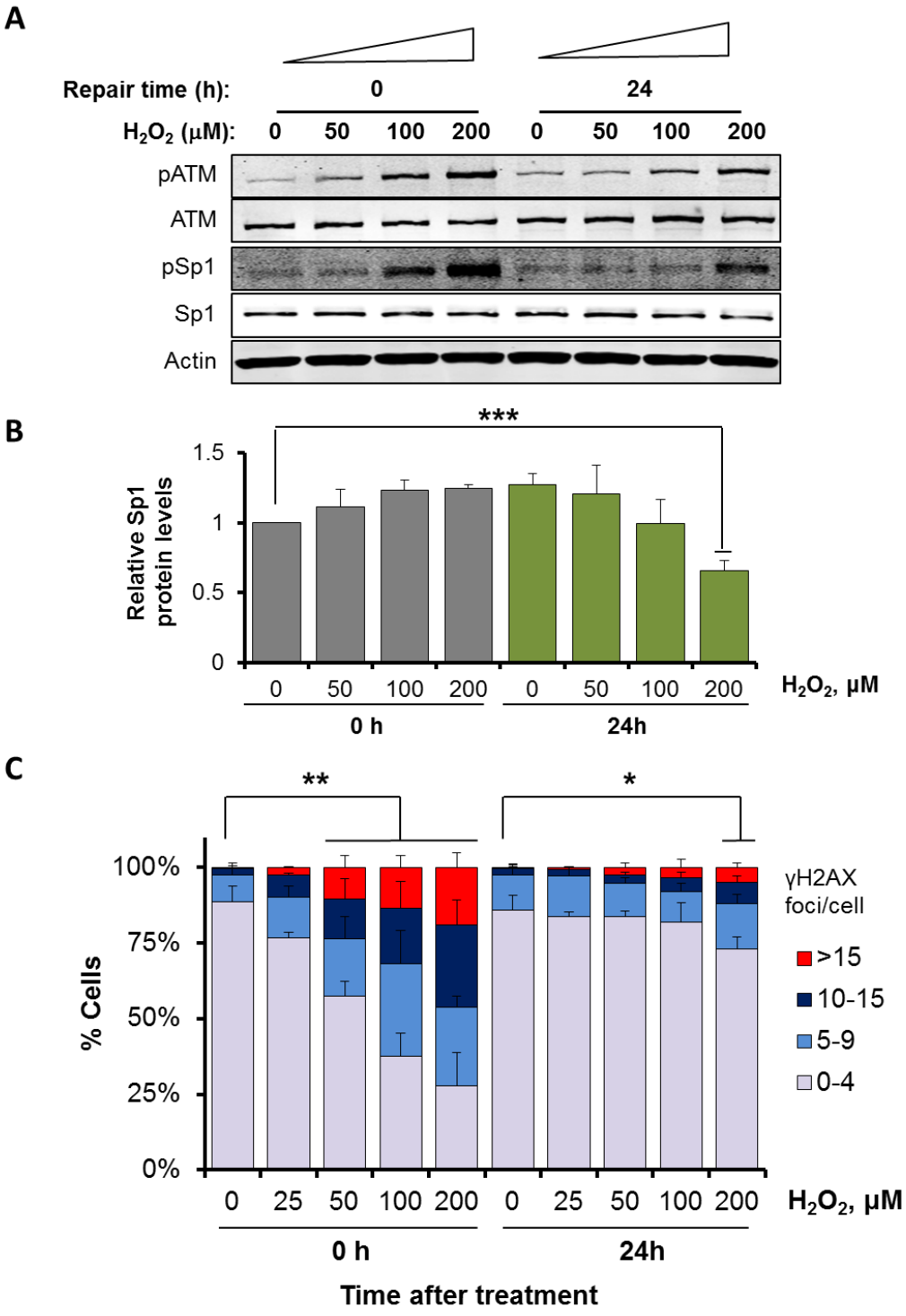

Figure 5.1 Unrepaired DNA strand breaks decrease Sp1 protein levels

(A) Representative Western blot analysis on TIG-1 cells shows dose-dependent induction of ATM phosphorylation and Sp1 Ser101 phosphorylation upon treatment with H_2O_2 (50-100-200 μM, 1 h) and release for either 0 or 24 h. Total Sp1 protein levels are decreased at 24 h release with the highest dose of H_2O_2. Actin was used as loading control. Left hand side of blot (0 h repair) is the same as Figure 4.1 with the addition of total Sp1 protein levels.**(B)** Quantification of total Sp1 levels assessed in panel (A), confirming that Sp1 levels are decreased after 24 h release with the highest dose of H_2O_2. **(C)** Quantification of γH2AX foci in TIG-1 cells treated as in (A). Foci were scored using high-throughput microscopy and at least 500 cells/condition were analysed. The histogram shows foci distribution as average ± SD from three independent experiments and confirms persistency of DNA strand breaks at the highest H_2O_2 dose 24 hours after treatment. Statistics were calculated comparing the populations with 0-4 foci/cell. *:p<0.05; **:p<0.01; ***:p<0.001

To further understand the consequence of Ser101 phosphorylation we investigated the fate of Sp1 protein levels under conditions of persistent DNA damage induced by prolonged exposure to the DNA damaging agents shown in Figure 2.1. Interestingly, after treatment with alkylating agent MMS, a time-dependent reduction in total Sp1 protein levels was observed (Figure 5.2 panel A, left hand side). Similarly, time course analysis of H_2O_2 treatment revealed that Sp1 protein levels were decreased after 24 h treatment. This was in contrast to the earlier points analysed in the time course, where no decrease in Sp1 protein levels was evident (Figure 5.2 panel A, right hand side). This further supported the hypothesis that destabilisation of Sp1 is linked to the load of persistent unrepaired DNA strand breaks. Furthermore, treatment with either topoisomerase poisons camptothecin or etoposide (Figure 5.2, panel B), also led to a decrease in Sp1 protein levels. In addition, cisplatin (Figure 5.2, panel C), or zeocin (Figure 5.2, panel D) treatment, which both triggered robust Sp1 phosphorylation, also caused a reduction in Sp1 protein levels. Taken together, these data indicate that Sp1 levels decrease in conditions when the repair capacity of cells is overwhelmed and unrepaired DNA strand breaks persist.

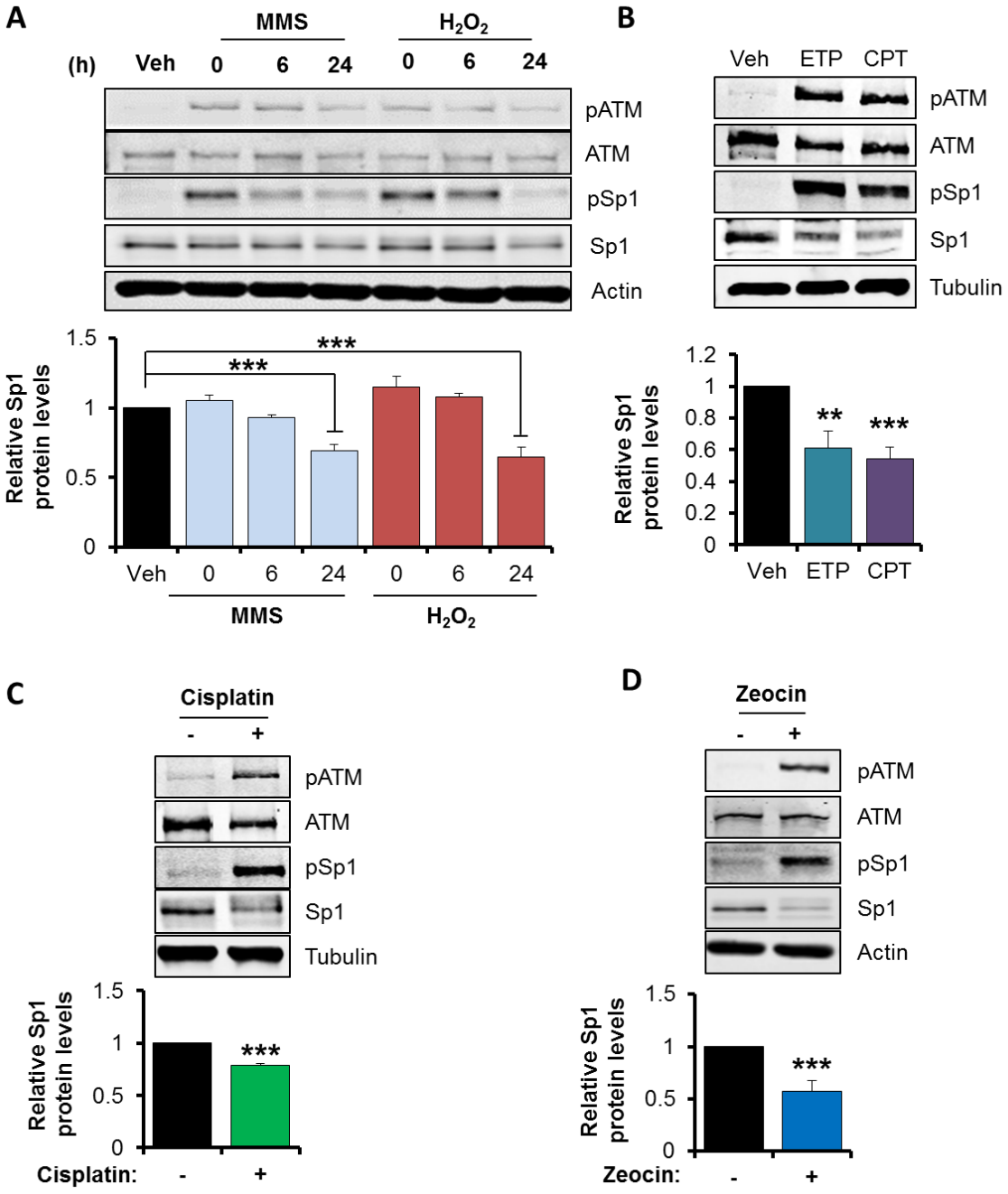

Figure 5.2 Persistent DNA damage leads to Sp1 downregulation

(A) Representative time course analysis on TIG-1 cells treated with either MMS (1 mM, 3 h) or H_2O_2 (200 μM, 1 h) and release for times indicated. Sp1 protein levels are decreased at 24 h recovery with both agents. **(B)** Representative Western blot analysis on TIG-1 cells after treatment with either etoposide (ETP) (10 μM, 6 h) or camptothecin (CPT) (10 μM, 6 h) showing decrease in total Sp1 protein levels. **(C)** Representative Western blot analysis on TIG-1 cells shows decreased total Sp1 protein levels after treatment with cisplatin (100 μM, 6 h). **(D)** Representative Western blot analysis on TIG-1 cells showing treatment with zeocin (50 μg/mL, 24 h) leads to decreased Sp1 protein levels. Panel (C) is the same as Figure 4.1 panel (E), In all panels, densitometric analysis of total Sp1 levels is reported below the blot with either actin or tubulin used as a loading control. Data are expressed as mean ± SD from at least three independent experiments **:p<0.01; ***:p<0.001.

5.2 Sp1 protein stability after persistent DNA strand breaks

Post translational modification of a protein can have a variety of consequences; as we observed correlation between Sp1 phosphorylation and its downregulation, we postulated that Ser101 phosphorylation might trigger destabilisation of the transcription factor. To examine this hypothesis, we investigated whether the observed reduction was proteasome-dependent. During 24 h treatment with zeocin, we found that the decrease in Sp1 protein levels was first evident at 16 h (Figure 5.3, panel A). Therefore, in an attempt to prevent Sp1 degradation, we co-incubated zeocin-treated cells with proteasome inhibitor MG132 after 16 h of zeocin treatment and measured Sp1 protein levels. Importantly, we observed that treatment with MG132 completely prevented a downregulation in Sp1 protein levels (Figure 5.3, panels B and C). This suggested that the reduction in Sp1 total protein in response to persistent DNA strand breaks was due to its increased proteolysis.

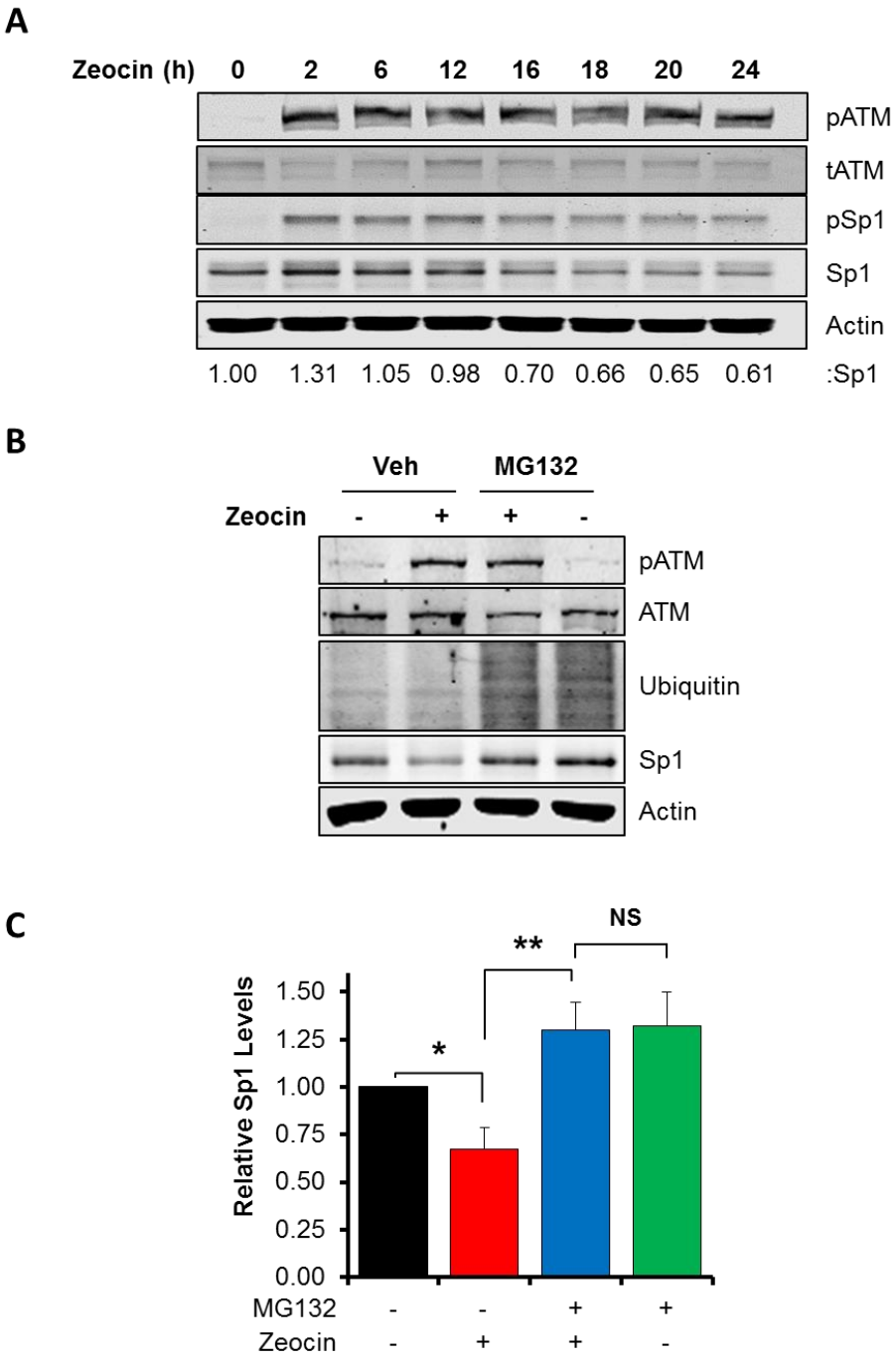

Figure 5.3 Persistent DNA damage induces Sp1 proteasomal degradation

(A) Representative time course analysis on TIG-1 cells treated with zeocin (50 µg/mL). Decreased Sp1 protein levels are observed after 16 h treatment. Quantification is reported below the blot. **(B)** Representative Western blot analysis on TIG-1 cells showing that Sp1 downregulation upon zeocin treatment (50 µg/mL, 22h) can be prevented by inhibition of proteasomal activity with MG132 (10 µM, 6 h added after 16 h zeocin). The presence of ubiquitin shows efficacy of MG132 treatment. **(C)** Densitometric quantification of data presented in panel (B). Data are expressed as mean ± SD from three independent experiments and confirm that Sp1 downregulation upon zeocin is proteasome-dependent. NS: Not significant. *:p<0.05; **:p<0.01. Actin was used as a loading control.

5.3 Sp1 stability is ATM-dependent in response to persistent DNA strand breaks

To understand if Sp1 stability was linked to ATM and Ser101 phosphorylation, we investigated whether inhibition of ATM kinase activity could prevent degradation of Sp1 protein. To this end, we incubated TIG-1 fibroblasts with zeocin in the presence or absence of an ATM inhibitor (KU-60019). Interestingly, we found that inhibition of ATM prevented the reduction in Sp1 protein levels seen with zeocin (Figure 5.4, panels A and B) indicating that ATM kinase activity is important for the destabilisation of Sp1 in response to persistent DNA damage.

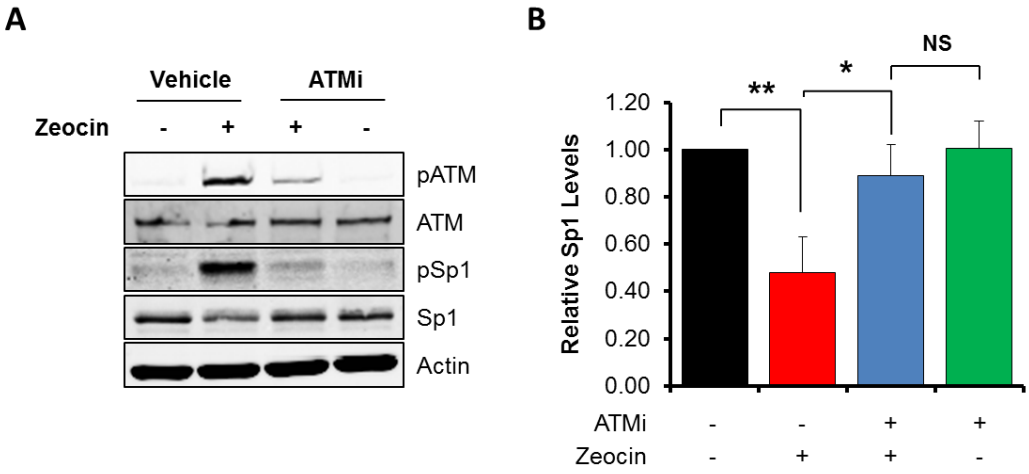

Figure 5.4 DNA damage-induced Sp1 degradation is dependent on ATM activity

(A) Representative Western blot analysis on TIG-1 cells showing decreased Sp1 protein levels upon zeocin treatment (50 µg/mL, 24h) and recovery upon co-incubation with an ATM inhibitor (ATMi – KU60019 10 µM, 24 h). **(B)** Densitometric quantification of data presented in panel (A). Data are expressed as mean ± SD from at least three independent experiments and confirm that Sp1 downregulation upon zeocin treatment is dependent on ATM activity *:p<0.05; **:p<0.01. Actin was used as a loading control.

5.4 Persistent DNA damage affects XRCC1 transcription in an ATM-dependent manner

Given our interest in understanding the role of Sp1 in regulating BER, we postulated that *XRCC1* transcription might also be affected by persistent DNA lesions. Consistent with the role of Sp1 in the modulation of *XRCC1* transcription, we observed a decrease in *XRCC1* mRNA levels when TIG-1 cells were treated with etoposide, camptothecin, zeocin or H_2O_2 (Figure 5.5). This further confirmed our hypothesis that Sp1 is involved in XRCC1 transcriptional regulation. Furthermore, the decrease in the levels of *XRCC1* transcript in response to zeocin could also be partially prevented by the addition of an ATM inhibitor (Figure 5.6, panel A), suggesting that the loss of Sp1 in these experimental conditions also had a functional impact on XRCC1. In addition to this, we also observed a decrease in *XRCC1* mRNA levels after persistent H_2O_2 treatment, which was also partially rescued by co-incubation with an ATM inhibitor (Figure 5.6, panel B). Taken together, these data show that ATM kinase activity is important for regulating Sp1 protein levels in response to persistent DNA strand breaks, suggesting that Ser101 phosphorylation by ATM may negatively affect Sp1 protein stability. This, in turn, has a negative effect on XRCC1 transcription as we observed that decreased Sp1 protein levels in response to persistent DNA strand breaks were associated with a reduction in XRCC1 mRNA levels.

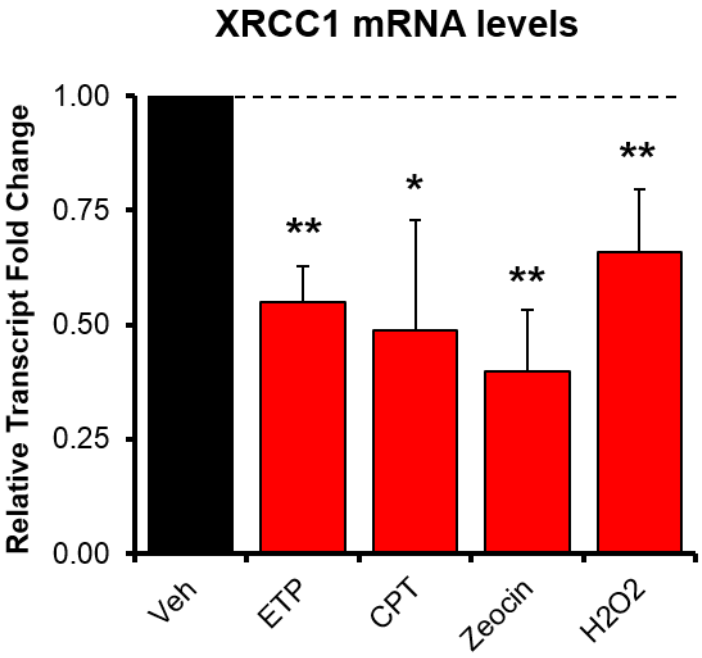

Figure 5.5 *XRCC1* transcription is decreased after persistent DNA damage

qPCR analysis showing decreased *XRCC1* transcript levels in TIG-1 cells after treatment with the indicated DNA damaging agents. Treatments were carried out as indicated in Figure 5.2: Etoposide (ETP) 10 μM, 6 h, camptothecin (CPT) 10 μM, 6 h, zeocin 50 μg/mL 24 h, H_2O_2 200 μM 1 h before 24 h recovery. Data are presented as mean ± SD from three independent experiments. GAPDH was used as endogenous control. *:p<0.05, **:p<0.01.

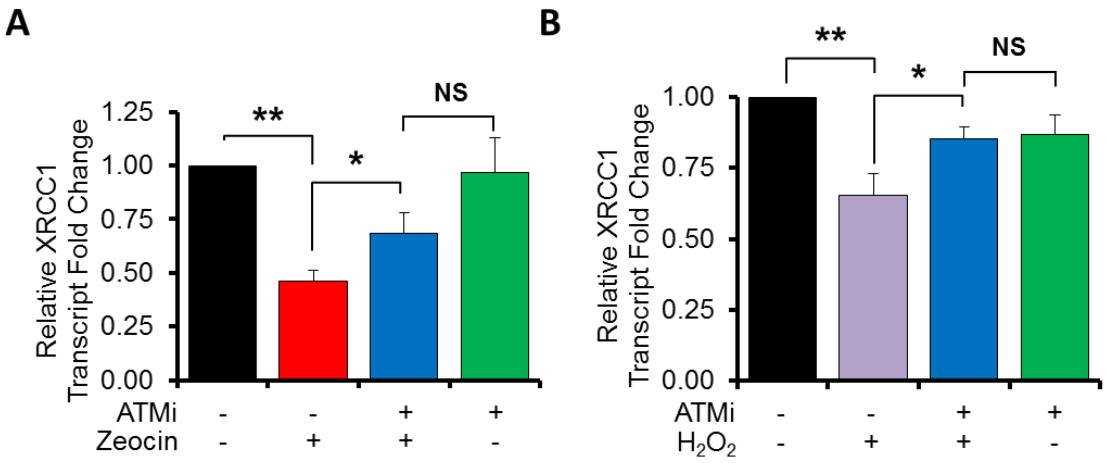

Figure 5.6 DNA-damage induced reduction in *XRCC1* transcription is dependent on ATM activity

(A) qPCR analysis showing decreased XRCC1 transcript levels upon treatment with zeocin (50 μg/mL, 72 h) and recovery upon co-incubation with an ATM inhibitor (10 μM, 72 h). **(B)** Histogram showing XRCC1 transcript levels upon H_2O_2 treatment (125 μM, 72 h). Decreased XRCC1 mRNA levels are observed with H_2O_2 and recovery is observed upon co-incubation with an ATM inhibitor (10 μM, 72 h). Data are presented as mean ± SD from three independent experiments. GAPDH was used as endogenous control. *p:<0.05, **:p<0.01.

5.5 Connectivity map analysis

Given our hypothesis that persistent DNA strand breaks can lead to down regulation of Sp1 protein levels and decreased XRCC1 expression, we were interested whether this phenomenon can be generalised to include other agents that cause ATM activation. We therefore used the Connectivity Map (cmap) resource (*https://portals.broadinstitute.org/cmap/*) to investigate if ATM activation correlating with downregulation of Sp1 and BER is a broader occurrence that is not just restricted to DNA damage. Cmap collects expression data obtained from human cells cultured in the presence of bioactive compounds. The resource enables the user to identify patterns between specific drugs and gene expression changes. To this end, we generated a gene expression signature of pseudo-ATM activation and BER downregulation. We designated the BER genes *XRCC1* and *APEX1* as components expected to decrease, and *CDKN1A* as a gene expected to increase, given that ATM activation is often associated with p21-dependent cell cycle delay (Zhou and Elledge, 2000). Using the tool we interrogated the cmap database and compiled a list of drugs whose treatment resulted in this particular gene expression profile (Figure 5.7, Table 5.1). We then checked Sp1 protein levels after cells were treated with compounds that produced highly significant enrichment scores.

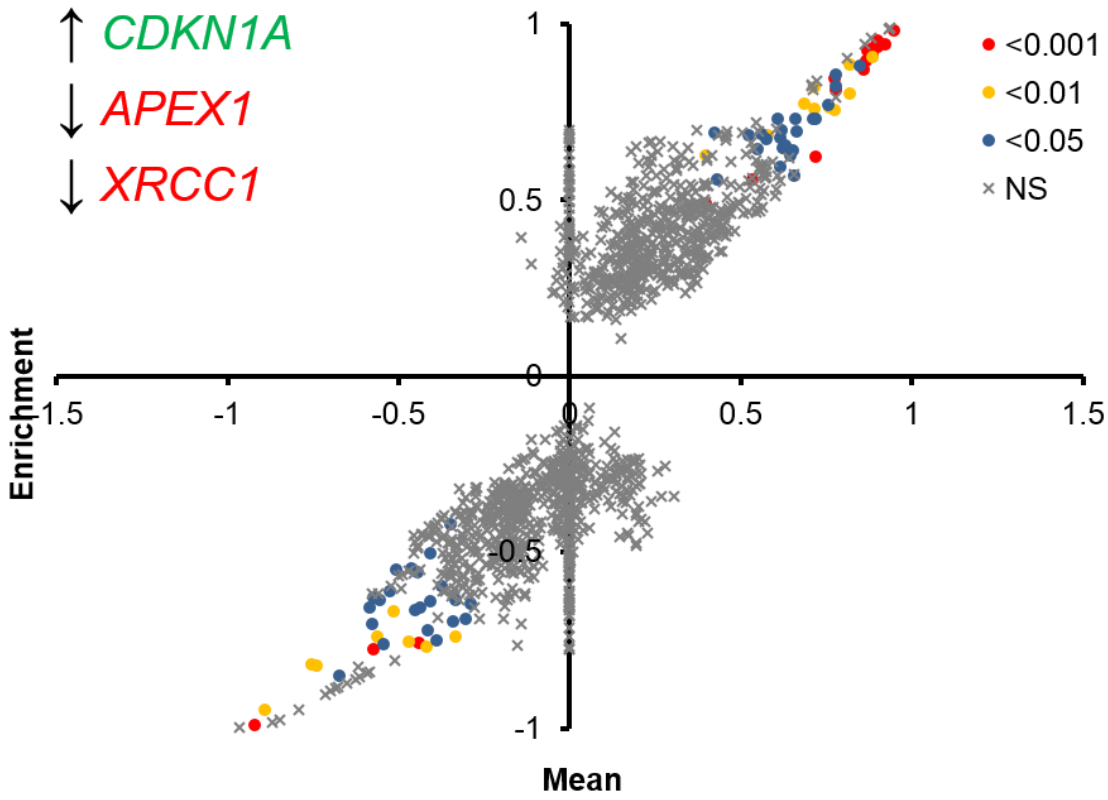

Figure 5.7 Connectivity map analysis

Scatter plot showing output from the connectivity map (cmap) analysis. The Connectivity Map dataset contains genome-wide microarray expression data for 453 different treatment and vehicle control pairs, using 164 distinct small molecules in three different cell lines. The tool builds a hypothetical gene expression signature based on down-regulated and up-regulated genes ("down tag" and "up tag", respectively). In this analysis, the "down tag" included *XRCC1* and *APEX1* whereas the "up tag" contained p21 (*CDKN1A*), in order to mimic conditions of ATM activation. Enrichment of the induced (p21) and repressed (XRCC1 and APEX1) genes for each treatment was estimated using the Kolmogorov-Smirnov statistic, and an overall connectivity score for the signature was generated. Positive enrichment values (top right) denote small molecules predicted to produce an expression signature consistent with the query signature (i.e. ATM activation). Negative enrichment values (bottom left) indicate compounds expected to affect genes in the query signature in the opposite manner (i.e. no ATM activation therefore no upregulation of p21 and no downregulation of *XRCC1* or *APEX1*). Compounds giving statistically significant positive or negative enrichment are highlighted using different colours. Mean enrichment is used to show spread of positive and negative enrichment scores.

Table 5.1 Output from connectivity map analysis

Table shows a subset of small molecules with the most significant p values highlighted in **Figure 5.7.** Compounds are sorted according to their proposed function. "n" denotes the number of experimental repeats that the enrichment score is calculated from. Small molecules in green have positive enrichment scores whereas those in red have negative enrichment scores.

NAME	MEAN	n	ENRICHMENT	P-VALUE	FUNCTION
Carmustine	0.871	3	0.923	0.00094	Alkylating agents
Lomustine	0.56	4	0.686	0.02063	
Camptothecin	0.819	3	0.886	0.00298	Topoisomerase inhibitors
Irinotecan	0.777	3	0.824	0.01092	
Etoposide	0.62	4	0.697	0.01753	
Hycanthone	0.714	4	0.822	0.00167	APE1 inhibitors
Gossypol	0.396	6	0.627	0.00842	
Myricetin	0.71	4	0.731	0.01044	
Trichostatin A	0.718	182	0.623	0	HDAC inhibitors
Scriptaid	0.948	3	0.983	0.00004	
Vorinostat	0.533	12	0.557	0.00042	
Sulfasalazine	0.779	5	0.814	0.00056	Affect redox homeostasis
Piperlongumine	0.849	2	0.88	0.02891	
Rifabutin	0.884	3	0.906	0.00174	HSP90 inhibitor
Ciclopirox	0.424	4	0.692	0.01866	Replication stress
Theobromine	-0.741	4	-0.823	0.00189	Vasodilation
Sulindac	-0.463	7	-0.547	0.01683	Anti-inflammatory

Considering that ATM is activated by DNA damage, it was unsurprising that many of the top positive hits were agents known to activate the DDR. This included classes of compounds such as alkylating agents and topoisomerase inhibitors shown earlier in in this thesis to negatively affect expression of Sp1 and *XRCC1* (Figure 5.2 and Figure 5.5). Consistent with this, irinotecan, which is another topoisomerase inhibitor, also induced downregulation of Sp1 and XRCC1 protein levels (Figure 5.8, panel A) and induction of p21 concomitant with ATM activation.

Interestingly, the cmap analysis also identified other classes of compound inducing gene expression changes consistent with our signature, which warranted further investigation. This included APE1 inhibitors (Table 5.1). Therefore, we investigated if APE1 inhibition affected Sp1 levels. Importantly, treatment of TIG-1 cells with APE1 inhibitor Comp III resulted in a reduction in Sp1 levels and a corresponding decrease in XRCC1 levels (Figure 5.8, panel B). Furthermore, Comp III treatment led to increased p21 expression and ATM phosphorylation, consistent with our pseudo-ATM activation gene expression signature (Figure 5.8, panel B). A number of histone deacetylase inhibitors (HDAC) were also identified in the cmap analysis (Table 5.1). Interestingly, two different studies have shown that treatment with HDAC inhibitors leads to a reduction in Sp1 protein levels. For instance, Hedrick and colleagues showed that vorinostat and panobinostat, another HDAC inhibitor, decreased Sp1 protein levels in rhabdomyosarcoma cell lines (Hedrick et al., 2015). Furthermore, Chen et al. found trichostatin A reduced Sp1 expression in epidermoid carcinoma cells (Chen et al., 2008a). In addition to this, it has been reported that piperlongumine, another small molecule with a positive connectivity score, down regulates Sp1 expression in a ROS-dependent manner (Karki et al., 2017); these findings are all in line with the output from the cmap analysis.

Conversely, by selecting two drugs that gave negative connectivity scores, we observed a lack of effect on Sp1. Treatment with the anti-inflammatory sulindac, for example, did not decrease Sp1 or XRCC1 protein levels (Figure 5.8, panel C). Similarly, treatment of TIG-1 fibroblasts with the alkaloid theobromine resulted in no change to the protein levels of Sp1 or XRCC1 (Figure 5.8, panel D). Importantly, treatment with neither sulindac nor theobromine led to p21

induction or measurable ATM autophosphorylation (Figure 5.8, panels C and D), further supporting our hypothesis that ATM activation is important for the downregulation of Sp1.

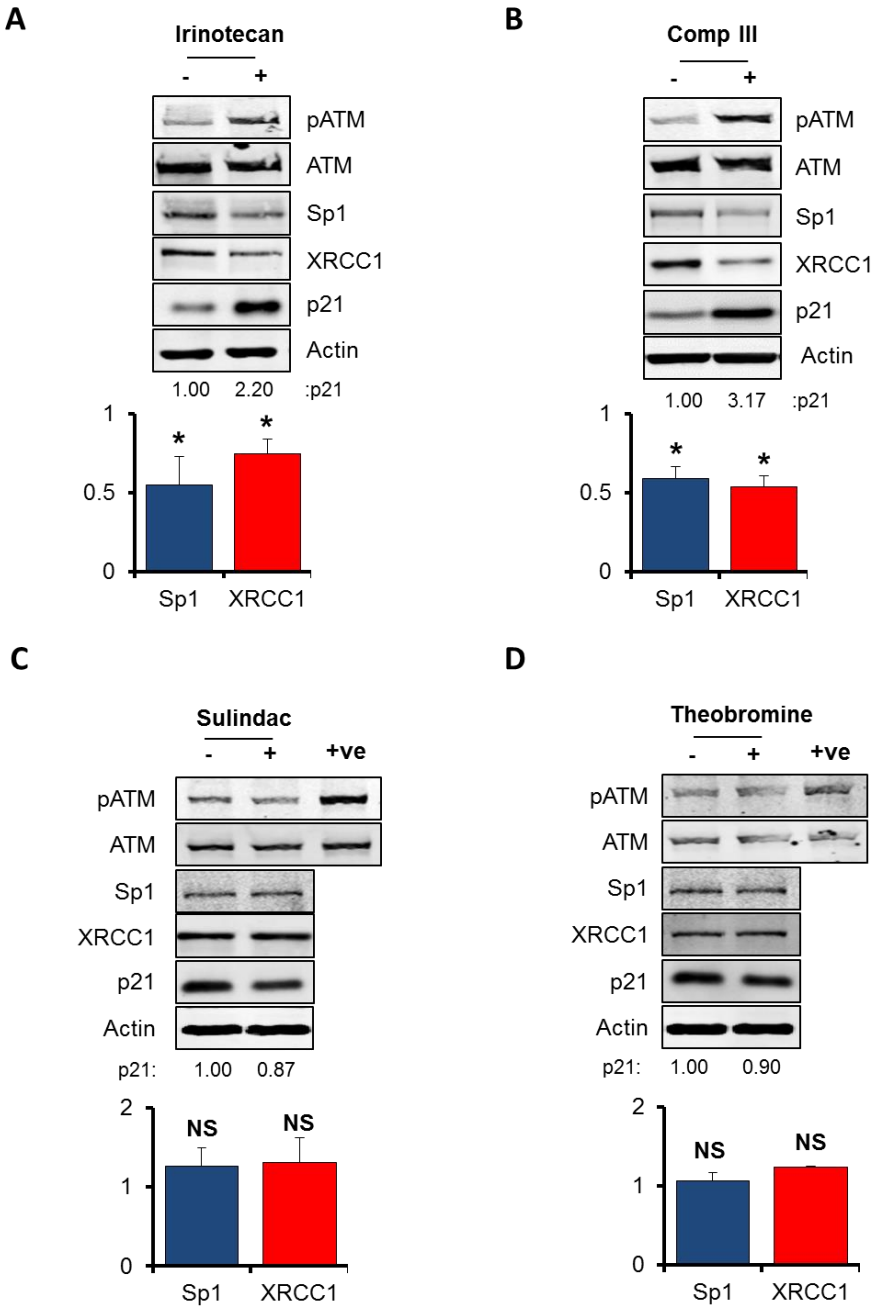

Figure 5.8 Validation of hits from Connectivity Map analysis.

Positive hits show Sp1 downregulation whereas negative hits do not affect Sp1 protein levels. **(A)** Representative Western blot on TIG-1 cells treated with Irinotecan (100 μM, 24 h) showing decreased Sp1 protein levels. Decreased XRCC1 protein levels concomitant with p21 induction and ATM activation are consistent with the cmap gene expression signature. **(B)** Representative Western blot on TIG-1 cells treated with APE1 inhibitor III (Comp III) (10 μM, 24 h) shows a reduction in Sp1 and XRCC1 protein levels as well as induction of p21. ATM activation is confirmed with Ser1981 phosphorylation. **(C)** Representative Western blotting analysis on TIG-1 cells treated with sulindac (10 μM, 24 h). Sulindac produced a negative enrichment score and this is confirmed by unchanged Sp1 and XRCC1 protein levels. No induction of p21 was present, and ATM phosphorylation was not observed **(D)** Representative Western blotting analysis after treatment of TIG-1 cells with alkaloid theobromine (10 μM, 24 h). Sp1 and XRCC1 protein levels were unchanged, and p21 was not induced. Absence of ATM activation further confirms the negative enrichment score. In all panels, actin was used as a loading control and densitometric quantification of Sp1 and XRCC1 protein levels are presented below each blot. Data are expressed as mean ± SD from at least three independent experiments. NS: not significant; *:$p<0.05$; **:$p<0.01$. +ve denotes positive control for ATM activation (TIG-1 cells treated with 200 μM H_2O_2, 1 h).

5.6 Sp1 is downregulated by accumulation of endogenous DNA damage

Having established that Sp1 and *XRCC1* levels are affected by exogenous sources of DNA damage, we then assessed if the same system operates in response to accumulation of endogenous DNA damage. BER is a very robust DNA repair pathway; therefore, accumulation of endogenous DNA damage is not expected to occur under normal circumstances. However, reductions in the cellular BER capacity can overwhelm BER, resulting in DNA damage accumulation. Changes to the BER capacity occur during physiological processes such as tissue differentiation (Narciso et al., 2007, Bauer et al., 2011, Weissman et al., 2007). This implies that a system responding to the accumulation of endogenous DNA damage may be particularly relevant for cell functionality. Additionally, polymorphisms in BER genes may negatively affect their activity leading to an impairment in BER (Karahalil et al., 2012).

In order to observe adjustments in response to accumulation of endogenous DNA damage, we employed a model of siRNA-mediated knock downs and investigated the effect on Sp1 protein levels. Specifically, we depleted cells of FEN-1, Lig III, PNKP, TDP-1 or XRCC1 (Figure 5.9, panels A, B, C, D and E) which are all involved in the processing of lesions during SSBR. Depletion of any of these proteins should slow down repair of endogenous lesions, resulting in accumulation of DNA damage (Orlando et al., 2014, Markkanen et al., 2015, Khoronenkova and Dianov, 2015, Poletto et al., 2016). Interestingly knock down in TIG-1 cells of all of these factors resulted in increased ATM phosphorylation concomitant with a reduction in total Sp1 protein levels (Figure 5.9, panels A, B,

C, D and E). Validation of TDP-1 knock down is given in (Figure 5.9, panel G). Knock down of XPG endonuclease was used as a negative control in these experiments. Although XPG is involved in DNA repair, we hypothesised that its knock down in TIG-1 cells would not greatly increase the levels of endogenous DNA damage; XPG is a component of NER, which is involved in the repair of UV-induced DNA damage and DNA cross-links. Spontaneous occurrence of these lesions is likely to be rarer than SSBs, which are typically considered the most common DNA lesion experienced by cells (Lindahl, 1993, Caldecott, 2008, Tubbs and Nussenzweig, 2017). Consistently, XPG-depleted cells did not show any ATM activation and Sp1 levels did not change in response to XPG knock-down (Figure 5.9, panel F), further suggesting the existence of a correlation between DNA damage load and Sp1 levels. However, these findings are in contrast to a report demonstrating that XPG depletion did increase levels of endogenous DNA damage (Trego et al., 2016). Trego and colleagues demonstrated that XPG depletion increased DSB and micronuclei formation, in the absence of exogenous DNA damage. This, however, was reported to be independent of XPG function in NER as depletion of XPA or XPC, did not produce similar outcomes (Trego et al., 2016). As knockdown in our system was not complete (Figure 5.9, panel F), it is possible that the remaining levels of XPG protein were sufficient to prevent the accumulation of endogenous DNA damage; this could be addressed further using an alkaline comet assay. Moreover, as knockdown of NER component XPC does not increase endogenous DNA damage or the levels of DNA-protein crosslinks, (Trego et al., 2016, Vaz et al., 2016) XPC depletion would have been a better negative control.

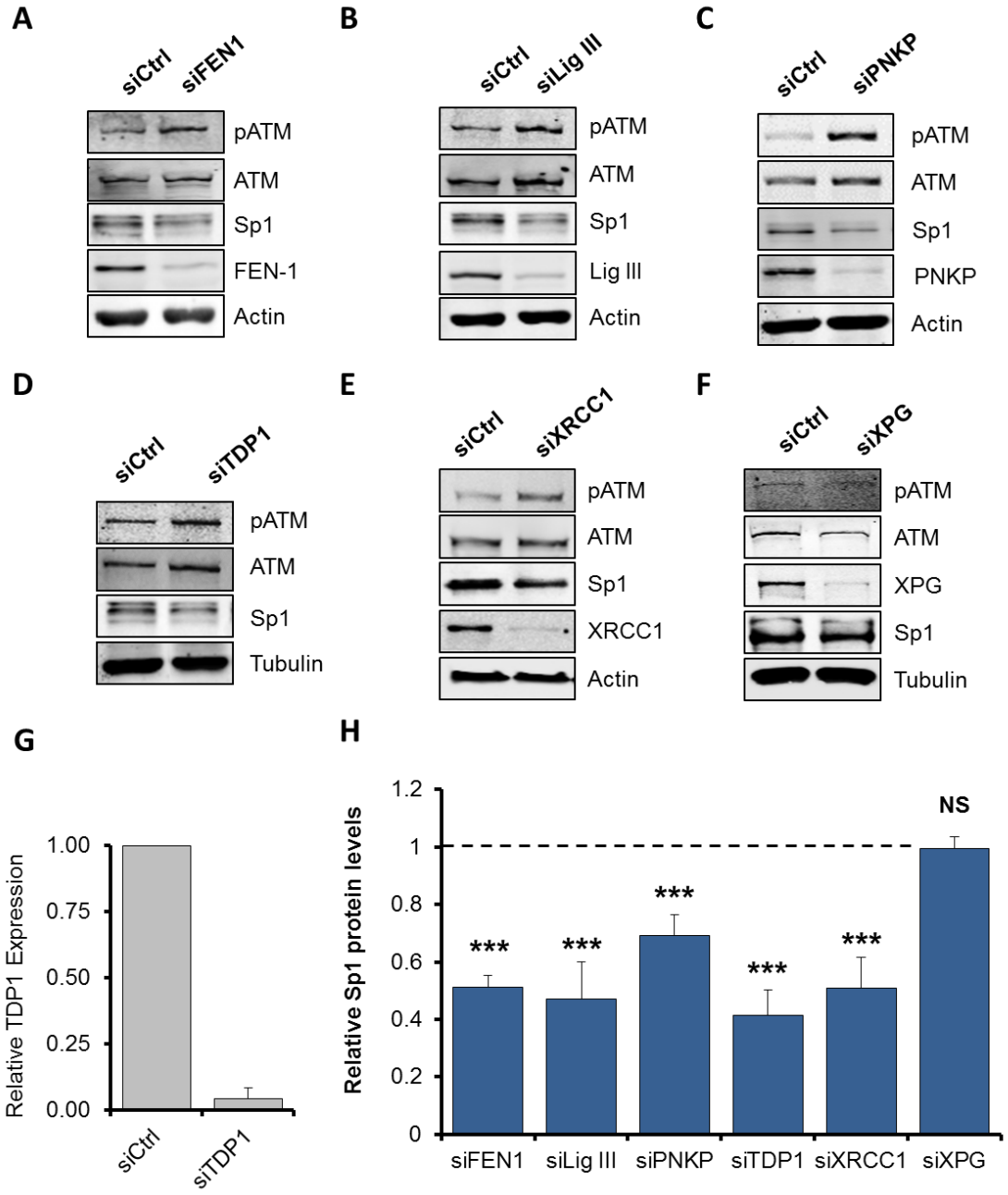

Figure 5.9 Accumulation of endogenous DNA damage affects Sp1 protein levels

Panels **(A-E)** Representative Western blot analyses on TIG-1 cells after siRNA-mediated knock down of different DNA repair proteins. Sp1 protein levels are decreased after knock down of **(A)** FEN-1, **(B)** Lig III, **(C)** PNKP, **(D)** TDP-1 and **(E)** XRCC1, and Ser1981 phosphorylation of ATM is present. **(F)** Depletion of XPG using siRNA is used as a negative control as Sp1 levels are unchanged and ATM phosphorylation is not present. In (A-F), actin or tubulin was used as a loading control. **(G)** qPCR analyses showing validation of TDP-1 siRNA. B2M and GAPDH were used as endogenous controls. **(H)** Densitometric analysis of total Sp1 protein levels panels (A-F) after knock down of different DNA repair proteins. Data are presented as mean ± SD from at least three independent experiments, relative to Sp1 levels in siCtrl-treated cells. NS: not significant; ***:p<0.001. siCtrl: non-targeting siRNA control.

As a further proof of concept, we also investigated what happens to *XRCC1* transcription under conditions of increased endogenous DNA damage. Importantly, concomitant with a reduction in Sp1 protein levels, we observed a reduction in *XRCC1* transcription upon siRNA-mediated knock down of PNKP, FEN-1 or TDP-1 (Figure 5.10, panel A). Conversely, knock down of XPG, which did not decrease Sp1 protein levels, did not lead to any statistically significant change in *XRCC1* levels (Figure 5.10, panel B). Taken together, these data suggest that Sp1 can also be downregulated in response to persistent endogenous DNA damage, and that this has the functional consequence of decreasing transcription of the *XRCC1* gene.

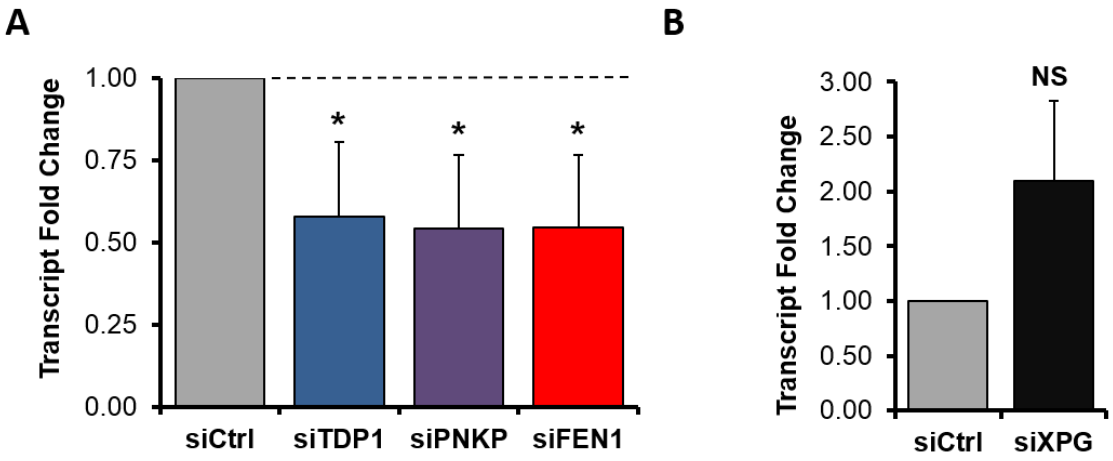

Figure 5.10 Increased endogenous DNA damage decreases *XRCC1* transcription

(A) qPCR analysis showing decreased *XRCC1* transcript levels in TIG-1 cells after knock down of different DNA repair genes, following the pattern of Sp1 protein levels. GAPDH and B2M were used as endogenous controls **(B)** qPCR analysis showing no significant change in *XRCC1* transcript levels in TIG-1 cells after knock down of NER factor XPG. Data are expressed as mean ± SD from three independent experiments. GAPDH and B2M were used as endogenous controls.

In summary, these data suggest the existence of a mechanism by which persistent unrepaired DNA strand breaks results in the downregulation of Sp1 protein levels. Consequently, persistent DNA damage has a negative effect on

BER expression. These findings are summarised in the schematic reported in Figure 5.11. We observed that ATM phosphorylates Sp1 in response to DNA damage. We hypothesise that if the level of DNA damage is above the threshold at which the cell can cope, phosphorylation of Sp1 will persist due to the presence of unrepaired DNA strand breaks. Ultimately, this leads to degradation of Sp1 protein in a proteasome-dependent manner. The loss of Sp1 then negatively impacts BER through reduced transcription of *XRCC1*. Unexpectedly, therefore, persistent DNA damage can lead to the downregulation of DNA repair. Although this demonstrates that BER can be modulated by the cellular DNA damage load, downregulation in response to persistent DNA damage is seemingly counterintuitive. However, we believe that this may have functional significance, as explained in the next chapter. Decreasing the efficiency of BER under circumstances when unrepaired DNA lesions are already present will likely contribute to further accumulation in DNA damage, and eventually lead to genomic instability. Nevertheless, given that maintenance of genome stability is paramount for cell survival, control mechanisms likely ensure that cells with excessive levels of DNA damage are rapidly identified and dealt with appropriately. Therefore, exacerbating the DNA damage load in a cell with persistent unrepaired DNA damage might constitute a mechanism to ensure the prompt identification of genetically unstable cells. This hypothesis spurred us to investigate the reasons behind Sp1 degradation and the downregulation of XRCC1 to understand why cells might decrease the efficiency of pathways able to address the unrepaired DNA damage

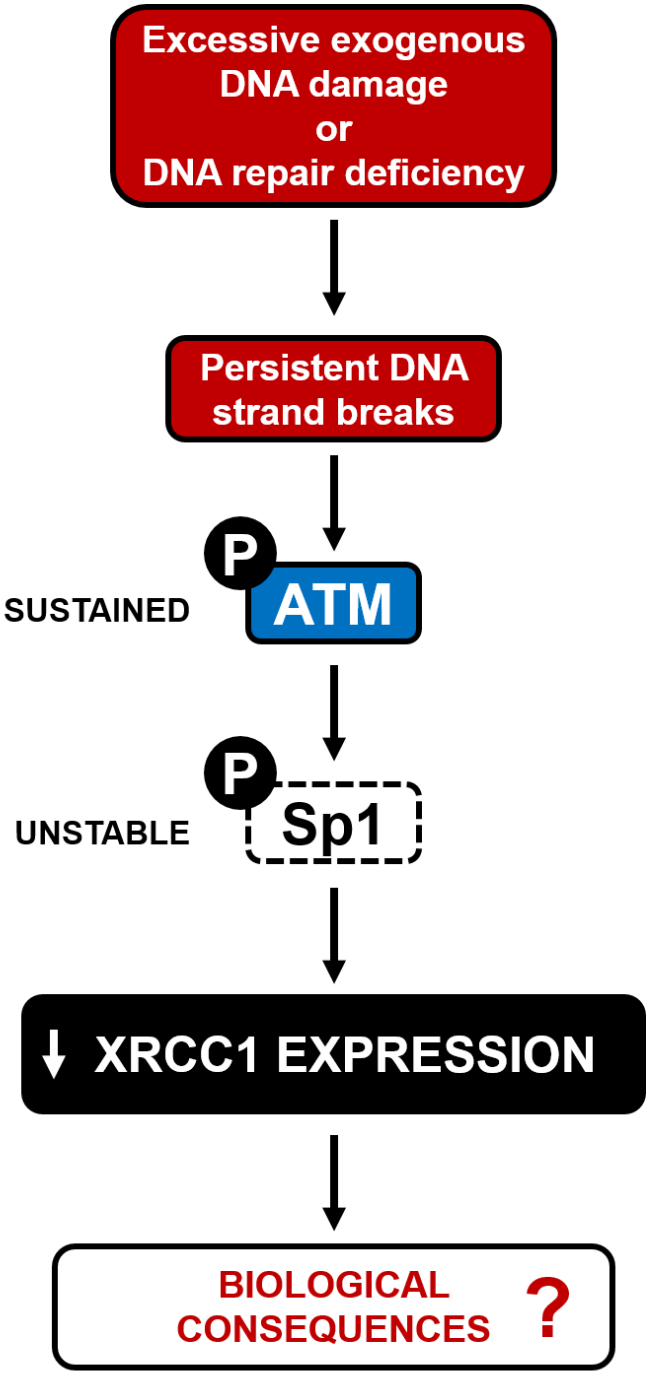

Figure 5.11 Working model.

ATM is autophosphorylated and activated in response to DNA damage of endogenous and/or exogenous origin. Activated ATM directly phosphorylates Sp1 at Ser101. Sustained Ser101 phosphorylation due to the presence of unrepaired DNA damage leads to destabilisation of Sp1 protein levels. This is due to proteasomal degradation of Sp1 occurring in an ATM-dependent manner. Loss of Sp1 has a negative impact on BER due to decreased XRCC1 expression.

6 Biological consequences of Sp1 depletion

In order to test our hypothesis that downregulation of Sp1 and XRCC1 after persistent DNA strand breaks has functional significance, we aimed to further characterise the effect of lack of Sp1 in our model of normal human fibroblasts.

6.1 Sp1-depleted cells show cell cycle delay and accumulation of spontaneous DNA damage

Firstly, we assessed the cell cycle profile of TIG-1 cells treated with Sp1-targeting siRNAs. Interestingly, we observed that Sp1 depletion leads to a significant increase in G_0/G_1-phase cells, and a concomitant decrease in S-phase cells, compared to the control siRNA-treated cells (Figure 6.1, panel A). Importantly, this finding was observed using two different siRNA sequences, thereby reducing the likelihood that this was an off-target effect of the siRNA sequence. Furthermore, Western blotting analysed revealed that siRNA-mediated depletion of Sp1 also led to an increase in p53 and p21 expression (Figure 6.1, panel B). This suggests that Sp1 depletion enforces a p53/p21-dependent cell cycle delay.

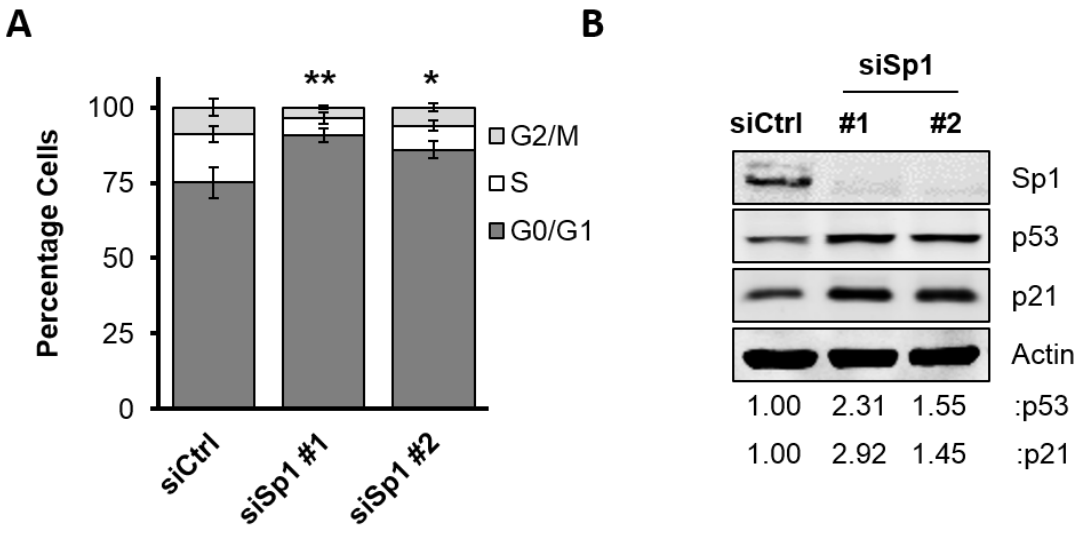

Figure 6.1 Cell cycle analysis in Sp1 depleted cells

(A) Cell cycle analysis on TIG-1 cells measured 72 h after siRNA-mediated Sp1 depletion. Sp1 depletion using two different siRNA sequences results in a G_0/G_1 cell cycle delay. Data are presented as mean % cells +/- SD from three independent experiments. Statistical significance compares G_0/G_1 percentages between siCtrl- and siSp1-treated cells. **(B**) Representative Western blot showing depletion of Sp1 leads to accumulation of p53 and p21 protein. Actin was used as a loading control. Quantifications are reported below the blot. *:p<0.05, **:p<0.01.

In order to investigate why loss of Sp1 resulted in a cell cycle delay, and given our previously identified role for Sp1 in *XRCC1* regulation, we assessed the level of DNA damage in Sp1-depleted cells using comet assays. We observed, using the alkaline comet assay, that depletion of Sp1 led to a significant increase in relative DNA damage (Figure 6.2, panel A). Importantly, these comet assays were carried out in the absence of external stimuli indicating that the observed increase in DNA damage was due to the accumulation of endogenous lesions which would ordinarily be repaired through BER. Representative images of Sp1-depleted cells from the comet analysis show a larger comet tail compared to control cells (Figure 6.2, panel B). Moreover, the amount of damage present in the tail is similar to that observed in XRCC1 knock down cells (Poletto et al., 2016) suggesting that the effect of Sp1 depletion could, at least partly, be

explained by a decrease in XRCC1 protein levels. It is important to note, however, that this version of the comet assay detects alkaline-sensitive lesions, which includes both DNA single and double strand breaks. Therefore, to further characterise the damage arising from Sp1 depletion, we used the neutral comet assay. In contrast to the alkaline comet assays, neutral comet assays detect mainly DSBs. Surprisingly, loss of Sp1 did not result in a detectable increase in the amount of DSBs detected compared to control cells (Figure 6.2, panel C). This finding was verified using two different siRNA sequences against Sp1. Representative images from the analysis show that both siCtrl- and siSp1 cells do not show accumulation of DNA in the comet tail, in contrast to H_2O_2 treated cells (Figure 6.2, panel D). In these experiments, H_2O_2 treatment was used as a positive control for the induction of DSBs. We speculate that the absence of detectable DSBs in Sp1-depleted cells may be explained by the cell cycle delay shown in Figure 4.1. Although we have demonstrated that cells lacking Sp1 experience increased endogenous DNA damage (Figure 6.2, panel B), functional cell cycle checkpoints likely ensure that the cells do not enter S-phase and replicate any SSBs into DSBs. Taken together, these findings indicate that Sp1 depletion decreases cellular capacity to repair endogenous DNA lesions in the form of SSBs.

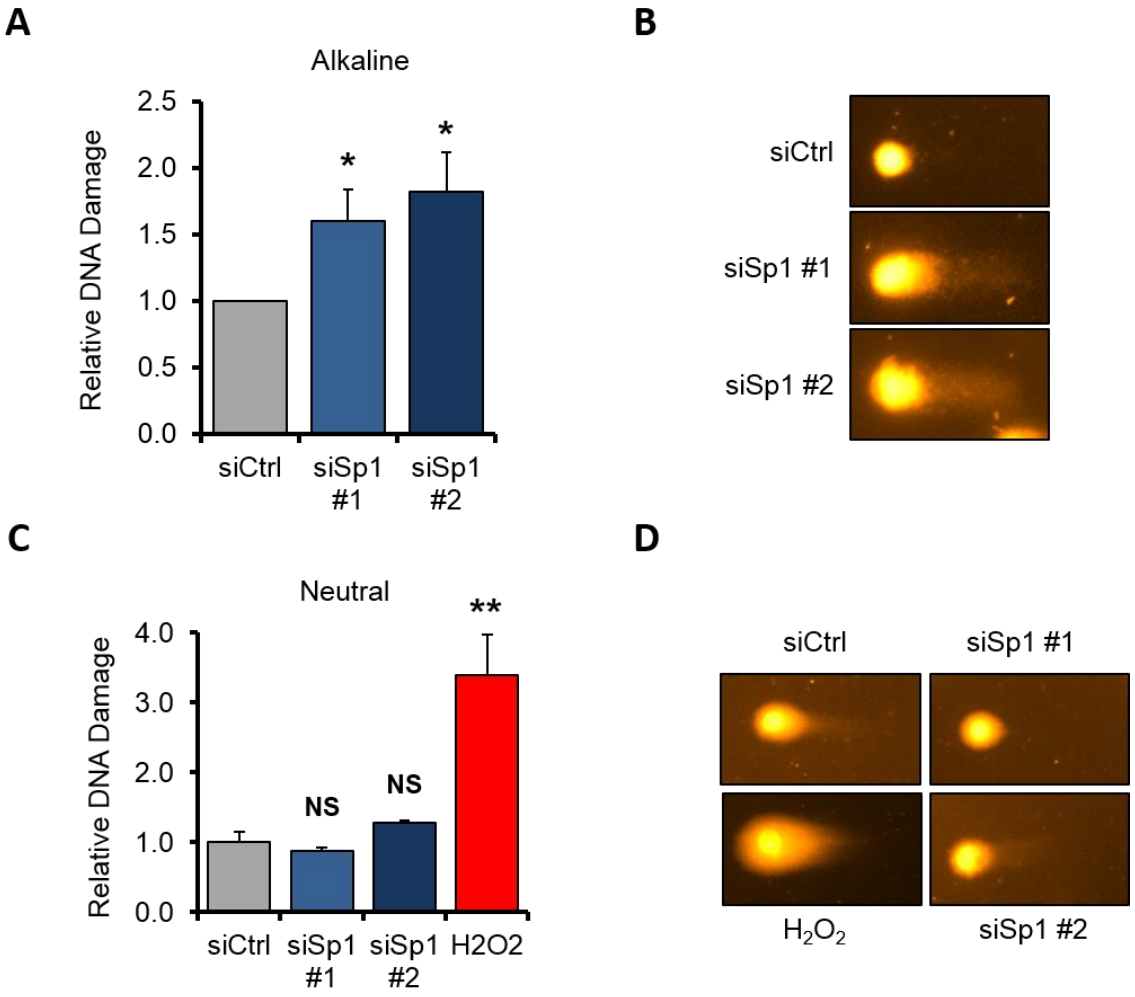

Figure 6.2 Sp1 depletion increases endogenous DNA damage

(A) Alkaline comet assay on TIG-1 cells harvested at 72 h after Sp1 depletion shows accumulation of DNA damage (N=3) **(B)** Representative images from alkaline comet assay shows increased tail DNA in Sp1 depleted cells compared with control cells. **(C)** Neutral comet assay on TIG-1 cells harvested at 72 h after Sp1 depletion shows no accumulation of DNA damage. H_2O_2 (200 µM, 10 min) is used as a positive control. (N=3) **(D)** Representative images from neutral comet assay shows absence of tail DNA in control and Sp1 depleted cells. H_2O_2 treated cells show presence of tail DNA. Data are reported as mean ± SD from the indicated number (N) of independent experiments. NS: Not significant. *:p<0.05, **:p<0.01.

6.2 Sp1-depleted cells have a reduced DNA repair capacity

Given that Sp1 depletion impacts the expression of XRCC1, we investigated whether BER efficiency is adversely affected by the loss of Sp1. Using an *in vitro* BER assay, we checked the ability of Sp1-depleted nuclear cell extracts to ligate a nicked oligonucleotide substrate. We observed that loss of Sp1 resulted in a moderate, but statistically significant reduction in nick ligation activity, compared to control cells (Figure 6.3, panel A). Given that Sp1 depletion does not completely abrogate XRCC1 expression, which is required to stabilise Lig III, it is not surprising that the observed ligation impairment is not as severe as in XRCC1-depleted cells, which were used as a positive control in the same assay (Figure 6.3, panel A). A representative *in vitro* ligation assay shows impaired substrate to product conversion in Sp1 depleted cells compared to the control (Figure 6.3, panel B). This indicated that cells lacking Sp1 have a reduced capacity to carry out BER.

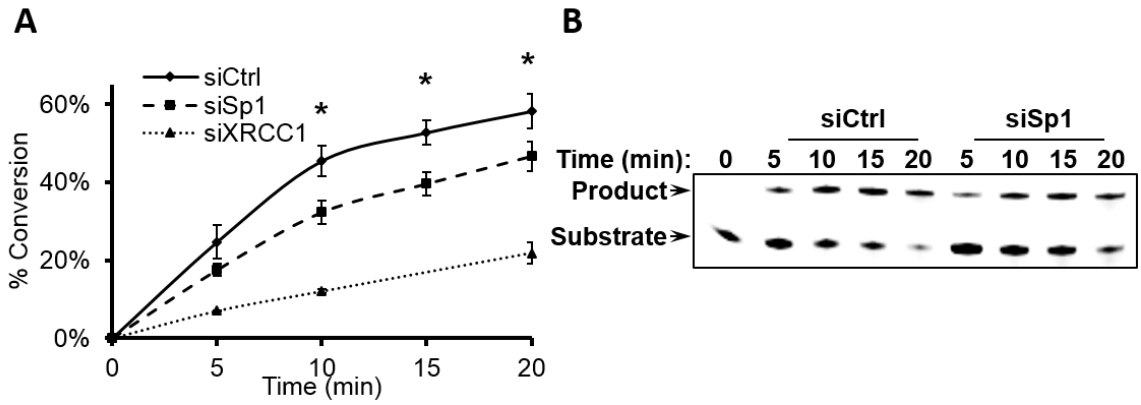

Figure 6.3 Depletion of Sp1 impairs DNA single strand break repair efficiency *in vitro.*

In vitro assay measuring the nick ligation activity in Sp1- and XRCC1-depleted nuclear cell extracts. Sp1 down regulation impairs cellular nick ligation efficiency Data are reported as mean % substrate to product conversion ± SD from three independent experiments. XRCC1 knock down is used as positive control for ligation impairment. *:p<0.05. **(B)** Representative *in vitro* ligation assay showing impaired substrate to product conversion in siSp1 TIG-1 cells compared to control cells.

To further characterise Sp1-depleted cells, we also investigated the double strand break repair capacity of TIG-1 cells after Sp1 knock down. Previous studies had demonstrated that knock down of Sp1 increases cellular sensitivity to DNA damaging agents (Olofsson et al., 2007, Iwahori et al., 2008). Consistent with these findings, we observed that cells treated with Sp1 targeting siRNA were defective in repairing zeocin-induced DNA double strand breaks (Figure 6.4). We scored 53BP1 foci as a proxy for DBSs in TIG-1 cells after treatment with zeocin and subsequent repair. The repair assay revealed significantly higher residual 53BP1 foci in siSp1 cells compared to the siCtrl cells at every repair time point investigated (Figure 6.4). This suggested that loss of Sp1 also negatively impacts DSB repair.

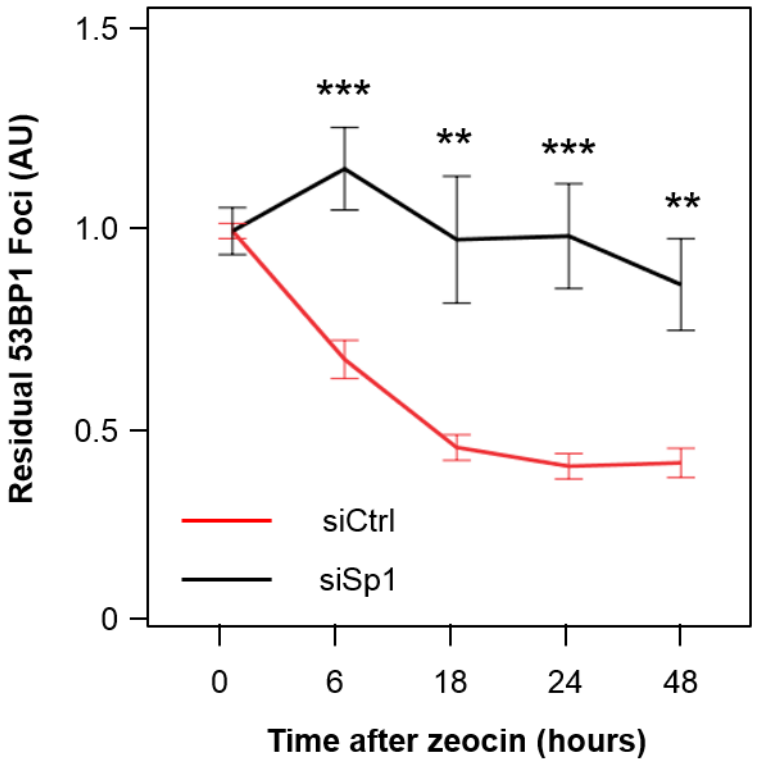

Figure 6.4 Loss of Sp1 impairs DSB repair.

Double strand break repair assay. Sp1-depleted TIG-1 cells were treated with zeocin (50 µg/mL, 1 h) and allowed to repair for times indicated. High residual 53BP1 foci in Sp1-depleted cells indicate impairment in double strand break repair. Foci were scored using high-throughput microscopy and at least 500 cells/condition were analysed. Results are expressed as mean ± SD from three independent experiments. **:p<0.01, **:p<0.001.

In order to further investigate the repair capacity of Sp1-depleted cells, we used reporter-based reactivation assays (a reaction scheme can be found in Appendix I (Figure 9.7, panel A). The recovery of luciferase activity of a heat-depurinated luciferase plasmid was used to determine the repair efficiency of control and Sp1 knock down TIG-1 cells. Heat depurination should result in DNA damage that is almost exclusively repaired through BER, although the presence of DSBs in the luciferase plasmid occurring as a consequence of clustered AP sites, cannot be completely ruled out. Consistent with the DNA repair impairment previously measured in Sp1-depleted cells, we observed significantly decreased repair in

Sp1-depleted cells at both 48 and 72 h after plasmid transfection, compared to control cells (Figure 6.5). siRNA-mediated knock down of different BER components was used as a positive control for the reactivation assays. Importantly, loss of APE1, Lig III or XRCC1, was also associated with a decreased recovery in luciferase expression compared to control cells (Appendix I Figure 9.7, panel C). These data further demonstrate that TIG-1 cells lacking Sp1 experience a reduced DNA repair capacity.

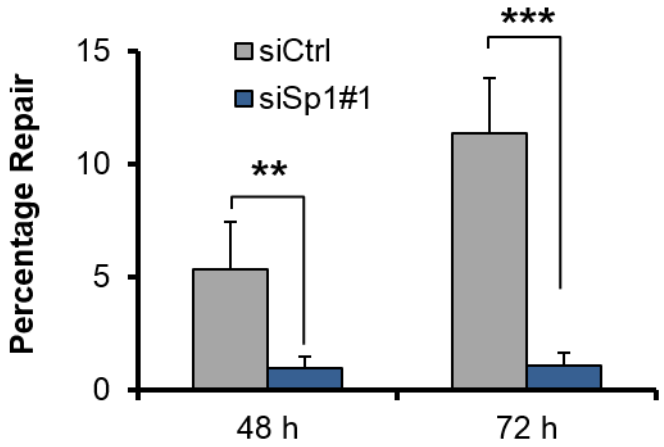

Figure 6.5 Reporter-based DNA repair assays

Cells were transfected with a heat-depurinated luciferase plasmid and repair efficiency was evaluated by measuring the recovery of luciferase expression over time. Sp1 depletion impairs DNA repair capacity. The histogram reports mean luciferase activity of damaged luciferase plasmid (relative to that of an intact luciferase plasmid) ± SD from six independent replicates. **:p<0.01, ***:p<0.001. Co-transfection of a GFP plasmid was used to normalise for transfection efficiency.

In order to understand the observed repair deficiency in Sp1 knock down cells, we investigated the mRNA levels of proteins involved in either single or double strand break repair (Figure 6.6). Consistent with previous findings, *APEX1* (Poletto et al., 2016) and *XRCC1* (Figure 3.2) transcript levels were decreased after Sp1 depletion. Similarly, mRNA levels of Pol β, another BER component, were also significantly reduced. Interestingly, we also found the transcription of

genes involved in DSB repair to be negatively affected by Sp1 depletion. For example, Ku70 (*XRCC6*), Ku80 (*XRCC5*), XRCC4 and DNA Ligase IV (*LIG4*), which are involved in NHEJ, significantly decreased after Sp1 knock down. Additionally, Sp1 depletion also resulted in a significant reduction in mRNA levels of HR pathway component Rad51. It should be noted, however, that only mRNA and not protein levels of these NHEJ and HR components were checked after Sp1 knock down. It is therefore unknown whether these changes translate to modulations at the protein level. Taken together, however, these data indicate that TIG-1 cells lacking Sp1 have a decreased repair capacity for both double and single strand breaks concomitant with an increase in the basal level of endogenous DNA damage.

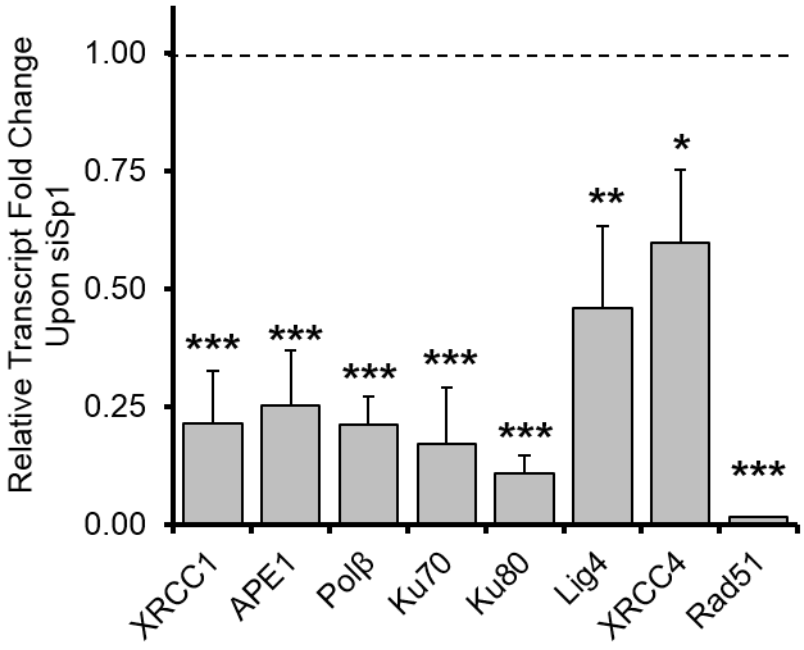

Figure 6.6 Sp1 depletion suppresses transcription of DNA repair genes

qPCR analysis showing reduction in transcript levels of genes from both SSBR and DSBR in TIG-1 cells depleted of Sp1. Results are expressed as mean ± SD from four independent experiments. B2M and GAPDH were used as endogenous controls. *:p<0.05, **:p<0.01, ***:p<0.001.

6.3 Cells lacking Sp1 show pro-apoptotic markers

We have established thus far that persistent DNA damage leads to downregulation of Sp1 in an ATM-dependent manner. Furthermore, we have demonstrated that cells lacking Sp1 have a decreased ability to repair both single and double strand breaks. In line with our hypothesis, this suggests that downregulation of DNA repair triggered by persistent DNA lesions is likely to have functional significance. In particular, we hypothesised that exacerbating DNA damage through impairing DNA repair might amplify a signal to encourage the identification of cells with persistent DNA strand breaks. Many factors can influence the choice between growth-arrest and apoptosis in response to unrepaired DNA damage. We further hypothesised that downregulating Sp1 might be a method to amplify a signal that allows cells with persistent DNA strand breaks to be flagged for irreversible elimination. This idea is supported by previous studies indicating that can ATM promote programmed cell death (Westphal et al., 1997, Duchaud et al., 1996). Moreover, it has been shown deficiency in BER can lead to apoptosis via a mechanism involving the accumulation of DNA strand breaks (Roos and Kaina, 2013, Ochs et al., 1999). In order to assess this hypothesis, therefore, we carried out a number of different experiments.

Firstly, we were struck by the change in fibroblast morphology in conditions when Sp1 levels are decreased; for example, upon siRNA-mediated Sp1 depletion or after zeocin treatment. Both conditions led to noticeable cell shrinkage, as assessed through α-tubulin staining of the cytoskeleton (Figure 6.7). Critically, this morphological change was found to be dependent on ATM kinase activity, as

ATM inhibition in the presence of zeocin was able to completely restore TIG-1 cell morphology (Figure 6.7).

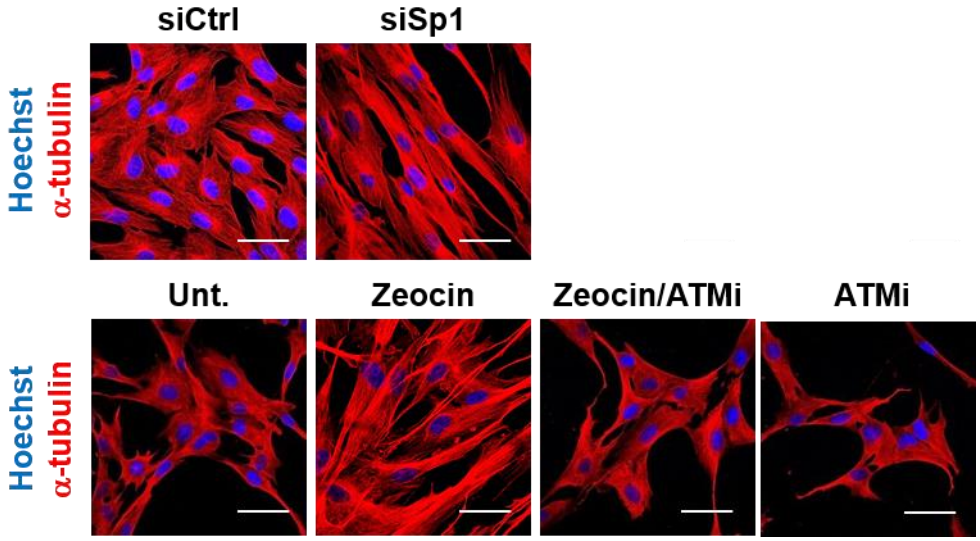

Figure 6.7 siRNA- or DNA damage-mediated depletion of Sp1 leads to cytoskeleton contraction in TIG-1 cells

Representative micrographs from TIG-1 cells treated with either control siRNA (siCtrl), Sp1-targeting siRNA (siSp1), or zeocin (50 µg/mL, 24 h). An ATM inhibitor (Ku-60019 10 µM) was added where indicated. Both siSp1 and zeocin treatments result in cytoskeleton contraction, as assessed by α-tubulin staining (red). This phenotype is reversed by ATM inhibition. Hoechst (blue) was used to stain nuclei. Scale bars 50 µm.

Cell shrinkage is a very early morphological change that occurs in apoptotic fibroblasts (Moodley et al., 2004). Therefore, we investigated whether cells expressing low levels of Sp1 might be in a pre-apoptotic state. Analysis using qPCR revealed that transcriptional upregulation of pro-apoptotic genes *BAX* and *PUMA (BBC3)* occurred in both Sp1 knock down TIG-1 cells (Figure 6.8, panel A) and cells treated with zeocin (Figure 6.8, panel B). Interestingly, the upregulation of pro-apoptotic genes in response to zeocin treatment could be prevented through inhibition of ATM kinase activity (Figure 6.8, panel B). This suggested that ATM could be important for the coordination of this process.

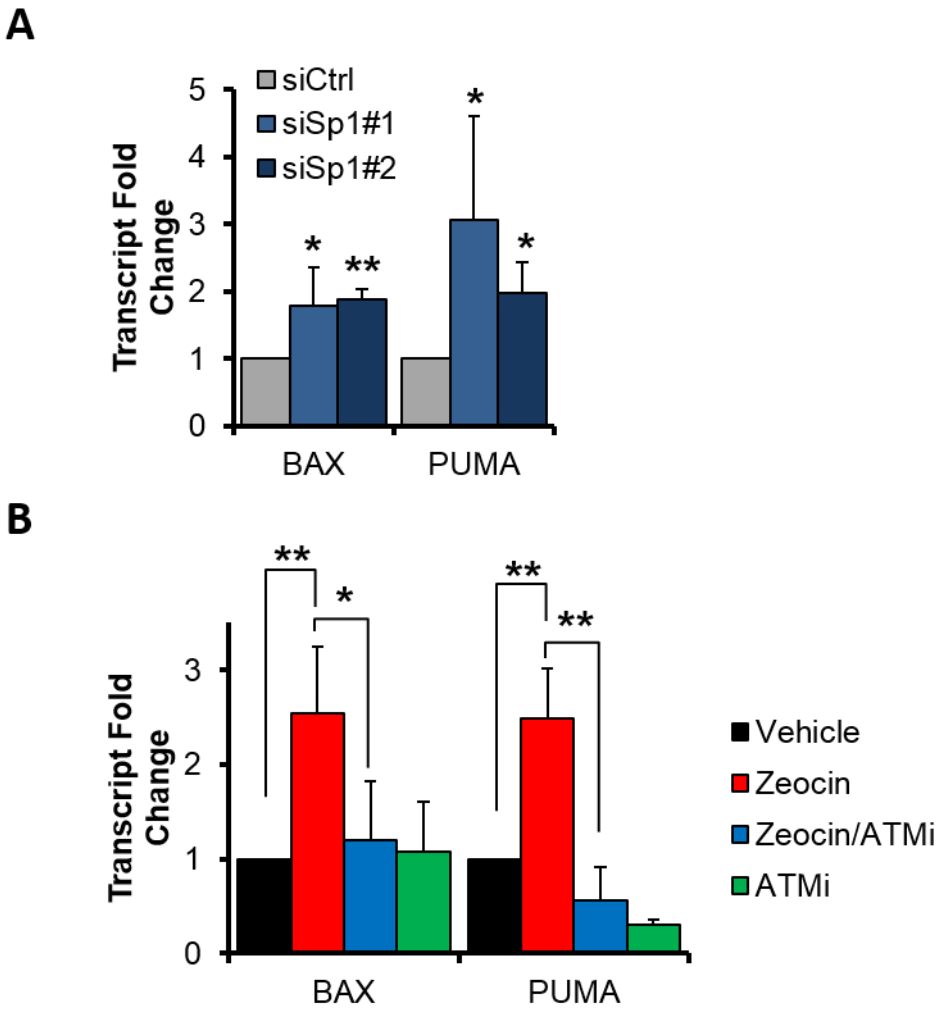

Figure 6.8 Sp1 depletion upregulates pro-apoptotic genes

(A) qPCR analysis assessing transcription of the indicated pro-apoptotic genes in TIG-1 cells treated with either a control siRNA (siCtrl) or an Sp1-targeting siRNA (siSp1). Loss of Sp1 leads to transcriptional upregulation of pro-apoptotic genes *BAX* and PUMA *(BBC3)*. **(B)** qPCR analysis assessing transcription of the indicated pro-apoptotic genes in TIG-1 cells treated with zeocin (50 µg/mL 24 h). An ATM inhibitor (Ku-60019 10 µM, 24 h) was added where indicated. Zeocin induces expression of pro-apoptotic genes *BAX* and PUMA (*BBC3)* which can be prevented by inhibition of ATM kinase activity. Results are expressed as mean ± SD from three independent experiments. B2M and GAPDH were used as endogenous controls. *:p<0.05; **:p<0.01.

6.4 Loss of Sp1 sensitises cells to pro-apoptotic agents

To further investigate whether Sp1-depleted cells were in a pre-apoptotic state, we checked their sensitivity to different apoptosis-inducing agents. Interestingly, Sp1-depleted cells showed significantly reduced viability when challenged with the pro-apoptotic agent navitoclax, a BH3 mimetic (Figure 6.9, panel A), as well as other apoptosis-inducing agents including staurosporine (Figure 6.9, panel B) and midostaurin (Figure 6.9, panel C). In fact, the higher sensitivity of Sp1 knock down cells to these compounds was accompanied by increased induction of full-blown apoptosis, measured by caspase 3/7 activation assays (Figure 6.10, panels A, B and C). In addition to this, annexin V/propidium iodide staining showed that navitoclax treatment resulted in a significantly higher percentage of apoptotic Sp1-depleted cells compared with control cells (Figure 6.11, panels A and B). Furthermore, in line with the caspase 3/7 activation assays, navitoclax treatment induced cleavage of caspase 3 detectable by Western blotting, specifically in Sp1 knock down cells (Figure 6.12). Moreover, Sp1-depleted TIG-1 cells expressed higher protein levels of apoptosis regulator BAX (Figure 6.12), consistent with the qPCR data (Figure 6.8, panel A). Interestingly, BAX underwent cleavage specifically in Sp1 knock down cells challenged with navitoclax (Figure 6.12); BAX cleavage has previously been proposed to boost apoptosis (Wood and Newcomb, 2000, Cao et al., 2003). Taken together, we conclude that cells expressing low levels of Sp1 are primed to undergo apoptosis, which can be triggered by the presence of external pro-apoptotic stimuli.

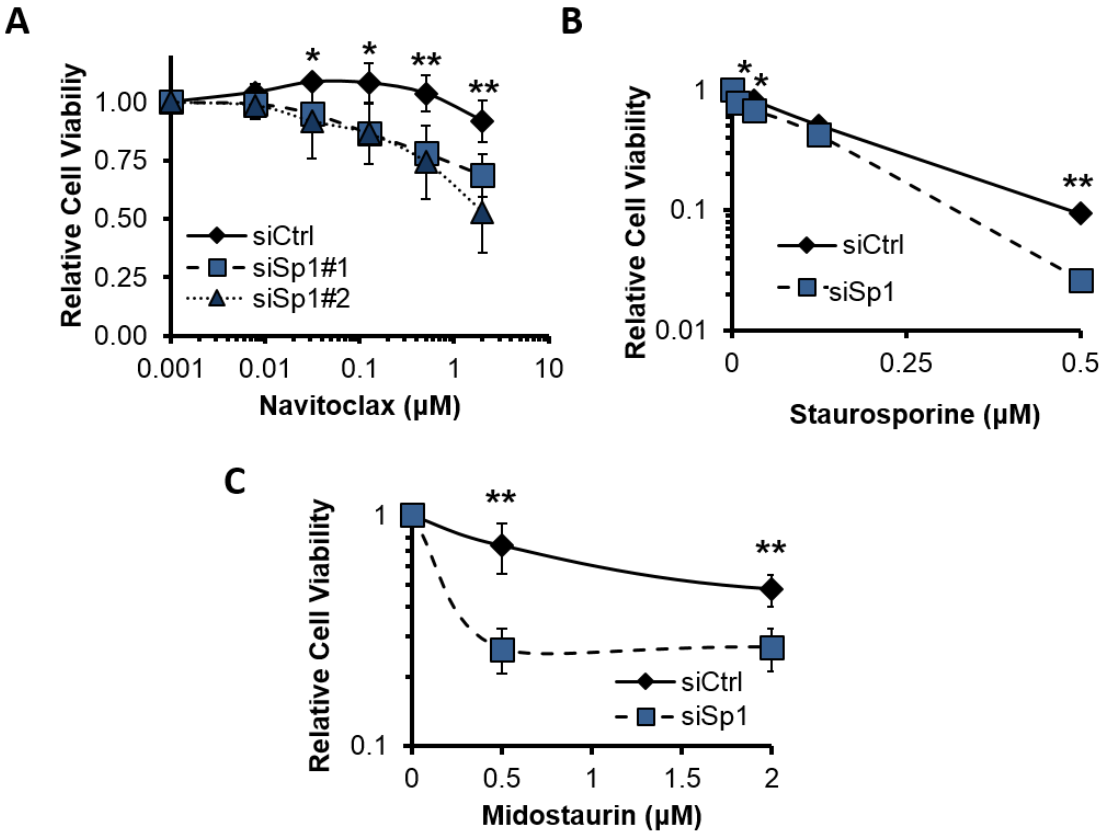

Figure 6.9 Loss of Sp1 sensitises cells to pro-apoptotic compounds

(A) Sensitivity of TIG-1 cells depleted of Sp1 to navitoclax. Cells were treated with increasing doses of navitoclax 48 h after siRNA transfection. Cell viability was measured using resazurin 48 h after treatment (N=7). **(B)** Sensitivity of TIG-1 cells to staurosporine upon treatment with the indicated siRNA. Cells were treated with increasing doses of staurosporine 48 h after siRNA transfection. Cell viability was measured using resazurin 24 h after treatment (N=3). **(C)** Sensitivity of TIG-1 cells to midostaurin upon treatment with the indicated siRNA. Cells were treated with increasing doses of midostaurin 48 h after siRNA transfection. Cell viability was measured using resazurin 48 h after treatment (N=4). Data are reported as mean ± SD from the indicated number (N) of independent experiments *:p<0.05; **:p<0.01.

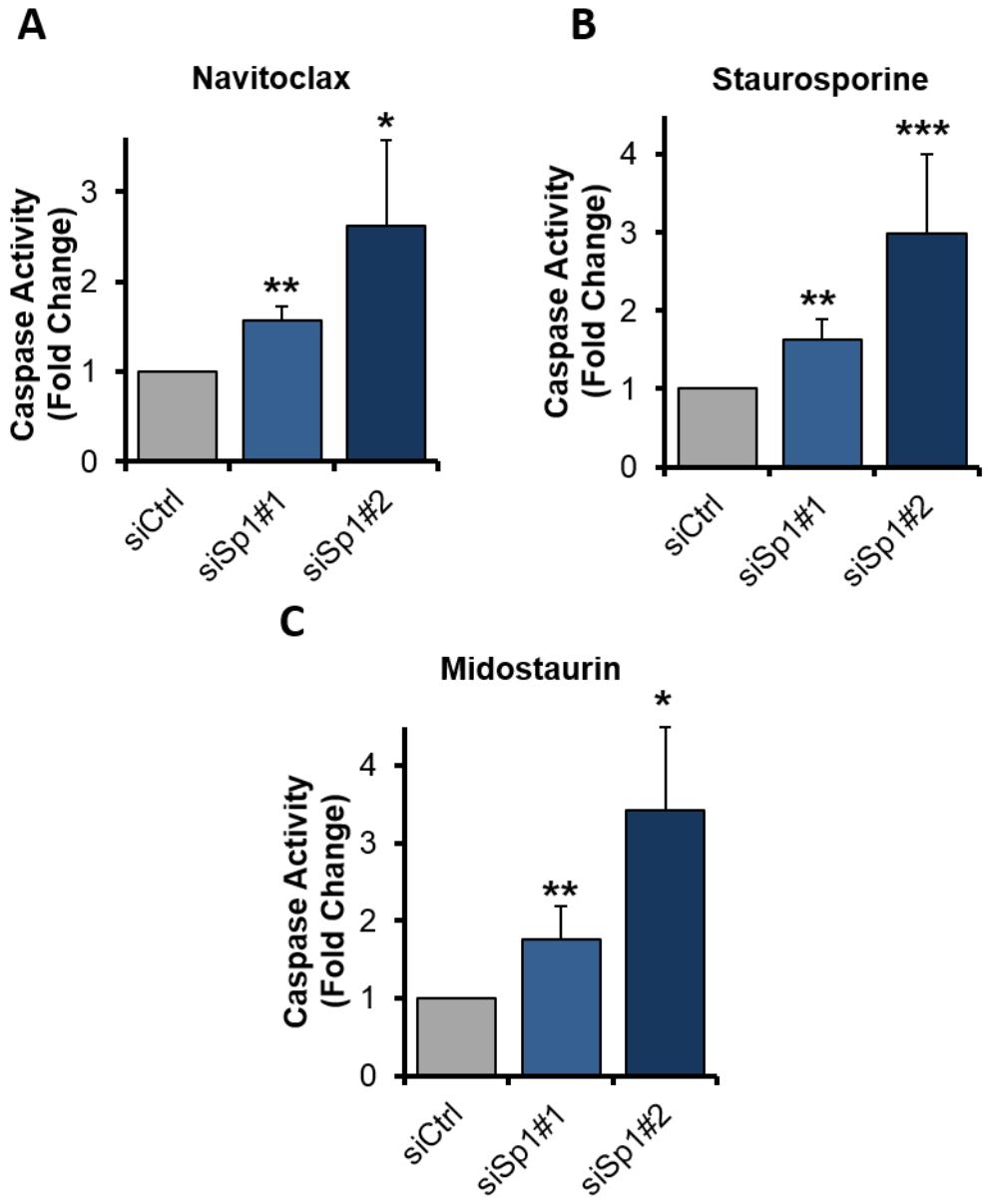

Figure 6.10 Loss of Sp1 sensitises cells to pro-apoptotic compounds

(A) Caspase 3/7 activity assay showing increased caspase activity in cells treated with navitoclax (2 μM, 48 h). Bars report the fold change in caspase activity above the vehicle upon navitoclax treatment, relative to siCtrl-treated cells (N=4). **(B)** Caspase 3/7 activation in TIG-1 cells upon treatment with the indicated siRNA and exposure to staurosporine (125 nM, 24 h) (N=4). **(C)** Caspase 3/7 activation in TIG-1 cells upon treatment with the indicated siRNA and exposure to midostaurin (8 μM, 48 h) (N=4). Data are reported as mean ± SD from the indicated number (N) of independent experiments *:p<0.05; **:p<0.01; ***:p<0.001

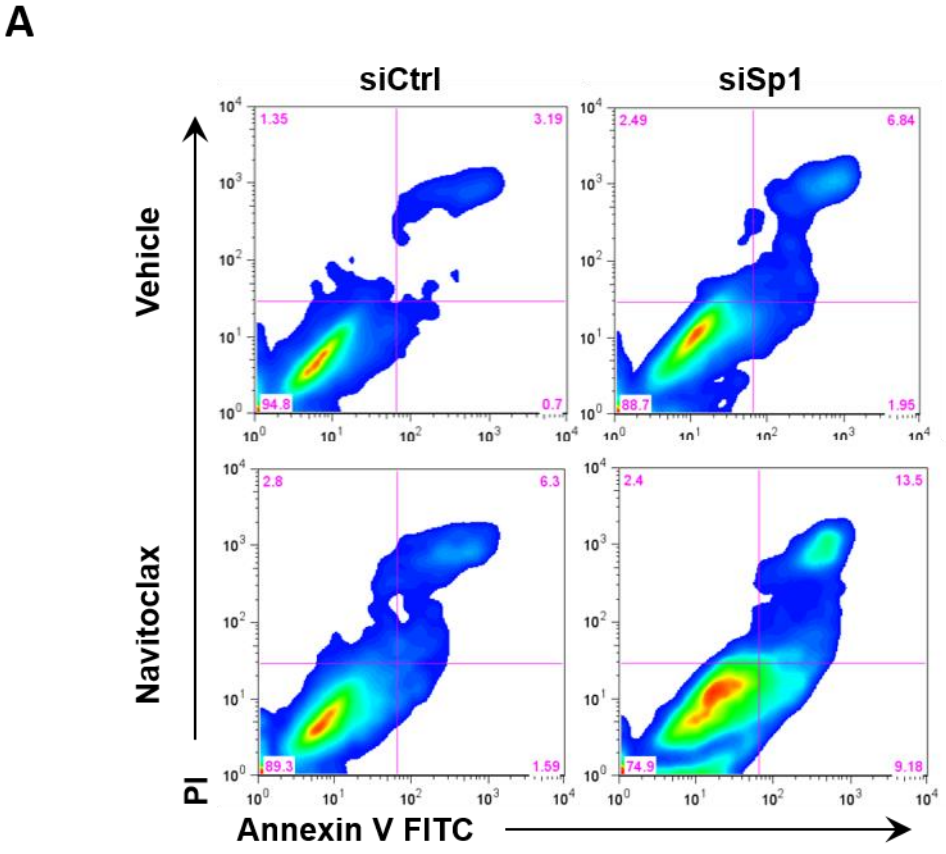

Figure 6.11 Sp1 depletion primes TIG-1 cells to apoptosis

(A) Representative density plot showing increased early- and late-apoptotic cells (bottom right and top right quadrant, respectively) in TIG-1 cells depleted of Sp1 upon treatment with navitoclax. Cells were treated with navitoclax (2 μM, 48 h) and subjected to Annexin V/PI staining and FACS analysis. **(B)** Quantification of the data shown in panel **(A)** demonstrating increased apoptosis in cells depleted of Sp1 upon treatment with navitoclax. Data are presented as mean ± SD from four independent experiments. *:p<0.05.

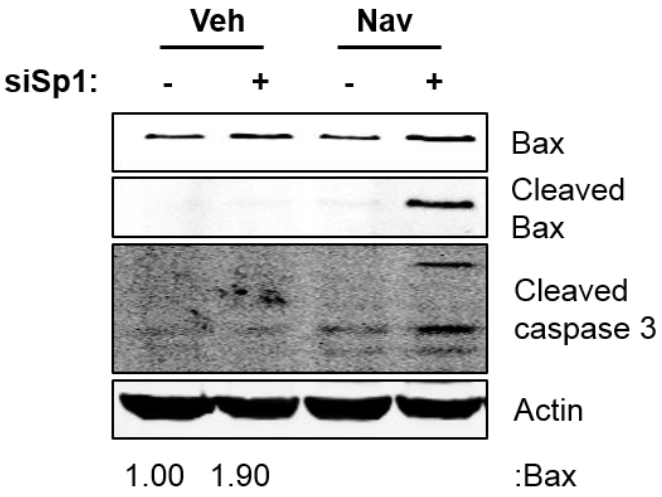

Figure 6.12 Sp1 depletion upregulates pro-apoptotic proteins and sensitises TIG-1 cells to apoptosis.

Representative Western blot analysis on TIG-1 cells showing cleaved caspase 3 and BAX in cells depleted of Sp1 upon treatment with navitoclax (2 μM, 48 h added 48 h after siRNA transfection). BAX protein levels are increased by Sp1 depletion. Navitoclax induces BAX and caspase 3 cleavage only in Sp1-depleted cells Actin is used as a loading control. Quantification of total BAX protein is reported below the blot.

6.5 Loss of Sp1 sensitises cells to elimination by the innate immune system

At the cellular level, our data suggest that the downregulation of Sp1 in response to persistent DNA damage is associated with the priming of these cells to elimination. Within a whole organism, however, the removal of genetically unstable cells is carried out by the innate immune system. Specifically, natural killer (NK) cells have been shown to respond to oncogenic stress and DNA damage in target cells through recognition of a number of cell surface ligands such as NKG2D and DNAM1 (Cerboni et al., 2014). Interestingly, in several cases the upregulation of these activating ligands has been proposed to be dependent on the ATM/ATR signalling axis (Gasser et al., 2005, Raulet and Guerra, 2009, Cerboni et al., 2014). Therefore we hypothesised that fibroblasts experiencing persistent DNA damage and expressing low levels of Sp1 might be

particularly susceptible to recognition and elimination by NK cells. Consistent with this idea, co-culture experiments revealed that Sp1 knock down TIG-1 cells were significantly more susceptible to cell death induced by NK cells (Figure 6.13), compared to control cells. This finding was reproduced using two different Sp1 siRNA sequences.

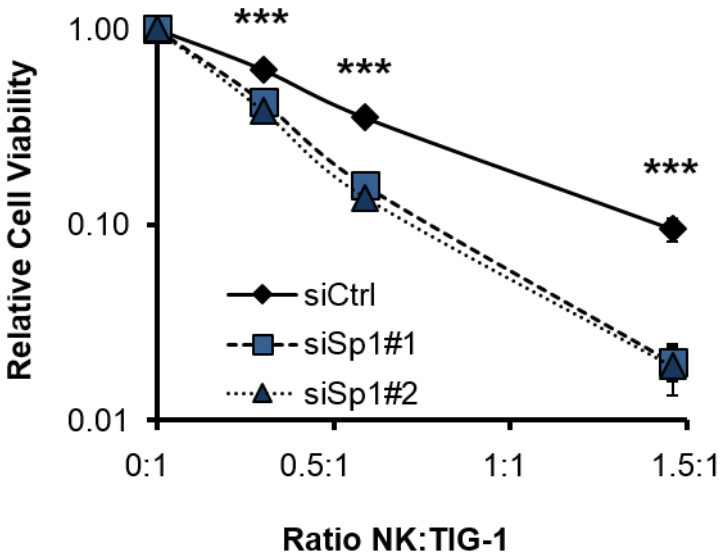

Figure 6.13 Loss of Sp1 sensitises TIG-1 cells to elimination by innate immune cells

Survival of TIG-1 fibroblasts upon incubation with NK cells. TIG-1 cells were treated with the indicated siRNA for 48 h before co-culture with NK cells at the indicated ratio for 24 h. Cell viability was measured using resazurin. Data show representative dose-response curves obtained in three independent biological replicates. ***:p<0.001.

Furthermore, zeocin-treated fibroblasts were also more vulnerable to elimination by NK cells, in comparison to untreated cells (Figure 6.14). Crucially, this susceptibility was found to be dependent on ATM kinase activity as ATM inhibition was able to completely restore fibroblast sensitivity to NK cell-mediated killing (Figure 6.14). These experiments suggest not only that Sp1 downregulation is an important factor that determines sensitivity to elimination by NK cells, but also that hypersensitivity to elimination is dictated by ATM activity.

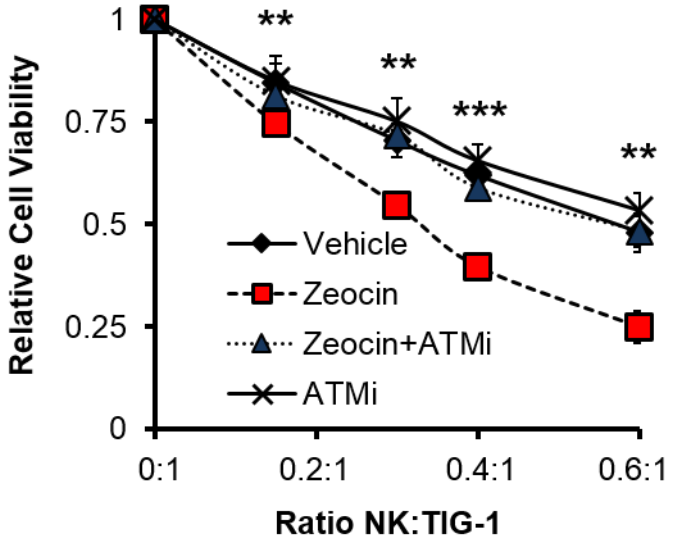

Figure 6.14 DNA damage-mediated depletion of Sp1 sensitises TIG-1 cells to elimination by innate immune cells.

TIG-1 cells were treated with zeocin (50 µg/mL) for 48 h, an ATM inhibitor (KU-60019, 10 µM) was added where indicated. Co-culture with NK cells at the indicated ratio for 14 h shows sensitivity of zeocin-treated TIG-1 cells and recovery upon co-incubation with ATM inhibitor. Data are presented as mean ± SD three independent experiments. **:p<0.01; ***:p<0.001.

In conclusion, we have demonstrated that loss of Sp1, either through siRNA-mediated knock down, or as a result of persistent DNA damage, has a negative impact on cells, particularly with respect to the DNA damage response. We have shown that cells lacking Sp1 are deficient in both SSB and DSB repair. Moreover, we have demonstrated that the absence of Sp1 is associated with increased susceptibility to pro-apoptotic agents, suggesting that cells lacking Sp1 are primed towards elimination. This can occur either through apoptosis or by the action of innate immune cells. These findings are consistent with our hypothesis that downregulation of Sp1 by persistent DNA strand breaks can promote the removal of cells with high levels of unrepaired DNA lesions.

7 Discussion

SSBs and base lesions are among the most common types of DNA damage that cells experience, posing a serious threat to both survival and genome stability. The inherent instability of the DNA molecule, in combination with the generation of by-products from cellular metabolism, fosters an environment where cells suffer continual endogenous DNA damage. The BER pathway, which copes with the majority of these types of damage, is therefore one of the most important cellular defences against genome instability.

It is clear that different tissues experience varying levels of DNA damage. For example, the high levels of metabolism in the brain are associated with increased levels of ROS (Wilson et al., 2011). This raises the question as to whether BER is able to adapt and respond to fluctuations in the level of endogenous DNA damage. A compelling mechanism has been described for varying BER protein levels in response to endogenous DNA damage. The model put forward by Parsons et al. suggests that BER components not actively engaged in repair are degraded through the proteasome system (Parsons et al., 2008, Parsons et al., 2009, Parsons et al., 2011). However, this mechanism describes only the acute response at the protein level to minor fluctuations in the DNA damage load. In contrast, it is not clear whether BER is also modulated at the transcriptional level. For example, several early studies suggested that mRNA production of BER components, including Pol β, could be induced in response to acute genotoxic stress (Fornace et al., 1989). However, these findings could not be replicated in later studies (Inoue et al., 2004), resulting in the general assumption that BER is not modulated by acute DNA damage, at least at the level of transcription. It is

important to note, however, that these studies focussed on the short-term response to DNA damage, rather than longer-term adjustments to variations in the cellular DNA damage load. Therefore, in this study, we sought to understand how BER can adapt long-term to a changing cellular environment. We were interested in how BER can be modulated by the cellular DNA damage load. Specifically, we sought to understand how the DNA damage load is sensed, and how this can be communicated to the BER pathway to adjust repair capacity. Given that loss of XRCC1 negatively affects BER efficiency (Fan et al., 2007, Horton et al., 2008), we hypothesised that modulations of XRCC1 might have repercussions throughout the whole BER pathway. Therefore, we investigated regulation of BER by the cellular load of DNA damage through studying transcriptional regulation of central scaffold protein XRCC1.

7.1 Sp1 regulates *XRCC1* transcription

The first part of this thesis describes the novel finding that transcription factor Sp1 is important for controlling the expression of core BER scaffold protein XRCC1. By using a combination of cell-based and *in vitro* assays, we demonstrated that Sp1 regulates transcription of the *XRCC1* gene under basal conditions. Furthermore, through bioinformatics we were able to identify a putative binding site for Sp1 in the *XRCC1* promoter that was highly conserved in mammals. Importantly, Sp1 was able to directly bind this site *in vitro* (Figure 3.7 and Figure 3.9), suggesting an evolutionarily important role for Sp1 in controlling *XRCC1* transcription.

Prior to this study, transcription factors E2F1 and FOXM1 had separately been proposed to modulate XRCC1 levels (Tan et al., 2007, Chen et al., 2008b). However, in our experimental model, knock down of either of these transcription factors did not affect XRCC1 mRNA levels. It is tempting to speculate that the differences we observed are due to variations in the cell models used. Both studies, for example, used cancer cells and MEFs as cell models, in contrast to the normal human fibroblasts used in this research. We have previously demonstrated that the coordination of BER is dysregulated in transformed cells (Poletto et al., 2016) which may explain the discrepancies between the studies. Alternatively, it is also possible that XRCC1 expression may be regulated in a context-dependent manner by other transcription factors in addition to Sp1.

Curiously, this is not the first study to connect Sp1 activity with the modulation of BER genes. For example, several studies have linked Sp1 with *APEX1* expression (Zaky et al., 2008, Bocangel et al., 2009, Poletto et al., 2016). Sp1 binding sites in the *MPG* promoter have also been identified (Christmann and Kaina, 2013) and Sp1-dependent regulation of the *POLD* promoter has been reported (Li and Lee, 2001). In addition to XRCC1, we also observed a decrease in Pol β mRNA after Sp1 depletion (Figure 6.6). Taken together, these indicate that a large number of BER genes can be regulated by Sp1. It is therefore tempting to speculate whether Sp1 may play a role in BER homeostasis through modulating gene expression across the whole BER pathway. This is in line with a pleiotropic role in BER for Sp1 in combination with p53 which has recently been proposed by Antoniali and colleagues (Antoniali et al., 2015).

7.2 Ser101 phosphorylation by ATM promotes Sp1 degradation in response to persistent DNA damage

As alluded to earlier, the wider aim of this thesis was to investigate how BER adapts to variations in the DNA damage load. In particular, we were interested in how cells can sense the level of endogenous DNA damage, and elicit the appropriate response downstream during BER. For several reasons the protein kinase ATM was a strong candidate for coordinating such a mechanism. Firstly, our lab has previously shown that ATM can be activated by accumulation of endogenous DNA lesions that would ordinarily be repaired through the BER pathway (Khoronenkova and Dianov, 2015). Furthermore, it has previously been shown that Sp1 undergoes ATM-dependent modification in response to DNA damage (Olofsson et al., 2007, Iwahori et al., 2008, Tan and Khachigian, 2009). Therefore, we hypothesised that Sp1 might form the link between DNA damage and BER, acting as a transducer between ATM activation and modulation of BER activity, potentially through *XRCC1* transcription. Therefore, this spurred us to investigate a role for ATM in greater detail, particularly with respect to Sp1 modification.

In order to address DNA damage-dependent regulation of BER, we focussed on the modulation of Sp1 in response to DNA damage. While some studies had previously shown that Sp1 was phosphorylated at Ser101 in an ATM-dependent manner upon genotoxic insult, (Olofsson et al., 2007, Iwahori et al., 2008) the direct involvement of ATM in the phosphorylation event was never demonstrated. By methodically investigating major DDR kinases, we were able to show that other DDR-related PIKK family members were not involved in Ser101

phosphorylation in response to DNA damage, nor were downstream kinases CHK1 and CHK2 (Figure 4.2). Using *in vitro* kinase assays, we confirmed that ATM directly phosphorylates Sp1 at Ser101. A previous study highlighted Ser56 as another residue phosphorylated in an ATM-dependent manner after DNA damage (Iwahori et al., 2008). However, the use of a phospho-S/TQ antibody in our *in vitro* kinase assays showed that Ser101 was the only canonical ATM site that underwent phosphorylation by ATM directly. Nevertheless, this does not rule out that another kinase acting downstream of ATM may be responsible for Ser56 phosphorylation *in vivo*. In fact, it has previously been suggested that Ser101 may act as a priming site for phosphorylation at other Sp1 residues (Beishline et al., 2012); our data would support this hypothesis.

There is much evidence in the literature that post translational modifications, particularly phosphorylation, are important for modulating Sp1 function. For instance, Ser59 phosphorylation is associated with increased transcriptional activity (Fojas de Borja et al., 2001, Kim and Lim, 2009) whereas Thr739 phosphorylation is important for Sp1 stability (Chuang et al., 2008, Wei et al., 2009). With respect to Ser101 phosphorylation, however, its precise molecular function remains more uncertain. For example, Ser101 phosphorylation has been linked to a non-transcriptional role for Sp1 in DNA repair (Beishline et al., 2012), as phosphorylated Sp1 was shown to co-localise with ATM at sites of DNA damage, independent of its DNA binding domain (Iwahori et al., 2008, Beishline et al., 2012). In this case, the authors hypothesised that the recruitment of phosphorylated Sp1 to DSBs might promote DNA repair. Although our data do not allow us to speculate on a specific role for Ser101-phosphorylated Sp1 at DSBs, this is the first study that has linked Ser101 phosphorylation with

modulation of Sp1 protein stability. We observed destabilisation of Sp1 protein in response to unrepaired DNA damage (Figure 5.1). This occurred concomitantly with persistent Ser101 phosphorylation (Figure 5.2), and crucially, was dependent on ATM activity (Figure 5.4). Furthermore, Sp1 destabilisation was not DNA damage-type specific, suggesting that downregulation of Sp1 might be a general cellular response to genotoxic stress (Figure 5.2). In fact, given that Sp1 destabilisation in response to H_2O_2 or zeocin did not occur immediately after the genotoxic insult, but only when DNA damage persisted, it is tempting to speculate that this mechanism depends on the cellular load of unrepaired DNA strand breaks. The earlier studies that identified Ser101 phosphorylation did not observe destabilisation of Sp1. However, those particular studies were carried out by treating cells for significantly shorter time periods than were used in this thesis (Olofsson et al., 2007, Iwahori et al., 2008, Beishline et al., 2012). Evidence presented here, on the other hand, indicates a time-dependency for Sp1 downregulation to occur, which supports the hypothesis that Sp1 destabilisation is related to the cellular load of unrepaired DNA strand breaks (Figure 5.2 panel A and Figure 5.3, panel A). Consequently, our findings do not preclude a potentially separate role for phosphorylated Sp1 recruited to DSBs during the short-term response to DNA damage. Instead, we propose that Sp1 degradation occurs only when the cellular repair capacity is exceeded and DNA damage persists. Moreover, it is interesting that we also observed downregulation of Sp1 in BER-depleted cells (Figure 5.9). Given that loss of BER factors is associated with accumulation of endogenously-generated DNA damage (Markkanen et al., 2015, Khoronenkova and Dianov, 2015, Poletto et al., 2016), this further suggests a correlation between Sp1 destabilisation and the

cellular DNA damage load. Furthermore, the evidence that Sp1 downregulation occurs in response to endogenous DNA damage suggests that this mechanism is important under physiological conditions.

As well as dependence on ATM activity, we also showed that Sp1 destabilisation is proteasome-mediated. DNA damage-induced Sp1 degradation, for example, could be prevented by the addition of a proteasome inhibitor (Figure 5.3). Although evidence suggests that Sp1 degradation is mediated through the proteasome in this study, the precise mechanism is unclear and further study is required. For example, Sp1 over-expression experiments using wild-type or Ser101 mutant proteins would provide further insight into the role of Ser101 phosphorylation in this mechanism. However, the TIG-1 fibroblasts we used respond very poorly to plasmid transfection and such experiments were not possible in this study. In addition, it is not certain whether Sp1 degradation is linked to ubiquitylation of the protein. At present, no residues in Sp1 that can be directly ubiquitylated have been identified (Beishline and Azizkhan-Clifford, 2015). Given our findings that Sp1 regulates components of the BER pathway, we hypothesised that E3 ligases known to be important for BER modulation, might be involved in Sp1 regulation. Such BER-related E3 ligases include CHIP, Cul1, Cul4, Mule and Ubr3 (Edmonds and Parsons, 2014). In particular, previous work from our lab demonstrated that Mule regulates Pol β turnover (Parsons et al., 2008, Parsons et al., 2009). Our attempts to prevent Sp1 degradation by targeting Mule with siRNA, however, were unsuccessful (data not shown). The potential role for other BER-related E3 ligases in Sp1 regulation therefore warrants additional study. In support of ubiquitin-mediated degradation, mimicking glucose starvation has previously been shown to induce Sp1

degradation. This was mediated through Sp1 interaction with β-transducin repeat-containing protein (β-TRCP), a substrate-recognition component of SCF ubiquitin ligase complexes (Wei et al., 2009). Moreover, this is in line with the hypothesis that Sp1 degradation is a cellular stress response, especially given that glucose deprivation is associated with increased genomic instability (Dai et al., 2013). Sp1 stability can also be negatively affected by SUMOylation at K16 (Wang et al., 2011). Therefore, it would be interesting to investigate whether Sp1 can be SUMOylated in response to persistent DNA damage, and if this is dependent on Ser101 phosphorylation.

7.3 Loss of Sp1 dysregulates DNA repair

To understand why persistent DNA damage resulted in Sp1 destabilisation, we investigated the cellular consequences of reduced levels of Sp1. In recent years, increasing evidence has emerged in support of a role for Sp1 in DNA repair. In addition to the proposed regulation of BER genes alluded to earlier, it has also been shown that cells lacking Sp1 are impaired in DSB repair. Specifically, depletion of Sp1 was shown to inhibit repair of site-specific DNA breaks (Olofsson et al., 2007), and lack of Sp1 was associated with decreased survival following exposure to exogenous agents (Olofsson et al., 2007, Iwahori et al., 2008). Here, however, we expand our knowledge of the DNA repair impairment experienced by cells lacking Sp1. For example, we show for the first time that lack of Sp1 negatively impacts the repair of endogenous DNA lesions normally dealt with by BER (Figure 6.3), leading to the accumulation of SSBs (Figure 6.2, panel A). A deficiency in BER is not necessarily surprising given the proposed transcriptional role for Sp1 in regulating numerous BER components, including XRCC1. Furthermore, XRCC1 forms important interactions with many members of the BER pathway, and is required to stabilise Ligase III protein levels (Nash et al., 1997, Cappelli et al., 1997). Consistent with previous findings, we also observed impaired DSB repair associated with a lack of Sp1. Furthermore, we demonstrated transcriptional downregulation of wide number of DNA repair genes operating in SSB- and DSB-repair pathways, suggesting that loss of Sp1 has wide-ranging effects on DNA repair. Given our findings that Sp1 is degraded in response to persistent unrepaired DNA strand breaks, we hypothesise that destabilisation of Sp1 contributes to further accumulation of DNA damage. This

exacerbates the cellular DNA damage load by generating a vicious cycle whereby the initial induction of DNA damage ultimately escalates the level of DNA lesions due to the impaired ability to repair DNA.

7.4 Loss of Sp1 promotes fibroblast elimination

Our data demonstrate that loss of Sp1 is associated with increased sensitivity to cell elimination, either through apoptosis, or by components of the innate immune system. Sp1 has previously been linked to regulating the expression of cellular components involved in apoptosis (Grande et al., 2012, Hirose and Horvitz, 2013, Li et al., 2014) and supports our findings that loss of Sp1 leads to upregulation of pro-apoptotic genes *BAX (BBC3)* and *PUMA*. This suggests that a lack of Sp1 drives fibroblasts towards apoptosis (Figure 6.8). It has previously been published that apoptosis can be induced in keloid-derived fibroblasts by the staurosporine derivative midostaurin (Nakazono-Kusaba et al., 2004). Interestingly, our lab has also shown that depletion of XRCC1 in fibroblasts causes sensitivity to midostaurin (unpublished data). Given that Sp1 is clearly important for regulating BER, particularly XRCC1, it is therefore reasonable that loss of Sp1 also leads to sensitivity to apoptosis-inducing agents, as we observed (Figure 6.9).

Within a whole organism, on the other hand, additional mechanisms to ensure the removal of genetically unstable cells also exist. For example, immune surveillance by components of the innate immune system, mainly through recruitment of NK cells, eliminates stressed and dangerous cells before they can

cause harm (Iannello and Raulet, 2013). Immune surveillance therefore constitutes an important defence against tumourigenesis. Moreover, the DDR is crucial to this process by alerting the immune system to cells with DNA damage or oncogenic stress (Gasser et al., 2005). In line with this, we found that cells lacking Sp1, which have higher levels of endogenous DNA strand breaks, were more sensitive to NK cells (Figure 6.13 and Figure 6.14). Importantly, this suggests that our findings are of relevance *in vivo*.

It is hypothesised that upregulation of ligands for the cytotoxic NK cell receptor NKG2D (NK group 2, member D) (Gasser et al., 2005), is the major mechanism in stressed cells to alert NK cells. Interestingly, this is proposed to be mediated by ATM/ATR signalling (Gasser et al., 2005, Soriani et al., 2009, Cerboni et al., 2014). Consistently, we found that inhibition of ATM kinase activity prevented fibroblast sensitivity to NK cells after DNA damage. Taken together, these data indicate that exacerbating the DNA damage load through degradation of Sp1 also promotes cell elimination through increased recognition by NK cells. We hypothesise that this might occur in an ATM-dependent manner due to upregulation of NKG2D ligands in fibroblasts experiencing persistent DNA damage, although validation of this idea will require further study.

Our data support a model whereby promoting cell death through continued accumulation of DNA strand breaks represents an anti-tumourigenic response within organisms to ensure the prompt elimination of cells with excessive DNA damage. In support of this hypothesis, induction of NK-activating ligands has been associated with the DDR in cancer cells (Waldhauer and Steinle, 2008,

Cerboni et al., 2014), where constitutive DDR activation occurs even in early neoplastic lesions (Bartkova et al., 2005, Gorgoulis et al., 2005). Furthermore, evasion of immune surveillance through shedding of NKG2D ligands is now an increasingly recognised mechanism used by cancer cells to prevent their recognition by the immune system (Shatnyeva et al., 2015). In conclusion, our data demonstrate that loss of Sp1 is detrimental to cells, both with respect to DNA repair but also their survival. This suggests that ATM-mediated Sp1 regulation might be an important determinant for cell fate outcomes.

7.5 Proposed Model

The experiments described in this thesis have allowed us to gather insights into the regulation of BER in response to the cellular load of DNA damage. We believe our data are supportive of a model whereby accumulation in DNA damage can actually lead to a downregulation in BER. Although somewhat counterintuitive, we believe that this is a defensive mechanism to ensure the elimination of cells with persistent DNA damage. Ordinarily, BER is a robust pathway; endogenously-generated DNA lesions, and even short bursts of genotoxin exposure are rapidly repaired (Fortini et al., 2000, Orlando et al., 2014) as the capacity of the pathway is sufficient for the level of DNA damage existing under physiological conditions. Moreover, there is no convincing evidence that BER can be induced in response to acute genotoxic stress; reproducible changes in BER gene expression level are not easily observable upon DNA damage (Christmann and Kaina, 2013). This is presumably due to the highly efficient buffering capacity of the pathway. Under normal circumstances,

therefore, BER prevents the accumulation of SSBs, thereby limiting the spontaneous formation of DSBs. However, the BER capacity may not always be sufficient to cope with the level of DNA lesions, leading to the presence of persistent unrepaired DNA strand breaks. This may occur as a result of acute genotoxic insult overwhelming the pathway, or when BER itself is impaired. Changes affecting the BER capacity are not unheard of, occurring ordinarily during physiological processes such as tissue differentiation (Narciso et al., 2007, Weissman et al., 2007, Bauer et al., 2011) or as a result of polymorphisms in BER genes, which may negatively affect their activity (Karahalil et al., 2012). Under such circumstances when BER is unable to promptly repair endogenous DNA lesions, this leads to an accumulation of unrepaired DNA damage, (Khoronenkova and Dianov, 2015, Poletto et al., 2016, Higo et al., 2017) which will ultimately result in genomic instability. Given the importance of preserving genome stability, it is safe to assume that control mechanisms must be in place to promote the elimination of genetically unstable cells. This thesis therefore proposes a mechanism to explain how physiological populations of healthy cells are maintained in an organism, by detecting and eliminating cells that have repair defects, partly through modulating cellular BER capacity. This is summarised below in Figure 7.1.

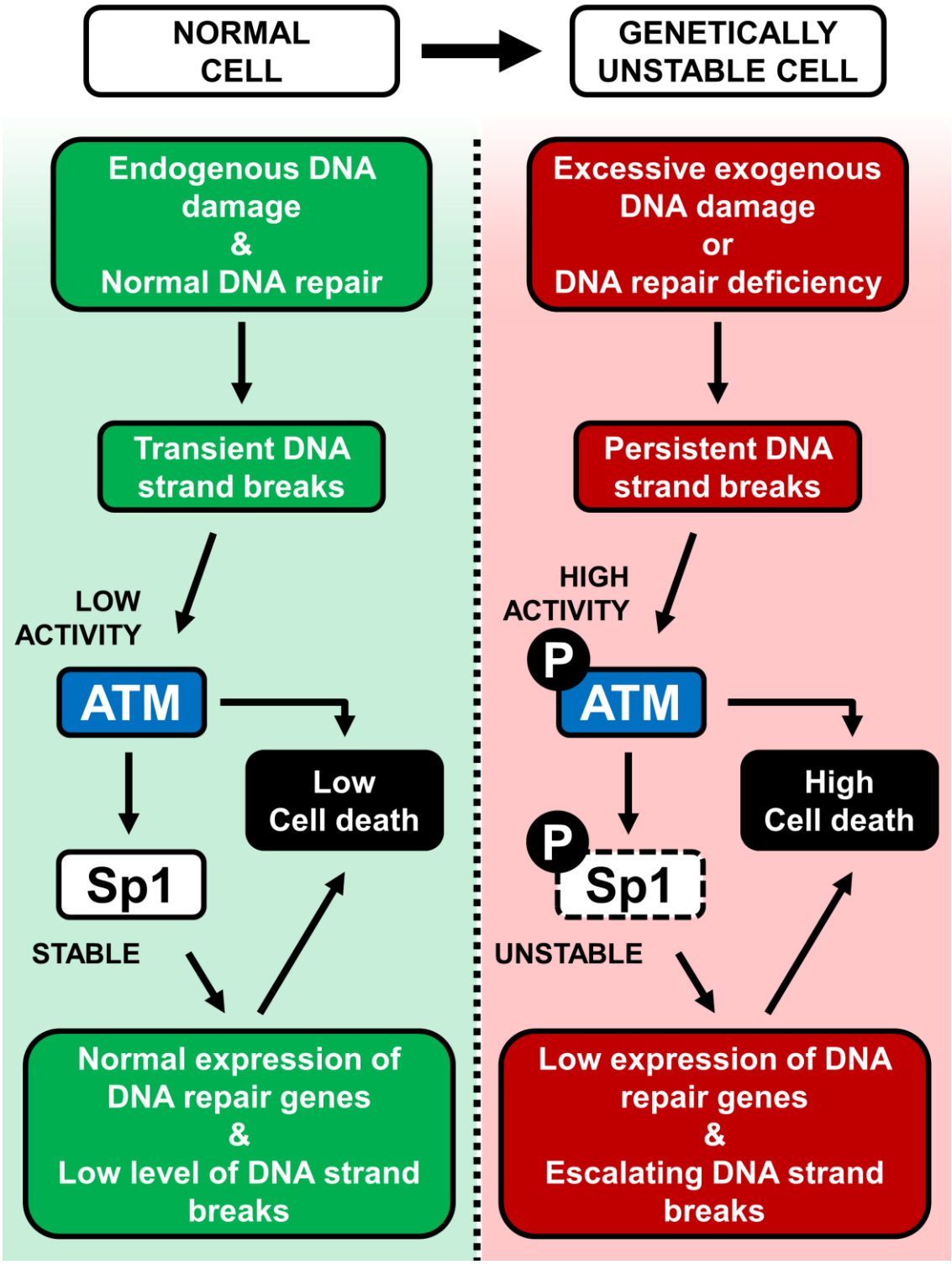

Figure 7.1 Proposed model. ATM/Sp1 cross-talk primes cells bearing persistent DNA damage to elimination

Left: in a normal cell, endogenous DNA damage is kept under control by DNA repair mechanisms. Low ATM activity ensures stable Sp1 protein levels, promoting efficient expression of DNA repair genes and low levels of cell death. *Right:* genetically unstable cells can arise under condition of excessive exogenous DNA damage, or DNA repair deficiency. This leads to persistent DNA damage and ATM activation, which triggers Sp1 phosphorylation and degradation. In this situation the cellular load of DNA damage is aggravated by decreased expression of DNA repair genes, leading to further ATM phosphorylation, activation of a pro-apoptotic cascade and cell death.

We propose that downregulation of transcription factor Sp1 in response to a signal radiating from ATM under conditions of persistent DNA damage, contributes to the elimination of genetically unstable and potentially pre-cancerous cells. We note that in unstressed cells, the level of ATM phosphorylation is very low. Correspondingly, Ser101 phosphorylation is barely detectable (Figure 5.1 and Figure 5.2) and Sp1 protein levels are stable. Under these circumstances, basal transcription of *XRCC1* and consequently, BER capacity, are sufficient to cope with the load of endogenous DNA lesions. This means that DNA damage is repaired, and genome stability is maintained (Figure 7.1, left hand side).

However, under conditions where persistent DNA strand breaks are present, the cellular response differs. In these cases, ATM becomes activated and phosphorylates Sp1. If ATM and Sp1 phosphorylation persists as a result of the accumulation of unrepaired DNA strand breaks, this ultimately leads to Sp1 degradation in a proteasome-dependent manner (Figure 5.3). This causes downregulation of *XRCC1* transcription and destabilisation of Lig III, which limits BER capacity (Figure 6.3 and Figure 6.5). DSB repair is also impaired (Figure 6.4), probably as a consequence of Sp1 downregulation. Consequently, reduction in Sp1 levels leads to a self-accelerating cycle of further DNA damage accumulation as DNA repair is compromised (Figure 7.1, right hand side). Ultimately, the escalation in DNA damage levels leads to the elimination of these cells. This can occur through apoptosis, as a result of upregulation in pro-apoptotic genes (Figure 6.8 and Figure 6.12), or by the action of cells in the innate immune system, for example through NK cells. We propose that this scenario represents a response that occurs during excessive acute DNA

damage, arising for example from exogenous mutagen exposure, or as a result of BER failure. Furthermore, we suggest that exacerbating DNA damage in cells by exceeding their repair capacity represents a pro-survival mechanism that ensures the removal of cells with persistent DNA damage. This helps to promote genome stability and prevent tumourigenesis by eliminating cells that are genetically compromised. It also demonstrates that cells can modulate their DNA repair capacity, in particular BER, in response to the accumulation of DNA strand breaks.

It is also of note that we observed downregulation of Sp1 in response to accumulation in endogenous DNA damage (Figure 5.9). This supports our hypothesis that this mechanism is actually a physiological response operating generally in organisms to combat genetic instability, rather than an atypical response occurring only as a result of excessive DNA damage induced by exogenous agents.

The consequences of DNA damage are inexorably linked to the severity of the lesion. It is still unknown, however, how cells transition between pro-survival and pro-death responses as a consequence of unrepairable DNA lesions. It is clearly a very complex mechanism as pro-survival and pro-death mechanisms can be activated via the same signalling proteins and pathways. For example, p53 both promotes cell cycle delay and apoptosis. Therefore, DNA damage thresholds must be critical for determining survival/death outcomes, particularly as repair capacity varies between cell types. Moreover, Roos et al., hypothesise that the phosphorylation status of p53 and the levels of anti-apoptosis proteins such as survivin are key determinants of cellular fate (Roos et al., 2016). How cells recognise the *persistence* of DNA damage, however, remains an intriguing open

question. Clearly sustained DDR signalling shifts the balance of pro-survival/death signalling towards cell death outcomes but it is uncertain exactly how this is achieved. However, there are various lines of evidence that the ATM kinase, acting as a central DDR coordinator, has an important role in the process. For example, ATM has been shown to promote apoptosis in neurons with excessive DNA damage (Lee et al., 2001). Furthermore, emerging evidence has linked ATM with promoting immune surveillance (Cerboni et al., 2014). The role of other strand break sensors such as Parp1, in promoting cell elimination should also be considered, however; persistent PAR signalling can induce parthanatos, an additional form of programmed cell death (Iyama and Wilson, 2013). Therefore, a potential role for Parp1 in this mechanism deserves further attention.

Our study supports a role for ATM in the pro-survival/pro-death decision-making process through ATM-dependent destabilisation of transcription factor Sp1. Of note, it has been reported that ATM undergoes selective pressure for inactivation in cancer (Negrini et al., 2010). Furthermore, loss of ATM correlates with the onset of different malignancies (Feng et al., 2015, Paull, 2015, Russell et al., 2015). This suggests that genetically unstable cells might escape this control mechanism through inactivation of ATM function, leading to the emergence of cancer. Furthermore, it is tempting to speculate that the cancer predisposition observed in A-T patients might be exacerbated by the absence of this escape mechanism.

Interestingly, the downregulation of DNA repair, specifically BER, under conditions of increased DNA damage has previously been observed in another cellular context. Higo and colleagues reported the decreased expression of SSB

repair genes including *Xrcc1* and *Lig3* in the cardiomyocytes of mice with heart failure, despite the presence of ROS and accumulation of DNA damage. In this case, the authors propose a model whereby the downregulation of DNA repair in these post-mitotic cells is an inbuilt survival mechanism to preserve organ function, at the expense of DNA damage accumulation (Higo et al., 2017). This study is in line with our hypothesis that decreased DNA repair can be a pro-survival mechanism within organisms.

In addition to this, the existence of a mechanism involving the downregulation of DNA repair in order to promote cell killing has recently been proposed (Ponath and Kaina, 2017). Ponath and Kaina suggested that monocytes, which express low levels of BER components, could be eliminated by their differentiated counterpart macrophages when these cells generate a ROS burst during the inflammatory response. In this case, the mechanism is proposed to protect healthy cells from excessive damage from ROS by limiting the numbers of macrophages in tissues (Ponath and Kaina, 2017). Importantly, this study provides evidence that organisms can deliberately exceed the repair capacity in subpopulations of cells in order to promote their elimination. This supports the idea that downregulation of DNA repair can be used to help maintain healthy cell populations.

7.6 Overall summary

The findings in this thesis, as outlined in the model described in Figure 7.1, bring together the following observations:

a) *ATM is a sensor for endogenous DNA damage*. A growing body of evidence has shown that ATM responds to ROS as well as DNA strand breaks of endogenous origin, phosphorylating a number of downstream targets (Guo et al., 2010b, Chou et al., 2015, Khoronenkova and Dianov, 2015, Higo et al., 2017);

b) *Sp1 is phosphorylated in response to DNA damage*. Specifically, previous studies identified Ser101 as a DNA damage-responsive modification site on Sp1, potentially phosphorylated in an ATM-dependent manner (Olofsson et al., 2007, Iwahori et al., 2008, Hau et al., 2015). The functional significance of this modification, however, had never been fully explored; we propose that persistent Ser101 phosphorylation promotes Sp1 degradation.

c) *Sp1 can modulate BER levels*. A number of studies have observed a link between Sp1 activity and modulation of BER gene expression (Zaky et al., 2008, Bocangel et al., 2009, Antoniali et al., 2015, Poletto et al., 2016). We report that Sp1 is important for regulating central scaffold protein XRCC1.

d) *BER is downregulated in response to persistent unrepaired DNA strand breaks.* We previously observed a correlation between BER components and the DNA damage load but the signal modulating this was unknown (Poletto et al., 2016). We now provide evidence of a mechanism linking BER expression with the load of DNA damage through ATM-dependent modulation of Sp1 stability. Furthermore, loss of Sp1 leads to accumulation of endogenous DNA damage.

e) *Loss of Sp1 primes cells to elimination.* Sp1 has been shown to regulate the expression of many different genes including cellular components involved in apoptosis (Grande et al., 2012, Hirose and Horvitz, 2013, Li et al., 2014). We show that lack of Sp1 upregulates pro-apoptotic genes and renders cells susceptible to pro-apoptotic agents or elimination by innate immune cells.

We believe that these findings form part of a physiological mechanism operating to promote organism survival by ensuring the removal of dangerous cells that are accumulating DNA damage. It also demonstrates that BER capacity can be directly affected by the cellular load of DNA damage.

7.7 Future work

There are a number of areas in this study that warrant further investigation.

Firstly, it would be interesting to investigate the mechanism that leads to Sp1 degradation in finer detail. Specifically, it would be beneficial to understand how Ser101 phosphorylation promotes Sp1 degradation. Given the importance of post translational modifications in modulating Sp1 stability, it is tempting to speculate that Ser101 phosphorylation might stimulate destabilisation by promoting other post translational modifications such as ubiquitylation or SUMOylation. Previous evidence suggests that Sp1 ubiquitin-mediated degradation might be mediated by phosphorylation at its C-terminus, for example (Wei et al., 2009). This is consistent with the idea that Ser101 phosphorylation could act as a priming site for further Sp1 modification. It remains to be determined, however, whether any Sp1 residues can be ubiquitylated. Degradation mediated through SUMOylation is also worth consideration. Several studies have already linked SUMOylation at K16 with Sp1 destabilisation (Spengler and Brattain, 2006, Chuang et al., 2008, Wang et al., 2011). Therefore, it would be interesting to establish if Ser101 phosphorylation can

stimulate Sp1 degradation through SUMOylation. In addition to the post translational modification, identification of the E3 ligase complex that might modify Sp1 after DNA damage would also further our understanding of the mechanism behind Sp1 degradation. For instance, the β-TrCP component of SCF ubiquitin ligase complexes has previously been shown to interact with Sp1 (Wei et al., 2009). Furthermore, the SUMO-dependent Sp1 destabilisation described above is reported to require Sp1 interaction with Ring finger protein 4 (RNF4) (Wang et al., 2011). These considerations suggest the need to further study whether Ser101 phosphorylation can affect Sp1 SUMOylation or ubiquitylation, and whether β-TrCP or RNF4 might be important for Sp1 degradation after persistent DNA damage.

It would also be interesting to understand the molecular mechanism that leads to NK-mediated elimination of fibroblasts lacking Sp1. We hypothesised that the increased recognition by NK cells is mediated by ATM-dependent upregulation of NKG2D ligands. Firstly, it would be interesting to establish which NKG2D ligands become upregulated in these cells. Well-studied NKG2D ligands include members of the MHC class I-like proteins family such as MICA, MICB, ULPBP1-6, which are usually present at low or undetectable levels in unstressed cells (Cerboni et al., 2014). Interestingly, transcriptional regulation of some of these ligands has been reported to be dependent on general transcription factors including Sp1. In fact, analysis of the MICA promoter identified putative Sp1 binding sites (Venkataraman et al., 2007). Secondly, in support of the role for ATM in this mechanism, it would be of interest to establish if expression of any NKG2D ligand is dependent on ATM in our cellular context.

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9 Appendix I

9.1 Protein expression and purification

To overexpress full-length recombinant Sp1 protein, a pET-28a plasmid expressing $His_{(6)}$-tagged recombinant Sp1 was generated as described in section 2.24.2. Recombinant Sp1 S101A was obtained through site-directed mutagenesis of the wild-type pET28a-Sp1 plasmid using primers Sp1S101A_F and Sp1S101A_R. Sp1 protein expression was carried out in Rosetta™ *E. coli* cells. Protein expression was induced with 1 mM Isopropyl β-D-1-thiogalactopyranoside (IPTG) at OD_{600} 0.8-1 for 3 h at 30°C. As overexpressed recombinant Sp1 was poorly soluble, purification of the protein had to be carefully optimised (data not shown). Preparative purification was carried out under denaturing conditions, followed by refolding of the protein. Recombinant Sp1 was purified by Fast Protein Liquid Chromatography (FPLC) using an ÄKTA FPLC (Amersham Biosciences) equipped with a HisTrap™HP column (GE Healthcare). All stages of purification were carried out in the presence of 1 mM β-Mercaptoethanol and 1 mM phenylmethylsulfonyl fluoride (PMSF). After protein induction cells were harvested by centrifugation and lysed in 6 M guanidine hydrochloride, 20 mM sodium phosphate pH 7.4, 500 mM NaCl, and protease inhibitors (aprotinin, chymostatin, leupeptin and pepstatin, all 1 µg/mL). Sp1 protein was eluted over an imidazole gradient in elution buffer containing 6 M guanidine hydrochloride, 20 mM sodium phosphate pH 7.4, 500 mM NaCl, 500 mM imidazole. (Figure 9.1 and Figure 9.2) Recombinant Sp1 was then refolded over 96 h by sequential dialysis against 10 mM Tris-HCl pH 7.5, 200 mM NaCl, 50 µM $ZnSO_4$, 0.4 M L-Arginine, 5% glycerol for the first 48 h, followed by 10 mM

Tris-HCl pH 7.5, 200 mM NaCl, 5% glycerol for a further 48 h. This protocol yielded a substantially enriched recombinant protein preparation, as assessed by Coomassie staining (Figure 9.3, panels A and B). Despite several attempts, however, the recombinant protein could not be further purified as Sp1 was extremely unstable. Given that both the wild-type and mutant forms of Sp1 gave consistently similar banding patterns and purification profiles (Figure 9.1, panel A; Figure 9.2, panel A; Figure 9.3, panels A and B), we decided to use these enriched protein fractions for the downstream *in vitro* assays. After dialysis, protein concentration was quantified using a Nanodrop2000. Predicted molecular weight and extinction coefficient were estimated using the ProtParam tool from *Expasy.org*. Recombinant Sp1 was stored in 10 mM Tris-HCl pH 7.5, 200 mM NaCl, 10% glycerol.

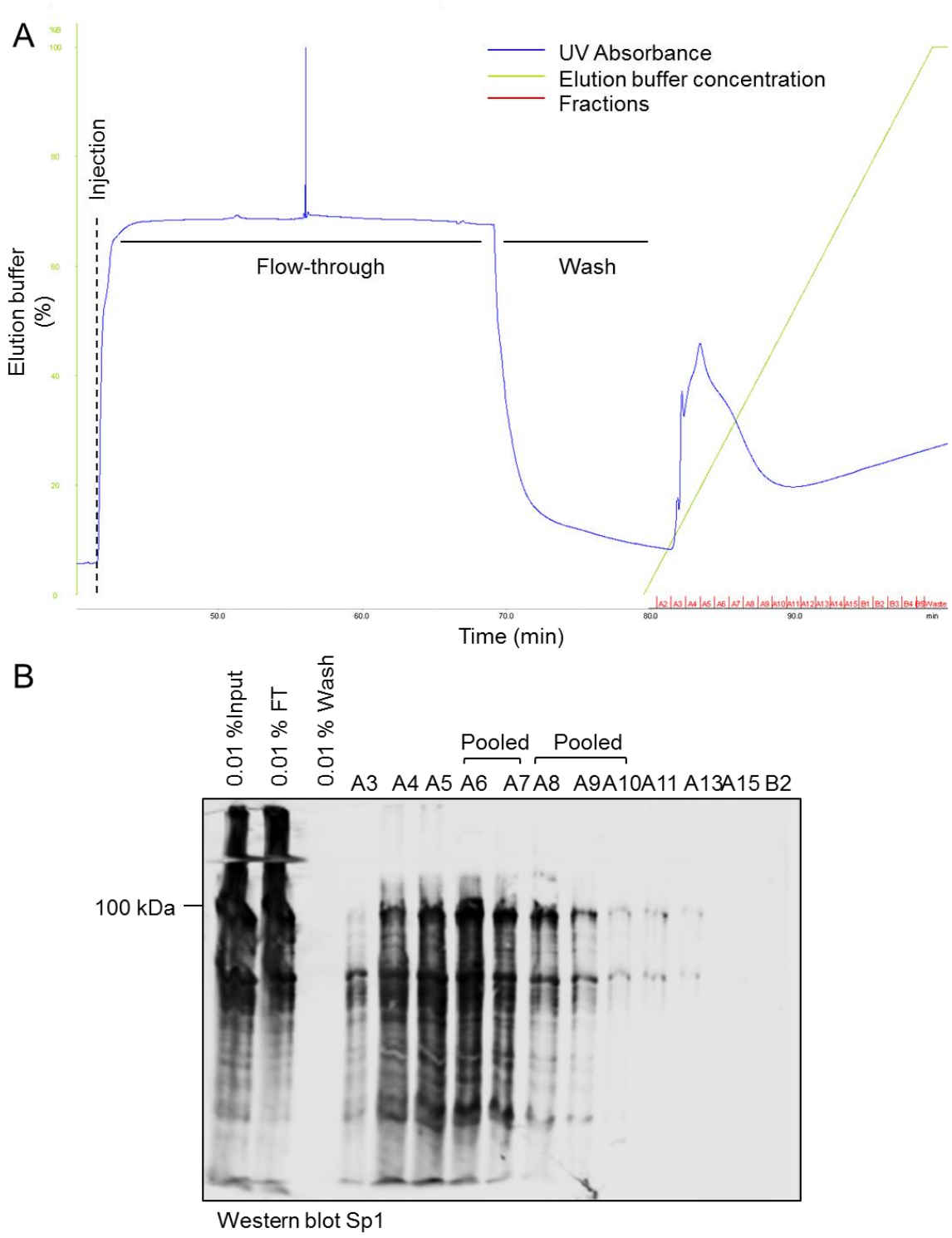

Figure 9.1 Wild-type Sp1 protein purification

(A) Chromatogram from HisTrap™ HP column. Protein was followed during the purification by measuring UV absorbance at 280 nm (blue line). After injection, flow-through equal to the volume of loaded cell lysate was collected, followed by a ten column volume washing step, which was also collected. Sp1 was then eluted from the column using an increasing concentration of imidazole-containing elution buffer (green line). **(B)** Western blot showing the fractions of interest from the purification. Each lane represents 0.2% of each total fraction. Fractions A6 and A7 in addition to fractions A8, A9 and A10 were pooled separately for dialysis. FT: flow through.

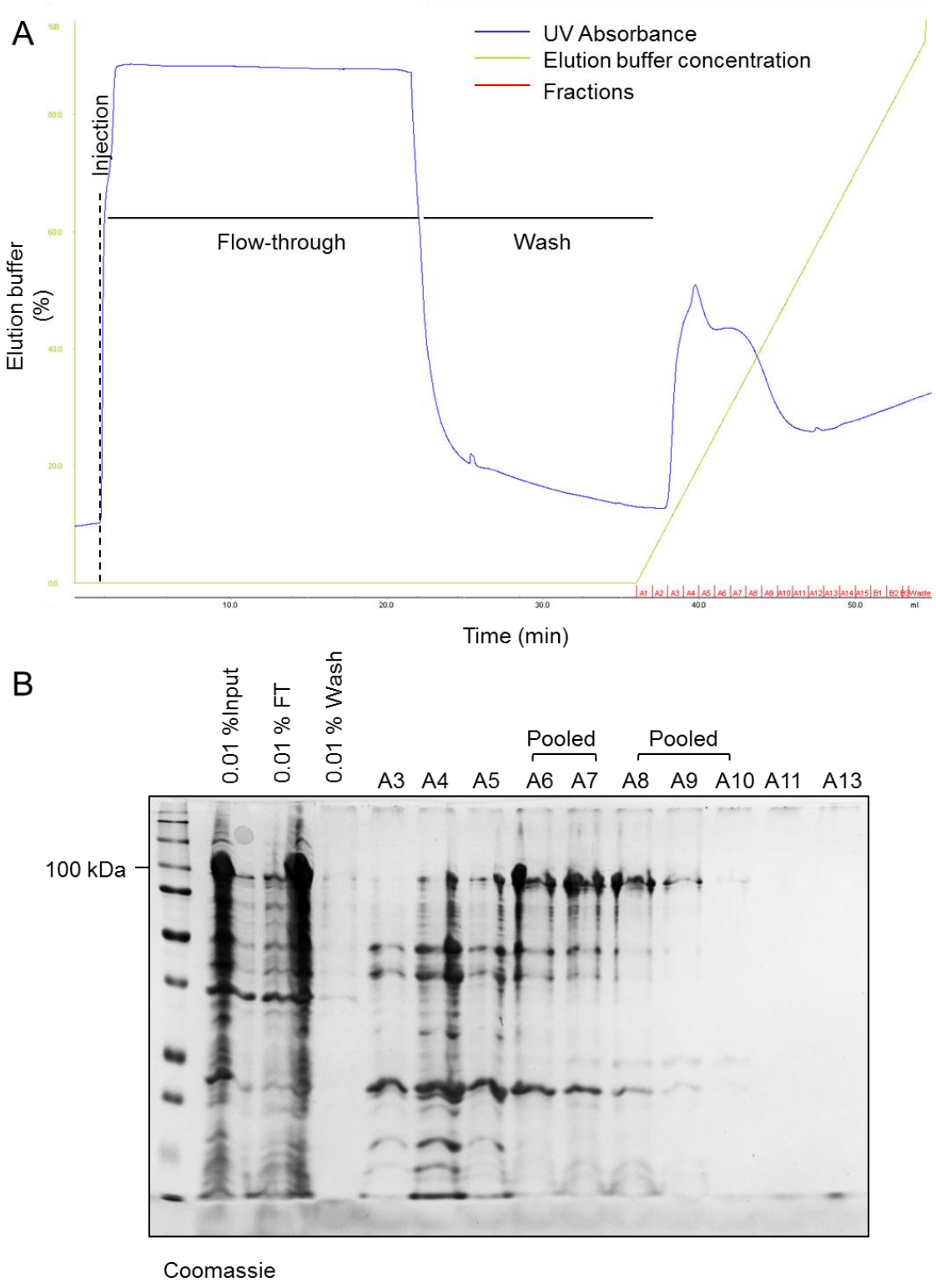

Figure 9.2 Sp1 S101A protein purification

(A) Chromatogram from HisTrap™ HP column. Protein was followed during the purification by measuring UV absorbance at 280 nm (blue line). After injection, flow-through equal to the volume of loaded cell lysate was collected, followed by a ten column volume washing step, which was also collected. Sp1 S101A was then eluted from the column using an increasing concentration of imidazole-containing elution buffer (green line). **(B)** Coomassie showing the fractions of interest from the purification. Each lane represents 0.2% of each total fraction. Fractions A6 and A7 in addition to fractions A8, A9 and A10 were pooled separately for dialysis. FT: flow through.

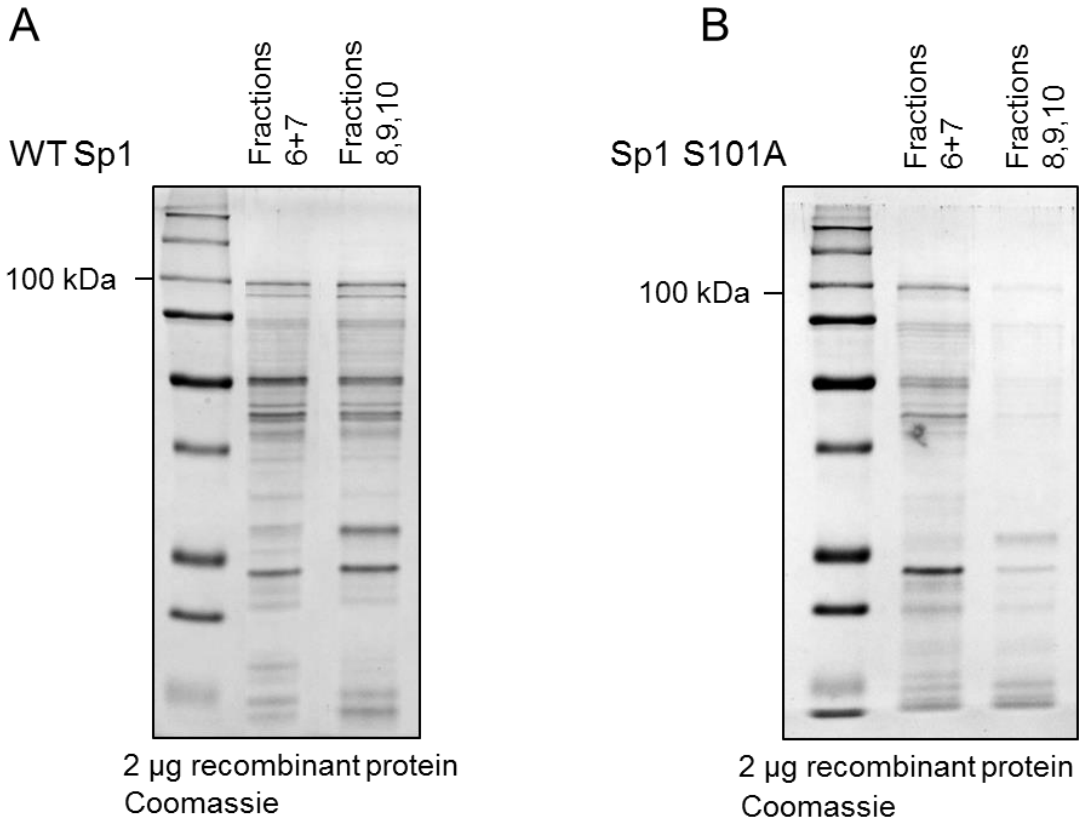

Figure 9.3 Wild-type and S101A Sp1 protein after dialysis

(A) Coomassie staining of wild-type Sp1 fractions. (B) Coomassie staining of S101A Sp1 fractions**.**

9.2 pGL3-XRCC1

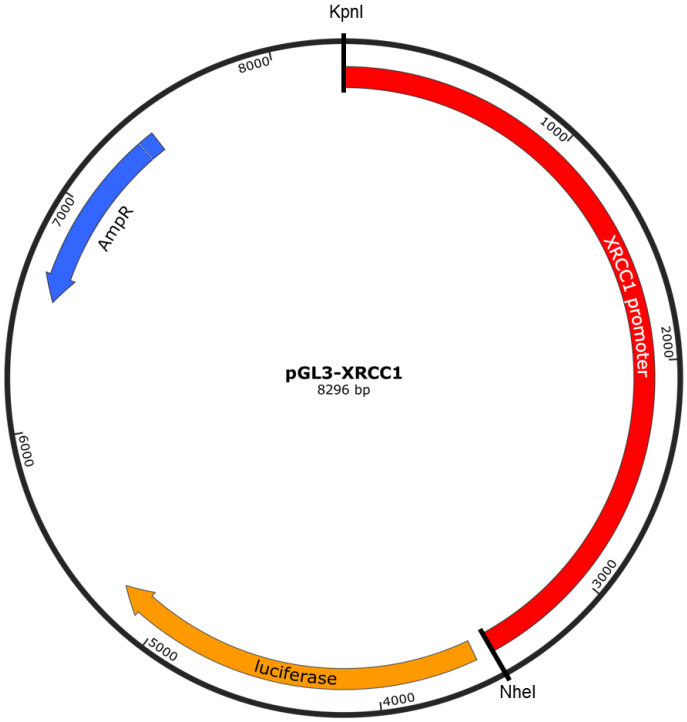

Figure 9.4 Plasmid map of *pGL3-XRCC1*

A 3.6 kb portion of the *XRCC1* promoter (red) is cloned upstream of the luciferase gene (orange) using KpnI and NheI sites present in the multiple cloning site. The vector has ampicillin resistance (blue).

9.3 ChIP optimisation

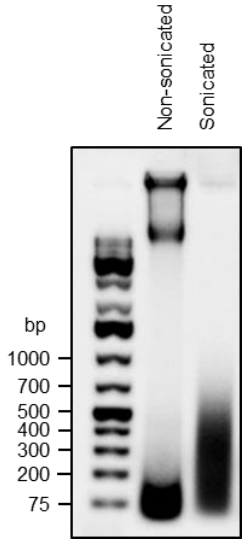

Figure 9.5 Validation of sonication efficiency in ChIP experiments

Chromatin was sheared using a Bioruptor® (Diagenode).The average size of sonicated DNA fragments is approximately 200-400 bp.

9.4 EMSA reaction scheme

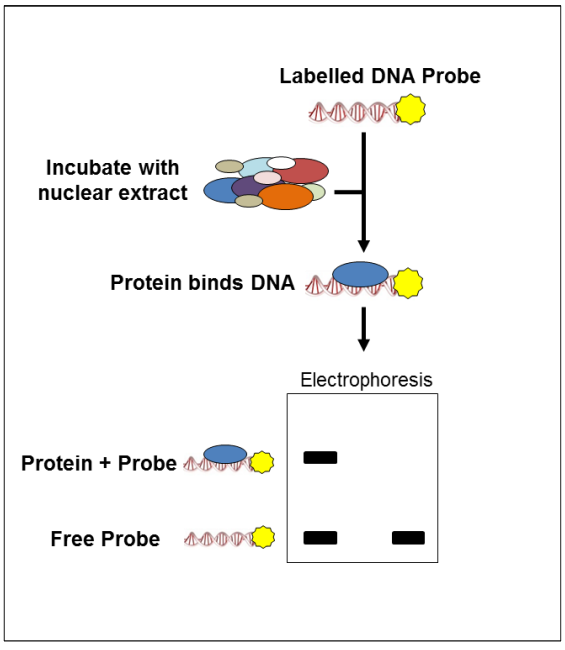

Figure 9.6 EMSA reaction scheme

Fluorescently labelled DNA probe is incubated with nuclear extract, allowing proteins to bind to probe. The reaction is then separated by gel electrophoresis; protein:DNA complexes migrate more slowly than the free linear unbound probe leading to detection of a shift.

9.5 Reactivation assay optimisation

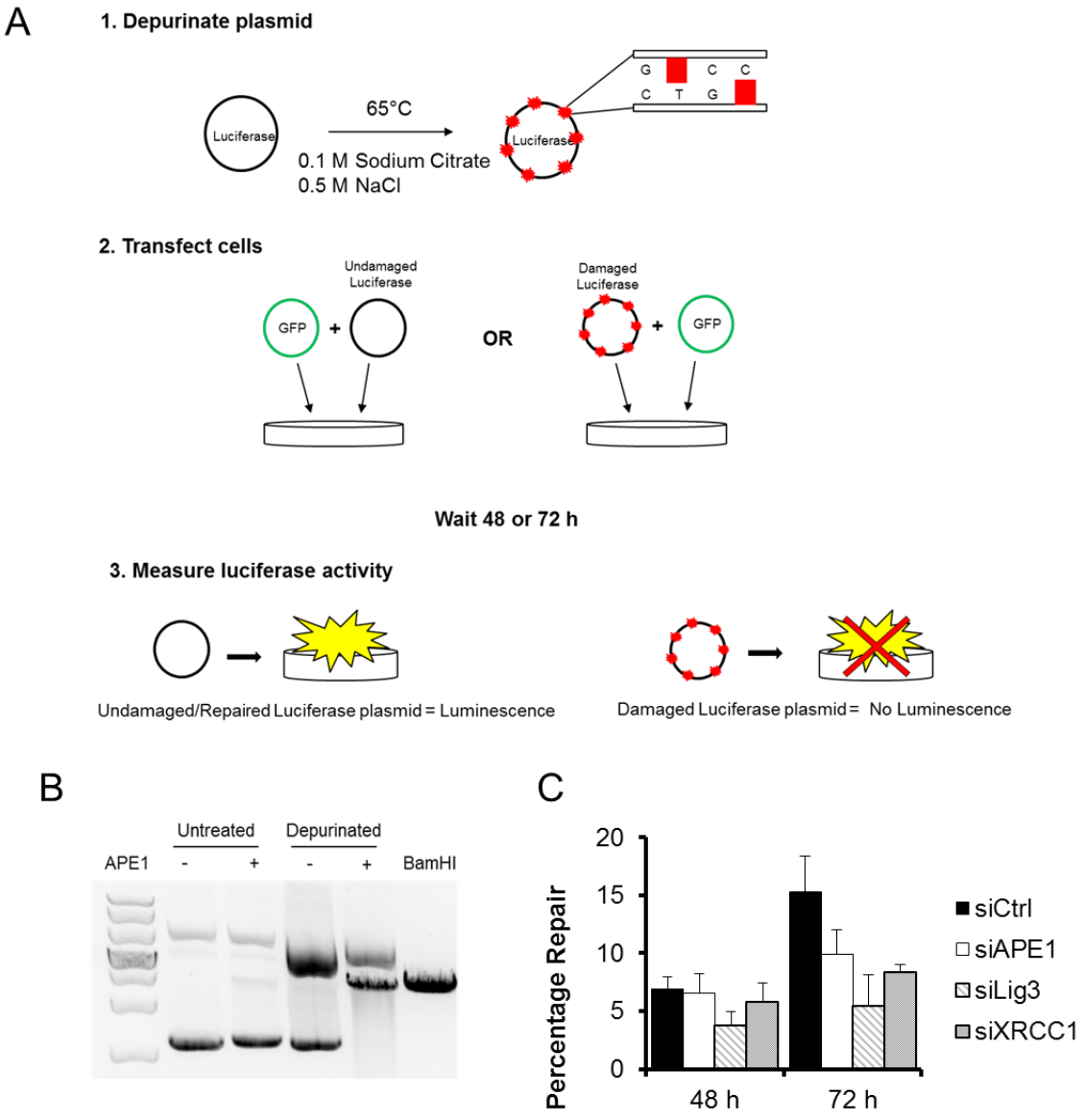

Figure 9.7 Reactivation assays.

(A) Reaction scheme for reactivation assays. pCMV-RL was depurinated as described in section 2.21.1. TIG-1 cells were transfected with either damaged or undamaged pCMV-RL in combination with pEGFP-N1 (encoding for GFP, to correct for bias in transfection efficiency). After 48 or 72 h, luciferase activity was measured. Detection of luciferase activity indicates repair of damaged plasmid. **(B)** Agarose gel analysis of heat-depurinated pCMV-RL reporter plasmid. Digestion of the damaged plasmid with recombinant APE1 results in the conversion of the plasmid into nicked and linear DNA, confirming the presence of AP sites. A BamHI-linearised plasmid was used as a marker to determine the migration of the linear vector. **(C)** Positive controls for reporter assay show that repair of heat-depurinated plasmid is impaired in TIG-1 cells after siRNA-mediated knock down of BER components. Percentage repair at 72 h is lower in APE1, Lig III or XRCC1 depleted cells, compared to siCtrl-treated cells. Histogram shows mean luciferase activity of damaged luciferase plasmid (relative to intact luciferase plasmid). The histogram reports mean ± SD of four technical replicates.

10 Appendix II

10.1 Primers

Table 10.1 Primers for qPCR

PRIMER	PRIMER SEQUENCE (5' TO 3')
APE1	For: CGGACAAGGAAGGGTACAGT
	Rev: CAAATTCAGCCACAATCACC
B2M	For: ATGTCTCGCTCCGTGGCCTTA
	Rev: ATCTTGGGCTGTGACAAAGTC
BAX	For: AGCTTCTTGGTGGACGCAT
	Rev: CAGAGGCGGGGTTTCATC
FOXM1	For: GGAGCAGCGACAGGTTAAGG
	Rev: GTTGATGGCGAATTGTATCATGG
GAPDH	For: AGCCACATCGCTCAGACAC
	Rev: GCCCAATACGACCAAATCC
Ku70	For: GTTGATGCCTCCAAGGCTATG
	Rev: CCCCTTAAACTGGTCAAGCTCTA
Ku80	For: TCCAAAAACTGTTCGATGTGA
	Rev: TGGAAGTGTGAATCCTGCTG
Ligase IV	For: TTTCTTCATCTTTGGGGCAG
	Rev: GAAGGCATCTGGTAAGCTCG
Pol β	For: AGTACACCATCCGTCCCTTG
	Rev: AAAGATGTCTTTTTCACTATCCACTG
PUMA (*BBC3*)	For: GTAAGGGCAGGAGTCCCAT
	Rev: GACGACCTCAACGCACAGTA
Rad51	For: TATCCAGGACATCACTGCCA
	Rev: GGTGAAGGAAAGGCCATGTA
SF3B2	For: CTGGCAACACAGTGAAGAGC
	Rev: GGAATGGAGACCCCTGAACT
TDP-1	For: ATGGTGATAAGCGAGAGGCT
	Rev: CGGAGGCCTTCTTCATAGAG
XRCC1	For: CTGGGACCGGGTCAAAAT
	Rev: CAAGCCAAAGGGGGAGTC
XRCC4	For: TGCTCCTTTTTCGACGTCTC
	Rev: TGCAAAGAAATCTTGGGACAG
APEX1 NegCtrl (ChIP)	For: GTGGCAGAGGTAAGGTGTTG
	Rev: CATCACCGAGCTGTTATCCTAG
DHFR (ChIP)	For: TCGCCTGCACAAATAGGGAC
	Rev: AGAACGCGCGGTCAAGTTT
XRCC1_1 (ChIP)	For: AGGGTTCGGACGTCATT
	Rev: CTCGGGCCTTTCAAACC
XRCC1_2 (ChIP)	For: TGGGAAGGCAGAACCAGAATC
	Rev: GGGAAGGTGAGAAAACAGGGTG

Table 10.2 Primers for cloning

PRIMER	PRIMER SEQUENCE (5' TO 3')	FUNCTION
Sp1_S101A_F	GCCACACAACTTGCACAGGGTGCCAATGGC	Introducing S101A mutation into pET28a-Sp1
Sp1_S101A_R	GCCATTGGCACCCTGTGCAAGTTGTGTGGC	
KpnI_XRCC1_F	ATTGGTACCATGATGGTCTTTCCCTCTTTCC	Cloning *XRCC1* promoter into pGL3 vector
NheI_XRCC1_R	ACGGCTAGCCTAATTCCCTCACGTCTTCCAAC	
EcoRI_Sp1_F	GCATCGAATTCATGAGCGACCAAGATCACTC	Sub-cloning Sp1cDNA into pET28a-Sp1
EcoRI_Sp1_R	CGTAGGAATTCTCAGAAGCCATTGCCACTG	

10.2 siRNA sequences

Table 10.3 siRNA sequences

TARGET	SEQUENCE (5' TO 3')
APE1	AGAAAGGUUUGGAUUGGGU
ATR	AACCUCCGUGAUGUUGCUUGA
Chk1	GGCUUGGCAACAGUAUUUCGGUAUA
Chk2	AUUGCACUGUCACUAAGCA
DNA-PKcs	CUUUAUGGUGGCCAUGGAG
E2F1	CGCUAUGAGACCUCACUGA
FEN1	GUUCUCUGAGGAGCGAAUC
FOXM1	GCCAAUCGUUCUCUGACAGAA
Ku80	GAAGUUCUGUCACAGCUGAUU
LigIII	AACUGCAACCCAGAUGAUAUG
PNKP	CACACUGUAUUUGGUCAAU
Sp1 #1	AACAGCGUUUCUGCAGCUACC
Sp1 #2	GGUAGCUCUAAGUUUUGAU
Sp1 #3	UAUUUGACCAGAACCAUCC
TDP-1	CUAGACAGUUUCAAAGUG
XPG	GGGAAGAUCCUGGCUGUUG
XRCC1	AGGGAAGAGGAAGUUGGAU

10.3 Antibodies

Table 10.4 Primary Antibodies

TARGET	ANTIBODY
53BP1	A300-272A – Bethyl Laboratories
Actin	ab6276 – Abcam
APE1	NB100-101 – Novus Biologicals
ATM	A1106 – Sigma
ATR	ab2905 – Abcam
BAX	#5023 – Cell Signaling Technology
CHK1	sc-8408 – Santa Cruz
CHK2	05-649 – Millipore
Cleaved caspase 3 (Asp 175)	#9661 – Cell Signaling Technology
Control Rabbit IgG	sc-2027 – Santa Cruz
DNA-PKcs	sc-9051 – Santa Cruz
E2F1	sc-193 – Santa Cruz
FEN1	Made in house
Ku80	ab3715 – Abcam
LigIII	GTX70145 – Genetex
p21	#2941 – Cell Signaling Technology
p53	sc-126 – Santa Cruz
pATM (Ser1981)	ab81292 – Abcam
PNKP	Kind gift from Prof Michael Weinfeld (University of Alberta)
pS/pT QG	#6966 – Cell Signaling Technology
pSp1 (Ser101)	39757 – Active Motif
Sp1	07-645 – Millipore
Ubiquitin	sc-9133 – Santa Cruz
XPG	HPA050374 - Sigma
XRCC1	MS-1393-P0 Thermo Fisher Scientific
α-Tubulin	T6199 – Sigma
γH2AX	05-636 – Millipore

Table 10.5 Secondary Antibodies

SECONDARY ANTIBODY	SOURCE
AlexaFluor 680 Mouse	A21057 – Thermo Scientific
AlexaFluor 680 Rabbit	A21109 – Thermo Scientific
IR Dye 800 Mouse	926-32210 – Li-Cor Biosciences
IR Dye 800 Rabbit	926-32211 – Li-Cor Biosciences
AlexaFluor 488 Rabbit	A32731 – Thermo Scientific

11 Appendix III Publications

During my DPhil, my work has contributed to the following publications:

FLETCHER, S. C., GROU, C. P., LEGRAND, A. J., CHEN, X., SODERSTROM, K., POLETTO, M. & DIANOV, G. L. 2018. Sp1 phosphorylation by ATM downregulates BER and promotes cell elimination in response to persistent DNA damage. *Nucleic Acids Res*, 46, 1834-1846.

POLETTO, M., YANG, D., **FLETCHER, S. C**., VENDRELL, I., FISCHER, R., LEGRAND, A. J. & DIANOV, G. L. 2017. Modulation of proteostasis counteracts oxidative stress and affects DNA base excision repair capacity in ATM-deficient cells. *Nucleic Acids Res,* 45, 10042-10055.

POLETTO, M., LEGRAND, A. J., **FLETCHER, S. C**. & DIANOV, G. L. 2016. p53 coordinates base excision repair to prevent genomic instability. *Nucleic Acids Res,* 44**,** 3165-75.