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The effect of Gadd45α on DNA-bound PCNA in response to UVB.

by

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ABSTRACT

Proliferating cell nuclear antigen (PCNA) is involved in many cellular processes, but is most clearly defined in DNA replication. Growth arrest and DNA-damage inducible protein 45 alpha (Gadd45α) also has many cellular functions. Gadd45α's role in nucleotide excision repair (NER), while unclear, has been attributed to interaction with PCNA. To further elucidate the role of this interaction we analysed rapidly DNA-bound PCNA in *Gadd45α*$^{+/+}$ (wild type) and *Gadd45α*$^{-/-}$ (knock out) MEFs after UVB. To do so, I developed a novel method for assessing DNA-bound proteins in intact MEFs by flow cytometry. Results were confirmed by immunofluorescent microscopy and immunoblotting. By all methods, there was no early PCNA response in the absence of Gadd45α, while in wild type cells, an increase of DNA-bound PCNA seen at 1h, begins to drop by 4 hours. Our data suggest that Gadd45α is a strong regulator of the early PCNA NER response.

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TABLE OF CONTENTS

Chapter 1 ♦ Literature Review 1

Ultraviolet Radiation 1

Sources and effects of UV radiation *1*

UV Radiation and Skin Cancer *4*

DNA Damage Response 10

Cellular Responses to DNA Damage *10*

DNA Repair *13*

Nucleotide Excision Repair *14*

PCNA 20

PCNA and Repair *24*

Gadd45α 27

Molecular Regulation of Gadd45α *27*

Gadd45α and Cancer *36*

Gadd45α mice *38*

Multiple Roles of Gadd45α *39*

Gadd45α and NER *43*

Summary and Goals 45

Chapter 2 ♦ Materials and Methods 47

Gadd45α Mouse Colony 47

Creation of MEFs *48*

Cell culture 49

Immunofluorescence Methods (Confocal Microscopy and Flow Cytometry) *49*

DNA-bound Western Blotting *50*

UVB irradiation 50

Immunofluorescent Microscopy 52

Method Development: Flow Cytometry 53

Flow cytometry 55

Cell lysis / Quantification 57

SDS-PAGE gel electrophoresis / Western blots 58

Statistics 59

Chapter 3 ♦ Gadd45α and DNA-bound PCNA: Immunofluorescent Analysis.... 60

Introduction 60

Immunofluorescent Microscopy Results 60

Flow Cytometry Results 64

Work-up of Flow Cytometry Method *64*

Flow Cytometry Results *70*

Conclusions 73

Chapter 4 ♦ Gadd45α and DNA-bound PCNA and p21:
Western Blot Analysis 75

Introduction 75

Results 77

Conclusions 79

Chapter 5 ♦ Discussion and Future Directions 80

LIST OF TABLES

Table 2-1: Experimental Design 51

Table 3-1: Flow cytometry analysis of PCNA 65

LIST OF FIGURES

Figure 1-1: Ultraviolet light spectrum 3

Figure 1-2: Ultraviolet light atmospheric penetration 3

Figure 1-3: Chemical formation of UV-induced DNA photoproducts 5

Figure 1-4: Development of Human Squamous Cell Carcinoma 9

Figure 1-5: Histology of NMSC 10

Figure 1-6: DNA damage response 12

Figure 1-7: Nucleotide Excision Repair Pathways 19

Figure 1-8: Crystal structure of human PCNA. 22

Figure 1-9: JNK and p38 MAPK Pathways 30

Figure 1-10: NF-κB Pathway 34

Figure 2-1: Genotyping gel. 48

Figure 3-1: Examples of IFM images 62

Figure 3-2: Graph of DNA-bound PCNA in *Gadd45α* wt and ko MEFs in response to UVB damage assayed by Immunofluorescent Microscopy (IFM) 63

Figure 3-3: NP-40 treatment of MEFs for PCNA IFM 66

Figure 3-4: Triton X-100 Titrations; 15 minute Permeabilization Treatment 68

Figure 3-5: Triton X-100 Titrations; 2 hour Permeabilization Treatment 69

Figure 3-6: Triton X-100 Titrations; 2 hour Permeabilization Treatment 70

Figure 3-7: Graph of DNA-bound PCNA in *Gadd45α* wt and ko MEFs in response to UVB damage assayed by Flow Cytometry (FC) 72

Figure 3-8: Comparison of Immunofluorescent Microscopy (IFM) and Flow Cytometry (FC) Graphs of DNA-bound PCNA in *Gadd45α* wt and ko MEFs in response to UVB damage. 73

Figure 4-1: DNA-bound Western Blot 78

LIST OF NOMENCLATURE, SYMBOLS, AND ABBREVIATIONS

6-4 PP	6-4 photoproduct
A, C, G, T	adenosine, cytosine, guanine, thymine (DNA bases)
BCC	basal cell carcinoma
BER	base excision repair
bp	base pair
BRCA	breast cancer gene or protein
CDK	cyclin dependent kinase
Cisplatin	*cis*-diamminedichloroplatinum(II)
CPD	cyclobutane pyrimidine dimer
CS	Cockayne syndrome
FC	flow cytometry
Gadd45α	growth arrest and DNA damage inducible 45 alpha
GGR	global genomic repair
HHR23	human homologue of RAD23
IκB	Inhibitor of NF-κB
IDC	Invasive ductal carcinoma
IFM	immunofluorescent microscopy
IKK	IκB kinase
IR	ionizing radiation
JNK	Jun N-terminal kinase
kDa	kilodalton
LOH	loss of heterozygosity

MAPK	mitogen-activated protein kinase
MAPKK	mitogen-activated protein kinase kinase
MAPKKK	mitogen-activated protein kinase kinase kinase
MEF	mouse embryonic fibroblast
MMR	mismatch repair
MMS	methyl methanesulfonate
MNNG	N-methyl-N-nitro-N-nitrosoguanidine
NER	nucleotide excision repair
NF-κB	nuclear factor κB
NMSC	non-melanoma skin cancer
NP-40	nonidet P-40
PCNA	proliferating cell nuclear antigen
PI3K	phosphoinositide 3-kinase
PKC	protein kinase C
SCC	squamous cell carcinoma
TCR	transcription coupled repair
TMZ	temozolomide
TNFR	Tumour necrosis factor receptor
TTD	trichothiodystrophy
UV	ultraviolet
UV-DDB	UV DNA damage binding
XP	xeroderma pigmentosum

Chapter 1 ♦Literature Review

Ultraviolet Radiation

Skin Cancer is a major health issue in Canada. An estimated 78,000 cases of non-melanoma skin cancer (NMSC), which includes squamous cell carcinoma (SCC) and basal cell carcinoma (BCC), were diagnosed in 2005 (National Cancer Institute of Canada, 2005). This represents a huge problem to our society. Indeed, the incidence of skin cancer has increased by two-thirds since 1990 making NMSC the most commonly diagnosed cancer in Canada (National Cancer Institute of Canada, 2005). The ozone layer plays a vital role in skin cancer prevention. It is present in the earth's stratosphere and filters harmful radiation emitted by the sun, including UV light. It is thought that the depletion of the ozone layer, which allows an increasing amount of harmful UVB rays to penetrate to the earth's surface is the cause of this increase in incidence of NMSC (World Health Organization, 2005). This study will review the effects of UV radiation and the mechanisms responsible for repairing the damage caused by UV.

SOURCES AND EFFECTS OF UV RADIATION

Ultraviolet (UV) radiation is vital to life on earth. The human skin uses UV in Vitamin D production. Additionally, UV light is used to treat various skin

diseases, such as psoriasis, vitiligo and cutaneous T-cell lymphoma (Craddock, KJ et al., 2004). However, UV radiation has deleterious effects, including blindness, aging, decreased immune function, gene mutation and skin cancer (Craddock, KJ et al., 2004; Matsumura, Y and Ananthaswamy, HN, 2002). UV is part of the electromagnetic spectrum in the range of 200 – 400nm and the UV spectrum is divided into three regions, UVA (315 – 400nm), UVB (280 – 315nm) and UVC (200 – 280nm) (Figure 1-1) (Soehnge, H et al., 1997). UVC, the highest energy UV, is largely absorbed by the earth's atmosphere and is blocked from reaching the earth's surface. It is found in germicidal UV lamps installed in laminar flow hoods for germicidal purposes. UVB is an intermediate energy source and is incompletely absorbed by the ozone layer, with 10% of the emitted UVB reaching the earth's surface. People who use tanning beds should be aware that traces of UVB are found in the lights used in these beds. UVB is absorbed by DNA, and is well known to play a major role in the pathogenesis of skin cancer. The low energy UVA completely penetrates the ozone layer (Figure 1-2) and is primary source of light used in tanning beds. Although it is not absorbed by DNA, UVA can cause oxidative DNA damage (World Health Organization, 2005).

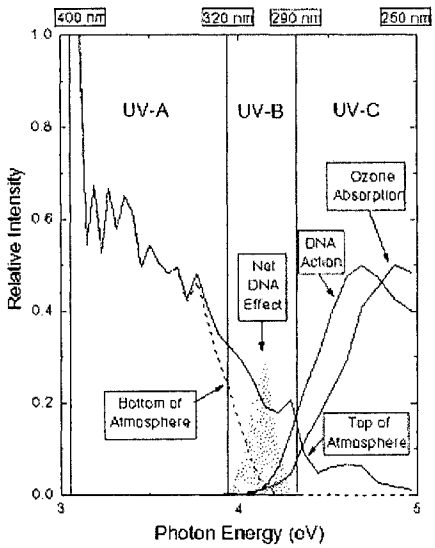

Figure 1-1: Ultraviolet light spectrum

UVC (200-280nm) has the highest energy but is blocked by the ozone layer and does not reach the earth's surface. UVB (280-320nm) is important in skin tumourigenesis and is 1000 times more effective at causing skin erythema than UVA (320-400nm). (www.phys.ksu.edu/gene/e3f3.html).

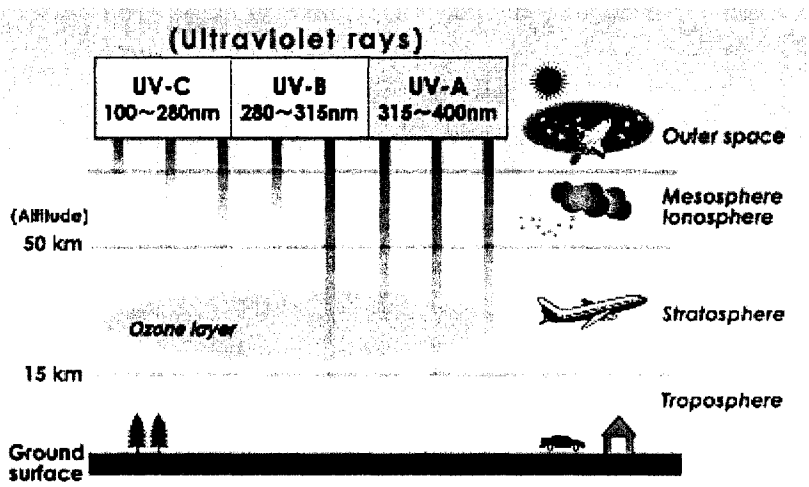

Figure 1-2: Ultraviolet light atmospheric penetration

Representation of the penetrational abilities of UV light. Shorter wavelengths (UVC and some UVB) are absorbed by the ozone, while the longer wavelengths reach the earth's surface. UVA completely penetrates the ozone layer and is present at ground level. (Center for Global Environmental Research: www-cger2.nies.go.jp/ moni-e/ozone/uv/uv01.html)

UV Radiation and Skin Cancer

UVA and UVB damage DNA via different mechanisms. UVA produces reactive oxygen species such as hydroxyl radicals and singlet oxygen through energy transfer from an excited photosensitizer or lipid peroxidation (Kvam, E and Tyrrell, RM, 1997). These molecules are responsible for creating single-strand DNA breaks, DNA-protein crosslinks, and altered DNA bases (Melnikova, VO and Ananthaswamy, HN, 2005). Because of its inability to be absorbed by DNA, UVA was not thought to be carcinogenic. However, animal studies have shown that exposure to high levels of UVA over extended periods will result in skin cancer (Strickland, PT, 1986; Zigman, S et al., 1976). Of note, this is comparable to regular use of tanning beds. DNA maximally absorbs UV light in the range of 245 – 290nm (Soehnge, H et al., 1997), within the UVB-UVC wavelengths. The absorption of UVB and UVC by DNA alters the bonds in pyrmidine bases, either cytosine (C) or thymine (T), producing two bulky DNA lesions, cyclobutane pyrmidine dimers (CPDs), and (6-4) pyrmidine-pyrmidone photoproducts (6-4PPs) (Figure 1-3). CPDs are the result of the formation of two bonds between C-4 and C-5 carbons of any two adjacent pyrmidines, either TT, CC or TC, saturating the double bonds and producing a four-membered ring. Formation of a 6-4PP results from a single bond between C-4 and C-6 position of two adjacent pyrmidines, either CT or CC residues (Figure 1-3) (Matsumura, Y and Ananthaswamy, HN, 2002). Both mutations are bulky and result in bending of the DNA backbone.

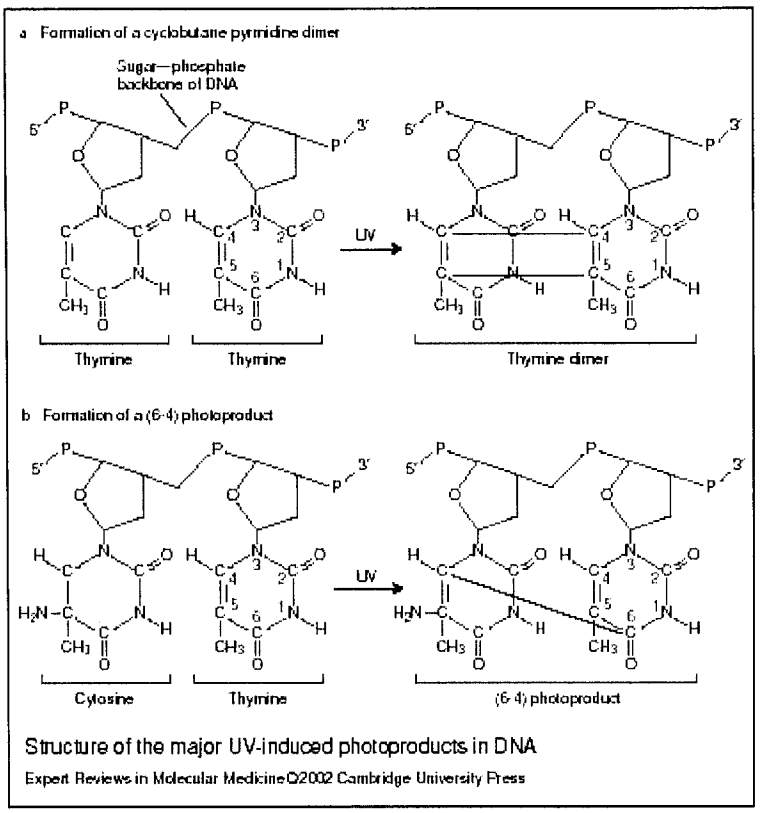

Figure 1-3: Chemical formation of UV-induced DNA photoproducts

a) Formation of a cyclobutane pyrmidine dimer after UV radiation (thymine dimer illustrated). Energy from UVB forms two bonds between C-4 and C-5 carbons of two adjacent pyrmidines, TT or CC, and becomes saturated to produce a four-membered ring and a bend in the DNA backbone. b) Formation of a (6-4) pyrmidine pyrmidone photoproduct after UV radiation. UV energy forms a single bond between C-4 and C-6 position of two adjacent pyrmidines, either CT or CC residues (Matsumura, Y and Ananthaswamy, HN, 2002).

UVB is the most deleterious form of UV light, which is supported by several veins of evidence. First of all, UVC does not reach the earth's surface so is not considered a major source of mutagenic DNA damage. Secondly, UVB light is more than 1000 times more efficient at causing erythema, or sunburn, than UVA (Parrish, JA et al., 1982). Since erythema is an indicator of DNA damage in the epidermis of the skin, this indicates that UVB is 1000 times more effective at

causing DNA damage than UVA. Finally, more than 50% of UVB rays penetrate into the epidermis, the epithelial layer of the skin (Bruls, WA et al., 1984). It has been shown that repeated exposure to UVB, even at doses too low to cause sunburn, are capable of causing a build-up of CPDs in human keratinocytes (Chouinard, N et al., 2001). 6-4PPs do not contribute significantly to tumourigenesis as they occur six times less frequently (Matsumura, Y and Ananthaswamy, HN, 2002) and are repaired at least five times faster than CPDs (Balajee, AS and Bohr, VA, 2000).

Unrepaired DNA damage can lead to DNA polymerase stalling causing fragmented DNA or mutations in the DNA strand if the damage base is mispaired. Although there are species differences between humans and other mammals, the nucleotide excision repair (NER) pathway, which is responsible for repairing CPDs and 6-4PPs, is highly conserved. Homologues of the NER proteins are present in prokaryotes and eukaryotes. Yeast, hamster and mouse models are often used in studying this pathway (Balajee, AS and Bohr, VA, 2000; de Boer, J and Hoeijmakers, JH, 2000; Friedberg, EC, 2001). Both CPDs and 6-4PPs occur solely in regions with tandem pyrmidine residues, which are "hot spots" for UV-induced DNA damage and mutations (Brash, DE, 1988; Mitchell, DL et al., 1992; Sage, E, 1993). Since purines do not have C-4 or C-5 carbons, they are not mutated by UV. Both lesions cause bends in the DNA backbone, or

helix distortion, and can cause mutations in the DNA sequence if they are not repaired.

If a damaged DNA base, such as a CPD or 6-4PP is not repaired before DNA replication, mutations in the sequence may result from inappropriate base pairing. During replication, many proteins assemble to copy DNA into identical daughter strands, thus conserving the correct DNA sequence. Replication Factor C (RFC) loads PCNA onto DNA. PCNA acts as a scaffold for other proteins, such as DNA polymerases that actively copy DNA (Khlimankov, DY et al., 2004). The "A-rule" has been suggested as an explanation for the characteristic mutations seen with UV-induced DNA damage. This rule states that when DNA polymerase δ or ε encounter a UV-lesion they will, by default, insert an adenosine (A) opposite the bases they cannot interpret (Matsumura, Y and Ananthaswamy, HN, 2002). Because A pairs with T, there is no mutation with a thymine dimer. However, when there is a CC CPD, a CC to TT transition occurs when the default A bases are inserted across from the Cs, instead of the correct guanine (G) bases. In the case of the 6-4PP between a pyrmidine and a C in the 3' position, the 5' base is interpreted correctly but the 3' C is paired with an A, resulting in a C to T mutation (Matsumura, Y and Ananthaswamy, HN, 2002).

The presence of unrepaired UV lesions can lead to skin cancer, which is a major health concern world wide. Skin cancer can be divided into two major types,

malignant melanoma and NMSC, which, in turn, is composed of two diseases, SCC and BCC. Melanoma is very invasive, with a high mortality rate. Malignant melanoma originates from epidermal melanocytes and can occur in any tissue that has these cells, although it is most commonly found in the skin (Cummins, DL et al., 2006). Of the estimated 14,900 cancers (excluding NMSCs) reported in Canada in 2005, 4,400 were melanoma, with 20% of the cases resulting in death (National Cancer Institute of Canada, 2005). The mortality rate of NMSC is far lower, as many of the tumours can be removed by surgery (National Cancer Institute of Canada, 2005), however these tumours should not regarded as indolent. SCC are invasive, with as many as 10% of the tumours metastasizing (Gross, DJ and Waner, M, 1992; Moller, R et al., 1979). In 2000 in the US, there were 1500 deaths from SCC, which was equivalent to the deaths caused by Hodgkin's disease (Stratton, SP et al., 2000). BCC is rarely fatal, but it can be locally destructive and causes considerable morbidity (Stratton, SP et al., 2000).

The progression to SSC has many stages, which are illustrated in Figure 1-4. Chronic exposure, even at suberythemal doses, or doses that are too low to cause sunburn, (Chouinard, N et al., 2001), leads to the accumulation of CPDs and eventually leads the precancerous lesions of dysplasia and actinic keratosis. From here, some of the cells will progress to SCC (Melnikova, VO and Ananthaswamy, HN, 2005) (Figure 1-5). In comparison, there is no pre-

cancerous stage for BCC (Figure 1-5). BCCs arise from cell layers deeper than SCCs, in the interfollicular basal calls, hair follicles or sebaceous glands (Hussein, MR, 2005).

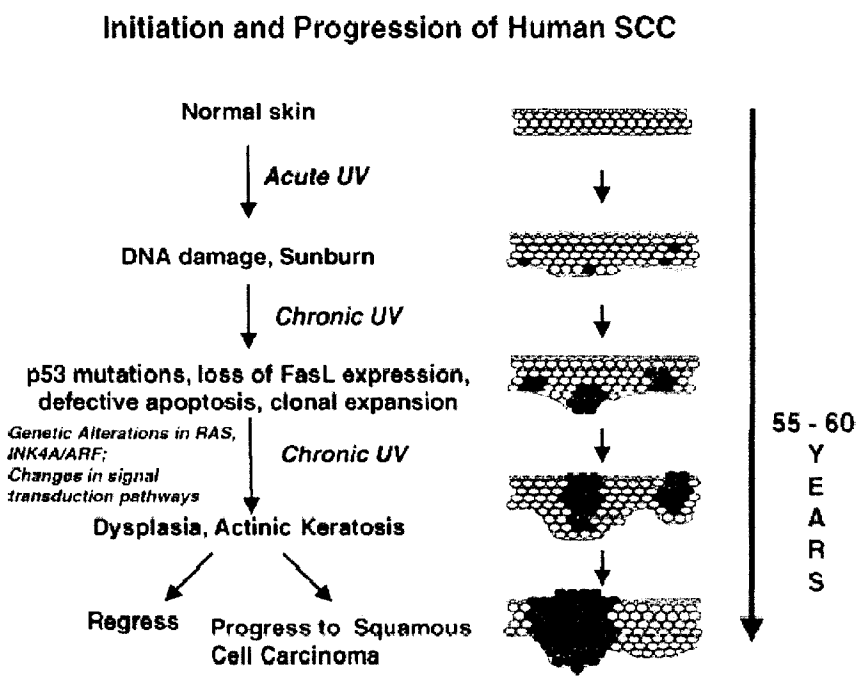

Figure 1-4: Development of Human Squamous Cell Carcinoma

UVB induction of DNA damage and apoptosis in keratinocytes. Accumulation of CPDs and defects in DNA repair and replication lead to accumulation of p53 mutations, loss of FasL expression, and resistance to apoptosis. With repeated exposure to UVB, the p53 mutant cells undergo clonal expansion and accumulate additional gene mutations. While some clones develop into actinic keratosis, others progress to SCC (Melnikova, VO and Ananthaswamy, HN, 2005).

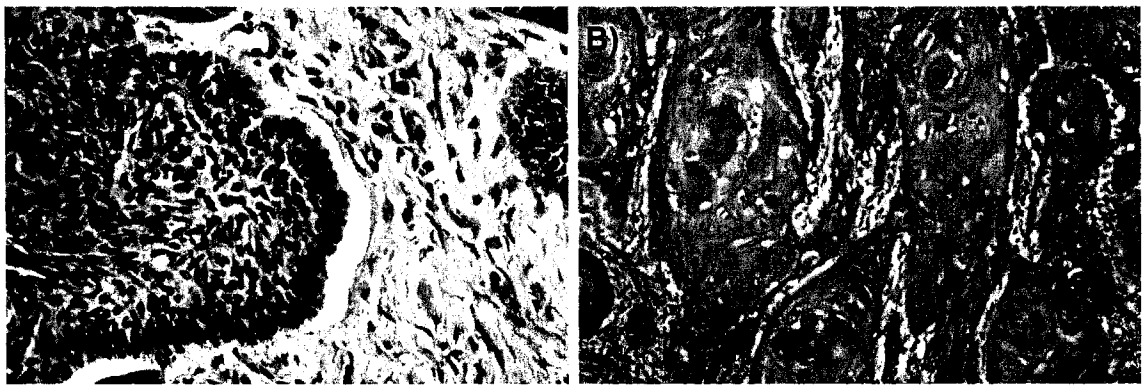

Figure 1-5: Histology of NMSC

A) High power H&E staining of a basal cell carcinoma. Typical of BCCs, undifferentiated basaloid cells form irregular nodules. The cells display no tendency to mature toward squamous cells. There is a clearing between the tumour cells and the surrounding connective tissue. B) High power H&E staining of a squamous cell carcinoma. Characteristic features of SCC include nests of polygonal cells with moderate amounts of cytoplasm. Towards the center of the nests the cells may lose nuclei to form "keratin pearls", which can be seen at the top right. From (Washingtion, Uo, 2006) www.pathology.washington.edu/about/education/gallery/skin.php

DNA Damage Response

Cellular Responses to DNA Damage

Cells are continually exposed to internal and external pressures, such as toxins, and mutagens like UV or chemotherapy agents, that result in DNA damage. The cell must repair the DNA damage to maintain the genome. Signalling pathways exist for important cellular functions, such as cell cycle control, programmed cell death (apoptosis), and the DNA damage response, which are all essential to organism survival.

The proper cellular response to DNA damage is an essential process. Zhou and Elledge, 2000 illustrate this process in Figure 1-6. When a cell is exposed to DNA damaging agents or replication stress, sensor proteins are the first to respond. They detect the damage and initiate transducer proteins which relay the message to effector proteins. Effector proteins make critical decisions on how the cell will proceed. The cell has the option to repair the DNA damage, undergo cell cycle arrest, proceed with transcription to assist in those pathways, or, if the damage is too severe, to undergo apoptosis (Zhou, BB and Elledge, SJ, 2000) (Figure 1-6). If the cell does not respond properly to this damage, deleterious consequences may occur.

All of the responses of DNA damage are important to organism survival. Transcription regulates the DNA damage response as certain key proteins are up-regulated in response to DNA damage. Cell cycle arrest provides time to repair the DNA damage, while apoptosis destroys the cell if damage is too great to repair. If these processes do not work together, or suffer disregulation, diseases, such as cancer, can result.

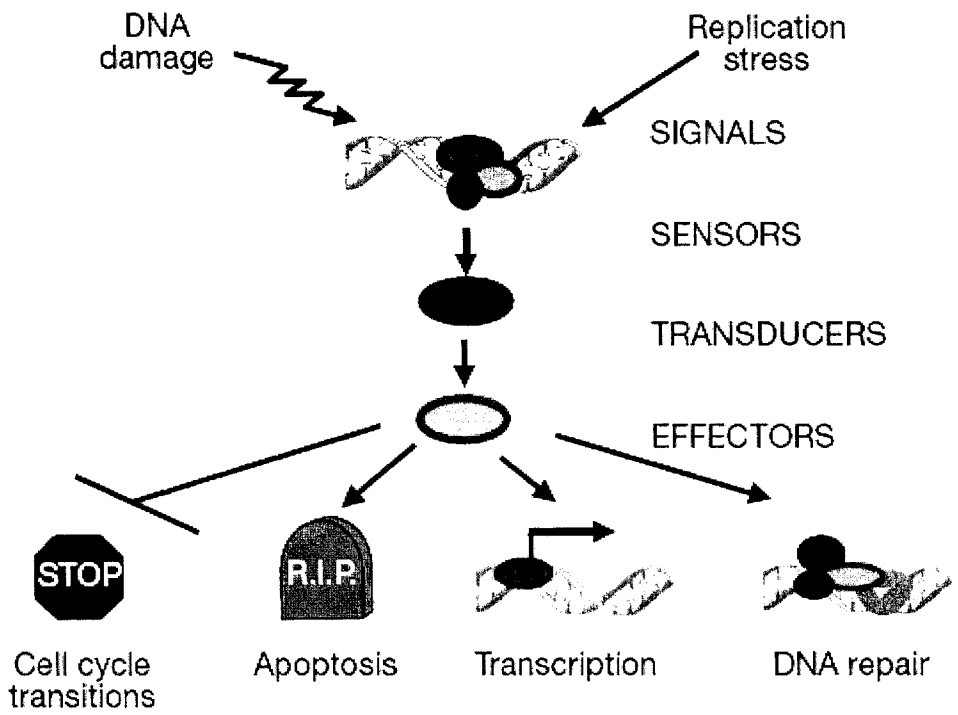

Figure 1-6: DNA damage response

Signalling cascade from DNA damage or replication stress. Sensing of the DNA lesions leads to signal transduction, resulting in cellular changes such as changes in transcription, apoptosis, cell cycle arrest and DNA repair (Zhou, BB and Elledge, SJ, 2000).

It has been shown that UVB damage regulates the apoptotic response in human keratinocytes (Maeda, T et al., 2001), as well as many other cellular functions. One of the major players in the UV damage response is p53, a tumour suppressor protein with at least 7 functional properties. p53 can activate 20 promoters, repress an additional 26 enhancers or promoters, and interact with at least 35 cellular and viral proteins (Hussein, MR, 2005). The importance of p53 in the UV response is highlighted by the fact that 35% of all *p53* mutations in skin cancers are transitions in dipyrmidine sequences, which are classic UV-induced

mutations (Pfeifer, GP et al., 2005). UVB causes post-translational stabilization of the *p53* gene, and the build-up of p53 protein transcribes downstream p53 "effector" proteins to control the DNA damage response. Examples of this include p21, a potent cyclin-dependent kinase (CDK) inhibitor that regulates the G_1 cell cycle checkpoint, growth arrest and DNA-damage inducible protein 45 alpha (Gadd45α) which stimulates nucleotide excision repair (NER) and Mdm2 which binds p53 and blocks the p53 transcriptional response (Maeda, T et al., 2005). p53 is also involved in the apoptotic response. When exposed to UVB-irradiation, differentiating keratinocytes will undergo apoptosis, while basal keratinocytes enter DNA repair (Tron, VA et al., 1999). In addition, inactivation of p53 in mouse keratinocytes causes a reduction in the number of apoptotic cells after UV-irradiation (Ziegler, A et al., 1994). There is evidence that p53 is not involved in apoptosis in the skin (Chaturvedi, V et al., 2005; Tron, VA et al., 1999). Since apoptosis is the cause of the severe side effects of many cancer treatments, this has important clinical implications; potential treatments could safely involve induction of the p53 pathway, without risking induction of apoptosis.

DNA REPAIR

There are multiple DNA repair pathways that respond to different forms of DNA damage. Base excision repair (BER) removes most kinds of DNA damage, including modified or damaged bases, removal of purine bases and single strand

breaks (Sharova, NP, 2005) and is associated with oxidative damage (Smith, ML and Seo, YR, 2002). Mismatch repair (MMR) responds to base mismatches and small insertion or deletion loops resulting from post-replicative errors, while repair of double-strand breaks, commonly caused by ionizing radiation or mechanical stress, is performed by recombination repair or non-homologous end-joining (Sharova, NP, 2005). Chemical agents such as chemotherapy drugs like cisplatin, psoralen and mitomycin, result in DNA cross-links which are repaired by cross-link repair, and UV-induced DNA-lesions (CPDs and 6-4PPs) are removed by NER (Lehmann, AR, 1995; Matsumura, Y and Ananthaswamy, HN, 2002). Recent evidence shows the existence of DNA-polymerases that are able to bypass irremovable bases and can either preserve DNA sequence or result in mutation depending on the type of damage and the resulting bypass mechanism (Sharova, NP, 2005). The mechanisms of the repair pathways differ greatly; while BER excises damaged bases as free bases and MMR removes mispaired bases as single nucleotides, NER removes an oligonucleotide fragment of ~25-30 nucleotides and resynthesized the DNA (de Boer, J and Hoeijmakers, JH, 2000; Friedberg, EC, 2001).

NUCLEOTIDE EXCISION REPAIR

NER repairs bulky DNA lesions including UV-induced photoproducts. Nearly all forms of life are exposed to UV. Consequentially, UV has driven the evolution of DNA repair pathways in many organisms (Cleaver, JE et al., 2001). The NER

pathway has developed in both the prokaryotic and eukaryotic kingdoms to deal with UV-induced damage, showing the importance of this mechanism (Cleaver, JE et al., 2001). It is the most studied (Cleaver, JE et al., 2001; Zhan, Q et al., 1999) and arguably the most complex DNA-repair pathway, involving as many as 30 different genes (Tannock, IF, Hill, Richard P., 1998) It is also the most diverse repair pathway as it removes many types of structurally unrelated DNA lesions (de Boer, J and Hoeijmakers, JH, 2000).

Disruptions of the NER pathway in humans results in three human syndromes characterized by extreme photosensitivity and a wide array of neurological disorders (de Boer, J and Hoeijmakers, JH, 2000). Patients with Cockayne syndrome (CS) and trichothiodystrophy (TTD) both display these symptoms, while individuals with xeroderma pigmentosum (XP) have more severe phenotypes (Balajee, AS and Bohr, VA, 2000; Riedl, T et al., 2003). XP was the first disorder described as resulting from a deficiency in NER (Cleaver, JE, 1968). Along with sun sensitivity and neurological symptoms, patients with XP have a marked predisposition to cancer arising from exposure to UV light (Cleaver, JE, 1968). The genetic complexity of these diseases was established by fusing cells from different patients to examine the phenotypic correction, or complementation. This is known as cell-fusion (Friedberg, EC, 2001). Cell-fusion experiments have identified seven complementation groups of proteins from patients with XP (XPA to XPG), two from CS patients (CSA and CSB), three from patients with

combined XP and CS (XPB, XPD and XPG) and three in patients with TTD (TTDA, XPB and XPD) (de Boer, J and Hoeijmakers, JH, 2000).

Each complementation group represents a different genetic mutation and is associated with different severity of symptoms. For example, XP patients with XPA, XPB, XPD and XPG display an extreme NER deficiency, associated with more severe neurological defects while those with XPE display the mildest defect (Batty, DP and Wood, RD, 2000; Friedberg, EC, 2001; Kraemer, KH et al., 1975; van Hoffen, A et al., 2003). It is interesting that a different genetic mutation of the same gene is able to produce a different disease. Individuals with mutations in XPB, XPD and XPG will develop XP or XP/CS depending on the mutation in XPB, XPD, or XPG. Additionally, TTD can result from a different mutation in XPB or XPD. Considering the vast differences in phenotype and severity of the three diseases, the additional cancer predisposition for XP, and the other abnormalities of CS (skeletal abnormalities) and TTD (brittle hair) it is remarkable that they are all caused by deficiencies of NER proteins.

The NER pathway is composed of multiple proteins reacting in a specific, ordered fashion to recognize, remove and replace damaged nucleotides (Figure 1-7). The two branches of NER, global genomic repair (GGR) and transcription coupled repair (TCR), differ depending on the recognition signal. PCNA is integral to NER and is involved in the resynthesis step of both pathways.

Damage recognition the GGR pathway involves the binding of XPC to the damage site. RAD23, a protein involved in NER in yeast, has two human homologues, human homologue for RAD23A or RAD23B (HHR23A or HHR23B). Both HHR23A and HHR23B are thought to interact with XPC, although when extracted from human cells, only HHR23B co-immunoprecipitates with XPC (Batty, DP and Wood, RD, 2000; Sugasawa, K et al., 1998; Wood, RD, 1999). The presence of UV-DNA damage binding (UV-DDB) protein complex is the first step in GGR signalling (Bohr, VA et al., 1985). UV-DDB is composed of the XPE protein p48 and p127, which is also found in association with proteins involved with transcription (Batty, DP and Wood, RD, 2000; van Hoffen, A et al., 2003). UV-DDB is probably an accessory complex; it enhances XPC-HHR23B binding but is not essential (Aboussekhra, A et al., 1995; Bessho, T et al., 1997; Kazantsev, A et al., 1996). This is also supported by the fact that XPE cells display the mildest NER defect (Batty, DP and Wood, RD, 2000) with a relatively high repair capacity (van Hoffen, A et al., 2003). The role of UV-DDB depends on the type of damage and may play a bigger role in the removal of 6-4PPs (Abramic, M et al., 1991; Keeney, S et al., 1993), which cause more distortion of the helical backbone (Balajee, AS and Bohr, VA, 2000; de Boer, J and Hoeijmakers, JH, 2000). The second NER pathway, TCR, is activated solely by damage on the transcribed strand of DNA and is driven by transcription (van Hoffen, A et al., 2003). Since damage on the non-transcribed strand is not recognized by TCR and GGR is able to repair damage on both strands of DNA,

GGR may overrule TCR (van Hoffen, A et al., 1995). TCR is initiated when RNA polymerase II (RNApolII) is arrested at the damage site resulting in protein displacement. CSB is part of the RNApolII elongation complex (Tantin, D, 1998; Tantin, D et al., 1997) and is likely involved, along with CSA in downstream signalling during elongation only (Balajee, AS and Bohr, VA, 2000). Transcription factor IIH (TFIIH) is involved in the initiation step of transcription, as well as NER, and is released during elongation (Balajee, AS and Bohr, VA, 2000). There is evidence that CPDs occurring within 50 bases of the initiation sites of different genes are repaired in the absence of CSB and CSA in yeast (Tijsterman, M and Brouwer, J, 1999). This may be due to the presence of TFIIH at the initiation site eliminating the need for its re-recruitment by CSA and CSB (Balajee, AS and Bohr, VA, 2000).

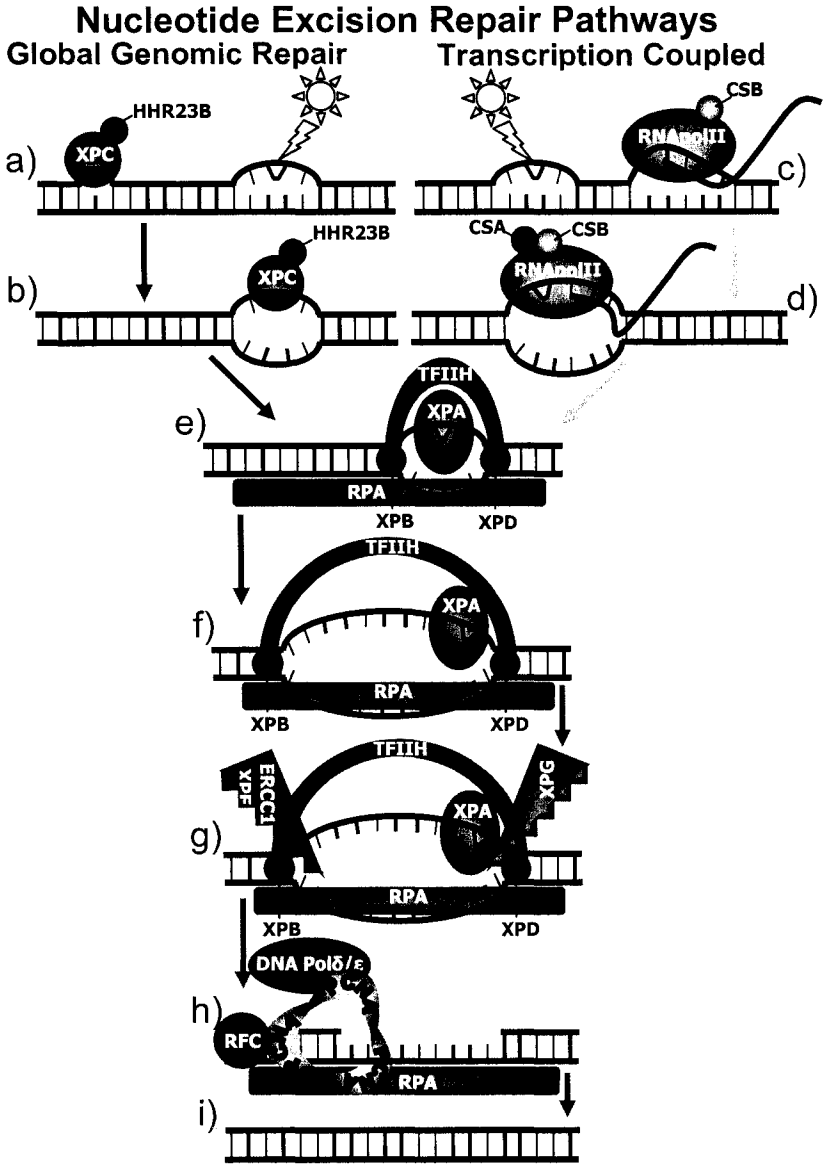

Figure 1-7: Nucleotide Excision Repair Pathways

Schematic Diagram of Global Genomic Repair (GGR) and Transcription Coupled Repair (TCR). During GGR, UV damage (pyrmidine dimer shown) (a) is recognized by XPC-HHR23B (b) possibly with the assistance of DDB. In TCR, UV damage is upstream (c) of RNApolII (with CSB) and is recognized during transcription (d). After recognition the two pathways converge when XPA, TFIIH, XPB and XPD are recruited to the damage site (e). The TFIIH:XPB:XPD complex unzips the DNA (f) to allow an ERCC1:XPF and XPG to excise the damaged strand of DNA (g). PCNA, RFC and DNApolδ or ε are recruited (h) and fill in the missing bases to complete a damage-free DNA strand (i) which is ligated by DNA ligase I (not shown).

After damage recognition XPA verifies the damage and the rest of the NER complex assembles. XPA recruits TFIIH, whose six subunits facilitate the unwinding of DNA with the XPB and XPD helicases, generating sites for DNA incision (Friedberg, EC, 2001). After unwinding, endonucleases are recruited to incise and excise the damaged DNA. XPG cleaves the DNA 3' to the DNA damage site, while the ERCC1-XPF heterodimer cuts the 5' end of the damage (Friedberg, EC, 2001). Replication protein A (RPA) stabilizes the process. Although the oligonucleotide is roughly 30 nucleotides in length, the damaged site is closer to 3' incision (Friedberg, EC, 2001).

Following excision, proliferating cell nuclear antigen (PCNA), replication factor C (RFC), RPA and DNA polymerase (DNApol) δ or ε assemble to fill in the missing bases (Shivji, MK et al., 1995). DNApolδ is normally associated with PCNA replication, but there is evidence that DNApolε is more efficient in NER (Shivji, MK et al., 1995). Finally DNA ligase I replaces DNApol on PCNA (Riva, F et al., 2004) to ligate the repaired DNA (Friedberg, EC, 2001; Shivji, MK et al., 1995).

PCNA

Proliferating cell nuclear antigen (PCNA) autoantibodies were first discovered in a proportion of patients with systemic lupus erythematosus (Miyachi, K et al.,

1978). This autoantibody was found to be present only in proliferating cells and was detected with multiple immunological techniques.

PCNA often acts in a p53-dependent manner (Paunesku, T et al., 2001) and is involved in many cellular processes through protein interactions, including DNA replication (Bravo, R and Celis, JE, 1980), repair and cell cycle control (Loor, G et al., 1997). PCNA has no enzymatic activity but forms a homotrimeric protein (Figure 1-8) that freely slides along double stranded DNA (Kelman, Z, 1997; Krishna, TS et al., 1994) acting as a scaffold. The importance of PCNA is evident as homologues have been found in eukaryotes, archaea, bacteria and viruses (Roberts, JA et al., 2003; Warbrick, E, 2000). Many proteins that interact with PCNA have a conserved PCNA binding motif (Jonsson, ZO et al., 1998) that has been extensively studied in the cyclin-dependent kinase inhibitor p21 (Warbrick, E, 2000). Since many proteins interact with PCNA through this site, and each PCNA homotrimer is able to bind up to three proteins, there is competition for the sites, and regulation must exist. However, it is not understood how a cell regulates protein binding to PCNA. Other proteins bind to PCNA outside of the conserved binding domain, including Gadd45α and Gadd45β (MyD118) (Vairapandi, M et al., 2000).

Figure 1-8: Crystal structure of human PCNA.

The three monomers are highlighted by different colour combinations; blue, green and red-orange. The disk-shape allows PCNA to act as a sliding clamp along DNA. From www.rscb.ord/pbd

Perhaps PCNA's major role is as the processivity clamp for DNA polymerase δ and ε, functioning as a tether between the catalytic unit and the DNA template (Ducoux, M et al., 2001; Kelman, Z, 1997). This interaction is vital to both DNA replication and repair. PCNA binds to DNA with the C-terminus facing toward the direction of DNA synthesis (Jonsson, ZO et al., 1998). It interacts with other replication proteins, including DNA ligase I (Riva, F et al., 2004), Fen1, a eukaryotic 5' endonuclease, and RFC, which assembles PCNA onto the DNA strand (Warbrick, E, 2000). However, PCNA also has many other roles in the cell, which is evident by the large number of proteins that interact with PCNA. XPG has a conserved PCNA-binding motif and may recruit PCNA to the replication phase of NER (Warbrick, E, 2000). The MMR proteins MSH3 and MSH6 both have the conserved PCNA-binding motif, and it has been theorized

that PCNA may be involved in strand differentiation; the presence of PCNA at the replication fork identifies the direction of DNA synthesis, which distinguishes the two strands (Warbrick, E, 2000). PCNA also interacts with proteins involved in post-replication processing and DNA helicases (Warbrick, E, 2000). PCNA interacts with p21 and a number of cyclins (Jonsson, ZO and Hubscher, U, 1997; Prosperi, E et al., 1994; Riva, F et al., 2004) indicating a role in the cell cycle. There is also evidence that PCNA hinders the negative growth control of Gadd45α or Gadd45β. Ectopic expression of Gadd45α or Gadd45β lacking the PCNA binding domains suppressed cell growth or induced apoptosis more efficiently than full length protein (Vairapandi, M et al., 2000).

PCNA antibodies have been used extensively to study PCNA staining patterns, with clone PC10 being the most widely used for immunofluorescence. Different patterns of PCNA exist in different stages of the cell cycle, with nuclei extracted with Nonidet P-40 (NP-40) showing PCNA positivity mainly in S-phase nuclei (Wilson, GD et al., 1992). While nuclei in S-phase have a high proportion of phosphorylated PCNA, cyclin D1 and cyclin A were bound to DNA with PCNA primarily at the G_1/S phase boundary, possibly preparing for DNA replication (Prosperi, E et al., 1994). It has been suggested that DNA-bound PCNA trimers during S-phase are organized into pools that selectively bind different partners, such as DNApolδ or DNA ligase I, which bind in a mutually exclusive manner (Riva, F et al., 2004).

PCNA AND REPAIR

PCNA was first extracted as an excision repair protein more than a decade ago (Nichols, AF and Sancar, A, 1992; Shivji, KK et al., 1992). It forms repair complexes in response to ionizing radiation (IR) (Karmakar, P et al., 2001), and UV (Aboussekhra, A and Wood, RD, 1995; Balajee, AS et al., 1998; Shivji, KK et al., 1992) and has been implicated in steps preceding resynthesis in MMR (Umar, A et al., 1996).

Initial work with PCNA antibodies led to the discovery of many different staining patterns, especially with clone PC10. To eliminate interference from non-DNA bound PCNA, treatment with detergent prior to fixation has been widely used (Miura, M, 1999). Two detergents are commonly used; NP-40 (Prosperi, E et al., 1994) and Triton X-100 (Aboussekhra, A and Wood, RD, 1995). Triton X-100 leaves only proteins that are so tightly bound to DNA that they cannot be released with high-salt extractions (Bravo, R and Macdonald-Bravo, H, 1987).

The formation of PCNA repair complex is dependent on both the TCR and GGR pathways and that immunoprecipitated-PCNA binds many parts of the NER pathways, including XPA, p53, RPA p34 subunit and TFIIH (Balajee, AS et al., 1998). The balance between replication and repair is likely maintained by altering the PCNA protein complex. PCNA binding to ING1 increases tenfold in

response to UV and is inhibited by p21. This UV-damage associated interaction induces apoptosis and indicates that ING1 contributes to shifting the PCNA response to repair and ultimately cell death in response to UV (Scott, M et al., 2001).

PCNA, like many proteins, undergoes post-translational modifications such as phosphorylation (Prosperi, E et al., 1994), ubiquitination (Kirkpatrick, DS et al., 2005), acetylation (Naryzhny, SN and Lee, H, 2004) and sumoylation (Papouli, E et al., 2005). These modifications act to regulate its diverse array of functions and localization (Naryzhny, SN and Lee, H, 2004). There are three isoforms of PCNA with different levels of acetylation. Co-immunoprecipitation studies show that p300 and histone deacetylase (HDAC1) are pulled down by PCNA. This suggests that these two proteins are responsible for the acetylation and deacetylation PCNA respectively. Moderately acetylated PCNA is found in all subcellular compartments of cells that are actively dividing, while the highly acetylated (acidic) form is found primarily in the nucleus. Deacetylated (basic) PCNA is the primary form found in nucleoplasm of dividing cells and prohibits PCNA from binding DNA polymerase β and δ (Naryzhny, SN and Lee, H, 2004). DNA-bound PCNA in S-phase cells is highly phosphorylated. Phosphorylation of PCNA likely precedes DNA-binding (Prosperi, E et al., 1994). Mono-ubiquitination of PCNA is a reversible process that is linked to DNA repair function (Huang, TT et al., 2006). This process is required for accumulation and

stability at DNA damage sites (Solomon, DA et al., 2004) and leads to translesion synthesis (Huang, TT et al., 2006; Papouli, E et al., 2005). As translesion synthesis is potentially dangerous, there are multiple processes that regulate this process. Ubiquitin specific protease 1 (USP1), a deubiquitinating enzyme, removes ubiquitin from PCNA and is inactivated by UV irradiation (Huang, TT et al., 2006). Ubiquitination and sumoylation of PCNA occur at the same site. Ubiquitination of PCNA promotes DNA-damage bypass, while sumoylation, which occurs independently of DNA damage, prevents translesion synthesis by interacting with Srs2p, a DNA helicase that suppresses unscheduled homologous recombination rather than competition between SUMO and ubiquitin for PNCA (Papouli, E et al., 2005).

PCNA is bound to DNA longer during repair than in replication and that mono-ubiquitination regulates the time that PCNA spends at replication sites. PCNA is bound to repair sites during all stages of cell cycle, but disappears at early S-phase and reappears at distinct foci in late S phase (Essers, J et al., 2005). There are different binding affinities of PCNA for DNA replication and repair and that the mobile pool of PCNA in the nucleus allows for dynamic assembly of PCNA at different sites (Essers, J et al., 2005). PCNA forms distinct pools used for either replication or repair. The mono-ubiquitinated DNA-bound PCNA forms repair foci with γH2AX, RPA and Rad51 and is not required for or involved in initiation of DNA replication (Szuts, D et al., 2005).

Gadd45α

Growth arrest and DNA damage inducible gene 45α (*Gadd45α*) is a member of a family of genes that are activated in response to various DNA damaging agents, such as UV, IR and methyl methanesulfonate (MMS) and media starvation. It was first described in 1989 (Fornace, AJ, Jr. et al., 1989) and is thought to be a nuclear protein (Carrier, F et al., 1994) that binds to a variety of proteins. Its three dimensional structure is yet to be determined. Gadd45α self-associates *in vitro* and *in vivo* and interacts with the other Gadd45 family proteins, Gadd45β and Gadd45γ (CR6) (Kovalsky, O et al., 2001). It responds to many types of DNA-damage signals, contains x-ray responsive elements (Daino, K et al., 2003) and is induced by UV in a p53-dependent and independent manner (Maeda, T et al., 2003).

Molecular Regulation of Gadd45α

Gadd45α is induced by different pathways in response to different damaging agents. Gadd45α is activated by p53 in response to IR, but, unlike the majority of p53-regulated proteins, is often activated in a p53-independent manner in response to UV damage (Fan, W et al., 2002; Jin, S et al., 2001; Maeda, T et al., 2003; Ohtani-Fujita, N et al., 1998; Takahashi, S et al., 2001; Tong, T et al.,

2001). Although DNA damage is required for activation of Gadd45α by p53 (Xiao, G et al., 2000), p53 is able to up-regulate Gadd45α in the absence of direct DNA-binding, acting to stimulate the promoter through protein-protein interactions (Zhan, Q et al., 1998). PRMT1, p300 and CARM1 have been are cofactors in the induction of Gadd45α in response to p53 expression and UV damage (An, W et al., 2004). YY1 has also been shown to bind to a subset of p53-responsive genes, including *Gadd45α* and *p21*, and may assist in regulating how the cells respond to p53 activation (Yakovleva, T et al., 2004). In addition to p53, Gadd45α is activated by the overexpression of BRCA1 through transcriptional upregulation (Jin, S et al., 2000)

Gene transcription is essential for protein expression. Gene promoters contain certain sequences (motifs) that allow specific transcription factors to bind and transcribe genes. For example, the Oct-1 transcription factor binds to the OCT-1 motif, while the NF-YA transcription factor binds to the CAAT motif. *Gadd45α* contains OCT-1 and CAAT motifs which play an important role in its activation (Fan, W et al., 2002). The OCT-1 motif is involved in the p53-independent activation of the *Gadd45α* promoter in response to UV irradiation (Takahashi, S et al., 2001) and both motifs allow *Gadd45α* to be activated in p53-independent manner by BRCA1 (Fan, W et al., 2002), histone deacetylase inhibitor (Hirose, T et al., 2003), Δ^{12}-prostaglandin J_2 (Ohtani-Fujita, N et al., 1998) through interaction with the transcription factors NF-YA and Oct-1 (Jin, S et al., 2001).

There is evidence that the mitogen activated protein kinase pathways help to regulate the expression of Gadd45α in response to UV damage through the OCT-1 and CAAT motifs. Inhibition of Jun N-terminal kinase (JNK) and ERK kinase drastically decreases the induction of Gadd45α after UV damage, but is not affected by the inhibition of p38 kinase. Additionally, expression of JNK1, Raf-1 and MEK1 results in strong activation of the *Gadd45α* promoter. This occurs in a p53-independent manner through the interaction with the OCT-1 and CAAT motifs (Tong, T et al., 2001).

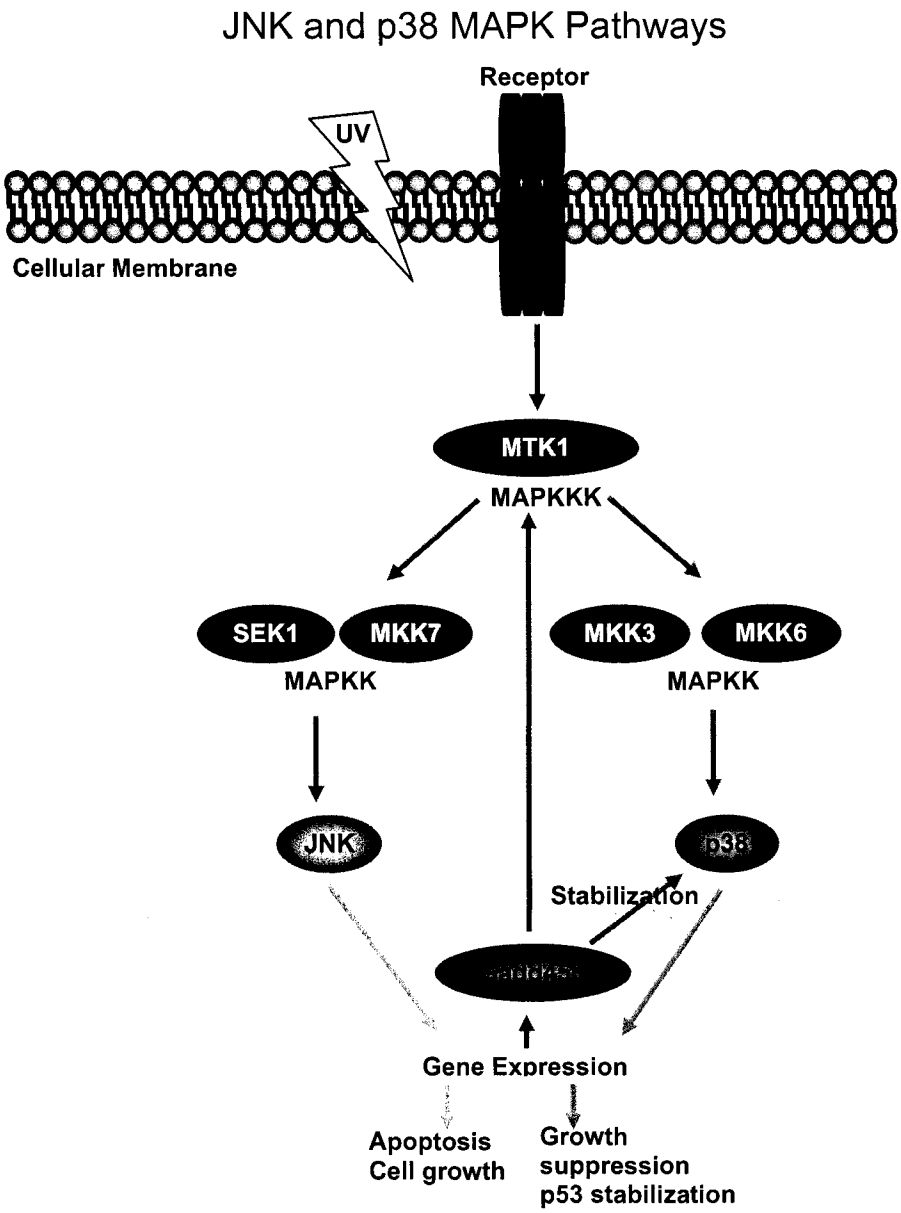

Figure 1-9: JNK and p38 MAPK Pathways

There are three major MAPK pathways; ERK (not shown), JNK and p38. After receptor stimulation, mitogen activated protein kinase kinase kinases (MAPKKKs) phosphorylate and activate mitogen activated protein kinase kinases (MAPKKs) which phosphorylate and activate mitogen activated protein kinases (MAPKs) (ERK, JNK or p38) which result in various cellular responses. JNK and p38 are initiated in response to various stimuli including UV and inflammatory cytokines. Some of these are the same stimuli that activate the NF-κB pathway (Figure 1-10). MEKK4 (MTK1), a MAPKKK activates the MAPKKs in both the JNK pathway (SEK1 and MKK7) and the p38 pathway (MKK3 and MKK6).

Many cellular pathways assist in the regulation of the Gadd45α protein, although the details of many of these remain unclear. It is known that the cell cycle affects expression of Gadd45α. In response to *p53* or *BRCA1* expression Gadd45α is transiently down-regulated during G_0/G_1, and is present at its highest levels during S-phase (Kettenhofen, R et al., 2001). There is evidence that the phosphoinositide 3-kinase (PI3K)-Atk pathway regulates Gadd45α through induction of the FOXO3a transcription factor. Atk is a serine-threonine kinase (also called protein kinase B) that is activated by receptor tyrosine kinases. It phosphorylates and inhibits FOXO family forkhead transcription factors, of which FOXO3a has been shown to up-regulate Gadd45α in response to UV (Tran, H et al., 2002). By inhibiting FOXO3a activity, Atk assists to inhibit the transcription of Gadd45α. Overexpression of FOXO3a results in a G_1 cell-cycle checkpoint and upregulates the expression of many genes involved in the DNA damage response at the G_2M checkpoint, including Gadd45α (Tran, H et al., 2002).

Another signalling pathway that regulates Gadd45α expression is the nuclear factor κB (NF-κB) pathway. NF-κB is a transcription factor and a vital protein in the stress-induced cell response, including apoptosis (Chen, F et al., 2001). Heterodimers of NF-κB family proteins, which includes Rel(c-Rel), RelA(p65), RelB, NF-κB1(p100/p50) and NF-κB(p105/p52) form the active component of

NF-κB (Papa, S et al., 2004; Schneider, G et al., 2006). In the cytoplasm, the NF-κB dimers are sequestered by the inhibitor of NF-κB (IκB) which keep the protein inactive (Papa, S et al., 2004; Schneider, G et al., 2006). The NF-κB pathway (Figure 1-10) has been linked to in tumourigenesis, cancer cell survival, apoptosis, invasion and metastasis (Papa, S et al., 2004; Schneider, G et al., 2006; Zerbini, LF et al., 2004). NF-κB is activated by stress signals, including UVB (Zerbini, LF et al., 2004) and translocates to the nucleus to transcribe various proteins. Post-transcriptional stabilization of *Gadd45α* mRNA occurs in response to the inhibition of NF-κB (Zheng, X et al., 2005). Inhibition of NF-κB in vitro inhibits cell proliferation and induces apoptosis. Similarly, in vivo obstruction of NF-κB abolished tumour formation in SCID mice (Zerbini, LF et al., 2004). Loss of NF-κB alters the expression of Gadd45 proteins, displaying an increased expression of Gadd45α and γ and decreased expression of Gadd45β. A connection between NF-κB and Gadd45α exists in c-Myc, since this transcription factor is a target gene for NF-κB and is able to suppress the Gadd45α promoter through a polymerase II recruitment mechanism (Barsyte-Lovejoy, D et al., 2004). Blockage of NF-κB reduces of c-Myc, while activation of NF-κB leads to increased levels of c-Myc and a reduction of Gadd45α promoter activity (Zerbini, LF et al., 2004). Assays using *IKKβ*$^{-/-}$ MEFs, which have inactive NF-κB, display an increase of *Gadd45α* mRNA of 3 to 4 times in knock-out cells over wild-type cells, and an increase of reactive oxygen species. Although these cells display no change in the Gadd45α regulatory proteins Atk, FoxO3a, p53 or c-Myc,

nucleolin, an mRNA stabilizing protein, was shown to bind to *Gadd45α* mRNA in increased levels in the knock-out cells using immunoprecipitation. This indicates that inhibition of NF-κB regulates *Gadd45α* mRNA through post-translational modifications rather than transcription in some cell types (Zheng, X et al., 2005).

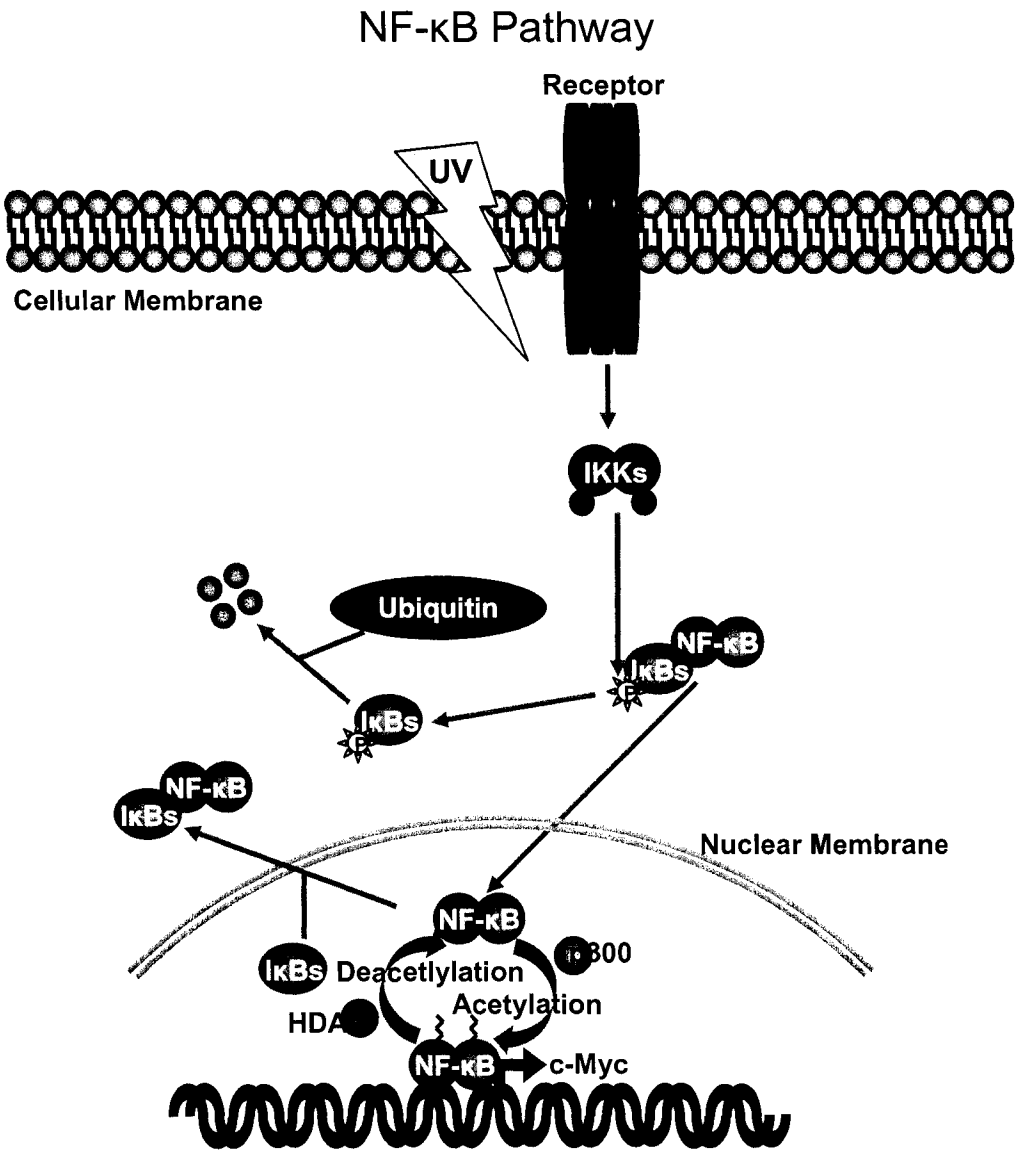

Figure 1-10: NF-κB Pathway

Schematic diagram of the NF-κB pathway. NF-κB is complexed with IκB in the cytoplasm, inhibiting its function. Receptor stimulation leads to IκB kinases (IKKs) phosphorylating IκB releasing it from NF-κB and inducing its ubiquitin-dependent degradation. Activated NF-κB translocates to the nucleus where it is stabilized by acetylation and transcribes various genes, such as c-Myc. Deacetylation of NF-κB allows IκB to bind, inactivating it and translocating to the cytoplasm.

There is evidence of crosstalk between the NF-κB and JNK mitogen activated protein kinase MAPK pathways. The three major MAPK pathways respond to different cellular signals (Figure 1-9). JNK is stimulated by UV and inflammatory cytokines through the tumour necrosis factor receptor (TNFR) similar to the NF-κB pathway (Papa, S et al., 2004). Stimulation of Gadd45α and γ in response to NF-κB inhibition is essential for JNK activation and induction of apoptosis (Zerbini, LF et al., 2004). NF-κB deficient cells can be rescued from TNF-α-induced apoptosis by inhibition of JNK signalling (De Smaele, E et al., 2001; Javelaud, D and Besancon, F, 2001; Tang, G et al., 2001). Similarly, when JNKK2/MKK7 is knocked-out, TNF-α-induced apoptosis is abolished in RelA-null cells (Deng, Y et al., 2003). In addition, when NF-κB is suppressed, JNK is persistently induced by TNF-α, which results in apoptosis (De Smaele, E et al., 2001; Javelaud, D and Besancon, F, 2001; Tang, G et al., 2001).

Further regulation of Gadd45α occurs in post-translational modifications. Gadd45α is ubiquitinated in an epidermal growth factor-dependent manner, while PKCδ results in Gadd45α deubiquitination and stabilization (Leung, CH et al., 2001). B23, a nucleolar phosphoprotein involved in ribosomal assembly, centrosome duplication, cell proliferation and transcriptional regulation, regulates the nuclear translocation of Gadd45α and is involved in the Gadd45α-induced G_2/M cell cycle checkpoint (Gao, H et al., 2005). In addition nucleolin binds to Gadd45α and stabilizes *Gadd45α* mRNA (Zheng, X et al., 2005). Although

regulation of Gadd45α is still not completely understood, it is clear that the process is very complex and occurs on many levels.

GADD45α AND CANCER

It has been hypothesized that loss of Gadd45α may substitute for loss of p53 in *p53* wild type tumours. One study of 24 tumour cell lines including 11 with wild type *p53* and found that *Gadd45α* sequence was not mutated in any of these cell lines (Campomenosi, P and Hall, PA, 2000). Another study evaluated 59 breast carcinomas for *Gadd45α* mutations. These carcinomas consisted of 8 with mutated *BRCA1*, 7 with *BRCA2* mutations and 9 with mutations in *p53*. No mutations for *Gadd45α* sequence were found and only 19 of the 59 displayed loss of heterozygosity (LOH) (Sensi, E et al., 2004). More recently hypermethylation of the *Gadd45α* promoter has been described in breast cancer (Wang, W et al., 2005) and prostate cancer (Wang, Y et al., 2005). Meningiomas have been analysed for *Gadd45α* mutations. No mutations or proteins levels were altered in either the 19 meningiomas with LOH or the 20 meningiomas without LOH suggesting that Gadd45α is not involved in meningioma tumourigenesis (Piaskowski, S et al., 2005). Invasive ductal carcinomas (IDC) of the pancreas were analyzed for *p53* and *Gadd45α* expression. A significant proportion of IDC displayed *Gadd45α* mutations and that expression of Gadd45α had no effect on the efficacy of adjuvant chemotherapy. Furthermore, in *p53* wild

type tumours, the presence of *Gadd45α* correlated with decreased survival than those without *Gadd45α*. These data suggest that *Gadd45α* can be a predictor of patient survival in resectable pancreatic IDCs (Yamasawa, K et al., 2002). These data suggest, that although *Gadd45α* sequence is not mutated in most cancers, downregulation of protein expression may occur through promoter methylation and that suppression of protein expression may play a role in tumour progression.

Breast carcinoma has also been shown to have an increased level of *Gadd45α* both in tumour samples and tumour cell lines (Schneider, G et al., 2006). A mouse model of this tumour, which is null for *p53*, over-expresses *Gadd45α*, indicating that *Gadd45α* functions in the neoplastic process. In these tumours, the addition of a super-IκB, which abolishes NF-κB activity, *Gadd45α* mRNA and protein are down-regulated (Schneider, G et al., 2006), in contrast to the accepted mechanism where decreased levels of NF-κB results in increased levels of Gadd45α (Zerbini, LF et al., 2004; Zheng, X et al., 2005). Additionally, the knock-down of Gadd45α results in increased apoptosis and decreased proliferation, indicating that Gadd45α no longer inhibits growth, but has acquired a prosurvival function. These results are highly tissue specific, and are not present in other cellular backgrounds (Schneider, G et al., 2006). High levels of *Gadd45α* have also been reported in other cancer cell lines and tumour samples including acute myelocytic leukemia (Casas, S et al., 2003), colon cancer (Scott,

DW et al., 2005) and gastric cancer (Napieralski, R et al., 2005), and was negatively associated with response to therapy and survival in gastric carcinoma (Napieralski, R et al., 2005). These increased levels may arise from mutated *p53* which leads to disregulation of p53-activated proteins (Hildesheim, J and Fornace, AJ, Jr., 2002; Yamasawa, K et al., 2002) and altered change in the function of these proteins, including Gadd45α (Schneider, G et al., 2006).

GADD45α MICE

Mice lacking the *Gadd45α* gene were developed through homologous recombination. These mice display a phenotype that is somewhat similar to p53 mice, including genomic instability, characterized by aneuploidy, chromosome aberrations, gene amplification, centrosome amplification, and increased radiation carcinogenesis (Hollander, MC and Fornace, AJ, Jr., 2002; Hollander, MC et al., 1999). Genomic instability is accompanied by abnormal mitosis, cytokinesis and growth control (Hollander, MC et al., 1999) and is coupled with defects in the S-phase cell cycle checkpoint (Hollander, MC et al., 2005). These mice also have an increased sensitivity to chemical carcinogenesis, which is linked to an increased mutation frequency and decreased repair (Hollander, MC et al., 2001) and display increased homologous recombination during development, which leads to DNA deletions and mutation (Bishop, AJ et al., 2003; Reliene, R and Schiestl, RH, 2003). This highlights the importance of

Gadd45α in maintaining genomic stability in the p53 pathway. Additionally, *Gadd45α* knock-out mice develop a lupus-like syndrome (Salvador, JM et al., 2002) and spontaneously activate the alternative p38 pathway in T-cells. This activation is abrogated by addition of Gadd45α, suggesting that Gadd45α inhibits this pathway in murine T-cells (Salvador, JM et al., 2005). Gadd45α-deficient mice have a pronounced decrease in GGR, similar to that of the p53-deficient mice (Smith, ML et al., 2000), indicating a role for Gadd45α in NER.

MULTIPLE ROLES OF GADD45α

The roles of Gadd45α vary depending on the type of damage or cell stress. Since Gadd45α deficient mice display defective NER, Gadd45α has been extensively studied in the UV-damage response. Gadd45α has many roles in the cell, as is evident by the diverse phenotype of the *Gadd45α*$^{-/-}$ mice. The role of Gadd45α in apoptosis is controversial. UV induces apoptosis in *Gadd45α* deficient MEFs (Maeda, T et al., 2002) and that microinjection of Gadd45α into human fibroblasts could not induce apoptosis, in contrast to microinjection of p53, which caused apoptosis (Wang, XW et al., 1999). Additionally, hematopoietic cells from Gadd45α- or Gadd45β-deficient mice were more sensitive to UVC- and chemotherapy-induced apoptosis, suggesting that these proteins are antiapoptotic in function (Gupta, M et al., 2005). Other evidence suggests that the presence of Gadd45α promotes apoptosis. One study shows

that Gadd45α protects against skin tumours by promoting apoptosis in vivo (Hildesheim, J et al., 2002). It causes the cytoskeleton to release Bim and translocates it to the mitochondria to induce apoptosis (Tong, T et al., 2005).

Many of the functions of Gadd45α are due to interactions with the p38 and JNK MAPK pathways (Figure 1-9). BRCA1 activation of Gadd45α results in JNK- or p38 dependent cell death (Harkin, DP et al., 1999; Takekawa, M and Saito, H, 1998). Yeast-two-hybrid assays verify that Gadd45α, β and γ interact with MTK1 (or MEKK4), a human MAPKKK that activates both p38 and JNK, by binding to the autoinhibitory domain of MTK1 and releasing autoinhibition (Mita, H et al., 2002; Takekawa, M and Saito, H, 1998). The Gadd45 proteins can activate MTK1 both in vitro and in vivo in mouse models (Takekawa, M and Saito, H, 1998). Following activation, MTK1 binds and activates MKK6 and results in activation of the JNK and p38 MAPK pathways (Figure 1-9) and apoptosis (Mita, H et al., 2002; Takekawa, M and Saito, H, 1998). Both p38 and JNK are activated by MMS, UV and, to a small extent, IR. These sources of DNA damage activate p53 and upregulate Gadd45α expression (Takekawa, M and Saito, H, 1998), indicating that Gadd45α may play an important role in the BRCA1-induced apoptotic pathway. However, Gadd45α-deficient MEFs do not display altered levels of p38 or JNK in response to genotoxic stress indicating that the absence of Gadd45α may not affect JNK and p38 MAPK activities (Wang, X et al., 1999). In these cells it is possible that Gadd45β(MyD118) or

Gadd45γ(CR6) could compensate for the lack of Gadd45α in the MAPK pathways (Sheikh, MS et al., 2000). p53-dependent activation of Gadd45α results in apoptosis in response to the methyltransferase inhibitor 5-aza-cytidine (Schneider-Stock, R et al., 2005), which demonstrates that Gadd45α can induce apoptosis even in the absence of UV light.

Gadd45α has roles in the MAPK pathways in addition to apoptosis resulting from interaction with MTK1. The ERK and JNK, but not the p38 pathways, are induced by the overexpression of H-ras in Gadd45α deficient MEFs, which correlates with a loss of H-ras-induced cell cycle arrest in the absence of Gadd45α (Bulavin, DV et al., 2003). This is strengthened by the fact that p38, but not JNK or ERK complexes with the Gadd45α proteins (Bulavin, DV et al., 2003). Gadd45α is a target and positive modulator of p38 (Hildesheim, J et al., 2002) and stabilizes p53 levels through interaction with p38, in a positive feedback loop (Jin, S et al., 2003). Inhibition of p38 in a Gadd45α proficient background results in deregulation of p53 activation (Bulavin, DV et al., 2003). Through the p38 pathway, Gadd45α acts as a negative regulator of ΔNp63α, a homologue of p53 that is predominantly expressed in basal keratinocytes (Hildesheim, J et al., 2004). ΔNp63α acts as a dominant-negative factor blocking the expression of multiple p53-effector genes and blocks the expression of adenomatous polyposis coli (APC) destruction complex. APC targets free cytoplasmic β-catenin for degradation, preventing it from translocating to the

nucleus and acting in a bipartite transcription factor complex. The targets of this complex includes the matrix metalloproteinases (MMPs) which regulate proliferation, metastasis and cell migration (Hildesheim, J et al., 2004). Loss of either Gadd45α or p38 prevents ΔNp63α suppression, GSK3β dephosphorylation and β-catenin degradation after UV treatment (Hildesheim, J et al., 2004). Gadd45α directly interacts with PP2A and GSK3β, components of the APC complex, and promotes dephosphorylation and activation of GSK3β, resulting in activation of the APC complex in vitro. Gadd45α-null keratinocytes display MMP expression and increased mobility in vitro and accelerated wound closure in vivo (Hildesheim, J et al., 2004). Additionally Gadd45α and p38 are essential in shuttling of APC between the nucleus and cytoplasm. p38 associates and modulates both casein kinase 2, which promotes APC nuclear import and protein kinase A which blocks APC nuclear import. Lack of p38, in response to inhibitors or in Gadd45α-null cells, causes a disruption APC import and in increased levels of β-catenin, indicating a role for Gadd45α, through the p38 MAPK pathway, in regulation of β-catenin (Hildesheim, J et al., 2005). Loss of Gadd45α triggers increased levels of β-catenin, through loss of active APC complex, and leads to upregulation of MMPs. High levels of MMPs can lead to uncontrolled proliferation, metastasis, cell migration, which are vital processes in the oncogenic process (Hildesheim, J et al., 2005).

One of the roles of Gadd45α in response to UV is modulation of the cell cycle. Gadd45α interacts with CR6-interacting factor 1 (CRIF1) to regulate the G_1/S cell cycle checkpoint (Chung, HK et al., 2003) and interacts with BRCA1 to maintain centrosome duplication and genetic stability (Wang, X et al., 2004). While overexpression of Gadd45α disrupts the subcellular localization of cyclin B1 to cause G_2M arrest (Jin, S et al., 2002), it has been shown that Gadd45α does not interact with cyclin B1 directly, but interacts with Cdc2, inhibiting the Cdc2/Cylclin B1 complex, resulting in growth suppression (Jin, S et al., 2000; Zhan, Q et al., 1999). Gadd45α-proficient cells can to inhibit Cdc2 and sequester Cdc2 in the cytoplasm to induce a G_2M check while Gadd45α-deficient cells have a defective G_2M checkpoint (Maeda, T et al., 2002). BRCA1 activation of Gadd45α results in induction of G_2/M checkpoint in response to the antimicrotubule agents Taxol and Vincristine (Mullan, PB et al., 2001). Additional evidence for a role of Gadd45α in cell cycle control exists in the fact that it interacts with p21 (Zhao, H et al., 2000), a cyclin-dependent kinase inhibitor that causes G_1 and G_2 arrest in p53-deficient cells (Cayrol, C et al., 1998).

GADD45α AND NER

The role of Gadd45α in NER has yet to be defined. Gadd45α can bind histones and modulate the accessibility of DNA on damaged chromatin (Carrier, F et al., 1999), however it is also thought that its effect on NER is related to interactions with PCNA (Smith, ML et al., 1994). It has been known for years that Gadd45α

interacts with PCNA (Smith, ML et al., 1994). In a very preliminary study, it was shown that Gadd45α is able to inhibit entry into S-phase and stimulate NER in vitro, binding to PCNA (Smith, ML et al., 1994). Gadd45α does not have a conserved PCNA binding domain, but Gadd45α binds tightly to residues 1-20, 61-80 and 196-215 on PCNA and is potentially able to bind in a stoichiometry of 2 Gadd45α molecules for each PCNA monomer (Hall, PA et al., 1995). Another study revealed that Gadd45α has a PCNA interacting domain at residues 137-165, and that Gadd45α binds to the N-terminal (1-46) and middle (100-127) regions of PCNA. In the same study ectopic expression of a Gadd45α N-terminal peptide, which lacked the PCNA binding domain, repressed colony formation and induced apoptosis more efficiently than full-length protein, while the C-terminal peptide, containing the PCNA binding domain, displayed decreased colony repression and apoptosis compared to full length protein. These results indicate that interaction of Gadd45α with PCNA impedes the negative growth control functions of Gadd45α (Vairapandi, M et al., 2000). There is evidence that, even though they do not share binding sites, Gadd45α and p21 compete for binding to PCNA, and while p21 disrupts PCNA trimerization, Gadd45α has no effect (Chen, IT et al., 1995). Although it is not well described, there is evidence that PCNA accumulation in the nucleus is defective in Gadd45α-deficient cells post UVB damage (Smith, ML et al., 2000). In Gadd45α-deficient keratinocytes, the additional loss of p21 restores capacity for NER (Maeda, T et al., 2005). This finding supports Gadd45α as a recruiter of PCNA to damage sites (Smith, ML et

al., 2000). Additionally, in Gadd45α-deficeint mouse keratinocytes, p21 levels are increased (Maeda, T et al., 2005) and may serve to inhibit loading of PCNA onto damage sites, resulting in a decreased capacity for NER in these cells. If Gadd45α and p21 compete for PCNA accessibility and p21 inhibits NER when bound to PCNA, the loss of Gadd45α could leave p21 free to inhibit PCNA binding to DNA in the resynthesis step of NER. Additional loss of p21 could relieve the inhibitory effect of p21 on NER.

Summary and Goals

It is apparent that Gadd45α has many roles in cellular homeostatsis. With all the recent research into the roles of Gadd45α, it is still unclear how it is involved in NER. It is known that Gadd45α is involved in NER through interaction with PCNA, but there is very little known about this relationship. It is the goal of this project to look at how Gadd45α affects DNA-bound PCNA. Because of a lack of reliable antibodies to Gadd45α, it is very difficult to directly assess the role of Gadd45α in various processes. In this study we will be using mouse embryonic fibroblasts (MEFs) proficient and deficient in *Gadd45α* to evaluate the effects of Gadd45α on DNA-bound PCNA. We will use confocal microscopy to evaluate the percent of DNA-bound PCNA positive cells in *Gadd45α*$^{+/+}$ and *Gadd45α* $^{-/-}$ MEFs. Since it is known that the Triton-X 100 resistant, (or DNA-bound) portion of PCNA is required for NER (Bouayadi, K et al., 1997), a novel method for

evaluating DNA-bound proteins in intact cells by flow cytometry using Triton X-100 was developed to replicate the confocal results and provide a faster, easier, less subjective method. Finally, DNA-bound PCNA and p21 were evaluated by western blotting.

Chapter 2 ♦Materials and Methods

Gadd45α Mouse Colony

Mice deficient in *Gadd45α*, previously generated by gene targeting (Hollander, MC et al., 1999), were obtained from Dr. Albert Fornace Jr, NCIC. Mice were earnotched at approximately 4 weeks of age for mouse identification and genotyping. Biopsies were digested in 100μL STE buffer (50mM Tris-HCl, 10mM EDTA, 100mM NaCl, 0.1% SDS) and 6μL Proteinase K (120μg) incubated overnight at 55°C. The reaction was stopped by incubating at 95°C for 10 minutes. Genotyping was performed as previously described (Hollander, MC et al., 1999) with modifications. The PCR mixture contained 1X PCR buffer without magnesium (Invitrogen, Burlington, ON, Canada), 1.5μL $MgCl_2$ (Invitrogen, Burlington, ON, Canada), 2.0μL dNTPs (Invitrogen, Burlington, ON, Canada), 0.25μL primer CH215 (2.5pmol), 0.25μL primer CH217 (2.5pmol), 0.5μL primer CH216 (5.0 pmol), 1.0μL Taq polymerase (in house), 3μL 3/100 dilution of tissue digestion, and dH_2O to 25μL. Primers CH216 (5'-GAA GAC CTA GAC AGC ACG GTT-3') and CH215 (5'-CCT CGT CTT ACC TCT GCA CAA-3') amplify the 324bp wildtype DNA fragment, and the primers CH216 and CH217 (5'-GCA TGC TCC AGA CTG CCT T-3') amplify the 161bp mutant DNA fragment. The PCR machine was run on the following settings: 94°C for 180s, 94°C for 30s, 62°C for 30s, 72°C for 30s and cycling back to the second step (94°C for 30s) 38 times and hold at 4°C. Along with a 1kb Plus DNA ladder (Invitrogen, Burlington, ON,

Canada), PCR products were run on a 2% (w/v) agarose TAE gel with ethidium bromide gel and visualized with UV light (Figure 2-1).

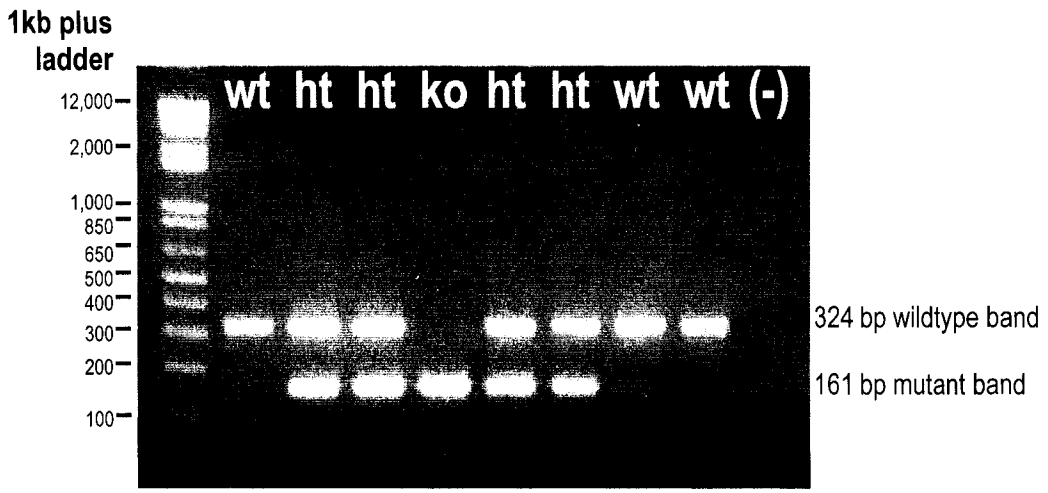

Figure 2-1: Genotyping gel.

Typical *Gadd45α* genotyping 2% agarose gel with ethidium bromide. Wildtype band migrates to 324 bp, while the mutant (knockout) band migrates to 161 bp. The genotypes of the DNA samples are as indicated on the gel wildtype (wt), heterozygote (ht) or knockout (ko).

CREATION OF MEFS

Primary mouse embryonic fibroblasts (MEFs) were created as follows. Embryos at E13.5 were removed from pregnant females, rinsed in phosphate buffered saline (PBS), separated from placental tissue and rinsed twice more. Embryos were minced with frosted slides in 0.25% trypsin-EDTA (Sigma-Aldrich, Oakville, ON, Canada) and incubated at 37°C for 5 minutes and neutralized with Dublecco's modified eagle medium (DMEM) (Sigma-Aldrich, Oakville, ON, Canada) with 20% fetal bovine serum (FBS). The cell suspension was passed

through a 21 gauge needle to further separate the cells and allowed to settle for 10 minutes. Supernatant was cultured in DMEM with 20% FBS and 1% penicillin-streptomycin (P/S), incubated at 37°C and 5% CO_2, for one passage and then frozen 10% DMSO to -80°C at a rate of -1°C/min before plunged into liquid nitrogen for long-term storage. After thawing, DMSO was removed from the MEFs by spinning and cells were resuspended and plated in DMEM with 20% FBS and 1% P/S. The pellet was digested in STE and Proteinase K and genotyped as described above. Experiments were done on cells on the 3rd or 4th passage.

Cell culture

Immunofluorescence Methods (Confocal Microscopy and Flow Cytometry)

Primary *Gadd45α*$^{+/+}$ and *Gadd45α*$^{-/-}$ MEFs were grown in p100 plates in phenol-red free DMEM (Sigma-Aldrich, Oakville, ON, Canada) supplemented with 20% FBS (Hyclone, Logan, UT, USA), 1% P/S (Invitrogen, Burlington, ON, Canada) and 584.68mg/L L-glutamine (Sigma-Aldrich, Oakville, ON, Canada) and incubated at 37°C and 5% CO_2. This media was chosen to eliminate autofluorescence due to indicator presence. Cells were split by washing with PBS and treated trypsin-EDTA and incubated at 37°C for 5 minutes. Trypsin was neutralized with 10% FBS and the suspension was transferred to a 15mL conical tube. The plates were rinsed twice with PBS, and the washes were added to the

tubes. Cells were centrifuged at 1600rpm for 5 minutes to remove any indicator present in the trypsin, resuspended in phenol-red free DMEM with FBS, P/S and L-glycine, and plated onto new plates. If plating was for an experiment, cells were counted on a Z2 Dual Threshold Coulter Counter (Beckman-Coulter, Mississauga, ON, Canada) and were plated for 75 – 80% confluency on the appropriate plate or slide.

DNA-BOUND WESTERN BLOTTING

Primary *Gadd45α*$^{+/+}$ and *Gadd45α*$^{-/-}$ MEFs were grown in p100 plates (Fisher Scientific, Ottawa, ON, Canada) in DMEM supplemented with 20% FBS and 1% P/S and incubated at 37°C and 5% CO_2. Cells were split by washing with PBS and treated trypsin-EDTA and incubated at 37°C for 5 minutes. Trypsin was neutralized with 10% FBS and cells were plated onto new plates. If plating was for an experiment, cells were counted on a Z2 Dual Threshold Coulter Counter (Beckman-Coulter, Mississauga, ON, Canada) and were plated for 75 – 80% confluency on the p100 plates.

UVB irradiation

Plates or slides were washed twice with PBS and exposed to 200J/m^2 UVB (290-320nm) from a bank of six unfiltered UVB bulbs (FS20T12/UVB-BP, Light

Sources Inc., Orange CT), with the lids on. Following UVB irradiation, the correct DMEM with FBS, P/S (and L-glutamine, if required) was added. To measure the time needed to reach the correct dose of UVB, the culture dish lids were placed on top of an IL1700 radiometer with a SED 240/UVB-1/W detector (International Light, Newburyport, MA, USA). The intensity of the UVB source penetrating the culture dish lid was measured and used to calculate exposure time to obtain the correct dosage. Culture dish lids filtered out contaminating UVC radiation (Baross-Francis, A et al., 1998). Plates or slides were incubated at 37°C with 5% CO_2 until the appropriate timepoint as described in Table 2-1.

		Doses and Timepoints		
Method	**Genotypes**	**0J/m^2**	**200J/m^2**	**Controls**
a) Immunofluorescent Microscopy	*Gadd45α*$^{+/+}$ *Gadd45α*$^{-/-}$	0h	0.5h, 0.75h, 1h, 2h, 4h	Total, Negative both (200J/m^2 1h)
b) Flow Cytometry	*Gadd45α*$^{+/+}$ *Gadd45α*$^{-/-}$	0h	0.5h, 0.75h, 1h, 2h, 4h	Plain, Negative both (200J/m^2 1h)
c) Western Blotting	*Gadd45α*$^{+/+}$ *Gadd45α*$^{-/-}$	0h	0.5h, 0.75h, 1h, 2h, 4h	Untreated, Amido Black stain (load)

Table 2-1: Experimental Design

Outline of UV dosage, timepoints and controls used in all experiments.

a) Immunofluorescent Microscopy. Controls include a slide stained for total PCNA (positive control), and a negative control to judge the amount of background staining.
b) Flow cytometry. Controls include a Plain Cell control to judge cellular integrity, and a negative control to eliminate background staining.
c) Western Blotting. Controls include the untreated (0J/m^2 0h) sample to standardize response and the Amido Black stain to control for loading.

Immunofluorescent Microscopy

Method adapted from (Aboussekhra, A and Wood, RD, 1995). To show the presence of DNA-bound PCNA, primary G*add45α*$^{+/+}$ and *Gadd45α*$^{-/-}$ MEFs were plated on 2-well slides (VWR, Mississauga, ON, Canada) coated with poly-L-lysine (#P-5899, Sigma-Aldrich, Oakville, ON, Canada) and treated with 200J/m^2 as described above. All washes were performed with PBS and were very gentle to minimize cell loss. At the appropriate timepoints, (Table 2-1) slides were gently washed two times with PBS and permeabilized with antibody buffer (PBS with 1% BSA and 0.2mg/mL EDTA) with 0.5% Triton X-100 for 15 minutes at 4°C to remove PCNA that is not bound to DNA. Slides were washed three times and fixed in 100% ice cold methanol at -80°C. Total control slides were fixed prior to permeablization. After washing three times, slides were incubated on a rocker for 1 hour at room temperature with primary PCNA antibody (PC10, #555566, BD Pharmingen, Mississauga, ON, Canada) diluted to 1:100 in antibody buffer. Antibody buffer without primary antibody was used on negative control slides. Cells were washed three times and incubated on a rocker for 1 hour at room temperature with secondary goat-anti-mouse IgG2a-Alexa 488 antibody (#A21131, Molecular Probes/Invitrogen, Burlington, ON, Canada) diluted to 1:100 in antibody buffer. Cells were washed three times, wells were removed and slides were coverslipped with Vectashield (H-1200, Vector Laboratories Inc, Gurlingame, CA, USA). Slides were sealed with nail polish and blinded by a lab member. Slides were examined on Zeiss Axiovert 100M microscope coupled

with a Zeiss LSM510 laser scanning system (Carl Zeiss, Germany). Images were taken with a 40X plan-neofluar., n.a. 1.4 or 40X Plan-Neofluar n.a. 1.30) with a zoom factor of 1. Alexa-488 and Dapi staining was visualized with an Argon laser (ex: 488nm em) and a Coherent Mira-2 photon laser (ex: 780nm) respectively. Emission data were collected with LP560 and BP 390-465IR filters. Optical sections of field of 230.3µm x 230.3µm x 0.9µm with scanned and displayed as 512 x512 pixel images. Each slide was photographed repeatedly until a minimum of 200 cells were imaged. Images were counted for total number of cells (DAPI) and DNA-bound PCNA (Alexa 488) using Zeiss Image-pro software (Carl Zeiss, Germany). A minimum of 200 cells per genotype per timepoint were counted and numbers were imported into Microsoft Excel (Microsoft, Mississauga, ON, Canada) and un-blinded. This experiment was performed in triplicate.

Method Development: Flow Cytometry

Wild type MEFs were grown on p60 plates (Fisher Scientific, Ottawa, ON, Canada), trypsinized and stored at -80°C in freezing media. Cells were thawed and treated with various concentrations of BSA and EDTA to find concentrations that eliminated cell agglutination. Final wash buffer consisted of PBS with 1% BSA and 0.2 mg/mL EDTA. Additional cells were fixed with 100% ice cold

methanol and 4% formaldehyde. As the methanol caused cell agglutination, formaldehyde was used in the final method.

Triton X-100 titrations were performed to eliminate cell destruction while eliminating non-DNA-bound PCNA. Cells were grown on glass coverslips (Fisher Scientific, Ottawa, ON, Canada) for confocal microscopy and p60 plates for flow cytometry. Cells on p60s were trypsinized and stored at -80°C in freezing media and thawed prior to experiments. Cells on coverslips were permeabilized immediately, while one coverslip was fixed with ice-cold methanol for 10 minutes at -20°C prior to permeablization. Various concentrations of Triton X-100 (0.5%, 0.4%, 0.3%, 0.2%, 0.1%, 0.05%) in wash buffer were incubated for 15 minutes at 4°C with the flow cytometry and confocal samples. Confocal coverslips were then fixed with ice-cold methanol, washed, incubated with antibodies and mounted on slides as described in the Immunofluorescent Microsocopy section. The only difference was that the cells grown on coverslips were mounted on slides rather than having the cells on the slides mounted with coverslips. Samples were viewed on the Zeiss Axiovert microscope as described above and analysed for elimination of non-DNA-bound PCNA. Flow cytometry samples were treated with the Triton X-100 titrations above in wash buffer for 15 minutes washed with 10mL wash buffer and spun at 300g for 5 minutes. Solution was aspirated from cells which were then fixed with 4% formaldehyde for 5 minutes and washed with 10mL wash buffer, spun at 300g for 5 minutes. Solution was

aspirated off and cells were resuspended in wash buffer and run on the FACS Calibur flow cytometer (Becton Dickenson, San Jose, CA, USA) to evaluate cell integrity with side scatter (SSC) and forward scatter (FSC). When the 15 minute incubations were not sufficient to maintain cellular integrity and eliminate non-DNA-bound PCNA, the experiments were repeated with 2 hour and overnight incubations at 4°C using various Triton X-100 concentrations (0.5%, 0.05%, 0.04%, 0.03%, 0.02%, 0.01%). It was determined that a permeablization buffer consisting of 0.03% Triton X-100 in wash buffer effectively eliminates non-DNA-bound PCNA while maintaining cellular integrity.

Flow cytometry

Gadd45α$^{+/+}$ and *Gadd45α*$^{-/-}$ MEFs were plated on p60 plates (Fisher Scientific, Ottawa, ON, Canada) and treated with 200J/m^2 as described above. At the appropriate timepoints (Table 2-1), plates were washed two times with PBS and treated trypsin-EDTA and incubated at 37°C for 5 minutes. Trypsin was neutralized with 10% FBS and the suspension was transferred to a 15mL conical tube. The plates were washed twice with PBS, and the washes were added to the tubes. Cells were pelleted at 1600rpm for 5 minutes, resuspended in 250µL of freezing media (phenol-red free DMEM with FBS, P/S, L-glycine and 10% DMSO) and stored at -80°C. Cell pellets were thawed and resuspended in wash buffer (PBS with 1% BSA and 0.2mg/mL EDTA) containing 0.03% Triton X-100

for 2 hours at 4°C to remove PCNA not bound to DNA. The plain control was incubated in wash buffer without Triton X-100 to maintain intact cells. All washes were performed by adding 10mL of wash buffer and centrifuging at 300g for 5 minutes. Cells were washed once and fixed in 4% formaldehyde for 5 minutes at room temperature. Cells were washed once and resuspended in primary PCNA antibody PC10, #555566, BD Pharmingen, Mississauga, ON, Canada) diluted to 1:100 in wash buffer and incubated for 1 hour at room temperature with occasional agitation. Negative control and plain control tubes were incubated with wash buffer without primary antibody. Cells were washed three times and incubated with secondary goat anti-mouse IgG2a-Alexa 488 antibody (#A21131, Molecular Probes/Invitrogen, Burlington, ON, Canada) diluted to 1:100 in wash buffer for 1 hour at room temperature with occasional agitation. Plain control was incubated in wash buffer without secondary antibody. Cells were washed three times, resuspended in 600µL wash buffer and taken to a FACS Calibur flow cytometer (Becton Dickenson, San Jose, CA, USA) with a 488nm laser; Alexa488 was measured with a bandpass filter at 530/30nm. 20,000 cells were counted on all samples and analysis was performed using CellQuest software (Becton Dickenson, San Jose, CA, USA). After background fluorescence was eliminated using the negative control, numbers were entered into Microsoft Excel to generate percent positivity. This experiment was performed in triplicate.

Cell lysis / Quantification

Method adapted from (Savio, M et al., 1996). Following the experimental design (Table 2-1), cells were trypsinized, pelleted and frozen at -80°C in 1.5mL microcentrifuge tubes. Cell pellets were resuspended in 200µL hypotonic lysis buffer (10mM Tris-HCl pH 7.4, 2.5mM $MgCl_2$, 1x protease inhibitor (Complete Mini, Roche, Laval, PQ, Canada), 0.5% Triton X-100) and incubated on ice for 8 minutes. Lysed cells were spun at 14,000g for 5 minutes at 4°C and lysates (soluble or non-DNA-bound proteins) were removed to new tube, but were not used in the experiments. Pellets were resuspended in 100µL digestion buffer (10mM Tris-HCl pH 7.4, 10mM NaCl, 5mM $MgCl_2$, and 1x protease inhibitor) containing 400U DNAse I (Invitrogen, Burlington, ON, Canada). The digestion was completed at 37°C for 1h and stopped by adding EDTA to a final concentration of 10mM and chilling on ice. Samples were cleared by centrifugation at 14,000g for 5 minutes and supernatant (insoluble or DNA-bound proteins) was removed to a fresh tube and stored at -80°C. Protein concentrations were determined using a detergent compatible protein assay (BioRadDC, Hercules, CA).

SDS-PAGE gel electrophoresis / Western blots

Equal amounts (20-30µg) of DNA-bound protein were separated by discontinuous SDS-PAGE gels (7.5% stacking gel, 12% separating gel) using standard protocols. Proteins were transferred to 0.45µm Immobilon-P PVDF membrane (Fisher Scientific, Pittsburgh, PA) in Tris/glycine buffer. Membranes were blocked in 4% skim milk in TBST (20mM Tris, 137mM NaCl, 0.001% Tween20, pH 7.6), rocked at room temperature for 1 h. Primary antibodies incubated in 4% skim milk in TBST according to manufacturer's instructions, rocked at 4°C overnight at the following concentrations: PCNA (#610115, BD Transduction Laboratories, Mississauga, ON, Canada) 1:5000, p21 (#556431, BD Pharmingen, Mississauga, ON, Canada) 1:1000. After washing 3 times for 5 minutes with TBST, membranes were incubated with IgG HRP-conjugated goat-anti-mouse secondary antibody (#554002, BD Pharmingen, Mississauga, ON) at a 1:2500 dilution for 1 hour rocking at room temperature. Following, secondary antibody, membranes were washed with TBST 3 times for 5 minutes. Following washing, the blots were incubated in luminal solution (recipe) for 5 minutes and exposed to autoradiograph film for 10 seconds to 5 minutes. Densitometry was performed using Quantity One software (Bio-Rad, Mississauga, ON, Canada). Protein levels were normalized back to loading control (amido black stain) and a ratio calculated using normalized values comparing all timepoints to *Gadd45α*$^{+/+}$ untreated protein.

Statistics

All statistics were performed on Microsoft Excel. The student t-test was used with unequal variance for all statistical analyses. One-tailed analysis was used to test the hypothesis that the *Gadd45α*$^{+/+}$ MEFs have a higher level of DNA-bound PCNA than the *Gadd45α*$^{-/-}$ samples at each timepoint on both the immunofluorescent microscopy (IFM) and flow cytometry (FC) data. The UV response was evaluated using a one-tailed analysis to test the hypothesis that there was more DNA-bound PCNA at 1h post-UVB than in the untreated cells for each genotype by the IFM and FC methods. The hypothesis that there was no difference in the response curves of the two genotypes was also tested using a two-tailed test on the IFM and FC methods. A two-tailed analysis tested the hypothesis that there was no difference in the response curves between the IFM and FC methods to determine the validity of the FC method.

Chapter 3 ♦Gadd45α and DNA-bound PCNA: Immunofluorescent Analysis.

Introduction

PCNA plays a major role in many cellular functions, including DNA repair. PCNA binds to DNA very early in response to UV damage, with a peak at 15 – 30 minutes post UVC-irradiation (Savio, M et al., 1996). Early binding of PCNA to DNA post UVC-damage is indicative of NER by immunofluorescent microscopy (Aboussekhra, A and Wood, RD, 1995). Gadd45α plays a role in NER, presumably through interactions with PCNA, although this remains unclear (Smith, ML et al., 1994). We propose that the loss of Gadd45α will decrease the amount of DNA-bound PCNA in response of UVB. To test this hypothesis, we analyzed the early PCNA-response to 200J/m^2 of UVB in *Gadd45α*$^{+/+}$ and *Gadd45α*$^{-/-}$ MEFs by immunofluorescent microscopy. To confirm our results, we developed a method for assessing DNA-bound PCNA positivity in intact cells by flow cytometry.

Immunofluorescent Microscopy Results

To evaluate the effect of Gadd45α on DNA-bound PCNA, immunofluorescent microscopy was performed as previously described (Aboussekhra, A and Wood, RD, 1995) with some modifications. The PCNA response in *Gadd45α*$^{+/+}$ and

Gadd45α$^{-/-}$ MEFs was evaluated by counting the total number of cells and the number of PCNA positive cells at various timepoints post-UV irradiation. The percentages of PCNA positive cells were compared at each timepoint and graphed (Figure 3-2). There is a statistically significant increase from 28% baseline positivity to 35% positivity of DNA-bound PCNA at 1h post-UVB irradiation in the *Gadd45α*$^{+/+}$ MEFs (p-value <0.05). In contrast, there is no PCNA response in the *Gadd45α*$^{-/-}$ MEFs. In addition, when the 1h induction of PCNA was compared with each genotype, the *Gadd45α*$^{+/+}$ MEFs had a statistically significant response (p-value <0.05) while the *Gadd45α*$^{-/-}$ MEFs did not (p-value 0.15). When the response curves of the *Gadd45α*$^{+/+}$ and *Gadd45α*$^{-/-}$ MEFs are compared, there is a statistical difference with a p-value <0.00001. Previous studies show that there is an increased level of total p21 in Gadd45α-deficient keratinocytes (Maeda, T et al., 2005). This could result in growth suppression in the Gadd45α-deficient cells leading to a lower level of PCNA, as is seen in the IFM results.

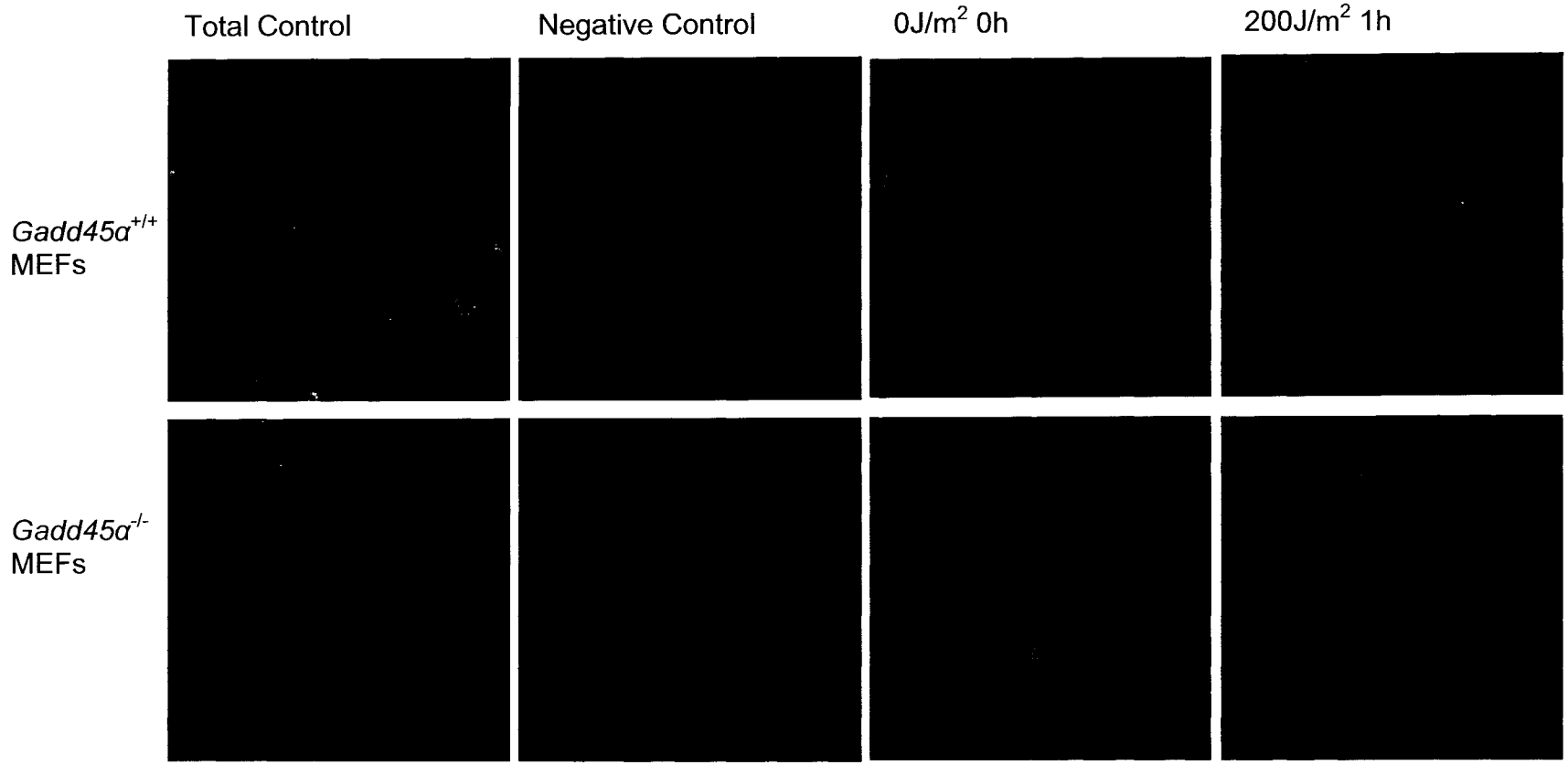

Figure 3-1: Examples of IFM images

Photomicrographs of immunofluorescent microscopy of DNA-bound PCNA pre- and post-irradiation with 200J/m^2 UVB. Images of 200 cells per timepoint were taken and total cells, represented by DAPI staining (blue), and DNA-bound PCNA positive cells, as indicated by Alexa-488 secondary staining (green) were counted on blinded samples. After blinding was removed, percentage of PCNA positive cells was calculated. The experiment was performed in triplicate. The red arrows indicate positive cells. The presence of background staining made these slides difficult to count, but was unavoidable as we were using an anti-mouse secondary antibody on mouse cells.

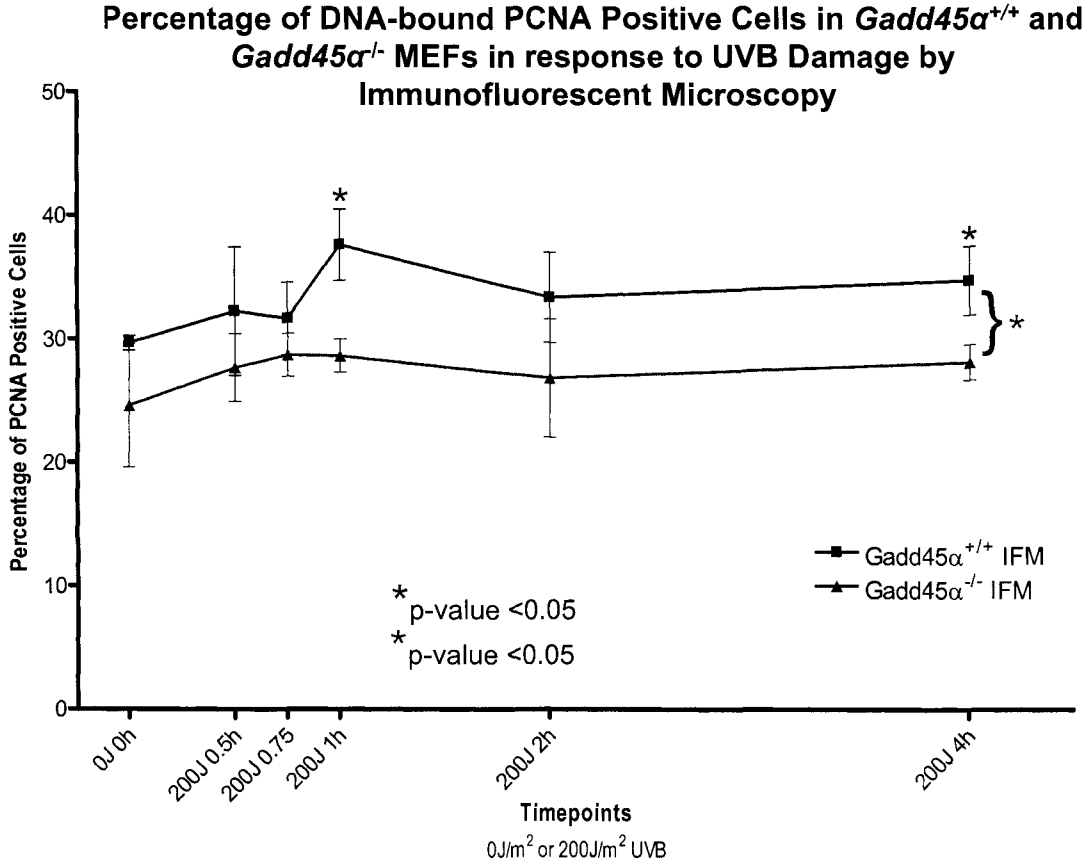

Figure 3-2: Graph of DNA-bound PCNA in *Gadd45α* wt and ko MEFs in response to UVB damage assayed by Immunofluorescent Microscopy (IFM)

Gadd45α$^{+/+}$ and *Gadd45α*$^{-/-}$ MEFs on glass slides were treated with 200J/m^2 of UVB, harvested and permeabilized to remove non-DNA-bound PCNA. Slides were stained with anti-PCNA and Alexa 488-conjugated secondary antibody and counterstained with DAPI. Slides were imaged on a confocal microscope and total cells were counted based on DAPI staining and PCNA positive cells were counted based on Alexa 488 staining. Statistical difference with a p-values of <0.05 is apparent at 1h and 4h after UVB-irradiation between *Gadd45α*$^{+/+}$ and *Gadd45α*$^{-/-}$ MEFs. The *Gadd45α*$^{+/+}$ MEFs have an increase of DNA-bound PCNA at 1h post-UVB decreasing by 4h. *Gadd45α*$^{-/-}$ MEFs do not display a PCNA response to UVB irradiation. The response curves of the *Gadd45α*$^{+/+}$ and *Gadd45α*$^{-/-}$ MEFs are statistically different with a p-value of <0.05. Graph represents mean and SD of three experiments.

There are problems associated with this method. It is extremely time consuming taking more than 20h at a microscope to image the cells and a similar amount of time to manually count the cells on a computer. Additionally, there is background staining as we used mouse cells with a goat-anti-mouse secondary antibody. The manual assessment of PCNA staining leads to a limited total number of cells counted, and a certain degree of subjectivity. Because of these limitations, we wanted to convert this method to be run on a flow cytometry to increase the number of cells counted and decrease interference of background staining.

Flow Cytometry Results

Work-up of Flow Cytometry Method

The work-up of the IFM method for flow cytometry was a challenging technical feat. First of all, MEFs have a tendency to agglutinate. The original wash buffer, PBS, caused complete aggregation of the MEFs which prevented them from being run on the flow cytometer. EDTA and BSA were added and adjusted to obtain an optimal wash buffer. Different fixatives were tried as well. 100% ice cold methanol caused agglutination of the MEFs while 4% formaldehyde did not (data not shown).

Flow cytometry of PCNA has been reported, (Table 3-1) however, in these studies NP-40 was used as a permeablization agent, and the extracts were primarily nuclei extracts. When we applied the use of NP-40 to our confocal method during work-up, a lot of interfering staining was observed (Figure 3-3) decreasing our confidence in using this method. In addition, we wanted to keep the method as similar as possible to the IFM and, to do so, used Triton X-100 as the permeabilizing agent. The first experiments included the "standard" dose of Triton X-100 at 0.05% as used in the IFM experiments. However, we were unable to analyze the cells as the nuclei were too small to analyze on the FACS Calibur, so we were forced to develop a method to utilize intact cells.

CELL TYPE	ANTIBODY	NUCLEI/ WHOLE CELL	LYSIS METHOD	DETERGENT	REFERENCE
Chinese hamster fibroblasts	PC10	Nuclei	hypotonic	NP-40	(Wilson, GD et al., 1992)
Human embryonic lung fibroblasts	Not indicated	Nuclei	hypotonic	NP-40	(Prosperi, E et al., 1994)
Human fibroblasts (skin)	PC10	Not indicated	hypotonic	NP-40	(Prosperi, E et al., 1993)

Table 3-1: Flow cytometry analysis of PCNA

Previous studies that looked at PCNA by flow cytometry. Note that all the studies used NP-40 as the permeabilizing detergent, and the majority were analyzing nuclei instead of intact cells. Not all of the information was present in the papers as is specified by "not indicated" in the table.

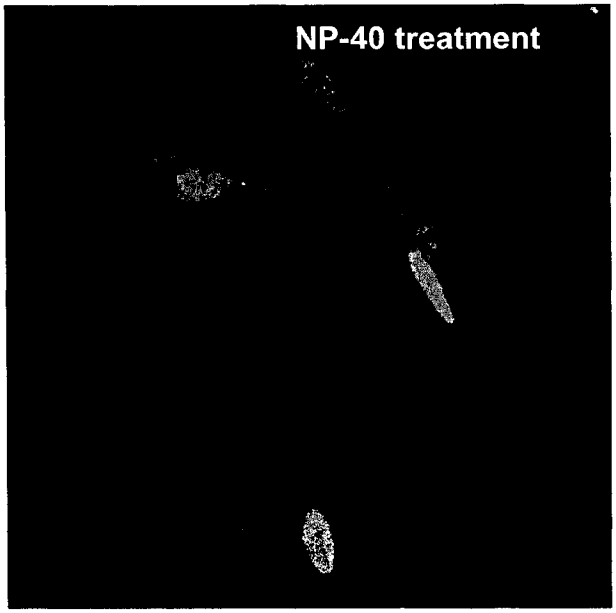

Figure 3-3: NP-40 treatment of MEFs for PCNA IFM

Since NP-40 has been extensively used for permeablization for flow cytometry, we tried using it instead of Triton X-100 for IFM. This photomicrograph depicts PCNA positive (green) cells and total (blue) cells. Because of the unusual background fluorescence with NP-40, so it was not used for our flow cytometry method.

As intact cells were required for flow cytometry a percentage of Triton X-100 that would maintain cellular integrity and eliminate non-DNA-bound PCNA was required. Titrations of Triton X-100 were assessed by both IFM, to evaluate presence of non-DNA-bound PCNA, and flow cytometry to assess cellular integrity. The first experiments used a 15 minute incubation with various amounts of Triton X-100 and viewed by confocal microscopy (Figure 3-4) and flow cytometry (data not shown). The 15 minute incubation was not sufficient to eliminate non-DNA-bound PCNA at levels low enough to maintain cellular integrity, as the 0.05% titration IFM image displayed PCNA positivity in all cells,

similar to the total PCNA control. An additional experiment with a 2 hour incubation with Triton X-100 was performed. Analysis by confocal microscopy (Figure 3-5) revealed that a concentration of Triton X-100 as low as 0.01% was sufficient to eliminate non-DNA-bound PCNA. As flow cytometry showed that Triton X-100 as high as 0.03% could maintain cellular integrity with a 2 hour incubation

Figure 3-6), we opted to use 0.03% Triton X-100 for the DNA-bound flow cytometry method.

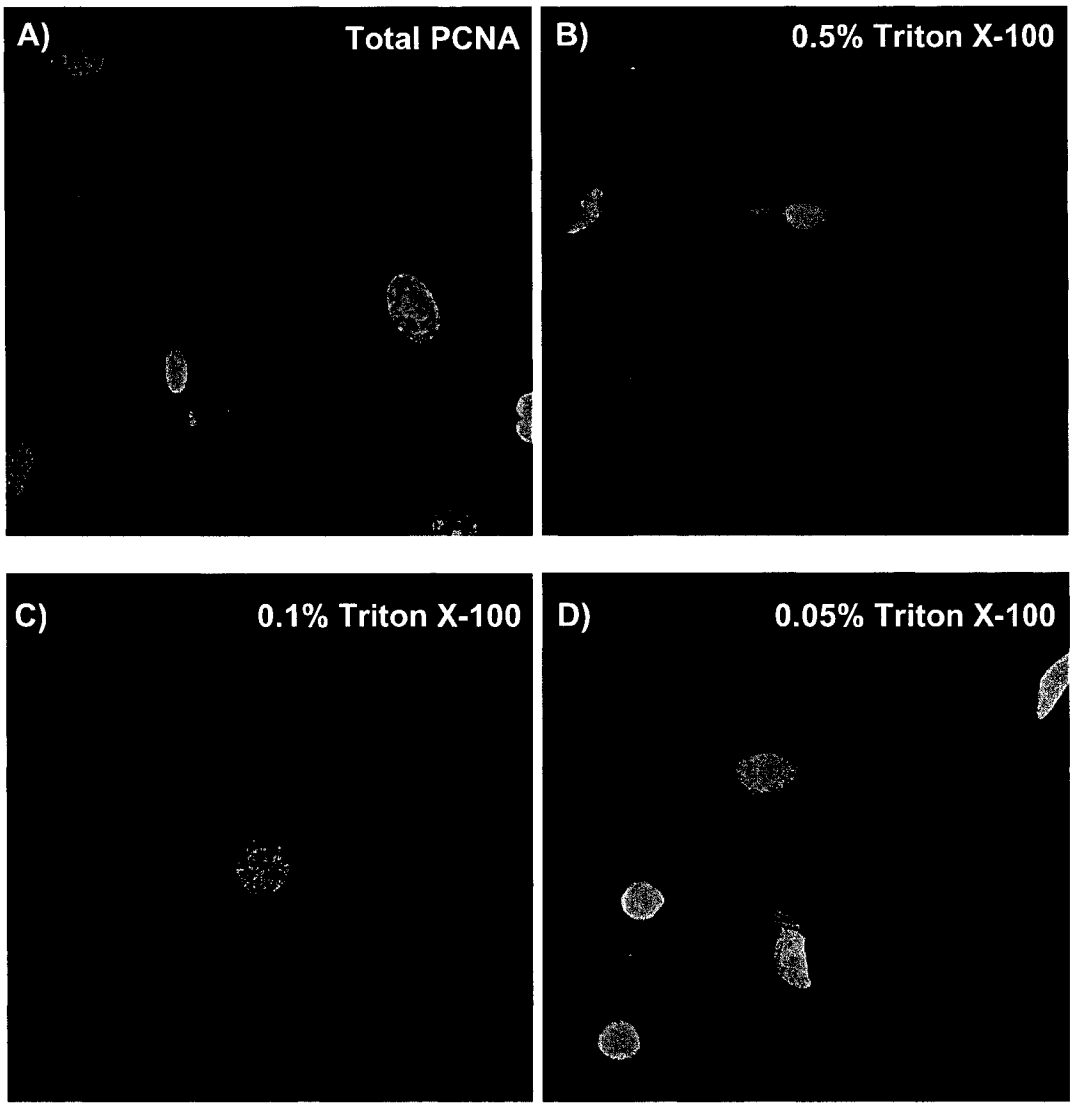

Figure 3-4: Triton X-100 Titrations; 15 minute Permeabilization Treatment

Photomicrographs of MEFs were grown on glass coverslips, permeabilized with various Triton X-100 titrations for 15 minutes at 4°C and stained with PCNA (green) counterstained with DAPI (blue). A) Total PCNA: Cells were fixed prior to permeabilization. All cells are positive for PCNA. B) 0.5% Triton X-100 treatment. This is the standard treatment used for confocal microscopy. Not all of the cells exhibit PCNA positivity, displaying the elimination of non-DNA-bound PCNA. C) 0.1% Triton X-100 treatment. Results are similar to the 0.5% treatment; only some of the cells display PCNA positivity, indicating the elimination of non-DNA-bound PCNA. D) 0.05% Triton X-100 treatment. All cells are positive for PCNA. Non-DNA-bound PCNA has not been eliminated. This treatment is not sufficient for elimination of non-DNA bound PCNA.

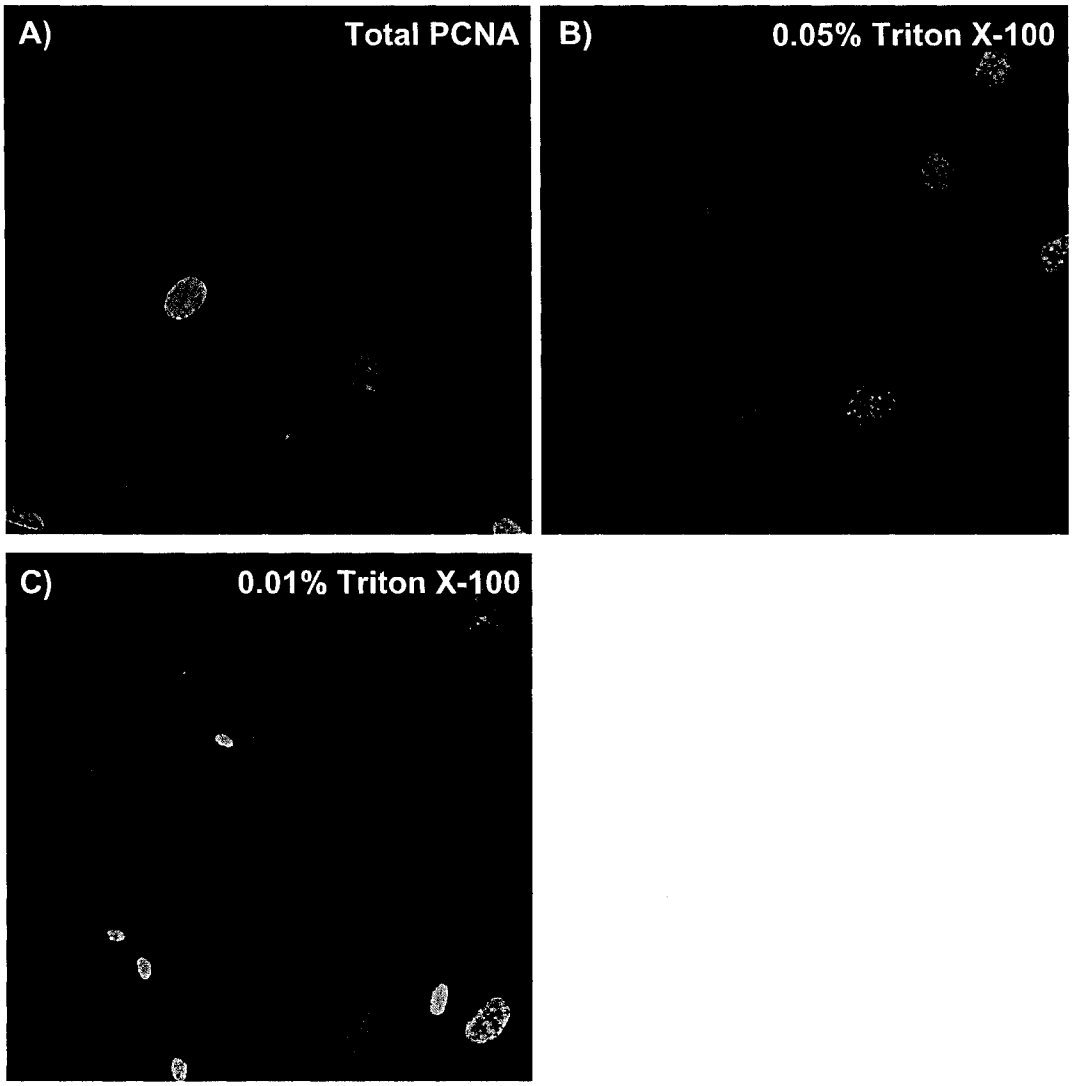

Figure 3-5: Triton X-100 Titrations; 2 hour Permeabilization Treatment

Photomicrographs of MEFs were grown on glass coverslips, permeabilized with various Triton X-100 titrations for 2 hours at 4°C and stained with PCNA (green) counterstained with DAPI (blue). A) Total PCNA: Cells were fixed prior to permeabilization. All cells are positive for PCNA. B) 0.05% Triton X-100 treatment. Not all of the cells display PCNA positivity, demonstrating the elimination of non-DNA-bound PCNA. C) 0.01% Triton X-100 treatment. Results are similar to the 0.05% treatment; only some of the cells display PCNA positivity, indicating the elimination of non-DNA-bound PCNA. Triton X-100 concentrations between 0.05% and 0.01% incubated for 2 hours are sufficient for eliminating non-DNA-bound PCNA.

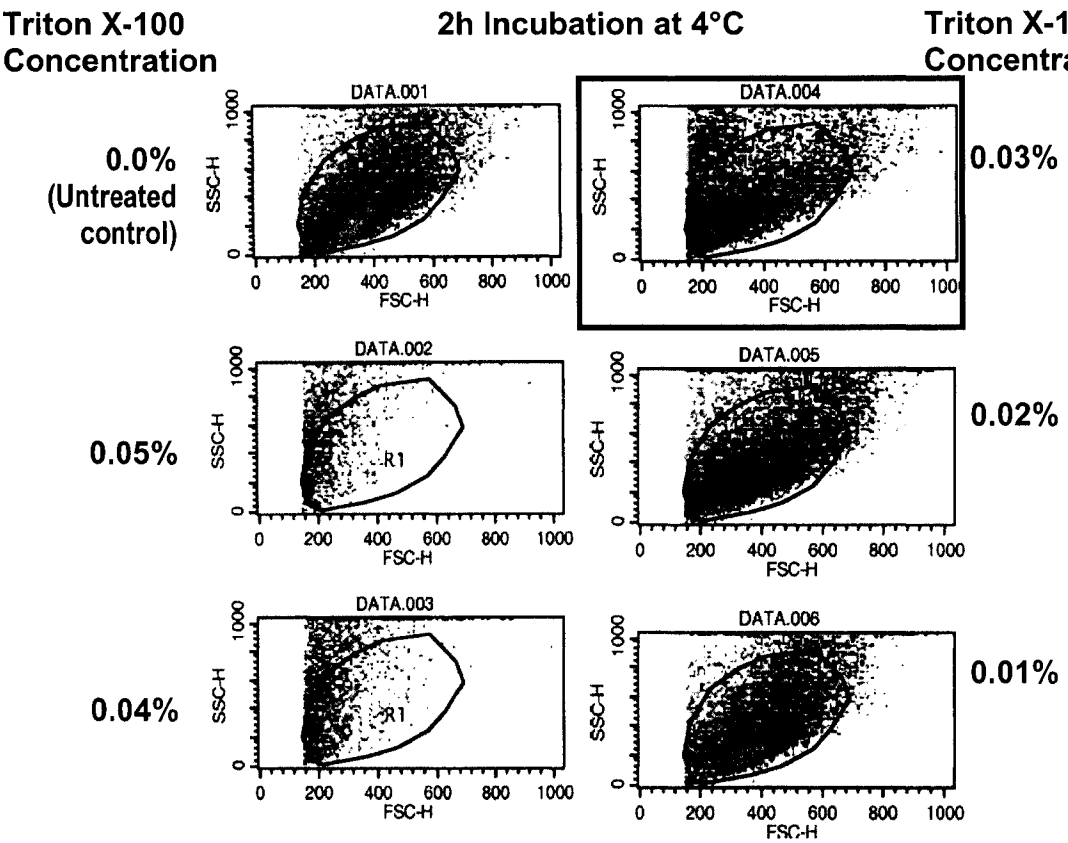

Figure 3-6: Triton X-100 Titrations; 2 hour Permeabilization Treatment

MEFs were treated with Triton X-100 titrations for 2 hours at 4°C and were run on the FACS Calibur for Side Scatter (SSC) and Forward Scatter (FSC) to evaluate cellular integrity. An untreated control (no Triton X-100) was used to assess intact cells. The intact cells were gated (shown by the black circle labelled R1) and any cells falling in this region are considered intact. Fragmented cells display a more vertical presentation as is evident in the 0.05% and 0.04% Triton X-100 concentrations. Triton X-100 concentrations between 0.01% and 0.03% incubated for 2 hours can maintain cellular integrity. The 0.03% Triton X-100 was used for all following experiments as indicated by the blue box.

FLOW CYTOMETRY RESULTS

The new method for assessing DNA-bound proteins by flow cytometry was performed on *Gadd45α*$^{+/+}$ and *Gadd45α*$^{-/-}$ MEFs in triplicate. Background fluorescence was removed by gating and the percentage of PCNA positive cells

was determined. Similar to the IFM results, the *Gadd45α*$^{+/+}$ MEFs displayed an increase from 29% baseline to 34% positivity of DNA-bound PCNA at 1h post-UVB irradiation with 200J/m^2 (Figure 3-7), however this increase was not statistically significant. However, when statistical analyses were run between the *Gadd45α*$^{+/+}$ and *Gadd45α*$^{-/-}$ MEFs at each timepoint, the *Gadd45α*$^{+/+}$ MEFs had a statistically higher amount of DNA-bound PCNA at 0.5h and 1h post-UVB (p-value <0.05). Similar to the IFM method, the *Gadd45α*$^{-/-}$ cells do not have an early PCNA response. The response curves of the *Gadd45α*$^{+/+}$ and *Gadd45α*$^{-/-}$ MEFs were statistically different with a p-value <0.05.

To validate the results of the flow cytometry method, they were compared back to the from the original IFM results. Both sets of results were graphed and statistical analysis using a two-tiered t-test was performed. When the two graphs are superimposed the similarities of the two sets of results are striking (Figure 3-8). There was no statistical difference between the IFM and FC PCNA response curve in either the *Gadd45α*$^{+/+}$ (p-value=0.20) or *Gadd45α*$^{-/-}$ (p-value=0.52) MEFs. This suggests that the two methods achieve similar results, and that the FC method for analyzing DNA-bound proteins can be used in place of the IFM method.

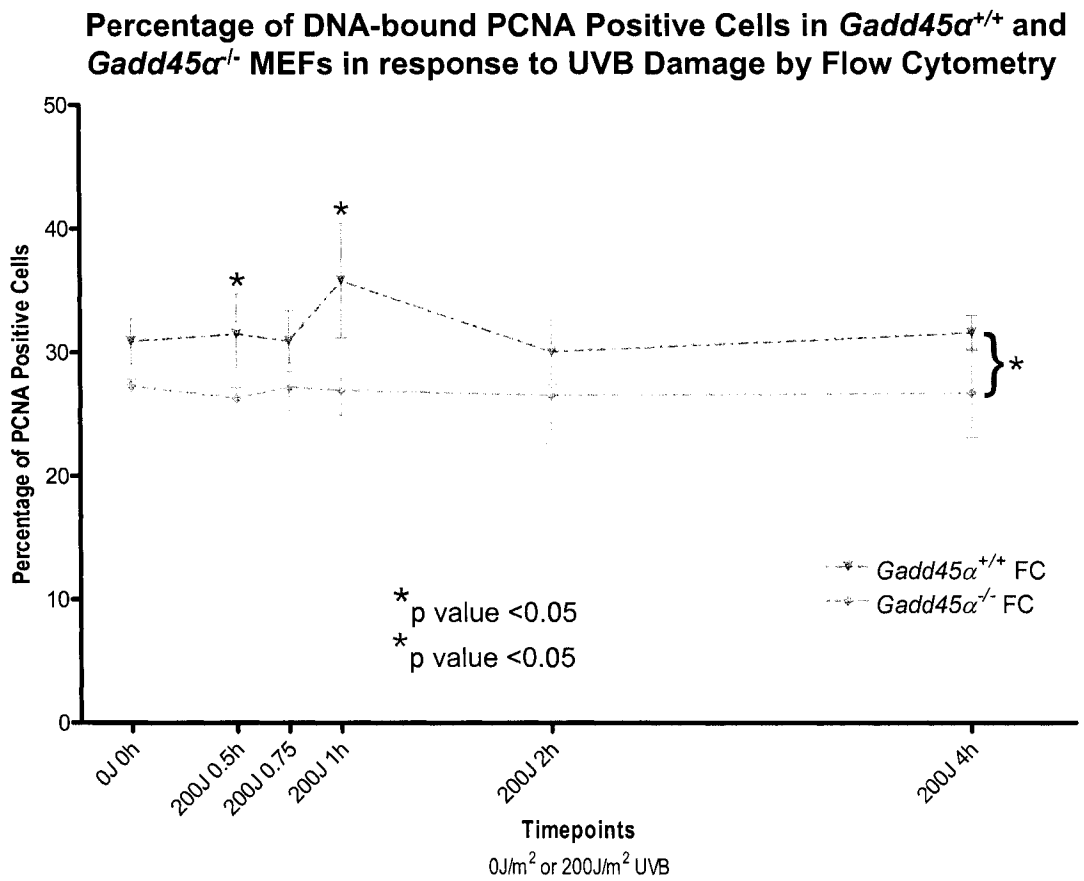

Figure 3-7: Graph of DNA-bound PCNA in *Gadd45α* wt and ko MEFs in response to UVB damage assayed by Flow Cytometry (FC).

Gadd45α$^{+/+}$ and *Gadd45α*$^{-/-}$ MEFs were treated with 200J/m^2 of UVB, harvested and permeabilized to remove non-DNA-bound PCNA. Cells were stained with anti-PCNA and Alexa 488-conjugated secondary antibody and run on a FACS Calibur. After eliminating background staining, the percentage of DNA-bound PCNA positive cells was determined. No statistical difference was seen at individual timepoints between *Gadd45α*$^{+/+}$ and *Gadd45α*$^{-/-}$ MEFs. The *Gadd45α*$^{+/+}$ MEFs have a moderate increase of DNA-bound PCNA at 1h post-treatment with 200J/m^2 of UVB and are decreasing by 4h. *Gadd45α*$^{-/-}$ MEFs do not display a PCNA response to UVB damage. The response curves of the *Gadd45α*$^{+/+}$ and *Gadd45α*$^{-/-}$ MEFs are statistically different with a p-value of <0.00001. Graph represents mean and SD of three experiments.

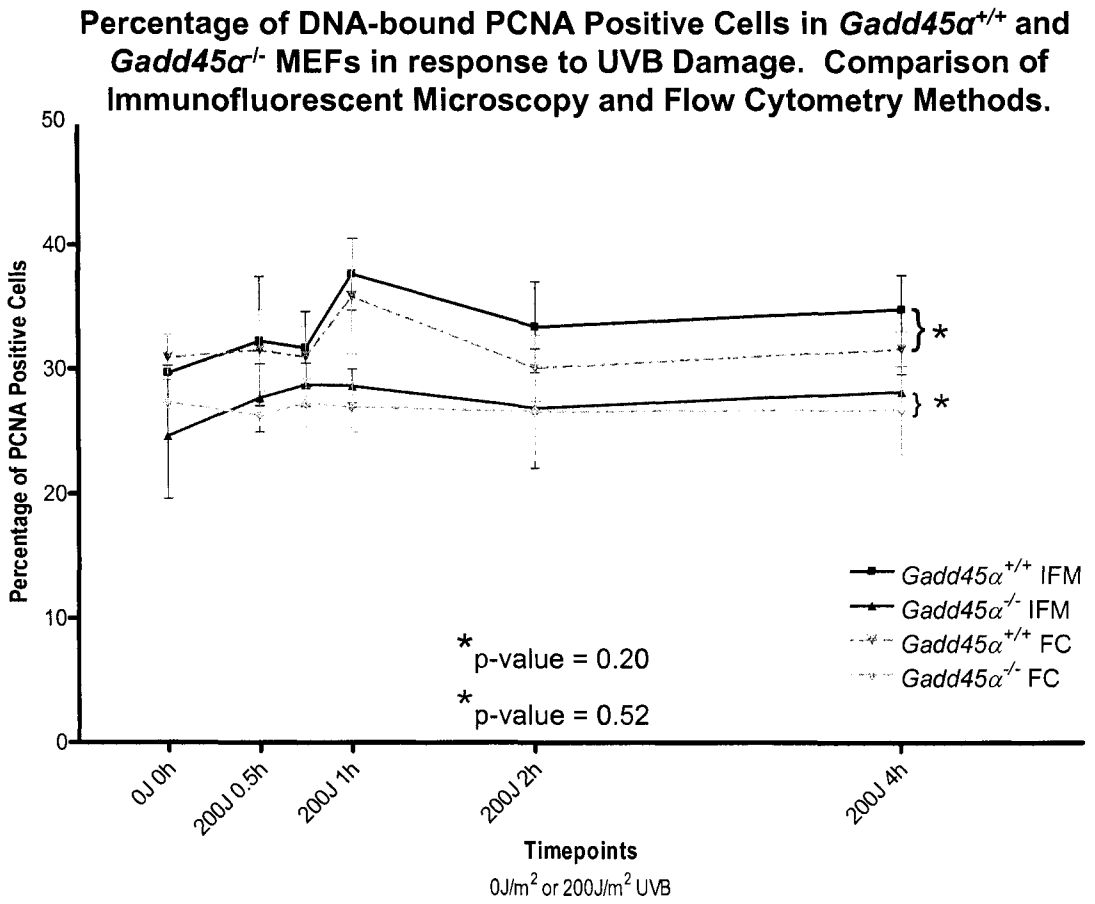

Figure 3-8: Comparison of Immunofluorescent Microscopy (IFM) and Flow Cytometry (FC) Graphs of DNA-bound PCNA in *Gadd45α* wt and ko MEFs in response to UVB damage.

The new method for evaluating DNA-bound PCNA by flow cytometry and the standard method of immunofluorescence microscopy were graphed together and compared by statistical analysis. There is no statistical difference between the IFM and FC wt response curves or the IFM and FC ko response curves, indicating that the methods are comparable.

Conclusions

Our results show that there is an early PCNA-response after 200J/m^2 of UVB-damage in wild type MEFs. The response is rapid, with a peak of 35% of cells

with PCNA-induction at 1h post UVB exposure, and a decline to baseline of 28% by 2h, and is confirmed by both immunofluorescent microscopy and flow cytometry. In contrast, both methods showed that there is no early PCNA-response in the *Gadd45α* knock-out MEFs. As indicated previously, the presence of increased levels of p21 in Gadd45α-deficient mouse cells may result in growth suppression in these cells. This could lead to the decreased levels of PCNA seen in the absence of Gadd45α. Our results indicate that Gadd45α is largely responsible for the early PCNA DNA binding event post UVB as verified by two independent methods. This correlates well with previous data that indicates that Gadd45α may play a role in recruiting PCNA onto DNA (Smith, ML et al., 2000). Since it has been shown that the early PCNA response is indicative of NER (Aboussekhra, A and Wood, RD, 1995), the lack of PCNA response in the Gadd45α-deficient cells may indicate a loss of NER.

Chapter 4 ♦Gadd45α and DNA-bound PCNA and p21: Western Blot Analysis

Introduction

The interaction of p21 with PCNA is perhaps the most extensively studied PCNA interaction (Warbrick, E, 2000). p21, a cyclin-dependent kinase inhibitor, was first described in 1993 (Harper, JW et al., 1993). It is a p53-dependent protein that is involved in many cellular functions including the G_1 and G_2 cell cycle checkpoints, quiescence, terminal differentiation (Maeda, T et al., 2002; Stivala, LA and Prosperi, E, 2004) and apoptosis (Dong, C et al., 2005).

While there is general agreement that the interaction of p21 with PCNA inhibits DNA-replication (Li, R et al., 1994; Pan, ZQ et al., 1995; Shivji, MK et al., 1994; Waga, S et al., 1994), there is conflicting evidence on how the interaction of p21 with PCNA affects DNA repair. Inhibition of PCNA functions has been indicated by many pathways. In vitro, p21 prevents PCNA-dependent replication by interfering with many other PCNA-protein interactions including RFC (Oku, T et al., 1998; Waga, S and Stillman, B, 1998) DNApolδ (Podust, VN et al., 1995), and FEN1 (Chen, U et al., 1996). p21 can also interfere with the interaction of PCNA with DNA repair factors involved in NER (Gary, R et al., 1997; Maga, G et al., 2004), which effectively inhibits the resynthesis step of NER (Pan, ZQ et al., 1995; Shivji, MK et al., 1998). One of the groups that demonstrated that p21 has no effect on PCNA-regulated DNA-repair (Shivji, MK et al., 1994), has more

recently shown that p21 can block polymerase binding to PCNA, effectively inhibiting NER (Shivji, MK et al., 1998). p21-null cells display DNA-bound PCNA at a similar level, but for a longer time than in the wild type cells. These same cells display defective NER, indicating a role for p21 in NER at a point after PCNA resynthesis (Stivala, LA et al., 2001). Additional evidence indicates that binding of p21 to DNA coincides with the removal of PCNA from DNA repair sites (Savio, M et al., 1996; Stivala, LA et al., 2001). This dichotomy of NER is also true for in vivo studies, with some in favour of p21-inhibition (Cooper, MP et al., 1999), and some against it (Li, R et al., 1996). In vivo and in vitro studies show that p21 interacts with PCNA during post-damage events and, although, the presence of p21 does not inhibit PCNA-dependent NER, it does inhibit replication (Li, R et al., 1996; Li, R et al., 1994).

Ubiquitin plays an important role in the p21-PCNA interaction. Increased PCNA-ubiquitination occurs in response to UV irradiation, hydroxyurea, aphidicolin and MMS, all of which down-regulate p21 expression (Soria, G et al., 2006). p21 inhibits the ubiquitination of PCNA, which is necessary for adequate DNA-repair function of PCNA (Soria, G et al., 2006), indicating that p21 is inhibitive of DNA repair. Additionally, high levels of p21, as well as association of p21 with PCNA have been shown to protect p21 from proteasomal degradation (Cayrol, C and Ducommun, B, 1998).

To further elucidate the role of Gadd45α on DNA-bound PCNA and to confirm our IFM and FC data, we ran DNA-bound western blots and probed with PCNA and p21. We altered the method of (Savio, M et al., 1996) to use Triton X-100 instead of NP-40 in the hypotonic lysis.

Results

In this study the *Gadd45α*$^{-/-}$ MEFs have a higher level of DNA-bound p21 in untreated cells (Figure 4-1). This is complementary to previous experiments in our lab that show increased levels of whole-cell p21 in *Gadd45α*$^{-/-}$ murine keratinocytes (Maeda, T et al., 2005). At 1h post-UVB p21 levels are increased in the *Gadd45α*$^{-/-}$ MEFs, but by 4h this relationship reverses with the *Gadd45α*$^{+/+}$ MEFs showing increased levels of p21. *Gadd45α*$^{+/+}$ and *Gadd45α*$^{-/-}$ MEFs display a similar DNA-bound PCNA response in untreated cells (0J/m^2) and at 0.5h. At 0.75h, 1.0h, 2.0h and 4.0h, the *Gadd45α*$^{+/+}$ cells have higher levels of PCNA over the *Gadd45α*$^{-/-}$ MEFs. The initial increased levels of p21 in the *Gadd45α*$^{-/-}$ MEFs indicates growth inhibition and correlates with decreased PCNA in these cells, while the *Gadd45α*$^{+/+}$ MEFs display lower initial levels of p21 with increased levels of PCNA, indicating a role of Gadd45α in regulating the early DNA-binding response of PCNA and in inhibition of p21 DNA-binding.

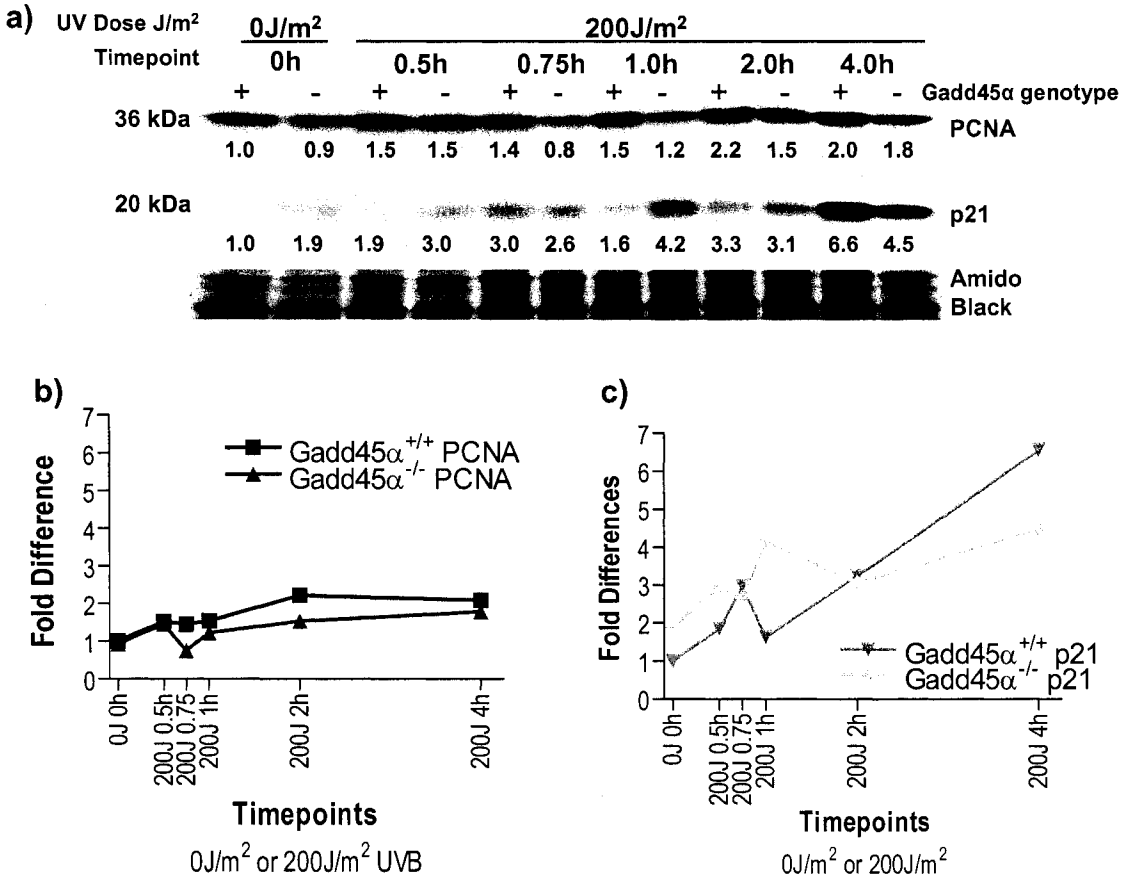

Figure 4-1: DNA-bound Western Blot

Response of DNA-bound PCNA and p21 in *Gadd45α*$^{+/+}$ and *Gadd45α*$^{-/-}$ MEFs in response to UVB-irradiation. *Gadd45α*$^{+/+}$ and *Gadd45α*$^{-/-}$ MEFs were treated with 200J/m^2 of UVB, harvested, treated to remove non-DNA-bound PCNA and digested with DNAse I to release DNA-bound proteins. Proteins were run on a 12% SDS-PAGE gel, probed with anti-PCNA, anti-p21 and anti-HRP secondary antibodies and exposed to film. All lanes were normalized to amido black staining for loading and standardized to untreated *Gadd45α*$^{+/+}$ samples. (a) Western Blot image. (b and c) Graphs of the PCNA (b) and p21 (c) western blot data for easier visualization of trends. DNA-bound PCNA increases in the *Gadd45α*$^{+/+}$ MEFs to a maximum at 2h. *Gadd45α*$^{-/-}$ MEFs do not display an early PCNA response but increase at 4h post-UVB damage. Experiment was performed in triplicate, but graphs are representative of a single experiment due to lack of standardization between blots.

Conclusions

If Gadd45α and p21 compete for PCNA accessibility (Chen, IT et al., 1995) and p21 inhibits NER when bound to PCNA (Cooper, MP et al., 1999), the loss of Gadd45α would leave p21 free to inhibit the resynthesis step of NER. This hypothesis may explain the increased levels of p21 and decreased PCNA in the *Gadd45α*-deficient cells at early timepoints, indicating our results are consistent with ineffective NER in the absence of *Gadd45α*. Our *Gadd45α*-proficient MEFs display lower initial levels of p21 with increased levels of PCNA. These results, taken with those in the *Gadd45α*-deficient cells, indicate a role for Gadd45α in regulating the early DNA-binding response of PCNA and in inhibition of p21 DNA-binding. Our findings support a role for Gadd45α in competing with p21 for PCNA binding (Chen, IT et al., 1995). The increase of p21 in the *Gadd45α*-proficient cells at 2h may represent the disassembly of PCNA at DNA repair sites as previously reported (Savio, M et al., 1996).

Chapter 5 ♦Discussion and Future Directions

The cellular response to DNA damage is vital to organism survival. Multiple proteins collaborate to generate different cellular pathways to determine cellular outcome. The functions of many of the proteins that orchestrate these pathways have yet to be elucidated. Gadd45α is known to play important roles in numerous responses to DNA damage, including cell cycle arrest, apoptosis and NER.

PCNA has many functions in response to various stresses and cellular signals, including DNA repair. PCNA is bound to DNA very soon after UV damage, with a peak at 15 – 30 minutes post UVC-irradiation (Savio, M et al., 1996). Early binding of PCNA to DNA is indicative of NER by immunofluorescent microscopy (Aboussekhra, A and Wood, RD, 1995). Evidence provided by *Gadd45α*-null cells prove that Gadd45α plays a role in NER (Smith, ML et al., 2000), presumably through interactions with PCNA, although these interactions remain unclear (Smith, ML et al., 1994). To elucidate the effect of Gadd45α on DNA-bound PCNA, we analyzed *Gadd45α*$^{+/+}$ and *Gadd45α*$^{-/-}$ MEFs for the early UVB-induced PCNA-response by immunofluorescent microscopy and confirmed our results by a novel method for viewing DNA-bound PCNA by flow cytometry in intact cells.

The immunofluorescent microscopy data indicates that there is no early PCNA-response in the absence of Gadd45α. Although the response in the *Gadd45α*$^{+/+}$ MEFs is not large, it is significantly greater than the knockout cell lines (Figure 3-2). These data are confirmed by the flow cytometry results (Figure 3-7). Although previous data show an increase of PCNA maximally at 30 minutes post UV damage (Savio, M et al., 1996), we show a maximal increase at 1h. This finding may be due to the difference in cell type and UV type; Savio et al. used 20J/m^2 UVC on human fibroblasts, where as we use 200J/m^2 UVB on MEFs. Our data indicate that the presence of Gadd45α enables PCNA to bind more readily to DNA at early time-points after UV damage.

The p21-PCNA interaction is one of the most studied of the PCNA interactions (Warbrick, E, 2000). There is controversy on how this interaction affects replication and repair. Some studies indicate that the binding of p21 to PCNA inhibits replication while some studies indicate that p21 inhibits DNA repair (Pan, ZQ et al., 1995; Savio, M et al., 1996) and replication (Li, R et al., 1994; Maga, G et al., 2004; Shivji, MK et al., 1994; Waga, S et al., 1994), while others disagree (Li, R et al., 1996; Li, R et al., 1994; Shivji, MK et al., 1994). Although there is controversy in the function of the p21-PCNA interaction, we analysed the DNA-bound protein in *Gadd45α*$^{+/+}$ and *Gadd45α*$^{-/-}$ MEFs for the presence of p21 and PCNA. While previous studies used NP-40 in the hypotonic lysis, we found that

NP-40 did not eliminate DNA-bound proteins. We used Triton X-100 to keep the method as similar as possible to our immunofluorescence studies.

The *Gadd45α*-deficient MEFs displayed no PCNA response to UVB irradiation, confirming our immunofluorescent studies. We found a constitutively increased amount of DNA-bound p21 in the untreated *Gadd45α*-deficient MEFs compared to the *Gadd45α*-proficient cells, which corroborates previous studies that found a higher amount of whole cell p21 protein in *Gadd45α*-deficient over *Gadd45α*-proficient murine keratinocytes (Maeda, T et al., 2005). In these cells, the p21 levels stay elevated throughout the first hour post-UVB treatment. By the 4h timepoint, the p21 levels *Gadd45α*-proficient MEFs have increased above those of the *Gadd45α*-deficient MEFs.

Previous studies have suggested that Gadd45α and p21 compete for PCNA accessibility (Chen, IT et al., 1995) and that p21 inhibits NER when bound to PCNA (Cooper, MP et al., 1999). These studies indicate the loss of Gadd45α would leave p21 free to inhibit the resynthesis step of NER and may partly explain the increased levels of p21 and decreased PCNA in the *Gadd45α*-deficient cells at early timepoints. Our *Gadd45α*-proficient MEFs display lower initial levels of p21 with increased levels of PCNA. These results, taken with those in the *Gadd45α*-deficient cells, indicate a role for Gadd45α in regulating the early DNA-binding response of PCNA and in inhibition of p21 DNA-binding.

Previous evidence indicates that p21 binds to DNA at later timepoints and functions to remove PCNA from DNA-repair sites (Savio, M et al., 1996). The Western blotting data supports this; the increase of p21 at 2h to 4h in our *Gadd45α*-proficient cells may be functioning to remove the PCNA, which decreases after the 2h timepoint. This data also supports a role for Gadd45α in competing with p21 for PCNA binding (Chen, IT et al., 1995). The peak increase timepoint is different from our immunofluorescence data, but the two methods are measuring different things. The immunofluorescence studies measured the percentage of cells that displayed DNA-bound positivity, while the western method is measuring the total amount of PCNA bound to DNA.

Since there is such debate about the role of p21 in PCNA-dependent DNA repair, the DNA-bound western blots could be used to further elucidate the role of both p21 and Gadd45α on the DNA-bound PCNA. Repeating these experiments with additional antibodies, such as FEN1 or other replication or repair specific antibodies should elucidate the role the increase in p21 is playing in our *Gadd45α*$^{-/-}$ MEFs. All of our results indicate that Gadd45α is involved in early DNA-binding of PCNA. As PCNA is monoubiquitinated in repair (Huang, TT et al., 2006; Solomon, DA et al., 2004), it would be interesting to determine the presence of ubiquitin on the DNA-bound Western blots. Additionally, our experiments should be repeated on different mouse genotypes, including *p21-*

knock out, *p53*-knock out, *Gadd45α/p21*-double knock out, and *Gadd45α/p53*-double knock out in both MEFs and keratinocytes, to illuminate the roles the different combinations of protein removal. To see the effect of the lack of Gadd45α on DNA-bound PCNA in human cells, this experiment should be performed on primary human keratinocytes with *Gadd45α* knocked-down by siRNA.

The development of the flow cytometry method was an important development in my thesis. Previous methods used NP-40 to lyse the cells and rid them of non-DNA bound proteins (Prosperi, E et al., 1994; Prosperi, E et al., 1993; Wilson, GD et al., 1992). However, here NP-40 lysis prior to in immunofluorescence microscopy resulted in unusual staining, coupled with the inability to remove non-DNA bound protein (Figure 3-3). Additionally, flow cytometry was unable to analyze nuclear extracts due to the small size of our MEF nuclei. These problems led to the analysis of DNA-bound proteins in intact cells using Triton X-100 as the permeabilizing agent. We demonstrate, for the first time, that it is possible to use a lower level of Triton X-100 with a longer incubation to effectively remove all soluble proteins (Figure 3-5) and leave the cells intact (Figure 3-6). When this method is compared to the standard immunofluorescent microscopy method, the curves are remarkably similar with no statistical difference between the two methods (Figure 3-8).

Flow cytometry has many benefits over immunofluorescent microscopy. First, the cell treatment and collection is quicker and easier. Cells can be frozen and used later allowing all timepoints to be processed simultaneously. The staining and fixation protocol is more rapid and many more cells are counted. Future applications of this method include using additional antibodies to help elucidate the PCNA response as replication or repair. These antibodies could include p21 and FEN1. Additionally, to get a more physiologic view, this experiment should be repeated on mouse keratinocytes, or on human keratinocytes that have *Gadd45α* knocked down by siRNA. To further clarify the role of various proteins in this process, different mouse colonies can be used including *p21*-knock out, *p53*-knock out, *Gadd45α/p21*-double knock out, and *Gadd45α/p53*-double knock out, which can be used with both fibroblasts and keratinocytes using this method.

In summary, we developed a novel method of analysing DNA-bound PCNA in intact cells by flow cytometry. Using this method, along with established methods of studying DNA-bound PCNA by immunofluorescent microscopy and Western blotting, we have established a potential role for Gadd45α in regulating the early DNA-binding events of PCNA. The flow cytometry method could be used to analyse additional proteins to further elucidate this or other pathways. The broad application of this method to nuclear protein analysis should prove to be a very useful tool.

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