The Role of Ubiquitylation in Regulating Apurinic/Apyrimidinic Endonuclease 1

A thesis submitted for the degree of Doctor of Philosophy

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ABSTRACT

Apurinic/apyrimidinic endonuclease 1 (APE1) is a key DNA repair factor involved in the DNA base excision repair (BER) pathway that is required for the maintenance of genome stability. In this pathway, APE1 cleaves DNA at an abasic site to generate a DNA single strand break, allowing for repair completion by a DNA polymerase and a DNA ligase. High levels of APE1 have been observed in multiple cancer types however it is not understood if this contributes to cancer onset and development. What is known is that these cancers tend to display increased resistance to DNA damaging treatments and APE1 is therefore considered a key target for inhibition in the treatment of APE1-overexpressing cancers. Considering the relevance of modulating APE1 levels in disease and cancer treatment, very little is known about how cellular APE1 levels are regulated. Our lab has previously shown that the levels of the BER factors Pol β, XRCC1 and DNA Lig IIIα are regulated by ubiquitylation-mediated proteasomal degradation. The aim of this doctoral thesis was therefore to determine if ubiquitylation also regulates APE1 stability in cells. I present evidence that APE1 is ubiquitylated in cells and have identified the UBR3 E3 ligase that is responsible for this activity. Using mouse embryonic fibroblasts generated from *Ubr3* knockout mice, I demonstrate that UBR3 regulates APE1 cellular levels. I furthermore show that a loss of cellular UBR3 leads to the formation of DNA double strand breaks and genome instability.

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ABBREVIATIONS

(6-4) PP	Pyrimidine-pyrimidone (6-4) photoproducts
•OH	Hydroxyl radical
5'- AMP	5'- adenosine monophosphate
5'- dRP	5'- deoxyribosephosphate
8-oxoG	7,8-dihydro-8-oxoguanine
AD	Alzheimer's disease
ALS	Amyotrophic lateral sclerosis
AOA1	Ataxia-oculomotor apraxia 1
AP site	Abasic site, apurinic/apyrimidinic sites
AP-1	Activator protein 1
APE1	Apurinic/Apyrimidinic endonuclease 1
APEX1	DNA- (apurinic or apyrimidinic site) lyase
APTX	Aprataxin
ARF-BP1	Alternate reading frame binding protein 1
ATM	Ataxia telangiectasia mutated
ATR	Ataxia telangiectasia and Rad3 related
BARD1	BRCA1 associated RING domain 1
BER	Base excision repair
BRCA1	Breast cancer 1
BRCA2	Breast cancer 2
BSA	Bovine serum albumin
cAMP	Cyclic adenosine monophosphate
CDK5	Cyclin-dependent kinase 5
CHIP	Carboxy terminus of Hsc70-interacting protein
CKII	Casein kinase II
CPD	Cyclobutane pyrimidine dimers

CREB	cAMP response element-binding
CT	Cycle threshold
CtIP	C-terminal binding protein-interacting protein
DAPI	4’,6-diamidino-2-phenylindole
DMEM	Dulbecco’s modified eagle medium
DNA	Deoxyribonuleic acid
DNA DSB	DNA double strand break
DNA SSB	DNA single strand break
DNALigIV	DNA ligase IV
DNA-PKcs	DNA-dependent protein kinase, catalytic subunit
dNTP	Deoxyribonucleotide triphosphate
DUB	Deubiquitylation enzyme
EDTA	Ethylenediaminetetraacetic acid
ERCC1	Excision repair cross-complementing rodent repair deficiency, complementation group 1
Exo1	Exonuclease 1
ExoA	Exonuclease A
ExoIII	Exonuclease III
FA	Fanconi anemia
FAAP24	Fanconi anemia associated protein of 24 kDa
FAN1	Fanconi associated nuclease 1
FANC	Fanconi anemia complementation group
FEN1	Flap endonuclease 1
FITC	Fluorescein isothiocyanate
FOXO4	Transcription factor
H2A	Histone H2A
H2AX	Histone H2A family member X
H_2O	Water
H_2O_2	Dihydrogen peroxide
HAP-1	Human apurinic/apyrimidinic endonuclease 1

HD	Huntington’s disease
HDAC	Histone deacetylase
HECT	Homology to E6-AP C-terminus
HIF-1α	Hypoxia inducible factor 1 alpha
HNPCC	Hereditary non-polyposis colorectal carcinoma
hnRNP-L	Heterogeneous nuclear ribonucleoprotein L
HR	Homologous recombination
HRE	Hypoxic response element
ICL	Interstrand cross-links
IPTG	Isopropyl β-D-1-thiogalactopyranoside
kDa	Kilodalton
LB	Lysogeny broth
Lig I	DNA ligase I
Lig IIIα	DNA ligase III alpha
MCSZ	Microcephaly with Seizures
MDM2	Murine double minute
MEF	Mouse embryonic fibroblast
MG132	Carbobenzoxy-Leu-Leu-leucinal (proteasome inhibitor)
MGMT	O6-methylguanine transferase
MI	Microsatellite instability
MMR	Mismatch repair pathway
MMS	Methyl methanesulfonate
mRNA	Messenger ribonucleic acid
MS	Multiple Sclerosis
Mule	Mcl-1 ubiquitin ligase
MUTYH	mutY homolog
NBS1	Nijmegen breakage syndrome 1
nCaRE	Negative calcium response elements
NEIL1	Nei endonuclease VIII-like 1
NEIL2	Nei endonuclease VIII-like 2

NEM	N-ethylmaleimide
NER	Nucleotide excision repair pathway
NF-κB	Nuclear factor kappa B
NHEJ	Non-homologous end joining
NPM1	Nucleophosmin
O_2	Oxygen
ODD	Oxygen dependent degron
OGG1	8-oxoguanine DNA glycosylase 1
$^{-}$OH	Hydroxide ion
p14-ARF	p14 alternate reading frame
p300	Histone acetyltransferase p300
p53	Protein 53
PARP1	Poly [ADP-ribose] polymerase 1
PBS	Phosphate-buffered saline
PCNA	Proliferating cell nuclear antigen
PCR	Polymerase chain reaction
PCV	Packed cell volume
PD	Parkinson’s disease
pI	Isoelectric point
PIP	PCNA interacting protein
PMSF	Phenyl methyl sulfonyl fluoride
PNKP	Polynucleotide kinase-phosphatase
Pol δ	Polymerase delta
Pol ε	Polymerase epsilon
Pol η	Polymerase eta
Pol ι	Polymerase iota
Pol κ	Polymerase kappa
Pol β	DNA polymerase beta
PTH	Parathyroid hormone
PVDF	Polyvinylidene difluoride

Ref-1	Redox enhancing factor 1
RFC	Replication factor C
RING	Really interesting new gene
RNA	Ribonucleic acid
ROS	Reactive oxygen species
RPA	Replication protein A
rpm	Revolutions per minute
RQ	Relative quantity
SCAN1	Spinocerebellar ataxia with axonal neuropathy-1
SDS	Sodium dodecyl sulfate
SIRT1	Sirtuin 1
Sp1	Specificity protein 1
SSBR	Single strand break repair
ssDNA	Single stranded DNA
TDG	Thymine DNA glycosylase
TDP1	Tyrosyl-DNA phosphodiesterase 1
TFIIH	Transcription factor IIH
TLS	Translesion synthesis pathway
TOP1	DNA topoisomerase 1
TSA	Trichostatin A
UbcH	Ubiquitin-conjugating enzyme
UBD	Ubiquitin binding domain
UBR3	Ubiquitin protein ligase E3 component n-recognin 3
UDG	Uracil DNA glycosylase
UPP	Ubiquitin proteasome pathway
USP	Ubiquitin specific protease
UV	Ultraviolet
WCE	Whole cell extract
XRCC1	X-ray cross complementing protein 1

Chapter 1

INTRODUCTION

1.1 GENOME STABILITY

A cell is the simplest unit of a self-sufficient living organism. To survive, it must respond appropriately to stimuli that arise both from its external environment and from within the cell. Such responses are primarily elicited through the interaction and activity of proteins that are present in cells. These interactions are highly regulated and are often grouped in to protein interaction networks or pathways that represent a single response. In reality, there is an immense amount of cross-talk between related pathways and the complexity of running a cell can be measured by the 20,000 or so proteins that are required to do so.

Cells have to produce their own proteins. A protein in its simplest form is linear, made up of repeating units of amino acids of which there are 20 that can be considered proteinogenic (standard amino acids). The information required by cells to reproducibly make proteins is stored in genes that are present in the form of deoxyribonucleic acid (DNA). The human genome project identified approximately 20,000-25,000 of such genes in human DNA (Consortium, 2004). As with proteins, DNA is linear and made up of repeating units known as bases, of which there are only four; cytosine, guanine, adenine and thymine. A single amino acid is coded for by three bases and this code is generally ‘read’ uninterrupted to produce a string of amino acids that forms the primary protein structure. Therefore, a permanent change in the sequence of the bases in a particular gene, known as a mutation, can affect the sequence of the amino acids of its protein product. Changes in the amino acid sequence of a protein can alter the proteins

structure, capacity for interaction with other proteins, activity and regulation. Depending on the role of the protein, this can diminish the cells ability to respond appropriately to certain stimuli and in some cases, multiple response pathways can be affected. Mutations arise primarily through damage that is inflicted on to DNA. It is imperative that the cell removes or repairs this damage before a mutation arises. Indeed there are more than 150 cellular proteins that are dedicated to the removal of damage on DNA (Wood et al., 2005).

Cancer is a key example of a disease caused primarily by changes in the DNA sequence. In cancer cells, key proteins of pathways that are involved in cellular proliferation and survival are dysfunctional due to abnormalities caused by mutations in their own genes or the genes of its regulators. Since cancer will affect 1:3 people throughout their lifetimes, there is a large research sector dedicated to understanding the key factors that lead to the onset and progression of cancer cells. An even larger number of studies are dedicated to improving the treatments for cancer. Currently, the leading non-invasive treatments, radiation therapy and chemotherapy, are based on their ability to severely damage DNA for triggering cancer cell death. The DNA repair field is consequently an important field of research for the study of both cancer development and cancer response to treatments. Additionally, manipulation of DNA repair is a major research area of interest for improving the effects of the current DNA damaging treatments for cancers.

Both the sources of DNA damage and the repair pathways responsible for their repair will be briefly discussed in this general introduction chapter. A few gaps in the current knowledge will also be highlighted at the end of the chapter to fully introduce the rationale behind the work presented in this doctoral thesis.

1.1.1 Deoxyribonucleic acid

Deoxyribonuleic acid, DNA, is a helical macromolecule that presents itself in the form of two hydrogen-bonded DNA polymers that run alongside each other in an anti-parallel fashion (Watson and Crick, 1953). The basic structure of each of these polymers lies upon a repetitive pentose sugar-phosphate backbone, for which each pentose sugar-phosphate unit supports one of the four nucleobases, adenine, guanine, cytosine or thymine. The nucleobases have a heterocyclic structure and are classified according to the number of rings within that structure; the purines, adenine and guanine, have two rings whereas the pyrimidines, cytosine and thymine, have only one ring **(Figure 1.1)**. Nucleobases, when bound to the first carbon of the pentose sugar, 2'-deoxyribose by means of a covalent glycosidic bond forms the respective 2'-deoxyribonucleoside. A 2'-deoxyribonucleoside containing one or more phosphates on the 5' position of the pentose sugar is known as a 2'-deoxyribonucleotide and is the single unit of the DNA polymer **(Figure 1.2)**.

A DNA polymer is created when a phosphodiester bond is generated between the 5'-phosphate group of the pentose sugar of one unit and the 3'-hydroxyl group of the pentose sugar of another unit. This bonding occurs only in the 5' to 3' direction and this has major implications not only for DNA synthesis, but also for its translation in to mRNA, the key messenger molecule that ultimately allows for the DNA code to be translated in to a protein form. The pentose sugar-phosphate backbone of a DNA polymer is highly negatively charged and subsequently hydrophilic, whereas the planar surfaces of the nucleobases are rather hydrophobic. It is therefore favourable at a cellular pH for the nucleobases of two DNA polymers to generate hydrogen bonds between each other to form duplex DNA. Due to the structure of the bases and the rigid supporting structure provided by the negative charge on the backbone, cytosine will hydrogen bond only to guanine (3 hydrogen bonds) and adenine will bind only to thymine (2 hydrogen bonds) as shown in **Figure 1.3**. This consistent complementation is

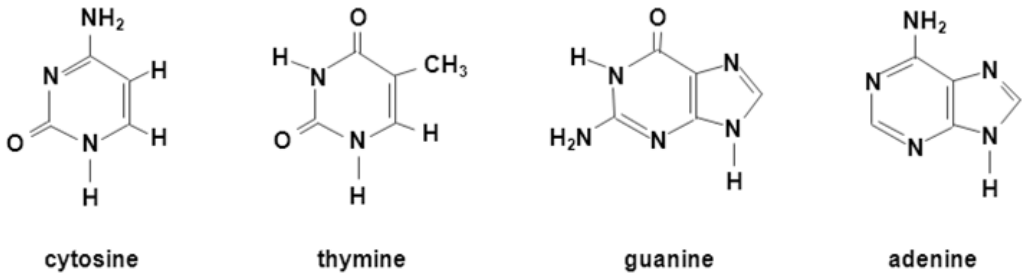

Figure 1.1: The chemical structure of the DNA bases cytosine, thymine, guanine and adenine. The pyrimidines, cytosine and thymine, possess a single ring structure whereas the structure of the purines, guanine and thymine, contain two rings.

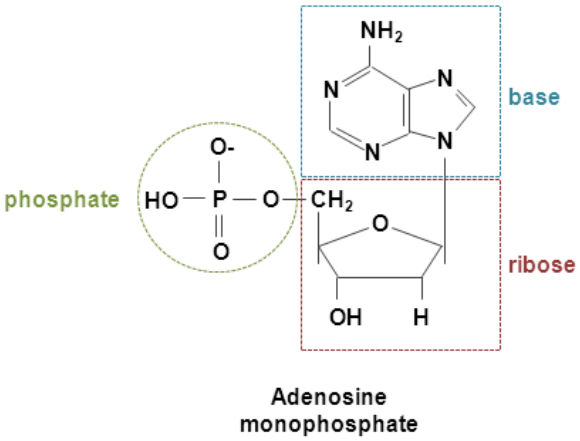

Figure 1.2: The chemical structure of adenosine monophosphate. The chemical structure of adenosine monophosphate. A glycosidic bond links N9 of the adenine base to C1 of the 2'-deoxyribose sugar. An ester bond links the phosphate group to C5 of the ribose structure.

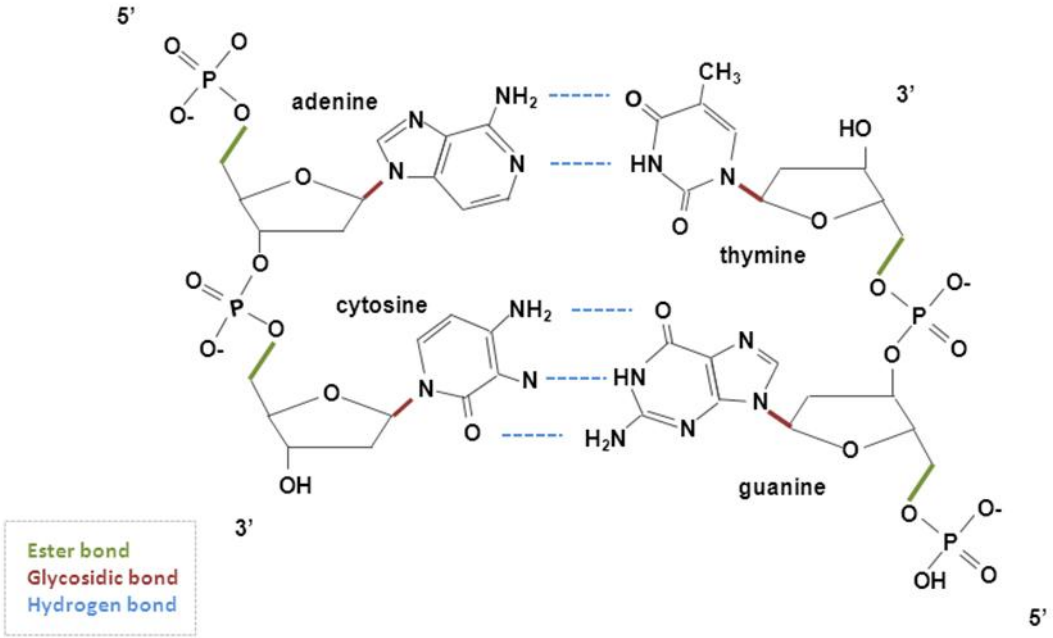

Figure 1.3: The chemical structure of duplex DNA. Two polymers (only two units each shown) are bound to each other through hydrogen bonding between the bases. Only two hydrogen bonds form between adenine and thymine whereas three hydrogen bonds form between cytosine and guanine. The DNA base is linked to the 2'-deoxyribose sugar through a glycosidic bond whereas two units of a single DNA polymer are joined through a phosphodiester bond.

precisely what allows for the preservation of the sequence throughout the replication of DNA, a process necessary for passing on genetic information to a daughter cell during cellular division. Though this hydrogen bonding between polymers provides a degree of stability for duplex DNA, the high stability of DNA is additionally due to the hydrophobic stacking interaction that occurs between the planar surfaces of the bases of the polymers. This stacking conformation, together with the negative charge provided by the DNA backbone ultimately allow for the iconic helical structure of duplex DNA.

In eukaryotic cells, DNA is stored in the form of a nucleoprotein known as chromatin (**Figure 1.4**). In chromatin, DNA is carefully wrapped around highly positively charged proteins known as histones. These histones further arrange themselves in to nucleosomes that allow for the formation of a 30 nm fibre of chromatin (Luger et al., 1997). The chromatin fibre bundles together around the nuclear matrix to produce chromosomes that have distinctive 'arm' structures. This allows for the tight packaging of DNA which has been shown to be important for regulating processes that occur on DNA such as transcription, DNA replication and DNA repair. Tight packaging of DNA furthermore reduces its exposed surface area, providing a physical level of protection from DNA damaging factors present in the cellular environment.

1.1.2 DNA damage

DNA is a highly stable molecule, an attribute that is imperative for preserving the genetic code throughout cellular and organismal generations. It is however necessary that DNA is also versatile, possessing a molecular structure that promotes chemical interactions and structural rearrangements to allow for vital cellular activities on DNA such as transcription, replication, recombination and repair. The ability of DNA to be modified as such however, makes it susceptible to undesirable chemical and physical attack at various points on DNA to include the bases, the deoxyribose sugar and the glycosidic and phosphodiester bonds. Indeed, a large array of lesions that threaten the

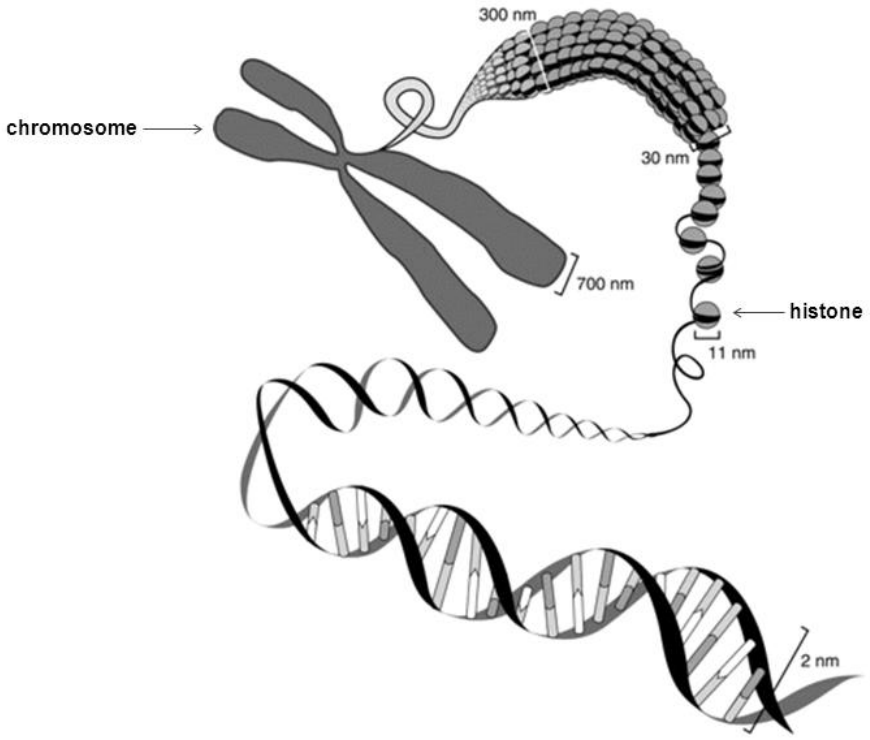

Figure 1.4: DNA packaging in to chromatin. DNA is wrapped around histone proteins and together arrange themselves in to a 30 nm fibre of chromatin. This chromatin fibre wraps itself around a nuclear matrix to form a 300 nm fibre that further 'bunches' in to the characteristic structure of chromosomes. Modified image taken from Weier, 2001.

integrity of the genetic code can be introduced on to DNA by both endogenous and exogenous sources of damage.

1.1.2.1 Endogenous damage

Endogenous damage encompasses lesions generated on DNA as a result of DNA interactants produced within the cell. Most of these endogenously generated lesions result from the interaction of DNA with water and oxygen from the cellular environment. Indeed there are many points on DNA that are susceptible to hydrolytic and oxidative attack. Errors in DNA synthesis, and in the cellular process that promotes tortional relaxation of DNA can also introduce damage on to DNA.

1.1.2.1.1 *Hydrolysis*

The cell utilises multiple enzymes that are capable of mediating controlled hydrolysis reactions on DNA. These enzymes include the exonucleases and endonucleases that hydrolyse the phosphodiester bond of DNA to generate a nick in the DNA backbone during proofreading and DNA repair processes. Uncontrolled hydrolysis of the phosphodiester bond produces a nick or single strand break in DNA. DNA single strand breaks can interfere with both transcription and replication processes on DNA**.** Furthermore, DNA single strand breaks that are not repaired efficiently allow for the formation of the toxic DNA double strand break at replication forks (Kuzminov, 2001). Unrepaired DNA double strand breaks can lead to gross chromosomal rearrangements that promote mutagenesis, as well as cell cycle arrest and cell death (Reviewed in Khanna and Jackson, 2001).

Random hydrolytic attack of the glycosidic bond linking the pentose sugar to guanine and adenine can result in the release of these bases from DNA (depurination). This leaves an abasic site (AP site) on DNA and it is estimated that approximately

10,000 apurinic sites are generated per cell per day (Lindahl and Nyberg, 1972). Hydrolytic cleavage of the glycosidic bond between the sugar and the pyrimidines (depyrimidination), cytosine and thymine, is much less common with only approximately 500 apyrimidinic sites being generated per cell per day (Lindahl and Karlstrom, 1973). If not repaired, an abasic site can result in problematic DNA synthesis by template error and ultimately mutagenesis (Guillet and Boiteux, 2002, Loeb et al., 1986, Yu et al., 2003).

Hydrolytic cleavage of the exocyclic -NH_2 group (deamination) of cytosine, adenine and guanine results in the formation of uracil, hypoxanthine and xanthine, respectively **(Figure 1.5).** Uracil preferentially base-pairs with adenine instead of guanine and hypoxanthine preferentially base-pairs with cytosine instead of thymine (Duncan and Miller, 1980). If not repaired in time, a single round of replication will allow for the preferred base insertion and a second round of replication will allow for a permanent and unrecognisable alteration to DNA, a mutation. Xanthine on the other hand has no binding preference and its presence in DNA can result in problematic DNA synthesis and replication arrest.

1.1.2.1.2 *Oxidation*

Most macromolecules in cells, including DNA, are subject to continuous chemical attack by endogenously generated reactive oxygen species (ROS) (Lindahl and Nyberg, 1972). ROS in a variety of forms are continuously produced in cells as a bi-product of normal cellular processes such as respiration and lipid peroxidation. Additionally ROS is generated upon alteration or deterioration of O_2, H_2O and H_2O_2 that is initiated by interactions with both endogenous and exogenous factors. For instance, the Fenton reaction uses Fe^{2+} to transform H_2O_2 that has low affinity for interaction on DNA into the highly reactive hydroxyl radical (•OH) and hydroxide ion ($^{-}$OH), both of which can interact with and damage DNA (Imlay et al., 1988).

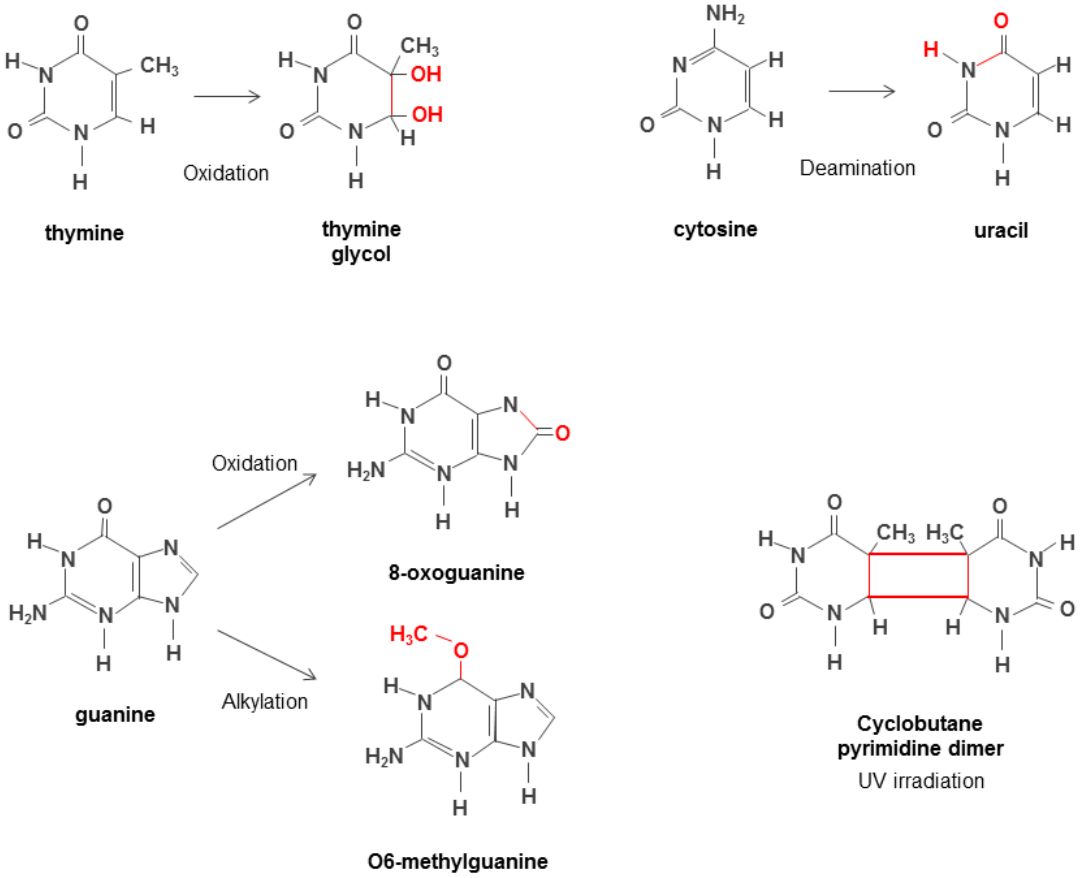

Figure 1.5: Typical base damages inflicted on to DNA bases. Oxidative damage to DNA can allow for the formation of thymine glycol that preferentially binds guanine instead of adenine and 8-oxoguanine that preferentially binds adenine instead of cytosine. Deamination of cytosine can allow for the formation of uracil that preferentially binds to adenine instead of guanine. Alkylation at the O6 position of guanine generates O6-methylguanine that is potentially mutagenic and cytotoxic. The most common lesion induced by UV irradiation is the cyclobutane pyrimidine dimer that is generated due to the formation of an obstructive link between two pyrimidines of the same DNA polymer.

The interaction of ROS with DNA is known to allow for the formation of at least 80 different types of DNA lesions (Reviewed in Dizdaroglu, 2012). These damages are mostly a result of hydrogen abstraction and double bond additions to either the nucleobases or the deoxyribose sugar of DNA. The 7,8-dihydro-8-oxoguanine (8-oxoG) lesion that forms as a result of saturation of the imidazole ring is one of the most common oxidative lesions in cells occurring at a rate of approximately 100 lesions per cell per day (Friedberg, 2006). It is the result of a double bond disruption between N7 and C8 of guanine and addition of oxygen to C8 **(Figure 1.5).** 8-oxoG preferentially binds adenine instead of cytosine and if not repaired efficiently introduces a transversion mutation in to DNA upon replication (Cheng et al., 1992).

1.1.2.1.3 *Errors in DNA synthesis*

During DNA synthesis, a DNA polymerase is responsible for the insertion of a new base opposite to the template strand. Though it is rare, a polymerase can introduce an incorrect base to form a base mismatch. The major replicative polymerases, Pol ε and Pol δ, consequently possess intrinsic 3' to 5' exonuclease activity that allows for the excision of the incorrectly inserted base (Reviewed in Lange et al., 2011). Unsurprisingly, loss of this exonuclease-mediated proofreading mechanism of polymerases through a loss-of-function mutation results in an increased rate of mutagenesis and cancer susceptibility in mice (Goldsby et al., 2002, Uchimura et al., 2009, Albertson et al., 2009). Though the majority of mismatches are recognised and removed immediately, some mismatches may evade removal or repair, thus increasing the probability of forming permanent mutations in DNA that is achieved at the point of DNA replication.

1.1.2.1.4 *Topoisomerases*

DNA topoisomerase 1 (TOP1) promotes the tortional relaxation of DNA and as such, eases the progression of processes such as transcription and DNA replication (Reviewed in Wang, 2002). TOP1 achieves this by nicking DNA to generate a DNA single strand break. TOP1 remains covalently bound to the DNA single strand break until the nick is resealed before dissociating from DNA with the help of its interaction partner, tyrosyl-DNA phosphodiesterase 1 (TDP1) (Yang et al., 1996). Collision of the TOP1-DNA complex with a replication fork or a transcription complex can result in the persistence of these DNA single strand breaks, and in a temporal manner, increases the likelihood for DNA double strand break formation and replication fork collapse.

1.1.2.2 Exogenous damage

There are various environmental and man-made exogenous sources of damage that either directly or indirectly affects the stability of DNA. In some cases, non-reactive exogenous chemicals such as some of those found in cigarette smoke are converted in to reactive agents that can go on to damage DNA.

1.1.2.2.1 *Alkylation*

Most alkylating agents are man-made and there are additionally some naturally occurring exogenous sources such as methyl chloride that is released in to the environment by algae. DNA alkylating agents can further be generated within the cell as a bi-product of processing both endogenous metabolites and exogenous chemicals.

Alkylating agents are electrophilic compounds that covalently attach reactive groups to the nucleophilic centres on macromolecules (Reviewed in Fu et al., 2012). They can either be classed as monofunctional that is capable of transferring only one reactive group to the target nucleophilic centre or bifunctional that is capable of

transferring two reactive groups. DNA possesses a multitude of sites for inappropriate alkylation to include both the cyclic and exocyclic nitrogens and to a lesser extent the oxygens of all the bases. Alkylations on DNA can have various effects from changes in base pairing preferences of the affected base to the loss of a base (depurination/depyrimidination) to leave an AP site on DNA. AP sites are obstructive during DNA synthesis, providing an opportunity for mutagenesis and stalling of the replication fork if not repaired efficiently (Guillet and Boiteux, 2002, Loeb et al., 1986, Yu et al., 2003).

Bifunctional alkylating agents further have the ability to form an interstrand cross-link between the two polynucleotide chains of duplex DNA (Reviewed in Fu et al., 2012). It does this by attacking two nucleophilic centers, one on each strand. This effectively covalently locks the two strands together, blocking processes such as transcription and replication that require separation of the two strands of duplex DNA. Due to the potential of alkylating agents to severely damage DNA at high concentrations, alkylating agents are currently used as chemotherapeutic agents in the treatment of some types of cancers. Some of these chemotherapy agents include mitomycin C, cisplatin, dacarbazine and procarbazine.

1.1.2.2.2 *Ionising radiation*

Ionising radiation involves the transfer of energy to a target molecule, thus allowing for a change in the energy state of that molecule. This energy is transferred in the form of electromagnetic waves or particles from various sources. Natural occurring sources include cosmic rays from space and radioactive material from the earth such as radon. Man-made sources of radiation come from radioisotopes that are used in nuclear fission reactions for the making of nuclear power, X-rays that are often used for medical examinations and particle accelerators that are used primarily for research in particle physics and for radiotherapy in the treatment of cancers. The mechanism for energy

transfer varies according to the source. Alpha and beta particles form physical radiation that tends to be fast moving protons, electrons and neutrons that have high energy levels but are only capable of transducing small distances. It is this type of radiation that is primarily used in radiotherapy for the treatment of cancers. Gamma rays on the other hand are a type of electromagnetic radiation that has lower energy but is capable of moving through even thick walls and is primarily a result of the radioactive decay of atomic nuclei.

Irradiation can generate damage on DNA both directly and indirectly and the extent and type of damage produced is dependent on both the source and dose of radiation. Direct absorption of energy by the bases and sugar is apparent however the majority of lesions are generated through indirect mechanisms whereby energy is transferred to the water, oxygen and other molecules surrounding DNA (Riley, 1994). This results in the formation of reactive species and excited molecules that are capable of introducing damage on to DNA to include oxidative lesions, AP sites, interstrand cross-links, DNA single strand breaks and DNA double strand breaks (Ward, 1988). The damages are often localised to a particular region on DNA and it is not uncommon that damages occur in clusters upon irradiation (Goodhead, 1994). Irradiation-induced DNA double strand breaks for instance are formed primarily through the close proximity of two single strand breaks within DNA. Though these DNA double strand breaks amount to less than 5% of the total amount of lesion types generated by irradiation, it is accountable for the majority of cell death observed in response to irradiation, a factor that is key to its use as a treatment for cancers (Ward, 2000, Ward, 1990). The diverse means by which irradiation inflicts damage on to DNA furthermore allows for the generation of a multitude of lesion types such as DNA termini containing a 3'-phosphate or 3'-phosphoglycolate (Bertoncini and Meneghini, 1995, Henner et al., 1982). Such groups interfere with repair and must be processed to leave a 3'-hydroxyl group that is required for repair completion.

1.1.2.2.3 *Ultraviolet radiation*

Ultraviolet radiation (UV) is a major source of DNA damage from the environment (Reviewed in Yang, 2011). UV-A, UV-B and UV-C rays are emitted from the sun, though the majority of these rays are excluded from the earth's atmosphere by the ozone. Damage inflicted by UV occurs primarily on the pyrimidine bases of DNA. Such lesions include cyclobutane pyrimidine dimers (CPD), pyrimidine-pyrimidone (6-4) photoproducts ((6-4) PP) and less commonly lesions such as cytosine hydrates and thymine glycols **(Figure 1.5)**. CPDs form when a 4-member ring structure results from the saturation of the C5, C6 double bonds of two pyrimidines that are adjacent to each other. The (6-4) PP lesion results from the interaction of the C6 of the 5' pyrimidine with the C4 of the 3' pyrimidine. This lesion is more likely to form between two cytosines whereas the CPDs tend to form between two thymines. Both lesions create distortions in the DNA structure that can lead to complications during DNA replication and repair, ultimately allowing for DNA single strand break formation, DNA-protein cross-linkages and mutagenesis if not repaired efficiently (Reviewed in Yang, 2011). Indirect damage caused through photosensitisation reactions can also damage other bases, such as the oxidation of guanine to form 8-oxoG lesions.

1.1.2.2.4 *Metabolic activation of exogenous chemicals*

There are various metabolic reactions within cells that produce intermediates and products that are capable of damaging DNA. A good example of this occurs in the process of biotransformation. The first phase of defense against foreign substances or xenobiotics within cells is carried out through biotransformation (Reviewed in Juchau et al., 1998). This process allows for the conversion of fat soluble molecules in to water soluble molecules that can ultimately be excreted in urine. This involves the addition of charged groups on to the xenobiotic molecule through oxidation reactions. This is mediated by enzymes such as the cytochrome p450s and the glutathione S-

transferases. The addition of charge on to a molecule can promote its chemical activity and these reactions are subsequently limited to specialized organelles. The majority of xenobiotics are thus safely removed from the cell. In some cases however, the charged intermediates may ‘leak’ out into the cellular milieu and interact with DNA, thus forming lesions. The highly toxic aflatoxins and the majority of chemicals produced by cigarette smoke are thought to be ‘activated’ through such a biotransformation process, allowing them to interact with and damage DNA.

1.2 DNA REPAIR

The cellular response to DNA damage varies mostly according to the type of damage inflicted, the extent of damage and also the cell cycle stage of the affected cell. In the majority of cases, the cell will attempt to repair the damage using the most suitable damage-specific repair process. This may involve cell cycle cessation prior to repair completion through checkpoint activation (Reviewed in Jackson and Bartek, 2009). This serves to prevent both mutagenesis and other damage-related errors from occurring during processes such as DNA replication. Finally, in the case where the inflicted damage is too excessive for repair, the cell will initiate apoptosis, or programmed cell death. All these cellular responses to DNA damage reduce mutagenesis rates which may affect cellular activity, but more importantly, preserves the genetic script that is passed on to the daughter cell during cellular division.

In this section, I will briefly introduce the damage-specific DNA repair pathways utilised by cells to maintain DNA integrity. The mechanisms for lesion detection, removal and repair completion will be introduced for each of the known DNA repair pathways. The consequence of pathway dysfunction and relationship to disease will also be discussed.

1.2.1 Reversal repair

Potentially harmful additions on to DNA can sometimes be removed without the need for altering the physical structure of DNA. Such processes fall in to a category of DNA repair known as reversal repair whereby the damage is simply ‘reversed’ through direct removal. Cells have evolved these mechanisms of repair for only a small number of lesions to include some UV irradiation induced damages and some forms of alkylated bases.

CPDs and (6-4) PPs that are the result of UV damage can be reversed in a light-dependent manner. This is carried out by the CPD photolyase and (6-4) photolyase enzymes that are capable of harbouring energy from light to split the bonds of these obstructive damages (Reviewed in Yang, 2011). The α-ketoglutarate-dependent dioxygenase enzymes of the AlkB homologue (ALKBH) family are utilised in the direct reversal of DNA N-alkyl damages. This is achieved through simple hydroxylation of the methyl group that is subsequently released as formaldehyde, leaving behind an intact, normal base (Reviewed in Fu et al., 2012).

The O6-methylguanine lesion that preferentially binds thymine instead of cytosine is also repaired through direct reversal **(Figure 1.5)** (Reviewed in Fu et al., 2012). This lesion is highly cytotoxic since the cell additionally attempts to replace thymine with cytosine however if the methylated guanine is not repaired in time, thymine will once again replace cytosine. Futile cycles of base replacement eventually trigger p53-mediated cell death and to avoid this catastrophic outcome, cells possess an enzyme that specifically removes the methyl group from the O6 position of guanine. This enzyme, O6-methylguanine transferase (MGMT), covalently attaches the methyl group on to its own cysteine, thus restoring the normal binding affinity of guanine. In fact, increased MGMT cellular levels are thought to be a key mechanism that promotes tumour resistance to the chemotherapy drug, temozolomide, for which O6-methylguanine is the primary cytotoxic lesion produced.

1.2.2 Nucleotide excision repair

The nucleotide excision repair pathway (NER) is an excision repair pathway that is responsible for the removal of DNA distorting lesions such as the UV irradiation-induced CPDs and (6-4) PPs (Reviewed in Diderich et al., 2011). It also removes damage generated by benzopyrene, cisplatin and aflatoxins and furthermore repairs some mismatched bases and some forms of oxidative lesions. There are two sub-

pathways within NER that are utilised depending on the transcriptional activity at the damaged site **(Figure 1.6)**. Global genome repair (GGR) occurs at transcriptionally inactive and active regions of DNA whereas transcription-coupled repair (TCR) provides a quick response repair mechanism at transcriptionally active regions of DNA. The GGR and TCR pathways were identified and characterised by the study of patients suffering from the rare autosomal recessive disorders, Xeroderma pigmentosa (XP) and Cockayne syndrome (CS), respectively (Reviewed in Diderich et al., 2011). The clinical manifestations of these diseases include differential degrees of skin and eye hypersensitivity to UV light, susceptibility to skin cancer and neurological defects. A large number of mutated genes were identified in these patients and these genes were eventually shown to be involved in the NER pathways. The NER factors were consequently named according to the disorder that they were identified in.

In global genome repair, damage recognition is achieved by the XP-C and HR23B complex which constantly scans DNA for large structural distortions (Sugasawa et al., 1998). Upon damage recognition, XP-A coordinates the recruitment of the transcription factor IIH (TFIIH) complex that includes the XP-B and XP-D helicases that unwinds approximately 25 nucleotides surrounding the damage (Coin et al., 2007). The XPF-ERCC1 endonuclease heterodimer subsequently cleaves the DNA backbone 22-24 nucleotides 5' to the lesion whilst the XPG endonuclease cuts 5 nucleotides 3' to the lesion (Mu et al., 1997, Sijbers et al., 1996, O'Donovan et al., 1994). A 27-29 nucleotide fragment containing the damage is released, the missing nucleotides replaced by replicative polymerases delta and epsilon (Pol δ and Pol ε) and the nick in the DNA backbone sealed using a DNA ligase (Ogi and Lehmann, 2006, Moser et al., 2007). In transcription-coupled repair, damage recognition by XP-C and HR23B is disposable since this is instead achieved through identification of a stalled RNA polymerase transcription complex at the damage site. The CSA and CSB proteins identify the stalled complex, separating it from the damage site to allow for excision repair as per global

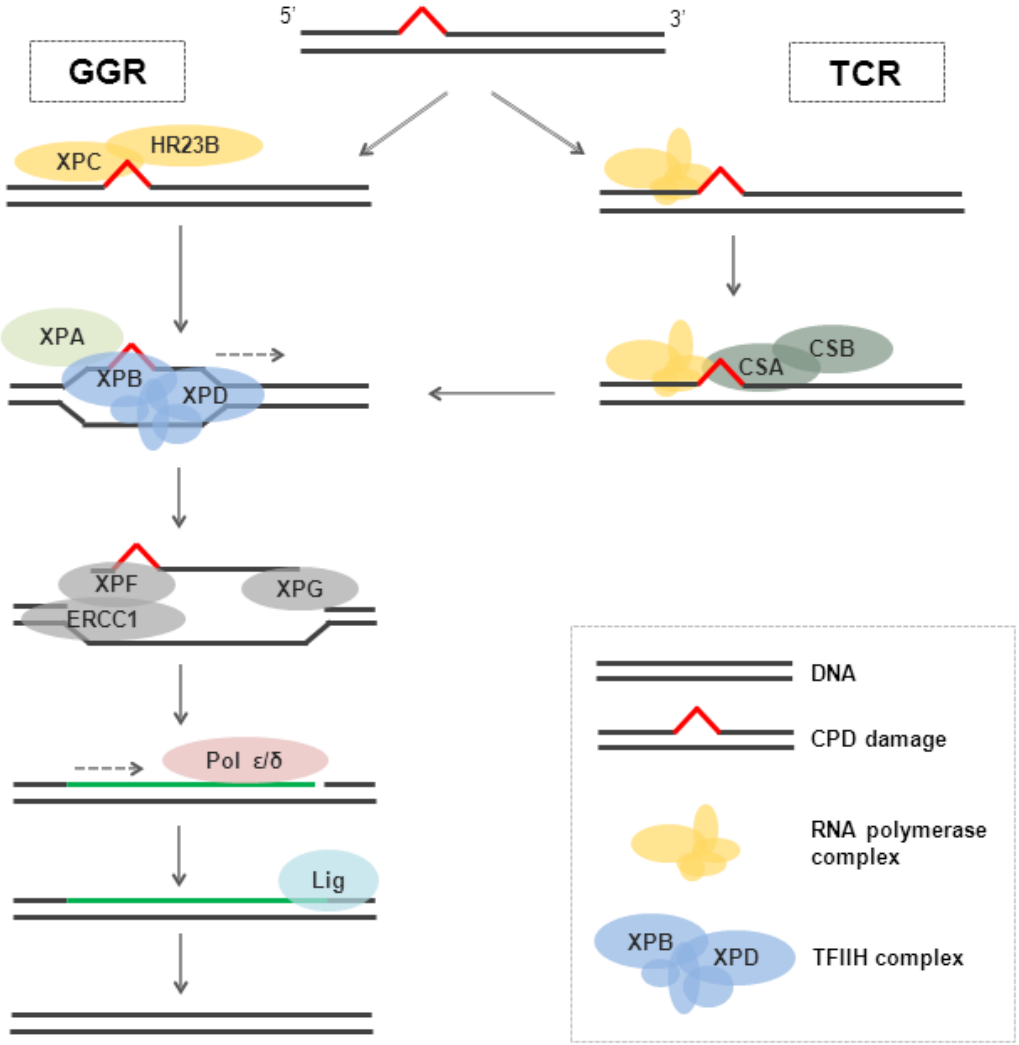

Figure 1.6: The nucleotide excision repair pathway. The UV irradiation-induced CPD lesion is repaired according to the global genome repair pathway (GGR) or the transcription-coupled repair pathway (TCR) as part of NER. In GGR, damage is recognised by the XPC-HR23B complex. XPA recruits the TFIIH transcription complex that contains the DNA helicases XPB and XPD that unwind approximately 25 nucleotides surrounding the lesion. The XPF-ERCC1 and XPG endonucleases cleave the strand that contains the CPD damage and repair is completed by Pol δ and Pol ε mediated DNA synthesis and ligation. In TCR, damage is recognized instead by the stalling of the RNA polymerase complex and the CSA-CSB complex coordinates the access of the TFIIH complex for repair completion according to GGR. Adapted from Diderich, 2011.

genome repair with the recruitment of TFIIH (Troelstra et al., 1992, Henning et al., 1995). Upon completion of repair, the RNA polymerase transcription complex can continue synthesising RNA

1.2.3 Mismatch repair

The mismatch repair pathway (MMR) repairs base mismatches that occur primarily during DNA synthesis and also by agents that modify bases to affect their base binding affinities. MMR additionally repairs insertion/deletion loops generated through slippage of the replicative machinery. Mismatches pose a threat to DNA integrity since they can allow for the formation of permanent changes to the DNA sequence if not repaired prior to DNA replication. In fact, MMR-deficient tumours have a 100-1000 fold increase in mutation rates compared to normal cells (Reviewed in Martin et al., 2010). The importance of the MMR pathway in preventing mutagenesis is further highlighted in sufferers of Lynch syndrome that is caused by inherited inactivating mutations in multiple MMR genes. This disease is characterised by the early onset of hereditary non-polyposis colorectal carcinoma, HNPCC. Since a loss of MMR results in microsatellite instability (MI), MI detection tests are used as a diagnostic tool for characterising the MMR status of tumours (Hampel et al., 2005).

Two major heterodimer components, the MutS heterodimers (MutSα and MutSβ) and the MutL heterodimers (MutLα, MutLβ and MutLγ) regulate MMR activity. The MutS heterodimer recognises a mismatch generated during DNA synthesis by identifying a distortion in DNA (Drotschmann et al., 2001). Upon binding to the mismatch, the MutS heterodimer recruits multiple MutL heterodimers to the mismatch site. The MutL heterodimer has intrinsic endonuclease activity that is activated upon binding with the replication factors, proliferating cell nuclear antigen (PCNA) and replication factor C (RFC) (Kadyrov et al., 2006). This allows the MutL heterodimer to generate a nick in the DNA backbone at the replication machinery site. Exonuclease 1, Exo1, then removes

the stretch of nucleotides on the newly synthesized strand, past the mismatch and stops on encountering a separate nick at the start of the Okazaki fragment that has been generated during replication (Genschel et al., 2002). An alternative model suggests that the second nick is also generated by the MutL heterodimer (Hombauer et al., 2011). The protective factor, replication protein A (RPA) coats the exposed single stranded region until repair is completed by the replicative polymerases and ligases (Ramilo et al., 2002).

1.2.4 Double strand break repair

The DNA double strand break lesion is a highly toxic lesion that can trigger cell death if not repaired efficiently. It is in fact the major cause of irradiation-induced cell death despite representing only a small percentage (< 5%) of the lesions generated on DNA by irradiation (Ward, 2000, Ward, 1990). The DNA double strand break repair pathways are subsequently major targets for inhibition in promoting the cell killing effects of irradiation in the treatment of tumours. In mammalian cells, DNA double strand breaks can be repaired by either non-homologous end joining (NHEJ) or homologous recombination (HR) (Reviewed in Hartlerode and Scully, 2009).

1.2.4.1 *Non-homologous end joining*

The non-homologous end joining pathway simply rejoins two DNA ends that have been created as a result of a DNA double strand break and therefore can function throughout the cell cycle **(Figure 1.7)**. At a DNA double strand break site, break recognition is carried out by the Ku70/Ku80 heterodimers that bind to both ends of the break and subsequently recruit the kinase DNA-PKcs to the damage site (Cary et al., 1997, Walker et al., 2001, Yaneva et al., 1997). This allows for a complex cellular response to the presence of damage, such as cell cycle check point activation that aims in part to protect DNA from damage-induced replication errors (Yaneva et al., 1997

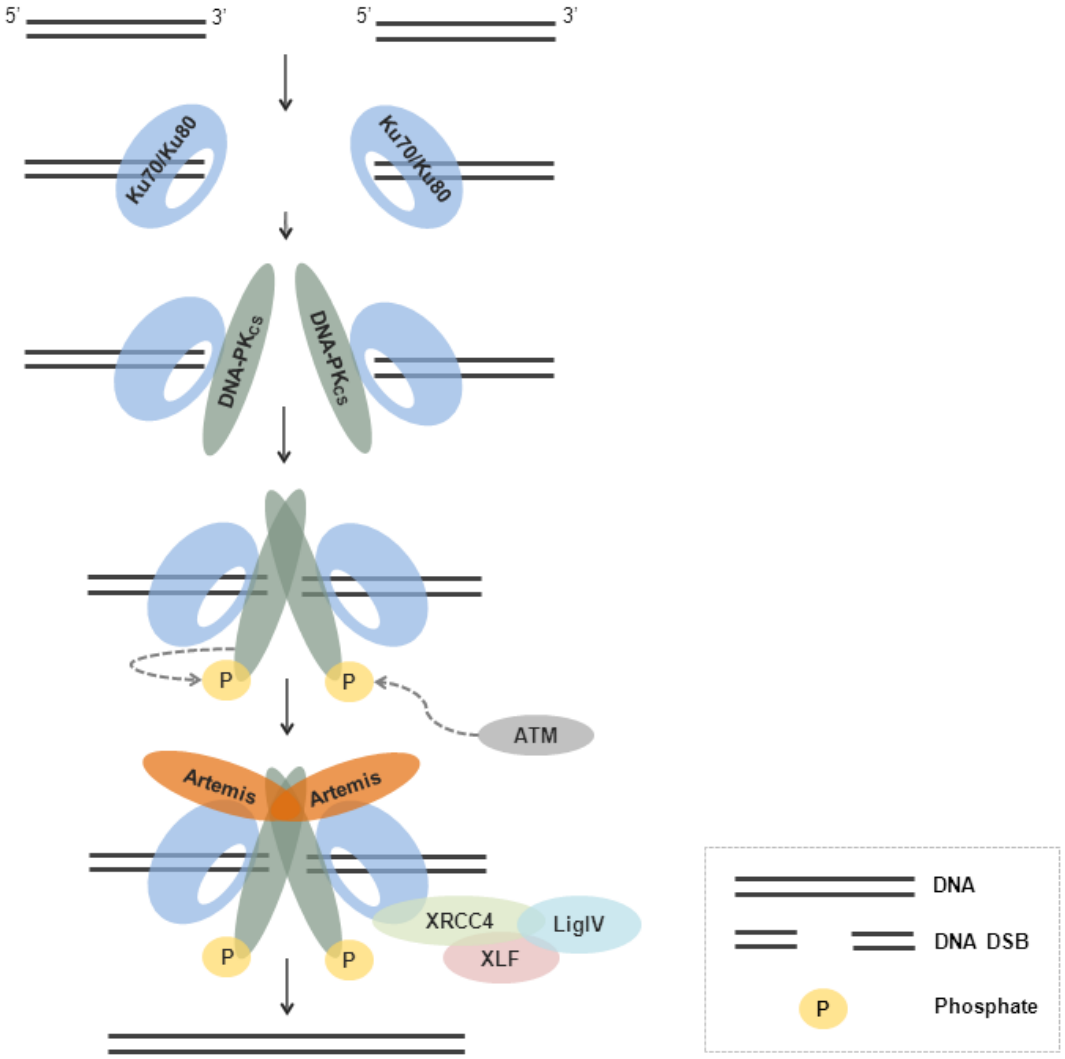

Figure 1.7: The non-homologous end joining pathway. DNA double strand breaks are identified by the Ku70/Ku80 heterodimer. The catalytic subunit of the kinase DNA-PK mediates a response to recruit factors such as the end processing factor Artemis that allows for repair completion by the XRCC4-DNA LigIV-XLF complex. Adapted from Hartlerode, 2009.

Gottlieb and Jackson, 1993). The Mre11-Rad50-Nbs1 (MRN) complex is also recruited and together, the factors allow for close association of the two DNA ends (DeFazio et al., 2002, Williams et al., 2008). Finally, the end processing factor Artemis binds DNA-PKcs and using both exonuclease and endonuclease activity generates DNA ends that are congruent for ligation by the XRCC4-DNA Lig IV-XLF complex (Grawunder et al., 1997, Nick McElhinny et al., 2000, Ma et al., 2002, Goodarzi et al., 2006). Extensive DNA end processing however can result in small end deletions prior to religation and the NHEJ pathway is therefore considered to be relatively error-prone (Reviewed in Hartlerode and Scully, 2009).

1.2.4.2 *Homologous recombination*

DNA double strand break repair can also be carried out by homologous recombination (HR) that utilises the intact sister chromatid as a template for repair, and as such, proves to be virtually error-free compared to NHEJ **(Figure 1.8)**. The requirement for a sister chromatid means that HR can only be carried out during the S and G2 stages of the cell cycle where two copies of DNA are available. In HR, DNA double strand breaks are recognised by the MRN complex that activates and recruits ataxia telangiectasia mutated, ATM, to the damage site (Hopfner et al., 2002, Williams et al., 2008, Moreno-Herrero et al., 2005). Activated ATM phosphorylates a multitude of factors such as the histone H2A variant, H2AX that are involved in eliciting a cellular response to the presence of DNA double strand breaks (Savic et al., 2009, Matsuoka et al., 2007). Such cellular responses include chromatin remodeling and checkpoint activation. MRN together with factors such as CtIP, BRCA1 and BARD1 resect and process the 5'-ends of the DNA double strand break to leave a 3'-overhang that can invade the template sister chromatid with the help of factors such as Rad51 and BRCA2 (Sung and Klein, 2006, Sartori et al., 2007, Yun and Hiom, 2009, Sharan et al., 1997). There are various

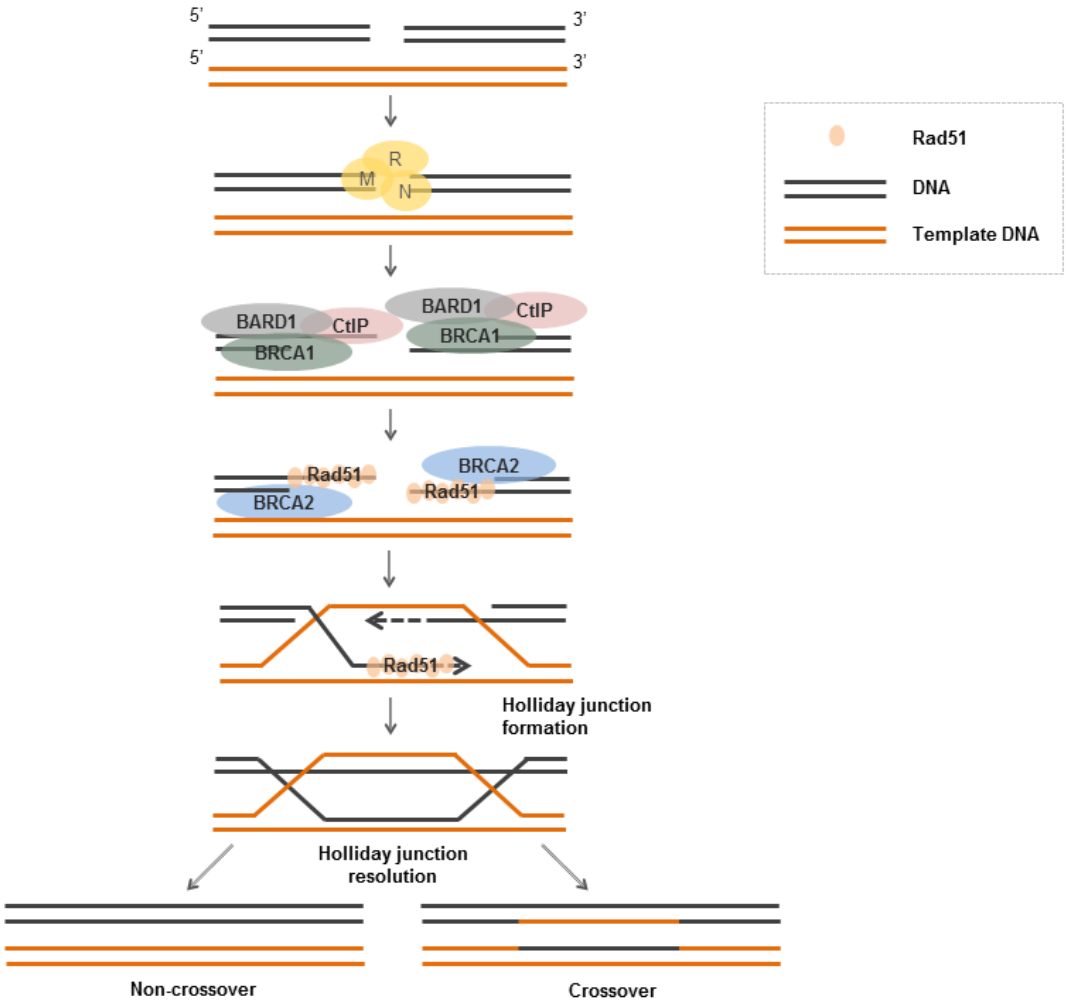

Figure 1.8: The homologous recombination pathway. This pathway for DNA double strand break repair requires a sister chromatid for repair completion. DNA DSBs are recognised by the MRN complex that recruits various strand end processing factors such as CtIP that trim the DNA ends in the 5' to 3' direction. Various factors such as BRCA1 and BARD1 assist in this process. Once processed, sister chromatid strand invasion is mediated by Rad51 and BRCA2 for DNA synthesis, ultimately leading to the formation of two Holliday junctions that are further resolved to generate either non-crossover or crossover products of repair. Adapted from Hartlerode, 2009.

models for strand invasion and copying of the template that ultimately lead to crossover or non-crossover products of repair.

1.2.5 DNA damage tolerance: translesion synthesis

The translesion synthesis (TLS) pathway allows the replication machinery to bypass damage by replacing the replicative polymerase with one that can accommodate the damaged base in to its active site. The mammalian bypass polymerases fall under the Y family of polymerases and include Pol η, Pol ι, and Pol κ, amongst others (Reviewed in Sale et al., 2012). Each polymerase has a preference for a lesion type that it is able to bypass relatively error-free. For instance, Pol η can synthesise DNA past CPD adducts whereas Pol κ preferentially synthesises past benzo[a]pyrene-G adducts and some types of methylated bases (Ohashi et al., 2000, Johnson et al., 1999b).

Replication machinery that has stalled upon encountering damage allows for increased exposure of single stranded DNA at the replication fork. The E3 ligase, Rad18, binds to the exposed ssDNA and together with the E2 enzyme, Rad6, is able to monoubiquitylate the replication factor PCNA at the stalled replication fork (Hoege et al., 2002, Stelter and Ulrich, 2003, Kannouche et al., 2004, Davies et al., 2008). Monoubiquitylation of PCNA enhances its interaction with translesion polymerases that possess both PCNA interacting motifs (PIP boxes) and ubiquitin binding zinc finger domains (UBZs) (Naryzhny, 2008, Bienko et al., 2005, Plosky et al., 2006, Bomar et al., 2010). Upon lesion bypass, PCNA is deubiquitylated by ubiquitin specific protease 1, USP1, allowing PCNA to replace the bypass polymerase with a replication polymerase (Huang et al., 2006).

Loss of translesion synthesis increases mutation rates since defective polymerases tend to be replaced by polymerases that are less suited for a particular damage type. Mutational inactivation of Pol η for instance causes xeroderma

pigmentosum variant disease and those afflicted experience high mutation rates in response to UV irradiation, and consequently suffer a much increased risk of developing skin cancers (Masutani et al., 1999, Johnson et al., 1999a).

1.2.6 Fanconi anemia pathway

Interstrand cross-links (ICLs) are highly toxic lesions that covalently lock together the two strands of DNA, thus obstructing DNA processes such as replication and transcription. The repair of ICLs is carried out by the combined efforts of the nucleotide excision repair (NER), homologous recombination (HR) and translesion synthesis (TLS) pathways and the interplay between these three DNA repair pathways is coordinated by the Fanconi anemia pathway (Kuraoka et al., 2000, Niedernhofer et al., 2004, Niedzwiedz et al., 2004, Prasher et al., 2005, Yamamoto et al., 2005). A loss of normal function in any of the FA pathway players manifests clinically as Fanconi Anemia, a disease highlighted by increased susceptibility to the development of blood cancer and solid tumours as well as increased sensitivity to DNA cross-linking agents (Alter, 1996, Auerbach, 1988, Auerbach and Wolman, 1976, Moldovan and D'Andrea, 2009). Indeed, the mutations identified in FA patients proved a useful tool in understanding the FA pathway and for the identification of most of the FA proteins (FANC proteins) that were subsequently named after the disease.

ICLs block replication to trigger the DNA damage response, activating the S-phase checkpoint kinase ATR, and promoting the recruitment of various homologous recombination factors such as Rad51 and BRCA1 to the blocked replication fork (Reviewed in Dianov et al., 2011). Activated ATR furthermore phosphorylates the FANCD2-FANCI complex, as such making it a substrate for monoubiquitylation by the E3 ligase FANCL in complex with the FA core complex (FANCA, FANCB, FANCC, FANCE, FANCF, FANCG, FANCL and FANCM) (Ishiai et al., 2008, Cole et al., 2010, Gurtan et al., 2006, Machida et al., 2006, Meetei et al., 2003). The FA associated protein

FAAP24 binds this core complex to DNA at repair foci allowing it to interact with the FAN1 nuclease. FAN1, together with XPF-ERCC1, cuts the DNA backbone of one strand allowing for the release or 'unhooking' of the ICL on one of the DNA strands (Bhagwat et al., 2009, Kratz et al., 2010, Liu et al., 2010, MacKay et al., 2010, Smogorzewska et al., 2010). Another mechanism for cutting the DNA backbone was shown to be mediated by the Pso2/SNM1 family exonucleases (Barber et al., 2005, Wang et al., 2011, Senegerova et al., 2012). Translesion synthesis polymerases then replace the missing nucleotides on the freed strand, whereas the strand containing the unhooked lesion is thought to be repaired by either the TLS or HR pathways, though this is currently not well understood (Reviewed in Sengerova et al., 2011).

1.3 BASE EXCISION REPAIR

The base excision repair (BER) pathway is an excision repair pathway responsible for the removal of non-bulky, non-distorting and minor alterations in DNA bases generated through base oxidations, base alkylations as well as spontaneous base substitutions (deaminations). It also repairs induced or spontaneously generated apurinic/apyrimidinic sites, (AP sites) as well as DNA single strand breaks. The repair of DNA single strand breaks is alternatively referred to as single strand break repair (SSBR) in literature and differs from BER only in its mechanism of damage recognition and DNA end processing. A loss in the correct regulation of the BER pathway or BER functionality has been observed in multiple tumour types and neurodegenerative diseases, and complete loss of the key BER players has proven to be embryonic lethal in mice (Xanthoudakis et al., 1996, Sugo et al., 2000, Tebbs et al., 1999, Puebla-Osorio et al., 2006). This is highly indicative of the importance of this pathway in the maintenance of genome stability and cellular survival.

Briefly, the BER pathway progresses according to the following sequence of events; upon damage identification, a damaged base is excised, the DNA backbone cleaved and the exposed strands processed prior to base insertion and DNA backbone ligation for repair completion **(Figure 1.9).** There are variations within each repair step that is dependent on the damage type being processed and these will be pointed out throughout this section. Though not fully understood, the coordination and regulation of the BER steps will also be discussed, as well as the role that BER plays in disease.

1.3.1 Damage recognition and base excision

The recognition and removal of a damaged base is achieved by the damage-specific DNA glycosylases **(Figure 1.9).** There are currently 11 known mammalian DNA

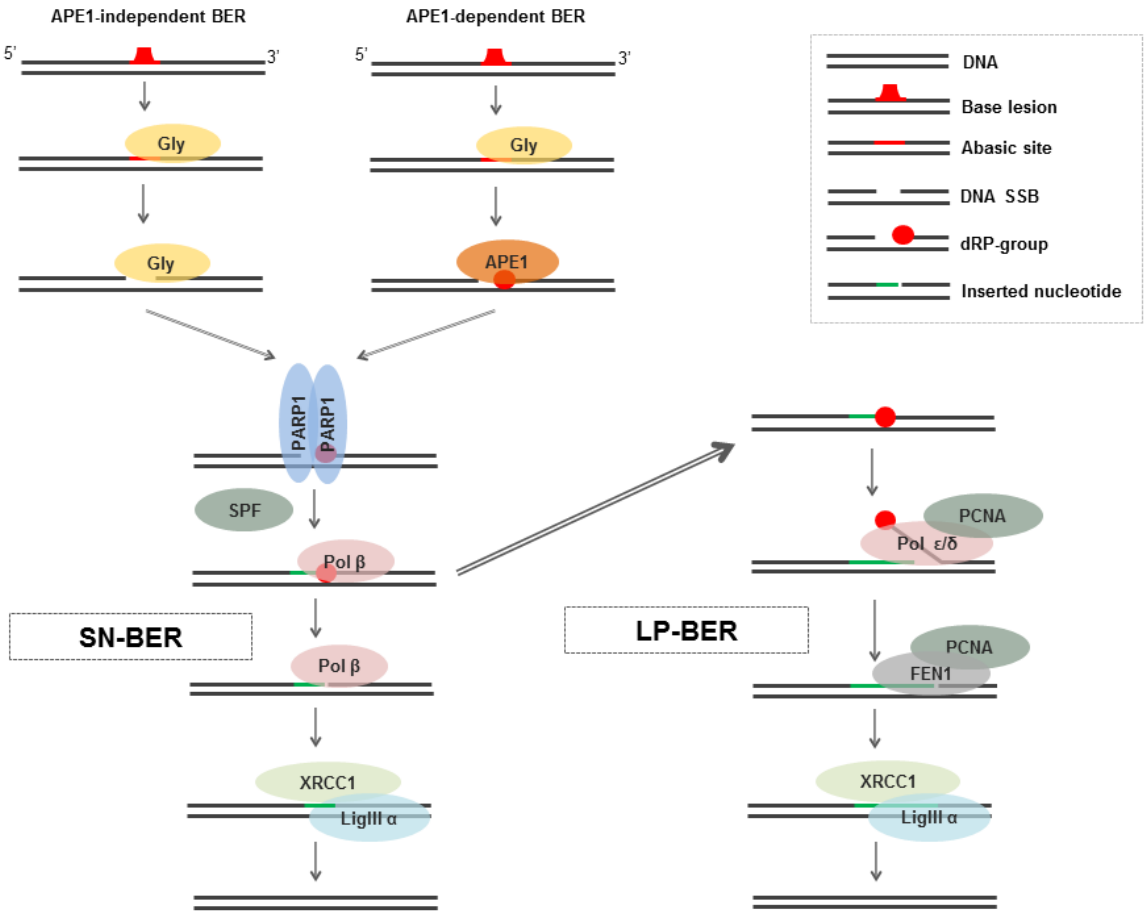

Figure 1.9: The base excision repair pathway. BER repairs small non-distorting base lesions, abasic sites and DNA single strand breaks. Base damage is identified and excised by a damage specific DNA glycosylase. Some bi-functional glycosylases can cleave the phosphodiester backbone (APE1-independent BER) however most backbone cleavage is achieved by the endonuclease APE1 to leave a DNA SSB and a 5'-dRP group (APE1-dependent BER). PARP1 recognises and protects the ends of DNA SSBs. It also recruits strand end processing factors such as PNKP, APTX and TDP1 as well as Pol β to the repair site. In single nucleotide (SN) BER, Pol β removes the 5'-dRP group and inserts the appropriate nucleotide for repair completion by the XRCC1-Lig IIIα complex. In cases where Pol β is unable to process 5'-dRP groups, repair proceeds according to long-patch (LP) BER. Pol β inserts the nucleotide and is replaced by Pol ε/δ in complex with PCNA that inserts 2-10 nucleotides by displacing a 'flap' of nucleotides. This 'flap' is cleaved away by FEN1 and repair is completed by PCNA and Lig I. Adapted from Dianov, 2011.

glycosylases that each recognise either a single lesion type or a small number of structurally similar lesions **(Table 1.1)** (Reviewed in Jacobs and Schar, 2012). There are two types of glycosylases, the monofunctional glycosylases that simply excise a damaged base and the bifunctional glycosylases that additionally cleave the DNA backbone upon base removal.

It is thought that DNA glycosylases continuously 'scan' DNA for base lesions (Berg et al., 1981). Weak interaction of the glycosylase with DNA allows it to slide along the DNA length until it encounters a damage for which it has a greater binding affinity (Berg et al., 1981). These stronger glycosylase-lesion specific interactions induce the flipping of the base away from the DNA helix thus exposing the base completely to the glycosylase for full discrimination (Slupphaug et al., 1996). Nucleophilic attack of the C1'-N glycosidic bond by the glycosylase promotes the release of the damaged base from the sugar phosphate DNA backbone to create an AP site (Mol et al., 1995, Slupphaug et al., 1996). The bifunctional glycosylases additionally possess β-lyase activity that allows for the hydrolytic attack of the DNA backbone 3' to the abasic site (Sun et al., 1995, Nash et al., 1997). This β-elimination reaction produces a DNA single strand break containing a 5'-phosphate and a 3'-α,β unsaturated aldehyde (Mazumder et al., 1991). The bifunctional glycosylases nei endonuclease VIII-like 1, 2 and 3 (NEIL1, NEIL2 and NEIL3) also cleave 5' to the abasic site by means of a δ-elimination reaction, releasing the deoxyribose group from the AP site such that a 3'-phosphate and a 5'-phosphate group remain (Takao et al., 2002).

1.3.2 DNA backbone incision

Cleavage of the DNA backbone at an abasic site is necessary for inserting the replacement nucleotide for completion of repair. Though the bifunctional glycosylases are capable of cleaving the DNA backbone, the majority of abasic sites are processed by the endonuclease apurinic/apyrimidinic endonuclease 1, APE1 (Demple et al., 1991

	Glycosylase	Substrate
Mono-functional	UNG	Uracil, 5-OHU
	hSMUG1	Uracil from ssDNA, 5-hydroxymethyluracil
	MBD4	Uracil or thymine opposite guanine
	TDG	Uracil or thymine opposite guanine
	MYH	Adenine opposite 8-oxoG
	MPG/AAG	3-methyladenine, 7-methylguanine, hypoxanthine
Bi-functional	OGG1	8-oxoG opposite cytosine, FapyG
	hNTH1	TG, CG, DHU, 5-OHC, 5-OHU, FapyG
	NEIL1	DHU, DHT, TG, 5-OHC, 5-OHU, 8-oxoG, FapyG, FapyA
	NEIL2	5-OHU, 5-OHC, 8-oxoG, FapyG, FapyA
	NEIL3	FapyG, FapyA

Table 1.1: Substrates of the DNA glycosylases. 5-OHU: 5-hydroxyuracil; 8-oxoG: 7,8 dihydro 8-oxoguanine; FapyG: 2,6-diamino-4-hydroxy-5-formamidopyrimidine; TG: thymine glycol; CG: cytosine glycol; DHU: dihydroxyuracil; DHT: dihydroxythymine; 5-OHC: 5-hydroxycytosine; OHU: 5-hydroxyuracil; FapyA: 4,6-diamino-5-formamidopyrimidine. Adapted from Jacobs, 2012.

Robson and Hickson, 1991). Upon removal of a damaged base, some glycosylases remain bound to the AP site and this is thought to protect the AP site from further damage and additionally, to recruit both APE1 and the scaffold protein, X-ray cross complementing protein 1, XRCC1 to the repair site (Parikh et al., 1998, Waters et al., 1999, Hardeland et al., 2000, Hill et al., 2001, Pope et al., 2002, Marsin et al., 2003, Campalans et al., 2005). APE1 can displace some of the glycosylases from an AP site to carry out its primary role of cleaving the DNA backbone 5' to the AP site by hydrolytic cleavage of the phosphodiester bond **(Figure 1.9)** (Parikh et al., 1998, Waters et al., 1999, Hill et al., 2001, Demple and Harrison, 1994, Barzilay and Hickson, 1995). This generates a DNA single strand break containing a 3'-hydroxyl group and a 5'-deoxyribosephosphate moiety (5'-dRP). If APE1 is released from its repair product prior to the binding of the next repair enzyme, DNA polymerase beta (Pol β), then the abundant DNA end protecting factor, poly [ADP-ribose] polymerase 1 (PARP1) binds to the DNA ends with high affinity **(Figure 1.9)** (de Murcia and Menissier de Murcia, 1994, Parsons et al., 2005b, Woodhouse et al., 2008). Through its ability to generate poly-ADP ribose (PAR) chains on itself and other proteins, PARP1 is additionally able to recruit downstream BER factors that have PAR-binding motifs to the repair site for repair continuation (Pleschke et al., 2000, El-Khamisy et al., 2003, Okano et al., 2003, Caldecott et al., 1996).

1.3.3 DNA end processing of BER SSB intermediates

An incoming nucleotide can only be inserted into a nucleotide gap that contains a 3'-hydroxyl and 5'-phosphate group. Both APE1 and the bifunctional glycosylases generate nucleotide gaps that lack one or more of these groups. DNA end processing factors ensure that the correct groups are present at the DNA ends. The 3'-phosphate groups generated by the β,δ-elimination activities of the glycosylases NEIL1, NEIL2 and NEIL3 are processed by the 3'-phosphatase activity of polynucleotide kinase-

phosphatase (PNKP), though this activity can also be achieved to a lesser extent by APE1 (Jilani et al., 1999, Habraken and Verly, 1988, Wiederhold et al., 2004). APE1 also processes 3'-α,β unsaturated aldehydes that are generated as a result of glycosylase-mediated β-elimination reactions (Izumi et al., 2000). The 5'-dRP group that is generated by APE1 endonuclease activity is removed by the dRP lyase activity of DNA polymerase beta, Pol β, prior to Pol β-mediated nucleotide insertion into the repair gap (Podlutsky et al., 2001b, Allinson et al., 2001).

1.3.4 DNA end processing of DNA SSB damage

DNA single strand breaks (SSB) are also generated by various endogenous and exogenous DNA damaging sources. These single strand breaks are recognised and initially protected from further damage by PARP1 and repaired by the latter stages of the BER pathway (de Murcia and Menissier de Murcia, 1994, Parsons et al., 2005b, Woodhouse et al., 2008). They often possess groups at the DNA ends that are not conducive with repair completion and multiple factors are involved in processing such ends prior to initiating repair (Reviewed in Caldecott, 2008). The DNA end processing factors that process the ends of the BER single strand break repair intermediate are also able to process the ends of these alternatively generated DNA SSBs. Additional modifications include 3'-phosphoglycolate that is processed by the 3'-phosphodiesterase activity of APE1 and also to a lesser extent by tyrosyl-DNA phosphodiesterase 1, TDP1 (Suh et al., 1997, Parsons et al., 2004, Zhou et al., 2005). The 5'-hydroxyl termini present at a blocked TOP1-DNA SSB intermediate is processed by the 5'-kinase activity of PNKP to generate a 5'-phosphate group (Reviewed in Caldecott, 2008). Finally, abortive ligase activity generates a 5'-AMP group that must first be removed by aprataxin, APTX, prior to repair completion (Ahel et al., 2006).

1.3.5 Nucleotide gap filling and DNA ligation

Pol β is recruited to the repair site through direct interaction with either APE1, PARP1 or directly to the DNA SSB (Bennett et al., 1997, Dantzer et al., 2000). Upon DNA end processing, repair proceeds according to either single nucleotide BER or long patch BER.

1.3.5.1 *Single nucleotide BER*

Using its dRP lyase activity, Pol β removes the 5'-dRP moiety at the nucleotide gap through a β-elimination reaction to create the conventional 3'-hydroxyl and 5'-phosphate groups required for nucleotide insertion and sealing of the DNA backbone (Matsumoto and Kim, 1995, Allinson et al., 2001). Using its polymerase activity, Pol β inserts the appropriate nucleotide whilst DNA ligase III alpha (Lig IIIα) complexed to XRCC1 catalyses the sealing of the DNA backbone thus completing repair **(Figure 1.9)** (Podlutsky et al., 2001b, Allinson et al., 2001, Caldecott et al., 1994, Cappelli et al., 1997)

1.3.5.2 *Long patch BER*

In some cases, Pol β is unable to remove the 5'-dRP moiety and in this case, repair continues with the alternative long patch BER pathway. A reduced or oxidised dRP moiety for instance cannot be processed by Pol β lyase activity however Pol β is still capable of inserting the nucleotide in to the nucleotide gap (Podlutsky et al., 2001a, Nakamura et al., 2000, Dianov et al., 1999). Upon nucleotide insertion at the 3'-end, DNA polymerases δ/ε replace Pol β, and with the help of PCNA, insert 2-10 nucleotides from the 3'-end of the repair gap **(Figure 1.9)** (Klungland and Lindahl, 1997, Fortini et al., 1998, Stucki et al., 1998). This action displaces approximately 2-10 nucleotides, generating a 'flap' that is then cleaved by Flap endonuclease 1, FEN1. DNA ligase I (Lig

I) finally seals the remaining nick in the DNA backbone to complete DNA repair (Kim et al., 1998).

1.3.6 Coordination of BER

Due to the variation in the base lesions and DNA SSB ends that are processed by the BER pathway, it is difficult to presume that this pathway is strictly coordinated through preassembled DNA repair protein complexes. Indeed, attempts to purify repair complexes using chromatography techniques were relatively unsuccessful and this indicates that such complexes either do not exist in the cell or those that do exist, are at the very least based on transient interactions (Reviewed in Dianov and Parsons, 2007). However the danger posed by the persistence of the repair intermediates such as abasic sites and DNA SSBs suggests that the BER pathway must be well coordinated and likely tailored to specific DNA damage types. The most widely accepted model for the coordination of the individual repair steps of BER is therefore that of 'passing the baton' (Mol et al., 2000, Wilson and Kunkel, 2000).

In the 'passing the baton' model, enzyme-substrate affinities allow the repair enzyme to remain bound to its repair product until it is displaced by the next repair enzyme in the pathway (Mol et al., 2000, Wilson and Kunkel, 2000). This transfer of substrates is thought to be mediated through direct interactions between the repair proteins. Indeed, multiple studies have shown that interactions exist between the BER factors, though these studies were primarily performed using immunoprecipitation techniques that do not provide concrete proof for a direct cellular interaction (Reviewed in Dianov and Parsons, 2007). The sequence in which the substrate is passed on is therefore not well understood.

Current evidence for the coordination of single nucleotide BER indicates that APE1 is capable of displacing the DNA glycosylase at an abasic site or single strand

break for further processing (Parikh et al., 1998, Hill et al., 2001, Waters et al., 1999). BER is thought to progress according to one of the following models. The first model ('passing the baton') suggests that APE1 remains bound to its DNA SSB repair intermediate and is only released upon direct interaction with Pol β that then inserts the replacement nucleotide. The second model does not follow the 'passing the baton' ideal, however shares some similarities **(Figure 1.10)**. Using cross-linked repair assays, our lab has previously shown that APE1 more likely dissociates from its DNA SSB repair intermediate prior to the binding of Pol β to the repair site (Parsons et al., 2005a). Exposure of a DNA SSB leaves it prone to further damage and this 'APE1 dissociation' model therefore includes the rapid binding of the abundant DNA end binding factor, PARP1, at the DNA SSB repair intermediate. The presence of PARP1 at the DNA ends is thought to protect the DNA ends from further damage until Pol β is recruited (Parsons et al., 2005b). Indeed, it was previously demonstrated by El-Khamisy *et al* that PARP1 is required for the efficient formation of XRCC1 repair foci at sites of oxidative damage repair induced by hydrogen peroxide (El-Khamisy et al., 2003). Finally, the efficiency of Pol β activity and overall repair was shown to increase significantly in the presence of the XRCC1-Lig IIIα complex suggesting that the interaction between Pol β and the pre-existing XRCC1-Lig IIIα complex is necessary for efficient repair completion (Caldecott et al., 1996, Dianova et al., 2004, Parsons et al., 2005a).

The two models described here are hugely simplified and it is likely that multiple combinations of interactions occur to provide efficient repair according to the lesion type being processed. At the core of the repair process, it appears that the scaffold protein XRCC1 plays a major role in stabilising such interactions ultimately to promote activity and indeed, it has been shown to interact with multiple BER factors including APE1, Pol β, PNKP, PARP1 and Lig IIIα (Caldecott et al., 1994, Caldecott et al., 1995, Caldecott et al., 1996, Campalans et al., 2005, Masson et al., 1998, Whitehouse et al., 2001, Vidal et al., 2001).

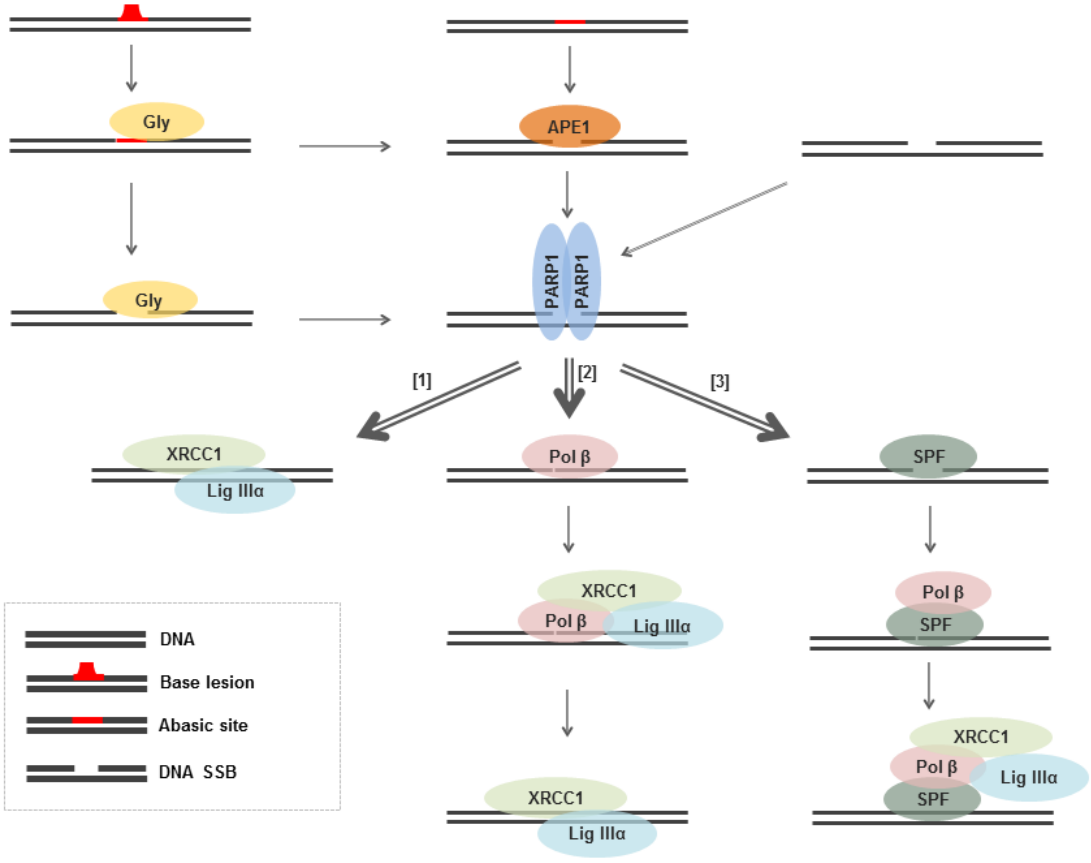

Figure 1.10: The APE1 dissociation model of base excision repair coordination. A DNA SSB can be generated by bi-functional glycosylases and APE1 during BER or as a result of direct damage on to DNA. For DNA SSBs generated by APE1, it is thought that APE1 dissociates prior to the binding of Pol β to the repair site. PARP1 identifies this DNA SSB as well as other types of DNA SSBs and allows for the recruitment of various factors involved in frank strand break repair [1], BER repair completion [2] or strand end processing for BER repair completion [3]. SPF: strand end processing factor. Adapted from Parsons, 2005b and Dianov, 2007.

1.3.7 Consequences of BER dysregulation

The base excision repair pathway is responsible for the repair of a large number of endogenously generated damage on DNA. It is therefore not surprising that mice engineered to lack the key base excision repair factors APE1, Pol β, XRCC1 and DNA Lig IIIα die during embryogenesis (Xanthoudakis et al., 1996, Sugo et al., 2000, Tebbs et al., 1999, Puebla-Osorio et al., 2006). In comparison, mice lacking the DNA glycosylases tended to be viable and this is likely due to the overlapping specificities of the glycosylases for some base lesion types (Reviewed in Jacobs and Schar, 2012). Mice that lack the glycosylases and heterozygous mice that have reduced expression of the key repair factors APE1, Pol β and XRCC1 accumulate damage and repair intermediates that correlate with increased hypersensitivity to DNA damaging agents, mutagenesis and compromised genome stability (Jacobs and Schar, 2012, Huamani et al., 2004, Meira et al., 2001, Unnikrishnan et al., 2009, Cabelof et al., 2006, Allen et al., 2008, McNeill et al., 2011, Saribasak et al., 2011). This is undoubtedly due to reduced BER repair activity in these mice. Due to the potential toxicity of the repair intermediates generated during base excision repair, overexpression of some glycosylases and APE1 can also promote genome instability whereas overexpression of Pol β primarily results in mutagenesis that is linked to erratic and incorrect base insertion during repair (Reviewed in Wallace et al., 2012)**.** These data point to the importance of fine-tuning the regulation of the individual BER players, not only according to the damage present in cells but also according to the availability of the other BER players. Indeed, a loss of such fine-tuned regulation of BER activity and the individual BER players have been implicated in cancer and neurodegenerative diseases.

1.3.7.1 *BER in neurodegenerative disease*

Neuronal cells tend to possess high metabolic rates and consequently these cells experience high levels of oxidative stress generated by ROS. The BER activity and

levels of some BER proteins such as APE1, XRCC1, Lig IIIα and PNKP are subsequently higher in these cells (Reviewed in Wilson and McNeill, 2007). For a large number of neuronal diseases, an excess of oxidative DNA lesions were observed and the BER activity responsible for the repair of these lesions tended to be much lower compared to the same tissue type of non-affected control subjects (Bogdanov et al., 2000, Kisby et al., 1997, Lovell et al., 2000, Ferrante et al., 1997). This inverse correlation also increased with the more advanced disease cases. There have therefore been a large number of studies in to the role of BER in the onset and/or progression of various neurodegenerative diseases including Alzheimer's disease (AD), Parkinson's disease (PD), Huntington's disease (HD), multiple sclerosis (MS) and amyotrophic lateral sclerosis (ALS). Studies that attempted to link common polymorphisms in the genes for the key BER factors OGG1, XRCC1, APE1 and PARP1 to neurodegenerative disease susceptibility proved mostly unsuccessful in identifying a BER determinant for the onset of the aforementioned neurological diseases. An association was established only for ALS in a meta-analysis of four separate studies, patients of which had a significantly higher incidence of APE1 Asp148Glu (Hayward et al., 1999, Greenway et al., 2004, Tomkins et al., 2000, Coppede et al., 2010). Both XRCC1 Arg399Gln and OGG1 Ser326Cys polymorphisms were also over-represented in ALS patients, however more studies are required to confirm this (Reviewed in Coppede, 2011).

The only BER factors that have been well characterised and linked directly to neurological disease are those that are involved in DNA single strand end processing. Mutations in TDP1, APTX and PNKP lead to well characterised congenital disorders with symptoms that are predominantly restricted to the nervous system (Reviewed in Caldecott, 2008, El-Khamisy and Caldecott, 2007, Reynolds et al., 2012). The progressive neurodegenerative disease spinocerebellar ataxia with axonal neuropathy-1 (SCAN1) that is characterised primarily by a loss of sensation at the extremities of the body and loss of independent walking is caused by a His493Arg mutation in TDP1 that

impairs DNA single strand break repair (Takashima et al., 2002, El-Khamisy et al., 2005). It was found that this mutant form of TDP1 had reduced DNA end processing ability, thus interfering with the speed of processing of TOP1-initiated breaks (Interthal et al., 2005, El-Khamisy et al., 2005). Another neurodegenerative disease, ataxia-oculomotor apraxia 1, AOA1 is caused by mutation of APTX and is characterised by a loss in the patients ability to coordinate muscular movements (Date et al., 2001, Moreira et al., 2001, El-Khamisy et al., 2009). More recently identified PNKP mutations were found to be the cause of reduced DNA single strand break repair in yet another neurological disorder, Microcephaly with Seizures (MCSZ), sufferers of which experience slow development, microcephaly and seizures (Shen et al., 2010, Reynolds et al., 2012). These diseases all suggest that DNA end processing during SSBR, though not necessary for cellular survival, is an important step of the BER pathway that is required for the efficiency of repair.

1.3.7.2 *BER in cancer*

A large number of lesions repaired by the BER pathway have the potential to generate somatic mutations that can ultimately contribute to carcinogenesis and the evolution of a cancer cell. Furthermore, BER repairs DNA lesions generated by cancer treatments such as radiation and chemotherapies and as such can influence patient response to these treatments. The BER pathway is therefore a key target for drug design in the treatment of cancers, particularly to reduce the repair of damage inflicted by DNA damaging agents used for the treatment of cancers.

Alterations in the level, localisation and activity of BER players have been observed in various tumour types however it is not known if these alterations are causative or consequential in tumourigenesis (Reviewed in Wallace et al., 2012). The overexpression of OGG1, APE1, PARP1, XRCC1 and Pol β have been noted in some cancers, and it is thought that this overexpression may promote the repair of damage

induced by key cancer treatments such as radiotherapy and chemotherapies, thereby promoting tumour resistance to these treatments. Inhibitors for some glycosylases, APE1, PARP1, PNKP and Pol β are therefore currently under investigation (Kelley et al., 2012, Tell et al., 2010, Srinivasan et al., 2012). PARP1 inhibitors that are currently in clinical trial have so far proven to be the most promising inhibitor (Reviewed in Mason et al., 2012). These inhibitors prevent the release of PARP1 from a DNA SSB thereby reducing the efficiency of repair completion. Unrepaired DNA SSBs can be converted in to the highly toxic DNA DSB lesion at the point of replication, making PARP1 inhibition a suitable concomitant treatment for both radiation and SSB-inducing chemotherapy treatments (Kuzminov, 2001).

Reduction in the activity of BER players has also been observed in some cancers. For instance, cytoplasmic localisation of APE1 that was observed in some cancer types may indirectly reduce the repair activity of APE1 (Lee et al., 2012, Wu et al., 2010, Al-Attar et al., 2010). Polymorphisms in the BER factors OGG1, NEIL1 and MUTYH that display reduced repair activity have also been identified in some cancers (Reviewed in Wallace et al., 2012). A reduction in BER activity is likely to promote tumour evolution since it increases the probability of mutagenesis, a key factor that promotes tumour development.

1.3.8 Regulation of BER activity

The base excision repair pathway has previously been described as a 'housekeeping' repair pathway. BER activity on DNA is likely to be constant due to the frequency of endogenously occurring lesions that it repairs. Regulation of BER activity is therefore not as dramatic as that of some other repair pathways that repair rarely occurring lesions such as interstrand cross-links and DNA double strand breaks. For this reason, it is thought that the BER repair pathway is regulated to a steady-state level throughout a cells lifetime. This involves constant tailoring of the availability of the BER

players in response to even slight changes in cellular requirement. This is ultimately achieved through a fine balance of transcriptional expression for making more BER factors when it is needed, and proteasomal degradation of any excess BER factors that are no longer required by the cell **(Figure 1.11)**. Such a balance needs to exist according to the amount of damage present on DNA, but more importantly according to the availabilities of the other BER factors since both overexpression and underexpression of any one BER enzyme can result in incapacitated repair and genome instability (Reviewed in Wallace et al., 2012).

The most supportive example of the steady-state regulation theory for the BER pathway is that of Pol β regulation. Pol β expression in response to alkylating agents is carried out by the binding of the CREB1 transcription factor to the regulatory cAMP response element at the Pol β gene promoter (He et al., 2003, Narayan et al., 1996). The removal of excessive amounts of Pol β through proteasomal degradation is promoted through the ubiquitylation of Pol β by the E3 ligases Mule/ARF-BP1 and carboxy terminus of Hsc70-interacting protein (CHIP) (Parsons et al., 2008, Parsons et al., 2009). Loss of Pol β ubiquitylation in cells results in high levels of Pol β that correlate with increased repair of hydrogen peroxide damage (Parsons et al., 2009). The Mule binding factor, p14 alternate reading frame, p14-ARF that normally inhibits Mule activity was also shown to regulate Mule activity in response to DNA damage (Chen et al., 2005). Loss of p14-ARF results in increased Mule-mediated Pol β ubiquitylation and proteasomal degradation in cells, leading to reduced repair of hydrogen peroxide induced damage (Parsons et al., 2009). Furthermore, Pol β that was ubiquitylated could be deubiquitylated by the deubiquitylation enzyme, ubiquitin specific protease 47 (USP47) (Parsons et al., 2011). Indeed, a loss of USP47 results in lower levels of Pol β in cells, reduced repair capacity with accumulation of unrepaired damage and decreased cellular viability in response to DNA damaging agents (Parsons et al., 2011).

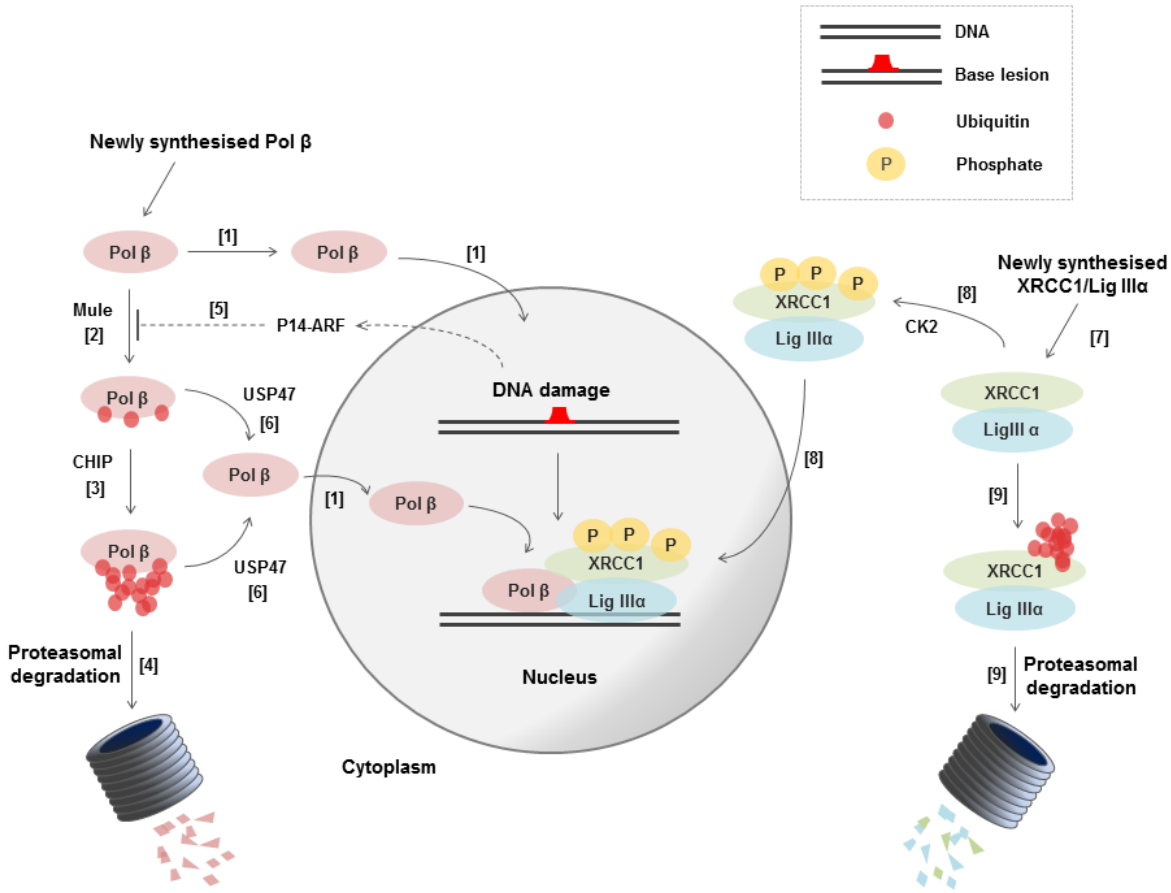

Figure 1.11: Steady-state regulation of base excision repair. The steady-state levels of Pol β, XRCC1 and DNA Lig IIIα are regulated by ubiquitylation and proteasomal degradation. Newly synthesised Pol β is transferred to the nucleus where it can carry out its DNA repair role [1]. Excess Pol β that is not required for repair is monoubiquitylated by the E3 ligase Mule at three sites [2] and further polyubiquitylated by the E3 ligase CHIP [3] to promote Pol β proteasomal degradation [4]. The Mule binding factor p14-ARF binds Mule in response to damage, thus preventing Mule-mediated ubiquitylation of Pol β [5], ultimately increasing the cellular pool of Pol β that is available for repair. Pol β that is already ubiquitylated can additionally be deubiquitylated by the DUB USP47 [6], once again generating Pol β for repair. Similarly, newly synthesised XRCC1 and Lig IIIα form a complex [7] that is available for repair. XRCC1 phosphorylation by CKII promotes this repair activity [8]. Non-phosphorylated XRCC1/Lig IIIα complexes tend to be less stable since they are prone to being ubiquitylated for degradation by the proteasome [9]. Adapted from Dianov, 2011.

Similarly to Pol β, the stability of XRCC1 was also shown to be regulated by proteasomal degradation that is dependent on XRCC1 phosphorylation mediated by casein kinase II (CKII) **(Figure 1.11)** (Parsons et al., 2010). Since the interaction of XRCC1 with DNA Lig IIIα stabilizes DNA Lig IIIα in cells, a loss of CKII-mediated XRCC1 phosphorylation also affects the stability of cellular DNA Lig IIIα (Parsons et al., 2010, Caldecott et al., 1995)

At the start of this DPhil project, it was not clear whether a role for ubiquitylation-mediated proteasomal degradation existed for regulating the availability of the key BER enzyme, APE1. APE1 produces the potentially toxic DNA SSB as a repair intermediate and its coordination within the 'dissociation model' of BER coordination presumes that it is released from its repair product irrespective of the availabilities of downstream BER factors (Parsons et al., 2005a). Excess APE1 in relation to other BER factors could therefore pose a threat to genome stability and we consequently believe that APE1 availability is also likely to be tightly regulated in cells. APE1 expression has been reported to be induced in response to oxidative damage however there is no known mechanism for the removal of excess cellular APE1 (Ramana et al., 1998, Grosch et al., 1998). Though APE1 was shown to be *in vitro* ubiquitylated by the murine double minute (MDM2) E3 ligase, the cellular role of APE1 ubiquitylation could not be determined (Busso et al., 2009). Since ubiquitylation-mediated proteasomal degradation plays a major role in regulating the availability of the BER factors for repair, it would be interesting to further explore the possibility that APE1 levels are also regulated by ubiquitylation in cells.

Indeed very little has been observed for alternative mechanisms of regulation for the BER pathway, though post-translational modifications such as phosphorylation and acetylation have been shown to fine-tune the activity, interaction, spatial regulation and stability of some of the BER proteins. For instance, cyclin-dependent kinase 5 (CDK5)-mediated phosphorylation of APE1 in neuronal cells reduces its endonuclease activity

whereas deacetylation of APE1 by the class III histone deacetylase, sirtuin 1 (SIRT1), enhances APE1 interaction with XRCC1, thus stimulating repair (Huang et al., 2010, Yamamori et al., 2010).

1.4 APE1

APE1, short for apurinic/apyrimidinic endonuclease 1 and alternatively referred to as Ref-1 (redox enhancing factor 1), HAP-1 (human apurinic/apyrimidinic endonuclease 1) and APEX1, is a multifunctional protein involved in maintaining genome stability and cellular survival. There are two major independent functions of APE1. It is involved within the BER pathway as an endonuclease with additional DNA end processing capabilities. Additionally, APE1 is a redox regulator of various transcription factors that are involved in cellular growth, proliferation and survival (Reveiwed in (Bhakat et al., 2009) and (Kelley et al., 2012). The importance of APE1 in cells is highlighted by the fact that APE1 null mice die during embryogenesis and unsurprisingly, a dysregulation of APE1 in cells is implicated in both tumourigenesis and neurodegenerative diseases (Xanthoudakis et al., 1996). It is not well understood if APE1 dysregulation in these diseases is causative or consequential. APE1 overexpression in tumour cells has also been linked to increased tumour resistance to both chemotherapy and radiation therapy (Naidu et al., 2010, Wang et al., 2009, Robertson et al., 2001). APE1 is therefore a key target of interest for inhibition in the treatment of APE1 overexpressing tumours. There are currently gaps in the understanding of the regulation of the individual functions of APE1 and furthermore, APE1 regulation within the context of BER regulation is not well understood. Such knowledge will allow us to understand better the roles of APE1 in disease onset and progression and to identify potential issues relating to the targeting of such an important factor for inhibition in both tumour cells and normal cells.

1.4.1 APE1 protein

APE1 was first discovered as a repair protein in 1991 following the isolation and analysis of its cDNA. It was subsequently shown to share sequence similarity with the E. coli exonuclease III, ExoIII, the *S. pneumoniae* exonuclease A, ExoA, as well as bovine and Drosophila DNA repair proteins (Robson and Hickson, 1991). At the time, this level of conservation suggested that the enzymatic activity of APE1 is more than likely necessary for normal cellular functioning. Indeed, a loss of APE1 in mice results in embryonic lethality and attempts to generate an APE1 null cell line have been unsuccessful (Xanthoudakis et al., 1996)

The human APE1 protein (Acc. # NP_542380.1) is approximately 35.5 kDa in size with 318 amino acids that can be separated in to two functional regions **(Figure 1.12)** (Xanthoudakis et al., 1994). The N-terminal 127 amino acids form the redox domain of human APE1 with cysteines 65 and 93 implicated in this role (Walker et al., 1993, Georgiadis et al., 2008, Luo et al., 2008). The C-terminal region of human APE1 is linked to DNA binding and endonuclease activity, the latter of which requires at least residues H309 and D283 (Masuda et al., 1998). The endonuclease domain of APE1 is very well conserved however the N-terminal redox domain is less so. APE1 also contains a regulatory region at its N-terminal 61 residues that form a flexible and exposed tail that has within it nuclear localisation signals at residue 1-7 and 8-13 (Jackson et al., 2005). This N-terminal regulatory region of APE1 is however completely lacking in most prokaryotic homologues of APE1 and poorly conserved in eukaryotes (Fantini et al., 2010). This pattern of homology most likely reflects the evolutionary acquisition of the separate functions of APE1. Indeed prokaryotic homologues lack redox function and the N-terminal tail is currently understood to be vital for the regulation of the compartmental localisation of APE1 in eukaryotic cells.

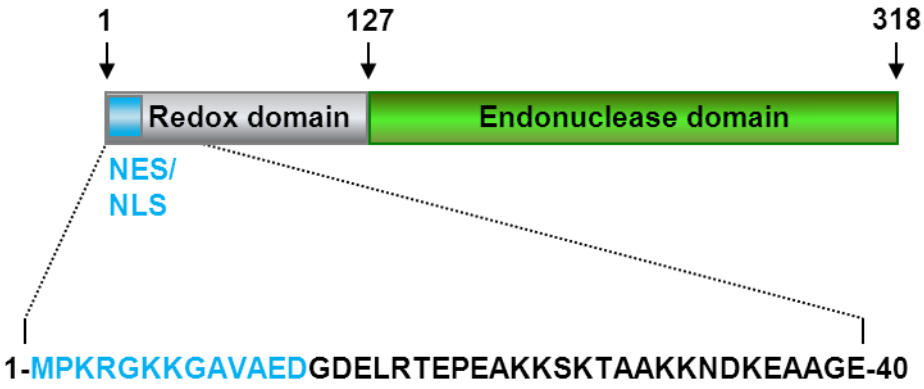

Figure 1.12: Representation of APE1 protein. The redox domain spans the first 127 residues of APE1. The N-terminal 61 residues of APE1 form a flexible tail that has within it a nuclear export signal and a nuclear localisation signal at residues 1-7 and 8-13. The endonuclease domain spans residues 127-318.

1.4.2 APE1 cellular roles

APE1 has multiple distinct roles within the cell. It is directly involved in DNA repair as part of the BER pathway and furthermore plays additional roles in repair coordination and maintenance of repair fidelity. In an unrelated role, APE1 promotes the DNA binding capabilities of various transcription factors involved in the regulation of cellular processes such as apoptosis, cellular growth and proliferation. APE1 therefore hugely impacts on genome integrity and cellular survival.

1.4.2.1 *APE in BER*

APE1 is the primary endonuclease within the BER pathway and is involved in processing abasic sites (AP sites) that occur at a frequency of 10,000 per cell per day (Lindahl and Nyberg, 1972). AP sites are sites on DNA that lack a base but possess an intact phosphodiester backbone. Such sites are generated either as a repair intermediate during BER by glycosylases that remove damaged bases or through depurination and depyrimidination events that occur either spontaneously or are induced by alkylating agents, for example. AP sites are highly mutagenic and accumulation of unrepaired AP sites can prevent efficient DNA replication and in some cases promote cell death (Guillet and Boiteux, 2002, Loeb et al., 1986, Yu et al., 2003). Indeed, APE1 heterozygous mice show increased numbers of AP sites, increased mutagenesis and hypersensitivity to DNA damaging agents (Huamani et al., 2004, Meira et al., 2001, Unnikrishnan et al., 2009).

During BER, APE1 cleaves the DNA backbone 5’ to the AP site, thus allowing for nucleotide insertion and repair completion by a polymerase and DNA ligase (Demple and Harrison, 1994, Barzilay and Hickson, 1995). APE1 also displays weak 3’-phosphodiesterase and 3’-phosphatase activities, allowing for the removal of the 3’-phosphoglycolate and 3’-phosphate residues, respectively that inhibit extension by DNA

polymerases (Chou and Cheng, 2002, Parsons et al., 2004, Parsons et al., 2005c). APE1 also aids in the coordination of base excision repair. Binding of APE1 to the DNA glycosylases TDG and UDG promotes their catalytic turnover and release from the AP site intermediate for further processing by APE1 (Guillet and Boiteux, 2002, Loeb et al., 1986, Yu et al., 2003). APE1 bound to its cleaved DNA product was also reported to interact directly with Pol β, stimulating the dRP-lyase activity of Pol β prior to releasing itself from the repair site (Wong and Demple, 2004). APE1 role in pathway coordination and DNA end processing therefore allows for efficient BER progression, inefficiency of which increases the probability of mutagenesis, cell cycle arrest and cell death.

APE1 also possesses 3'-exonuclease activity that is able to remove 3'-nucleotides that have been damaged or misincorporated by error-prone polymerases during both DNA repair and replication (Chou and Cheng, 2002). This suggests that APE1 exonuclease activity is a distinct function that allows APE1 to proofread during both DNA replication and DNA repair, thereby impacting positively on the fidelity of these processes.

1.4.2.2 *APE1 in transcription*

In a very distinct role to repair but equally as important, APE1 acts as a redox factor to various transcription factors such as AP-1, p53, HIF-1α, the p50 subunit of NF-κB, CREB/ATF, Pax5 and Pax8 (Reviewed in Bhakat et al., 2009). In its reduced state, APE1 is able to reduce the oxidised forms of these transcription factors, thus enabling them to bind promoter sites on DNA. This allows for the transcription of target genes that are involved in processes such as the immune response, apoptosis, cell cycle maintenance, cellular proliferation and differentiation.

APE1 has also been shown to be directly involved in transcription complexes and the best known example is that of the hypoxia inducible transcriptional complex involving

APE1, p300, HIF-1α, STAT2 and ATF/CREB (Ziel et al., 2004, Gray et al., 2005). This complex binds to the hypoxic response element (HRE) of the vascular endothelial growth factor (VEGF) promoter allowing for its expression (Ziel et al., 2004). Hypoxic regions of tissue, or regions of low oxygen levels, stimulate the expression of VEGF through this transcriptional complex to allow for the formation of blood vessels. In fact, many angiogenic tumours overexpress VEGF and furthermore evasion of hypoxia-induced apoptosis in hypoxic tumours is mediated by HIF-1α (Reviewed in Amini et al., 2012). As part of this transcriptional complex, APE1 therefore allows for the survival of hypoxic tumour cells and is consequently a therapeutic target of interest for the treatment of this tumour type.

APE1 was also implicated as a transacting factor that is capable of binding to negative calcium response elements (nCaRE) found at the promoters of various genes including the rennin and parathyroid genes, thus preventing their expression in response to calcium influx signaling (Okazaki et al., 1994, Bhakat et al., 2003a, Kuninger et al., 2002, Sengupta et al., 2012, Fuchs et al., 2003). In fact, APE1 complexed to heterogeneous nuclear ribonucleoprotein L (hnRNP-L) was shown to also repress its own expression by binding to the nCaRE-B2 regulatory sequence of the APE1 promoter (Kuninger et al., 2002).

1.4.2.3 *APE1 in apoptosis and maintenance of RNA*

Other than APE1s indirect role in the transcriptional control of apoptosis, cleavage of the N-terminal regulatory domain of APE1 has also been shown to primarily encourage apoptotic events. A caspase-3-mediated cleavage product of APE1 lacking residues 1-35, AN34 (Apoptosis Nuclease Mr 34,000), possessed increased exonuclease activity that allowed for DNA fragmentation in response to apoptotic signals (Yoshida et al., 2003). Alternative cleavage of the N-terminal 31 residues of APE1 by Granzyme A and Granzyme K was also involved in mediating a response to apoptosis

triggered by an immune response (Fan et al., 2003, Guo et al., 2008). Guo *et al* additionally showed that APE1 cleavage by Granzyme K resulted in an accumulation of reactive oxygen species within cells that could mediate cell death.

APE1 is furthermore implicated in the maintenance of RNA quality, RNA processing and ribosome biogenesis. APE1 is capable of processing AP sites in single stranded DNA and it was shown that this activity is comparable to the activity of APE1 on dsDNA (Berquist et al., 2008). This ssDNA processing ability of APE1 was further linked to the cleansing of mRNA products that are damaged, which if not repaired or destroyed completely by endonucleases such as APE1, can switch off protein synthesis and interrupt the codon reading capabilities of affected tRNA molecules (Korennykh et al., 2007, Cochella and Green, 2005). APE1 was also shown to directly interact with many factors involved in ribosome biogenesis to include nucleophosmin (NPM1), and it was shown that APE1 interaction with NPM1 through its N-terminal 33 residues allowed for its localisation to the nucleolus (Vascotto et al., 2009).

1.4.3 Consequences of APE1 dysregulation

The multiple functions of APE1 in repair and regulation of transcription factor activity means that APE1 is important for the maintenance of genome integrity and cellular growth, proliferation and survival. Indeed, APE1 null mice die during embryogenesis and heterozygous mice that display reduced levels of APE1 show increased mutational rates in both the liver and the spleen, poorer BER activity in the brain and liver, increased sensitivity to oxidative damage and increased spontaneous micro-tumour formation (Huamani et al., 2004, Meira et al., 2001, Xanthoudakis et al., 1996, Unnikrishnan et al., 2009). APE1 that was overexpressed, albeit in an XRCC1 null cell line, showed an increase in genomic instability and increased sensitivity to an alkylating agent presumably induced through the inefficient repair and accumulation of the APE1 DNA SSB repair intermediate (Sossou et al., 2005). It is unsurprising therefore

that a dysregulation of APE1 activity has been noted in various tumours and neurodegenerative diseases, both of which are linked heavily to a loss of faithful DNA repair.

1.4.3.1 *APE1 in neurodegenerative disease*

The role of APE1 in the onset and progression of neurodegenerative diseases is currently not understood. High levels of ROS were linked to reduced overall BER activity in neuropathologies such as Huntington's disease (HD), Parkinson's disease (PD), Alzheimer's disease (AD), multiple sclerosis (MS) and amyotrophic lateral sclerosis (ALS) (Reviewed in Hegde et al., 2012). Unrepaired ROS-mediated DNA damage is thought to be at least partially responsible for the neuronal cell death observed for these diseases. Indeed, a loss of APE1 was shown to stimulate neuronal cell death (Huang et al., 2010). The kinase CDK5 together with its activating partner p35 was shown to phosphorylate APE1 at residues S54 and T233 in neuronal cells, thus reducing its endonuclease activity (Huang et al., 2010). Increased CDK5/p35 stability and activity have been observed in both AD and PD and it is now believed that the role of CDK5/p35 in these diseases is mediated by its ability to reduce APE1 repair activity (Smith et al., 2003, Cruz and Tsai, 2004, Huang et al., 2010). Indeed, high levels of phosphorylated APE1 were observed in brain samples obtained from both AD and PD patients (Huang et al., 2010). The polymorphic variant of APE1, Asp148Glu, also has reduced endonuclease activity and was correlated to the incidence of ALS in a large meta-analysis of four separate studies (Hayward et al., 1999, Greenway et al., 2004, Tomkins et al., 2000, Coppede et al., 2010). It is still not understood whether reduced APE1 activity directly contributes to the onset of these diseases or if it is simply a consequence of disease progression.

1.4.3.2 *APE in cancer*

Altered cellular localisation of APE1 has been observed in cancers and both predominant cytoplasmic and nuclear localisation of APE1 has been associated with cancer aggressiveness (Lee et al., 2012, Wu et al., 2010, Al-Attar et al., 2010). For instance, lung tumour cells that possessed mostly cytoplasmic localisation of APE1 displayed increased NF-κB activity that positively correlated with tumour aggressiveness (Wu et al., 2010). Additionally, strong nuclear localisation of APE1 in ovarian cancer cells obtained from patients was reported to reduce the positive effects of tumour debulking surgery and for both ovarian and gastroesophageal tumours, nuclear localisation of APE1 was also correlated to poor patient survival (Al-Attar et al., 2010).

Increased levels of APE1 were also observed in several tumour types to include gliomas, multiple myelomas, pancreatic cancer, ovarian cancer and prostate cancer (Fishel et al., 2011, Moore et al., 2000, Xie et al., 2010, Bobola et al., 2001, Kelley et al., 2001). Such an increase in APE1 has been associated with increased cellular potential for proliferation and angiogenesis, and it is widely thought that the repair mediated by APE1 endonuclease activity in APE1 overexpressing tumour cells promotes resistance to DNA damaging treatments such as irradiation and some types of chemotherapy (Naidu et al., 2010, Wang et al., 2009, Robertson et al., 2001). APE1 inhibition is therefore currently considered a suitable treatment for resensitising tumour cells to the cell killing effects of such treatments (Kelley et al., 2012, Tell et al., 2010, Madhusudan et al., 2005).

As with the dysregulation of APE1 localisation in cancers, it is not entirely clear which of the two major roles of APE1 account for the effects of excessive amounts of APE1 in tumour cells. However APE1's role in promoting the DNA binding abilities of transcription factors such as p53, HIF-1α, AP-1 and NF-κB can also indirectly promote DNA damage repair since many of these transcription factors regulate the expression of genes for DNA repair proteins that are involved in multiple DNA repair pathways. These

transcription factors additionally promote the expression of genes involved in growth, metastasis and stress response. Since cells within a tumour are frequently heterogeneous in nature, the potential for APE1 to impact on various aspects pertaining to tumour development and tumour sensitivity to treatment makes it a very attractive target for inhibition.

Current research in to the efficacy of APE1 inhibitors for the treatment of cancers is at an early stage. Due to the multi-disciplinary involvement of APE1 in regulating factors involved in cancer progression and treatment response, APE1 inhibitors that separately target both the APE1 redox function and the APE1 endonuclease function are currently under investigation (Kelley et al., 2012, Tell et al., 2010, Madhusudan et al., 2005). The APE1 redox function inhibitor E3330 that was shown to reduce the activity of the transcription factors NF-κB, AP-1 and HIF-1α in pancreatic cancer cells shows the most promise since this inhibitor promotes a block in pancreatic cancer cell proliferation and migration (Fishel et al., 2011, Kelley et al., 2012). This compound was shown to be specific to APE1, did not affect normal cells negatively and various low IC-50 analogues are currently under study, making it a promising drug for use in patients (Kelley et al., 2011, Nyland et al., 2010, Kelley et al., 2012). Multiple inhibitors of APE1 endonuclease activity were recently isolated in a high-throughput screen and these inhibitors are currently undergoing confirmation testing (Simeonov et al., 2009). The more advanced study in to the indirect inhibitor of APE1 endonuclease activity, methoxyamine that covalently binds to abasic sites thus preventing APE1 access to the repair site, is currently in phase I clinical trials for treating advanced cancers such as malignant melanoma and lung cancer (TRACON Pharmaceuticals, Inc.).

1.4.4 Regulation of APE1

The regulation of APE1 activity and localisation within cells is not well understood. The fact that APE1 possesses multiple independent roles produces an

additional complication in elucidating the mechanisms regulating the individual functions of APE1 in cells. As mentioned previously, dysregulation of APE1 localisation, levels and activity are implicated in both tumours and neurodegenerative diseases and APE1 is therefore being investigated as a target for inhibition. A full understanding of the regulation of the individual functions of APE1 will allow us to better understand the role of APE1 in disease and additionally will help us to predict the effects that inhibition may have on both tumour and normal cells. Such an understanding will further allow us to generate a link between the regulation of APE1 activity and the regulation of the BER pathway that is currently not well understood.

1.4.4.1 *Transcriptional regulation*

The 2.6 kb APE1 gene contains a CpG island immediately upstream that has recognition sequences for various transcription factors including Sp1, USF, AP-1 and CREB (Akiyama et al., 1994, Harrison et al., 1995). The transcriptional expression of APE1 is poorly characterised, however it was reported to be cell cycle-dependent, peaking in early S phase and is induced in a CREB dependent manner in response to oxidative stress (Grosch et al., 1998, Grosch and Kaina, 1999, Fung et al., 2001, Ramana et al., 1998). Both APE1 itself and p53 have been shown to repress APE1 expression through binding the APE1 nCaREB2 negative regulatory element and Sp1 promoter, respectively (Izumi et al., 1996, Zaky et al., 2008). HIF-1α was furthermore shown to downregulate the production of APE1 mRNA in hypoxic conditions (Loboda et al., 2009). Since APE1 allows for activation of both the HIF-1α and p53 transcription factors, it is likely that their ability to repress APE1 expression is part of a negative feedback loop to switch off their own activation. It is feasible to suggest that APE1 may regulate its own protein levels by such a feedback loop as well.

1.4.4.2 *Cellular localisation*

APE1 has been observed in both the nucleus and the cytoplasm of cells. The role of APE1 in the nucleus is rather obviously linked to its primary DNA repair role however the role for APE1 cytoplasmic localisation is less well understood. In terms of cellular localisation, APE1 possesses two nuclear import signals at its N-terminal tail (residues 1-7 and residues 8-13) and has been shown to localise to the nucleus in response to calcium mobilisation and various ROS-mediated stresses such as hypoxia and inflammation (Jackson et al., 2005). Furthermore inhibition of nuclear export mediators resulted in the accumulation of nuclear APE1 and this was again dependent on the N-terminal residues of APE1 (Jackson et al., 2005). This suggests that a nuclear export signal is also likely to be present on the APE1 N-terminal tail.

1.4.4.3 *Post-translational modification*

Post-translational modifications including phosphorylation, acetylation, S-nitrosation, S-glutathionylation and ubiquitylation have been described for APE1. APE1 phosphorylation by CKII was reported to abolish APE1 endonuclease activity in one study (Yacoub et al., 1997). This was not shown to be the case in a second study that instead suggested CKII enhanced APE1 redox regulation of the AP-1 transcription factor (Fritz and Kaina, 1999). APE1 *in vitro* redox activity was further shown to be enhanced on phosphorylation by protein kinase C, PKC (Hsieh et al., 2001). A more recent study that generates a link between APE1 phosphorylation and the diseases AD and PD shows that the CDK5 kinase and its activating factor p35 phosphorylate APE1 at residues S54 and T233 (Huang et al., 2010). This phosphorylation reduces APE1 endonuclease activity and as such was shown to encourage neurotoxin-induced neuronal death (Huang et al., 2010).

Acetylation of APE1 at its N-terminal tail lysines K6 and K7 by the histone acetyl transferase p300 enhanced the binding of APE1 to the NCaRE sequence in the promoter of the parathyroid hormone (PTH) gene thus repressing PTH expression (Bhakat et al., 2003a). The class III HDAC, SIRT1 was subsequently shown to allow for the removal of acetyl groups from lysines K6 and K7 in response to genotoxic stress (Yamamori et al., 2010). Furthermore, it was shown that SIRT1 knockdown by siRNA resulted in increased amounts of abasic sites and reduced APE1 interaction with XRCC1, thus interfering with the recruitment of APE1 to sites of DNA damage (Yamamori et al., 2010, Vidal et al., 2001). Class I and class II HDACS may also be involved in deacetylating APE1 since inhibition of the class I and II HDACS by treatment with trichostatin A (TSA) allowed for accumulation of acetylated APE1 in cells (Bhakat et al., 2003a, Fantini et al., 2008). This effect however could also be a result of indirect regulation.

More recently it was shown that APE1 is also S-glutathionylated at residue C99 that is implicated in the redox activity of APE1 however this modification was reported to instead reduce APE1 endonuclease activity (Kim et al., 2011). On the other hand, S-nitrosation of APE1 at residues C93 and C310 that are implicated in APE1 endonuclease activity promoted APE1 cytoplasmic localisation and redox activity (Qu et al., 2007, Wu et al., 2010).

Finally, APE1 was also shown to be *in vitro* ubiquitylated by the E3 ligase, MDM2, however the role of ubiquitylation in the regulation of cellular APE1 could not be determined (Busso et al., 2009). Since ubiquitylation-mediated proteasomal degradation plays a major role in the regulation of overall BER activity, the aim for this doctoral project was to determine if APE1 levels are also regulated in such a manner. Knowledge on the regulation of APE1 levels in cells will not only allow us to understand the potential cellular effects of APE1 inhibition in normal cells, it will also allow us to understand the regulation of APE1 in the context of BER regulation and disease.

1.5 UBIQUITYLATION

Ubiquitylation is the process by which ubiquitin monomers or polymers are conjugated on to proteins. Modification of proteins by ubiquitin can regulate protein stability, localisation and protein-protein interactions to effect changes in protein activity and function within a multitude of cellular processes. Such an influence on cellular activity requires the ubiquitylation process to be rather specific in terms of both targeting substrates and directing the outcome of ubiquitylation. Indeed, ubiquitylation is a regulatory process possessing multiple attributes that allow it to carry out controlled protein regulation.

1.5.1 The ubiquitylation process

Ubiquitylation involves the addition of the highly conserved 76 amino acid ubiquitin molecule on to a lysine residue of a target protein through a series of enzymatic reactions involving an E1 activating enzyme, an E2 conjugating enzyme and an E3 ubiquitin ligase **(Figure 1.13)** (Reviewed in Ye and Rape, 2009). Firstly, the E1 activating enzyme forms a thiol ester bond between its conserved internal cysteine residue and the C-terminal G76 residue of ubiquitin in an ATP-dependent manner. This activates the ubiquitin molecule, allowing it to be transferred on to the next ubiquitylation enzyme, the E2 conjugating enzyme. The E1 activating enzyme bound to activated ubiquitin interacts directly with the E2 conjugating enzyme, allowing for the formation of a thiol ester bond between the C-terminal G76 residue of activated ubiquitin and the catalytic cysteine of the E2 enzyme. The E2 enzyme conjugated to ubiquitin then binds an E3 ligase, thus allowing for either the transfer of the activated ubiquitin molecule directly on to the lysine acceptor residue on an E3-bound target protein or on to the E3 for E3-mediated ubiquitin transfer on to the target protein. This generates an amide

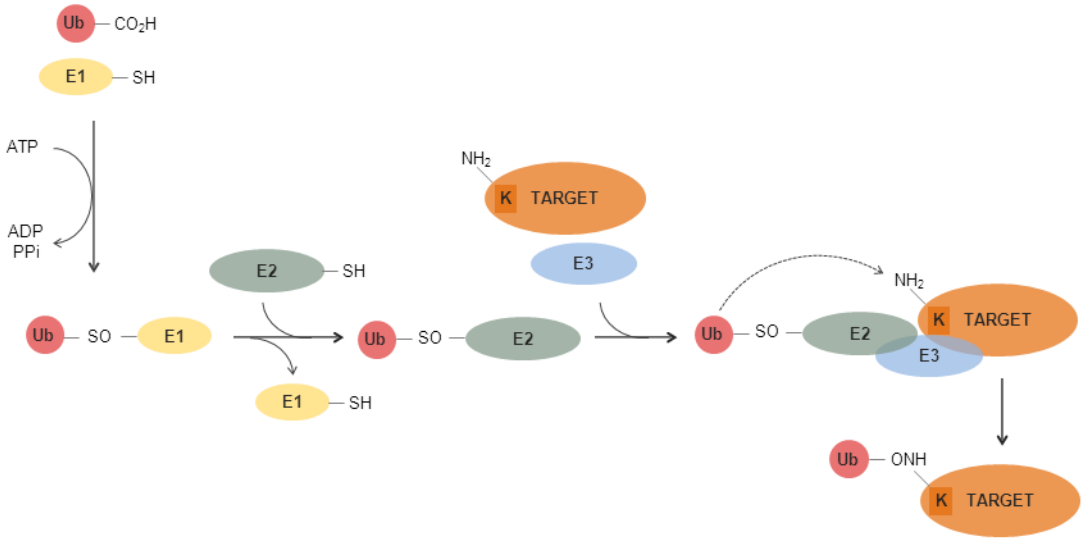

Figure 1.13: The ubiquitylation process. The ubiquitylation process is mediated primarily through the actions of an E1 activating enzyme, an E2 conjugating enzyme and an E3 ligase. An energy-dependent reaction allows for the formation of a thiol ester bond between an ubiquitin molecule and the E1 activating enzyme. The activated ubiquitin molecule is subsequently transferred on to an E2 enzyme, again through the formation of a thiol ester bond. The E2-ubiquitin complex interacts with an E3 ligase and this ultimately allows for the transfer of the ubiquitin molecule on to a lysine residue present on a substrate protein. This generates an amide isopeptide bond between the ε-amino group of the side chain of an acceptor lysine residue and the C-terminal G76 residue of ubiquitin.

isopeptide bond between the ε-amino group of the side chain of an acceptor lysine residue on the substrate and the C-terminal G76 residue of ubiquitin**.** Successive addition of ubiquitin molecules on to acceptor lysines of pre-existing ubiquitin moieties on a substrate results in substrate polyubiquitylation, whereas a single ubiquitin addition on to a substrate is known as monoubiquitylation.

The manner in which ubiquitin is transferred on to the substrate in the final step of the ubiquitylation process is dependent on the class of E3 ligase mediating the transfer. There are three primary classes of E3 ligases characterised by the HECT, RING and U-box domains (Reviewed in Ardley and Robinson, 2005, Metzger et al., 2012). HECT domain E3 ligases conjugate ubiquitin on to an internal cysteine for subsequent transfer on to its target substrate. RING domain E3 ligases act more as a scaffold protein, allowing for the transfer of the activated ubiquitin molecule directly from the E2 conjugating enzyme on to the substrate and the U-box E3 ligases have been shown to additionally elongate pre-existing ubiquitin chains on substrates.

1.5.2 Specificity of the ubiquitylation process

Ubiquitylation is involved in regulating many cellular processes and therefore needs to be highly specific in terms of substrate selection. Substrate specificity is provided primarily by the E2-E3 complex as well as signals on substrates that mark them for ubiquitylation by E3 ligases.

1.5.2.1 *E2-E3 complex*

There are over 30 E2 conjugating enzymes and more than 1,000 E3 ligases encoded by human DNA. Substrate specificity is provided primarily by the E3 ligase since a single E3 ubiquitin ligase targets either one or a small group of substrate proteins for ubiquitylation. Furthermore, some E3 ligases can interact with multiple E2's

to expand its specificity in terms of substrate selection. It is the cellular regulation of the activities of each of the individual E2 enzymes and E3 ligases that ultimately permits for the immense regulatory power held by the ubiquitylation process in cells.

1.5.2.2 *Substrate markers*

E3 ligases target substrates for ubiquitylation by the identification of markers that are either inherent to the substrate or generated on the substrate through post-translational modification. The most common markers are degrons that promote substrate ubiquitylation for subsequent proteasomal degradation. Cellular proteins may possess one of many types of degrons. The most common degrons are generated according to the N-end rule pathway. This pathway generates destabilising N-terminal residues (N-degrons) as a target for recognition and binding by E3s that have N-recognin sequences (Bachmair et al., 1986, Glickman and Ciechanover, 2002, Mogk et al., 2007, Tasaki et al., 2009). Post-translation modification of proteins in response to a cellular signal can also complete a degron in some cases. For instance, the hypoxia inducible factor 1 alpha, HIF-1α, transcription factor that is induced and activated in response to hypoxia is hydroxylated in normoxic conditions to form an oxygen-dependent degron (ODD) (Ivan et al., 2001). This degron is identified specifically by the Von Hippel-Lindau (VHL) subunit of an E3 ligase complex that promotes HIF-1α ubiquitylation for proteasomal degradation (Ivan et al., 2001, Jaakkola et al., 2001). Similarly, phosphorylation of a protein can result in the formation of a phosphodegron. The SCFSkp2/Cks1 E3 ligase for instance recognises phosphorylated p27 in the cell cycle regulating p27/Cdk2/E complex to promote p27 ubiquitylation and degradation (Tsvetkov et al., 1999, Nguyen et al., 1999). Additionally, partially degraded proteins or misfolded proteins can reveal an otherwise hidden degron.

1.5.3 Cellular role of protein ubiquitylation

Protein ubiquitylation can effect changes in protein stability, localisation and protein-protein interaction. The outcome of protein ubiquitylation varies primarily according to the type of ubiquitin chain added on to a substrate which in turn is mediated by the E2-E3 ligase complex (Reviewed in Nagy and Dikic, 2010). The E2-E3 ligase complex therefore is the primary factor dictating the outcome of ubiquitylation.

Ubiquitin is a highly conserved 76 amino acid protein that has within it seven lysines at positions K6, K11, K27, K29, K33, K48 and K63. Polyubiquitylation involves the addition of ubiquitin on to the lysine residues of another ubiquitin moiety or ubiquitin chain already present on the substrate and this can be added on to any of the seven lysines. The linkage type is a major factor regulating the outcome of protein ubiquitylation (Reviewed in Dikic et al., 2009). For instance, K48-linked ubiquitin chains tend to target a protein for proteasomal degradation whereas K63-linked chains play a major role in signal transduction through its ability to alter protein interactions with other proteins that contain ubiquitin binding domains (UBDs).

1.5.3.1 *Proteasomal degradation*

The most common outcome of protein ubiquitylation is protein targeting for degradation through the highly conserved ubiquitin proteasomal pathway (Hershko and Ciechanover, 1982). In this pathway, the 26S proteasome is responsible for the identification and subsequent cleavage of ubiquitylated proteins. The 26S proteasome is a hollow barrel-shaped structure that has a 19S regulatory unit at either or both ends of the barrel with two alpha and 2 beta subunits making up the 20S unit in the middle.

Polyubiquitylated proteins that contain the K48 ubiquitin linkage chain are the main substrates for proteasomal degradation. The identification of ubiquitylated proteins for proteasomal degradation is carried out by ubiquitin receptors and factors present in

the 19S subunit of the proteasome. These receptors and factors possess ubiquitin binding domains (UBDs) that can bind to ubiquitylated proteins (Reviewed in Dikic et al., 2009). Once bound to the 19S proteasomal subunit, the tertiary structure of the targeted ubiquitylated protein is unfolded in an ATP-dependent fashion and deubiquitylation enzymes that form part of the 19S subunit allow for the removal and recycling of the ubiquitin units. The processed peptide is subsequently weaved through the hollow catalytic core of the proteasome where it is cleaved in to many small peptides by the catalytic activity of the proteasome beta subunits. This degradation process not only rids the cell of an unwanted protein, it reintroduces ubiquitin monomers and amino acids back in to the cellular milieu for reuse.

Proteasomal degradation is a key process regulating the availability and consequently the activity of proteins in cells. A significant role for this process has more recently been identified in the regulation of multiple DNA repair processes to include BER (Reviewed in Dianov et al., 2011). Our lab has recently shown that the availability of key BER factors XRCC1, Pol β and DNA Lig IIIα are regulated through proteasomal degradation (Parsons et al., 2008, Parsons et al., 2009, Parsons et al., 2010, Parsons et al., 2011). The E3 ligases Mule and CHIP were identified as cellular E3 ligases responsible for the ubiquitylation of Pol β (Parsons et al., 2008, Parsons et al., 2009). Loss of this regulatory mechanism results in increased levels of Pol β in cells (Parsons et al., 2008, Parsons et al., 2009). High levels of Pol β in cells have been shown to induce mutagenesis and therefore regulation of the availability of Pol β is necessary for the correct regulation and functioning of the DNA base excision repair pathway, a key pathway involved in maintaining genome stability (Chan et al., 2007, Canitrot et al., 1998, Tan et al., 2005, Luo et al., 2012).

1.5.3.2 *Protein-protein interaction*

Ubiquitylation of a protein can promote its interaction with other proteins that have an ubiquitin binding domain (UBD). There are more than 20 families of UBD's that allow proteins to interact with other proteins modified by ubiquitin depending on the ubiquitin chain linkage type and chain length (Reviewed in Husnjak and Dikic, 2012). Additionally, a UBD-containing protein usually also possesses an additional domain allowing for its direct interaction with the protein that is ubiquitylated to provide interaction specificity. Indeed, the receptors and factors of the 19S proteasome subunit that recognise ubiquitylated proteins for processing contain primarily UBDs that are capable of identifying K48-linked polyubiquitin chains (Reviewed in Husnjak and Dikic, 2012).

Protein interactions mediated through ubiquitylation and UBDs also play an important role outside of protein degradation. Interaction between ubiquitylated factors and UBD-containing proteins was shown to be of great importance in signal transduction, for instance in promoting the recruitment of DNA double strand break repair factors to a repair site (Reviewed in Hofmann, 2009). The recruitment of BRCA1, an important DNA double strand break repair factor, to a repair site on DNA is mediated by the actions of the E2 ubiquitin conjugating enzyme 13 (UBC13), the E3 ligases RING finger protein 8 (RNF8) and RNF168 (Huen et al., 2007, Kolas et al., 2007, Mailand et al., 2007, Doil et al., 2009, Stewart et al., 2009, Wang and Elledge, 2007). The binding of RNF8 to ATM-phosphorylated MDC1 at a DNA double strand break site recruits the UBC13 E2 ligase. UBC13 together with RNF8 ubiquitylate the histones H2A and H2AX. The E3 ligase RNF168 subsequently binds to the ubiquitin modification on these histones through its UBD, motif interacting with ubiquitin (MIU). The RNF168 E3 ligase further amplifies the ubiquitin signal through polyubiquitylation to attract the binding of receptor associated protein 80 (RAP80) to the ubiquitin modification through its UBD, the ubiquitin interaction motif (UIM). RAP80 subsequently binds the phosphorylated

scaffold protein abraxas that binds to and stabilises BRCA1, as well as other DSB repair factors, at the repair site (Wang et al., 2007). Interactions mediated through ubiquitin are therefore vital for DSB signalling and repair completion.

Monoubiquitylation of a protein can also promote protein interactions through monoubiquitin binding UBDs. During translesion synthesis, on encountering a lesion, PCNA is monoubiquitylated at K164 by the E3 ligase RAD18 combined with the E2 enzyme Rad6 (Kannouche and Lehmann, 2004, Kannouche et al., 2004, Hoege et al., 2002, Stelter and Ulrich, 2003). This promotes a PCNA switch from replicative polymerases to translesion polymerases that contain the UBDs, ubiquitin binding zinc finger (UBZ) and ubiquitin binding motif (UBM) (Acharya et al., 2008). This polymerase switch allows the replication system to bypass the damage error-free.

1.5.3.3 *Protein cellular localisation*

Ubiquitylation of proteins can also modulate the localisation of proteins within cellular compartments. Monoubiquitylation of the transcription factor FOXO4 in response to oxidative stress allows for FOXO4 localisation from the cytoplasm to the nucleus without any changes in FOXO4 levels (van der Horst et al., 2006). Similarly, p53 ubiquitylation by the E3 ligase MSL2 promotes p53 localisation to the cytoplasm without affecting p53 stability (Kruse and Gu, 2009).

1.5.4 Deubiquitylation

The removal of ubiquitin moieties from proteins, termed deubiquitylation, also provides an additional level of protein regulation (Reviewed in Reyes-Turcu et al., 2009). As with ubiquitylation, this process is highly specific and is mediated by the activity of isopeptidases or deubiquitylation enzymes (DUBs). Indeed, more than 90 DUBs are encoded by the human genome, each belonging to one of the five DUB families that

exist, the largest of which is the ubiquitin-specific protease (USP) family that currently has approximately 55 cysteine protease members. Each DUB displays specificity for the different types of ubiquitin chains present on proteins and additionally for the substrate itself. Such specificities are important since deubiquitylation plays a major role in regulating several cellular processes including DNA repair and chromatin remodelling. The DUB ubiquitin-specific protease 3, USP3, for instance is implicated in switching off ubiquitin-mediated signalling during DNA double strand break repair by removing ubiquitin moieties attached to histones (Nicassio et al., 2007). DUBs are also particularly important within the ubiquitin proteasome pathway for the recycling of ubiquitin units prior to peptide cleavage by the proteasome (Reviewed in Weissman et al., 2011). Other than its role within the UPP, deubiquitylation of a protein can also promote the cellular stability of a protein since ubiquitylated proteins that are targeted for degradation can be deubiquitylated prior to proteasomal processing, thus reintroducing these proteins back into the cellular pool. This was shown to be the case for the ubiquitylated form of the base excision repair enzyme Pol β that is deubiquitylated by ubiquitin-specific protease 47, USP47, to allow for fine-tuned regulation of its availability in cells (Parsons et al., 2011).

1.6 PROJECT AIMS

The involvement of the key BER enzyme, APE1, in processes such as DNA repair, cellular proliferation and survival make it a desirable target for inhibition in the treatment of diseases such as cancer. Since dysregulation of the levels of APE1 as part of the BER pathway are known to have detrimental effects on genome stability, it is of major interest to determine the cellular mechanisms regulating APE1 cellular levels. This will not only allow us to understand the potential cellular effects of APE1 inhibition in normal cells, it will also allow us to understand the regulation of APE1 in the context of BER regulation and related diseases. Modulation of the availability of some of the BER repair enzymes appears to be through a fine balance of transcriptional expression and proteasomal degradation. Ubiquitylation-mediated proteasomal degradation has thus far been shown to negatively regulate the availabilities of the key BER enzymes XRCC1, DNA Lig IIIα and Pol β for repair, however there is no known role for ubiquitylation in regulating APE1 levels in cells.

The study presented in this thesis therefore aims to determine the role of ubiquitylation in regulating APE1 in cells. To carry out this study, the following key questions were asked to guide the progress of the research.

1. Can we observe a naturally occurring ubiquitylated form of APE1 in human cells?

2. If cellular APE1 is ubiquitylated in cells, what is the cellular E3 ligase that specifically mediates this activity?

3. Using the E3 ligase as a research tool, are we able to determine the effect of modulating APE1 ubiquitylation on cellular APE1?

4. Given the role of APE1 in the maintenance of genome stability, does the E3 ligase equally play a role in maintaining genome integrity?

Chapter 2

MATERIALS AND METHODS

2.1 MATERIALS

2.1.1 Reagents

General laboratory reagents and chemicals were obtained from Sigma Aldrich (Poole, UK).

2.1.2 Antibodies

Human APE1 (polyclonal) and PARP1 (polyclonal) antibodies were purified in house (Dianova et al., 2001) and UBR3 antibodies were generated as previously described (Tasaki et al., 2007). Antibodies against APE1/APEX (13B8E5C2), ubiquitin (Ubi-1), beta-actin (AC-15), hsp70 (BRM-22), Lig IIIα (6G9), Pol β (polyclonal), GFP (polyclonal) and histone H3 (polyclonal) were purchased from Abcam (Cambridge, UK). Antibodies against p14-ARF (polyclonal) and 53BP1 (polyclonal) were obtained from Bethyl Laboratories (Montgomery, USA). Alpha-tubulin (DM1A) antibodies were purchased from Sigma-Aldrich (Gillingham, UK), MDM2 (SMP14) and CDK5 (polyclonal) antibodies were obtained from Santa Cruz (California, USA), FLAG M2 (L5) antibodies were obtained from Agilent Technologies (Stockport, UK) and γH2AX (JBW301) antibodies were purchased from Millipore (Watford, UK). The secondary antibodies, AlexaFluor 680/800 nm goat anti-rabbit and Alexafluor 680/800 nm goat anti-mouse polyclonal antibodies were obtained from Molecular Probes, Invitrogen (Paisley, UK).

The reactivity of the antibodies and the dilutions used for Western blotting and immunostaining are further summarised in **Table 2.1**.

2.1.3 Cells

HeLa, HCT116 and U2OS cells were cultured as a monolayer in Dulbeccos Modified Eagle Medium (DMEM) containing GlutaMAX, 1mg/ml D-glucose and pyruvate (Invitrogen, Paisley, UK) further supplemented with 10% foetal bovine serum purchased from Gibco (Invitrogen, Paisley, UK). The *Ubr3* +/+ and *Ubr3* -/- mouse embryonic fibroblasts (MEFs) were cultured as a monolayer in DMEM containing GlutaMAX, 4.5 mg/ml D-glucose and pyruvate (Invitrogen, Paisley, UK) supplemented with 10% foetal bovine serum (Gibco, Invitrogen, Paisley, UK) and 1% L-glutamine. All cells were incubated at 37 °C in a 5% CO_2 incubator. The *Ubr3* +/+ and *Ubr3* -/- MEFs were isolated from *Ubr3* +/+ and *Ubr3* -/- mouse embryos as recently described (Tasaki et al., 2007). Briefly, embryonic day 13.5 embryos (E13.5) were generated in the 129SvImJ/C57BL/6 genetic background and selected for primary MEF isolation. The MEFs were immortalised by repeated culture of the primary MEFs over a time period of 2 months by plating approximately 1.5 x 10^6 cells onto a 10 cm^2 plate every 3-5 days.

2.1.4 Plasmids and proteins

Histidine-tagged APE1 protein was generated from the pET14b plasmid containing the *Ape1* gene insert that was kindly provided by Prof. Ian Hickson (University of Oxford). Mutant *Ape1* was generated from this plasmid by site directed mutagenesis (described in section 2.2.11). Plasmids were expressed in Rosetta expression bacteria from Stratagene (La Jolla, CA, USA) and the proteins purified using Ni-NTA agarose beads (Qiagen, Crawley, UK) following the manufacturer's instructions. Mouse *Ape1* (*Apex*) in pQE30 plasmid was kindly provided by Dr. Nakabeppu

Antibody	Reactivity (Isotype)	Clone	Dilution
anti-53BP1	Rabbit (IgG)	Polyclonal	1:300 (IS)
anti-β-actin	Mouse (IgG1)	AC-15	1:10,000 (WB)
anti-APE1 (Human)	Rabbit	Polyclonal	1:10,000 (WB) 1:300 (IS)
anti-APE1 (Mouse)	Mouse (IgG2b)	13B8E5C2	1:5,000 (WB) 1:300 (IS)
anti-CDK5	Rabbit (IgG)	Polyclonal	1:1,000 (WB)
anti-FLAG M2	Mouse (IgG1)	L5	1:2000 (WB)
anti-GFP	Rabbit (IgG)	Polyclonal	1:1000 (WB)
anti-γ-H2AX	Mouse (IgG1)	JBW301	1:2,000 (WB)
anti-histone H3	Rabbit (IgG)	Polyclonal	1:1,000 (WB)
anti-hsp70	Mouse (IgG1)	BRM-22	1:5,000 (WB)
anti-Lig IIIα	Mouse (IgG1)	6G9	1:1000 (WB)
anti-MDM2	Mouse (IgG1)	SMP14	1:1,000 (WB)
anti-p14-ARF	Rabbit (IgG)	Polyclonal	1:2,000 (WB)
anti-PARP1	Rabbit (IgG)	Polyclonal	1:5000 (WB)
anti-Pol β	Mouse (IgG)	Polyclonal	1:1000 (WB)
anti-α-tubulin	Mouse (IgG1)	DM1A	1:10,000 (WB)
anti-ubiquitin	Mouse (IgG1)	Ubi-1	1:5,000 (WB)
anti-UBR3	Rabbit	Polyclonal	1:1,000 (WB) 1:300 (IS)
AlexaFluor 680/800 nm	Rabbit	Polyclonal	1:10,000
AlexaFluor 680/800 nm	Mouse	Polyclonal	1:10,000

Table 2.1. Antibodies used in this study. Reactivity, isotype, clonality and dilutions used for Western blotting (WB) and Immunostaining (IS) are shown.

(Tominaga et al., 2004). The *Apex* gene insert was copied from the bacterial expression plasmid pQE30 by PCR using the primers; Forward: 5'-GTACGAATCCATGCCAAAGCGGGGAAAGAAAGC-3'; Reverse: 5'-GTACCTCGAGC AGTGCTAGGTAAAGGGTGATGGGAC-3' that additionally contain overhangs coding for the restriction sites, EcoR1 and Xho1. Using these restriction site overhangs, the *Apex* gene was subcloned into the mammalian expression vector, pCMV3Tag3a plasmid (Agilent Technologies, Stockport, UK). A high copy number vector was used to express N-terminal FLAG-tagged mouse UBR3 protein for FLAG immunoprecipitation from the PADH1 promoter in the SC295 *S. cerevisiae* strain (MATa, GAL4 GAL80 ura3-52, leu2-3,112 reg1-501 gal1 pep4-3) as previously described (Tasaki et al., 2007). Ubiquitin, E1 activating enzyme and the individual E2 conjugating enzymes, UbcH2, UbcH3, UbcH5a, UbcH5b, UbcH5c, UbcH6, UbcH7, UbcH8 and UbcH10 were bought from Boston Biochemicals (Cambridge, Boston).

2.2 METHODS

2.2.1 Whole cell extracts

Cells were grown on 10 cm^2 dishes and at the appropriate confluency or time-point, were washed twice in ice-cold phosphate-buffered saline (PBS). They were then scraped in ice-cold PBS and pelleted by centrifugation in an Eppendorf centrifuge 5804R (A-4-44 rotor) at 1500 rpm at 4 °C for 5 min, re-suspended in 1 ml ice-cold PBS and centrifuged in a Eppendorf centrifuge 5417R (A-8-11 rotor) at 1500 rpm at 4 °C for 5 min. Whole cell extracts were prepared from the pellets using Tanaka's method (Tanaka et al., 1992). Briefly, cells were resuspended in one packed cell volume (PCV) of buffer I containing 10 mM Tris-HCl (pH 7.8), 200 mM KCl, 1 µg/ml of each of the protease inhibitors pepstatin, aprotinin, chymostatin and leupeptin, all from Roche Diagnostics Ltd (Lewes,UK), and 1 mM PMSF. Two PCVs of buffer II containing 10 mM Tris-HCl (pH 7.8), 600 mM KCl, 40% glycerol, 0.1 mM EDTA and 0.2% Nonidet P-40, 1 µg/ml of each

of the protease inhibitors pepstatin, aprotinin, chymostatin and leupeptin and 1 mM PMSF was then added and the suspension mixed thoroughly by rocking for 30 min at 4 °C. The cell lysate was then centrifuged in a Beckman Coultier Optima MAX-XP Ultracentrifuge (TLA-55 rotor) at 40,000 rpm at 4 °C for 20 min and the supernatant was carefully collected, aliquoted and stored at -80 °C for further use.

2.2.2 Cell fractionation

Cells were fractionated into cytoplasmic and nuclear fractions as previously described (Woodhouse et al., 2008). Briefly, cell pellets were resuspended in 2 PCV of Buffer I (20 mM Tris-HCl pH 7.4, 2.5 mM $MgCl_2$, 0.5% Nonidet P-40) containing 1 mM PMSF and 1 µg/ml each of aprotinin, pepstatin, chymostatin and leupeptin. The suspension was incubated on ice for 10 min before centrifuging in an Eppendorf centrifuge 5417R (A-8-11 rotor) at 10,000 rpm for 2 min. The supernatant was collected as the cytoplasmic fraction (C). Two PCV of buffer II (20 mM PO_4 pH 8.0, 0.5 M NaCl, 1 mM EDTA, 0.75% Triton X-100, 10% glycerol and 5 mM $MgCl_2$) containing 1 mM PMSF, 1 µg/ml each of aprotinin, pepstatin, chymostatin and leupeptin, and was added to the nuclear pellet and incubated on ice for 10 min before centrifuging again at 10,000 rpm for 2 min. The supernatant was collected as the nuclear fraction (N). Protein concentrations were determined using the Bradford assay (described in section 2.2.3) and the extracts stored at -80 °C. For the identification of the ubiquitylated form of APE1, the deubiquitylating enzyme inhibitor NEM was also added to buffers at a final concentration of 1 mM.

2.2.3 Protein concentration: Bradford assay

Protein concentrations were measured by the Bradford assay using Bio-Rad Protein assay reagent (Bio-Rad, Hemel Hempstead, UK) for measurement of the

absorbance reading at an optical density of 595 nm using a UV spectrophotomer. A BSA protein standard (0.2 mg/ml) was used as a reference for calculation of the protein concentration.

2.2.4 Western blotting

Proteins were separated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE). Protein samples were diluted in loading buffer (25 mM Tris, pH 6.8, 2.5% mercaptoethanol, 1% SDS, 5% glycerol, 1 mM EDTA and 0.05 mg/ml bromophenol blue) and denatured at 95 °C for 5 min before loading on to a 1.5 mm 10% SDS-polyacrylamide gel (Invitrogen, Paisley, UK). The All-Blue Precision Plus Protein Standards (Bio-Rad Ltd., Hercules, CA, USA) was used as a protein marker ranging 10-250 kDa in size. Electrophoresis was carried out at 125 V for 2 hr in SDS-PAGE running buffer (25 mM Tris, pH 8.3, 192 mM glycine, 0.1% SDS). The separated proteins were then transferred onto an Immobilon-FL polyvinylidene difluoride (PVDF) membrane, 0.45 µm pore size (Millipore, Billerica, MA, USA), at 25 V for 1.5 hr in SDS-PAGE transfer buffer (25 mM Tris, pH 8.3, 192 mM glycine, 20% methanol). The membrane was blocked in Odyssey Blocking Buffer (LI-COR Biosciences, Cambridge, UK) diluted 1:1 in PBS for 1 hr at room temperature. The primary antibodies were diluted according to **Table 2.1** in to Odyssey Blocking Buffer diluted 1:1 in PBS and containing 0.1% Tween-20 and the membranes were incubated therein overnight at 4 °C. The membrane was washed three times in PBS containing 0.1% Tween-20 and then incubated for 1 hr in a similarly diluted secondary antibody. The membranes were again washed three times for 5 min in PBS with 0.1% Tween-20 and visualised using the Odyssey image analysis system (Li-cor Biosciences, Cambridge, UK).

2.2.5 Immunostaining

Cell monolayers were grown on coverslips to sub-confluent levels. The cells were fixed on to the coverslips by incubating with 4% paraformaldehyde for 30 min at room temperature. Each coverslip was washed three times with PBS (Sigma, Gillingham, UK) and the cells made permeable by treating with 0.2% (w/v) Triton X-100 for 10 min at 4 °C. The coverslips were subsequently blocked at room temperature with 2% (w/v) BSA - Fraction V for 1 h before incubating overnight with primary antibodies against APE1, UBR3 or 53BP1 diluted 1:300 in BSA. The coverslips were washed three times with PBS and incubated in secondary antibodies Alexa Fluor 488 $F(ab')_2$ fragment of goat anti-rabbit or anti-mouse (Invitrogen, Paisley, UK) for 1 hr. The coverslips were mounted on to slides using DAPI stain-containing Vectasheild hard set mounting media (Vector Laboratories, Peterborough, UK) prior to imaging on a BioRad confocal microscope using a 488 nm (FITC) argon laser lines for excitation.

2.2.6 Purification of ubiquitylated APE1

HeLa WCE was prepared from a 20 g HeLa pellet (Cilbiotech, Mons, Belgium) as described above and dialysed overnight against Buffer A (50 mM Tris pH 8.0, 1 mM EDTA, 5% glycerol, 1 mM DTT, 1 mM protease inhibitor PMSF, 1 mM deubiquitylating enzyme inhibitor NEM) containing 150 mM KCl. The dialysate was clarified by centrifugation using a Beckman Avanti J-25 centrifuge (JA-25.50 rotor) at 25,000 rpm for 20 min at 4 °C and subsequent filtering through 0.45 µm filters. The clarified dialysate was passed over a 250 ml Phosphocellulose cation exchange column pre-equilibrated with Buffer A containing 150 mM KCl. The bound proteins were eluted with Buffer A containing 1000 mM KCl, and concentrated using Vivaflow 50 and a 10 kDa MWCO PES membrane module (Sartorius Stedim, Aubagne, France). The resulting solution was concentrated and buffer exchanged in to Buffer A containing 50 mM KCl before further fractionation on a Mono-Q anion exchange column (GE Healthcare, Little

Chalfont, UK) equilibrated with Buffer A containing 50 mM KCl. The column was washed with 5 column volumes of Buffer A containing 50 mM KCl and bound proteins eluted using a gradient of Buffer A containing 50 mM KCl against Buffer A containing 1000 mM KCl. The flow-through was collected and further fractionated on a Mono-S cation exchange column (GE Healthcare, Little Chalfont, UK) pre-equilibrated with Buffer A containing 50 mM KCl. The column was washed with 5 column volumes of Buffer A containing 50 mM KCl and the proteins eluted using a gradient of Buffer A containing 50 mM KCl against Buffer A containing 1000 mM KCl. After each elution, the collected protein peak fractions were separated by 10% SDS-PAGE, transferred on to a PVDF membrane and analysed by Western blotting using APE1 or ubiquitin antibodies for protein identification.

2.2.7 Purification of E3 ligase activity

HeLa cytoplasmic extract was prepared from a 20 ml pellet (Cilbiotech, Mons, Belgium) as described previously (described in section 2.2.2) and dialysed overnight against Buffer A (50 mM Tris pH 8.0, 1 mM EDTA, 5% glycerol, 1 mM DTT, 1 mM PMSF) containing 150 mM KCl. The dialysate was clarified by centrifugation at 25,000 rpm for 20 min at 4 °C and subsequent filtering through 0.45 µm filters. The clarified dialysate was passed over a 250 ml Phosphocellulose cation exchange column pre-equilibrated with Buffer A containing 150 mM KCl and 1 mM NEM. The flow-through was collected and concentrated using Vivaflow 50 and a 10 kDa MWCO PES membrane module (Sartorius Stedim, Aubagne, France). It was further diluted to a final concentration of 75 mM KCl and loaded on to a 20 ml HiLoad Mono-Q Sepharose anion exchange column (GE Healthcare, Little Chalfont, UK). The column was washed with 5 column volumes of Buffer A containing 50 mM KCl and the proteins eluted using a gradient of Buffer A containing 50 mM KCl against Buffer B containing 1000 mM KCl. The fractions eluted at around 350 mM KCl that had ubiquitylation activity were pooled

and concentrated to 1 ml using Amicon Ultra-15 filter units (Millipore, Watford, UK) and fractionated over a Superose-12 size exclusion column (GE Healthcare, Little Chalfont, UK) pre-equilibrated with Buffer A containing 150 mM KCl. Active fractions identified in the 200 kDa to 400 kDa size range were pooled and diluted to 50 mM KCl before loading on to a 1 ml Mono Q HR 5/5 anion exchange column (GE Healthcare, Little Chalfont, UK) pre-equilibrated in buffer A containing 50 mM KCl. The column was washed with 5 column volumes of Buffer A containing 50 mM KCl and the proteins eluted using a gradient of Buffer A containing 50 mM KCl against Buffer B containing 1000 mM KCl. At each purification stage, the ubiquitylating activity of each fraction on APE1 was determined by *in vitro* ubiquitylation of recombinant histidine-tagged APE1 using the individual fractions as the E3 source. Active fractions were pooled for further purification and the active fractions from the Mono Q HR 5/5 column were analysed by tandem mass spectrometry for protein identification as previously described (Parsons et al., 2009).

2.2.8 *In vitro* ubiquitylation assay

The *in vitro* ubiquitylation assay was carried out as previously described (Parsons et al., 2008). His-tagged APE1 (100 ng) was ubiquitylated *in vitro* using a reaction mix of 15 µl final volume containing 0.7 pmol E1 activating enzyme, 9.5 pmol E2 enzyme mix or as indicated, E3 source as indicated, and 0.6 pmol of either wild-type ubiquitin or mutant ubiquitin that is unable to form polyubiquitin chains (Boston Biochem, Cambridge, USA), 4 mM ATP in ubiquitylation buffer containing 25 mM Tris-HCl pH 8, 5 mM $MgCl_2$, 200 µM $CaCl_2$, 1 mM DTT and 10 µM MG-132. The reaction mix was incubated at 30 °C for 1 hr and terminated by the addition of 3X SDS PAGE sample buffer. Proteins were separated by SDS-PAGE and analysis was carried out further by Western blotting.

2.2.9 APE1 ubiquitylation site identification

The 35 N-terminal amino acids of the APE1 protein were cleaved by trypsinisation. 8 µg of APE1 was incubated in buffer containing 50 mM Tris-HCl (pH 7.4), 50 mM KCl, 10 mM MgCl2, 1 M urea and 0.5 µg of trypsin for 2 hr at 23 °C. Trypsin and the N-terminal cleaved product were removed by buffer exchanging the reaction three times into RWDB buffer using the Amicon Ultra-4 filter units (Millipore, Watford, UK). The N-terminal cleaved APE1 was as such subjected to an *in vitro* ubiquitylation assay for analysis by Western blotting. The reaction for full length APE1 *in vitro* ubiquitylated by UBR3 was separated by 10% SDS-PAGE and the gel stained with InstantBlue Coomassie blue stain (Expedeon Protein Solutions, Harston, UK). The band correlating to monoubiquitylated APE1 was excised, digested and trypsinised before analysis by tandem mass spectrometry. Mass spectrometry was used for identification of the sites of ubiquitylation on APE1 as previously described (Parsons et al., 2009).

2.2.10 Mass spectrometry

Both the E3 ubiquitin ligase active fractions obtained following chromatography and the *in vitro* APE1 monoubiquitylated bands that were excised from the 10% polyacrylamide gel were sent to Dr. Benedikt Kessler's mass spectrometry lab based at the University of Oxford for identification of protein and ubiquitylation sites, respectively. There, the samples were subject to digestion by trypsinisation and LC-MS/MS tandem mass spectrometry was used to analyse the digested material using either a high capacity iontrap (HCTplusTM, Bruker Daltonics, Bremen, Germany) or a nanoAcquity UPLC system coupled to a quadrupole time-offlight (QTOFpremierTM) tandem mass spectrometer (Waters, Milford, USA). The LC-MS/MS mass spectrometry results were analysed according to published guidelines (Taylor and Goodlett, 2005) and the MS/MS spectra were compared against the SwissProt database (release version 54.0, 07/2007) using Mascot version 2.2 (MatrixScience, London, UK).

2.2.11 Site-directed mutagenesis

The QuikChange Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA, USA) was used to carry out site directed-mutagenesis and the primers obtained from Eurogentec (Southampton, UK) that were used are listed in **Table 2.2**. The primers were purified on a non-denaturing polyacrylamide gel before use. The PCR reaction mix was prepared using 1X reaction buffer provided in the kit, 5% DMSO, 35 ng plasmid containing the full length gene, 125 ng each of purified forward and reverse primers, 25 µM dNTP and made up to a 50 µl reaction volume using deionised water. PfuTurbo DNA polymerase (2.5 U) provided with the kit was added to each mix and the mix placed into a thermal cycler programmed at 95 °C for 30 s followed by 18 cycles of 95 °C for 30 s, 55 °C for 1 min and 68 °C for 12 min. Upon completion, 10 U DpnI was added and the mix incubated at 37 °C for 2 h. Competent XL1 cells (Stratagene, La Jolla, CA, USA) were transformed with the final mutagenesis reactions according to the manufacturer instructions and plated on to LB-agar (Merck Biosciences, Nottingham, UK) plates containing suitable antibiotics obtained from VWR International Ltd (Poole, UK). The antibiotic resistant colonies were selected, expanded in 7 ml LB (Merck Biosciences, Nottingham, UK) containing suitable selection antibiotics and the plasmids purified using the Qiaprep Miniprep kit (Qiagen, Crawley, UK) according to the manufacturer's protocol.

2.2.12 DNA purification

Plasmids were purified from 7 ml of transformed bacterial culture using the Qiaprep Miniprep kit (Qiagen, Crawley, UK) according to the manufacturer's protocol. Plasmid DNA concentrations were determined using the NanoDrop ND-1000 Spectrophotometer (software version V3.5.2) at a wavelength of 230 nm.

APE1 Mut.	SDM primer sequence
K7R	For. 5'-GCCGAAGCGTGGGAAAAGGGGAGCCGTGGC-3' Rev. 5'-GCCACCGCTCCCCTTTTCCCACGCTTCGGC-3'
K24R	For. 5'-GACAGAGCCAGAGGCCAGGAAGAGTAAGAC-3' Rev. 5'-GTCTTACTCTTCCTGGCCTCTGGCTCTGTC-3'
K35R	For. 5'-GCCGCAAAGAAAAATGACAGAGAGGCAGCAGGAGAGGGCCCAGC-3' Rev. 5'-GCTGGGCCCTCTCCTGCTGCCTCTCTGTCATTTTTCTTTGCGGC-3'
K6-7R	For. 5'-GCCGAAGCGTGGGAGAAGGGGAGCGGTGGC-3' Rev. 5'-GCCACCGCTCCCCTTCTCCCACGCTTCGGC-3'
K24-27R	For. 5'-GCCAGAGGCCAGGAGGAGTAGGACGGCCGCAAAGAAAAATGACAAAGAGGC-3' Rev. 5'-GCCTCTTTGTCATTTTTCTTTGCGGCCGTCCTACTCCTCCTGGCCTCTGGC-3'
K31-32R	For. 5'-GCCAAGAAGAGTAAGACGGCCGCAAGGAGAAATGACAAAGAGGCAGC-3' Rev. 5'-GCTGCCTCTTTGTCATTTCTCCTTGCGGCCGTCTTACTCTTCTTGGC-3'
K31-35R	For. 5'-GGACGGCCGCAAGGAGAAATGACAGAGAGGCAGCAGGAGAGGGCCCAGC-3' Rev. 5'-GCTGGGCCCTCTCCTGCTGCCTCTCTGTCATTTCTCCTTGCGGCCGTCC-3'
T233A	For. 5'-GAATGCTGGCTTCGCGCCACAAGAGCG-3' Rev. 5'-CGCTCTTGTGGCGCGAAGCCAGCATTC-3'
T233E	For. 5'-GAATGCTGGCTTCGAGCCACAAGAGCGCCAAG-3' Rev. 5'-CTTGGCGCTCTTGTGGCTCGAAGCCAGCATTC-3'

Table 2.2: Site-directed mutagenesis primers used in this study.

2.2.13 Protein expression

Bacterial expression plasmid gene inserts were expressed in bacterial Rossetta cells (Stratagene, La Jolla, CA, USA). 10 ng purified plasmid was mixed with 40 µl electrocompetent Rossetta cells on ice and electroporated at 0.5 V. The cells were resuspended into 1 ml SOB and grown for 1 hr at 37 °C before spreading on to an LB agar (Merck Biosciences, Nottingham, UK) plate containing the suitable selection antibiotics (VWR International Ltd, Poole, UK) for overnight growth. Successfully transformed colonies were grown overnight in 5 ml LB (Merck Biosciences, Nottingham, UK) containing suitable selection antibiotics, diluted in 50 ml LB and left to grow at 37 °C. At OD 600 = 0.5, 1 mM IPTG (VWR International Ltd, Poole, UK) was added to the culture and the culture incubated for approximately 2 hr at 37 °C to induce protein expression. The Rossetta cells were subsequently pelleted for protein purification and stored at -80 °C.

2.2.14 Ni-NTA affinity purification

Histidine-tagged APE1 and mutants were purified from expression bacterial pellets on Ni-NTA agarose (Qiagen, Crawley, UK) following the manufacturer's instructions. Briefly, bacterial pellets were re-suspended in lysis buffer containing 20 mM Tris-HCl, pH 8.0, 0.5 M NaCl, 10 mM imidazole and supplemented with 1 mM PMSF and 1 µg/ml each of the protease inhibitors chymostatin, apoprotin, pepstatin and leupeptin (Roche Diagnostics Ltd, Lewes, UK). The cells were further disrupted by the addition of lysozyme (Sigma, Gillingham, UK) and shearing through sonication. The lysate was clarified by centrifugation in a Beckman Coultier Optima L-100 XP Ultracentrifuge (SW40Ti rotor) at 40,000 rpm for 10 min and filtering through 0.45 µM filters. The clarified lysate was incubated with Ni-NTA agarose for 2 hr at 4 °C. The beads were washed thrice with lysis buffer, centrifuging at 1200 rpm for 2 min using an Eppendorf centrifuge 5415D (F45-24-11 rotor) to allow for separation of beads from buffer. The

bound proteins were eluted using elution buffer containing 20 mM Tris-HCl, pH 8.0, 0.5 M NaCl, 250 mM imidazole and the resulting elute was dialysed overnight against buffer containing 50 mM Hepes pH 7.8, 50 mM KCl, 1 mM EDTA, 10% glycerol. The purity was analysed using the Experion Electrophoresis System (Bio-Rad, Hemel Hempstead, UK) and the protein solution aliquoted and stored at -80 °C. All steps were performed on ice or at 4 °C.

2.2.15 Reverse transcriptase and real-time PCR

For both reverse transcriptase and real-time PCR, total mRNA was purified from cells using the RNeasy kit (Qiagen, Crawley, UK) according to the manufacturer's protocol. The mRNA concentration was determined using the NanoDrop ND-1000 Spectrophotometer (software version V3.5.2) at a wavelength of OD 230 nm. The SuperScript RT-PCR system (Invitrogen, Paisley, UK) was further used to generate cDNA from the purified mRNA according to the manufacturer's protocol. For reverse transcriptase PCR analysis, the cDNA for APE1, UBR3 and actin was amplified by PCR using primers obtained from Eurogentec (Southampton, UK) as summarised in **Table 2.3**. The PCR conditions were set for 1 cycle at 95 °C for 5 min, 25 cycles (or 30 cycles for UBR3) at 95 °C for 30 sec, 55 °C for 20 sec and 72 °C for 20 sec, and 1 cycle at 72 °C for 5 min. The amplification products were analysed by 2% agarose gel electrophoresis containing SYBR Green (Invitrogen, Paisley, UK) and imaged using the Molecular Imager FX system (Bio-Rad, Hemel Hempstead, UK).

For real-time PCR analysis, the Taqman SYBR green kit was used for cDNA amplification and data collection using the 7500 FastReal-Time PCR system (Applied Biosystems, Paisley, UK), software version v.2.0.3. Software analysis of the run provided the cycle threshold (CT value) and further calculations according to the

Gene (mouse)	GenBank Accession	RT-PCR primer sequence	Amplicon location
Actin	NM_007393.3	For. 5'-GGAGGGGGTTGAGGTGTT-3' Rev. 5'-TGTGCACTTTTATTGGTCTCAAG-3'	1803 -1872
APEX	NM_009687.2	For. 5'-TCCTTCTCGGTTTTCTTTGC-3' Rev. 5'-CGGGGAAGAACCCAAGTC-3'	174 - 242
GAPDH	NM_008084.2	For. 5'-CTGGCACTGCACAAGAAGAT-3' Rev. 5'-GGGTTCCTATAAATACGGACTGC-3'	13 - 88
p14-ARF	NM_009877.2	For. 5'-GGGTTTTCTTGGTGAAGTTCG-3' Rev. 5'-TTGCCCATCATCATCACCT-3'	158 - 303
PARP1	NM_007415.2	For. 5'-AGGCCGCCTACTCTATCCTC-3' Rev. 5'-GATTCACTGCTGCCTTGAGA-3'	2179 - 2239
Pol β	NM_011130.2	For. 5'-CTCGAGTTACTGGCATTGGAC-3' Rev. 5'-TCTTAATTCCTTCATCTACAAACTTCC-3'	365 - 424
UBR3	NM_001081548.2	For. 5'-CCACACTGGACACGACTTCA-3' Rev. 5'-GGGAATATTTGAACTTGATTTAATCTG-3'	508 - 647

Table 2.3: Primers used for both real-time and reverse transcriptase PCRs.

equations below were carried out to determine the relative quantities of mRNA (RQ value), thus enabling statistical analysis across experiments.

$$\Delta CT = CT_{gene} - CT_{reference\ gene}$$

$$\Delta\Delta CT = \Delta CT_{control} - \Delta CT_{treatment}$$

$$RQ = 2^{\Delta\Delta CT}$$

2.2.16 Alkaline COMET assay

The Alkaline COMET assay was used to determine repair kinetics as described previously (Parsons et al., 2009, Parsons et al., 2011). *Ubr3* +/+ and *Ubr3* -/- MEF cells in suspension (250 µl) were treated with MMS or irradiation and kept on ice. The cells were resuspended in 1 ml 1% low melt agarose prior to adding on to a slide pre-coated with 800 µl set 1% agarose and covering with a coverslip. The slides were kept on ice for 5 min to set the agarose and subsequently placed in to a 37 °C humidified incubator for the length of repair time. At the repair time-point the coverslips were removed and slides were placed in to coplin jars containing fresh cold lysis buffer (2.5 M NaCL, 100 mM EDTA, 10 mM Tris base, pH adjusted to pH 8.0 using NaOH). Lysis was carried out for 1 hr at 4 °C. Slides were transferred to a COMET-20 electrophoresis tank and covered completely with cold freshly prepared electrophoresis buffer (300 mM NaOH, 1 mM EDTA, 1% DMSO, pH > 13.0) for 30 min. Single cell gel electrophoresis (SCGE) was subsequently carried out at 25 V and adjusted to 300 mA. The slides were subsequently removed, covered in 1 ml neutralisation buffer (Tris-HCl, pH 8.0) thrice and dried overnight. The slides were rehydrated in H_2O for 30 min and 1 ml SYBR Gold (1:10,000 diluted in H_2O, pH 8.0) was added for 30 min. Coverslips were placed on to slides and the slides dried in darkness. Slides were imaged on a Nikon Exclipse 90i

microscope and data (% tail DNA intensities) generated using the Kinetic Imaging Komet 5.5 software.

2.2.17 Clonogenic survival assay

Ubr3 +/+ and *Ubr3* -/- MEF cells were plated on to 10 cm^2 dishes at a density of 300 cells/plate and allowed to adhere overnight. The cells were treated with varying amounts of the DNA damaging agent methyl methanesulphonate, MMS, (Sigma-Aldrich, Gillingham, UK) for 45 min. The MMS-containing media was removed and the plates washed twice with PBS before replacing with fresh media. The plates were incubated at 37 °C, 5% CO_2 for approximately 7 days until colonies were clearly visible for counting. The media was removed from the plates, the plates washed once with PBS and prior to fixing and staining with methylene blue for 1 hr. The dye was removed and the plates washed in water, dried and colonies counted using a colony counter. The surviving fraction was calculated as the number of colonies in treatment plate divided by the number of colonies in the untreated control plate.

2.2.18 Plasmid transfection

Cells were transfected using Lipofectamine 2000 transfection reagent (Invitrogen, Paisley, UK) according to the manufacturer's protocol. Briefly, 10 cm^2 dishes containing adhered cells were prepared for plasmid transfection. Per plate, 10 µl lipofectamine was pre-incubated in 0.5 ml media without FBS for 5 min. This was added to 0.5 ml non-FBS media containing the desired amount of plasmid for transfection, mixed and incubated further for 20 min. 5 ml of media containing FBS was added to the mix at the end of the incubation period and placed onto plates containing adhered cells. The plates were incubated at 37 °C for 6 hr, the media washed off and replaced with media containing

FBS. The cells were harvested at the 24 hr transfection time-point unless otherwise stated.

2.2.19 mRNA silencing

Protein levels in cells were down-regulated by siRNA-mediated mRNA depletion. siRNA primers for MDM2 and mouse APE1 were ordered from Eurogentec (Southampton, UK) and their sequences shown in **Table 2.4**. The siRNA sequences were delivered in to cells using Lipofectamine 2000 RNAiMAX siRNA reagent (Invitrogen, Paisley, UK) according to the manufacturer's protocol. Briefly, 10 cm^2 dishes containing adhered cells were prepared for siRNA delivery. Per plate, 10 µl RNAiMAX was pre-incubated in 0.5 ml media without FBS for 5 min. This was added to 0.5 ml non-FBS media containing the desired amount of siRNA primer added, mixed and incubated further for 20 min. 5 ml of media containing FBS was added to the mix at the end of the incubation period and placed onto plates containing adhered cells. The plates were incubated at 37 °C for 6 hr, the media washed off and replaced with media containing FBS. The cells were harvested at the 72 hr treatment time-point unless otherwise stated.

2.2.20 AP site incision assay

A Cy5/FAM labelled uracil substrate (Eurogentec, Southampton, UK) was prepared by annealing 30 pmol 5'-FAM-CAATAGAGTAACACGG**U**CGACCAGTC CCTGCCAATC-3' with 60 pmol 5'-GATTGGCAGGGACTGGTCGGCCGTGTT ACTCTATTG-3' in 100 µl TE buffer, heated at 95 °C for 5 min and slow cooled to room temperature. The AP site was prepared just before use by incubating the substrate with UDG for 30 min at 37 °C and then purifying the DNA using a Biospin P30 column (Biorad, Hemel Hempstead, UK). The AP site substrate was then incubated with indicated amounts of the endonuclease under investigation (APE1, $APE1_{T233E}$) in 10 µl

Gene	GenBank Accession	siRNA sequence
APE1 (Human)	NM_001641.2	Seq. 1 For. 5'-AAUGACAAAGAGGCAGCAGG-3' Seq. 1 Rev. 5'-CCUGCUGCCUCUUUGUCAUU-3' Seq. 2 For. 5'-AACCUGCCACACUCAAGAUC-3' Seq. 2 Rev. 5'-GAUCUUGAGUGUGGCAGGUU-3'
APE1 (Mouse)	NM_009687.2	Seq. 1 For. 5'-GAAGCACCAGAUAUCUUGU-3' Seq. 1 Rev. 5'-ACAAGAUAUCUGGUGCUUC-3' Seq. 2 For. 5'-AGAAAGGUUUGGAUUGGGU-3' Seq. 2 Rev. 5'-ACCCAAUCCAAACCUUUCU-3'
CDK5	NM_004935	Seq. 1 For. 5'-CCAAGCUGCCAGACUAUAA-3' Seq. 1 Rev. 5'-UUAUAGUCUGGCAGCUUGG-3'
MDM2	NM_002392.4	Seq. 1 For. 5'-AAGCCAUUGCUUUUGAAGUUA-3' Seq. 1 Rev. 5'-UAACUUCAAAAGCAAUGGCUU-3' Seq. 2 For. 5'-UAGGAAUUUAGACAACCUGAA-3' Seq. 2 Rev. 5'-UUCAGGUUGUCUAAAUUCCUA-3'

Table 2.4: Silencing RNAs used in this study. The MDM2 sequence 1 (Dornan 2004) and sequence 2 (Jin 2003) have been previously published.

buffer containing 50 mM HEPES-KOH (pH 7.8), 50 mM KCl, 10 mM $MgCl_2$, 0.5 mM EDTA, 1 mM DTT, 8.5% glycerol for 20 min at 37 °C. The reactions were stopped by incubation on ice with 9.5 mg/ml $NaBH_4$ for 10 min prior to the addition of formamide loading dye. The reactions were analysed by 20% PAGE and fluorescence imaging using the Molecular Imager FX system (Bio-Rad, Hemel Hempstead, UK).

Chapter 3

RESULTS I

APE1 is subject to ubiquitylation in human cells

3.1 INTRODUCTION

APE1 is a key DNA repair enzyme involved in the base excision repair pathway of DNA repair. This repair pathway is indispensable for the maintenance of genome stability. It is a housekeeping pathway that is continuously active throughout a cells life, repairing thousands of endogenously generated DNA lesions on a daily basis within each cell. These lesions are potentially mutagenic in dividing cells, and obstructive to cellular processes on DNA in non-dividing cells. Due to the continuous requirement for the BER pathway throughout a cells lifetime, it is thought that the BER pathway should be regulated to a steady-state level corresponding to the amount of endogenous damage present on DNA in cells to provide efficient DNA repair. To this end, we believe that regulation of the BER pathway occurs by modulating the availability of the BER enzymes for repair rather than by mechanisms that switch the pathway on or off. Indeed, both overexpression and underexpression of key BER enzymes such as APE1 and Pol β have been shown to negatively affect genome stability (Reviewed in Wallace et al., 2012).

In recent years our lab has focussed on determining the mechanisms that govern the availability of the various BER enzymes for repair. Ubiquitylation-mediated proteasomal degradation is the prevailing process in cells that removes excessive amounts of protein. Our lab has previously shown that the levels of the BER enzymes Pol β, XRCC1 and DNA Lig IIIα are regulated by ubiquitylation (Parsons et al., 2008,

Parsons et al., 2009, Parsons et al., 2010, Parsons et al., 2011). Pol β ubiquitylation by the E3 ligase enzymes CHIP and Mule promotes its proteasomal degradation whereas deubiquitylation of ubiquitylated Pol β by the deubiquitylation enzyme USP47 can reintroduce Pol β in to the cellular pool for DNA repair (Parsons et al., 2008, Parsons et al., 2009). Loss of Pol β ubiquitylation results in an increase in Pol β levels and repair activity whereas loss of deubiquitylation produces the opposite effect. It is clear that ubiquitylation and deubiquitylation fine-tune the levels of Pol β in cells.

A role for ubiquitylation in regulating APE1 cellular levels has not yet been identified. As part of its repair role in BER, APE1 cleaves the DNA backbone at an abasic site to generate a single nucleotide gap that is required for nucleotide insertion and backbone ligation by BER polymerases and ligases, respectively. As with most BER factors, both overexpression and underexpression of APE1 promotes genome instability and it is therefore of interest to understand the regulation of APE1 levels in cells. Since we have shown that the levels of the other key BER factors are regulated by ubiquitylation, the aim of this doctoral thesis is to determine if APE1 levels are also regulated by ubiquitylation-mediated proteasomal degradation. To validate the feasibility of undertaking such a study, the aim of this first results chapter is to determine if APE1 is ubiquitylated in cells at all.

3.2 RESULTS

3.2.1 Higher molecular weight APE1 bands are present in the cytoplasm of HeLa cells.

To identify a role for ubiquitylation in regulating APE1 in cells, it was of importance to firstly demonstrate that APE1 is modified by ubiquitin in cells. A single ubiquitin molecule has a molecular weight of 8.5 kDa. Since APE1 is 35.5 kDa in size, a single ubiquitin addition on to APE1 should increase the molecular weight of APE1 to

approximately 44 kDa. Similarly, two ubiquitin additions on to APE1 should increase its molecular weight to approximately 52.5 kDa, and so on. Since ubiquitin is linked to its substrate through a covalent bond, it is possible to observe the higher molecular weight ubiquitylated forms of APE1 by SDS-PAGE and Western blotting analysis using APE1 and ubiquitin antibodies. This is of course dependent on the cross-reactivity of these antibodies for the ubiquitylated form of APE1. To show that such a higher molecular weight band exists for APE1 in human cells, HeLa cell extracts were analysed for such bands using SDS-PAGE and Western blotting. It was expected that if ubiquitylation promotes APE1 degradation, then this higher molecular weight form of APE1 would be much less stable. To increase the probability of identifying the ubiquitylated form of APE1, HeLa cells were fractionated in to cytoplasmic and nuclear fractions for initial analysis. It was observed in various studies that APE1 localisation within a cell varies according to cell type and cellular situation with some cell types showing mostly nuclear localisation of APE1 whereas others have shown both cytoplasmic and nuclear localisation (Fantini et al., 2010). In order to determine the visual spread of APE1 within HeLa cells, HeLa cells were fixed on to slides and immunostained with both APE1 antibodies (green) and the nuclear marker, DAPI (blue) followed by imaging on a BioRad confocal microscope **(Figure 3.1)**. It is apparent that APE1 is localised to both cellular compartments, though it is present at a higher concentration in the nucleus of these HeLa cells. The ubiquitylated form of APE1 in HeLa cells could therefore be present in either cellular compartment. I consequently fractionated HeLa cells in to cytoplasmic and nuclear extracts that were prepared in the presence of 1 mM MG132 (proteasomal inhibitor) and 1 mM NEM (deubiquitylating enzyme inhibitor) in order to prevent proteasomal degradation of APE1 as well as preserve the ubiquitylated form of APE1. The extracts were separated by 10% SDS-PAGE and analysed by Western blotting using antibodies against APE1, the cytoplasmic marker tubulin and the nuclear protein p14-ARF **(Figure 3.2)**. The distribution of the fractionation controls, tubulin and p14-ARF, imply that the cells are suitably fractionated in to cytoplasmic and nuclear fractions.

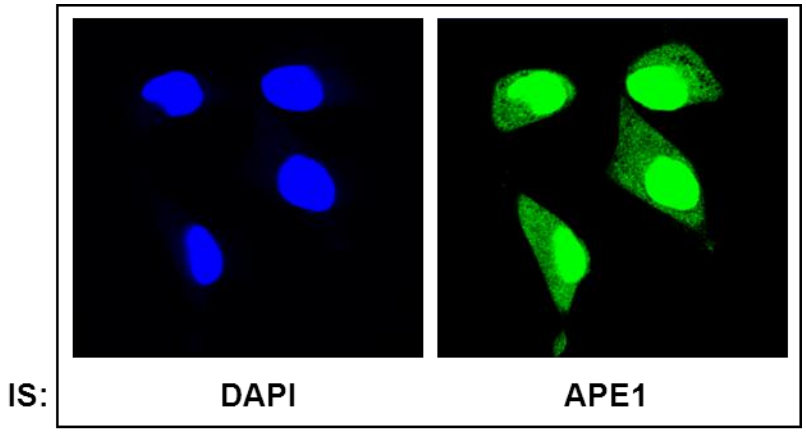

Figure 3.1: APE1 is localised to both the cytoplasm and the nucleus. HeLa cells were fixed on to a slide and stained with the nuclear marker DAPI (blue) or immunostained with antibodies to APE1 (green). Images were taken using a BioRad confocal microscope.

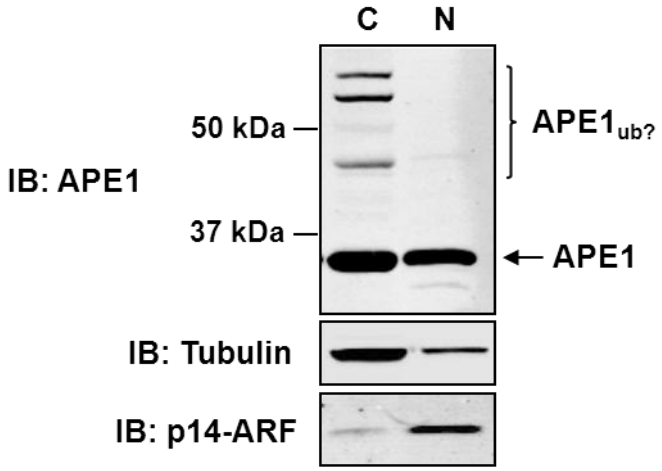

Figure 3.2: Higher molecular weight forms of APE1 are present in the cytoplasmic fraction of HeLa cells. HeLa cells were fractionated in to cytoplasmic (C) and nuclear (N) fractions in the presence of a protease inhibitor cocktail, PMSF (1 mM), the proteasomal inhibitor MG132 (100 nM) and the deubiquitylation enzyme inhibitor NEM (1 mM). The fractions were separated by 10% SDS-PAGE, transferred on to a PVDF membrane by Western blotting and immunoblotted with antibodies against APE1, the cytoplasmic marker tubulin and the nuclear marker p14-ARF. Molecular weight markers (kDa), unmodified APE1 (APE1) and higher molecular weight APE1 that may be ubiquitylated ($APE1_{ub?}$) are indicated.

The APE1 antibodies do not only pick up a band of ~ 35 kDa that represents unmodified APE1 in both fractions, they also additionally pick up higher molecular weight bands that match the expected size for ubiquitylated APE1. Unlike for unmodified APE1, these higher molecular weight bands were observed almost entirely in the cytoplasmic fraction of HeLa cells.

Since it is not unusual for antibodies to pick up non-specific bands, it was of importance to firstly identify which of these higher molecular weight bands is APE1-specific. Using small interfering RNAs (siRNA) to prevent the translation of mRNA in to protein, thus reducing protein levels, it is possible to identify bands that are specific to a particular protein by observing their cellular levels. siRNA sequences targeting APE1 mRNA were introduced in to HeLa cells using the RNAiMax lipofectamine reagent and the cells harvested 72 hr after treatment. Whole cell extracts were prepared from these cells and separated by 10% SDS-PAGE prior to Western blotting analysis using antibodies against APE1 and actin, a loading control (**Figure 3.3**). The low exposure image for APE1 that shows a loss of APE1 compared to the control cells confirm that the siRNA treatment was successful. In the high exposure image for APE1, only the intensity of the ~ 46 kDa band is reduced for APE1 siRNA treated cells compared to the untreated cells. This band is likely to be an APE1-specific band.

3.2.2 Cellular APE1 is modified by ubiquitin in cells.

Though the size of the ~ 46 kDa APE1 band observed in **Figure 3.3** suggests that this band could represent APE1 modified by one or two ubiquitin moieties, this band may not contain any ubiquitin at all. It would be ideal to simply immunoblot the membrane in **Figure 3.3** with ubiquitin antibodies to show that the higher molecular weight bands observed with APE1 antibodies also contain ubiquitin. Unfortunately this is not possible since a large number of proteins present in both the nucleus and cytoplasm can be modified by ubiquitin. I therefore decided to separate the ~ 46 kDa APE1 away

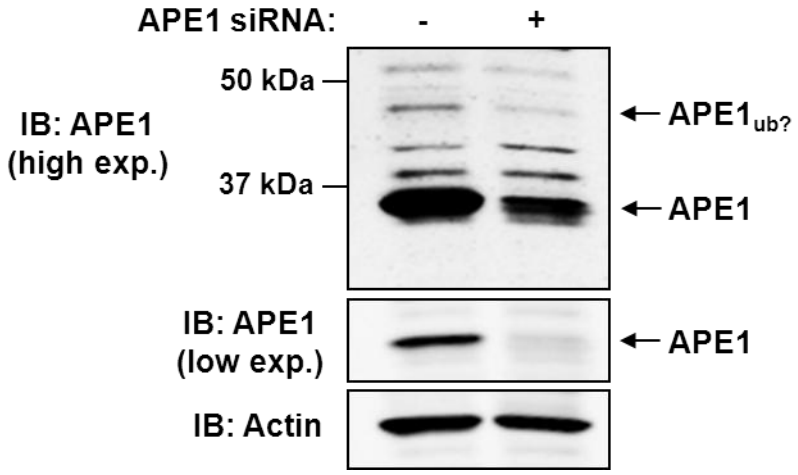

Figure 3.3: The ~ 46 kDa higher molecular weight band is APE1-specific. Silencing RNA sequences targeting human APE1 mRNA were introduced in to HeLa cells using the RNAiMax lipofectamine reagent. After 72 hr treatment, the cells were harvested, whole cell extracts prepared and the extracts separated by 10% SDS-PAGE, transferred on to a PVDF membrane and analysed by Western blotting using antibodies against APE1 and the loading control actin. Both high (high exp.) and low (low exp.) exposure images were produced for the APE1 analysis. Molecular weight markers (kDa), unmodified APE1 (APE1) and higher molecular weight APE1 that may be ubiquitylated ($APE1_{ub?}$) are indicated.

from other proteins such that, on analysis by Western blotting, it would be possible to confidently show that this band also contains ubiquitin. To do this, whole cell extracts were generated from a 20 g HeLa pellet in the presence of 1 mM NEM (deubiquitylating enzyme inhibitor) and 1 mM PMSF (protease inhibitor), clarified and separated over a series of ionic exchange columns according to the scheme shown in **Figure 3.4**. Both the Phosphocellulose and Mono-S columns are 'negatively' charged columns and therefore tend to bind 'positively' charged proteins whereas the Mono-Q column is 'positively' charged and binds 'negatively' charged proteins. There is a disparity between the degree of difference in charge that is dependent on the pH of the solution in which the proteins are suspended. The isoelectric point of APE1 is 8.33 and at the pH of the buffers used in the purification process, the net charge on APE1 is positive and therefore it is expected to bind to both the negative Phosphocellulose and Mono-S columns.

Clarified HeLa whole cell extracts were passed over the Phosphocellulose cation exchange column to allow for protein binding. The column was washed with buffer containing 150 mM KCl, the unbound proteins collected (flow-through) and the bound proteins eluted using step elutions of buffer containing 300 mM KCl, 700 mM KCl and 1000 mM KCl. The three elutes and the flow-through were separated by 10% SDS PAGE and analysed by Western blotting using APE1 antibodies (**Figure 3.5**). The ~ 46 kDa APE1 band was present in both the 300 mM KCl and 700 mM KCl elutes (depicted $APE1_{ub?}$, **Figure 3.5**) that were subsequently combined for further purification on the positively charged Mono-Q anion exchange column. Once loaded, the column was washed with buffer containing 50 mM KCl and the unbound proteins were collected (flow-through). The bound proteins were further collected in to fractions eluted with a gradient of buffer containing 50 mM KCl to 1 M KCl. The wash flow-through and the fractions A7 to C3 that span the elution gradient were separated by 10% SDS-PAGE and analysed by Western blotting using APE1 antibodies (**Figure 3.6**). Unsurprisingly, unmodified APE1 as well as the ~ 46 kDa APE1 band did not bind to this positively

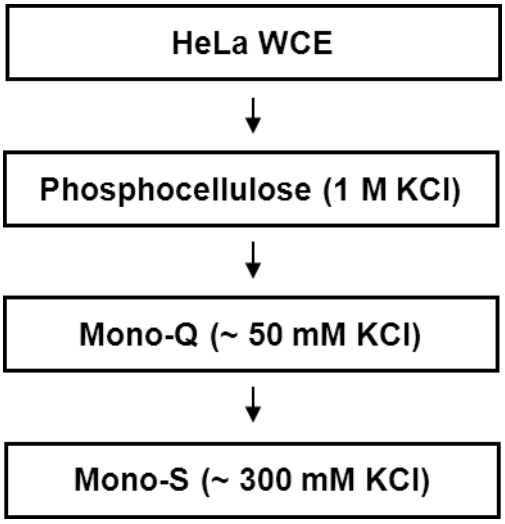

Figure 3.4: Scheme used for the purification of ubiquitylated APE1 from HeLa cells. Whole cell extracts (WCE) were prepared from a 20 g HeLa pellet and dialysed overnight in to buffer containing 150 mM KCl. The clarified dialysate was passed over a pre-equilibrated Phosphocellulose cation exchange column and proteins step eluted using buffer containing 150 mM to1000 mM KCl. The higher molecular weight APE1 band of ~ 46 kDa identified by Western blot analysis was eluted using 1 M KCl and fractions containing this form of APE1 were concentrated and buffer exchanged in to buffer containing 50 mM KCl prior to separation on the pre-equilibrated Mono-Q anion exchange column. The ~ 46 kDa APE1 band was identified by Western blotting in the flow-through that was obtained by washing the Mono-Q column with buffer containing 50 mM KCl. The flow-through was separated further on a pre-equilibrated Mono-S cation exchange column and proteins eluted using a gradient of buffer containing 50 mM KCl to 1000 mM KCl. The ~ 46 kDa APE1 band was identified by Western blotting in fractions eluted with approximately 300 mM KCl.

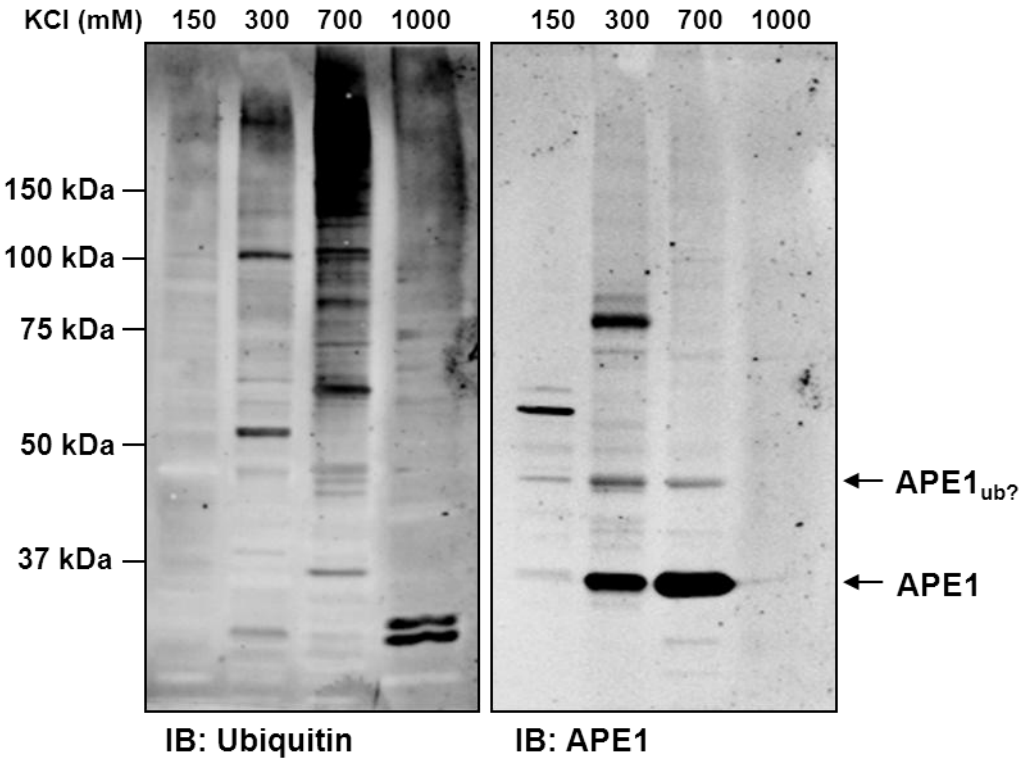

Figure 3.5: Purification of the ~ 46 kDa APE1 protein: Phosphocellulose column. Whole cell extracts (WCE) were prepared from a 20 g HeLa pellet and dialysed overnight in to buffer containing 1 mM deubiquitylating enzyme inhibitor NEM and 1 mM protease inhibitor PMSF. The clarified dialysate was passed over a pre-equilibrated Phosphocellulose cation exchange column and proteins step eluted using buffer containing 150 mM, 300 mM, 700 mM and 1 M KCl. The elutes were separated by 10% SDS-PAGE and analysed by Western blotting using APE1 and ubiquitin antibodies. Molecular weight markers (kDa), unmodified APE1 (APE1) and the ~ 46 kDa APE1 protein that may be ubiquitylated ($APE1_{ub?}$) are indicated.

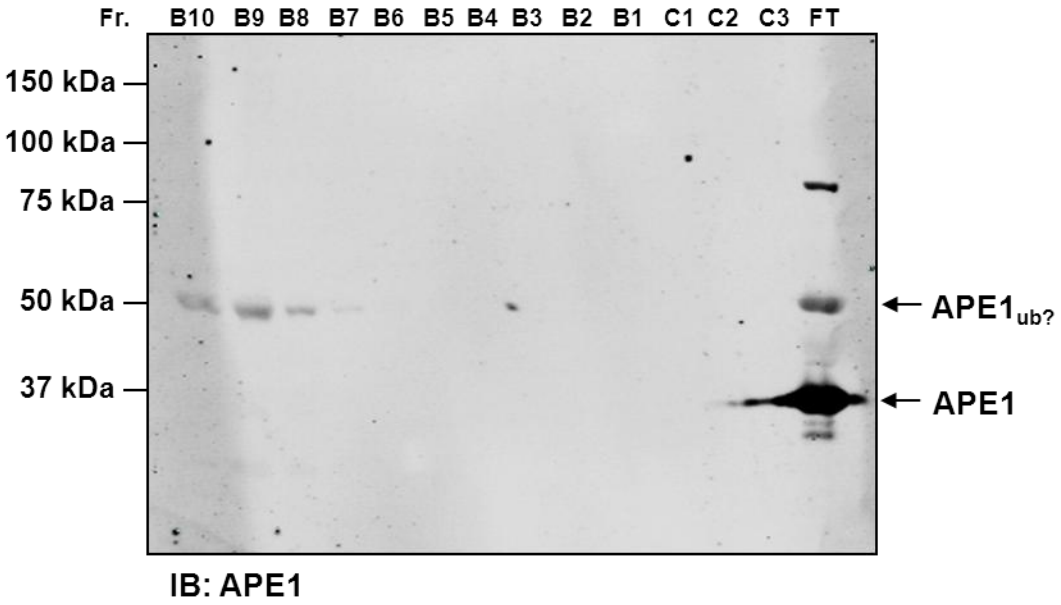

Figure 3.6: Purification of the ~ 46 kDa APE1 protein: Mono-Q column. The fractions obtained from the Phosphocellulose cation exchange column that contained higher molecular weight APE1 of ~ 46 kDa was passed over a Mono-Q anion exchange column. The column was washed with buffer containing 50 mM KCl and the flow-through (FT) collected. The bound proteins were eluted with a gradient of 50-1000 mM KCL containing buffer. The flow-through and the protein peak fractions B10-C3 collected from the Mono-Q column were separated by 10% SDS-PAGE, transferred on to a PVDF membrane and immunoblotted with APE1 antibodies. Molecular weight markers (kDa), unmodified APE1 (APE1) and higher molecular weight APE1 of ~ 46 kDa that may be ubiquitylated ($APE1_{ub?}$) are indicated.

charged column and were found to be present in the wash flow-through. The flow-through from the Mono-Q column was subsequently passed over the negatively charged Mono-S cation exchange column, the column washed with buffer containing 50 mM KCl and protein elution carried out using a gradient of buffer containing 50 mM KCl to 1 M KCl. The protein peak fractions A7 - A15 that were collected were separated by 10% SDS-PAGE and analysed by Western blotting using APE1 and ubiquitin antibodies (**Figure 3.7**). The ~ 46 kDa APE1 band is present across fractions A8 - A15 and for each fraction, both APE1 and ubiquitin antibodies pick up this band. Both blots have sufficiently low background specificities and furthermore, the intensities of the APE1 and ubiquitin bands are comparable across the fractions suggesting that the bands are very likely to be ubiquitylated APE1.

To be certain that the ~ 46 kDa APE1 band is APE1 modified by ubiquitin, APE1 immunoprecipitation was carried out on the A9 fraction collected from the Mono-S column. Both the input and the immunoprecipitate were separated by 10% SDS-PAGE and analysed by Western blotting using both APE1 and ubiquitin antibodies (**Figure 3.8**). The immunoprecipitation appears to be somewhat inefficient in terms of immunoprecipitating the ~ 46 kDa APE1 band and this is likely due to a higher affinity of the APE1 antibodies for the unmodified APE1 that is also present in these fractions (depicted APE1, **Figure 3.8**). We were however able to immunoprecipitate a small amount of the ~ 46 kDa APE1 band and furthermore showed that it also cross-reacts with ubiquitin antibodies (**Figure 3.8**, right panel). We therefore conclude that the ~ 46 kDa APE1 band identified in HeLa cells is APE1 modified by ubiquitin.

3.3 RESULTS SUMMARY

I have shown in this results chapter that endogenously ubiquitylated APE1 is present in the cytoplasm of HeLa cells and therefore conclude that APE1 is likely to be regulated in cells by ubiquitylation. The observation that ubiquitylated APE1 is present

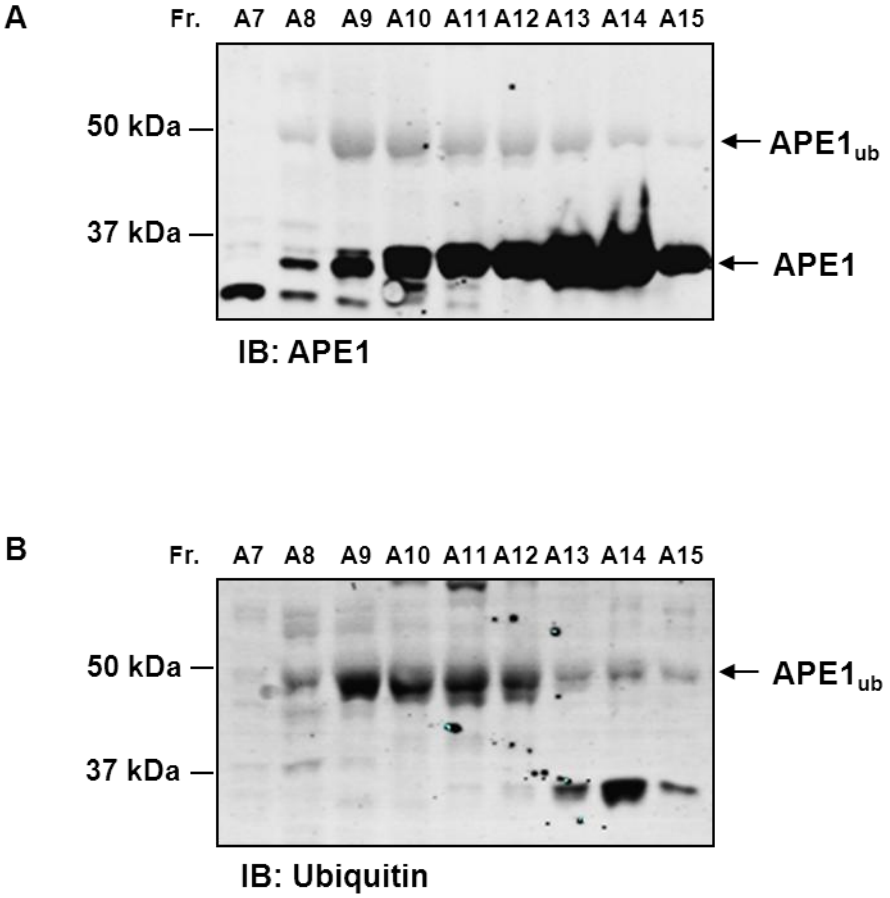

Figure 3.7: Purification of the ~ 46 kDa APE1 protein: Mono-S column. The flow-through obtained from the Mono-Q column that contained the ~ 46 kDa APE1 band was passed over a Mono-S cation exchange column. The bound proteins were eluted with a gradient of buffer containing 50 mM – 1000 mM KCL. Protein peak fractions A7 – A15 collected from the Mono-S column were separated by 10% SDS-PAGE, transferred on to a PVDF membrane and immunoblotted separately with APE1 antibodies (**A**) and ubiquitin antibodies (**B**). Molecular weight markers (kDa), unmodified APE1 (APE1) and ubiquitylated APE1 ($APE1_{ub}$) are indicated.

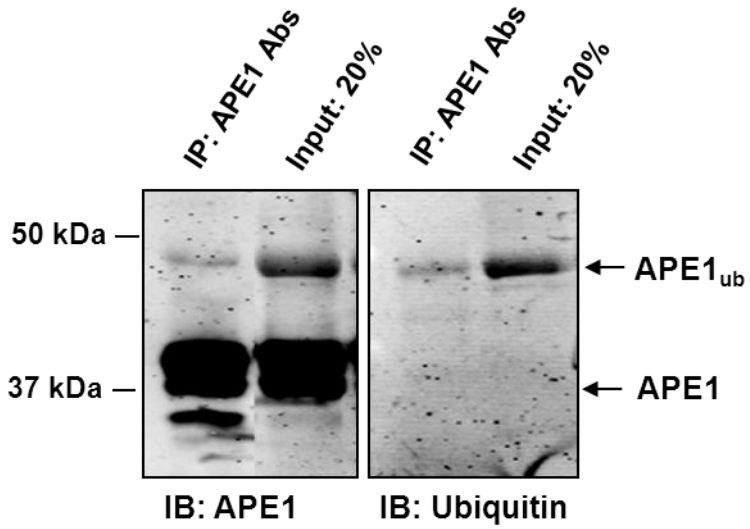

Figure 3.8: The ~ 46 kDa APE1 protein contains ubiquitin. APE1 was immunoprecipitated away from the peak monoubiquitylated APE1-containing Mono-S fraction (A9) using APE1 antibodies. The resulting pull-down was analysed by 10% SDS–PAGE and immunoblotting using antibodies against either APE1 (left panel) or ubiquitin (right panel). Molecular weight markers (kDa), unmodified APE1 (APE1) and ubiquitylated APE1 ($APE1_{ub}$) are indicated.

only in the cytoplasm suggests that at the very least, APE1 ubiquitylation prevents APE1 localisation to the nucleus and thus may indirectly prevent APE1 repair activity on DNA through spatial regulation.

Our lab has previously determined that the levels of the BER enzymes Pol β, XRCC1 and DNA Lig IIIα are regulated by ubiquitylation (Parsons et al., 2008, Parsons et al., 2009, Parsons et al., 2010, Parsons et al., 2011). This regulation by ubiquitylation fits with our model that suggests the BER pathway is regulated to a steady-state level through fine-tuned modulation of BER enzyme availability throughout a cells lifetime. Other than regulating protein stability in cells, ubiquitylation can additionally regulate protein localisation, activity and protein-protein or protein-DNA interactions (Dikic et al., 2009). Since we have shown that ubiquitylated APE1 is present in cells, we conclude that it is feasible to fully investigate the role of ubiquitylation in regulating cellular APE1.

Chapter 4

RESULTS II

APE1 is ubiquitylated by the cellular E3 ligase UBR3

4.1 INTRODUCTION

In the previous chapter, we showed that ubiquitylated APE1 exists in the cytoplasm of HeLa cells suggesting that APE1 is regulated by ubiquitylation in cells. Identification of the mechanism that allows for APE1 ubiquitylation in cells will allow us to fully study the role of ubiquitylation in regulating cellular APE1.

Ubiquitylation involves the addition of ubiquitin on to a protein and is mediated by the actions of three enzymes; an E1 activating enzyme, an E2 conjugating enzyme and an E3 ligase that promotes the covalent binding of ubiquitin on to the target substrate. Modification of proteins by ubiquitin can alter protein stability, localisation, activity and protein-protein or protein-DNA interactions (Dikic et al., 2009). In this capacity, protein ubiquitylation is a major cellular process that regulates cellular activity. It is therefore critical that the ubiquitylation process is highly specific in terms of substrate targeting and this specificity is provided primarily by the E3 ligase that is involved in substrate recognition. Indeed there are over a thousand E3 ligases encoded by the human genome, each of which recognises a single substrate or a small group of substrates (Reviewed in Ardley and Robinson, 2005, Metzger et al., 2012). Due to the immense influence of the E3 ligase on the outcome of protein ubiquitylation, this chapter focusses on identifying the cellular E3 ligase that is responsible for ubiquitylating APE1 in cells. Modulation of the E3 ligase specific for APE1 ubiquitylation will serve as a powerful tool

for determining the role that ubiquitylation plays in regulating APE1 activity, localisation, stability and protein or DNA interaction. In this chapter, we therefore aim to identify the E3 ligase responsible for APE1 ubiquitylation in cells.

4.2 RESULTS

4.2.1 An APE1 ubiquitylating activity is present in purified HeLa fractions.

An understanding of the direct mechanism allowing for APE1 ubiquitylation in cells will aid in determining the role of ubiquitylation in regulating APE1 stability. This involves identifying the cellular E3 ligase that allows for APE1 ubiquitylation in cells. Since there are over a 1000 E3 ligases present in cells, it is necessary to first purify the cellular ubiquitylating activity against APE1 away from other cellular E3 ligases. This was achieved by combining standard protein purification methods carried out according to the scheme in **Figure 4.1** and an in house developed *in vitro* ubiquitylation assay for APE1. To identify which of the purified protein peak fractions for each purification step that contained ubiquitylating activity against APE1, the fractions were used as the E3 source in an *in vitro* ubiquitylation assay containing 0.7 pmol E1 activating enzyme, 9.5 pmol total for a mixture of E2's (UbcH2, UbcH3, UbcH5a, UbcH5b, UbcH5c, UbcH6, UbcH7, UbcH8, UbcH10), 0.6 pmol ubiquitin and 2.8 pmol recombinant Histidine-tagged human APE1 as the substrate. The assay reactions were carried out at 30 °C for 30 minutes and the completed reactions were separated by 10% SDS-PAGE and analysed by Western blotting using APE1 antibodies. The fractions that allowed for the formation of higher molecular weight APE1 bands that correspond to the addition of ubiquitin (8.5 kDa) were identified as active fractions that are likely to contain the cellular E3 ligase responsible for APE1 ubiquitylation.

The purification process was initiated by fractionating a 20 g HeLa cell pellet in to cytoplasmic and nuclear extracts for use as the E3 source in an *in vitro* ubiquitylation

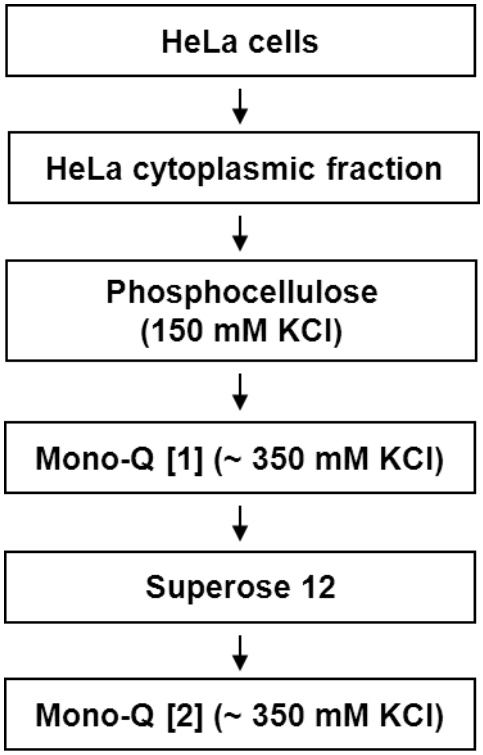

Figure 4.1: Scheme used for the purification of the APE1 ubiquitylating activity from HeLa cells. HeLa cytoplasmic extract was prepared from a 20 g HeLa pellet and passed over a pre-equilibrated Phosphocellulose cation exchange column. The proteins were eluted using buffer containing KCl and the APE1 ubiquitylating activity of the collected fractions was determined by using the fractions as an E3 source in an *in vitro* ubiquitylation assay against APE1. APE1 ubiquitylating activity was identified in fractions eluted at 150 mM KCl. The active fractions were separated further on a Mono-Q anion exchange column [1], eluted using a gradient of buffer containing KCl and the collected fractions subjected to an *in vitro* ubiquitylation assay against APE1. APE1 ubiquitylating activity was identified in fractions eluted at ~ 350 mM KCl. The active fractions were consequently separated on a Superose 12 size exclusion column and the collected fractions subjected to an *in vitro* ubiquitylation assay against APE1. APE1 ubiquitylating activity was observed in fractions containing proteins that were approximately 200 kDa to 400 kDa in size. These fractions were further separated on a Mono-Q anion exchange column [2] and the eluted fractions subjected to an *in vitro* ubiquitylation assay against APE1. APE1 ubiquitylation activity was identified in fractions eluted with ~ 350 mM KCl.

assay against recombinant His-APE1, as described previously. The completed reactions were analysed by 10% SDS-PAGE and Western blotting using APE1 antibodies (**Figure 4.2**). Faint higher molecular weight APE1 bands (depicted $APE1_{ub}$, **Figure 4.2**) were observed for reactions using the cytoplasmic fraction as an E3 source, but not for the nuclear fraction. This cytoplasmic activity is in keeping with our finding that the ubiquitylated form of APE1 is present only in the cytoplasm of HeLa cells. The cytoplasmic fraction of HeLa cells was consequently passed over a Phosphocellulose cation exchange column that preferentially binds positively charged proteins. The column was washed with buffer containing 150 mM KCl to remove unbound proteins as the flow-through. The bound proteins were step eluted using buffer containing 300 mM KCl, 700 mM KCl and 1000 mM KCl. Both the flow-through and the eluted fractions were subjected to an *in vitro* ubiquitylation assay for recombinant His-APE1. The completed reactions were separated on a 10% SDS-PAGE and analysed by Western blotting using APE1 antibodies (**Figure 4.3**). Higher molecular weight bands for APE1 were observed only in the reaction that used the flow-through as the E3 source in an *in vitro* ubiquitylation assay against APE1. This suggests that the E3 ligase that allows for APE1 ubiquitylation is present in the flow-through and is likely to have a pI lower than 8.0 that corresponds to the pH of the wash buffer.

The flow-through from the Phosphocellulose column was subsequently passed over a 20 ml Mono-Q anion exchange column. The column was washed using 50 mM KCl buffer and the bound proteins eluted in to fractions using a gradient of buffer containing 50 mM KCl to 1 M KCl. The fractions within the protein peaks were subjected to an *in vitro* ubiquitylation assay against recombinant His-APE1. The completed reactions were separated by 10% SDS-PAGE and analysed by Western blotting using APE1 antibodies (**Figure 4.4**). Higher molecular weight APE1 bands that correspond to ubiquitylated His-APE1 (depicted $APE1_{ub}$, **Figure 4.4**) were eluted with buffer containing ~ 350 KCl, generating a peak of

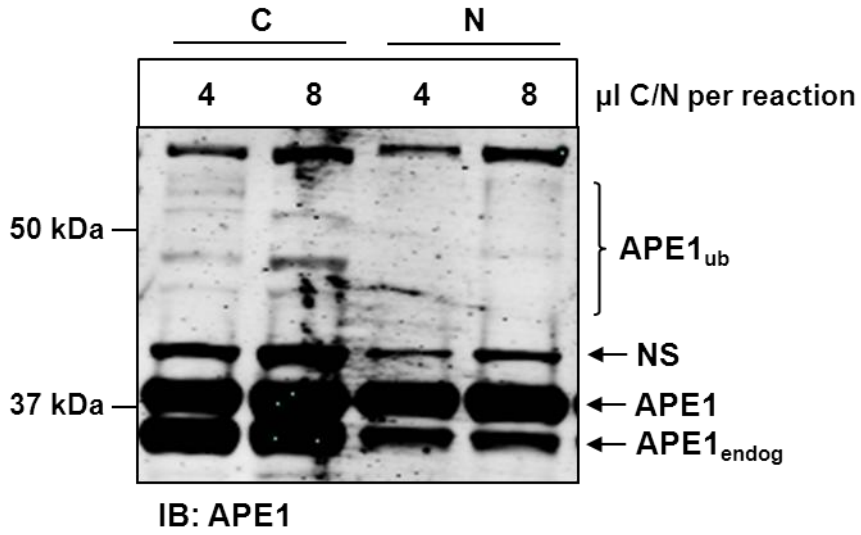

Figure 4.2: APE1 ubiquitylating activity is present in the cytoplasmic fraction of HeLa cells. HeLa nuclear (N) and cytoplasmic (C) extracts were prepared and either 4 µl or 8 µl used to *in vitro* ubiquitylate recombinant His-tagged APE1 (2.8 pmol) in the presence of E1 (0.7 pmol), ubiquitin (0.6 pmol) and an E2 mix (9.5 pmol). The completed reactions were separated by 10% SDS-PAGE and analysed by transferring on to a PVDF membrane and immunoblotting with APE1 antibodies. Molecular weight markers (kDa), cellular APE1 ($APE1_{endog}$), unmodified His-tagged APE1 (APE1), ubiquitylated His-tagged APE1 ($APE1_{ub}$) and non-specific bands (NS) are indicated.

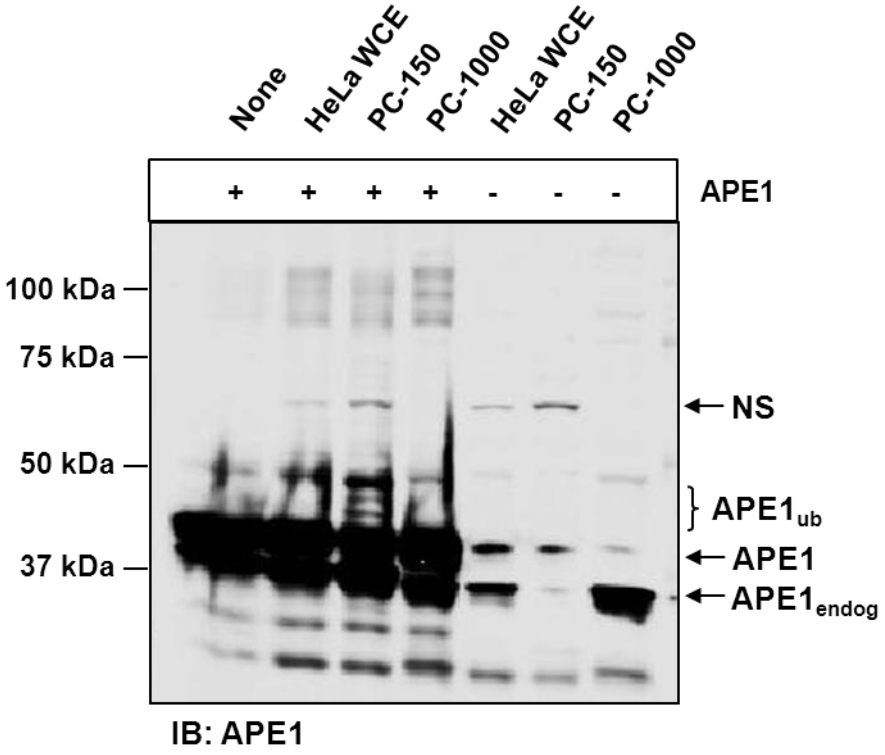

Figure 4.3: Purification of APE1 ubiquitylating activity from HeLa cells: Phosphocellulose column. HeLa cytoplasmic extract was separated on a Phosphocellulose cation exchange column. The unbound proteins (PC-150) were collected by washing the column with buffer containing 150 mM KCl. The bound proteins were eluted with buffer containing 1000 mM KCl (PC-1000). HeLa whole cell extract (WCE) and both the PC-150 and PC-1000 fractions were used as the E3 source in an *in vitro* ubiquitylation assay containing recombinant His-tagged APE1 (2.8 pmol), E1 (0.7 pmol), ubiquitin (0.6 pmol) and an E2 mix (9.5 pmol). Control reactions lacking His-tagged APE1 are also shown in the last three lanes. The completed reactions were separated by 10% SDS-PAGE and analysed by Western blotting using antibodies against APE1. Molecular weight markers (kDa), cellular APE1 ($APE1_{endog}$), unmodified His-tagged APE1 (APE1), ubiquitylated His-tagged APE1 ($APE1_{ub}$) and non-specific bands (NS) are indicated.

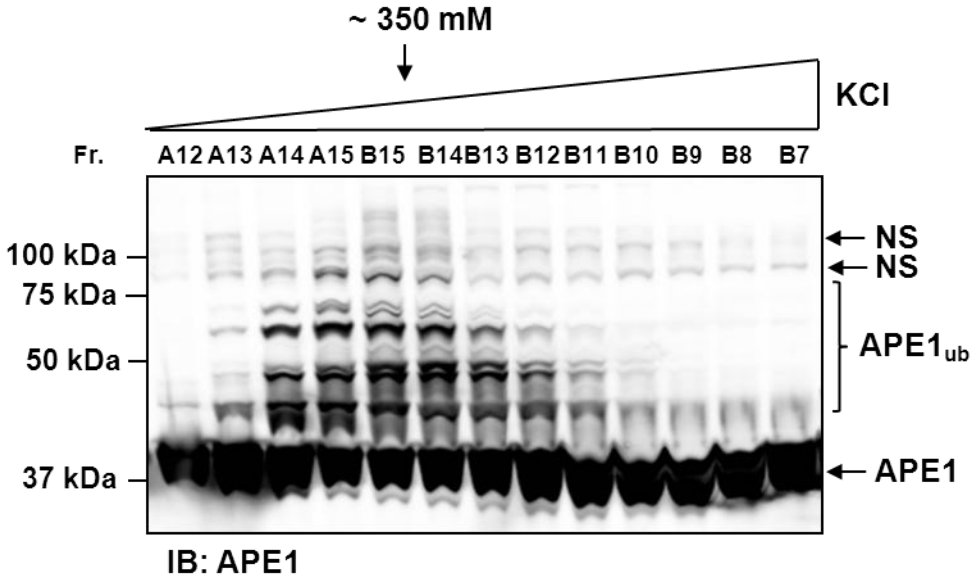

Figure 4.4: Purification of APE1 ubiquitylating activity from HeLa cells: Mono-Q column [1]. The flow-through collected from the Phosphocellulose column that had APE1 ubiquitylating activity was separated on the Mono-Q anion exchange column. Protein peak fractions A12 - B7 were used as the E3 source in an *in vitro* ubiquitylation assay containing recombinant His-tagged APE1 (2.8 pmol), E1 (0.7 pmol), ubiquitin (0.6 pmol) and an E2 mix (9.5 pmol). The completed reactions were separated by 10% SDS-PAGE and analysed by Western blotting using antibodies against APE1. Molecular weight markers (kDa), non-specific bands (NS), unmodified His-tagged APE1 (APE1) and ubiquitylated APE1 ($APE1_{ub}$) are indicated. The fractions containing the peak APE1 ubiquitylating activity were eluted with buffer containing approximately 350 mM KCl as indicated.

activity between fractions A14-B10. These fractions are likely to contain an E3 ligase that at the very least is capable of *in vitro* ubiquitylating recombinant His-APE1. Bands that do not fit in to the activity peak are most probably non-specific bands and are depicted NS in **Figure 4.4**.

The peak activity fractions collected from the Mono-Q column (A14-B10) were pooled and further separated over the Superose 12 size exclusion column. This column separates proteins based on their physical size. Not only does this column provide further purification of the APE1 ubiquitylating activity, it will also provide an approximate molecular weight of the E3 ligase present in the subsequent active fractions. The pooled fractions were passed over the Superose 12 column and fractions collected. The protein peak fractions (**Appendix I**) were subjected to an *in vitro* ubiquitylation assay against His-APE1. The completed reactions were separated by 10% SDS-PAGE and analysed by Western blotting using APE1 antibodies (**Figure 4.5**). A peak of ubiquitylation activity was observed between fractions B14 and B6. Bands that are not specific to the ubiquitylating activity on APE1 are indicated as NS in **Figure 4.5**. The B14 fraction contained proteins with an estimated molecular weight of 400 kDa whereas fraction B6 contained proteins of approximately 200 kDa in size. The E3 ligase responsible for the *in vitro* ubiquitylation of His-APE1 is therefore approximately 200 kDa to 400 kDa in size.

The active fractions B14 - B6 from the Superose 12 column were combined and passed over a smaller Mono-Q anion exchange column that has a higher resolution than the previous 20 ml Mono-Q anion exchange column used. The column was washed with buffer containing 50 mM KCl and the bound proteins eluted in to fractions using a gradient of buffer containing 50 mM KCl to 1 M KCl. The protein peak fractions were used as the E3 ligase source in an *in vitro* ubiquitylation assay against His-APE1. The completed reactions were separated by 10% SDS-PAGE and analysed by Western blotting using APE1 antibodies (**Figure 4.6**). An APE1 ubiquitylation activity peak was observed in fractions B3 to C2 that were eluted with buffer containing ~ 350 mM KCl.

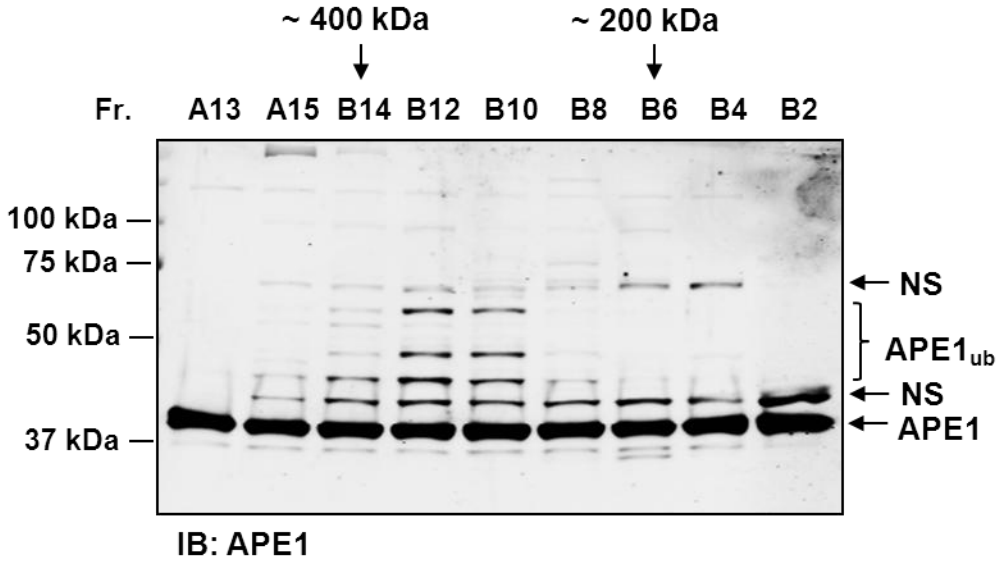

Figure 4.5: Purification of APE1 ubiquitylating activity from HeLa cells: Superose 12 column. Fractions from the Mono-Q column that were shown to be capable of *in vitro* ubiquitylating APE1 were combined and the proteins therein separated further by size over the Superose 12 size exclusion column. Protein peak fractions A13 - B2 were used as the E3 source in an *in vitro* ubiquitylation assay containing recombinant APE1 (2.8 pmol), E1 (0.7 pmol), ubiquitin (0.6 pmol) and an E2 mix (9.5 pmol). The completed reactions were separated by 10% SDS-PAGE and analysed by Western blotting using antibodies against APE1. Molecular weight markers (kDa), non-specific bands (NS), unmodified His-tagged APE1 (APE1) and ubiquitylated APE1 ($APE1_{ub}$) are indicated. Furthermore, the peak APE1 ubiquitylating activity is present in fractions containing proteins that are approximately 200 – 400 kDa in size, as indicated.

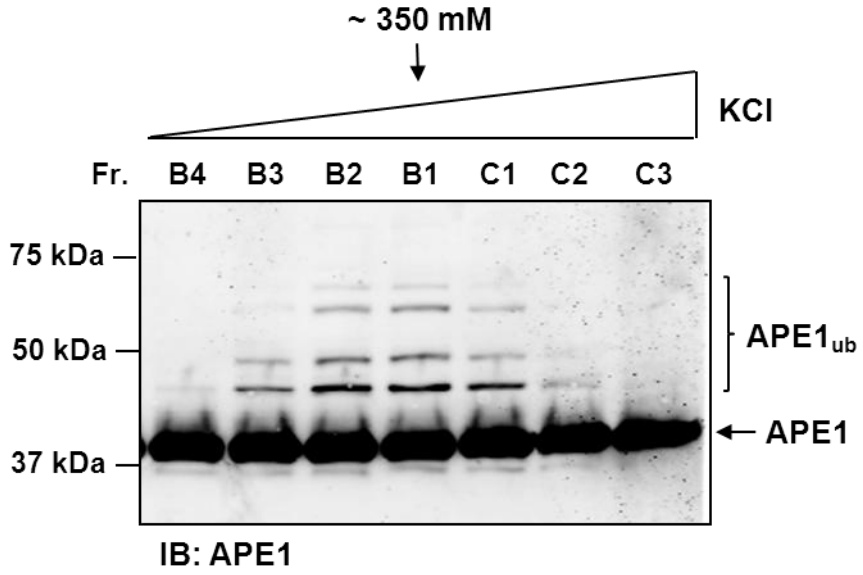

Figure 4.6: Purification of APE1 ubiquitylating activity from HeLa cells: Mono-Q column [2]. Fractions containing APE1 ubiquitylating activity that were purified from the Superose 12 column were combined and separated over a Mono-Q anion exchange column. Protein peak fractions B4 - C3 were used as the E3 source in an *in vitro* ubiquitylation assay containing recombinant His-tagged APE1 (2.8 pmol), E1 (0.7 pmol), ubiquitin (0.6 pmol) and an E2 mix (9.5 pmol). The completed reactions were separated by 10% SDS-PAGE and analysed by Western blotting using antibodies against APE1. Molecular weight markers (kDa), unmodified His-tagged APE1 (APE1) and ubiquitylated APE1 ($APE1_{ub}$) are indicated. The fractions containing the peak APE1 ubiquitylating activity were eluted with buffer containing approximately 350 mM KCl, as indicated.

The non-specific bands observed for active fractions obtained from previous columns are no longer present in these reactions. There is however a marked decrease in the APE1 ubiquitylating activity for these fractions and this is potentially due to natural degradation or loss of activity of the E3 ligase present in the purified active fractions. The peak activity fractions of this final Mono-Q column were therefore analysed for protein content by mass spectrometry analysis to identify the E3 ligase therein.

4.2.2 Mass spectrometry analysis reveals the presence of the E3 ligase UBR3 in the purified active fractions.

Since we were completely unaware of the E3 ligase specific for APE1, mass spectrometry analysis was used to determine which proteins were present in the peak APE1 ubiquitylating activity fractions (B2-B1) collected from the final Mono-Q column. The mass spectrometry analysis was carried out by the service provided by Dr. Benedikt Kessler's lab based at the University of Oxford. There, the proteins present in the two fractions were subjected to trypsinisation for peptide formation and the peptides analysed by LC-MS/MS tandem mass spectrometry. For protein identification, the LC-MS/MS spectra provided a list of identified peptides that were compared against the SwissProt database using Mascot version 2.2, to provide a list of proteins that correspond to the peptides identified within the trypsinised fractions. The mass spectrometry analysis of the trypsinised peak activity fractions from the final Mono-Q column revealed an overwhelming presence of seven separate peptides that belong to the E3 ligase, ubiquitin protein ligase E3 component n-recognin 3, UBR3, and these peptides are highlighted in the amino acid sequence of UBR3 in **Figure 4.7** and further summarised in **Table 4.1**. An example of the % ion count map for the peptide SSSNIPCVPK obtained from the mass spectrometry analysis is shown in **Appendix II**.

Figure 4.7: UBR3 peptides identified by mass spectrometry analysis of APE1 ubiquitylating HeLa fractions. HeLa protein fractions B1 and B2 that possess APE1 ubiquitylating activity purified by the Mono-Q [2] column were analysed by mass spectrometry for protein identification. The fractions were trypsinised and subject to tandem mass spectrometry analysis. By comparing the results to the Mascot protein database, seven peptide sequences belonging to the E3 ligase UBR3 were identified within the fractions and these peptides are underlined and highlighted in red within the full protein sequence for UBR3.

Position	Peptide sequence	Score
164 - 180	SQAGGACDCGDSNVMR	28
164 - 190	SQAGGACDCGDSNVMRESGFCKRHQIK	11
191 – 200	SSSNIPCVPK	22/11
398 – 431	MVTFLLNMLPDQEYKVAFTKTFVQHYAFIMKTLK	2
1044 – 1064	KKFQEIINRSSSEANQVVRPK	6
1418 – 1424	EMESVMK	12
1467 - 1480	GGNLCSGGASTAGK	12

Table 4.1: UBR3 peptides identified by mass spectrometry analysis of APE1-ubiquitylating HeLa fractions. Peptide position in the UBR3 protein sequence and the scores obtained for mass spectrometry are shown.

4.2.3 UBR3 is responsible for the APE1 ubiquitylating activity of the purified active fractions.

The E3 ligase, UBR3, has been identified in the purified fractions that contain APE1 ubiquitylating activity. There are more than 1000 cellular E3 ligases and it is possible that the active fractions contain another E3 ligase that is responsible for APE1 ubiquitylation but that was not identified by the mass spectrometry. The next step was therefore to confirm that UBR3 is indeed responsible for ubiquitylating APE1. To confirm the presence of UBR3 in the peak activity fractions, the fractions B4 - C3 obtained from the final Mono-Q column were separated by 10% SDS-PAGE and analysed by Western blotting using UBR3 antibodies (top panel, **Figure 4.8**). UBR3 is a 213 kDa protein and indeed, a band of this approximate size is present in the peak activity fractions. The levels of UBR3 present across the peak activity fractions furthermore correlate with the propensity of these fractions to *in vitro* ubiquitylate APE1 (lower panel, **Figure 4.8**). To show that UBR3 is responsible for the APE1 ubiquitylating activity exhibited by these active fractions, UBR3 was immunodepleted from the active fractions obtained from the Superose 12 column using UBR3 antibodies or XRCC1 antibodies (mock) attached to beads. The UBR3-immunodepleted fraction (UBR3 ID) and the mock immunodepleted fraction (mock ID) were subsequently used as the E3 ligase source in an *in vitro* ubiquitylation assay against His-APE1. The completed reactions were separated by 10% SDS-PAGE and analysed by Western blotting using APE1 and UBR3 antibodies (**Figure 4.9**). UBR3 is present in the mock ID reaction but not in the UBR3 ID reaction suggesting that UBR3 was successfully immunodepleted away from the peak activity fraction. The results show that the higher molecular weight APE1 bands present in the mock ID reaction are no longer present in the UBR3-ID reaction. The non-specific bands observed previously for the Superose 12 peak activity fraction however are still present in the UBR3-ID reaction (depicted NS, **Figure 4.9**). These findings demonstrate that

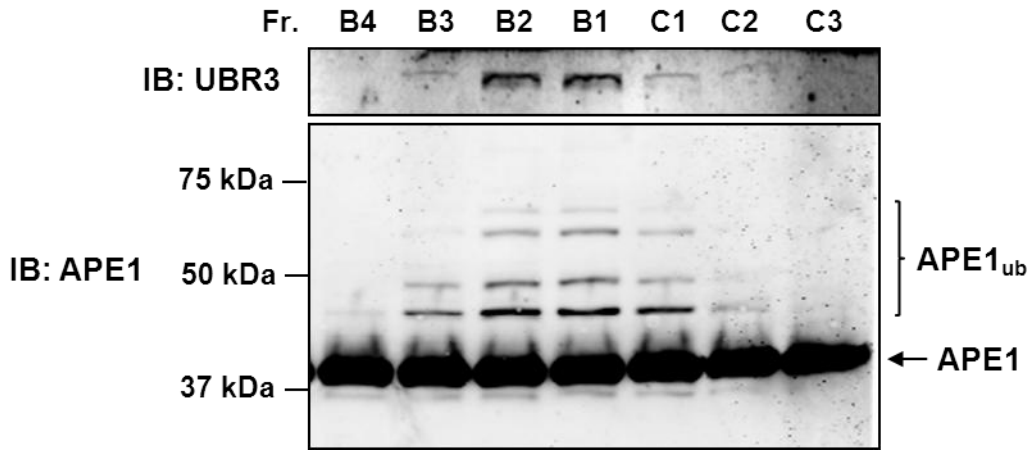

Figure 4.8: UBR3 levels correlate with APE1 ubiquitylating activity. Upper panel: The protein peak fractions B4 - C3 purified on the Mono-Q column [2] that displayed APE1 ubiquitylating activity were separated by 10% SDS-PAGE and analysed by Western blotting using antibodies against UBR3. **Lower panel:** Protein peak fractions B4 - C3 purified from the final Mono-Q [2] column were used as the E3 source in an *in vitro* ubiquitylation assay containing recombinant His-tagged APE1 (2.8 pmol), E1 (0.7 pmol), ubiquitin (0.6 pmol) and an E2 mix (9.5 pmol). The completed reactions were separated by 10% SDS-PAGE and analysed by Western blotting using antibodies against APE1. Molecular weight markers (kDa), unmodified His-tagged APE1 (APE1) and ubiquitylated APE1 ($APE1_{ub}$) are indicated.

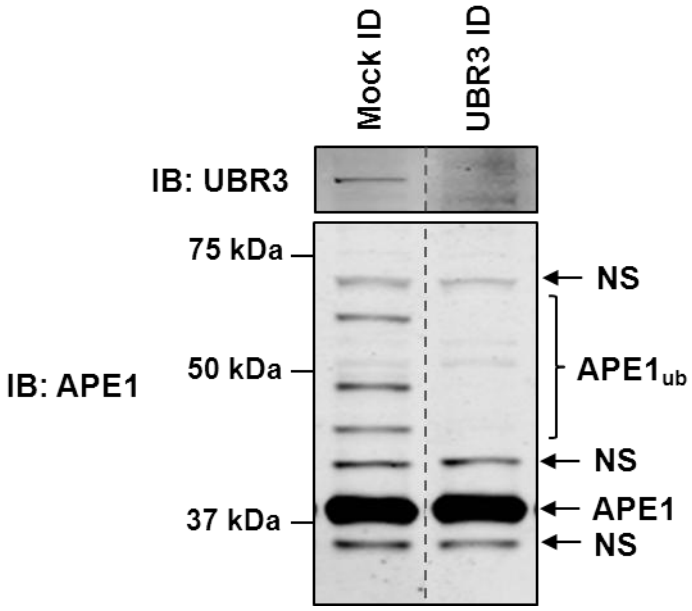

Figure 4.9: UBR3 immunodepletion results in a loss of APE1 ubiquitylating activity. Fraction B12 from the Superose 12 size exclusion column was mock immunodepleted using XRCC1 antibodies attached to beads or UBR3 immunodepleted using UBR3 antibodies attached to beads. The mock immunodepleted and UBR3 immunodepleted fractions were used as the E3 source in an *in vitro* ubiquitylation assay containing recombinant His-tagged APE1 (2.8 pmol), E1 (0.7 pmol), ubiquitin (0.6 pmol) and an E2 mix (9.5 pmol). The completed reactions were separated by 10% SDS-PAGE and analysed by Western blotting using antibodies against APE1 (lower panel) and UBR3 (top panel). Molecular weight markers (kDa), non-specific bands (NS), unmodified His-tagged APE1 (APE1) and ubiquitylated APE1 ($APE1_{ub}$) are indicated. The dashed line indicates that the two lanes taken from the same image were placed next to each other.

UBR3 is likely to be the E3 ligase present in the purified active fraction that is responsible for *in vitro* ubiquitylating APE1.

4.2.4 UBR3 is capable of *in vitro* ubiquitylating APE1.

To further prove that UBR3 is indeed capable of *in vitro* ubiquitylating APE1, I wanted to show that recombinant UBR3 protein is also able to *in vitro* ubiquitylate APE1. Collaborations with Dr. Yong Tae Kwon and Dr. Takafumi Tasaki allowed us to access a purified recombinant FLAG- tagged mouse UBR3 protein. To determine the purity of the FLAG-tagged mouse UBR3 protein, it was separated by 10% SDS-PAGE and the gel stained with Coomassie blue (**Figure 4.10**). A dominant band is present at the expected molecular weight of FLAG-UBR3 (UBR3 = 213 kDa) at just above 200 kDa. However, a less intense band (13.2% of total) was also identified at ~ 85 kDa that could represent either a contaminating protein or a degradation product of FLAG-UBR3. With this in mind, an *in vitro* ubiquitylation assay was carried out on His-APE1 using the FLAG immunoprecipitate (further referred to as FLAG-UBR3) as the E3 source. The reactions were separated by 10% SDS-PAGE and analysed by Western blotting using APE1 and FLAG antibodies (**Figure 4.11**). The controls lacking His-APE1, FLAG-UBR3 or E2/ubiquitin reveal that at least the higher molecular weight bands are FLAG-UBR3 dependent. Indeed, the intensity of these bands correlates with the amount of FLAG-UBR3 present in the reaction. Interestingly, a band that is likely to correspond to monoubiquitylated APE1 (depicted $APE1_{monoub}$, **Figure 4.11**) is present in a reaction lacking FLAG-UBR3 only (Lane 2, **Figure 4.11**). Some E2 conjugating enzymes are able to add ubiquitin directly on to a substrate without the need for an E3 ligase (Hoeller et al., 2007). It is possible that the E2 enzyme required by UBR3 for APE1 ubiquitylation can independently *in vitro* monoubiquitylate APE1.

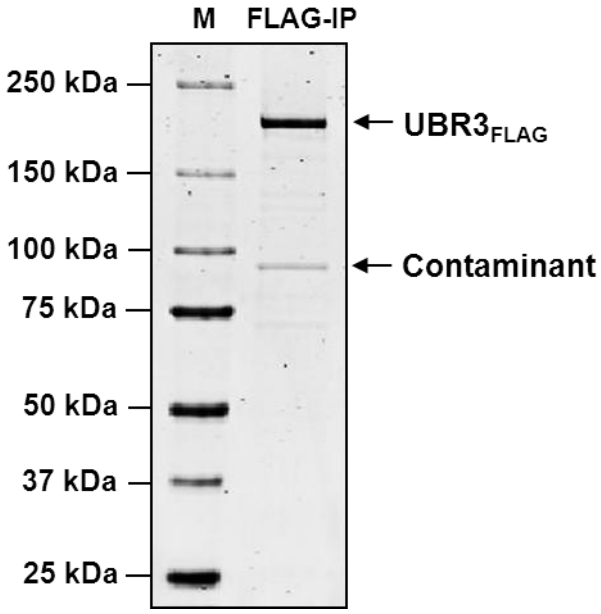

Figure 4.10: Purified recombinant FLAG-UBR3 purity. Recombinant N-terminal FLAG-tagged UBR3 and protein markers (M) were separated by 10% SDS-PAGE and stained with Coomassie blue prior to imaging. Molecular weight markers (kDa), FLAG-tagged UBR3 ($UBR3_{FLAG}$) and a contaminant or degradation product is also indicated.

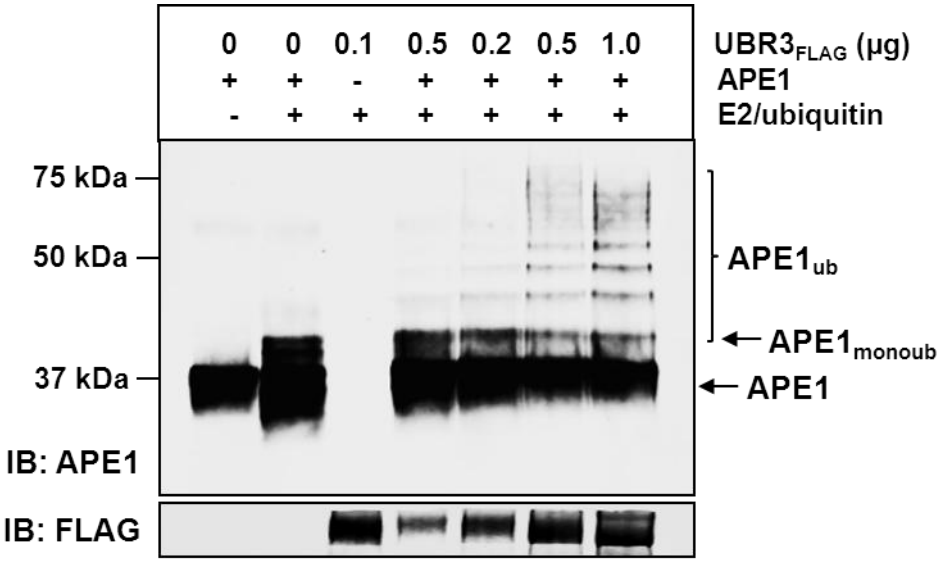

Figure 4.11: Recombinant UBR3 protein is able to *in vitro* ubiquitylate APE1. Indicated amounts of purified FLAG-tagged UBR3 ($UBR3_{FLAG}$) protein was used as the E3 source in an *in vitro* ubiquitylation assay containing the indicated amounts of recombinant His-tagged APE1 (2.8 pmol), E1 (0.7 pmol), ubiquitin (0.6 pmol) and an E2 mix (9.5 pmol). The completed reactions were separated by 10% SDS-PAGE and analysed by Western blotting using antibodies against APE1 (top panel) and FLAG (lower panel). Molecular weight markers (kDa), unmodified His-tagged APE1 (APE1) ubiquitylated APE1 ($APE1_{ub}$) and monoubiquitylated APE1 ($APE1_{monoub}$) are indicated.

4.2.5 The E2 enzyme UbcH2 is required for UBR3-mediated APE1 *in vitro* ubiquitylation.

Though the E3 ligase provides the majority of specificity for the ubiquitylation process, the E2 enzyme also provides some specificity. An understanding of the E2 conjugating enzyme that allows for APE1 ubiquitylation by UBR3 may provide further insight in to the cellular regulation of APE1. To determine which E2 conjugating enzyme is required by UBR3 to carry out its ubiquitylating activity on APE1, we performed an *in vitro* ubiquitylation assay on APE1 using UBR3 and the individual E2 enzymes that were present in the E2 mix used previously (UbcH2, UbcH3, UbcH5a, UbcH5b, UbcH5c, UbcH6, UbcH7, UbcH8, UbcH10). The reactions were separated by 10% SDS-PAGE and analysed by Western blotting using APE1 antibodies (**Figure 4.12**). Only UbcH2 allowed for APE1 *in vitro* ubiquitylation by UBR3 as determined by the appearance of higher molecular weight APE1 bands for this reaction.

4.2.6 UBR3 is localised to the cytoplasm of mouse embryonic fibroblasts

We initially observed that ubiquitylated APE1 is present only in the cytoplasm of HeLa cells and have now shown that the ubiquitylation activity against APE1 is also present in the cytoplasm of HeLa cells. In order to determine the localisation of UBR3 within cells, mouse embryonic fibroblast cells were fixed on to slides and immunostained with both UBR3 antibodies (green) and the nuclear marker, DAPI (blue) followed by imaging on a BioRad confocal microscope (**Figure 4.13**). Indeed, UBR3 is localised solely to the cytoplasm of these mouse embryonic fibroblasts suggesting that UBR3-mediated APE1 ubiquitylation is very likely to occur in the cytoplasm of cells.

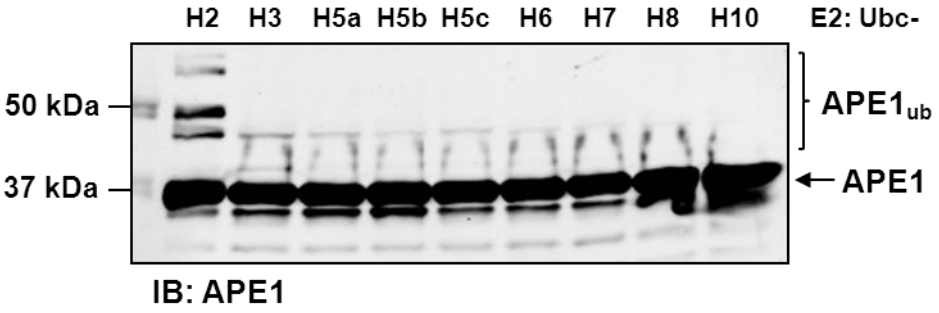

Figure 4.12: UBR3 uses the UbcH2 E2 activating enzyme to *in vitro* ubiquitylate APE1. The E2 enzymes UbcH2, UbcH3, UbcH5a, UbcH5b, UbcH5c, UbcH6, UbcH7, UbcH8 and UbcH10 were each (0.6 pmol) used in an *in vitro* ubiquitylation assay containing purified FLAG-tagged UBR3 protein (1 µg), recombinant His-tagged APE1 (2.8 pmol), E1 (0.7 pmol) and ubiquitin (0.6 pmol). The completed reactions were separated by 10% SDS-PAGE and analysed by Western blotting using antibodies against APE1. Molecular weight markers (kDa), unmodified His-tagged APE1 (APE1) and ubiquitylated APE1 ($APE1_{ub}$) are indicated.

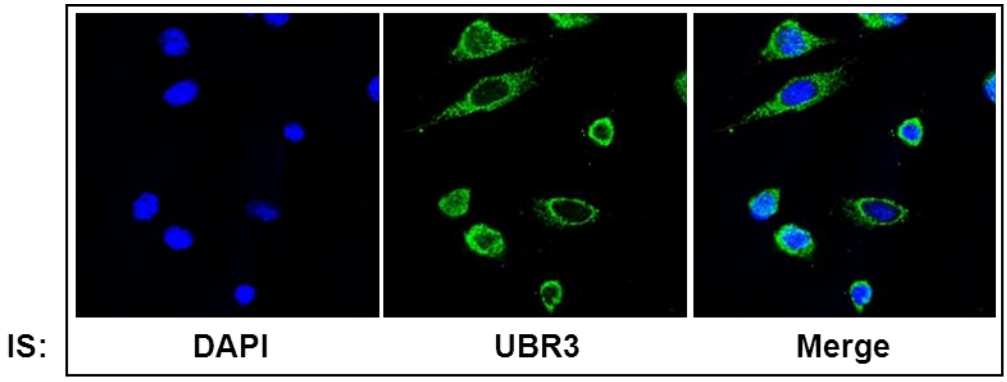

Figure 4.13: Cellular UBR3 is localised to the cytoplasm of mouse embryonic fibroblasts. Mouse embryonic fibroblasts were fixed on to slides and immunostained with the nuclear marker DAPI (blue) or antibodies against UBR3 (green). Images were taken using a Biorad confocal microscope.

4.3 RESULTS SUMMARY

In the previous chapter, ubiquitylated APE1 was identified in HeLa cells. Ubiquitylation is a major process in cells that has been shown to regulate cellular proteins, effecting changes in protein stability, localisation, activity and interactions. This process is mediated by three major enzymes, an E1 activating enzyme, an E2 conjugating enzyme and an E3 ligase. The E3 ligase is the key enzyme responsible for substrate recognition in the ubiquitylation process (Ardley and Robinson, 2005, Nagy and Dikic, 2010). It was therefore of interest to determine the E3 ligase that specifically ubiquitylates APE1 in cells since modulation of the E3 enzyme can be used as a research tool in studying the role of ubiquitylation in regulating cellular APE1.

Using column chromatography and an *in vitro* ubiquitylation assay for recombinant APE1, I was able to purify an APE1 ubiquitylating activity from HeLa cells. Mass spectrometry analysis of the purified active fraction revealed the presence of the E3 ligase, ubiquitin protein ligase E3 component n-recognin 3, UBR3. Indeed, UBR3 levels in the purified active fractions correlated with the APE1 ubiquitylating activity of these fractions. UBR3 immunodepletion from the active fractions resulted in a loss of APE1 ubiquitylating activity, suggesting that UBR3 is responsible for the APE1 ubiquitylating activity of these purified active fractions. Recombinant UBR3 was additionally capable of *in vitro* ubiquitylating APE1 and this activity was shown to be dependent on the E2 enzyme UbcH2. On a cellular level, UBR3 was found to be localised to the cytoplasm of cells which is also the cellular compartment in which ubiquitylated APE1 was identified within.

The findings presented in this chapter imply that UBR3 is the cellular E3 ligase that is responsible for ubiquitylating APE1 and this activity is likely to occur in the cytoplasmic compartment of cells. UBR3 belongs to a family of RING-domain containing E3 ligases characterised by the presence of the UBR box (Tasaki et al., 2005, Tasaki et al., 2007). There are no known cellular targets for UBR3 however the UBR-box

containing E3 ligases UBR1, UBR2, UBR4 and UBR5 have been shown to ubiquitylate substrates to promote their proteasomal degradation (Tasaki et al., 2007, Tasaki et al., 2005, Tasaki et al., 2009, Bachmair et al., 1986, Glickman and Ciechanover, 2002, Mogk et al., 2007). They achieve this by recognising N-terminal destabilising residues in their substrates according to the N-end rule pathway of substrate recognition, however it was shown that UBR3 is unlikely to recognise substrates according to this pathway (Tasaki et al., 2009). Based on this current understanding, it is not possible to presume that APE1 ubiquitylation by UBR3 will promote its proteasomal degradation.

In order to develop additional tools for studying the role of UBR3 in regulating cellular APE1, it was of interest to map the sites of UBR3-mediated ubiquitylation on APE1. Furthermore, an understanding of the type of ubiquitylation (monoubiquitylation or polyubiquitylation) that is imposed on APE1 will provide insight in to the role of UBR3 in regulating APE1 in cells. The next chapter therefore aims to characterise the ubiquitylating activity of UBR3 on APE1.

Chapter 5

RESULTS III

The E3 ubiquitin ligase UBR3 polyubiquitylates APE1 at its N-terminal tail

5.1 INTRODUCTION

In the previous chapter, I identified the cellular E3 ligase UBR3 that is able to ubiquitylate APE1. Since the E3 ligase provides most of the substrate specificity in the ubiquitylation process, modulation of the E3 ligase in cells can serve as a powerful tool for understanding the cellular role of the E3 ligase in regulating its substrate. In this chapter, I aim to first characterise the ubiquitylating activity of UBR3 on APE1 as this may provide further insight and research opportunities for the study of the role of ubiquitylation in regulating cellular APE1.

Lysine residues are the key acceptor sites for ubiquitin on substrates (Chau et al., 1989). Identification of such sites on a substrate can present an opportunity to create research tools for studying the effect of a loss of ubiquitylation on the regulation of APE1. Such tools include plasmids that encode a non-ubiquitylatable substrate for introduction in to cells for overexpression studies. A key aim of this chapter was therefore to identify the key ubiquitin acceptor sites on APE1 as mediated by the E3 ligase UBR3.

Both the E2 conjugating and E3 ligase activities additionally determine the extent of ubiquitylation that is carried out on a substrate (monoubiquitylation or polyubiquitylation) as well as the linkage type generated between ubiquitins of a polyubiquitin chain, both of which affect the cellular outcome of ubiquitylation (Ardley

and Robinson, 2005, Ye and Rape, 2009). For instance, monoubiquitylation is largely linked to altering protein localisation and interactions in cells whereas K48-linked polyubiquitin additions on to substrates are highly associated to substrate proteasomal degradation. The second aim of this chapter was therefore to characterise the type of ubiquitylation that is carried out on APE1 by UBR3 as this may provide further insight in to the likely role of UBR3-mediated ubiquitylation in regulating APE1.

5.2 RESULTS

5.2.1 APE1 is ubiquitylated at its N-terminal lysine residues

The UBR3 E3 ligase belongs to a family of UBR box-containing E3 ligases that are known to recognise destabilising N-terminal residues in their substrate (Tasaki et al., 2005, Tasaki et al., 2007). It was therefore of interest to determine which residues on APE1 are ubiquitylated by UBR3. Ubiquitin moeities are added on to lysine residues of proteins (Chau et al., 1989). It is possible to identify acceptor lysines for ubiquitin in proteins through mass spectrometry analysis of the ubiquitylated protein (Peng et al., 2003). To identify which sites on APE1 are ubiquitylated by UBR3, APE1 was *in vitro* ubiquitylated by UBR3 in an *in vitro* ubiquitylation assay and the completed reaction was separated by 10% SDS-PAGE. The proteins embedded in the gel were stained with Coomassie blue stain and the band corresponding to monoubiquitylated APE1 was subsequently identified and excised. The gel pieces were further analysed by the mass spectrometry service run by Dr. Benedict Kessler's lab (Summarised in **Figure 5.1**). The proteins contained within the gel pieces were extracted and subjected to trypsin treatment. Cleavage of a lysine containing an ubiquitin moiety by trypsin generates a glycine-glycine dipeptide of 114.1 Da that remains attached to the lysine side-chain (Peng et al., 2003). This increase in molecular weight compared to an unmodified lysine allows for the identification of lysine residues that are modified by ubiquitin when subjected to nano LC-MS/MS tandem mass spectrometry. The LC-MS/MS spectra

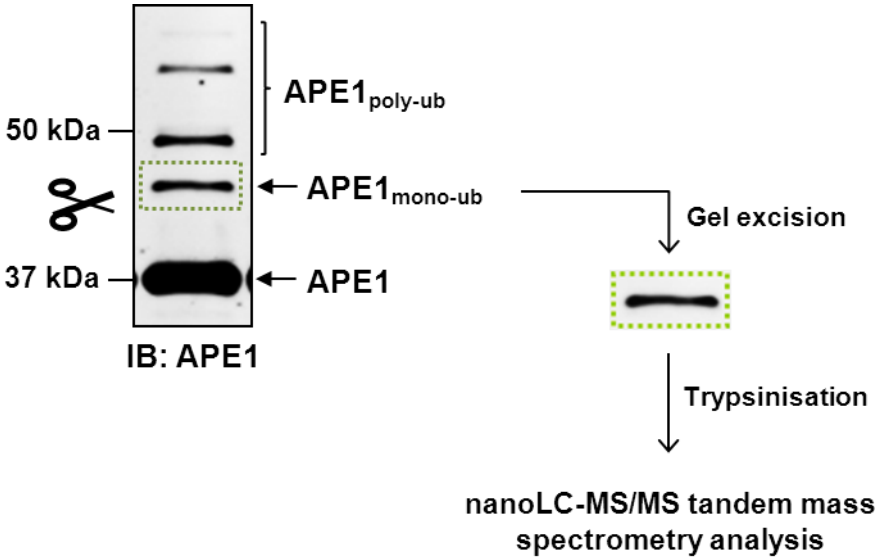

Figure 5.1: Mass spectrometry method for APE1 ubiquitylation site identification. Recombinant His-tagged APE1 (2.8 pmol) was *in vitro* ubiquitylated by UBR3 (1 µg) in a reaction containing E1 (0.7 pmol), ubiquitin (0.6 pmol) and an E2 mix (9.5 pmol). The completed reactions were separated by 10% SDS-PAGE and the gel stained with Coomassie blue stain. The band corresponding to monoubiquitylated APE1 was excised and the protein extracted and trypsinised for analysis by nanoLC-MS/MS tandem mass spectrometry.

results obtained showed that the lysine residues K7, K24, K25, K27, K32 and K35 of APE1 were modified by ubiquitin (**Figure 5.2** and **Appendix III**). These residues form part of the flexible and consequently exposed N-terminal tail of APE1 that is approximately 35 residues in length. To show that ubiquitylation of APE1 is indeed limited to its N-terminal 35 residues, APE1 protein that lacked the N-terminal 35 residues (APE1Δ1-35) was generated. This truncated APE1 protein was subsequently subjected to an *in vitro* ubiquitylation assay using UBR3 as the E3 ligase source. The completed reaction was separated by 10% SDS-PAGE and analysed by Western blotting using APE1 antibodies (**Figure 5.3**). The band representing ubiquitylated APE1 for the reaction carried out on full-length APE1 is no longer present for the reaction carried out on APEΔ1-35. This suggests that UBR3 ubiquitylates APE1 primarily on acceptor lysine residues present within its N-terminal 35 residues.

To further identify which sites in particular impact on the ability of APE1 to be ubiquitylated by UBR3, I also generated APE1 mutant protein that had N-terminal lysines replaced with arginine residues, an amino acid that is chemically similar to lysine but cannot be modified by ubiquitin. It is well known that an E3 ligase may *in vitro* ubiquitylate a neighbouring lysine if its preferential lysine is not available (Hou et al., 1994, Treier et al., 1994). Since the N-terminal region of APE1 is lysine rich, the sequences coding for three clusters of lysines (K6/K7, K24/K25/K27 and K31/K32/K35) were mutated either on their own or in combination within a histidine-tagged APE1 gene by site-directed mutagenesis. All the APE1 mutants used in this study were confirmed by gene sequencing and are summarised in **Figure 5.4**. The mutants were expressed in IPTG-induced Rossetta bacteria, lysates generated and the proteins purified by Ni-NTA purification. Wild-type full-length APE1 and mutant APE1 proteins were subsequently *in vitro* ubiquitylated by UBR3 and the completed reactions were separated by 10% SDS-PAGE and analysed by Western blotting using APE1 antibodies (**Figure 5.5**). As observed through the intensity of the higher molecular weight bands, the K24/25/27R

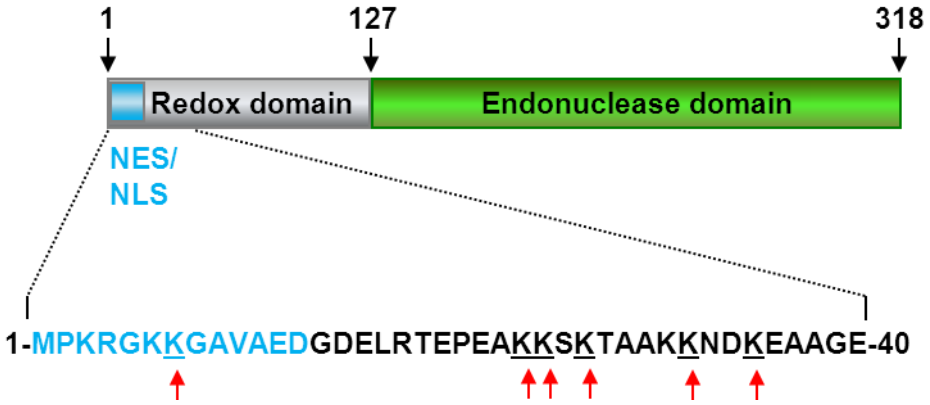

Figure 5.2: APE1 lysine residues K7, K24, K25, K27, K32 and K35 were identified as acceptor sites for ubiquitin. *In vitro* monoubiquitylated APE1 protein was subjected to nanoLC-MS/MS tandem mass spectrometry analysis for identification of APE1 residues modified by ubiquitin. The lysines K7, K24, K25, K27, K32, and K35 of the APE1 N-terminal flexible tail were identified as being modified by ubiquitin. The ubiquitin acceptor sites on APE1 are underlined and indicated by arrows in the depiction of the APE1 N-terminal residues. The nuclear localisation and export signals (NES/NLS highlighted in blue) are also present at the N-terminal tail that forms part of the redox activity domain of APE1 (residues 1-127).

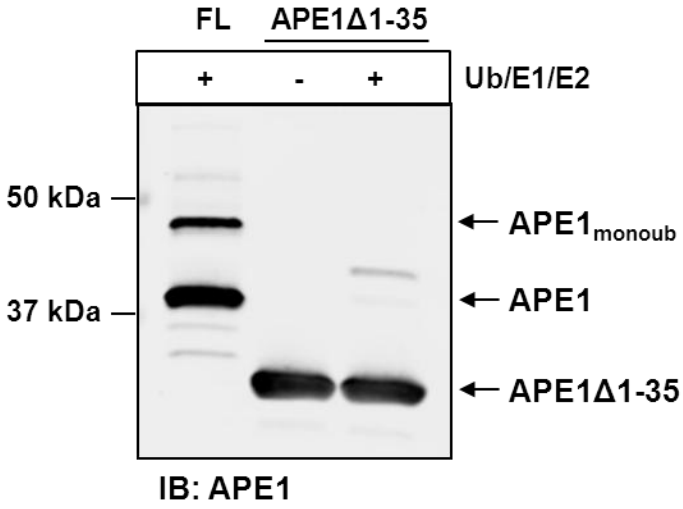

Figure 5.3: APE1 that lacks the N-terminal 35 residues is no longer ubiquitylated by UBR3. APE1 lacking the N-terminal 35 residues (APE1Δ1-35) was generated by trypsination at 23 °C for 2hr. Buffer exchanged APE1Δ1-35 and full-length (FL) APE1 were *in vitro* ubiquitylated by UBR3 (1 µg) in a reaction containing E1 (0.7 pmol), ubiquitin (0.6 pmol) and an E2 mix (9.5 pmol). A control reaction lacking ubiquitin and the E1/E2 mix was also carried out. The reactions were separated by 10% SDS-PAGE, transferred on to a PVDF membrane and immunoblotted with APE1 antibodies. Molecular weight markers (kDa), unmodified APE1 (APE1), monoubiquitylated APE1 ($APE1_{monoub}$) and unmodified N-terminal deleted APE1 (APE1Δ1-35) are indicated. **Acknowledgements**: Appreciation goes to Katherine Wright who generated the APE1Δ1-35 truncation protein and carried out this experiment.

Figure 5.4: Summary of APE1 mutants generated and used in the study to determine sites of ubiquitylation on APE1. The first forty residues of APE1 are depicted. APE1 lacking the N-terminal 35 residues (**[2]** = APE1Δ1-35) was generated by trypsinising full length APE1 at 23 °C for 2 hr. APE1 mutants were generated by site-directed mutagenesis in the bacterial expression vector pET14b containing the APE1 gene. All the mutants were confirmed by gene sequencing. The plasmids were subsequently expressed in Rossetta expression cells using 1 mM IPTG and the expressed APE1 protein was affinity purified using Ni-NTA agarose beads. The mutant protein generated from wild-type APE1 **[1]** include K6/7R **[3]**, K24/25/27R **[4]**, K31/32/35R **[5]**, K6/7R + K24/25/27R **[6]**, K6/7R + K31/32/35R **[7]**, K24/25/27R + K31/32/35R **[8]**, K6/7R + K24/25/27R + K31/32/35R **[9]**. The lysine to arginine mutations are highlighted in red and the ubiquitylation sites identified by nanoLC-MS/MS tandem mass spectrometry are underlined. The nuclear localisation and export signal present at the N-terminal tail is highlighted in blue. **Acknowledgements**: Appreciation goes to Katherine Wright who generated the APE1Δ1-35 truncation APE1 protein.

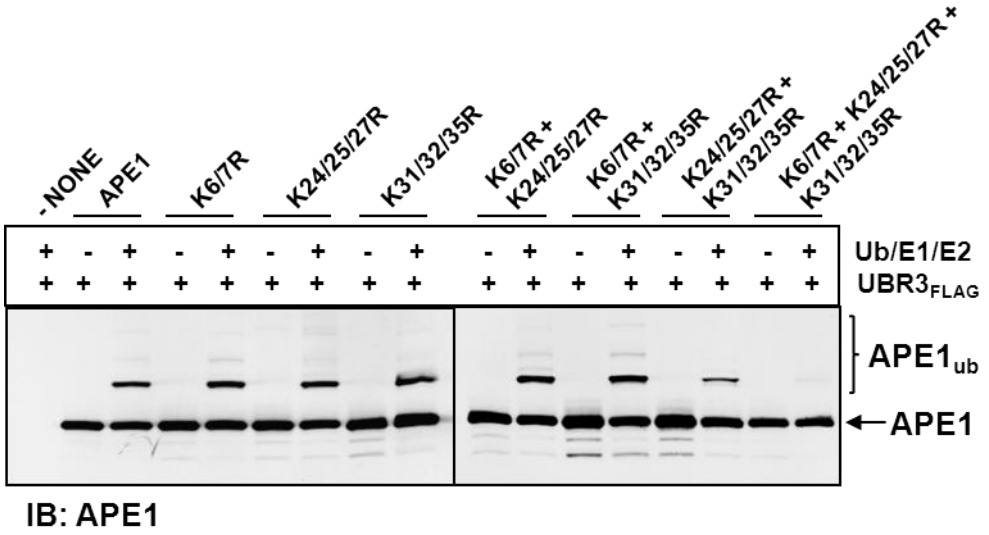

Figure 5.5: APE1 is *in vitro* ubiquitylated at multiple lysines on its N-terminal tail. Recombinant His-tagged wildtype APE1 (2.8 pmol) and APE1 lysine to arginine mutants (2.8 pmol) were *in vitro* ubiquitylated by 1 µg FLAG-tagged UBR3 ($UBR3_{FLAG}$) in a reaction containing E1 (0.7 pmol), ubiquitin (0.6 pmol) and an E2 mix (9.5 pmol) as indicated. For each APE1 protein, a control reaction lacking ubiquitin and the E1/E2 mix was also carried out. The reactions were separated by 10% SDS-PAGE, transferred on to a PVDF membrane and immunoblotted with APE1 antibodies. Molecular weight markers (kDa), unmodified APE1 (APE1) and ubiquitylated APE1 ($APE1_{ub}$) are indicated.

and K31/32/35R combination mutant was less ubiquitylated than the wild-type APE1 protein suggesting that these residues are likely to be the key residues required for APE1 ubiquitylation. However near total loss of UBR3-mediated APE1 ubiquitylation was achieved only when 8 of the 9 lysines in the APE1 N-terminal tail were mutated to arginines. This suggests that APE1 ubiquitylation by UBR3 is restricted to the N-terminal 35 lysine residues, however UBR3 does not appear to discriminate between the lysines present in this region.

5.2.2 APE1 is *in vitro* polyubiquitylated by UBR3

The level of ubiquitylation on a protein can dictate the outcome of ubiquitylation on that protein. Monoubiquitylation of a protein is often associated with altering protein-protein interactions whereas polyubiquitylation of a protein tends to target it for proteasomal degradation. The higher molecular weight bands observed for *in vitro* ubiquitylated APE1 could represent multiple single ubiquitin additions at multiple sites on the APE1 N-terminal tail or they could represent polyubiquitylated APE1. To determine the level of ubiquitylation on APE1, mutant ubiquitin lacking all seven lysines and thus unable to form polyubiquitin chains was used instead of wild-type ubiquitin in an *in vitro* ubiquitylation assay against APE1 using UBR3 as the E3 source. The reactions were separated by 10% SDS-PAGE and analysed by Western blotting using APE1 antibodies (**Figure 5.6**). Indeed, the intensity of the top three of the four higher molecular weight bands observed using wild-type ubiquitin is reduced when mutant ubiquitin was instead used in the reaction, showing that APE1 is polyubiquitylated at one site by UBR3. The results also show that the bands generated through UBR3 activity are ubiquitin-dependent and furthermore, the control reaction lacking APE1 produced no additional bands suggesting that the higher molecular weight bands are also APE1 dependent. These results suggest that APE1 can be *in vitro* ubiquitylated at multiple lysines on its N-

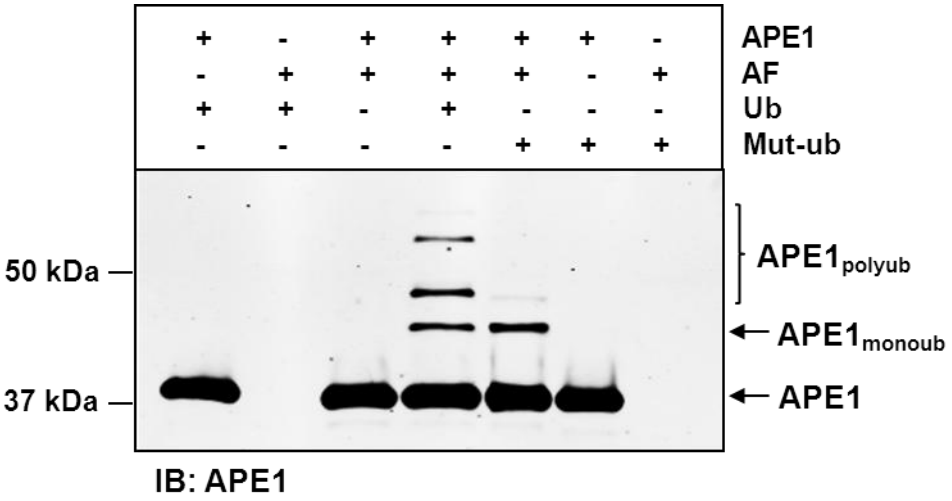

Figure 5.6: APE1 is polyubiquitylated by UBR3, primarily at a single site. As indicated, recombinant His-tagged APE1 (2.8 pmol) was *in vitro* ubiquitylated by a fraction containing APE1 ubiquitylating activity (AF) purified from the final Mono-Q anion exchange column using either wild-type ubiquitin (Ub) (0.6 nmol) or mutant ubiquitin (Mut-ub) (0.6 nmol) that is unable to form polyubiquitin chains. The reactions were carried out in the presence of E1 (0.7 pmol) and an E2 mix (9.5 pmol). The reactions were separated by 10% SDS-PAGE, transferred on to a PVDF membrane and immunoblotted with APE1 antibodies. Molecular weight markers (kDa), unmodified APE1 (APE1), monoubiquitylated APE1 ($APE1_{monoub}$) and polyubiquitylated APE1 ($APE1_{polyub}$) are indicated.

terminal tail, however *in vitro* ubiquitylation at one site prevents further ubiquitylation at another site for a single APE1 protein.

5.3 RESULTS SUMMARY

In this chapter, we have identified the sites of ubiquitylation on APE1 and furthermore show that APE1 is polyubiquitylated by UBR3. Mass spectrometry analysis of *in vitro* monoubiquitylated APE1 revealed that the N-terminal lysines K7, K24, K25, K27, K32 and K35 were modified by ubiquitin. APE1 lysine to arginine mutants generated by site-directed mutagenesis revealed that multiple lysines in the APE1 N-terminal 35 residues can be ubiquitylated by UBR3. I have however shown that APE1 is polyubiquitylated at a single lysine acceptor on APE1 suggesting that addition of a single ubiquitin unit on to any of the lysines of the APE1 N-terminal tail is likely to prevent further ubiquitylation of APE1 at another site.

It is becoming more apparent that the flexible N-terminal tail of APE1 acts as a regulatory domain of APE1. For instance, the N-terminal lysines 6, 7, 27, 31, 32 and 35 of APE1 were identified as targets for acetylation in cells to effect changes in both redox and endonuclease function (Bhakat et al., 2003a, Fantini et al., 2010, Poletto et al., 2012). The N-terminal tail of APE1 also interacts directly with XRCC1, CSB and NPM1 and APE1 nuclear localisation and export signals are present at residues 1-7 and 8-13 (Wong et al., 2007, Vidal et al., 2001, Vascotto et al., 2009, Fantini et al., 2010, Jackson et al., 2005). Taking in to consideration the various functions of the regulatory N-terminal tail and the large number of mutations required to generate a non-ubiquitylatable form of APE1, I conclude from this chapter that it is unrealistic to utilise research tools based on the ubiquitylation sites of APE1 for the study of the effect of ubiquitylation in regulating APE1 in cells. This is because such mutations may additionally effect various changes in APE1 cellular activity that is unrelated to its inability to be ubiquitylated in cells.

Though polyubiquitylation of a protein is largely linked to its proteasomal degradation, it has become increasingly obvious in recent years that modification of substrates by polyubiquitin monomers can additionally effect changes in protein interaction and activity. It is therefore not possible to assume that APE1 polyubiquitylation by UBR3 will lead to its proteasomal degradation in cells. In the next chapter, the aim was therefore to determine the cellular role for UBR3 in regulating APE1 using mouse embryonic fibroblasts that lack the first exon of the *Ubr3* gene.

Chapter 6

RESULTS IV

UBR3 regulates the steady-state levels of APE1 in cells and promotes genome stability

6.1 INTRODUCTION

APE1 is ubiquitylated in cells and we have identified a cellular E3 ligase, UBR3 that is responsible for *in vitro* polyubiquitylating APE1 at its N-terminal tail. Though polyubiquitylation of a protein is historically associated to its proteasomal degradation, it has become increasingly obvious in recent years that K63-linked polyubiquitin additions on to proteins can additionally effect changes in protein interaction and activity (Reviewed in Husnjak and Dikic, 2012). In this chapter I therefore aimed to identify the cellular role of UBR3 in regulating APE1.

UBR3 is a relatively unstudied E3 ligase that belongs to a family of RING-domain containing E3 ligases characterised by the UBR box (Tasaki et al., 2005, Tasaki et al., 2007). The UBR1, UBR2, UBR4 and UBR5 E3 ligases in this family were shown to recognise N-terminal destabilising residues in their substrates for ubiquitylation and subsequent proteasomal degradation (Tasaki et al., 2005, Tasaki et al., 2009, Bachmair et al., 1986, Glickman and Ciechanover, 2002, Mogk et al., 2007). There are no known substrates for UBR3 and previous studies have shown that UBR3 does not follow the conventional N-end rule pathway of substrate recognition. In fact, the only study that investigates the role of UBR3 in cells is that carried out by our collaborators Dr. Takafumi Tasaki and Dr. Yong Tae Kwon. They were able to generate *Ubr3* -/- mice for characterisation studies (Tasaki et al., 2007). *Ubr3* -/- mice that were initially generated

on a 129SvlmJ background died during embryogenesis. This suggests that UBR3 has a major role to play in embryonic development. On the other hand, *Ubr3* -/- mice generated on a C57BL/6 background survived development however these mice displayed impairments in suckling as well as in the sensory pathways for touch, smell, hearing and vision. Indeed, UBR3 is expressed at much higher levels in cells important for these processes as well as in other neuronal cells. However, this study gave no indication of the role of UBR3 in the maintenance of genome stability and the *Ubr3* -/- mice displayed no notable signs of tumourigenesis.

Through collaboration, I was able to obtain *Ubr3* +/+, *Ubr3* +/- and *Ubr3* -/- mouse embryonic fibroblasts (MEF) from Dr Takafumi Tasaki and Dr Yong Tae Kwon. Using these MEF cells as study tools, this results chapter aims to determine if a loss of UBR3 disturbs the normal regulation of APE1 and subsequently BER activity in these MEF cell lines.

6.2 RESULTS

6.2.1 *Ubr3* -/- MEF cells exhibit a two-fold increase in APE1 protein levels.

I have so far shown that APE1 is ubiquitylated in cells and can be *in vitro* polyubiquitylated by cellular UBR3. In order to assess the role of UBR3 in regulating cellular APE1, *Ubr3* +/+, *Ubr3* +/- and *Ubr3* -/- MEF cells generated by our collaborators Dr. Yong Tae Kwon and Dr. Takafumi Tasaki were utilised as key research tools. The *Ubr3* -/- MEFs were isolated from a mouse embryo in which the first exon of *Ubr3* was deleted (Tasaki et al., 2007). To show that UBR3 was no longer present in the *Ubr3* -/- MEFs, mRNA was purified from both *Ubr3* +/+ and *Ubr3* -/- MEFs and cDNA generated for further analysis by reverse transcriptase PCR using primers targeting the sequence for the first exon of mouse *Ubr3* and actin as a reaction control. The reactions were separated on a 2% agarose gel containing the nucleic acid dye, Sybr Green, prior to

imaging on the BioRad Molecular Imager FX (**Figure 6.1**). A band was obtained for amplified *Ubr3* for *Ubr3* +/+ suggesting that these MEFs do express the *Ubr3* gene. This band however was not present in *Ubr3* -/- MEFs suggesting that the *Ubr3* -/- MEFs indeed lack the exon 1 region of the *Ubr3* gene. This validates the use of these *Ubr3* +/+ and *Ubr3* -/- MEFs as tools in our study to investigate the role of UBR3 in regulating APE1.

UBR3 is part of an E3 ligase family that is known to promote proteasomal degradation of their substrates, thus regulating protein levels and stability in cells (Tasaki et al., 2005). To determine whether the cellular levels of APE1 differed between *Ubr3* +/+ MEFs and *Ubr3* -/- MEFs, whole cell extracts were generated from these two MEF cell lines and separated by 10% SDS-PAGE followed by Western blotting analysis using antibodies against APE1 and the loading control actin (**Figure 6.2**). The results show that APE1 protein levels are at least two-fold higher in *Ubr3* -/- MEFs compared to *Ubr3* +/+ MEFs (Students t-test: $p<0.001$). Further Western blotting analysis of the whole cell extracts for two independently generated clones for each cell line and an *Ubr3* +/- cell line show that APE1 levels are also increased in the *Ubr3* -/- MEF clones and also for the *Ubr3* +/- MEF cell line (**Figure 6.3**). Indeed, if UBR3 were responsible for the proteasomal degradation of APE1, one would expect that a loss of UBR3 should prevent APE1 degradation and thus allow for its accumulation in cells.

6.2.2 APE1 mRNA levels are not significantly higher in *Ubr3* -/- MEF cells

Cells are able to increase cellular protein content through the upregulation of gene expression. To determine whether the increase in APE1 protein in *Ubr3* -/- cells is due to transcriptional upregulation of APE1 gene expression, mRNA was purified from both *Ubr3* +/+ and *Ubr3* -/- MEFs and cDNA generated. The cDNA was subsequently used in a reverse transcriptase reaction using primers designed against APE1 cDNA and actin cDNA as a reaction control. The completed reactions were separated on a 2%

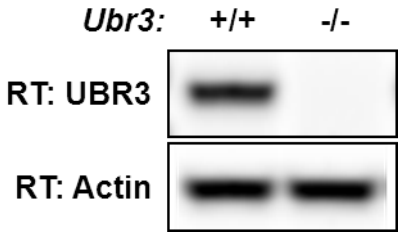

Figure 6.1: UBR3 mRNA is not present in the *Ubr3* -/- MEF cells. Reverse transcriptase was used to prepare cDNA from mRNA that was purified from *Ubr3* +/+ and *Ubr3* -/- MEF cells. The cDNA was subjected to PCR amplification using primers targeting exon 1 of *Ubr3* and *actin*. The reverse transcriptase PCR products were separated on a SYBR green-containing 2% agarose gel by electrophoresis and imaged on the Molecular Imager FX system.

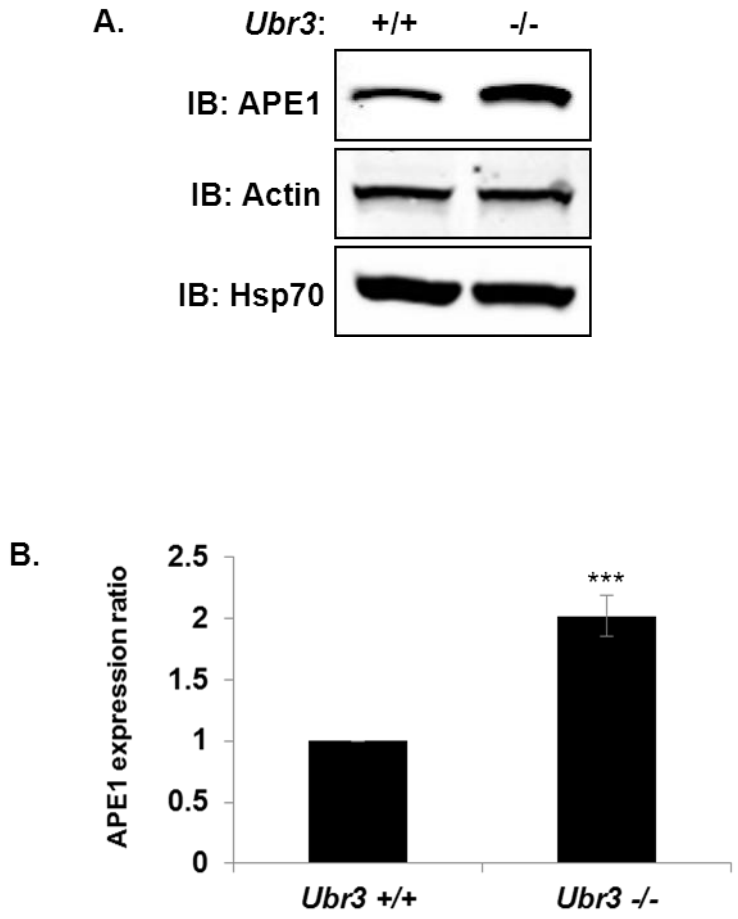

Figure 6.2: *Ubr3* -/- MEF cells exhibit a 2-fold increase in the levels of APE1 protein. A. WCEs were generated from *Ubr3* +/+ and *Ubr3* -/- MEF cells and the extracts separated by 10% SDS-PAGE followed by Western blotting analysis using antibodies against APE1 and the loading controls actin and Hsp70. **B.** Graphical representation of the APE1 expression ratios of *Ubr3* +/+ and *Ubr3* -/- MEF cells, showing standard deviations obtained for three independent experiments. Statistically significant results comparing APE1 expression ratios of *Ubr3* +/+ and *Ubr3* -/- cells are indicated as *** for P<0.001, as analysed by Student's t-test.

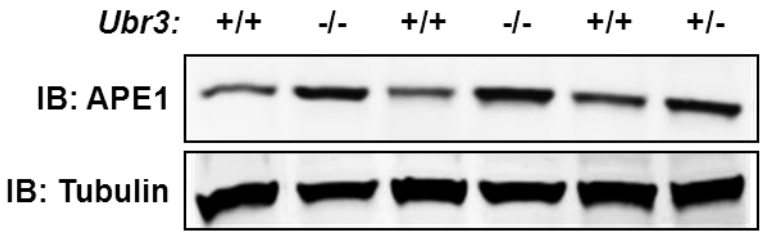

Figure 6.3: Two additional *Ubr3* -/- MEF clones and a *Ubr3* +/- MEF cell line display increased levels of APE1 protein. WCEs were generated from *Ubr3* +/+, *Ubr3* -/- and *Ubr3* -/+ MEF cells and separated by 10% SDS-PAGE followed by Western blotting analysis using antibodies against APE1 and the loading control, tubulin.

agarose gel containing the nucleic acid dye, Sybr Green, and imaged on the BioRad Molecular Imager FX (**Figure 6.4**). It is apparent from the results that there is no significant increase in the amount of APE1 mRNA obtained from the *Ubr3* -/- cell line compared to the amount of APE1 mRNA obtained from the *Ubr3* +/+ cell line. This finding suggests that there is no transcriptional upregulation of APE1 gene expression in the *Ubr3* -/- MEFs compared to the *Ubr3* +/+ MEFs. To more accurately determine the levels of APE1 mRNA present in these cells, quantitative real-time PCR was also carried out using cDNA obtained from both the *Ubr3* +/+ and *Ubr3* -/- MEF cell lines. The reactions were carried out using primers for APE1 cDNA and GAPDH cDNA that was used as a reaction control. Using SYBR-Green as a detection reagent, the PCR reactions were carried out on the 7500 FastReal-Time PCR system and the software (version v.2.0.3) used to determine the cycle thresholds (CT). The relative quantity of APE1 mRNA against GAPDH mRNA (RQ values) was calculated using the equation RQ = $2^{\Delta\Delta CT}$. The average RQ values for three experiments, each performed in triplicate, were plotted on a graph with standard errors highlighted (**Figure 6.5**). The results showed only a 1.3 ± 0.1 fold increase in APE1 mRNA levels in the *Ubr3* -/- MEFs. This increase is in contrast to a 2-fold increase in protein as previously observed by Western blotting. Taken together, the results for both reverse transcriptase and real-time PCRs suggest that APE1 gene expression is not significantly upregulated in the *Ubr3* -/- MEF cell line.

6.2.3 APE1 protein is more stable in *Ubr3* -/- MEF cells

The *Ubr3* -/- MEF cells have high levels of APE1 protein that is not due to increased transcriptional expression of the APE1 gene. This suggests that the levels of APE1 are being regulated on a post-translational level. Another major mechanism utilised by cells to regulate protein levels within cells is ubiquitylation-mediated protein

degradation. A loss in such a mechanism could also result in the accumulation of a protein within a cell. To better understand the cellular stability of APE1 in the *Ubr3* -/-

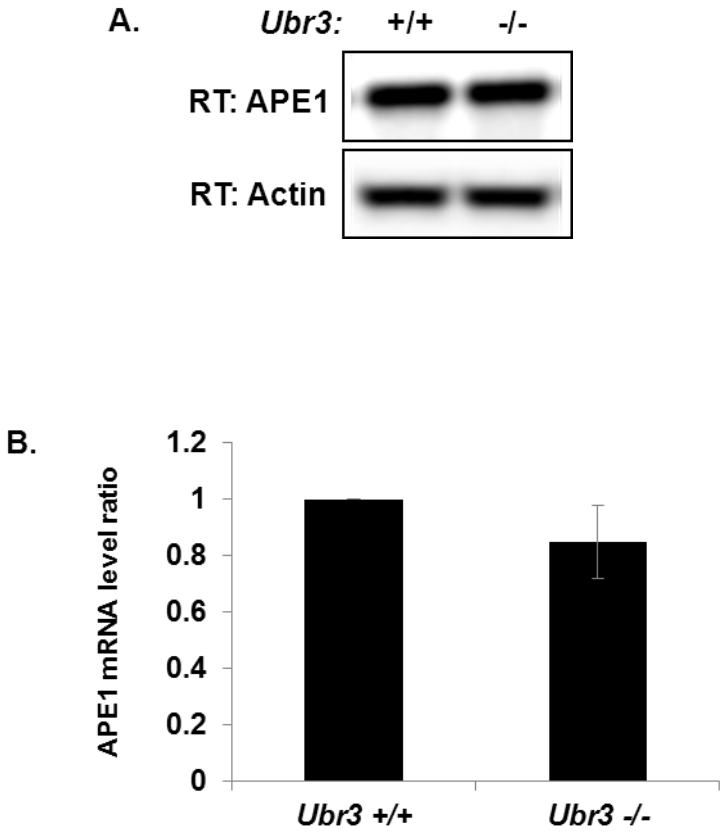

Figure 6.4: APE1 mRNA levels are not upregulated in *Ubr3* -/- MEF cells: Reverse transcription PCR. A. Reverse transcription PCR was used as a quantitative measure of mRNA levels. Reverse transcriptase was used to prepare cDNA from mRNA that was purified from *Ubr3* +/+ and *Ubr3* -/- MEF cells. The cDNA was subjected to PCR amplification using primers targeting APE1 and actin cDNA. The reverse transcriptase PCR products were separated on a SYBR green-containing 2% agarose gel by electrophoresis and imaged on the Molecular Imager FX system. **B.** Graphical representation of APE1 mRNA level ratios of *Ubr3* +/+ and *Ubr3* -/- MEF cells, showing standard deviations obtained for three independent experiments.

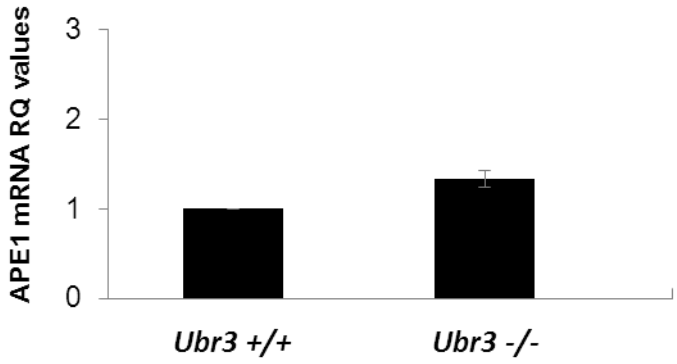

Figure 6.5: APE1 mRNA levels are not significantly upregulated in *Ubr3 -/-* MEF cells: Real-time PCR. A. Real-time PCR was used as a quantitative measure of mRNA levels. Reverse transcriptase was used to prepare cDNA from mRNA that was purified from *Ubr3* +/+ and *Ubr3* -/- MEF cells. The 7500 FastReal-Time PCR system was used to amplify the cDNA in a reaction containing SYBR-Green stain, polymerase and primers targeting APE1 and GAPDH cDNA. The amplification was tracked by recording the intensity of the SYBR green detection reagent. The cycle threshold (CT) data was produced by the system software and the relative quantity of mRNA, or RQ values, were calculated as $RQ = 2^{\Delta\Delta CT}$. Shown is a graphical representation of the APE1 mRNA RQ values for the *Ubr3* +/+ and *Ubr3* -/- MEF cells, showing standard deviations obtained for three independent experiments, each carried out in triplicate.

MEF cells compared to *Ubr3* +/+ cells, both cell lines were transfected with 0.5 µg of the pCMV3Tag3a mammalian expression plasmid encoding FLAG-tagged mouse APE1. This amount of plasmid is sub-optimal to ensure that APE1 is not expressed to its maximum capacity in cells after 24 hours of treatment, thus allowing us to observe subtle differences in the stability of transfected APE1 between the two cell lines (Optimisation of FLAG-APE1 expression in *Ubr3* +/+ MEF cells, **Appendix IV**). Plasmid expression was carried out for 24 hours, the cells collected and whole cell extracts prepared. The whole cell extracts were separated by 10% SDS-PAGE and analysed by Western blotting using antibodies against APE1 and tubulin (**Figure 6.6**). FLAG-APE1 is expressed at an approximate 2.5-fold increased level (Students t-test: $p<0.02$) in the *Ubr3* -/- MEF cell line compared to the *Ubr3* +/+ MEF cell line suggesting that FLAG-APE1 is more stable in the *Ubr3* -/- cell line.

To further investigate APE1 stability, siRNA sequences were designed to target mouse APE1 mRNA to allow for the repression of APE1 mRNA translation in to protein. If the mechanism allowing for APE1 protein degradation in cells is functional, and APE1 protein is not being produced by the cell, then APE1 that is already present in cells should be lost, though this is dependent on the inherent stability of the protein. *Ubr3* +/+ and *Ubr3* -/- cells were transfected with siRNA sequences targeting APE1 mRNA or GFP mRNA (negative control) using RNAiMax lipofectamine and incubated at 37 °C for 72 hours. Whole cell extracts were generated and separated by 10% SDS-PAGE and analysed by Western blotting using antibodies against APE1 and the loading control, tubulin (**Figure 6.7**). APE1 levels were reduced significantly (Students t-test: $p<0.001$) in the *Ubr3* +/+ MEF cells however there was no change in APE1 levels in the *Ubr3* -/- cells. This finding again suggests that APE1 is more stable in the *Ubr3* -/- MEF cells. It is possible that this gain of stability is due to a loss of UBR3-mediated APE1 ubiquitylation for subsequent proteasomal degradation.

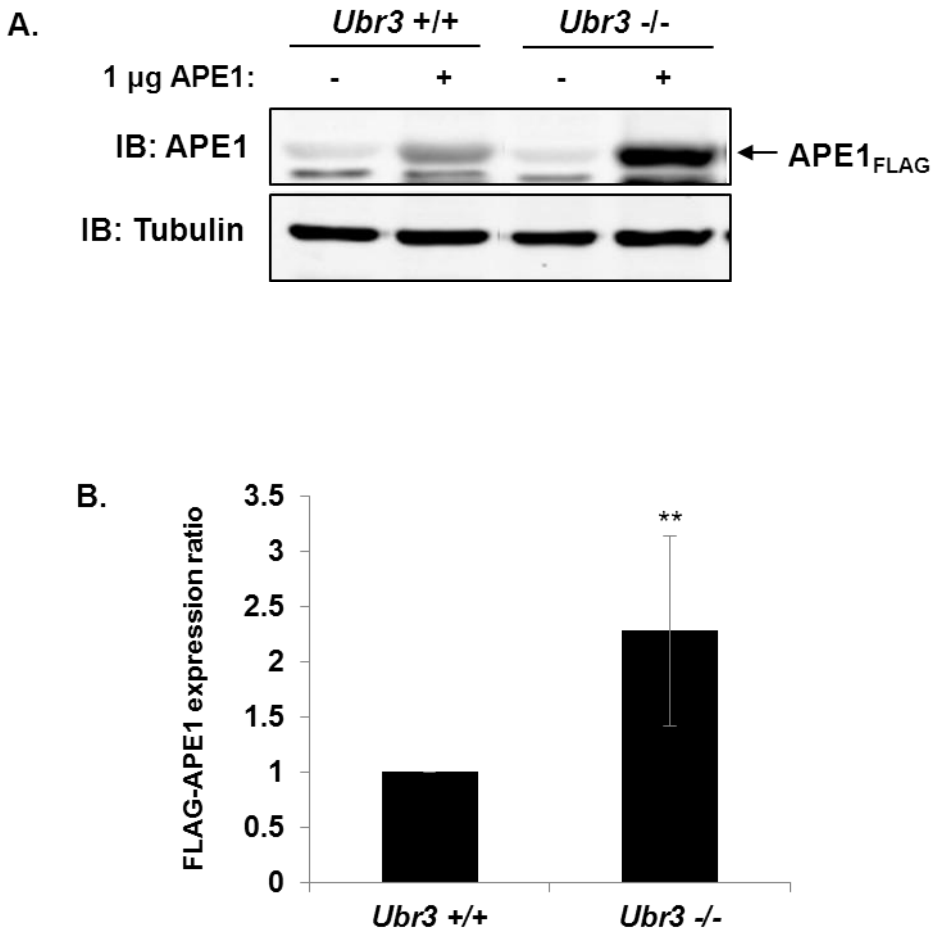

Figure 6.6: Transiently overexpressed mouse APE1 is more stable in *Ubr3* -/- MEF cells. A. pCMV3Tag3a (0.5 µg) plasmid encoding FLAG-tagged mouse APE1 was transfected in to *Ubr3* +/+ and *Ubr3* -/- MEF cells using lipofectamine 2000 transfection reagent and allowed to express for 24 hr. The cells were harvested, whole cell extracts were prepared and separated by 10% SDS-PAGE, followed by transferring on to a PVDF membrane and immunoblotting with antibodies against the FLAG tag and tubulin. **B.** Graphical representation of the FLAG-APE1 expression ratios of *Ubr3* +/+ and *Ubr3* -/- MEF cells, showing standard deviations obtained for three independent experiments. Statistically significant results comparing FLAG-APE1 expression ratios in *Ubr3* +/+ and *Ubr3* -/- cells are indicated as ** for $P<0.02$, as analysed by Student's t-test.

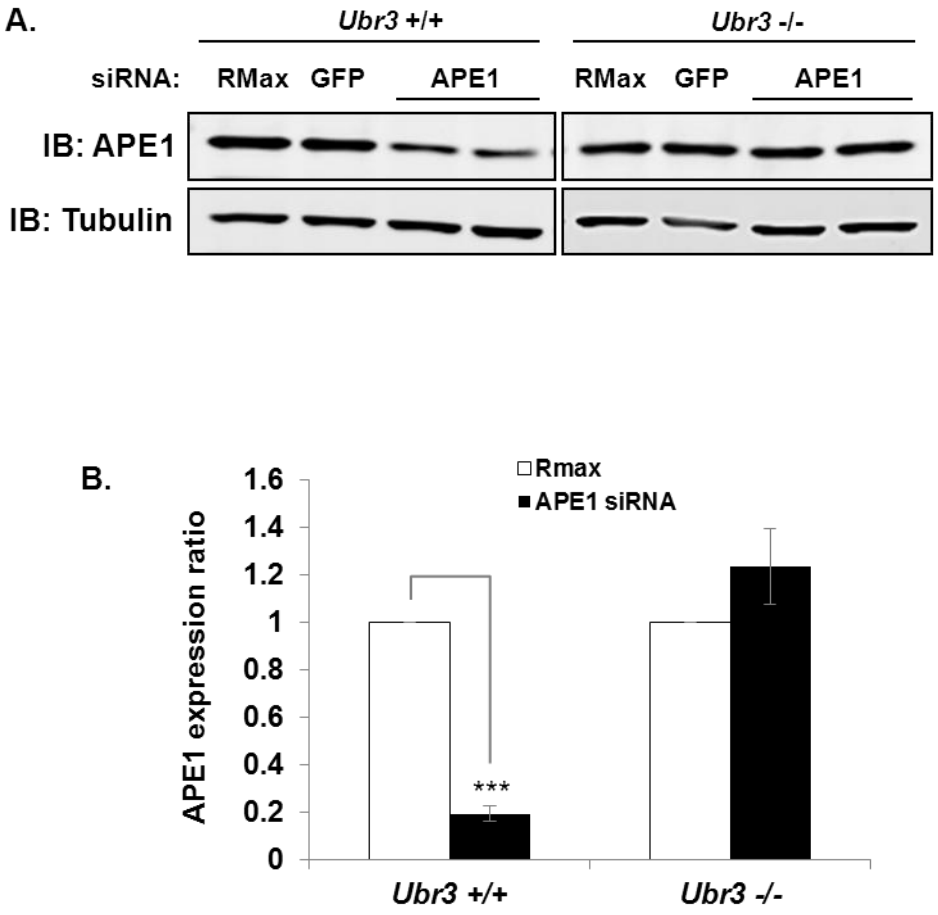

Figure 6.7: Cellular APE1 is more stable in *Ubr3* -/- MEF cells. A. Silencing sequences targeting mouse APE1 mRNA or non-targeting GFP (scramble siRNA) were introduced in to *Ubr3* +/+ and *Ubr3* -/- MEF cells using the RNAiMax lipofectamine reagent. After allowing 72 hr for silencing, the cells were harvested, whole cell extracts prepared and separated by 10% SDS-PAGE, followed by Western blotting analysis with antibodies against APE1 and tubulin. **B.** Graphical representation of the APE1 expression ratios of *Ubr3* +/+ and *Ubr3* -/- MEF cells treated with APE1 siRNA or the RNAiMax control treatment, showing standard deviations obtained for three independent experiments. Statistically significant results comparing APE1 expression ratios in *Ubr3* +/+ and *Ubr3* -/- cells are indicated as *** for P<0.001, as analysed by Student's t-test.

6.2.4 *Ubr3 -/-* MEF cells do not display altered cellular localisation of APE1

Ubiquitylation can also affect the cellular localisation of a protein. To investigate whether a loss of UBR3-mediated APE1 ubiquitylation effects a change in APE1 localisation, nuclear and cytoplasmic extracts were generated for each of the *Ubr3* +/+ and *Ubr3* -/- MEF cell lines. The extracts were separated by 10% SDS-PAGE and analysed by Western blotting using antibodies against APE1 and the cytoplasmic marker tubulin (**Figure 6.8**). The localisation of APE1 is equivalent for the *Ubr3* +/+ MEF and *Ubr3* -/- MEF cell lines with 30.4% present in the nuclear fraction for the *Ubr3* +/+ MEF compared to 31.9% for the *Ubr3* -/- cell line. To visually show that APE1 localisation is not impaired in *Ubr3* -/- MEFs, *Ubr3* +/+ and *Ubr3* -/- MEF cells were fixed on to slides and immunostained with APE1 antibodies (green) and the nuclear marker DAPI. The slides were imaged using a BioRad confocal microscope (**Figure 6.9**). Though there appears to be a stronger overall APE1 signal coming from the *Ubr3* -/- MEF cells, it is clear that APE1 is localised to both the nucleus and the cytoplasm for each cell line and that the localisation pattern is comparable. APE1 ubiquitylation by UBR3 is therefore unlikely to be a major regulator of the cellular localisation of APE1 though it is important to realise that total APE1 is being analysed in these experiments, and not ubiquitylated APE1 which was previously identified in the cytoplasm of HeLa cells.

6.2.5 *Ubr3 -/-* MEF cells repair MMS and IR-induced damage more efficiently

A high level of APE1 in some tumour types is thought to allow for the increased repair of chemotherapy and radiation induced damage, thereby promoting tumour resistance to such treatments (Naidu et al., 2010, Wang et al., 2009, Robertson et al., 2001). It was therefore of interest to investigate the repair capacity of the *Ubr3* -/- cells that have higher levels of APE1 compared to the *Ubr3* +/+ cells. The alkaline COMET assay is a widely used technique for determining the DNA repair kinetics of cells since it allows for relatively sensitive detection and quantification of both DNA single and

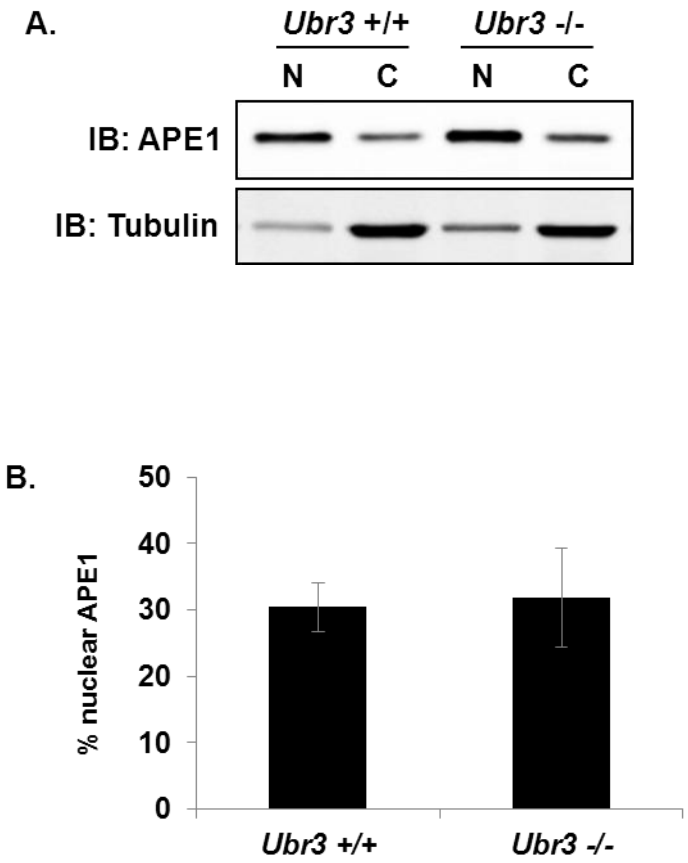

Figure 6.8: *Ubr3* -/- MEF cells do not display altered cellular localisation of APE1: Western blot. A. Nuclear (N) and cytoplasmic (C) extracts were generated from *Ubr3* +/+ and *Ubr3* -/- MEF cells and separated by 10% SDS-PAGE followed by Western blotting analysis using antibodies against APE1 and the cytoplasmic marker tubulin. **B.** Graphical representation of the % nuclear APE1 in *Ubr3* +/+ and *Ubr3* -/- MEF cells, showing standard deviations obtained for three independent experiments.

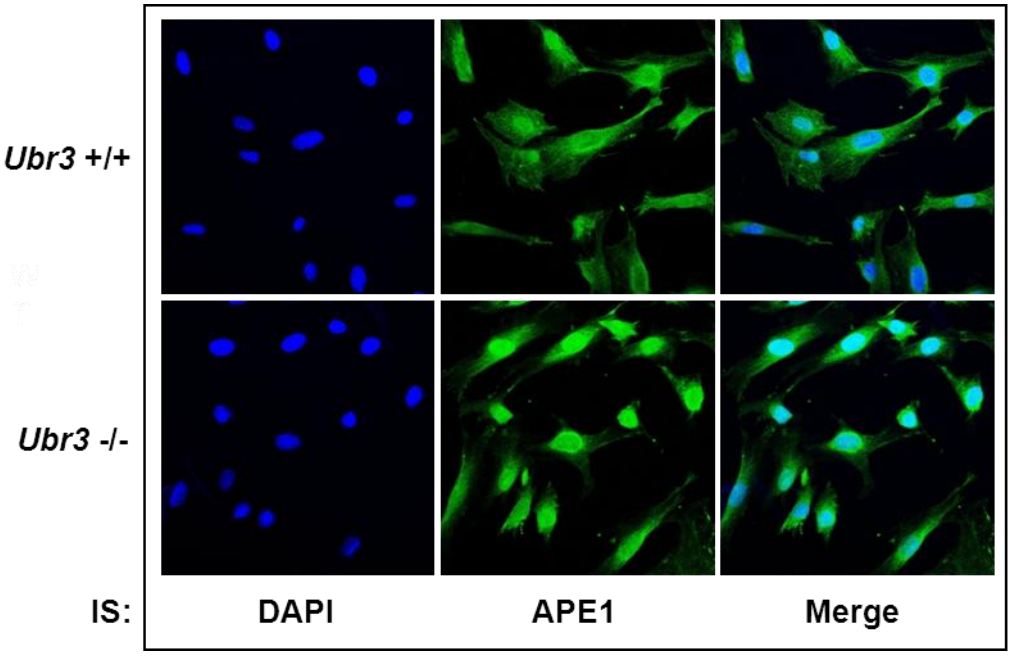

Figure 6.9: *Ubr3* -/- MEF cells do not display altered cellular localisation of APE1: Immunostaining. *Ubr3* +/+ and *Ubr3* -/- MEF cells were fixed on to a slide and stained with the nuclear marker DAPI (blue) or immunostained with APE1 antibodies (green). Images were taken using a Biorad confocal microscope and merged.

double-strand breaks as well as alkali labile sites such as abasic sites (Described in Parsons et al., 2009, Parsons et al., 2011). This is calculated as the DNA tail intensity (%) that is a direct measure of the amount of DNA strand breaks present in a cell since this tail is generated upon electrophoretic separation of fragmented DNA. Using this assay we firstly determined the optimal dose for both MMS (250 µM) and irradiation (6 Gy) treatment of *Ubr3* +/+ and *Ubr3* -/- MEF cells that generated % tail DNA intensities of no more than 50%. MMS induces base lesions that are repaired primarily through APE1 whereas irradiation induces a mixture of base lesions, DNA single strand breaks and DNA double strand breaks that are repaired by multiple pathways including the base excision repair pathway (Lundin et al., 2005, Ward, 2000). *Ubr3* +/+ and *Ubr3* -/- MEF cells were subsequently treated with 6 Gy irradiation or 250 µM MMS and the cells were suspended in 1% low melt agarose prior to spreading on to duplicate slides. The slides were placed in to a 37 °C incubator for the indicated repair time (up to 4 hours for MMS treatment and up to 2 hours for irradiation treatment) prior to lysing and separation using a specialised electrophoresis unit. Upon separation the slides were stained with SYBR-Gold staining, imaged using the Nikon Eclipse 90i microscope and data collected using the Kinetic Imaging Komet 5.5 software. The % tail DNA intensities for 50 cells per slide (100 cells per condition) were measured, averaged and normalised for each of the repair time-points (**Figure 6.10**). For the irradiation treated cells (**Figure 6.10, A**), the *Ubr3* -/- MEF cells displayed significantly increased repair rates at time-points of 20 minutes (Students t-test: $p<0.05$), 30 minutes (Students t-test: $p<0.02$), 60 minutes (Students t-test: $p<0.05$) and 120 minutes (Students t-test: $p<0.05$). For the MMS treated cells (**Figure 6.10, B**), the *Ubr3* -/- MEF cells displayed significantly increased repair rates at time-points of 120 minutes (Students t-test: $p<0.02$) and 180 minutes (Students t-test: $p<0.05$). These results suggest that the *Ubr3* -/- MEF cells do indeed repair DNA damage inflicted by both MMS and irradiation treatments more efficiently than the *Ubr3* +/+ MEF cells.

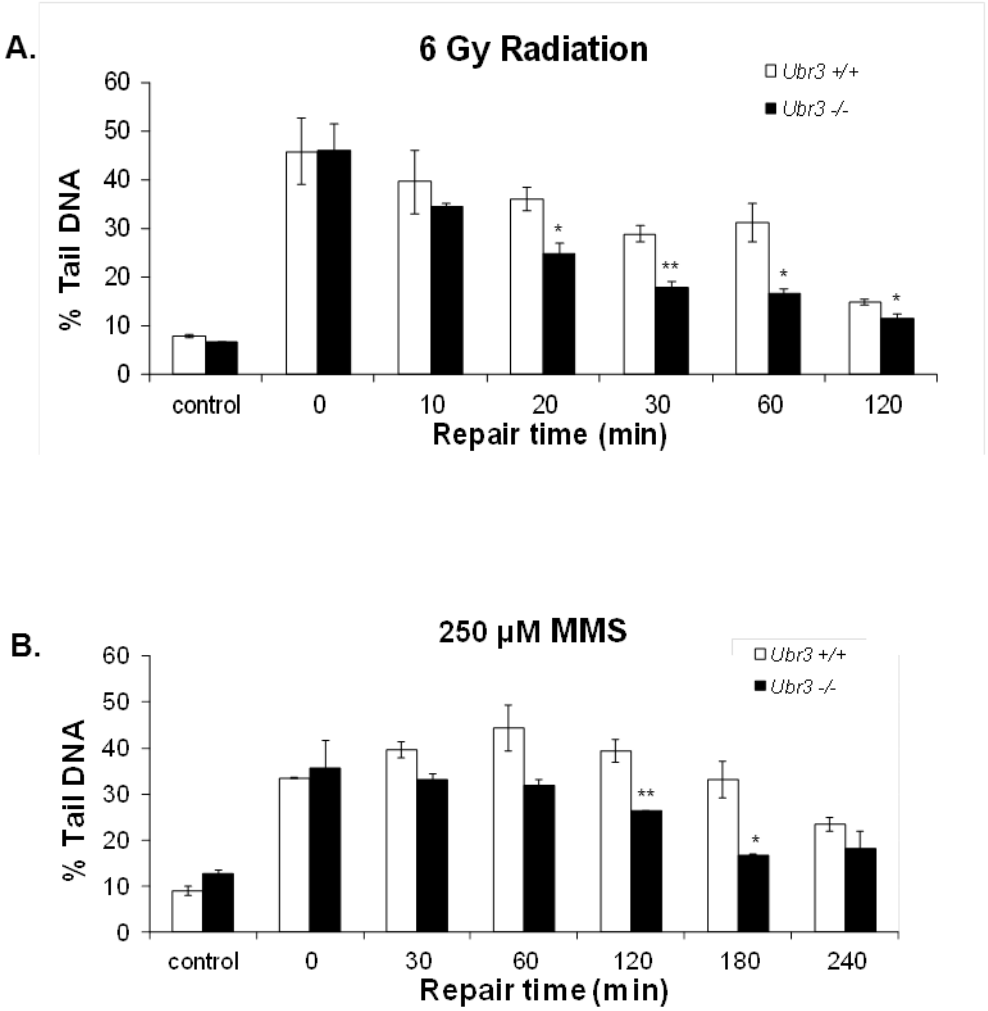

Figure 6.10: *Ubr3* -/- MEF cells repair MMS and irradiation damage more efficiently. An alkaline COMET assay was used to determine repair kinetics. *Ubr3* +/+ and *Ubr3* -/- MEF cells were treated with either 6 Gy irradiation (**A**) or 250 µM MMS (**B**). The cells were embedded in 1% agarose on slides and lysed at the indicated repair time-points. The DNA was separated by single cell gel electrophoresis, stained with SYBR Gold and imaged using a Nikon 90i microscope. The % tail DNA intensities for 100 cells were obtained from 2 slides each and the graphical representation with standard deviations is shown for one representative experiment. Statistically significant results comparing the % tail of *Ubr3* +/+ and *Ubr3* -/- cells are indicated as ** for P<0.02 or * for P<0.05, as analysed by Student's t-test.

Though the increased repair activity observed in the *Ubr3* -/- MEF cells could be related to the higher levels of APE1 in these cells, these cells would additionally require upregulated downstream BER activity for repair completion. It was therefore of interest to determine the levels of downstream BER factors in these cells compared to the *Ubr3* +/+ MEF cells. To determine whether the cellular levels of the downstream BER repair factors differed between *Ubr3* +/+ MEFs and *Ubr3* -/- MEFs, whole cell extracts were generated from these two MEF cell lines and separated by 10% SDS-PAGE followed by Western blotting analysis using antibodies against the BER factors PARP1, Pol β, Lig IIIα, the negative regulator for Pol β ubiquitylation, p14-ARF and the loading control, actin (**Figure 6.11, A**). There appears to be a moderate increase in the levels of both PARP1 and Pol β but no changes in the levels of DNA Lig IIIα. The levels of p14-ARF that negatively regulates Pol β ubiquitylation is significantly increased in the *Ubr3* -/- cells and this is the most likely explanation for the increase in Pol β levels in these cells.

To understand better the mechanisms by which PARP1, Pol β and p14-ARF are upregulated in the *Ubr3* -/- cells, the mRNA levels for these factors were also analysed by reverse transcriptase PCR. cDNA was generated from mRNA that was purified from both *Ubr3* +/+ and *Ubr3* -/- MEFs and the cDNA was subsequently used in a reverse transcriptase reaction using primers designed against the cDNA for PARP1, Pol β, p14-ARF and actin as a reaction control. The completed reactions were separated on a 2% agarose gel containing the nucleic acid dye, Sybr Green, and imaged on the BioRad Molecular Imager FX (**Figure 6.11, B**). There appears to be a moderate increase in PARP1 mRNA levels and a significant increase in p14-ARF mRNA levels in the *Ubr3* -/- MEF cells that may explain the increased protein levels exhibited by these cells. There is however no change in Pol β mRNA levels. The noted increase in Pol β protein levels in the *Ubr3* -/- cells is likely due to the increase in p14-ARF that binds to the E3 ligase Mule, thus inhibiting its ability to ubiquitylate Pol β and subsequently increasing Pol β cellular stability by preventing its proteasomal degradation (Parsons et al., 2009). It

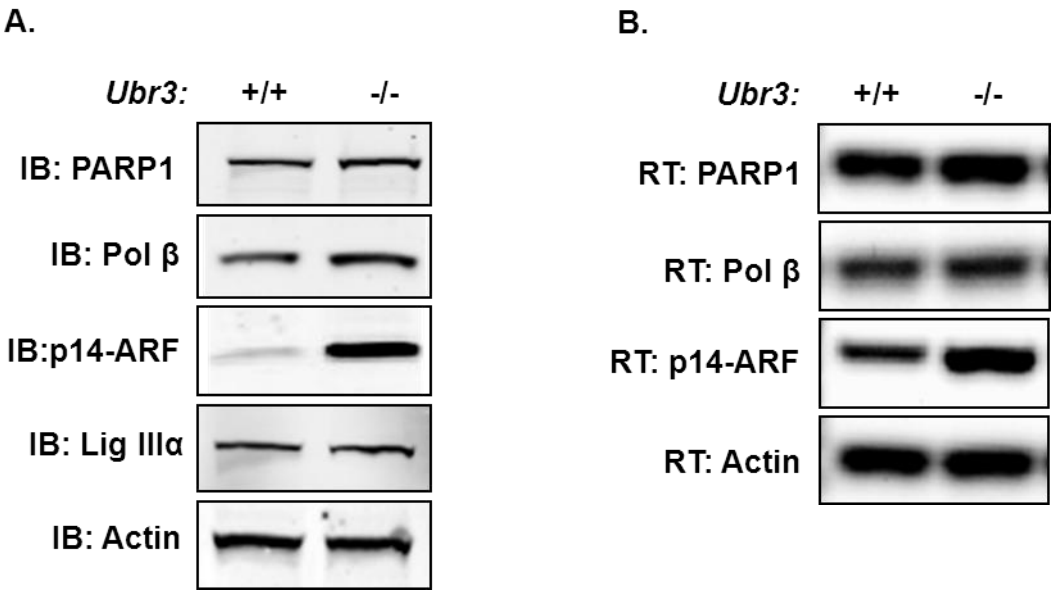

Figure 6.11: Protein and mRNA levels of BER factors in the *Ubr3* +/+ MEF and *Ubr3* -/- MEF cells. A. WCEs were generated from *Ubr3* +/+ MEF and *Ubr3* -/- MEF cells and separated by 10% SDS-PAGE followed by Western blotting analysis using antibodies against PARP1, Pol β, p14-ARF, Lig IIIα and the loading control, actin. **B.** Reverse transcription PCR was used as a measure of mRNA levels. Reverse transcriptase was used to prepare cDNA from mRNA that was purified from *Ubr3* +/+ and *Ubr3* -/- MEF cells. The cDNA was subjected to PCR amplification using primers targeting cDNA for PARP1, Pol β, p14-ARF and actin. The reverse transcriptase PCR products were separated on a SYBR green-containing 2% agarose gel by electrophoresis and imaged on the Molecular Imager FX system.

appears therefore that the *Ubr3* -/- cells may have altered their downstream BER activity in response to the increase in APE1-mediated repair activity.

6.2.6 *Ubr3* -/- cells display hallmarks of genome instability and exhibit high levels of DNA double strand breaks

The images generated for DAPI immunostaining of *Ubr3* +/+ and *Ubr3* -/- MEF cells (**Figure 6.12**) highlighted an irregularity in the *Ubr3* -/- cell line. These cells displayed micronuclei (Indicated by arrows in **Figure 6.12, A**), irregularly shaped nuclei and nuclei of varying sizes (**Figure 6.12, B**) whereas the *Ubr3* +/+ cells did not. All of these irregularities have previously been identified as hallmarks of genome instability (Gisselsson et al., 2001, Fenech et al., 1999). This observation suggests that the *Ubr3* -/- MEF cells may experience additional genome instability compared to the *Ubr3* +/+ MEF cells.

The repair intermediate generated by APE1 is a DNA single strand break. DNA SSBs that are not repaired efficiently can allow for the formation of DNA double strand breaks (Kuzminov, 2001). It is therefore possible that excessive amounts of APE1 in the *Ubr3* -/- cells can allow for the formation of DNA DSBs that may contribute to the genome instability observed in these cells. To determine the numbers of endogenously generated DNA DSBs present in the *Ubr3* +/+ and *Ubr3* -/- cells, the cells were fixed on to slides and immunostained with antibodies against 53BP1, a well characterised marker of DNA DSB repair foci and the nuclear marker DAPI (Huyen et al., 2004). The slides were imaged on a BioRad confocal microscope and representative images are shown (**Figure 6.13, A**). The total number of repair foci for 300 cells per cell line was counted across three independently set up slides. The ratio of foci number was calculated, plotted and the standard deviations for the ratio obtained from the three slides are shown (**Figure 6.13, B**). The results show that there is approximately 2.5-fold more 53BP1 repair foci present in *Ubr3* -/- MEF cells compared to *Ubr3* +/+ cells (Students t-

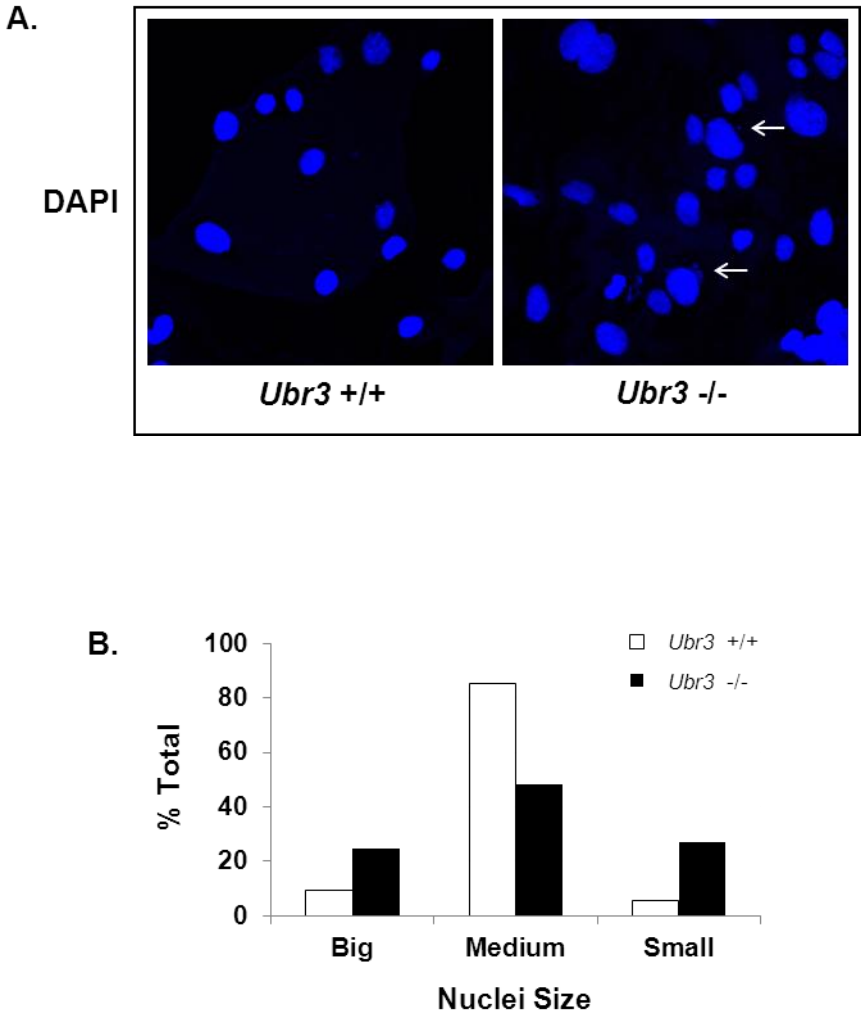

Figure 6.12: The nucleus of *Ubr3* -/- MEF cells are irregularly shaped and sized. A. *Ubr3* +/+ and *Ubr3* -/- MEF cells were fixed on to a slide and stained with the nuclear marker DAPI (blue). Images were taken using a Biorad confocal microscope. The arrows point out the presence of micronuclei in the *Ubr3* -/- cells. **B.** 100 DAPI stained nuclei per cell line for *Ubr3* +/+ and *Ubr3* -/- MEF cells were categorised according to size (large, medium or small) and the distribution (% of total) is shown in the graph.

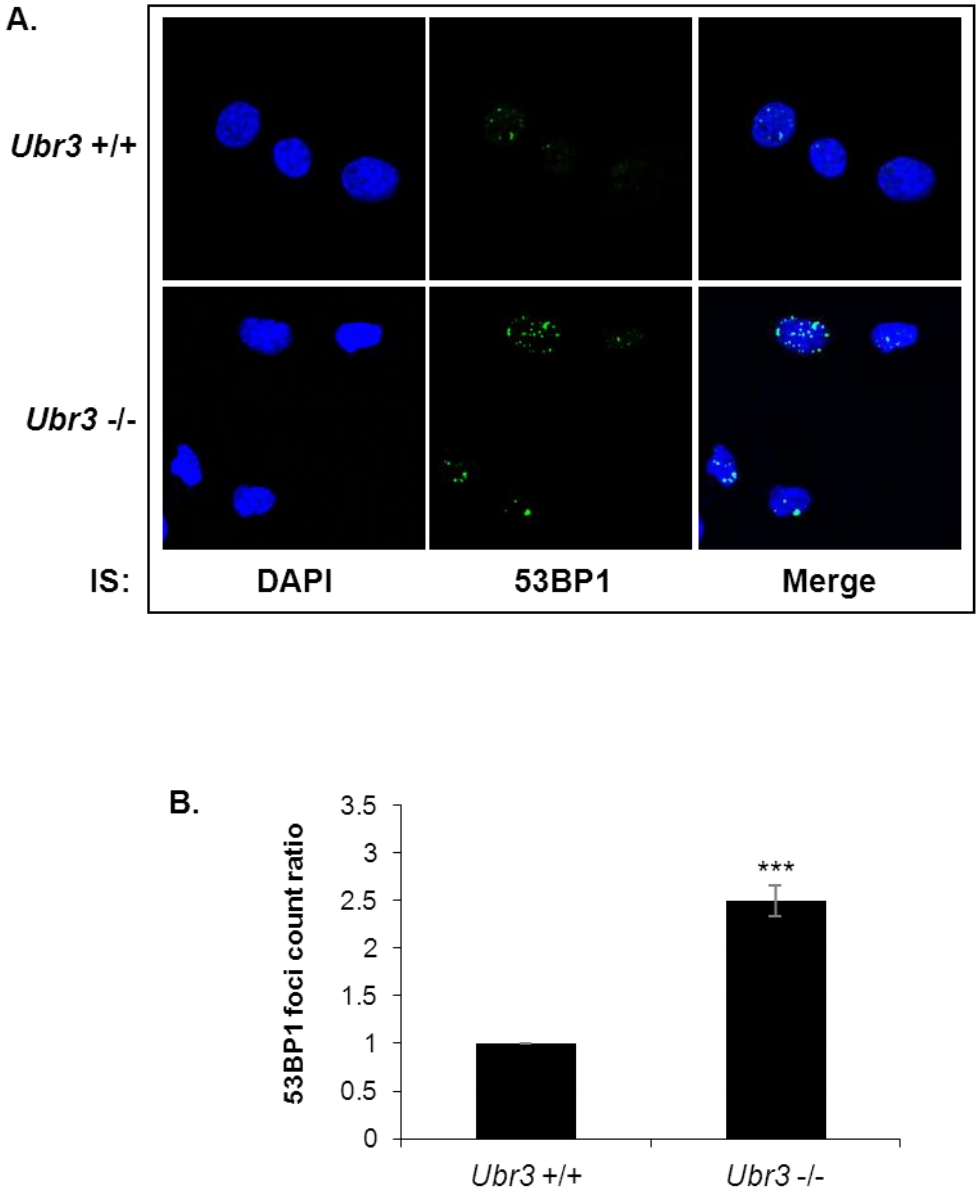

Figure 6.13: *Ubr3* -/- MEF cells have high numbers of 53BP1 repair foci, a marker for DNA double strand breaks. A. Untreated *Ubr3* +/+ and *Ubr3* -/- MEF cells were fixed on to a slide and stained with the nuclear marker DAPI (blue) or immunostained with antibodies against the DNA double strand break repair foci marker 53BP1 (green). Images were taken using a Biorad confocal microscope and merged. **B.** Graphical representation of the 53BP1 foci count ratio of *Ubr3* +/+ and *Ubr3* -/- MEF cells, showing standard deviations obtained for three independent experiments. Statistically significant results comparing 53BP1 foci count ratios in *Ubr3* +/+ and *Ubr3* -/- cells are indicated as *** for $P<0.001$, as analysed by Student's t-test.

test: p<0.001). To further show that the *Ubr3* -/- MEF cells contained high levels of DNA double strand breaks compared to the *Ubr3* +/+ MEF cells, whole cell extracts were generated for both cell lines and separated by 10% SDS-PAGE followed by Western blotting analysis using antibodies against the DNA DSB marker, phosphorylated H2AX (γH2AX) and actin that was used as a loading control (**Figure 6.14**) (Rogakou et al., 1999, Paull et al., 2000). The *Ubr3* -/- MEF cells exhibited a 6-fold increase in the amount of γH2AX compared to *Ubr3* +/+ cells (Students t-test: p<0.001), again suggesting that these cells have a greater number of endogenously generated DNA DSBs.

6.2.7 Excessive amounts of APE1 induce the formation of DNA double strand breaks

To show that high levels of APE1 could be responsible for the increased numbers of endogenously generated DNA double strand breaks in *Ubr3* -/- MEF cells, 1 μg of the mammalian expression plasmid pQCXIH encoding FLAG-tagged human APE1 was transfected in to HeLa cells using Lipofectamine transfection reagent. APE1 gene expression was allowed for 24 hours and whole cell extracts prepared. The whole cell extracts were separated by 10% SDS-PAGE and analysed by Western blotting using antibodies against APE1, γH2AX and tubulin (**Figure 6.15**). FLAG-APE1 was overexpressed to ~ 50% of endogenous APE1 levels and cells overexpressing APE1 displayed an approximately 8-fold increase in the levels of γH2AX (Students t-test: p<0.001). It is possible therefore that the increased APE1 levels observed in *Ubr3* -/- MEF cells is responsible for the increased numbers of DNA DSBs present in these cells.

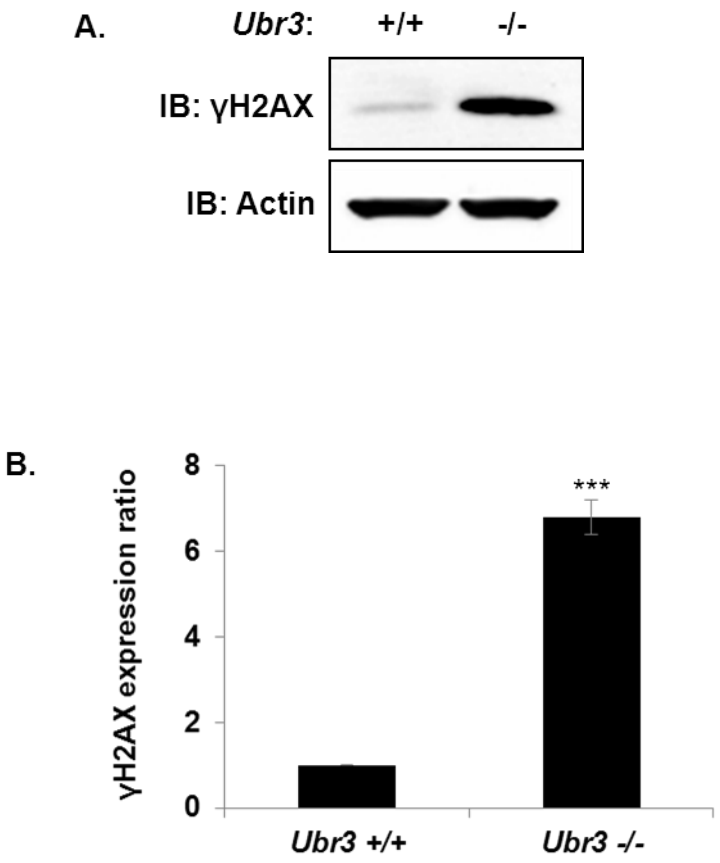

Figure 6.14: *Ubr3* -/- MEF cells display high levels of the DNA double strand break marker, γH2AX. A. Whole cell extracts generated from *Ubr3* +/+ and *Ubr3* -/- MEF cells were separated by 4%-16% SDS-PAGE, transferred on to a PVDF membrane by Western blotting and immunoblotted with antibodies against γH2AX or actin. **B.** Graphical representation of the γH2AX expression ratio of *Ubr3* +/+ and *Ubr3* -/- MEF cells, showing standard deviations obtained for three independent experiments. Statistically significant results comparing γH2AX expression ratios in *Ubr3* +/+ and *Ubr3* -/- cells are indicated as *** for $P<0.001$, as analysed by Student's t-test.

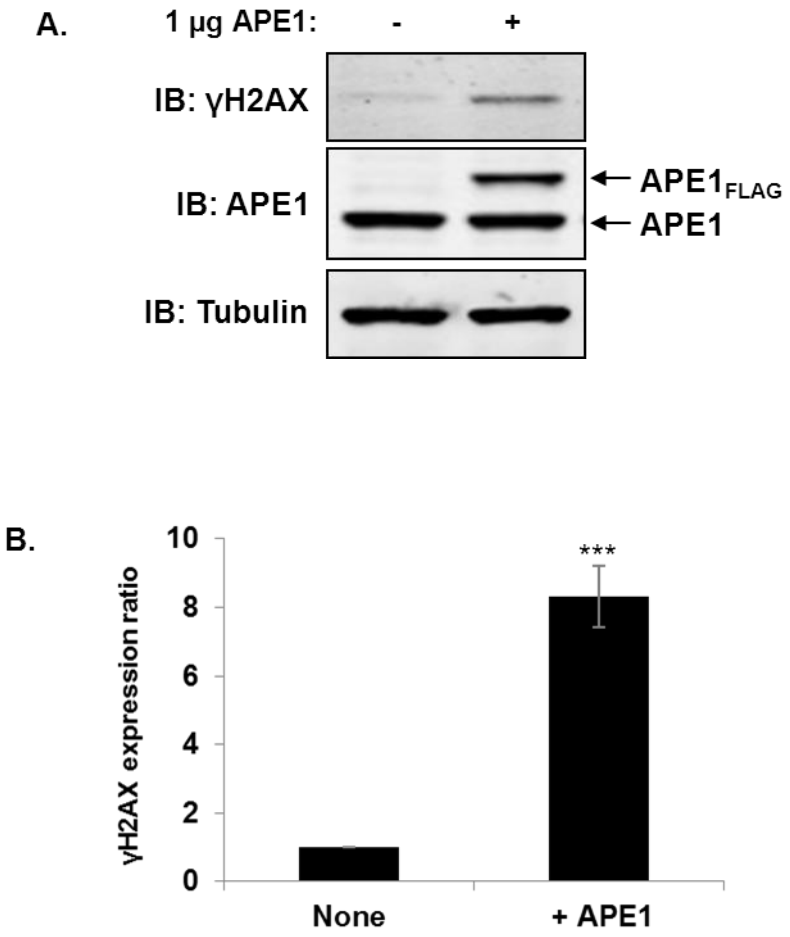

Figure 6.15: HeLa cells transiently expressing FLAG-tagged APE1 display high levels of the DNA double strand break marker, γH2AX. A. HeLa cells were transiently transfected with 1 µg of the pQCXIH plasmid encoding FLAG-tagged APE1 using lipofectamine 2000 transfection reagent. The control and APE1 overexpressing HeLa cells were harvested after 24 hr and whole cell extracts prepared for separation by 10% SDS-PAGE and analysis by Western blotting using antibodies against the DNA double strand break marker γH2AX, APE1 or tubulin. FLAG-tagged APE1 ($APE1_{FLAG}$) and endogenous APE1 (APE1) are indicated. **B.** Graphical representation of the γH2AX expression ratio of non-transfected HeLa (None) and APE1 overexpressing (+ APE1) HeLa cells, showing standard deviations obtained for three independent experiments. Statistically significant results comparing γH2AX expression ratios in these cells are indicated as *** for $P<0.001$, as analysed by Student's t-test.

6.2.8 *Ubr3 -/-* MEF cells are more sensitive to MMS treatment

DNA double strand breaks are very toxic lesions that trigger cell cycle arrest and cell death if not repaired efficiently. In fact, less than 5% of lesions generated by irradiation are DNA DSBs, yet these lesions are the main cause of cellular death in irradiated cells (Ward, 2000, Ward, 1990). One would therefore expect that a greater number of DNA DSBs would allow for increased cellular sensitivity to DNA damaging agents. It was therefore of interest to determine the sensitivity of *Ubr3* -/- cells to DNA damaging agents, particularly those that involve direct repair by APE1. MMS is a DNA damaging agent that generates base lesions that are repaired primarily by the base excision repair pathway. A clonogenic survival assay was carried out to determine the sensitivity of *Ubr3* +/+ and *Ubr3* -/- MEF cells to MMS treatment. *Ubr3* +/+ and *Ubr3* -/- MEF cells that were plated at a low density (300 cells / 10 cm^2 dish) were treated with increasing concentrations of MMS and left to expand over a period of 7-9 days until the colonies were visible by eye. The colonies were stained with methylene blue stain and counted. The surviving fraction for each MMS concentration was calculated (number of colonies for treatment / number of colonies for control) and the results obtained for three independent experiments were plotted on a logarithmic scale graph (**Figure 6.16**). The surviving fraction was significantly lower for the MMS treated *Ubr3* -/- MEF cells compared to the *Ubr3* +/+ MEF cells at the treatment dose of 2 mM MMS (Students t-test: $p<0.02$) suggesting that the *Ubr3* -/- MEF cells are more sensitive to MMS treatment.

6.3 RESULTS SUMMARY

In the previous chapter I showed that cellular UBR3 *in vitro* polyubiquitylates APE1 primarily at a single site on its N-terminal tail. Since polyubiquitylation of a protein can mediate multiple cellular outcomes, this chapter aimed to understand the role of UBR3-mediated ubiquitylation in regulating APE1 in cells. To achieve this, mouse

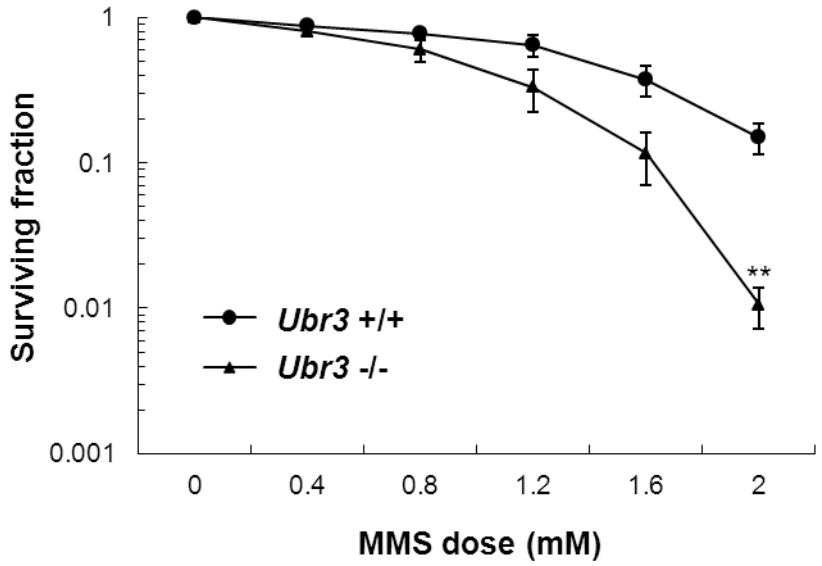

Figure 6.16: *Ubr3* -/- MEF cells are more sensitive to MMS treatment. *Ubr3* +/+ and *Ubr3* -/- MEF cells seeded at a density of 300 cells per 10 cm^2 plate were treated with indicated amounts of MMS for 45 minutes. The plates were washed, the media replaced and cells allowed to grow for 7 days. The surviving colonies were stained with methylene blue, counted and the surviving fraction calculated as a ratio to the untreated control. Three independent experiments, each performed in triplicate were carried out, the results pooled and depicted in the representative graph on a logarithmic scale. The standard errors across the three experiments are shown and statistically significant results comparing the surviving fractions of *Ubr3* +/+ and *Ubr3* -/- cells are indicated as ** for $P<0.02$, as analysed by Student's t-test.

embryonic fibroblasts that lack the first coding exon of the *Ubr3* gene were used as a key study tool.

UBR3 belongs to a family of E3 ligases that regulate protein stability in cells and therefore the stability of APE1 in *Ubr3* -/- MEF cells was of primary interest. Indeed, *Ubr3* -/- MEF cells displayed a two-fold increase in APE1 protein that was not due to upregulated mRNA levels. I subsequently showed that APE1 overexpressed in *Ubr3* -/- MEF cells is more stable compared to APE1 overexpressed in *Ubr3* +/+ MEF cells. Furthermore, using siRNA technology to silence the translation of APE1 mRNA in to protein, I was able to show that *Ubr3* -/- cells are unable to remove APE1 protein already present in cells whereas a decrease in APE1 protein levels was observed in *Ubr3* +/+ MEF cells. The increased stability of APE1 in the *Ubr3* -/- MEF cells suggest that they lack the mechanism regulating the cellular degradation of APE1 and this is likely to involve APE1 polyubiquitylation by UBR3.

APE1 is an important DNA repair protein involved in maintaining genome integrity and its overexpression in cancer cells has been linked to radiation and chemotherapy resistance, presumably through higher APE1 repair activity (Naidu et al., 2010, Wang et al., 2009, Robertson et al., 2001). The *Ubr3* -/- MEF cells that contain higher levels of APE1 are indeed more efficient at repairing damage induced by both MMS and irradiation, though we also show that these cells display increased levels of the key BER factors PARP1 and Pol β that appear to be related to non-degradation related mechanisms of upregulation. Despite the increased repair activity exhibited by *Ubr3* -/- MEF cells, irregularities that indicated these cells suffer from genome instability were also observed. Indeed, untreated *Ubr3* -/- MEF cells possessed 2.5-fold more 53BP1 repair foci and a 6-fold increase in γH2AX, both of which are markers for DNA double strand breaks. To show that this increase in the levels of DNA DSBs could be related to increased APE1-mediated single strand break formation, overexpression of APE1 in HeLa cells also allowed for the formation of DNA DSBs. DNA DSBs are highly

toxic lesions and we subsequently found that the *Ubr3* -/- MEF cells were more sensitive to the DNA damaging agent MMS.

The findings of this chapter allow us to conclude that cellular APE1 stability is regulated by the E3 ligase UBR3. A loss of this regulatory mechanism not only impacts on overall base excision repair activity, it also promotes genome instability.

Chapter 7

DISCUSSION

7.1 OVERVIEW

APE1 is an essential enzyme involved in the base excision repair (BER) pathway. It also has an unrelated role in regulating the activity of transcription factors such as NF-κB and p53 through modulation of their redox status (Reviewed in Bhakat et al., 2009). The importance of APE1 in maintaining genome stability, as well as in regulating cellular proliferation and survival, is reflected in APE1 null mice that die during embryogenesis (Xanthoudakis et al., 1996). Altered levels of APE1 were observed in some cancer types and neurodegenerative diseases, though it is not clear if these changes promote disease onset or if it is simply a consequence of disease progression (Wang et al., 2004, Kelley et al., 2001, Moore et al., 2000, Herring et al., 1998, Hegde et al., 2012). APE1 overexpression in some cancers was further linked to the onset of radiation and chemotherapy resistance that is thought to be due to increased repair efficiency mediated by increased APE1 activity related to its levels (Naidu et al., 2010, Wang et al., 2009, Robertson et al., 2001). As a result, APE1 is considered a key target for drug design and APE1 inhibitors are currently under study for the treatment of APE1 overexpressing tumours (Kelley et al., 2012, Tell et al., 2010, Madhusudan et al., 2005).

The complete mechanism allowing for the regulation of APE1 cellular levels and activity within the BER pathway is not well understood. The current model for the regulation of BER activity involves the maintenance of a cellular pool of BER repair enzymes that is continuously available for repair. Our lab has previously shown that the availability of the BER repair factors XRCC1, Lig IIIα and Pol β is regulated by

ubiquitylation-dependent proteasomal degradation (Parsons et al., 2008, Parsons et al., 2009, Parsons et al., 2010, Parsons et al., 2011). In the APE1 dissociation model of BER coordination, APE1 is released from its repair product regardless of the availability of downstream BER factors at the repair site, leaving a DNA single strand break that is subsequently identified and protected by PARP1 until repair continues. This BER coordination model suggests that overexpression of APE1 can have detrimental effects on genome integrity, affecting ultimately genome stability and cellular survival. It also suggests that the levels of APE1 must be tightly regulated according to the availability of other BER proteins. It was therefore of interest to determine if APE1 stability in particular, is also regulated by ubiquitylation-mediated proteasomal degradation in cells. The results presented in this doctoral thesis show that ubiquitylated APE1 is present in the cytoplasm of HeLa cells. We have identified the E2 activating enzyme UbcH2 and the cytoplasmic E3 ligase UBR3 that allow for APE1 ubiquitylation in these cells. This ubiquitylation is carried out on the N-terminal lysine residues of APE1 and was shown to primarily regulate APE1 stability in cells since *Ubr3 -/-* MEF cells displayed a two-fold increase in APE1 cellular levels. The *Ubr3 -/-* MEF cells also exhibited signs of genome instability and increased numbers of endogenously generated DNA double strand breaks. I finally demonstrated that overexpression of APE1 in HeLa cells can also induce the formation of DNA double strand breaks.

7.2 APE1 UBIQUITYLATION BY UBR3

A role for ubiquitylation in regulating cellular APE1 was previously unknown. A recent report by Busso *et al* proposed that APE1 is ubiquitylated by the RING domain-containing E3 ligase MDM2, however the cellular role for MDM2-mediated ubiquitylation of APE1 was not determined (Busso et al., 2009). Busso *et al* demonstrated this finding using *in vitro* ubiquitylation assays as well as overexpression studies in cells. We were able to confirm that recombinant MDM2 is capable of *in vitro* ubiquitylating APE1

(**Appendix V**) however downmodulation of MDM2 levels through siRNA treatment did not produce the expected increase in endogenous APE1 protein levels (**Appendix VI**). Additionally, cellular MDM2 (~ 90 kDa isoform) levels did not correlate to the ubiquitylation activity purified from HeLa cells (**Appendix VII**). It is possible that the purification process resulted in a loss of MDM2 activity through protein degradation or unfolding. It is also possible that APE1 is ubiquitylated by MDM2 only in certain cellular situations such that MDM2 may require a signal or modification on APE1 for APE1 identification or perhaps MDM2 needs to be modified itself. The overexpression and immunoprecipitation studies by Busso *et al* however show that cellular APE1 is ubiquitylated upon overexpression of MDM2 and ubiquitin (Busso et al., 2009). Though overexpression studies are somewhat artificial, it is not possible to entirely rule out MDM2 as an E3 ligase that is capable of ubiquitylating APE1 in cells.

The findings presented in this doctoral thesis implicate another RING domain-containing E3 ligase in regulating cellular APE1. Using column chromatography purification in combination with an *in vitro* ubiquitylation assay against APE1, I was able to purify the cytoplasmic RING domain-containing E3 ligase, UBR3 that is capable of ubiquitylating APE1. UBR3 belongs to a family of ubiquitin E3 ligases characterised by the UBR box (Tasaki et al., 2005, Tasaki et al., 2007). The UBR1, UBR2, UBR4 and UBR5 E3 ligases in this family were shown to recognise N-terminal destabilising residues in their substrates leading to subsequent substrate ubiquitylation and proteasomal degradation (Tasaki et al., 2005, Tasaki et al., 2009, Bachmair et al., 1986, Glickman and Ciechanover, 2002, Mogk et al., 2007). This mechanism of substrate recognition for degradation has been designated as the N-end rule pathway that involves the conversion of a stable N-terminal residue on a substrate in to one that is recognised as unstable (Suzuki and Varshavsky, 1999, Mogk et al., 2007). This is achieved primarily by cleavage of the first N-terminal residue to reveal an ‘unstable’ second residue that may require further modification by acetylation or arginylation to be

identified for ubiquitylation. Previous studies using a library of engineered peptides could not confirm that UBR3 recognises its substrates through the N-end rule pathway (Tasaki et al., 2009). Furthermore the second residue of APE1's N-terminal tail (1-Met-Pro-Lys-3), proline, is a stabilising residue according to the N-end rule pathway of protein degradation. Though we have shown that UBR3 ubiquitylates APE at its N-terminal lysines, UBR3 is unlikely to recognise APE1 as a substrate according to the N-end rule pathway. It would be interesting to determine what triggers APE1 recognition by UBR3 for ubiquitylation. Such knowledge will not only allow us to understand the cellular circumstance in which APE1 is targeted for ubiquitylation by UBR3, it will also aid in the identification of alternative substrates for UBR3.

Characterisation of the ubiquitylation activity of UBR3 on APE1 revealed that APE1 is polyubiquitylated by UBR3 at its N-terminal lysine residues. Consistent with the major role of protein polyubiquitylation in regulating protein stability, we demonstrated that *Ubr3 -/-* MEF cells exhibited a two-fold increase in APE1 protein levels with no concomitant increase in APE1 mRNA levels. In relating the higher levels of APE1 to increased APE1 cellular stability, APE1 that was overexpressed in the *Ubr3 -/-* cells was more stable compared to APE1 overexpressed in the *Ubr3* +/+ cells. Moreover, loss of APE1 mRNA translation through siRNA treatment did not allow for a loss of APE1 protein already present in the *Ubr3 -/-* MEF cells, whereas APE1 levels were reduced for the *Ubr3* +/+ cells. Collectively, these results suggest that *Ubr3 -/-* MEF cells are not able to degrade cellular APE1 and that UBR3-mediated ubiquitylation of APE1 most likely signals APE1 for proteasomal degradation in cells. To further consolidate our findings, we attempted to overexpress and transiently knock down UBR3 in cells however we were unable to achieve either result. It is unclear why these experiments were unsuccessful. For instance, there is no evidence that UBR3 is capable of auto-ubiquitylating itself since this would explain the lack of UBR3 protein in cells transfected with the UBR3-containing expression vector.

APE1 is the first substrate identified for UBR3-mediated ubiquitylation. In fact, the only other study that focusses primarily on UBR3 is one that involves phenotypic characterisation of mice that lack the UBR3 gene. *Ubr3 -/-* mice generated in the 129SvlmJ background died during embryogenesis and *Ubr3 -/-* mice generated in the C57BL/6 background that survived development, showed impairments in the sensory pathways for touch, smell, hearing and vision (Tasaki et al., 2007). Indeed, mouse cells involved in the sensory pathways as well as other neuronal cells exhibited high levels of UBR3. The embryonic lethality observed in the 129SvlmJ background mice suggest that UBR3 plays a vital role during development. APE1 was also shown to be necessary for embryonic development since APE1 null mice die during embryogenesis (Xanthoudakis et al., 1996). Additionally, overexpression of APE1 in XRCC1-deficient CHO cells resulted in an accumulation of abasic sites and an increase in genome instability (Sossou et al., 2005). Since we show that UBR3 regulates the cellular levels of the multifunctional APE1 protein that is involved in DNA repair as well as regulation of cellular growth, proliferation and survival, it is possible that a loss of UBR3-mediated regulation of APE1 is responsible for defective development in the *Ubr3 -/-* 129SvlmJ mice. Alternatively, an unknown cellular function of UBR3 may also be responsible for the lethality observed in *Ubr3 -/-* 129SvlmJ background mice.

7.3 APE1 UBIQUITYLATION AT ITS N-TERMINAL RESIDUES

Mass spectrometry analysis of monoubiquitylated APE1 revealed that APE1 is ubiquitylated by UBR3 at multiple lysines on its N-terminal tail to include K7, K24, K25, K27, K32 and K35. UBR3-mediated *in vitro* ubiquitylation of APE1 mutants that had lysines changed to non-ubiquitylatable arginine residues revealed that most lysines of the N-terminal tail can be *in vitro* ubiquitylated by APE1, though K24, K25, K27, K31, K32 and K35 appear to accept ubiquitin with greater affinity than K6 and K7. We also showed that APE1 is polyubiquitylated at a single site in an *in vitro* ubiquitylation assay

carried out using mutant ubiquitin that is unable to form polyubiquitin chains. These results together suggest that UBR3 does not have a preference for which lysine on the APE1 N-terminal tail it ubiquitylates, however ubiquitylation of APE1 at one site inhibits further ubiquitylation of APE1 at another site.

It is becoming increasingly apparent that the N-terminal tail of APE1 acts as a regulatory domain of APE1, controlling its cellular localisation as well as its endonuclease and redox activities. The N-terminal 42 residues of APE1 form a flexible and exposed tail whereas residues 43 - 318 of APE1 make up a tight globular structure (Strauss and Holt, 1998). These N-terminal 42 residues of APE1 are highly conserved in mammals but poorly conserved in other eukaryotes and almost entirely lacking in prokaryotes (Fantini et al., 2010). This is a reflection on the requirement for regulatory mechanisms within the context of the evolution of the multiple functions of APE1 since prokaryotes lack redox activity and a need for compartmental regulation. In mammals, all the lysines of the N-terminal tail are completely conserved and only lysines K24, K25 and K27 seem to be highly conserved between for instance *Homo sapiens* and other eukaryotes such as *Xenopus laevis* and *Caenorhabditis elegans* (Fantini et al., 2010).

Lysines K6, K7, K27, K31, K32 and K35 have been reported to be modified by acetylation (Bhakat et al., 2003a, Fantini et al., 2010, Poletto et al., 2012). The human acetyl transferase, p300, acetylates lysines K6 and K7 to promote APE1 redox activity (Bhakat et al., 2003a). Acetylation at lysines K27, K31, K32 and K35 on the other hand was shown to increase APE1 endonuclease activity (Fantini et al., 2010). Since acetylation of APE1 is primarily involved in promoting APE1 cellular functions it is not unlikely that APE1 modified by acetylation prevents its recognition and consequent ubiquitylation by UBR3. Equally, ubiquitin additions on to the lysines of APE1's N-terminal tail may prevent its acetylation. It would be of interest to investigate whether APE1 deacetylation promotes APE1 degradation since deacetylation of these sites on APE1 will expose target lysines for ubiquitylation.

We have also shown that the ubiquitylated form of APE1 is present only in the cytoplasm and that the UBR3 E3 ligase is a cytoplasmic E3 ligase. This finding suggests that the ubiquitylation of APE1 is likely to occur in the cytoplasm and furthermore that ubiquitylation of APE1 prevent it's localisation to the nucleus, thus indirectly impairing APE1 DNA repair activity. We have shown that lysines K6 and K7 are sites of ubiquitylation on APE1 and indeed these lysines form part of the nuclear localisation signal located at residues 1 - 7 on the APE1 N-terminal tail (Jackson et al., 2005). Close proximity to a second nuclear localisation signal at residues 8 - 13 may further explain the loss of nuclear localisation of ubiquitylated APE1 (Jackson et al., 2005)

Ubiquitylation of APE1 at its N-terminal tail may additionally affect it's interaction with several factors including XRCC1, NPM1 and CSB that are known to interact directly with the APE1 N-terminal tail (Wong et al., 2007, Vidal et al., 2001, Vascotto et al., 2009, Fantini et al., 2010). Reduced or inhibited interaction of APE1 with these factors may prevent the respective cellular outcomes of these interactions.

7.4 APE1 UBIQUITYLATION IN BER

The BER pathway is constantly required by cells for the repair of endogenously generated lesions. We therefore believe that this pathway is regulated to a steady-state level rather than through a mechanism that switches the pathway on or off. This involves fine-tuned regulation of the availability of the individual BER players, likely according to the amount of damage present on DNA. This regulation is largely achieved through a balance of gene expression for the making of BER factors and ubiquitylation-mediated proteasomal degradation of excess BER factors. The BER factors Pol β, XRCC1 and DNA Lig IIIα were all shown to be regulated in this manner (Parsons et al., 2010, Parsons et al., 2009, Parsons et al., 2008). Until now, this was not shown for APE1, a key BER enzyme that generates DNA single strand breaks as a repair intermediate. The APE1 dissociation model of BER coordination suggests that APE1 is released from its

repair product irrespective of the availability or binding of downstream repair factors at the repair site. Excessive amounts of APE1 could therefore damage DNA since excessive amounts of DNA SSBs increase the probability of DNA double strand break formation on collision with replication forks (Kuzminov, 2001). This suggests that the levels of APE1 must also be tightly coordinated and this was the primary rationale for investigating the role of ubiquitylation in regulating APE1 levels in cells.

I now show for the first time in this study that the availability of APE1 is also regulated by ubiquitylation-mediated proteasomal degradation and I have identified the E3 ligase UBR3 that specifically ubiquitylates APE1 in cells. This finding supports the current model for the steady-state regulation of the BER pathway in cells. It furthermore implicates UBR3 as a major regulatory factor involved in maintaining BER activity.

Cells that lack UBR3 protein displayed increased repair rates upon treatment with the DNA alkylating agent, MMS. MMS induces base damages that are almost entirely repaired through APE1 as part of BER (Lundin et al., 2005). The increased repair observed in these cells is likely due to the excessive amounts of APE1 present in these cells. At a first glance, this result appears to suggest that APE1 is ordinarily a rate limiting factor of the BER pathway. Though this is not necessarily false, it is more likely that the increased repair rate is additionally due to upregulated downstream BER activity. Excessive amounts of APE1 may process abasic sites at a faster rate, generating too much of the potentially toxic DNA single strand break repair intermediate at a given time. In order to cope with the excessive DNA single strand breaks, it is likely that the activities of the remaining BER factors are upregulated in response, thus allowing for the observed increased rate of repair completion. Indeed we were able to show that both PARP1 and Pol β levels are also increased in the *Ubr3* -/- cells. In essence, due to the danger posed to the cell by unrepaired BER repair intermediates each step is likely to be ‘rate limiting’. This would suggest that the amount of BER proteins present in cells is not only regulated according to the levels of damage present

on DNA, they are also regulated in accordance with the availability of other BER factors. The regulatory mechanisms of the individual BER factors are therefore likely to share a common regulatory signal and it would be interesting to determine if there is indeed a shared cellular signal that ultimately allows for the modulation of the availability of BER factors, particularly in response to DNA damage.

7.5 APE1 UBIQUITYLATION IN THE MAINTENANCE OF GENOME STABILITY

The *Ubr3 -/-* MEF cells displayed irregularly shaped nuclei and micronuclei that are hallmarks of genome instability (Gisselsson et al., 2001, Fenech et al., 1999). Other than APE1, there are no known cellular targets for UBR3 in cells. It is therefore difficult to explain why a loss of UBR3 may promote genome instability. We have been able to show that *Ubr3 -/-* MEF cells contain high levels of γH2AX and increased numbers of 53BP1 foci, both of which are markers for the presence of DNA double strand breaks (Rogakou et al., 1999, Paull et al., 2000, Huyen et al., 2004). DNA DSBs can trigger cell cycle arrest and cell death if not repaired efficiently (Reviewed in Khanna and Jackson, 2001). Additionally, both misrepair as well as 'correct' repair (through the error-prone NHEJ pathway) of DNA DSBs can result in chromosomal rearrangements that may tamper with genome integrity. It is possible therefore that these DNA DSBs are the cause for the genome instability observed in the *Ubr3 -/-* MEF cells.

APE1 processes abasic sites in cells to generate DNA single strand breaks as a repair intermediate (Reviewed in Barzilay and Hickson, 1995). Unrepaired DNA SSBs can be converted in to DNA DSBs upon collision with a replication fork in dividing cells (Kuzminov, 2001). It is possible that excessive DNA SSBs generated by APE1 can in this way allow for the formation of DNA DSBs if it is not repaired efficiently by downstream repair factors. To corroborate this theory, I was able to show that overexpression of APE1 in HeLa cells induced the formation of DNA DSBs, as observed through increased levels of γH2AX. Since I show in this report that a loss of UBR3 from

cells results in high levels of cellular APE1, it is possible that the increased DNA DSBs observed in *Ubr3 -/-* MEF cells is a direct result of the increased amounts of APE1 present in these cells. To prove this, it would be interesting to compare the levels of genome instability in cells that lack *Ubr3* alone to those that lack *Ubr3* and possess reduced levels of APE1. A reduction in genome instability in the latter would provide a direct link between UBR3-mediated APE1 regulation and the instability noted in the *Ubr3* -/- MEF cells. For such a study, it is important to note that a complete *Ape1* knockout would prove embryonic lethal and a partial knock-down from normal APE1 levels has been shown to promote genome instability (Xanthoudakis et al., 1996, Huamani et al., 2004). For the proposed experiment, it is therefore very important to reduce APE1 protein levels in the *Ubr3* -/- mice to the same level as it is in the *Ubr3* +/+ mice. This may be achieved by generating *Ubr3* -/- mice that are heterozygous for *Ape1*. Alternatively, the genome instability observed in the Ubr3 -/- MEF cells could be due to a UBR3 function unrelated to APE1 though this is difficult to scrutinize as there are no other known cellular targets for UBR3.

Interestingly, the 129SvlmJ background *Ubr3 -/-* mice died during embryogenesis (Tasaki et al., 2007). The reduced ability of the *Ubr3 -/-* cells to maintain genome integrity may explain the embryonic lethality observed in these mice. *Ubr3 -/-* mice generated in the C57BL/6 background were viable however, and did not display any obvious signs of tumourigenesis, a well-documented feature of mice that are compromised in their ability to maintain genome integrity. At the time of the UBR3 mouse study, there were no known functions attributed to UBR3 and the observations made were not necessarily aimed at identifying features related to compromised genome integrity. It would therefore be of interest to carry out further observations in these mice, keeping in mind this newly identified role of UBR3 in regulating APE1. Furthermore, it would be interesting to better relate the genetic backgrounds of both the

129SvlmJ and C57BL/6 background mice to the potential outcome of compromised DNA repair.

7.6 APE1 UBIQUITYLATION IN DISEASE AND DRUG TARGETING

Overexpression of APE1 was observed in multiple tumour types such as osteosarcomas, prostate, cervical and ovarian cancers, however its role in tumourigenesis and tumour progression is not well understood (Wang et al., 2004, Kelley et al., 2001, Moore et al., 2000, Herring et al., 1998). The most popular notion is that since cancer cells experience high levels of endogenous damage, APE1 expression is upregulated for the purpose of repair, thereby promoting the survival and progression of cancer cells. In support of this notion, the APE1 overexpressing *Ubr3* -/- cells used in this study displayed increased repair of both MMS and IR treatments. However, they also exhibited increased levels of DNA double strand breaks and signs of genome instability. This ability of APE1 to promote genome instability provides the cancer cell with the potential to accumulate mutations and subsequently evolutionary survival advantages, thus allowing it to develop a more aggressive phenotype. As such, it is therefore also possible for an overexpression of APE1 to contribute to the development of a pre-cancerous cell. Since a loss of UBR3 promotes an increase in the cellular levels of APE1, it would be of interest to characterise the levels and activities of UBR3 and APE1 in a panel of tumour cells that normally exhibit high levels of APE1, with tumour grade taken in to consideration. Such an association study would provide insight in to the mechanisms by which APE1 is upregulated in these cells and thus may shed light on the question concerning whether high levels of APE1 in tumour cells may be causative or consequential in tumour onset and development.

In relation to its increased ability to repair DNA damage, APE1 overexpression in tumour cells was associated with reduced sensitivity to chemotherapy and radiation therapy (Naidu et al., 2010, Wang et al., 2009, Robertson et al., 2001). Inhibitors of

APE1 endonuclease activity are consequently being investigated as an adjuvant treatment to both chemotherapy and radiation therapy (Kelley et al., 2012, Tell et al., 2010). Although APE1 inhibition does indeed sensitise cells to radiation therapy and chemotherapy in preliminary studies, APE1 inhibition may also cause irreversible damage to normal cells (Raffoul et al., 2007, Xiang et al., 2008, Huamani et al., 2004).

We have shown in this study that overexpression of APE1 promotes the formation of the toxic DNA DSB lesion, presumably through inefficient downstream repair of DNA single strand breaks generated by excess APE1. If unrepaired, DNA DSBs can cause cell cycle arrest and cell death (Reviewed in Khanna and Jackson, 2001). Indeed, radiation therapy that is the most widely used non-invasive treatment for cancer, promotes cancer cell death mainly through its ability to induce DNA DSBs.

The option to take advantage of the DNA DSB-inducing capability of APE1 overexpressing tumours should be of interest for further study. This prospect is particularly exciting since it would allow us to capitalise on an inherent defect that is present only in the tumour cells of a patient. This would be more favourable than inhibiting APE1 endonuclease activity which is likely to damage normal cells. Taking in to consideration my finding that APE1 overexpressing *Ubr3* -/- MEF cells repair damage more efficiently, likely due to the collaborative repair efforts of both APE1 and upregulated BER activity, inhibition of the downstream BER repair activity may prove to be a suitable treatment alternative to APE1 inhibition. For instance, DNA SSB repair can be inhibited by PARP1 inhibition (Rouleau et al., 2010) and inhibition of PARP1 in APE1 overexpressing tumours could ultimately result in an increased rate of DNA SSB accumulation. DNA SSBs can be converted in to DNA DSBs that can cause cell death if not repaired efficiently (Kuzminov, 2001). The DNA DSB repair capacity of cells is therefore also an important consideration for such a treatment design since upregulated repair of DNA DSBs may negate the cell killing effects of PARP1 inhibition. Tumour

characterisation of DNA DSB repair capacity for individual patient tumours is therefore crucial for selecting patients that are likely to respond well to such a treatment plan.

It is also noteworthy to mention that inhibitors of APE1 redox activity that do not reduce APE1 endonuclease activity are not to be included in the aforementioned proposal as these two functions of APE1 are very much unrelated. Though I propose that caution is required in the use of APE1 endonuclease inhibitors for the treatment of cancer, I believe that APE1 redox inhibitors should remain a stronghold in the efforts to treat APE1 overexpressing tumours.

7.7 FUTURE WORK

The identification of UBR3 as the E3 ligase promoting APE1 ubiquitylation is simply a stepping stone for understanding the overall regulation of the levels and activity of both APE1 and BER in cells. The aim of future projects should be to fully decipher the mechanisms by which APE1 levels are regulated. For instance, it is not known whether APE1 is a cellular substrate for deubiqutylation. Alongside transcription and protein degradation, deubiquitylation is yet another major cellular process that allows for the regulation of cellular protein stability and it has already been shown to be of importance in the regulation of Pol β levels and overall BER activity (Parsons et al., 2011). It would be of interest to determine whether APE1 is deubiquitylated in cells and if a loss of such a regulatory mechanism will also impact on APE1 and overall BER activity in cells.

More directly related to the work presented in this doctoral thesis is the question of how the APE1 ubiquitylating activity of UBR3 itself is regulated. The E3 ligase UBR3 is required for the regulation of cellular APE1 levels but when and how does UBR3 recognise APE1 as a substrate for ubiquitylation and subsequent degradation? APE1 is very stable in cells under normal conditions. Treatment of cells with the proteasomal inhibitor MG132 or the mRNA translation inhibitor cycloheximide do not effect changes

in APE1 cellular levels (**Appendix VIII**). Taking in to consideration the time-frames possible for these experiments, this observation is not particularly surprising since cells continuously require APE1 as part of the BER pathway and maintaining a well-managed pool of APE1 is likely to be less energy-consuming than continuously making and degrading APE1. Our findings however show that APE1 degradation is required for maintaining genome stability, suggesting that APE1 degradation is necessary for the regulation of BER activity in cells. Due to the high stability of APE1 in cells, it is likely that APE1 degradation takes place in response to a specific cellular signal or situation.

We are currently unaware of the cellular situation that promotes APE1 ubiquitylation. It is possible that APE1 produced in response to high levels of damage is degraded upon repair completion, however we have been unable to observe a change in the levels of APE1 in response to high levels of damage despite such observations having been published by others previously (Grosch et al., 1998, Grosch and Kaina, 1999, Fung et al., 2001, Ramana et al., 1998). An alternative proposal is that APE1 levels are regulated in a cell cycle-dependent manner. The repair intermediate for APE1 is a DNA single strand break. APE1 activity could therefore pose a threat to the integrity of DNA during the S phase of the cell cycle in which DNA is being replicated since collision of a replication fork with a DNA SSB can generate the toxic DNA DSB (Kuzminov, 2001). It is possible therefore that degradation of APE1 is carried out by the cell during S phase to reduce the overall APE1 endonuclease activity during DNA replication. A study in to the changes in the levels of APE1 and APE1 ubiquitylation throughout the various cell cycle stages would address this hypothesis.

Cellular responses to changes in their environment are largely mediated through post-translational modification of proteins. This can effect changes in protein activity, localisation, stability and protein-protein or protein-DNA interactions. Post-translational modification of APE1, UBR3, or both may allow for APE1 identification by UBR3 and

subsequent ubiquitylation. There are no known post-translational modifications for UBR3 and an in-depth study will be required to further pursue this hypothesis.

APE1 on the other hand is known to be modified by acetylation, phosphorylation, S-glutathionylation and S-nitrosation (Bhakat et al., 2003b, Kim et al., 2011, Sengupta et al., 2011, Qu et al., 2007, Poletto et al., 2012, Huang et al., 2010). It is feasible to assume that a post-translational modification on APE1 that prevents its activities or promotes its localisation to the cytoplasm may also mark it for identification and ubiquitylation by UBR3. A 2010 paper by Huang *et al* showed that phosphorylation of APE1 from neuronal cells at residue T233 by the kinase CDK5 and its activating partner p35 reduces APE1 endonuclease activity (Huang et al., 2010). We have confirmed this (**Appendix IX**) and have since determined that the APE1 T233E phosphomimic mutant is less stable on transfection in to cells, and that this loss of stability is due to proteasomal degradation (**Appendix X**). The APE1 T233E phosphomimic mutant protein is additionally more readily *in vitro* ubiquitylated by UBR3 compared to wild-type APE1 protein (**Appendix XI**). I was however unable to show that CDK5 was responsible for changing APE1 levels in cells (**Appendix XII**). This link between the APE1 T233E phosphomimic mutant and APE1 ubiquitylation was recently published by Izumi *et al* however they have not identified the kinase responsible but have instead suggested that CDK5 is the likely kinase mediating APE1 phosphorylation in cells (Busso et al., 2011).

Identification of the cellular kinase that phosphorylates APE1 at site T233 may provide insight in to the cellular situation that promotes APE1 and BER regulation by UBR3. As with the work presented in this doctoral thesis, such a study would involve purification of a phosphorylating activity from human cells that specifically phosphorylates APE1 at site T233. Active fractions will be identified in an *in vitro* kinase assay against APE1 using the purified fractions as the kinase source. Since we are interested in a specific phosphorylation site, an APE1 T233A non-phosphorylatable mutant protein substrate will be used as a negative control. Additionally, antibodies

designed specifically against APE1 phosphorylated at site T233 would be of great use during Western blotting analysis for identifying bands that are due to APE1 phosphorylation specifically at site T233. Analysis of a highly purified active fraction by mass spectrometry will reveal candidate kinases that can be further tested for activity on APE1. This could be achieved by purifying the kinases and using them directly in *in vitro* kinase assays, immunodepleting the active fraction of the individual kinases to confirm a loss of activity from that active fraction. The activity of the kinase can be further analysed in cells through overexpression and knockdown studies. Such studies will also provide insight in to the role of the kinase activity in cells.

A full understanding of both the mechanisms and the cellular signals that promote the regulation of APE1 levels in cells will allow us to better dissect the role of APE1 dysregulation in disease and subsequently its suitability as a drug target. For instance, it would be a more comprehensive study to associate levels of APE1 in tumours with not just UBR3, but also the kinase and deubiquitylating enzyme that may additionally promote changes in the cellular levels of APE1.

7.8 CONCLUSIVE SUMMARY

I present in this doctoral thesis evidence for the regulation of the stability of APE1 through ubiquitylation-mediated proteasomal degradation (**Figure 7.1**). This ubiquitylation activity is carried out by the UbcH2 E2 conjugating enzyme and the UBR3 E3 ligase. A loss of UBR3 increases cellular APE1 levels and promotes the formation of the toxic DNA double strand break and genome instability. This is likely related to it's role in regulating APE1 levels since APE1 overexpression also promotes the formation of DNA double strand breaks. I therefore conclude that the regulation of APE1 levels by UBR3 plays a major role in the regulation of cellular BER activity for the maintenance of genome stability.

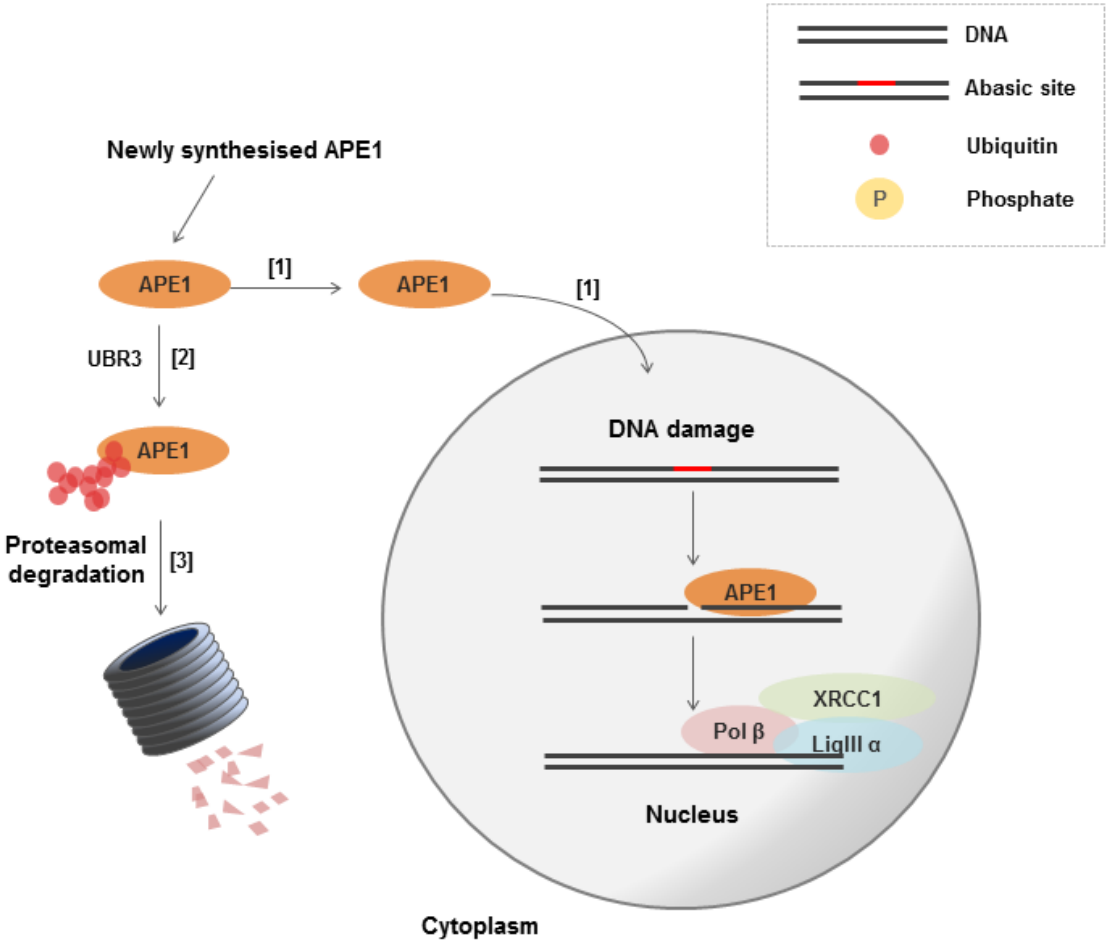

Figure 7.1: Steady-state regulation of APE1 cellular levels. Newly synthesised APE1 is used for repair [1] and any excess of APE1 is recognised for ubiquitylation by UBR3 [2] for its subsequent proteasomal degradation [3].

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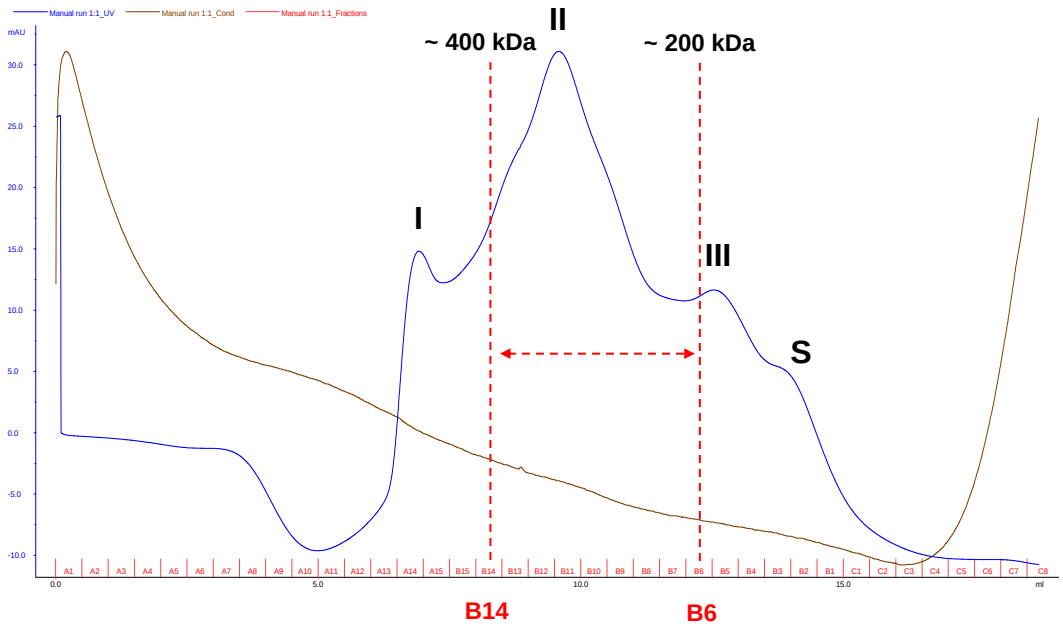
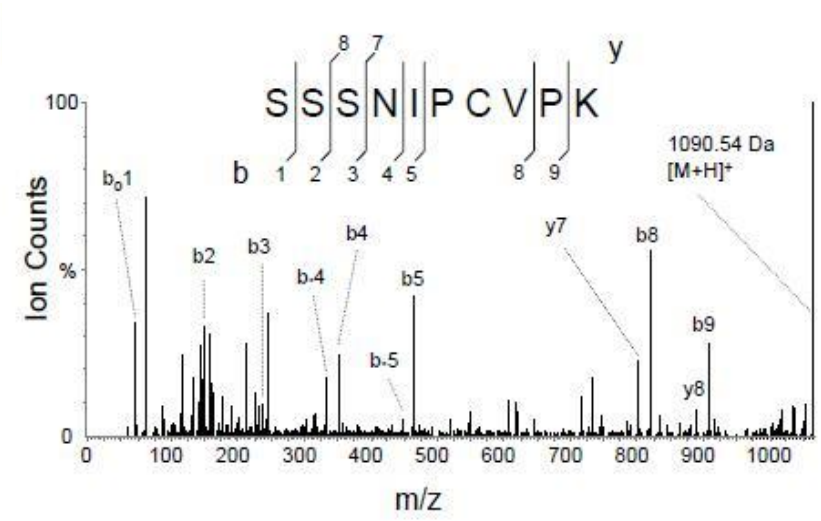
APPENDICES

APPENDIX I



Example of protein content analysis for fractions purified by gel filtration on the Superose 12 column. The fractions A14 to B10 that displayed APE1 ubiquitylating activity obtained from the Mono-Q [1] column were pooled and separated in to fractions (X axis) according to approximate size on the gel filtration Superose 12 column. Shown in blue is the analysis of protein content for elution fractions obtained from the Superose 12 column, recorded at UV 280 nm. Three peaks and a shoulder are present and indicated as I, II, III, S. Fractions B14 (~ 400 kDa) to B6 (~ 200 kDa) were shown to contain APE1 ubiquitylating activity and are indicated as the space between the arrows.

APPENDIX II



LC-MS/MS spectra obtained for the UBR3 peptide SSSNIPCVPK. LC-MS/MS mass spectrometry analysis of the B1 and B2 fractions of the final Mono-Q [2] column that contained APE1 ubiquitylating activity revealed the presence of the peptide, SSSNIPCVPK. Using Mascot and the SwissProt database, the peptide identified (SSSNIPCVPK) was shown to belong to the E3 ligase, UBR3.

APPENDIX III

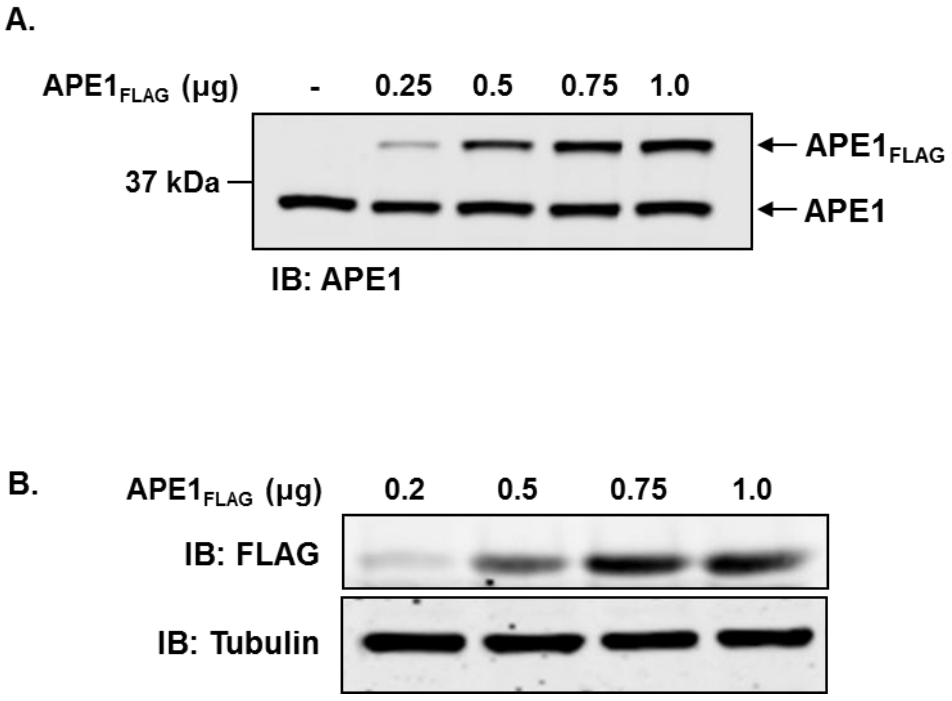
Start - End	Observed	Mr(expt)	Mr(calc)	Delta	Miss	Sequence
8 - 18	566.3570	1130.6994	1130.5204	0.1791	1	K.GAVAEDGDELR.T (Ions score 48)
18 - 31	829.9960	1657.9774	1657.9111	0.0664	4	L.RTEPEAKKSKTAAK.K GlyGly (K) (Ions score 15)
36 - 52	893.9000	1785.7854	1785.8057	-0.0202	2	K.EAAGEGPALYEDPPDQK.T (Ions score 47)

Start - End	Observed	Mr(expt)	Mr(calc)	Delta	Miss	Sequence
7 - 18	630.5170	1259.0194	1258.6153	0.4041	1	K.KGAVAEDGDELR.T (Ions score 70)
7 - 18	687.5970	1373.1794	1372.6582	0.5212	1	K.KGAVAEDGDELR.T GlyGly (K) (Ions score 103)
7 - 24	958.1170	1914.2194	1913.9330	0.2864	2	K.KGAVAEDGDELRTEPEAK.K (Ions score 72)
8 - 18	566.3590	1130.7034	1130.5204	0.1831	0	K.GAVAEDGDELR.T (Ions score 72)
8 - 18	1131.7280	1130.7207	1130.5204	0.2004	0	K.GAVAEDGDELR.T (Ions score 45)
8 - 24	894.2260	1786.4374	1785.8381	0.5994	1	K.GAVAEDGDELRTEPEAK.K (Ions score 77)
8 - 25	1015.2330	2028.4514	2027.9759	0.4755	2	K.GAVAEDGDELRTEPEAKK.S GlyGly (K) (Ions score 41)
32 - 52	1136.6960	2271.3774	2271.0655	0.3120	2	K.KNDKEAAGEGPALYEDPPDQK.T (Ions score 26)
32 - 52	1193.7000	2385.3854	2385.1084	0.2771	2	K.KNDKEAAGEGPALYEDPPDQK.T GlyGly (K) (Ions score 33)

966	829.9960	1657.9774	1657.9111	0.0664	4	15	1.1e+02	4	L.RTEPEAKKSKTAAK.K + GlyGly (K)

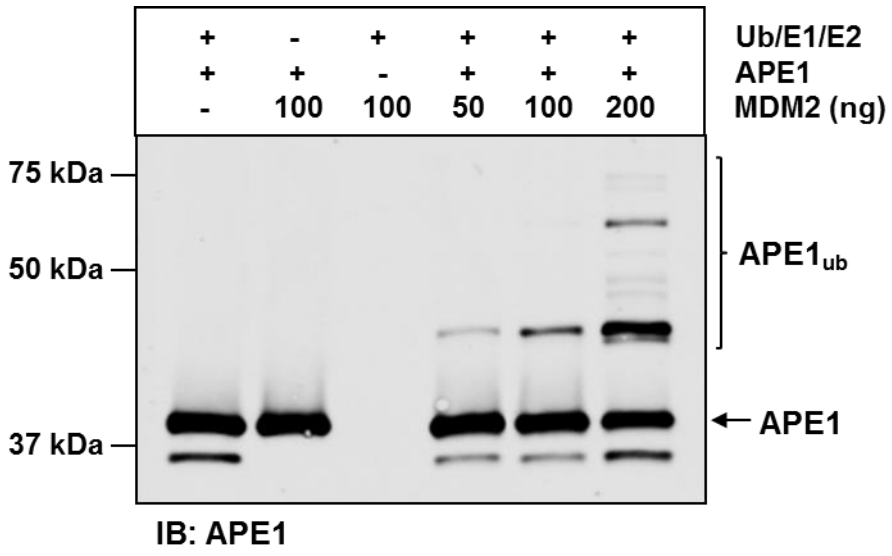
APE1 lysine residues K7, K24, K25, K27, K32 and K35 were shown to be modified by ubiquitin. *In vitro* monoubiquitylated APE1 protein was first trypsinised and then subjected to nanoLC-MS/MS tandem mass spectrometry analysis for identification of APE1 residues modified by ubiquitin. Cleavage of a ubiquitin moeity away from a lysine residue generates a glycine-glycine dipeptide that is idenfitied by its 114.1 Da increase in mass. The LC-MS/MS spectra results are displayed above as produced by Mascot showing peptides that contain lysines modified by the glycine-glycine dipeptide. Lysine K7, K24, K25, K27, K32, and K35 of the APE1 N-terminal flexible tail were as such identified as being modified by ubiquitin.

APPENDIX IV



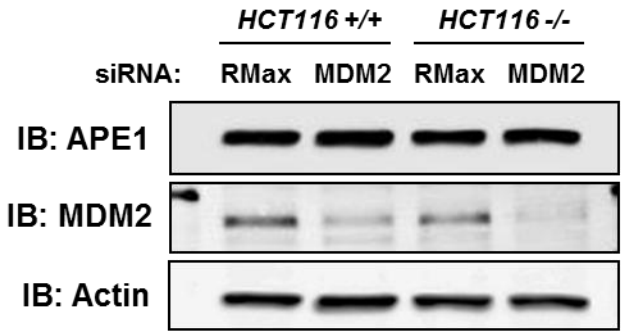
Optimisation of FLAG-APE1 expression in HeLa and *Ubr3* +/+ MEF cells. A. HeLa cells were transiently transfected with 0.25 µg, 0.5 µg, 0.75 µg and 1 µg of the pQCXIH plasmid encoding FLAG-tagged APE1 using lipofectamine 2000 transfection reagent. The cells were harvested after 24 hr and whole cell extracts prepared for separation by 10% SDS-PAGE and analysis by Western blotting using antibodies against APE1. Molecular weight markers (kDa), FLAG-tagged APE1 ($APE1_{FLAG}$) and endogenous APE1 (APE1) are indicated. **B.** *Ubr3* +/+ cells were transiently transfected with 0.2 µg, 0.5 µg, 0.7 µg and 1 µg of the pCMV3Tag3a plasmid encoding FLAG-tagged mouse APE1 using lipofectamine 2000 transfection reagent. The cells were harvested after 24 hr and whole cell extracts prepared for separation by 10% SDS-PAGE and analysis by Western blotting using antibodies against APE1.

APPENDIX V



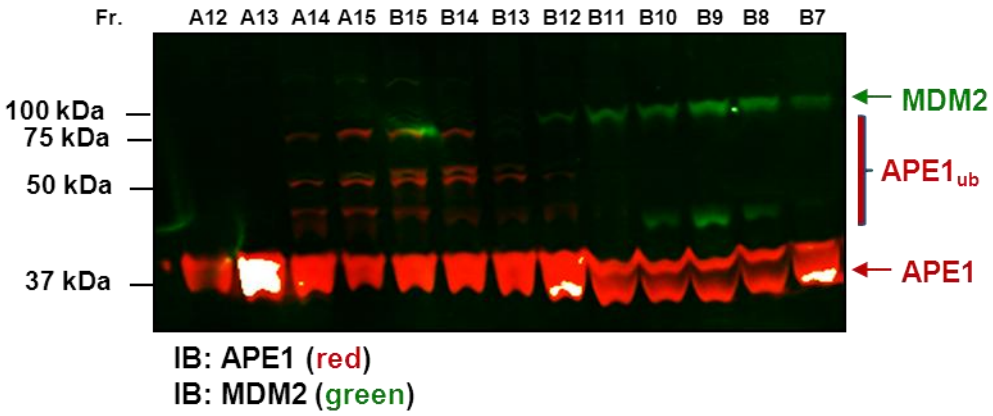
MDM2 can *in vitro* ubiquitylate APE1. Indicated amounts of recombinant MDM2 protein was used as the E3 source in an *in vitro* ubiquitylation assay containing recombinant His-tagged APE1 (2.8 pmol), E1 (0.7 pmol), ubiquitin (0.6 pmol) and an E2 mix (9.5 pmol). The completed reactions were separated by 10% SDS-PAGE and analysed by Western blotting using antibodies against APE1. Molecular weight markers (kDa), unmodified His-tagged APE1 (APE1) and ubiquitylated APE1 ($APE1_{ub}$) are indicated.

APPENDIX VI



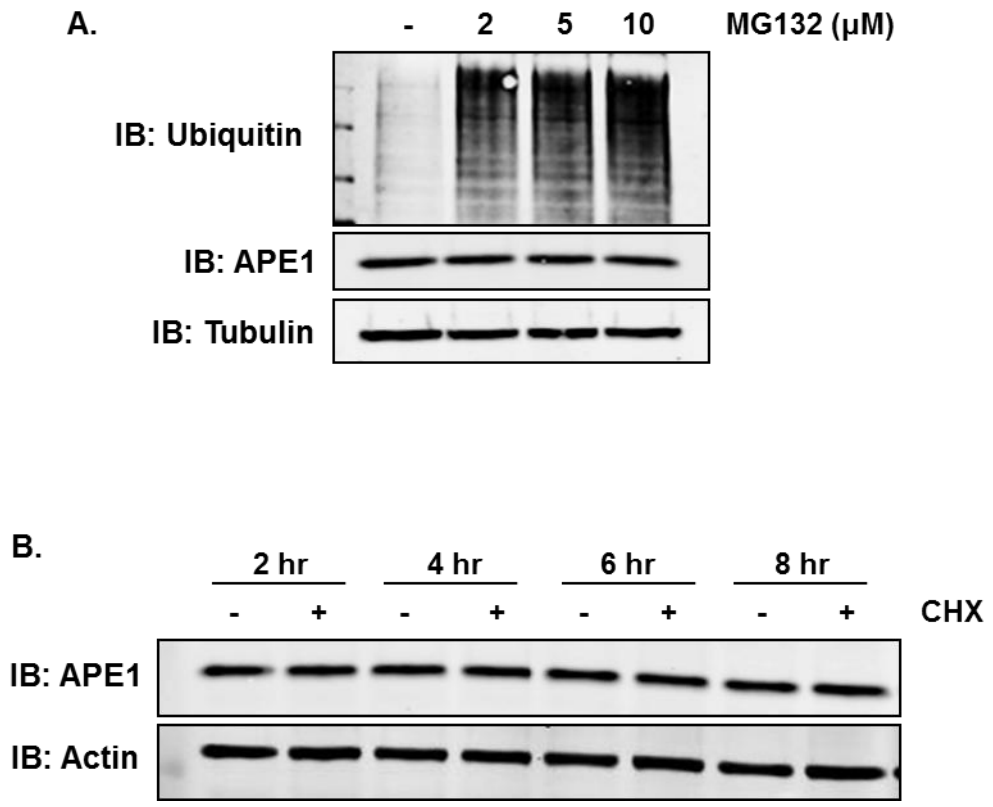
MDM2 knockdown in HCT116 +/+ and HCT116 -/- cells does not affect APE1 protein levels. Silencing sequences targeting MDM2 mRNA were introduced in to HCT116 +/+ and HCT116 -/- cells using the RNAiMax lipofectamine reagent that was additionally used in the control lacking MDM2 siRNA (Rmax). After allowing 72 hr for silencing, the cells were harvested, whole cell extracts prepared and separated by 10% SDS-PAGE, followed by Western blotting analysis with antibodies against APE1, MDM2 and the loading control, actin.

APPENDIX VII



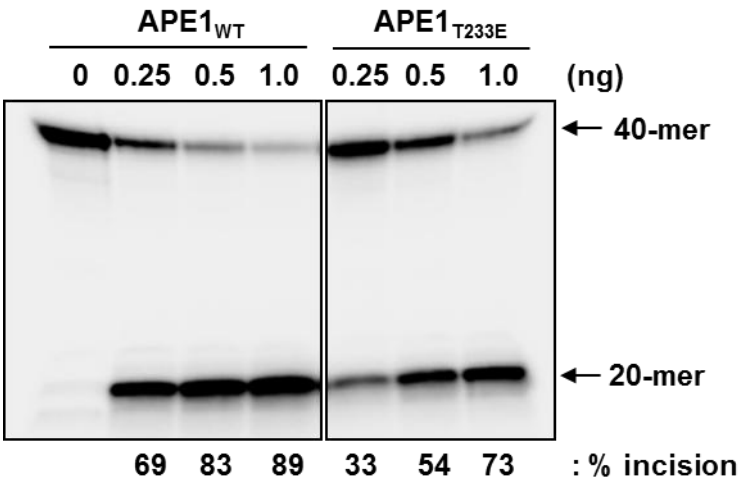
MDM2 levels do not correlate with APE1 ubiquitylation activity. An *in vitro* ubiquitylation assay was carried out on recombinant APE1 using fractions A12 – B7 that were purified by the Mono-Q [1] column as the E3 ligase source. The reactions were incubated for 30 minutes at 37 °C before separation by 10% SDS-PAGE and transferring on to a PVDF membrane. The membrane was analysed by Western blotting using antibodies against APE1 (red) or MDM2 (green). Molecular weight markers (kDa), MDM2, unmodified His-tagged APE1 (APE1) and ubiquitylated APE1 ($APE1_{ub}$) are indicated.

APPENDIX VIII



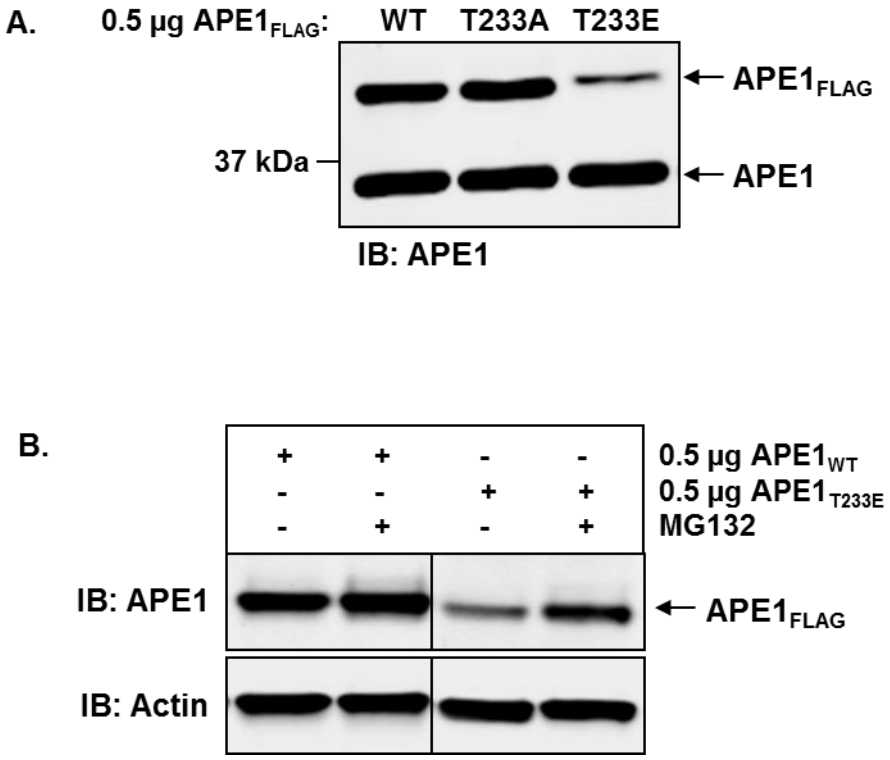
APE1 is inherently a stable cellular protein A. HeLa cells were treated with the indicated amounts of the proteasomal inhibitor, MG132 suspended in DMSO for 17 hours. The untreated DMSO control is shown. The cells were harvested and separated by 10% SDS-PAGE, followed by Western blotting analysis with antibodies against APE1, the positive control, ubiquitin and the loading control, tubulin. **B.** Mouse embryonic fibroblasts were treated with 50 μg/ml of the mRNA translation inhibitor cycloheximide (CHX) suspended in DMSO for the indicated lengths of time. Untreated DMSO controls are shown. The cells were harvested and separated by 10% SDS-PAGE, followed by Western blotting analysis with antibodies against APE1 and the loading control, actin.

APPENDIX IX



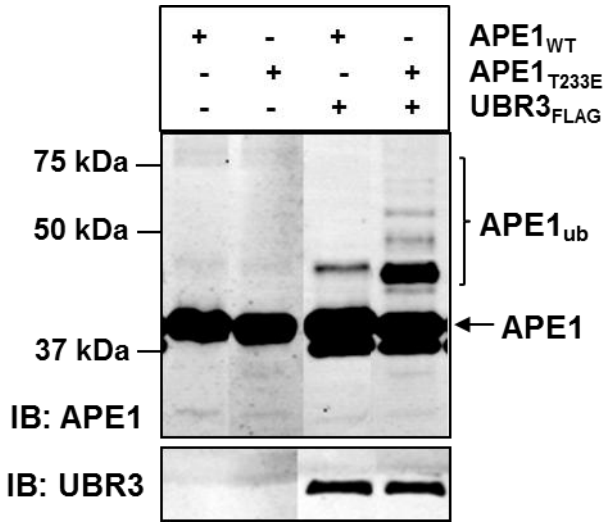
The T233E phosphorylation mimic APE1 mutant has lower AP-site incision activity. A 5'-FAM labelled 40-mer duplex oligonucleotide containing an abasic site was used as the substrate for AP endonuclease activity by the indicated amounts (ng) of wild-type APE1 protein and T233E phosphorylation mimic mutant APE1. The reactions were carried out for 20 min and separated on a 20% (w/v) denaturing polyacrylamide gel and analysed by fluorescence imaging.

APPENDIX X



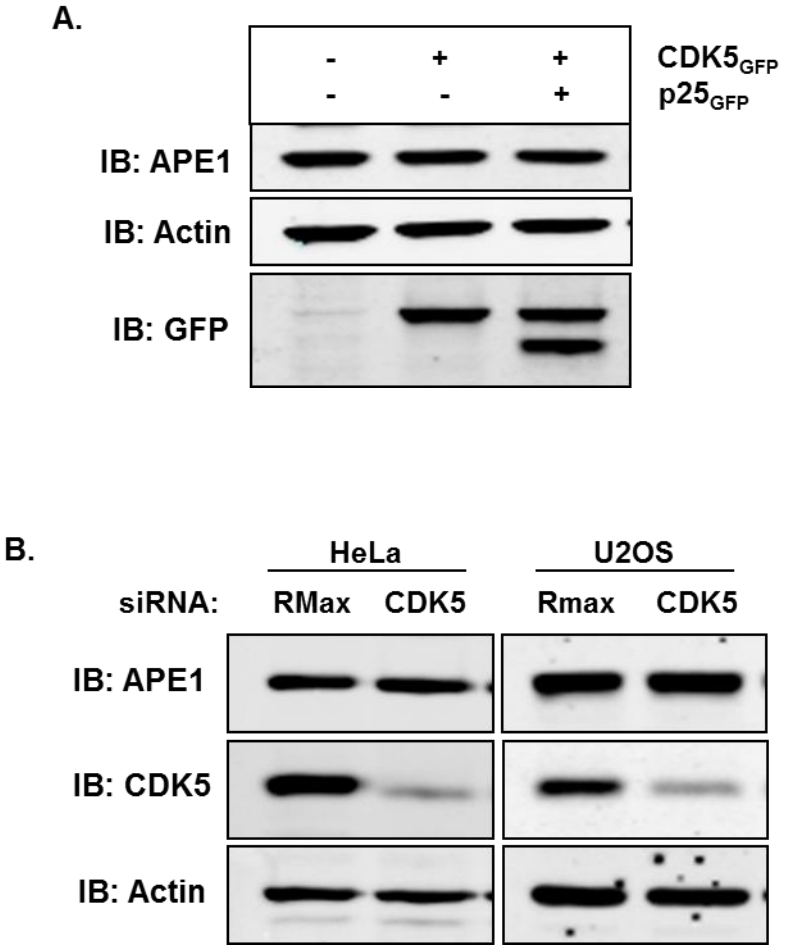
Transiently expressed T233E phosphorylation mimic APE1 mutant is less stable in HeLa cells A. HeLa cells were transiently transfected with 0.5 µg of plasmid encoding FLAG-tagged wild-type APE1, T233A non-phosphorylatable APE1 mutant and T233E phosphorylation mimic APE1 mutant using lipofectamine 2000 transfection reagent. The cells were harvested after 24 hr and whole cell extracts prepared for separation by 10% SDS-PAGE and analysis by Western blotting using antibodies against APE1. Molecular weight markers (kDa) are indicated. **B.** HeLa cells were transiently transfected with 0.5 µg of plasmid encoding FLAG-tagged wild-type APE1 and the T233E phosphorylation mimic APE1 mutant using lipofectamine 2000 transfection reagent. The cells were additionally treated with 10 µM of the proteasomal inhibitor MG132 suspended in DMSO 9 hr post-transfection and the treatment carried out for 15 hr. The non-treated DMSO control is shown. The cells were harvested and whole cell extracts prepared for separation by 10% SDS-PAGE and analysis by Western blotting using antibodies against APE1 and the loading control, actin.

APPENDIX XI



Recombinant UBR3 protein *in vitro* ubiquitylates the T233E phosphorylation mimic APE1 mutant more efficiently. Low amounts (200 ng) of purified FLAG-tagged UBR3 ($UBR3_{FLAG}$) protein was used as the E3 source in an *in vitro* ubiquitylation assay containing either wild-type recombinant His-tagged APE1 (2.8 pmol) or T233E phosphorylation mimic APE1 mutant protein (2.8 pmol) and E1 (0.7 pmol), ubiquitin (0.6 pmol) and an E2 mix (9.5 pmol). The completed reactions were separated by 10% SDS-PAGE and analysed by Western blotting using antibodies against APE1 (top panel) and FLAG (lower panel). Molecular weight markers (kDa), unmodified His-tagged APE1 proteins (APE1) and ubiquitylated APE1 ($APE1_{ub}$) are indicated.

APPENDIX XII



Modulation of CDK5 levels in HeLa cells does not alter the levels of APE1 protein. A. HeLa cells were transiently transfected with 1 µg of plasmid encoding GFP-tagged CDK5 and GFP-tagged p25, a co-activator of CDK5 activity using lipofectamine 2000 transfection reagent. The cells were harvested after 24 hr and whole cell extracts prepared for separation by 10% SDS-PAGE and analysis by Western blotting using antibodies against APE1, GFP and the loading control, actin **B.** Silencing sequences (20 nM) targeting CDK5 mRNA were introduced in to HeLa and U2OS cells using the RNAiMax lipofectamine reagent. After allowing 72 hr for silencing, the cells were harvested, whole cell extracts prepared and separated by 10% SDS-PAGE, followed by Western blotting analysis with antibodies against APE1, CDK5 and the loading control, actin.

LICENSES

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Ubiquitin ligase UBR3 regulates cellular levels of the essential DNA repair protein APE1 and is required for genome stability

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ABSTRACT

APE1 (Ref-1) is an essential human protein involved in DNA damage repair and regulation of transcription. Although the cellular functions and biochemical properties of APE1 are well characterized, the mechanism involved in regulation of the cellular levels of this important DNA repair/transcriptional regulation enzyme, remains poorly understood. Using an *in vitro* ubiquitylation assay, we have now purified the human E3 ubiquitin ligase UBR3 as a major activity that polyubiquitylates APE1 at multiple lysine residues clustered on the N-terminal tail. We further show that a knockout of the *Ubr3* gene in mouse embryonic fibroblasts leads to an up-regulation of the cellular levels of APE1 protein and subsequent genomic instability. These data propose an important role for UBR3 in the control of the steady state levels of APE1 and consequently error free DNA repair.

INTRODUCTION

Genome instability is the major cause of many human diseases, including cancer. One of the major sources of genome instability is the loss of a DNA base due to hydrolytic attack of the glycosylic bond linking DNA bases to the sugar phosphate backbone of the DNA molecule (1). These abasic sites (apurinic/apyrimidinic, AP sites) in DNA can also arise as an intermediate product during excision repair of various DNA base lesions. It has been estimated that every single human cell repairs about 10 000 AP sites per day (2) and this highlights the importance of the proteins involved in the repair of this cytotoxic and mutagenic lesion. Human AP endonuclease-1 (APE1, also known as Ref-1) initiates repair of AP sites by incising the phosphodiester bond 5′ to the AP site and generating a DNA single-strand break (SSB) containing a 5′-sugar phosphate residue (3,4). This SSB is further repaired by DNA polymerase β and XRCC1–DNA ligase IIIα complex. DNA polymerase β removes the 5′-sugar phosphate residue and adds one undamaged nucleotide into the repair gap. Finally, XRCC1–DNA ligase IIIα complex seals the DNA ends thus accomplishing the repair process (5). Although being a small protein (36 kDa), APE1 has multiple other enzymatic activities, including 3′-phosphatase, 3′-phosphodiesterase and 3′- to 5′-exonuclease activities specific for internal nicks and gaps in DNA (6). It has also been shown to catalyse the release of 3′-phosphoglycolate moieties that block the repair of DNA SSBs (7–10). All these enzymatic activities of APE1 are important for the repair of DNA lesions induced by oxidative stress and ionising radiation. In addition, APE1 has an important role as a reductive activator of a number of transcription factors, including p53, AP-1, NF-κB and HIF-1α, in response to oxidative stress (11).

APE1 is known to be absolutely required for cellular survival since deletion of APE1 in mice is embryonic

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lethal (12–14) and stable cell lines lacking the protein have not been established, again pointing to a critical role for APE1. Down-regulation of APE1 by RNA interference (15,16) and the generation of conditional *Ape1* null mice (16) confirmed that AP endonuclease activity is essential for mammalian cell viability and plays a central role in endogenous and induced DNA damage processing. Consequently, controlling the cellular levels of APE1 protein is very important as both over- or under-expression of APE1 leads to genomic instability (17–19) and is frequently found in cancer cells (11,20–23) and neurological disorders [(24) and references within].

We have previously demonstrated that the cellular amounts of DNA polymerase β and XRCC1–DNA ligase IIIα, involved in repair of SSBs generated by APE1, are tuned to the amount of DNA damage through regulated proteasomal degradation. This is controlled by the E3 ubiquitin ligases Mule, CHIP and the DNA damage signalling protein and tumour suppressor ARF (25,26). However the mechanism controlling APE1 levels is unknown. Assuming that APE1 levels are also controlled by ubiquitylation, we first demonstrated the existence of ubiquitylated APE1 in human cells and then developed an *in vitro* ubiquitylation assay for APE1. Using this assay, we purified UBR3 as the major human E3 ubiquitin ligase targeting APE1 for ubiquitylation, and demonstrate that UBR3 controls the cellular levels of APE1 and is required for genome stability.

MATERIALS AND METHODS

Antibodies

Polyclonal APE1 antibodies were purified as described (27). APE1 mouse monoclonal antibodies for identification of mouse APE1 (APEX) were purchased from Abcam (Cambridge, UK). ARF and 53BP1 antibodies were purchased from Bethyl Laboratories (Montgomery, USA), tubulin antibodies were from Sigma-Aldrich (Gillingham, UK), Mdm2 antibodies were from Santa Cruz (California, USA) and γ-H2AX antibodies were from Millipore (Watford, UK). Actin, hsp70 and ubiquitin antibodies were purchased from Abcam (Cambridge, UK). UBR3 antibodies were purified as recently described (28).

Plasmids and proteins

Ubiquitin, E1 and E2 enzymes were purchased from Boston Biochemicals (Cambridge, USA). pET14b plasmid encoding human APE1 protein was kindly provided by Prof. Hickson and APE1 mutants were constructed using the 'QuikChange' protocol (Stratagene, Amsterdam, Holland) and confirmed by sequencing. Human histidine-tagged APE1 was purified by HisTrap HP column chromatography (GE Healthcare, Little Chalfont, UK). Plasmid encoding mouse APE1 protein (APEX) was kindly provided by Dr Nakabeppu and the *Apex* gene was re-cloned into pCMV3Tag3a plasmid (Agilent Technologies, Stockport, UK). N-terminal FLAG-tagged mouse UBR3 (UBR3) was expressed in *Saccharomyces cerevisiae* SC295 (*MATa, GAL4 GAL80 ura3-52, leu2-3,112 reg1-501 gal1 pep4-3*) from the P_{ADH1} promoter in a high copy vector as described previously (28). To purify UBR3 protein, the yeast lysate containing UBR3 was mixed with anti-Flag M2 agarose beads (Sigma, Gillingham, UK) and after washing the beads, the bound UBR3 protein was eluted using FLAG peptide-containing elution buffer (200 μg/ml). Purified UBR3 protein was dialyzed against PBS, concentrated and stored at −80°C until required.

Establishment and immortalization of Ubr3$^{-/-}$ mouse embryonic fibroblasts

Construction and characterization of *Ubr3*$^{-/-}$ mouse was recently described (28). Primary mouse embryonic fibroblasts (MEFs) were established from wild-type and *Ubr3*$^{-/-}$ embryos at E13.5 in the 129SvImJ/C57BL/6 background as previously described (29). Immortalized MEFs were established from primary MEFs by repeated cell cultures over ∼2 months (∼1.5×10^6 cells onto a 10 cm^2 plate every 3–5 days).

Whole-cell extracts

Whole-cell extracts were prepared by Tanaka's method (30). Briefly, cells were resuspended in one packed cell volume of buffer containing 10 mM Tris–HCl (pH 7.8), 200 mM KCl, 1 μg/ml of each protease inhibitor (pepstatin, aprotinin, chymostatin and leupeptin), 1 mM DTT and 1 mM PMSF. Two packed cell volumes of buffer containing 10 mM Tris–HCl (pH 7.8), 600 mM KCl, 40% glycerol, 0.1 mM EDTA and 0.2% Nonidet P-40 was then added and mixed thoroughly before rocking the cell suspension for 2 h at 4°C. The cell lysate was then centrifuged at 40 000 rpm at 4°C for 20 min and the supernatant was collected, aliquoted and stored at −80°C.

Cell fractionation

Cell fractionation was performed as recently described (31). Briefly, cell pellets were resuspended in two packed cell volumes of buffer containing 20 mM Tris–HCl pH 7.4, 2.5 mM $MgCl_2$, 0.5% (v/v) Nonidet P-40, 1 mM PMSF, 1 mM DTT and 1 μg/ml each of aprotinin, pepstatin, chymostatin and leupeptin, and incubated on ice for 10 min. Following centrifugation at 10 000 rpm for 2 min, the supernatant containing cytoplasmic proteins (C) was collected. The nuclear pellet was similarly extracted with two packed cell volumes of buffer containing 20 mM Tris–HCl pH 8.0, 0.5 M KCl, 1 mM EDTA, 0.75% (v/v) Triton X-100, 10% (v/v) glycerol, 1 mM PMSF, 1 mM DTT and 1 μg/ml each of aprotinin, pepstatin, chymostatin and leupeptin and the supernatant containing nuclear proteins (**N**) was collected. For identification of ubiquitylated APE1 bands from HeLa cells, 1 mM NEM was also added to each of the buffers prior to extractions.

Western blots

Western blots were performed by standard procedure as recommended by the vendor (Novex, San Diego, USA). Blots were visualized using the Odyssey image analysis system (Li-cor Biosciences, Cambridge, UK).

In vitro ubiquitylation assay

In vitro ubiquitylation was performed as recently described (25). Briefly, assays were performed in a 15 µl reaction volume in the presence of E1 activating enzyme (0.7 pmol), the indicated E2 conjugating enzymes (9.5 pmol) and ubiquitin (0.6 pmol) or mutant ubiquitin unable to form polyubiquitin chains (Boston Biochem, Cambridge, USA) in buffer containing 25 mM Tris–HCl (pH 8.0), 4 mM ATP, 5 mM $MgCl_2$, 200 µM $CaCl_2$, 1 mM DTT, 10 µM MG-132 for 1 h at 30°C. SDS–PAGE sample buffer 25 mM Tris-HCl (pH 6.8), 2.5 % beta-mercaptoethanol, 1 % SDS, 5 % glycerol, 1 mM EDTA, 0.15 mg/ml bromophenol blue) was added, the samples were heated for 5 min at 95°C prior to separation of the proteins on a 10% SDS–polyacrylamide gel, followed by transfer to a PVDF membrane and immunoblot analysis with the indicated antibodies.

Partial purification of monoubiquitylated APE1

Whole-cell extracts from 20 g HeLa cells was prepared as described above and dialysed against Buffer A (50 mM Tris, pH 8.0, 1 mM EDTA, 5% glycerol, 1 mM DTT, 1 mM PMSF) containing 150 mM KCl and 1 mM NEM. The dialysate was clarified by centrifugation at 25 000 rpm for 20 min at 4°C, filtered through 0.45 -µm filters and fractionated by Phosphocellulose chromatography using Buffer A containing 150 mM KCl and 1 mM NEM. Proteins were eluted using Buffer A containing 1 M KCl and 1 mM NEM, concentrated and diluted to 50 mM KCl and fractionated by Mono-Q chromatography and the flow-through was collected. Proteins were then fractionated by Mono-S chromatography using Buffer A containing 50 mM KCl and proteins eluted using a linear gradient of 50–1000 mM KCl. Fractions were analysed by 10% SDS–PAGE and immunoblotting with APE1 or ubiquitin antibodies.

Mapping and identification of ubiquitylation sites

Cleavage of the first 35 amino acids from the APE1 protein for *in vitro* ubiquitylation assays was achieved by treating APE1 (8 µg) with 0.5 µg of trypsin in buffer containing 50 mM Tris–HCl (pH 7.4), 50 mM KCl, 10 mM $MgCl_2$ and 1 M urea for 2 h at 23°C. APE1 was then buffer exchanged three times using Amicon Ultra-4 filter units (Millipore, Watford, UK) to remove trypsin and the N-terminal tail of APE1, prior to incubation in the *in vitro* ubiquitylation assay. For identification of ubiquitylation sites, full-length APE1 protein was ubiquitylated *in vitro* using UbcH2 E2 conjugating enzyme as described above, separated by SDS–PAGE and the Coomassie stained band corresponding to monoubiquitylated APE1 was excised, digested with trypsin and analysed by tandem mass spectrometry as recently described (26).

Purification of the E3 ubiquitin ligase for APE1

Cytoplasmic proteins were isolated from 20 g HeLa cells (Cilbiotech, Mons, Belgium) as described above and dialysed against Buffer A (50 mM Tris, pH 8.0, 1 mM EDTA, 5% glycerol, 1 mM DTT, 1 mM PMSF) containing 150 mM KCl. The dialysate was clarified by centrifugation at 25 000 rpm for 20 min at 4°C, filtered through 0.45 -µm filters and applied to a Phosphocellulose column using Buffer A containing 150 mM KCl and the flow-through was collected (PC-FI). PC-FI was concentrated using Vivaflow 50 and 10 kDa MWCO PES membrane modules (Sartorius Stedim, Surrey, UK), diluted 2-fold to achieve a final concentration of 75-mM KCl in the fraction and then added to a 20 ml HiLoad Mono-Q Sepharose column (GE Healthcare, Little Chalfont, UK), washed with Buffer A containing 50 mM KCl and proteins eluted using a linear gradient from 50 to 1000 mM KCl. Active fractions were pooled, concentrated using Amicon Ultra-15 filter units (Millipore, Watford, UK) and loaded onto a Superose-12 column (GE Healthcare, Little Chalfont, UK) in Buffer A containing 150 mM KCl. Active fractions were pooled, diluted to 50 mM KCl and loaded onto a 1 ml Mono Q HR 5/5 column (GE Healthcare, Little Chalfont, UK) in buffer A containing 50 mM KCl. Proteins were eluted using a linear gradient of 50–1000 mM KCl. At each chromatography stage, aliquots of the fractions were analysed for E3 ubiquitin ligase activity using APE1 as a substrate and active fractions pooled for the next chromatography step. Active fractions from the final Mono-Q chromatography stage were analysed by tandem mass spectrometry to identify candidate E3 ubiquitin ligases, as recently described (26).

Plasmid transfection

Cells were transfected using Lipofectamine 2000 transfection reagent (Invitrogen, Paisley, UK) according to the manufacturer's protocol.

RT–PCR analysis

RNA was prepared from *Ubr3* wild-type and knockout MEFs using the RNeasy kit (Qiagen, Crawley, UK) and cDNA was subsequently generated using the SuperScript RT–PCR system (Invitrogen, Paisley, UK). UBR3, APE1 and actin was amplified using the following primers: 5′-GGGAATATTTGAACTTGATTTAATCTG-3′ and 5′-CCACACTGGACACGACTTCA-3′; 5′-CGGGGAAGAACCCAAGTC-3′ and 5′-TCCTTCTCGGTTTTCTTTGC-3′; 5′-GGAGGGGGTTGAGGTGTT-3′ and 5′-TGTGCACTTTTATTGGTCTCAAG-3′, respectively. Cycling conditions of one cycle at 95°C for 5 min, 25 cycles (or 30 cycles for UBR3) at 95°C for 30 s, 55°C for 20 s and 72°C for 20 s, and one cycle at 72°C for 5 min. PCR products were separated by 2% agarose gel electrophoresis containing SYBR Green (Invitrogen, Paisley, UK) and analysed using the Molecular Imager FX system (Bio-Rad, Hemel Hempstead, UK).

Clonogenic survival assay

Ubr3 wild-type and knockout MEFs were seeded in triplicate onto 10-cm^2 dishes at a density of 300 cells/plate and allowed to adhere for 16 h. The cells were then treated

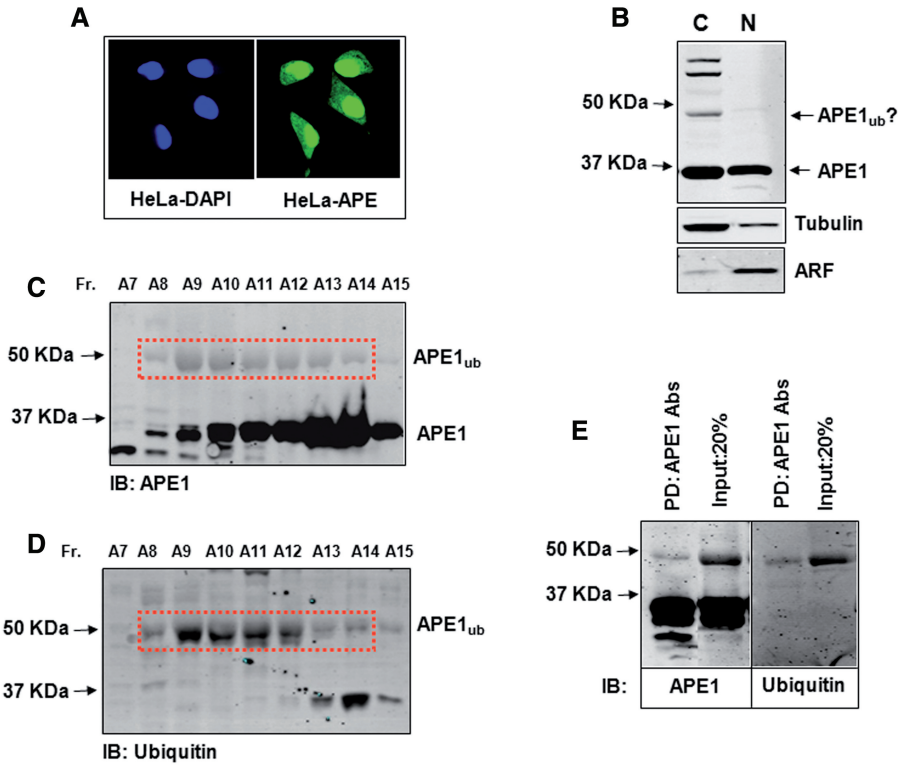

Figure 1. Purification of ubiquitylated APE1 from human cells. (**A**) HeLa cells were fixed and immunostained with DAPI and APE1 antibodies demonstrating cytoplasmic and nuclear localization of APE1. (**B**) Cytoplasmic and nuclear fractions prepared from HeLa cells were analysed by 10% SDS–PAGE and immunoblotting using the indicated antibodies. Three bands cross-reacting with APE1 antibodies, in addition to the 36 kDa native form of APE1, were present in the cytoplasmic fraction and were predicted to be ubiquitylated APE1. (**C** and **D**) Analysis of Mono-S chromatography fractions purified from HeLa cells analysed by 10% SDS–PAGE and immunoblotting using either (C) APE1 or (**D**) ubiquitin antibodies. (**E**) The peak monoubiquitylated APE1 containing Mono-S fraction (A9) was immunodepleted using APE1 antibodies and the pull-down was then analysed by 10% SDS–PAGE and immunoblotting using either APE1 (left panel) or ubiquitin (right panel) antibodies. Molecular weight markers are indicated on the left hand side of appropriate figures and the positions of monoubiquitylated APE1 ($APE1_{ub}$) are shown.

with methyl methanesulphonate (MMS; Sigma-Aldrich, Gillingham, UK) for 45 min, the cells were washed twice with PBS, the media replaced and the cells allowed to form colonies over 7 days. Colonies were stained using methylene blue and subsequently counted. The surviving fraction was calculated by normalizing against the colony numbers achieved for the untreated dishes.

Immunostaining

Subconfluent cell monolayers on coverslips were fixed with 4% paraformaldehyde for 30 min at room temperature, the cells were permeabilized with 0.2% (w/v) Triton X-100 for 10 min at 4°C and blocked at room temperature with 2% (w/v) BSA—Fraction V for 1 h. The coverslips were then incubated overnight at 4°C with primary antibodies raised against APE1 or 53BP1 diluted 1:300 in BSA. The coverslips were washed and incubated in secondary antibodies, Alexa Fluor 488 $F(ab')_2$ fragment of goat anti-rabbit or anti-mouse (Invitrogen, Paisley, UK) for 1 h. Slides were then mounted using Vectashield hard set mounting media containing 4,6-diamidino-2-phenylindole (DAPI; Vector Laboratories, Peterborough, UK) and imaged using the BioRad MRC 600 confocal microscope and 488 nm (FITC) argon laser lines for excitation.

RESULTS

APE1 ubiquitylation in living cells

In HeLa cells, the concentration of APE1 protein is much higher in the nucleus, although a substantial amount can also be detected in the cytoplasm (Figure 1A). Taking into account that the volume of the cytoplasm is ~8-fold larger than the volume of the nucleus, the absolute amount of APE1 that can be extracted into cytoplasmic and nuclear fractions is approximately the same (Figure 1B). To demonstrate that APE1 ubiquitylation occurs in living cells, we performed cell fractionation in the presence of *N*-ethylmaleimide (NEM), which is an effective inhibitor of deubiquitylation enzymes. We found that in HeLa cell extracts prepared with NEM, in addition to APE itself, immunoblotting with APE1-specific antibodies detected at least three extra protein bands with a molecular weight of between 45 and 60 kDa, but these were only observed in the cytoplasmic fraction (Figure 1B). Since the molecular weight of monoubiquitylated APE1 is expected to be ~44 kDa, the protein with a molecular weight just <50 kDa that cross-reacted with the APE1 antibodies was considered as a potential candidate for monoubiquitylated APE1 ($APE1_{ub}$, Figure 1B, indicated by arrow). To provide evidence that this protein was $APE1_{ub}$, we

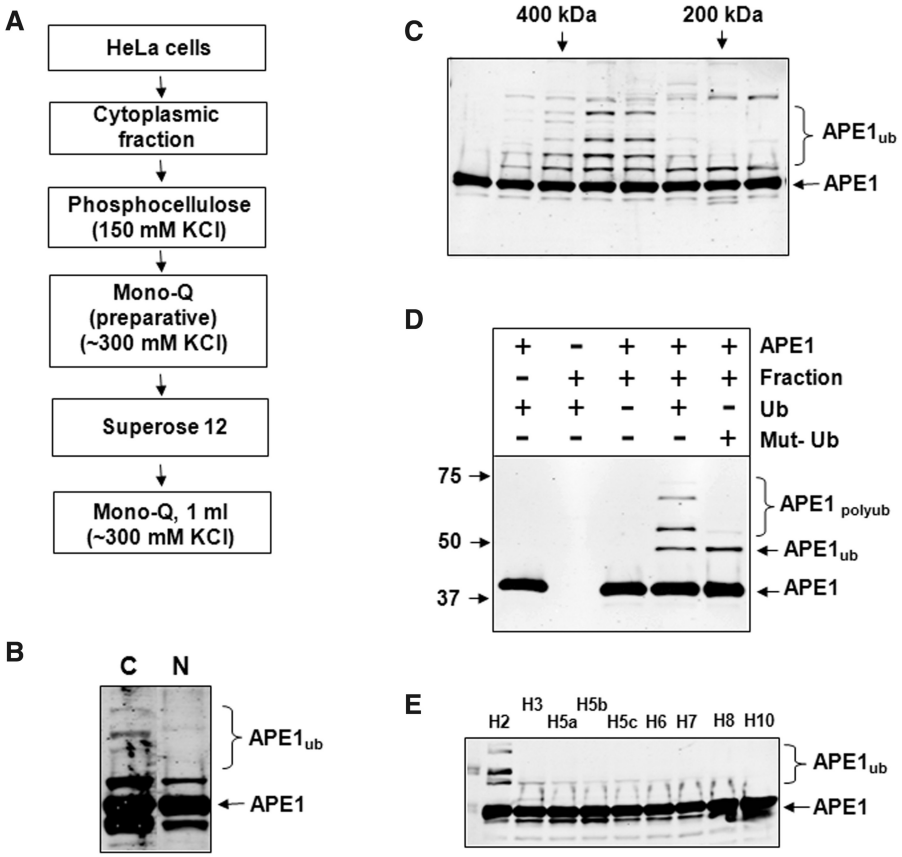

Figure 2. Purification of the E3 ubiquitin ligase for APE1. (**A**) Purification scheme for the isolation of the E3 ubiquitin ligase for APE1 from HeLa cells. (**B**) Cytoplasmic and nuclear fractions (8 µg) were prepared from HeLa cells and analysed for *in vitro* ubiquitylation activity for APE1 (2.8 pmol) in the presence of E1 (0.7 pmol), ubiquitin (0.6 nmol) and various E2 enzymes (9.5 pmol). Reactions were separated by 10% SDS–PAGE and analysed by immunoblotting using APE1 antibodies. The major ubiquitylation activity against APE1 can be observed in the cytoplasmic fraction. (**C**) *In vitro* ubiquitylation of APE1 (2.8 pmol) by Superose 12 fractions purified from HeLa cells in the presence of E1 (0.7 pmol), ubiquitin (0.6 nmol) and various E2 enzymes (9.5 pmol). Reactions were separated by 10% SDS–PAGE and analysed by immunoblotting using APE1 antibodies. E3 ubiquitin ligase activity for APE1 corresponds to a protein of molecular weight between 200 and 400 kDa. (**D**) *In vitro* ubiquitylation of APE1 (2.8 pmol) by an active fraction purified from HeLa cells in the presence of E1 (0.7 pmol), various E2 enzymes (9.5 pmol) and ubiquitin (Ub; 0.6 nmol) or mutant ubiquitin (Mut-Ub) unable to form polyubiquitin chains. (**E**) *In vitro* ubiquitylation of APE1 (2.8 pmol) by an active fraction purified from HeLa cells in the presence of E1 (0.7 pmol), ubiquitin (Ub; 0.6 nmol) and various E2 enzymes (9.5 pmol each). Reactions (D and E) were separated by 10% SDS–PAGE and analysed by immunoblotting using APE1 antibodies. Molecular weight markers are indicated on the left hand side of appropriate figures and the positions of ubiquitylated APE1 ($APE1_{ub}$) are shown.

partially purified this protein from a cytoplasmic extract of HeLa cells by sequential Phosphocellulose, Mono-Q and Mono-S chromatographies and monitored fractions with APE1 antibodies. Fractions containing the ~44–50 KDa protein cross-reacting with the APE1 antibodies were then probed with antibodies directed against ubiquitin. We found that fractions containing the ~44–50 kDa protein cross-reacted with both APE1 (Figure 1C) and ubiquitin (Figure 1D) specific antibodies. Furthermore, after precipitation of this protein with APE1 antibodies, the precipitated protein still cross-reacted with both APE1 and ubiquitin antibodies (Figure 1E). We thus conclude that this ~44-kDa protein is monoubiquitylated APE1.

UBR3 is an E3 ubiquitin ligase for APE1

Having established that APE1 ubiquitylation occurs in living cells, we pursued purification of an E3 ubiquitin ligase activity for APE1 from human cell extracts using a series of chromatography columns (Figure 2A) and an *in vitro* ubiquitylation assay. We first observed that the majority of APE1 E3 ubiquitin ligase activity was detected in the cytoplasm (Figure 2B) and that the activity became very pronounced after gel-filtration over a Superose 12 column (Figure 2C). From this column, we determined that the E3 ubiquitin ligase for APE1 was ~200–400 kDa. We further demonstrated that *in vitro* ubiquitylation of recombinant APE1 is dependent on the presence of both active fraction and ubiquitin (Figure 2D, fourth lane) and further established, using wild-type and mutant ubiquitin unable to form polyubiquitin chains, that recombinant APE1 is polyubiquitylated since we observed only a single product of ubiquitylation using mutant ubiquitin (Figure 2D, last lane). We also screened nine ubiquitin E2 conjugating enzymes for their ability to mediate ubiquitylation of APE1 and discovered that the ubiquitylation reaction is specific for UbcH2 (human homologue of yeast Ubc8, Figure 2E). Proteins

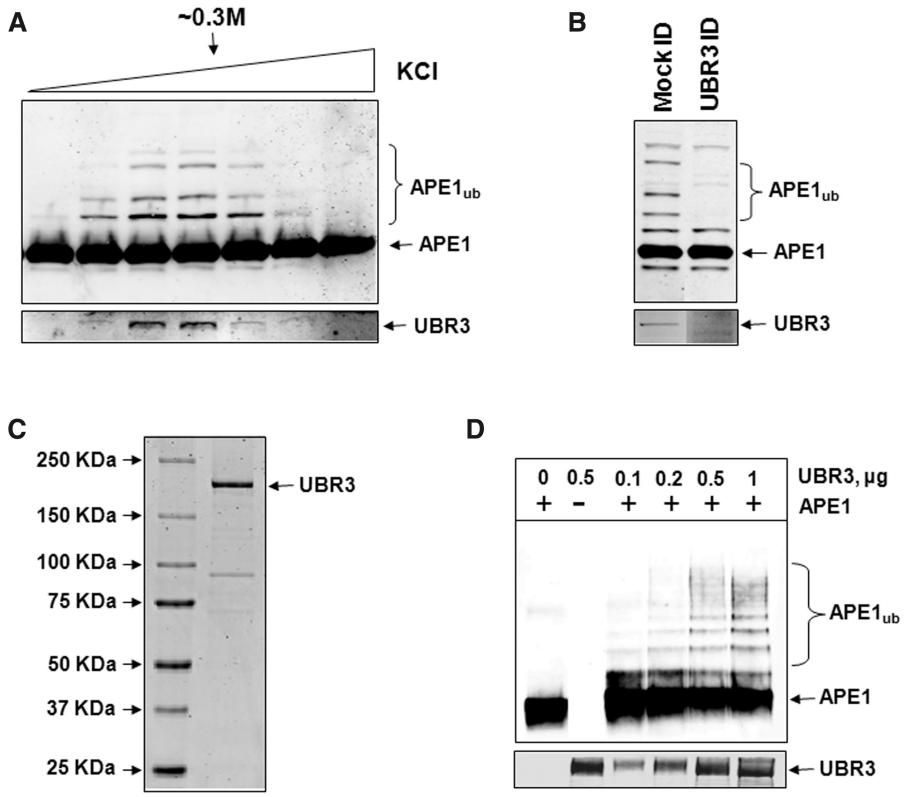

Figure 3. UBR3 is the E3 ubiquitin ligase for APE1. (**A**) *In vitro* ubiquitylation of APE1 (2.8 pmol) by the final Mono-Q chromatography fractions purified from HeLa cells in the presence of E1 (0.7 pmol), UbcH2 (10.5 pmol) and ubiquitin (0.6 nmol) were analysed by 10% SDS–PAGE and immunoblotting using APE1 antibodies. Fractions containing APE1 ubiquitylation activity were shown to correlate with the presence of UBR3 protein detected by immunoblotting (lower panel). (**B**) The peak APE1 ubiquitylation activity containing fraction from the final Mono-Q chromatography was mock immunodepleted and immunodepleted with UBR3 antibodies. The fraction was subsequently analysed for *in vitro* ubiquitylation of APE1 (2.8 pmol) in the presence of E1 (0.7 pmol), UbcH2 (10.5 pmol) and ubiquitin (0.6 nmol) by 10% SDS–PAGE and immunoblotting using APE1 antibodies (upper panel). Furthermore, the pull-down was analysed by 10% SDS–PAGE and immunoblotting using UBR3 antibodies (lower panel). (**C**) N-terminal Flag-tagged mouse UBR3 was expressed in *S. cerevisiae*, purified and analysed by 4–12% SDS–PAGE and Coomassie staining. (**D**) Purified Flag-tagged mouse UBR3 was analysed for *in vitro* ubiquitylation of APE1 (2.8 pmol) in the presence of E1 (0.7 pmol), UbcH2 (10.5 pmol) and ubiquitin (0.6 nmol) by 10% SDS–PAGE and immunoblotting using APE1 antibodies and UBR3 antibodies (lower panel). Molecular weight markers are indicated on the left hand side of appropriate figures and the positions of ubiquitylated APE1 ($APE1_{ub}$) are shown.

from active fractions from the final purification step (Mono-Q column) were subjected to nanoLC–MS/MS tandem mass spectrometry, which revealed UBR3 as a potential E3 ubiquitin ligase involved in UbcH2-dependent APE1 ubiquitylation (Supplementary Figure S1). Indeed, when active fractions were tested for the presence of UBR3, we found a strong correlation between APE1 ubiquitylation activity in these active fractions (Figure 3A, upper panel) and the amount of UBR3 protein detected by immunoblotting (Figure 3A, lower panel). To confirm that UBR3 is responsible for APE1 ubiquitylation in the Mono-Q fractions, we immunoprecipitated UBR3 away from active fractions (Figure 3B, lower panel) and demonstrated that UBR3-depleted fractions had significantly reduced ubiquitylation activity (Figure 3B, upper panel). We next purified recombinant mouse UBR3 protein (Figure 3C) and found that it was able to polyubiquitylate APE1 in an *in vitro* ubiquitylation system reconstituted with purified enzymes (Figure 3D).

It has recently been reported that Mdm2 is involved in APE1 ubiquitylation (33). Although we found that recombinant Mdm2 is able to ubiquitylate APE1 in an *in vitro* ubiquitylation assay (Supplementary Figure S2), we discovered that the major ubiquitylation activity for APE1 purified from HeLa cells did not co-purify with Mdm2 protein (Supplementary Figure S3). We thus conclude that UBR3 is the major human E3 ubiquitin ligase involved in polyubiquitylation of APE1.

UBR3 ubiquitylates several lysines at the N-terminus of APE1

APE1 protein contains nine lysines within the 35 amino acid N-terminal region (Figure 4A). To test whether these residues are the sites of ubiquitylation, we cleaved off this N-terminal region by controlled trypsin hydrolysis. We demonstrated that in contrast to the full length protein (~32% of the substrate is ubiquitylated), truncated APE1 lacking the first 35 amino acids from the N-terminus cannot be efficiently ubiquitylated by a UBR3-containing fraction purified from HeLa cells (~4% of the substrate is ubiquitylated, Figure 4B). In support of this finding, nanoLC–MS/MS tandem mass spectrometry of *in vitro* ubiquitylated full length APE1

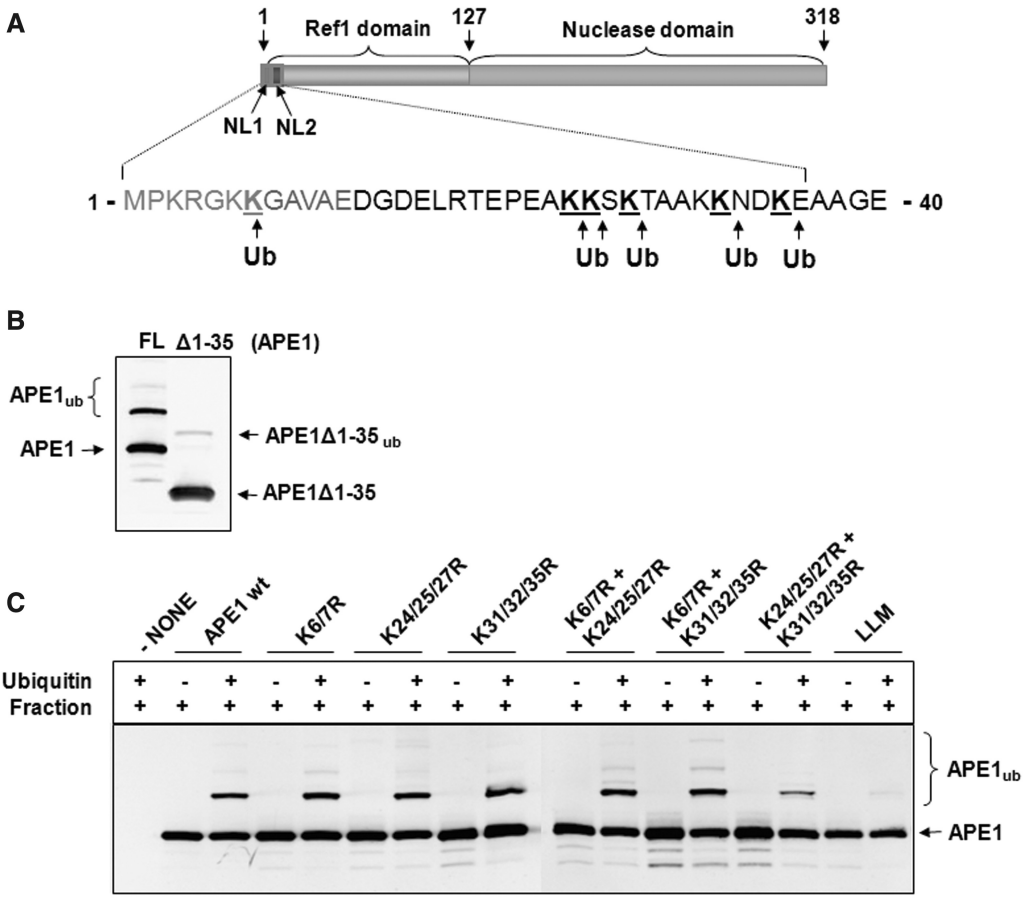

Figure 4. Identification of UBR3 ubiquitylation sites within APE1. (**A**) Schematic diagram of the protein structure of APE1 showing the nuclear localization sequences (NL1 and NL2), Ref-1 domain and nuclease domain. Major sites (K7/K24/K25/K27/K32/K35) of ubiquitylation by UBR3 identified by mass spectrometry are shown. (**B**) An N-terminal truncation of APE1 lacking the first 35 amino acids (APE1Δ1-35) was analysed for *in vitro* ubiquitylation, in comparison to full length protein (FL), by an active fraction purified from HeLa cells in the presence of E1 (0.7 pmol), UbcH2 (10.5 pmol) and ubiquitin (0.6 nmol) by 10% SDS–PAGE and immunoblotting using APE1 antibodies. (**C**) Wild-type protein (WT) and APE1 mutants, in which the indicated lysines were mutated to arginines, were analysed for *in vitro* ubiquitylation by an active fraction purified from HeLa cells in the presence of E1 (0.7 pmol), UbcH2 (10.5 pmol) and ubiquitin (0.6 nmol) by 10% SDS–PAGE and immunoblotting using APE1 antibodies.

identified lysines 7, 24, 25, 27, 32 and 35 as targets for ubiquitylation. At least for an *in vitro* reaction, it seems that these lysines are interchangeable ubiquitylation targets for UBR3, since replacement of individual lysines or combinations of two to three lysines with arginines did not significantly reduce ubiquitylation (Figure 4C). The ubiquitylation of APE1 by UBR3 only significantly dropped when the majority of lysines (K24, K25, K27, K31, K32 and K35) were replaced with arginines and was completely abolished in the Lysine Less Mutant (LLM), that was constructed by replacement of all but K3 within the first 35 amino acids of APE1 (Figure 4C, last lane).

UBR3 knockout cells have increased levels of APE1 and show genome instability

Ubr3-knockout mice have been generated and have previously been shown to have a genetic background-dependent lethality, indicating an essential role for this gene (28). To assess the role of UBR3 in controlling APE1 levels, we prepared cell extracts from wild-type and *Ubr3*-knockout MEFs and found ~2-fold increase in APE1 levels in *Ubr3*-knockout cells (Figure 5A). To exclude that the increase in APE1 levels was due to clonal variability, we further analysed two more independent *Ubr3*-knockout cell lines in comparison to three independent wild-type cell lines and found that the *Ubr3*-knockout cells always contained higher APE1 levels (Figure 5B). Interestingly, a cell line generated from a *Ubr3*-heterozygous mouse also contained higher protein levels of APE1 (Figure 5B). To further demonstrate that APE1 stability depends on UBR3, we transfected plasmids expressing mouse AP-endonuclease 1 (APEX) into wild-type or *Ubr3*-knockout cells. The level of APEX expression was ~5-fold higher in *Ubr3*-knockout cells compared to wild-type cells (Figure 5C). RT–PCR of the RNA purified from *Ubr3*-knockout cells clearly indicated that the levels of *ape1* mRNA in *Ubr3*-wild-type and *Ubr3*-knockout cells is approximately the same

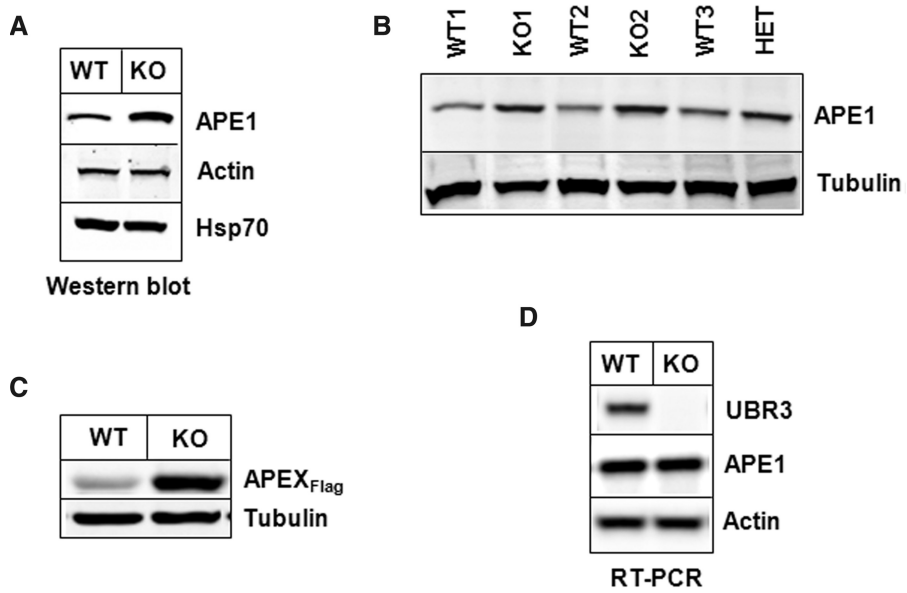

Figure 5. Cells from *Ubr3*-knockout mice contain elevated levels of APE1 protein. (**A** and **B**) Whole cell extracts from MEFs from several wild-type (WT), *Ubr3*-knockout (KO) and heterozygous mice (HET) were prepared and analysed by 10% SDS–PAGE and immunoblotting using the indicated antibodies (**C**) Wild-type and *Ubr3*-knockout MEFs cells were grown on 10-cm^2 dishes for 24 h to 70–80% confluency and then transfected with equal amounts of mouse Flag-tagged APE1 (APEX$_{Flag}$) expression plasmid. After 24 h, cells were pelleted by centrifugation, whole cell extracts were prepared and analysed by 10% SDS–PAGE and western blotting with the antibodies indicated. (**D**) RNA was prepared from wild-type and *Ubr3*-knockout MEFs and the levels of APE1, UBR3 and actin mRNA analysed by RT–PCR.

(Figure 5D) and shows that modulation of APE1 is at the protein level. These data were further confirmed using real-time PCR that showed only a 1.3 ± 0.1-fold increase in *ape1* mRNA levels in *Ubr3*-knockout cells, in contrast to an at least 2-fold increase in protein level (Supplementary Figure S4).

We also noticed that the nuclei of *Ubr3*-knockout cells have significant variations in size and shape, in comparison to wild-type cells, that is an indicator of genetically unstable cells (Figure 6A and B), (33). We hypothesized that elevated levels of APE1 in *Ubr3*-knockout cells leads to genomic instability through the imbalance of DNA repair and the generation of excessive SSBs that in turn will be converted into DNA double-strand breaks during replication. In support of this hypothesis, we observed a higher number of spontaneously formed 53BP1 foci (Figure 6C and D), as well as higher levels of histone H2AX phosphorylation in *Ubr3*-knockout cells compared to wild-type cells (Figure 6E), indicating increased levels of endogenously generated DNA double-strand breaks. If our model is correct, then over-expression of APE1 itself should generate DNA strand breaks by misbalancing the repair of endogenous DNA lesions. Indeed, over-expression of APE1 in HeLa cells induced formation of DNA double-strand breaks as detected by increased phosphorylation of histone H2AX (Figure 6F). Imbalance of DNA repair in *Ubr3*-knockout cells should also lead to increased formation of DNA strand breaks after mutagen treatment and these can then increase cell toxicity. Indeed, using clonogenic survival assays we were able to show that *Ubr3* knockout cells, in contrast to the wild-type cells, are more susceptible to the cell killing effects of the DNA alkylating agent MMS (Figure 6G), that generates base lesions that are almost entirely dependent on APE1 for their repair.

DISCUSSION

APE1 is an essential protein that possesses multiple activities involved in DNA repair and it also operates as a redox factor activating a number of transcription factors, including NF-κB, AP-1 and p53 (34). Several studies have addressed the transcriptional regulation of APE1 however, it is also clear that post-translational modifications of APE1 play a major role in protein activity, stability and localization, although very little is known about the mechanisms involved [reviewed in (35)].

In this study, we investigated the role of ubiquitylation in the regulation of the steady state levels of APE1 protein and identify both the E2 ubiquitin conjugating enzyme, UbcH2 and the E3 ubiquitin ligase, UBR3, as the major enzymes involved. Previously, it was suggested that ubiquitylation of APE1 is accomplished by Mdm2 (32). However, even though we found that Mdm2 can ubiquitylate APE1 *in vitro*, we were not able to demonstrate that Mdm2 purified from human cell extracts possessed a significant ubiquitylation activity for APE1. Nevertheless, we cannot completely rule out that Mdm2 may serve as a minor backup E3 ubiquitin ligase for APE1 in the absence of UBR3. There is also a possibility that Mdm2, as a major regulator of p53, is involved in transcriptional regulation of APE1 since p53 was implicated in the negative regulation of APE1 transcription (36).

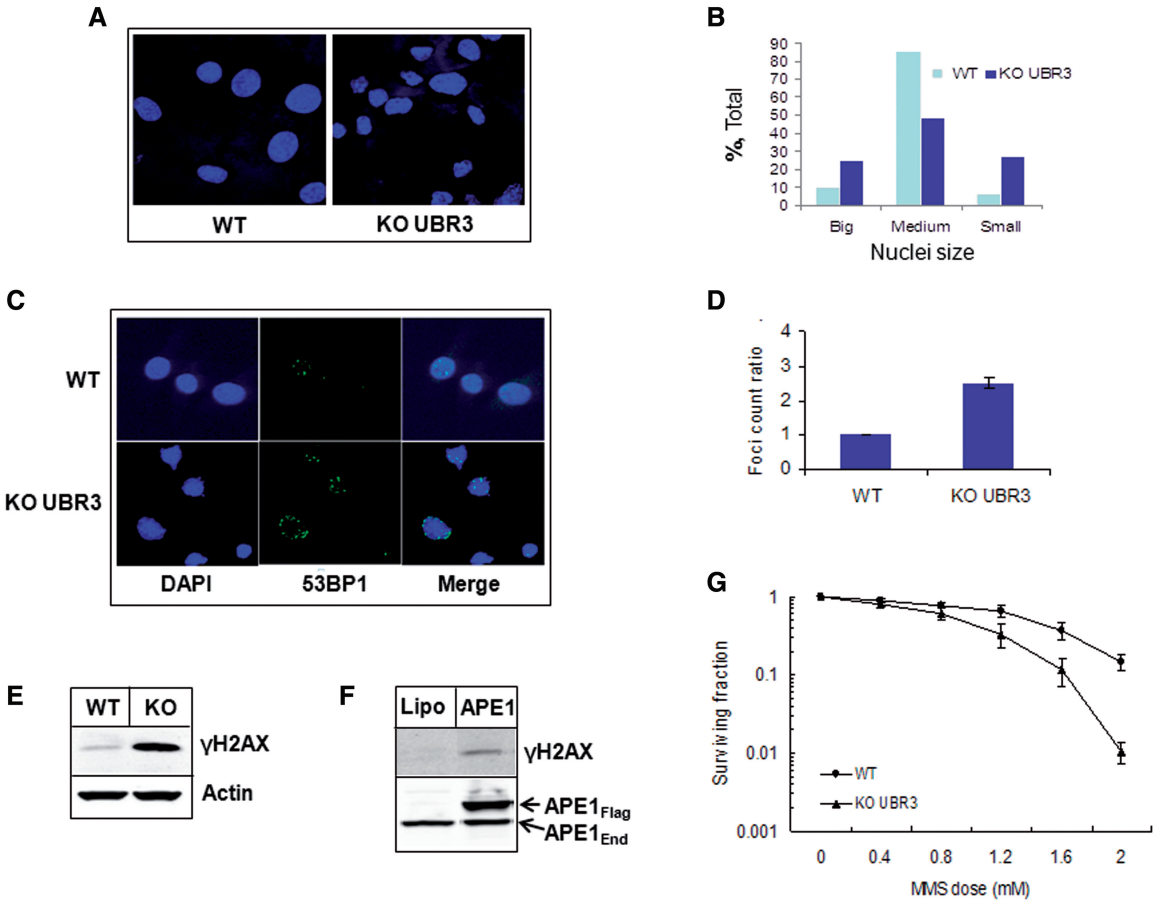

Figure 6. Cells from *Ubr3*-knockout mice are genetically instable. **(A** and **C)** MEFs from wild-type and *Ubr3*-knockout mice were fixed and immunostained with the indicated antibodies and stained with DAPI. **(B)** Graphic presentation of nuclei size distribution in wild-type and *Ubr3*-knockout fibroblasts (500 cell nuclei were evaluated). **(D)** Graphic representation of 53BP1 foci ratios calculated from three individual slides (53BP1 foci were counted from 100 cells/slide). **(E)** Whole-cell extracts from MEFs from wild-type (WT) and *Ubr3*-knockout (KO) mice were prepared and analysed by 10% SDS–PAGE and immunoblotting using the indicated antibodies. **(F)** HeLa cells were grown on 10-cm^2 dishes for 24 h to 70–80% confluency and then transfected with human Flag-tagged APE1 ($APE1_{Flag}$) expression plasmid. After 24 h cells were pelleted by centrifugation, whole cell extracts were prepared and analysed by 10% SDS–PAGE and western blotting with the antibodies indicated. **(G)** MEFs from wild-type and *Ubr3*-knockout mice were grown on 10-cm^2 dishes in triplicate and treated with the indicated concentrations of MMS for 45 min and colonies allowed to grow for 7 days. The graph represents the mean surviving fraction normalized against the untreated controls from three independent experiments and the standard errors are shown.

We have previously identified the mouse *Ubr3* gene as one of the ubiquitin ligase family members characterized by the UBR box (28,29). The UBR box is a signature unique to all known recognition E3 components (UBR1, UBR2, UBR4 and UBR5) of the mammalian N-end rule pathway, called *N*-recognins [reviewed in (37)]. The UBR box of mouse UBR1 and UBR2 has been shown to function as a substrate recognition domain required for type-1 (basic; Arg, Lys and His) and type-2 (bulky hydrophobic; Phe, Tyr, Trp, Leu and Ile) destabilizing N-terminal residues of the N-end rule pathway (29,38). Although UBR3 shares several domains and sequence similarities with UBR1 (25% identity, 51% similarity) and UBR2 (25% identity, 48% similarity), UBR3 apparently does not exhibit *N*-recognin activities, suggesting that UBR3 may have a function outside the conventional N-end rule pathway. However, very little is known about its substrates and cellular functions. We now demonstrate that UBR3 is involved in regulation of the steady state levels of APE1 protein by ubiquitylating its N-terminal lysines, thus stimulating its proteasomal degradation. Since APE1 has the N-terminal Met-Pro-Lys sequence and there is no evidence that APE1 is cleaved into a C-terminal fragment bearing a destabilizing N-terminal residue, it appears that UBR3 recognizes APE1 through an alternative degradation signal.

In our studies to demonstrate the effect of ubiquitylation on APE1, we used MEFs generated from mice lacking exon 1 of *Ubr3* and show that a loss of *Ubr3* results in an increased level of APE1. We also show that there is no increase in *ape1* mRNA levels suggesting that this difference in APE1 protein level is due to post-translational regulation. It is still unclear whether accumulation of APE1 in *Ubr3* knockout fibroblasts is due to a lack of mono- or polyubiquitylation, although based on the *in vitro* ubiquitylation data the latter seems more probable and since due to the very high stability of APE1 protein (turnover time >16 h) neither cycloheximide nor

MG132 treatment experiments can be performed. In spite of many attempts, we were also unable to transiently over-express or efficiently knockdown UBR3 in mammalian cells. Although the mechanism preventing UBR3 over-expression is unclear, most probably an excess of UBR3 causes cell toxicity.

While common sense suggests that over-expression of APE1 should result in faster DNA repair, recent data from several laboratories indicates that an uncoordinated increase in one of the repair enzymes of the base excision repair (BER) pathway leads to misbalanced DNA repair and genome instability (39). In the case of excessive APE1, the repair intermediate that would accumulate would be DNA SSBs that arise during BER of damaged DNA bases or direct incision of abasic sites (5). If the amount of SSBs generated by APE1 exceed the repair capacity of the subsequent steps of BER (gap filling by DNA polymerase β and DNA ligation by XRCC1–DNA ligase IIIα complex), these SSBs can then be converted into DNA double-strand breaks during replication. Accumulation of DNA double-strand breaks may result in genomic instability and increased susceptibility to the cell killing effects of DNA damaging agents. The extent to which these events occur would of course be dependent on the cell type affected, since up-regulated downstream BER activity may compensate for the effects of APE1 over-expression and the ultimate effect on cellular survival is furthermore dependent on the double-strand break repair capacity of the affected cell. For the *Ubr3*-knockout cells, which contain higher protein levels of APE1 than the corresponding wild-type cells, we observed the hypothesized effects. First, we found that *Ubr3*-knockout cells exhibit a significant variation in the size and shape of the nucleus, that is a well-known hallmark of genomic instability (33). We next demonstrated that *Ubr3*-knockout cells had a higher number of spontaneous 53BP1 foci and increased levels of γH2AX in response to endogenously generated damage, both indicators for the presence of DNA double-strand breaks (40). We show that this increase is likely due to APE1 over-expression since transient over-expression of APE1 in HeLa cells also show an increase in the levels of γH2AX. Finally, we demonstrate that *Ubr3*-knockout cells are more susceptible to the cell killing effects of the DNA alkylating agent MMS, that generates base damages which are almost entirely dependent on APE1 for their repair.

It appears that precise tuning of the levels of DNA repair enzymes involved in the BER pathway is required for genome stability. Even a moderate misbalance of the equilibrium between BER proteins may lead to accumulation of DNA double-strand breaks and results in genomic instability. This conclusion is supported by previous observations that cell lines stably over-expressing APE1 show genomic instability (41). *Ubr3*-knockout mice were able to be generated depending on the genetic background, which results in early embryonic lethality or neonatal lethality caused by a number of pathologies, although the reason for this at that time remained unclear (28). Our current study may partially explain the observed phenotype of *Ubr3*-knockout mice and it is quite possible that the different abilities of the 1239vImJ and C57BL/6 mice, used to generate the *Ubr3*-knockout mice, to tolerate DNA double-strand breaks may be one of the reasons for the differential effects of the knockout.

In summary, we propose that newly synthesized cytoplasmic APE1 is directly transferred to the nucleus, where it is able to participate in DNA repair. However if the level of APE1 exceeds the amount required for DNA damage repair, then APE1 is ubiquitylated by UBR3 and becomes a target for proteasomal degradation. An important question that remains unanswered is how cells would know whether they need more or less APE1 and to make the decision whether or not to ubiquitylate APE1. In other words, how is the E3 ubiquitin ligase activity of UBR3 on APE1 regulated? It is possible that other post-translational modifications of APE1, including recently reported phosphorylation (42) and acetylation (43), may modulate its stability, activity or subcellular localization in response to DNA damage by controlling UBR3 ubiquitylation and we are currently investigating this hypothesis.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

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REVIEW

Regulation of DNA Repair by Ubiquitylation

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Abstract—Cellular DNA repair is a frontline system that is responsible for maintaining genome integrity and thus preventing premature aging and cancer by repairing DNA lesions and strand breaks caused by endogenous and exogenous mutagens. However, it is also the principal cellular system in cancer cells that counteracts the killing effect of the major cancer treatments, e.g. chemotherapy and ionizing radiation. Although it is clear that an individual's DNA repair capacity varies, the mechanisms involved in the regulation of repair systems that are responsible for such variations are only just emerging. This knowledge gap is impeding the finding of new cancer therapy targets and the development of novel treatment strategies. In recent years the vital role of post-translational modifications of DNA repair proteins, including ubiquitylation and phosphorylation, has been uncovered. This review will cover recent progress in our understanding of the role of ubiquitylation in the regulation of DNA repair.

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Key words: DNA repair pathways, regulation of DNA repair systems, post-translational protein modification, ubiquitylation

REGULATION OF BASE EXCISION REPAIR

Base excision repair (BER) is initiated by damage specific DNA glycosylases that release the corrupted base by hydrolysis of the N-glycosylic bond linking the DNA base to the sugar phosphate backbone (Scheme 1). The arising abasic site (AP-site) is further processed by AP-endonuclease 1 (APE1) that cleaves the phosphodiester bond 5′ to the AP-site, generating a DNA single strand break (SSB) with a 5′-sugar phosphate. This SSB is then repaired by a DNA repair complex that includes DNA polymerase β (Pol β), XRCC1, and DNA ligase IIIα (Lig III). Pol β possesses AP lyase activity that removes the 5′-sugar phosphate and also, functioning as a DNA polymerase, adds one nucleotide to the 3′-end of the arising single-nucleotide gap. Finally, Lig III seals the DNA ends, therefore accomplishing DNA repair (reviewed in [1]). This pathway is commonly referred to as the short patch BER pathway, through which cells are accomplishing the majority of repair (Scheme 1, left branch). However, if the 5′-sugar phosphate is resistant to cleavage by Pol β, then a switch to Pol δ/ε occurs that adds 2-8 more nucleotides into the repair gap, therefore generating a flap structure that is removed by flap endonuclease-1 (FEN-1) in a PCNA-dependent manner. DNA ligase I (Lig I) then seals the remaining nick in the DNA backbone, and this process is commonly referred to as long patch BER [2, 3] (Scheme 1, right branch).

To support error-free transcription and replication, BER proteins should be present in adequate amounts to be able to promptly repair DNA. Indeed, mutations affecting the amounts or enzymatic activities of these proteins increase genome instability and reduce cell viability in response to DNA damage [4-10]. On the other hand, the amount of BER enzymes should be tightly controlled since when overproduced, BER enzymes may affect other DNA transactions and also lead to genome instability and cancer [11, 12]. The number of DNA lesions in human cells originates from the chemical instability of the DNA molecule itself, but also depends on cellular metabolism and exposure to exogenous mutagens [13].

A combination of these factors leads to variations in DNA damage levels, and BER should be responsive to the changing environment and indeed, as it has been recently demonstrated, the amount of BER enzymes present within a cell at any time (the steady-state level of BER

Abbreviations: AP-site, abasic site (apurinic/apyrimidinic); BER, base excision repair; Lig III, DNA ligase III; NER, nucleotide excision repair.

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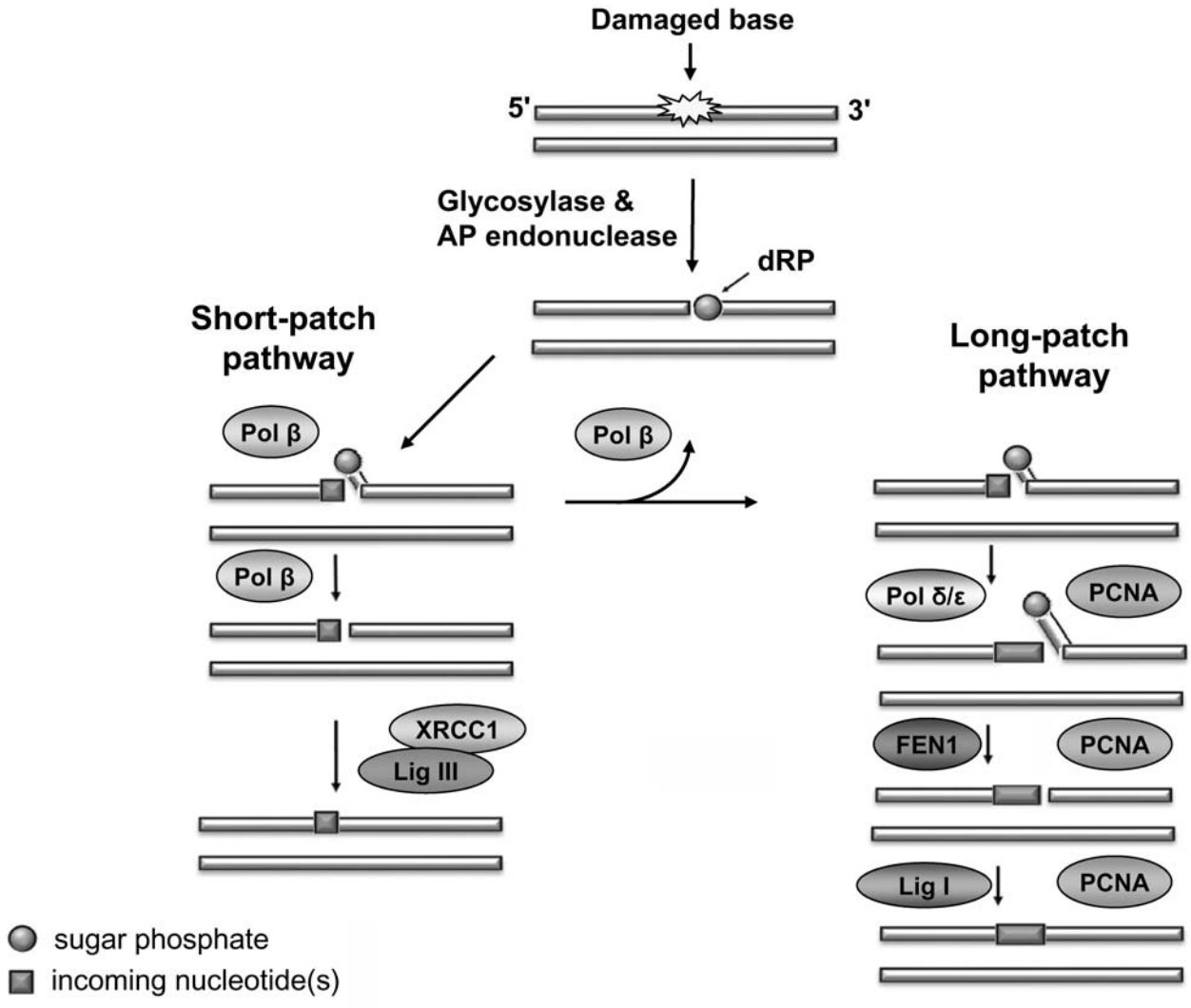
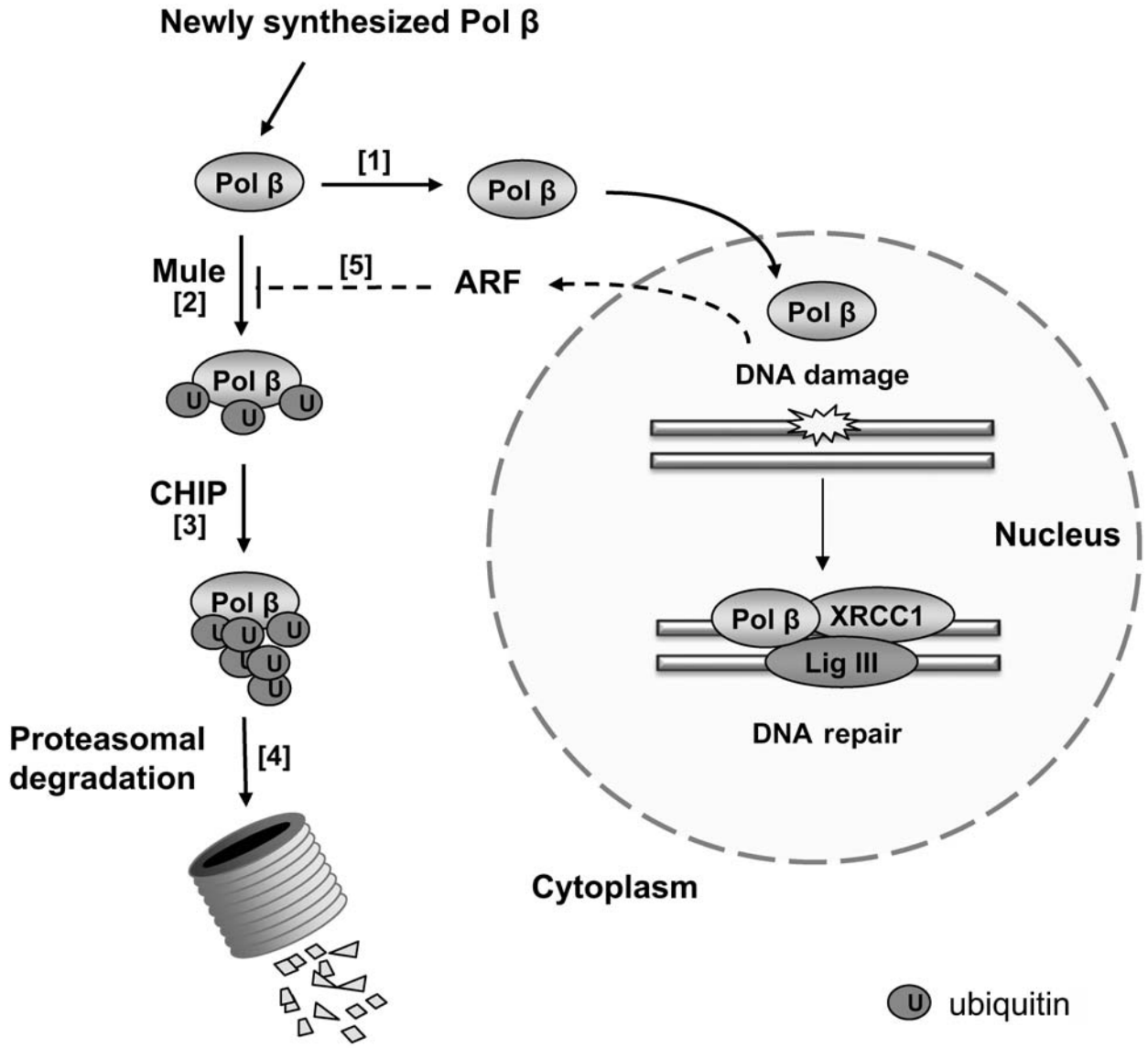


BER pathways

Scheme 1

enzymes) is tightly regulated and is linked to the number of DNA lesions [14]. This is achieved by controlling the nuclear pool of three major BER enzymes (XRCC1, Lig III, and Pol β) through targeted proteasomal degradation. Proteins targeted for degradation are marked with a chain of ubiquitin (Ub, small 76 amino acid protein) molecules. Ubiquitylation consists of conjugation of Ub, via its C terminus, onto the ε-amino group of a lysine residue of the substrate protein and is achieved by a cascade of ubiquitin-activating enzymes. First, Ub is activated through an ATP-dependent reaction by an ubiquitin-activating enzyme (E1) to form an E1–Ub thioester. Second, the activated Ub is delivered to a ubiquitin-conjugating enzyme (E2) and finally, a complex is formed between the E2–Ub thioester, the target protein, and a ubiquitin ligase (E3) that conjugates Ub to the protein. Polyubiquitylated proteins are recognized by the 26S proteasome that unfolds the protein, removes the polyubiquitin chains, and degrades the protein (for review see [15]).

If the levels of BER proteins exceed the level of DNA lesions, then the excessive BER enzymes are ubiquitylated and thus labeled for proteasomal degradation. For example, sequential ubiquitylation of Pol β by the E3 ubiquitin ligases Mule (ARF-BP1) and CHIP leads to Pol β degradation and down regulates its steady state level [16]. However, when more Pol β is required for DNA repair, Mule activity is down regulated by ARF protein [17, 18], whose release is modulated in response to DNA damage [19]. Release of ARF and Mule inhibition leads to accumulation of Pol β and increased DNA repair (Scheme 2). However, as well as inhibiting E3 ubiquitin ligase activities, in many cases the steady state level of proteins is also controlled by an opposing activity. In this scenario, ubiquitylation leading to protein degradation is counteracted by deubiquitylation, primarily by ubiquitin



Regulation of base excision repair by Mule, CHIP, and USP47. Depending on the amount of Mule, newly synthesized Pol β is either directly transferred to the nucleus [1] or ubiquitylated by Mule [2]. Ubiquitylated Pol β is then a target for CHIP-mediated polyubiquitylation [3] and subsequent degradation by the proteasome [4]. However, if DNA damage is detected, the activity of Mule is inhibited by ARF [5], which then generates more active Pol β that is able to enter the nucleus to participate in DNA repair

Scheme 2

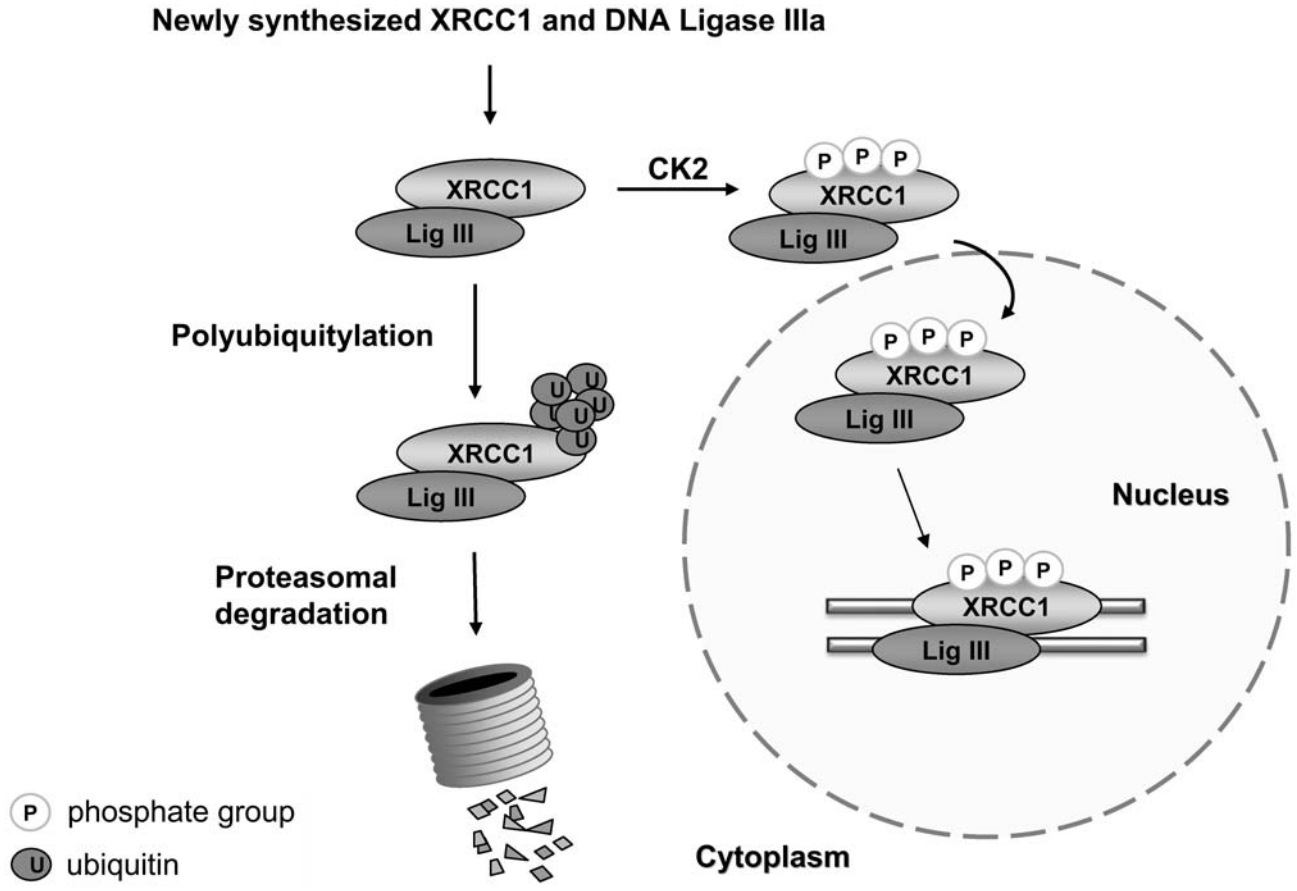
specific proteases (USPs) that are the major class of deubiquitylation enzymes. There are several examples of the role of USPs in the DNA damage response (for review see [20, 21]).

Cellular signaling at the molecular level (that involves regulating protein activity and/or affinity to their substrates, protein–protein interactions, and cellular localization) is accomplished by different post-translational modifications and, in addition to ubiquitylation, phosphorylation plays an important role [22]. Phosphorylation of proteins by one of an estimated 500 protein kinases existing in mammalian cells involves the transfer of the γ-phosphate of an ATP molecule onto the target protein. Phosphorylation plays an important role in regulating a wide range of different cellular processes, including the DNA damage response and DNA repair, but also phosphorylation can promote or inhibit ubiquitylation that can also effect protein degradation and protein trafficking. Phosphorylation of XRCC1 by casein kinase 2 (CK2) is one of the best examples of the role of phosphorylation in BER. XRCC1 is central to the BER process since it operates as a scaffold protein supporting multiple protein–protein interactions required for DNA repair. XRCC1 is a 69 kDa protein comprising several domains – an N-terminal domain (NTD) that binds both nicked DNA [23] and Pol β [24], a central breast cancer gene 1

C-terminal domain (BRCT I) that interacts with poly(ADP-ribose) polymerases (PARP-1 and PARP-2 [25, 26]), and a C-terminal BRCT II domain that binds Lig III [27].

In addition to this, XRCC1 is also known to interact with several other proteins including APE1 [28], polynucleotide kinase (PNK) [29, 30], and proliferating cell nuclear antigen (PCNA) [31]. The cellular importance of XRCC1 is highlighted by the fact that a deficiency in mice is embryonic lethal, and cell lines lacking XRCC1 are defective in the repair of SSBs and sensitive to alkylating agents such as methylmethanesulfonate, as well as other DNA-damaging agents [32]. XRCC1 has various phosphorylation sites within the protein. DNA damage-dependent phosphorylation of XRCC1 by DNA-dependent protein kinase at serine 371 [33] and, more recently, Chk2-dependent phosphorylation of XRCC1 at threonine 284 in response to DNA damage have been observed [34]. However, the biological role of these phosphorylations is unclear. Additionally, between the two BRCT domains is a region (amino acids 403-538) that is known to be phosphorylated *in vitro* by CK2 [29, 35, 36].

It was recently demonstrated that the cytoplasmic form of CK2 is the major protein kinase activity involved in phosphorylation of XRCC1 in human cell extracts and that XRCC1 phosphorylation is required for XRCC1–Lig III complex stability [37]. It was also shown that XRCC1–Lig III complex containing mutant XRCC1, in which CK2 phosphorylation sites have been mutated, is unstable and subject to proteasomal degradation. Accordingly, a knockdown of CK2 by siRNA results in both reduced XRCC1 phosphorylation and stability, which also leads to a reduced amount of Lig III and accumulation of DNA strand breaks [37] (Scheme 3). Most probably, the majority of BER proteins are regulated by phosphorylation and ubiquitylation. There are some indications that APE1 [38] and PARP-1 [39] are regulated by ubiquitylation in addition to uracil DNA glycosylase [40]. However, more research is required to identify the entire mechanism and proteins involved in the regulation of BER.



Model for regulation of XRCC1–Lig III complex stability. The majority of newly synthesized XRCC1 is phosphorylated by CK2 before or after formation of a complex with Lig III. The XRCC1–Lig III complex stabilized by phosphorylation is then translocated into the nucleus and can participate in DNA repair. Alternatively, if XRCC1 is in excess, the newly synthesized protein is either not phosphorylated or is dephosphorylated, which then makes it susceptible to ubiquitylation and is subsequently degraded by the proteasome

Scheme 3

NUCLEOTIDE EXCISION REPAIR

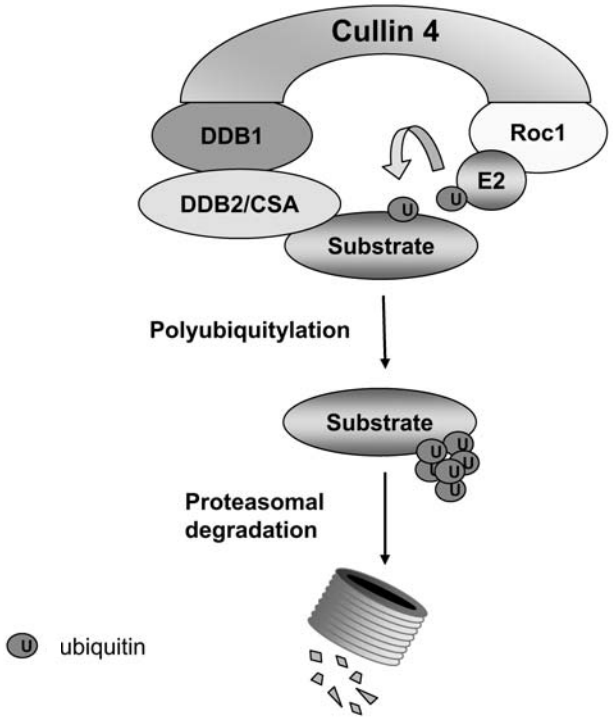
Nucleotide excision repair (NER) is a DNA repair pathway for the removal of bulky DNA adducts, such as cyclobutane pyrimidine dimers (CPDs) and 6-4 photoproducts (64PPs), induced by UV irradiation and chemical mutagens. There are at least two distinct pathways of NER, namely transcription coupled repair (TCR) and global genome repair (GGR). TCR operates predominantly in the repair of lesions occurring on actively transcribed regions of the DNA, whilst GGR can repair lesions throughout the genome. These pathways also differ by the way in which the DNA damage is sensed. TCR occurs as a result of a stalled RNA polymerase due to blockage of transcription elongation by the DNA lesion, and damage recognition is further aided by the Cockayne syndrome proteins, CSA and CSB. In contrast, GGR is initiated by the binding of the xeroderma pigmentosum C/HR23B (XPC/HR23B) complex and the heterodimer of UV-damaged DNA binding proteins 1 and 2 (DDB1 and DDB2) that play essential roles in DNA damage sensing.

Following DNA damage recognition, both pathways of NER are thought to involve the same proteins and mechanism of repair, which involves the multiprotein complex transcription factor IIH (TFIIH), XPA and RPA that are involved in damage verification, the endonucleases ERCC1-XPF and XPG that incise the damaged strand, and a DNA polymerase and DNA ligase that fill and seal the gap, respectively. Abnormalities in the NER pathway give rise to the human disorders xeroderma pigmentosum and Cockayne syndrome that are characterized by severe UV sensitivity and neurological defects [41].

Interestingly, certain proteins required for both TCR and GGR are also components of E3 ubiquitin ligase complexes that are involved in protein ubiquitylation. There are two known protein complexes involved in GGR and TCR, and both of them contain a scaffold protein cullin-4 (Cul4A or Cul4B) and also DNA damage binding protein 1 (DDB1) (Scheme 4). The crystal structure of the Cul4A–DDB1 complex revealed that the N terminus of Cul4 interacts with DDB1, whereas the C terminus interacts with two other proteins, regulator of cullins-1 (Roc1) and an E2 conjugating enzyme [42]. To target this ubiquitin ligase complex to specific substrates, it also includes one of a number of WD-40 repeat proteins, providing specificity for ubiquitylation [43]. Examples of these WD-40 repeat protein adaptors are DDB2 and CSA that are involved in GGR and TCR, respectively [44]. The role of DDB1 in this complex is to mediate interaction between the ubiquitin ligase scaffold protein Cul4 and WD-40 repeat proteins that provide interaction with various substrates. Not surprisingly, a knockout of DDB1 in mice is embryonic lethal [45]. However, Cul4A conditional knockout mice have recently been found to be viable and healthy although Cul4B, which is a close homolog of Cul4A, may be compensating for the loss of Cul4A [46].

During TCR, RNA polymerase II is stalled by the presence of DNA damage, which then results in the recruitment of CSA and CSB proteins, which in turn recruit other NER proteins. It was recently proposed that the role of the Cul4A–Roc1–DDB1–CSA ubiquitin ligase complex in TCR is ubiquitylation of CSB that stimulates its proteasomal degradation and removal from DNA damage when recruitment of NER factors is accomplished [47, 48]. However, it is not clear whether this degradation is important for TCR. Interestingly, it was recently found that the CSB protein contains a ubiquitin binding domain, and it was proposed that the ubiquitylation activity of the Cul4A–Roc1–DDB1–CSA complex may be required for ubiquitylation of some TCR proteins and thus promote CSB recruitment [49]. Therefore, more studies are required to further establish the role of the CSA-containing ubiquitin ligase, particularly in TCR.

A similar role is assigned to the ubiquitylation activity of the Cul4A–Roc1–DDB1–DDB2 ubiquitin ligase complex. It has been proposed that after binding to the UV induced DNA lesions and initiation of GGR by recruitment of the required NER factors to DNA damage sites, the complex is subsequently removed from the DNA by self-ubiquitylation and subsequent degradation [50-52]. There may be other targets of the Cul4A–Roc1–



Structure of Cullin 4 containing ubiquitin ligases

Scheme 4

DDB1–DDB2 ubiquitin ligase complex, so therefore the DDB heterodimer may also promote the ubiquitylation of cellular proteins in association with Cul4A, which may have other consequences apart from protein degradation. Indeed, the DDB2-containing ubiquitin ligase complex has also been shown to ubiquitylate histones that may be involved in chromatin remodeling to allow access of NER repair factors to the DNA lesion [53, 54]. Specifically, ubiquitylation of H3 and H4 has been shown to occur by the Cul4A–Roc1–DDB1 ubiquitin ligase complex in response to UV irradiation, and it is thought that this causes a disruption of the interaction of histones with DNA and enhances the recruitment of XPC to the UV DNA damaged site, therefore providing a link between chromatin remodeling and DNA repair in response to UV irradiation [54].

DOUBLE STRAND BREAK REPAIR AND CHROMATIN REMODELING

In mammalian cells, genomic DNA is wrapped around a histone octamer, consisting of histones H2A, H2B, H3, and H4, to form nucleosomes that are further packaged to form chromatin. Thus, damaged DNA is masked and is not readily available for DNA repair. Remodeling (opening and closing) of chromatin is thought to occur by post-translational modifications on the N-terminal tails of the histones, which is required for several cellular processes including DNA repair. Histone ubiquitylation is one such modification, and although its precise role in chromatin remodeling and the effect on different DNA repair pathways remains to be studied in detail, increasing information is becoming available regarding the role of histone ubiquitylation during double strand break repair.

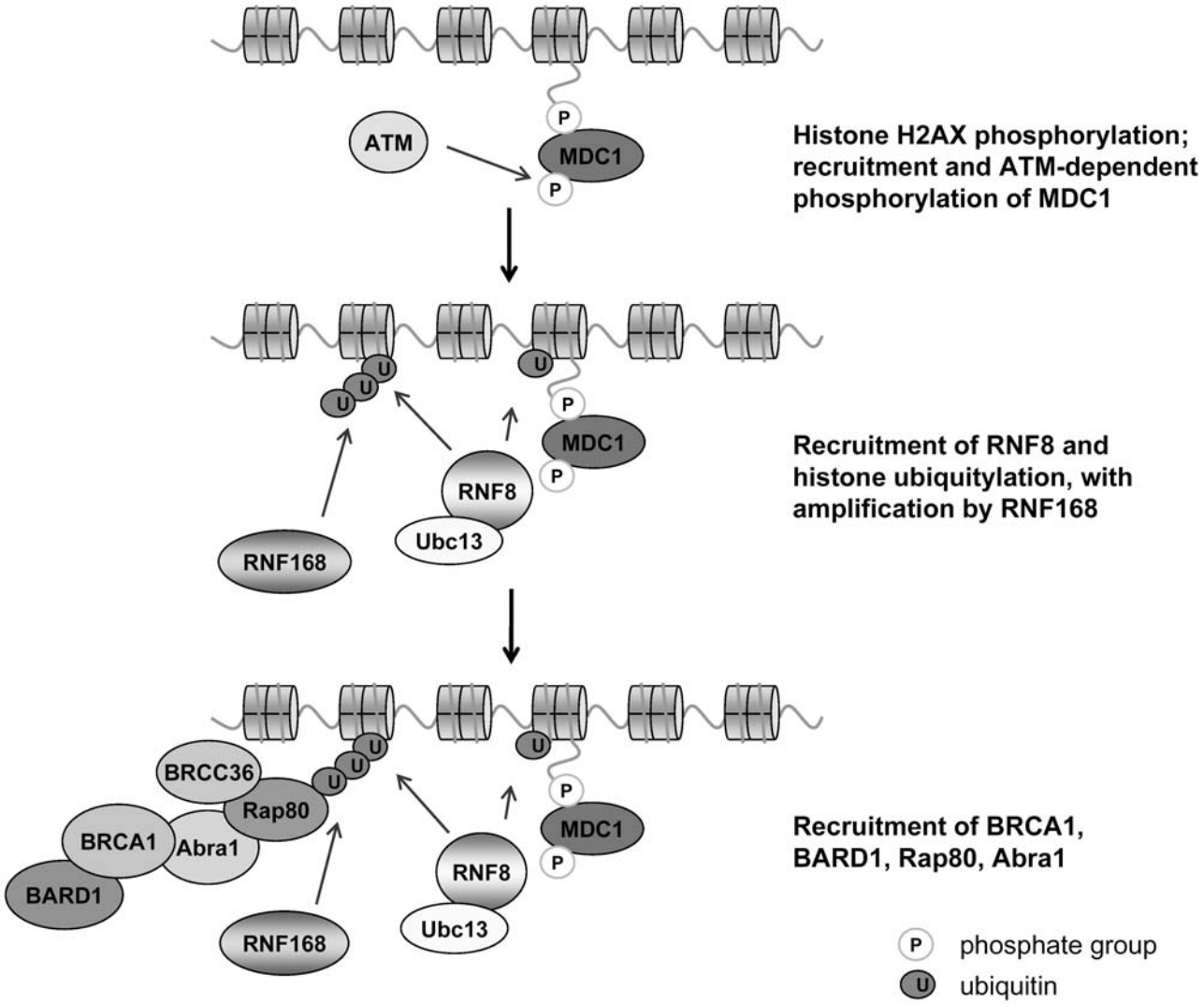
DNA double strand breaks (DSBs) are potentially toxic lesions that can arise from various endogenous and exogenous sources, such as collapsed replication forks and ionizing radiation, and the cellular response to DSBs involves a battery of proteins that sense the DNA damage, cause cell cycle arrest through activation of checkpoint proteins, and also recruit DNA repair enzymes.

An early event in the repair of DSBs is the ATM-dependent phosphorylation of the histone H2A variant, H2AX, and the subsequent recruitment of the mediator of DNA damage checkpoint 1 (MDC1). ATM subsequently phosphorylates MDC1, which is consequently recognized by a complex consisting of the E3 ubiquitin ligase RNF8 and the E2 conjugating enzyme Ubc13, which then monoubiquitylates γ-H2AX and H2A. Ubiquitylation of γ-H2AX by RNF8/Ubc13 has also been shown to be amplified by another ubiquitin ligase, RNF168, a protein that is mutated in RIDDLE syndrome patients who are immunodeficient and radiosensitive due to defective DSB repair [55, 56]. Histone γ-H2AX polyubiquitylation stimulates recruitment of Rap80, Abra1, 53BP1, BRCA1, and other proteins required for DSB repair [57-60] (Scheme 5). Since ubiquitylation of γ-H2AX and H2A is involved in recruitment of cell cycle checkpoint and DNA repair proteins in response to DSBs, this event should be reversible following the repair of the DNA damage and should be catalyzed by deubiquitylation enzymes. Indeed, Rap80 is also known to form a complex with BRCC36, a deubiquitylation enzyme that is thought to reverse the ubiquitylation caused by RNF8/Ubc13, and therefore these two protein complexes possibly modulate the levels of ubiquitylation events occurring at DSBs [61]. However, USP3 has also been shown to deubiquitylate H2A, although a direct role for USP3 following the repair of DSBs requires further investigation [62].

While thought to be specific for double strand break repair in response to ionizing radiation, H2A ubiquitylation has also been observed in response to UV irradiation. Using cells stably expressing green fluorescing protein (GFP)-tagged ubiquitin, monoubiquitylation of H2A following UV irradiation by the ubiquitin ligase Ring2 has been observed and was dependent on both the presence of a functional NER process and on the DNA damage signaling protein kinase ATR [63]. However, it is thought that Ring2 actually regulates the nuclear ubiquitin pool, and therefore the observed decrease in H2A ubiquitylation following Ring2 knockdown was thought to be an indirect effect. Subsequently, the same authors demonstrated that H2A ubiquitylation following UV irradiation was actually performed by the Ubc13/RNF8 ubiquitin ligase complex [64]. The recruitment of Ubc13/RNF8 following UV irradiation, similarly to ionizing radiation, also occurs via interaction with MDC1. This appears to suggest that the cellular response to UV and ionizing radiation may involve the same pathway of H2A ubiquitylation that provides a universal signal of DNA damage detection. This proposed mechanism would in fact recruit a plethora of DNA repair enzymes, and following DNA damage detection only a subset of these enzymes would actually be used to repair the DNA lesion. Ubiquitin ligase activity on histones within chromatin has also been demonstrated for Mule [65], Cul4–DDB–Roc1 [54], Mdm2 [66], and Bre1 [67] E3 ubiquitin ligases, although the biological role of these ubiquitylation events is unclear.

UBIQUITYLATION IN THE FANCONI ANEMIA PATHWAY

The diverse roles for ubiquitylation in the regulation of DNA repair certainly come to light in the example of the Fanconi anemia (FA) pathway. This pathway is involved in the coordination of the repair of DNA interstrand cross-links (ICLs), a toxic lesion that locks together the two strands of DNA, blocking both DNA replica-

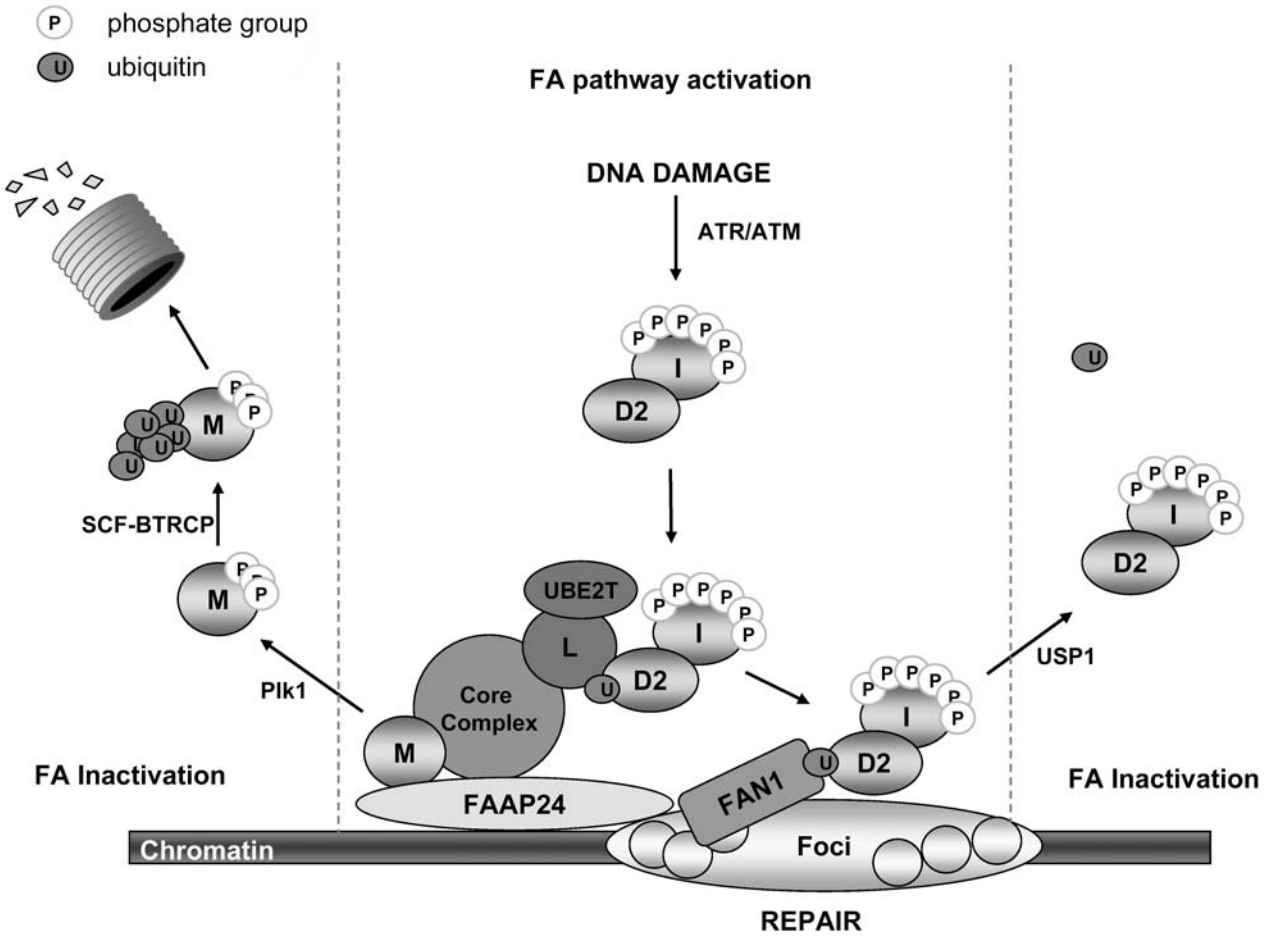


Model for regulation of DNA double strand break repair

Scheme 5

tion and transcription. These lesions are repaired through multiple repair pathways, primarily the NER, translesion synthesis (TLS), and homologous recombination (HR) pathways, and it is well accepted that the FA pathway is necessary for the coordination of these repair events [68-72]. Indeed, a loss of normal function in any of the 13 FA pathway genes (FANCA, FANCB, FANCC, FANCD1, FANCD2, FANCE, FANCF, FANCG, FANCI, FANCJ, FANCL, FANCM, and FANCN) manifests clinically as Fanconi anemia, a disease highlighted by the patient's hypersensitivity to ICLs and susceptibility to the development of blood cancer and solid tumors [73-76]. The FA pathway is activated only during the S-phase of the cell cycle in response to the presence of ICLs, and is thought to incur deleterious effects if active at other points in the cell cycle. It is therefore not surprising that it appears to be a highly regulated pathway with multiple mechanisms allowing for its transient activity. These include phosphorylation-dependent ubiquitylation affecting protein localization and pathway activation, as well as deubiquitylation- and ubiquitylation-dependent degradation allowing for pathway inactivation.

FA pathway activation in response to the presence of ICLs is ultimately dependent on the monoubiquitylation status of the FANCD2–FANCI complex, more importantly that of FANCD2. This monoubiquitylation is triggered by ATR-mediated phosphorylation of FANCI at six sites in response to DNA damage [77]. FANCI phosphorylation appears to stimulate FANCD2 monoubiquitylation, through FANCD2–FANCI recruitment to the E3 ligase complex that consists of the PHD domain E3 ligase, FANCL, the E2 conjugating enzyme, UBE2T, and a multisubunit core complex of eight FA proteins (FANCA, FANCB, FANCC, FANCE, FANCF, FANCG, FANCL,



Mechanisms of Fanconi anemia pathway regulation

Scheme 6

FANCM) and two FA-associated proteins FAAP24 and FAA100 [78-81]. Upon FANCD2 monoubiquitylation at residue K561, the FANCD2– FANCI complex localizes to repair foci on DNA containing HR and TLS factors such as Rad51, BRCA1, PCNA, and γ-H2AX [82-85] (Scheme 6).

Recently it was shown that monoubiquitylated FANCD2 at repair foci is necessary for the recruitment of the newly identified FAN1 nuclease through direct interaction with the N-terminal ubiquitin binding zinc finger of FAN1 (UBZ domain) [86-89]. Furthermore, FAN1 exonuclease/endonuclease activity was implicated in the HR repair step of ICL repair and deemed necessary for repair completion. The monoubiquitin moiety on FANCD2 is therefore essential for FA pathway functionality through its direct interaction with ubiquitin binding domains on repair factors in the repair of the toxic ICL lesion.

The availability of the core complex factors provides a level of regulation for the FA pathway, as a loss of any of the ten core complex factors reduces either the level of FANCD2–FANCI monoubiquitylation or repair through recruitment to sites of damage. FANCM for instance dimerizes with FAAP24, and despite being associated with the core complex is not necessary for core complex contribution to FANCD2 monoubiquitylation [90, 91]. The importance of the FANCM–FAAP24 complex rather lies in its ability to recognize sites of DNA damage, consequently recruiting the core complex to where it is needed on chromatin [91, 92]. Interestingly, post-replication and repair, FANCM is hyperphosphorylated by polo-like kinase, Plk1, and this modification results in the formation of a phosphodegron, consequently leading to FANCM degradation by the β-TRCP component of the SCF ubiquitin E3 ligase [93]. In the absence of FANCM, the core complex is no longer recruited to DNA, and this ensures that the pathway is active only during S phase.

Another major source of negative regulation for the FA pathway comes with the discovery that monoubiquitylation of FANCD2 can be reversed leading to FANCD2–FANCI complex dissociation from DNA and

consequent inactivation of the FA pathway [20]. This is carried out by the deubiquitylation enzyme, USP1, complexed to UAF1 (USP1 associated factor 1) [20, 94]. This complex has been shown to deubiquitylate FANCD2–FANCI in the absence of DNA damage, thereby preventing FA pathway activity. To complement this regulatory role, in the presence of DNA damage USP1 transcriptional expression is switched off, and it has also been shown to induce its own degradation through endo-proteolytic cleavage at a conserved internal diglycine motif (Gly-Gly), thus ensuring that monoubiquitylated FANCD2 remains available for repair [94, 95].

It is clear that ubiquitylation and deubiquitylation are critical events involved in the cellular responses to DNA damage through different DNA repair pathways. However, further studies are required to advance our understanding of the orchestration of the events occurring at the sites of DNA damage.

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