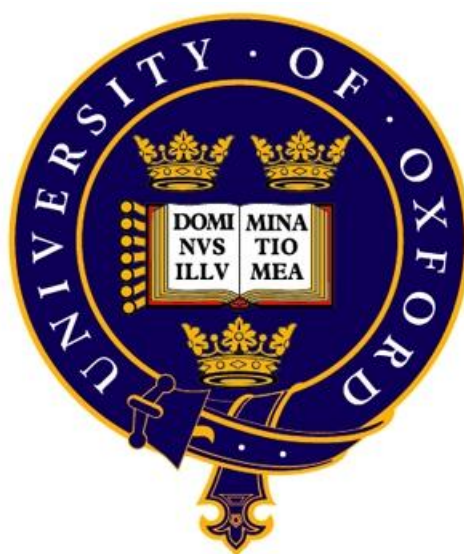


Base Excision Repair of Radiation-Induced DNA Damage in Mammalian Cells

A thesis submitted for the degree of Doctor of Philosophy



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Abstract

A specific feature of ionising radiation is the formation of clustered DNA damage, where two or more lesions form within one to two helical turns of the DNA induced by a single radiation track. The complexity of ionising radiation-induced DNA damage increases with increasing ionisation density and it has been shown that complex DNA damage has reduced efficiency of repairability. In mammalian cells, base excision repair (BER) is the predominant pathway for the repair of non-DSB clustered DNA lesions and is split into two sub-pathways known as short patch (SP) BER and long patch (LP) BER. SP-BER is the predominant pathway, especially in the repair of isolated DNA lesions. However, LP-BER is thought to play a greater role in the repair of radiation-induced clustered lesions. In this study, cell lines were generated stably expressing the fluorescently tagged BER proteins, XRCC1-YFP (marker for SP-BER) or FEN1-GFP (marker for LP-BER). The recruitment and loss of XRCC1-YFP and FEN1-GFP to sites of DNA damage induced by both ultrasoft X-ray (USX), a form of low linear energy transfer (LET) radiation, and near infrared (NIR) laser microbeam irradiation ('mimic' high LET radiation) was visualised in real-time and the decay kinetics of the fluorescently-tagged proteins determined. The half-life of fluorescence decay of FEN1-GFP following USX irradiation was longer than that of XRCC1-YFP, indicating that LP-BER is a slower process than SP-BER. Additionally, the fluorescence decay of XRCC1-YFP after NIR laser microbeam irradiation was fitted by bi-exponential decays with a fast component and a slow component, reflecting the involvement of XRCC1 in the repair of different types of DNA damage. In contrast to USX irradiation, where the XRCC1-YFP fluorescence decay reached background levels by 20 min, XRCC1-YFP still persisted at some of the NIR laser induced DNA damage sites even after 4 hours. This is consistent with the fact that the laser induces more complex damage that presents a major challenge to the repair proteins, persisting for much longer than the simple damage caused by low LET USX irradiation. Persistent, unrepaired DNA damage can potentially lead to mutations and replication-induced DSBs if it persists into S-phase. PARP1 inhibition reduced the recruitment of XRCC1 to DNA damage sites. However, a considerable amount of XRCC1 was still detected at the DNA damage sites, leading to the conclusion that there is a subset of DNA damage that requires XRCC1 but not PARP1 for repair. Understanding how clustered damage is repaired by the BER pathway can aid the design of future therapies which can be used in combination with radiotherapy to enhance the radiosensitisation effect. Knockdown of FEN1 was investigated and found to radiosensitise A549 (adenocarcinoma) cells, possibly as a result of an excess of unrepaired radiation-induced lesions requiring LP-BER for repair, although FEN1 knockdown alone induced cell death in non-cancerous BEAS-2B cells.

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List of abbreviations

° – degrees

°C – degrees Celsius

8-oxoG – 8-oxo-7,8-dihydroguanine

α – alpha

A – adenine

ADP – adenosine diphosphate

AIF – apoptosis inducing factor

ALC1 – amplified in liver cancer 1

AP site – apurinic/apyrimidinic site

APE1 – AP endonuclease 1

ATP – adenosine triphosphate

BEEM – bronchial epithelial cell basal medium

BEGM – bronchial epithelial growth medium

BER – base excision repair

BLM – Bloom syndrome protein

bp – base pair

BRCA1 – breast cancer type 1

BRCT - BRCA1 carboxyl-terminal

BrdU – bromodeoxyuridine

BSA – bovine serum albumin

C – cytosine

Cdk2 – cyclin-dependent kinase 2

CHO – Chinese hamster ovary

CK2 - casein kinase 2

cm – centimetre

Cy - cyanine

DAPI – 4',6-diamidino-2-phenylindole

dH₂O – distilled H₂O

DHT – dihydrothymine

DMEM – Dulbecco's modified Eagle's medium

DMSO – dimethyl sulphoxide

DNA – deoxyribonucleic acid

dRP – deoxyribophosphate

DSB – double strand break

ECACC – European Collection of Cell Cultures

E. coli – *Escherichia coli*

EDTA – ethylene diamine tetracetic acid

EGTA – ethylene glycol tetracetic acid

EMS – ethyl methanesulfonate

EndoG – endonuclease G

FACS – fluorescence activated cell sorting

FBS – foetal bovine serum

FEN1 – flap endonuclease 1

FITC - fluorescein isothiocyanate

fs – femtosecond (s)

FSG – fish skin gelatin

G418 – geneticin

γ – gamma

g – g-force

G – guanine

GFP – green fluorescent protein

GIROB – Gray Institute for Radiation Oncology and Biology

Gy - gray

h – hour (s)

HAT – histone acetyltransferase

HDAC – histone deacetylase

HeNe – helium/neon

hNTH1 – human homolog of endonuclease III

H₂O₂ – hydrogen peroxide

HR – homologous recombination

KCl – potassium chloride

kDa – kilo Dalton

keV – kiloelectron volt

kV - kilovolt

l – litre

LB – Luria-Bertani

LET – linear energy transfer

Lig I – DNA ligase I

Lig III – DNA ligase III

LP-BER – long patch BER

M – molar

MEF – mouse embryonic fibroblasts

MeV – megaelectron volt

mg – milligram

MHz – mega hertz

min – minute (s)

ml – millilitre

mm - millimetre

mM – millimolar

mmol – millimole

MPG – methylpurine DNA glycosylase

ms – millisecond

mW - milliwatt

µg – microgram

µl – microlitre

µm - micrometre

µM – micromolar

MMS – methyl methanesulfonate

MRN – Mre11-Rad50-Nbs1 complex

mV – millivolt

NaB – sodium butyrate

NaCl – sodium chloride

NAD – nicotinamide adenine dinucleotide

Na₂EDTA – disodium ethylenediamine tetraacetate

NAM - nicotinamide

Na₃VO₄ – sodium orthovanadate

NEIL1- Nei endonuclease VIII-like 1

NEIL2- Nei endonuclease VIII-like 2

ng – nanogram

NHEJ – non-homologous end joining

NIR – near infrared

nm – nanometre

nM – nanomolar

NTH1 - endonuclease III-like protein 1

mSv - millisievert

OD – optical density

OGG1 - 8-oxoguanine DNA glycosylase

•OH – hydroxyl radical

PAGE – polyacrylamide gel electrophoresis

PAR – poly(ADP-ribose)

PARG – poly(ADP-ribose) glycohydrolase

PARP – poly(ADP-ribose) polymerase

PBS – phosphate buffered saline

PCNA – proliferating cell nuclear antigen

PE – plating efficiency

PFA - paraformaldehyde

PMSF - phenylmethylsulphonyl fluoride

PNK – polynucleotide kinase

PNKP – polynucleotide kinase 3'-phosphatase

Pol β – polymerase beta

Pol δ/ε – polymerase delta/epsilon

RBE – relative biological effectiveness

rcf – relative centrifugal force

RNA – ribonucleic acid

ROS – reactive oxygen species

RPA – replication protein A

rpm – revolutions per minute

s – second (s)

SCE – sister chromatid exchange

S. cerevisiae – *Saccharomyces cerevisiae*

SDS – sodium dodecyl sulphate

SER – survival enhancement ratio

SF – surviving fraction

siRNA – small interfering RNA

SMUG1 - single-strand-selective monofunctional uracil DNA glycosylase 1

SOB – super optimal broth

SP-BER – short patch BER

SSB – single strand break

SSBR – single strand break repair

T – thymine

TBE – Tris borate EDTA buffer

TBS-T – Tris buffered saline and Tween 20

TDG - thymine/uracil mismatch glycosylase

TDP1 – tyrosyl-DNA phosphodiesterase 1

TE – Tris-EDTA buffer

TEMED – N,N,N',N'-tetramethylethylenediamine

Tg – thymine glycol

THF – tetrahydrofuran

Ti:Sa – titanium:sapphire

Tris HCl – Tris hydrochloride

TSA – Trichostatin A

UDG – uracil DNA glycosylase

UNG2 – uracil DNA glycosylase 2

USX – ultrasoft X-ray

UV - ultraviolet

V – volt

W – watt

WRN – Werner syndrome protein

XRCC1 – X-ray cross-complementing protein 1

YFP – yellow fluorescent protein

Title

Abstract

Acknowledgements

List of abbreviations

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Chapter 1: Introduction

1.1 Overview

Ionising radiation is known to induce clustered DNA damage, which is defined as two or more lesions located within one to two helical turns of DNA, and has been experimentally detected in both plasmid DNA and mammalian cells (Gulston *et al*, 2002, Sutherland *et al*, 2000). Lesions found within clustered damage sites include double strand breaks (DSBs), single strand breaks (SSBs), apurinic/apyrimidinic (AP sites) and base lesions (Goodhead, 1994, Sutherland *et al*, 2000, Ward, 1994). Monte Carlo track structure simulation and experimental data has shown that the yields of non-DSB clustered damage sites are more than four times the yield of DSBs (Brenner and Ward, 1992, Gulston *et al*, 2002, Sutherland *et al*, 2000). The majority of the non-DSB damage is repaired by the base excision repair (BER) pathway. It has been hypothesised that clustered damage sites are more difficult to repair than isolated lesions (Goodhead, 1994, Ward, 1994). This has been demonstrated both *in vitro* (David-Cordonnier *et al*, 2002, David-Cordonnier *et al*, 2000, Harrison *et al*, 1999, Lomax *et al*, 2002, Mourgues *et al*, 2007) and *in vivo* in *Escherichia coli* (D'Souza and Harrison, 2003, Malyarchuk *et al*, 2003, Malyarchuk *et al*, 2004, Pearson *et al*, 2004, Shikazono *et al*, 2006), yeast (Kozmin *et al*, 2009) and mammalian cells (Gulston *et al*, 2004, Malyarchuk *et al*, 2008, Peddi *et al*, 2008). It has also been established that the complexity of DNA damage increases with increasing ionisation density (Goodhead, 1994, Nikjoo *et al*, 1998, Ottolenghi *et al*, 1997).

Therefore, the purpose of this study was to investigate the repair kinetics of simple and clustered DNA damage in mammalian cells at DNA damage sites induced by

different types of radiation, using fluorescently-tagged BER proteins as markers. Further to this, the effect of poly(ADP-ribose) polymerase (PARP) inhibition on the recruitment of the BER scaffold protein, XRCC1, to sites of radiation-induced DNA damage and the effect of histone deacetylase (HDAC) inhibitors, which cause relaxation of chromatin, on the real time recruitment and loss of XRCC1 to radiation-induced DNA damage sites in mammalian cells was investigated. There are two sub-pathways of BER known as short-patch (SP) and long-patch (LP) BER (Fortini and Dogliotti, 2007). SP-BER is thought to be the predominant pathway in mammalian cells but as the complexity of the damage increases LP-BER is thought to increase in importance (Byrne *et al*, 2009, Cunliffe *et al*, 2007, Imoto *et al*, 2008, Lomax *et al*, 2004a). Additionally, cancerous cells often exhibit defects in DNA repair pathways. It was therefore of interest to investigate the radiosensitising effect of knocking down a key LP-BER protein in both cancerous and non-cancerous cell lines since this pathway is less frequently used to repair endogenous DNA damage.

1.2 Non-DSB DNA damage

Although DNA is most stable in its double stranded helical form, it is a dynamic structure that frequently exists in intermediate forms during replication, repair and recombination. DNA is often subject to insult from a variety of endogenous and exogenous DNA damaging agents. The presence of DNA damage can initiate transient arrest of the cell cycle by a DNA damage checkpoint (Sancar *et al*, 2004), and prolonged inhibition of DNA metabolism causes stalling of transcription, replication or chromosome segregation which is potentially cytotoxic by triggering apoptosis (Rich *et*

al, 2010, Roos and Kaina, 2012). If DNA is misrepaired a heritable change or mutation in the genome may occur.

Base lesions and single strand breaks are the most common form of DNA damage occurring in the human genome with approximately 10^4 spontaneous SSBs occurring in each cell every day (Lindahl, 1993). DNA can also spontaneously decay under normal physiological temperatures and pH through hydrolysis or deamination (Lindahl, 1993, Lindahl and Nyberg, 1972, Loeb & Preston, 1986). Reactive oxygen species (ROS) produced during normal aerobic metabolism can interact with and damage DNA, giving rise to a variety of lesions (Ames *et al*, 1993). It is estimated that mitochondria leak around 2×10^{10} superoxide and hydrogen peroxide (H_2O_2) molecules per day which can interact with the cellular contents to form highly reactive hydroxyl radicals (Ames *et al*, 1993, Bjelland and Seeberg, 2003). Apurinic/aprimidinic (AP) sites arise when DNA undergoes hydrolytic attack on the N-glycosyl bond. Guanine has the lowest oxidation potential of the four bases and is therefore the most readily oxidised. Hydrolytic deamination of cytosine to form uracil, and oxidation of guanine to form 8-oxo-7,8-dihydroguanine (8-oxoG) are the main premutagenic lesions formed in cycling cells (Lindahl, 1993, Wallace, 2002). The cell has developed pathways to ensure efficient repair of such damage, predominantly the base excision repair (BER) pathway (see section 1.5)

Additionally, environmental mutagens such as ionising radiation, UV light, and genotoxic chemicals such as those found in cigarette smoke, can cause a variety of DNA lesions including base lesions, SSBs, DSBs, AP sites and DNA-protein crosslinks (Friedberg *et al*, 1995). For example, oxides of nitrogen in cigarette smoke cause

oxidation of macromolecules including DNA and their high concentrations also deplete existing levels of antioxidants (Ames *et al*, 1993, Frei *et al*, 1991). Ionising radiation produces the same types of lesions as endogenous DNA damage but the spatial distribution of the lesions is unique to ionising radiation, as described in the following sections. Eleven types of base damage induced from exposure to low LET ionising radiation have been detected in mammalian cells (Cadet *et al*, 2003) and are shown in Figure 1.1. Thymine glycol is the most commonly produced base lesion by sparsely ionising radiation (Pouget *et al*, 2002).

Double strand breaks (DSBs) are also formed as a result of ionising radiation, which can lead to chromosome aberrations, deletions and mutagenesis, and it has been suggested that as little as one DSB can be lethal to a cell (Olive, 1998). DSBs are predominantly repaired via classical non-homologous end-joining (C-NHEJ) or homologous recombination (HR) (reviewed in van Gent *et al*, 2001). C-NHEJ is active throughout the cell cycle and the key components are the Ku70/Ku80 heterodimer, DNA-PKcs and the XRCC4-Ligase IV complex, which directly ligate two DSB ends and is generally an error-prone process. However, it has been shown that complex DSBs consisting of partially cohesive three base 3' overhangs with 3'-phosphoglycolate termini and a single base gap in each strand can be accurately repaired by C-NHEJ, although the repair is dependent on the presence of Ku, XRCC4 and DNA Ligase IV (Chen *et al*, 2001, Lee *et al*, 2003). HR operates in late S and G2 phase of the cell cycle and relies on a homologous chromosome for use as a DNA template, resulting in error-free repair. It has been demonstrated that HR plays a key role in the repair of

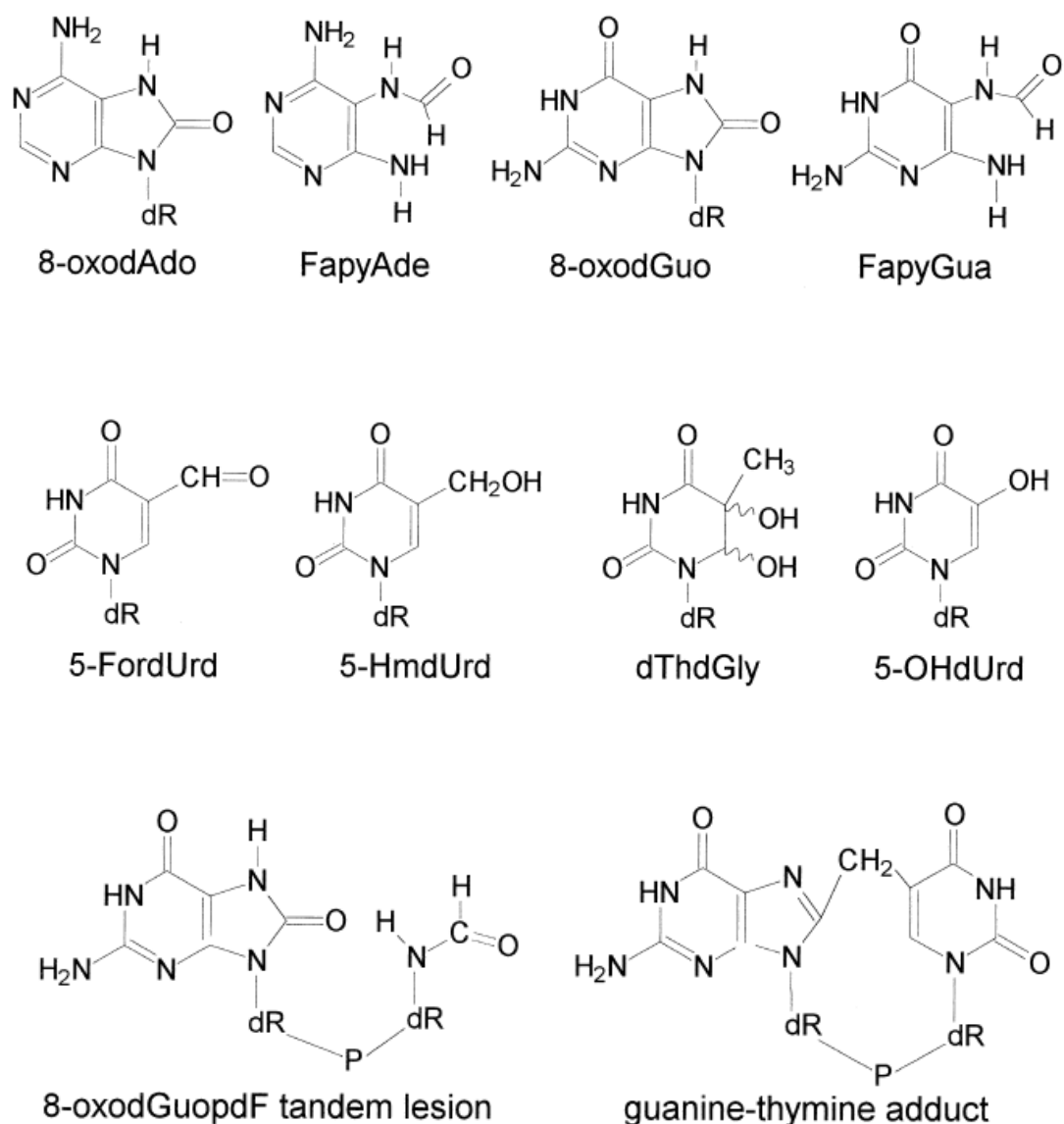


Figure 1.1: Structure of base lesions induced within mammalian cells. Using HPLC-MS/MS techniques, the types of base damage induced in mammalian cells after irradiation were detected and the structures of the lesions are illustrated (obtained from Cadet *et al*, 2002).

replication-induced DSBs (Lundin *et al*, 2003, Michel *et al*, 2001). However, this thesis will focus on the induction and repair of non-DSB radiation-induced DNA damage.

1.3 Ionising Radiation

It is estimated that the average exposure to background ionising radiation in the UK is 2.7 mSv per year (Watson *et al*, 2005). The sources of this radiation are summarised in Figure 1.2 and generally originate from natural sources (e.g. Radon, found within the earth's crust), enhanced natural sources (e.g. air travel, as exposure to cosmic rays increases at higher altitudes) and man-made sources (e.g. radio diagnostics). It can be seen from Figure 1.2 that the vast majority of exposure to radiation occurs through natural sources, which indicates that each individual is at approximately the same risk. Only when external factors such as occupation or medical usage are considered does the risk change between individuals, not taking into account variations in lifestyle or genetic factors.

Ionising radiation is generated when unstable radioactive isotopes release energy to achieve a stable energy state. The energy released varies depending on the radioactive source. It can be in the form of electromagnetic radiation (γ -rays) or particulate radiation (e.g. α - & β -particles). Electromagnetic radiation is composed of photons, and the photons released can interact with biological molecules and water with the effect dependent on the wavelength of the radiation. For example, in the visible range of the electromagnetic spectrum, there is little effect on DNA and proteins because the photons are not absorbed to produce electronic states. However, in the UV range, the photons are absorbed directly by the biological molecule to produce electronic excited states that can vigorously react and change chemically. As the wavelength

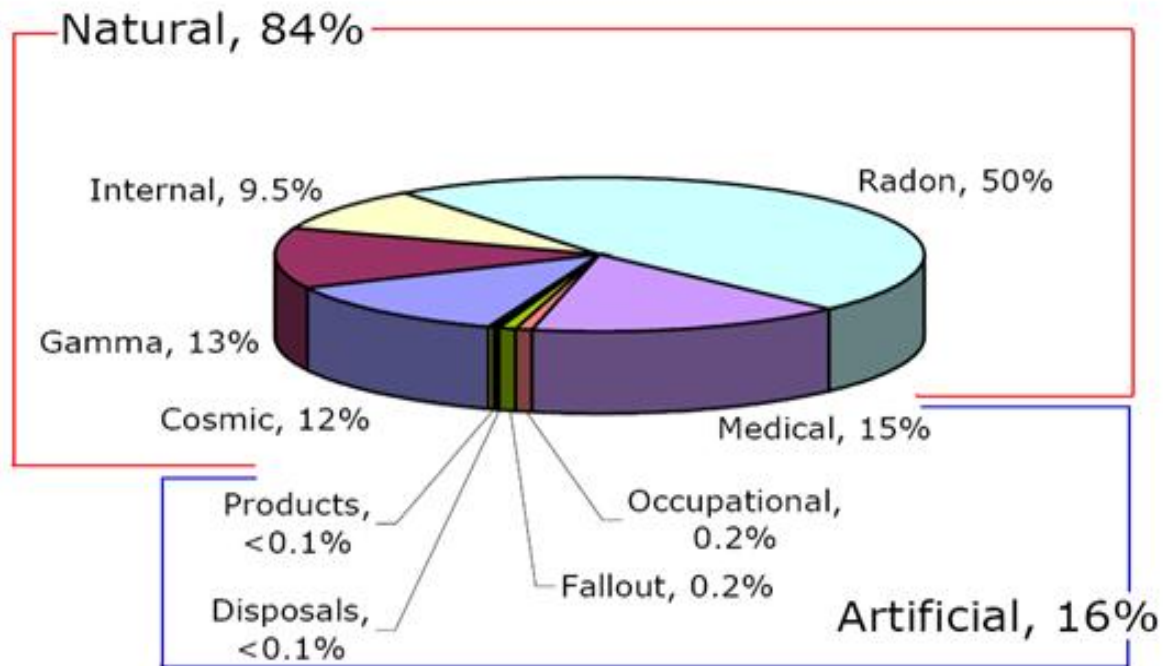


Figure 1.2: The relative contribution of various natural and artificial sources of ionising radiation to the average annual dose received by the UK population (2.7 mSv). The categories indicated on the above pie chart include the following sources:

Cosmic: Radiation from extraterrestrial sources relevant at ground level and from average air travel per year.

Gamma: Terrestrial gamma radiation from the ground and buildings.

Internal: Intakes of radionuclides occurring in food and drink (excluding radon).

Radon: Exposure derived from indoor radon gas concentrations resulting from natural radon emitted from the ground.

Medical: Radiology and nuclear medicine, patient exposure from procedures such as X-ray.

Occupational: Exposure in the place of work, including medical workers, airline workers, and miners.

Products: Consumer products, e.g. smoke alarms.

Disposals: Discharges of radioactive waste.

Fallout: Resulting from nuclear weapons testing and nuclear accidents (e.g. Chernobyl).

(Information obtained from HPA Review 2005 (Watson *et al.* 2005))

decreases into the range of X-rays and γ -rays, the energy of the photons becomes significant enough to cause the release of core electrons from excited atoms and this process is known as ionisation (Hall, 1994).

X-rays were first identified in 1895 by Wilhelm Conrad Röntgen and this was followed by the discovery of natural radioactivity a few months later by Henry Becquerel. In 1886, Emil Grubbé treated an advanced ulcerated breast cancer with X-rays (Bernier *et al*, 2004, Hall, 1994, Grubbé, 1933). In 1889, Marie Curie discovered radium as a natural source of high energy photons or γ -rays and it was then used for 20 years as the only source of γ -rays for the treatment of cancer (Bernier *et al*, 2004).

Ionising radiation does not deposit its energy uniformly. Instead, the energy is localised along tracks in a pattern that is dependent upon the radiation quality. The quality of radiation is defined by its linear energy transfer (LET). This is measured by the amount of energy deposited along a unit length of a radiation track. Additionally, ionising radiation may be directly ionising or indirectly ionising. Direct ionisation occurs when the radiation deposits energy directly onto DNA, causing ionisation of the DNA. α -particles cause mainly direct ionisation of DNA as they traverse the cell, and are known as a type of high linear energy transfer (LET) radiation (Monson *et al*, 2006). X-rays and γ -rays are sparsely ionising, meaning that neither deposit high amounts of energy along their track length, and therefore only cause few direct ionisations of DNA as they traverse the cell (forms of low LET radiation). X-rays and γ -rays exert a lot of their damage on DNA via indirect ionisation. Indirect ionisation occurs when the radiation interacts with the surrounding molecules as it traverses the cell and deposits some of its energy to cause ionisation of the molecule and ejection of an electron. The

ionised molecule or radical product and the ejected electron may interact and bring about a change in the DNA, termed secondary events (Hall, 1994). Water is the most common molecule to be ionised and result in secondary events, in a process called radiolysis ($\text{H}_2\text{O} \rightarrow \text{H}_2\text{O}^+ + \text{e}^-$). Both products are reactive as the ion radical can react further with water to produce a reactive hydroxyl radical ($\text{H}_2\text{O} + \text{H}_2\text{O}^+ \rightarrow \text{H}_3\text{O}^+ + \bullet\text{OH}$), while the electron can cause reductive damage. Hydroxyl radicals ($\bullet\text{OH}$) can interact with DNA within ~ 4 nm from where they were formed (Roots and Okada, 1975). The $\bullet\text{OH}$ resulting from water radiolysis contributes considerably to the levels of DNA damage reported in a cell after exposure to sparsely ionising radiation such as γ -radiation (Fielden, 1991, Purkayastha *et al*, 2005). In support of this, several studies have noticed a decrease in damage caused by exposure to ionising radiation in radical-scavenging conditions in both isolated DNA (Sutherland *et al*, 2001) and irradiated cells (Chapman *et al*, 1979, deLara *et al*, 1995). When fully hydrated, each nucleotide is surrounded by 20 water molecules, leading to a high possibility that following traversal of the cell by a radiation track, water may be hydrolysed. It has been known for a long time that addition of $\bullet\text{OH}$ on to C8 of guanine results in the formation of 8-oxoG (Kasai *et al*, 1984). Interestingly, Bergeron *et al* (2010) have more recently shown that addition of $\bullet\text{OH}$ to the C8 of guanine only accounts for $\sim 5\%$ of 8-oxoG generated following γ -irradiation of an aqueous aerated DNA solution. 45% of the 8-oxoG produced is formed following an electron transfer reaction, and the remaining 50% is found in tandem with another lesion, which is a result of peroxy addition on to the C8 of the purine base from an adjacent pyrimidine (Bergeron *et al*, 2010). The base excision repair of more than 40% of the 8-oxoG present in these tandem lesions was

inhibited (see section 1.9 for more information on the repair of clustered DNA damage).

Figure 1.3 shows an example of tracks inducing direct and indirect ionisations caused by low and high LET radiation. The energy emitted by ionising radiation consists of spurs, blobs and short tracks. 95% of the energy generated by X and γ rays is in the form of spurs, which involve three ion pairs and have a diameter of approximately 4 nm (Mozumder and Magee, 1966). In the case of high LET radiation, blobs are more common, which are considerably larger at 7 nm and contain 12 ion pairs. The diameter of both spurs and blobs is larger than that of the DNA double helix and, including the diffusion distance of free radicals produced, damage may typically occur over a distance of 20 base pairs from a single radiation track. Figure 1.4 depicts the typical yields of DNA damage induced following irradiation of a mammalian cell with 1 Gy of sparsely ionising radiation.

The DNA damage lesions induced by ionising radiation are chemically indistinguishable from those induced in cells by normal aerobic metabolism (O'Neill and Wardman, 2009, see also section 1.2). However, the difference between endogenously induced damage and that caused by ionising radiation is the spatial distribution of the damage. Endogenous damage generally arises as isolated lesions whereas ionising radiation-induced damage is in the form of clustered DNA damage due to energy being deposited unevenly along the radiation tracks (Goodhead *et al*, 1993, Ward, 1988).

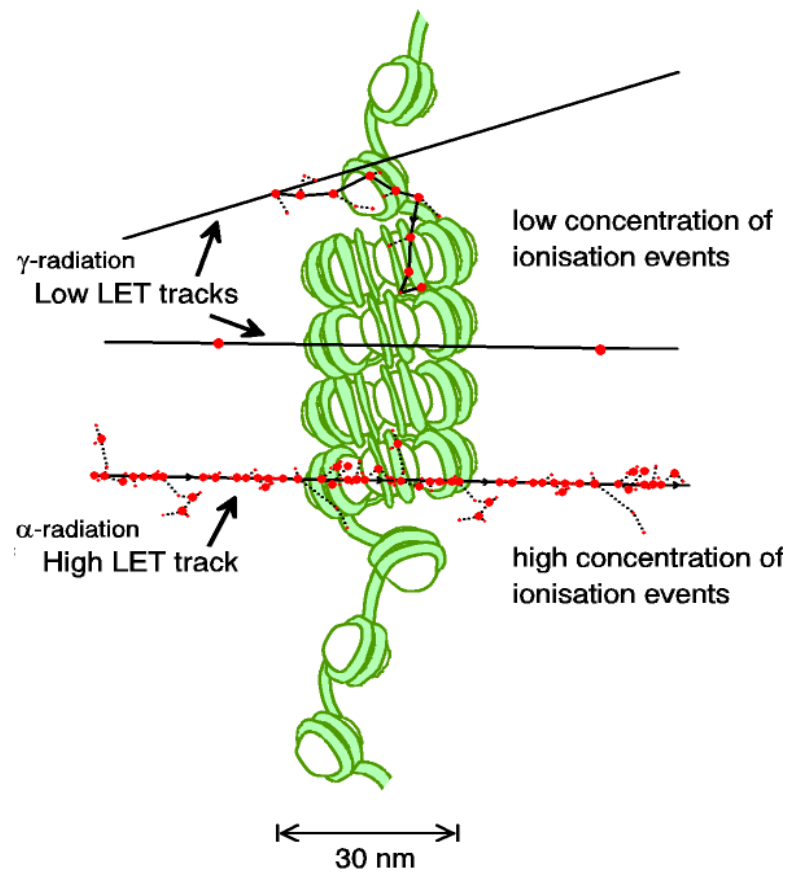


Figure 1.3: Schematic representation depicting the ionisation events of both high and low LET radiation along their respective tracks. Each ionisation event is represented by a red dot, either as a direct effect of the radiation along the track, or as a result of a secondary ionisation event. α radiation has an increased number of ionisation events, compared to low LET radiation, along its track per unit length, leading to it being termed high LET. Such a high level of ionisation events along the track structure are not seen in low LET radiation. Of the two tracks depicting low LET radiation, the lower track illustrates the number of direct ionisation events along the track structure, with the upper track showing the secondary electrons. It is the distribution of these secondary electrons that primarily cause clustered DNA damage. (Adapted from Goodhead, 1994).

Radiation	Low-LET	Electron track-ends	High-LET
Tracks in nucleus	1000	1100	2
Ionisation in nucleus	100,000	100,000	100,000
Ionisation in DNA	1500	1500	1500
Excitation in DNA	1500	1500	1500
Base damage	10^4	10^4	10^4
DNA SSB	850	500	450
8-Hydroxyadenine	700	-	-
Hydrogen peroxide	5×10^5	-	-
RBE for DNA DSB	~1	~1	~1
PCC breaks: initial	6	-	12
PCC breaks: 8 h	<1	-	4
DNA protein crosslink	150	-	-
Chromosome aberration	0.3	-	2.5
Dicentric	0.1	-	0.8
Complex aberration	10%	20%	45%
Chromosome instability	<10%	50%	40%
HPRT mutation	10^{-6}	10^{-5}	10^{-5}
Lethal lesions	0.5	1.1	2.6
Cell inactivation	30%	60%	85%

Figure 1.4: The table gives a quantitative summary of the typical effects of 1 Gy of different types of ionising radiation to a mammalian cell (Nikjoo *et al*, 1998). SSB – single strand break; DSB – double strand break; RBE – relative biological effectiveness; PCC – prematurely condensed chromosomes; HPRT – hypoxanthine-guanine phosphoribosyl transferase.

1.4 Clustered DNA damage

Clustered DNA damage, also known as “(local) multiply damaged sites” (Ward, 1981), is defined as two or more lesions located within one to two helical turns of DNA by passage of a single radiation track (Goodhead, 1994, Ward, 1994). Clustered DNA damage sites can be classed as either DSB or non-DSB clusters (Figure 1.5). Depending on the LET of the radiation, several DSB formed are often termed “dirty” DSBs due to base lesions and/or SSBs being located in close proximity to the break termini as predicted from track structure and determined experimentally (Datta *et al*, 2005, Nikjoo *et al*, 2001). Indirect evidence for ‘dirty’ DSB came from the differences in the rate of repair of cellular DSB (Jenner *et al* 1992). Non-DSB clustered DNA damage consists of SSBs, base lesions and AP sites (Goodhead, 1994, Ward, 1994), which are all also generated endogenously as isolated lesions and repaired by the base excision repair (BER) pathway (Chaudhry & Weinfeld, 1997, David-Cordonnier *et al*, 2001, David-Cordonnier *et al*, 2002, Harrison *et al*, 1999). Experimental evidence confirmed the formation of non-DSB clusters in mammalian cells by ionising radiation (Sutherland *et al*, 2000, Gulston *et al*, 2002). Oxidative damage including non-DSB clustered damage sites induced by ionising radiation is the focus of this thesis.

It is conceivable for clustered damage sites to be formed endogenously by ROS attack on DNA, but this happens very rarely and is thought to be insignificant in comparison to the high numbers of clustered damage sites formed in DNA after exposure to ionising radiation (Bennett *et al*, 2004, Sutherland *et al*, 2003). However, the incidence of endogenous clustered damage sites has been found to be higher in cancer cells than

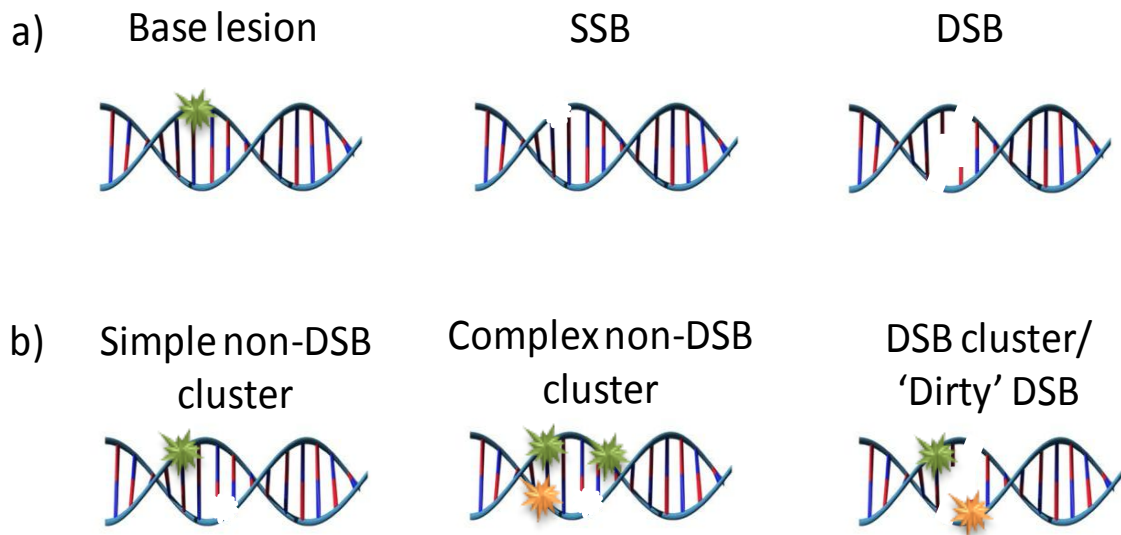


Figure 1.5: Schematic depicting non-DSB and DSB lesions induced in DNA. (a) Depicts isolated lesions that can be generated endogenously (e.g. by ROS) or by ionising radiation. (b) Representative cartoon of non-DSB and DSB clustered DNA damage induced by ionising radiation. The first two clusters illustrate an example of simple and complex non-DSB clusters which contain a SSB and two different types of base lesions, shown by the green and orange stars. The right cluster depicts a DSB with associated base lesions, known as a DSB cluster, or 'dirty' DSB due to the close proximity of the base lesion to the break termini.

in normal cells (Gollapalle *et al*, 2007). Clustered DNA damage sites can also be formed by radiomimetic drugs such as bleomycin (Povirk, 1996).

Biophysical (Monte Carlo) modelling studies of the effect of ionising radiation on DNA have established that the complexity of DNA damage increases with increasing ionisation density and LET (Goodhead, 1994, Nikjoo *et al*, 1998, Ottolenghi *et al*, 1997). Low LET radiation (such as γ -radiation) generates a low concentration of ionisation events where ~70% of the energy is sparsely deposited along the tracks causing isolated lesions (Figure 1.3). However, 30-40% of the energy is produced as low-energy electrons that form short secondary electron tracks (delta tracks) that result in clustering of the ionisation events and, therefore, clustered damage sites (Figure 1.3). With high LET radiation (such as α -radiation) there is a high concentration of ionisation events along the radiation track and, therefore, 90% of the DNA damage is clustered with greater complexity than for sparsely ionising radiation (Figure 1.3). It has been shown that approximately 40% of DSBs induced by low LET radiation have associated SSBs or base damage, whereas this increases to more than 90% for high LET radiation (Datta *et al*, 2006, Nikjoo *et al*, 1999).

Additionally, free radicals arising from radiolysis of water were also considered during biophysical modelling studies, as these are a major source of damage induction, with the $\bullet\text{OH}$ being a major cause of DNA strand breakage (Milligan *et al*, 1996). Ottolenghi *et al* (1997) took into account the diffusion of radicals after irradiation of hydrated DNA at distances characteristic to inside a cell and found that the direct effects of radiation exposure are dominant with high LET radiation whereas when low LET radiation is used the relative contribution of secondary electrons (indirect effect) is

more significant. Confirmation of this came from a number of studies involving γ -irradiated isolated DNA, where it was shown that the yield of DNA strand breaks depended upon the scavenging capabilities of the aqueous solution in which the DNA was irradiated (Krisch *et al*, 1991, Milligan *et al*, 1993, Prise *et al*, 1993). Consistent with this finding, if the scavenging capability of the solution is decreased, the yields of SSBs and DSBs increase with both high and low LET (Fulford *et al*, 2001). The formation of clustered DNA damage by ionising radiation in mammalian cells has now been confirmed, including the fact that more clustered DNA damage is induced as the LET is increased (Georgakilas, 2008, Gulston *et al*, 2002, Sutherland *et al*, 2000, Sutherland *et al*, 2002). The yield of non-DSB damage is ~4-8 times greater than that of prompt DSBs, although this may be an under-estimation as only clusters containing bistranded lesions are detected. Non-DSB damage is generally repaired by the base excision repair pathway.

1.5 BER

Base modifications (e.g. 8-oxo-7, 8-dihydroguanine (8-oxoG), thymine glycols, uracil etc), abasic sites and DNA single-strand breaks present in clustered damage sites, as well as those caused by endogenous processes, are repaired by the base excision repair (BER) and single strand break repair (SSBR) pathways (Chaudhry and Weinfeld, 1997, David-Cordonnier *et al*, 2000, David-Cordonnier *et al*, 2001, David-Cordonnier *et al*, 2002, Harrison *et al*, 1999) and reviewed in Eccles *et al*, 2011, Georgakilas *et al*, 2008, Sage and Harrison, 2011. The BER and SSBR pathways have been reviewed in Dianov *et al*, 2001, Lindahl and Wood, 1999, Wilson and Bohr, 2007, Zharkov, 2008. BER is initiated with damage recognition and base excision by a DNA glycosylase

specific to the type of base lesion, resulting in the formation of an AP site (Krokan *et al*, 1997, Nilsen and Krokan, 2001). DNA glycosylases bind to DNA and probe for damaged bases by flipping bases out from the DNA double helix (McCullough *et al*, 1999). There are multiple DNA glycosylases in mammalian cells, each with their own substrate specificity (Figure 1.6, Robertson *et al*, 2009). DNA glycosylases are classified as either monofunctional or bifunctional. Monofunctional glycosylases hydrolyse N-glycosidic bonds to generate AP sites. Human monofunctional glycosylases include thymine/uracil mismatch glycosylase (TDG) (Neddermann *et al*, 1996) and single-strand-selective monofunctional uracil DNA glycosylase 1 (SMUG1) (Haushalter *et al*, 1999). Bifunctional glycosylases possess an intrinsic lyase activity, cleaving the DNA at the resultant AP sites (Mitra *et al*, 1997) and include the human homolog of endonuclease III (hNTH1) (Aspinwall *et al*, 1997, Ikeda *et al*, 1991) which removes oxidised pyrimidines. 8-oxoguanine DNA glycosylase (hOGG1) (Radicella *et al*, 1997), which removes oxidised purines, is classified as a bifunctional glycosylase but acts as a monofunctional glycosylase as it has a very weak lyase activity and requires AP endonuclease (APE1) (Vidal *et al*, 2001b). The more recently identified human DNA glycosylases endonuclease VIII-like 1 (hNEIL1) and endonuclease VIII-like 2 (hNEIL2) (Bandaru *et al*, 2002, Hazra *et al*, 2002a, Hazra *et al*, 2002b, Takao *et al*, 2002, Morland *et al*, 2002) generate DNA strand breaks with 3' phosphate termini, the removal of which requires polynucleotide kinase (PNK) rather than APE1 (Wiederhold *et al*, 2004).

The DNA backbone is cleaved 5' to the AP site by APE1 or 3' to the AP site by an AP lyase (Robertson *et al*, 2009). If the backbone is cleaved by APE1, termini consisting of a 3'-hydroxyl (3'OH) and a 5'-deoxyribose-5-phosphate (5'dRP) are generated (Wilson

Enzyme	Enzymatic activities
UNG	uracil
SMUG1	uracil, 5-OH-meU
TDG	T, U and ethenoC (CpG sites)
MBD4	T and U opposite G (CpG sites)
MUTYH	A opposite 8-oxoG
OGG1	8-oxoG, fapyG
NTHL1	Tg, fapyG, DHU, 5-OHU, 5-OHC
NEIL1	as NTH1 and fapyA, 8-oxoG
NEIL2	overlap with NTH1/NEIL1
NEIL3	unknown
MPG	3-meA, hypoxanthine, ethenoA

Figure 1.6: DNA glycosylases (human nomenclature) and their substrates. Table information taken from Robertson *et al*, 2009.

and Barsky, 2001). DNA polymerase β (pol β) then catalyses the release of the 5'dRP from the incised AP site (Matsumoto and Kim, 1995) prior to insertion of a single nucleotide (Singhal *et al*, 1995). The AP lyase activity associated with bifunctional DNA glycosylases creates a nick containing a 3' sugar moiety (either a 3' α , β unsaturated aldehyde or 3' phosphate end group) which requires further processing by APE1 (Dempfle & Harrison, 1994) prior to nucleotide insertion by pol β . An APE1-independent BER pathway has also been discovered, involving the mammalian DNA glycosylases/AP lyases NEIL1 and NEIL2, that relies on PNK for end processing (Wiederhold *et al*, 2004).

Repair proceeds via two sub-pathways, short patch (SP) and long patch (LP) BER. The main difference between the two pathways is the number of nucleotides in the repair patch (Fortini and Dogliotti, 2007). During SP-BER, thought to be the predominant pathway, a single nucleotide is incorporated into the DNA by pol β and the nick is sealed by DNA ligase III α in a complex with X-ray repair cross complementing 1 (XRCC1) (Cappelli *et al*, 1997). The scaffold protein XRCC1 is present throughout SP-BER and is one of the first proteins recruited to the nick generated by the action of a glycosylase or APE1 (Campalans *et al*, 2005, Vidal *et al*, 2001). LP-BER involves the synthesis of a repair patch containing two or more nucleotides. The first nucleotide is inserted by pol β and the subsequent nucleotides are inserted by pol δ/ϵ (Podlutsky *et al*, 2001). A section of DNA is displaced into a flap structure that is then processed by flap endonuclease 1 (FEN1) and the nick is sealed by DNA ligase I, which has been found to be responsible for limiting the repair patch length (Pascucci *et al*, 1999, Sattler *et al*, 2003). Proliferating cell nuclear antigen (PCNA) binds to and enhances the

activity of FEN1 (Klungland and Lindahl, 1997, Hutton *et al*, 2008). Pol β and FEN1 have been shown to interact (Liu *et al*, 2005) and this has led to the suggestion that pol β can carry out strand displacement in LP-BER. It has been shown that FEN1 can promote strand displacement synthesis by pol β and pol β in turn can promote FEN1 cleavage of the displaced strand (Balakrishnan *et al*, 2009). The Rad9-Rad1-Hus1 (9-1-1) complex has also been hypothesised to be involved in LP-BER by stimulating APE1 and pol β (Gembka *et al*, 2007). An overview of the two BER sub-pathways can be seen in Figure 1.7.

LP-BER is required in situations where the AP site contains a sugar-phosphate group that is altered in a way that prevents repair by the SP-BER proteins (Klungland and Lindahl, 1997, Liu *et al*, 2008), including reduced or oxidised AP sites (DeMott *et al*, 2002, Sung *et al*, 2005). Additionally, it is thought that the switch from SP-BER to LP-BER might depend on the relative ATP concentration near the AP site (Petermann *et al*, 2003). When the ATP levels are low, XRCC1 is thought to promote strand displacement by pol β to initiate LP-BER, which is able to generate ATP from poly(ADP-ribose), whereas when the ATP concentration is high, Ligase III promotes SP-BER by preventing strand displacement synthesis and promoting ligation (Petermann *et al*, 2003, Robertson *et al*, 2009).

During BER, DNA repair substrates are thought to be passed on in a coordinated manner from one protein to the next preventing the intermediates from triggering cell cycle arrest or cell death pathways (Parikh *et al*, 1999, Prasad *et al*, 2011, Wilson and Kunkel, 2000). Evidence of this includes channelling of the DNA substrate from an AP site through to a ligated product during SP-BER (Prasad *et al*, 2010), although the same

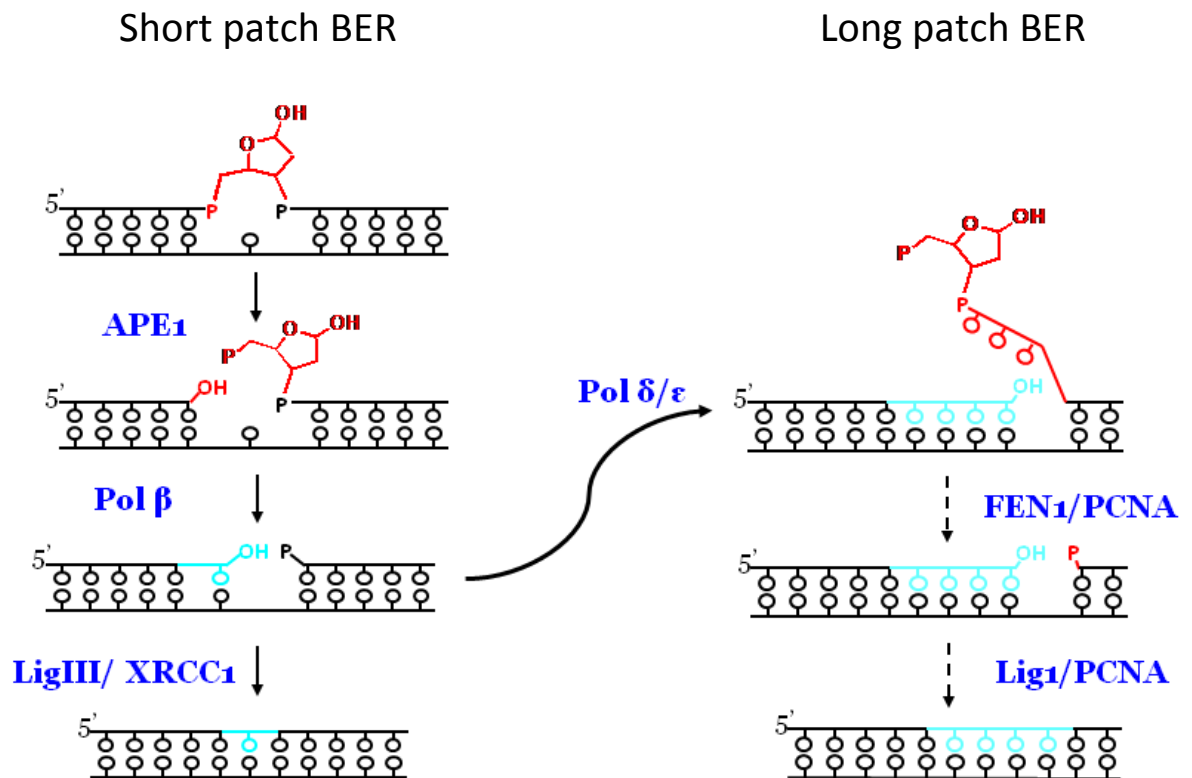


Figure 1.7: Diagrammatic representation of BER and the two sub-pathways; short patch (SP) and long patch (LP) BER. The left hand side depicts the SP-BER pathway, with the initial lesion shown here as an AP site (base damage would have been removed prior to this by a damage-specific glycosylase). Incision of the AP site by APE1 is followed by incorporation of the relevant nucleotide by pol β and removal of the 5' dRP moiety. The gap is then sealed by lig III. However, if pol β stalls or is inhibited in some way, there is a handover to pol δ/ε which are then able to add up to 10 bases into the break site, creating a flap of displaced DNA that is cleaved by FEN1 in a PCNA dependent manner. The gap is sealed by ligase I in this instance.

group found that FEN1 has to recognise and bind to the LP-BER flap structure intermediate rather than the intermediate being channelled straight from pol β to FEN1. Balakrishnan *et al* (2009), however, suggest that LP-BER proteins are part of a multienzyme complex, and function in a defined sequential manner (Balakrishnan *et al*, 2009). This theory has recently been strengthened by crystal structures of FEN1 with DNA from which the authors propose that FEN1 binds DNA exposed in the pol β complex and displaces pol β to access the flap structure, resulting in a direct handoff which prevents exposure of the potentially dangerous intermediate (Tsutakawa *et al*, 2011).

1.5.1 SSBR

Single strand break repair (SSBR) is thought to resemble BER after the initial step of damage recognition and base removal (Zharkov, 2008). PARP1 detects and binds to SSBs which results in PAR synthesis and recruitment of the scaffold protein XRCC1. XRCC1 can then recruit and enhance the activity of the remainder of the proteins required for repair (see sections 1.6 and 1.8). To enable gap-filling and ligation, the SSB termini must consist of a 3'-hydroxyl and 5'-phosphate. Radiation-induced SSBs often require processing prior to ligation due to the 3' terminus containing blocking end groups such as phosphate or phosphoglycolate (Henner *et al*, 1982). 3'-phosphate end groups are processed by polynucleotide kinase 3'-phosphatase (PNKP) (Jilani *et al*, 1999, Karimi-Busheri *et al*, 1999, Whitehouse *et al*, 2001) and 3'-phosphoglycolate groups are processed by APE1 (Chen *et al*, 1991, Henner *et al*, 1982, Izumi *et al*, 2000, Parsons *et al*, 2004, Winters *et al*, 1992, Winters *et al*, 1994).

1.6 XRCC1

X-ray repair cross complementing protein 1 (XRCC1) is a non-enzymatic scaffold protein that can interact with and enhance the activity of many DNA repair proteins (Caldecott, 2003). XRCC1^{-/-} mice are embryonic lethal (Tebbs *et al*, 1999) and four Chinese hamster ovary (CHO) cell lines (EM7, EM9, EMC11 and EMC12) deficient in XRCC1 were found to be sensitive to a range of DNA damaging agents including alkylating agents, H₂O₂ and ionising radiation, resulting in an increase in sister chromatid exchange (SCE) and a reduced ability to repair SSBs (Caldecott *et al*, 1995, Caldecott *et al*, 2001, Shen *et al*, 1998, Thompson *et al*, 1982, Thompson *et al*, 1990, Zdzienicka *et al*, 1992). XRCC1 is now known to be involved in SSB repair and BER, and can interact with many proteins in these pathways including the DNA glycosylases MPG, NEIL2, NTH1, OGG1, UNG2 (Akbari *et al*, 2010, Campalans *et al*, 2005, Marsin *et al*, 2003), APE1 (Vidal *et al*, 2001), DNA Pol β (Caldecott *et al*, 1996), Ligase III α (Caldecott *et al*, 1994), PARP1 (Masson *et al*, 1998), PARP2 (Schreiber *et al*, 2002), PCNA (Fan *et al*, 2004) and PNK (Whitehouse *et al*, 2001). The fact that XRCC1 can interact with all these proteins indicates that it is involved throughout BER and it has been shown to stimulate the activities of these proteins. For example, it helps stimulate the 3' dRPase activity of APE1 (Vidal *et al*, 2001) as well as the 3'-phosphatase activity of PNK (Whitehouse *et al*, 2001). In addition, phosphorylation of XRCC1 by casein kinase 2 (CK2) stimulates the binding of XRCC1 to PNK (Loizou *et al*, 2004). More recently, it has been found that CK2 is the main protein kinase that phosphorylates XRCC1 and that XRCC1 phosphorylation is required for XRCC1-Ligase III complex stability (Parsons *et al*,

2010). Additionally, CK2 phosphorylation of XRCC1 reduces the affinity of XRCC1 for DNA (Ström *et al*, 2011b).

XRCC1 has been shown to bind to both nicked and gapped DNA by its N-terminal domain, which is also the same domain with which it interacts with Pol β (Mani *et al*, 2004, Marintchev *et al*, 2000). XRCC1 has two BRCA1 carboxyl-terminal (BRCT) phospho-protein interaction domains, BRCT1 and BRCT2, which are important for many of its protein interactions and recruitment to DNA damage (Caldecott, 2003, Kubota and Horiuchi, 2003). The stable interaction between XRCC1 and Ligase III α , which is required for the stability of Ligase III (Moore *et al*, 2000), is facilitated through the BRCT2 domain (Caldecott *et al*, 1994, Cappelli *et al*, 1997) and is also involved in XRCC1/Ligase III α heterodimer formation (Cuneo *et al*, 2011). The BRCT1 domain mediates interaction between XRCC1 molecules enabling them to form dimers and oligomers, and the XRCC1/Ligase III α complex is a heterotetramer consisting of two molecules of each protein (Beernick *et al*, 2005). The central BRCT1 domain of XRCC1 has also been shown to be important throughout the cell cycle where it is essential for recruitment of XRCC1 to sites of DNA damage and replication, and for cell survival following treatment with the alkylating agent MMS (Hanssen-Bauer *et al*, 2012, Kubota and Horiuchi, 2003, Taylor *et al*, 2002). The BRCT1 domain contains a binding site for PAR which aids the interaction of XRCC1 with both PARP1 and PARP2 (Masson *et al*, 1998, Pleschke *et al*, 2000, Schreiber *et al*, 2002). The three most common single nucleotide polymorphisms (SNPs) of XRCC1 found in humans are located in the region comprising the nuclear localisation signal and BRCT1 domains, and these have been associated with an increased incidence of specific types of cancer (Hanssen-Bauer *et*

al, 2012). XRCC1 also interacts with aprataxin and tyrosyl-DNA phosphodiesterase (TDP1) and deficiency in either of these proteins is thought to result in defective SSBR and accumulation of oxidative DNA damage leading to neurodegenerative disease (Ahel *et al*, 2006, El-Khamisy *et al*, 2005, Hirano *et al*, 2007, Plo *et al*, 2003).

XRCC1 has also been implicated in DSB repair as X-ray-induced DSBs showed a lower rate of rejoining in XRCC1 deficient CHO cells (Nocentini, 1999, Schwartz *et al*, 1987) although this was thought to be due to unrepaired SSBs being encountered at replication forks resulting in replication-induced DSBs, and the higher frequency of SCE and genetic deletions in these cells was also put down to SSBs persisting into S phase (Caldecott, 2003).

However, more recently it has been shown that XRCC1 is involved in a back-up NHEJ pathway, along with PARP1 and DNA ligase III, which is only used when Classical NHEJ (C-NHEJ) is deficient (Audebert *et al*, 2004, Wang *et al*, 2006). Levy *et al* (2006) found that the BRCT1 domain of XRCC1 can be phosphorylated by the NHEJ protein DNA-PK to cause XRCC1 dimer dissociation and that phosphorylation of p53 by DNA-PK can be stimulated by XRCC1 (Levy *et al*, 2006). An S phase specific role has been suggested for XRCC1 (Fan *et al*, 2004, Kubota and Horiuchi, 2003, Taylor *et al*, 2000, Taylor *et al*, 2002). Taylor *et al* (2000), observed S phase-specific XRCC1 foci, which they suggested were connected to RAD51 and DNA recombination. Fan *et al* (2004) proposed that XRCC1 is recruited by PCNA into strand break repair complexes that are associated with DNA replication machines. Additionally, XRCC1 has been proposed to have a role in the repair of uracil at replication foci. The uracil DNA glycosylase UNG2 is responsible for post-replicative removal of mis-incorporated dUMP during replication

(Otterlei *et al*, 1999) and the catalytic domain of UNG2 directly interacts with the nuclear localisation signal region of XRCC1 (Akbari *et al*, 2010). Complexes containing XRCC1 and UNG2 can perform complete repair of uracil and these proteins only colocalise during S-phase (Akbari *et al*, 2010).

Apart from Ligase III α , which is constitutively present in a complex with XRCC1, XRCC1 was thought to interact with various repair proteins sequentially at the site of DNA damage (Caldecott, 2001, Caldecott, 2003, Whitehouse *et al*, 2001). However, it has since been suggested that XRCC1 can be found in complexes containing several different repair proteins (Akbari *et al*, 2010, Fan *et al*, 2004, Luo *et al*, 2004, Hanssen-Bauer *et al*, 2011). Luo *et al* (2004) reported the existence of two preformed XRCC1-containing complexes in HeLa cells. The first containing the SSBR proteins Ligase III, Pol β and PNK, and a second novel complex consisting of Ligase III and aprataxin (Luo *et al*, 2004). It was very recently proposed that XRCC1 can be found in complexes comprised of different proteins depending on the type of damage being repaired and the cell cycle phase (Akbari *et al*, 2010, Hanssen-Bauer *et al*, 2011). It has been suggested that there are three types of XRCC1 repair complexes (Akbari *et al*, 2010, Hanssen-Bauer *et al*, 2011). The first is constitutive or “classic” BER/SSBR complexes which are present throughout the cell cycle to repair either endogenous DNA damage or low levels of exogenously induced DNA damage, consisting of the initiating DNA glycosylase, APE1, Pol β , PNK, XRCC1 and Ligase III. The second type of complexes additionally contain the LP-BER proteins PCNA and FEN1, and are thought to be needed when higher levels of DNA damage have been induced. Finally, the third types of complexes are thought to just be involved at replication, and can be divided further into pre-replicative BER

complexes (additionally containing UNG2, Pol δ and Ligase I), and post-replicative BER complexes (containing more Ligase III, Pol β , PARP1 and PNK (Akbari *et al*, 2010, Hanssen-Bauer *et al*, 2011)). Another recent observation is that DNA Ligase III is dispensable for nuclear DNA repair, suggesting that XRCC1 dependent DNA repair could utilise other DNA ligases such as Ligase I (Gao *et al*, 2011, Simsek *et al*, 2011, Sleeth *et al*, 2004).

1.7 FEN1

Flap endonuclease 1 (FEN1) is a multifunctional structure-specific nuclease. FEN1 can act either as an endonuclease where it specifically recognises double-stranded DNA with a 5'-unannealed flap and makes an endonucleolytic cleavage at the base of the flap or as an exonuclease where it degrades nucleotides from a nick or a gap progressively (Harrington & Lieber, 1994, Murante *et al*, 1994). More recently, FEN1 was also shown to possess a gap endonuclease (GEN) activity (Zheng *et al*, 2005). FEN1^{-/-} mice are embryonic lethal (Kucherlapati *et al*, 2002, Larsen *et al*, 2003). This is likely due to the well characterised roles of FEN1 in DNA repair and recombination (Lieber *et al*, 1997, Liu *et al*, 2004).

FEN1 has been shown to have a critical role in Okazaki fragment maturation (Bambara *et al*, 1997, Goulian *et al*, 1990, Lieber *et al*, 1997, Lui *et al*, 2004, Waga *et al*, 1994). During DNA replication, the lagging DNA strand is generated as RNA-initiated discontinuous segments called Okazaki fragments. Prior to ligation of the Okazaki fragments, the RNA primer must be displaced into a 5' single-stranded flap that is then removed by FEN1, leaving a nick ready for ligation by DNA ligase I (Bambara *et al*, 1997, Liu *et al*, 2004). This essential role in replication explains why FEN1 is highly

expressed in proliferative tissues (Kim *et al*, 2000, Kleppa *et al*, 2012, Otto *et al*, 2001, Warbrick *et al*, 1998).

The role of FEN1 in LP-BER is also well established, where it cleaves the 5'-flap generated by DNA polymerase strand-displacement in preparation for ligation of the resulting nick by DNA ligase I (Fortini and Dogliotti, 2007, Klungland and Lindahl, 1997). Klungland & Lindahl (1997) used purified proteins to show that FEN1 is essential for repair of a reduced AP site as well as γ -ray-induced oxidised AP sites in plasmid DNA. It had already been shown that yeast expressing mutant RAD27, the *S. cerevisiae* homologue of FEN1, exhibit hypersensitivity to the methylating agent MMS (Reagan *et al*, 1995, Sommers *et al*, 1995). The first *in vivo* evidence that FEN1 was involved in DNA repair was when FEN1^{-/-} DT40 chicken cells were found to be sensitive to methylating agents and hydrogen peroxide (Matsuzaki *et al*, 2002). Shibata and Nakamura (2002) also discovered that human carcinoma cell lines expressing a dominant-negative FEN1 mutant protein are also hypersensitive to MMS. Asagoshi *et al* (2010) have shown using FEN1 null and wild-type DT40 chicken cell extracts that LP-BER is defective in the absence of FEN1, providing evidence that FEN1 is needed for this repair pathway in mammalian cells.

FEN1 has been reported to interact with at least 30 proteins (Shen *et al*, 2005) including PCNA, RPA, Pol β , Pol δ , APE1, WRN, BLM, endonuclease/helicase DNA2, endonuclease G (Endo G), HIV integrase and p300. Guo *et al*, (2008a) have mapped the sites of FEN1 interaction for five of these proteins that have roles in DNA replication and repair. Via its interaction with PCNA, FEN1 is recruited to repair sites for LP-BER and replication foci for RNA primer removal where PCNA also stimulates the cleavage

activity of FEN1 (Chen *et al*, 1996, Li *et al*, 1995, Wu *et al*, 1996). PCNA has been implicated in coordinating the sequential action of DNA Pol δ , FEN1 and DNA Ligase I and disruption of its interaction with FEN1 impairs Okazaki fragment ligation (Levin *et al*, 2000, Zheng *et al*, 2007). It has also been shown that the PCNA binding site of FEN1 is required for viability (Larsen *et al*, 2008, Zheng *et al*, 2007).

FEN1 is localised to the nucleus during S phase of the cell cycle or in response to DNA damage (Qiu *et al*, 2001, Shibata & Nakamura, 2002) and has been shown to accumulate in the nucleoli before migrating into the nuclear plasma upon UV irradiation and phosphorylation (Guo *et al*, 2008b). Post-translational modifications are important in regulating the activity of FEN1. FEN1 is acetylated by p300 in response to UV irradiation (Hasan *et al*, 2001), methylated to suppress phosphorylation and facilitate PCNA binding (Guo *et al*, 2010), and phosphorylated in late S phase by Cdk2-cyclin E which results in dissociation of FEN1 from PCNA (Henneke *et al*, 2003).

FEN1 has been shown to have several other functions in addition to its role in DNA replication and LP-BER. FEN1 is required for HR between homologous sequences with nonhomologous tails at the DNA break ends (Kikuchi *et al*, 2005, Negritto *et al*, 2001) and has even been implicated in NHEJ in situations where there is a 5' flap at the end of one of the DSB ends to be rejoined (Wu *et al*, 1999). In response to stalled replication forks, the Werner syndrome protein (WRN) forms a complex with FEN1 and activates its gap-dependent endonuclease activity to initiate break-induced recombination (Zheng *et al*, 2005). During the process of apoptosis, FEN1 interacts with Endo G nuclease to degrade genomic DNA (Parrish *et al*, 2003). FEN1 is also found

in the mitochondria where it cooperates with DNA2 nuclease to process DNA intermediate structures during mitochondrial DNA replication and repair (Duxin *et al*, 2009, Kalifa *et al*, 2009, Liu *et al*, 2008, Zheng *et al*, 2008). Additionally, FEN1 has a role in both telomere maintenance (Parenteau and Wellinger, 1999, Parenteau and Wellinger, 2002, Saharia *et al*, 2008, Vallur and Maizels, 2010) and prevention of repeat expansion (Freudenreich *et al*, 1998, Gordenin *et al*, 1997).

1.8 PARP

Poly(ADP-ribose) polymerase (PARP) was first purified in the 1970s (Okayama *et al*, 1977, Yamada *et al*, 1971) as the enzyme that catalyses synthesis of nuclear poly(ADP-ribose) (PAR) polymers, and soon after was found to be activated by DNA strand breaks (Benjamin and Gill, 1980). PAR polymers are generated by PARP cleaving nicotinamide adenine dinucleotide (NAD⁺) and adding it onto various acceptors including histones, transcription factors and DNA repair proteins but most importantly itself (Durkacz *et al*, 1980, Burkle, 2005). PARP binds as a homodimer enabling each monomer to automodify the other (Mendoza-Alvarez and Alvarez-Gonzalez, 1993). Once the PAR chains have reached a certain length, the negative charge that they carry repels PARP from the DNA (Ferro and Olivera, 1982). PAR has a short half-life and is degraded by poly(ADP-ribose) glycohydrolase (PARG) and possibly ARH3, so that PARP is once again free to bind to DNA strand breaks (Desnoyers *et al*, 1995, Fisher *et al*, 2007, Oka *et al*, 2006, Ueda *et al*, 1972).

There are 17 known PARPs in mammalian cells (Ame *et al*, 2004) that all share a conserved catalytic domain containing the PARP signature motif, but only PARP1 and PARP2 are known to respond to DNA damage (Ame *et al*, 1999, Benjamin & Gill, 1980,

Durkacz *et al*, 1980, Menissier de Murcia *et al*, 1989). Nuclear ADP-ribosylation is also required in many other processes including regulation of mitosis, cellular differentiation and proliferation, regulation of telomere length and cell death (Hassa *et al*, 2006). PARP1 is the most abundant (1×10^6 molecules per cell (Woodhouse and Dianov, 2008)) and studied member of the PARP family as it accounts for the majority of cellular PARP activity (Shieh *et al*, 1998). PARP1 has an N-terminal DNA-binding domain which contains two zinc-fingers important for binding to DNA strand breaks (Gradwohl *et al*, 1990), a central automodification domain containing specific glutamate and lysine residues that accept ADP-ribose (Altmeyer *et al*, 2009, Tao *et al*, 2009) as well as a BRCT motif (Bork *et al*, 1997) allowing PARP to interact with other DNA damage proteins such as XRCC1 (Caldecott *et al*, 1996, El-Khamisy *et al*, 2003, Masson *et al*, 1998), and a C-terminal catalytic domain which transfers ADP-ribose subunits from NAD^+ to protein acceptors (Chambon *et al*, 1963, Hayashi *et al*, 1983, Kameshita *et al*, 1984, Ogata *et al*, 1981). More recently, a third zinc-binding domain has been discovered that is thought to mediate interdomain contacts to help couple the DNA-binding and catalytic functions of PARP1 (Langelier *et al*, 2008).

1.8.1 Roles of PARP1

Upon binding to DNA strand breaks, PARP1 becomes activated and rapidly forms PAR chains (levels increase up to 500 fold following DNA damage and PARP1 activation (Benjamin and Gill, 1980, Menissier de Murcia *et al*, 1989)) to which many proteins can be recruited. Some of these proteins bind directly to the PAR chains on PARP1 itself (e.g. XRCC1 (El-Khamisy *et al*, 2003, Masson *et al*, 1998), ALC1 (Gottschalk *et al*, 2009), histone macroH2A1.1 (Timiniszky *et al*, 2009)) whereas others bind to PAR chains on

proteins PARylated by PARP1 (Gagne *et al*, 2008), implicating PARP1 activity in a range of cellular processes.

The most studied role of PARP1 is in the detection of SSBs following DNA damage and subsequent recruitment of proteins involved in the SSBR and BER pathways. Durkacz *et al* were the first group to show the involvement of PARP1 in BER by demonstrating that PARP1 inhibition prevented rejoining of DNA strand breaks caused by dimethyl sulphate and enhanced cytotoxicity (Durkacz *et al*, 1980). The idea of a role of PARP1 in BER was strengthened when it was discovered that PARP^{-/-} mice are hypersensitive to DNA damaging agents (Menissier de Murcia *et al*, 1997, Masutani *et al*, 1999, Wang *et al*, 1995) and that PARP1 interacts with the BER proteins DNA Ligase III α (Leppard *et al*, 2003), DNA Pol β (Prasad *et al*, 2001), PCNA (Frouin *et al*, 2003) and XRCC1 (Caldecott *et al*, 1996, El-Khamisy *et al*, 2003, Masson *et al*, 1998). It has since been found that PARP1 can recruit XRCC1 to DNA damage sites via its PAR chains and that PARP inhibition can prevent the recruitment of XRCC1 to SSBs (El-Khamisy *et al*, 2003, Godon *et al*, 2008, Lan *et al*, 2004, Okano *et al*, 2003). Satoh and Lindahl found that the repair of a γ -irradiated plasmid containing a SSB was stimulated by the addition of NAD⁺ and retarded by the PARP inhibitor 3-aminobenzamide, indicating that PARP1 is involved in the repair of the SSB (Satoh and Lindahl, 1992, Hodgkins *et al*, 1996). However, PARP1 depleted extracts still repaired the γ -irradiated DNA, leading to the first indication that PARP1 binds to and protects SSBs but is not actually required for DNA repair (Satoh and Lindahl, 1992). Several other groups have suggested that PARP1 is not essential for repair (Godon *et al*, 2008, Lindahl *et al*, 1995, Noël *et al*, 2003, Vodenicharov *et al*, 2000). In fact, it is now thought that PARP1's main function in DNA

repair is to protect excessive SSBs caused by DNA damage until repair proteins are available to carry out repair (Parsons *et al*, 2005, Satoh & Lindahl, 1997, Woodhouse *et al*, 2008).

PARP1 hyperactivation leads to extended PAR synthesis and depletion of NAD⁺ levels (Berger *et al*, 1986, Carson *et al*, 1988, Juarez-Salinas *et al*, 1979), which can trigger a PARP-dependent form of cell death known as “Parthanatos” that is distinct from apoptosis, necrosis and autophagy, and requires AIF (apoptosis inducing factor) to induce DNA fragmentation and chromatin condensation leading to caspase-independent cell death (Andrabi *et al*, 2006, Wang *et al*, 2009, Ying *et al*, 2005). Additionally, cellular ATP levels may be exhausted trying to replenish the cytosolic NAD⁺ pool, which can cause necrosis (Berger *et al*, 1986, Carson *et al*, 1988, Zong *et al*, 2004). This can particularly be a problem in the case of post-ischaemic damage in the heart or brain which causes excessive DNA damage resulting in high levels of PAR and depletion of NAD⁺ and ATP, inducing cell death (Endres *et al*, 1997, Zhang *et al*, 2002). In fact, PARP inhibition and knockout has been shown to protect against neuronal and cardiac cell death as a result of ischaemia (Eliasson *et al*, 1997, Outeiro *et al*, 2007, Yamazaki *et al*, 2011). Additionally, hyperactivation of PARP1 has been connected with an inflammatory response associated with multiple organ failure and PARP inhibitors are being investigated for use in the treatment of arthritis, diabetes and shock (Cuzzocrea, 2005, Peralta-Leal *et al*, 2009).

PARP1 has been implicated in the local modulation of chromatin compaction as it can recruit histone macroH2A1.1 which binds to PAR chains via its macrodomain (Timinszky *et al*, 2009) and ALC1 (amplified in liver cancer 1) which is a chromatin

remodelling enzyme that becomes activated after PAR binding (Gottshalk *et al*, 2009). PARP1 has also been proposed to regulate H1 binding to chromatin (Ferra and Olivera, 1982, Krishnakumar *et al*, 2008). As well as modulating chromatin structure, PARP1 might regulate transcription by altering DNA methylation patterns and acting as a co-regulator of transcription factors (Caiafa *et al*, 2009, Kraus *et al*, 2008). Finally, PARP1 deficiency can alter the expression of many genes involved in cell cycle control and stress response (Frizzell *et al*, 2009, Simbulan-Rosenthal *et al*, 2000).

1.8.2 PARP2

PARP2 lacks the central automodification domain but shares ~70% homology with the catalytic domain of PARP1 and provides residual activity (~15%) in the absence of PARP1 (Shieh *et al*, 1998). PARP2 can also interact with BER proteins such as XRCC1, DNA Pol β and DNA Ligase III (Ame *et al*, 1999, Schreiber *et al*, 2002). PARP2 can heterodimerise with PARP1 but PARP2 recognises different targets within DNA than PARP1, as PARP2 recognises gaps or flap-structures rather than SSBs (Schreiber *et al*, 2002, Yelamos *et al*, 2008). While PARP1 and PARP2 knockout mice are viable (Menissier de Murcia *et al*, 1997, Menissier de Murcia *et al*, 2003, Masutani *et al*, 1999, Wang *et al*, 1995), double PARP1 and PARP2 knockout mice are embryonically lethal (Huber *et al*, 2004), indicating a role of PARP2 in genomic integrity and cell survival. However, PARP2 only contributes 5-10% of the total PARP activity in response to DNA damage (Schreiber *et al*, 2002) and siRNA depletion of PARP2 only has a minor impact on SSBR (Fisher *et al*, 2007). Mortusewicz *et al* showed that PARP1 and PARP2 accumulate with different kinetics at laser-induced DNA damaged sites where PARP1 accumulated fast and transiently and PARP2 showed a delayed and persistent

accumulation, suggesting a later role for PARP2 in DNA repair (Mortusewicz *et al*, 2007). Unique roles for PARP2 have been indicated in genome surveillance, spermatogenesis, adipogenesis and T cell development (Yelamos *et al*, 2008). Most PARP1 inhibitors also inhibit PARP2, due to the structural homology between PARP1 and PARP2, although one group has described selective PARP2 inhibitors to further investigate the role of this enzyme (Ishida *et al*, 2006).

1.8.3 PARP1/2 inhibitors

Inhibition of PARP began over 30 years ago with analogues mimicking the nicotinamide component of NAD⁺, the most popular of these first generation PARP inhibitors was 3-aminobenzamide (Durkacz *et al*, 1980, Satoh & Lindahl, 1992). PARP inhibitors work by inhibiting the catalytic activity of PARP so that PARP can still bind to SSBs but cannot form PAR chains. This prevents recruitment of DNA repair proteins to the PAR chains and results in PARP remaining bound to the strand break, as the negatively charged PAR chains are required for repulsion of PARP from DNA once they reach a certain length. Initially, PARP inhibitors were developed for use in combination with existing chemotherapy agents or radiotherapy to potentiate their effects rather than as single agents, as PARP function in the absence of extensive DNA damage is not essential for cell survival (Menissier de Murcia *et al*, 1997, Masutani *et al*, 1999, Wang *et al*, 1995). In human cell lines, the PARP inhibitors NU1025 and NU1085 were found to enhance the cytotoxicity of various DNA damaging agents (MTIC, bleomycin, temozolomide, topotecan) and γ -irradiation (Bowman *et al*, 1998, Delaney *et al*, 2000). The tricyclic benzimidazole class of PARP inhibitors were also found to potentiate the cytotoxicity of both temozolomide and topotecan (Skalitzky *et al*, 2003, White *et al*,

2000). Taking this further, substantial chemosensitisation and radiosensitisation was seen with PARP inhibitors in tumour xenographs (Calabrese *et al*, 2003, Calabrese *et al*, 2004, Miknyoczki *et al*, 2003). Additionally, a combination of a DNA-PK inhibitor (NU7026) with the PARP inhibitor AG14361 caused increased sensitisation of cell lines to ionising radiation (Veuger *et al*, 2003, Veuger *et al*, 2004). The approach of a combination of PARP inhibitors with another therapy is still being used and it has recently been shown that PARP inhibitors can increase the radiosensitivity of head and neck tumours (Nowsheen *et al*, 2011), lymphomas (Schaefer *et al*, 2011) and lung tumour xenografts (Senra *et al*, 2011). Two phase I trials are currently investigating combinations of ABT-888 and radiation with whole brain radiation in brain metastases and with chest wall/nodal irradiation in patients with inflammatory or loco-regionally recurrent breast cancer (Davar *et al*, 2012).

An important breakthrough was made in 2005 when it was shown by two groups that PARP inhibition in BRCA-deficient cells causes enhanced cell death as a result of “synthetic lethality” (Bryant *et al*, 2005, Farmer *et al*, 2005). Synthetic lethality arises when mutations in two genes have little or no effect individually but the combination results in cell death (Dobzhansky, 1946). Bryant *et al* (2005) found that low concentrations of the PARP inhibitors NU1025 and AG14361 were extremely cytotoxic to the BRCA2-deficient cell line V-C8, when compared with the V79 wild-type cell line or the V-C8 cell line complimented with BRCA2. A similar effect was seen in the MCF7 and MDA-MB-231 cell lines when BRCA2 was depleted. Finally, they showed that BRCA2-deficient tumours in a mouse xenograft model are susceptible to treatment with the PARP inhibitor AG14361 alone (Bryant *et al*, 2005). Similarly, Farmer and

colleagues (2005) found that siRNA depletion of PARP1 causes a reduction in clonogenic survival in BRCA1- and BRCA2-deficient cells compared with wild-type cells. They also found that BRCA1- or BRCA2-deficient cells are extremely sensitive to PARP inhibition compared with heterozygous mutant or wild-type cells. Depletion of BRCA1 using siRNA in MCF7 cells induced sensitivity to PARP inhibitors, and PARP inhibition reduced the formation of tumours from BRCA2-deficient cells but had no effect on tumours from wild-type cells (Farmer *et al*, 2005). Both groups proposed a model where PARP inhibition prevents/retards repair of SSBs and these SSBs then persist until replication where they cause stalled replication forks which can collapse into replication-induced DSBs. Repair of these strand breaks would normally occur by homologous recombination (HR). However, in the absence of the HR proteins BRCA1 and BRCA2, these replication-induced DSBs may persist and result in cell death. In HR deficient cells, the more error-prone NHEJ can step in but is not involved in repair of replication-induced DSB. Disabling NHEJ has been found to rescue the lethality of PARP inhibition or PARP1 knockdown in cell lines lacking BRCA1 or BRCA2 (Patel *et al*, 2011), implying that NHEJ has to be proficient for the cytotoxic effect of PARP inhibitors in HR deficient cells, potentially due to the error-prone nature of NHEJ.

BRCA1 and BRCA2 gene mutations are usually hereditary (Venkitaraman, 2002) and are only rarely mutated in sporadic cancers (Futreal *et al*, 1994, Lancaster *et al*, 1996, Merajver *et al*, 1995). However, a subset of breast and ovarian basal-type sporadic cancers have been found to share many traits with familial BRCA1 breast tumours (Hedenfalk *et al*, 20010, Jazaeri *et al*, 2002, Sorlie *et al*, 2003) and have been described as having a BRCA-phenotype or “BRCAness” (Turner *et al*, 2004). Sporadic tumours

with a BRCA-phenotype have defects in homologous recombination such as hypermethylation of the BRCA1 promoter, decreased expression of other HR proteins (e.g. RAD51, ATM, MRE11-RAD50-NBS1 (MRN) complex) and PTEN deficiency (McCabe *et al*, 2006, Mendes-Pereira *et al*, 2009, Sessa, 2011). The two main types of cancer found to exhibit “BRCAness” are serous ovarian cancers (Sessa, 2011, Thrall *et al*, 2006) and triple-negative breast cancer (O’Shaughnessy *et al*, 2011). A lot of work has been done on these two subtypes of cancer to see if PARP inhibitors are a viable option for therapy and one promising combination is the use of the PARP inhibitor Iniparib in combination with gemcitabine and carboplatin for the treatment of triple-negative breast cancer (Alli *et al*, 2009, Hastak *et al*, 2010, O’Shaughnessy *et al*, 2011).

There are currently nine PARP inhibitors in various stages of clinical trials (Davar *et al*, 2012, Rouleau *et al*, 2010, Underhill *et al*, 2010) which are listed in Table 1.1. Seven are being used in cancer therapy, either as single agents or in combination with chemotherapy or radiation (Table 1.1). BSI-201 (Iniparib) is in phase III clinical trials, AZD2281 (Olaparib), AG-014966 and ABT-888 are in phase II clinical trials and MK-4827, CEP-9722 and E7016 are in phase I clinical trials. The use of INO-1001 and MP-124 is being explored as neuro- or cardiac-protectants from ischaemic insults (Davar *et al*, 2012, Radovits *et al*, 2007, Szabo *et al*, 2005). However, there is concern over the long-term use of PARP inhibitors due to their roles in other cellular processes such as regulation of transcription, replication fork restart, inflammation and cell death (Patel and Kaufmann, 2010) and one study has shown that the combination of PARP1 and p53 knockout in mice increased cancer incidence (Tong *et al*, 2003).

Table 1.1: PARP inhibitors in development.

Name	Current Development Stage
AG-014699/ PF-01367338 (Rucaparib)	Phase II - single agent in BRCA-associated breast or ovarian cancer. Phase I - combination with chemotherapy in advanced solid tumors.
AZD2281 (Olaparib)	Phase I/II - singly or combination with chemotherapy in various cancer types including breast, ovarian and colorectal cancers.
ABT-888 (Veliparib)	Phase II - combination with chemotherapy in various cancer types including breast cancer, colorectal cancer, glioblastoma multiforme (GBM) and melanoma. Phase I: combination with radiation.
BSI-201 (Iniparib)	Phase III - gemcitabine and $\pm$ BSI-201 in breast and lung cancers. Phase I/II - single agent or combination with chemotherapy in various cancer types including glioma and ovarian cancer.
MK-4827	Phase I - single agent; combination with carboplatin-containing regimens.
CEP-9722	Phase I - combination with temozolomide in advanced solid tumors.
E7016 (GPI-21016)	Phase I - combination with temozolomide in advanced solid tumors.
INO-1001	Phase II in cardiovascular disease. Phase I - combination with temozolomide in melanoma (completed) without further investigation in oncology.
LT-673	Preclinical.
MP-124	Phase I in acute ischemic stroke.
NMS-P118	Preclinical.
XAV939	Preclinical, highly selective against PARP-5 (tankyrase).

Information taken from Davar *et al*, 2012.

1.9 Repair of clustered DNA damage

It was hypothesised by both Goodhead (1994) and Ward (1994) that the repair of radiation-induced clustered damage sites would be more difficult than the isolated lesions induced endogenously, due to the close proximity of the lesions within the sites, especially as enhanced mutagenesis, carcinogenesis and lethality is seen with increasing ionisation density and hence increasing complexity of clustered damage sites (O'Neill and Wardman, 2009). Additionally, approximately 2.6 million SSBs must be induced by hydrogen peroxide (H_2O_2) to achieve the same level of cell inactivation as the approximately 1000 SSBs induced by one gray of ionising radiation (Ward *et al*, 1985).

Several *in vitro* studies have been performed using short synthetic oligonucleotides designed with known DNA lesions placed at specific locations. The repair of these substrates was investigated either by reconstituting the BER pathway with purified proteins or by using nuclear or whole cell mammalian extracts. It was found that the efficiency of repair of an AP site or SSB is reduced when it is within five bases of a base lesion, and that the extent of the reduction in repair is dependent on the base lesion, with 8-oxo-7,8-dihydroguanine (8-oxoG) and thymine glycol (Tg) lesions exhibiting a greater reduction in repair than 5,6-dihydrothymine (DHT) lesions (Bellon *et al*, 2009, Budworth and Dianov, 2003, Byrne *et al*, 2009, David-Cordonnier *et al*, 2001, David-Cordonnier *et al*, 2002, Eccles *et al*, 2011, Harrison *et al*, 1999, Lomax *et al*, 2004a, Lomax *et al*, 2004b). Additionally, bistranded clustered damaged sites have the potential to give rise to DSB, if both lesions within the cluster are excised prior to repair of one of the lesions. AP/AP bistranded clusters have been shown to form DSB

when they are in a 3' orientation to each other or if they are more than three bases apart when in a 5' orientation (Chaudhry and Weinfeld, 1997, David-Cordonnier *et al*, 2002, Eccles *et al*, 2010, Lomax *et al*, 2004b, Paap *et al*, 2008). If the AP sites are less than three bases apart in the 5' orientation then incision of one AP site inhibits incision of the second AP site (Chaudhry and Weinfeld, 1997, David-Cordonnier *et al*, 2002, Lomax *et al*, 2004b).

It has therefore been shown that the repair of clustered DNA damage lesions is retarded when compared to the same lesions present in isolation. The extent of retardation is dependent on the type of lesions within the cluster, the distance between the lesions and the orientation of the lesions to each other (David-Cordonnier *et al*, 2001, David-Cordonnier *et al*, 2002, Eccles *et al*, 2011, Lomax *et al*, 2002, Malyarchuk *et al*, 2003). The mutability of clustered lesions in an *E. coli* reporter system has also been investigated and it was shown that lesions in the cluster become highly mutagenic (D'Souza & Harrison, 2003, Malyarchuk *et al*, 2003, Pearson *et al*, 2004, Shikazono *et al*, 2006). The majority of this work has been undertaken on bistranded lesions (Chaudhry & Weinfeld, 1997, David-Cordonnier *et al*, 2000, Harrison *et al*, 1999, Lomax *et al*, 2004a, Lomax *et al*, 2004b) but tandem lesions (two closely spaced lesions within about five bases on the same DNA strand) are also induced by ionising radiation (Box *et al*, 1998) and are retarded in their repair in a similar way to bistranded (Budworth *et al*, 2005, Cuniffe *et al*, 2007, Lomax *et al*, 2005). More recently, it has been shown that depending on the types of lesions in the cluster, the cluster may be highly mutagenic or lead to DSBs (Bellon *et al*, 2009, Eccles *et al*, 2009, Eot-Houllier *et al*, 2005). Interestingly, XRCC1 was found to be important for the repair

of an AP site in close proximity to a base lesion (8-oxoG or DHT), as immunodepletion of XRCC1 slows down the initial rate of repair of the AP site, whereas the absence of XRCC1 had no influence on the repair of an SSB in similar clustered damaged sites (Byrne *et al*, 2009, Mourgues *et al*, 2007). Specifically, it has been shown that DNA glycosylases (David-Cordonnier *et al*, 2000, David-Cordonnier *et al*, 2001), the XRCC1/Ligase III complex (Lomax *et al*, 2004a), FEN1 (Budworth *et al*, 2002) and Ligase I (Mourgues *et al*, 2007) have reduced repair activities when acting on lesions within a clustered DNA damage site.

It has now been demonstrated that the lifetime of non-DSB clustered damage induced in mammalian cells by ionising radiation is substantially longer than that for isolated lesions (Magnander *et al*, 2010). Clustered damage sites can be detected in mice 20 weeks post-irradiation with X-rays (Gollapalle *et al*, 2007). Additionally, the complexity of the clustered damage has been shown to increase with increasing LET in mammalian cells and this can have an additional effect on the lifetime of the clustered damage. For example, Asaithamby *et al* found that following high LET radiation, DSBs still persisted after 48 h, indicating that the additional complexity of these breaks made them harder for the cell to repair than more simple breaks (Asaithamby *et al*, 2010), as proposed earlier by Jenner *et al* (1992). The enhanced mutability of clustered damage has also been verified in mammalian cells and yeast (Kozmin *et al*, 2009, Malyarchuk *et al*, 2008). Despite extensive DSB formation as a result of lesion processing within a bacterial system, the same was not seen after transformation of plasmids containing bistranded clustered uracils into HeLa cells (Malyarchuk and Harrison, 2005). Plasmids were able to be repaired in this system and there was no

significant increase in deletions, implying that repair occurs in an essentially error-free manner. It was suggested that human cells have developed a system of reducing the number of SSB repair intermediates, which could lead to DSBs. Within mammalian cells, persistent SSBs pose a serious threat to the genomic stability as they may be converted to replication-induced DSBs (Kuzminov, 2001) which may lead to increased recombination amongst sister chromatids or chromosome aberrations (Pascucci *et al*, 2005, Thompson *et al*, 1982). Therefore, the lifetime extension of clustered lesions increases the likelihood of lesions persisting until replication, increasing the potential for mutation or cell death (Gulston *et al*, 2004, Harper *et al*, 2010). Clustered DNA damage arising as a result of ionising radiation has the disadvantage of causing mutations in normal tissue, potentially leading to cancer, but also the advantage of inducing cell death in tumour cells.

1.10 Near Infrared (NIR) laser microirradiation for real time studies in mammalian cells

Radiation microbeams are defined as subcellular probes that use ionising radiation to induce cellular damage (Daudin *et al*, 2006, Folkard *et al*, 1997, Pallon and Malmqvist, 1994, Prise *et al*, 1994, Sheng *et al*, 2009). Cellular damage can also be induced by lasers using either single photons or multiple-photon absorption processes to study the recruitment of repair proteins to sites of DNA damage in mammalian cells.

In 1931, Maria Göppert-Mayer proposed that multiple photons of light may be absorbed simultaneously to excite a molecule (Göppert-Mayer, 1931). Multiphoton laser microirradiation has been reported to induce a whole range of DNA lesions including base damage, SSBs, DSBs, and cyclobutane pyrimidine dimers (CPDs) and has

enabled many studies into the repair of these lesions (Harper *et al*, 2008, King and Oh, 2004, Kong *et al*, 2009, König *et al*, 2001, Mari *et al*, 2006, Meldrum *et al*, 2003, Reynolds *et al*, 2012, Tirlapur *et al*, 2001, Uematsu *et al*, 2007). It has also been shown that multiphoton excitation of cancer cells can induce reproductive cell death (reviewed in König, 2000).

In this study, a NIR multiphoton laser microbeam was produced using 180 fs pulses from a titanium:sapphire (Ti:Sa) laser operating at 730 nm (1.7 eV) (for information on the set-up see section 2.6.4 and Figure 2.2). For multiphoton processes to occur, a molecule must interact with a sufficient number of photons to generate the excited state. As the wavelength increases, the energy of the photon decreases. This means that multiphoton excitation of a molecule by two photons using a NIR laser microbeam at 730 nm is equivalent in energy to a single photon from a 365 nm (3.4 eV) UV source. The irradiations in this study were performed in the presence of Hoescht dye which absorbs at 365 nm resulting in similar photochemical processes as those generated by conventional UV irradiation when two photons are simultaneously absorbed by the dye. DNA absorbs maximally in the range of 200-290 nm so multiphoton excitation of DNA would require absorption of three photons at a wavelength of 730 nm to equal the energy of a single photon from a 243 nm (4.9 eV) source. There are many advantages of using this NIR laser microirradiation setup for DNA repair studies in mammalian cells. Firstly, the energy produced by NIR light is not significantly absorbed by biological tissues at a wavelength of 730 nm. Therefore the damage will only be induced at the focal spot of the laser microbeam, which in this case is an area of $\sim 400 \text{ nm}^3$ and can be focused in a defined area of the nucleus, unlike UV irradiation where

damage is induced along the irradiation path, or gamma irradiation where the whole cell is irradiated (Figure 1.8). The laser microbeam setup consists of a multiphoton laser scanning system built into an adapted commercial fluorescence confocal microscope (Botchway *et al*, 2012), which allows the cells to be visualised and imaged before and after the irradiation without having to move the sample. Additionally, the irradiation time is so short (damage can be induced in nanoseconds and enough cells for an experiment can be irradiated in less than 10 seconds) that cells can be maintained at 37 °C during the irradiation without repair of the induced lesions taking place.

NIR laser microbeam irradiation mimics the DNA damage induced by both complex ionising and UV irradiation (Botchway *et al*, 2012). It has been shown that hydroxyl radicals can be produced using this system enabling its use to induce oxidative stress (Botchway *et al*, 2007). Previous studies using the NIR multiphoton laser microbeam setup used in this thesis (Botchway *et al*, 2010, Botchway *et al*, 2012) have shown that SSBs, DSBs and CPDs in the presence of Hoechst dye are produced following NIR laser microirradiation (Harper *et al*, 2008, Meldrum *et al*, 2003, Reynolds *et al*, 2012) and these studies have mainly focused on the repair of DSBs. Harper *et al* (2008) demonstrated that the NIR laser microbeam can induce damage with repair kinetics similar to that induced after both low and high LET radiation. DSBs found to be similar to those induced after low LET irradiation were repaired within 4 h post-irradiation and the DSBs that are still present 24 h after irradiation were thought to be similar to the more complex and difficult to repair lesions induced after high-LET radiation (Leatherbarrow *et al*, 2006, Karlsson and Stenerlow, 2004). In addition to this,

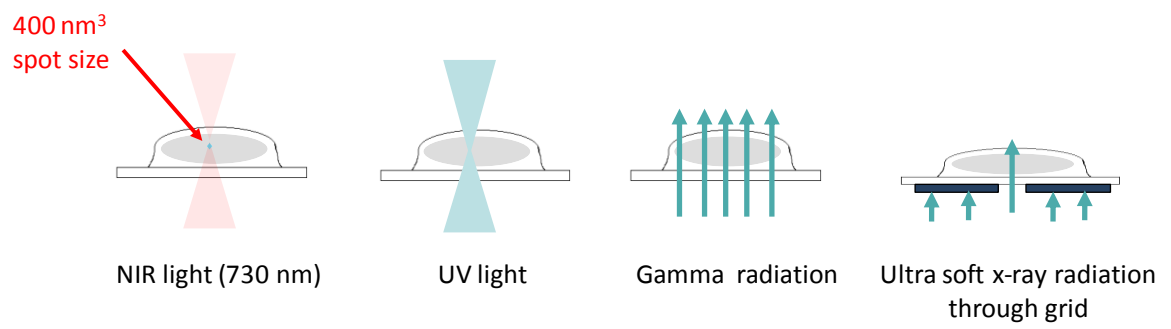


Figure 1.8: Schematic diagram showing the radiation path of different sources of radiation through a mammalian cell.

persistent DSBs arising at later times post-laser irradiation were seen and it was concluded that these DSBs were due to replication-induced DSBs arising as a result of unrepaired SSBs and UV photoproducts persisting until replication (Harper *et al*, 2010). After 24 h, 40% of cells have persistent DSBs following NIR laser microbeam irradiation (Harper *et al*, 2008) indicating that a large proportion of the damage induced by the NIR laser is complex and therefore difficult to repair.

1.11 Ultrasoft X-ray irradiation

Ultrasoft X-ray irradiation shows all the characteristic features of low LET radiation (Goodhead *et al*, 1983) including rejoining kinetics of DSBs similar to ^{60}Co γ -rays (Botchway *et al*, 1997, deLara *et al*, 2001). However, ultrasoft X-rays have an indication of a higher residual yield (deLara *et al*, 2001). Ultrasoft X-rays are highly attenuated in air and can be shielded using a grid system to give defined tracks of DNA damage, unlike γ -irradiation which cannot be shielded, resulting in the damage being spread all over the cell (See Figure 1.8, also see section 2.6.3 and Figure 2.1 for USX irradiation setup information). Ultrasoft X-rays (0.1-5 keV) react with molecules within the cell by the photoelectric effect, producing photo- and Auger electrons of low, well-defined energy with the dimensions of the electron tracks being comparable to those of DNA (Botchway *et al*, 1997, deLara *et al*, 2001). Aluminium K (1.5 keV) ultrasoft X-rays were used in this study. The majority of DNA damage induced by low LET radiation is by secondary electron interactions occurring randomly in the cell (Hall and Garcia, 2006). Approximately 30% of the absorbed dose for low-LET radiation, such as hard x-rays and γ -rays, is deposited by these low energy secondary electrons (Nikjoo and Goodhead, 1991), similar to the energy deposition by ultrasoft X-rays. Since low

LET ultrasoft X-rays simulate the low energy secondary electrons produced with other low LET sources (e.g. γ -radiation and hard X-rays), they result in a slightly higher yield of clustered lesions (Botchway *et al*, 1997, deLara *et al*, 2001, Nikjoo and Goodhead, 1991). This is reflected in the observation that ultrasoft X-rays are generally more effective than hard x-rays or γ -rays for a range of biological endpoints for a given absorbed dose, including induction of chromosomal aberrations, mutations and cell killing (Goodhead, 1983, Goodhead, 1990, Hill, 2004). This increased sensitivity reflects the enriched low energy electron tracks by ultrasoft X-rays for the same dose of gamma-radiation.

For the ultrasoft X-ray irradiation set up used in this study (Figure 2.1), cells have to be cooled to 10 °C (see section 2.6.3) to prevent repair occurring during the irradiation time and then moved to a confocal microscope and warmed to 37 °C before imaging can occur. This could result in loss of detection of any initial fast repair processes. To overcome this limitation, van Oven *et al* (2009) have developed an ultrasoft X-ray multi-microbeam system designed to induce up to 10 DSBs per second at specific sites simultaneously within a large number of cell nuclei by using a nickel mesh filter with holes that are 2.2-3.5 μm in diameter, and plan to incorporate it into an upright confocal fluorescence microscopy system to allow real-time observation of fluorescently labelled repair proteins at DNA damage sites (van Oven *et al*, 2009), however, this set up was not available for the work in this thesis. The amounts of SSBs and DSBs induced by ionising radiation can be defined and quantified whereas UV or multiphoton laser microirradiation is difficult to control and reproduce between different experimental setups, and generates large amounts of UV-specific lesions as

well as other types of damage (Dinant *et al*, 2007, Kong *et al*, 2009). Therefore, the relatively well defined, quantifiable and biologically relevant spectrum of DNA damage induced by ultrasoft X-rays does have advantages over laser microirradiation techniques.

1.12 Influence of chromatin on DNA repair

Formation of radiation-induced DNA damage is thought to be influenced by the structural organisation of DNA (Falk *et al*, 2008, Magnander *et al*, 2010, Nygren *et al*, 1995, Radulescu *et al*, 2004, Xue *et al*, 1994). DNA is packaged into chromatin which is composed of nucleosomes, each of which consists of 146 base pairs of DNA wrapped around an octamer of the four core histones (H3, H4, H2A, and H2B) and one molecule of the linker histone H1 (Noll and Kornberg, 1977, Richmond *et al*, 1984). The N-terminal tails of histones extend outwards from the nucleosome and can be modified by acetylation, methylation, ubiquitylation and phosphorylation (Kouzarides, 2007, Spencer and Davie, 1999). Chromatin is packaged either in the form of euchromatin which represents open, gene rich, transcriptionally active regions or heterochromatin which represents condensed regions with low gene density and high levels of repetitive sequences. Histones within euchromatin are highly acetylated whereas histones within heterochromatin have low levels of acetylation (de Wit and van Steensel, 2009, Guenther *et al*, 2007, Peng and Karpen, 2008, Pokholok *et al*, 2005).

The number of both SSBs (Nygren *et al*, 1995) and DSBs (Radulescu *et al*, 2004) have been shown to increase when DNA is modified into a less compact structure. It has more recently been shown that chromatin structure can influence clustered DNA damage induction (Magnander *et al*, 2010). Low-LET radiation induced DNA damage

increases as the chromatin changes from a condensed to an open format. The chromatin state has a greater effect on clustered damage induction than prompt DSBs, with 200 times more clustered damages induced in the most unshielded structure compared with the most compacted, while only 140 times more DSBs were induced (Magnander *et al*, 2010). Additionally, depletion of free radical scavengers caused a 2-fold higher effect on clustered damage induction than for prompt DSBs (Magnander *et al*, 2010). Further, clustered lesions and SSBs are more affected by free-radical scavengers than prompt DSBs (Nygren *et al*, 1995, Sutherland *et al*, 2001). Therefore, the structure of chromatin appears to have a greater protective effect against lesions found in clusters than prompt DSBs. This is most likely due to the fact that SSBs and base lesions are induced by the indirect, radical-mediated effects of ionising radiation (Douki *et al*, 2006) and compaction of chromatin excludes water molecules, preventing the generation of potentially damaging $\bullet\text{OH}$ in close proximity to the DNA.

Chiu *et al* (1986) showed that transcriptionally active DNA regions were more susceptible to DNA damage than bulk DNA. It has also been shown that repair is more efficient in euchromatin compared with heterochromatic regions (Slijepcevic and Natarajan, 1994, Surralles *et al*, 1997). More recently, Falk *et al* (2008) demonstrated that although areas with high gene density are more susceptible to complex DNA damage, the repair of damage in these regions is also more efficient. The majority of DSBs (~85%) are repaired with fast kinetics in an ATM-independent manner whereas the remaining 15% are repaired with much slower kinetics, and their repair is thought to depend on ATM (Goodarzi *et al*, 2010, Iliakis *et al*, 1991, Jenner *et al*, 1992, Lobrich *et al*, 1995, Riballo *et al*, 2004). It was proposed that the slowly repairing DSB are

those that are complex. An alternative suggestion was put forward (Cowell *et al*, 2007, Goodarzi *et al*, 2009, Goodarzi *et al*, 2010) that the DSBs that repair with slow kinetics are located within heterochromatin, which impairs the access of repair kinases. Additionally, several groups have noticed that γ H2AX is not detected within heterochromatin at 15 min to 2 hours after radiation-induced DNA damage (Cowell *et al*, 2007, Falk *et al*, 2008, Goodarzi *et al*, 2008, Vasireddy *et al*, 2010). However, more recently it has been suggested by Jakob *et al* that DNA damage seen as γ H2AX within heterochromatin at early times is relocated to the edge of the heterochromatin for repair within 20 min of the damage being formed and that this process is aided by ATM-dependent local decondensation of the chromatin (Jakob *et al*, 2010).

1.13 Aims of project

The complexity of clustered damage sites increases as the ionisation density increases, and the repair of clustered lesions is slower than the repair of single lesions due to impairment of BER, which can lead to an enhanced number of persistent lesions. Therefore, the probability that these lesions will remain unrepaired at replication is increased, raising the potential for a higher mutation frequency or cytotoxicity. Whilst information using DNA substrates with defined damage is vital to our understanding of the details of radiation-induced DNA damage repair, it is also important to be able to confirm the results of these studies in the genomic DNA of mammalian cells. This was performed here by investigating the repair kinetics of radiation-induced DNA damage using the fluorescently-tagged BER proteins XRCC1-YFP and FEN1-GFP as markers of BER. Ultrasoft X-ray irradiation was used to simulate the damage caused by low-LET radiation and the NIR multiphoton laser microbeam was used to simulate more

complex damage. Previous work within the group has looked at the repair of DSBs by visualising the recruitment and loss of fluorescently-tagged NHEJ proteins in real time following different types of radiation (Reynolds *et al*, 2012). However, the majority of DNA damage induced by ionising radiation is in the form of non-DSB lesions with a lower estimate of the yield of non-DSB clustered damage being 4-8 times greater than that of prompt DSBs (Gulston *et al*, 2002, Sutherland *et al*, 2000, Sutherland *et al*, 2002). Therefore, in this thesis, the recruitment and loss of the BER proteins XRCC1-YFP and FEN1-GFP to sites of DNA damage induced by both ultrasoft X-ray irradiation and NIR laser microbeam irradiation was visualised in real time and quantified. PARP1 has been shown to recruit XRCC1 to sites of SSBs (Okano *et al*, 2003). Therefore, it was also of interest to investigate the recruitment of XRCC1 to sites of DNA damage containing a range of lesions induced by both low LET ultrasoft X-ray irradiation and the much more complex damage inducing NIR multiphoton laser microbeam irradiation in PARP inhibited cells. These methods of inducing damage have been compared with recent real time studies using monophotonic laser induction of damage. Additionally, due to the increase in DNA damage induced by ionising radiation when chromatin is modified into a less compact structure, the effect of histone deacetylase (HDAC) inhibitors, which cause relaxation of chromatin, on repair involving XRCC1 was investigated in real time following both ultrasoft X-ray and NIR laser microbeam irradiation. Finally, the effect of knocking down the key LP-BER protein FEN1 was investigated in cancerous (A549) and non-cancerous (BEAS-2B) cells to see whether this had an effect on radiosensitivity and whether there was a difference in the radiosensitivity between the two cell lines. The more complex non-DSB clustered damage induced by ionising radiation is thought to rely more heavily on

LP-BER for repair (Byrne *et al*, 2009, Lomax *et al*, 2004a, Cunniffe *et al*, 2007, Imoto *et al*, 2008), so if this pathway is deficient then some of the damage induced by radiation may persist until replication and result in mutations or collapsed replication forks and replication-induced DSBs, which if unrepaired can also lead to cell death.

Chapter 2: Materials and Methods

2.1 – Culturing of mammalian cells

Cells were cultured in the appropriate culture medium (see section 2.1.2) in T75 flasks with a growth area of 75 cm². All cells were maintained at 37°C with 5% CO₂ humidified air in an incubator (Thermo Scientific, Forma Steri-Cycle HEPA Class 100) until the culture reached 70-80% confluency, determined by visualising the cells using an inverted microscope (Nikon Eclipse TS100).

2.1.1 – Cell culture reagents

Table 2.1: Cell culture reagents used during this research.

Reagent	Supplier
Dulbecco's Modified Eagle's Medium (DMEM) with 4500 mg/L glucose and sodium bicarbonate, without L-glutamine and sodium pyruvate, liquid, sterile-filtered, suitable for cell culture	Sigma Aldrich
Nutrient Mixture F-12 Ham with sodium bicarbonate, without L-glutamine, liquid, sterile-filtered, suitable for cell culture	Sigma Aldrich
Bronchial Epithelial Cell Basal Medium (BEBM)	Lonza
BEGM Single Quot Kit Supplements and Growth Factors: Bovine Pituitary Extract (13 mg/ml), 2 ml; Hydrocortisone, 0.5 ml; Human Epidermal Growth Factor (3 µg/ml), 0.5 ml; Epinephrine, 0.5 ml; Transferrin (10 mg/ml), 0.5 ml; Insulin, 0.5 ml; Retinoic Acid, 0.5 ml; Triiodothyronine, 0.5 ml; GA-1000 (30 mg/ml Gentamicin and 15 µg/ml Amphotericin), 0.5 ml.	Lonza
Foetal Bovine Serum (FBS)	Invitrogen
L-glutamine solution, liquid (200 mM)	Invitrogen
Penicillin (5000 units/ml)/ streptomycin (5000 µg/ml), liquid	Invitrogen
Trypsin/ethylenediamine-tetraacetic acid (EDTA) x 10, liquid	Invitrogen
G418-sulphate solution (50 mg/ml)	PAA Laboratories
Cell lysis buffer (10 x): 20 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1 mM Na ₂ EDTA, 1 mM EGTA, 1% Triton, 2.5 mM sodium pyrophosphate, 1 mM beta-glycerophosphate, 1 mM Na ₃ VO ₄ , 1 µg/ml leupeptin.	Cell Signaling Technology
Dimethyl Sulphoxide (DMSO) Hybri-Max®, cell culture tested	Sigma Aldrich

2.1.2 – Cell lines

Table 2.2: Cell lines used during this research.

Cell line	Culture medium	Tissue of origin	Comments
EMC11	DMEM supplemented with 10% FBS, 100 units/ml penicillin, 100 µg/ml streptomycin and 2 mM L-glutamine.	Chinese hamster ovary	Derived from CHO cell line. Deficient in XRCC1. Kind gift from G. Dianov.
EMC11 cells with YFP tagged XRCC1	DMEM supplemented with 10% FBS, 100 units/ml penicillin, 100 µg/ml streptomycin, 400 µg/ml G418 and 2 mM L-glutamine.	Chinese hamster ovary	Derived from CHO cell line. Deficient in XRCC1. Stably transfected with YFP tagged XRCC1 (See section 2.3.2).
CHO-K1	Nutrient mixture F12 Ham supplemented with 10% FBS, 100 units/ml penicillin, 100 µg/ml streptomycin and 2 mM L-glutamine.	Chinese hamster ovary	Repair proficient. Maintained by GIROB.
CHO-K1 cells with GFP tagged FEN1	Nutrient mixture F12 Ham supplemented with 10% FBS, 100 units/ml penicillin, 100 µg/ml streptomycin, 400 µg/ml G418 and 2 mM L-glutamine.	Chinese hamster ovary	Repair proficient. Stably transfected with GFP tagged FEN1 (See section 2.3.2).
HeLa	DMEM supplemented with 10% FBS, 100 units/ml penicillin, 100 µg/ml streptomycin and 2 mM L-glutamine.	Human cervix carcinoma	Maintained by GIROB.
A549	DMEM supplemented with 10% FBS, 100 units/ml penicillin, 100 µg/ml streptomycin and 2 mM L-glutamine.	Human lung carcinoma	Kind gift from A. Ryan.
BEAS-2B	BEBM basal medium containing BEGM Single Quot Kit Supplements and Growth Factors.	Human bronchial epithelium, normal	Obtained from ECACC.

2.1.3 – Cell passage

Cells were passaged 2-3 times weekly to ensure an exponentially growing population. For routine cell passage, the culture medium was aspirated and the cells washed with 10 ml of PBS in deionised H₂O at 37°C and pH 8.0 to remove residual levels of Ca²⁺ and Mg²⁺ as well as FBS which is inhibitory to trypsin. Following washing, 1 ml of trypsin/EDTA (x 2) in PBS was added to a T75 flask and the cells were incubated for 3 min at 37°C. Trypsin hydrolyses the peptide bonds that form when adherent cells attach to the surface of a flask causing the cells to detach from the flask and to be released into solution. The cells were diluted with 9 ml of culture medium and 1 ml of this cell suspension was added to a T75 flask containing 12 ml of culture medium.

2.1.4 – Cryogenic preservation of mammalian cells

Cells in exponential growth were harvested as described in section 2.1.3. The number of cells in suspension was counted using a haemocytometer. Mammalian cells were cryogenically preserved at 1×10^6 cells per vial. Cells were centrifuged for 3 min at 300 relative centrifugal force (rcf) (g-force) at room temperature. The culture medium was aspirated and the cell pellet was re-suspended in 1 ml per vial of complete medium containing 10% v/v DMSO. The cryogenic tubes were placed in a cryo container with a 1°C/min cooling rate to prevent crystallisation within the cells and hence cell lysis through rapid freezing. The cells were maintained at -80°C for 24 h and transferred into liquid nitrogen for long-term storage.

2.1.5 – Cell plating for irradiation of cycling cell populations

For all γ -radiation (see section 2.6.1) and NIR laser microbeam (see section 2.6.4) experiments, cells were plated into 30 mm diameter glass walled, number 1 glass cover-slip based dishes. A549 and EMC11 XRCC1-YFP tagged cells were plated at 2×10^5 cells/dish whereas the CHO FEN1-GFP tagged cells were plated at 1.5×10^5 cells/dish in 3 ml of medium (for specific culture medium see section 2.1.2). Cells were incubated for 24 h prior to irradiation at 37°C and in 5% CO₂ humidified air during which a monolayer of adherent cells was formed. For α -particle (see section 2.6.2) and ultrasoft X-ray (see section 2.6.3) irradiation, cells were plated into 30 mm diameter glass walled, 0.9 μ m Mylar based dishes to minimise energy absorption and attenuation of the radiation which would occur through glass cover-slip based dishes. A549 and EMC11 XRCC1-YFP tagged cells were plated at 1×10^5 cells/dish whereas the CHO FEN1-GFP tagged cells were plated at 7.5×10^4 cells/dish in 3 ml of medium (see section 2.1.2) at 37°C. Cells were incubated for 48 h prior to irradiation at 37°C and in 5% CO₂ humidified air during which a monolayer of adherent cells was formed.

2.1.6 – Cell plating to obtain enhanced G1-phase cell populations prior to irradiation

For ultrasoft X-ray irradiation experiments involving cells arrested in G1-phase of the cell cycle, EMC11 XRCC1-YFP tagged cells were plated at 1×10^5 cells/dish into 30 mm diameter glass walled, 0.9 μ m Mylar based dishes in 3 ml of medium (see section 2.1.2). The cells were incubated for 24 h at 37°C and in 5% CO₂ humidified air. To serum starve the cells, the medium was removed and the cells were washed 3 times in PBS before adding 3 ml of medium containing 1% FBS. The cells were then incubated for 48 h at 37°C and in 5% CO₂ humidified air prior to irradiation. Flow cytometry

analysis (see section 2.11) was used to determine the percentage of cells in each cell cycle phase.

2.1.7 – Inhibitors

PARP inhibitor KU-0058684 (KuDOS) was stored at - 20°C in 100% DMSO at a stock concentration of 10 mM.

Sodium butyrate (B5887, Sigma), a class I and II HDAC inhibitor, was stored at - 20°C in dH₂O at a stock concentration of 500 mM.

Trichostatin A (T8552, Sigma), a class I and II HDAC inhibitor, was stored at - 20°C in 100% DMSO at a stock concentration of 250 µM.

Nicotinamide (N3376, Sigma), a sirtuin inhibitor, was stored at - 20°C in dH₂O at a stock concentration of 1 M.

2.2 – Amplification and purification of plasmid DNA

2.2.1 - Preparation of electrocompetent bacteria

5 ml Luria-Bertani (LB) broth (10 g/l peptone, 5 g/l yeast extract, 10 g/l NaCl, Merck) was inoculated with the DH5α strain of *E. coli* and incubated at 37°C in a shaking incubator (220 rpm, New Brunswick Scientific model innova 4230) for 16 h. 1 ml of bacterial culture was then added to two flasks each containing 100 ml LB broth and incubated at 37°C in a shaking incubator at 220 rpm for ~ 2 h until an OD₅₉₅ between 0.5 and 0.7 was reached. The culture was divided into 4 x 50 ml centrifuge tubes followed by centrifugation at 5000 x g at 4°C for 10 min. The supernatant was removed and each cell pellet was resuspended in 50 ml ice cold distilled H₂O followed by

centrifugation at 5000 x g at 4°C for 10 min. The wash step was repeated a total of four times. After the final wash the pellets were resuspended in 1 ml of 10% glycerol, flash frozen on dry ice in 60 µl aliquots and stored at -80°C.

2.2.2 – Transformation of electrocompetent bacteria

100 ng of plasmid DNA (pEYFP-XRCC1 or FEN1-GFP, see section 2.3) was incubated with a 60 µl aliquot of DH5α electrocompetent cells on ice for 10 min. The sample was transferred to a 0.1 cm electroporation cuvette (BioRad) and the bacteria were transformed by electroporation at 1.8 kV using a BioRad *E. coli* pulser with time constraints of ~ 5 ms, followed by immediate addition of 500 µl SOB (2% w/v tryptone, 0.5% w/v yeast extract, 10 mM NaCl, 2.5 mM KCl). The sample was transferred to a 15 ml centrifuge tube and incubated in a shaking incubator (220 rpm, New Brunswick Scientific model innova 4230) for 1 h at 37°C. The pEYFP-XRCC1 plasmid contains a kanamycin resistance cassette and the FEN1-GFP plasmid contains an ampicillin resistance cassette. Transformants were selected for by plating 150 µl of the SOB containing electroporated bacteria on to LB agar (Sigma) containing 50 µg/ml kanamycin or 100 µg/ml ampicillin and incubated at 37°C for 16 h.

2.2.3 – Amplification and purification of plasmid DNA

From the above transformations, a single colony was used to inoculate an aliquot of 5 ml LB broth containing either 50 µg/ml kanamycin or 100 µg/ml ampicillin and incubated at 37°C in a shaking incubator (220 rpm, New Brunswick Scientific model innova 4230) for 8 h. 1 ml of bacterial culture was added to two conical flasks each containing 200 ml LB broth containing either 50 µg/ml kanamycin or 100 µg/ml

ampicillin and incubated at 37°C in a shaking incubator (220 rpm, New Brunswick Scientific model innova 4230) for 16 h at 37°C. The bacteria were harvested by centrifugation at 6000 x g for 15 min at 4°C. The plasmid DNA was then purified using a QIAfilter Plasmid Maxi Kit (QIAGEN). Each bacterial pellet was resuspended in 10 ml Buffer P1. To lyse the bacterial cells, 10 ml Buffer P2 was added to the resuspended bacteria and mixed thoroughly by vigorously inverting 6 times. The suspension was then incubated at room temperature for 5 min. During the incubation, a QIAfilter cartridge was prepared by screwing the cap onto the outlet nozzle of the QIAfilter cartridge, which was then placed in the QIArack. 10 ml chilled Buffer P3 was added to the bacterial lysate and mixed immediately and thoroughly by vigorously inverting 6 times. This results in precipitation of the genomic DNA, proteins, cell debris and the detergent potassium dodecyl sulphate. The lysate was immediately poured into the barrel of the QIAfilter cartridge and incubated at room temperature for 10 min. This allows time for the precipitate to float and form a layer at the top of the solution. During the incubation, a QIAGEN-tip column was equilibrated by applying 10 ml Buffer QBT and allowing the column to empty by gravity flow. After the 10 min incubation, the cap was removed from the QIAfilter Cartridge outlet nozzle and the plunger gently inserted into the cartridge. The cell lysate was filtered into the previously equilibrated QIAGEN-tip column. The cleared lysate was then allowed to enter the resin by gravity flow. The QIAGEN-tip column was then washed twice with 30 ml Buffer QC. The DNA was eluted with 15 ml Buffer QF and collected in a 50 ml centrifuge tube.

The DNA was precipitated by adding 10.5 ml (0.7 volumes) room temperature isopropanol (Fisher Scientific) to the eluted DNA. This was mixed and immediately

centrifuged at 15,000 x g for 30 min at 4°C. The supernatant was carefully decanted to prevent disturbing the DNA pellet. The DNA pellet was then washed with 5 ml room temperature 70% ethanol (Fisher Scientific) and centrifuged at 15,000 x g for 10 min. The supernatant was again carefully decanted to prevent disturbing the DNA pellet. The 70% ethanol removes precipitated salt and replaces isopropanol with the more volatile ethanol, making the DNA easier to redissolve. The DNA pellet was air-dried for 15 min and then redissolved in 200 µl of Tris-EDTA buffer (10 mM Tris-HCl (Sigma), 1 mM EDTA (Sigma) in dH₂O, pH 8.0). The concentration of the plasmid DNA was measured using a spectrophotometer (GeneQuant, Amersham Biosciences) and then the DNA was stored at – 20°C.

2.3 – Transfection of mammalian cells with plasmid DNA

The pEYFP-XRCC1 plasmid was a generous gift from Dr G. Dianov. To create this plasmid, the XRCC1 gene was cloned into the multiple cloning site of the pEYFP-N1 vector (BD Biosciences Clontech). The FEN1-GFP plasmid was purchased from Origene (RG201785).

2.3.1 – Transient transfection

EMC11 cells were plated into 30 mm diameter glass walled, number 1 glass cover-slip based dishes at 7.5×10^4 cells/dish in 3 ml of antibiotic-free medium (for specific medium see section 2.1.1). The cells were then incubated for 24 h at 37°C and in 5% CO₂ humidified air prior to transfection. For each dish of cells to be transfected, 4 µg pEYFP-XRCC1 DNA was diluted in Opti-MEM I Reduced Serum Medium (Invitrogen, Life Technologies) to a total volume of 250 µl. 10 µl Lipofectamine™ 2000 (Invitrogen, Life

Technologies) was diluted in Opti-MEM I Reduced Serum Medium to a total volume of 250 µl. This was incubated at room temperature for 5 min. The diluted DNA was combined with the diluted Lipofectamine™ 2000 and incubated for 20 min at room temperature. The complexes were then added directly to the culture medium of the cells in the glass dishes. After 6 h the medium was removed from the cells, the cells were washed with PBS and 3 ml of complete culture medium (see section 2.1.1) was added to the cells. The cells were incubated at 37°C and 5% CO₂ humidified air for a further 18 h to allow for expression of the YFP-tagged XRCC1 before irradiation (see section 2.6) and/or visualisation using confocal microscopy (see section 2.9).

2.3.2 – Creation of stable cell lines

EMC11 (for creation of EMC11 XRCC1-YFP tagged stable cell line) or CHO (for creation of CHO FEN1-GFP tagged stable cell line) cells were plated into 2 x 60 mm petri dishes (430166, Corning) at 5×10^5 cells/dish in 5 ml of complete growth medium (see section 2.1.2). The cells were incubated for 24 h at 37°C and in 5% CO₂ humidified air. 5 µg of DNA (XRCC1-YFP or FEN1-GFP) was diluted in Opti-MEM I Reduced Serum Medium (Invitrogen, Life Technologies) to a total volume of 150 µl. 20 µl of SuperFect Transfection Reagent (QIAGEN) was added to the diluted DNA and incubated for 10 min at room temperature to allow the transfection-complex formation. During the incubation, the medium was removed from the cells in one of the 60 mm petri dishes and the cells were washed with PBS. After the incubation time, 1 ml of complete growth medium was added to the transfection complexes and mixed by pipetting up and down twice. The transfection complexes were then transferred to the cells in the 60 mm dish. The cells were incubated for 3 h at 37°C and in 5% CO₂ humidified air. The

medium containing the transfection complexes was then removed, the cells washed with PBS and 5 ml of complete growth medium was added to the cells. The cells were incubated for a further 24 h at 37°C and in 5% CO₂ humidified air. Both the transfected cells and the untransfected control cells were then split 1:10 into 5 x 60 mm petri dishes each in 5 ml complete growth medium containing 400 µg/ml G418 (PAA Laboratories). G418 (Geneticin) was used to select for cells that had been transfected with the XRCC1-YFP or FEN1-GFP plasmids as both plasmids contain a neomycin resistance gene which confers resistance to G418. The medium was changed in the dishes daily, taking care not to pipette directly on to the cells. After 1-2 weeks all the untransfected cells had died, leaving colonies of stable cells in the transfected dish. Dilution cloning was then performed. The cells were trypsinised and counted as described in sections 2.1.3 and 2.1.4, then serial diluted to 0.8 cells/well into 2 x 96-well plates in 125 µl of complete growth medium containing 400 µg/ml G418. For example, for 100,000 cells in 10 ml, 2 ml cell suspension was added to 8 ml medium (1:5 dilution) to give 20,000 cells, then repeated twice to give 800 cells. Finally, a 1 ml cell suspension was added to 9 ml medium (1:10 dilution) to give 80 cells in 10 ml. 100 µl of this final cell suspension was added to each well of a 96 well plate to average 0.8 cells/well. This dilution should provide 30-40% of wells with 1 cell/well.

After a few days, the wells were checked and marked if they contained a single colony of cells. Once the colonies were large enough, the colonies were moved using a pipette tip into 24-well plates. Then after a few more days, when the wells were confluent, the cells were washed with PBS and then removed from the plate surface using 100 µl Trypsin-EDTA (see section 2.1.1) at 37°C. Half of each colony was split into

a T25 flask (growth area of 25 cm²) and the other half into a 30 mm diameter glass walled, number 1 glass cover-slip based dish. Once 50 – 70% confluent, the cells were viewed using a Biorad Radiance 2000 confocal microscope coupled to a Nikon TE2000 microscope. An argon ion (488 nm) laser was used to excite EGFP tagged and YFP tagged DNA repair proteins in the respective cells. If the clone showed nuclear fluorescence then half of the cells in the T25 flask were cryogenically frozen as described in section 2.1.4 and the other half were seeded into a T75 flask to be tested further so that the most appropriate clone was selected (see section 3.2 for clone selection).

2.4 – Western blotting

2.4.1 Western blotting reagents

Table 2.3: Western blotting reagents used during this research.

Reagent	Components (Sigma-Aldrich)
Laemmli Buffer	50 mM Tris (pH 6.8), 2% SDS, 0.1% bromophenol blue, 10% glycerol in dH ₂ O
TBS-T	1% Tween, 137 mM NaCl, 2.7 mM KCl, 3.7 mM Trizma-Base in dH ₂ O
Running Buffer	2 mM Trizma-Base, 250 mM Glycine, 0.1% SDS in dH ₂ O

2.4.2 Preparation of cell lysate

Cells were harvested as described in Section 2.1.3. The cells in each T75 flask to be lysed were diluted in 5 ml culture medium and transferred to a 15 ml centrifuge tube. The number of cells in suspension was counted using a haemocytometer (see section 2.1.4). The cells were centrifuged for 5 min at 600 relative centrifugal force (rcf) (g-force) at room temperature. The culture medium was aspirated using a pipette and

the cell pellet was resuspended in cell lysis buffer (Cell Signaling Technology, see Section 2.1.1). 1 x cell lysis buffer was diluted from 10 x stock in deionised H₂O immediately before use and chilled to 4°C. 400 µl of 1 x buffer was used for every 10⁷ cells. The lysate was transferred to a microcentrifuge tube, briefly vortexed, and then centrifuged for 10 min at 15,700 x g at 4°C. The supernatant was then transferred to a fresh microcentrifuge tube and stored at – 20°C.

2.4.3 Determination of protein concentration

The protein concentration was measured by performing a Bradford assay. The Bradford assay, a colorimetric protein assay, is based on an absorbance shift of the dye Coomassie Brilliant Blue G-250 in which under acidic conditions the red form of the dye is converted into its blue form to bind to the protein being assayed. The binding of the protein stabilizes the blue form of the Coomassie dye; thus the amount of the complex present in solution is a measure for the protein concentration, and can be estimated by use of an absorbance reading as follows.

2 µl of each lysate to be tested was added to an individual cuvette and diluted to 40 µl in deionised H₂O. 40 µl deionised H₂O was added to a cuvette and 40 µl 0.2 mg/ml BSA to another cuvette, to be used as the control. BioRad Protein Assay Reagent (diluted 1:5 in deionised H₂O) was added to each cuvette to make a final volume of 1 ml and incubated at room temperature for 5 min. An absorbance reading was made for each sample at OD₅₉₅ using a UV spectrophotometer (Ultraspec 2000, Pharmacia Biotech). The deionised H₂O sample was used to set the reference and the 0.2 mg/ml BSA protein standard was used to calculate the concentrations of each sample in relation

to this standard. The following calculation was used to determine the concentration of each lysate:

$$\text{Concentration} = (0.2 \text{ mg/ml} / \text{Abs}_{595} \text{ BSA}) \times \text{Abs}_{595} \text{ Sample} \times \text{Dilution Factor}$$

The volume of the lysates was doubled using Laemmli buffer (see section 2.4.1) containing 10% β -mercaptoethanol (added immediately before use) and so the final concentration was half that determined above. The lysates were boiled for 5 min at 100°C to denature the proteins before being used for Western blotting.

2.4.3 Western blotting

A mini-PROTEAN 3 system (BioRad) was used to cast and run 10-well, 0.75 mm gels.

The following gel solution was made to pour two 12% acrylamide gels:

Table 2.4: Reagents used to make a 12% gel solution.

Reagent	Volume
Acrylamide/Bis-acrylamide 30%, 37.5:1 (Thistle Scientific)	4 ml
1 M Tris (Sigma-Aldrich, made up in dH ₂ O, pH 8.8)	3.75 ml
20% SDS (Sigma-Aldrich, made up in dH ₂ O)	37.5 μ l
dH ₂ O	2.19 ml
10% Ammonium persulphate (Sigma-Aldrich, made up in dH ₂ O)	66.6 μ l
$\geq 99.0\%$ N,N,N',N'-Tetramethylethylenediamine (Fluka, Sigma-Aldrich)	6.7 μ l

The gel solution was immediately added into the gap between the short and long glass plates clamped in the gel-casting apparatus (mini-PROTEAN 3, BioRad) to 1 cm from top of short plate. The gap was filled with isopropanol (Fisher Scientific) to remove any air bubbles and the gels were left to set for 40 min. The isopropanol was removed and the gels rinsed with dH₂O before drying with blotting paper. Stacking gel solution was made as follows:

Table 2.5: Reagents used to make the stacking gel solution.

Reagent	Volume
Acrylamide/Bis-acrylamide 30%, 37.5:1 (Thistle Scientific)	835 μ l
1 M Tris (Sigma-Aldrich, made up in dH ₂ O, pH 6.8)	625 μ l
20% SDS (Sigma-Aldrich, made up in dH ₂ O)	50 μ l
dH ₂ O	2.515 ml
10% Ammonium persulphate (Sigma-Aldrich, made up in dH ₂ O)	25 μ l
$\geq 99.0\%$ <i>N,N,N',N'</i> -Tetramethylethylenediamine (Fluka, Sigma-Aldrich)	6 μ l

Stacking gel mixture was added to fill the 1 cm gap at the top of the plates and a 10-well comb was inserted. This was left to set for 10 min. Once set, the comb was removed and the wells washed with dH₂O before transferring the gel to a gel tank (BioRad). The inner chamber was filled up to the top with running buffer (see section 2.4.1) and 200 ml running buffer was added to the outer chamber. The first lane of the gel was loaded with 3 μ l molecular weight marker (Prestained Protein Molecular Weight Marker, Fermentas), then the cell lysates (see sections 2.4.2 and 2.4.3) were loaded onto the gel with any spare wells being filled up with Laemmli buffer (see section 2.4.1). The gels were run at 125 V for 2-3 hours depending on the size of the protein to be detected (FEN1 2 hours, XRCC1 3 hours).

Proteins were transferred using an iBlot[®] 7-Minute Blotting System (Invitrogen) onto nitrocellulose (iBlot[®] transfer stack, Invitrogen). For FEN1 Program 3 was used for 5 min and for XRCC1 Program 3 was used for 7 min. The membrane was blocked in 1:1 Odyssey[®] Blocking Buffer (LI-COR): PBS for 1 h at room temperature. The blots were

then incubated with primary antibody (rabbit anti-FEN1 (ab17993, Abcam) 1:2000 dilution; mouse anti-XRCC1 (Ab-3 (Clone 144), Thermo Scientific) 1:1000 dilution; mouse anti-beta-actin (ab8226, Abcam) 1:500 dilution) in 1:1 Odyssey Blocking Buffer (LI-COR): TBS-T (see section 2.4.1) overnight at 4°C. The blots were washed 3 times with TBS-T (see section 2.4.1) for 5 min at room temperature followed by incubation with secondary antibody (goat anti-Rabbit IRDye® 800CW (LI-COR); goat anti-Mouse IRDye® 680 (LI-COR) diluted 1:10,000 in 1:1 Odyssey Blocking Buffer (LI-COR): TBS-T (see section 2.4.1) for 45 min at room temperature in the dark. The blots were washed another 3 times with TBS-T (see Section 1.4.1) for 5 min at room temperature. The blots were then scanned using the Odyssey® Sa Infrared Imaging System (LI-COR).

2.5 – siRNA

The FEN1 siRNA (s5103, Silencer Select, Applied Biosystems) and scrambled siRNA control (Negative Control, Silencer Select, Applied Biosystems) used in these experiments were diluted to a 5 µM stock in RNase free, sterile, dH₂O (Applied Biosystems).

For siRNA optimisation, cells were plated into two 6-well plates at 2×10^5 cells/well in 2 ml antibiotic free medium and incubated for 24 h at 37°C and in 5% CO₂ humidified air. For transfection, three 1.5 ml centrifuge tubes containing 15 µl of 5 µM stock FEN1 siRNA (for a 30 nM/well final concentration of siRNA), three 1.5 ml centrifuge tubes containing 7.5 µl of 5 µM stock FEN1 siRNA (for a 15 nM/well final concentration of siRNA) and three 1.5 ml centrifuge tubes containing 15 µl of 5 µM stock scrambled siRNA (for a 30 nM/well final concentration of siRNA) were each diluted in Opti-MEM I Reduced Serum Medium (Invitrogen, Life Technologies) to a total volume of 250 µl.

Ten 1.5 ml centrifuge tubes containing 5 μ l Lipofectamine™ 2000 (Invitrogen, Life Technologies) for the HeLa and A549 cells or 5 μ l Lipofectamine™ RNAiMAX (Invitrogen, Life Technologies) for the BEAS-2B cells were diluted in Opti-MEM I Reduced Serum Medium (Invitrogen, Life Technologies) to a total volume of 250 μ l. These were incubated at room temperature for 5 min. Each tube of diluted siRNA was combined with a tube of diluted Lipofectamine™ 2000/Lipofectamine™ RNAiMAX, and the final tube of diluted Lipofectamine™ 2000/Lipofectamine™ RNAiMAX had 250 μ l Opti-MEM I Reduced Serum Medium added to make the Lipofectamine only control. These were then incubated for 20 min at room temperature to form siRNA/Lipofectamine transfection complexes. The complexes were then added directly to the culture medium of the cells in the appropriate wells. After 6 h the medium was removed from the cells, the cells were then washed with PBS before 3 ml of complete culture medium (see section 2.1.1) was added to the cells. The cells were incubated at 37°C and 5% CO₂ humidified air until they were lysed (see section 2.4.2) at 24 h, 48 h or 72 h post-transfection.

For cell survival and RAD51 foci assays, cells were plated into T75 flasks at 1.5×10^6 cells/flask in 15 ml antibiotic free medium and incubated for 24 h at 37°C and in 5% CO₂ humidified air. For transfection, 56.3 μ l of 5 μ M stock siRNA was diluted in Opti-MEM I Reduced Serum Medium (Invitrogen, Life Technologies) to a total volume of 1875 μ l (so that there was a 15 nM final concentration of siRNA added to the cells in the flask). 37.5 μ l Lipofectamine™ 2000 (Invitrogen, Life Technologies) for the HeLa and A549 cells or Lipofectamine™ RNAiMAX (Invitrogen, Life Technologies) for the BEAS-2B cells was diluted in Opti-MEM I Reduced Serum Medium to a total volume of

1875 µl. This was incubated at room temperature for 5 min. The diluted siRNA was combined with the diluted Lipofectamine™ 2000/Lipofectamine™ RNAiMAX and incubated for 20 min at room temperature. The complexes were added directly to the culture medium of the cells in the flask. After 6 h the medium was removed from the cells, the cells were washed with PBS and 15 ml of complete culture medium (see section 2.1.1) was added to the cells. The cells were incubated at 37°C and 5% CO₂ humidified air for a further 30 h before seeding them for the clonogenic cell survival or RAD51 foci assays. The cells would then be γ-irradiated 48 h after siRNA transfection (optimised in section 6.2).

2.6 – Irradiation of mammalian cells

2.6.1 – Low LET γ-ray irradiation

Cells were plated as described in section 2.1.5 into 30 mm diameter glass walled, number 1 glass cover-slip based dishes. Cells were cooled to 10°C for 10 min prior to irradiation to slow cellular processes including DNA repair. Cells were irradiated at room temperature in the culture medium with ¹³⁷Caesium γ-rays (GSR D1 irradiator, Gamma-Service Medical GmbH) at a dose rate of 1.7 Gy min⁻¹ over a dose range of 0.5-10 Gy and placed on ice immediately after irradiation to prevent DNA repair. Following irradiation, the culture medium was replaced with medium pre-warmed to 37°C. Time 0 was recorded immediately following addition of warmed medium to the cells, and the cells were incubated at 37°C and in 5% CO₂ humidified air for the stated repair times. The cells were fixed for immunofluorescent staining as described in section 2.8.

2.6.2 – High LET alpha irradiation

Cells were plated as described in section 2.1.5 into 30 mm diameter glass walled, 0.9 μm Mylar based dishes. Cells were maintained at room temperature for 10 min prior to irradiation to prevent condensation forming on the Mylar base, which would absorb a small proportion of the energy from the α -particle. Irradiations were carried out at room temperature with ^{238}Pu α -particles ($120 \text{ keV } \mu\text{m}^{-1}$) at a 20° angle to the source. The irradiation point was determined as the position of the dish closest to the irradiation source. DNA damage produced by α -particles forms along distinct ‘tracks’ and little damage occurs outside of the track. This is due to the nature of the α -particle radiation, which produces very few δ -rays. The dose to the cells was calculated by measuring the dose directly through the Mylar base as the dose of the α -particle varies with distance from the source. The variation in the characteristics of the α -particles at varying distances from the irradiation point was calculated by Dr. M. Hill, as changes in energy occur as the distance the particle travels increases:

Table 2.6: Characteristics of high LET α -particles at 0 mm and 10 mm from the irradiation point.

	0 mm from irradiation point	10 mm from irradiation point
Entrance energy (MeV)	3.25	1.75
Incident LET (keV/ μm)	125	150
Range in sample (μm)	19	10

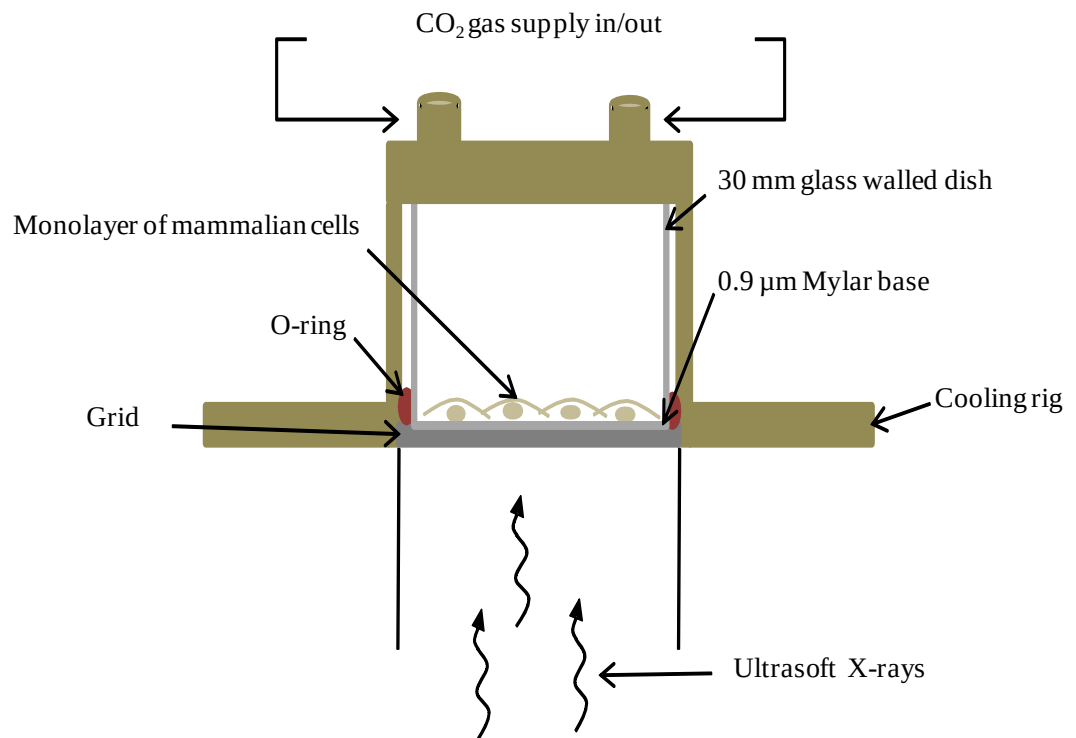
The range of the α -particle track also varies with increased distance from the irradiation point, e.g. 19 μm at 0 mm from the irradiation point and 10 μm at 10 mm from the irradiation point. At 10 mm, the α -particle will traverse a distance of 10 μm within a cell and therefore will only show as tracks across the entire nucleus in cells

less than 10 μm in diameter. Irradiation at 20° induces a change in the cellular morphology causing a reduction in the length of the α -particle track due to increased absorption of the energy. The α -particles do not traverse the entire cell as the energy is absorbed and are visualised as tracks of DNA damage within the nucleus. Following irradiation, the dishes were incubated at 37°C for the stated repair time and fixed for immunofluorescent staining as described in section 2.8.

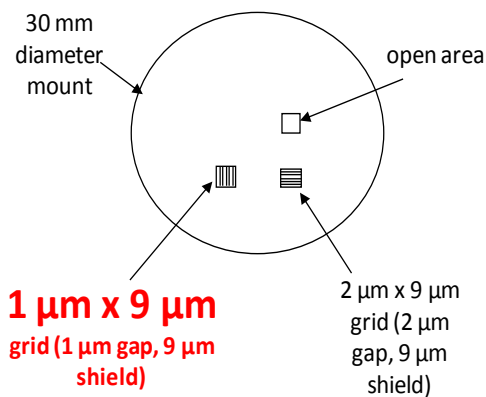
2.6.3 – Ultrasoft X-ray irradiation

The GIROB ultrasoft X-ray set up (Figure 2.1a) consists of a cold cathode discharge tube that has an aluminium foil transmission target producing Alk shell (1.49 keV) ultrasoft X-rays with a bremsstrahlung contamination of <1% (Botchway et al., 1997, de Lara et al., 2001). The X-rays are produced by low energy electron bombardment of the target and travel through a flight tube, which is continuously flushed with helium at atmospheric pressure to reduce debris X-ray transmission and reduce attenuation of low energy X-rays (Botchway et al., 1997, de Lara et al., 2001). Ultrasoft X-rays are highly attenuated in air at 99.9% compared with $\leq 2\%$ in helium. The ultrasoft X-ray set-up contained a dish holder directly above the ultrasoft X-ray source that was thermostatically controlled by a pumped water circulator. A 1 μm x 9 μm gold grid was used in these shielding experiments and was placed in the bottom of the dish holder (Figure 2.1b). A continuous flow of helium into the O-ring of the dish holder held the dish in position creating a gas seal. The dose rate was calculated by Dr. M. Hill using an air filled ion chamber with a 0.248 mg cm^{-2} window for the Alk shell ultrasoft X-rays. The ionisation current was measured before each experiment using an electrometer (Keithley 616) with an ion chamber window 1 mm behind an empty Mylar based dish.

(a)



(b)



(c)

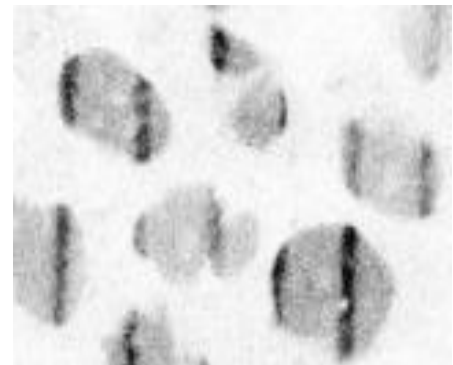


Figure 2.1: Ultrasoft X-ray irradiation. (a) Cartoon schematic of the ultrasoft X-ray set-up used for these experiments (Mark Hill). (b) Diagram of the gold grid used to shield the cells during irradiation. The ultrasoft X-rays can only pass through the gaps in the grid. The 1 μm by 9 μm grid was used. (c) Confocal image of EMC11 XRCC1-YFP tagged cells taken 1 min after irradiation with ultrasoft X-rays through the 1 μm by 9 μm grid. The black stripes represent XRCC1-YFP recruited to sites of DNA damage.

The dose rate was calculated with correction for variation in the ultrasoft X-ray intensity across the base of the Mylar dish. The nominal dose was 10 Gy min⁻¹.

Cells were plated (as described in sections 2.1.5 and 2.1.6) into dishes with a 0.9 µm Mylar base. Cells were cooled and maintained at 10°C before and during irradiation with Al_K shell ultrasoft X-rays and irradiated in culture medium at the stated dose through a 1 µm x 9 µm gold grid (Figure 2.1b). The irradiations using the 1 µm x 9 µm gold grid allowed the investigation of XRCC1-YFP and FEN1-GFP recruitment to low LET induced DNA damage above background fluorescence levels (Figure 2.1b and Figure 2.1c). Shielding experiments with the grid using hard X-rays, γ-radiation and α-particle radiation is difficult due to the highly penetrating nature of these radiations. The final nominal dose to the line produced via the grid is 1/10 the dose to the whole cell so that 100 Gy to the dish is equivalent to 10 Gy to the line (in the unshielded area). Following irradiation, culture medium was replaced with 3 ml of culture medium warmed to 37°C. Time 0 was recorded immediately following addition of warmed medium (37°C) to the cells and images were taken at the stated times post irradiation using confocal microscopy (Radiance Eclipse 2000, Bio-Rad) equipped with an argon ion laser at 488 nm, HeNe (helium/neon) laser at 543 nm and a second HeNe laser at 633 nm (for image collection see section 2.9.1). Throughout the real time imaging of XRCC1-YFP and FEN1-GFP following irradiation at 10°C, the cells were maintained at 37°C.

2.6.4 – NIR multiphoton laser microbeam irradiation

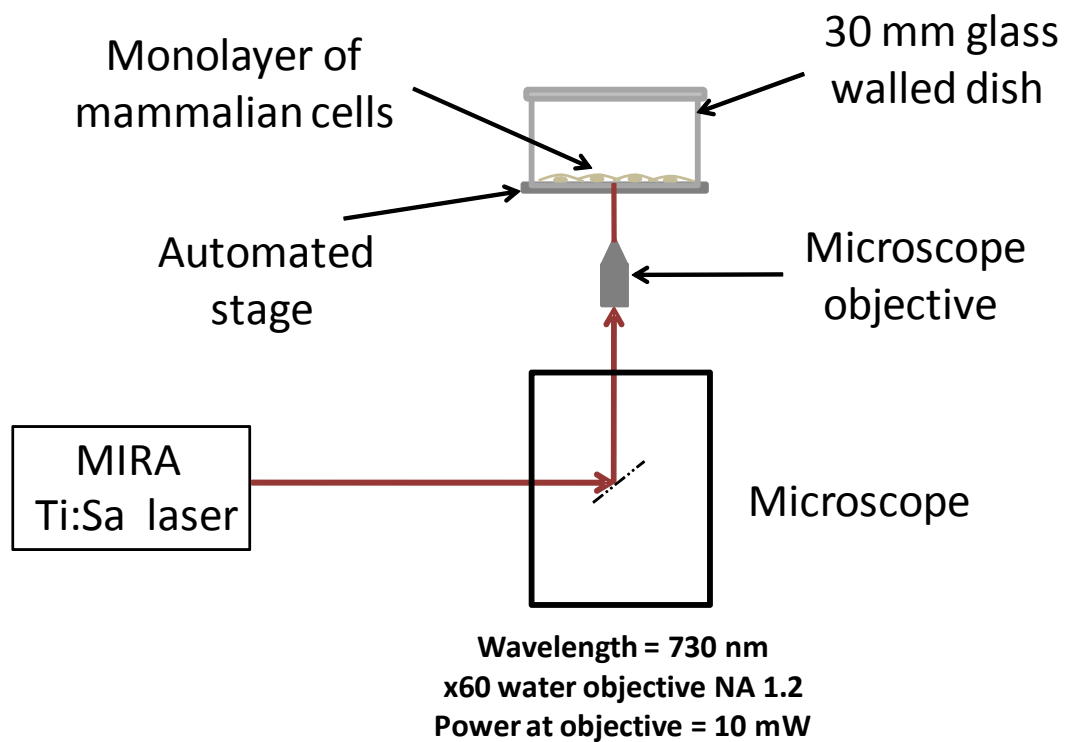
The laser microbeam (Harper *et al*, 2008) used in these experiments consists of a high-powered Ti:Sa (Titanium:Sapphire) laser (Mira 900, Coherent Lasers, UK) tuneable

between 680 - 1100 nm. The laser process is achieved by excitation of the Ti:Sa complex at approximately 530 nm to pump the molecules into the excited state. This is achieved by using a secondary laser source (Verdi V18, Coherent Lasers, solid-state Neodymium:Vanadate). The Ti:Sa laser produces ultra-short pulses with a repetition rate of 76 MHz and a pulse width of 180 femtoseconds. The Ti:Sa laser system can operate in either continuous wave or mode-locked. Continuous wave operation allows a large continuous wave to pass through the system whereas mode-locked systems work on a narrow beam at a specific frequency. The Ti:Sa system is a self mode-locking laser and mode-locking was used for these experiments. Mode-locking occurs when the longitudinal light waves within the laser cavity are simultaneously added to produce a coherent intense pulse. The laser is then passed through a series of lenses, mirrors, slits and neutral density filters into an Eclipse TE2000 (Nikon) inverted microscope (Figure 2.2a).

The laser beam was focused through a high numerical aperture microscope objective to induce two-photon excitation, as this requires the photons to be concentrated temporally and spatially. Enough photons must be produced within a short time frame to supply multiple photons within the focal point for two photons to be absorbed simultaneously by the same molecule. This is achieved with the fluence produced at peak powers of ultrafast mode-locked laser pulses. A wavelength of 730 nm was chosen as NIR light is transparent to mammalian cells and therefore no or negligible cellular damage would be produced through a one-photon excitation process.

Cells were plated as described in section 2.1.5 into 30 mm diameter glass walled, number 1 glass cover-slip based dishes. 10 µg/ml Hoechst 33258 dye (Sigma-Aldrich)

(a)



(b)

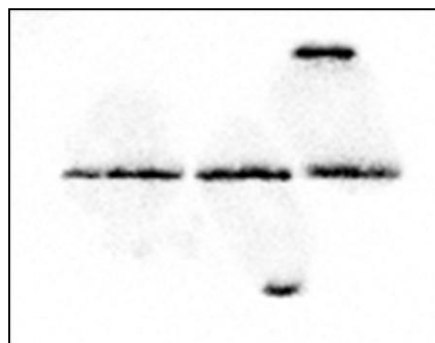


Figure 2.2: NIR multiphoton laser microbeam irradiation. (a) Cartoon schematic of the NIR multiphoton laser microbeam set-up used for these experiments (Stan Botchway, RAL). (b) Confocal image of EMC11 XRCC1-YFP tagged cells taken 1 min after NIR multiphoton laser microbeam irradiation. The black stripes represent XRCC1-YFP recruited to sites of DNA damage.

was added 10 min prior to irradiation at 37°C. Hoescht 33258 is a DNA intercalator and although Hoescht 33258 dye does not absorb at 730 nm, the high peak intensities together with the ultrashort pulsed light of the laser pulses allows multiphotons to be simultaneously absorbed resulting in excitation of the dye molecules. Hoescht 33258 dye was therefore incorporated into DNA to photochemically induce high levels of DNA damage. With peak optical absorbance of Hoescht 33258 at 365 nm, the major multiphoton processes using the 730 nm ultrashort pulsed laser methodology involves two-photon excitation. A red bandpass filter (RG610) was placed on top of the culture dish to prevent Hoechst dye from absorbing UV light from ambient light.

A software package, Wincommander, was used to set parameters for automated movement of the microscope stage over a small area in the X- and Y-plane over an area of 100 μm in the X-plane with a step size of 12 μm for the CHO cells and 16 μm for the EMC11 XRCC1-YFP tagged cells, as the nuclei of these cells were larger. Each movement of the stage was controlled individually using the Wincommander software allowing for more precise irradiation of a small number of cells and faster image collection (see Figure 2.2b for an example image of XRCC1-YFP recruitment to DNA damage sites induced by NIR multiphoton laser microbeam irradiation in EMC11 XRCC1-YFP tagged cells).

Cells were maintained at 37°C throughout the irradiation using a temperature control chamber connected to a pumped water circulator (Neslab RTE7 Digital One, Thermo Scientific). The time between irradiation of the cells and image collection was limited to a few seconds as the microscope used for irradiation with the NIR laser microbeam was also used for confocal imaging (see section 2.9.1). The laser was set to a

wavelength of 730 nm and a nominal power of 10 mW measured through the x 40 air, numerical aperture 0.95, microscope objective using a power meter (Molelectron, Power Max with a 500 PM10 head). Cells were irradiated in culture medium using a x 60 water, numerical aperture 1.2, microscope objective. This was used to finely focus the laser within the centre of the nucleus of individual cells and the higher magnification allowed more accurate data collection during confocal microscopy due to the higher resolving power (numerical aperture 1.2) than with the x 40 (numerical aperture 0.9) objective. Time 0 was recorded immediately following irradiation of the cells (less than 10 s) and images were collected at the stated times following irradiation using confocal microscopy (EC1, Nikon) equipped with an argon ion laser at 488 nm and HeNe (helium/neon) laser at 543 nm (for image collection see section 2.9.1).

2.7 – Clonogenic cell survival

Clonogenic survival assays were performed to determine survival following low LET γ -ray irradiation. Cells were seeded into 60 mm petri dishes (430166, Corning), at pre-optimised cell numbers (see table 2.7), in triplicate for each dose in 5 ml culture medium (for specific culture medium see section 2.1.2). Cell numbers seeded per dish were chosen so that there were a countable number of colonies at the end of the assay. Too few colonies would reduce statistical significance, while too many would mean that they could not be counted properly because they tend to merge in to one another. CHO, EMC11 and EMC11 XRCC1-YFP tagged cells were incubated for 8 h at 37°C and in 5% CO₂ humidified air prior to irradiation. A549 and BEAS-2B cells were incubated for 15 h at 37°C and in 5% CO₂ humidified air prior to irradiation. The cells

were irradiated with $^{137}\text{Caesium}$ γ -rays (see section 2.6.1). Six doses were used in the range of 0-10 Gy as illustrated in table 2.3. After irradiation, cells were incubated at 37°C and in 5% CO_2 humidified air until the surviving cells produced macroscopic colonies that could be readily counted. A countable colony is defined as a group of 50 or more cells originating from a single cell. The medium was removed from the petri dishes and the cells were washed with PBS. 1 ml of crystal violet stain (0.5% in ethanol) was added to each dish and incubated at room temperature for 30 min. The stain was removed and the dishes were rinsed with tap water then left to dry. Colonies were identified and counted using a colony counter (Gallenkamp). The plating efficiency (PE) for each cell line was calculated using the following equation applied to the non-irradiated control:

$$\text{PE} = \frac{\text{number of colonies counted}}{\text{number of cells seeded}} \times 100$$

This PE was used to calculate a surviving fraction (SF) for each irradiated population, allowing for the plating efficiency of the non-irradiated controls:

$$\text{SF} = \frac{\text{number of colonies counted}}{\text{number of cells seeded} \times (\text{PE}/100)}$$

The cell survival data was plotted as the logarithm of the surviving fraction versus dose.

Table 2.7 – Seeding densities for each radiation dose and cell line used in the clonogenic survival assay.

Dose (Gy)	Number of cells plated				
	CHO	EMC11	EMC11 XRCC1-YFP	A549	BEAS-2B
0	100	150	100	100	150
0.5	100	300	100	100	150
1	150	500	200	150	200
2	200	1000	300	300	300
5	500	3000	500	750	750
10	5000	10000	5000	7500	7500

2.8 – Immunocytochemistry for labelling proteins with fluorescent molecules post irradiation

2.8.1 – Labelling of XRCC1 and γ H2AX

Following incubation at 37°C for the required repair times post irradiation the cells were washed with PBS then fixed in 1 ml of 3% paraformaldehyde (Sigma-Aldrich) in PBS at 4°C for 30 min. The cells were then washed 3 times with 1 ml PBS for 5 min at room temperature and then permeabilised with 500 μ l of 1% Triton X-100 (Sigma-Aldrich) in PBS for 10 min at room temperature. The cells were washed a further 3 times with 1 ml PBS for 5 min at room temperature and then blocked for 1 h at room temperature in 500 μ l of 1% BSA (Sigma-Aldrich) and 1% FSG (Sigma-Aldrich) in PBS. Cellular proteins were labelled by addition of 200 μ l of the primary antibody (mouse anti-XRCC1 (ab1838, Abcam) diluted as 1:100, rabbit anti- γ H2A.X (phospho S139,

ab11174, Abcam) diluted as 1:500) in 1% BSA and 1% FSG in PBS overnight at 4°C. The cells were washed 3 times with 1 ml PBS for 5 min at room temperature followed by the addition of 200 µl of secondary antibody (goat anti-mouse FITC labelled (Jackson ImmunoResearch Laboratories), goat anti-rabbit Cy5 labelled (Millipore)) diluted 1:100 in 1% BSA and 1% FSG in PBS overnight at 4°C in the dark. Cells were further washed 3 times with 1 ml PBS for 5 min at room temperature. A drop of Vectorshield® mounting medium containing DAPI (Vector Laboratories) was added to each dish and the cells were covered with a 25 mm diameter number 1 glass cover-slip. Cells were viewed using a laser scanning Biorad Radiance 2000 confocal microscope (BioRad) with a x 40 oil objective (see section 2.9.2).

2.8.2 – Labelling of RAD51 and Cyclin A

Following incubation at 37°C for the required repair times post irradiation the cells were washed with PBS then fixed in 1 ml of 3% paraformaldehyde (Sigma-Aldrich) in PBS-T (0.1% Triton X-100 in PBS) for 15 min at room temperature. The cells were then washed twice with 1 ml PBS for 10 min at room temperature and then permeabilised with 500 µl of 0.3% Triton X-100 (Sigma-Aldrich) in PBS for 10 min at room temperature. The cells were then blocked with 3% BSA in PBS for 40 min at room temperature. Cellular proteins were labelled by addition of 200 µl of the primary antibody (rabbit anti-RAD51 (sc8349, Santa Cruz), mouse anti-cyclin A (UPC391, Vector Laboratories) diluted 1:50 in 3% BSA in PBS, overnight at 4°C. Cells were washed 3 times with 1 ml PBS for 10 min at room temperature followed by the addition of 200 µl of secondary antibody (goat anti-mouse FITC labelled (Jackson ImmunoResearch Laboratories), goat anti-rabbit Cy5 labelled (Millipore)) diluted 1:100 in 3% BSA in PBS

overnight at 4°C in the dark. Cells were further washed 3 times with 1 ml PBS for 10 min at room temperature. A drop of Vectorshield® mounting medium containing DAPI (Vector Laboratories) was added to each dish and the cells were covered with a 25 mm diameter number 1 glass cover-slip. Cells were viewed using a laser scanning Biorad Radiance 2000 confocal microscope (BioRad) with a x 40 oil objective (see section 2.9.2).

2.9 – Microscopy

Visualisation of cellular proteins was carried out using confocal microscopy.

2.9.1 – Visualisation of proteins in real time

In the real time NIR laser microbeam irradiation, cells containing a fluorescently tagged protein were laser irradiated in the presence of Hoescht 33258 dye at 37°C and simultaneously imaged using a Nikon Eclipse C1 confocal microscope coupled to a Nikon TE2000 microscope. Following ultrasoft X-ray irradiation, fluorescently tagged proteins in cells were viewed using a Biorad Radiance 2000 confocal microscope coupled to a Nikon TE2000 microscope. An argon ion (488 nm) laser was used to excite GFP tagged and YFP tagged DNA repair proteins in the respective cells. The pinhole and gain of the confocal microscope was adjusted to obtain optimal fluorescence without saturation of the pixels. The images were collected using three Kalman filtered scans. Kalman filter averages a number of scans thus increasing the signal to noise ratio, resulting in a less grainy image. Images were collected at the stated times following initial radiation with a minimum of 10 cells visualised for analysis per experiment (for analysis see section 2.10).

2.9.2 – Visualisation of proteins in fixed cells

Fixed cells and immunofluorescently labelled proteins were viewed using a laser scanning Biorad Radiance 2000 confocal microscope coupled to a Nikon TE2000 microscope. The confocal microscope was equipped with a 405 nm laser to view DAPI labelled DNA, an argon ion (488 nm) laser to view FITC/GFP/YFP labelled proteins, HeNe (543 nm) laser to view Cy3 labelled proteins and a HeNe (633 nm) laser to view Cy5 labelled proteins. The pinhole and gain of the microscope were adjusted using a control sample to obtain optimal fluorescence without saturation of the pixels as a standard set-up procedure. The images were collected by separate laser scans to prevent bleed through from the fluorophores and using three Kalman filtered scans. A minimum of 3 images were collected per sample containing approximately 100 cells. For γ -radiation, the number of foci/nucleus was determined using ImageJ software (National Institutes of Health).

2.10 – Quantification of protein intensity from real time confocal images

Real time measurements of protein recruitment and loss were determined by collecting images in real time using confocal microscopy until the relative fluorescence reached minimal levels and was not able to be accurately measured. The confocal microscope images of recruitment and loss of proteins in real time were analysed by measuring the intensity of the fluorescently tagged protein of interest using Quantity One[®] software. The real time experiments looked at the fluorescence intensity of the protein along a radiation track, within one individual cell over the repair time course, with a minimum of 10 cells analysed per experiment. In an individual cell, the intensity along the track was measured by selecting the entire track at each time point post

irradiation. The nuclear background intensity was determined by selecting an unirradiated area within the cell nucleus (Figure 2.3a). The intensity of the protein along the track was calculated by subtracting the nuclear background from the intensity of the track. The intensity of the track minus the nuclear background was calculated for every time point collected post irradiation. The relative intensity of the track for each cell was normalised to the maximum fluorescence intensity determined at earlier times (maximum relative fluorescence of 1). This was to minimise variations due to differences in fluorescence within different cells in the same experiment and across experiments. The average fluorescence of the cells was then calculated and this was then normalised to the maximum fluorescence intensity determined at earlier times to give a representation of the loss of relative fluorescence of a population of cells. A minimum of three experiments were carried out and the data is expressed as the mean together with the standard error of the mean.

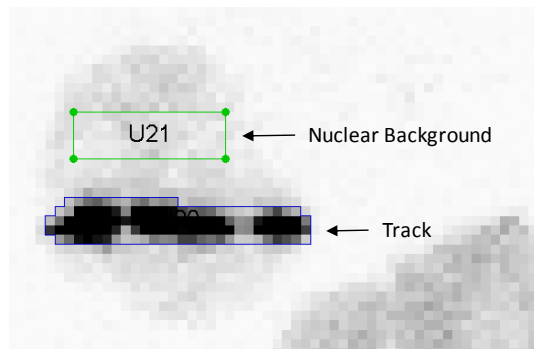
The kinetic analysis of the normalised data was carried out using Origin® software (Figure 2.3b). The exponential decay fits were initially fitted using either simplified single exponential decay:

$$y = y_0 + Ae^{-x/t}$$

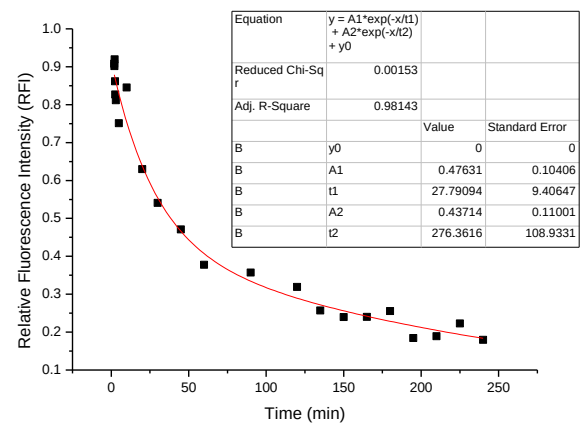
or bi-exponential decays:

$$y = y_0 + Ae^{-x/t_1} + Ae^{-x/t_2}$$

(a)



(b)



(c)

$$\text{Amplitude for reaction 1} = \frac{A1}{(A1 + A2 + Y0)}$$

$$\text{Amplitude for reaction 2} = 1 - \frac{A1}{(A1 + A2 + Y0)}$$

$$\text{Rate constant (k)} = \frac{1}{T1 \text{ or } T2}$$

$$\text{Half life} = \frac{\ln 2}{k}$$

Figure 2.3: Analysis of the real time recruitment and loss of fluorescently tagged proteins. (a) Quantity One® analysis of the intensity of proteins recruited in real time following irradiation. The tracks were selected along with a nuclear background reading and the intensity in the selected area was calculated. (b) Kinetic analysis of the loss of relative fluorescence from the normalised data using Origin®. (c) Equations for calculating the amplitude of each reaction, the rate constant of each reaction and the half life of the exponential decays.

assuming in the first instance that the fluorescence decays to background levels at longer times. This provided an initial estimate of the lifetime and enabled a judgement to be made as to whether a bi-exponential fit is more appropriate. During the fitting process, data that appeared to have residual levels of protein fluorescence (Y_0) at the latest time point analysed, had the Y_0 values set to a residual in 0.1 increments to determine the best fit for the exponential decay. Each fit was analysed according to the R-Squared (R^2) value (R^2 should be 1 for a perfect fit), and the error on the half life, as well as the amplitude, to determine the accuracy of the exponential decay fit. A double exponential decay fit was applied if a single exponential fit could not be achieved using the same principles as above by comparing the accuracy of each fit. The rate constant for the reaction, and for bi-exponential decay the amplitude percentage for each component, was then calculated (Figure 2.3c).

2.11 – Determination of cell cycle phase by flow cytometry analysis

Cells were plated as described in section 2.1.6 into 30 mm diameter glass walled, 0.9 μm Mylar based dishes with four dishes used for each flow cytometry sample. Cells were pulse labelled with 10 μM BrdU (Invitrogen, Life Technologies) for the final 30 min in culture at 37°C prior to fixation. BrdU is only incorporated into cycling cells and therefore will only label S-phase cells. Cells were harvested as described previously in section 2.1.3 followed by centrifugation at 600 rcf for 5 min at 4°C. The medium was aspirated then the cell pellet was resuspended in 100 μl PBS (residue left in tube after discarding the supernatant). The cells were fixed in 500 μl ice cold 70% ethanol added dropwise (to avoid clumping) while vortexing. Cells were then incubated at 4°C for a minimum of 30 min. The fixative was removed by centrifugation at 600 rcf for 5 min at 4°C followed by addition of 1 ml 2 M hydrochloric acid at room temperature for 20

min. The reaction was stopped by the addition of 2 ml PBS at room temperature followed by centrifugation at 600 rcf for 5 min at room temperature. The pellet was resuspended in 1 ml 2% FBS in PBS followed by centrifugation at 600 rcf for 5 min at room temperature. The pellet was resuspended in 100 µl mouse anti-BrdU monoclonal antibody (347580, BD Biosciences) diluted 1:100 in 2% FBS in PBS and incubated for 90 min at room temperature. 1 ml 2% FBS in PBS was added followed by centrifugation at 600 rcf for 5 min. The pellet was resuspended in 100 µl of goat anti-mouse AlexaFluor 488 antibody diluted 1:200 in 2% FBS in PBS and incubated for 60 min at room temperature in the dark. Cells were washed in 1 ml PBS followed by centrifugation at 600 rcf for 5 min. Cells were resuspended in 500 µl PBS containing 5 µl of a 1 mg/ml stock of propidium iodide at room temperature and analysed using a Becton Dickinson FACS Sort. The percentage of cells in each cell cycle phase was calculated using Cell QuestTM software (Becton Dickinson).

Chapter 3: Effect of different types of radiation on recruitment and loss of BER proteins at DNA damage sites in mammalian cells

3.1 Introduction

As discussed in detail in Chapter 1, the complexity of DNA damage is influenced by what is causing the damage, such as the type of radiation, and can affect the repair processes of the damage. Endogenous DNA damage is generated by free radicals that arise as by-products of oxidative metabolism. The damage is in the form of isolated lesions that can be readily repaired. In contrast, ionising radiation produces clustered DNA damage where two or more lesions are located within one or two helical turns of DNA by passage of a single radiation track. Lesions within clusters are the same as those induced endogenously, typically strand breaks, abasic sites or oxidised bases (Lomax *et al*, 2002, Shikazono *et al*, 2009, Weinfeld *et al*, 2001), but repair of lesions within clustered damage sites can be compromised (Byrne *et al*, 2009, Chaudry and Weinfeld, 1997, David-Cordonnier *et al*, 2001, David-Cordonnier *et al*, 2002, David-Cordonnier *et al*, 2000, Harrison *et al*, 1999, Lomax *et al*, 2004a, Lomax *et al*, 2004b, Peddi *et al*, 2008) and this can potentially lead to mutations and replication-induced DSBs if the damage persists into S-phase (Bellon *et al*, 2009, Budworth *et al*, 2005, D'Souza and Harrison, 2003, Eccles *et al*, 2009, Harrison *et al*, 2006, Pearson *et al*, 2004, Malyarchuk *et al*, 2003, Shikazono *et al*, 2006).

The yield and complexity of clustered DNA damage can be related to the LET of the radiation (more detail in Chapter 1). Low LET radiation (such as γ -radiation) generates a low concentration of ionisation events where ~70% of the energy is sparsely deposited along the radiation tracks causing isolated lesions that can be readily

repaired. However, 30-40% of the energy is produced as low-energy electrons that form short secondary electron tracks (delta tracks) that result in clustering of the ionisation events and, therefore, more complex damage. With high LET radiation (such as α -radiation) there is a high concentration of ionisation events along the radiation track and, therefore, 90% of the DNA damage is clustered, and the complexity of some of the clusters is greater than those produced by low LET radiation.

The BER pathway recognises and repairs base modifications, AP sites and DNA single-strand breaks present in clustered damage sites as well as those caused by endogenous processes (Dianov *et al*, 2001, Lindahl and Wood, 1999, Wilson and Bohr, 2007, Zharkov, 2008). BER is initiated with damage recognition and base excision by a DNA glycosylase and subsequent incision of the phosphodiester backbone 5' of the AP site by an AP endonuclease, generating 3'-hydroxyl and 5'-deoxyribose-5-phosphate (5'dRP) termini. A single nucleotide is incorporated into the DNA by Pol β and repair is completed by DNA ligase III α in complex with XRCC1. XRCC1 has also been shown to have an early role in the pathway where it forms a molecular scaffold for recruitment of the other proteins involved in SP-BER (Campalans *et al*, 2005).

SP-BER is the most commonly used BER pathway in mammalian cells but LP-BER is required in situations where the AP site contains a sugar-phosphate group that is altered in a way that prevents repair by the SP-BER proteins (Klungland and Lindahl, 1997, Liu *et al*, 2008). LP-BER involves the synthesis of a repair patch containing two or more nucleotides. Following insertion of a base by Pol β there is a polymerase switch to Pol δ/ϵ , which insert up to 10 nucleotides, displacing a section of DNA into a flap structure that is then processed by FEN-1, before the nick is sealed by DNA ligase I.

PCNA binds to and enhances the activity of FEN-1 (Klungland and Lindahl, 1997 and Hutton *et al*, 2008). FEN-1 has been shown to interact with Pol β (Liu *et al*, 2005) and can promote strand displacement synthesis by Pol β (Balakrishnan *et al*, 2009). Pol β in turn promotes FEN-1 cleavage of the displaced strand (Balakrishnan *et al*, 2009).

As the rate of repair of clusters is slower than that for repair of single lesions (Eccles *et al*, 2009, Harrison *et al*, 1999, Lomax *et al*, 2004a, Lomax *et al*, 2004b), and the damage complexity increases as the ionisation density increases, the aim of this part of my project is to investigate the repair kinetics of simple and clustered DNA damage induced by different types of radiation using the SP-BER protein XRCC1 and the LP-BER protein FEN1 as markers of the two pathways. To do this, the recruitment and loss of these BER proteins, in real time, to DNA damage sites induced by different types of radiation was investigated.

3.2 Creation of cell lines stably expressing XRCC1-YFP or FEN1-GFP

To enable investigation of the recruitment and loss of XRCC1 in real time to sites of DNA damage, cells were transfected with a plasmid encoding a YFP-tagged form of XRCC1 (see section 2.3). EMC11 cells were chosen as they are a Chinese Hamster Ovary (CHO) cell line deficient in XRCC1 (Zdzienicka *et al*, 1992). The deficiency is caused by a C390Y substitution which results in XRCC1 protein instability (Berquist *et al*, 2010). Therefore, in the absence of endogenous XRCC1, only YFP-tagged XRCC1 will be recruited to DNA damage sites in real time experiments, allowing it to be visualised and quantified.

Initially, EMC11 cells were transiently transfected with the pEYFP-XRCC1 plasmid (see Section 2.3.1). The advantage of transient transfection is that the cells can be used for experiments 24 h post-transfection. However, when imaged on a confocal microscope, it was found that only ~ 20% of cells are successfully transfected based on the relative number of DAPI stained cells to green fluorescent cells (Figure 3.1a). Further, the majority of green fluorescent cells over express the YFP-tagged XRCC1 protein (Figure 3.1a). Therefore, an EMC11 cell line stably transfected with XRCC1-YFP was created (see section 2.3.2). The EMC11 XRCC1-YFP clone selected has a similar level of XRCC1-YFP expression in every cell (Figure 3.1b) and the expression is much lower than that seen in the majority of transiently transfected cells (Figure 3.1a).

Cell lysates from CHO cells, EMC11 XRCC1-YFP stably transfected cells and EMC11 cells were immunoblotted for XRCC1 and XRCC1-YFP (Figure 3.1c). This was done to verify that the fluorescence detected in the cell nuclei using confocal microscopy (Figure 3.1b) was YFP-tagged XRCC1 and to compare the level of XRCC1-YFP in the clone to endogenous levels of XRCC1 in the parental CHO cell line. In addition it was confirmed that untransfected EMC11 cells do not express endogenous XRCC1 protein. As expected, there is an endogenous XRCC1 band in the CHO cell lysate lane but not in the EMC11 cell lysate lane, confirming that EMC11 cells are deficient in XRCC1 (Zdzienicka *et al*, 1992). The XRCC1-YFP band migrates higher than the endogenous XRCC1 band due to the 27 kDa YFP tag, as seen in the EMC11 XRCC1-YFP cell lysate lane (Figure 3.1c). The XRCC1-YFP band appears to be slightly more intense than the endogenous XRCC1 in the CHO cell lysate lane but does not appear to be obviously over expressed (Figure 3.1c). An antibody to GFP (also detects YFP-tagged proteins)

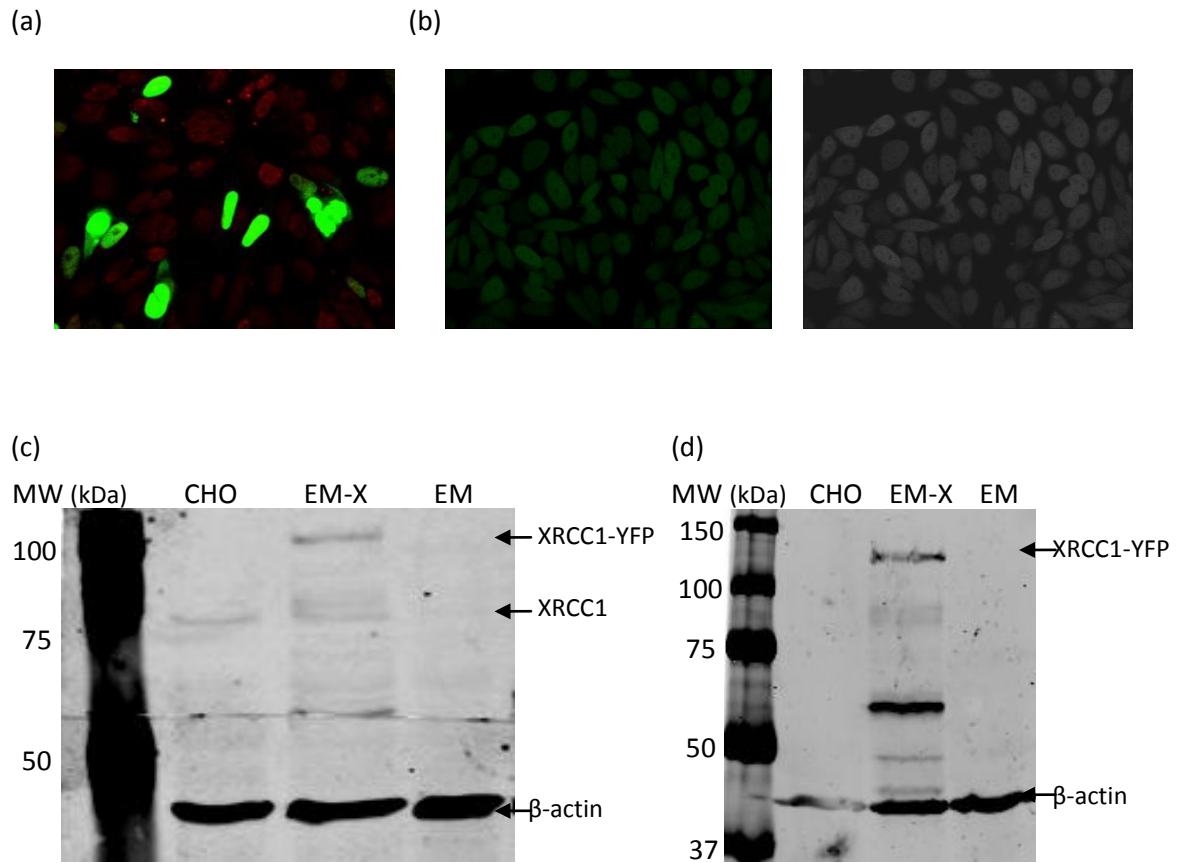


Figure 3.1: Generation of a cell line stably expressing XRCC1-YFP. (a) Confocal microscope image of EMC11 cells transiently transfected with XRCC1-YFP. The red fluorescence represents DAPI stained nuclei, the green fluorescence represents cell nuclei expressing XRCC1-YFP. (b) Confocal microscope image of the selected clone of EMC11 cells stably transfected with XRCC1-YFP (left image). The right image shows DAPI stained nuclei. (c) Western blot showing the levels of XRCC1 and XRCC1-YFP in cell lysates from CHO cells, EMC11 cells stably transfected with XRCC1-YFP (EM-X) and EMC11 cells (EM). β -actin was used as a loading control. (d) Western blot of the same cell lysates used in (c), showing all bands detected by the GFP antibody. β -actin was used as a loading control.

was used to establish that the bands seen in the EMC11 XRCC1-YFP cell lysate lane at approximately the same position as endogenous XRCC1 in the CHO cell lysate lane (Figure 3.1c) were due to expression of XRCC1-YFP rather than endogenous XRCC1 (Figure 3.1d).

To test the functionality of the XRCC1-YFP tagged protein in the stably transfected EMC11 cell line, a clonogenic cell survival assay was performed (see section 2.7). This assay compared the radiosensitivity of the EMC11 XRCC1-YFP stable clone with the XRCC1 deficient EMC11 cells and CHO cells, which have endogenous XRCC1. As can be seen in Figure 3.2, the EMC11 cells stably expressing XRCC1-YFP have a higher survival rate at all doses of γ -radiation used compared to that for the EMC11 (XRCC1 deficient) cells. The survival enhancement ratio (SER) at a surviving fraction of 0.1 is 1.3 between the EMC11 and EMC11 XRCC1-YFP cell lines, showing that XRCC1-YFP is functioning in the EMC11 XRCC1-YFP stable clone. The SER is 1.2-1.3 between the CHO and EMC11 XRCC1-YFP cell lines, with the CHO cells being less radiosensitive than the EMC11 XRCC1-YFP cells. This difference in survival may be due to an effect of the YFP tag on the exogenously introduced XRCC1. In addition, genetic abnormalities in the EMC11 cells that occurred before stable transfection of XRCC1-YFP may have affected their cell survival after γ -radiation. The SER between the CHO and EMC11 cells is 1.7.

A cell line stably expressing FEN1-GFP was also generated (see section 2.3.2). Cells that are completely deficient in FEN1 are not available, therefore CHO cells were used to make the results more comparable with the XRCC1-YFP cell line, as the EMC11 cells are derived from CHO cells. Figure 3.3a shows confocal microscopy images of three clones that have been stably transfected with FEN1-GFP. These clones were chosen to

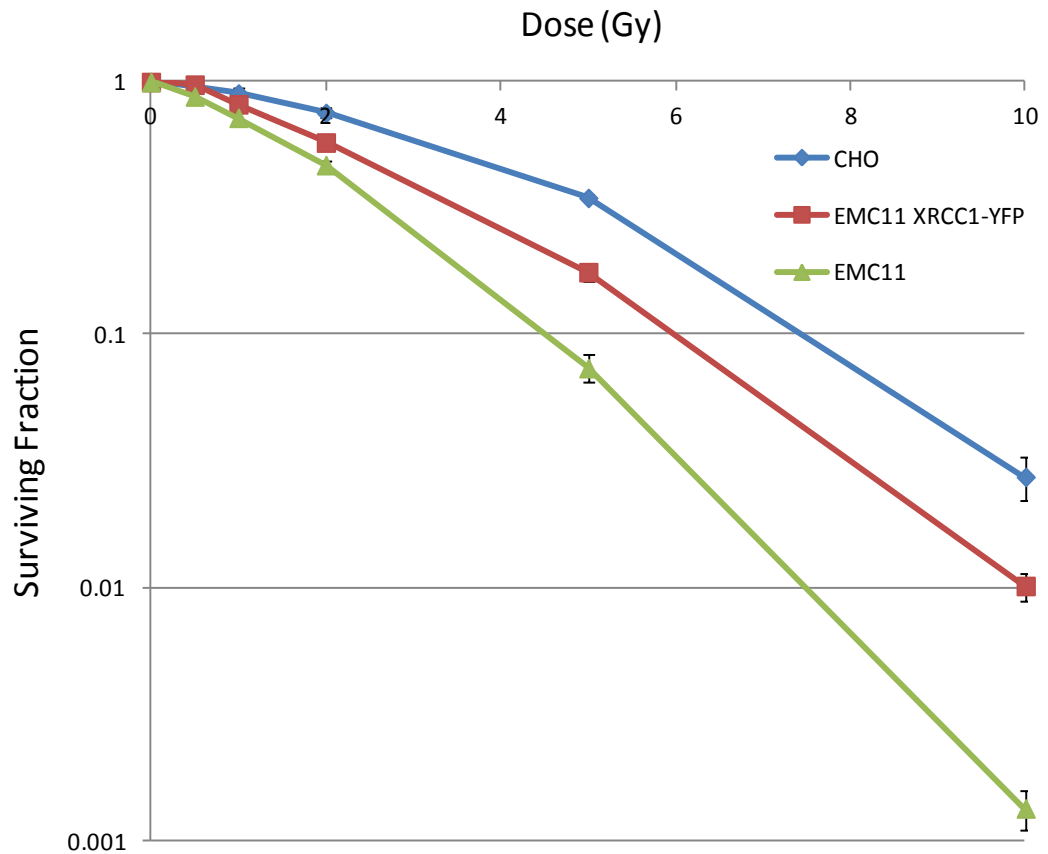


Figure 3.2: Clonogenic cell survival assay comparing the cell survival of CHO, EMC11 and EMC11 cells stably transfected with XRCC1-YFP after increasing doses of γ -irradiation. The graph represents the mean of 3 independent experiments $\pm$ SEM. EMC11 cells stably expressing XRCC1-YFP have a higher survival rate at all doses used compared to untransfected EMC11 (XRCC1 deficient) cells. This provides evidence for the functionality of XRCC1-YFP in the stably transfected EMC11 cells.

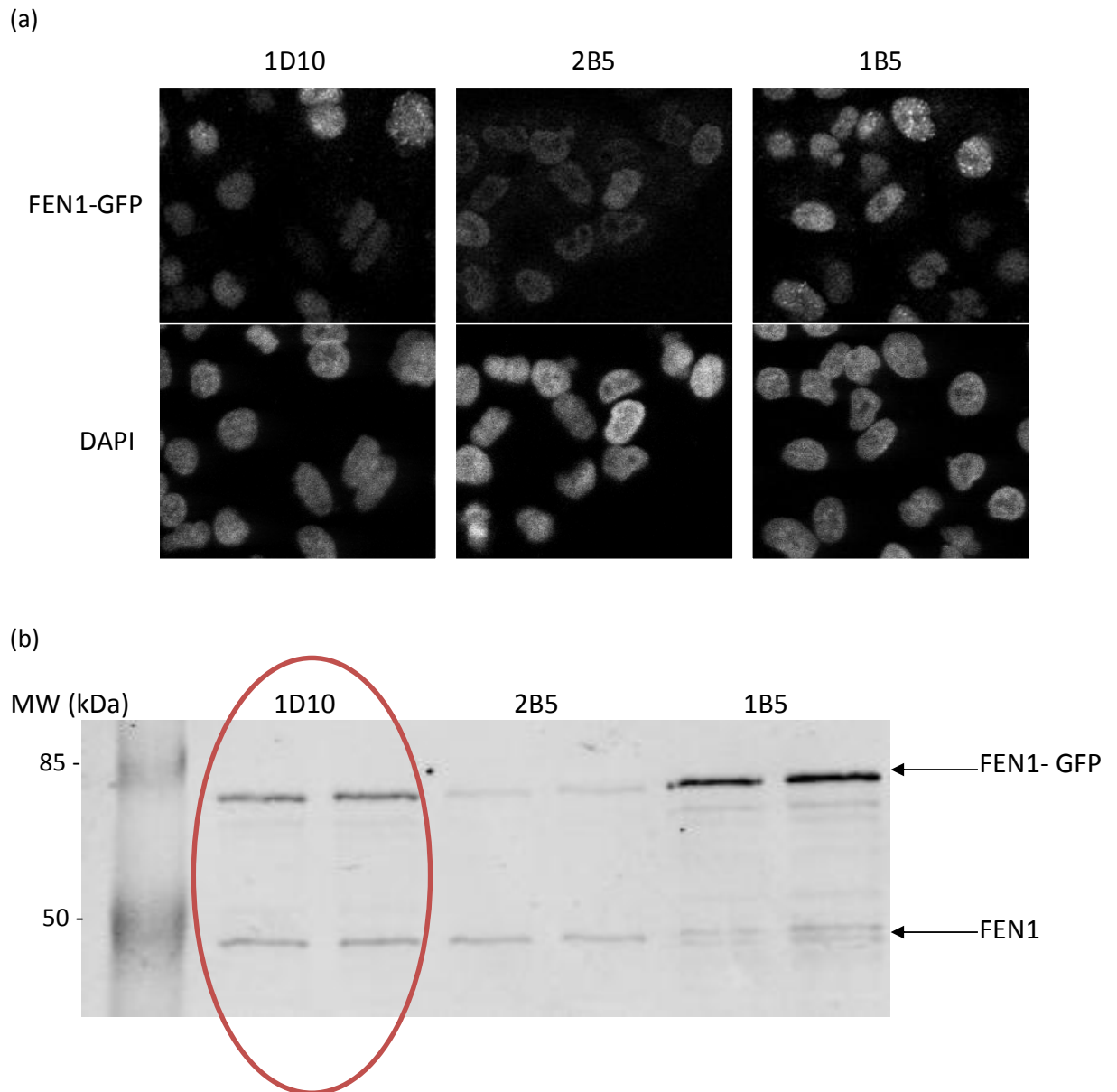


Figure 3.3: Selection of a cell line stably expressing FEN1-GFP. The confocal microscope images in (a) represent three clones of CHO cells stably expressing FEN1-GFP that were selected due to the FEN1-GFP expression being localised to the nucleus and consistent across all cells in the clone. Cell lysates from these clones were immunoblotted for FEN1 in (b) to compare FEN1-GFP levels relative to endogenous FEN1. The 1D10 clone was selected for use in experiments due to the FEN1-GFP levels in this clone being the most similar to endogenous FEN1, when compared with the other two clones. All cell lysates were loaded in duplicate.

be tested further as they show nuclear expression of FEN1-GFP which appears to be expressed at an even level across all the cells in each clone (Figure 3.3a). Not all clones surviving selection with G418 (see section 2.3.2) have nuclear expression of FEN1-GFP. Many show perinuclear expression of FEN1-GFP or an equal expression of FEN1-GFP in both the nucleus and cytoplasm, indicating that the FEN1-GFP protein in these cells is not functioning as expected. Lysates were made from each of the clones shown in Figure 3.3a and these were immunoblotted for FEN1 (Figure 3.3b) to compare FEN1-GFP levels relative to that of endogenous FEN1. The 1D10 clone was selected for use in experiments as the FEN1-GFP levels in this clone are similar to endogenous FEN1. Clone 2B5 under expresses FEN1-GFP and clone 1B5 over expresses FEN1-GFP. Over expression of FEN1 has been shown to cause DSBs (Vispé *et al*, 2003).

3.3 Investigation of the recruitment and loss of BER proteins at DNA damage sites induced in mammalian cells by different types of radiation

Once the XRCC1-YFP and FEN1-GFP stable cell lines had been generated, they were irradiated with different types of radiation that varied in LET and the complexity of DNA damage formed.

γ -radiation is a form of low LET radiation where ~70% of the damage is in the form of isolated lesions. ¹³⁷Caesium γ -rays (see section 2.6.1) were used to irradiate both the EMC11 XRCC1-YFP tagged and CHO FEN1-GFP tagged cell lines. Cells were plated into glass dishes (as in section 2.1.5) 24 h prior to irradiation with 1 Gy or 10 Gy and fixed 2 min, 5 min, 15 min and 30 min following irradiation. Cells were stained for γ H2AX (see section 2.8.1), a marker of DSB, followed by imaging for γ H2AX and XRCC1-YFP using confocal microscopy (see section 2.9.2). H2AX is phosphorylated (γ H2AX) after

formation of DSBs and many molecules, often thousands, of H2AX become phosphorylated for each DSB. This makes it easy to detect γ H2AX using an antibody to γ H2AX, seen as γ H2AX nuclear foci. γ -H2AX foci was detected at all doses and time points tested, with the intensity and number of γ -H2AX foci increasing with time and dose (Figures 3.4 and 3.5) indicating that DNA damage has been induced. In contrast, XRCC1-YFP (Figure 3.4) or FEN1-GFP (Figure 3.5) were not detected as nuclear foci above background levels at any of the doses or time points tested. Therefore, ¹³⁷Caesium γ -ray irradiation could not be used as a form of low LET radiation to investigate recruitment and loss of XRCC1-YFP and FEN1-GFP at DNA damage sites in mammalian cells.

²³⁸Plutonium α -particles (see section 2.6.2) were used as a high LET source of ionising radiation, where ~90% of the lesions formed are clustered. Both the EMC11 XRCC1-YFP tagged and CHO FEN1-GFP tagged cell lines were irradiated with α -particles using a dose of 1 Gy and the foci detected 5 min post-irradiation (Figure 3.6). Cells were stained for γ -H2AX (see section 2.8.1) and γ -H2AX, XRCC1-YFP and FEN1-GFP were imaged using confocal microscopy (see section 2.9.2). The arrows show γ -H2AX localised to DNA damage tracks caused by the alpha-particle irradiation (Figure 3.6). XRCC1-YFP (Figure 3.6a) or FEN1-GFP (Figure 3.6b) foci tracks cannot be seen to localise to DNA damage induced by 1 Gy of α -particles above nuclear background levels. Therefore, ²³⁸Plutonium α -particle irradiation could not be used to investigate XRCC1-YFP and FEN1-GFP recruitment and loss at DNA damage sites in mammalian cells following high LET radiation.

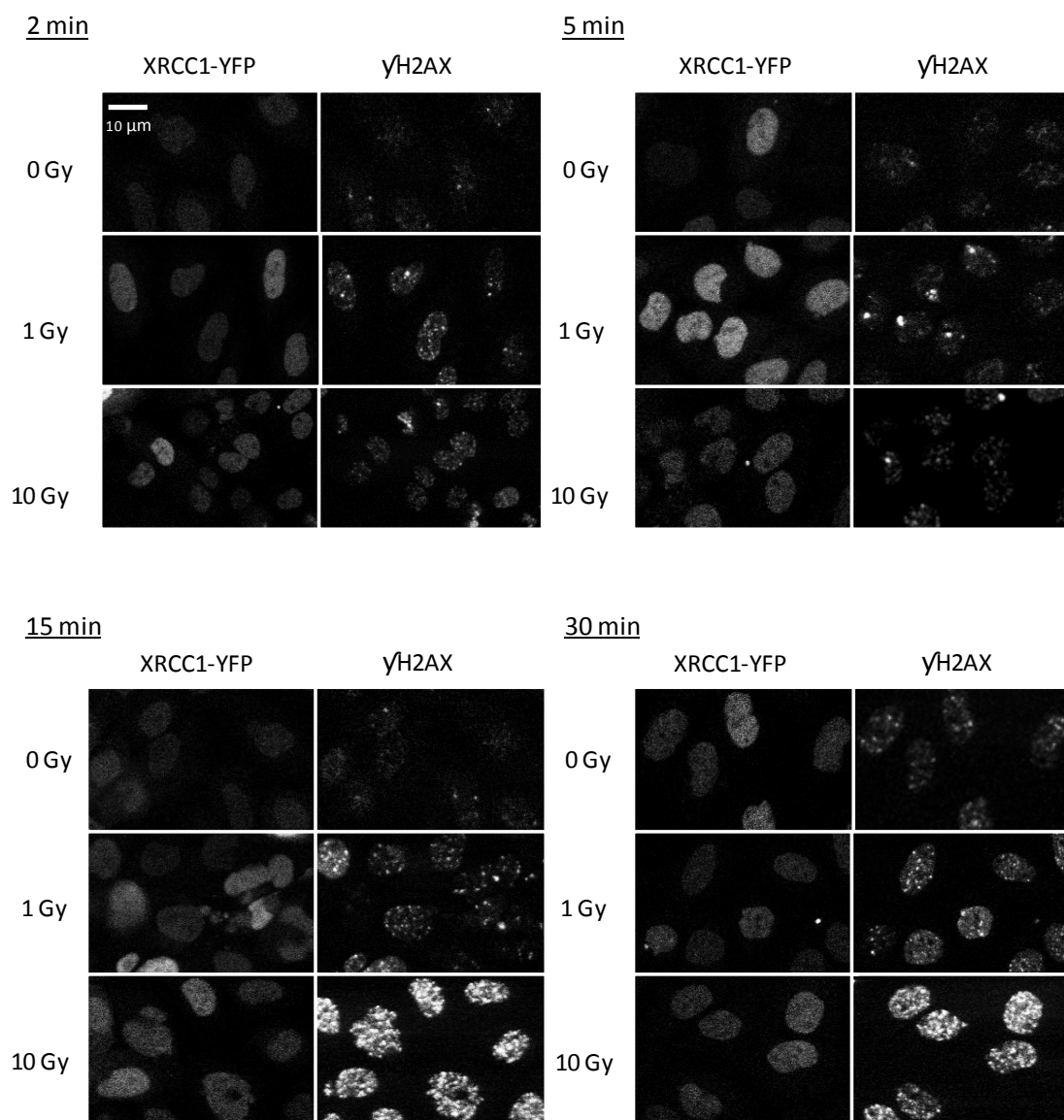


Figure 3.4: ¹³⁷Caesium γ-ray irradiation of EMC11 XRCC1-YFP tagged cells. Cells were irradiated at the specified doses, incubated at 37°C in 5% CO₂ humidified air and fixed in 3% paraformaldehyde after the specified repair times. Cells were stained for γ-H2AX formation. Confocal microscope images were taken to detect localisation of XRCC1-YFP and γ-H2AX at DNA damage in the cell nuclei. The intensity and number of γ-H2AX foci increase with time and dose whereas XRCC1-YFP foci were not detected above background levels at any of the times or doses used.

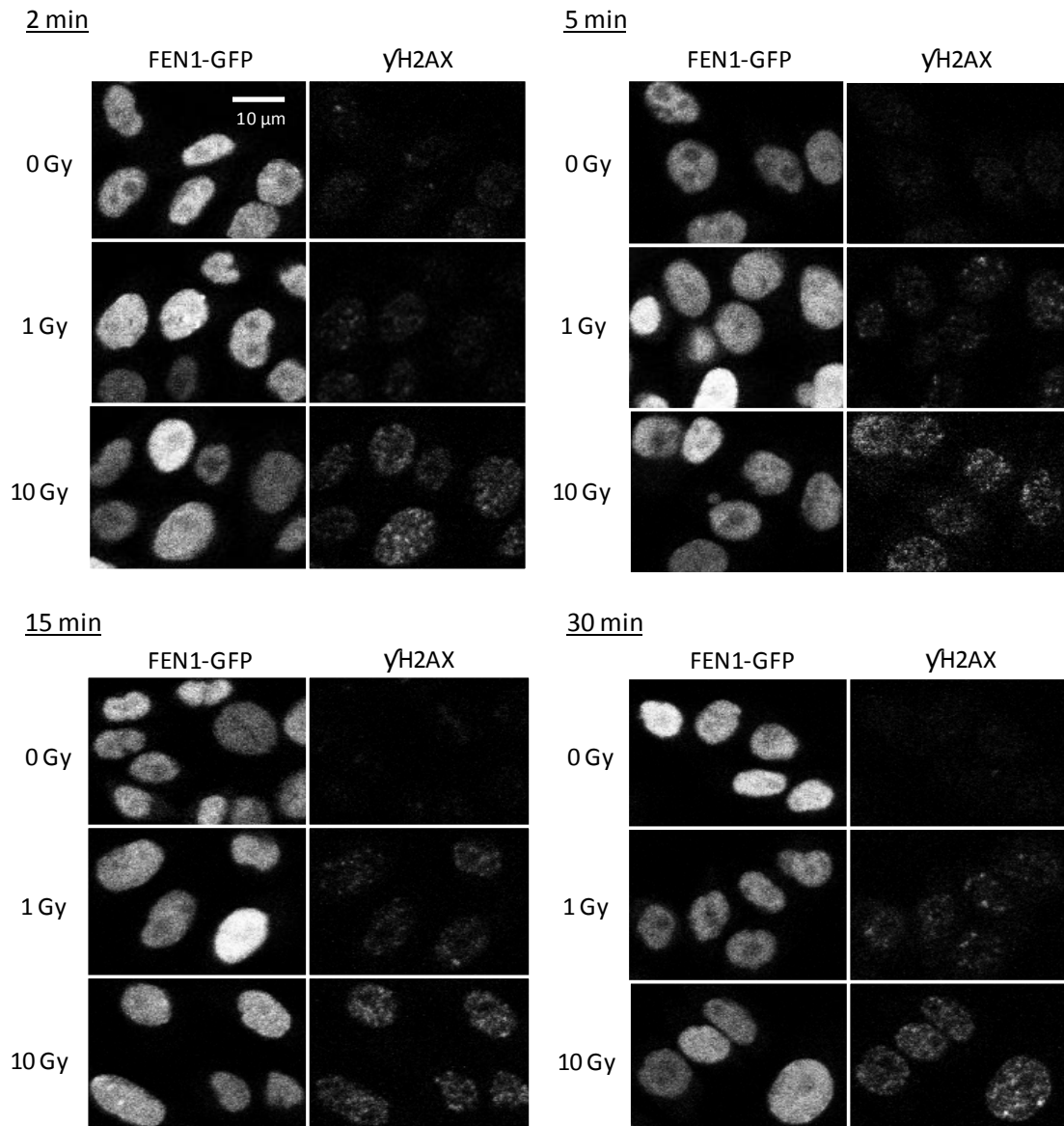


Figure 3.5: ¹³⁷Caesium γ-ray irradiation of CHO FEN1-GFP tagged cells. Cells were irradiated at the specified doses, incubated at 37°C in 5% CO₂ humidified air and fixed in 3% paraformaldehyde after the specified repair time. Cells were stained for γ-H2AX formation. Confocal microscope images were taken to detect localisation of FEN1-GFP and γ-H2AX in the cell nuclei. The intensity and number of γ-H2AX foci increase with time and dose whereas no FEN1-GFP foci can be detected above background levels at any of the times or doses used.

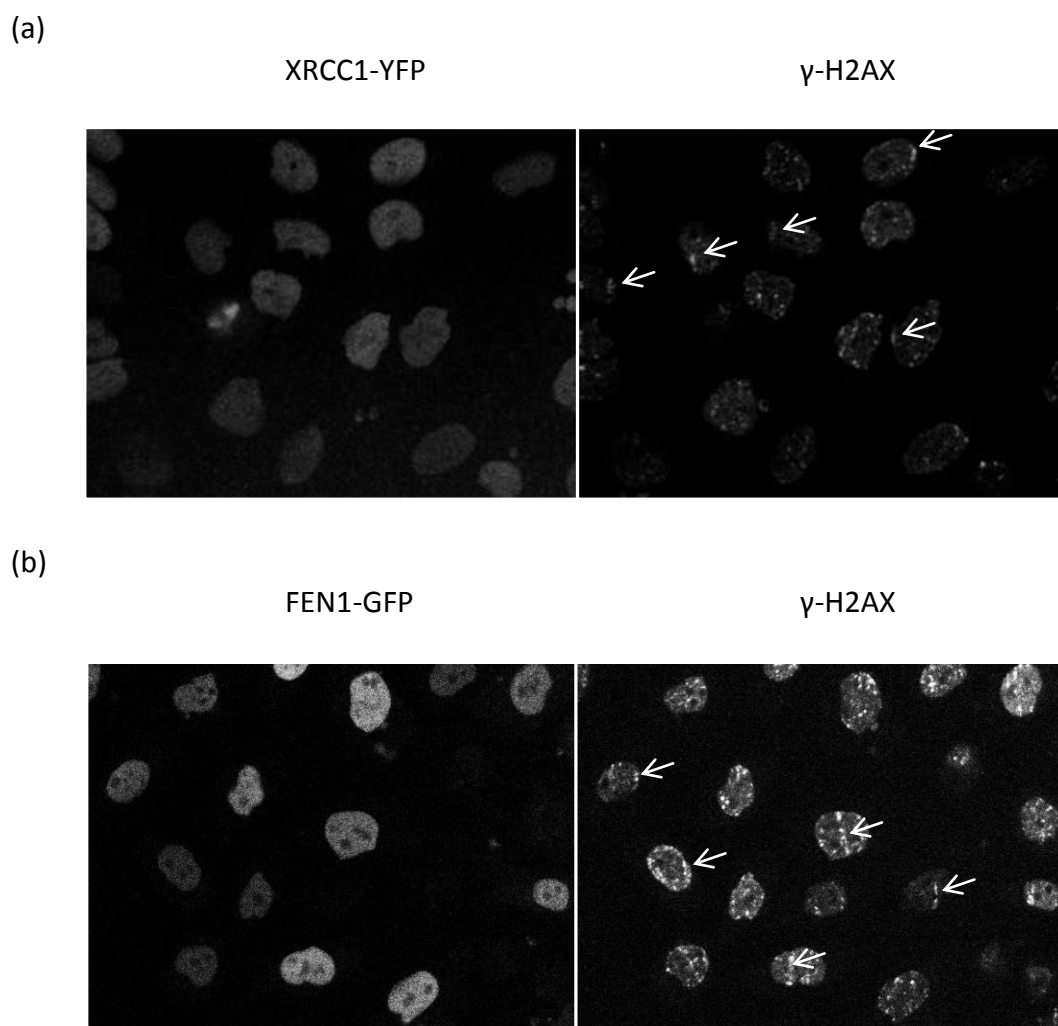


Figure 3.6. ^{238}Pu alpha-particle irradiation of (a) EMC11 XRCC1-YFP and (b) CHO FEN1-GFP tagged cells. Cells were irradiated with 1 Gy alpha-particles, incubated at 37°C in 5% CO_2 humidified air and fixed in 3% paraformaldehyde 5 min after irradiation. Cells were stained for γ -H2AX. Confocal images were taken to detect the localisation of XRCC1-YFP or FEN1-GFP and γ -H2AX in the cell nuclei. The arrows show γ -H2AX localised to DNA damage tracks caused by the alpha-particle irradiation. XRCC1-YFP or FEN1-GFP cannot be seen to localise to DNA damage tracks above nuclear background levels.

As $^{137}\text{Caesium}$ γ -rays could not be used as a source of low LET radiation, the use of ultrasoft X-ray irradiation as an alternative for low LET radiation (see section 2.6.3 and Figure 2.1) was investigated. The majority of the DNA damage induced by low LET radiation is by secondary electron interactions and all of the energy deposited by ultrasoft X-rays is in the form of low energy electrons, giving rise to secondary electron tracks and to some extent clustering of DNA lesions. Importantly for us, ultrasoft X-rays are highly attenuated in air and can be shielded using a grid system giving defined tracks of DNA damage (see Figure 2.1), unlike $^{137}\text{Caesium}$ γ -ray irradiation where the whole cell is irradiated (see Chapter 1 for more detail).

EMC11 XRCC1-YFP tagged and FEN1-GFP tagged cells were irradiated with increasing doses of ultrasoft X-rays. Cells were plated into 0.9 μm Mylar based dishes, as described in section 2.1.5, 48 h prior to irradiation. Due to the low dose rate, cells were irradiated at 10°C to prevent repair occurring during the irradiation time (1-50 min depending on the dose). Following irradiation, the culture medium was replaced with medium pre-warmed to 37°C and the cells were incubated for 2 min before being fixed. Cells were stained for γH2AX formation and γH2AX , XRCC1-YFP and FEN1-GFP were imaged using a confocal microscope. Both XRCC1-YFP and FEN1-GFP are recruited to tracks of DNA damage caused by ultrasoft X-ray irradiation (Figure 3.7 and Figure 3.8). The intensity of the γH2AX recruitment to the DNA damage tracks decreases from 135 Gy to 1.35 Gy but is still clearly visible at 1.35 Gy. Similarly, the intensity of XRCC1-YFP recruitment to the DNA damage tracks also decreases with dose but is just visible after 5.4 Gy. 27 Gy was chosen as the dose for future XRCC1-YFP real time recruitment and loss experiments using ultrasoft X-ray irradiation, as it is

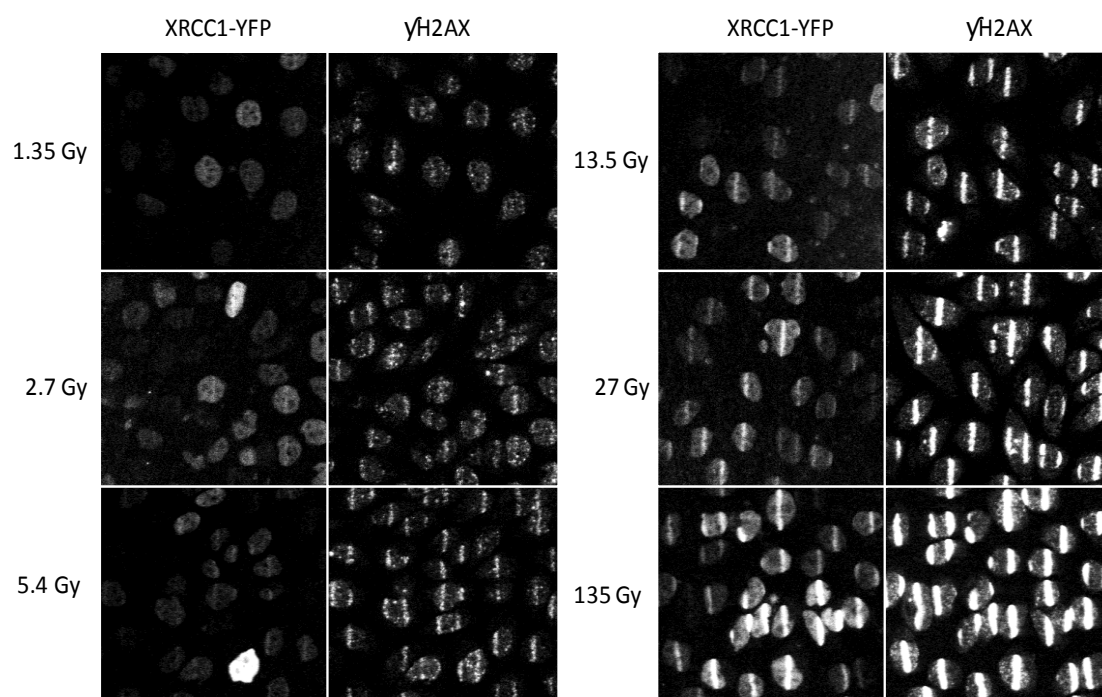


Figure 3.7: Ultrasoft X-ray dose dependency for XRCC1-YFP and γ H2AX in cycling EMC11 XRCC1-YFP tagged cells growing on 0.9 μ m Mylar and irradiated at 10°C through a 1 μ m x 9 μ m grid. Culture medium was replaced with medium pre-warmed to 37°C and cells were incubated for 2 min in 5% CO₂ humidified air and fixed in 3% paraformaldehyde. Cells were stained for γ H2AX formation and imaged using a confocal microscope.

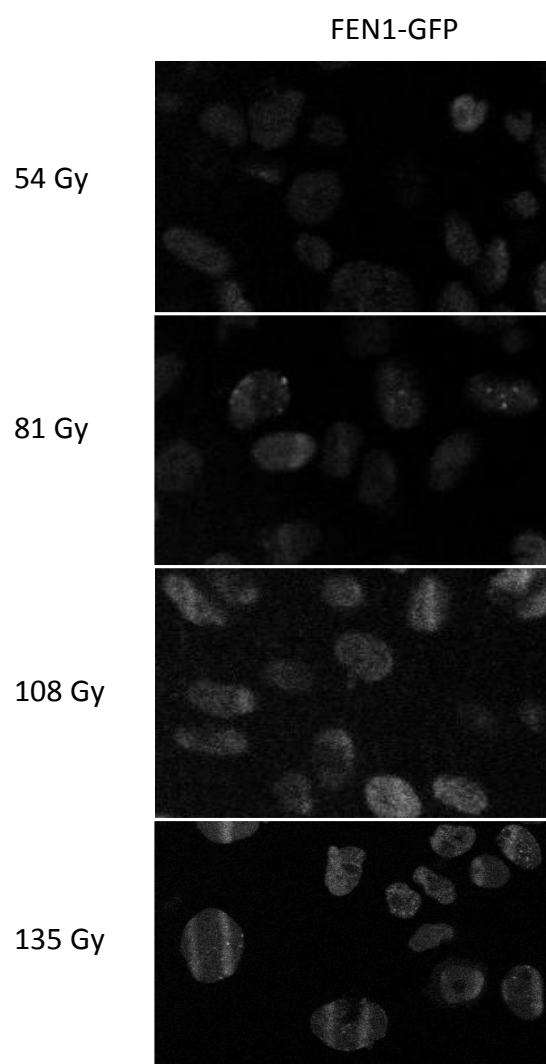


Figure 3.8: Ultrasoft X-ray dose dependency for FEN1-GFP in cycling CHO FEN1-GFP tagged cells growing on 0.9 μm Mylar and irradiated at 10°C through a 1 μm x 9 μm grid. Culture medium was replaced with medium pre-warmed to 37°C and cells were incubated for 2 min in 5% CO₂ humidified air and fixed in 3% paraformaldehyde. Cells were imaged using a confocal microscope.

possible to quantify the foci tracks accurately without the risk of saturation effects of the detection system. 135 Gy was chosen as the dose for future FEN1-GFP real time recruitment and loss experiments using ultrasoft X-ray irradiation, as it is the lowest dose where FEN1-GFP recruitment can be seen clearly. FEN1-GFP recruitment can be seen after 108 Gy or 81 Gy but at these doses FEN1-GFP is difficult to quantify accurately. There is no visible recruitment of FEN1-GFP after 54 Gy.

NIR multiphoton laser microbeam irradiation (see section 2.6.4 and Figure 2.2) was investigated as an alternative to ²³⁸Plutonium α -particle irradiation as it is proposed to induce a larger fraction of complex damage (Botchway *et al*, 2010, Harper *et al*, 2008, Konig *et al*, 1995, Lan *et al*, 2004, Mari *et al*, 2006, Meldrum *et al*, 2003, Niioka *et al*, 2008, Tirlapur *et al*, 2001) relative to that produced by ultrasoft X-ray irradiation. As discussed in more detail in Chapter 1, the energy produced by NIR light is not significantly absorbed by biological tissues at a wavelength of 730 nm. On its own, 730 nm NIR light energy can't cause complex damage to the cell because the energy decreases as the wavelength increases. Therefore, multiphotons must be used, e.g. two photons at a wavelength of 730 nm have the equivalent energy of 1 photon at 365 nm (in the UV range). The other advantage of multiphotons is that the damage will only occur at the focal point of the laser as this is the only point where multiphoton processes can occur (Botchway *et al*, 2010, Meldrum *et al*, 2003, Diaspro *et al*, 2006). The cells also contain Hoescht dye which absorbs at 365 nm to cause oxidative damage. Thus, damage will only be induced at a 400 nm³ spot size in the nucleus which can be manipulated to cause defined tracks of DNA damage (Harper *et al*, 2008). Both XRCC1-YFP and FEN1-GFP were found to be recruited to DNA damage tracks

caused by NIR multiphoton laser microbeam irradiation at 37°C, in contrast to the lower temperature used for ultrasoft X-ray irradiation (Figure 3.9 and Figure 3.10).

An assessment of laser power dependency was carried out on the EMC11 XRCC1-YFP tagged cells (Figure 3.9) where the cells were irradiated with increasing laser power at 37°C and a wavelength of 730 nm, through a x60 water objective in the presence of 10 µM Hoescht 33258. Previous studies have shown that Hoescht 33258 dye increases the level of DNA damage and cytotoxicity when irradiated with UV light through multiphoton irradiation with NIR laser light (Dinant *et al*, 2007, Murga *et al*, 2007, Singh and Krishna, 2005). Confocal microscope images were taken 2 min following irradiation as the preliminary experiments indicated that XRCC1 is recruited within this time. The intensity of the lines of XRCC1-YFP above 10 mW (15 mW and 20 mW) are similar to that at 10 mW indicating potential saturation, which would not allow accurate quantification and analysis. The intensity is markedly decreased at 5 mW and the lines of XRCC1-YFP are barely detectable at 1 mW (Figure 3.9). Therefore, a power of 10 mW was chosen as the power for XRCC1-YFP real time recruitment and loss experiments using NIR multiphoton laser microbeam irradiation.

A laser power dependency was also carried out on the CHO FEN1-GFP tagged cells (Figure 3.10). Confocal microscope images were taken 5 min following irradiation. The later time point for imaging was used because it had been noted in preliminary irradiations that FEN1-GFP recruitment takes approximately 5 min, and is slower than the time for recruitment of XRCC1. Again, a power of 10 mW was chosen as the power for FEN1-GFP real time recruitment and loss experiments using NIR multiphoton laser microbeam irradiation since the lines of FEN1-GFP are clearly detectable at this power.

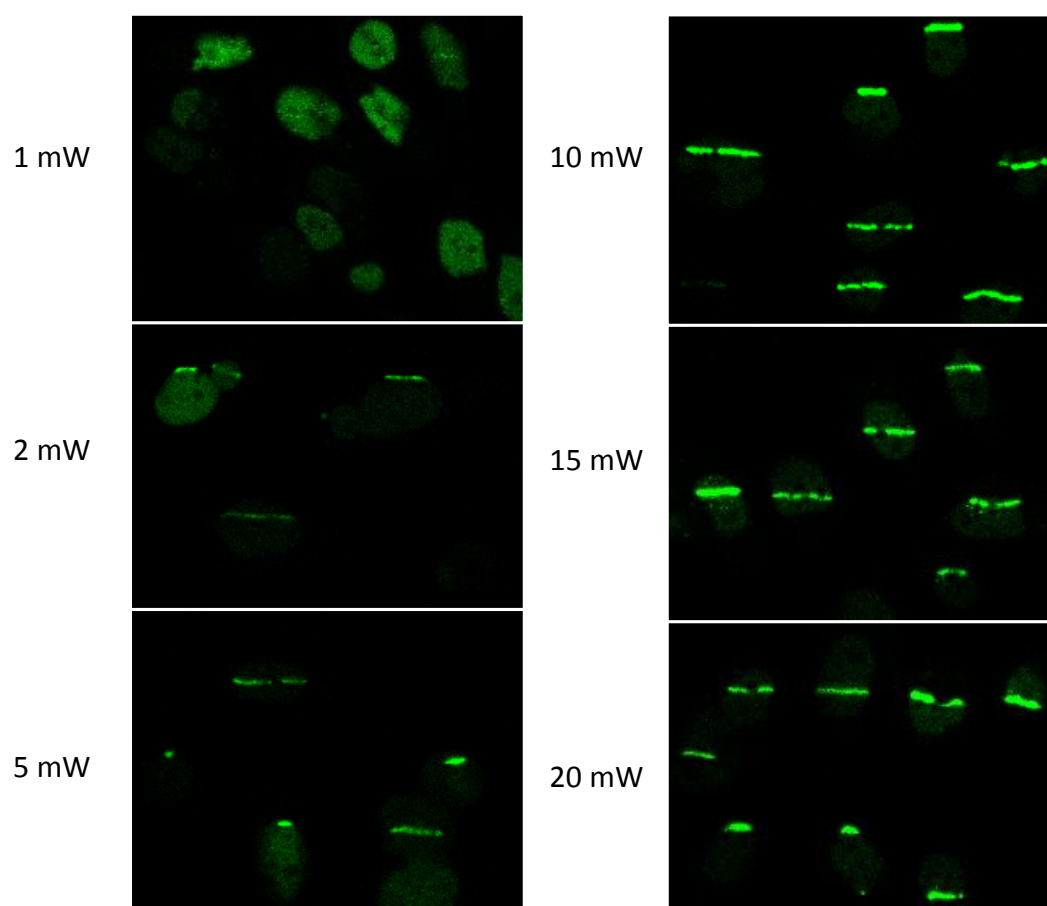


Figure 3.9: NIR laser microbeam power dependency for the recruitment of XRCC1-YFP in cycling EMC11 XRCC1-YFP tagged cells imaged in real time, following irradiation at the specified powers at 37°C, 730 nm, using a x60 water objective, in the presence of 10 μ M Hoescht 33258. Confocal microscope images were taken 2 min following irradiation.

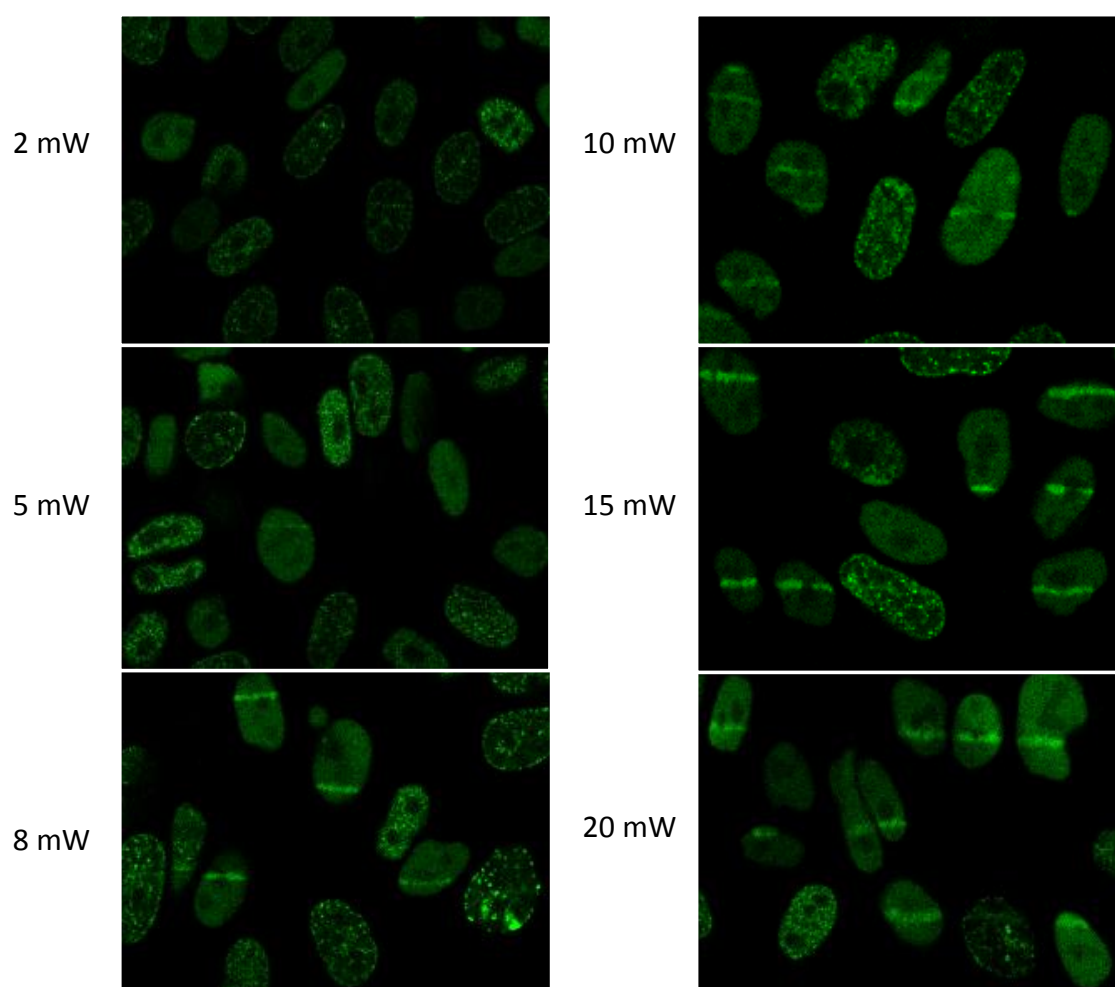


Figure 3.10: NIR laser microbeam power dependency for the recruitment of FEN1-GFP in cycling CHO FEN1-GFP tagged cells imaged in real time, following irradiation at the specified powers at 37°C, 730 nm, using a x60 water objective, in the presence of 10 μ M Hoescht 33258. Confocal microscope images were taken 5 min following irradiation.

In addition, FEN1-GFP and XRCC1-YFP recruitment and loss following laser microbeam irradiation will be easier to compare.

Two distinct distributions of FEN1-GFP in the nuclei of the CHO FEN1-GFP tagged cells can be clearly seen in Figure 3.10. Some of the nuclei show only diffuse nuclear fluorescence whereas other nuclei show both diffuse and focal GFP nuclear signal. This has also been seen by another group (Solovjeva *et al*, 2005) and may represent FEN1-GFP localisation to replication foci in S-phase cells.

3.4 Kinetics of recruitment and loss of BER proteins at DNA damage sites in mammalian cells following ultrasoft X-ray irradiation

Following the optimisation of the irradiation doses, the kinetics of the recruitment and loss of XRCC1-YFP and FEN1-GFP at DNA damage sites in mammalian cells were determined after ultrasoft X-ray irradiation with a dose of 27 Gy and 135 Gy, respectively.

Figure 3.11 represents the results of three independent experiments showing the real time recruitment and loss of XRCC1-YFP following ultrasoft X-ray irradiation (see section 2.6.3) of cycling EMC11 XRCC1-YFP cells. XRCC1-YFP recruitment peaks at the first time point imaged (2 min) and decreases to background levels by 20-30 min. The fluorescence decay of XRCC1-YFP is fitted by a single exponential decay with a half-life of $4 (\pm 1)$ min (Figure 3.11). In contrast, the recruitment of FEN1-GFP peaks at around 3 min and decreases to background levels by 30-40 min (Figure 3.12). The fluorescence decay of FEN1-GFP is fitted by a single exponential decay with a half-life of $6 (\pm 1)$ min, which is longer than that determined with XRCC1-YFP. The single exponential decay of

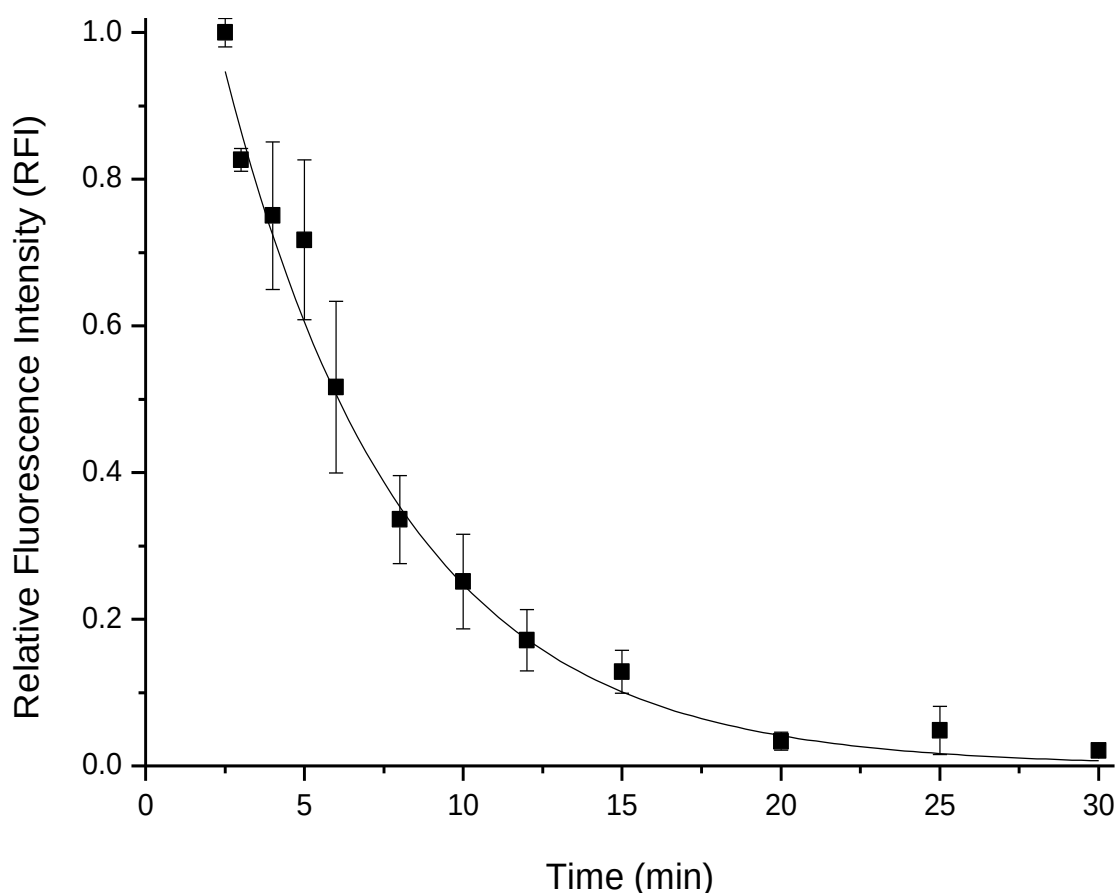


Figure 3.11: Real time recruitment and loss of XRCC1-YFP following ultrasoft X-ray irradiation of cycling EMC11 XRCC1-YFP cells with a dose of 27 Gy. Each point represents the normalised relative fluorescence intensity of the foci track minus the nuclear background, in a minimum of 10 cells per experiment. The graph represents the mean of 3 independent experiments $\pm$ SEM. Kinetic analysis of the loss of relative fluorescence from the normalised data was performed using Origin[®] for each independent experiment. The average for all 3 experiments was then calculated. The fluorescence decay of XRCC1-YFP is fitted by a single exponential decay with a half-life of $4 (\pm 1)$ min.

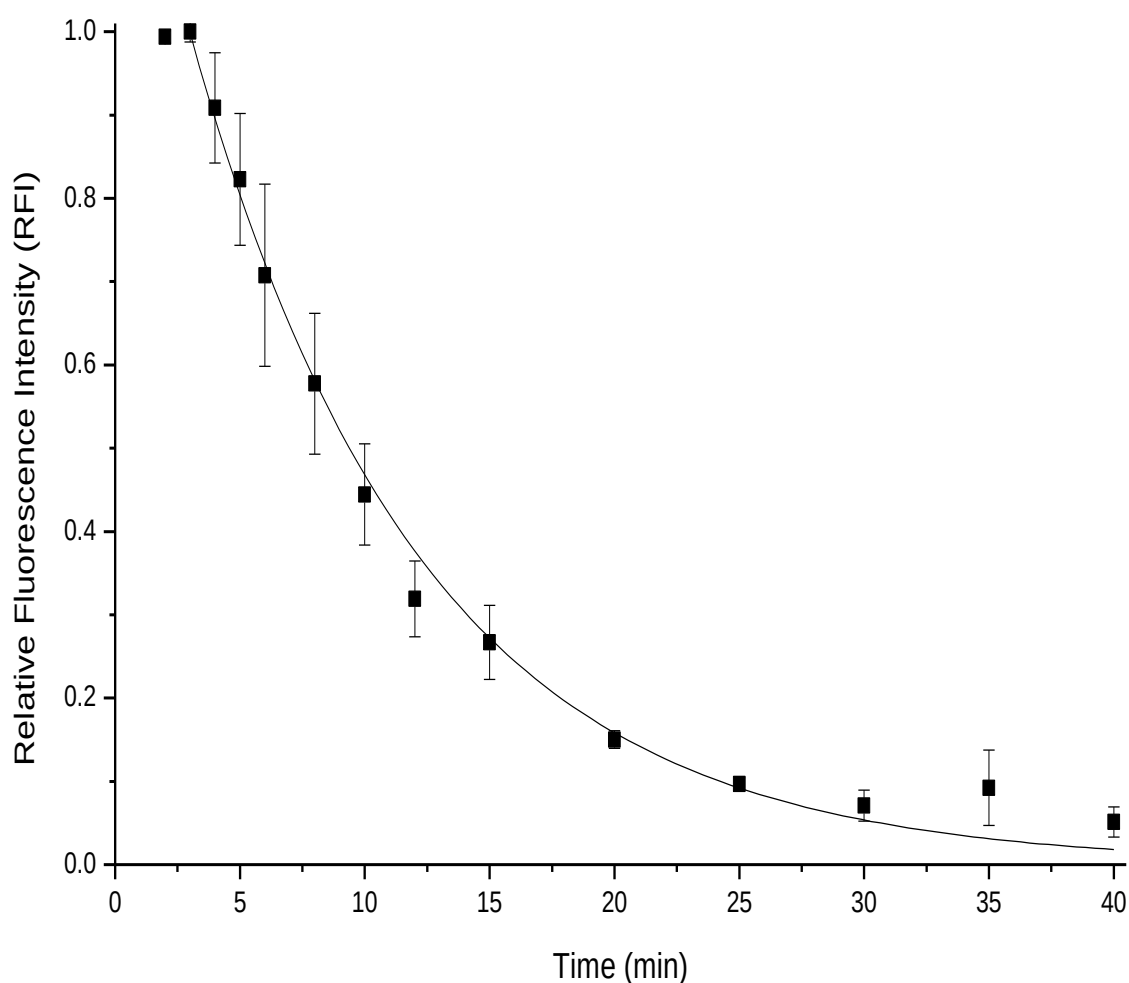


Figure 3.12: Real time recruitment and loss of FEN1-GFP following ultrasoft X-ray irradiation of cycling CHO FEN1-GFP cells with a dose of 135 Gy. Each point represents the normalised relative fluorescence intensity of the foci track minus the nuclear background, in a minimum of 10 cells per experiment. The graph represents the mean of 3 independent experiments $\pm$ SEM. Kinetic analysis of the loss of relative fluorescence from the normalised data was performed using Origin[®] for each independent experiment. The average for all 3 experiments was then calculated. The fluorescence decay of FEN1-GFP is fitted by a single exponential decay with a half-life of $6 (\pm 1)$ min.

these BER proteins is suggested to reflect the more simple damage caused by ultrasoft X-ray irradiation that is relatively easy to repair.

3.5 Kinetics of recruitment and loss of BER proteins at DNA damage sites in mammalian cells following NIR multiphoton laser microbeam irradiation

Using the power optimised in section 3.3, the kinetics of the recruitment and loss of XRCC1-YFP and FEN1-GFP at DNA damage sites in mammalian cells were investigated following NIR multiphoton laser microbeam irradiation at a power of 10 mW.

NIR multiphoton laser microbeam irradiation produces a higher proportion of complex damage than ultrasoft X-ray irradiation so should result in different kinetic profiles for loss of XRCC1-YFP and FEN1-GFP fluorescence at DNA damage sites. Figure 3.13 represents the results of three independent experiments looking at the real time recruitment and loss of XRCC1-YFP following NIR laser microbeam irradiation (see section 2.6.4) of cycling EMC11 XRCC1-YFP cells. XRCC1-YFP recruitment peaks at around 1 min after irradiation and the fluorescence decay was followed for 4 hours. The fluorescence decay of XRCC1-YFP is fitted by bi-exponential decays with half-lives of $15 (\pm 9)$ min and $153 (\pm 35)$ min. At 4 hours $\sim 20\%$ of the XRCC1-YFP remains at the DNA damage sites.

Three independent experiments looking at the real time recruitment and loss of FEN1-GFP following NIR laser microbeam irradiation of cycling CHO FEN1-GFP cells were also carried out. Unfortunately, it was not possible to calculate the half life of the fluorescence decay of FEN1-GFP following NIR laser microbeam irradiation. FEN1-GFP can be seen faintly recruited to DNA damage tracks at 1 min. Recruitment peaks at

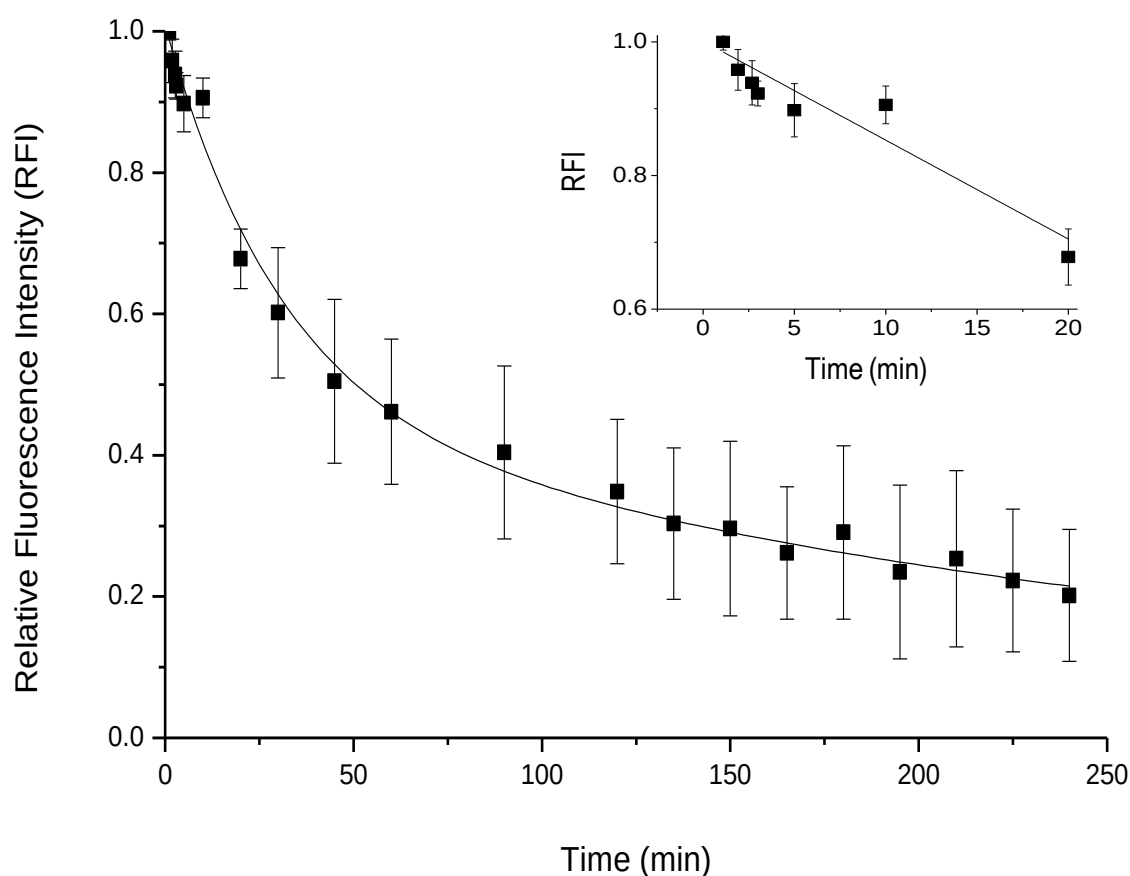


Figure 3.13: Real time recruitment and loss of XRCC1-YFP following laser microbeam irradiation of cycling EMC11 XRCC1-YFP cells with a power of 10 mW. The inset represents the data expanded to show the first 20 min in more detail. Each point represents the normalised relative fluorescence intensity of the foci track minus the nuclear background, in a minimum of 10 cells per experiment. The graph represents the mean of 3 independent experiments $\pm$ SEM. Kinetic analysis of the loss of relative fluorescence from the normalised data was performed using Origin[®] for each independent experiment. The average for all 3 experiments was then calculated. The fluorescence decay of XRCC1-YFP is fitted by bi-exponential decays with half-lives of 15 ($\pm$ 9) min and 153 ($\pm$ 35) min.

10 min and this level of FEN1-GFP intensity persists at 30 min (Figure 3.14a and Figure 3.14b). By 60 min, the lines start to break up into large foci-like structures which persist to at least 180 min (Figure 3.14a).

3.6 Cell cycle effects on XRCC1-YFP recruitment and loss at DNA damage sites in mammalian cells

It has previously been shown that XRCC1 can interact with PCNA at replication foci in S-phase (Fan *et al*, 2004), which has led to the suggestion that XRCC1 has additional roles in S-phase. To see if there is a difference in the kinetics of XRCC1 recruitment and loss in G1 phase cells compared to cells in S-phase, EMC11 XRCC1-YFP tagged cells were serum starved (see section 2.1.6) to reduce the number of cells in S-phase and enhance the population of cells in G1, before irradiating with ultrasoft X-rays (see section 2.6.3). Exponentially growing cells were also irradiated at the same time for comparison.

Before undertaking the irradiations, the amount of time for serum starvation required before irradiation was optimised using flow cytometry analysis (see section 2.10). Cells were plated and serum starved (as described in section 2.1.6) in growth medium containing 1% Fetal Bovine Serum (FBS), with four 0.9 µm Mylar dishes seeded for each sample. Cells were harvested after 24 h, 48 h and 72 h of serum starvation (see section 2.10). Additionally, four 0.9 µm Mylar dishes were seeded under normal growth conditions as in section 2.1.5 and harvested after 48 h (see section 2.10) to be used as the exponentially growing control sample. Figure 3.15 shows the number of cells in each phase of the cell cycle for the control and serum starved samples.

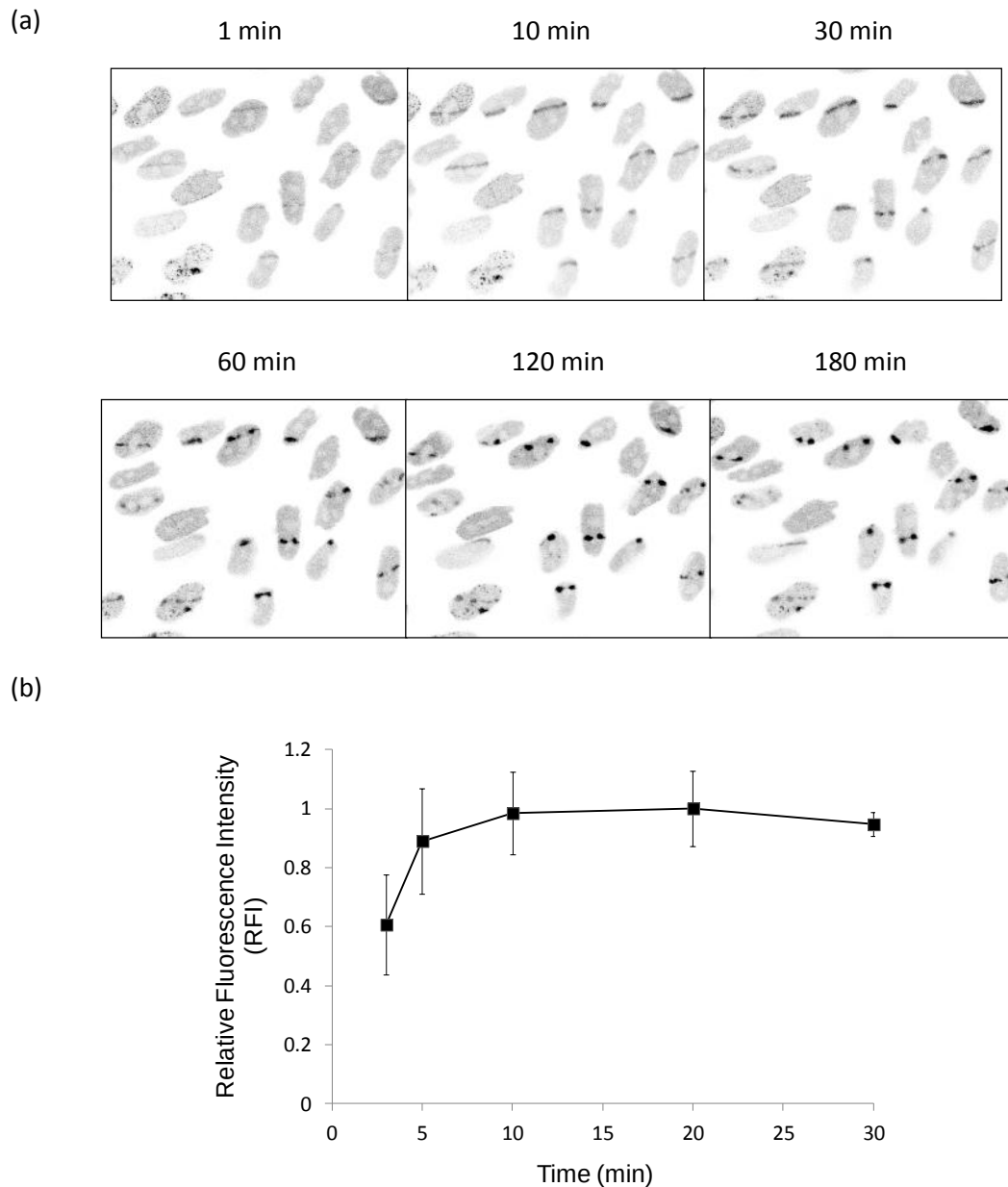
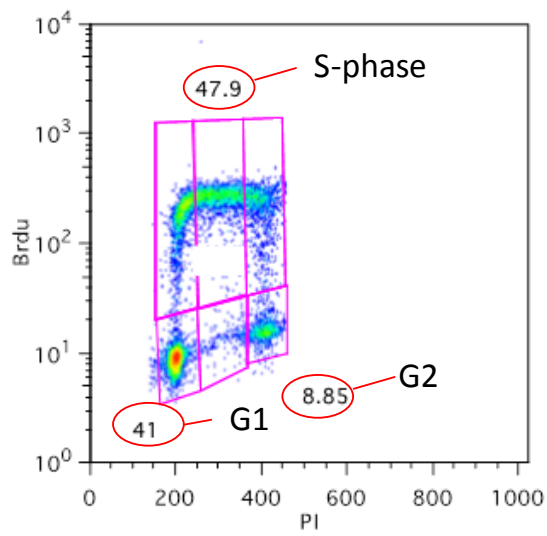
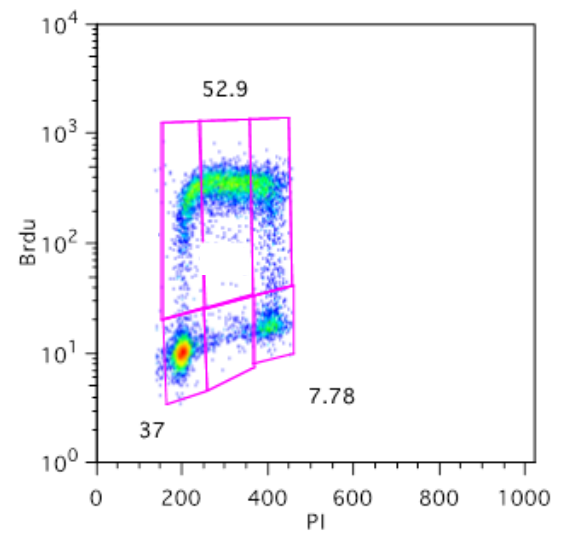


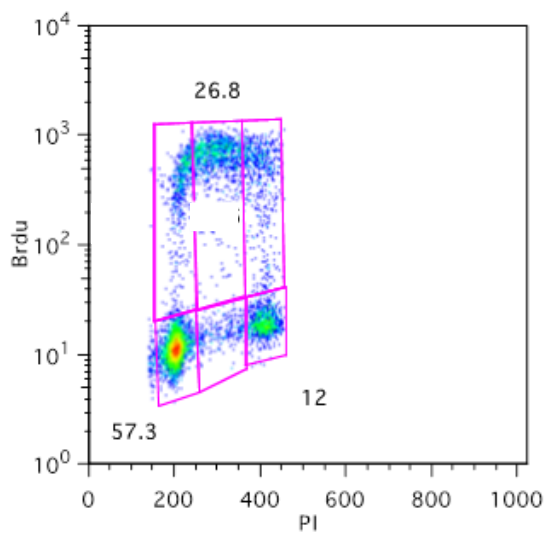
Figure 3.14: Real time recruitment and loss of FEN1-GFP following laser microbeam irradiation of cycling CHO FEN1-GFP cells. (a) Confocal microscope images of FEN1-GFP recruitment to DNA damage tracks caused by laser microbeam irradiation taken at specific timepoints after irradiation. (b) The graph shows the data for the first 30 min and represents the mean of 3 independent experiments $\pm$ SEM. Each point represents the normalised relative fluorescence intensity of the foci track minus the nuclear background, in a minimum of 10 cells per experiment.



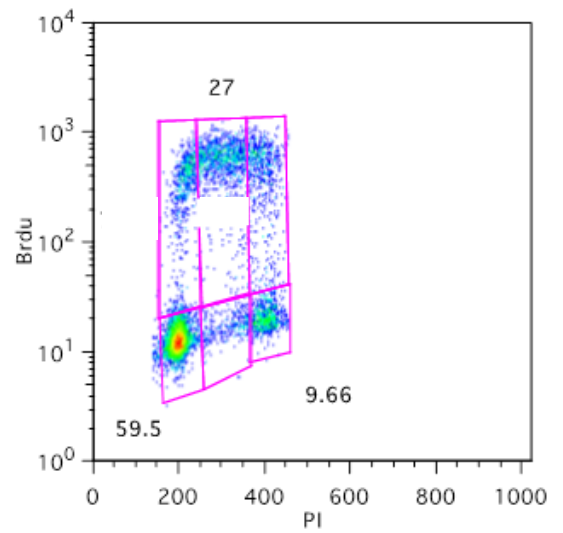
Control



24 h serum starved



48 h serum starved



72 h serum starved

Figure 3.15: Determination of cell cycle phase by flow cytometry analysis. The control sample consists of cells harvested after 48 h growth in complete culture medium on 0.9 μm Mylar.

Serum starvation does not seem to have had an influence on cell cycle phase compared to control cells after 24 h. However, after 48 h of serum starvation the number of cells in G1 phase has increased 1.4 fold and the number of cells in S-phase has decreased 1.8 fold. There was a similar fold difference seen after 72 h of serum starvation, resulting in 48 h serum starvation being chosen for the irradiations.

Figure 3.16 represents the results of three independent experiments showing the real time recruitment and loss of XRCC1-YFP following ultrasoft X-ray irradiation (see section 2.6.3) of exponentially growing and serum starved EMC11 XRCC1-YFP cells. The fluorescence decay of XRCC1-YFP is fitted by a single exponential decay for both exponential and serum starved cells with a half-life of $5 (\pm 1)$ min for exponential cells and a half-life of $8 (\pm 4)$ min for serum starved cells. Taking in to account the errors, there does not appear to be an obvious difference in the fluorescence decay kinetics of XRCC1-YFP between exponentially growing and serum starved cells after ultrasoft X-ray irradiation, although the level of residual fluorescence intensity of XRCC1-YFP at 35-40 min may be greater with serum starved cells. A more pronounced difference may have been seen if there was a higher proportion of serum starved cells in G1 phase compared to those in the exponential control group (Figure 3.15).

3.7 Discussion

In this Chapter, the aim was to investigate the repair kinetics of simple and clustered DNA damage by investigating the recruitment and loss of the SP-BER protein XRCC1 and the LP-BER protein FEN1 to DNA damage sites induced by different types of radiation, which produce different proportions of simple relative to complex DNA damage.

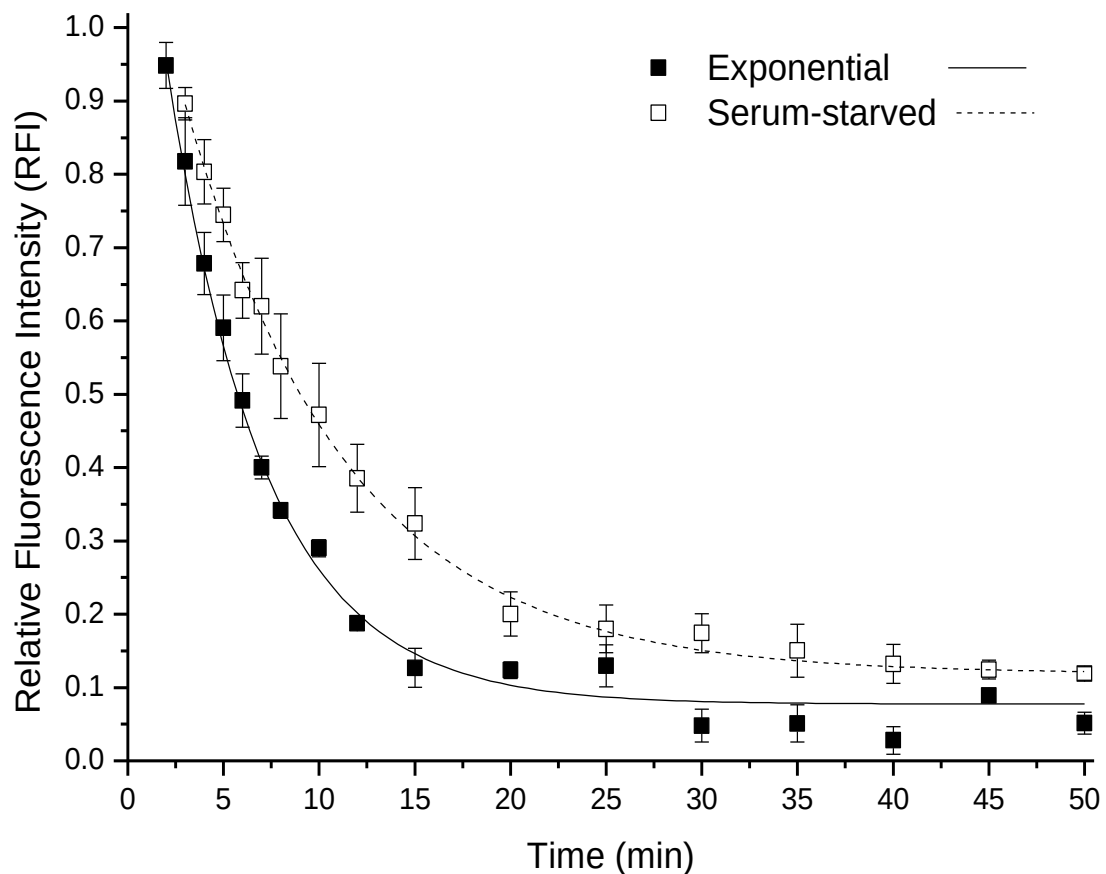


Figure 3.16: Real time recruitment and loss of XRCC1-YFP following ultrasoft X-ray irradiation of exponential and serum starved EMC11 XRCC1-YFP cells with a dose of 27 Gy. Each point represents the normalised relative fluorescence intensity of the foci track minus the nuclear background, in a minimum of 10 cells per experiment. The graph represents the mean of 3 independent experiments $\pm$ SEM. Kinetic analysis of the loss of relative fluorescence from the normalised data was performed using Origin® for each independent experiment. The average for all 3 experiments was then calculated. The fluorescence decay of XRCC1-YFP is fitted by a single exponential decay for both exponential and serum starved cells with a half-life of $5 (\pm 1)$ min for exponential cells and a half-life of $8 (\pm 4)$ min for serum starved cells.

Cell lines stably expressing XRCC1-YFP and FEN1-GFP protein were generated and specific clones were selected that had an equal level of expression of the fluorescently tagged protein in every cell and did not significantly over express the tagged protein when compared to endogenous levels of the same protein (Figure 3.1 and Figure 3.3). EMC11 cells, which were used to make the XRCC1-YFP expressing stable cell line, were chosen as they are XRCC1 deficient (Zdzienicka *et al*, 1992). The advantage of this is that XRCC1 recruited to DNA damage sites in real time experiments could be visualised and quantified, as it is in the form of YFP-tagged XRCC1. This also enabled the function of the XRCC1-YFP protein in the stable clone to be tested by comparing its radiosensitivity with that of XRCC1 deficient EMC11 cells and CHO cells, which have endogenous XRCC1, and are the cell line from which the EMC11 cells were originally derived. The XRCC1-YFP protein appeared to be functional in the clone as it is less radiosensitive compared to the untransfected EMC11 cells, with an SER of 1.3 (Figure 3.2). FEN1 deficient cells are not available, most likely due to its essential role in replication, so the CHO FEN1-GFP clone generated had endogenous FEN1, although a clone was deliberately selected to have a level of FEN1-GFP similar to that of the endogenous FEN1 (Figure 3.3).

Two different types of radiation were used that vary in LET and the complexity of DNA damage induced. ¹³⁷Caesium γ-rays were initially used as a form of low LET radiation and ²³⁸Plutonium α-particles as a form of high LET radiation. However, neither XRCC1-YFP nor FEN1-GFP recruitment could be seen above nuclear background levels after either ¹³⁷Caesium γ-ray irradiation (Figure 3.4 and Figure 3.5) or ²³⁸Plutonium α-particle irradiation (Figure 3.6). However, ultrasoft X-ray and NIR laser microbeam

irradiation were found to be good sources of low and 'mimic' high LET radiation, respectively. The majority of the DNA damage induced by low LET radiation is by secondary electron interactions and all of the energy deposited by ultrasoft X-rays is in the form of low energy electrons, giving rise to secondary electron tracks, which induce both simple damage together with a lower level of clustered DNA damage. Also, unlike γ -radiation where the whole cell is irradiated, ultrasoft X-rays are highly attenuated in air and can therefore be shielded using a grid system to produce defined tracks of DNA damage. This meant that when the EMC11 XRCC1-YFP tagged cells and the CHO FEN1-GFP tagged cells are irradiated with ultrasoft X-rays together with the grid (Figure 2.1), XRCC1-YFP (Figure 3.7) and FEN1-GFP (Figure 3.8) are recruited to tracks of DNA damage. NIR multiphoton laser microbeam irradiation was used as this has been shown to induce mainly complex damage and fewer simple DNA damage changes. The laser can be focused to a 400 nm^3 spot size so that with rastering, defined tracks of DNA damage are produced in the nucleus of the cell (Figure 2.2). Both XRCC1-YFP (Figure 3.9) and FEN1-GFP (Figure 3.10) recruitment to DNA damage tracks is seen after NIR multiphoton laser microbeam irradiation.

The fluorescence decay of XRCC1-YFP and FEN1-GFP after ultrasoft X-ray irradiation (Figure 3.11 and Figure 3.12) was fitted by a single exponential decay with a half-life of $4 (\pm 1)$ min and $6 (\pm 1)$ min, respectively. The fluorescence decay of XRCC1-YFP after NIR multiphoton laser microbeam irradiation (Figure 3.13) was fitted by bi-exponential decays with half-lives of $15 (\pm 9)$ min and $153 (\pm 35)$ min. In contrast to ultrasoft X-ray irradiation, where the XRCC1-YFP fluorescence decay approaches background levels by 20 min (Figure 3.11), XRCC1-YFP still persists at some of the DNA damage sites even

after 4 hours (Figure 3.13). These differences in the lifetime of XRCC1 at DNA damage sites are consistent with the fact that the laser induces more complex damage that persists for much longer than the simple damage caused by low LET ultrasoft X-ray irradiation.

Previous work by Dikomey and Franzke (Dikomey and Franzke, 1986) has shown that with CHO cells irradiated using X-rays, the repair kinetics of the resulting single strand breaks (SSB) could be explained in terms of three exponential components with half-lives of 2 min, 17 min and 200 min. They suggested that the decay of SSB with a half-life of 2 min represents the repair of simple SSB, whereas that with a half-life of 17 min represented the repair of complex SSB. A small proportion of SSB that are very complex was proposed to account for the half-life of 200 min. The relative contributions of the different types of SSB was determined to be 70, 25 and 5%. Based on the observations of Dikomey and Franzke (Dikomey and Franzke, 1986), the loss of XRCC1-YFP fluorescence with a half-life after ultrasoft X-ray irradiation of ~ 4 min is proposed to represent mainly simple SSB. As XRCC1 is involved with ligase III in the final stages of ligation during BER, the rate of repair in real time of USX-induced SSB is similar to that determined for SSB when detected at the DNA level using alkaline elution methods (Dikomey and Franzke, 1986). The two lifetimes determined with the NIR multiphoton laser microbeam are consistent with the two types of SSB with slower repair as seen by Dikomey and Franzke and represent more complex SSB. Therefore, the ultrasoft X-ray low-LET radiation induces simpler SSB that can be repaired more easily than the more complex SSB predominantly induced by the NIR multiphoton laser microbeam. More recently, Olive and colleagues have demonstrated that DNA

repair in SCCVII murine tumour cells and macrophages after 15 Gy X-ray irradiation measured using the comet assay, had a half-life of 5 min and most of the cells had completed repair by 30 min (Olive *et al*, 2012), consistent with the XRCC1-YFP fluorescence decay after ultrasoft X-ray irradiation presented here (Figure 3.11). Additionally, since the cells have functional Ku present, a major protein involved in NHEJ, the observations with XRCC1 cannot represent the back-up NHEJ pathway for repair of DSB. It has been shown that the back-up NHEJ pathway occurs in cells deficient in Ku (Wang *et al*, 2006). Based on the kinetics for loss of XRCC1 fluorescence at DNA damage, DSB are not involved as they decay at longer times than measured here. With the NIR laser-induced DNA damage, it is proposed that the SSB are mainly chemically complex, e.g. other lesions are present close to the SSB ends but on either strand.

It is proposed that SSB are produced with different levels of chemical complexity by the different radiation types, and it is inferred that they are repaired with different efficiencies by BER. Arguments were presented above that other pathways involving NHEJ of DSB are not involved. Consistent with the repair efficiency being dependent on the chemical complexity of SSB are the biochemical studies using oligonucleotides containing synthetic SSB with varying degrees of chemical complexity. Lesions close to SSB ends but on the same strand (Parsons *et al*, 2005b) or bistrandedly opposed to the SSB (Eccles *et al*, 2011) have been shown using purified BER proteins or cell extracts to have reduced repair efficiency. It has recently been shown that clustered damage sites are long lived in mammalian cells (Magnander *et al*, 2010). As a consequence of impaired BER of clustered damaged sites, the lifetime of the composite lesions is

lengthened such that the probability that they remain unrepaired at replication is increased. Thus, the potential for a higher mutation frequency or cytotoxicity arising from the clustered lesions is increased.

After ultrasoft X-ray irradiation, the simple lesions and SSB are repaired mainly by SP-BER whereas FEN1 is involved in LP-BER. XRCC1 and FEN1 are recruited within 2 or 3 minutes post-irradiation to DNA damage, respectively. This difference in recruitment times most likely reflects the fact that XRCC1 is recruited very early in the BER pathway (Campalans *et al*, 2005), whereas FEN1 is recruited after strand displacement following the inability of Pol β to process an oxidised or reduced AP site (Klungland and Lindahl, 1997, Liu *et al*, 2008). The longer half-life seen for FEN1-GFP compared to XRCC1-YFP could indicate that LP-BER is a slower process than SP-BER, especially as several bases are inserted during LP-BER and base addition is predicted to be the slowest step in BER (Sokhansanj *et al*, 2002). Unfortunately, the half-life of the FEN1-GFP decay could not be calculated following NIR laser microbeam irradiation due to the FEN1-GFP breaking up into large foci-like structures around 60 min which were still present at 180 min (Figure 3.14a). However, the relative fluorescence intensity of FEN1-GFP was calculated for the first 30 min (Figure 3.14b), and was found to increase up until 10 min where it appeared to plateau for the remainder of the 30 min analysed.

Finally, it was investigated whether XRCC1-YFP recruitment and loss at DNA damage sites induced by ultrasoft X-ray irradiation is dependent on the phase of the cell cycle by comparing cells in an enhanced G1 phase compared to exponentially growing cells. To achieve an enhanced G1 population the cells were serum starved prior to irradiation (see section 2.1.6 and Figure 3.15) which led to a 1.4 fold increase in G1

phase cells and a 1.8 fold decrease in S-phase cells when compared to exponentially growing cells (Figure 3.15). The fluorescence decay of XRCC1-YFP shows no obvious difference between exponentially growing and enhanced G1 phase (serum starved) cells after ultrasoft X-ray irradiation. This implies that XRCC1 is involved in the same DNA repair processes of irradiation-induced SSB during all stages of the cell cycle. Two other groups have shown that repair of SSBs is unaffected by cell cycle phase (Graubmann & Dikomey, 1983, Olive *et al*, 2012). However, it is also possible that there wasn't a great enough enhancement of G1 phase cells in the irradiated population and this may have to be investigated further.

In conclusion the findings with XRCC1 and FEN1 are consistent with their recruitment to SSB of different chemical complexity. The dynamics of repair of the SSB determined from loss of XRCC1 from SSBs emphasise the influence of the SSB complexity on the efficiency of repair in living mammalian cells.

Chapter 4: Effect of PARP inhibition on XRCC1 recruitment to sites of DNA damage

4.1 Introduction

PARP1 is a molecular nick sensor that can detect and bind to DNA strand breaks. The DNA-binding domain of PARP1 contains two zinc fingers that are important for binding of PARP1 to strand breaks (Menissier de Murcia *et al*, 1989, Gradwohl *et al*, 1990). PARP1 binds as a homodimer that once bound to DNA strand breaks becomes activated (Benjamin & Gill, 1980, Durkacz *et al*, 1980) and begins a process of automodification (Mendoza-Alvarez and Alvarez-Gonzalez, 1993). The automodification domain of PARP1 contains specific glutamate and lysine residues to which ADP-ribose can bind, allowing PARP1 to poly(ADP-ribosyl)ate itself (Altmeyer *et al*, 2009, Tao *et al*, 2009). PARP1's C-terminal domain sequentially transfers ADP-ribose subunits from NAD⁺ onto itself (Chambon *et al*, 1963, Ogata *et al*, 1981) and other protein acceptors to form poly(ADP-ribose) (PAR) chains (Hayashi *et al*, 1983, Kameshita *et al*, 1984). PAR chains can function to recruit DNA repair proteins. XRCC1 is one of the proteins recruited by the PAR chains of PARP1, where it can then perform its role as a scaffold protein in recruiting other DNA repair proteins required for repair of the strand break (El-Khamisy *et al*, 2003, Masson *et al*, 1998). In addition to recruitment of XRCC1 after DNA damage recognition, PARP1 has been found to interact directly with other SSB and BER proteins, including DNA ligase III α (Leppard *et al*, 2003) DNA Pol β (Prasad *et al*, 2001) and PCNA (Frouin *et al*, 2003). Once the PAR chains have reached a certain length, the negative charge they carry repels PARP1 from the DNA, enabling access of other proteins (Ferro and Olivera, 1982). Poly(ADP-ribose) glycohydrolase (PARG) removes PAR from PARP1, enabling PARP1 to recognise

and bind to strand breaks again (Desnoyers *et al*, 1995, Fisher *et al*, 2007, Oka *et al*, 2006, Ueda *et al*, 1972).

The first indication that PARP1 has a role in DNA repair was when it was shown to be activated after treatment with alkylating agents and γ -radiation (Durkacz *et al*, 1980). Cellular PAR levels have been found to increase up to 500-fold following DNA damage induction (Benjamin & Gill, 1980, Menissier de Murcia *et al*, 1989). Mouse models in which the PARP1 gene has been deleted show hypersensitivity to γ -irradiation and alkylating agents, high genomic instability, accumulation of DNA strand breaks and impaired DNA repair (Menissier de Murcia *et al*, 1997, Masutani *et al*, 1999, Wang *et al*, 1995). However, these knockout mice do not have any defects in viability, fertility or development, which is in contrast to mouse models in which other BER proteins have been deleted (DNA Pol β , XRCC1, DNA ligase III α), that result in embryonic lethality (Gu *et al*, 1994, Puebla-Osorio *et al*, 2006, Tebbs *et al*, 1999). A couple of groups have shown using PARP1^{-/-} cell lines and extracts that BER is impaired (Dantzer *et al*, 1999 and Trucco *et al*, 1998) and it has been suggested that PARP1 has a role in LP-BER (Dantzer *et al*, 2000, LePage *et al*, 2003, Prasad *et al*, 2001).

Apart from SSBR and BER, PARP1 has been implicated in several other DNA processes including NHEJ (Mitchell *et al*, 2009, Veuger *et al*, 2004), back-up NHEJ (Audebert *et al*, 2004, Wang *et al*, 2006), regulation of transcription (Frizzell *et al*, 2009, Kraus, 2008, Krishnakumar *et al*, 2008 and Simbulan-Rosenthal *et al*, 2000) and recruitment of MRE11 to help restart stalled replication forks (Bryant *et al*, 2009, Sugimura *et al*, 2008, Yang *et al*, 2004). However, the exact role of PARP1 in DNA repair is still unclear and there is conflicting evidence as to whether PARP1 is actually needed for repair

(Lindahl *et al*, 1995, Noël *et al*, 2003), especially as cells with PARP1 knocked down can still efficiently repair SSBs (Godon *et al*, 2008, Vodenicharov *et al*, 2000) and the presence of PARP1 can even reduce the repair kinetics (Allinson *et al*, 2003, Parsons *et al*, 2005). In fact, it has been suggested that rather than having a direct role in repair of DNA damage, PARP1 protects the strand breaks until repair proteins can be recruited (Parsons *et al*, 2005, Satoh & Lindahl, 1997, Woodhouse *et al*, 2008).

It has been observed that following hydrogen peroxide treatment, PAR nuclear foci rapidly form followed shortly by colocalisation of XRCC1 (El-Khamisy *et al*, 2003, Okano *et al*, 2003). El-Khamisy and colleagues also found that mutating the BRCT1 domain of XRCC1 (that binds to PAR chains formed by PARP1) or using PARP^{-/-} cells decreased the recruitment of XRCC1 to DNA damage foci (El-Khamisy *et al*, 2003). Several groups have also shown that in the presence of a PARP inhibitor, XRCC1 is not recruited to DNA damage (Godon *et al*, 2008, Lan *et al*, 2004, Okano *et al*, 2003). The PARP inhibitor KU-0058684 (Loh *et al*, 2005) used in this Chapter allows PARP1 to bind to strand breaks but prevents the formation of the PAR chains thus XRCC1 cannot be recruited to these breaks and PARP1 remains bound to the breaks, blocking their repair.

In Chapter 3, the kinetics of recruitment and loss of XRCC1 at DNA damage sites was investigated using different types of radiation, and it was found that XRCC1 is involved in the repair of both simple and complex non-DSB DNA damage. Therefore, it was of interest to investigate whether inhibition of PARP affected recruitment of XRCC1 to lesions generated by both ultrasoft X-ray and NIR laser microbeam irradiation by

looking at the recruitment of XRCC1-YFP to DNA damage sites in the presence of a PARP inhibitor in real time.

4.2 PARP inhibition causes a reduction in the level of recruitment of XRCC1 to sites of

DNA damage

The recruitment and loss of XRCC1 to DNA damage sites in the presence of PARP inhibitor was first examined after ultrasoft X-ray irradiation (see section 2.6.3). EMC11 XRCC1-YFP tagged cells were treated with either 250 nM PARP inhibitor (KU-0058684) or the equivalent volume of DMSO for 1 h prior to ultrasoft X-ray irradiation. The fluorescence intensity of XRCC1-YFP peaks at 1 min in the DMSO control cells whereas the maximal fluorescence is slightly delayed in the PARP inhibited cells, peaking at 3 min (Figure 4.1a). The kinetics of XRCC1-YFP fluorescence decay are the same in both the PARP inhibitor treated and DMSO control cells with a half-life of $4 (\pm 1)$ min for both (Figure 4.1). However, there is much less XRCC1-YFP recruited to the DNA damage sites in the PARP inhibitor treated cells compared to the DMSO treated cells (Figure 4.1b and Figure 4.3a). The actual fluorescence intensity at the peak in PARP inhibitor treated cells is ~40% of the maximal fluorescence intensity in the DMSO control cells (Figure 4.1b). When this experiment was repeated with NIR multiphoton laser microbeam irradiation (see section 2.6.4), the kinetics of the fast component of repair seen as loss of XRCC1-YFP fluorescence at damage sites induced by laser microbeam irradiation are similar between the PARP inhibited and the DMSO control cells. Interestingly, a slow component of loss of XRCC1-YFP fluorescence was not seen in cells treated with the PARP inhibitor (Figure 4.2). The fluorescence decay of XRCC1-YFP was fitted using bi-exponential kinetics for the DMSO control cells with the half-

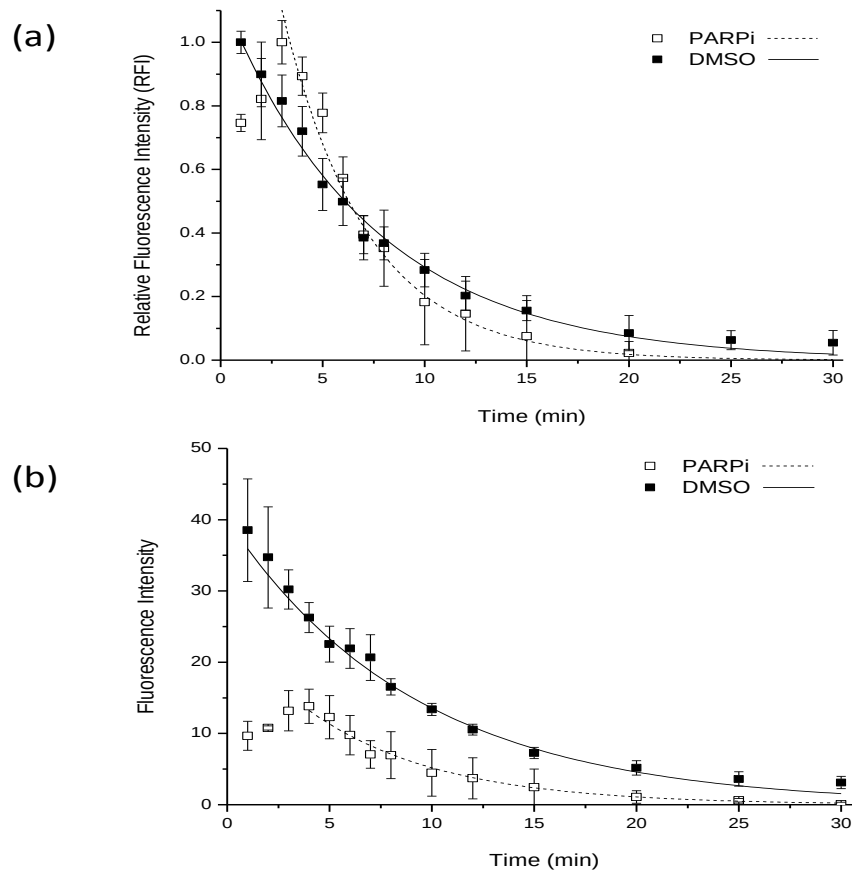


Figure 4.1: Real time recruitment and loss of XRCC1-YFP following 135 Gy ultrasoft X-ray irradiation of EMC11 XRCC1-YFP cells, incubated with 250 nM PARPi for 1 hour prior to irradiation. The cells were imaged on the Bioradiance confocal microscope. Each point represents in (a) the normalised relative fluorescence intensity or (b) the actual fluorescence intensity of the foci track minus the nuclear background in a minimum of 10 cells per experiment. The graphs represent the mean of 3 independent experiments $\pm$ SEM. Kinetic analysis of the loss of relative fluorescence from the normalised data was performed using Origin[®] for each independent experiment. The average for all 3 experiments was then calculated. The fluorescence decay of XRCC1-YFP is fitted by a single exponential decay with a half-life of $4 (\pm 1)$ min for both DMSO control and PARP inhibitor treated cells.

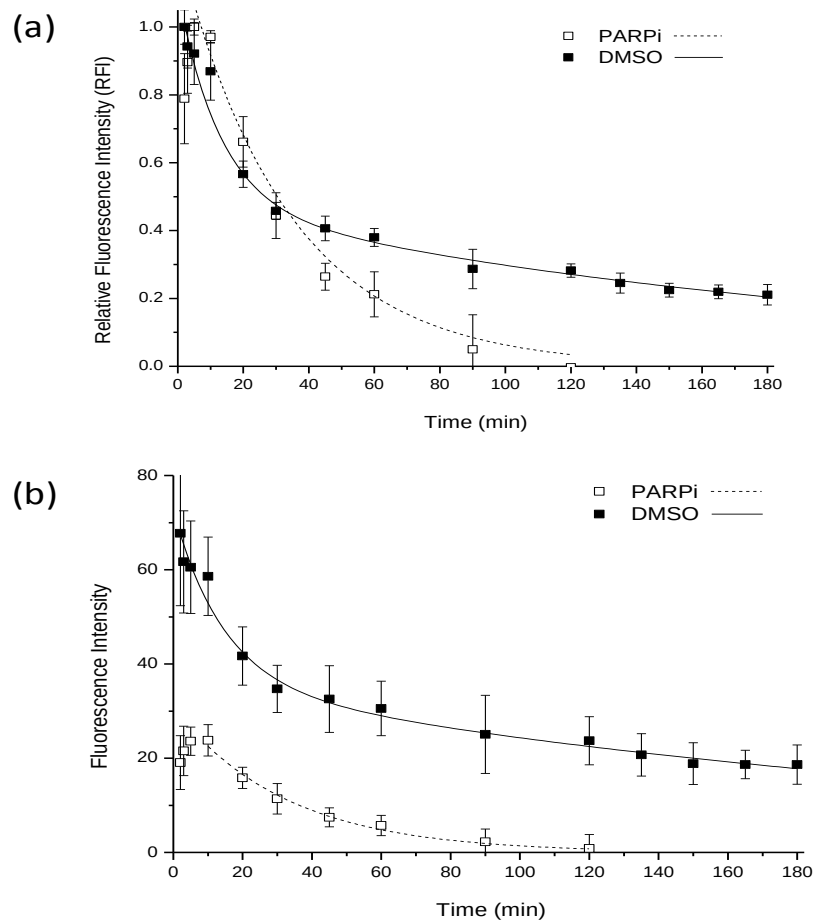


Figure 4.2: Real time recruitment and loss of XRCC1-YFP following NIR laser microbeam irradiation of EMC11 XRCC1-YFP cells incubated with 250 nM PARPi for 1 hour prior to irradiation. Each point represents in (a) the normalised relative fluorescence intensity or (b) the actual fluorescence intensity of the foci track minus the nuclear background in a minimum of 10 cells per experiment. The graphs represent the mean of 3 independent experiments $\pm$ SEM. Kinetic analysis of the loss of relative fluorescence from the normalised data was performed using Origin[®] for each independent experiment. The average for all 3 experiments was then calculated. The fluorescence decay of XRCC1-YFP is fitted by bi-exponential decays for the DMSO control cells with half-lives of $8 (\pm 4)$ min & $144 (\pm 23)$ min and a single exponential decay for the PARP inhibitor treated cells with a half life of $18 (\pm 2)$ min.

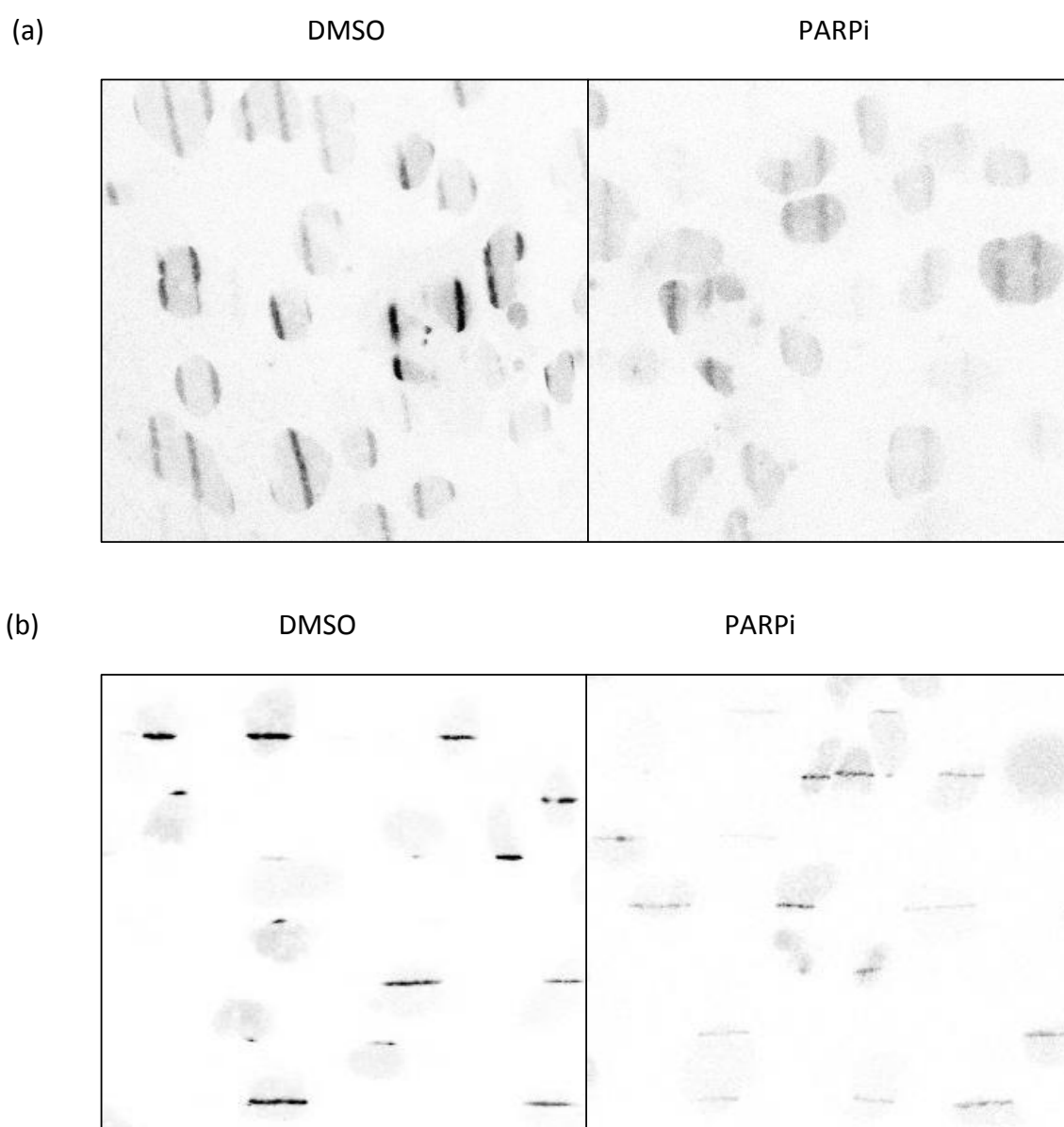


Figure 4.3: Representative images of irradiated EMC11 XRCC1-YFP tagged cells treated with DMSO or 250 nM PARP inhibitor (PARPi) for 1 h prior to irradiation. (a) Confocal microscope images taken 1 min following 135 Gy ultrasoft X-ray irradiation. (b) Confocal microscope images taken 3 min following NIR laser microbeam irradiation. The black stripes represent XRCC1-YFP recruitment to DNA damage sites induced by the irradiation.

lives of the two processes being $8 (\pm 4)$ min & $144 (\pm 23)$ min whereas a single exponential decay with a half life of $18 (\pm 2)$ min fitted the loss of XRCC1-YFP fluorescence in the PARP inhibitor treated cells. Similar to the results seen after ultrasoft X-ray irradiation, there is a delay in maximal XRCC1-YFP fluorescence in the PARP inhibited cells compared to the DMSO control cells, where the fluorescence intensity of XRCC1-YFP peaks at around 2 min in the control cells and at 5-10 min in the PARP inhibitor treated cells (Figure 4.2). Further, the level of fluorescence of XRCC1-YFP due to its recruitment to DNA damage sites in PARP inhibited cells is ~40% of the level recruited in DMSO control cells based on the maximal fluorescence levels (Figure 4.2b and Figure 4.3b).

4.3 A subset of DNA damage lesions requires XRCC1 but not PARP1 for repair

Previously, several groups have shown that when PARP1 is inhibited, XRCC1 cannot be recruited to sites of DNA damage (Godon *et al*, 2008, Lan *et al*, 2004, Okano *et al*, 2003). The fact that some XRCC1-YFP could still be recruited to DNA damage sites in PARP inhibited cells after ultrasoft X-ray (Figure 4.1) and NIR laser microbeam (Figure 4.2) irradiation was unexpected. To confirm that this was not due to incomplete inhibition of PARP1, a titration was performed where EMC11 XRCC1-YFP tagged cells were treated with increasing concentrations (100 – 3000 nM) of PARP inhibitor for 1 h prior to ultrasoft X-ray irradiation. The kinetics of recruitment and loss of XRCC1-YFP were followed for 20 min after irradiation. It was found that XRCC1-YFP is recruited to DNA damage sites at all concentrations used and that the fluorescence decay kinetics are the same at all concentrations (data not shown). The fluorescence intensity of XRCC1-YFP at 5 min is dependent on the concentration of PARP inhibitor (Figure 4.4).

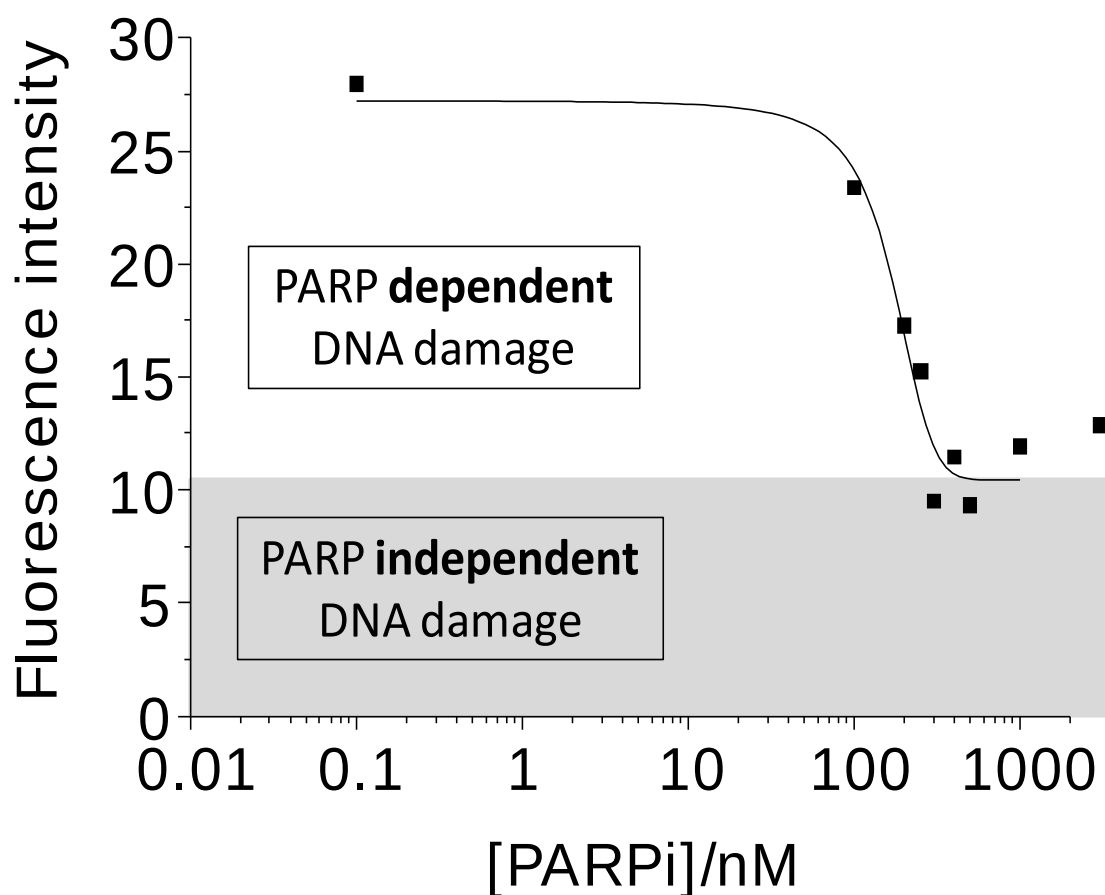


Figure 4.4: PARP inhibitor titration. EMC11 XRCC1-YFP cells were treated with increasing concentrations of PARP inhibitor for 1 h prior to 135 Gy ultrasoft X-ray irradiation. The intensity of XRCC1-YFP at 5 min post-irradiation is plotted for each concentration of PARP inhibitor. The XRCC1-YFP fluorescence intensity plateaus out from 300 nM indicating that ~ 40% of XRCC1 can still be recruited to DNA damage sites when PARP is completely inhibited. Therefore, there is a subset of DNA damage requiring XRCC1 for repair that is PARP independent.

From Figure 4.4 the IC_{50} of XRCC1 recruitment was determined to be ~ 200 nM. At concentrations of the PARP inhibitor greater than 300 nM, the level of fluorescence of XRCC1-YFP decreases to a constant level independent of the concentration of the PARP inhibitor (Figure 4.4). From this dependence when PARP is inhibited, XRCC1 is still recruited to some DNA damage sites from which it is suggested that a subset of DNA damage requires XRCC1 but not PARP1 for repair.

4.4 The effect of PARP inhibition on XRCC1 recruitment to damage in different phases of the cell cycle

In Chapter 3 (section 3.6), the recruitment and loss of XRCC1-YFP was investigated in both exponentially growing and serum-starved (enhanced G_1 phase) cells. The decay kinetics of the fluorescence of XRCC1-YFP were found to be similar in both exponential and serum-starved cells (Figure 3.16). To examine whether PARP inhibition has a different effect on XRCC1-YFP recruitment and loss in cycling (exponentially growing) cells compared to serum-starved (enhanced G_1 phase) cells, both exponentially growing and serum-starved cells were treated with PARP inhibitor or the equivalent volume of DMSO 1 h prior to 135 Gy ultrasoft X-ray irradiation. The kinetics of loss of XRCC1-YFP from the DNA damage sites are similar for all four conditions with half-lives of $9 (\pm 3)$ min for exponentially growing PARP inhibited cells, $12 (\pm 2)$ min for exponentially growing DMSO treated cells, $10 (\pm 3)$ min for serum-starved PARP inhibited cells and $13 (\pm 2)$ min for serum-starved DMSO treated cells (Figure 4.5).

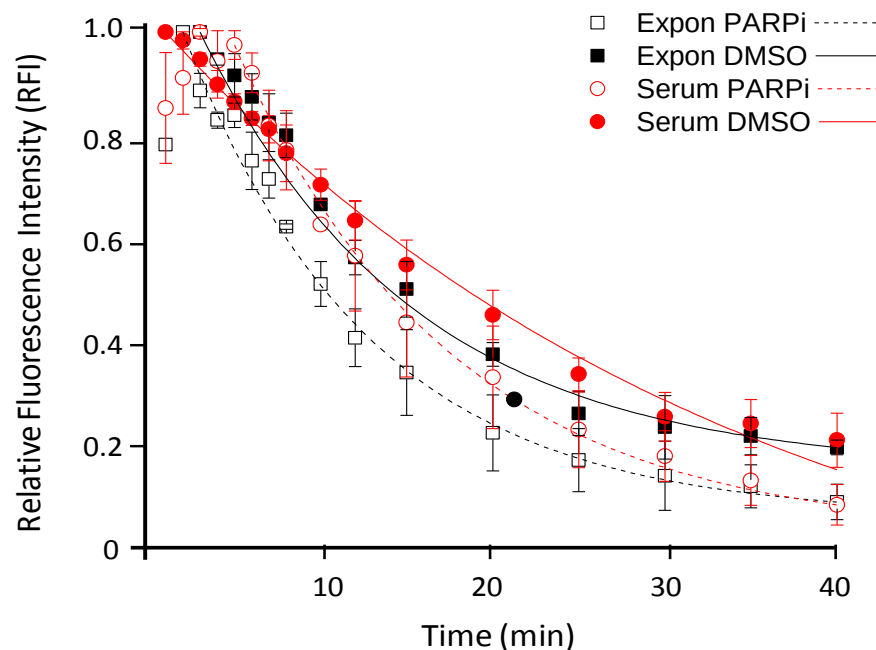


Figure 4.5: Real time recruitment and loss of XRCC1-YFP following 135 Gy ultrasoft X-ray irradiation of EMC11 XRCC1-YFP cells, incubated with 250 nM PARP inhibitor (PARPi) or the equivalent volume of DMSO for 1 hour prior to irradiation. The cells were imaged on the Bioradiance confocal microscope. Each point represents the normalised relative fluorescence intensity of the foci track minus the nuclear background in a minimum of 10 cells per experiment. The graphs represent the mean of 3 independent experiments $\pm$ SEM. Kinetic analysis of the loss of relative fluorescence from the normalised data was performed using Origin[®] for each independent experiment. The average for all 3 experiments was then calculated. The fluorescence decay of XRCC1-YFP is fitted by a single exponential decay with a half-lives of $9 (\pm 3)$ min for exponentially growing (expon) PARPi treated cells, $12 (\pm 2)$ min for exponentially growing DMSO treated cells, $10 (\pm 3)$ min for serum starved (serum) PARPi treated cells and $13 (\pm 2)$ min for serum starved DMSO treated cells.

4.5 Discussion

PARP inhibition has been found to cause synthetic lethality when used in homologous recombination deficient cell lines and human tumours (Bryant *et al*, 2005, Farmer *et al*, 2005, Fong *et al*, 2009). Synthetic lethality occurs when two nonessential proteins are lost or inhibited simultaneously, resulting in cell death. Cell death does not occur if either protein is present and functional (Dobzhansky, 1946). In the presence of PARP inhibitor, PARP1 can bind to SSBs but cannot form PAR chains, preventing recruitment of other repair proteins and importantly the dissociation of PARP1 from the SSB. This persistence of PARP1 at SSB sites is thought to result in the persistence of the SSB which, if in S-phase cells, increases the probability of encountering a replication fork, where it can lead to a replication-induced DSB. Homologous recombination (HR) can repair these types of DSB but in cells where HR is deficient, the DSBs persist and result in cell death (Roos & Kaina, 2012, Underhill *et al*, 2011). PARP inhibitors are currently being used in clinical trials as single agents targeting BRCA1/BRCA2 deficient tumours or tumours that exhibit “BRCAness” which have defects in homologous recombination due to dysfunctional BRCA1/2 from epigenetic modification or deficiency in other proteins involved in homologous recombination (Tan *et al*, 2008, Turner *et al*, 2004), or in combination with ionising radiation (Davar *et al*, 2012) or chemotherapy (e.g. Temozolomide (Bedikian *et al*, 2009, Plummer *et al*, 2008)). Many studies have been undertaken to investigate PARP1, although the exact role of PARP1 in DNA repair is still under debate (see section 4.1).

Here, it has been shown that XRCC1-YFP recruitment to DNA damage sites induced by ultrasoft X-ray irradiation (Figure 4.1) or NIR laser microirradiation (Figure 4.2) is

substantially reduced in the presence of the PARP inhibitor (also seen in the confocal images in Figure 4.3). This is most likely due to the inability of PARP to form PAR chains once it has bound to SSBs as it has been shown that PARP is unable to recruit DNA repair proteins (such as XRCC1) or to dissociate from the SSB, blocking access of other proteins (as described in Mortusewicz *et al*, 2007). However, interestingly, the recruitment of XRCC1-YFP to damage sites in the PARP inhibited cells (maximal fluorescence is ~40% of that seen in uninhibited cells) was still seen but at drastically reduced levels and with delayed kinetics as compared to non-PARP-inhibited cells (Figure 4.1 and Figure 4.2). One explanation for the delay in and lower levels of recruitment of XRCC1 to damage sites in PARP inhibited cells compared to uninhibited PARP proficient cells (Figure 4.1 and Figure 4.2) is the recruitment of PARP to sites of damage (e.g. SSBs) occurs with faster kinetics than glycosylases, such as Nth and OGG1 which are recruited to base lesions, especially as PARP is very abundant in mammalian cells (average of 1×10^6 molecules per cell (Woodhouse and Dianov, 2008)). Several groups have looked at the recruitment of XRCC1 to DNA damage by PARP1 by using PARP inhibitors in combination with various DNA damage-inducing agents (Godon *et al*, 2008, Lan *et al*, 2004, Okano *et al*, 2003, Ström *et al*, 2011a). Okano *et al*, (2003) used cells expressing UV damage endonuclease (UVDE) to induce SSBs at specific sites in the nucleus by irradiating the cells with UV through tiny pores in membrane filters. In the absence of PARP inhibitor they observed PAR recruitment to the SSBs at 2 min which decreased substantially by 10 min and was back to background levels by 30 min. In contrast to the results in this Chapter and in Chapter 3, they did not see much of a decrease in the levels of XRCC1 at the DNA damage sites, even after 30 min. Also, contrary to the findings in this thesis, they did not observe PAR or XRCC1 at the DNA

damage sites in the presence of the PARP inhibitor DIQ (Okano *et al*, 2003). The difference between the data presented by Okano *et al* (2003) and the data presented in Chapter 3 is that the damage is almost entirely SSBs rather than the diversity of DNA damage lesions induced after ultrasoft X-ray irradiation and NIR multiphoton laser microirradiation (Botchway *et al*, 2010, Harper *et al*, 2008, Yokoya *et al*, 2009). However, in support of the diversity of DNA damage lesions produced by USX and NIR multiphoton laser microirradiation (Figures 4.1 and 4.2), it has been shown that following 365 nm UVA irradiation recruitment of GFP-tagged XRCC1 was still seen in the presence of the PARP inhibitor DIQ (~40 % of that recruited in control cells) and the peak fluorescence intensity of GFP-XRCC1 is slightly delayed in the PARP inhibited cells (Lan *et al*, 2004). Also, Mortusewicz *et al* (2007) using 405 nm laser microirradiation to induce damage at preselected subnuclear sites followed the recruitment of GFP-XRCC1 in PARP^{-/-} MEFs for 5 min post-irradiation. They found that the level of recruitment of XRCC1 is substantially reduced in the PARP^{-/-} MEFs, reflecting the finding that PAR chains aid in the recruitment of XRCC1 (El-Khamisy *et al*, 2003, Masson *et al*, 1998). These findings are compatible with the findings in this chapter (Figure 4.2), when the maximal fluorescence of GFP-tagged XRCC1 in the wild-type MEFs peaks at around 2 min, whereas in the PARP^{-/-} cells GFP-XRCC1 has not peaked by the last time point taken at 5 min when ~30% of the maximal recruitment in the wild-type MEFs was seen (Mortusewicz *et al*, 2007). Finally, Godon *et al*, (2008) used 405 nm laser microirradiation to generate localised damage in the nucleus of HeLa cells. They found that in the presence of PARP inhibitor a pronounced decrease was seen in the level of YFP-tagged XRCC1 recruitment. Similarly, when calculating the difference between the maximum intensities of XRCC1-YFP recruitment, the

recruitment of XRCC1 in PARP inhibited cells is ~40% of that seen in uninhibited cells (Godon *et al*, 2008). Therefore, using several approaches, the suggestion that the recruitment of XRCC1 in the presence of PARP inhibitor is reduced but not absent is consistent with the findings using ultrasoft X-ray irradiation and NIR multiphoton laser microirradiation. Previous studies have focused on the decrease of XRCC1 recruitment and not why some XRCC1 is still recruited in the presence of PARP inhibitor.

To confirm that the XRCC1-YFP recruitment seen in Figure 4.1 is not due to incomplete inhibition of PARP, a PARP titration was carried out in EMC11 XRCC1-YFP tagged cells where cells were incubated with increasing concentrations of PARP inhibitor (100-3000nM) for 1 h prior to ultrasoft X-ray irradiation. The intensity of XRCC1-YFP at 5 min post-irradiation was calculated for each PARP inhibitor concentration and plotted on a log scale (Figure 4.4). It showed that no further decrease occurs in XRCC1-YFP recruitment on increasing the PARP inhibitor concentration by 10 fold (300-3000 nM), indicating that ~40% XRCC1-YFP is still recruited even in the presence of PARP inhibitor (Figure 4.4). Therefore, it is suggested that two subsets of lesions are induced by ultrasoft X-ray irradiation and that XRCC1 is recruited to both sub-sets of radiation-induced DNA damage; those that require PARP for the recruitment of XRCC1 and those that XRCC1 can be recruited to without the involvement of PARP. One proposal is that the lesions which require PARP binding for XRCC1 recruitment (PARP-dependent) are directly induced SSBs; this proposal would be consistent with the lack of recruitment of XRCC1 seen by Okano *et al*, (2003). The PARP-independent lesions are proposed to be base lesions which are excised by glycosylases resulting in the formation of SSB that may then recruit XRCC1, allowing BER to progress in a coordinated manner. XRCC1

recruitment may then minimise exposure of SSB BER intermediates to PARP. XRCC1 has been shown to interact with several DNA glycosylases involved in BER such as OGG1 (Marsin *et al*, 2003), MPG, NEIL1 and hNTH1 (Campalans *et al*, 2005).

From the yields of DNA damage calculated for the different types of lesions produced after γ -irradiation, Pouget *et al* (2002) calculated that 45% of the damage is in the form of SSBs and 55% in the form of base lesions. As it has consistently been shown in our results (Figures 4.1, 4.2 and 4.4) as well as results from other groups (Godon *et al*, 2008, Lan *et al*, 2004), ~40% of XRCC1-dependent lesions are PARP-independent. This implies that SSBs alone cannot be the only form of sub-lesions requiring PARP for repair. Ström *et al* proposed a BER model where a subset of BER SSB intermediates become uncoupled during repair and are then bound by PARP1 which if inhibited is trapped on these SSB intermediates, preventing further repair (Ström *et al*, 2011a). Alternatively, based on the numbers of different types of DNA damage generated per cell per Gray in mammalian cells, calculated by Cadet *et al*, (2008), it is proposed that the PARP-dependent damage requiring XRCC1 includes not only SSBs but also purine base damage, whereas the PARP-independent damage but requiring XRCC1 is mainly pyrimidine base damage (Figure 4.6). The glycosylases which excise either purine or pyrimidine damage tend to have different specificities. Evidence to support this includes the fact that in the presence of a PARP inhibitor, the kinetics of recruitment and loss of the glycosylase Nth, which excises pyrimidine damage generally, at the sites of damage is unaffected (Lan *et al*, 2004). Additionally OGG1 has recently been shown to bind to PARP-1 through its BRCT domain and this interaction is enhanced by oxidative stress (Noren-Hooten *et al*, 2011). The types of SSB arising from excision of

(a)

<u>Lesions/cell/Gy in mammalian cells</u>		
5,6-thymine glycol (Tg)	582	} Pyrimidine (888)
5-(hydroxymethyl)-2'-deoxyuridine	174	
5-formyl-2'-deoxyuridine	132	
FapyG	234	} Purine (354)
8-oxoguanine	120	
SSB	1000	
Cadet <i>et al.</i> , Acc. Chem. Res., 41 1075 (2008)		

(b)

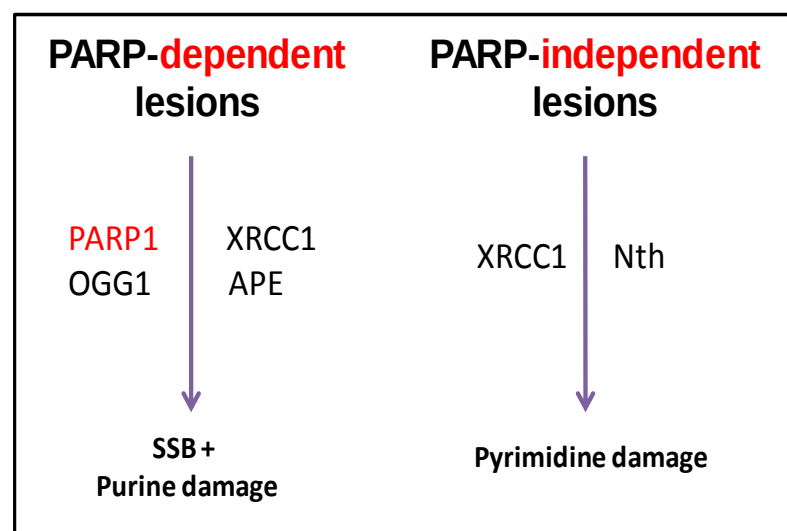


Figure 4.6: Types of damage repaired by the PARP-dependent and PARP-independent pathways. (a) Number of each type of lesion induced per Gray per mammalian cell. Pyrimidine damage represents 40% of the total non-DSB damage which could be repaired by XRCC1. (b) Scheme showing the types of proteins that could be involved in repair of PARP-dependent and PARP-independent lesions and what those lesions could represent.

damaged bases by Nth or OGG1 are different, as Nth has a β -lyase activity whereas OGG1 is stimulated by AP-endonuclease (Vidal *et al*, 2001b). The 'SSB' intermediates induced by OGG1/AP-endonuclease may be accessible to PARP whereas the 'SSB' intermediate induced by Nth remains 'hidden' during recruitment of DNA Pol β . To explore this proposal on the different DNA damage types, more research needs to be undertaken to investigate XRCC1 recruitment in the presence of PARP inhibitor after induction of specific types of DNA damage, as the damage induced by both ultrasoft X-ray and NIR laser irradiation produce a broad spectrum of damage types (Botchway *et al*, 2010, Cadet *et al* 2008, Harper *et al*, 2008, Yokoya *et al*, 2009).

Another interesting observation was that the slow component of repair after NIR laser microirradiation was not observed in the presence of PARP inhibitor (Figure 4.2). This may be due to the recruitment and/or persistence of XRCC1 at later times being too low to detect above background levels. However, an alternative explanation is that PARP is also involved in the repair of complex damage. Complex damage persists for longer than simple damage and during delayed repair, the BER intermediates may become uncoupled from the BER proteins, allowing PARP to bind to the now exposed SSBs and therefore preventing recruitment of XRCC1.

As the experiments reported in this study were performed using cells that are proficient in classical nonhomologous end joining (C-NHEJ), it is proposed that the kinetics of repair involving XRCC1-YFP in these cells represents the repair of base lesions and not DSB repair by the back-up NHEJ (B-NHEJ) pathway, which has been shown to not significantly contribute to DSB repair when the Ku proteins involved in C-

NHEJ are present (Audebert *et al*, 2004, Perrault *et al*, 2004, Reynolds *et al*, 2012, Wang *et al*, 2003).

The effect of cell cycle on the loss of fluorescence of XRCC1-YFP at damage sites was explored in exponentially growing and serum-starved (enhanced G₁ phase) cells following PARP inhibition (Figure 4.5). Similar to the results seen in Figure 3.16, no difference was observed in the decay kinetics on cell cycle, with or without PARP inhibitor. This implies that the PARP-dependent damage also recruits XRCC1 throughout the cell cycle.

In conclusion, inhibition of PARP substantially reduces the amount of XRCC1 recruited to DNA damage sites induced by both ultrasoft X-ray and NIR laser microbeam irradiation. However, even when PARP is inhibited, ~40% of XRCC1-dependent lesions can still recruit XRCC1. Therefore, there is a subset of XRCC1-dependent lesions that do not require PARP1 for repair.

Chapter 5: Effect of HDAC inhibitors on BER

5.1 Introduction

Genomic DNA in mammalian cells is packaged into the nucleus in the form of chromatin (Kornberg, 1977). Chromatin is composed of nucleosomes, each of which consists of 146 base pairs of DNA wrapped around an octamer of the four core histones (H3, H4, H2A, and H2B) and one molecule of the linker histone H1 (Noll and Kornberg, 1977, Richmond *et al*, 1984). These histones can be modified via processes such as phosphorylation, methylation, ubiquitylation and acetylation (Kouzarides, 2007, Spencer and Davie, 1999). Acetylation of histones is regulated by the opposing actions of histone acetyltransferases (HATs) and histone deacetylases (HDACs) that work by adding and removing acetyl groups from lysine residues on the N-terminal tails of the histones (Waterborg, 2002). Acetylation of histones causes chromatin remodelling by loosening of the contact between the histones and DNA (Garcia-Ramirez *et al*, 1995, Norton *et al*, 1989, Shogren-Knaak *et al*, 2006, Tse *et al*, 1998, Wang *et al*, 2001) and is also associated with transcriptional activation (Benson *et al*, 2006, Hansen *et al*, 1998, Nightingale *et al*, 1998, Wang *et al*, 2000). Conversely, deacetylation of histone tails results in transcriptional repression through chromatin condensation (Kemp *et al*, 2005, Koprinarova and Russev, 2008, Lee and Workman, 2007, Misteli and Soutoglou, 2009). Non histone proteins can also be reversibly acetylated, and this can affect the activity and stability of these proteins (Glozak *et al*, 2005).

There are currently 18 known HDACs in human cells that fall into four classes. Class I is comprised of the ubiquitously expressed HDACs 1, 2, 3 and 8 and are mainly located in

the nucleus (Glozak and Seto, 2007, Yang and Seto, 2008). Class II are mainly cytoplasmic and include HDACs 4-7, 9 and 10 (Yang and Seto, 2008). HDAC class III consists of Sirtuins 1-7 which can be both nuclear and cytoplasmic (Haigis and Sinclair, 2010). Unlike the other three of the four classes of HDACs that have been identified in humans, which are zinc-dependent, class III HDACs depend on nicotinamide adenine dinucleotide (NAD⁺) for their catalytic activity (Lawson, 2010). HDAC11 is the only member of class IV (Gao *et al*, 2002) and is thought to be involved in the regulation of interleukin-10 expression (Lian *et al*, 2012).

Excessive deacetylation of histones has been linked to cancer as this can block the transcription of a variety of tumour regulatory genes (Glozak and Seto, 2007). Broad-specificity HDAC inhibitors are currently in clinical trials as they are known to selectively kill cancer cells by preventing cell proliferation, promoting differentiation and inducing apoptosis (Bertrand, 2010, Camphausen and Tofilon, 2007, Glozak and Seto, 2007, Smith and Workman, 2009). HDAC inhibitors have also been trialled in combination with other cancer therapies, including radiotherapy (Dokmanovic *et al*, 2007, Koprionova *et al*, 2011, Rasheed *et al*, 2007).

The first known HDAC inhibitor was sodium butyrate (NaB) (Boffa *et al*, 1978, Candido *et al*, 1978, Prasad, 1980, Sealy and Chalkley, 1978, Vidali *et al*, 1978). Sodium butyrate is a short-chain fatty acid that can inhibit class I and class II HDACs and has often been used to investigate the role of histone acetylation in chromatin structure and function (Davie, 2003, Hendzel *et al*, 1991). Sodium butyrate has been shown to affect many cell processes including differentiation, proliferation, motility, induction of cell cycle arrest and apoptosis (Mühlethaler-Mottet *et al*, 2008) although the mechanisms are

poorly understood (Xu *et al*, 2007). Microarray assays of global gene expression profiles have shown that sodium butyrate significantly regulates the expression of over 450 genes in bovine kidney epithelial cells (Li and Li, 2006) and over 10,000 genes in human epithelial cells (Daly and Shirazi-Beechey, 2006). Trichostatin A (TSA) has also been identified as a class I and II HDAC inhibitor that acts by inhibiting cell cycle progression, inducing differentiation and modifying histones (Ailenberg and Silverman, 2002, Kemp *et al*, 2005, Monneret, 2005, Noh *et al*, 2009, Yoshida *et al*, 1990, Yoshida *et al*, 1995). Class III HDAC inhibitors include nicotinamide (NAM), which is the endogenous inhibitor of sirtuins (formed by NAD^+ during catalysis) and can control sirtuin activity through a negative feedback loop (Bitterman *et al*, 2002, Lawson, 2010).

Several groups have used HDAC inhibitors to investigate the participation of HDACs in DNA double strand break repair (Koprinarova *et al*, 2011, Miller *et al*, 2010, Robert *et al*, 2011) and HDACs have been found recruited to nuclear repair foci after DNA damage (Kao *et al*, 2003, Miller *et al*, 2010). However, little work has been done on the effect of HDAC inhibitors on the BER pathway, although it is known that some BER proteins can be acetylated or deacetylated by HATs and HDACs (Bhakat *et al*, 2003, Busso *et al*, 2010, Hasan *et al*, 2001, Hasan *et al*, 2002, Yamamori *et al*, 2010). Therefore, the effect of different HDAC inhibitors on BER was looked at by investigating, in real time, the recruitment and loss of XRCC1 and FEN1 at sites of radiation-induced DNA damage in mammalian cells.

5.2 Effect of HDAC inhibitors on the recruitment and loss of fluorescently-tagged BER proteins, in real time, at radiation-induced DNA damage sites

EMC11 XRCC1-YFP tagged cells were treated with 2 mM sodium butyrate (NaB) for 24 h prior to NIR laser microbeam irradiation (see section 2.6.4) or ultrasoft X-ray irradiation (see section 2.6.3), or 400 nM TSA for 15 h prior to ultrasoft X-ray irradiation. NaB and TSA are both class I and II HDAC inhibitors. The kinetics of the fluorescence decay of XRCC1-YFP to DNA damage sites were calculated for both NaB/TSA treated and untreated cells. The half-life of the fast component of XRCC1 fluorescence decay is approximately 7 fold slower after NaB treatment than in control cells after NIR laser microbeam irradiation, whereas the half-life of the slow component is not obviously affected by NaB treatment (Figure 5.1). Similarly, there is an increase in the XRCC1-YFP fluorescence decay half life after ultrasoft X-ray irradiation in NaB and TSA treated cells compared to untreated cells (Figure 5.2 and Figure 5.3) of approximately 3 fold and 2 fold, respectively.

To investigate whether class III HDACs also have an effect on XRCC1-YFP recruitment and loss at radiation-induced DNA damage sites in mammalian cells, EMC11 XRCC1-YFP tagged cells were treated with 5 mM nicotinamide (NAM) for 16 h prior to ultrasoft X-ray irradiation. In contrast to the class I and II HDAC inhibitors, there was an approximately 1.5 fold decrease in the half life of XRCC1-YFP fluorescence decay in NAM treated cells compared to untreated control cells (Figure 5.4).

Importantly, it was noticed that there was an increase in overall nuclear XRCC1-YFP fluorescence in the NaB and TSA treated cells compared to the controls (Figure 5.5)

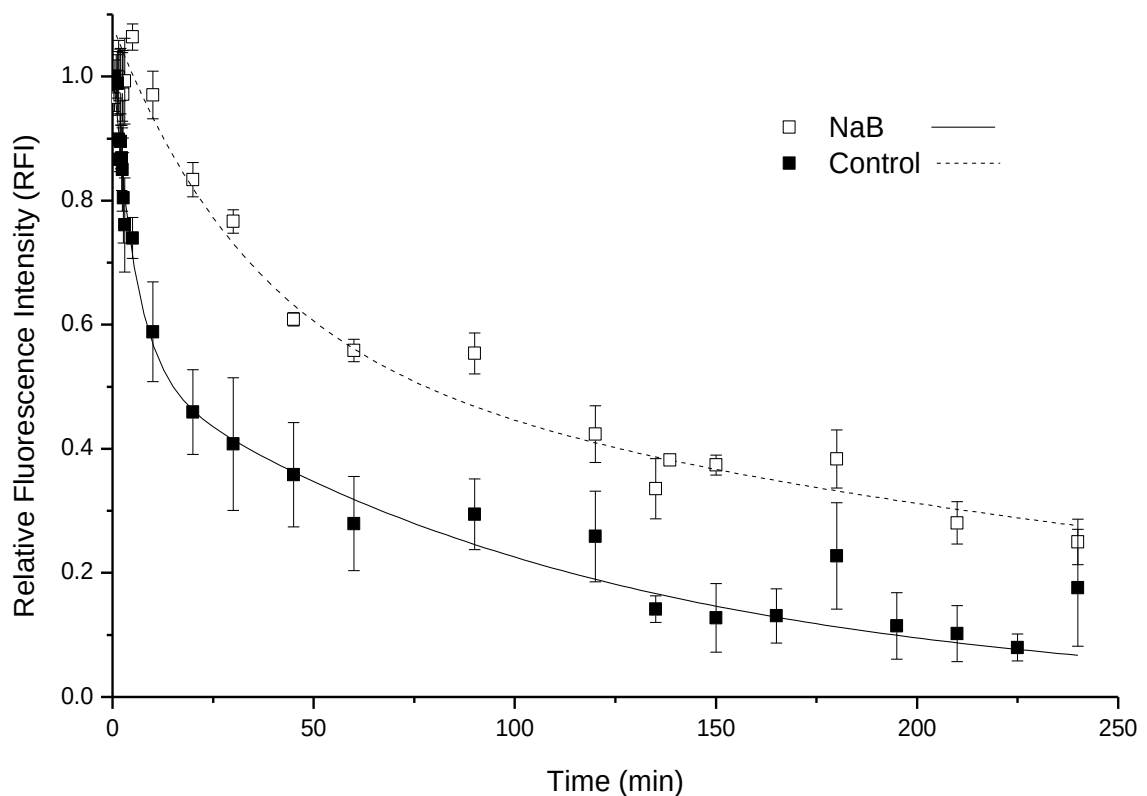


Figure 5.1: Real time recruitment and loss of XRCC1-YFP following laser microbeam irradiation of EMC11 XRCC1-YFP cells incubated with 2mM NaB for 24 h prior to irradiation. Each point represents the normalised relative fluorescence intensity of the foci track minus the nuclear background in a minimum of 10 cells per experiment. The graph represents the mean of 3 independent experiments $\pm$ SEM. Kinetic analysis of the loss of relative fluorescence from the normalised data was performed using Origin[®] for each independent experiment. The average for all 3 experiments was then calculated. The fluorescence decay of XRCC1-YFP is fitted by bi-exponential decays for both the control cells (half-lives of $4 (\pm 3)$ min & $167 (\pm 111)$ min) and the NaB treated cells (half lives of $27 (\pm 7)$ min & $211 (\pm 16)$ min).

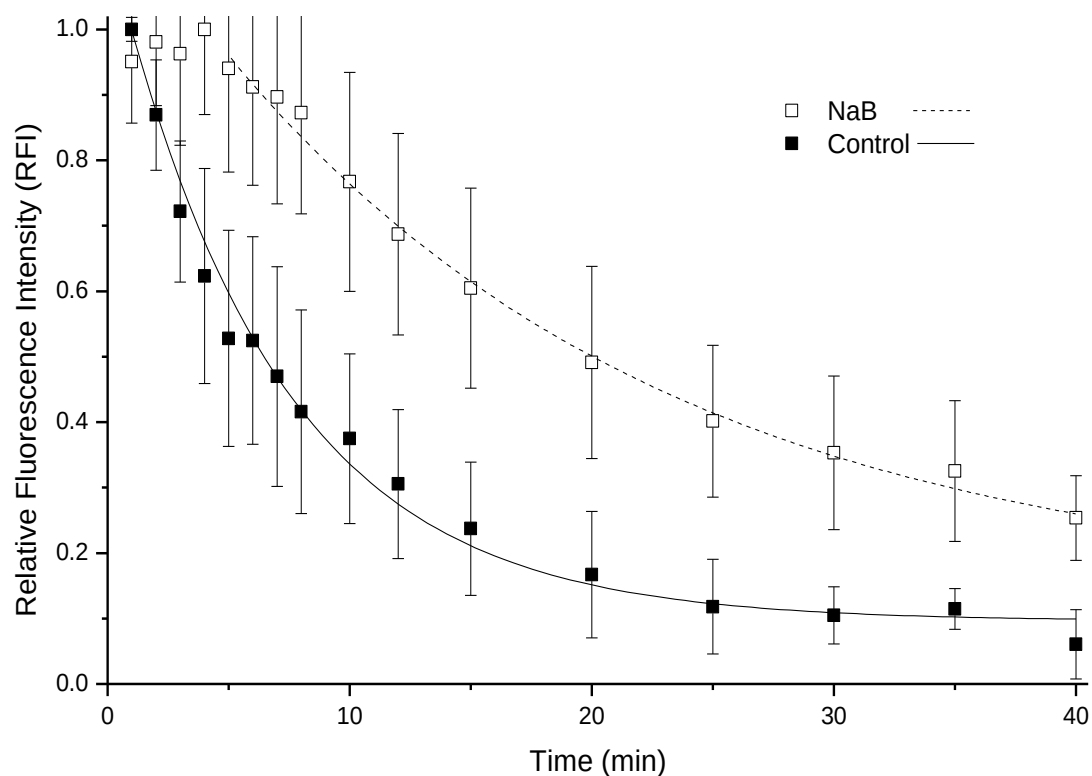


Figure 5.2: Real time recruitment and loss of XRCC1-YFP following 27 Gy ultrasoft X-ray irradiation of EMC11 XRCC1-YFP cells, incubated with 2 mM NaB for 24 h prior to irradiation. The cells were imaged on the Bioradiance confocal microscope. Each point represents the normalised relative fluorescence intensity of the foci track minus the nuclear background in a minimum of 10 cells per experiment. The graph represents the mean of 3 independent experiments $\pm$ SEM. Kinetic analysis of the loss of relative fluorescence from the normalised data was performed using Origin[®] for each independent experiment. The average for all 3 experiments was then calculated. The fluorescence decay of XRCC1-YFP is fitted by a single exponential decay for both control and NaB treated cells with a half-life of $7 (\pm 5)$ min for the control cells and a half-life of $24 (\pm 14)$ min for the NaB treated cells.

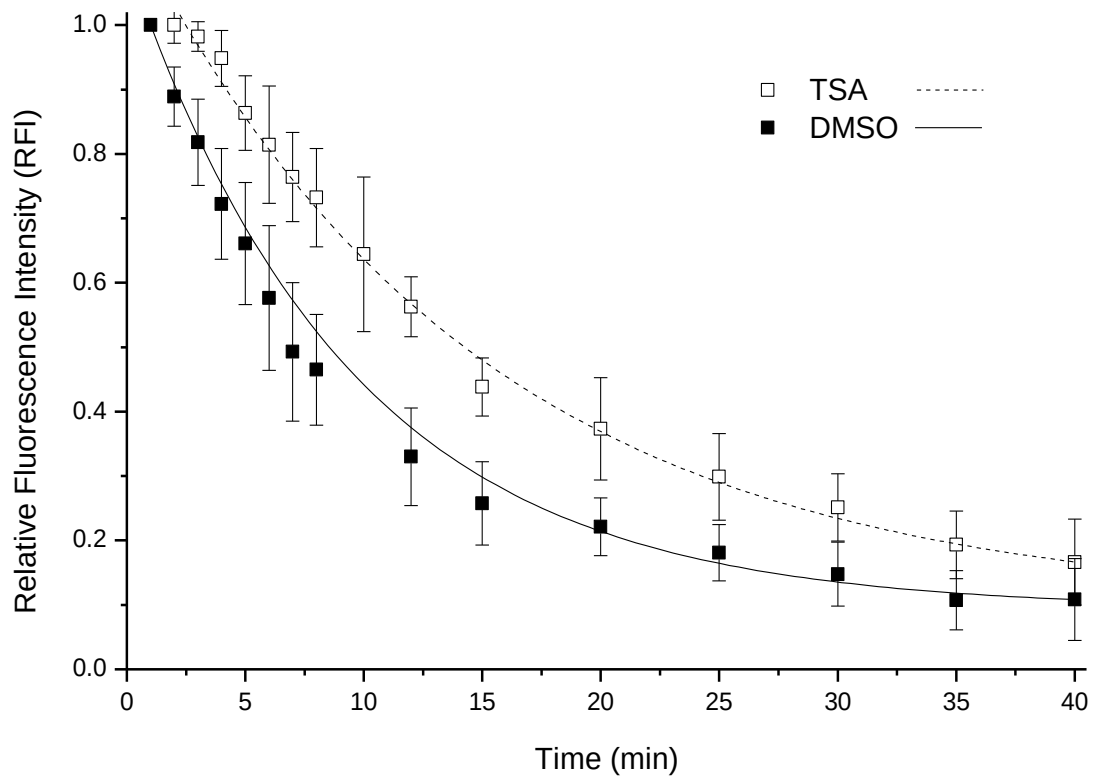


Figure 5.3: Real time recruitment and loss of XRCC1-YFP following 27 Gy ultrasoft X-ray irradiation of EMC11 XRCC1-YFP cells, incubated with 400 nM TSA for 15 h prior to irradiation. The cells were imaged on the Bioradiance confocal microscope. Each point represents the normalised relative fluorescence intensity of the foci track minus the nuclear background in a minimum of 10 cells per experiment. The graph represents the mean of 3 independent experiments $\pm$ SEM. Kinetic analysis of the loss of relative fluorescence from the normalised data was performed using Origin[®] for each independent experiment. The average for all 3 experiments was then calculated. The fluorescence decay of XRCC1-YFP is fitted by a single exponential decay for both control and TSA treated cells with a half-life of $6 (\pm 2)$ min for the control cells and a half-life of $11 (\pm 3)$ min for the TSA treated cells.

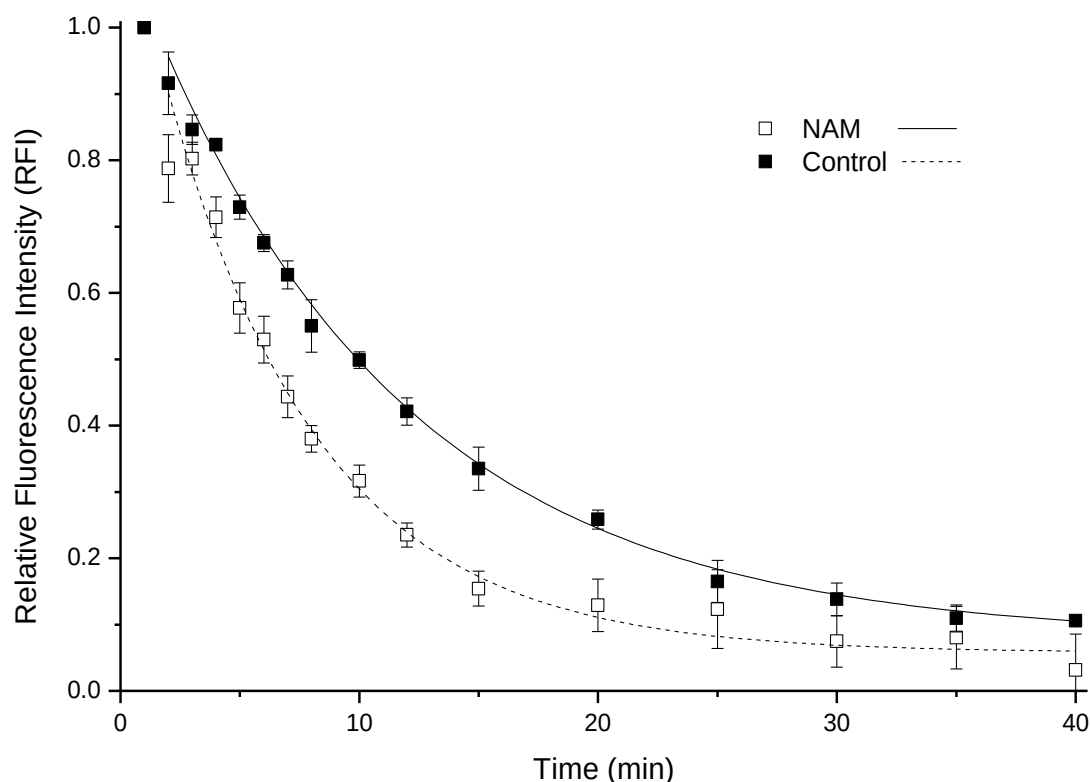


Figure 5.4: Real time recruitment and loss of XRCC1-YFP following 27 Gy ultrasoft X-ray irradiation of EMC11 XRCC1-YFP cells, incubated with 5 mM NAM for 16 h prior to irradiation. The cells were imaged on the Bioradiance confocal microscope. Each point represents the normalised relative fluorescence intensity of the foci track minus the nuclear background in a minimum of 10 cells per experiment. The graph represents the mean of 3 independent experiments $\pm$ SEM. Kinetic analysis of the loss of relative fluorescence from the normalised data was performed using Origin[®] for each independent experiment. The average for all 3 experiments was then calculated. The fluorescence decay of XRCC1-YFP is fitted by a single exponential decay for both control and NAM treated cells with a half-life of $10 (\pm 1)$ min for the control cells and a half-life of $6 (\pm 1)$ min for the NAM treated cells.

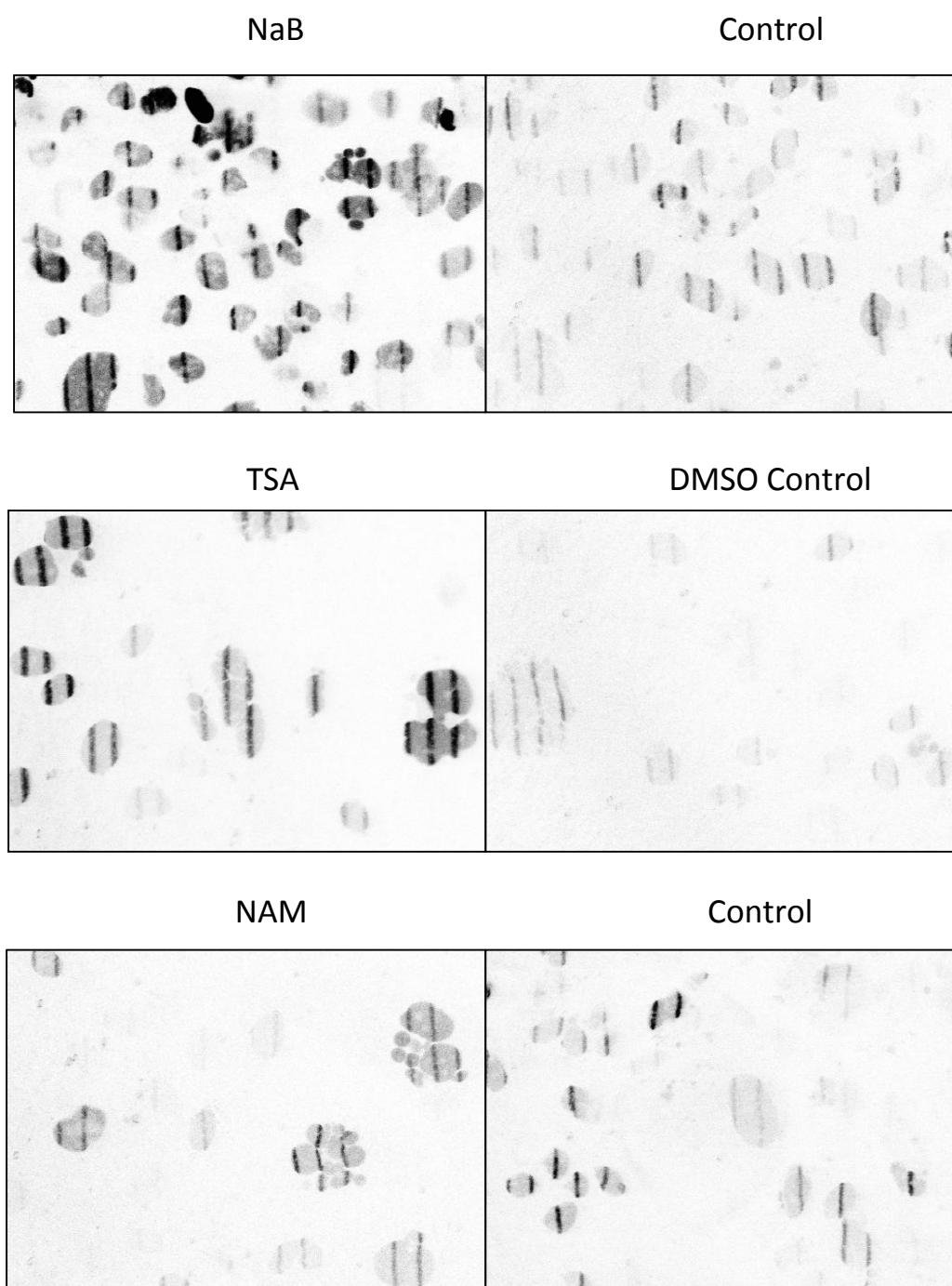


Figure 5.5: Confocal microscope images of ultrasoft X-ray irradiated (27 Gy) EMC11 XRCC1-YFP tagged cells treated with HDAC inhibitors (2mM NaB for 24 h, 400 nM TSA for 15 h or 5 mM NAM for 16 h) compared to untreated control cells imaged on the same day. Images were taken 2 min following irradiation. TSA was solubilised in DMSO and therefore an equal volume of DMSO was added to the control cells in the second panel.

which was also seen in the CHO FEN1-GFP cells after NaB treatment, so it was decided to investigate this further.

5.3 BER protein levels in mammalian cells following HDAC inhibitor treatment

It is already known that HATs and HDACs can affect transcription of many genes (see section 5.1) and an increase in nuclear fluorescence was seen in the EMC11 XRCC1-YFP tagged and CHO FEN1-GFP tagged cells after HDAC inhibitor treatment, particularly NaB (Figure 5.5). Therefore, the levels of BER proteins in the EMC11 XRCC1-YFP and CHO FEN1-GFP tagged cells after HDAC inhibitor treatment was investigated using western blotting (see section 2.4). As can be seen in Figure 5.6, the level of endogenous FEN1, APE1 and Pol β remained the same in the EMC11 XRCC1-YFP tagged cells with or without HDAC inhibitor treatment. However, there was a huge increase in the expression of XRCC1-YFP after NaB treatment. There was also a smaller but detectable increase in the expression of XRCC1-YFP after TSA treatment and a slight increase after NAM treatment (Figure 5.6). Similarly, the endogenous levels of FEN1, XRCC1, APE1 and Pol β in CHO FEN1-GFP cells remained the same after NaB treatment when compared to untreated control cells (Figure 5.7), whereas FEN1-GFP was clearly overexpressed after NaB treatment.

5.4 Recruitment and loss of endogenous XRCC1 in mammalian cells after sodium butyrate treatment

The effect of NaB on endogenous XRCC1 was investigated to show whether the persistence of XRCC1-YFP at sites of radiation-induced DNA damage seen after NaB treatment of EMC11 XRCC1-YFP tagged cells in section 5.2 (Figure 5.1 and Figure 5.2)

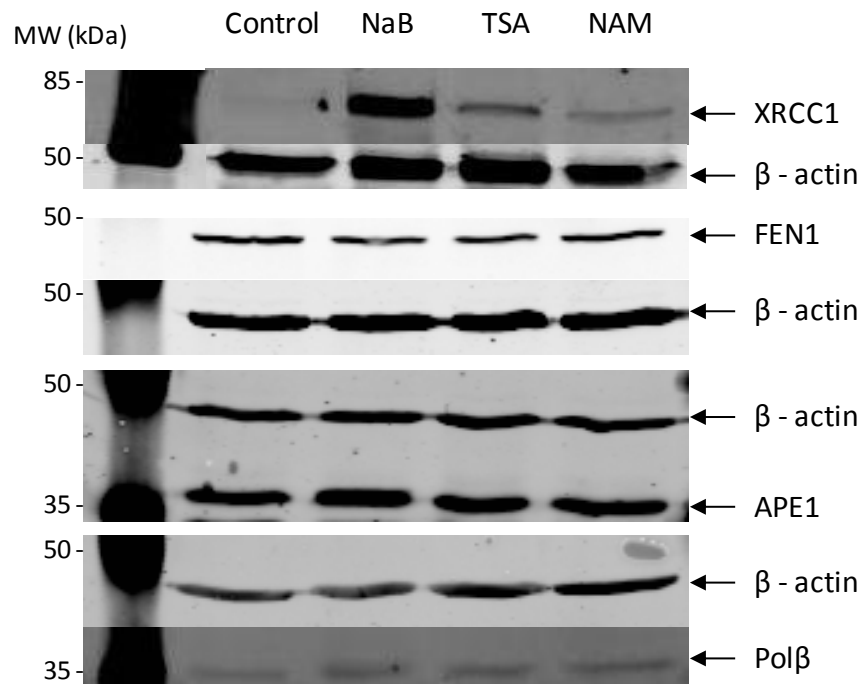


Figure 5.6: Western blot showing the levels of XRCC1-YFP, FEN1, APE1 and Pol β in untreated EMC11 XRCC1-YFP tagged cell lysate compared to NaB (2mM, 24h), TSA (400 nM, 15h), and NAM (5 mM, 16h) treated EMC11 XRCC1-YFP tagged cell lysates. β -actin was used as a loading control.

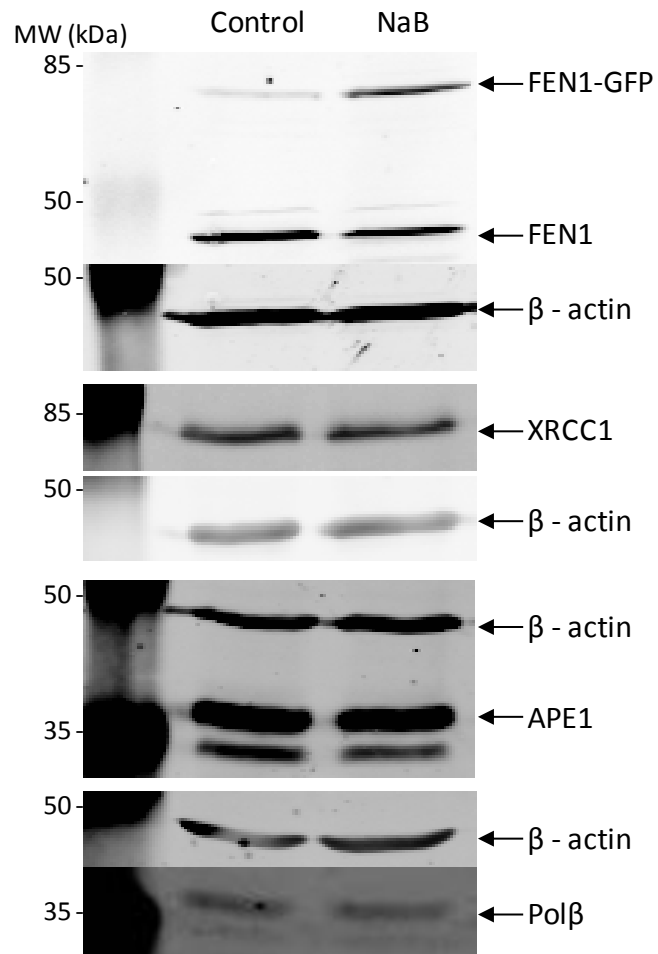


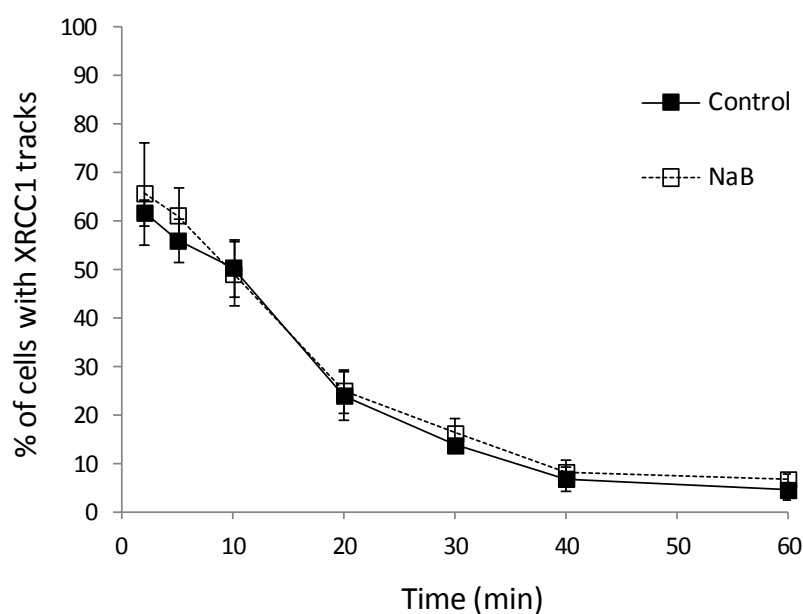
Figure 5.7: Western blot comparing the levels of FEN1-GFP, FEN1, XRCC1, APE1 and Pol β in untreated CHO FEN1-GFP tagged cell lysate with NaB (2mM, 24h) treated CHO FEN1-GFP tagged cell lysate. β -actin was used as a loading control.

was due to overexpression of XRCC1-YFP rather than the inhibition of deacetylation of cellular proteins induced by NaB. CHO cells untreated or treated with 2mM NaB for 24 h were irradiated with ultrasoft X-rays and fixed at specified times after irradiation. Cells were co-stained for XRCC1 and γ -H2AX (see section 2.8.1). XRCC1 and γ -H2AX were imaged using confocal microscopy (as described in section 2.9.2). γ -H2AX was used as a control for cells with ultrasoft X-ray induced DNA damage. The total number of cells and the number of cells with XRCC1 present at DNA damage sites were counted for each time point in both the untreated and NaB treated CHO cells using ImageJ software. The percentage of cells with XRCC1 recruited to DNA damage tracks compared to the total number of cells at each time point was calculated. As can be seen from the graph in Figure 5.8, there was no detectable difference in XRCC1 persistence in untreated or NaB treated cells, indicating that the difference in XRCC1-YFP kinetics seen in section 5.2 was due to overexpression of XRCC1-YFP caused by NaB treatment.

5.5 Discussion

HDAC inhibitors have previously been used to investigate the involvement of HDACs in DSB repair (Koprinarova *et al*, 2011, Miller *et al*, 2010, Robert *et al*, 2011) and it was found that inhibition of HDAC activity can impede both NHEJ and HR (Koprinarova *et al*, 2011, Miller *et al*, 2010). This was initially thought to be surprising as hyperacetylation of histones leads to chromatin relaxation and therefore increases the accessibility of the DNA to repair factors. However, it was hypothesised that in mammalian cells rapid deacetylation of histones following DSB induction condenses the chromatin to keep the broken ends in close proximity to each other, aiding

(a)



(b)

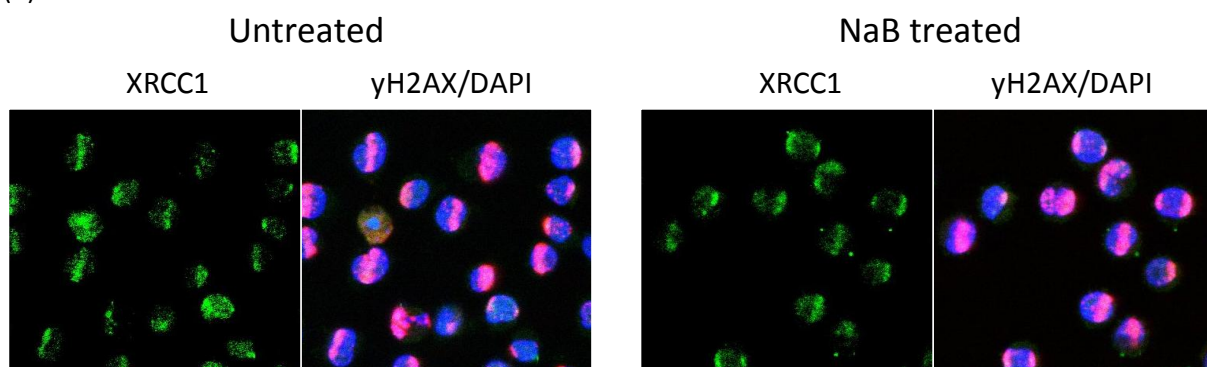


Figure 5.8: (a) Graph showing the percentage of cells that have endogenous XRCC1 recruited to DNA damage tracks induced by USX (27 Gy) over time in untreated or NaB treated (2mM, 24 h) CHO cells. A minimum of 100 cells were counted for each timepoint. XRCC1 was detected immunohistochemically using XRCC1 antibody. The graph represents the mean of 3 independent experiments $\pm$ SEM. (b) Confocal images of cells fixed 5 min following USX irradiation. Green represents XRCC1 staining, pink represents γ H2AX staining and blue represents DAPI staining of the cell nuclei.

accurate repair (Koprinarova *et al*, 2011, Soutoglou *et al*, 2007). Miller *et al* take this a step further by suggesting that after DSB induction, acetylation of histones occurs in a biphasic manner, with rapid deacetylation occurring to promote NHEJ, followed by histone acetylation to enhance HR (Miller *et al*, 2010). In contrast, DNA excision repair in UV-irradiated cells is enhanced when histones H3 and H4 are hyperacetylated (Dresler, 1985, Smerdon *et al*, 1982).

The model where histone acetylation leads to transcriptional activation through chromatin relaxation and histone deacetylation results in chromatin compaction and transcriptional repression is now thought to be too simplified, especially as microarray assays following sodium butyrate treatment show that most of the genes affected by sodium butyrate are actually repressed (Li and Li, 2006, Daly and Shirazi-Beechey, 2006). Histones are not the only proteins to be acetylated or deacetylated by HATs and HDACs e.g. SIRT1 has been found to deacetylate p53 (Luo *et al*, 2001). Interestingly, it has been shown that some BER proteins can be acetylated or deacetylated by HATs and HDACs (Bhakat *et al*, 2003, Busso *et al*, 2010, Hasan *et al*, 2001, Hasan *et al*, 2002, Yamamori *et al*, 2010) indicating that HDAC inhibition could interfere with the BER pathway. The transcriptional coactivator p300 has been shown to acetylate APE1 (Bhakat *et al*, 2003) and APE1 can be deacetylated by both HDAC1 (Busso *et al*, 2010) and SIRT1 (Yamamori *et al*, 2010). Yamamori *et al* also showed that inhibition of SIRT1 by nicotinamide prevents association of APE1 with XRCC1. Additionally, p300 acetylation appears to have contrasting effects on the various BER proteins. Acetylation of FEN1 by p300 considerably reduced FEN1's DNA binding and nuclease activity (Hasan *et al*, 2001), the dRP-lyase activity of Pol β was impaired after p300

acetylation (Hasan *et al*, 2002) and acetylation of NEIL1 by p300 inhibits its glycosylase activity (Bhakat *et al*, 2004). Whereas acetylation of the glycosylase OGG1 stimulates its glycosylase activity (Bhakat *et al*, 2006) and acetylated Werner protein (WRN) enhances Pol β -mediated strand displacement and stimulates LP-BER (Mufuoglu *et al*, 2008).

It was decided to investigate whether inhibition of class I and II or class III HDACs had an effect on the recruitment and loss of the BER proteins XRCC1 and FEN1 at sites of DNA damage induced by NIR laser microbeam or ultrasoft X-ray irradiation. NaB treatment resulted in a 7 fold increase in the half life of the fast component of XRCC1-YFP fluorescence decay after NIR laser microbeam irradiation (Figure 5.1) and a 3 fold increase in the XRCC1-YFP fluorescence decay half life after ultrasoft X-ray irradiation (Figure 5.2). Similarly, a 2 fold increase in the XRCC1-YFP fluorescence decay half-life after ultrasoft X-ray irradiation was seen with TSA treatment, another class I and II HDAC inhibitor (Figure 5.3). This was initially thought to be due to chromatin relaxation causing more DNA to be exposed and vulnerable to free-radical attack, causing more damage and at the same time increasing the clustering of the damage, resulting in retardation of repair. It has been shown that the yield of non-DSB lesions is greater in relaxed chromatin than condensed chromatin and the relative increase in lesions between the two forms of chromatin is greater than that with DSBs (Magnander *et al*, 2010, Nygren *et al*, 1995). On the other hand this could be seen as a surprising result as it has been shown that although more damage is induced in euchromatin than heterochromatin (i.e relaxed versus condensed chromatin), the repair is more efficient in euchromatin compared with heterochromatic regions

(Surralles et al, 1997). Jakob *et al* have also shown the opposite results to us where although the recruitment of GFP-tagged XRCC1 was the same in both heterochromatic and euchromatic regions, the decay kinetics were slower for the condensed, heterochromatic regions (Jakob *et al*, 2011).

Additionally, an increase in overall nuclear fluorescence was seen in the EMC11 XRCC1-YFP tagged cells after NaB and TSA treatment when compared to untreated control cells (Figure 5.5) indicating that these HDAC inhibitors can upregulate expression of the XRCC1-YFP protein. The levels of both endogenous and fluorescently tagged XRCC1 and FEN1 proteins in the stable clones after treatment with HDAC inhibitors were examined and compared to untreated cell lysates (Figure 5.6 and Figure 5.7). It was found that the HDAC inhibitors increased the level of the fluorescently tagged XRCC1 and FEN1 proteins, whereas the level of endogenous XRCC1 and FEN1 remain the same with and without HDAC inhibitor treatment as do the levels of APE1 and Pol β . This implied that the HDAC inhibitors were promoting expression of the exogenously introduced fluorescently-tagged proteins rather than having any influence on the expression of the endogenous BER proteins. This is thought to be due to the promoters used to express the fluorescently tagged proteins. The plasmid encoding the XRCC1-YFP gene uses a CMV immediate early promoter and a CMV promoter is also used for transcription of the FEN1-GFP gene. It has been shown that NaB can enhance expression from the CMV major immediate early promoter in a defective adenovirus vector (Wilkinson and Akrigg, 1992) and so this seems a likely explanation. Therefore, it would be best to repeat this work with plasmids encoding the endogenous promoters of XRCC1 and FEN1.

It was confirmed that the longer fluorescence decay half life of XRCC1-YFP seen after NaB treatment was due to overexpression of XRCC1-YFP rather than an effect of NaB on the endogenous BER pathway, as no difference in the persistence of endogenous XRCC1 was detected after ultrasoft X-ray irradiation of NaB treated CHO cells when compared to untreated cells (Figure 5.8). One possible reason for the longer half life seen after overexpression of XRCC1-YFP is that the intensity of XRCC1-YFP at the DNA damage tracks was so great that loss of XRCC1-YFP could not be detected at early timepoints due to pixel saturation during analysis.

Interestingly, despite a slight increase in XRCC1-YFP expression after NAM treatment (Figure 5.6), there was an approximately 1.5 fold decrease in the half-life of XRCC1-YFP fluorescence decay in NAM treated cells compared to control cells (Figure 5.4). NAM has been shown to prevent the association of APE1 and XRCC1 (Yamamori *et al*, 2010), so the shorter half life seen in Figure 5.4 could be due to the fact that XRCC1 isn't being recruited to APE1 sites but is still being recruited to DNA damage not requiring processing by APE1. This requires further investigation.

Chapter 6: Investigating the radiosensitisation effect of knocking down LP-BER proteins in mammalian cells

6.1 Introduction

LP-BER is required when 5' termini of breaks are resistant to β -elimination by Pol β , e.g. when an AP site contains a sugar phosphate group that has been modified (i.e. oxidised or reduced) (Klungland & Lindahl, 1997). IR can induce various oxidised AP sites (Xue & Greenberg, 2007), sugar lesions arising from either C1' or C2' oxidation of 2'-deoxyribose of nucleotides that have been initially cleaved on the 5' side by APE1 (DeMott *et al*, 2002, Greenberg *et al*, 2004, Sung *et al*, 2005). In the absence of LP-BER, the C4' oxidised AP site 2-deoxyribonolactone can form covalent cross-links with Pol β (Sung *et al*, 2005), that can lead to chromosomal aberrations and cell death. The key steps in LP-BER are displacement of a section of DNA into a flap structure by DNA pol δ/ϵ that is then subsequently processed by FEN1, followed by ligation of the resulting nick by DNA ligase I.

Ionising radiation results in clustered DNA damage and repair of this damage is more heavily reliant on the LP-BER pathway than isolated lesions (Byrne *et al*, 2009, Cunniffe *et al*, 2007, Imoto *et al*, 2008, Lomax *et al*, 2004a). Thus elimination of the LP-BER pathway could increase the probability of some lesions within a cluster remaining unrepaired and persisting until S phase, leading to stalled replication forks to give replication-induced DSBs (Harper *et al*, 2010, Saleh-Gohari *et al*, 2005). To investigate this, FEN-1, one of the key LP-BER proteins, has been knocked down by RNA interference in mammalian cells and the radiosensitivity of the cells determined using a clonogenic cell survival assay. Clonogenic cell survival assays detect the proportion of

cells that have retained the ability to replicate indefinitely after treatments that can cause reproductive death, such as ionising radiation (Franken *et al*, 2006). A549 (lung adenocarcinoma) and BEAS-2B (normal bronchial epithelium) cells were used to see if a difference could be seen in radiosensitivity between cancerous (A549) and non-cancerous (BEAS-2B) cells. The majority of cancer cells exhibit genomic instability that if enhanced by knocking down key repair proteins may result in cell death (Komarova *et al*, 2002, McManus *et al*, 2009). McManus *et al* (2009) have shown that knockdown of RAD54B causes chromosome instability in mammalian cells and that knockdown of FEN1 in RAD54-deficient human colorectal cancer cells causes synthetic lethality. It has also been shown that a DNA ligase I inhibitor causes a higher level of cytotoxicity in cancerous cells compared to a cell line established from normal breast epithelium (Chen *et al*, 2008). Additionally, inhibition of FEN1 blocked LP-BER resulting in enhanced cytotoxicity of the DNA-alkylating agent Temozolomide in colon cancer cells (Panda *et al*, 2009).

6.2 siRNA optimisation

Initially, HeLa cells were used to determine if FEN1 siRNA caused sufficient knockdown of FEN1 protein, and to establish how long following siRNA transfection the knockdown occurs, how long the knockdown persists and what concentration of siRNA is required. HeLa cells were selected as they are a human cell line often used for siRNA transfection and have previously been used by several groups for FEN1 knockdown using RNA interference (Liu *et al*, 2008, Sampathi *et al*, 2009, Schultz-Norton *et al*, 2007).

It is important to establish when and for how long the FEN1 protein is knocked down to optimise the time for irradiation during the cell survival assay. To study the influence of LP-BER on the survival of cells following IR, it is important that FEN1 is knocked down at the time of irradiation and during the repair processes. The manufacturer's recommended siRNA concentration is 30 nM, so this concentration was used to establish the time frame of FEN1 knockdown in HeLa cells (see section 2.5 for the siRNA protocol). FEN1 knockdown occurs by 24 h post-transfection with siRNA and remains knocked down at 72 h (Figure 6.1a). By 120 h the level of FEN1 protein has started to come back up and approaches control levels at 144 h (Figure 6.1a). Cells transfected with only Lipofectamine™ 2000 or a scrambled siRNA were used as controls to show that the knockdown seen was due to the FEN1 siRNA specifically knocking down FEN1 protein, rather than cytotoxicity caused by the transfection reagent or a non-specific effect caused by introducing siRNA into the cells.

Next, the optimum siRNA concentration for FEN1 siRNA knockdown in HeLa cells was determined. 30 nM FEN1 siRNA achieved knockdown in the time course experiment (Figure 6.1a) and so this was used as the maximum concentration. A siRNA titration from 1 nM to 30 nM was performed to establish the concentration of siRNA that gives good transfection efficiency but is as low as possible, to minimise non-specific effects and cytotoxicity. 48 h was chosen as a suitable time to lyse the transfected cells using information from the time course experiment (Figure 6.1a). FEN1 is knocked down to a similar level at concentrations of 30 nM, 25 nM, 20 nM & 15 nM FEN1 siRNA (Figure 6.1b). FEN1 levels are still low at a concentration of 10 nM FEN1 siRNA but start to increase again from 5 nM down to 1 nM siRNA (Figure 6.1b).

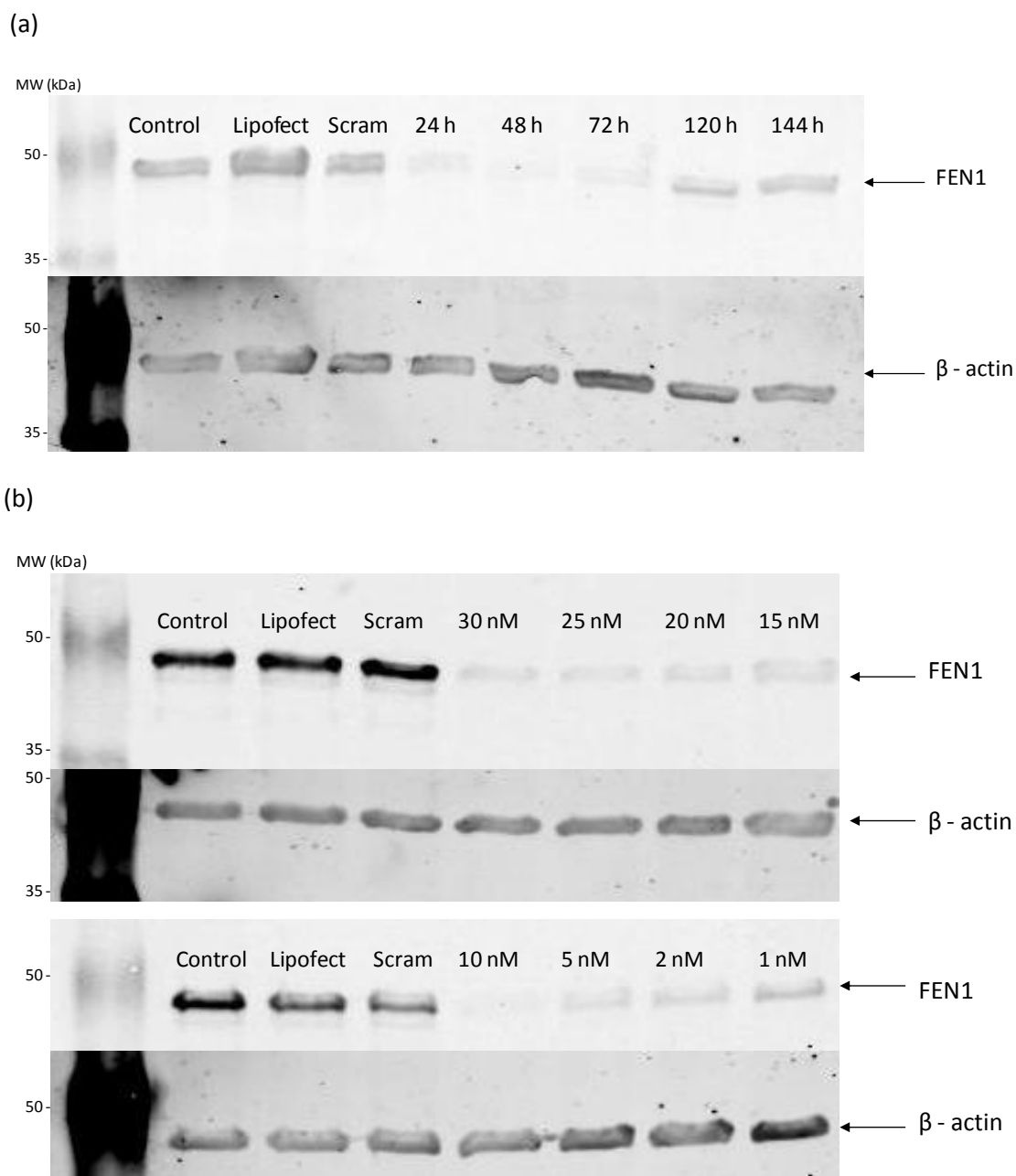


Figure 6.1: FEN1 siRNA optimisation in HeLa cells. Western blots showing the relative amount of FEN1 in HeLa cell lysates made (a) at specified time points after transfection with 30 nM FEN1 siRNA or (b) 48 h after transfection with decreasing concentrations of FEN1 siRNA, compared to untransfected control cells (control), cells transfected with Lipofectamine™ 2000 alone (Lipofect) or 30 nM scrambled siRNA (Scram). The control lysates in (a) were lysed at the same time as the 24 h FEN1 siRNA lysate. β -actin was used as a loading control.

Using the information gained from the time course and titration experiments performed with FEN1 siRNA in HeLa cells (Figure 6.1), FEN1 siRNA optimisation was achieved in the A549 cells. Although FEN1 is knocked down after 24 h, it is knocked down to a greater extent by 48 h and is still at maximum knockdown after 72 h (Figure 6.2). There does not seem to be a difference between the two concentrations of FEN1 siRNA used (15 nM and 30 nM) so a concentration of 15 nM was chosen to be used for future experiments to minimise any potential non-specific or cytotoxic effects caused by introducing the siRNA to the cells. 48 h was also selected as the optimal time for future experiments as at this time FEN1 is maximally knocked down and remains knocked down 24 h later. This persistence of knockdown is important for looking at the effects of FEN1 knockdown on DNA repair after irradiation. It was decided not to use a shRNA-expressing vector for constant knockdown of FEN1 due to FEN1's role in other pathways (e.g. replication) which may result in masking the effect on repair of radiation-induced DNA damage.

It was also important to check that trypsinisation and re-plating of the cells following transfection with FEN1 siRNA did not affect the knockdown of FEN1. This is necessary because the protocol for the cell survival assay requires that the cells are trypsinised and seeded into 60 mm petri dishes 30 h post-transfection with siRNA and then irradiated 15 h after seeding (see section 2.7 for protocol). Therefore, cells were trypsinised 30 h after transfection with FEN1 siRNA, along with untransfected control cells. Half were lysed (see section 2.4.2) and the other half were re-plated then lysed 15 hours later. Both the HeLa and A549 cells showed knocked down levels of FEN1 at the time of trypsinisation and 15 h after re-plating, at the time when the cells would

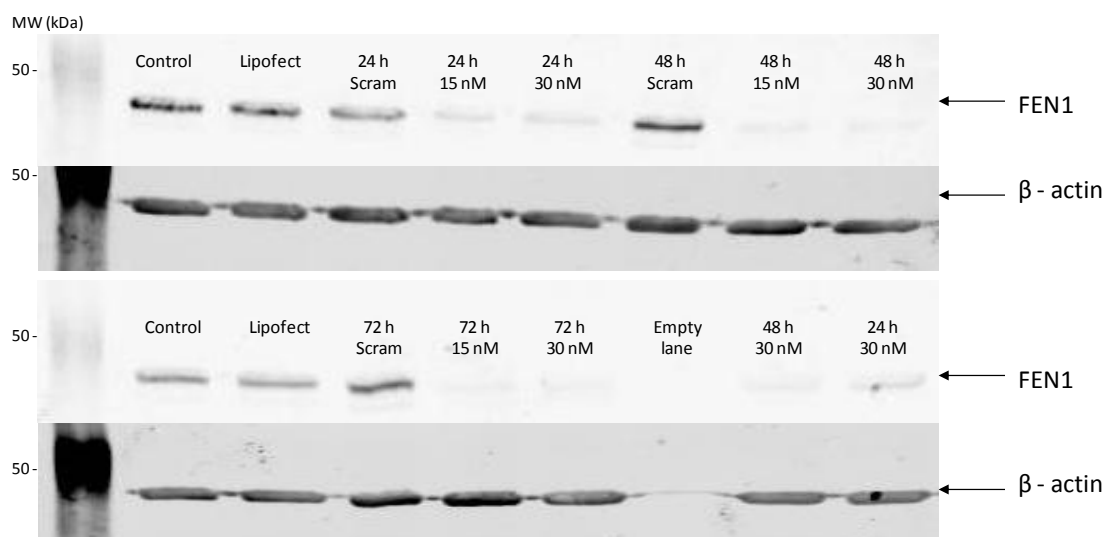


Figure 6.2: FEN1 siRNA optimisation in A549 cells. Western blots showing the relative amount of FEN1 in A549 cell lysates made at specified time points after transfection with 15 nM or 30 nM of FEN1 siRNA compared to untransfected control cells (control), cells transfected with Lipofectamine™ 2000 alone (Lipofect) or 30 nM scrambled siRNA (Scram). The 24 h and 48 h 30 nM FEN1 siRNA lysates were repeated on the second blot to compare with the 72 h lysates. β -actin was used as a loading control.

be irradiated (Figure 6.3).

FEN1 siRNA optimisation was also carried out on the BEAS-2B cells. Lipofectamine™2000 was found to be an ineffective transfection reagent (data not shown) but successful transfection was achieved with Lipofectamine™ RNAiMAX. Again, a concentration of 15 nM FEN1 siRNA and an irradiation time of 48 h post-transfection were considered to be the most suitable for future cell survival experiments (Figure 6.4).

For clonogenic cell survival assays it is necessary to plate different cell numbers depending on the cell line and radiation dose. Ideally, there should be 50-100 colonies per dish. Too few colonies would reduce statistical significance, while too many could not be counted accurately because they tend to merge in to one another. Therefore, the number of cells plated to achieve the required number of colonies had to be optimised for both the A549 and BEAS-2B cell lines. Table 6.1 shows the number of cells required to seed for each dose of irradiation for an optimal cell survival assay, irradiating 15 h after seeding and fixing 10 days following IR.

Table 6.1: Numbers of cells seeded per plate for each dose in the cell survival assay.

Dose	A549 cells seeded	BEAS-2B cells seeded
0	100	150
0.5	100	150
1	150	200
2	300	300
5	750	750
10	7500	7500

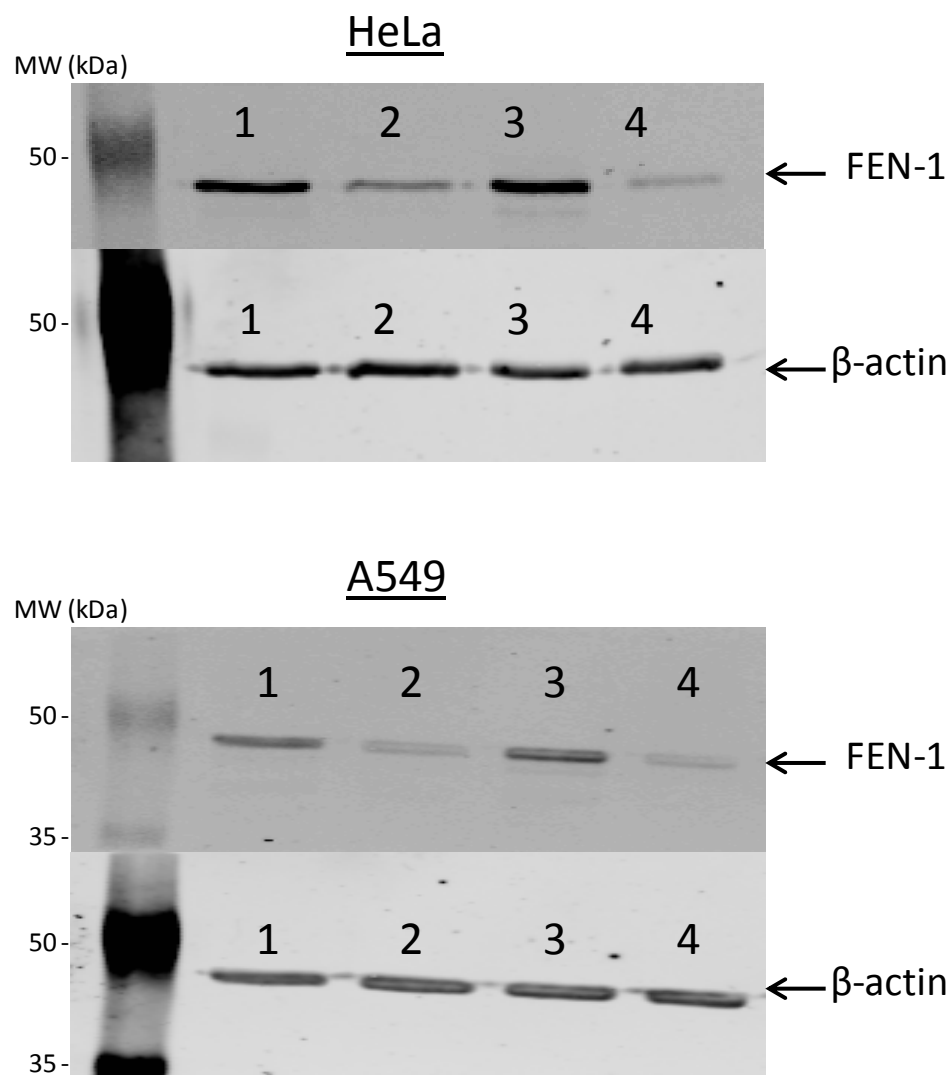


Figure 6.3: Test to confirm that FEN1 is still knocked down in HeLa and A549 cells after trypsinisation and re-plating. Lane 1 contains the untransfected control cell lysate made at the time of trypsinisation. Lane 2 contains the lysate of the FEN1 siRNA transfected cells made at the time of trypsinisation (30 h after transfection with 15 nM FEN1 siRNA). Lane 3 contains the untransfected control cell lysate made 15 h after trypsinisation and re-plating. Lane 4 contains the lysate of the FEN1 siRNA transfected cells made 15 h after trypsinisation and re-plating. The same loading pattern was used for both cell lines. β-actin was used as a loading control.

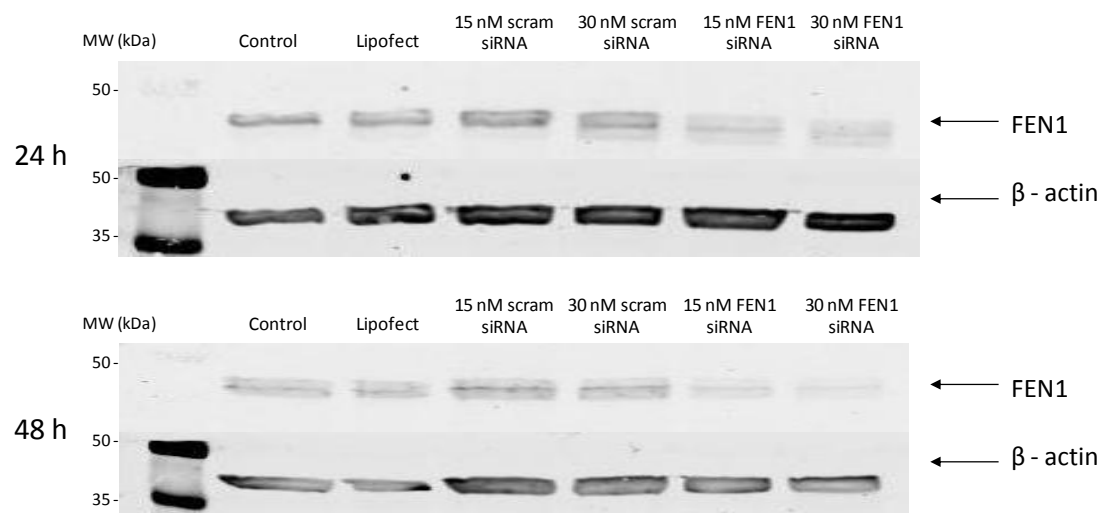


Figure 6.4: FEN1 siRNA optimisation in BEAS-2B cells. Western blots showing the relative amount of FEN1 in BEAS-2B cell lysates made at specified time points after transfection with 15 nM or 30 nM of FEN1 siRNA compared to untransfected control cells (control), cells transfected with Lipofectamine™ RNAiMAX alone (Lipofect) or scrambled siRNA (Scram). β-actin was used as a loading control.

6.3 Radiosensitivity of A549 and BEAS-2B cells after FEN1 knockdown

Once the siRNA transfection and the cell numbers for the clonogenic cell survival assay had been optimised, the radiosensitivity of A549 cells after FEN1 knockdown was investigated. As can be seen from Figure 6.5a, FEN1 knockdown causes radiosensitivity of the A549 cells when compared to cells transfected with scrambled siRNA and untransfected cells. The sensitisation enhancement ratio (SER) between the FEN1 siRNA and scrambled siRNA transfected cells at 10 % survival is 1.2. In contrast, FEN1 knockdown in BEAS-2B cells appears to be lethal to the cells as no colonies were formed, even in the unirradiated dishes. The lethality is due to the FEN1 knockdown rather than the siRNA transfection method as the radiosensitivity of BEAS-2B cells transfected with scrambled siRNA 48 h prior to irradiation was very similar to untransfected BEAS-2B cells (Figure 6.6a). A sample of cells transfected with FEN1 siRNA were seeded into T75 flasks at the same time as the cells that were seeded into 60 mm dishes prior to irradiation, and lysed at the time of irradiation to check the levels of FEN1 protein. FEN1 was knocked down as seen in all the FEN1 siRNA transfected lysates for all three repeat experiments in both the A549 and BEAS-2B cells when compared with the scrambled siRNA transfected and untransfected cell lysates (Figure 6.5b and Figure 6.6b).

6.4 Persistence of RAD51 in A549 cells after FEN1 knockdown

The possibility that inhibition of LP-BER causes increased levels of radiation-induced DNA damage to persist to replication, potentially causing replication-induced DSBs and stalled replication forks, was explored by staining for RAD51 foci in control and FEN1 knocked down A549 cells at various times post γ -irradiation. Cells were seeded into 30

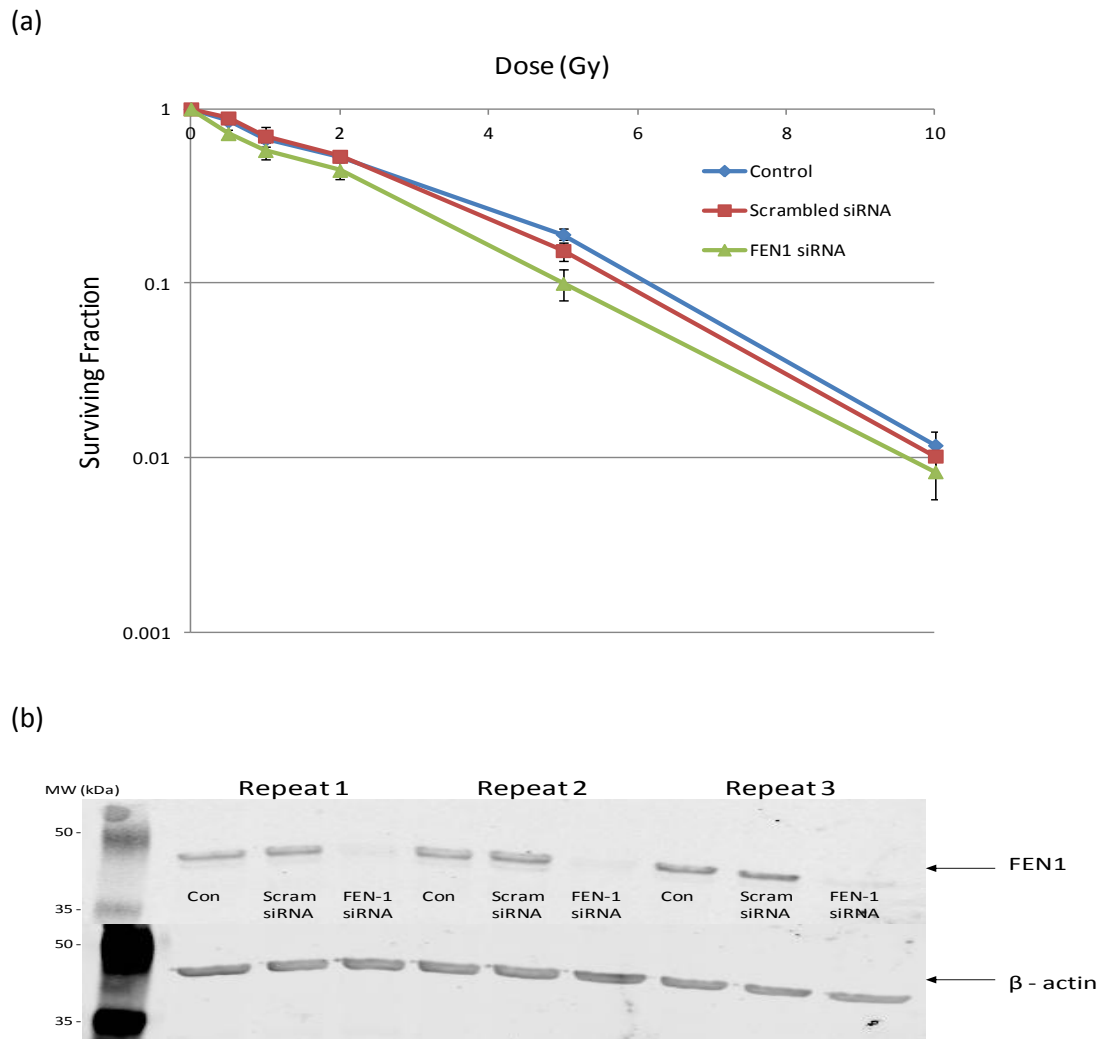
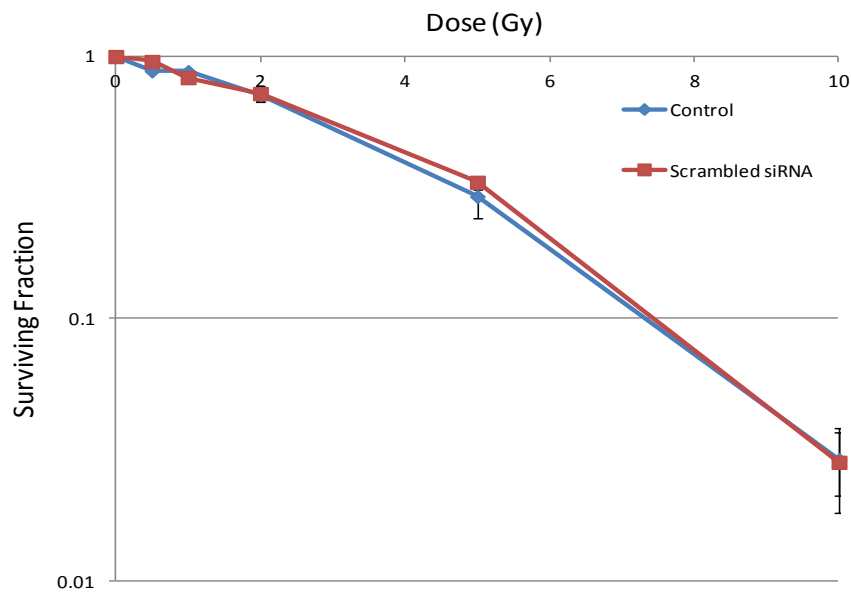


Figure 6.5: FEN1 knock down radiosensitises A549 cells. (a) Clonogenic cell survival assay comparing the radiosensitivity of A549 cells transfected with FEN1 siRNA or scrambled siRNA for 48 h prior to irradiation with untransfected A549 cells. The graph represents the mean of 3 independent experiments $\pm$ SEM. SER = 1.2 at 10% survival between the FEN1 siRNA and scrambled siRNA transfected A549 cells. (b) Western blot showing the relative amount of FEN1 in A549 cell lysates made at the time of irradiation for each of the 3 repeats. FEN1 is clearly knocked down in the cells transfected with FEN1 siRNA compared to the untransfected and scrambled siRNA controls. β -actin was used as a loading control.

(a)



(b)

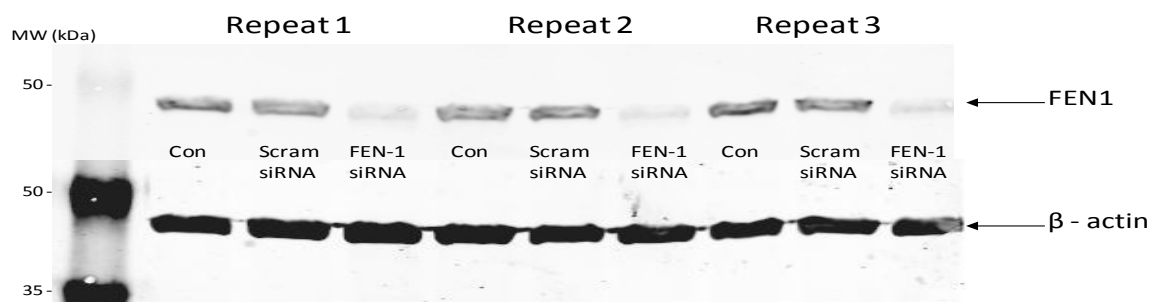


Figure 6.6: FEN1 knock down is lethal to BEAS-2B cells. (a) Clonogenic cell survival assay comparing the radiosensitivity of untransfected BEAS-2B cells with BEAS-2B cells transfected with scrambled siRNA for 48 h prior to irradiation. There is no data shown for FEN1 siRNA as this was lethal to the cells. The graph represents the mean of 3 independent experiments $\pm$ SEM. The difference in survival is insignificant indicating that the siRNA transfection method was not lethal to the BEAS-2B cells. (b) Western blot showing the relative amount of FEN1 in BEAS-2B cell lysates made at the time of irradiation for each of the 3 repeats. FEN1 is clearly knocked down in the cells transfected with FEN1 siRNA compared to the untransfected and scrambled siRNA controls. β -actin was used as a loading control.

mm glass dishes 30 h after siRNA transfection (see section 2.5) and then irradiated with 10 Gy γ -irradiation 15 h later (see section 2.6.1). Cells were incubated at 37 °C and in 5 % CO₂ humidified air for the specified repair times (1 h, 2 h, 3 h, 4 h, 6 h, 8 h, 10 h, 12 h & 24 h), fixed, stained for RAD51 and Cyclin A and imaged using confocal microscopy (see sections 2.8.2 and 2.9.2). ImageJ software was used for analysis. DAPI was used to stain the nuclei of the cells to determine the total cell count. Cyclin A is a marker for cells in S and G2 phase (Pagano *et al*, 1992, Pines & Hunter, 1991). Staining for Cyclin A, rather than S phase-specific BrdU, will show if any RAD51 foci are formed in non-S phase cells and enable us to reliably count these foci too, as some of the replication-induced DSBs may persist from S phase into early G2 phase where they arrest until repair is complete (DiPaola, 2002). As expected, all of the RAD51 foci were detected in Cyclin A stained cells, as homologous recombination occurs in S and G2 phases of the cell cycle. No obvious difference was seen in the number of RAD51 foci per RAD51 positive cell between the control and FEN1 siRNA transfected A549 cells at any of the time points investigated (Figure 6.7). However, there is a pronounced increase from 3 h in the average number of RAD51 foci per total number of cells in the FEN1 siRNA transfected cells compared to control cells, although the numbers are again comparable with control cells by 12 h (Figure 6.7). This indicates that there are more cells with RAD51 foci in the cells depleted of FEN1 from 3 h, potentially due to cells arresting to repair the replication-induced DSBs that have arisen from persistent radiation-induced DNA damage that wasn't repaired as a result of a deficiency in the LP-BER pathway.

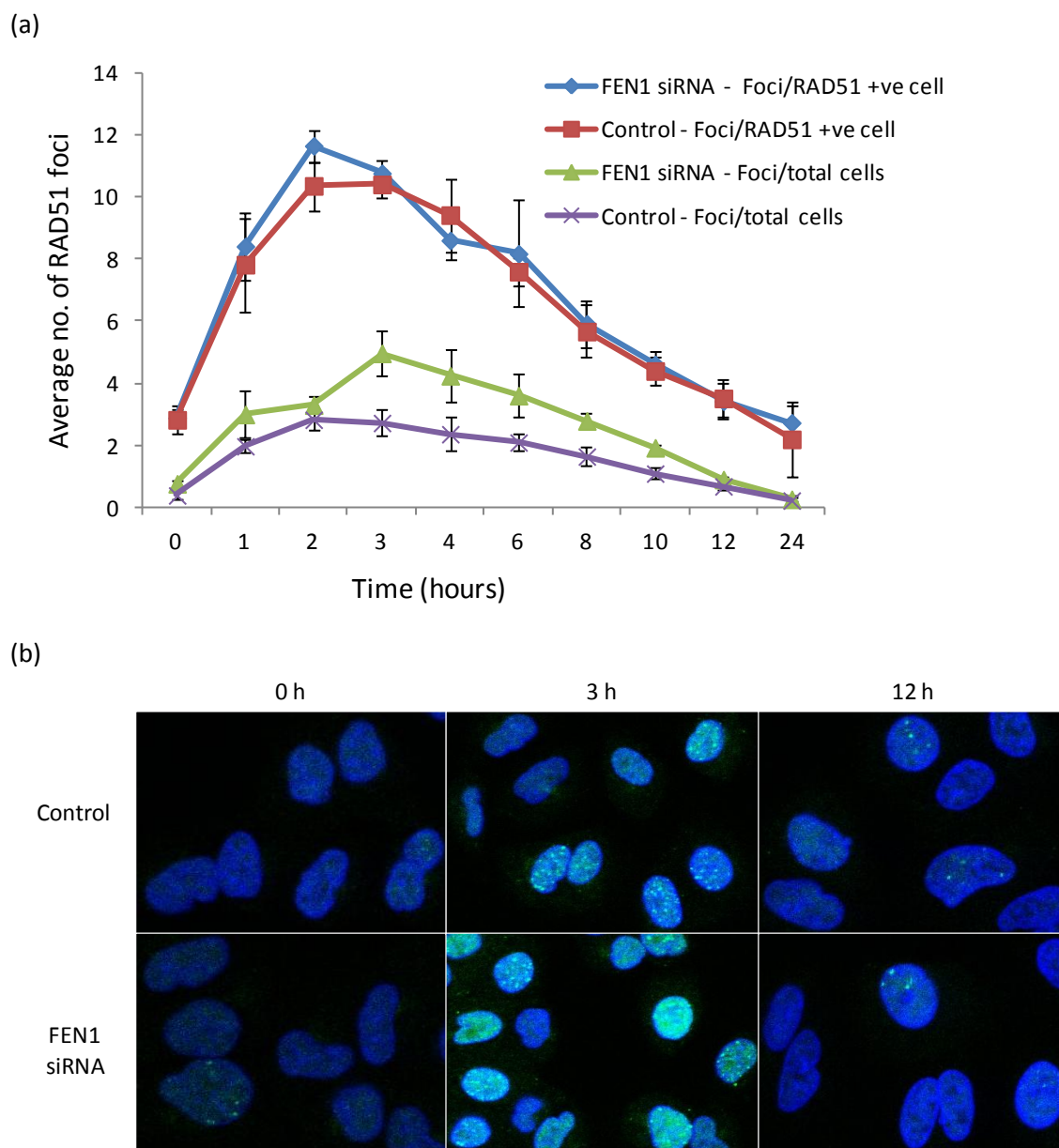


Figure 6.7: Detection of RAD51 foci in A549 cells transfected with FEN1 siRNA compared to untransfected A549 cells (Control). A549 cells were transfected with FEN1 siRNA 45 h prior to 10 Gy γ -irradiation. Cells were fixed and stained for RAD51 at the specified timepoints post-irradiation. Foci were counted using ImageJ software. A minimum of 100 cells per time point were analysed. (a) Graph representing the mean of 3 independent experiments $\pm$ SEM. (b) Confocal images of A549 cells fixed at the specified timepoints post-irradiation and stained for RAD51 (green) and DAPI (blue).

6.5 Discussion

FEN1, a key member of the LP-BER pathway, protein levels were knocked down using RNA interference in mammalian cells to investigate whether the cells show increased radiosensitivity, since clustered DNA damage, in part, requires the LP-BER pathway for repair (Byrne *et al*, 2009, Lomax *et al*, 2004a, Cunniffe *et al*, 2007, Imoto *et al*, 2008) and the lethal and mutagenic effects of IR are thought to result from incompletely or incorrectly repaired DNA lesions (Lindahl and Wood, 1999).

It was found that FEN1 knockdown does radiosensitise A549 (lung adenocarcinoma) cells when compared to the same cells transfected with scrambled siRNA and untransfected cells (Figure 6.5a). The SER at 10% survival is 1.2. This is comparable to the 1.3 SER value at 10% survival in A549 cells treated with the PARP inhibitor Olaparib (Senra *et al*, 2011), which is being considered for clinical trials in combination with radiotherapy. However, FEN1 knockdown in the normal bronchial epithelial BEAS-2B cells is lethal to the cells even before γ -irradiation. The lethality was proven to be due to FEN1 knockdown rather than the siRNA transfection process as cells transfected with scrambled siRNA showed similar radiosensitivity to the untransfected cells (Figure 6.6a). This in contrast to the result seen by Chen and colleagues, who demonstrated that cancerous (MCF7) cells showed increased radiosensitivity in the presence of a DNA ligase I inhibitor, whereas the normal breast epithelium (MCF10A) cell line was unaffected (Chen *et al*, 2008). The fact that FEN1 knockdown alone is lethal to BEAS-2B cells may be related to the other roles FEN1 has in cells. The 5'-flap structure that is recognised and cleaved by FEN1 in LP-BER is also a common intermediate in DNA replication and recombination (Bambara *et al*, 1997, Kikuchi *et al*, 2005, Liu *et al*, 2004,

Waga et al, 1994). In particular, the role of FEN1 in Okazaki fragment processing is well established, where it is required to remove the RNA primers from the fragments prior to ligation by DNA ligase I (Bambara *et al*, 1997, Waga et al, 1994). FEN1^{-/-} mice do not survive past early embryogenesis (Larsen *et al*, 2003) and deficiencies in FEN1 can cause genomic instability and tumorigenesis (Kucherlapati *et al*, 2002). The absence of FEN1 in BEAS-2B cells may trigger checkpoint activation and signalling pathways that result in cell cycle arrest and apoptosis (Roos and Kaina, 2012). Interestingly, it appears as if A549 cells have adapted to be able to replicate when FEN1 is knocked down in non-stressed conditions. However, following induction of clustered damage induced by irradiation, some lesions that require LP-BER will not be repaired, leading to additional replication-induced DSBs and chromosomal instability that may be enough to trigger cell inactivation and result in the increased radiosensitivity seen in A549 cells (Figure 6.5a).

It has been shown that repair of clustered DNA damage can be retarded (Byrne *et al*, 2009, Chaudry and Weinfeld, 1997, David-Cordonnier *et al*, 2001, David-Cordonnier *et al*, 2002, David-Cordonnier *et al*, 2000, Harrison *et al*, 1999, Lomax *et al*, 2004a, Lomax *et al*, 2004b, Peddi *et al*, 2008) and that this damage can sometimes persist until replication, causing stalled replication forks and replication-induced DSBs (Harper *et al*, 2008, Harper *et al*, 2010, Saleh-Gohari *et al*, 2005). It was hypothesised that by knocking down FEN1 and therefore substantially reducing the efficiency of LP-BER, even more lesions could persist until replication after irradiation causing an increase in stalled replication forks and replication-induced DSBs. This was investigated by counting the number of RAD51 foci in control and FEN1 knocked down A549 cells after

10 Gy γ -irradiation over a time course of 24 h (Figure 6.7). The HR protein RAD51 promotes strand invasion and homologous pairing between two DNA duplexes and colocalises with DNA damage signalling and repair proteins (Baumann *et al*, 1996, Gupta *et al*, 1997, Paull *et al*, 2000). It is expressed during S and G2 phases of the cell cycle where it can be found to localise to sites of DSBs (vanVeelen *et al*, 2005, Yamamoto *et al*, 1996) and is therefore used here as a marker of replication-induced DSBs. No difference was seen in the number of RAD51 foci per RAD51 positive cell between the control and FEN1 knockdown cells but the number of cells with RAD51 foci is greater from 3h in the FEN1 knockdown cells compared to the control cells, although by 12 h the levels are similar in both control and FEN1 knockdown cells. This implies that at low levels of FEN1 there is an increase in the number of cells that have replication-induced DSBs. The delay in the repair of replication-induced DSBs can result in both chromosomal aberrations and cell inactivation. ATR can recognise the replication-induced DSBs and cause a signal cascade that results in cell cycle arrest to give the cell time to repair the damage. However, if DNA repair cannot be achieved, the DSBs and DNA blocking lesions will persist and constant DNA damage signalling can eventually trigger apoptosis (Roos and Kaina, 2012). The return in the number of RAD51 positive cells to control levels by 12 h could reflect an increase in cell killing due to the persistent replication-induced DSBs.

It has been shown that cells can become blocked in the G2/M phase post-irradiation and that some cells can be released from the G2/M checkpoint with unrepaired clustered damage resulting in a range of chromosome aberrations (Asaithamby *et al*, 2011, Deckbar *et al*, 2007, Harper *et al*, 2008, Terzoudi *et al*, 2005). Cells can

sometimes use specialised DNA polymerases (including Pol η , Pol κ , Pol ι and Rev-1) to bypass unrepaired lesions during replication in a process called translesion synthesis (Chang and Cimprich, 2009, Lehmann *et al*, 2002, Lindahl & Wood, 1999) but this process is error-prone and can lead to mutations.

Shibata and Nakamura saw a prolonged S phase delay after MMS treatment in cells expressing a nuclease-defective dominant-negative FEN1 (Shibata and Nakamura, 2002). However, they did not see an effect after IR, although the cells they used also contained endogenous FEN1. S phase may also be prolonged by FEN1 deficiency due to the role of FEN1 in processing stalled replication forks, as it has been suggested that WRN and FEN1 function together to process DNA structures associated with replication forks (Sharma *et al*, 2004, Zheng *et al*, 2005). Another group used a DT40 chicken cell line with a homozygous deletion of the FEN1 gene and observed a delay in the progression of the FEN1 null cells through S/G2 phase (possibly due to the role of FEN1 in replication) and this delay was further increased after oxidative stress, highlighting the role of FEN1 in DNA repair (Asagoshi *et al*, 2010).

During S phase, repair by homologous recombination is used to restart DNA replication forks that have been blocked by DNA lesions, or those that have collapsed into replication-induced DSBs when SSBs are encountered (Thompson and Hinz, 2009, Thompson and Jones, 2010). Several groups have shown that inhibiting PARP1 in HR-deficient cells causes a massive increase in cell death (Bryant *et al*, 2005, Farmer *et al*, 2005, McCabe *et al*, 2006). Further, a synthetic lethal effect is seen by combining FEN1 knockdown with chromosomal instability caused by RAD54B deficiency (McManus *et al*, 2009). Additionally, A549 cells expressing a dominant negative truncated Pol β

protein, which lacks the polymerase domain but can still bind DNA, show only a small increase in radiosensitivity whereas the same truncated Pol β protein in HR-deficient cells substantially increased this radiosensitivity (Neijenhuis et al, 2010). Therefore, it would be interesting to see if knocking down FEN1 in HR-deficient cells increases the radiosensitisation effect further. Additionally, together with the reports on stalled repair of DNA damage, these observations on FEN1 are consistent with stalled repair in S-phase cells.

In conclusion, A549 lung adenocarcinoma cells can replicate in the absence of FEN1, whereas normal lung epithelial BEAS-2B cells cannot, possibly due to triggering of signalling pathways that are defective in the cancerous cells. However, knocking down FEN1 results in radiosensitisation of A549 cells. This may in part reflect that the repair of some of the clustered damage caused by ionising radiation requires fully functional LP-BER. Therefore, in the absence of LP-BER, these unrepaired lesions persist until replication, resulting in stalled forks and replication-induced DSBs which can lead to chromosomal aberrations and cell inactivation. This radiosensitisation effect may be increased further if combined with other repair deficiencies.

Chapter 7: General Discussion

The overall aim of this project was to increase the understanding of base excision repair of radiation-induced DNA damage including clustered damage sites of varying complexity in mammalian cells.

Clustered DNA damage is thought to be a unique signature of ionising radiation where two or more lesions are formed within one to two helical turns of DNA by passage of a single radiation track (Goodhead, 1994, Ward, 1994). The complexity of clustered DNA damage sites increases with the ionisation density and LET of the radiation (Goodhead, 1994, Nikjoo *et al*, 1998, Ottolenghi *et al*, 1997). Clustered damage can be split into non-DSB clustered sites, comprising of SSBs, AP sites and base lesions (Goodhead, 1994, Sutherland *et al*, 2000, Ward, 1994), and “dirty” DSBs which are DSBs that have other types of DNA lesions in close proximity (Datta *et al*, 2005, Nikjoo *et al*, 2001). Radiation-induced non-DSB clusters are formed in mammalian cells with a frequency of 4-8 times that of prompt DSBs (Georgakilas, 2008, Gulston *et al*, 2002, Sutherland *et al*, 2000, Sutherland *et al*, 2002). Lesions within these clustered damage sites have been found to be more difficult to repair than isolated lesions formed endogenously (Byrne *et al*, 2009, Chaudry and Weinfeld, 1997, David-Cordonnier *et al*, 2001, David-Cordonnier *et al*, 2002, David-Cordonnier *et al*, 2000, Harrison *et al*, 1999, Lomax *et al*, 2004a, Lomax *et al*, 2004b, Peddi *et al*, 2008) and this can potentially lead to mutations and replication-induced DSBs if the damage persists into S-phase (Bellon *et al*, 2009, Budworth *et al*, 2005, D’Souza and Harrison, 2003, Eccles *et al*, 2009, Harrison *et al*, 2006, Pearson *et al*, 2004, Malyarchuk *et al*, 2003, Shikazono *et al*, 2006). Additionally, chromatin structure is thought to play a role in the induction of

clustered DNA damage with the amount of clustered DNA damage increasing as the compaction of the chromatin decreases (Magnander *et al*, 2010). The main pathway that repairs non-DSB clustered lesions is the base excision repair pathway (Dianov *et al*, 2001, Lindahl and Wood, 1999, Wilson and Bohr, 2007, Zharkov, 2008). The BER pathway is split into two sub-pathways, short-patch BER and long-patch BER, and differ in the number of bases inserted by the repair polymerase (Fortini and Dogliotti, 2007). SP-BER is the predominant pathway, especially in the repair of isolated DNA lesions. However, LP-BER is thought to play a greater role in the repair of radiation-induced clustered lesions.

Cell lines were generated stably expressing the fluorescently tagged BER proteins, XRCC1-YFP or FEN1-GFP. The scaffold protein XRCC1 was chosen as a marker for SP-BER and the structure-specific nuclease FEN1 was chosen as a marker for LP-BER. Two different types of radiation, ultrasoft X-ray and NIR multiphoton laser microbeam, that vary in LET and the complexity of DNA damage induced, were used to irradiate the cell lines. Ultrasoft X-ray irradiation is a form of low LET radiation that deposits all of its energy in the form of low energy electrons, giving rise to secondary electron tracks that induce both simple damage together with a lower level of clustered DNA damage (Botchway *et al*, 1997, deLara *et al*, 2001). NIR multiphoton laser microbeam irradiation has been shown to induce mainly complex damage and fewer simple DNA lesions (Botchway *et al*, 2012, Harper *et al*, 2008, Meldrum *et al*, 2003). The recruitment and loss of XRCC1-YFP and FEN1-GFP to sites of DNA damage induced by both ultrasoft X-ray and NIR laser microbeam irradiation was visualised in real-time and the decay kinetics of the fluorescently-tagged proteins determined. The

fluorescence decay of XRCC1-YFP and FEN1-GFP after ultrasoft X-ray irradiation (Figure 3.11 and Figure 3.12) was fitted by a single exponential decay with a half-life of $4 (\pm 1)$ min and $6 (\pm 1)$ min, respectively. XRCC1 is a scaffold protein that is known to enhance the activity of several of the BER proteins and is thought to be involved in the BER process from the beginning, where it interacts with DNA glycosylases (Campalans et al, 2005), through to the end where its complex partner ligase III α performs the final step of ligation (Cappelli *et al*, 1997). Therefore, the half-life of XRCC1-YFP decay is likely to reflect the half-life of repair of damage repaired by the SP-BER pathway. It is of some debate as to whether FEN1 is part of a multiprotein complex along with other LP-BER proteins (Balakrishnan *et al*, 2009) or whether it only binds to DNA once strand displacement has taken place to cleave the resulting flap structure (Prasad *et al*, 2010). It is suggested from the later maximal recruitment of FEN1 compared to XRCC1 (FEN1-GFP fluorescence intensity peaked at 3 min whereas XRCC1 maximal fluorescence intensity was at the first time point imaged at 2 min) following ultrasoft X-ray irradiation that FEN1 is recruited to DNA damage after repair has begun, possibly once the polymerase has performed strand displacement. Additionally, the longer half-life seen for FEN1-GFP decay compared to XRCC1-YFP indicate that LP-BER is a slower process than SP-BER, maybe due to the more complex lesions processed by LP-BER together with the additional processes required to extend the flap and remove it (Klungland and Lindahl, 1997, Liu *et al*, 2008). A five times higher dose of ultrasoft X-ray irradiation was required to detect FEN1-GFP at damage sites in comparison to XRCC1-YFP, which may also reflect the greater requirement for FEN1 with increasing damage complexity. Similarly the maximal recruitment of FEN1-GFP was several minutes later than that of XRCC1-YFP following NIR multiphoton laser microbeam

irradiation (Figure 3.13 and Figure 3.14). It was not possible to determine the decay kinetics of FEN1-YFP following NIR laser microirradiation due to the FEN1-GFP moving into large foci-like structures by 60 min post-irradiation which persist until at least 180 min (Figure 3.14a).

In contrast to ultrasoft X-ray irradiation, where the XRCC1-YFP fluorescence decay approaches background levels by 20 min (Figure 3.11), ~ 20 % of the maximal level of XRCC1-YFP recruited to the NIR laser microirradiation-induced DNA damage still persists at 4 hours post-irradiation (Figure 3.13). The fluorescence decay of XRCC1-YFP after NIR multiphoton laser microbeam irradiation (Figure 3.13) was fitted by bi-exponential decays with half-lives of $15 (\pm 9)$ min and $153 (\pm 35)$ min. Comparing these results with those of Dikomey and Franzke (1986) it was concluded that the relatively short half-life (4 min) of loss of XRCC1-YFP fluorescence after ultrasoft X-ray irradiation represents the repair of mainly simple SSB/damage, the lifetime of the fast component (15 min) determined with the NIR multiphoton laser microbeam represents more complex damage than that seen with USX that takes longer to repair, and the very long half-life seen (~150 min) following NIR laser irradiation represents very complex damage that presents a major challenge to the repair proteins (Dikomey and Franzke, 1986). Recently, Asaithamby *et al* have also suggested that long lasting damage (unrepairable after 24 h) is due to the extremely complex DNA damage (Asaithamby *et al*, 2011) rather than the idea suggested previously that persistent DNA damage is located in heterochromatin and is therefore inaccessible to repair proteins (Goodarzi *et al*, 2010).

The recruitment of fluorescently-tagged XRCC1 to sites of DNA damage has been investigated by other groups, but using different types of laser microirradiation (Lan *et al*, 2004, Mortusewicz *et al*, 2007, Figure 7.1). In addition to the findings with ultrasoft X-ray and NIR laser microbeam irradiation in this study, it is clear that the lifetime of XRCC1 at sites of DNA damage depends on the types of damage and density of the damage induced i.e. radiation inducing a higher density of DNA damage leads to a greater persistence of XRCC1 at the DNA damage sites. Additionally, it can be seen in Figure 7.1 that the lifetime of XRCC1 at radiation-induced damage is very different between the different types of lasers. This is most clearly seen when comparing the induction of damage by NIR 730 nm laser and 365 nm pulse laser. With the 365 nm laser XRCC1-GFP persists at the damage sites for at least 60 min post-irradiation (Lan *et al*, 2004), whereas with the 730 nm laser more than half of the XRCC1-YFP has dissociated from the DNA damage by 60 min (Figure 7.1). Similarly, a difference in kinetics of XRCC1-RFP recruitment is observed following irradiation with 405 nm laser light compared with those in our study. For example, recruitment of XRCC1-RFP peaks at ~3 min following 405 nm laser irradiation and persists at this maximal level for 5 min (Mortusewicz *et al*, 2007), whereas following 730 nm laser irradiation XRCC1-YFP recruitment has peaked by 2 min and XRCC1 levels are decreasing by 5 min. In comparison with ultrasoft X-ray irradiation, when more than half of the XRCC1-YFP has dissociated from the DNA damage sites, the findings using 365 or 405 nm light (Lan *et al*, 2004, Mortusewicz *et al*, 2007) imply that XRCC1 is recruited and persists at the DNA damage induced. This again may reflect the density of damage and types of damage induced by the different forms of radiation. The advantage of NIR 730 nm laser microirradiation is that the cellular contents are transparent to 730 nm laser light

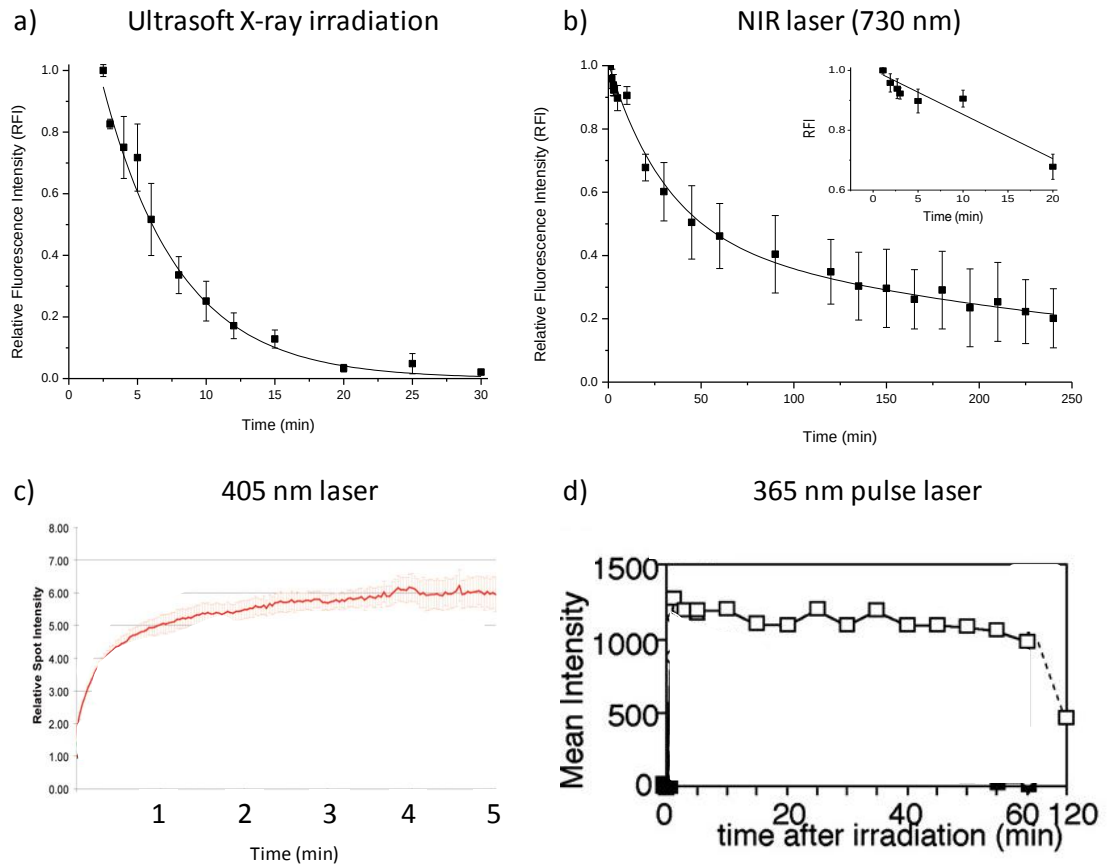


Figure 7.1: Variability of lifetime of XRCC1 after different types of damage-induction. The top two graphs represent XRCC1-YFP fluorescence decay following a) ultrasoft X-ray and b) NIR (730 nm) multiphoton laser microbeam induced damage. The graph in c) is taken from Mortusewicz *et al* (2007) and shows recruitment of XRCC1-RFP in MEFs following microirradiation with a 405 nm diode laser. The graph in d) represents XRCC1-GFP dissociation kinetics following irradiation of HeLa cells using a 365 nm pulse laser (Lan *et al*, 2004). These graphs clearly show that there is variability in the damage induced between the lasers. In addition these graphs show that a higher density of damage is generated with laser microirradiation compared to low-LET ultrasoft X-ray irradiation as seen by the longer decay times and persistence of XRCC1 at the damage sites.

and therefore damage is only induced at the focal point (400 nm^3) of the laser beam within the cell nucleus, which is where the multiphoton processes occur (for more detail see section 1.10). In contrast, damage will occur along the whole path of the 405 nm and 365 nm laser beams, damaging other components of the cell in addition to the area in the nucleus that the laser beam is focused on. Additionally, a raster scanning program was used for NIR 730 nm laser microirradiation, which means that a defined track of damage is induced across the nucleus of a cell in nanoseconds, reducing the potential for saturation of repair processes, which is in contrast to the 405 nm and 365 nm laser beams where the beam was focused on the same area of the nucleus ($1\text{ }\mu\text{m}$ diameter) for 1 s, potentially leading to more damage than the cell repair pathways can cope with (Lan *et al*, 2004, Mortusewicz *et al*, 2007). Alternatively, other types of damage to that induced by ionising radiation (USX) are potentially produced by the monophotonic laser methods.

PARP1 detects and binds to SSBs where it becomes activated and auto-modifies itself to form PAR chains which can be used to recruit other proteins, including XRCC1 (El-Khamisy *et al*, 2003, Masson *et al*, 1998). Once the PAR chains have reached a certain length their negative charge repels PARP1 from the DNA, allowing the now recruited repair proteins to complete repair of the SSB (Satoh & Lindahl, 1997). PARP inhibition allows PARP1 to detect and bind to the SSBs but prevents the formation of PAR chains which, in turn, is thought to prevent both PARP1 recruitment of repair proteins such as XRCC1 and dissociation of PARP1 from the SSB (Loh *et al*, 2005). This leads to persistence of SSBs and if they encounter a replication fork this may result in stalled replication forks and a replication-induced DSB. These replication forks are either

repaired by homologous recombination (Lundin *et al*, 2003, Michel *et al*, 2001) or if unrepaired, induce signalling cascades that result in cell death (Roos and Kaina, 2012). For this reason, PARP inhibitors have been shown to cause synthetic lethality in tumour cells that have a deficiency in homologous recombination (Bryant *et al*, 2005, Farmer *et al*, 2005) and are currently in clinical trials (Davar *et al*, 2012, Rouleau *et al*, 2010, Underhill *et al*, 2010). Several groups have shown that inhibition of PARP1 inhibits recruitment of XRCC1 to radiation-induced DNA damage (Godon *et al*, 2008, Lan *et al*, 2004, Okano *et al*, 2003). In this study, the recruitment of XRCC1-YFP following ultrasoft X-ray and NIR laser microbeam irradiation in the presence of the PARP1/2 inhibitor KU-0058684 (Loh *et al*, 2005) was investigated. It was found that for ultrasoft X-ray irradiation and the fast component following NIR laser microbeam irradiation the decay kinetics of XRCC1-YFP were the same with and without the PARP inhibitor (Figure 4.1a and Figure 4.2a). However, a substantial reduction was seen in the level of XRCC1 recruited to the DNA damage sites (Figure 4.1b and Figure 4.2b) reflecting the role of PARP1 in the recruitment of XRCC1 to DNA damage. However a proportion of DNA damage sites still recruit XRCC1 despite PARP inhibition (Figure 4.1b and Figure 4.2b). The recruitment of XRCC1-YFP to DNA damage sites following ultrasoft X-ray irradiation was determined after treatment with increasing concentrations of PARP inhibitor. When PARP1 is completely inhibited, XRCC1 is still recruited to ~40% of the damage sites. It was therefore concluded that XRCC1 is recruited to two subsets of lesions induced by ultrasoft X-ray irradiation; those that require PARP1 for the recruitment of XRCC1 and those that XRCC1 can be recruited to without the involvement of PARP1. The next challenge is to determine which lesions fall into the two subtypes. It is likely that the PARP1-dependent subtype includes SSBs,

due to the high affinity of PARP1 for SSBs and the fact that Okano *et al*, (2003) did not see any XRCC1 recruitment following PARP inhibition after what was thought to be purely SSB damage. It is also possible that the PARP1-independent lesions are those that are detected by glycosylases that can recruit XRCC1 directly, as XRCC1 has been shown to interact with several glycosylases (MPG, NEIL1 and hNTH1 (Campalans *et al*, 2005) and OGG1 (Marsin *et al*, 2003)). BER then proceeds in a sequential manner with the intermediate substrates being passed from one protein to the other without exposure of a SSB intermediate (Prasad *et al*, 2010). However, Cadet *et al* have shown that only 45% of the total non-DSB lesions induced per cell per Gray consists of SSBs (Cadet *et al*, 2008, Figure 4.6a). If ~60% of the ultrasoft X-ray-induced DNA damage requiring XRCC1 is PARP1-dependent, this leaves 15% of PARP1-dependent lesions unaccounted for. It is possible that these PARP1-dependent lesions are BER-intermediates that become uncoupled from the BER pathway and that PARP1 binds to them to protect them and aid in recruitment of XRCC1 to continue repair (Ström *et al*, 2011). Cadet *et al* (2008) determined that 15% of lesions generated/cell/Gy are purine lesions, and it is possible that the repair of these purine lesions is PARP1-dependent, in addition to SSBs. The remaining 40% of lesions induced represent pyrimidine damage (Cadet *et al*, 2008) and could account for the subset of lesions requiring XRCC1 for repair in a PARP1-independent manner (Figure 4.6). This hypothesis is supported by the fact that the recruitment of the DNA glycosylase NTH1, which is specific for detection of pyrimidine damage, is not affected by PARP inhibition following 365 nm laser microirradiation (Lan *et al*, 2004). Additionally, an interaction between OGG1 and PARP1 has been detected that increases following oxidative stress (Noren-Hooten *et al*, 2011). Although both OGG1 and NTH1 are bifunctional glycosylases, it has been

shown that APE1 can stimulate OGG1 glycosylase activity which results in bypass of the lyase activity of OGG1 (Vidal *et al*, 2001b). It is therefore possible that PARP1 can bind to the intermediate generated by OGG1/APE1 whereas the intermediate generated by NTH1 is not exposed and can be passed straight to Pol β .

One way of determining whether oxidised purines (e.g. 8-oxoG) require both PARP1 and XRCC1 for repair is to use the photosensitiser Ro19-8022 in combination with 405 nm laser irradiation. Ro19-8022 is a photosensitiser that gives rise to a DNA damage profile similar to that seen with singlet oxygen (Will *et al*, 1999). Singlet oxygen has been shown to interact preferentially with guanine (Muller *et al*, 1990, Ravanat *et al*, 2000, Ravanat *et al*, 2004) and the formation of oxidised pyrimidines, AP sites and SSBs is very low (Schultz *et al*, 2000). Therefore, it would be interesting to monitor the recruitment of XRCC1-YFP in PARP inhibited cells following 405 nm laser irradiation with and without Ro19-8022 treatment. If there is not a substantial increase in XRCC1-YFP recruitment to 405 nm laser-induced damage following Ro19-8022 treatment then it strengthens the proposal that repair of oxidised purines is PARP1-dependent. Conversely, if a substantial increase in XRCC1-YFP recruitment is seen following Ro19-8022 treatment then it would imply that the repair of oxidised purines, and potentially base lesions in general, is PARP1-independent. Very recently, the amount and accumulation rate of FEN1-YFP in PARP inhibited MEFs was found to be reduced at DNA damage sites following pulsed 800 nm multiphoton laser irradiation compared to uninhibited cells (Kleppa *et al*, 2012), although the opposite effect was seen by Hanssen-Bauer *et al* (2011) using different PARP inhibitors. Therefore, it would be interesting to see if there is any effect of PARP inhibition on FEN1 recruitment to DNA

damage sites induced by ultrasoft X-ray and NIR multiphoton laser microbeam irradiation with the CHO FEN1-GFP tagged cells used in this thesis.

It has been suggested that XRCC1 has an S phase specific role (Fan *et al*, 2004, Kubota and Horiuchi, 2003, Taylor *et al*, 2000, Taylor *et al*, 2002). Therefore, the decay kinetics of XRCC1-YFP in enhanced G1 (serum-starved) cells compared to exponentially growing cells was investigated and it was found that there was no difference in the decay kinetics between the two conditions, although there was a higher residual fluorescence intensity of XRCC1-YFP in the serum-starved cells at 35-40 min when compared to the exponentially growing cells (Figure 3.16). It is possible that the residual XRCC1 in the enhanced G1 cells could represent more complex persistent damage which in S-phase can be repaired by additional repair pathways such as homologous recombination (Lundin *et al*, 2003, Michel *et al*, 2001), replication-coupled SSBR (Caldecott, 2008) or pre- and post-replicative BER (Akbari *et al*, 2010, Hanssen-Bauer *et al*, 2011). The effect of PARP inhibition on XRCC1-YFP recruitment and loss during the different cell cycle phases was also investigated and it was found that the kinetics of loss of XRCC1-YFP from DNA damage sites was the same in both enhanced G1 and exponentially growing cells in the presence of PARP inhibitor (Figure 4.5). Therefore, PARP-dependent damage also recruits XRCC1 throughout the cell cycle. However, it is also possible that a more pronounced effect would have been seen between S phase and G1 phase cells if there had been a greater enhancement of cells in G1 phase than that seen in the serum-starved cells (Figure 3.15) and this may have to be investigated further. Another method used to obtain G₀/G1 populations is isoleucine deprivation, and lovastatin can be used to obtain cells arrested in early G1

(O'Connor and Jackman, 1995). Alternatively, a double thymidine block could be used to arrest the cells at G1/S phase. This can be followed by addition of nocodazole (a mitotic inhibitor) to block cells in G2/M. Flow cytometric analysis can be used to monitor progression of cells from G1/S to G2/M, allowing calculation of the timing for when cells are in specific cell cycle phases (Humphrey and Brooks, 2005).

DNA is packaged into chromatin which is either in the form of euchromatin (open, gene rich, transcriptionally active regions) or heterochromatin (condensed regions with low gene density and high levels of repetitive sequences), and the level of both SSBs (Nygren *et al*, 1995) and DSBs (Radulescu *et al*, 2004) has been shown to increase when DNA is modified into a less compact structure. De-condensation of DNA may allow access to DNA which may lead to higher levels of damage. For instance, it has also been demonstrated that as the chromatin structure changes from a condensed to an open format, the level of clustered DNA damage induced by low-LET radiation increases (Magnander *et al*, 2010). Chromatin relaxation can occur following acetylation of the histone tails, which causes loosening of the contact between the histones and DNA (Garcia-Ramirez *et al*, 1995, Norton *et al*, 1989, Shogren-Knaak *et al*, 2006, Tse *et al*, 1998, Wang *et al*, 2001). Acetylation of histones is regulated by the opposing actions of histone acetyltransferases (HATs) and histone deacetylases (HDACs) that work by adding and removing acetyl groups from lysine residues on the N-terminal tails of the histones (Waterborg, 2002). Therefore, HDAC inhibitors should result in relaxation of chromatin, potentially leading to higher levels of damage induction following irradiation. It has also been shown that some BER proteins can be acetylated or deacetylated by HATs and HDACs (Bhakat *et al*, 2003, Busso *et al*, 2010,

Hasan *et al*, 2001, Hasan *et al*, 2002, Yamamori *et al*, 2010) indicating that HDAC inhibition could interfere with the BER pathway. Following treatment of XRCC1-YFP or FEN1-GFP tagged cells with the class I and class II HDAC inhibitors NaB or TSA prior to ultrasoft X-ray it was found that expression of both XRCC1-YFP and FEN1-GFP is substantially enhanced by NaB and to a lesser extent TSA (Figure 5.5, Figure 5.6 and Figure 5.7), whereas levels of endogenous BER proteins were unaffected. In addition NaB did not detectably affect the persistence of endogenous XRCC1 at sites of ultrasoft X-ray induced DNA damage. Therefore, it was concluded that the 2-3 fold increase in the half-lives of XRCC1-YFP fluorescence decay seen following NaB and TSA treatment were artefacts of induction of the CMV promoter present on the XRCC1-YFP plasmid, by these inhibitors. This work will need to be repeated with plasmids containing the endogenous promoters for FEN1 and XRCC1. An interesting result was seen following treatment of EMC11 XRCC1-YFP tagged cells with the Class III HDAC (Sirtuin) inhibitor nicotinamide. In Figure 5.6 it can be seen that nicotinamide slightly increases the expression of XRCC1-YFP. However, following ultrasoft X-ray irradiation of nicotinamide treated EMC11 XRCC1-YFP tagged cells, the half-life of XRCC1-YFP decay is actually slightly shorter (1.5 fold) than that in the untreated cells (Figure 5.4). This may be because inhibition of SIRT1 by nicotinamide prevents association of APE1 with XRCC1 (Yamamori *et al*, 2010) so that XRCC1 cannot be recruited to APE1 sites but can be recruited to DNA damage that does not require processing by APE1. This hypothesis needs further investigation, potentially by using an APE1 inhibitor (e.g. 7-nitroindole-2-carboxylic acid (NCA) or lucanthone) to see whether the recruitment of XRCC1-YFP following ultrasoft X-ray irradiation is similarly affected.

LP-BER is required when 5' termini of breaks are resistant to β -elimination by Pol β , e.g. when an AP site contains a sugar phosphate group that has been oxidised or reduced (Klungland & Lindahl, 1997). Clustered damage induced by ionising radiation relies more heavily on LP-BER than isolated lesions for repair (Byrne *et al*, 2009, Lomax *et al*, 2004a, Cunliffe *et al*, 2007, Imoto *et al*, 2008). The majority of cancer cells exhibit genomic instability that if enhanced by knocking down key repair proteins may result in cell death (Komarova *et al*, 2002, McManus *et al*, 2009). Therefore, the effect of knockdown of the key LP-BER protein FEN1 on radiosensitivity of cancerous (A549) and non-cancerous (BEAS-2B) cells was investigated to see if cancerous cells are more susceptible to radiation-induced cell death as a result of FEN1 deficiency and whether this could be potentially exploited for cancer therapy. FEN1 knockdown alone was lethal to the non-cancerous BEAS-2B cells. This could be due either to the important role of FEN1 in replication or as a result of an accumulation of flap-structure intermediates that could trigger cell death pathways (Liu *et al*, 2004, Roos and Kaina, 2012). A549 cells survived FEN1 knockdown and may have developed ways of evading the cell death pathways induced by an accumulation of unprocessed BER and replication intermediates. However, radiosensitivity was seen in FEN1 depleted A549 cells when compared to FEN1 proficient cells (Figure 6.5a). This may be a result of an excess of unrepaired radiation-induced lesions that require LP-BER for repair persisting until replication and then resulting in stalled replication forks and replication-induced DSBs (Kuzminov, 2001). Replication-induced DSBs can generally be repaired by homologous recombination (Lundin *et al*, 2003, Michel *et al*, 2001). Excessive numbers of unrepaired lesions and, therefore, replication-induced DSBs may result in saturation of the HR pathway, leading to genetic instability and cell death. Thus, knockdown of

FEN1 does increase radiosensitivity of the lung adenocarcinoma A549 cell line but a wider range of cancerous cell lines would have to be tested to see if they show a similar effect. Additionally, the lethality seen in the normal lung epithelial BEAS-2B cells would mean that any therapy involving knockdown of FEN1 would have to be specifically delivered to the tumour cells.

Ionising radiation is an important and effective cancer therapy due to cell death induced as a result of DSB formation. In addition to prompt DSB, ionising radiation also results in non-DSB clustered damage that can be converted to DSB during lesion processing (Gulston *et al*, 2004, Eccles *et al*, 2010) or due to inefficient lesion repair resulting in replication induced DSB (Harper *et al*, 2010). Therefore proteins in the BER pathway could be potential targets to increase the efficacy of radiotherapy by forcing more non-DSB clustered sites to form DSB. However, non-DSB clusters are induced at a 4-8 higher rate than prompt DSB (Gulston *et al*, 2002, Sutherland *et al*, 2002) and at environmental levels of radiation exposure (1-5 mGy) one DSB is induced per 20-100 cells whereas one non-DSB clustered damage site is predicted to be induced every 5-20 cells (Eccles *et al*, 2011). Therefore, there will be cells that contain a non-DSB clustered damaged site but no DSB and thus are likely to survive an insult of low dose ionising radiation. Cells containing only non-DSB clustered damage have potential for mutagenesis and the induction of tumourigenesis following the mis-incorporation of bases via mis-repair or translesion synthesis at replication (Lehman, 2002) due to the lifetime extension of lesions within the non-DSB cluster. Cells containing DSB can trigger cell death pathways (Roos and Kaina, 2012) thus eliminating the damaged cell.

The induction of clustered DNA damage by ionising radiation can be seen as a double-edged sword as at low doses, such as those at environmental levels or those received by normal tissue during radiotherapy, it can cause persistent lesions that can result in mutations and potentially carcinogenesis, whereas it can also be used in cancer therapy to induce tumour cell death. Understanding how clustered damage is repaired by the BER pathway can aid the design of future therapies which can be used in combination with radiotherapy to enhance the radiosensitisation effect, or it can help to define thresholds by establishing how much radiation exposure can be tolerated.

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