The Role of Brm in Non-Melanoma Skin Cancer Progression

Andrew William Farrell

Department of Dermatology
Central Clinical School
Faculty of Medicine
The University of Sydney

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REFERENCES
Statement of Originality

I hereby declare that this thesis is my own work, performed in the Discipline of Dermatology, Faculty of Medicine, The University of Sydney, Australia. It contains no material previously published or written by another person except where due reference has been given in the text. It contains no material which has been submitted or accepted for any other degree at any other university or educational institution. All statistical analysis was performed by myself. The Introduction (Chapter 1) contains sections of text taken from a review paper completely written by myself that was published in 2011 (Farrell, A.W., Halliday, G.M. & Lyons, J.G. 2011. Chromatin structure following UV-induced DNA damage-repair or death? Int J Mol Sci, 12, 8063-85).

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  Awarded November, 2014

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  Awarded November, 2011
Abstract

The role of Brm in non-melanoma skin cancer progression

**Background:** Australia has the highest incidence of non-melanoma skin cancer (NMSC) in the world. These cancers are predominantly caused by exposure to ultraviolet radiation (UVR) in sunlight. Brm is an ATPase subunit that drives chromatin remodelling via the SWI/SNF complex. Thus, Brm is able to regulate access to transcription factors and repair enzymes to DNA, in addition to cell division. The loss of Brm has been observed in several cancer types, where its loss correlates with decreased patient survival. The loss of Brm has been identified in NMSC, but not pre-malignant lesions, suggesting Brm plays a role in the progression of a benign skin lesion into a malignant skin cancer. Brm-deficient mice also harbour excessive levels of epidermal hyperplasia as well as heightened tumour incidence following UVR as compared to mice with wildtype Brm. Moreover, a novel hotspot mutation of Brm (Q203K) has also been discovered in 17% of NMSC studied, which was the first mutation of Brm discovered in any type of cancer.

**Aims:** This thesis aims to study the function of the Q203K mutation as compared to the loss of Brm in several key aspects of keratinocyte responses to UV-induced DNA damage: proliferation, cell cycle regulation, UVR-induced cell death and DNA repair.

**Methods:** The loss of Brm was studied with keratinocytes grown *ex vivo* from neonatal Brm-/- mice and compared to keratinocytes grown from Brm+/+ mice. Further, a human keratinocyte cell line, HaCaT was also studied, in combination with Brm miRNAs to knockdown Brm expression in these cells. The overexpression of either wildtype or Q203K Brm was also studied in HaCaTs. These cells were treated with UV, and their responses were observed using assays for cell growth, cell death, cell cycle regulation and DNA repair.

**Results:** In neonatal mouse keratinocytes, as well as HaCaT cells, the loss of Brm expression led to an altered cellular response to UVR. Cells lacking Brm exhibited increased proliferative potential following UVR due to a reduced time spent in
G1 cell cycle arrest. Further, Brm-deficient cells displayed increased formation of UVR-induced cyclobutane pyrimidine dimers (CPDs) in the dark. Interestingly, when Brm was knocked down in the immortalised human keratinocyte cell line HaCaT, these cells were not as sensitive to the loss of Brm in combination with UVR, and showed less prominent effects on both cell cycle regulation and CPD formation. It was hypothesised that primary neonatal keratinocytes were more sensitive to UVR due to these cells being naïve, whereas HaCaT cells already exhibit several UVR-induced mutations, such as that of p53. Therefore, the loss of Brm in mouse neonatal keratinocytes was more catastrophic to the cell. Lastly, the mutation of Q203K did not lead to increased cellular sensitivity to UVR, suggesting the presence of this mutation in NMSC was most likely non-functional. However, increasing levels of Brm in HaCaT cells did lead to enhanced levels of cell viability at high density, which the Q203K mutation was able to reverse, suggesting Q203K does display some functionality, and thus may play a role in other functions not studied in this thesis.

**Conclusions:** The loss of Brm but not the Q203K mutant in combination with UVR is enough to cause increased cellular sensitivity in the cell, leading to the proliferation of cells whilst harbouring DNA damage via defective cell cycle checkpoints. This would likely lead to heightened levels of mutations in the cell, and increased carcinogenesis downstream, as previously witnessed in Brm-deficient mice. The Q203K mutation was not comparable to the loss of Brm, and this thesis suggests that the Q203K mutation in NMSC is not likely to contribute to increased carcinogenesis, however further testing would have to be conducted to fully determine this.
<table>
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<td>6-4PPs</td>
<td>6-4 Photoproducts</td>
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<tr>
<td>8-oxo-dG</td>
<td>8-oxo-deoxyguanidine</td>
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<tr>
<td>Ac</td>
<td>Acetylation</td>
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<td>AK</td>
<td>Actinic Keratosis</td>
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<td>Amp</td>
<td>Ampicillin</td>
</tr>
<tr>
<td>ARID1A/B</td>
<td>AT-Rich Interactive Domain Containing Protein A/B</td>
</tr>
<tr>
<td>ATM</td>
<td>Ataxia-Telangiectasia-Mutated</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine 5’-Triphosphate</td>
</tr>
<tr>
<td>ATR</td>
<td>Ataxia-Telangiectasia-Rad Related</td>
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<tr>
<td>BAF</td>
<td>Brg1-Associated Factor</td>
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<tr>
<td>BCC</td>
<td>Basal Cell Carcinoma</td>
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<tr>
<td>BER</td>
<td>Base Excision Repair</td>
</tr>
<tr>
<td>BPE</td>
<td>Bovine Pituitary Extract</td>
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<td>BRD7</td>
<td>Bromodomain-Containing 7</td>
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<td>BrdU</td>
<td>5-Bromo-2’-Deoxyuridine</td>
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<td>Brg1</td>
<td>Brahma-Related Gene 1/SMARCA4</td>
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<td>Brm</td>
<td>Brahma/SMARCA2</td>
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<tr>
<td>Bsd</td>
<td>Blasticidin</td>
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<tr>
<td>CDK</td>
<td>Cyclin-Dependent Kinase</td>
</tr>
<tr>
<td>CHD</td>
<td>Chromodomain Helicase/ATPase DNA Binding Protein</td>
</tr>
<tr>
<td>CPD</td>
<td>Cyclobutane Pyrimidine Dimer</td>
</tr>
<tr>
<td>CO₂</td>
<td>Carbon Dioxide</td>
</tr>
<tr>
<td>CRC</td>
<td>Chromatin Remodeling Complex</td>
</tr>
<tr>
<td>CSA/B</td>
<td>Cockayne Syndrome Protein A/B</td>
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<tr>
<td>DAPI</td>
<td>4’,6-Diamidino-2-Phenylindole Dihydrochloride</td>
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<td>DBP</td>
<td>DNA-binding Protein</td>
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<tr>
<td>DDB1/2</td>
<td>DNA Damage Binding Protein 1/2</td>
</tr>
<tr>
<td>DHPLC</td>
<td>Denaturing High-Performance Liquid Chromatography</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic Acid</td>
</tr>
<tr>
<td>DNA-PK</td>
<td>DNA-Damage Dependent Protein Kinase</td>
</tr>
<tr>
<td>DSB</td>
<td>Double Strand Break</td>
</tr>
<tr>
<td>DTT</td>
<td>Dithiothreitol</td>
</tr>
<tr>
<td>E2F</td>
<td>E2 factor</td>
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<tr>
<td>E. coli</td>
<td><em>Escherichia coli</em></td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediaminetetraacetic Acid</td>
</tr>
<tr>
<td>EdU</td>
<td>5-ethynyl-2'-deoxyuridine</td>
</tr>
<tr>
<td>EGF</td>
<td>Epidermal Growth Factor</td>
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<tr>
<td>EGFP</td>
<td>Enhanced Green Fluorescent Protein</td>
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<tr>
<td>EtOH</td>
<td>Ethanol</td>
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<tr>
<td>EYFP</td>
<td>Enhanced Yellow Fluorescent Protein</td>
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<tr>
<td>FBS</td>
<td>Foetal Bovine Serum</td>
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<tr>
<td>GADD45a</td>
<td>Growth arrest and DNA damage-inducible protein 45-alpha</td>
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<tr>
<td>GGR</td>
<td>Global Genome Repair</td>
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<tr>
<td>H3</td>
<td>Histone-H3</td>
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<tr>
<td>H2AX</td>
<td>H2A histone variant H2AX</td>
</tr>
<tr>
<td>HDAC</td>
<td>Histone Deacetylase Complex</td>
</tr>
<tr>
<td>hOGG1</td>
<td>Human 8-oxoguanine DNA glycosylase-1</td>
</tr>
<tr>
<td>HR</td>
<td>Homologous Recombination</td>
</tr>
<tr>
<td>INO</td>
<td>Inositol</td>
</tr>
<tr>
<td>ISWI</td>
<td>Imitation of Switch</td>
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<tr>
<td>K</td>
<td>Lysine</td>
</tr>
<tr>
<td>LDH</td>
<td>Lactate Dehydrogenase</td>
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<td>Me</td>
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mRNA  Messenger RNA
miRNA/miR  MicroRNA
MNK  Murine Neonatal Keratinocyte
NaCl  Sodium Chloride
NER  Nucleotide Excision Repair
NHEJ  Non-Homologous End Joining
NMSC  Non-Melanoma Skin Cancer
NO  Nitric Oxide
P  Phosphorylation
pBAF  Polybromo Brg1-Associated Factor
PBS  Phosphate-Buffered Saline
PcG  Polycomb Group
PCR  Polymerase Chain Reaction
PIKK  Phosphatidylinositol-3 Kinase-Like Kinase
PRC2  Polycomb Repressive Complex 2
Q  Glutamine
Rb  Retinoblastoma Protein
RFC  Replication Factor C
RNA  Ribonucleic Acid
ROS  Reactive Oxygen Species
RT-PCR  Real-Time Polymerase Chain Reaction
SCAI  Suppressor of Cancer Cell Invasion
SCC  Squamous Cell Carcinoma
SEM  Standard Error of Mean
ssUV  Solar Simulated Ultraviolet
SWI/SNF  Switch/Sucrose Non-Fermenting
TCR  Transcription Couple Repair
<table>
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<tr>
<th>Abbreviation</th>
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<tr>
<td>TGF</td>
<td>Tumour Growth Factor</td>
</tr>
<tr>
<td>TFIIH</td>
<td>Transcription Factor IIH</td>
</tr>
<tr>
<td>Ub</td>
<td>Ubiquitylation</td>
</tr>
<tr>
<td>UDS</td>
<td>Unscheduled DNA Synthesis</td>
</tr>
<tr>
<td>UV</td>
<td>Ultraviolet</td>
</tr>
<tr>
<td>UVR</td>
<td>Ultraviolet Radiation</td>
</tr>
<tr>
<td>XP</td>
<td>Xeroderma Pigmentosum</td>
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Chapter 1: Introduction

1.1. Structure of human skin

The skin is the largest organ of the human body, where its principal function is to act as a barrier. This includes protection of the underlying tissues from various environmental factors, such as ultraviolet (UV) radiation, infections, trauma and harmful chemicals; as well as maintaining normal body temperature. The skin is also involved in the production of the steroid vitamin D₃, storage of lipid nutrients, excretion of salts, water and organic wastes, as well as sensory reception of touch, pressure, temperature and pain stimuli (Martini, 2004). Skin consists of two main layers: the epidermis (outer layer), which is composed of stratified squamous epithelium; and the dermis (inner layer) which is made of collagen-rich connective tissue, that provides strength and flexibility to skin. Unlike the epidermis, it contains blood vessels, nerve fibres and lymphatic vessels. It also contains other cells such as fibroblasts, and cells of the immune system. Below the dermis is another layer of connective tissue, called the hypodermis, which contains various amounts of adipose tissue. Also present in skin are appendages such as the sweat glands, hairs and sebaceous glands (Ross et al. 1989, Fig. 1.1).
Figure 1.1: The structure and major components of the skin. From University of Waikato, www.sciencelearn.org.nz.

1.1.1. The human epidermis

The human epidermis is a stratified squamous epithelium, which consists of proliferating basal and differentiated suprabasal keratinocytes. Although these keratinocytes predominate, other cells such as melanocytes, Langerhans cells and Merkel cells coexist with them in the epidermis. In humans, it is thinnest on the eyelids and thickest on the palms and soles of the feet. The epidermis consists of 4 or 5 layers, depending on the site of skin which include, from bottom to top, the stratum basale, the stratum spinosum, the stratum granulosum, the stratum
lucidum (does not exist in thin skin) and the stratum corneum (Chu et al. 2003) (Fig 1.2).

Figure 1.2: The various layers of the epidermis. Ref:

1.1.2. Dynamics of the human epidermis

In some tissues such as skin and mucosa, differentiated cells have a short life span, undergoing continuous and rapid divisions derived from a small group of cells named ‘stem cells’. These stem cells have two characteristics, firstly, they undergo self-renewal by division and they also produce daughter or ‘progenitor’ cells
(Spillane et al. 2007). In the skin, these cells are located in the basal layer of the epidermis, which is attached to the basement membrane. Before terminal differentiation, a stem cell undergoes 5-6 mitoses which results in a group of cells known as transit-amplifying cells, which have limited proliferative potential (Watt, 2001). These transit-amplifying cells finally undergo terminal differentiation and create the outer layer of the epidermis (Fig 1.3).

**Figure 1.3: Stem cell division.** A single stem cell (S) divides to produce one stem cell and a transit-amplifying cell (T). After three rounds of division, the progeny of the transit-amplifying cell withdraw from the cell cycle and undergo terminal differentiation (TD). *From Watt, 2001.*

There are three main factors that affect the cell kinetics in the human epidermis. These include cell division, differentiation and death. All proliferating cells go through a cell cycle which includes mitosis (M), interphase (G1), active DNA synthesis (S) and a short resting or pre-mitotic phase (G2) (Fig 1.4; Lloyd et al. 1982; Shaefer, 1998). Some basal keratinocytes, however, may remain in a dormant state called the G0 phase and re-enter the cell cycle when required. In keratinocytes, the cell cycle is mainly regulated in the G1 phase. Enzymes named cyclin-dependent kinases (CDKs) are known to regulate cell cycle progression. These CDKs become
activated upon binding to their appropriate cyclin partners, many of which are responsible for transitions between stages of the cell cycle (Gniadecki, 1998). During G1 progression, CDK complexes are activated, which have many downstream targets such as proteins from the retinoblastoma (Rb) family and transcription factors from the E2 factor (E2F) family, which form complexes and act as transcriptional repressors (Sardet et al. 1995).

Figure 1.4: The eukaryotic cell cycle. All cells progress through this cycle and a disturbance in this cycle can lead to many cellular defects. Image from Purves et al. 2004.
1.2. Non-melanoma skin cancer

1.2.1. Skin cancer in humans

Skin cancer is a disorder occurring from the result of malignant transformation and division of keratinocytes or melanocytes causing non-melanoma skin cancer (NMSC) and melanoma respectively. Other cell types can give rise to rarer types of NMSC. NMSC is the most prevalent skin cancer in Caucasian populations. It is estimated that 2-3 million cases of NMSC occur worldwide each year. This incidence varies, and is highest in Caucasians (Narayanan et al. 2010). Although these numbers are already high, they are expected to double within the next 30 years (Rhee et al. 2007). In Australia, non-melanoma skin cancer is three times more common than all other carcinomas combined and costs the Australian healthcare system approximately $500 million per year (Cancer Council Australia Clinical Practice Guide NMSC, 2008). An estimated 440,000 Australians are treated for non-melanoma skin cancers each year, while in 2011, there were 543 deaths resulting from non-melanoma skin cancer in Australia (Cancer Council Australia, 2012).

Non-melanoma skin cancer is predominantly made up of basal cell carcinoma (BCC) and squamous cell carcinoma (SCC) in a ratio of approximately 4:1 (Ridky, 2007). Other types of NMSC arising from other cell types in the skin exist, such as lymphocytes, vascular endothelial cells, Merkel cells and mesenchymal stromal cells, however these are rare. These cancers are usually found in heavily sun-exposed areas, in particular the head and neck regions.
BCC is a slow growing skin cancer with low metastatic potential that originates from keratinocytes in the epidermis. While metastasis is rare, BCCs can invade into local tissues causing considerable damage. They do not always originate in the basal layer of the epidermis but are named basal due to their basal-cell-like appearance (Neville et al. 2007; Rubin et al. 2005). SCC is a malignant tumour arising from keratinocytes in the epidermis, skin appendages and other stratified squamous mucosa. SCCs usually develop in benign epidermal lesions, most commonly in actinic keratoses (AK) or ‘sun spots’ (Czarnecki et al. 2002; Alam et al. 2001). AKs are scaly or crusty lesions that occur on sun-exposed skin. A small proportion (10%) of AKs progress to become an invasive SCC. SCC is known to carry a >10-fold higher risk of metastasis and mortality as compared to BCC (Fuchs and Marmur, 2007).

1.2.2. Risk factors for skin cancer

NMSC can occur when skin cells are damaged. The most common damaging factor is ultraviolet radiation (UVR). NMSC is directly related to the amount of UVR received and inversely proportional to the ‘degree of skin pigmentation’. Between 95-99% of skin cancers in Australia are caused by exposure to UVR from the sun (Staples et al. 2006). BCC has been linked to UVR exposure and severe sunburns in childhood and adolescence. Intense intermittent exposure to the sun is associated with a higher risk than a similar degree of continuous exposure (Rubin et al. 2005). Lifestyle changes over the last few decades have led to an increase in exposure to
sunlight via increased sunbathing habits and outdoor activities, which has led to an increase in skin cancers worldwide. There are several other factors that increase the risk for NMSC. These include use of tanning salons, family history of skin cancer, fair skin or red hair colour, a tendency to burn instead of tanning, immunosuppression, large numbers of moles on the body and geographical location (Han et al. 1996). Individuals with a prior history of AK have an increased risk of developing subsequent lesions as compared to individuals with no history of these lesions (Raasch and Buettner, 2002).

The relative risk of NMSC is three times higher for people who grew up in areas associated with high levels of UVR from the sun as compared to those that move to these areas later in life. From this, it can be concluded that there is a dose-related relationship between sun exposure and NMSC, particularly those who receive large dose of UVR in early life (Narayanan, 2010).

1.2.3. UVR and UV-induced mutations

In 1801, Johann Ritter discovered the UV region of the solar spectrum. In this discovery, he found that some form of energy in the dark portion beyond violet caused chemical action (Ritter, 1801). The primary feature that characterizes the properties of each region of the solar spectrum is its wavelength. Below 100nm are X-rays and γ-radiation. Above 400nm lies visible light, infrared, microwave and radiowaves. UVR is between these wavelengths, ranging between 100nm and 400nm. This is further broken down into UVA (320-400nm), UVB (290-320nm) and
UVC (100-290nm) wavebands. UVC is mostly absorbed by the ozone layer in the atmosphere and does not reach the Earth’s surface. However, both UVA and UVB penetrate through the ozone layer. At the surface of the Earth, UVA comprises about 95% of solar UV energy, the remainder being the UVB fraction (Narayanan, 2010).

When UV reaches the skin, UVB radiation is absorbed mostly by the upper epidermal layers, whereas UVA penetrates deeper into the skin, reaching the basal layer of the epidermis (Fig 1.5; Matsunaga et al. 1991). Both UVA and UVB are known to induce DNA mutations. UVB in particular is heavily genotoxic through direct absorption by DNA. UVB causes characteristic G:C to A:T DNA mutations (Drobetsky et al. 1995; Robert et al. 1996).
Figure 1.5: The spectrum of UV light and wavelength-dependent penetration into the skin. UVC is completely blocked by the ozone layer, however UVB is able to penetrate into the epidermis and UVA is able to penetrate deeper into the dermis. From Gupta et al. 2007.

In 1960, Beukers and colleagues identified a UV-induced modification of DNA bases. They established that thymine bases formed cyclobutane pyrimidine dimers (CPD) following UVC irradiation (Fig 1.6). It was subsequently found that CPDs were induced by UVC and UVB radiation between neighbouring pyrimidine bases in DNA.
strands. Further studies also found another DNA lesion induced by UVR, at di-pyrimidine sites, the 6-4 photoproduct (6-4PP) (Fig 1.6). 6-4PPs comprise 20-30% of DNA damage produced in nucleosomal DNA. However, CPDs comprise 70-80% of DNA damage and induce a bend or kink in the DNA helix (Suquet and Smerdon, 1993). CPDs are also repaired more slowly than 6-4PPs, which may be due to CPDs being produced in nucleosomal DNA, whereas 6-4PPs are easier to access in the linker DNA (Palomera-Sanchez and Zurita, 2011). A CPD occurs via the formation of a four-membrane ring structure, resulting from the saturation of the pyrimidine 5,6 double bonds. These dimers are able to theoretically form several configurations: cis-syn, cis-anti, trans-syn and trans-anti. In a usual double-stranded DNA form, CPDs are generally in the cis-syn form (De Gruijl, 2000).

Although UVA has also been shown to form pyrimidine dimers (Rochette et al. 2003; Mouret et al. 2006), it requires much more energy than UVB to incur this damage. However, UVA causes greater levels of oxidative damage than UVB, and has been shown to cause increased 8-oxo-deoxyguanidine (8-oxo-dG) lesions, which result from the oxidation of guanine moieties (Dahle and Kavm, 2003).
Figure 1.6: Structures of cyclobutane dimer (CPD) and pyrimidine pyrimidinone dimer (6-4PP). From Jiang et al. 1997.

Another type of DNA damage is double-strand breaks (DSBs), in which both strands of the DNA helix are severed, which can lead to genome rearrangements. DSBs are known to be more hazardous than other DNA lesions such as 6-4PPs and CPDs, however, they require much larger doses of UVR (Papamichos-Chronakis and Peterson, 2013). Upon DNA damage via UVR, there are several possible outcomes. Firstly, if the DNA damage is minimal, the lesion will be repaired completely and the cell can continue in its normal state. If the lesion is more substantial and thus repair is not possible, the cell will enter into cell cycle arrest and activation of apoptosis processes, and the cell will die. However, if the level of DNA damage is too high, and the cell is not correctly repaired or successfully killed via apoptosis, a wrong nucleotide can be incorporated in the next round of transcription, establishing a DNA mutation (De Gruijl, 2000).
1.3. DNA repair

To counteract DNA lesions, eukaryotes have evolved a number of processes that make it possible for the cell to recover from damage. The inherent biological tendency is to maintain genetic homeostasis. To accomplish this, DNA damage induces cellular responses leading to cell cycle arrest. A cell can repair or tolerate the DNA damage, or the cell that harbours damage can be removed from the population via induced death. Diverse DNA repair pathways have been conserved in a large number of species as they have evolved over time to repair different forms of lesions (Horakova et al. 2010).

The nucleotide excision repair (NER) pathway is primarily involved in removing helix-distorting lesions such as CPDs and 6-4PPs induced by UVR (Riedl et al. 2003). NER employs a method called unscheduled DNA synthesis (UDS) to fill gaps left following excision of the oligonucleotides containing the dimers (De Gruijl, 2000). Xeroderma pigmentosum (XP) patients are known to lack this form of repair, and thus run a dramatically increased risk of skin cancers (Kraemer et al. 1997).

Upon UVR, cells are arrested in the G1 and G2 phases of the cell cycle, both of which are regulated by the tumour suppressor p53 (Decraene et al. 2001). In eukaryotic cells, the NER pathway is subdivided into two pathways: global genome repair (GGR), which repairs lesions located anywhere within the genome, and transcription coupled repair (TCR) which repairs genes in the transcribed strand of active genes (Hanawalt, 2002)(Fig 1.7). In mammals, congenital defects in GGR and TCR lead to an increased sensitivity towards DNA damaging agents such as UV and
disorders such as Cockayne’s Syndrome (de Boer and Hoeijmakers, 2000). Both pathways have a core set of repair factors involved, but have specific factors involved in the initial stages of recognition. GGR is initiated by the activation of several DNA-damage binding proteins, most importantly the XPC-HR23B centrin-2 complex and/or the DDB1-DDB2 (DNA damage binding protein 1 and 2) heterodimer complex (Palomera-Sanchez and Zurita, 2011). Chromatin remodelling complexes such as SWI/SNF (switch/sucrose non-fermenting) are able to recruit checkpoint kinases such as ataxia-telangiectasia-Rad related (ATR) and ataxia-telangiectasia-mutated (ATM), influencing further recruitment of repair factors such as XP group C (XPC) (Ray et al. 2009). TCR of the transcribed strands is initiated by two distinct damage recognition mechanisms. Both the adenosine 5’-triphosphate (ATP)-dependent chromatin remodelling protein Cockayne Syndrome protein B (CSB) and the WD40 domain containing protein Cockayne Syndrome protein A (CSA) are recruited to the site of damage (Henning et al. 1995). TCR is then theorised to be initiated by the abrogation of RNA polymerase II during transcription (Fousteri et al. 2006).

Subsequent steps are performed by a common set of NER factors that are shared by GGR and TCR. This involves the unwinding of the DNA duplex at the sites of damage by XP group B and D helicases (XPB and XPD), which are subunits of the transcription factor IIH (TFIIH) complex (Schaeffer et al. 1993; Evans et al. 1997). XPA is then recruited to stabilise the repair complex and to orient the dual incision of the DNA lesion by two structure-specific endonucleases, XPG and ERCC1-XPF, leading to the removal of a section of single stranded DNA with a gap of 25-30
nucleotides. This gap is then filled by DNA synthesis and ligation via DNA polymerase δ/ε, replication factor C (RFC), PCNA, RPA and DNA ligase I (Fig. 1.7; O’Donovan et al. 1994; Matsunaga et al. 1995).

Figure 1.7: The mechanisms of NER following UV radiation. ⌀ = photolesion. Photolesions are recognised by both TCR and GGR, both of which have specific recognition factors involved. These two pathways then integrate, with several repair proteins assembling at the site of damage, unwinding the DNA, and orienting the excision of the photolesion. This is then followed by new DNA synthesis and ligation. From Farrell et al. 2011.
Another form of repair, named base excision repair (BER), is responsible for removing small, single base non-helix-distorting oxidative lesions from DNA, commonly induced by UVA radiation (Liu et al. 2007). Highly reactive oxygen radicals that can attack DNA strands induce these oxidative lesions. Of all of these oxidative lesions, 8-oxo-dG lesions are the most prevalent and are predominantly repaired by the human 8-oxoguanine DNA glycosylase-1 (hOGG1) enzyme via the BER pathway, the function of which is dependent on CSB (Javeri et al. 2011).

More severe DNA damage can also form double strand breaks (DSBs). DSBs are repaired by the appropriate pathway, depending on the cell cycle phase. DSBs that occur in the G1 phase are mainly repaired by non-homologous end joining (NHEJ). However, cells in G2 or S phase are generally repaired by homologous recombination (HR) (Papamichos-Chroankis and Peterson, 2013). Upon the formation of a DNA lesion, the cell activates DNA repair pathways, as well as signals for checkpoint activation to arrest the cell cycle. The three phosphatidylinositol-3 kinase-like kinases (PIKKs), ATM, ATR and DNA-damage-dependent protein kinase (DNA-PK) are first activated, signalling the presence of a lesion to cell cycle machinery. Although all of these recognise DNA lesions, ATM and DNA-PK mainly function after ionizing radiation, whereas ATR mainly responds to UV irradiation (Ward and Shen, 2001; Stiff et al. 2004). These in turn phosphorylate p53, either leading to the activation of cell cycle arrest proteins such as p21 and Chk2 or pro-apoptotic proteins such as Bax and various caspases (Solier and Pommier, 2009). If the DNA damage is too high, the cell is pushed into apoptosis. If apoptosis fails, the damaged cell may undergo cellular proliferation. A defect in this process can
contribute to oncogenic transformation, leading to cancer development.

1.4. When a cell can’t be repaired - apoptosis

If DNA damage is too severe to be able to be adequately repaired, it may become necessary for the cell to undergo voluntary cell death, also known as apoptosis. Apoptosis is an essential process of the cell involved in maintaining homeostatic control of cell number and eliminating cells capable of inflicting harm to an organism. When a cell has DNA damage, this causes the blockage of DNA replication, leading to the collapse of replication forks and lesion formation, inducing apoptosis. This causes the cell to shrink and the chromatin to condense, degrade and separate into individual bodies, a phenomenon known as membrane blebbing (Yuan, 1996). A family of cysteine-dependent aspartate-directed proteases, named caspases, are responsible for the proteolytic cleavage of cellular proteins, internucleosomal DNA fragmentation and propagation of death signalling. This leads to the engulfment of these apoptotic bodies by neighbouring macrophages and dendritic cells (Vermuelen et al. 2005). The process of apoptosis is different to other forms of cell death such as necrosis, which is a premature passive process that results in the breakdown of the cell membrane in response to environmental stress (Jin and El-Deiry, 2005).
1.5. Chromatin, The SWI/SNF Complex and Cancer

1.5.1. Chromatin

Eukaryotes have a vast amount of DNA that they need to store. In order to deal with this issue, they have evolved a mechanism whereby they compact their DNA into repeating arrays of nucleosomes, comprised of 146bp of DNA wrapped around an octamer of histone proteins – a tetramer of histone H3 and H4 flanked by two H2A and H2B dimers, otherwise known as chromatin (Luger et al. 1997). A fifth histone protein, H1, promotes chromatin structure of a higher order by facilitating the compaction of nearby nucleosomes at the entry and exit points. ‘Linker DNA’ connects these core proteins, which is typically 30-50bp in length (Kornberg and Lorch, 1999). Chromatin has been subdivided into two functionally distinct classes. Firstly, euchromatin which is decondensed in the interphase of the cell cycle and contains most of the actively transcribed genes, and secondly heterochromatin which remains condensed throughout the whole cell cycle and contains mostly inactive genes (Horakova et al. 2010; Verzijlbergen et al. 2009). Each core histone contains a globular domain and an N-terminal tail protruding from the nucleosome. The nucleosome is in a constant dynamic equilibrium between a fully wrapped state and a collection of partially unwrapped states. This unwrapping transiently exposes buried DNA sites for access. However, the conversion between these states is dynamic and rapid. Any process that requires access to DNA such as replication, transcription or DNA repair must therefore overcome this chromatin structural barrier (Li et al. 2005). In order to gain access to DNA, cells employ two main
cellular machineries: histone acetylases and chromatin remodelling complexes. Histone acetylases add acetyl groups to the N-terminal tails of histones that protrude from the nucleosome core. The modifications themselves vary, depending on the specific event required such as transcriptional activation, silencing and histone deposition (Smith and Peterson, 2005). Modifications can be reversed via histone deacetylases, which have a negative effect on transcription (Fig. 1.8; Muchardt and Yaniv, 2003).
Figure 1.8: Genetic information in eukaryotes in compacted into nucleosomes. These nucleosomes consist of a DNA helix wound around core histones, forming a ‘bead on a string’ structure as seen here. This is then folded into higher order chromatin. There are two states of chromatin – active and condensed. If the chromatin is in an accessible state, proteins can be recruited, allowing downstream cellular functions. This chromatin state is directly influenced by post-transcriptional modifications of the N-terminal histones, including acetylation (Ac), phosphorylation (P), poly (ADP)-ribosylation, ubiquitylation (Ub) and methylation (Me), which each play a varying role in chromatin maintenance. From Sparmann and van Lohuizen, 2006.
The association of DNA with histones in chromatin prevents DNA repair proteins from accessing photolesions. However, this barrier may be overcome if the DNA lesions change the structural properties of nucleosomes, promoting enzymes to repair these lesions. The cell has several mechanisms by which chromatin can be manipulated to access DNA. These include ATP-dependent chromatin remodeling complexes, incorporation of histone variants into the nucleosome and covalent histone modifications (Vaquero et al. 2003). During interphase, the relaxed chromatin of the eukaryotic cell undergoes a distinct change, resulting in the formation of a highly condensed mitotic chromosome.

UVB radiation is known to directly affect chromatin structure. Although chromatin is known to undergo conformational changes following DNA damage and during apoptosis, the direct evidence of this is limited and more research is required into UV effects on chromatin structure. The maintenance of genomic stability following UV-induced DNA damage in eukaryotic cells is a complex process, in which histone modifications and nucleosome remodelling via ATP-dependent chromatin remodelling complexes are tightly regulated. Exposure to UVR triggers the DNA damage response, prompting the remodelling of chromatin via its condensation (relaxation). This relaxation enhances the accessibility of DNA damage sites to repair machinery. From here, activation of checkpoint pathways arrests cell cycle progression, thus preventing transfer of damage to new cells (Ahn et al. 2004). From here, the cell undergoes repair and the DNA lesion is removed.
1.5.2. Chromatin remodelling complexes

Gene regulation is heavily regulated at various levels to ensure the transcriptome of the cell is appropriate for its developmental stage and cell type. The state of chromatin in which a gene is embedded determines its expression levels to a large extent. The activation or repression of transcription is accomplished by recruitment of various chromatin-associated multi-subunit protein complexes that combine both histone-modifying and chromatin-modifying activities. Nucleosomal structure is the platform for which many variations, modifications and binding of proteins all impact DNA and cell functions (Smith and Peterson, 2005). A number of chromatin remodelling complexes use ATP to alter chromatin structure, making the DNA accessible to the proteins that bind chromatin and regulate cellular processes. These chromatin-remodelling factors are important in the link between chromatin structure and cellular processes such as transcription, replication, recombination, aging, repair, cell cycle control, death signalling and responses to external stimuli such as UVR. DNA repair requires access to all DNA base pairs, and recombination requires access to long stretches of DNA. Hence, nucleosomes must be mobilised or ejected to provide rapid access (Chakravarthy et al. 2005).

ATP-dependent chromatin remodelling complexes (CRCs) are divided into 4 families, including the ISWI (imitation of switch), SWI/SNF, INO (inositol) and CHD (chromodomain helicase/ATPase DNA binding protein) families. All of these CRCs are important in the chromatin remodelling process, and exhibit mechanistic differences (Fig. 1.9; Imbalzano and Xiao, 2004). Within each of these CRCs is a
helicase-like subunit of the SWI2/SNF2 family of SNF2 ATPases. These complexes are large proteins that contain a number of domains including bromodomains, chromodomains, pleckstrin homology domains, SANT domains and AT hook regions. These domains play a role in the stabilisation of the interaction between the CRC and histones and/or DNA (Boyer et. al 2000). CRCs contact both DNA and histones, allowing for nucleosome repositioning or removal in an ATP-dependent fashion. Although they are known to play a role in transcription, recently many of these CRCs have been found to play key roles in the repair of DSBs in yeast, functioning by allowing access of repair proteins and critical histone modifications at the DSB site following DNA damage (Imbalzano and Xiao, 2004).

Core histones are mostly globular molecules, although they also possess an unstructured N-terminal tail where many post-translational modifications occur (Vaquero et al. 2003). Phosphorylation, ubiquitylation and methylation of proteins play a primary role in the DNA damage response by facilitating access of various repair proteins to DNA breaks, as well as cell signalling and promoting chromosomal stability. Acetylation plays an important role downstream of these modifications, important for cell cycle progression and the stabilization of chromatin post DNA damage (Altaf et al. 2007). In human cells, CRCs have been found to play important and various roles in cell differentiation, development and tumour suppression (Huang et al. 2003). For example, SWI/SNF has been found to be necessary for the development of bone by osteoblast differentiation (Young et al. 2005) as well as for the development of neural tissue (Seo et al. 2005).

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Figure 1.9. The four subfamilies of the human SWI/SNF complex (green) contain well-characterised complexes (red). Each one of these complexes contains at least one ATPase subunit (yellow), which mediate interactions with other epigenetic factors. From Tang et al. 2010.

Although the complete mechanism by which CRCs remodel chromatin is not yet completely elucidated, 2 possible mechanisms have been proposed. One mechanism involves the twisting of DNA over nucleosomes in a cork screw-like motion. This remodelling activity applies torsional force to DNA and alters its twist on the surface of the nucleosome histone core. Although this mechanism is a common characteristic of some chromatin remodelling, experiments using branched DNA, DNA nicks or beads attached to certain positions within nucleosomes indicate
that this mechanism is unlikely to be a major mechanism by which SWI/SNF mobilizes nucleosomes (Owen-Hughes, 2003). Current evidence supports the other major class of mechanism, which involves the formation of a bulge or loop on the surface of the histone octamer. The DNA at the edge of the nucleosome has been found to be more loosely associated with the core histone octamer than DNA at the centre of the nucleosome (Polach and Widom, 1995). Disassociation of DNA at this site as well as at a different site downstream would result in the formation of a loop of DNA that extends from the nucleosome. Such loops could then propagate around the core histone octamer in a similar fashion to the mechanism employed by RNA polymerases (Fig 1.10; Studitsky et al. 2004; Halliday et al. 2009). Recent evidence using atomistic molecular dynamics simulations, which allow the detailed structure and dynamic of nucleosomes to be assessed, also support the loop theory. This evidence suggested a key role of DNA kinking within loops, allowing the relaxation of DNA bending strain to be coupled with improved DNA-core interactions. Further, the loop position was found to influence the dynamics of DNA at the extremities of the nucleosome (Pasi and Lavery, 2016).
Figure 1.10: The model for nucleosome mobilisation via the SWI/SNF complex using the bulge mechanism. DNA (black) is wound around the core histone octamer (blue) (A, B). The SWI/SNF complex (red) binds to the DNA, inducing the formation of a loop or bulge of DNA (C, D). This loop is then propagated around the core histone octamer, with no net change in energy (E). The loop of DNA is then released, resulting in a nucleosome that has been mobilised along the DNA strand (F). From Halliday et al. 2009.

1.5.3. The SWI/SNF complex

The human SWI/SNF complex is a large, highly conserved, multi-subunit complex that is approximately 2 MDa in size. Components of the SWI/SNF complex were originally identified in screens for genes that regulate mating-type switching (SWI) and sucrose non-fermenting (SNF) phenotypes in yeast (Neigeborn et al. 1984; Stern et al. 1984; Breeden et al. 1987). The components of SWI/SNF consist of 9-12 subunits depending on the species, comprising a large multisubunit complex
that possesses ATP-dependent nucleosome remodelling activity in vitro (Smith et al. 2003). These subunits have a molecular mass of ~2 MDa in mammals and 1.14 MDa in yeast (Euskirchen et al. 2012). The development of the SWI/SNF complex over time between species reflects an increasing complexity of chromatin via evolution. Higher eukaryotes exhibit an increased genome size, the presence of DNA methylation and more complex genetic organisation. Thus, the major components of the SWI/SNF complex have been conserved over time. This is likely to preserve overall shape and chromatin remodelling activity, but could also be due to a need for the addition of components that harbour more specialised or tissue-specific roles (Tang et al. 2010).

In all SWI/SNF complexes, there is an ATPase subunit, being either Brm (Brahma) or Brg1 (Brahma-related gene-1). Brg1 and Brm are both highly conserved ATPases of the mammalian SWI/SNF complexes. The mammalian complex is also referred to as BAF. In official nomenclature, the genes encoding BAF subunits are called SWI/ SNF-related, matrix-associated, actin-dependent regulator of chromatin (SMARC) genes (Ring et al. 1998). In mammals there are two major subfamilies of the SWI/SNF complex, BAF (Brg1-associated factor) and pBAF (polybromo Brg1-associated factor) (Fig. 1.11). These two subfamilies are similar in their subunit composition, sharing 8 subunits in total (Brg1, BAF170, BAF155, BAF60A, BAF57, BAF53, β-actin and BAF47). However, BAF contains BAF250A and BAF250B, whilst pBAF contains BAF180 and BAF200 (Wilson and Roberts, 2011). An important difference between these two subfamilies is that only pBAF is able to facilitate ligand-dependent transcriptional activation by nuclear receptors in vitro.
These mammalian SWI/SNF complexes are always comprised of several highly conserved core subunits, including one of two mutually exclusive catalytic ATPase subunits; either BRM (also known as SMARCA2 – not present in pBAF) or BRG1 (also known as SMARCA4), as well as a set of other core subunits; SNF5 (also known as Ini1), BAF155 and BAF170. Other various subunits are also present and these are thought to regulate lineage-specific functions of the complexes. These include the BAF53, BAF60, BAF57, BAF45 and β-actin subunits, which are all shared by both BAF and pBAF. The complexes vary in that BAF contains one of either AT-rich interactive domain containing protein 1A (ARID1A) and ARID1B subunits, whereas the BAF180, BAF200 and bromodomain-containing 7 (BRD7) subunits are exclusively present in pBAF complexes (Table 1; Wilson and Roberts, 2011).
Table 1: The various subunits of the human SWI/SNF complex and their functions.

<table>
<thead>
<tr>
<th>Subunit</th>
<th>Gene</th>
<th>Size (kDa)</th>
<th>Function</th>
</tr>
</thead>
<tbody>
<tr>
<td>BRG1</td>
<td>SMARCA4</td>
<td>190</td>
<td>Core subunit, ATPase activity, transcription regulator</td>
</tr>
<tr>
<td>BRM</td>
<td>SMARCA2</td>
<td>190</td>
<td>BAF core subunit, ATPase activity, transcription regulator</td>
</tr>
<tr>
<td>BAF155</td>
<td>SMARCC1</td>
<td>155</td>
<td>Actin dependent regulator of chromatin, highly homologous to BAF170</td>
</tr>
<tr>
<td>BAF170</td>
<td>SMARCC2</td>
<td>170</td>
<td>Actin dependent regulator of chromatin, highly homologous to BAF155</td>
</tr>
<tr>
<td>BAF180</td>
<td>PBRM1</td>
<td>180</td>
<td>Contains 5 bromodomains, negative regulator of cell proliferation</td>
</tr>
<tr>
<td>BAF60A</td>
<td>SMARCD1</td>
<td>60</td>
<td>Actin dependent regulator of chromatin, interacts with transcription factors</td>
</tr>
<tr>
<td>BAF60B</td>
<td>SMARCD2</td>
<td>60</td>
<td>Actin dependent regulator of chromatin, interacts with transcription factors</td>
</tr>
<tr>
<td>Gene</td>
<td>Protein Name</td>
<td>Position</td>
<td>Function</td>
</tr>
<tr>
<td>-------------</td>
<td>--------------</td>
<td>----------</td>
<td>--------------------------------------------------------------------------</td>
</tr>
<tr>
<td>BAF60C</td>
<td>SMARCD3</td>
<td>60</td>
<td>Actin dependent regulator of chromatin, interacts with transcription factors</td>
</tr>
<tr>
<td>BAF57</td>
<td>SMARCE1</td>
<td>57</td>
<td>Binds the minor groove of DNA, interacts with transcription factors</td>
</tr>
<tr>
<td>BAF53A</td>
<td>ACTL6A</td>
<td>53</td>
<td>Chromatin association, enhances ATPase activity</td>
</tr>
<tr>
<td>BAF53B</td>
<td>ACTL6B</td>
<td>53</td>
<td>Chromatin association, enhances ATPase activity</td>
</tr>
<tr>
<td>BAF47/SNF5</td>
<td>SMARCB1</td>
<td>47</td>
<td>Binds BAF155 and BAF170 directly, important in cell proliferation and differentiation</td>
</tr>
<tr>
<td>BAF250A</td>
<td>ARID1A</td>
<td>250</td>
<td>Contains ARID DNA binding element, subunit of BAF</td>
</tr>
<tr>
<td>BAF250B</td>
<td>ARID1B</td>
<td>250</td>
<td>Contains ARID DNA binding element, subunit of BAF</td>
</tr>
<tr>
<td>BAF200</td>
<td>ARID2</td>
<td>200</td>
<td>Contains ARID DNA binding element, component of pBAF</td>
</tr>
<tr>
<td>β-actin</td>
<td>ACTB</td>
<td>45</td>
<td>Required together with BAF53 for maximum ATPase activity of BRM/BRG1</td>
</tr>
</tbody>
</table>
1.5.4. SWI/SNF and DNA structure

SWI/SNF is known to play a role in the alteration of nucleosomal structure by utilising ATP hydrolysis. A genetic screen for suppressive mutations of SWI/SNF phenotypes identified various histone and chromatin components, suggesting SWI/SNF was involved in histone binding and chromatin organization (Winston and Carlson, 1992). Since then, it has been shown that SWI/SNF is recruited to chromatin, where it hydrolyses ATP and uses this energy to remodel nucleosomes (Hirschorn et al. 1992). Although SWI/SNF contains between 9-12 subunits, only the core subunits (Brg1 or Brm, SNF5, BAF155 and BAF170) are required in vitro to remodel nucleosomes at a rate comparable to the entire complex (Phelan et al. 1999). Epigenetic associations also contribute to SWI/SNF and chromatin associations. For instance, the bromodomains found in Brm and Brg1, as well as several other subunits, are known to recognise acetylated lysines in histones, contributing to chromatin structure and organisation (Singh et al. 2007). All SWI/SNF subunits comprise protein-protein or DNA binding domains, thus all are important for chromatin targeting and the remodelling process.

Chromatin dynamics also have a profound influence on transcriptional regulation. Evidence that SWI/SNF was involved in transcriptional regulation came when microarray analysis in yeast showed that approximately 5% of all yeast genes were regulated by SWI/SNF at the transcriptional level. This data also showed that SWI/SNF acts as not only an activator of transcription, but also a repressor (Sudarsanam et al. 2000). The activity of SWI/SNF results in many biochemical
outcomes, including transient exposure of nucleosomal DNA and nucleosome movement in the form of sliding and displacement (Clapier and Cairns, 2009). The Brm and Brg1 proteins employ the energy of ATP hydrolysis to weaken the interaction between histone core particles and DNA. This results in localised disruption of DNA-histone contacts or mobilization of the nucleosomes on the chromatin fibre (Fig 1.12). This allows for contact and binding of various transcription factors to specific sites of DNA, thus mediating activation or repression of expression, dependent on the transcription factor. Thus, SWI/SNF is able to regulate the expression of many genes. The fact that SWI/SNF is able to regulate so many genes is likely the primary reason why it plays a pivotal role in development and the prevention of various diseases. The inactivation of core SWI/SNF subunits such as Brg1 and SNF5, lead to altered chromatin remodelling patterns, with disruptions in specific nucleosome patterning, combined with a loss of overall nucleosome occupancy at a large number of promoters (Tolstorukov et al. 2013).
Figure 1.12: The various effects of nucleosome remodelling. (a) Remodellers (in green) such as SWI/SNF assist in the chromatin assembly by moving deposited histone octamers, generating additional room for histone deposition. (b) DNA-binding proteins (DBP) become accessible via nucleosomal repositioning, ejection or unwrapping. (c) Nucleosome content is modified by dimer replacement containing a histone variant or through dimer ejection. From Clapier and Cairns, 2009.

1.5.5. SWI/SNF, Brm and the cell cycle

Recent evidence has shown that the SWI/SNF complex plays an important role in many cellular events in various human tissues. This link is likely due to the
large number of genes that are transcriptionally activated by SWI/SNF, such as p53, EZH2, E2F and beta-catenin. Mammalian SWI/SNF complexes are integral in the dynamic transcriptional regulation of a large array of genes, including cell cycle regulators, signalling proteins, molecules involved in cell architecture and adhesion to the extracellular matrix (Krebner et al. 2013).

The ATPase subunit of SWI/SNF, Brm, was first identified as one of the trithorax group proteins, which are known to antagonize the function of polycomb group (PcG) proteins (Tamkun et al. 1992). Based on homology with *Drosophila* BRM, two genes from mammals were cloned encoding the Swi2/Snf2 homologs Brm and Brg1. Upon fusion with a DNA-binding domain, both of these activated transcription (Muchardt and Yaniv, 1993). Further, trans-heterozygote Brm mutant flies display homeotic transformations similar to those provoked by reduced expression of homeotic genes. Fully homozygous Brm mutants die as unhatched larvae, indicating that the function of this protein is not limited to homeotic gene expression (Tamkun et al. 1992). Further, expression of a dominant-negative Brm allele caused peripheral nervous system defects and decreased viability (Elfring et al. 1998).

The two ATPase subunits of SWI/SNF, Brm and Brg1, employ the energy of ATP hydrolysis to weaken the interaction between histones and DNA. This can eventuate in either localised disruption of the DNA-histone contacts or mobilisation of the nucleosomes on the chromatin fibre (Fig 1.12). This facilitates the binding of various transcription factors downstream that can access specific sites on the DNA,
thus mediating either activation or repression of gene expression. Therefore, it is unsurprising that Brm and Brg1 are known to be master regulators of the genome (Murchardt and Yaniv, 1993). Both Brm and Brg1 contain a bromodomain, a motif found in several transcription factors. There is a large degree of homology between the two subunits, showing 86% genetic similarity (Murchardt and Yaniv, 1993). The expression of Brm and Brg1 varies in normal tissue. Brg1 is found in tissues constantly undergoing proliferation or self-renewal; however, Brm is expressed in tissues not routinely undergoing proliferation such as brain, liver, fibromuscular stroma and epithelial cells (Reisman et al. 2005). Several cell line models are used in Brm/Brg1 studies, in particular the cervix carcinoma-derived cells C33A and the human adrenal carcinoma line SW13, which contain either very low or undetectable levels of both subunits (Murchardt and Yaniv, 1993). Further screening has shown many other cell lines exhibiting Brm and Brg1 mutations or loss. Observation of the SWI/SNF complex in HeLa cells at varying phases of the cell cycle has shown that Brg1 and Brm are phosphorylated at the G2/M transition (Muchardt et al. 1996). Following this, Brm but not Brg1, was targeted for degradation and was only resynthesised following exit from mitosis. This finding suggested that accumulated levels of Brm can be specifically regulated upon re-entry to the cell cycle (Muchardt et al. 1996). Further to this, it has been shown that Brm levels are high in cells arrested in G0 by serum starvation, but rapidly drop following addition of serum (Reyes et al. 1998). Other observations also suggest a link between increased cell growth and low levels of Brm. In cells transformed by various oncogenes, the level of Brm was found to be drastically reduced, suggesting that these oncogenes are
targeting Brm (Muchardt et al. 1998). Furthermore, Brm re-expression in KRAS-transformed cells partially reversed the transformed phenotype and substantially slowed growth (Muchardt et al. 1998). Together, these findings show that increased Brm expression promotes the entry of cells into G1/G0 cell cycle arrest, while the loss of Brm seems to correlate with a defect in cell cycle control, leading to oncogenic transformation. Some human tumour lines exhibit mutations of Brg1, and reintroduction of this protein into these lines reverses their transformed phenotype (Wong et al. 2000). Moreover, regular SW13 cells treated with low-dose UV fail to maintain a pause at the G1/S boundary. However, when Brg1 was re-expressed in these cells, this pause was maintained and the cells were much less sensitive to UV. Therefore there is a checkpoint deficiency in SW13 cells in response to UV that can be corrected by the re-expression of Brg1 (Gong et al. 2008).

The most thoroughly documented link between SWI/SNF and the regulation of the cell cycle concerns the interaction between Brm/Brg1 and the retinoblastoma protein (Rb) (Dunaief et al. 1994). Rb is one of the major cell cycle regulators in control of the transition of the cell cycle from G1 to S-phase as well as the progression through this phase. Both Brm and Brg1 have been shown to interact with the tumour suppressor Rb gene product. A major target of the Rb complex is the transcription factor, E2F1, which induces the S-phase of the cell cycle. However, to mediate complete G1 cell cycle arrest, Rb also requires the presence of Brm/Brg1, via direct interaction (Strobeck et al. 2000; Liu et al. 2004). Altogether, Rb and Brm cooperate to suppress the activity of E2F1, in order to induce G1 arrest (Trouche et al. 1997). Serum starvation has a decreased effect on Brm -/- fibroblasts, suggesting
that Brm is vital for a proper cell cycle arrest response. Further to this, these cells also spend twice as long in the G2-M phase, suggesting Brm-/- cells exit less rapidly from G2/M phase compared to cells with normal Brm levels (Coisy-Quivy et al. 2006). Intriguingly, in many studies, the inactivation of SWI/SNF is considered to be an alternative method for inactivating the Rb pathway. It is very likely that the disruption of the Rb pathway in many tumour types is caused by the loss of SWI/SNF activity (Marquez et al. 2014). Although it is not entirely clear what respective roles Brm and Brg1 play in Rb-mediated growth arrest, it is clear that both proteins are able to cooperate with Rb upon overexpression (Strober et al. 1996).

The inactivation of Brg1 by homologous recombination in mice is embryonic lethal at a preimplantation stage, and tumours are found in heterozygous mice (Bultman et al. 2000). In contrast, the inactivation of the Brm gene by homologous recombination in mice does not show any developmental phenotypic results, except for an average 14% weight increase in Brm homozygous mutants as compared to their wildtype littermates (Reyes et al. 1998). The loss of Brm in these mice also led to a marked increase in Brg1 expression, suggesting the two subunits are able to compensate for one another. Due to the increase in weight seen in Brm-/- mice, the growth of mouse embryo fibroblasts was tested. It was found that Brm-/- cells had a marked increase in cell proliferation, as well as increased cells in both S-phase and G2-M phase. Further to this, Brm-/- cells exhibited a defective G1 arrest phase following treatment with 10J/m² UVB irradiation, suggesting that Brm plays a key role in cellular proliferation and cell cycle control (Reyes et al. 1999). These findings
suggest that while Brm may have a regular effect on cellular proliferation, when in combination with carcinogens such as UVR, this effect is further exacerbated, leading to increased tumour development.

SWI/SNF has also been shown to interact with the tumour suppressor protein, p53. p53 is highly conserved, and works as a central factor in cellular stress responses such as apoptosis, cell cycle arrest, DNA repair and oxidative stress (Levine, 1997). In 2002, Lee and colleagues discovered that several subunits of the SWI/SNF complex, including Brg1 and SNF5, associate with p53 in vitro and in vivo (Lee et al. 2002). The transcriptional activity of p53 was further enhanced when expression plasmids encoding SNF5 and Brg1 were co-transfected with p53. In contrast, cells exhibiting dominant negative SNF5 and Brg1 substantially inhibited p53-dependent transcription. Further, dominant negative SNF5 and Brg1 were able to prevent p53-mediated apoptosis and growth arrest. This finding implied that SWI/SNF is necessary for p53 transcription and activity such as apoptosis and growth arrest. In 2007, Xu and colleagues also found that Brm plays a key role in the activity of p53 in many cell models, especially cells that are deficient in Brg1. In MCF7 cells, knockdown of Brg1 but not Brm impaired p21 induction. However, in Brg1-deficient H1299 cells, p21 induction was also decreased by Brm knockdown (Xu et al. 2007). Likewise, if Brg1 was knocked down in MCF7 cells, Brm was again necessary for p21 induction. In contrast, Brm and Brg1 were not found to play a role in p53-mediated induction of MDM2. This data showed that Brm and Brg1 differentially regulate a subset of p53 target genes (Xu et al. 2007). Together, this data shows that SWI/SNF is an important regulator of cellular responses. It does
this by allowing the access of many genes to DNA, allowing the downstream transcription of various genes, enhancing the ability of the cell to adequately respond to carcinogens such as UVR via cell cycle responses.

1.5.6. SWI/SNF, Brm and DNA repair

Recent studies show that damage recognition and the selection of appropriate repair machinery is highly dependent on the structure of chromatin surrounding the DNA damage lesions. In particular, the compaction/condensation of chromatin and the presence of active transcription prior to damage appear to be important in the selection of an appropriate repair pathway (Aymard et al. 2014). Previous experiments utilising reconstituted nucleosomes containing DNA with 6-4PPs or CPDs showed that the presence of the lesions did not affect the reconstitution of the nucleosomes in vitro, however it did lead to the dynamic unwrapping of chromatin leading to a relaxed state (Duan and Smerdon, 2010). This suggests that UV damage causes changes in the conformation of the nucleosome, thus allowing access to factors that change the chromatin structure, permitting entry of various factors involved in DNA repair.

Previously, it has been reported that SWI/SNF stimulates the removal of 6-4PPs, but not CPDs from nucleosomal DNA (Hara and Sancar, 2003). Other studies have shown that the damage-recognition heterodimer Rad4-Rad23 in yeast links with SWI/SNF (Gong et al. 2006). Rad4-Rad23 recognises the DNA lesion, which then recruits SWI/SNF to the lesion in order to facilitate NER. Furthermore,
SWI/SNF inactivation led to a slower removal of CPDs from the silent HML locus in yeast (Gong et al. 2006). The Brm/Brg1 deficient cell line SW13 is known to be very sensitive to the effects of UV radiation (Gong et al. 2008). However, the re-expression of Brg1 in these cells makes them drastically more resistant to UV radiation. In SW13+Brg1 cells, there was a large decrease in apoptotic cells in comparison to unreconstituted SW13 cells. Moreover, the repair of CPDs in SW13+Brg1 cells was much higher over time as compared to normal SW13 cells, suggesting that functional SWI/SNF is necessary for adequate CPD removal (Gong et al. 2008). However, the re-expression of Brg1 in SW13 cells did not affect the repair rate of 6-4PPs. SW13+Brg1 cells exhibited vastly increased levels of the P53-regulated proteins P21 and GADD45 following UV-induced damage, suggesting that SWI/SNF promotes apoptosis via induction of these two cell cycle control proteins (Gong et al. 2008). Moreover, p21 expression was upregulated in SW13+Brg1 cells. In agreement with these results, it was found in human normal cells with Brg1 knockdown that the removal of UV-induced CPDs but not 6-4PPs was significantly affected (Zhao et al. 2009). Unfortunately the role of Brm was not tested in these cells, however it is possible that if Brm were altered it would have played a similar role to alteration of Brg1.

SWI/SNF is also known to interact directly with BRCA1, where it plays a role in BRCA1-mediated DNA repair (Bochar et al. 2000). The BRCA1 tumour suppressor protein is mutated in approximately 90% of familial breast cancers and 50% of inherited breast and ovarian cancers. The BRCA1 protein is important for DNA repair and is a co-activator of p53-mediated transcription. The inactivation of
BRCA1 leads to genetic instability (Muchardt and Yaniv, 2001). The role of BRCA1 in UV damage has also been studied. In response to UV radiation, BRCA1 interacts with ATR, and also contributes to the recruitment of the RPA complex to damaged DNA, suggesting BRCA1 plays a role in UV-induced DNA repair (Tibbetts et al. 2000; Kranz et al. 2008). Moreover, in HeLa cells, active Brg1 and ATR are required for the efficient recruitment of BRCA1 to UV-induced CPD sites. The depletion of Brg1 in HeLa cells also led to a decrease of BRCA1 and RPA phosphorylation in response to UV irradiation (Zhang et al. 2013). These data suggest that SWI/SNF modulates BRCA1-mediated repair in response to UV irradiation by regulating ATR/ATM activation.

The SWI/SNF complex binds to H2A histone variant H2AX (γ-H2AX) at Serine-139 on the C-terminal tail, which serves as a binding site for repair and checkpoint proteins, allowing for DNA repair events to be facilitated. Knockout of the SWI/SNF complex results in inefficient CPD removal and increased DNA sensitivity following DNA damage (Chai et al. 2005; Gong et al. 2008; Park et al. 2006). Therefore, the SWI/SNF complex promotes H2AX phosphorylation by directly acting on chromatin. Lee and colleagues found that the catalytic ATPase subunit of SWI/SNF, Brg1, binds to γ-H2AX nucleosomes by interacting with acetylated histone 3 through its bromodomain (Lee et al. 2010). When Brg1 was deleted, they found that this caused a significant defect in the repair of DSBs. If SWI/SNF or Brg1 are inactivated, the cells became highly susceptible to DNA-damage induced apoptosis due to a prolonged activation of p53 (Park et al. 2009; Lee et al. 2010).
It should also be noted that SNF5, another subunit of the SWI/SNF complex, is important in the DNA damage response. Knockout of SNF5 led to a hypersensitive DNA damage response, and also an aberrant apoptotic effect and change in p53 activity, leading to a proliferative defect in cells (Kratzmeier et al. 2000; Nag et al. 2009). Other studies have also shown that SNF5 interacts with UV damage recognition factor XPC and co-localises with XPC at the damage site. SNF5 deficiency led to a defect in downstream ATM and H2AX phosphorylation (Goebel et al. 2007).

Collectively, these data show that ATP-dependent CRCs form a cooperative feedback activation loop with various repair factors and checkpoint proteins. This heavily influences the repair of both single and double stranded DNA lesions. Further, upon inefficient DNA repair, SWI/SNF can influence the apoptotic response of a cell. This response is especially critical in the response to UVR. However, if there is a defect in apoptosis, it is likely that the cell will proliferate while harbouring a mutation or DNA lesion, leading to further progeny with defects. The accumulation of these mutations and defects are likely to lead to oncogenic transformation.

1.5.7. The role of SWI/SNF and Brm in cancer

Although early evidence such as the loss of SWI/SNF subunits in cancer cells suggested that SWI/SNF may play a role in cancer, the first definitive evidence that SWI/SNF played an involvement in human cancer came in the late 1990's, when it was discovered that SNF5 mutations were present in a large majority of malignant rhabdoid tumours, a rare but highly aggressive type of cancer in children (Versteeg
et al. 1998). This was followed by studies using mice with SNF5 deletion, which found that SNF5-/ mice die at early embryonic stage, and 30% of SNF5 heterozygotes develop tumours in brain and soft tissues, resembling rhabdoid tumours. These tumours also lose the SNF5 wildtype allele (Roberts et al. 2000; Klochendler-Yeivin, 2000).

Since the novel discovery of the connection between SNF5 and rhabdoid tumours, the next clue that SWI/SNF played a role in cancer was in 2004 when frequent mutations of Brg1 in lung cancer were first observed (Medina et al. 2004). Following these findings, sequencing allowed for the widespread analysis of SWI/SNF in a variety of cancers. Whole-exome analysis has found SWI/SNF to be mutated at high frequency, implicating a spectrum of SWI/SNF subunits as playing a role in 18 cancer types (Shain and Pollack, 2013). Collectively, SWI/SNF mutations are known to occur in approximately 19-20% of human cancers, which is a similar total to p53, which is known to be the single-most mutated tumour suppressor gene (Fig 1.13; Kadoch et al. 2013). The highest mutation rates were in ovarian clear cell carcinoma (75%), clear cell renal cell carcinoma (57%), hepatocellular carcinoma (40%), gastric cancer (36%), melanoma (34%), and pancreatic cancer (26%)(Kadoch et al. 2013). SWI/SNF is also subject to more deleterious mutations (frameshift, nonsense, rearrangement, splice-site and missense-damaging mutations) in comparison to other frequently mutated genes (Fig 1.14; Shain and Pollack, 2013). This suggests that SWI/SNF mutations are likely driver mutations. Interestingly, almost all studies show only one highly mutated SWI/SNF subunit per cancer type. Some of these subunits such as Brg1 and ARID1A are mutated more
frequently than others, which suggests that some subunits may be preferentially targeted (Shain and Pollack, 2013). However, it is clear that there is an array of different SWI/SNF mutations seen in human cancer, giving rise to a spectrum of different tumour types. The mechanism by which each mutation of each individual subunit promotes tumourigenesis, as well as the function of each SWI/SNF mutation is now an active field of scientific investigation. It has been found that SWI/SNF is highly mutated in melanoma. In 29 studied cases, 17 mutations struck SWI/SNF subunits, including the mutation of ARID1A (n=5), Brg1 (n=4), SNF5 (n=3), ARID2 (n=3), Brm (n=1) and BAF155 (n=1) (Shain and Pollack, 2013). This included a homozygous mutation in ARID2 and three mutations targeting SNF5 in the same sample. Interestingly, Brg1 expression is increased in primary and metastatic melanoma. The knockdown of Brg1 in melanoma cell lines results in significantly reduced cell proliferative ability (Lin et al. 2010). It has now been shown that SWI/SNF interacts with SOX10 and MITF, leading to the activation of melanocyte and Schwann-cell specific genes, which are necessary for the promotion of melanoma tumourigenesis (Mehrota et al. 2014). These observations conflict with the fact that SWI/SNF is commonly mutated in melanoma, however it seems that Brm and Brg1 are rarely disrupted, suggesting that these mutations may compromise SWI/SNF activity but not completely ablate it.
Figure 1.13: The wide spectrum of SWI/SNF mutants in human cancers. 18 tumour types were surveyed, and the corresponding results were plotted on a bar graph. This study encompassed 24 whole-exome studies, together spanning 18 diverse cancer types. Ovarian, renal cell and hepatocellular cancers in particular show a large percentage of SWI/SNF mutations. Altogether this shows an average of 19% of tumours harbour a SWI/SNF mutation. From Shain and Pollack, 2013.
Figure 1.14: The frequency distribution for types of SWI/SNF mutations as compared to all genes. Note that SWI/SNF mutations are more prone to deleterious mutations. *From Shain and Pollack, 2013.*

As previously mentioned, SWI/SNF can bind to the Rb protein, facilitating repression of its target genes. The downregulation of Rb can lead to the further inactivation of genes such as p16 and E2F, leading to an altered cellular proliferation pattern. Further, SWI/SNF also interacts with Myc, both as an activator and a repressor (Helming et al. 2014). SWI/SNF is also implicated in the regulation of
many cell cycle genes, including the INK4A-ARF locus, p16, p21, p53, cMyc, cyclin D and cyclin E, and has been shown to directly regulate several of these genes (Ruijtenberg and van den Heuvel, 2016). For example, reintroduction of SNF5 into tumour cells results in the upregulation of p15 and p16 expression and the arrest of cell division (Wilson et al. 2010). Down-regulation of SWI/SNF also leads to the loss of p21 expression, while reexpression of SWI/SNF in malignant rhabdoid tumour cells induces p21 levels, independently of p53 (Kuwahara et al. 2010). Further, loss of SWI/SNF subunits also leads to the elevated levels of the Polycomb gene, EZH2. In a genetically engineered in vivo tumour model, inactivation of EZH2 completely blocked tumour onset driven by SNF5 loss (Wilson et al. 2010). In total, SWI/SNF is known to be able to bind to the promoters of roughly one-third of all genes, making SWI/SNF a master regulator of the genome (Tolstorukov et al. 2013). Thus, it is not surprising that SWI/SNF plays a role in tumour suppression in so many cancers.

As a core subunit of SWI/SNF, Brg1 has been reported to be silenced, deleted or mutated in an array of cancers, including many cancer cell lines, as well as primary tumours (Bartlett et al. 2011). Brg1 knockout mice are embryonic lethal, and a small percentage of Brg1 heterozygous mice develop breast cancers (Bultman et al. 2000). Brg1 is frequently mutated in lung cancer, where it is found to be mutated in over 30% of lung cancer cell lines and is also lost in 10-50% of lung cancer specimens (Reisman et al. 2003). Brg1 mutations have also been identified in Burkitt lymphoma, medulloblastoma and esophageal cancer (Wu et al. 2012).
Brm is also mutated in human cancers, however these are more rarely found as compared to Brg1 mutations. In a mouse model in which lung tumours were induced by exposure to carbamate ethyl, inactivation of one or both Brm alleles led to a significant increase in the number of tumours (Glaros et al. 2007). Brm protein is also absent in human lung, gastric and prostate cancers (Oike et al. 2014). Further, low Brm expression in non-small-cell lung cancer correlates with a poorer prognosis, whereas co-expression of both Brm and Brg1 had the best prognostic outcome (Reisman et al. 2003; Fukuoka et al. 2004). Brm protein is also reduced in 67% of human gastric cancer specimens (Yamamichi et al. 2007). In one study a clear correlation between Brm deficiency and the histologic appearance of gastric malignancy was observed (Yamamichi et al. 2007). Brm is also implicated in prostate cancer development. In a mouse model, the loss of Brm was found to induce hyperplasia of the prostatic epithelia (Shen et al. 2008). Further, a significant decrease in Brm expression was observed in human prostate tumours. The loss of Brm expression was found to deregulate E2F1 activation, conferring a proliferative advantage, thus driving oncogenic transformation (Shen et al. 2008). Moreover, in Brm expression screens of various tumours, Glaros and colleagues found that this protein was also lost in approximately 15% of transitional cell bladder cancers, 9% of esophageal adenocarcinoma, 15% of breast cancers and 10-22% of ovarian cancers (Glaros et al. 2007). Recent research has also shown that concomitant loss of Brm and Brg1 is a hallmark for small cell carcinoma of the ovary and that Brm polymorphisms are present in pancreatic cancer, which were associated with poorer survival (Jelinic et al. 2016; Segedi et al. 2016). According to the most recent TCGA data, Brm was further found to be mutated in 3/49 SCC samples,
6/39 SCC samples, 29/818 malignant melanoma and 2/17 desmoplastic malignant melanoma (Sharpe et al. 2015; Pickering et al. 2014, Sanborn et al. 2015; Shain et al. 2015). This evidence shows that both Brm and Brg1 are important tumour suppressor genes, which are frequently lost or mutated in a variety of cancers.

Recently, it was shown that several subunits of SWI/SNF, including Brm, interact with SCAI (suppressor of cancer cell invasion), a protein that inhibits the invasive migration of human tumour cells through the control of MAL/SRF signalling. When Brm was silenced via siRNA in human cancer cells, this abolished the transcriptional activity of SCAI, indicating that a functional SWI/SNF complex is necessary for SCAI to suppress promoter activity. Further to this, when Brm was silenced in addition to SCAI, this led to an increase in cell invasion into 3D matrigel matrices. This data indicated that SCAI and SWI/SNF interact to control gene expression in human cancer cells to regulate invasive cell migration (Krebner et al. 2013).

Although Brg1 and Brm share high degrees of homology and overlapping functions, other observations suggest they may play different roles in cancer. It has also been found that depletion of Brm in Brg1-mutant lung tumours is synthetically lethal, meaning that while depletion of Brg1 alone is not lethal to the cell, the depletion of both Brm and Brg1 together are lethal (Oike et al. 2013). In 2013, Oike and colleagues screened siRNA against a panel of Brg1 mutant lung cancer cell lines, and found that the further inhibition of Brm significantly decreased cell viability compared with control siRNA, where Brg1 wildtype cells were unaffected (Oike et al. 2013). Another group studied this same relationship, and found that the inactivation of Brg1 led to greater incorporation of Brm into
the SWI/SNF complex. By screening 165 cancer cell lines, Brm was found to be the main essential gene for cell survival, even more so than p53, in Brg1 mutant cell lines (Wilson et al. 2014). The dependence on Brm in these cell lines was due to the loss of Brg1 leading to a greater dependence on Brm signalling. Moreover, depleted Brm in Brg1-mutant cell lines leads to cell cycle arrest and induction of senescence. It was proposed that the synthetic lethality might be explained by paralog insufficiency, where a loss of one family member leads to a critical dependence of paralogous subunits (Hoffman et al. 2014). This concept of paralogous insufficiency in SWI/SNF is not unique to just Brg1 and Brm. It has also been found that in cancers with ARID1A mutations, ARID1B is especially vulnerable and is the main gene necessary for the survival of ARID1A-deficient cancer lines and primary cells (Helming et al. 2014).

Similar to Brg1, Brm is silenced in a spectrum of solid tumour types, at similar rates to the frequency of KRAS and HER2 alterations. However, unlike the majority of tumour suppressor genes that are irreversibly mutated, Brm is reversibly suppressed. Therefore this makes Brm a much more suitable candidate for therapy, as it can be reactivated. Broad-acting histone deacetylase complex (HDAC) inhibitors such as suberoylanilide hydroxamic acid, trichostatin A and butyrate are able to readily induce Brm expression in Brm-deficient cell lines (Glaros et al. 2007). Unfortunately however, these compounds were also found to inactivate Brm via C-terminal acetylation, thereby impeding the use of these compounds to restore Brm function clinically (Kahali et al. 2014). Restoring Brm expression may be a valuable clinical target in several tumour types, especially in Brg1-deficient cancers. However, it is still not clear which HDAC inhibitors could be useful for this purpose. It is likely that currently
available compounds may not be suitable, as most are relatively nonspecific and inhibit many other HDACs. This means newer compounds that specifically target one or a small subset of HDACs may be needed in the future.

The evidence clearly shows that the SWI/SNF complex serves as a tumour suppressor, in an array of cancers. Studies in the future will further elucidate the molecular mechanisms behind the relationship between aberrant chromatin regulation and oncogenesis. These findings could contribute to the establishment of personalised treatment, depending on the genetic profiles of the tumours.

1.5.8. SWI/SNF and non-melanoma skin cancer

In 2009, a hotspot mutation of Brm in non-melanoma skin cancer was discovered (Moloney et al. 2009). A genetic analysis of Brm in human non-melanoma skin cancers was conducted, including the controls for normal skin and precancerous AK lesions. Genes from a total of 27 Caucasian patients were screened for Brm sequence variants, using denaturing high-performance liquid chromatography (DHPLC). Previously untreated skin cancers scheduled for excision, AK and normal skin biopsies were collected. Three Brm coding exons and intron-exon junctions, 26% of the transcript length, were chosen based on essential function. The same single-base substitution of exon 4 was found in two of six BCCs and one of ten SCCs but not in any normal skin samples or AKs. In total, the Q203K missense mutation was found in 17% of studied NMSCs and occurred in a highly conserved region of the Brm gene. This substitution consisted of a G:C to T:A transversion, resulting in the substitution of a glutamine by lysine at codon 203 (Q203K) (Moloney et al. 2009; Fig 1.15). The amino acid
change seen here results in a change from a neutral to a positive amino acid. Thus it was considered likely that this mutation could alter protein function. However, the effect of the Q203K mutation is still to be elucidated.

Figure 1.15: A DHPLC chromatograph confirming the Q203K mutation in Brm exon 4 present in SCC as compared to the wildtype Brm sequence in blood. From Moloney et al. 2009.

Furthermore, in 2011, it was found that a loss of expression of both Brm and Brg1 was associated with progression of benign skin lesions into invasive skin cancers (Bock et al. 2011). This was discovered by a microarray analysis of human skin cancers, which found that Brm mRNA was decreased in SCC as compared with AK and normal skin. The level of Brg1 however was not significantly altered. When protein expression was tested in normal skin, AK, SCC and BCC, both Brm and Brg1 were found to be significantly reduced in 100% of the BCC and SCC samples tested as compared to normal skin and AK, suggesting both of these subunits play a role in the progression of skin cancers (Bock et al.)
This study was the first to suggest that Brm and Brg1 play a role as tumour suppressors in human skin cancer, as well as the first to suggest that UVR may be a driver in SWI/SNF loss, promoting oncogenesis.

This finding led to further studies looking at the effect of UV on mice with a homozygous Brm allele loss. Brm-/- or Brm+/+ mice on a C57BL/6 background were irradiated with UV over a course of 25 weeks (Halliday et al. 2012). In these experiments, a low dose of UV was used so that there were few skin tumours in the wildtype mice. By the end of monitoring, it was found that there was a significant increase of skin cancers in Brm-/- mice, as compared to their wildtype littermate controls (Halliday et al. 2012; Fig 1.16). Brm+/− mice did not differ significantly from the controls, suggesting a full loss of Brm was necessary to observe the functional effect of Brm loss.
Figure 1.16: Brm knockout mice have increased incidence of UV radiation induced skin tumours. (a) UVR did not have a pronounced effect on both Brm+/- and wildtype mice, however, there was a noticeable increase in tumours in Brm-/- mice at the end of the study. (b) The effect on p53+/- on skin tumours was also pronounced, however, a further knockout of Brm did not increase tumour incidence. From Halliday et al. 2012.
Interestingly, the loss of a single allele of p53 (p53+/-), did not exacerbate the skin tumour rate, however a single allele loss of p53 did cause an increase in average tumour diameter over time in combination with a single or double allele loss of Brm (Halliday et al. 2012; Fig 1.16; Fig 1.17). A study using the full loss of p53 in mice was not possible as they develop spontaneous tumours by an average of 4.5 months (Donehower, 1996).

Figure 1.17: Single and double allele losses of Brm increase the average diameter and growth-rate of UVR-induced skin cancers in mice with a loss of a single p53 allele. From Halliday et al. 2012.
Moreover, it was also found that the loss of Brm prevented UV radiation induced immunosuppression (Halliday et al. 2012). This was tested by a contact sensitivity assay in the mice. As expected, it was found that UV radiation caused the suppression of both systemic and local contact sensitivity in wildtype mice, but this was not the case for Brm-/- mice, which were not found to have this immune suppression response. Brm+/- mice showed a similar level of immunosuppression to the wildtype mice, indicating that a single allele of Brm is enough to mediate immunosuppression in response to UVR (Halliday et al, 2012). Interestingly, this study also showed that Brm-/- mice have increased UVR-induced ocular tumours as compared to their wildtype littermates.

Another study in 2014 further expanded upon these results. Firstly, it was found that Brm-/- mice irradiated for 25 weeks had a significantly increased level of epidermal hyperplasia, as compared to Brm+/+ mice (Hassan et al. 2014). The Brm-/- mice were shown to have more than twice the epidermal thickness as compared to the Brm+/+ mice. Ki-67+ staining also confirmed that Brm-/- mice had a significantly higher level of cell divisions as compared to Brm+/+ mice. This increase was seen in animals irradiated for both 2 and 25 weeks, indicating that the loss of Brm does not take long to have a functional effect when paired with a carcinogen such as UVR (Hassan et al. 2014).

1.6. The HaCaT model to study skin cancer

The HaCaT cell model has been used extensively for functional studies in the past including the work presented in this thesis. This human keratinocyte derived cell line was first studied by Boukamp and colleagues (Boukamp et al.
1988) and has been used by many others following this. HaCaT cells possess UV-type specific mutations in both alleles of the p53 gene, and carry chromosomal aberrations characteristically seen in SCCs (Boukamp et al. 1997). Therefore, HaCaT cells have become a common model to study the functional consequences of the overexpression and suppression of genes commonly associated with non-melanoma skin cancer progression.

1.7. Aims and hypothesis of this study

From the understanding of the current literature, it is clear that SWI/SNF and Brm/Brg1 play a fundamental role in the maintenance of the cellular environment, affecting the activation and inactivation of various target genes, and thus playing a pivotal role in the prevention of cancer development. Although it has been shown recently that Brm plays a role in NMSC, the evidence for this is still very limited and much more needs to be done to understand this relationship. The development of effective strategies for the prevention of NMSC requires a better understanding of the molecular events that occur in the transition from a benign lesion to a malignant skin cancer. Therefore, a further understanding of the events such as the loss of the Brm subunit from an AK to a malignant NMSC is very important if there are to be better treatment options or cures in the future.

Previous work in my laboratory has shown that Brm-/- mice are more prone to NMSC following UVR, as compared to Brm+/+ mice. Mice with Brm knockout also exhibit increased epidermal thickness and Ki-67+ proliferating cells following UVR as compared to Brm+/+ mice. The appearance of a mutation
in human NMSC (Q203K) also suggests that there may be molecular events occurring in order to change or inhibit SWI/SNF function in NMSC.

The aim of this project is to further understand the role of Brm and the Q203K mutation in NMSC by conducting functional studies. Firstly, the effects of the loss of Brm will be tested in murine neonatal keratinocytes. This will be conducted by the culturing of keratinocytes from both Brm-/- and Brm+/+ neonatal mice and investigating their response to UVR. Further, the loss of Brm, as well as the overexpression of the Q203K mutation and normal Brm will be studied in HaCaT cells by the transfection of these cells with Brm microRNA (miRNA) or a wildtype or Q203K mutant Brm cDNA sequence. These cells will then be irradiated with a set dose of UVR, composed of both UVA and UVB, in order to test the cellular responses to UVR. Cellular events including cell cycle arrest, cell proliferation, cell death and DNA repair will all be tested as it is known that these events all play a large role in oncogenic transformation and as reviewed in this chapter, there is evidence that Brm influences these cellular responses. A defect in any of these key events can result in carcinogenesis.

Firstly, I will test whether the loss or mutation of Brm can affect cell proliferation. This will be completed by the use of a CytoTOX-ONE Homogenous Membrane Integrity Assay. This fluorometric based assay is useful as it is able to test not only the amount of live cells (via cell lysis) but also dead cells. It is a rapid, fluorescent measure of lactate dehydrogenase (LDH) release from cells with a damaged membrane.

Following this, I will use an on the plate technique to look at the cell cycle response following UV, which will be tested at various time points in situ. For this assay I will use a 5-ethynyl-2’-deoxyuridine (EdU) stain. This stain labels
replicating DNA (S-phase) cells. For further clarity, I will also use a phospho-
histone H3 (Ser10) antibody stain, which will allow me to count the number of
cells in M-phase (mitosis). This approach is a novel method, and will be
measured on the plate.

Lastly, I will study how Brm affects DNA repair in NMSC. This will be done
with the use of a commonly used CPD antibody to measure DNA damage. I will
look at CPD formation at varying time points, from 15 minutes up to 24 hours.
This will allow me to track how the cells repair the CPDs over time. I will also
assess total DNA repair with the use of a unscheduled DNA synthesis (UDS) assay.

The maintenance of genomic stability following UV-induced DNA damage
in eukaryotes is a complex process, in which chromatin-remodelling complexes
such as SWI/SNF are tightly regulated. Thus it will be interesting to see what
occurs when a core subunit of the SWI/SNF complex, such as Brm is lost or
mutated. It is hypothesised that the loss of Brm will affect the cellular machinery,
which could in turn affect cellular proliferation, cell cycle response and DNA
repair of these cells in combination with UVR. It will be interesting to see if the
loss of Brm and the effect of the Q203K mutation play similar or different roles in
NMSC.
Chapter 2: Materials and Methods

2.1. Brm-/− and Brm+/+ mice and their housing

Brm−/− and Brm+/+ mice were on a C57BL/6 background and were bred and housed at the University of Sydney animal house. These mice were backcrossed over at least 10 generations to ensure genetic similarity. Brm−/− mice used in our studies have been shown to lack functional BRM protein and were first investigated by Reyes and colleagues (1998). This study was carried out in strict accordance with the recommendations in the Australian code of practice for the care and use of animals for scientific purposes by the National Health and Medical Research Council of Australia. The protocol was approved by the Committee on the Ethics of Animal Experiments of the University of Sydney (Permit Number: K14/3-2012/3/5718). All efforts were made to minimize suffering. Mice were supplied with standard gamma-irradiated chow (Specialty Feeds, Glen Forrest, WA, Australia) and autoclaved water ad libitum. Their diet was supplemented with autoclaved sunflower seeds twice weekly. Mice were housed in groups of 2 (male and female breeders) in individually ventilated cages. All cages contained autoclaved compressed paper for bedding, as well as hand torn paper towel provided as nesting material. An autoclaved plastic dome and toilet rolls were provided as environmental enrichment items. The cages were changed once weekly. Breeding pairs of both Brm+/+ and Brm−/− mice were set up at approximately 8 weeks of age, and following birth of pups, at 1 day of age, the pups were removed and used to grow neonatal keratinocytes for experiments as described below. No adult mice were used for experiments in this study. The room in which the animals were housed operated on an
automatic 12 hour light-dark cycle. The room was air-conditioned with a constant temperature of 21°C.

2.1.1. Isolation of keratinocytes from neonatal mice

Neonatal primary murine keratinocytes were grown in keratinocyte-SFM (Life Technologies, Carlsbad, CA, USA). Keratinocyte-SFM (KSF) is optimised for the growth and maintenance of human keratinocytes and other types of epithelial cells without the need for a feeder layer of cells. KSF is a complete serum-free medium when supplemented with human recombinant Epidermal Growth Factor (EGF) and Bovine Pituitary Extract (BPE) at the time of use. Brm-/- or Brm+/+ mice of one day of age were decapitated, the head discarded and the body immersed in 70% ethanol (EtOH) for 5 min, then rinsed in sterile Dulbecco’s Phosphate Buffered Saline (DPBS) with 2% antibiotic/antimycotic (Life Technologies) (anti-anti) three times. The body was then placed in a sterile 10cm petri dish on ice in a sterile biohazard hood. Hind legs were amputated above the prominent joint and forelegs above the prominent joint and the tail was cut off. 5mm of the tail was kept for genotyping. The skin was then cut along the midline of the back all the way to the front and the skin gently prised from the midline and separated from the body using sterile forceps. The skin explant was washed twice in DPBS with antibiotics and antimycotics and placed dermis-side down in a sterile 35mm petri dish, spreading the skin so that edges were not folded over and then allowed to dry for 5 min, to flatten the explant. Skins were floated dermis-side down on 2 ml of 5U/mL dispase (Gibco, Invitrogen, Carlsbad, CA, USA) in KSF in a 6cm plate for 16 h at 4°C. A pipette was then used to carefully remove the liquid from the dish and then the skin was washed twice in
sterile DPBS. The epidermis was then mechanically separated from the dermis using sterile forceps and was placed in 1mL TrypLE Select (Life Technologies) for 20 min, while the dermis was discarded. Following the incubation, 1ml of defined trypsin inhibitor (Life Technologies) was added and scissors were used to cut the epidermis into small pieces. The skin solution was pipetted up and down 10 times through a 10ml pipette to ensure cell dissociation and then filtered through 70µm Nytex mesh (Falcon, Thermo Fisher) into a 50ml tube. The dish was rinsed with 5ml KSFM and added to the centrifuge tube. This was then centrifuged in a benchtop centrifuge at 300g for 5 min at room temperature and cells were seeded in KSFM as required onto a collagen-coated plate and incubated overnight in a humidified water-jacketed 37°C incubator with 5% CO₂ (Sanyo, North Sydney, NSW, Australia). Cells were always used immediately following culture and were not passaged nor frozen. To aid in the binding of the primary keratinocytes to the wells, all plates were coated with 10µg/cm² rat tail collagen type 1 (Thermo Fisher). The collagen was diluted in sterile 0.012M hydrochloric acid (Thermo Fisher). The collagen solution was added to the plate/well and incubated at room temperature for 1h. Following this, the plate was washed with sterile DPBS (Life Technologies) three times and then dried at room temperature prior to use.
Figure 2.1: Phase contrast photomicrograph of primary neonatal keratinocytes, grown for 24h on a collagen-coated plate. Objective = 20X, scale bar = 100μm.

2.1.2. Mouse tail DNA extraction

Approximately 5mm was removed from the end of the mouse tail and placed into an Eppendorf tube. 270μl of TP lysis buffer (50mM Tris-Cl, 100mM NaCl, 0.5% SDS, 5mM EDTA; pH 7.5) and 30μl of 20mg/ml Proteinase K (Sigma Aldrich, St Louis, MO, USA) was then added to the tube and the tubes were incubated at 37°C overnight. 120μl of 5M sodium chloride (NaCl) was then added and the tube was vortexed briefly. The tubes were centrifuged at 10000g for 10 min. Supernatant was transferred to a fresh tube, and 700μl 100% ethanol (EtOH) was added and incubated at room temperature for 5 min. Tubes were again centrifuged at 10000g for 5 min. The pellet was rinsed with 500μl 75% EtOH and centrifuged at 10000g for 5 min. DNA pellets were resuspended in 200μl TE buffer (10mM Tris, 1mM EDTA, pH 8.0).
2.1.3. PCR to ensure correct genotypes of mice

In order to ensure the Brm status of each mouse, all mice used for breeding in experiments were tested via PCR. The DNA from each mouse was tested with primers against both Brm wildtype and Brm knockout. The primers for Brm wildtype were (Forward: CCTGAGTCATTTGCTATAGCCTGTG; Reverse: CTGGACTGCCAGCTGCAGAG; 310bp), and the primers for Brm knockout were (Forward: CCTGAGTCATTTGCTATAGCCTGTG; Reverse: CATCGCCTTCTATCGCCTTC; 700bp). For each PCR reaction, 1µl of mouse DNA was mixed with 10µl Phusion® High-Fidelity DNA Polymerase (New England Biolabs, Ipswich, Massachusetts, USA), 0.5µl of each primer, and 8µl of deionised water in a PCR tube. Each PCR was run on a BioRad T100 Gradient Thermal Cycler (Biorad, Hercules, CA, USA). For each PCR the cycle was as follows: 98°C for 5 min; [98°C for 10 sec, 58°C for 30 sec, 72°C for 30 sec; x35 cycles], 72°C for 5 min. Each PCR product was added to 2µl of 5X DNA gel-loading dye (5mg/ml Orange G, 0.1M EDTA pH 8, 50% glycerol), separated by electrophoresis on a 1.5% agarose gel for 60 min at 60V at room temperature in 1X TAE buffer (Life Technologies), using a Biorad Power Pack 300 (BioRad) and Mini-Sub® Cell GT Cell (BioRad). The agarose gel containing the separated nucleic acid was visualised with SYBR-Safe DNA gel stain (Thermo Fisher) and UV light exposure (UV Transilluminator 2000, Bio-Rad).

2.2. Cell culture of HaCaT cells

HaCaT cells were provided by Prof. Rebecca Mason, Department of Physiology, The University of Sydney. HaCaT cells are immortalised human keratinocytes and have been used extensively for the study of skin cancer progression.
(Boukamp et al. 1988). For culture of the HaCaT cells, Dulbecco’s Modified Eagle Medium (DMEM) (Gibco, Thermo Fisher) supplemented with 10% foetal bovine serum (FBS; Hyclone, Thermo Fisher) (complete DMEM) was used. Cells were kept at 37°C in a 5% CO₂ incubator. At approximately 70-90% confluence, the cells were passaged by washing with DPBS twice and incubating with 0.1% trypsin-EDTA (Gibco, Thermo Fisher) until detachment occurred. The trypsin was then neutralised with 1.5X the volume of complete DMEM used and centrifuged at 300g for 5 min at room temperature. The supernatant was discarded and the cell pellet was resuspended with complete DMEM and the cells were plated at the required density for each plate, then incubated in a 37°C incubator with 5% CO₂.

During periods where the cells were not required, vials of HaCaT cells were routinely stored in liquid nitrogen. Following centrifugation, the cells were resuspended with complete DMEM also containing 10% DMSO, which acts as a cryoprotectant, and prevents the formation of ice crystals, which can lyse the cell during the thawing process. The cell suspension was placed in 2ml cryotubes (Thermo Fisher), and put in an isopropanol tank (Thermo Fisher), before being placed in a -80°C freezer. Following 2 weeks at -80°C, if the cells were not used they were permanently stored in liquid nitrogen. To thaw frozen cells, they were heated in a 37°C water bath, and the contents of the vial was resuspended in complete DMEM, and centrifuged as previously. The cell pellet was resuspended in 5ml complete DMEM, and transferred to a T25 flask (Corning, Corning, NY, USA). 24 hours following plating, the cell media was removed and replaced with fresh complete DMEM to remove any dead cells that did not survive the thawing process.
2.2.2. Viability assessment and counting of cells

For all experiments, both HaCaT cells and primary mouse neonatal keratinocytes (MNKs) were counted and assessed for viability with a Beckman Coulter Vi-CELL automated viability analyzer (Beckman Coulter Inc., Fullerton, CA, USA). The Vi-Cell utilises the widely used trypan blue dye exclusion method to determine cellular viability. It contains a liquid handling system and is fully automated and allows sample respiration, reagent handling, and subsequent instrument cleaning. Once the sample has been aspirated and mixed with trypan blue, it is pumped through the flow cell for imaging. The Vi-Cell takes 100 images and analyses these for a thoroughly accurate determination of cellular viability and number.

2.3. UV irradiation and spectral measurements

2.3.1. UV irradiation of cells using a UV solar simulator

Ultraviolet irradiations were performed using a 1000-watt xenon-arc Oriel Solar Simulator (Newport, Stratford, CT, USA) (Fig 2.2). This device provides high intensity irradiation that resembles the solar spectrum. The irradiation output is continuous, with a solar-like spectrum in a uniform collimated beam. Filters were used to create the solar-simulated spectrum. The filter used for these studies was a UVC block (280-400nm) used for solar simulated UV (ssUV) irradiations, meaning that the output consisted of UVA and UVB alone. The Oriel solar simulator is also connected to a digital power supply, as well as a timer, which allows for the accuracy in every given UV dose.
2.3.2. Radiometry and Spectroradiometry

In order to give accurate doses of UV in my experiments, both radiometric as well as spectroradiometric measurements were made. Where radiometry involves the measurement of total energy emitted by the radiating source over the entire optical spectrum, spectroradiometry measures the spectral content of the radiating source. In our laboratory, radiometric measurements are performed by a portable IL-1700 broadband radiometer (International Light, Newburyport, USA) (Fig 2.3).
However, as radiometers do not provide an absolute reading of energy they measure, they need to be calibrated against a more reliable and accurate measurement device. This provides a calibration factor, which was used in the calculation of time needed for irradiation to obtain specific UV doses. In order to do this, an Optronic OL-754 spectroradiometer (Optronic Laboratories Inc., Orlando, FL, USA) was used to calibrate the UV solar simulator to provide a reliable dose measurement and a well-defined UV output of the spectra used in the experiments. The spectroradiometer can be configured for automated measurements of the spectral output of radiating sources such as arc lamps and solar radiation. It is very important that the spectroradiometric measurement system has the appropriate input optics and that the system is properly calibrated against traceable lamps. It was calculated that the ssUV spectrum contained 91.3% UVA and 8.7% UVB. For the purpose of calibration, spectral irradiance generated by our simulator was measured by the OL-754 spectroradiometer. This measures the UV spectral output. The area under the
curve in the desired range (290-320nm for UVB and 320-400nm for UVA) was calculated which represents the total energy of the desired spectra. The output of the solar simulator is extremely similar to the solar output of the sun, mimicking its effects (Fig 2.4). Compared to sunlight, the ssUV spectra is a good match with no wavelengths below those of sunlight, but with slightly more low wavelength UVR and a correspondingly less high wavelength UVR (Poon et al. 2003). By dividing the IL-1700 broadband reading with this number, a constant can be calculated which is used in the calibration factor. This number corrects for the discrepancy between the broadband radiometer’s measurement and the real irradiance. For calculation of time needed for each dose with a particular spectrum, the actual dose was divided by the broadband reading multiplied by the calibration factor. Using this formula, all cells were given a dose of 4J/cm² of ssUV. For this, the cells were kept in the same amount of DPBS, and irradiated for the time calculated using the formula below. Following each UV dose, the DPBS was then removed and growth medium was added to the cells. Each day that these irradiations took place, the broadband reading was taken. Every 3 months, the lamp was calibrated as above and a new calibration factor was calculated.

\[
\text{Time (sec)} = \frac{\text{Dose}}{\text{Broadband reading } \times \text{ calibration factor}}
\]
Figure 2.4: The relative spectral irradiance emitted by the Oriel solar simulator using a UVC filter as compared to normal sunlight using the UVC blocking filter. Both sunlight and ssUV have been normalized to 350nm to correct for differences in intensity and therefore enable the shape of spectra to be seen.

2.4. The construction of plasmid DNA, transfection into HaCaT cells and analysis of transfected cells

2.4.1. Construction of Brm miRNAs and the Block-iT lentiviral system

In my experiments, miRNA gene knockdown of the Brm gene was performed using Invitrogen’s BLOCK-iT Lentiviral Pol II miRNA expression system. Briefly, five sets of two complementary nucleotides containing miRNA target sequences
against Brm were designed. These complementary sequences were aligned to create a double-stranded oligonucleotide. The oligos used can be seen below (Table 2.1).

**Table 2.1: Oligonucleotides used for miRNA expression.**

<table>
<thead>
<tr>
<th>Oligo Number</th>
<th>Sequence</th>
<th>Details, target sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>GL960</td>
<td>tgctgAATCTTTGAGGCCATTTGGTgttttgcaa ctgactgacACCACATGCTCAAAGATT</td>
<td>Forward oligo to use with GL961, Brm c5077-5097</td>
</tr>
<tr>
<td>GL961</td>
<td>cctgAATCTTTGAGGCATTTGGTgtcagtcaggtgc caaaccACCACATGCTCAAAGATTc</td>
<td>Reverse oligo, use with GL960</td>
</tr>
<tr>
<td>GL962</td>
<td>tgctgTGATGTATAGACAAAACGAGACTACGAGACAAAAGCATGCTCAAAGATT</td>
<td>Forward oligo to use with GL963, Brm c4961-4981</td>
</tr>
<tr>
<td>GL963</td>
<td>cctgTGATGTATAGACAAAACGAGACTACGAGACAAAAGCATGCTCAAAGATTc</td>
<td>Reverse oligo, use with GL962</td>
</tr>
<tr>
<td>GL964</td>
<td>tgctgTAGAATATATATACCCACACCAGTCAGTCCTACAGTATATTTCTA</td>
<td>Forward oligo to use with GL965, Brm c5137-5157</td>
</tr>
<tr>
<td>GL965</td>
<td>cctgTAGAATATATATACCCACACCAGTCAGTCCTACAGTATATTTCTA</td>
<td>Reverse oligo, use with GL964</td>
</tr>
<tr>
<td>GL966</td>
<td>tgctgTATAAGCTAAACTCTGAGCTCgttttggca ctgactgacGAGCTCAGTTTAGCTTATA</td>
<td>Forward oligo to use with GL967, Brm c647-667</td>
</tr>
<tr>
<td>GL967</td>
<td>cctgTATAAGCTAAACTCTGAGCTCgtcagtcaggtgc caaatcGAGCTCAGTTTAGCTTATAc</td>
<td>Reverse oligo, use with GL966</td>
</tr>
<tr>
<td>GL968</td>
<td>tgctgTTTGTCTGGCAGACTATCTCAAAGGTATTTGGCA ACTGACTAGTTAGTGCGAGACAAA</td>
<td>Forward oligo to use with GL969, Brm c4998-5018</td>
</tr>
<tr>
<td>GL969</td>
<td>cctgTTTGTCTGGCAGACTATCTCAAAGGTATTTGGCA ACTGACTAGTTAGTGCGAGACAAAc</td>
<td>Reverse oligo, use with GL968</td>
</tr>
</tbody>
</table>

The double-stranded construct was then incorporated into a donor vector (SPEFLB-ENTR-mEYFP-Bsal-miR) via ligation, in order to create a Brm miR expression plasmid. Each plasmid also contained a fluorescent protein open reading frame (EYFP) for visualisation, and ampicillin/blastcidin for antibiotic resistance.
selection of colonies/transfected cells in *E. coli* and HaCaT cells, respectively. Following this, the miR expression sequence was transferred into the pLenti6-V5-DEST vector with the use of the LR-clonase II mix (Invitrogen), in order to obtain a Brm miRNA lentiviral expression plasmid (Fig 2.5). Each plasmid was modified via the use of restriction enzymes, as each plasmid contained specific restriction sites enabling them to cut with restriction enzymes. Ligation of different segments of the plasmids were then performed in order to produce the desired plasmid (Fig 2.6). 1µg of each of the plasmid was digested at 37°C for 2 hours with 10U of restriction enzymes per 10μl reaction mixture in restriction buffer conditions recommended by the manufacturer (New England Biolabs, Ipswich, MA, USA). The digestion products were added to 5μl of 5X DNA gel-loading dye separated by electrophoresis on a 1% agarose gel for 60 min at 60V at room temperature in 1X TAE buffer (Life Technologies), using a Biorad Power Pack 300 (BioRad) and Mini-Sub® Cell GT Cell (BioRad). The agarose gel containing the separated nucleic acid was visualised with SYBR-Safe DNA gel stain (Thermo Fisher) and UV light exposure (UV Transilluminator 2000, BioRad). The DNA fragment was cut from the gel using a scalpel and purified using a QIAquick® Gel Extraction Kit (Qiagen, Valencia, CA, USA). The DNA was ligated using 3U/µl T4 DNA ligase (Promega, Fitchburg WI, USA) in 10X Ligation Buffer (Promega) at 4°C overnight. Following ligation of the new plasmid, each ligation reaction was transformed into One Shot® TOP10 Chemically Competent *E. coli* (Invitrogen), with the exception of lentiviral constructs containing the pLenti6-V5-DEST which used One Shot® Chemically Competent Stbl3 *E. coli* (Invitrogen), which reduced the frequency of homologous recombination of long terminal repeats found in lentiviral vectors. During this procedure the ligation mixture
was incorporated into the competent *E. coli* and then cultured on LB agar plates containing ampicillin overnight at 37°C. Single colonies were picked and cultured in 2ml Terrific Broth (Sigma) containing 30µg/ml ampicillin, with shaking at 225rpm overnight at 37°C. Minipreps of each plasmid were completed by the alkaline lysis method. Following overnight inoculation of cultures, they were centrifuged at 300g for 3 min. The supernatant was discarded and resuspended in 100µl Solution I (25mM TrisCl, pH 8, 10mM EDTA, 50mM glucose) and vortexed. 200µl of Solution II (0.2M NaOH, 1% SDS) was then added and allowed to lyse for 5 min. 150µl of Solution III (3M potassium acetate in 11.5% glacial acetic acid) was added, and placed on ice for 5 min. The tube was then centrifuged at 10000g for 3 min. The supernatant was transferred to a fresh tube and 800µl of 100% EtOH was added and incubated at room temperature for 2 min, followed by centrifugation at 10000g for 5 min. The supernatant was then removed and the DNA pellet was air dried for 5 min before resuspension in 100µl of TE buffer. Each plasmid was then checked via restrictive digests as previously discussed to ensure the correct plasmid was expanded. Positive clones were further expanded in a 100ml culture to make a midi-prep using a Qiagen Plasmid Midi Kit (Qiagen). Plasmid concentration and purity was measured using a spectrophotometer and the plasmid was stored at 4°C in TE buffer. Following construction, each plasmid was tested using a restriction enzyme digest (Table 2.2). Each plasmid used for transfection was also sent for sequencing (Macrogen, Seoul, South Korea) to ensure each plasmid contained the correct miRNA sequence.
Table 2.2: The screening of plasmids via restriction enzyme digests.

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Restriction Enzyme(s)</th>
<th>Size of products (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SPEFLB-C-mEYFP-His6</td>
<td>MluI + XbaI</td>
<td>2729+2445+1190+67</td>
</tr>
<tr>
<td>SPEFLB-C-mEYFP-His6^SpeI</td>
<td>Xhol + XbaI</td>
<td>5323+1032</td>
</tr>
<tr>
<td>SPEFLB2</td>
<td>EcoRV + AflIII</td>
<td>4739+829</td>
</tr>
<tr>
<td>SPEFLB-ENTR</td>
<td>AflIII</td>
<td>5067+851</td>
</tr>
<tr>
<td>SPEFLB-C-mEYFP-Bsal-miR</td>
<td>EcoRV + EcoRI</td>
<td>5680+922</td>
</tr>
<tr>
<td>SPEFLB-ENTR-mEYFP-Bsal-miR</td>
<td>PstI</td>
<td>4681+813+612+509+202</td>
</tr>
<tr>
<td>huBrm-miR-mEYFP-Bsal-miR</td>
<td>XbaI + HindIII</td>
<td>5855+991</td>
</tr>
<tr>
<td>huBrm-miR-mEYFP-Bsal-miR/pLenti6-V5-DEST</td>
<td>Stul</td>
<td>6312+1214+ 535</td>
</tr>
</tbody>
</table>

Figure 2.5: The outline of the Block-iT lentiviral miRNA expression system to create lentiviral expression clones. From Lentiviral Expression System manual (Invitrogen).
Figure 2.6: A construction map of the Brm knockout plasmids and their integration into lentiviral constructs.

When transfected into the virus producer cell line, HEK293T, the lentiviral expression plasmid is capable of producing a highly infective lentivirus containing the miRNA sequence that is capable of infecting mammalian cells (in this case, HaCaT cells). This therefore delivers a highly effective and targetted miRNA to the HaCaT cells for long-term knockdown of the Brm molecule. Following successful transduction of HaCaT cells with the miRNA of interest, the cells were selected with blasticidin, as well as sorted for EYFP fluorescence in order to purify only cells expressing the miRNAs.
A total of 5 plasmids containing various Brm miRNA constructs were constructed using this method, as well as a LacZ control construct, which was supplied by Invitrogen. These plasmids as well as their shortened name frequently used throughout this thesis in brackets are listed below. A graphical map of a control and Brm miRNA plasmid (Brm miR1) can also be seen below (Fig 2.7).

- LacZ-mEYFP-Bsa-miR/pLenti6-V5-DEST (LacZ miR)
- huBrm(960-961)-mEYFP-Bsa-miR/pLenti6-V5-DEST (Brm miR1)
- huBrm(962-963)-mEYFP-Bsa-miR/pLenti6-V5-DEST (Brm miR2)
- huBrm(964-965)-mEYFP-Bsa-miR/pLenti6-V5-DEST (Brm miR3)
- huBrm(966-967)-mEYFP-Bsa-miR/pLenti6-V5-DEST (Brm miR4)
- huBrm(968-969)-mEYFP-Bsa-miR/pLenti6-V5-DEST (Brm miR5)

**Figure 2.7:** Plasmid graphic maps of the final miRNA plasmids constructed for this thesis, also containing their restriction sites. Each plasmid contained a miRNA sequence, as well as EYFP for fluorescence and blasticidin (Bsd) and ampicillin (Amp) for antibiotic selection.
2.4.2. Construction of plasmids for overexpression of Brm and the Q203K Brm mutant

As well as designing miRNAs to knock down Brm expression, plasmids were also designed to overexpress Brm function, as well as the Brm mutant Q203K. As previously, these plasmids contained a fluorescent protein (in this case, mCherry), as well as blasticidin and spectinomycin for antibiotic selection. Unfortunately, due to the large insert sizes of these plasmids, they could not be transfected using lentiviral techniques, and thus were not inserted into lentiviral vectors. Instead, they were left in their original SPEFLB vectors. As previously, the final plasmids were created from starter plasmids that were digested via various restriction enzymes, and then ligated to other digested plasmids, in order to receive the desired plasmid as outlined previously (Fig 2.8). All plasmids were produced from *E. coli* and tested via restriction enzyme digests as previously discussed (Table 2.3). However, at all points spectinomycin was used in substitute for ampicillin. The construction maps, as well as the maps for these plasmids can be seen below (Figs 2.8 & Fig 2.9). A total of 3 plasmids were constructed for this part of the experiment. These are listed below:

- SPEFLB-ENTR-C-mCherryHA (mCherry control)
- huBrmS(Q203K)mCherryHA/SPEFLB-ENTR (Q203K)
- huBrmS-mCherryHA/SPEFLB-ENTR (Brm wildtype)
Table 2.3: The screening of plasmids using restriction enzyme digests.

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Restriction enzyme(s)</th>
<th>Size of products</th>
</tr>
</thead>
<tbody>
<tr>
<td>SPEFLB-ENTR-C-mCherryHA</td>
<td>PstI</td>
<td>4681+960+514+509</td>
</tr>
<tr>
<td>huBrmFLAG/ENTR-FL</td>
<td>AflII</td>
<td>4427+2917</td>
</tr>
<tr>
<td>huBrms(Q203K)FLAG/ENTR-FL</td>
<td>HindIII</td>
<td>6480+636</td>
</tr>
<tr>
<td>huBrms(Q203K)Cfus/ENTR-FL</td>
<td>NotI</td>
<td>4413+2661</td>
</tr>
<tr>
<td>huBrms(Q203K)mCherryHA/SPEFLB-ENTR</td>
<td>HindIII</td>
<td>10789+648</td>
</tr>
<tr>
<td></td>
<td>ClaI+XhoI</td>
<td>6821+4616</td>
</tr>
<tr>
<td>huBrmS-mCherryHA/SPEFLB-ENTR</td>
<td>HindIII</td>
<td>11437</td>
</tr>
<tr>
<td></td>
<td>ClaI+XhoI</td>
<td>6821+4616</td>
</tr>
</tbody>
</table>
Figure 2.8: A construction map of the Brm wildtype and Q203K plasmids, as well as the mCherry control. The final plasmids used in the study are highlighted.
Figure 2.9: The final plasmid maps of plasmids expressing the wildtype Brm gene, as well as the Q203K mutant. A control plasmid was also constructed to control for the mCherry fluorescent protein.
2.4.3. Sequencing of plasmid DNA

Following the successful construction of plasmids, each plasmid was sent for DNA sequencing (Macrogen) to confirm the correct miRNA sequence was created. A total of 20μl of 0.5μg/ul of plasmid DNA was sent in an Eppendorf tube, as well as 20μl of sequencing primer for each plasmid. For the Brm miRNAs, the primer sequence was TGGCCGTCGATCGTTTAAAG (forward). For the Brm mCherry constructs the primer sequence was TCGAGGAAAAGCCAAACCTGTA (forward). Following sequencing by Macrogen, all results were viewed with the use of ApE software (http://biologylabs.utah.edu/jorgensen/wayned/ape/) to confirm the correct sequences were present in each plasmid.

2.4.4. Transfection of HaCaT cells with miRNA constructs

300 mM phosphate buffer

195 mM Na₂HPO₄, 105 mM NaH₂PO₄, pH 7.05

2X HEPES buffer

140 mM NaCl, 50 mM HEPES, pH 7.05

All miRNA constructs were transduced using Invitrogen’s BLOCK-iT Lentiviral Pol II miRNA expression system, with modifications, as above. The use of this system allows the creation of a replication-incompetent HIV-1 based lentivirus using a producer cell line, HEK293T, that is used to deliver the gene of interest into dividing and non-dividing mammalian cells. These cells were normally cultured as per Section 2.2. HEK293T cells were plated at 1.5 × 10⁷ cells per
175cm² plate in complete DMEM and incubated overnight at 37°C. When cells had reached ~80% confluence, the cell medium was removed and replaced with fresh media containing 25µM chloroquine (Sigma), 1h prior to transfection. Packaging plasmids (pLP1, pLP2 and VSVG) were supplied by Invitrogen. These plasmids supply the helper functions as well as structural and replication proteins required to produce a recombinant lentivirus containing the gene of interest.

Table 2.4: The transfection of HEK293T cells with the use of the pLenti6 constructs as well as packaging plasmids.

<table>
<thead>
<tr>
<th>Plasmid DNA</th>
<th>Component</th>
<th>Conc (µg/µL)</th>
<th>T175</th>
</tr>
</thead>
<tbody>
<tr>
<td>pLenti6 plasmid</td>
<td>Vector/Rev</td>
<td>1.00</td>
<td>50 µl</td>
</tr>
<tr>
<td>pLP1</td>
<td>Tat</td>
<td>1.00</td>
<td>25 µl</td>
</tr>
<tr>
<td>pLP2</td>
<td>gag/pol</td>
<td>1.00</td>
<td>12.5 µl</td>
</tr>
<tr>
<td>pLP/VSVG</td>
<td>VSV-G envelope</td>
<td>1.00</td>
<td>15 µl</td>
</tr>
</tbody>
</table>

Solution A was prepared by combining 16.7 µL of 300 mM phosphate buffer and 3.33 ml 2x HEPES buffer. Solution B was prepared by combining 333 µl of 2.5 M CaCl₂, 3 ml of 1/10 TE buffer and plasmid DNA as in Table 2.2. Solution B was added dropwise to solution A whilst vortexing and incubated for 1 min at room temperature to allow DNA/calcium precipitation. The precipitate was added dropwise to the cells, and the plates incubated overnight at 37°C. At ~16h post-transfection, the medium was removed and replaced with 20 ml of OptiPRO
media (Invitrogen) supplemented with 2mM L-glutamine, 10% FBS and 5mM sodium butyrate (Sigma), to increase viral production. After an additional 24h, the virus containing supernatant was collected, briefly centrifuged and 0.45µm filtered (MillexHV Millipore; Billerica, MA, USA) to remove any cell debris. The transfection efficiency was confirmed qualitatively by viewing EYFP positive cells using an inverted fluorescence microscope (Leica Microsystems, Wetzlar, Germany). A transfection efficiency of 20% or greater was acceptable to make sufficient levels of virus.

Viral supernatants were transferred to ultracentrifuge tubes (Beckman Coulter) on ice and spun at 100,000g for 2h at 4°C, in a Beckman L8-70M Ultracentrifuge using an SW28 rotor (Beckman Coulter). Following virus concentration, the supernatant was removed and the viral pellets were resuspended in a virus resuspension buffer (3% (w/v) lactose in PBS) (Sigma) at 1/100th of the original volume. Following overnight incubation on ice, the virus was resuspended and the virus was used to transduce mammalian cells.

In order to determine viral titre, HaCaT cells were plated at a density of 1 × 10⁵ cells/well in a 12 well plate containing complete DMEM and incubated overnight at 37°C to achieve ~80% confluence. Concentrated virus was then diluted 1:100 and 1:1000 in a final volume of 1ml DMEM with 2% FBS containing 8 µg/ml Polybrene® (Sigma). Plates were then incubated overnight at 37°C and media was replaced the following day. At 48h post transduction, cells were prepared and analysed by flow cytometry. Samples were washed, detached and centrifuged in 5 ml polystyrene round bottom tubes (BD Biosciences, San Jose, USA). Supernatant was discarded and cell pellets were resuspended in 200µL.
FACS buffer (0.5% BSA and 0.05% sodium azide in DPBS) and stored on ice. Fluorescent protein expression was determined using the BD FACSCanto flow cytometer (BD Biosciences) and analysed using FlowJo version 8.1 (Treestar). Viral titres were calculated according to the following formula:

\[
\text{Viral titre (transducing units/mL)} = \% \text{ fluorescence positive cells} \times 10^5 \text{ cells plated} \times \text{virus dilution factor.}
\]

Following the determination of each viral titre, in order to transduce the cells, HaCaT cells were cultured in a 6-well plate (2.5 \times 10^5 cells/well) in complete medium and incubated overnight at 37°C. After 24h (~80% confluency), media was removed and replaced with 2ml of fresh media containing 8\(\mu\)g/mL Polybrene® (Sigma) and \(\sim 5 \times 10^5\) transducing units of concentrated lentivirus. After an additional 48h, the cells were checked for transfection efficiency by EYFP fluorescence on a fluorescent microscope (Fig 2.10). Generally a transfection efficiency of 50% or greater was achieved. Following this, the cells were selected with 10\(\mu\)g/ml blasticidin (Sigma) in complete DMEM for 10 days in order to select for transfected cells. Media was replaced every 2 days to remove dead cells. Once positive cells reached approximately 50% confluence, as determined by fluorescence microscopy, cells were retrieved using trypsin/EDTA and the cells were sorted using flow cytometry. Cell sorting using flow cytometry was utilized to isolate the EYFP+ transfected cells containing miRNAs. This was done to achieve a highly pure population for subsequent experiments. Prior to sorting, 1.0 \times 10^6 transduced and blasticidin selected HaCaT cells were washed with DPBS and recovered by trypsinisation. The trypsin was neutralized by adding complete DMEM, and cells were pelleted by
centrifugation at 300g, then resuspended in PBS+10% FBS containing EDTA. The cells were then passed through a sterile 40µm cell strainer (Falcon) into a FACS tube. Sorting was performed based on the yellow fluorescence of the EYFP protein using a FACSARia cell-sorter (BD Biosciences) at The Centenary Institute, Sydney. Positively sorted cells were then expanded in culture and frozen down as previously discussed. The same subset of selected cells was used for all experiments.
2.4.5. Transfection of HaCaT cells with Brm wildtype and Q203K plasmids

Due to the size of some of the constructs, the technique of nucleofection was used, as lentiviral transduction was unsuccessful. Nucleofection is a transfection
technology, which uses electroporation that is pre-optimised for each cell line (Lonza, Basel, Switzerland). Electroporation uses an electrical pulse to the cell, which creates temporary pores in the cell membrane, thus allowing entry for plasmid DNA. Nucleofection delivers long-term transfection and high transfection efficiency into cells that are generally difficult to transfect. Briefly, a Nucleofector-2™ (Lonza) device was used, as well as a nucleofector kit for HaCaT cells (Lonza). This device has a pre-programmed protocol in its memory for use with HaCaT cells. Prior to nucleofection, HaCaT cells were cultured in a 6 well plate (2.5 × 10⁵ cells/well) in complete DMEM and incubated overnight at 37°C. Upon reaching 80% confluence, the cell medium was removed and cells were trypsinised and neutralised as previously. The cells were centrifuged and then counted using the Vicell cell counter. 2.5 × 10⁶ cells were resuspended in 100μl Nucleofector solution V, and added to a cuvette with 2.5μg of plasmid DNA. Nucleofector program U-020 was selected (HaCaT). Once the program was finished, 500μl of complete DMEM was added to the cuvette and gently transferred into a new 6-well plate and made up to 1.5ml per well. The cells were incubated in a humidified 37°C/5% CO₂ incubator for 2 days, they were then checked for mCherry expression by fluorescent microscopy. If cells were expressing mCherry, they were then selected with 10μg/ml blasticidin and collected via trypsinisation then sorted by flow cytometry as previously described. The same subset of selected cells was used for all experiments.
2.4.6. Confirmation of transfection by Western Blot

To confirm the levels of Brm as well as Brg1 following transfection, the protein levels of these molecules were tested via Western blot and unique antibodies that recognised these molecules. HaCaT cells were plated at 2.5 x 10⁵ per T25 flask. At confluence, the flask was placed on ice and washed with 1ml of cold DPBS and then decanted. The cells were then lysed using 100μl ice-cold RIPA buffer (0.5% deoxycholate, 150mM NaCl, 1% NP40, 50mM Tris pH 8.0, 0.1% SDS, 10% glycerol, 5mM EDTA, 20mM NaF) with 1μl protease inhibitor cocktail (Sigma) and the lysate was transferred to a microfuge tube and centrifuged at 10000g for 5 min. The pellet was discarded and the supernatant protein was used at 40μg per 7.5% SDS-PAGE gel.

The skin from Brm+/+ and Brm-/- mice was also tested for protein levels. As per section 2.2.1, the skin was taken and chopped up using sterile scissors. The skin then had RIPA lysate buffer added as above, and was incubated on ice for 30 min. The lysate was then centrifuged and used as above.

In order to make a 7.5% SDS Page gel, the following was used:

<table>
<thead>
<tr>
<th>Solution</th>
<th>Volume (ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>40% acrylamide/bisacrylamide (29:1) (BioRad)</td>
<td>1.875</td>
</tr>
<tr>
<td>2M TrisCl, pH 8.8 (BioRad)</td>
<td>2.000</td>
</tr>
<tr>
<td>10% SDS (Sigma)</td>
<td>0.100</td>
</tr>
<tr>
<td>H₂O</td>
<td>5.915</td>
</tr>
<tr>
<td>10% ammonium persulphate (Sigma)</td>
<td>0.100</td>
</tr>
<tr>
<td>TEMED (Sigma)</td>
<td>0.010</td>
</tr>
<tr>
<td>Total</td>
<td>10.000</td>
</tr>
</tbody>
</table>
The following was used to make the stacking layer (4%):

<table>
<thead>
<tr>
<th>Solution</th>
<th>Volume (ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>40% acrylamide/bisacrylamide (29:1) (BioRad)</td>
<td>0.750</td>
</tr>
<tr>
<td>0.5M TrisCl, pH 6.8 (BioRad)</td>
<td>0.900</td>
</tr>
<tr>
<td>10% SDS (Sigma)</td>
<td>0.075</td>
</tr>
<tr>
<td>H₂O</td>
<td>5.690</td>
</tr>
<tr>
<td>10% ammonium persulphate (Sigma)</td>
<td>0.075</td>
</tr>
<tr>
<td>TEMED (Sigma)</td>
<td>0.010</td>
</tr>
<tr>
<td>Total</td>
<td>7.500</td>
</tr>
</tbody>
</table>

Laemmli’s loading buffer (Biorad) and 40µg of protein were loaded per sample. Prior to loading, 50mM dithiothreitol was added to each lysate, and they were incubated at 95°C for 5 min to reduce the disulfide bonds of proteins. Protein concentration was measured using the Lowry’s protein assay (Biorad) and assessed with a spectrophotometer microplate reader set at an absorbance of 570nm. Gels were run in a Biorad miniprotean II cell apparatus (Biorad) on ice at a voltage of 100V until samples had passed the stacking layer. Past the stacking layer, the gel was run at 150V until the samples reached the end of the gel. Gel proteins were transferred to a PVDF membrane (EMD Millipore, Billerica, MA, USA). The PVDF membrane was placed on top of the SDS Page gel containing the proteins, with 3mm chromatography paper (Whatman, Maidstone, Kent, UK) placed on either side, followed by Scotchbrite padding on either side. The gel sandwich was placed in the apparatus containing transfer buffer (5mM Tris, 38.4mM glycine) with 10% methanol. The entire apparatus was placed in ice and transferred in a cold room overnight at a constant voltage of 30V. Membranes were then removed and incubated for 1 h with 20ml TBST (50mM Tris-Cl,
150mM NaCl, 0.05% Tween-20; pH 8.0) with 5% w/v skim milk powder (Diploma, Mount Waverley, Vic, Australia) to block non-specific protein binding sites. Membranes were washed with TBST and incubated with 5µg/ml of primary antibody and 0.5% skim milk powder in TBST for 3 h at room temperature. Primary antibodies used were rabbit anti-SMARCA2/Brm antibody – ChIP grade (ab#15597, Abcam, Cambridge, UK), rabbit anti-Brg1 antibody (ab #4081, Abcam) or rabbit anti-GAPDH (Sigma). Following incubation with primary antibody, the membranes were washed 5 times in TBST for 10 min. This was followed by incubation with 1µg/ml secondary anti-rabbit horseradish-peroxidase (HRP) antibody (Sigma) in 0.5% skim milk powder/TBST for 1 h at room temperature. Membranes were then washed 5 times for 10 min in TBST. Membranes were placed in plastic sample bags (PerkinElmer, Waltham, Massachusetts, USA) containing chemiluminescent reagents from an ECL Advance Western Blotting Kit (GE Healthcare, Fairfield, CT, USA). Images of the membranes were obtained by photography using a Syngene G:box (Syngene, Frederick, MD, USA). If required, Western blots were quantitated using ImageJ software and normalising the protein of interest to GAPDH.

2.4.7. Confirmation of miRNA efficiency by RT-PCR

In order to further confirm that Brm was knocked down in miRNAs, the cDNA of transfected HaCaT cells was assessed using primers specific against Brm. HaCaT cells were plated at 2.5 x 10^5 cells per T25 flask. After 3 days, cells were washed by replacing media with DPBS at 4°C. The DPBS was decanted and the cells were collected via a cell scraper (Thermo Fisher). The cells were collected from the
flask via pipette and transferred into an Eppendorf tube. RNA was extracted using an RNeasy kit (Qiagen) and cDNA was synthesized by reverse transcription using Superscript III™ Reverse Transcriptase (Life Technologies). Quantitative PCR was performed on the cDNA using the RotorGene 6000 real-time PCR machine (Corbett Robotics, San Francisco, CA, USA) using primers specific for Brm (Forward: CATGAGCCAGCCACACAG; Reverse: GCTGCAAGCCAGGCAACCTTT; 161bp). The housekeeping gene, GAPDH was used for normalisation (Forward CCACCATGGAAGGCTGGGCTC; reverse AGTGATGGGATGACTGTC; 241bp).

2.5. The CytoTOX-ONE Assay for measurement of cell growth and cell death

The CytoTOX-ONE assay Homogenous Membrane Integrity Assay (Promega, Madison, WI, USA) was used to measure both live and dead cells over a time course, giving an accurate measure of cell proliferation over time. This assay is a homogenous, fluorometric method for estimating the number of non-viable cells present in multi-well plates. Measurement of the leakage of components from the cytoplasm into the surrounding culture medium has been widely accepted as a valid method to estimate the number of non-viable cells. The CytoTOX-ONE assay utilises a rapid, fluorescent measure of the release of lactate dehydrogenase from cells with a damaged membrane. The CytoTOX-ONE reagent mix does not damage healthy cells; however, another advantage of this assay is that the media can be replaced (containing the dead cells), new media added, and a lysis buffer added to the cells, effectively lysing the cells and providing an accurate measurement of live cells. For this assay, cells were plated into clear 96-
well plates (avoiding edge wells) at the desired density (3.2 x 10^3 cells per well for HaCaT or 3.2 x 10^4 cells for MNKs) in the appropriate growth medium (complete DMEM for HaCaTs and KSFM for mouse keratinocytes) and grown for 24h in a 37°C/5% CO₂ incubator. More MNK cells were plated than HaCaT cells as these cells have a high cell death rate and grow much more slowly than HaCaT cells. These cell numbers were previously optimised for each experiment. The following day, the cell medium was aspirated, and the cells were washed twice with DPBS. The cells were then irradiated with or without 4J/cm² ssUV in DPBS, the DPBS removed, and growth medium was added. The cells were then incubated at 37°C/5% CO₂ for 24, 48, 72 or 96h post treatment with or without UV. One plate for each cell type was also used before UV (0 h) to use for normalization. After the desired incubation time, the plates were allowed to equilibrate at room temperature for 20 minutes. The media (containing the dead cells) was then moved to free wells on the plate and the cell medium in the initial wells was replaced with fresh growth medium. Four wells also had cell medium without cells measured as a control. The wells with live cells then had 2µl lysis buffer (9% Triton X-100) added to the media to lyse the live cells in these wells. The CytoTOX assay was then performed as indicated in the manufacturer’s instructions and the resulting fluorescence was measured on the FluoSTAR Omega microplate reader (BMG Labtech, Ortenberg, Germany) with an excitation of 594nm and emission of 590nm.
2.6. A cell cycle stain for S- and M-phase cells

2.6.1. Development of a cell cycle stain for cells bound to a plate

Materials:

Click-iT EdU Alexa Fluor 594 Imaging Kit (Life Technologies – C10339)

Phospho-Histone H3 (Ser10) (D2C8) XP Rabbit mAb (Alexa Fluor 488 Conjugate)
– (Cell Signaling #3465)

DAPI (4’,6-Diamidino-2-Phenylindole Dihydrochloride) – (Life Technologies – D1306).

Blocking buffer: 1x PBS/5% normal serum/0.3% Triton™ X-100)

Antibody Dilution Buffer: (1X PBS/1% BSA/0.3% Triton™ X-100)

Wash Buffer: DPBS (Invitrogen) + 3% BSA

Cell cycle progression was tested in both murine neonatal keratinocytes and HaCaTs following UV. Murine neonatal keratinocytes (3.0 x 10^5) or HaCaT cells (3.0 x 10^4) were plated in 24-well plates and incubated at 37°C/5% CO₂ for 24h. Following 24h incubation, the cell medium was removed and replaced with DPBS. Each plate was then irradiated with or without 4J/cm² ssUV as described previously, and incubated for 24, 48, 72 or 96h in complete KSFM (mouse keratinocytes) or complete DMEM (HaCaTs). Following their desired incubation time, the cells were fixed, stained and imaged as described below.

Generally to measure the cell cycle of cells, they are required in solution in order to run them through a flow cytometer. However, when cells receive UVR, they
become more stubborn to remove from the bottom of wells using conventional trypsinisation techniques. Therefore, this method was developed in order to avoid the need to detach the cells from the plate and therefore to avoid any bias in cells analysed due to incomplete detachment from the plate. Firstly, a 5-ethynyl-2'-deoxyuridine (EdU) assay (Click-iT EdU Alexa Fluor 594 Imaging Kit, Thermo Fisher) was used to measure S-phase cells. EdU is a thymidine anlaogue that is efficiently incorporated into new synthesised DNA and fluorescently labelled with a bright, photostable Alexa-Fluor dye in a fast, and highly specific reaction. The thymidine analog is readily detected by click chemistry, the covalent cross-linking of its ethynyl group with a fluorescent azide, which is small enough to freely diffuse through native tissues and DNA. Cells uniformly stained for EdU are in S-phase. A phospho-histone H3 (Ser10) rabbit monoclonal antibody (Cell Signaling, Danvers, Massachusetts, USA) assessed mitotic (M-phase) cells. The modification of histone-H3 is tightly correlated with chromosome condensation during both mitosis and meiosis (Hendzel et al. 1997). DAPI was used as a total nuclear stain.

Staining was all performed in situ on cells grown in 24-well plates and was a completely novel methodology developed for these experiments. In order to assess the validity of this experiment, murine neonatal keratinocytes were plated as normally for 24h. Following this, one plate had KSFM without ’serum’ and another had KSFM with ‘serum’ added for a further 24h. Although the keratinocyte-SFM does not contain serum as normally used in cell medium, it does contain bovine pituitary extract (BPE) as well as epidermal growth factor (EGF), which play a similar role to FBS in aiding cell growth. Thus by excluding these factors, it is possible to starve the cells in a similar fashion to a regular
serum starvation assay. Therefore, $3.0 \times 10^5$ per well of murine neonatal keratinocytes were mixed with KSFM medium, with or without BPE and EGF, and plated in a 24-well plate at $37^\circ C/5\%\ CO_2$ for 24h. One hour prior to the desired incubation time (23h), 10µM EdU was added to wells in 1ml KSFM and incubated at $37^\circ C/5\%\ CO_2$ for 1h. One control well was not treated with EdU (medium only) to correct for background staining. The cells were washed with 1ml DPBS thrice, and the cells were incubated in ice-cold 100% EtOH for 7 min at -20°C to fix the cells. The EtOH was then aspirated, and the wells were washed with wash buffer thrice. To eliminate the residual binding capacity of each well, each well had 1ml blocking buffer added and the wells were incubated for 1h at room temperature. The blocking buffer was then removed and 500µl anti-histone H3 antibody (1:200 in antibody dilution buffer) (Cell Signaling) was added to the wells. One well had 500µl rabbit IgG (1:200 in antibody dilution buffer) (Abcam) added as a control. All wells were incubated at room temperature for 2h in the dark. The antibodies were then aspirated and the wells washed with washing buffer thrice. Following this, the EdU reaction cocktail containing azide was mixed as per manufacturers instructions and 500µl was added to each well and incubated at room temperature for 30 min in the dark. The cocktail mix was then aspirated and the wells washed with wash buffer thrice. Lastly, the cells were incubated with 2µg/ml DAPI in DPBS for 10 min in the dark. DAPI was then removed and the wells were washed with DPBS thrice. Following this, each plate was imaged with the use of a Cytation-3 live cell imager (Biotek, Winooski, Vermont, USA) in the Bosch Institute Facilities at The University of Sydney.
2.6.2. Analysis of cell cycle data

Following image recovery, each image was analysed using ImageJ software. Each image set contained an image for DAPI, EdU and histone-H3. In order to gather the intensity of each of these per cell, firstly, a cell counting plugin supplied by A/Prof Guy Lyons was used to count DAPI cells, as seen below (Fig 2.11a, b). The plugin was set up to avoid smaller objects likely to be debris or dead cells, as well as larger objects that were likely to be multiple cells. Following the plugin counting each DAPI cell, a mask of this image was created in order to gather intensity readings for the EdU channel and histone-H3 channel (Fig 2.11c, d). Following this, an intensity reading for each channel was generated for each cell per image, and each cell intensity reading was compiled into a csv file (Microsoft Excel) and imported into FlowJo software (Treestar, Ashland, Oregon) in order to separate positively and negatively stained cells. Negative cells and their gates were determined by analysing the fluorescence of control stained cells, with anything above this intensity deemed positive. In order to confirm that only cells in S-phase were counted (uniformly brightly stained cells) and not cells undergoing unscheduled DNA synthesis (UDS) (punctate or speckled stained cells), each experiment had the cells undergoing UDS subtracted from the total by manually counting speckled cells in an average of five images per sample. This therefore left only cells undergoing S-phase. For each experiment, a total of 3000 cells per well were counted and used. Generally 3 wells were counted, giving a total cell count of 9000 per experiment.
Figure 2.11: The use of ImageJ to count cells and gather cell intensities for each channel. Firstly, a cell counting plugin was used to count individual DAPI cells (a and b), and this mask was used to gather cellular intensities of EdU staining (c) and histone-H3 staining (d). Objective = 20X, scale = 100µm.

2.7. DNA repair assays

2.7.1. The unscheduled DNA synthesis assay to measure overall DNA repair

Unscheduled DNA synthesis (UDS) due to DNA repair can be measured via the uptake of the thymidine analog, 5-ethynyl-2′-deoxyuridine (EdU), into the DNA of cells that are not in the normal phase of DNA synthesis (S-phase). This methodology was used previously in section 2.6, using an EdU kit (Thermo
Fisher). However, in this case the assay was used to measure punctate or speckled cells only (cells undergoing UDS). UDS was assayed as previously described (Nakagawa et al. 1998), except that in this case, EdU was used to stain cells instead of 5-bromo-2’-deoxyuridine (BrdU). The use of EdU is advantageous over BrdU staining, as it does not require the DNA to be denatured with harsh chemicals or heat. Murine neonatal keratinocytes were plated at 6.0 x 10^5 cells per well, or HaCaT cells were plated at 6.0 x 10^4 cells per well in a 4-well chamber slide and incubated overnight at 37°C/5% CO₂. Following exposure to 4J/cm² or sham-irradiation, cells were incubated with 10µM EdU and the azide reaction cocktail, and imaged as per section 2.6.1. ImageJ software was used to count total cells using DAPI, and then the number of cells undergoing UDS was quantified based on the appearance of punctate spots (grains) in the nuclei, in contrast to the uniformly intense staining of the nuclei, which underwent ordinary DNA synthesis during S-phase. Cells undergoing UDS were manually counted. All slides were blinded by covering treatment label with black tape by a third party to prevent experimental bias. Slides were only unblinded once analysis was complete. The total number of punctate cells were counted per image, and averaged against the total number of cells via DAPI, to calculate the total percentage of cells undergoing UDS. This was calculated in at least 5 images per sample, per experiment at 40X magnification.

2.7.2. Measurement of cyclobutane pyrimidine dimers (CPDs) to analyse UV-specific DNA lesions in mouse skin

As per section 2.1.1, both Brm+/+ and Brm-/- pups were culled at 1 day of age and their skins were removed. Each skin was then placed on filter paper
dampened with DPBS and stretched out. Each skin was then irradiated with 4J/cm² ssUV or sham-irradiated and the skins were placed in a tube containing complete DMEM and then placed at 37°C/5% CO₂ for a specific amount of time. For incubation times, the skin was cultured for 15 min, 30 min, 1h, 2h, 3h or 24h and then placed in 4% paraformaldehyde solution in PBS to fix the skins and stop further repair. This allowed me to observe the repair of UV-induced CPD lesions over time. Following fixation, each skin section was processed into paraffin blocks, and 10µm sections were cut onto Superfrost® Plus Gold Slides (Menzel-Gläser, Glasbearbeitungswerk GmbH & Co.) as described previously (Fischer et al. 2008). The slides were then deparaffinised, via processing them through alcohol gradients before bringing them to water and TBST to rehydrate the sections. Each slide then had the boundaries of the skin sections drawn with a Dako Pen (Dako, Glostrup, Denmark) to confine solutions to the skin sections. Each slide section had 200µl 1% Triton-X 100 in TBST added to prevent background staining, and the slides were incubated for 30 min at room temperature. All slides were then washed thrice with TBST for 5 min. 200µl of 20µg/ml proteinase-K (Sigma) was then added to each section and the slides were incubated at 37°C for 30 min. All slides were then washed thrice in TBST for 5 min. Each section then had 100µl 2M hydrochloric acid added for antigen retrieval, and the slide was incubated at room temperature for 15 min. 100µl of 50mM Tris base (pH 8.0) was added on top of this and incubated for a further 15 min. Again, slides were then washed thrice in TBST for 5 min. 200µl of serum free protein block (Dako) was then added to each slide to block unoccupied areas of the slide, and incubated at 37°C for 1h. The protein block was then poured off, and 200µl of 0.25µg/ml mouse anti-thymine dimer monoclonal antibody
(Kamiya Biomedical, Tukwila, Washington, USA) or 0.25µg/ml mouse IgG1 isotype control (Sigma) in serum-free antibody diluent (Dako) was added to each slide section and incubated at 37°C for 1h. All slides were then washed thrice with TBST for 5 min. 200µl of 0.5µg/ml IRDye® 680RD Goat anti-mouse IgG (Licor, Lincoln, Nebraska, USA) in antibody diluent was then added to the slides and incubated at 37°C for 30 min. All slides were then washed thrice with distilled water. Lastly, Vectashield anti-fade mounting medium with DAPI (Vector Laboratories, Burlingame, California, USA) was added to each section, and then slides were coverslipped and sealed with nail polish. The cells were visualised and imaged using a fluorescence microscope as per section 2.7.1. ImageJ software was used to analyse all images. The interfollicular epidermis of skin was traced using the software in the DAPI channel, which was used to make a mask, and the CPD intensity of each section was calculated. Each section also had the number of cells manually counted in the DAPI channel per epidermal section, in order to give a reading of the amount of CPD intensity per cell (Fig 2.13). A total of 5 images were measured per sample, per experiment, for a total of 15 images analysed per sample over 3 separate experiments.
Figure 2.13: Example of the measurement of CPD intensity in neonatal mouse skins. The epidermis was traced using ImageJ software on the DAPI channel (top), and a mask was created from this to measure the CPD intensity of the same section. The total number of cells in the DAPI channel was also manually counted in order to give a total CPD intensity/cell measure. Scale = 100μm.
2.7.3. Measurement of cyclobutane pyrimidine dimers (CPDs) to analyse UV-specific DNA lesions in HaCaT cells

CPD lesions were also measured *in vitro* in HaCaT cells. HaCaT cells were plated at 6.0 x 10⁴ cells per well of a 4-well chamber slide and incubated overnight at 37°C/5% CO₂. Following exposure to 4J/cm² or sham-irradiation, each sample was incubated further at 37°C/5% CO₂ for times of 15 min, 30 min, 1h, 2h, 3h or 24h. After the desired incubation time, each well was then fixed with 100% ice-cold EtOH at -20°C for 7 min. Following this, the slides were stained and imaged as per section 2.7.2. ImageJ software was used to analyse all images. As done previously in section 2.6.2, each DAPI image had the cell number counted with the use of a cell counting plugin, and a DAPI mask was used to measure the total CPD intensity per cell.
Chapter 3: Brm regulates cell cycle responses to UVR in neonatal murine skin

3.1. Introduction

The first part of this study aims to investigate the effect that Brm plays in the response to UVR, with the use of an *ex vivo* animal model. From previous animal work completed in my laboratory, it has already become clear that Brm is important in the cellular response to UVR. Work in this chapter used Brm knockout mice on a C57BL/6 background, which have a loss of both Brm alleles (Brm-/-) (Reyes et al. 1998). These animals are at all times compared to C57BL/6 mice that have both functioning alleles of Brm (Brm+/+). From long-term studies of these mice, we already know that Brm-/- mice suffer from increased tumour incidence following a UVR regime (Halliday et al. 2012). Further, mice irradiated with UVR for both 2 and 25 weeks show increased epidermal hyperplasia and increased levels of Ki-67+ proliferating cells (Hassan et al. 2014). To further understand the mechanistic reasons for the increased tumour incidence, and the role that Brm plays in skin cancer, cellular responses to UVR will be studied in primary cultured MNKs. By extracting the keratinocytes from neonatal mice (mice at 1 day of age) and culturing them in specialised media and coated wells, it is possible to use an *ex vivo* system to study the effect of Brm loss. Further, with the use of an Oriel solar simulator, the cells can be treated with solar simulated UV (ssUV) and their response to ssUV can be tracked. For CPD staining, full neonatal mouse skins will be irradiated with UVR, and CPD staining will be performed using immunohistochemistry. In order to understand how the loss of Brm impacts keratinocyte cells, the following will be
investigated - changes in cell proliferation, cell death, cell cycle regulation and DNA repair (UDS and CPD staining). These assays will help to gather information as to whether functional Brm is necessary for an adequate response to the damaging effects of UVR. It is hypothesised that Brm protects skin from UV radiation-induced damage, and thus the loss of Brm in an animal model will lead to cellular defects in responses to UVR.

3.2. Results

3.2.1. Growth of murine neonatal keratinocytes ex vivo

Although the methodology to grow primary MNKs has been performed before by other laboratories, this methodology had to at first be optimised for all experiments, as it had not been used in my laboratory. Firstly, a sufficient medium for optimal cell growth had to be determined. Previously, the most common medium for keratinocyte growth was SMEM (Sigma-Aldrich) +10% chelated (calcium removed) FBS. However, a more recent growth medium that is commonly used is keratinocyte-SFM (KSFM; Invitrogen), which contains lower calcium content (<0.1mM), which is important for keratinocyte growth and prevents growth of fibroblasts (which can contaminate epidermal cell suspensions). Both of these media were tested for optimised growth, with equal numbers of cells on a cell culture flask for 24h (Fig 3.1). It was noted that microscopically, SMEM+FBS had a much higher cell death rate than the KSFM medium. Further, there were more cells bound to the plate with the KSFM medium. It was also noticed that there were more round, cobblestone-like cells in the KSFM medium flask, the typical morphology of a keratinocyte. The
SMEM+FBS medium had more elongated, fibroblast-like cells. For the above reasons, the KSFM medium was used for all experiments.

![Figure 3.1: The growth of keratinocytes in both SMEM+FBS and KSFM. On the left, cells grown in SMEM+FBS, on the right, cells grown in keratinocyte-SFM. Note in the right panel there appeared to be more live cells and fewer dead cells based on morphological appearance. Objective = 20X, scale = 100μm.](image)

Both collagen and fibronectin are commonly used in primary keratinocyte culture to promote growth. Both of these were coated onto flasks at 10μg/cm², compared to lack of coating, to test whether this affected growth of MNKs (Fig 3.2). It was found that collagen and fibronectin both aided in the binding of the MNKs to the flasks. The coatings reduced the numbers of dead cells and increased bound cells. There did not appear to be a large difference between the collagen and fibronectin coated flasks. However, since collagen coating is easier and less expensive, this was chosen to use for all experiments, in combination with keratinocyte serum-free medium.
Figure 3.2: Collagen or fibronectin coating increases keratinocyte growth.
Primary MNKs were grown on collagen, fibronectin or without coating. The keratinocytes grew much better on collagen and fibronectin. Objective = 20X, scale = 100μm.

3.2.2. Validation of Brm status in C57BL/6 mice

All parent breeders used to produce mice in these experiments were tail tipped and genotyped (see Chapter 2.1.2 for further methodology) to ensure that the correct genotype was used. This was done with the use of specific primers for both the Brm wildtype allele and the Brm knockout allele. Using PCR, it is possible to tell apart these genotypes as Brm-/- mice only form a product with
the Brm knockout primers, and the Brm+/+ mice only form a product with the Brm wildtype primers. If a mouse contains a wildtype and knockout allele (Brm+/-), a product will be formed with both primers (Fig 3.3).

![Image](image_url)

**Figure 3.3: Genotyping of mice.** Examples of an agarose gel, stained with SYBR safe DNA gel stain. PCR products were obtained from running a PCR on mouse DNA with specific primers. The allele being detected is shown in the heading to each panel and the mouse genotype at the bottom of the gel, with the Brm wildtype allele being detected at 310bp, and the knockout allele being detected at 700bp. (M = marker; Hyperladder II).

Protein was also extracted from both Brm+/+ and Brm -/- murine skin. Using their corresponding antibodies, Brm and Brg1 protein was detected. It was found that that the intensity of the band corresponding to Brm protein was completely lost in Brm-/- mice as compared to Brm+/+ mice. It was also noted that Brg1 was upregulated in the Brm-/- mice as compared to the Brm+/+ mice (Fig 3.4). Brg1 was present in Brm+/+ skin, but its expression was low. These results were not unexpected, as the original paper describing Brm knockout mice
showed no Brm protein in knockout mice and showed that Brg1 was upregulated in total organ protein extracts (Reyes et al. 1998). However, the status of Brm and Brg1 in the skin was not examined in that paper.

![Western blot image showing decreased Brm and increased Brg1 protein in Brm-/- mouse skin.](image)

**Figure 3.4:** Brm-/- mouse skin shows decreased Brm protein but increased Brg1 protein. Protein was extracted from either Brm-/- or Brm+/+ mouse epidermis, and Brm/Brg1 were probed using antibodies on a Western blot. GAPDH was used as a loading control. Brm protein was reduced in Brm-/- mouse epidermis, as compared to Brm+/+. Brg1 protein was elevated in the Brm-/- mice, as compared to Brm+/+.

### 3.2.3. Brm-/- cells are more resistant to UVR-induced cell death

To find a suitable dose for all solar simulated UV (ssUV) experiments, several doses were tested on primary MNKs. The ssUV spectrum consists of both UVA (320-400nm) and UVB (290-320nm). All cells were irradiated using a 1000-Watt xenon-arc Oriel Solar Simulator (Newport, Stratford, CT. USA). Varying the time gave different doses of ssUV. The UV spectrum contained 91.3% UVA and 8.7% UVB. The goal of this experiment was to determine a dose of UVR
that was high enough to induce cell cycle arrest, without substantial levels of cell death in wildtype cells. With increasing dose, there was a microscopically observable effect on the cells 24 h after irradiation (data not shown). By 8J/cm², there were a large number of dead cells visible microscopically. At 2J/cm² and 4J/cm² the effect of UV on cell death was not as apparent, although there were fewer cells when compared to the unirradiated cells. The effect of various ssUV doses was also tested on cell viability of both Brm-/- and Brm+/+ MNKs. In order to do this, a CytoTOX-ONE Homogenous Membrane Integrity Assay (Promega) was used. This kit and the Vicell cell viability counter were previously compared, and the CytoTOX-ONE assay was found to be vastly more sensitive and accurate. Triton-X100 (9%) was added to the viable cells adhering to the plate, releasing LDH from the cell membrane upon death, giving an accurate measurement of live cell number. This LDH fluorescence in the media, or the supernatant from lysed cells was then measured on a FLUOstar Omega microplate reader.

As expected, as the UVR dose increased a respective reduction in cell viability was noted (Fig. 3.5). By 24h, the viability of cells without irradiation was already quite low, which is normal for primary cells (Brm+/+ at 79.5% and Brm-/- at 77%). In all unirradiated cells, the viability increased from 24 to 48h, and reduced at 72h, which is likely due to cells reaching confluence (Fig 3.5a). There was no significant difference in the viability of Brm-/- or Brm+/+ unirradiated cells over time (P=0.2047; 2-way ANOVA). In Brm+/+ cells, the viability decreased to 74.9% with 2J/cm² UVR, and 76.2%, 72.4% and 59.8% for doses of 4, 6 and 8J/cm² respectively. Importantly, it was found that both Brm-/- and Brm+/+ MNKs were able to recover from doses of 2 and 4J/cm², as the cells were able to continue growing up to 72h, and viability increased over time with
these doses (Fig 3.5b, c). However, larger doses of 6 and 8J/cm² led to decreased viability over time, suggesting these doses were too high for studying non-lethal effects (Fig 3.5d, e). This data suggests that primary MNKs should not be irradiated with a dose of ssUV exceeding 4J/cm². Following these experiments, it was concluded that 4J/cm² ssUV was the most suitable experimental dosage as there was not a large difference in cell viability between 2 and 4J/cm² in wildtype cells 24h post UV, whereas larger doses such as 6J and 8J/cm² caused larger decreases in cell viability over time. The 4J/cm² data also shows there is a delayed growth 24h after UV, indicative of a cell cycle arrest response. It was not known if a lower dose such as 2J/cm² would lead to significant levels of cell cycle arrest. Interestingly, with doses of 2, 4 and 6J/cm² ssUV, Brm-/- MNKs showed significantly increased resistance to UV, with higher viability readings over time course as compared to Brm+/+ cells (2J; P=0.0160, 4J; P=0.0003, 6J; P=0.0004; 2-way ANOVA). The largest dose of 8J/cm² did not confer a survival advantage for Brm-/- cells over Brm+/+ cells however (P=0.2045; 2-way ANOVA). Please note that this experiment was a single replicate experiment.
MNKs with doses of 2J (P=0.016), 4J (P=0.0003) and 6J (P=0.0004) but not doses above 4J/cm

4J/cm tested by a CytoTOX assay. Viability of cells was similar at 2 and 4J/cm², however, there was a noticeable decrease in viability with higher doses such as 6 and 8J/cm². MNKs were not able to recover effectively from doses above 4J/cm². Brm-/- MNKs had a survival advantage over Brm+/+ MNKs with doses of 2J (P=0.016), 4J (P=0.0003) and 6J (P=0.0004) but not
8J (P=0.2045). Graphs are indicative of 1 single experiment, with 8 wells of a 96-well plate measured (n=8). Mean ± SD plotted. 2-way ANOVA used.

3.2.4. Brm-/− MNKs grow faster than Brm+/+ MNKs following UVR

In these experiments, the effect of 4J/cm² ssUV on cell growth and death in Brm+/+ and Brm-/− MNKs was analysed. Previously optimised conditions were used. Either Brm+/+ or Brm-/− MNKs (3.2 x 10⁴) were grown in 96-well plates and left to incubate at 37°C/5% CO₂ for 24h. The cells were then irradiated with 4J/cm² ssUV or sham-irradiated and then cultured at 37°C/5% CO₂ for fixed periods of time (24, 48, 72 or 96h). The CytoTOX-ONE kit was used to quantitate the number of live and dead cells. The resulting fluorescence was read on a FLUOstar Omega microplate reader. Microscopic images of the cells were also taken 24h following treatment with or without 4J/cm² UVR, to assess morphology (Fig 3.6). The number of morphologically observable dead cells did not change noticeably following UVR treatment. As previously described, an overall decrease in cell number in both of the UVR treated groups as compared to the untreated groups was noted (Fig 3.7).
Figure 3.6: 4J/cm² leads to a decrease in cell numbers in MNKs. Microscopic images of neonatal MNKs, 24 hours before and after 4J/cm² ssUV. Visually, there was a decrease in cell number in the UVR-treated samples. Objective = 20X, scale = 100μm.

Treatment of MNKs with UVR led to a decrease in live cell number at all time points for both Brm+/- and Brm-/- MNKs. In both samples, there was a significant effect of UVR on cell growth over the time course (Brm+/-, P<0.0001; Brm-/-, P<0.0001; 2-way ANOVA) (Fig 3.7). The number of live cells in the untreated groups reached its highest at 72h, and plateaued around this time. This was due to the cells reaching confluence at this point. However, it was found in the UVR-treated group that these cells continued to grow up until the final time point, 96h. Further, the growth of UVR irradiated Brm-/- and Brm+/- MNKs differed (*P<0.0001; 2-way ANOVA) over the entire time course, with the exception of 24h (Fig 3.7). There were little differences between UVR-treated
Brm-/- and Brm+/+ cells at 24h, however by 48h (P=0.0002), 72h (P<0.0001) and 96h (P<0.0001; 2-way ANOVA) Brm-/- cultures had 1.35, 1.7 and 1.6-fold more cells than Brm+/+ respectively. Interestingly, even though there was not a large difference between the groups, without UV there was an overall significant difference between the Brm+/+ and Brm-/- cells (P=0.0028; 2-way ANOVA). At all times post 24h Brm+/+ cells were slightly more elevated than Brm-/- cells but this was only significantly different at one time point (48h; P=0.0006; 2-way ANOVA).

Figure 3.7: Brm-/- MNK numbers recover faster from damaging 4J/cm² UVR than Brm+/+ MNKs. Brm+/+ and Brm-/- MNKs were studied at 24, 48, 72 and 96h post UV with the use of the CytoTOX assay. Unirradiated cells grew at a steady rate and plateaued at 72h, due to reaching confluence in both Brm+/+ (red line) and Brm-/- (green line) cells. The growth of Brm-/- MNKs (purple dotted line) was significantly different to that of Brm+/+ MNKs (blue dotted line) following UV treatment (P<0.0001). Without UV
treatment, there was still a significant difference between the groups but this was not as pronounced (P=0.0028). Brm-/ - compared to Brm+/ + at time points of 0, 24, 48, 72 and 96h. Each time point represents mean ± SEM from of 2 independent experiments (8 wells per experiment; n=16). Two-way ANOVA used. Where the SEM is not obvious it is too small to be seen. “Cell proliferation index” = spectrophotometer reading at 590nm.

The effect of 4J/cm^2 UVR on cell viability was also assessed. UVR had a significant effect on the viability of primary MNKs in both Brm-/ - and Brm+/ + cells (both P<0.0001). Further, there was no significant difference between the viability of Brm-/ - and Brm+/ + MNKs in the unirradiated samples (P=0.4768)(Fig 3.8a). Interestingly, Brm-/ - MNKs generally had higher cell viability as compared to Brm+/ + MNKs when irradiated with 4J/cm^2 ssUV, which was significantly different over the 96h time course (P<0.0001) (Fig 3.8b). The increased viability of Brm-/ - UV-treated cells as compared to Brm+/ + was especially prominent at 48h (P=0.0127), 72h (P<0.0001) and 96h (P<0.0001; 2-way ANOVA) suggesting Brm-/ - cells had a survival advantage to UVR as compared to Brm+/ + cells.
Figure 3.8: The loss of Brm in primary MNKs increases cell viability following exposure to UVR. Brm-/- or Brm+/+ were (a) sham-irradiated or (b) irradiated with 4J/cm² UVR and their viability was tested at 24, 48, 72 or 96h with the use of a CytoTOX-ONE assay. UVR decreased viability of both Brm-/- and Brm+/+ cells (P<0.0001). There was no significant difference between unirradiated cells, however, the loss of Brm in MNKs led to increased cell viability in irradiated cells (P<0.0001). Each time point represents mean ± SEM from of 2 independent experiments (8 wells per experiment; n=16). Two-way ANOVA used. Where the SEM is not obvious it is too small to be seen.

3.2.5. Development of an on the plate technique to analyse cell cycle progression

Since Brm-/- MNKs were able to recover faster from the damaging effects of UVR, it was investigated whether this was due to Brm-/- cells having defective UVR-induced cell cycle arrest. To do this, 2 separate stains were used. Firstly, a 5-ethynyl-2'-deoxyuridine (EdU) assay was used to measure S-phase cells. Cells
that are uniformly stained for EdU are in S-phase. A phospho-histone H3 (Ser10) rabbit monoclonal antibody (Cell Signaling) assessed mitotic (M-phase) cells. DAPI was used as a total nuclear stain. Staining was all performed on the plate and was a novel methodology developed for these experiments. The purpose of developing this method was to avoid the need to detach the cells from the plate and therefore to avoid any bias in cells analysed due to incomplete detachment from the plate. More information on the development of this assay is available in Chapter 2.6.

This method was first tested by ‘serum starving’ the MNKs for 24h, fixing the cells and then comparing ‘serum starved’ to serum-treated cells. Although the KSFM does not contain FBS as normally used in cell medium, it does contain bovine pituitary extract (BPE) as well as epidermal growth factor (EGF), which play a similar role to FBS in aiding cell growth. Thus by excluding these factors, it is possible to starve the cells in a similar fashion to a regular serum starvation assay. The percentage of nuclei undergoing S-phase and M-phase was quantified by numbers of uniformly stained EdU and histone H3 positive nuclei respectively. These numbers were then compared to the total numbers of DAPI+ nuclei. For cell intensity controls, cells were stained with azide but not EdU as well as a rabbit IgG isotype control. Both EdU and H3 showed full nuclear staining. The number of both EdU- and H3-positive cells was drastically reduced in the serum-starved cells as compared to the cells that were not serum-starved. There was also an overall reduction in cell number in the serum-starved cells, consistent with the reduction in cycling cells (Fig 3.9).
Figure 3.9: Removal of BPE and EGF from keratinocyte cell media drastically reduces the number of S-phase (EdU, red) and M-phase cells (H3, green). Brm+/+ MNKs were stained with EdU/azide as well as a phospho-histone H3 antibody to assess S and M-phase cells respectively, and visualised on a fluorescent microscope. DAPI nuclear counterstain is in
blue. Visually there is a greater abundance of both S-phase and M-phase cells in the non-starved cells (right), hence validating the methodology. Merged image is of DAPI, H3 and EdU staining. All images were taken with a 20X objective, scale = 100μm.

To assess the percentages of both S- and M-phase cells, each image was analysed via ImageJ. By counting each DAPI+ cell and then using this as a mask, an intensity value for both the EdU and H3 channels of each cell were determined. This data was entered into a spreadsheet in a format that allowed its importation into FlowJo software, allowing each image to be studied similarly to a flow cytometry plot. This methodology is further described in Chapter 2.6.2. With the use of controls (a) as previously mentioned, negative cells could be identified and gated, with each cell above this intensity peak being considered positive. Firstly, the intensity of EdU was measured (S-phase) (Fig 3.10). There was approximately a 2-fold increase in EdU positive cells in the non-serum starved cells (c) as compared to the serum-starved cells (b), indicating that serum starvation in MNKs reduces the amount of S-phase cells.
Figure 3.10: Histogram plots show greater number of EdU positive cells in non-serum-starved MNKs as compared to serum-starved MNKs. Brm+/+ MNKs were stained with EdU/azide as well as DAPI and visualised on a Cytation 3 cell imager. Histogram plots of EdU positive cells in (a) control (b) no BPE/EGF and (c) +BPE/EGF treated groups. The no-EdU control treated cells were used to draw gates for negative cells. Note a large shift to the right on the X-axis (EdU) in cells treated with BPE/EGF (c) as compared to the serum-starved cells (b). Representative plots are shown of one experiment with approximately 2500 counted cells per culture.

Using the same methodology, the intensity of histone-H3 positive cells was also measured (Fig 3.11). All H3-negative cells were gated by the use of an isotype control (a), with anything brighter than this intensity peak being considered H3-positive. Similar to the shift in S-phase cells seen in Fig 3.18, there was an approximate 4-fold increase in H3-positive MNKs in the non-serum starved cells (c) as compared to serum starved (b).
Figure 3.11: Dot plots show greater number of histone-H3 positive cells in non-serum-starved cells as compared to serum-starved MNKs. MNKs were stained with phospho-histone H3 antibody as well as DAPI and visualised on a Cytation 3 cell imager. Dot plots of histone-H3 positive cells in (a) isotype control (b) no BPE/EGF and (c) +BPE/EGF treated groups. DAPI intensity is on the Y-axis, with histone H3 intensity on the X-axis. The isotype control treated cells were used to draw gates for negative cells. Note a large increase in positive cells in the BPE/EGF treated cells (c) as compared to (b). Representative plots are shown of one experiment with approximately 2500 counted cells per culture.

Lastly, both wells that were counted in the experiment were added together to find the total percentage of (a) EdU-positive cells and (b) H3-positive cells. The percentage of EdU-positive cells was significantly increased in the non-serum-starved cells (43.4%) as compared to the serum-starved cells (20.9%) (P=0.0014; paired t-test). The number of H3-positive cells drastically increased in the non-serum-starved cells (2.38%), and this was statistically significant as compared to the serum-starved cells (0.58%)(P=0.0043; paired t-test) (Fig 3.12).
Figure 3.12: Serum starvation of MNKs leads to a decrease of both S- and M-phase cells. The effect of serum starvation on MNKs on the percentage of both (a) EdU-positive and (b) H3-positive cells. There is a sharp decrease in both EdU and H3-positive cells following serum starvation. The percentage of EdU cells (P=0.0014), as well as the percentage of H3 cells (P=0.0043) was significantly decreased with serum starvation. Results are indicative of 2 separate experiments, with 2 wells and 2500 cells measured per sample (n=4). Mean + SD shown, Students t-test used.

3.2.6. The loss of Brm accelerates cell cycle progression of MNKs after UVR treatment

Following the serum starvation experiment confirming this methodology as valid, a further experiment to investigate the impact of UVR on Brm+/+ and Brm-/- MNKs was completed. The cell cycle assay was performed over various time points (24, 48, 72 and 96h), giving a thorough measure of how UVR affected the cell cycle of both Brm+/+ and Brm-/- MNKs over time. For this experiment, equal numbers of either Brm+/+ or Brm-/- MNKs were plated on 24-well plates and incubated at 37°C/5% CO₂ for 24h. The cells were then irradiated with 4J/cm² ssUV or sham-irradiated and cultured for different time periods. The cells
were then stained with EdU and an anti-histone H3 antibody. All plates were imaged using a Cytation-3 live cell imager. A total of 3 wells were sampled per experiment, including 1 well stained with isotype antibody (rabbit IgG), and without EdU but stained with azide as a further control. There was strong staining for both EdU and histone-H3 in both unirradiated samples. The pattern of EdU and H3 staining was noticeably reduced in the 24h Brm+/+ UV sample, compared to the Brm+/+ No UV. However, the reduction of S-and M-phase cells was less noticeable in the Brm-/- UV samples (Fig 3.13).
Figure 3.13: 4J/cm² UVR treatment of Brm+/+ but not Brm-/- MNKs decreases the number of EdU and histone-H3 stained cells. Primary Brm-/- and Brm+/+ MNKs were irradiated with ±4J/cm² ssUV and grown for 24h. Following 24h incubation, a reduction in EdU and H3 positive cells was seen in the Brm+/+ sample treated with UV, as compared to the untreated sample. This reduction was not as noticeable in the Brm-/- samples. All images were taken at 20x magnification, scale = 100µm. Each image is a merger of DAPI, H3 and EdU.

Images were analysed as described above for EdU (Fig 3.14) and H3 (Fig 3.15). In the 24h gate shown below (Fig 3.14), the EdU positive peak seen in the Brm+/+ No UV sample (c) was drastically reduced in the Brm+/+ UV sample (d) with the percentage of EdU-positive cells reducing by 32.5%. However, there was almost no decrease in the Brm-/- UV sample (b) compared to the Brm-/- No
UV sample (a), with UVR reducing the number of positive cells by only 0.3%. In the 24h gates for phospho-histone H3 (Fig 3.15), a large reduction in H3-positive cells can be seen in both UV samples (b, d) as compared to the No UV samples (a, c).

**Figure 3.14:** Brm+/+ MNKs but not Brm-/- MNKs show decreased S-phase cells 24h post 4J/cm² UVR. Histogram plots for EdU intensity in (a) Brm-/- No UV, (b) Brm-/- UV, (c) Brm+/+ No UV, and (d) Brm+/+ UV treated MNKs, 24h after treatment. As previously shown, control treated cells were used to identify the intensity of negative cells (EdU negative), and anything
above this threshold was considered positive for EdU (EdU positive). In the Brm+/+ UV treated samples (d), there is a reduction in EdU positive cells compared to its No UV control (c), as can be seen by the curve shifting to the left. However, this shift is not seen in Brm-/cells treated with UV (b), which has a similar curve to the No UV control (a). Representative plots are shown of one experiment with approximately 3000 counted cells per culture.
Figure 3.15: Both Brm-/- and Brm+/+ MNKs show a reduction in M-phase cells 24h post 4J/cm² UVR. Dot plots for histone H3 intensity in (a) Brm-/- No UV, (b) Brm-/- UV, (c) Brm+/+ No UV, and (d) Brm+/+ UV treated MNKs, 24h after treatment. DAPI intensity is on the Y-axis, with histone H3 intensity on the X-axis. Control treated cells were used to identify the intensity of negative cells, and anything above this threshold was considered positive for H3. In both the UV treated samples (b and d), there were less H3 positive cells as compared to the untreated samples (a and c). Representative plots are shown of one experiment with approximately 3000 counted cells per culture.
To analyse the effect of 4J/cm\(^2\) ssUV on the cell cycle of Brm+/+ and Brm-/- MNKs, a total of 3 experiments were compiled, using 2 experimental wells (n=6) and one isotype/no EdU control well per experiment. Each experiment was then entered into the flow cytometry program, FlowJo, and the total percentage of both EdU-positive cells (Fig 3.16) and H3-positive cells (Fig 3.17) were calculated. A complication is that EdU also stains cells undergoing unscheduled DNA synthesis (UDS) to repair DNA damage. However, cells undergoing UDS are less bright and show nuclear punctate staining, unlike S-phase cells, which show bright and uniform staining. In order to correct the data for S-phase cells, the total percentage of cells undergoing UDS was subtracted from each experiment. This was completed by manually counting the percentage of punctate stained EdU-positive cells and subtracting this from the total EdU-positive cells (UDS data is shown in Fig 3.23). In the No UV samples (Fig 3.16a), there was a gradual reduction in S-phase cells over time, with the highest percentages at 24h and the lowest percentages at 96h, presumably due to cell growth slowing as they reached confluence. There was no difference between unirradiated Brm-/- and Brm+/+ MNKs over the time course (P=0.7797; 2-way ANOVA). Irradiation with 4J/cm\(^2\) ssUV (Fig 3.16b) dramatically decreased S-phase Brm+/+ cells at all times except for 96h. However, irradiation of Brm-/- cells had a less striking effect. Over the course of the experiment, UV-irradiated Brm-/- cells had higher levels of S-phase cells than UV-irradiated Brm+/+ cells (P=0.0002; 2-way ANOVA), the gene effect lasting 72h. A similar effect was found for H3-cells (Fig 3.17). As for the EdU cells, the number of H3-stained cells was highest at 24h in the absence of UV, and this slowly fell over time, until 96h, when it was lowest for both Brm-/- and Brm+/+ cells (Fig 3.17a). Over the time
course, there was no significant difference in M-phase cells between the Brm−/− and Brm+/+ samples without UVR (P=0.4061; 2-way ANOVA). Irradiation with 4J/cm² UVR reduced the number of M-phase cells, in both Brm−/− and Brm+/+ cells at 24h (Fig 3.17b). Further, over time, both Brm−/− and Brm+/+ cells showed increased staining. Brm−/− cells had a peak of M-phase cells at 72h, whereas Brm+/+ cells peaked at 96h. Similar to the pattern of S-phase cells, when treated with UVR, Brm−/− MNKs had a significantly higher level of M-phase cells over the time course as compared to Brm+/+ cells (P=0.0005; 2-way ANOVA) (Fig 3.17b). The 96h time point was not included in the statistical analysis of the overall time course. This is because at 96h many wells had reached confluence, and thus the results at this time were due to the confluent culture conditions, rather than reflecting the effects of UVR.

![Graph](image.png)

**Figure 3.16:** Brm−/− MNKs undergo reduced UVR-induced cell cycle arrest and progress faster into S-phase following 4J/cm² ssUV than Brm+/+ MNKs. Primary neonatal keratinocytes were either sham-irradiated (a) or UV-irradiated (b), and further incubated for various time points. S-phase cells were determined by staining with EdU/azide and counting fully cells with a fully stained nucleus. Following UVR, Brm−/− MNKs had higher levels of S-
phase cells compared to Brm+/+ MNKs over the time course (*P=0.0002; b). However, without UVR, there was a high level of S-phase cells and no significant difference between the groups (P=0.7797; a). By 96h the cells became confluent and stopped growing due to the culture conditions as can be seen in the lack of proliferation in the control unirradiated cells. Hence results at this time were due to the confluent culture conditions rather than reflecting the response to UVR. Therefore these are shown for completeness but not included in the statistical analysis. Statistical analysis is Brm-/- compared to Brm+/+ at time points of 24, 48 and 72h. Each time point represents mean ± SEM from 3 independent experiments (2 wells per experiment; N=6). *P=0.0002; Two-way ANOVA used.
Figure 3.17: Brm-/- MNKs undergo reduced UVR-induced cell cycle arrest and progress faster into M-phase than Brm+/+ MNKs. Primary neonatal keratinocytes were either sham-irradiated (a) or UV-irradiated (b), and further incubated for various time points. M-phase cells were determined by staining with anti-histone H3 antibody and counting stained cells. Following UV, Brm-/- keratinocytes had higher numbers of M-phase cells as compared to Brm+/+ keratinocytes (*P=0.0005; b). However, without UVR, there was a high level of M-phase cells and no significant difference between the genotypes (P=0.4061; a). By 96h the cells became confluent and stopped growing due to the culture conditions as can be seen by the lack of proliferation in the control unirradiated cells. Hence results at this time were due to the confluent culture conditions rather than reflecting the response to UV and therefore are shown for completeness but not included in the statistical analysis. Brm-/- compared to Brm+/+ at time points of 24, 48 and 72h. Each time point represents mean ± SEM from 3 independent experiments (2 wells per experiment; N=6). *P=0.0005; Two-way ANOVA used.
Further information was obtained by examination of different comparisons between these groups. In Brm-/ cells, there was no significant effect of UVR on S-phase cells over time (P=0.6704; 2-way ANOVA) (Fig 3.18a), however the number of M-phase cells was reduced (P=0.0208; 2-way ANOVA) (Fig 3.18b). In contrast to Brm-/ cells, UVR significantly reduced the number of Brm+/ S-phase cells (P<0.0002; 2-way ANOVA; Fig 3.18c). Similarly to the effect on Brm-/ cells, UVR also significantly reduced M-phase Brm+/+ cells (P<0.0002; 2-way ANOVA) (Fig 3.18d). These results suggest that Brm is important for cells for progression through S-phase but not M-phase in response to UVR. Please note that these figures are the same data as plotted in Fig 3.16 and Fig 3.17, but plotted differently to facilitate comparison.
Figure 3.18: Brm is important for progression through S- but not M-phase in response to UVR. Primary neonatal Brm-/- (a, b) or Brm+/+ (c, d) keratinocytes were either sham-irradiated (a) or UV-irradiated (b), and further incubated for various time points. S-phase and M-phase cells were counted by staining with EdU/azide or an anti-histone H3 antibody and counting fully stained cells. In Brm-/- MNKs, UVR did not have a significant effect on S-phase cells (P=0.6704; a), however there was a significant effect on M-phase cells (‡P=0.0208; b). However, UVR had a significant effect on S-phase cells (*P<0.0002; c), as well as M-phase cells (*P<0.0002; d) in Brm+/+ MNKs. By 96h the cells became confluent and stopped growing due to the culture conditions as can be seen in the lack of proliferation in the control unirradiated cells. Hence results at this time were due to the
confluent culture conditions rather than reflecting the response to UV and therefore are shown for completeness but not included in the statistical analysis. No UV as compared to UV at time points of 24, 48 and 72h. Each time point represents mean ± SEM from 3 independent experiments (2 wells per experiment; n=6). Two-way ANOVA used. Data is the same as Fig 3.16 and 3.17 but with different comparisons.

Considering the role of Brm in UVR-induced cell cycle arrest, the percentage of S-phase cells at the earlier time point of 1h was investigated. UVR induced a decrease in S-phase cell numbers 1h post UVR in both Brm−/− and Brm+/+ cells (both P<0.0001; 1-way ANOVA; Fig 3.19). There was no significant difference between the percentage of S-phase Brm−/− and Brm+/+ MNKs with or without UV irradiation (P=0.9713 and P=0.9752; 1-way ANOVA). Therefore Brm does not appear to be required for entry into G1/G0 UV-induced cell cycle arrest but is involved in maintaining G1 arrest. Brm−/− cells are able to overcome cell cycle arrest much faster than Brm+/+ cells, as indicated by increased numbers of S- and M-phase cells at 24h (Fig 3.18).
Figure 3.19: UVR causes a significant decrease in S-phase cells 1h after UVR in both Brm-/- and Brm+/+ MNks. Primary neonatal Brm-/- and Brm+/+ keratinocytes were irradiated with or without UVR, and further incubated for 1h. S-phase cells were determined by staining with EdU/azide and counting uniformly stained cells. UVR significantly reduced S-phase cells in both Brm-/- and Brm+/+ cells (P<0.0001). There was no significant difference between Brm-/- and Brm+/+ unirradiated (P=0.9752) or irradiated cells (P=0.9713). Results are pooled from 3 separate experiments, 2 wells per experiment (n=6). Mean + SEM shown. One-way ANOVA used.

3.2.7. Brm loss increases levels of CPD lesions following UVR

To analyse the effect that Brm has on repair of DNA damage, 2 assays were used. Firstly, a method to analyse UDS was used. As with the cell cycle experiments, an EdU stain was used. UDS is observed as punctate or speckled cells, which is distinct from uniformly stained cells undergoing S-phase (Nakagawa et al. 1998; Fig 3.20). This methodology is further described in
Chapter 2.7.1. Briefly, equal numbers of either Brm+/+ or Brm-/- MNKs were plated on 4-well chamber slides and incubated at 37°C/5% CO₂ for 24h. The cells were then irradiated with or without 4J/cm² UVR and stained with EdU for 1h and then fixed. All slides were imaged using an Olympus BX51 microscope connected to a Jenoptik ProgRes MF camera and controlled with Image Pro Plus 7.0. Illumination was sourced from a xenon arc lamp. A total of 2 experimental wells were sampled per experiment, as well as 1 control well stained with azide but unprobed with EdU. The percentage of nuclei undergoing UDS was quantified by numbers of EdU stained punctate nuclei as compared to the total numbers of nuclei stained with DAPI. Due to all samples having to be measured manually, all slides were blinded and only unblinded following analysis.
Figure 3.20: An example of cells stained for UDS. Yellow arrowheads indicate cells undergoing S-phase. These cells show uniform and strong nuclear staining. Yellow arrowheads indicate cells undergoing UDS. In contrast, these cells have punctate or granular nuclear cell staining, which is less intense than S-phase cells (yellow arrowheads). Objective = 40X, scale = 100μm. This image is a merge of both DAPI and EdU staining.

After ssUV irradiation, repair of photodamage to nuclear DNA was calculated as the percentage of cells undergoing UDS. This was measured by counting the total number of punctate cells and comparing this to all cells stained with DAPI. Visually, when treated with UVR, there was an obvious decrease in intensely stained EdU-block nuclear positive cells (S-phase) in both Brm−/− and Brm+/+ MNKs. There was an increase in less intense, punctate stained cells, indicative of UDS (Fig 3.21).
Fig 3.21: UVR causes UDS in MNKs. DAPI and EdU labelled MNKs visualised by fluorescence microscopy. The untreated samples (No UV) show a large number of uniformly stained (S-phase) cells with few UDS cells (punctate staining). However, 1h post UVR in both Brm+/+ and Brm-/- cells, there is a decrease in S-phase cells and an increase in cells undergoing UDS. Objective = 40X, scale bar = 100μm. Each image is a merger of DAPI and EdU.

Using ImageJ software and the manual counting criteria as outlined in Fig 3.20, the percentage of nuclei undergoing UDS was determined in each treatment group. UVR treatment increased the percentage of UDS nuclei in both Brm-/- (P=0.0023) and Brm+/+ (P=0.0002) cells (Fig 3.22; 1-way ANOVA). This result was expected, as it is known that UVR induces DNA lesions, leading to a stimulation of DNA repair (de Gruijl et al. 2001). There was no significant
difference in the level of UDS in the unirradiated cells (Brm-/ vs Brm+/+, P=0.995; 1-way ANOVA). Similarly there was no significant difference in UDS in irradiated Brm+/+ compared to Brm-/ cells (P=0.5327; 1-way ANOVA).

Figure 3.22: UVR treatment leads to a significant increase in UDS 1h following UVR in both Brm-/ and Brm+/+ MNKs. Brm-/ or Brm+/+ MNKs were incubated for 1h post ±4J/cm² UVR and then stained with EdU/azide to determine the percentage of punctate stained EdU-positive cells (cells undergoing UDS). UVR treatment led to a significant increase of UDS in both Brm-/ (P=0.0023) and Brm+/+ (P=0.0002) MNKs. Results are pooled from 3 separate experiments, 2 wells per experiment (n=6). Mean + SEM shown. One-way ANOVA used.

UDS at 24h, 48h, 72 and 96h post treatment was also determined. In both Brm-/ and Brm+/+ unirradiated cells, the amount of UDS was highest at 24 and 48h. The percentage of cells undergoing UDS decreased rapidly by 72 and 96h. However, in the irradiated samples, the amount of UDS increased over time, where it was highest at 72 and 96h in both Brm+/+ and Brm-/ MNKs (Figure 3.23). There was no significant difference in the percentage of UDS positive cells in either the unirradiated cells (Brm-/ vs. Brm+/+, P=0.9651) or irradiated cells
(Brm-/ - vs. Brm+/+, P=0.3381) over the time course of the experiment. UVR was found to cause an altered UDS response in both Brm-/ - (P=0.0021; 2-way ANOVA) and Brm+/+ cells (P=0.0018; 2-way ANOVA). The amount of UDS in the unirradiated cells was significantly higher in the unirradiated cells at both 24 and 48h in both Brm-/ - and Brm+/+ cells (all P<0.0001; 2-way ANOVA). However, by 72h, the amount of UDS was higher in the UVR samples than in the No UV samples. This increase in UDS was statistically significant in Brm-/ - (P<0.0001; 2-way ANOVA) but not Brm+/+ cells (P=0.28; 2-way ANOVA). By 96h the amount of UDS was also higher in the UV samples of both Brm-/ - (P<0.0001; 2-way ANOVA) and Brm+/+ cells (P=0.0073; 2-way ANOVA). This result indicates that the majority of DNA repair following UVR is completed within 24h and that UVR reduces the amount of UDS at earlier time points. Further, it is important to note that the levels of UDS seen at 24, 48, 72 and 96h are much lower than that seen at 1h after UV-irradiation. In unirradiated cells the level of UDS at 1h and 24h was similar, however, in UVR-treated cells, the levels of UDS were approximately 7.7-fold lower in Brm-/ - cells and 11.8-fold lower in Brm+/+ cells. This indicates that MNKs are undergoing repair quite early after UVR, and are undergoing very little repair (less than unirradiated cells) by 24h. Brm+/+ cells also appear to recover faster from DNA repair than Brm-/ - cells.
Figure 3.23: The loss of Brm does not affect the percentage of cells in UDS in either (a) unirradiated or irradiated (b) cells at later time points. Brm−/− or Brm+/+ MNKs were incubated for 24, 48, 72 and 96h post ±4J/cm² UVR and then stained with EdU/azide to test for punctate stained EdU-positive cells (cells undergoing UDS). The loss of Brm did not affect the percentage of UDS in either (a) unirradiated (P=0.9651) or (b) irradiated (P=0.3381) cells. Results are indicative of 3 separate experiments, 2 wells measured per experiment (n=6). Mean ± SEM shown. Two-way ANOVA used.

UDS assesses the re-synthesis step of DNA repair but is not specific for UVR-induced DNA damage, as can be seen by the relatively high level of DNA repair in unirradiated cells, presumably due to factors such as reactive oxygen induced DNA damage in the cultures. To better assess whether a UVR-specific lesion was affected by the loss of Brm, both Brm+/+ and Brm−/− neonatal mouse skins were stained for CPDs following 4J/cm² UVR. Mouse pups at 1 day of age were euthanized and the skins were removed. The skins were irradiated with 4J/cm² UVR or sham-irradiated and cultured in medium for a set time for DNA repair to occur, before fixing the skins in 4% PFA to stop further repair. To trace CPD formation and repair over time the following times were used: 15 min, 30
min, 1h, 2h, 3h and 24h. The methodology for this is described in detail in Chapter 2.7.2.

Unirradiated Brm+/+ and Brm-/- skin samples generally had little/no staining for CPDs, as expected, and this staining was never higher than that of the isotype control. The amount of time spent in media following sham-irradiation also had no effect, and there was no CPD signal found at any of the time points analysed. However, when the skins were irradiated with UVR, a CPD signal was observable by 15 min. The CPD signal was visible in both the epidermis and dermis of the mouse skins. By 1h, the level of CPDs had increased even further in both Brm-/- and Brm+/+ skins (Fig 3.24). Over this time frame there was never a complete loss of CPD signal, indicating that even at 24h post UVR, CPD repair was not complete. Visually, it was noticeable in most samples that Brm-/- skins had a higher CPD signal at almost all time points following UVR, including at 24h.
Figure 3.24: 4J/cm² UVR induces CPDs in both Brm+/+ and Brm-/- murine neonatal skins that repair over time. Neonatal mouse skins were irradiated with 4J/cm² UVR or sham-irradiated and incubated in cell medium at 37°C/5% CO₂ for varying times before fixation. When stained with an anti-CPD antibody, the No UV samples did not show any CPD formation. However, the UVR-treated samples showed CPD formation as early as 15 min post UVR. The peak of CPD formation was around 1h in the UVR
samples, which reduced by 24h post UVR. Visually, a reduction in CPDs was noted in the majority of Brm+/+ samples by 24h, however Brm-/- skins did not show as marked a reduction. All images are at 20X magnification, scale = 100μm. DAPI staining (blue) detects all cells while red cells indicate CPD positive cells.

By image analysis of the epidermal area, the total CPD intensity per cell was calculated (see section 2.7.2). At all time points measured, the levels of CPDs were higher in UVR-treated samples than the No UV samples. There was no significant difference in the number of CPDs between unirradiated Brm-/- and Brm+/+ skins at any time point, or over the time course (P=0.7569). Further, the CPD intensity per cell was higher in all UVR-treated Brm-/- neonatal skins, compared to Brm+/+ skins (Fig 3.25). The only time point where the increase in CPDs in Brm-/- were not of statistical significance was at 15 min (P=0.8597) and 2h (P=0.9533), whereas the difference between UVR-treated skins was significant at 30m (P=0.0067), 1h (P=0.0075), 3h (P=0.0085) and 24h (P=0.0043; 2-way ANOVA). Interestingly, from 15 min to 1h, the level of CPDs did not increase in Brm+/+ skins, however, CPDs dramatically increased in Brm-/- skins. The amount of CPDs in UVR-treated Brm-/- skin was steady at 2h; and continued to increase up to 3h, and then slightly decreased by 24h. For Brm+/+ skin, the intensity of CPDs was found to increase up until 2h, but by 3h this had reduced. By 24h, this had reduced even further. Over the total time course of the experiment, it was found that Brm-/- had significantly higher levels of CPD staining per cell than Brm+/+ (P=0.0161; 2-way ANOVA) (Fig 3.25). These results suggest that the expression of Brm in C57/B6 mice is important to prevent excessive CPD lesions in response to UVR. The loss of Brm leads to
increased CPD formation, and therefore a higher rate of repair is needed in the same amount of time to compensate. Considering the levels of UDS are similar in Brm-/- and Brm+/+ cells, it is unlikely that they are able to compensate for this.

Figure 3.25: 4J/cm² UVR induces CPDs in both Brm+/+ and Brm-/- murine neonatal skins that repair over time. Neonatal mouse skins were irradiated with 4J/cm² UVR or sham-irradiated and incubated in cell medium at 37°C/5% CO₂ for varying times before fixation. When stained with an anti-CPD antibody, the No UV samples showed limited CPD formation. However, the UV samples showed CPD formation as early as 15min post UVR. The peak of CPD formation was around 1-2h in the UV samples, which reduced by 24h post UVR. Visually, a reduction in CPDs was noted in the majority of Brm+/+ samples by 3 and 24h, however Brm-/- skins showed a greater amount of CPDs at these times. Over the total time course of the experiment, it was found that Brm-/- had significantly higher levels of CPD staining per cell (P=0.0161). Results are indicative of 3 independent experiments, with 3 mice used total (n=3). Mean + SEM shown.
3.2.8. Results summary

With the use of a Brm-knockout ex vivo mouse model, it was found that the loss of Brm in these mice led to an increase in Brg1 expression. The loss of Brm and increase of Brg1 alone did not lead to any cellular abnormalities. However, following UVR, an increase in the growth rate of the cells, as well as a decrease in cell death was noted in Brm-/- cells. Further, upon assessment of cell cycle regulation, it was found that Brm-/- cells had increased levels of both S- and M-phase cells at 24, 48 and 72h post UVR, but not at 1h. This indicated that Brm-/- and Brm+/+ cells were undergoing initial G1/G0 cell cycle arrest in response to UVR, but Brm-/- cells were cycling through the G1-S transition faster than Brm+/+ cells. Moreover, although these cells contained similar levels of DNA repair, Brm-/- cells contained higher levels of UVR-specific DNA lesions at all times studied up to 24h, indicating that Brm-/- cells were cycling whilst harbouring significant levels of DNA photolesions.

3.3. Discussion

In this chapter, the role of the loss of Brm in neonatal mouse skin was assessed, with an aim to identify whether a loss of Brm conferred cellular changes in response to UVR. As an integral part of the SWI/SNF chromatin-remodelling complex, it is known that Brm plays an important role in the configuration of chromatin, and thus, can influence many cellular events. Brm is known to bind to the Rb complex, and Rb-dependent growth inhibition at the G0/G1 phase is dependent on its binding with Brm/Brg1 (Marquez et al. 2015). Active Brm/Brg1 is also vital for the transcription of the tumour suppressor p53,
which plays a vital role in cellular stress responses, such as apoptosis, cell cycle arrest, DNA repair and oxidative stress (Xu et al. 2007). Although SWI/SNF and Brm have long been implicated in the prevention of tumourigenesis, the role that Brm plays in non-melanoma skin cancer and particularly responses to UVR is still relatively unknown. Recently, it was shown that Brm-/- mice harbour more tumours following a 25-week UV-irradiation schedule as compared to their wildtype littermate controls (Halliday et al. 2012). Further, Brm-/- mice also harbour increased epidermal hyperplasia as compared to Brm+/+ mice following UV-irradiation. Ki-67+ staining also confirmed that UV-irradiated Brm-/- mice had significantly higher levels of cell division compared to Brm+/+ mice (Hassan et al. 2014). From these findings, it was important to analyse the cellular events in response to UVR, in order to determine the role that Brm plays in NMSC. Key responses to UVR such as cell proliferation, cell cycle regulation and DNA repair were all monitored, as these events all influence carcinogenesis. It was hypothesised that the loss of Brm would alter the cellular response to UVR, leading to an increase in cell proliferation, dysregulation of the cell cycle, and an altered DNA repair response.

3.3.1. Brg1 protein is upregulated in Brm knockout mouse skin

To ensure that Brm knockout mice were not expressing Brm protein, skin samples from Brm-/- and Brm+/+ mice were collected and their protein levels were analysed for Brm expression. As expected, Brm-/- mice did not express any detectable Brm, whereas Brm+/+ mice possessed high levels of Brm protein. An active form of either the closely related Brm or Brg1 is important for SWI/SNF
function (Euskirchen et al. 2012). The original paper studying Brm-knockout mice found these mice exhibited an increase in Brg1 expression in total organ extracts (Reyes et al. 1998). The majority of the literature supports that Brm and Brg1 are interchangeable, and can compensate for one another, however Brm-/- mice are completely viable, whereas Brg1-/- mice are embryonic lethal, suggesting varying roles for these molecules (Reyes et al. 1998; Bultman et al. 2000). Interestingly, ablation of Brg1 in the forming epidermis does not affect the proliferation and early differentiation of keratinocytes, but does induce severe skin permeability skin defects (Indra et al. 2005). However, this is in contrast to the absolute requirement of Brg1 for the proliferation of F9 carcinoma cells and the cells of pre-implantation embryos (Bultman et al. 2010; Sumi-Ichinose et al. 1997), therefore illustrating the cell type-specific differential requirement of Brg1-containing remodelling complexes in vivo.

In this study, the level of Brg1 protein was tested in the skin and it was found to be upregulated in Brm-/- mice as compared to Brm+/+ mice. Brg1 was present in the skin of Brm+/+ mice as well, suggesting that normal wild-type mice express both Brm and Brg1. In the literature it is mostly thought that Brg1 is able to compensate for a loss of Brm, and vice-versa (Strobeck et al. 2002; Gong et al. 2008). Moreover, Brm/Brg1-null mice show even greater epidermal defects in the suprabasal cells than Brg1-null alone mice, indicating Brm can partially substitute for Brg1 functions in these cells (Indra et al. 2005). This suggests that Brg1 may be upregulated in Brm-/- epidermis to compensate for these epidermal defects. However, evidence suggests that Brm and Brg1 are involved in the regulation of the expression of different sets of genes (Kadam and Emerson, 2003); therefore it is unlikely that one subunit is able to fully
compensate for the other. Interestingly, overexpression of Brg1 has been found to be associated with increased invasiveness in prostate cancers (Sun et al. 2007). However, increasing Brg1 in these tissues also lead to decreasing levels of Brm, suggesting that Brm but not Brg1 is a tumour suppressor gene in prostate cancer. However, the majority of the literature supports Brg1 playing a role in tumour suppression (Reisman et al. 2009). Therefore, any increase in Brg1 in these cells would be expected to play a tumour suppressive role, and not the contrary and therefore may partially counteract the loss of Brm.

3.3.2. The loss of Brm in MNKs decreases cell death and increases cell growth in response to UVR

An appropriate cellular response to UVR-induced damage is essential if a cell is to maintain homeostasis. DNA damage via UVR induces cellular recovery systems, which involve the activation of repair pathways, cell cycle arrest and apoptosis (Gentile et al. 2003). Generally, if a cell harbours excessive damage it is removed via apoptosis, however, various diseases including cancer can be caused by the suppression of programmed cell death, as well as uncontrolled proliferation (Thompson, 1995; Hanahan and Weinberg, 2011). The SWI/SNF complex is known to influence p53-mediated apoptosis, and the inactivation of Brm and Brg1 can leave cells highly susceptible to DNA-damage induced apoptosis due to prolonged activation of p53 (Park et al. 2009). Firstly, it was discovered that Brm-/ - cells have a significant survival advantage over Brm+/+ cells when irradiated with doses of 2, 4 or 6J/cm² ssUV, indicating that Brm is necessary for UVR-induced apoptosis activation in response to both low and high
doses of ssUV. This result was further confirmed in a time course with 4J/cm² ssUV, again indicating that Brm-/- cells had a survival advantage over Brm+/+ cells. This was especially prominent at later time points such as 48 and 72h, indicating that although the loss of Brm does not lead to immediate apoptotic evasion, it is likely necessary for UVR-induced apoptosis later in the cell cycle, possibly at the later G2/M phase. Interestingly, this finding is similar to that of Gong and colleagues (2008), who found that increasing Brg1 in Brm-null SW13 cells led to increased resistance to UVR-induced apoptosis (Gong et al. 2008). This result suggests that increased Brg1, but not decreased Brm in these MNKs may be responsible for the observed increased apoptotic evasion in response to UVR.

Brm has also long been implicated to play a role in cellular proliferation and the cell cycle. Brm specifically promotes entry of cells into G1/G0 cell cycle arrest, and is also phosphorylated at the G2/M transition (Muchardt et al. 1996; Muchardt et al. 1998). This study demonstrated that in MNKs, the loss of Brm led to increased cell growth in response to 4J/cm² UVR. Although both the Brm-/- and Brm+/+ MNKs initially responded similarly to UVR by reducing in cell number, the Brm-/- cells were found to recover more quickly from the damaging effects of UVR. Again, these results were more prominent at later time points, such as 48h and 72h, and by 96h Brm-/- cultures had double the number of cells of Brm+/+ cells, indicating that in addition to evading apoptosis, these cells were growing at twice the rate of Brm+/+ cells. Considering the timing of the results, it seems likely that the increased cell number is at least partly explained by the increased apoptotic evasion seen in Brm-/- cells. It is likely that many living Brm-/- cells may harbour increased DNA damage, since a major function of
apoptosis is to remove cells with irreparable DNA damage from the organ (Thompson, 1995). Brm is also known to be important for entry of a cell to the G1/G0 phase, while the loss of Brm can be attributed to oncogenic transformation (Das et al. 2007). This therefore suggests a clear growth-inhibitory role for Brm; however, it is most likely that the role of Brm depends on the context. For example, the loss of Brm in Brg1-deficient cell lines actually slows growth of the cell and leads to decreased cell viability, whereas the loss of Brm in Brg1 wildtype cells has minimal effect (Oike et al. 2013; Wilson et al. 2014). Generally, if DNA damage is more substantial and thus repair is not possible, the cell will enter into cell cycle arrest and activate apoptosis processes, leading to removal of the cell from the organ. However, if the DNA damage is insufficient to warrant apoptosis of the cell, yet cannot be repaired, a wrong nucleotide can be incorporated in the next round of transcription, establishing a DNA mutation (De Gruijl, 2000). Therefore, a combination of increased cellular growth and decreased cell death would almost certainly lead to downstream mutations in Brm-/- cells. Persistence of UVR-induced lesions such as CPDs leads to downstream UVR signature mutations, as seen in XP patients, who are extremely sensitive to UVR and sunburn, and therefore highly prone to UVR-induced skin cancer (Yarosh, 2004).

In order for a cancer cell to divide uncontrollably, the cell must not only hijack proliferative pathways, but must also evade cellular death pathways (Hanahan and Weinberg, 2011). Therefore, the acquired resistance to apoptosis and increased cellular growth as seen in UV-irradiated Brm-/- cells are both hallmarks of a cancer cell. It is likely that p53, and its target genes important for the apoptotic and cell cycle arrest response are being affected by the loss of Brm,
as Brm/Brg1 are known to form complexes with p53 (Lee et al. 2002). Further, loss of Brm and Brg1 can lead to decreased activation of p53 target genes as well as cell death (Xu et al. 2007; Naidu et al. 2009). The mutation of p53 is extremely common in aggressive cancers, and intriguingly, the percentage of SWI/SNF component mutants in cancer is strikingly similar to that of p53, indicating a tumour suppressive role for Brm and Brg1 in many cancer types (Shain and Pollack, 2012). Mutations of the p53 gene in cell lines leads to irregular cell growth, cell cycle and apoptotic responses, leading to the mutant cell harbouring increased growth and survival advantages, as well as G1 checkpoint deficiency (Rivlin et al. 2011). Mutation of p53 is common in the majority of cancers, including melanoma and NMSC (Brash et al. 1996; Carson et al. 2012).

Brm also forms complexes with the Rb complex, with the growth of cells reliant on Rb forming complexes with Brm and Brg1 (Dunaief et al. 1994). Since Brg1 is upregulated in Brm-null MNKs, it is likely that the upregulation of Brg1 is leading to apoptotic evasion, as also seen in UVR-treated SW13 cells (Gong et al. 2008), with the evasion of cell death leading to increased numbers of damaged cells. However, it is also possible that the loss of Brm alone is leading to downregulation or altered activation of molecules associated with cell growth and apoptosis, such as p53 and pRb, and thus also leading to an increased proliferative state. It is unknown if Brg1 would be able to compensate for the loss of Brm in this context, but previous literature suggests that Brg1 alone can form complexes with Rb and p53 (Dunaief et al. 1994; Naidu et al. 2009).

These results are in agreement with previous experiments conducted in my laboratory. Firstly, a UVR-carcinogenesis study on the same line of Brm-/mice used in my study found that following a 25-week UVR regime, Brm-/ mice
harboured significantly more tumours than Brm+/+ mice (Halliday et al. 2012). Interestingly, when Brm loss was in combination with a partial p53 loss (p53+/-), the skin tumour rate was not exacerbated above that of the partial p53 loss alone; however, there was an increased average tumour diameter over time. This indicated that Brm loss augments carcinogenesis, even with partially defective p53, however, this tumourigenesis was not completely independent of p53 status. Moreover, there was no correlation found between skin tumour growth rate and Brg1 mRNA levels, indicating that altering levels Brg1 are unlikely to be involved in UVR skin tumourigenesis. Another recent study also showed that 25-week irradiated Brm-/- mice have increased epidermal hyperplasia, as well as increased levels of Ki-67+ proliferating cells (Hassan et al. 2014). Increased cellular growth shortly following UVR would account for the increased epidermal hyperplasia seen in these mice. Hence, this result in combination in with the increased cellular growth and evasion of apoptotic cells encountered in this study combines to tell a story of why Brm-/- mice are harbouring more tumours than Brm+/+ mice following UVR.

3.3.3. Brm loss leads to a defective cell cycle response to UVR in MNKs

Previously, it was shown that the loss of Brm in combination with UVR led to an increased cellular growth rate. Therefore, it seems likely that a defect in cell cycle regulation is present in UVR-treated Brm-/- MNKs. UVR induces both G1 and G2 cell cycle arrest, with the G1 arrest caused via a p53-dependent mechanism, and G2 being p53-independent, instead involving a block in cdc25-dependent activation of cyclin dependent kinase (CDK) complexes (Lu and Lane,
Further, human keratinocytes undergo G2 phase cell cycle arrest for up to 48h post UVR, which is associated with p16 expression but no involvement with p53 (Pavey et al. 2001). SWI/SNF has been implicated in the regulation of many cell cycle genes, including the INK4A-ARF locus, p21, cMyc, as well as cyclin D and E (Ruijtenberg and van den Heuvel, 2016). Brm expression is also important for entry of cells into G1/G0 cell cycle arrest (Muchardt et al. 1998; Reyes et al. 1998). An adequate cell cycle response is vital for cell damage responses to UVR, in order to allow time for DNA repair to take place. Considering the length of the cell cycle of a human keratinocyte is approximately 24h (Cooper, 2000), and the fact that the unirradiated cell numbers doubled in mouse keratinocytes (Fig 3.7) in 24h, it can be assumed the cell cycle duration of a mouse keratinocyte is similar to that of a human keratinocyte, however, the effects of UVR on cell cycle regulation would slow this growth rate due to increased cell cycle arrest (Gentile et al. 2003).

With the use of a cell cycle staining assay, over the first 72h of the course of the experiment, the percentage of S-phase cells was significantly increased in UV-irradiated Brm-/- cells as compared to Brm+/+. Similarly, UVR-treated Brm-/- keratinocytes had a significant increase in M-phase cells compared to Brm+/+ keratinocytes over the time course. However, when the cells were unirradiated, there was almost no effect of Brm deletion on either S- or M-phase cells. This illustrates that, without a carcinogen such as UVR, the loss of Brm has neither growth advantage nor disadvantage. In order to understand the cell cycle kinetics of Brm-/- cells better, the amount of S-phase cells was also tested at 1h post UVR. Interestingly, it was found that both Brm-/- and Brm+/+ had a significant reduction (approximate 4-fold decrease) in S-phase cells at this time.
Collectively, these data indicated that both Brm-/- and Brm+/+ MNKs undergo G0/G1 cell cycle arrest shortly after irradiation with 4J/cm² UVR, indicating that Brm is not necessary for G0/G1 arrest. However, when Brm-/- MNKs are treated with UVR, the cells show an increased growth advantage over time as compared to cells with functional Brm. It is likely that increased Brg1 in Brm-/- cells could be compensating for the loss of Brm, enabling the cells to undergo G0/G1 arrest, considering expression of Brg1 enables Rb-mediated cell cycle arrest (Strobeck et al. 2000). This is evidenced by the fact that Brm is able to compensate for the loss of Brg1 in SW13 cells, and vice-versa, restoring Rb-mediated G1 cell cycle arrest (Strobeck et al. 2002). Therefore, it is highly likely if both Brm and Brg1 were deregulated in these cells, no arrest would occur whatsoever, as witnessed in the extremely UVR sensitive cell line SW13 that contain undetectable levels of both Brm and Brg1 (Gong et al. 2008). Thus, in Brm-/- MNKs, the upregulation of Brg1 may compensate for the loss of Brm, allowing G1 cell cycle arrest to take place. However, the cell cycle data indicates that Brm-/- cells exit G1 cell cycle arrest earlier than Brm+/+ cells, and enter S-phase as observed by their increase in S-phase cells at 24h. It is unlikely that the G2/M phase of these cells are also affected, as another paper has previously shown that the loss of Brm/Brg1 in combination with UVR leads to DNA damage without affecting the G2/M DNA damage checkpoint (Park et al. 2009). However, the fact that Brm-/- showed a significant increase in M-phase at 72h as compared to Brm+/+ MNKs suggests that Brm-/- cells may also be undergoing reduced G2 arrest as compared to Brm+/+ cells, or that the increased M-phase cell number is a consequence of the larger number of cells completing S-phase.
As well as being important for Rb-mediated cell cycle arrest, Brm and Brg1 are also known to form complexes with p53, a complex that is extremely important in the response to UVR (Xu et al. 2007). Upon UV-irradiation-induced DNA damage, levels of p53 increase and this leads to G1 cell cycle arrest. Further, loss of p53 leads to cells becoming resistant to UVR-induced G1 arrest (Geyer et al. 2000). In cell lines with inactive Brg1, Brm was necessary for p53-mediated induction of p21 in MCF7 cells (Xu et al. 2007). p21 is a well-known regulator of cell cycle arrest at the G1 and S-phase. Mutation of p53 has been shown in combination with the loss of Brm/Brg1 in ovarian hypercalcemic-type cancer (Jelinic et al. 2016), further suggesting that the loss of Brm/Brg1 can lead to dysregulation of p53. Although p53 and Rb are not directly linked, the pathways interact at several points such as through the p14ARF/INK4A locus, and can influence one another. Further, the majority of tumours show perturbation of growth regulation and cell cycle checkpoints via downregulation or mutation of both of these tumour suppressors (Bates et al. 1998). An inactive Rb complex also leads to the dysregulation of E2F, causing repression of several E2F-dependent genes involved in cell cycle progression such as cyclin A and cyclin E. Previous experiments have shown that the cooperation of Brm and Rb are vital for E2F regulation and for the induction of growth arrest (Trouche et al. 1997). Further, Brm loss in prostate confers a proliferative advantage via the dysregulation of E2F (Shen et al. 2008). Therefore, it seems likely that the loss of Brm in MNKs is leading to decreased or delayed p53 and Rb-mediated signalling in response to UVR, likely leading to delayed activation of target genes such as p21, E2F, cyclins and various other signals, leading to faster passage through the G1-phase into S-phase. It is likely that Brg1 is compensating for Brm in Brm-/-
cells, and forming complexes with Rb and p53, in order to initiate G0/G1 cell cycle arrest, however, Brg1 alone is likely not fully dispensable for Brm in these cells for complete Rb and p53-mediated cell cycle regulation. Each tissue system and cell type have different dependencies on various SWI/SNF subunits, hence a loss of Brm in skin keratinocytes may be more catastrophic to the cell than in other systems. However, as referenced by the increased epidermal hyperplasia and tumour incidence in UVR-treated Brm-/- mice (Halliday et al. 2012; Hassan et al. 2014), it seems that the loss of Brm in these mice in combination with UVR is sufficient to cause a cellular catastrophe, leading to increasing levels of cellular proliferation, decreased cell death, deregulation of cell cycle checkpoints, and hence, tumourigenesis. It is also expected that the reduced time Brm-/- cells spend in cell cycle arrest would leave them with less time to repair UVR-induced DNA lesions. This could lead to the accumulation of DNA damage, and downstream mutations, a prerequisite to malignancy, as mutations can lead to carcinogenesis downstream (Branzei and Foiani, 2008).

3.3.4. The loss of Brm in MNKs leads to increased levels of UVR-induced DNA lesions

The decreased time that Brm-/- cells are spending in G1 cell cycle arrest is likely to lead to decreased time available for repair. Further, Brm and Brg1 have previously been linked with the removal of UVR-induced DNA lesions, influencing the recruitment of repair factors (Gong et al. 2008; Zhao et al. 2009). In order to assess whether Brm-/- and Brm+/+ cells were undergoing similar levels of UVR-induced DNA repair, 2 assays were utilised. Firstly, overall DNA
repair via measurement of UDS was analysed. This is a direct DNA repair assay as it measures the amount of nucleotides incorporated into foci of repairing DNA, but does not assess specific photolesions. It was found that UVR significantly increased the levels of UDS 1h post UVR. However, the loss of Brm did not influence the level of UDS in either unirradiated or irradiated cells. Similarly at later time points such as 24h, the loss of Brm did not influence the percentage of cells undergoing UDS in unirradiated or irradiated cells. Therefore, the loss of Brm does not lead to an overall decrease in the level of DNA repair following UVR. Since UDS only analyses the DNA re-synthesis step of DNA repair (Kelly and Latimer, 2005), it was decided to also stain for CPDs over a time course during which DNA repair should occur, in order to analyse whether the loss of Brm influenced the appearance or repair of UVR-specific DNA lesions. The loss of Brm in MNKs led to an increased level of CPDs at all time points measured, indicating that although the loss of Brm does not influence overall DNA repair, the loss of Brm leads to increased levels of CPD lesions. Due to increased CPD accumulation, a higher rate of repair would be needed in the same amount of time. However, since UDS is similar in both cell types, Brm-/- cells are not able to fully compensate for the increase in CPD accrual, explaining why CPD lesions are still higher in Brm-/- than Brm+/+ epidermis at 24h.

Interestingly, both Brm-/- and Brm+/+ cells developed increased CPD formation in the dark after cessation of the UVR exposure, with Brm+/+ showing their peak of CPD formation at 2h, and Brm-/- cells at 3h, before both showing repair by 24h. CPDs formed long after the initial UVR treatments are referred to as “CPDs in the dark” (Premi et al. 2015). CPDs in the dark constitute half of all CPD formation in UVA and UVB-irradiated murine melanocytes. CPD in the dark
formation has been shown to involve melanin in the case of melanin containing cells. The increased formation of CPDs in the dark is believed to be related to increased levels of produced reactive oxygen species (ROS) such as nitric oxide (NO), superoxide and peroxynitrite, which degrade melanin to form melanin degradation products. In turn, these melanin products appear in the nucleus, and peroxynitrite is able to excite electrons in the melanin fragment to a triplet state that has the high energy of a UV photon. Eventually, this energy is transferred to DNA bases, forming CPD lesions (Premi et al. 2015). In the experiments described in this chapter, the keratinocytes isolated from murine skin may have contained enough melanin to induce CPDs in the dark via the mechanism described by Premi and colleagues. However CPD formation in the dark has also been observed to occur in keratinocyte cell suspensions unlikely to contain melanin (Gupta et al. 2007; Dixon et al. 2011). It has also been demonstrated previously that NO is involved in CPD formation in the dark in keratinocytes (Gupta et al. 2007). Thus it is possible that other pigments may also lead to CPD formation in the dark via alternate mechanisms.

Interestingly, the mutation of Brm/Brg1 subunits has been associated with increased cytotoxic stress and increased ROS sensitivity (Xia et al. 2007; Freeman et al. 2014). The loss of Brm and Brg1 in these studies led to increased sensitivity to DNA damaging agents such as deoxyrubicin, cisplatin and menadione, confirming that Brm is necessary for cell viability when exposed to ROS-producing agents (Xia et al. 2007; Freeman et al. 2014). Thus, it is likely that the loss of Brm is leading to altered molecular events, in turn leading to increased cellular sensitivity to UVR, and thus, increased CPD formation in the dark, possibly via ROS or NO production. The loss of Brm and upregulation of
Brg1 may also lead to the reorganisation of nucleosomes, altering expression of genes, concomitantly affecting UVR resistance to DNA damage or making the DNA more accessible to DNA damaging molecular events. SWI/SNF is important for the conformation of nucleosomal DNA, and thus the efficient repair of DNA lesions (Gaillard et al. 2003). Therefore the loss of Brm and the compensation of Brg1 could be leading to altered nucleosomal structure, and therefore, increased CPD formation.

Further, it is also likely that the reduced time that Brm-/- cells are spending in G1 cell cycle arrest is not allowing enough time for their DNA to be fully repaired, thus continuing to proliferate with DNA that is improperly repaired. DNA repair is tied closely with DNA replication and the cell cycle, and therefore, the time it takes to repair the damage is closely related to the time it takes for a cell to divide (Branzei and Foiani, 2008). By 24h, the bulk of irradiated Brm-/- cells had reached S-phase, whereas, Brm+/+ cells had not. This suggests that Brm-/- cells are prematurely exiting G1 phase and entering S-phase, whereas the majority of UVR-damaged Brm+/+ cells are still in G1-phase, repairing their DNA. This is also confirmed in the cell growth data, where Brm-/- cells show doubling from 0 to 48h, suggesting a complete cycle has been completed, whereas Brm+/+ cells only show a 1.3-fold increase in cell number from 0 to 48h, suggesting the majority of cells have not completed a full cycle by this stage. This is further confirmed in UVR-irradiated cells at 48h, which showed that Brm-/- cells have significantly higher levels of S-phase cells at this time point. Considering the increased level of CPDs in Brm-/- cells at 24h, this clearly indicates that Brm-/- cells are proliferating while still harbouring significant levels of DNA lesions. Further, evasion of apoptosis as seen in Brm-/-
cells would also likely be leading to higher levels of CPDs in these cells, as these cells would likely all be carrying increased DNA damage. This is supported by the literature, which have found SWI/SNF proteins are integral for a proper NER response (Hara and Sancar, 2003). For instance, SW13 cells are null for both Brm and Brg1 expression, and restoration of Brg1 in these cells led to an increase in the repair of CPD lesions over time (Gong et al. 2008). SW13 cells also fail to arrest at the G1/S phase after UVR, however, the re-expression of Brg1 into these cells reverses this. Growth arrest and DNA damage-inducible protein 45-alpha (GADD45a), an important repair protein, is upregulated in SW13 cells expressing Brg1 as compared to SW13 cells with no Brm/Brg1 (Gong et al. 2008). The loss of Brm/Brg1 in combination with UVR also leads to a defective NER response, via the impaired recruitment of downstream NER factors, XPG and PCNA (Zhao et al. 2009). It is likely that the phenomena seen in other cells exposed to UVR are quite similar to the pattern seen in MNKs in these experiments. Brm may be modulating checkpoint activation after UV damage via regulation of proteins such as p53/p21 and GADD45a, and the loss of Brm in these cells could be leading to a defect or delay in this checkpoint. Instead, Brm-/- cells are undergoing G1/G0 arrest and quickly passing through this checkpoint through to the G1-S phase, whilst harbouring unrepaired DNA damage. The increased expression of Brg1 in these cells may be enough to still cause G1 arrest, but may not be enough to mirror cells with full Brm expression. This would therefore leave cells with a smaller amount of time to repair the larger number of CPD lesions that form in the dark, even if the repair rate is unaltered by loss of Brm. An increased NER response would therefore be necessary in Brm-/- cells. However, repair is similar in both Brm-/- and Brm+/+ cells at 1h and 24h,
suggesting that Brm-/ cells do not show an increased NER response. CPD formation is thought to be especially important for the initiation of skin cancer, as it has been closely linked to generation of mutations in tumour suppressor genes, such as p53, that are expressed in many UVR-induced skin tumours (Weber, 2005). If the improperly repaired cell is then able to proliferate, this can lead to cell mutations and carcinogenesis (Alteri et al. 2008). A reduction in Brm could also be leading to a decreased response of the BRCA1 gene, by modulating ATR/ATM activation. Recently it was found that a reduction of Brg1 can impair the recruitment of BRCA1 to damage sites, and attenuates DNA damage induced BRCA1 phosphorylation (Zhang et al. 2014). A loss of Brm in this system may lead to a reduction in BRCA1 phosphorylation, leading to the prevention of ATR/ATM activation, and thus, an impaired NER response.

The combination of the events as described due to Brm loss in these cells would have a major effect on the cell. Although the loss of Brm and upregulation of Brg1 is still leading to initial p53 and Rb-mediated G0/G1 arrest, the lack of Brm permits early exit from G1 into S-phase. This means the cell would not have long enough to repair DNA lesions such as CPDs. The reduced time spent in cell cycle arrest may account for why the number of CPDs is much greater in Brm-/cells, however the loss of Brm could also be increasing ROS production or leading to greater sensitivity to ROS, thus causing further CPD formation in the dark. The increase in DNA damage, in combination with an increased proliferative rate due to faulty cell cycle checkpoints as well as a decreased apoptotic rate could lead to an increase in the mutation rate, and thus the expansion of cells with damaged DNA. The increased rate of formation of mutations could then accelerate the development of carcinogenesis (Rastogi et al. 2010). This is confirmed by
previous experiments in my laboratory that showed that Brm-/- mice have significantly higher number of tumours following 25 weeks of UV-irradiation (Halliday et al. 2012). These mice also showed increased epidermal hyperplasia and Ki-67+ proliferating cells after 2 weeks of UVR, suggesting that the loss of Brm in combination with UVR damage can have almost immediate consequences (Halliday et al. 2012; Hassan et al. 2014) (Fig 3.26).

**Figure 3.26:** A model for how the loss of Brm in murine skin leads to carcinogenesis. In a normal cell system (a)(Brm+/+), when a cell is irradiated with UVR, p53 and p21 combine with Brm and SWI/SNF in order to arrest the cell (G0/G1 arrest). This leads to the activation/recruitment of other factors such as Rb, E2F, GADD45a in order to cause an adequate cell cycle/repair response. DNA repair will be activated if the damage is manageable, or apoptosis if the DNA damage is too high. Eventually, with successful repair, the cell enters S-phase and then G2/M phase leading to normal cell division by approximately 72h. However, the loss of Brm (b)(Brm-/-) instead leads to the upregulation of Brg1 in the cell. The
upregulation of Brg1 is enough to cause initial G0/G1 arrest via p53 and Rb, however, it is not dispensable for Brm in G1 maintenance and instead, the cell quickly cycles through the G1-S phase, thereby cycling with unrepaired DNA lesions, reaching S-phase prior to 24h. The lack of Brm and increasing levels of Brg1 also leads to evasion of apoptosis, likely leading to the proliferation of cells with damaged DNA. Thus, Brm-/- cells are reaching cell division much faster than Brm+/+ cells, whilst still harbouring DNA damage, likely leading to mutations and carcinogenesis downstream as witnessed in UVR-treated Brm-/- mice.

3.4. Conclusion

With the use of a novel cell cycle staining methodology, and other techniques, it was found that Brm-/- MNKs respond to UVR in an alternative manner as compared to Brm+/+ MNKs. The loss of Brm led to an increase in Brg1 expression, increased cellular proliferation following UVR exposure, decreased cell death, as well as the prevention of cell cycle arrest, leading to increased levels of S- and M-phase cells post UV-irradiation. Brm-/- MNKs also harboured significantly more UVR-induced CPD lesions than Brm+/+ cells. The increase of CPD formation in the dark seen in Brm-/- cells also suggests that the loss of Brm is leading to increased ROS production or increased sensitivity to ROS. The lack of cell cycle arrest, increased proliferation, decreased cell death and increased CPD damage in these mice suggests that this is the reason why these mice harbour more tumours than mice with functional Brm following UV-irradiation. It is likely that the loss of Brm is leading to decreased recruitment of
important molecules such as p53 and Rb, which control cellular events such as cell cycle arrest and DNA repair. These findings support a Brm knockout phenotype that does not initially precipitate carcinogenesis, but rather a synergistic relationship with carcinogens such as UVR to yield tumours, similar to p53. This is supported by other studies such as that of Glaros and colleagues (2007) where lung cancers were induced by carbamate ethyl. When one or both Brm alleles were inactivated, this led to a significant increase in the number of lung tumours. It is also unknown how dependent Brm deficiency is on p53 status. The next chapter will address this by examining a cell line that is both deficient for Brm and contains p53 mutations as well as other mutations. These results indicate that the loss of Brm alone in combination with UVR is enough to cause defects in the cell, leading to increased proliferation, decreased cell death, a lack of cell cycle arrest and increased CPD formation. It is likely that these events together lead to an increase in tumourigenicity as seen in Brm-/- mice when treated with UVR.
Chapter 4: Brm regulates cellular proliferation in UV-irradiated human keratinocytes independent of p53

4.1. Introduction

The next part of this study follows up results from the previous chapter, using a transfected HaCaT cell model to study human keratinocytes that are non-tumourigenic but along the pathway of being transformed. HaCaT cells are derived from human keratinocytes. This immortalised cell line has been used extensively for the study of skin cancer progression, looking at the functional consequences of overexpression and suppression of a number of genes, in particular the tumour suppressor p53 (Lee et al. 1993; Boukamp et al. 1997; Chaturvedi et al. 1999; Muthusamy et al. 2013). HaCaT cells possess UVR-specific mutations in both alleles of the p53 gene and also carry chromosomal aberrations seen in SCCs (Lee et al. 1993; Boukamp et al. 1997). The treatment of HaCaT cells with UVB radiation leads to a total change in the expression levels of 192 genes, including genes involved in inhibition of cell growth, apoptosis, signal transduction, metabolism and transcription (Lee et al. 2005). These changes occur in a time-dependent fashion, similar to the events in primary cultured keratinocytes, reflecting the sophisticated events in the skin post UVR. This suggests that HaCaT cells are a good model to study cellular changes following UVR in cells that have commenced the process of transformation into an SCC. Although SWI/SNF has not been thoroughly studied in HaCaT cells, previous studies have shown that the knockout of the SWI/SNF gene, ARID1B in HaCaT cells leads to increased cell growth, consistent with SWI/SNF having a tumour-suppressive function in skin cancer. Interestingly however, the knockdown of
ARID1A or Brg1 did not affect HaCaT growth (Shain et al. 2012). Further, Brm and Brg1 protein were shown to be lost in two types of NMSC, including BCC and SCC but not AK, indicating that Brm and Brg1 are lost when cells progress from benign to a malignant tumour (Bock et al. 2011). These findings indicated that Brm and Brg1 might be novel tumour suppressor genes for NMSC.

In this study, five miRNAs were constructed in order to target Brm function. These miRNAs targetted key sequences of Brm, all likely to be vital for Brm function, thus downregulating Brm protein and mRNA. These Brm miRNAs were at all times compared to a LacZ control miRNA. All of these miRNAs were inserted in a vector using a Gateway BLOCK-iT lentiviral POLII miRNA system. Following successful transduction of HaCaT cells, each miRNA was tested for Brm/Brg1 protein levels. Following this, similar experiments to those described in Chapter 3 were conducted to assess responses to UVR, including cell growth, cell death, cell cycle regulation and DNA repair.

4.2. Results

4.2.1. HaCaT cells show a normal Brm sequence

In order to analyse the loss of Brm in HaCaT cells, it first had to be determined whether these cells contained a normal Brm sequence. Recently, a single-base substitution of Brm in exon 4 (Q203K) was discovered in SCC and BCC (Moloney et al. 2009). Therefore, it was important to ensure this mutation was not also present in HaCaT cells. DNA was extracted from HaCaT cells and the specific DNA sequence to be sequenced was constructed via the use of Brm specific primers and PCR as in Chapter 2. This DNA sequence was then sent for
sequencing in combination with specific forward and reverse primers. It was confirmed with both forward and reverse primers that the single-base substitution was not present in HaCaT cells, and that these cells contained normal Brm DNA (Fig 4.1).

![Figure 4.1: HaCaT cells contain a normal exon 4 Brm sequence. HaCaT cells were sequenced using exon 4 Brm specific primers in order to analyse whether these cells contained a previously discovered Q203K sequence. HaCaT cells did not contain the Q203K mutant nor did they contain any other detected Brm mutants.](image)

4.2.2. Gene knockdown using a lentiviral microRNA technique

HaCaT cells were chosen for this experiment as they are derived from normal human keratinocytes, and have been used to study skin cancer progression in the past. The study required a cell line with long-term knockdown,
and to do this the BLOCK-iT lentiviral transduction technique was most suitable, as this provides cells with long-term downregulation of the desired gene. This technique uses a plasmid containing a small insert that encodes a small hairpin precursor miRNA molecule designed to target the mRNA of the specified gene (in this case, Brm). The methodology of this is further explained in Chapter 2. Following successful transformation into the vector, each plasmid was sequenced using specific primers to assess if each miRNA was inserted correctly prior to use. The lentiviral kit also contains a miRNA control for LacZ, to use as a control to compare to other knockdowns. Following transformation of the competent E. coli with the plasmid constructs, containing the miRNA against Brm or LacZ, positive colonies were selected. The plasmid DNA constructs were then purified and transfected into producer HEK293T cells using a simple calcium phosphate transfection method. All plasmids contained both blasticidin resistance for antibiotic selection, as well as EYFP or EGFP enabling fluorescence visualisation. The 293T cells before and after transfection can be seen below (Fig 4.2).
Figure 4.2: The transfection of HEK293T cells with Brm miRNA containing EYFP. HEK293T cells were transfected using a calcium phosphate method. The panels on the left show cells imaged in bright field while images on the right show cells imaged for EYFP. As expected, untransfected cells show no fluorescent signal. However, in the transfected cells on the bottom, a large signal can be seen in the fluorescent image, indicating successful transfection. All images are objective = 20X, scale = 100μm.

Following positive transfection of the 293T cells containing the miRNAs, the 293T cells produce a lentivirus into the supernatant. At five days post transfection, this supernatant was tested in order to confirm that virus was present. Firstly, the viral titre of each supernatant was estimated, to ensure each
transduction was performed with similar levels of virus. It is important to have an estimation of the concentration of infectious forming units in the supernatant, as this measurement determines how much viral supernatant needs to be used in order to achieve a desired multiplicity of infection, which corresponds to the number of virions per cell in a transduction. Titration of the lentiviral stock is necessary to generate reproducible gene knockdown results. To perform the viral titration, 10-fold dilutions of the lentiviral stock were made (1:10 and 1:100) and different dilutions of virus were transduced into HaCaT cells in the presence of Polybrene. At four days post transduction, the HaCaT cells were removed from the flask and analysed using a LSRII 5 laser flow cytometer with the laser set at 515nm (EYFP excitation is at 514nm). All files were analysed using FlowJo software. As expected, in cells transduced with 1:100 virus, the transduction efficiency was much lower (10.1%) than in cells transduced with 1:10 virus (57.5%) (Fig 4.3). Following this, the lentiviral titre could be calculated for each viral supernatant using the formula as discussed in Chapter 2, to ensure each cell sample was transduced with the equal amounts of virus.
Figure 4.3: Viral titration using virus from transfected HEK293T cells into HaCaT cells. HaCaT cells were transduced with (a) no virus (b) 1:100 virus dilution or (c) 1:10 virus dilution. An increasing efficiency of transduction was noted with increasing virus dose (10.1% of cells transduced with 1:100 virus and 57.5% of cells transduced with 1:10 virus).

These plasmid constructs also contained a gene giving antibiotic resistance to blasticidin, to allow for antibiotic selection of positively transfected cells, as well as an EYFP fluorescent tag, to allow for visualisation and selection of positively transfected cells. Therefore at 2 days post transduction, 10μg/ml of blasticidin was added to the cell media. After 10 days, the blasticidin was removed and the cells were cultured in normal medium only (Fig 4.4). Following this, the cells were removed from the flasks and cells that were positive for EYFP fluorescence were selected with the use of a FACSaria cell-sorter (Fig 4.4; Fig 4.5). There were no noticeable morphological effects of the transduction of HaCaT cells with Brm miRNAs, with the exception of Brm miRNA 3 which had reduced growth following transduction. Therefore, the miR3 cell line was not
used in experiments. Following sorting for EYFP fluorescence, all cells were EYFP-positive, indicating that all cells were successfully transduced (Fig 4.4).

**Figure 4.4:** The transduction of HaCaT cells with lentivirus containing EYFP-tagged Brm miRNA constructs. Post transduction, HaCaT cells were treated with selective medium containing 10μg/ml blasticidin, to select for
transduced cells. To ensure all cells were transduced, the cells were then selected for EYFP by flow cytometry so that all cells were expressing EYFP.

Figure 4.5: The sorting of EYFP-positive transduced HaCaT cells. Firstly, cells were gated for side and forward scatter in order to eliminate cellular debris (a). The cells were then separated into EYFP positive and negative and only cells from this that were high in EYFP signal were collected (purple dots) (b). If cells did not have a high EYFP signal (blue dots) they were not collected. Following cell sorting, only highly pure transfected EYFP-positive cells were left.

4.2.3. Brm is successfully knocked down using Brm miRNAs in HaCaT cells

Following the collection of highly pure transduced HaCaT cells containing the miRNAs, both protein and RNA was collected from these cells in order to analyse Brm content in both control and Brm miRNA HaCaT cells. Firstly, the
level of Brm and Brg1 protein was analysed using a Western blot and antibodies specific to Brm and Brg1, as well as a housekeeping gene, GAPDH (Fig 4.6). Following incubation with primary antibody, an anti-rabbit IgG HRP antibody was used for visualisation. The level of Brm in untransduced HaCaT cells and LacZ miRNA cells was similar and quite visible. However, all 5 Brm miRNAs down-regulated Brm protein, especially miRNAs 2-5, which all showed undetectable Brm protein (100% reduction). Brm miRNA 1 showed a 0.3-fold downregulation of Brm. Interestingly, the level of Brg1 protein increased in all samples with the exception of Brm miR5. Brm miR1 cells showed a 2.5-fold increase in Brg1 protein, while Brm miR2 showed a 5.5-fold increase. Further, Brm miR3 showed a 1.8-fold increase and Brm miR4 showed a 2.6-fold increase in Brg1 protein levels. Interestingly however, Brm miR5 cells showed a 0.6-fold decrease in Brg1 protein (Fig 4.6).

In order to test whether the transduction of Brm miRNA was also successful at the transcription level, an RT-PCR was performed. RNA was collected and cDNA was synthesised from the RNA by reverse transcription in order to perform a real-time PCR on the level of Brm mRNA in the cells. Primers were used to directly amplify Brm signal. Only Brm miRNAs 2-5 were tested, as these cells all showed the most significant down-regulation of Brm protein. Brm mRNA was reduced in all 4 of the tested HaCaT lines transduced with Brm miRNAs as compared to the LacZ miRNA control. In Brm miR2 there was a 91% reduction in Brm mRNA, as well as reductions of 77%, 82% and 89% in miR3, miR4 and miR5 respectively (all P<0.0001; one-way ANOVA) (Fig 4.7).
Figure 4.6: Brm protein is downregulated in Brm miRNA transfected HaCaT cells as compared to LacZ miRNA. HaCaT = untransfected HaCaT, LacZ = LacZ miRNA HaCaT and miR1-miR5 correspond to 5 various Brm miRNAs. HaCaT cells were transduced with LacZ or Brm miRNA virus. Equal amounts of protein from these cells were loaded onto a 7.5% acrylamide gel. Gels were run at 120V and proteins were transferred to a PVDF membrane. Membranes were then stained with an anti-Brm antibody, anti-Brg1 antibody or anti-GAPDH, followed by secondary antibodies for visualisation. All blots were imaged using a Syngene G-box. The amount of Brm protein was much higher in the LacZ control miRNA than any of the Brm miRNAs, especially miRNAs 2-5, which showed no staining for Brm protein. The level of Brg1 was increased in Brm miRs 1-4 and reduced in Brm miR5.
Figure 4.7: The level of Brm miRNA is downregulated in all four HaCaT lines transduced with Brm miRNAs (miR 2-5). RNA was extracted from transduced HaCaT cells and cDNA was synthesised via reverse transcription. Quantitative PCR was performed on the cDNA using primers specific for Brm or LacZ. All results were normalised to GAPDH and then the fold difference in Brm was compared to the control (LacZ which was set as 1). All Brm miRNAs were significantly different to LacZ (P<0.0001). Data represents mean ± SEM. One-way ANOVA (n=4).

4.2.4. The knockdown of Brm in HaCaT cells increases cell growth following UVR

As in Chapter 3, the effect of 4J/cm² UVR on both cell growth and death was first tested on transduced HaCaT cells. For this initial experiment, 3 Brm miRNA cell lines as well as a LacZ miRNA control cell line were tested. The 3 miRNAs were Brm miRNA 2, 4 and 5. These 3 miRNAs were chosen as they gave...
the best knockdown of Brm protein and RNA and did not have an altered growth pattern compared to untransduced HaCaT cells. The same number of HaCaT cells were grown in 96-well plates and left to incubate at 37°C/5% CO₂-in-air for 24h. The cells were then irradiated with 4J/cm² UVR or sham-irradiated and then cultured at 37°C/5% CO₂ for fixed periods of time (24, 48, 72 or 96h). The CytoTOX-ONE kit was used to quantitate the number of live and dead cells. The resulting fluorescence was read on a FLUOstar Omega microplate reader. The fluorescence of each cell line over the time course was normalised to its reading at Day 0, to prevent any bias from cell plating differences, due to using several cell lines.

Unirradiated cells grew steadily over time, generally reaching confluence by 96h, when growth was halted. The knockdown of Brm in HaCaTs by any of the miRNAs did not significantly affect the growth of the cells over time, as none of the Brm miRNA cell lines were found to be significantly different to the LacZ miRNA or any of the other Brm miRNAs (Fig 4.8a). Irradiated cells generally had a stunt in growth following UVR treatment, but continued to grow up to 96h. All Brm miRs showed a trend of increased cell growth at 48, 72 and 96h as compared to the LacZ control. However, only the irradiated Brm miR4 was found to be significantly different to the LacZ miRNA control over the time course (P=0.0018; 2-way ANOVA), whereas Brm miR2 and miR5 were not (P=0.0868 and P=0.2019 respectively; 2-way ANOVA) (Fig 4.8b).
Figure 4.8: The knockdown of Brm in HaCaT cells affects growth in UVR treated cells but not unirradiated cells. HaCaT cells containing LacZ (green circles), Brm miR2 (red square), Brm miR4 (blue triangle) or Brm miR5 (purple diamond) were (a) sham-irradiated or received (b) 4J/cm² UVR. Their cell growth was then tested at 24, 48, 72 and 96h post treatment. There was no significant difference between cell lines in the growth of unirradiated cells. Although there was an observable trend in the growth of all Brm miRNA transduced cell lines following UVR, only Brm miR4 was found to be significantly different to the LacZ miRNA control cell line over the time course (P=0.0018), whereas Brm miR2 and miR5 were not. Each time point represents mean ± SEM from 3 independent experiments (8 independent cultures per experiment; n=24 independent cultures). Two-way ANOVA used. Where the SEM is not obvious it is too small to be seen.
When analysed for UVR effect on each cell line, rather than a comparison of the different cell lines to each other, it was found that UVR treatment led to a significant decrease in cell growth in the LacZ group (P<0.0001; 2-way ANOVA)(Fig 4.9a). However, although there was a UVR-induced decrease in cell growth in the miRNAs over time, UVR did not cause a significant decrease in growth of Brm miR2 (P=0.0619)(Fig 4.9b), miR4 (P=0.3565)(Fig 4.9c) or miR5 cells (P=0.0769; 2-way ANOVA)(Fig 4.9d). The difference in growth was most pronounced at later time points such as 48 and 72h. LacZ showed a significant difference in growth between unirradiated and irradiated cells at 48 (P<0.0001), 72 (P<0.0001) and 96h (P=0.0158; 2-way ANOVA), however, this was not the case with the cells containing Brm miRNAs, with no significant differences at these time points with the exception of Brm miRNA5 which showed a significant difference at 72h (P=0.018; 2-way ANOVA). These results suggest that the loss of Brm in each of the miRNA-transduced cells caused an increased cell growth following UVR treatment but did not alter growth in unirradiated cells.
Figure 4.9: The knockdown of Brm in HaCaT cells alters their growth in response to 4J/cm² UVR. HaCaT cells transduced with (a) LacZ miRNA, (b) Brm miRNA2, (c) Brm miRNA4 or (d) Brm miRNA5 were studied at 24, 48, 72 or 96h post ±4J/cm² UVR with the use of a CytoTOX-ONE assay to analyse cell growth. UVR treatment led to a significant decrease in cell growth in LacZ cells (P<0.0001) but not any of the Brm miRNA transduced cells. Each time point represents mean ± SEM from of 3 independent experiments (8 independent cultures per experiment; n=24). Two-way ANOVA used. Where the SEM is not obvious it is too small to be seen. Data is the same as Fig 4.8 with different comparisons.

The effect of UVR on the viability of the cells was also tested at the same time points as above. There was no significant difference between any of the unirradiated samples or irradiated samples, indicating that the loss of Brm in HaCaT cells did not impact the viability of cells with or without UVR (Fig 4.10).
Figure 4.10: The knockdown of Brm in HaCaT cells does not alter cell viability in unirradiated or irradiated cells. HaCaT cells containing LacZ (green circles), Brm miR2 (red square), Brm miR4 (blue triangle) or Brm miR5 (purple diamond) were (a) sham-irradiated or received (b) 4J/cm² UVR. The percentage of live cells over total cells was then calculated with the use of a CytoTOX-ONE assay to assess cell viability. The knockdown of Brm with 3 separate miRNAs did not significantly alter viability in either unirradiated or irradiated cells from the control. Each time point represents mean ± SEM from of 3 independent experiments (8 independent cultures per experiment; n=24). Two-way ANOVA used.
There was an overall trend of a small decrease in cell viability with UVR over time in all of the 4 cell lines, with the viability of all cell lines lower at both 24 and 48h in the irradiated samples. However, UVR did not cause a significant decrease in cell viability in LacZ cells (P=0.3153), Brm miR2 (P=0.7001) or Brm miR4 (P=0.1023; 2-way ANOVA). There was however a significant decrease in viability in Brm miR5 cells (P=0.0491; 2-way ANOVA) (Fig 4.11).

Figure 4.11: Only Brm miR5 cells showed a significant decrease in viability in response to 4J/cm² UVR. HaCaT cells transduced with (a) LacZ miRNA, (b) Brm miRNA2, (c) Brm miRNA4 or (d) Brm miRNA5 were studied at 24, 48, 72 or 96h post ±4J/cm² UVR with the use of a CytoTOX-ONE assay to analyse cell viability. UVR led to a decrease in viability at 24 and 48h in all cell lines however over the time course only Brm miR5 had a significantly lower viability in response to UVR (P=0.0491). Each time point represents mean ± SEM from of 3 independent experiments (8 independent wells per experiment; n=24). Two-way ANOVA used. Where the SEM is not obvious it is too small to be seen. The data is the same as Fig 4.10 but with different comparisons.
4.2.5. The knockdown of Brm in HaCaT cells leads to a dysfunctional cell cycle response to UVR

Following the result indicating that Brm miRNA transduced cells recover from UVR faster than the LacZ miRNA controls, the miRNA with the most significantly different response (Brm miR4) was chosen to use for other further experiments. Cell cycle was assayed at 24, 48, 72 and 96h post UVR, in order to analyse the effect that UVR was having on the cell cycle response over time. This experiment used the novel methodology described in Chapter 3, with an EdU stain to measure S-phase cells and a phospho-histone H3 antibody to measure M-phase cells. Briefly, equal numbers of either LacZ miRNA or Brm miRNA4 transduced HaCaT cells were plated in 24-well plates and incubated at 37°C/5% CO₂-in-air for 24h. The cells were then irradiated with 4J/cm² UVR or sham-irradiated and cultured for different time periods. The cells, remaining attached to the culture plate surface, were then stained with EdU and an anti-histone H3 antibody. All plates were imaged using a Cytation-3 live cell imager. A total of 3 experiments were conducted, with 4 independent cultures being analysed per experiment, including 1 stained with isotype antibody (Rabbit IgG), and without EdU but stained with azide as a further control (n=3 cultures per experiment, total of n=9). As in previous experiments, both EdU and H3 staining was nuclear and strongly stained both unirradiated samples. Interestingly, at 24h post UVR, there seemed to be a visual reduction in S-phase cells in the LacZ control but not the Brm miR cells (Fig 4.12).
Figure 4.12: 4J/cm² UVR reduces S-phase cell numbers in LacZ HaCaT cells but not Brm miR HaCaT cells 24h post UVR. LacZ or Brm miR transduced HaCaT cells were irradiated with ±4J/cm² UVR and grown for 24h before fixation and staining with EdU/azide and anti-H3 antibody. Following incubation, there appeared to be a reduction in S-phase cells in the LacZ miR cells, but not Brm miR cells. All images = 20X objective, scale = 100μm. Each image is a merger of DAPI (blue), H3 (green) and EdU (red).

As in Chapter 3, to assess the percentages of both S- and M-phase cells, each image was analysed by ImageJ and FlowJo software. This methodology is described in detail in Chapter 2. Firstly, the gates on the dot-plots for both EdU and histone-H3 were drawn with the use of controls, as illustrated in Chapter 2, allowing negative cells to be identified and gated (Fig 4.13).
Figure 4.13: Gating of control stained cells. Dot-plots of HaCaT cells were treated with azide but not EdU for the control of EdU (a) or a rabbit IgG isotype antibody for the control of phospho-histone H3 staining (b). These controls allowed gates to be drawn accurately for cells that were negative for EdU and H3. Thus anything with higher intensity was considered positive. Representative plots are shown of one experiment with approximately 3000 counted cells per culture.

Next, the number of EdU-positive cells was determined. In all samples, there was a reduction in EdU-positive cells in irradiated LacZ cells as compared to unirradiated cells (Fig 4.14a, b). However, this was not seen in irradiated Brm miR cells, which actually showed an increase in S-phase cells as compared to the unirradiated cells (Fig 4.14c, d). This suggested that LacZ cells were more affected by UVR at 24h than the Brm miR cells. Lastly, dot-plots for histone-H3 positive cells were also assessed. Generally, only a slight variation was found in either irradiated or unirradiated cells for either LacZ (Fig 4.15a, b) or Brm miR
(Fig 4.15c, d), suggesting that 4J/cm² UVR did not drastically alter M-phase cell numbers in either LacZ or Brm miR HaCaT cells.

Figure 4.14: LacZ miR but not Brm miR HaCaT cells show reduced EdU-positive cells following UVR. Dot-plots for EdU intensity in (a) LacZ miR unirradiated, (b) LacZ miR irradiated, (c) Brm miR unirradiated and (d) Brm miR irradiated HaCaT cells, 24h after treatment. In LacZ cells, there was a reduction of EdU-positive cells in the irradiated samples, however this was not seen in Brm miR cells, as irradiated Brm miR cells exhibited an increase in EdU-positive cells. Representative plots are shown of one experiment with approximately 3000 counted cells per culture.
Figure 4.15: 4J/cm² UVR does not alter mitotic cell numbers in either LacZ or Brm miR HaCaT cells. Dot-plots for phospho-histone H3 intensity in (a) LacZ miR unirradiated, (b) LacZ miR irradiated, (c) Brm miR unirradiated and (d) Brm miR irradiated HaCaT cells, 24h after treatment. Generally there was only a small amount of variance in positive cells in all samples, indicating that UVR did not drastically alter the number of M-phase cells in any of the samples. Representative plots are shown of one experiment with approximately 3000 counted cells per culture.
In order to analyse the full effect of 4J/cm² UVR on the cell cycle of both LacZ and Brm miR transduced HaCaT cells, a total of 3 experiments were compiled, using 3 independent experimental cultures per experiment (n=9). For each independent culture about 3,000 cells were counted and the average used as the result for that culture. Each experiment was then entered into the flow cytometry program, FlowJo, and the total percentage of both EdU-positive cells (Fig 4.16) and H3-positive cells (Fig 4.17) were calculated. As previously discussed, EdU also stains for cells undergoing UDS, therefore all cells undergoing UDS (punctate stained cells) were manually counted and these numbers were subtracted from the total number of positively stained EdU cells. This leaves only S-phase cells, which are uniformly stained throughout the nucleus and therefore the total percentage of S-phase cells was calculated. In the unirradiated cells, the percentage of S-phase cells was initially quite high at both 24 and 48h, however this reduced by 72h and was lowest at 96h, which is due to cells reaching confluence at this stage and thus slowing in growth (Fig 4.16a). There was no statistical difference in S-phase cells in unirradiated LacZ compared to unirradiated Brm miR cells over the time course (P=0.9212; 2-way ANOVA) or at any of the 4 time points measured, indicating that loss of Brm alone did not alter the cell cycle of HaCaT cells. Irradiation with 4J/cm² UVR reduced the number of S-phase cells at 24h in LacZ cells, but not in Brm miR cells, which had an increase in S-phase cells at 24h (Fig 4.16b). At 24h the number of S-phase cells in the irradiated samples was significantly higher in the Brm miR samples than in the LacZ samples (P<0.0001; 2-way ANOVA). However, by 48h, the growth of the Brm miR cells had slowed to similar levels to that of LacZ cells and there was no significant difference in their S-phase cell number (P=0.9998;
2-way ANOVA). This was similar at both 72 and 96h (P=0.9462 and P=0.6045 respectively; 2-way ANOVA), indicating that HaCaT cells could overcome cell cycle deficiencies post UVR quite quickly. Over the entire time course, the Brm miR cells were found to have significantly different numbers of S-phase cells compared to LacZ miR cells in response to UVR (P=0.0064; 2-way ANOVA) (Fig 4.16b). As in the cell cycle experiments in Chapter 3, the 96h time point was not included in the statistical analysis of the overall time course. This is because at 96h many cultures had reached confluence, and thus the results at this time were due to the confluent culture conditions, rather than reflecting the effects of UVR.

Figure 4.16: Brm miR HaCaT cells undergo reduced UVR-induced cell cycle arrest and progress faster into S-phase following 4J/cm² UVR than LacZ HaCaT cells. HaCaT cells transduced with either a LacZ miRNA (LacZ miR) or Brm miRNA (Brm miR) were irradiated with or without UVR, and further incubated for various time points. S-phase cells were determined by staining with EdU/azide and counting cells with a fully stained nucleus. There was no difference in the % of S-phase unirradiated cells between the groups (P=0.339; panel a), however, Brm miR cells recovered more quickly from UVR than LacZ cells and had an increased % of S-phase cells (P=0.0036; panel b). By 96h the cells became confluent and stopped
growing due to the culture conditions as can be seen by the lack of proliferation in the control unirradiated cells. Hence results at this time were due to the confluent culture conditions rather than reflecting the response to UVR and therefore are shown for completion but not included in the statistical analysis. No UV as compared to UV at time points of 24, 48 and 72h. Each time point represents mean ± SEM from 3 independent experiments (3 independent cultures per experiment; n=9). Two-way ANOVA used.

Similar to S-phase cells, the number of M-phase cells in unirradiated cultures was highest at 24h and slowly declined over time. Interestingly, it was also the highest in irradiated cells at 24h, and the number of mitotic cells decreased at later times. Further, there was no difference in M-phase cells in the unirradiated LacZ or Brm miR samples over the time course (P=0.293; 2-way ANOVA) or at any of the 4 time points measured (Fig 4.17a). The number of M-phase cells was slightly higher in Brm miR irradiated cells than in irradiated LacZ cells at 24 and 48h, however these increases were not statistically significant (P=0.6686 and P=0.8934; 2-way ANOVA). By 72h, LacZ cells had slightly higher numbers of mitotic cells compared to Brm miR, but this increase was not significant (P=0.8180; 2-way ANOVA). By 96h, the number of M-phase cells was quite low and were similar in numbers in the two cell lines (P>0.9999; 2-way ANOVA). There was no significant difference in the number of M-phase cells between LacZ miR and Brm miR cells over the entire time course (P=0.4557; 2-way ANOVA)(Fig 4.17b).
Figure 4.17: Brm miR HaCaT cells show similar levels of M-phase cells to LacZ miR cells in cells both unirradiated and irradiated with 4J/cm² UVR. HaCaT cells transduced with either a Brm miR or LacZ miRNA were sham-irradiated (a) or treated with UVR (b), and further incubated for various time points. M-phase cells were determined by staining with anti-histone H3 antibody and counting stained cells. There was no difference between the groups in the number of mitotic cells in unirradiated cells (P=0.2644) or irradiated cells (P=0.4557) over the time course. By 96h the cells became confluent and stopped growing due to the culture conditions as can be seen by the low number of M-phase cells in the control unirradiated cells. Hence results at this time were due to the confluent culture conditions rather than reflecting the response to UVR and therefore are shown for completion but not included in the statistical analysis. No UV as compared to UV at time points of 24, 48 and 72h. Each time point represents mean ± SEM from 3 independent experiments (3 independent cultures per experiment; n=9). Two-way ANOVA used.

For clarity, the results from the experiments described above were re-analysed for the effect of UVR within a cell line, rather than comparing the two cell lines to each other under the two conditions. UVR was not found to affect the
number of S-phase cells over the time course in LacZ cells (P=0.0586; 2-way ANOVA) (Fig 4.18a), however there was a highly significant decrease in S-phase cells in the UVR group at 24h (P<0.0001; 2-way ANOVA). By later time points the number of S-phase cells was similar in both unirradiated and irradiated cells (48h, P=0.8969; 72h, P=0.2945; 2-way ANOVA). By 96h, the number of S-phase cells was significantly higher in the UVR sample (P=0.0442; 2-way ANOVA), which is due to the unirradiated cells reaching confluence and thus having slowed growth. Further, 4J/cm² UVR did not change the number of M-phase cells in LacZ cells as compared to unirradiated cells over the time course (P=0.7088; 2-way ANOVA) (Fig 4.18b). In Brm miR cells, there was also no significant effect of UVR on S-phase cells over the time course (P=0.2076; 2-way ANOVA) (Fig 4.18c); however, UVR significantly increased S-phase cells compared to unirradiated cells at 24h (P=0.0119; 2-way ANOVA). At 48 and 72h the numbers of S-phase cells were similar, and by 96h the number of S-phase cells was higher in the UVR group (P=0.0026; 2-way ANOVA). Interestingly, although there were small increases in M-phase cells at all time points in UV-irradiated Brm miR compared to unirradiated cells, none of these increases were statistically significant (P=0.0699; 2-way ANOVA) (Fig 4.18d). Please note that these figures are the same data as plotted in Fig 4.16 and Fig 4.17, but plotted differently.
Figure 4.18: Brm miR cells recover faster from UVR in % of S- but not M-phase cells. HaCaT cells transduced with either LacZ miRNA (a, b) or Brm miRNA (c, d) were irradiated without (black) or with UVR (grey), and further incubated for various time points. S- and M-phase cells were counted by staining with EdU/azide or an anti-histone H3 antibody and counting fully stained cells. In LacZ cells, UVR did not have an overall significant effect on S-phase cells ($P=0.0586$; a), however UVR did significantly reduce S-phase cells at 24h ($P<0.0001$). UVR did not cause a change in LacZ miR M-phase cells ($P=0.7088$; b). In Brm miR cells, UVR did not have an overall effect on S-phase cells ($P=0.2076$; c), however, there was a significant increase in S-phase cells at 24h ($P=0.0119$). Further, UVR did not cause a change in mitosis in Brm miR cells ($P=0.0699$; d). By 96h the cells became confluent and stopped growing due to the culture
conditions as can be seen in the lack of proliferation in the control unirradiated cells. Hence results at this time were due to the confluent culture conditions rather than reflecting the response to UVR and therefore are shown for completion but not included in the statistical analysis. No UV as compared to UV at time points of 24, 48 and 72h. Each time point represents mean ± SEM from 3 independent experiments (3 independent cultures per experiment; n=9). Two-way ANOVA used. This figure is the same data as plotted in Fig 4.16 and Fig 4.17, but plotted differently to facilitate comparison.

Considering the difference of S-phase cells in LacZ as compared to Brm miR HaCaT cells at 24h post UVR, the percentage of S-phase cells was also calculated at an earlier time point (1h), in order to assess the cell cycle response immediately following UVR. UVR led to a reduction in S-phase cells at 1h post treatment in LacZ cells (P=0.0011; 1-way ANOVA). Although there was a negative trend in UV-irradiated Brm miR cells, this decrease in S-phase cells was not significant (P=0.1188; 1-way ANOVA) (Fig 4.19). Further, there was no significant difference between LacZ and Brm miR unirradiated cells (P>0.9999) or UV-irradiated cells (P=0.6356; 1-way ANOVA). This finding suggests that in HaCaT cells, Brm is at least partly required for entry into UVR-induced cell cycle arrest, as well as the maintenance of G1/G0 cell cycle arrest. HaCaT Brm miR cells are able to overcome cell cycle arrest much faster than LacZ miR cells, as indicated by increased S-phase numbers at 24h post UVR (Fig 4.16b).
Figure 4.19: UVR induces a significant reduction in LacZ miR but not Brm miR S-phase cells 1h post treatment. HaCaT cells transduced with LacZ miRNA or Brm miRNA were irradiated with (grey) or without UVR (black), and further incubated for 1h. S-phase cells were determined by staining with EdU/azide and counting uniformly stained cells. UVR induced a reduction of S-phase cells at 1h post treatment in both LacZ cells (P=0.0011) but not Brm miR cells (P=0.1188). There was no significant difference between LacZ and Brm miR unirradiated cells (P>0.9999) or UV-irradiated cells (P=0.6356). Results are pooled from 3 separate experiments, 2 independent cultures per experiment (n=6). Mean + SEM shown. One-way ANOVA used.

4.2.6. The knockdown of Brm in HaCaT cells increases the accumulation of UVR-specific CPD lesions

Lastly, the effect of Brm loss in HaCaT cells on DNA repair was assessed. As previously, 2 separate assays were used. Firstly, EdU staining was used to
analyse UDS. UDS is observed as punctate or speckled staining in the nucleus, which is distinct from uniformly stained cells undergoing S-phase (Nakagawa et al. 1998). The methodology is further described in Chapter 2. Briefly, equal numbers of either HaCaT cells transduced with LacZ or Brm miRNA were plated in 4-well chamber slides and incubated at 37°C/5% CO₂ for 24h. The cells were then irradiated with or without 4J/cm² UVR and incubated again at 37°C/5% CO₂ with cell medium containing EdU for 1h, and then stained and imaged as explained in Chapter 2. A total of 2 independent experimental cultures were conducted per experiment, as well as 1 control well stained with azide but unprobed with EdU, with a total of 3 experiments conducted (n=6). The percentage of nuclei undergoing UDS was quantified by manually counting the number of EdU stained punctate nuclei as compared to the total number of nuclei stained with DAPI. Due to all samples having to be measured manually, all slides were blinded and only unblinded following analysis. Following UV-irradiation, there was a significant increase in the number of cells undergoing UDS in both LacZ and Brm miR HaCaTs (Fig 4.20).
Figure 4.20: 4J/cm² UVR causes an increase in cells undergoing UDS in both LacZ and Brm miR HaCaT cells. DAPI and EdU labelled HaCaT cells visualised by fluorescent microscopy. The unirradiated samples (No UV) show a large number of cells with block stained nuclei (S-phase) with few UDS cells (speckled staining, green arrows). However, 1h post UVR, there was a decrease in S-phase cells and an increase in UDS (green arrows) in both LacZ and Brm miR cells. 40X objective, scale bar = 100μm. Each image is a merger of DAPI (blue) and EdU (red).

With the use of ImageJ software and manual counting as previously outlined, the percentage of cells undergoing UDS was determined in each treatment group. UVR treatment increased the percentage of UDS nuclei in both LacZ (P=0.0071) and Brm miR cells (P=0.0145; 1-way ANOVA). Although there was a decrease in the percentage of UDS cells in the Brm miR irradiated cells as
compared to LacZ cells of approximately 3%, this difference was not statistically significant (P=0.4143; 1-way ANOVA). There was also no difference in UDS between the unirradiated samples (P=0.2548; 1-way ANOVA) (Fig 4.21).

Figure 4.21: 4J/cm² UVR causes an increase in cells undergoing UDS in both LacZ and Brm miR HaCaT cells. HaCaT cells transduced with LacZ miRNA or Brm miRNA were irradiated with (grey) or without UVR (black), and further incubated for 1h and then stained with EdU/azide to determine the percentage of punctate stained EdU-positive cells (cells undergoing UDS). UVR led to an increase in UDS in both LacZ (P=0.0071) and Brm miR cells (P=0.0145). There was no significant difference between unirradiated (P=0.2548) or irradiated cells (P=0.4143). Results are pooled from 3 separate experiments, 2 independent cultures per experiment (n=6). Mean ± SEM shown. One-way ANOVA used.

Following measurements of UDS at 1h, the rate of UDS at 24, 48, 72 and 96h was also determined. In unirradiated cells, the level of UDS over the time course was fairly consistent, but declined slightly by 96h. There was no significant difference in UDS between LacZ and Brm miR unirradiated cells over
the time course (P=0.5964; 2-way ANOVA)(Fig 4.22a). In irradiated cells, the amount of UDS was highest at 24h, and declined over time to 96h, where it was at its lowest. There was no difference between the responses of LacZ and Brm miR cells over the time course (P=0.6914; 2-way ANOVA)(Fig 4.22b). Furthermore, UVR increased the rate of UDS in both LacZ miR (P=0.0016) and Brm miR cells (P=0.0006; 2-way ANOVA). At 24h, the amount of UDS in both LacZ and Brm miR cells was significantly higher in the UV-irradiated cells (both P<0.0001; 2-way ANOVA). However, at 48, 72 and 96h, the level of UDS was similar and there was no statistically significant effect of UVR at these later times. This result suggests that in HaCaT cells UVR-induced DNA repair was still occurring at 24h, however DNA repair following this was a response to DNA damage induced by the culture conditions and not due to UVR effects.
Figure 4.22: The loss of Brm in HaCaT cells does not affect UDS in either (a) unirradiated or (b) irradiated cells. LacZ or Brm miR HaCaT cells were incubated for various times post ±4J/cm² UVR and then stained with EdU/azide to determine the percentage of punctate stained EdU-positive cells (cells undergoing UDS). UVR led to an increase in UDS in both LacZ (P=0.0016) and Brm miR cells (P=0.0006). There was no significant difference in UDS between LacZ and Brm miR unirradiated (P=0.5964) or irradiated (P=0.6914) cells over the time course. Results are indicative of 3 separate experiments, 2 independent cultures analysed per experiment (n=6). Mean + SEM shown. Two-way repeated measures ANOVA used.

In the experiments described above, there was an increase in UDS at both 1h and 24h post UV-irradiation. However, since UDS does not specifically analyse UVR-induced DNA damage but also detects repair of other types of DNA damage, therefore having a high background, CPDs were also detected following 4J/cm² UVR irradiation. Both LacZ and Brm miR transduced HaCaT cells were plated at equal densities into 4-well chamber slides and grown for 24h at 37°C/5% CO₂. The cells were irradiated with 4J/cm² UVR or sham-irradiated and cultured in cell medium for a set time for DNA repair to occur, before fixing the cells in 4%
PFA to stop further repair. To trace CPD formation and repair over time the following times were used: 15 min, 30 min, 1h, 2h, 3h and 24h. All slides were imaged as previously, and all images were analysed using ImageJ software, as described in detail in Chapter 2.

As expected, unirradiated HaCaT cells showed little or no staining for CPDs, and their staining was never higher than the isotype control. The amount of time spent in growth medium also had no effect, as none of the unirradiated time points showed higher levels of CPDs than any other (Fig 4.23). Upon irradiation with UVR, there was a CPD signal apparent as early as 15min post UVR in both LacZ and Brm miR cells. By 1h the level of CPDs was reducing in LacZ miR cells but increased quite drastically in Brm miR cells. By 24h, the majority of the CPD signal had reduced heavily in both samples, however it never reached as low as the unirradiated samples (Fig 4.23).
Figure 4.23: 4J/cm² UVR induces CPDs in both LacZ and Brm miR HaCaT cells that repair over time. Transduced LacZ or Brm miR HaCaT cells were irradiated with UVR or sham-irradiated and incubated in cell medium at 37°C/5% CO₂ for various times. Unirradiated (No UV) samples did not show any staining for CPDs. However, UVR-treated samples showed CPD staining as early as 15 min after UVR. LacZ cells did not show a dramatic increase in CPDs by 1h; however, Brm miR cells appeared to show a larger increase in
CPDs at this time point. By 24h, the a CPD staining had drastically decreased, indicating CPD repair had occurred. Objective = 20X, scale = 100μm. DAPI staining (blue) detects all cells while red cells indicate CPD positive cells.

Following imaging, the total CPD intensity of each sample was measured. The cells were counted using ImageJ, with DAPI staining used as a mask to analyse the total CPD intensity per cell. This methodology is described in further detail in Chapter 2. In unirradiated cells, the amount of CPD staining was consistently low in both cell types and at all time points, indicating that none of these events influenced CPD formation. Over the time course, there was no significant difference between LacZ and Brm miR unirradiated cells (P=0.2731; 2-way ANOVA), and there was no significant difference at any time point measured. However, UVR induced CPDs as early as 15min in both LacZ and Brm miR cells. The level of CPDs in LacZ and Brm miR cells at both 15 min and 30 min was similar (P=0.9815 and P=0.7799; 2-way ANOVA). Interestingly however, Brm miR cells showed significantly increased levels of CPD in the dark formation up until 1h, whereas the level of CPDs decreased in LacZ miR cells, with Brm miR cells having significantly higher levels of CPD staining at this time point (P=0.001; 2-way ANOVA). By 2h post UVR, the LacZ miR cells again showed a significant decrease in CPDs. The level of CPDs in Brm miR cells also began declining at 2h, however they again showed a significantly increased level of CPDs as compared to LacZ miR cells (P=0.001; 2-way ANOVA). By 3h, LacZ cells only showed a small decrease in CPDs, indicating that the majority of CPD repair was likely completed by this time. Brm miR cells had another steady decrease in CPDs by 3h, indicating CPD repair was still occurring at this time. Although Brm
miR cells had a higher CPD intensity at 3h, it was not significantly different from LacZ miR cells (P=0.3976; 2-way ANOVA). By 24h, both LacZ and Brm miR cells showed their lowest CPD intensity over the experiment, indicating both cells had repaired over this time course. There was no significant difference between LacZ and Brm miR cells at this time point (P=0.6952; 2-way ANOVA). Lastly, Brm miR cells were found to have significantly higher levels of CPDs over the time course as compared to LacZ miR cells (P=0.0212; 2-way ANOVA)(Fig 4.24). These findings indicate that both LacZ and Brm miR HaCaT cells are able to repair CPDs from a UVR dose of 4J/cm², however, the loss of Brm in HaCaT cells leads to an increase in the intensity of CPDs formed in the dark, and therefore significantly higher levels of CPDs over the time course.
Figure 4.24: The loss of Brm leads to statistically significant increasing levels of CPDs in the dark following 4J/cm² UVR. Transduced LacZ or Brm miR cells were irradiated with 4J/cm² UVR or sham-irradiated and incubated in cell medium at 37°C/5% CO₂ for varying times before fixation. The unirradiated (No UV) samples showed limited (generally undetectable) CPD formation. However, the UVR samples showed CPD formation as early as 15 min post UVR. The peak of CPD formation was at 30 min in LacZ cells, which showed a decrease in lesions past this time point. However, in Brm miR cells, the peak of CPD formation was at 1h, and the cells recovered slowly from this time point. The level of CPDs in irradiated LacZ and Brm miR irradiated cells were found to be significantly different over the time course (P=0.0212). Results are pooled from 3 independent experiments, with 2 wells analysed per experiment (n=6). Mean + SEM shown. 2-way ANOVA used. Where the SEM is not obvious it is too small to be seen.
4.2.7. Results summary

The loss of Brm in the immortalised cell line, HaCaT, led to an observable increase in cell growth as compared to the loss of a control gene, LacZ following UVR. This was noted in all 3 Brm miRNAs tested, suggesting that Brm can influence cell growth post UVR, independent of p53 expression. However, there were no noted changes in cell viability, indicating that Brm does not affect apoptotic activation following UVR in these cells. Brm miR cells underwent reduced initial G1 arrest following UVR as compared to LacZ miR cells, as evidenced by a lack of decrease in S-phase cells at 1h post UVR. Further, Brm miR cells cycled significantly faster following UVR as compared to LacZ miR cells, exiting through the G1-S phase earlier, as evidenced by their increased levels of S-phase cells at 24h following UVR. Lastly, Brm miR cells also showed increased CPD in the dark formation at 1h and 2h following UVR as compared to LacZ miR cells, further supporting that the loss of Brm leads to increased ROS sensitivity, inducing significantly more CPDs. There was no change in DNA repair, and both cells repaired their DNA at a similar rate, indicating that Brm alone did not influence overall DNA repair in HaCaT cells, at least under the conditions tested in this thesis.

4.3. Discussion

Results from the previous chapter (Chapter 3) effectively showed that the loss of Brm in murine neonatal keratinocytes led to defects in their response to UVR, in particular, defective cell cycle arrest and increased CPD formation. Here I expand on these results to analyse the effect of the loss of Brm in human
keratinocytes with mutations in p53 and other undefined genes, to determine whether these results would translate to human cells early in the process of transforming into SCCs. Previously, both Brm and Brg1 have been shown to be downregulated in both SCC and BCC, but not in premalignant AK lesions, indicating that these subunits are downregulated in the progression of benign skin lesions into malignant lesions (Bock et al. 2011). However, the functional effects of the loss of these subunits in NMSC are unknown. This chapter investigated the role of Brm in NMSC, with the use of a miRNA transfection system to reduce Brm in HaCaT cells, which are commonly used as a model of a transformed but non-tumourigenic human keratinocyte cell. HaCaT cells possess UV-type specific mutations in both alleles of the p53 gene, and carry chromosomal aberrations characteristically seen in SCCs (Boukamp et al. 1997). Therefore, HaCaT cells have become a common model to study the functional consequences of the overexpression and suppression of genes commonly associated with NMSC progression. The goal of this chapter was to analyse whether the loss of Brm in a non-tumourigenic but highly mutated cell line in combination with UVR would have similar effects to the loss of Brm in a naïve cell such as a primary MNK.

4.3.1. Brm is successfully knocked down in HaCaT cells

Firstly, gene sequencing determined that HaCaT cells contained a wildtype Brm exon 4 sequence, validating the use of these cells for the loss of Brm expression experiments. Next, using lentiviral technology, Brm protein and mRNA was successfully knocked down in several HaCaT cell lines with high
efficiency, in particular Brm miRs 2-5, which showed complete reduction in Brm protein and respective reductions in Brm miRNA. Brm miRs 1-4 showed increases in Brg1 protein, which was especially prominent in Brm miR2. Interestingly however, Brm miR5 showed a reduction in Brg1 protein. This result suggests that Brg1 was compensating for the loss of Brm in HaCaT cells in Brm miR1-4, as was seen in Brm-/- C57BL/6 mice in Chapter 3. It is unknown however why Brg1 was reduced in Brm miR5. Because HaCaT cells normally express both Brm and Brg1, it is likely that altering the protein level of one ATPase leads to the upregulation of the other ATPase subunit, possibly in turn compensating for the others actions.

4.3.2. The loss of Brm in HaCaT cells increases cell growth in response to UVR

Following the successful knockdown of Brm protein and mRNA, the effect of the loss of Brm in HaCaT cells on responses to UVR was determined. Firstly, the effect on cell growth and death was tested on transduced HaCaT cells. For this experiment, 3 different miRNAs targeting various parts of the Brm gene were tested. These miRNAs (Brm miR 2, 4 and 5) were used as they caused the greatest level of Brm depletion in both protein and mRNA, and did not cause any detectable cellular effects post transduction in the absence of UVR. It was found that all unirradiated Brm miRNA transfected cells grew at a relatively similar rate, and none of the cells were found to be morphologically different from one another, confirming that the loss of Brm alone does not alter the growth rate of the cells. Upon exposure to UVR, all Brm miRNA transfected cells exhibited
increased growth as compared to LacZ miRNA cells, however, only one cell line (Brm miR4) was found to have a significantly increased growth rate as compared to LacZ miRNA HaCaT cells. In addition, it was found that UVR led to a statistically significant decrease in cell growth in LacZ miR cells over time, but this was not the case in any of the Brm miR cells, indicating that UVR induced growth arrest was not observed in the absence of Brm. Furthermore, UVR was found to lead to a decrease in cell viability in the Brm miR5 cell line, but not LacZ miR cells, nor the other 2 Brm miR cell lines studied. It is not known why UVR led to increased cell death in Brm miR5 cells more so than other cell lines, or why UVR had a lesser effect on cell growth in Brm miR4 cells as compared to other miRNA cell lines. Each of these individual miRNAs targetted various parts of the Brm gene, some of which may have been more vital to its function than others. It is also possible that each miRNA may have had slightly varying downstream molecular effects.

The reduction in cell growth with UVR, as well as the level of cell death was much less than what was witnessed in primary MNKs, suggesting that immortalised HaCaT cells are more resistant to the effects of UVR than primary MNKs. This may have to do with the cells previously having been exposed to UVR, which is likely since HaCaT cells show a mutation spectrum in p53 indicative of UVR-induced mutations (Lehman et al. 1993). It could alternatively be due to one or more of the multiple mutations in these cells. HaCaT cells also show reduced activation of p21, p15, p16 and p27 as compared to normal keratinocytes; however, they are still highly susceptible to UVR-induced apoptosis (Chaturevedi et al. 1999), suggesting apoptosis may function via alternative pathways in HaCaT cells. Other studies have also shown that in cell lines with
mutant p53, p53 and its target genes such as p21 and GADD45a can still be upregulated in response to genotoxic stress, especially prior to apoptosis, suggesting that p53 may still play a role in HaCaT cells (Henseleit et al. 1997; Gong and Almasan, 1999). This suggests that although p53 and other important cell cycle molecules are mutated in these cells, different variants of the mitogen activated protein kinase (MAPK) pathway are still being regulated; possibly mediating these changes post UVR. These molecular changes in HaCaT cells may also explain why these cells have such varying results to primary MNKs as seen in Chapter 3. It is possible that HaCaT cells are less dependent on SWI/SNF signalling since it is known that Brm/Brg1 cooperate with p53 to regulate cell cycle responses and apoptosis (Park et al. 2008; Naidu et al. 2009). Thus, a loss of p53 signalling in HaCaT cells may place less reliance on Brm/Brg1 expression in these cells. For example, in p53-deficient prostate carcinoma and osteosarcoma cells, p14ARF exclusively triggers expression of the pro-apoptotic Bax-homolog Bak, followed by release of cytochrome c, activation of caspases and subsequent fragmentation of genomic DNA (Muer et al. 2012). Therefore, even in the event of loss, alternative pathways can be triggered to induce cellular events.

Interestingly, Brm-/‐ MNKs showed increased apoptotic evasion following UVR, whereas Brm-null HaCaTs did not. The upregulation of Brg1 in SW13 cells has previously been associated with decreased apoptosis in these cells (Gong et al. 2008). It is possible that increased Brg1 expression in MNKs is leading to increased apoptotic evasion following UVR, however this process may be reliant on active p53, and thus why Brm-null HaCaT cells do not show an increase in apoptosis/cell death following UVR.
Nevertheless, the results presented in this chapter indicate that Brm regulates some responses to UVR even in the presence of the multiple mutations in HaCaT cells. These findings confirm results in UVR-treated Brm-/- mice with or without a loss of a single allele of p53 (p53+/+ or p53+/-). The loss of Brm in C57BL/6 mice as used in this thesis led to increased tumourigenicity following UVR treatment over 25 weeks, regardless of p53 status (Halliday et al. 2012). Further, the loss of Brm in these chronically irradiated mice also led to increased epidermal hyperplasia and Ki-67+ proliferating cells regardless of p53 status (Hassan et al. 2014). This confirms that Brm plays a role in NMSC regardless of p53 status. Therefore, the loss of Brm and Brg1 in NMSC such as BCC and SCC as previously observed (Bock et al. 2011), could be giving these malignant cells an additional growth advantage when exposed to UVR. Loss of Brm mRNA and protein has also previously been associated with carcinogenesis in other tumour types such as lung adenocarcinoma and gastric cancer, where its loss correlates with poorer survival (Reisman et al. 2003; Yamamichi et al. 2007). Cell division prior to the repair of DNA damage can lead to the incorporation of a mutation, therefore the increased cell division seen in Brm-/- mice and HaCaT cells would reduce available time for DNA repair. These events could have led to the increased photocarcinogenesis as previously observed in Brm-/- mice (Halliday et al. 2012).

4.3.3. Brm loss in HaCaT cells hastens cell cycle progression following UVR

As in the previous chapter, a cell cycle stain utilising EdU for S-phase cells and an anti-histone H3 antibody for M-phase was used. Firstly, at 1h post UVR, it
was found that UVR led to a significant reduction in S-phase cells in LacZ miR HaCaTs 1h post treatment. Interestingly, although there was a decreasing trend in S-phase cells with UVR, the decrease seen in Brm miR was not statistically significant 1h following UVR. Further, Brm miR cells actually exhibited a significant increase in S-phase cells 24h post UVR as compared to unirradiated cells, whereas LacZ cells exhibited a decrease in S-phase cells at 24h. There was no difference in M-phase cells in the UVR-treated groups, nor was there any differences in S- or M-phase cells in unirradiated cells. These results indicate that in HaCaT cells, UVR-induced entry to G0/G1 cell cycle arrest is at least partly Brm-dependent. Further, it is also crucial for maintenance of UVR-induced G1 arrest, as cells lacking Brm cycle through to S-phase faster than with intact Brm expression. It is likely that in this case, increased Brg1 expression in Brm miR cells is not enough to fully mediate neither initial Rb-mediated G1/G0 cell cycle arrest nor its maintenance in these cells post UVR. It is likely if these cells were depleted of both Brm and Brg1 that they would be insensitive to Rb-mediate cell cycle arrest, as seen in other cell systems, causing further proliferative dysfunction (Strobeck et al. 2002; Gong et al. 2008). These results are also in agreement with the earlier cell growth studies following UVR, as increasing cycling cells early in culture due to less time spent in cell cycle arrest would lead the witnessed increase in cell numbers.

Although p53 is mutated in HaCaT cells, LacZ control miR cells were still able to arrest at 1h post UVR, suggesting cell cycle arrest in this cell line is most likely p53-independent. In Brm+/- MNKs it is likely that p53 and Brm are forming a complex in order to facilitate normal cell cycle arrest, apoptosis and DNA repair responses, by promoting the phosphorylation of target genes such as
p21 and E2F1 (Ruijtenberg and van den Heuvel, 2016). However, with the p53 mutant present in HaCaT cells, the Brm/Brg1/p53 complex may no longer be viable, and thus HaCaT cells are relying on a variant pathway for cellular stabilisation following UVR damage. The loss of p53 in HaCaT cells may therefore place more dependence on Rb-mediated pathways. It is unlikely that both Rb and p53 is affected in HaCaT cells as these cells have previously been shown to express pRb and E2F upon growth arrest by TGF-β (Belbrahem et al. 1996). It is also possible that increased p38 and JNK, as witnessed in other HaCaTs irradiated with UVR, are compensating for the loss of p53 in these cells, eliciting similar cell cycle and apoptotic responses to p53 activation (Muthusamy and Piva, 2013). Both p38 and JNK are critical for the cell cycle regulation and apoptotic response of many cells in the response to DNA damage (Thornton and Rincon, 2009; Gutierrez et al. 2010). Further, SWI/SNF proteins have been shown to regulate or be regulated by the p38/JNK pathways previously (Simone et al. 2003; Jordan et al. 2013), suggesting Brm may also be able to regulate these proteins in HaCaT cells following UVR-induced DNA damage. Regardless, these results suggest that independent of p53, Brm plays a role in NMSC, with the loss of Brm in HaCaTs leading to both reduced activation of G1 arrest, as well as reduced time spent in G1 arrest following UVR. This is also supported by previous findings. For example, Brm-/- mouse embryonic fibroblasts have defective cell cycle arrest responses to UVR (Reyes et al. 1998). The observed increase in cells through the G1-S phase in Brm miR cells would reduce the time available for repair of the cells, and without an increase in apoptosis to clear these cells, this would be expected to increase UVR-induced mutagenesis and cellular sensitivity to UVR-induced carcinogenesis. G1
checkpoint defects are a common feature of many cancer cells, including melanoma and NMSC (Carson et al. 2012; Boukamp, 2005).

4.3.4. Brm loss in HaCaT cells leads to increased levels of CPDs in the dark

Previously, Brm/- MNKs were shown to have defective G1 arrest in response to UVR. Further, these cells were also shown to harbour increased CPD formation long after the initial UVR treatment, indicative of increased sensitivity to ROS species. Reduced cell cycle arrest in these cells would have also led to less time available to repair these increased lesions. Similar to MNKs, in UVR irradiated HaCaT cells, there was an increase in UDS at 1h after irradiation in all cells studied, confirming that these cells contain DNA repair mechanisms regardless of Brm status. CPDs were also investigated in these cells, and interestingly, Brm miR cells showed significantly higher levels of CPDs at 1h and 2h post UVR, indicative of increased CPD in the dark formation. There were however no significant differences in total CPDs at later stages, indicating that both Brm and LacZ miR were both undergoing significant levels of CPD repair following initial CPD induction, confirming the UDS results suggesting equal DNA repair in these cell lines. The increased formation of CPDs in the dark following UVR is believed to be related to increased levels of ROS species, eventually leading to energy transfer to DNA bases, and thus forming CPD lesions (Premi et al. 2015). Increased CPD in the dark formation has been shown in keratinocytes previously (Gupta et al. 2007; Dixon et al. 2011) as well as in UVR treated Brm/- MNKs in Chapter 3. The mutation of Brm, Brg1 and other SWI/SNF subunits has previously been implicated in increases in cytotoxic stress and increased ROS.
sensitivity (Xia et al. 2007; Freeman et al. 2014). For example, the mutation of Brm and Brg1 as well as other DNA repair genes can make cells especially sensitive to doxorubicin, a common chemotherapy agent (Xia et al. 2007), suggesting that Brm is necessary for genetic stability. Moreover, further studies also determined that the mutation of Brm and Brg1 makes cells especially sensitive to the oxidative stress agent menadione. Therefore, Brm is necessary for cell viability when exposed to ROS-generating agents (Freeman et al. 2014). Both UVA and UVB are able to stimulate the production of ROS in the skin (Heck et al. 2003; Nishimura et al. 2006), which has been linked with photoaging as well as photocarcinogenesis (Scharffetter-Kochanek et al. 1997). Importantly, SCC tumours show increases in UVR-induced mutations that are likely caused via ROS (Agar et al. 2004; Halliday et al. 2005). Therefore this suggests that the loss of SWI/SNF subunits such as Brm could be causing genetic instability, leading to increased ROS production or increased sensitivity to oxygen radicals, inducing increased CPD lesion formation downstream. The distribution of CPDs via sunlight provides a good match with skin cancer mutations, such as that of p53 in NMSC (Tornaletti et al. 1995). Increased CPD formation, especially with increasing UVR exposures, has been linked with photocarcinogenesis. Sites of mutation hotspots are known to coincide with nucleotide positions where the DNA repair process for removal of UVR-induced CPDs are particularly slow (Pfeifer and Besaratinia, 2012). Although the loss of Brm led to increased formation of dark CPDs, Brm does not seem to be linked to DNA repair in HaCaT cells. It is unlikely that the increased CPD formation seen at 1 and 2h is due to impaired cell cycle function in these cells, since the level of cell cycle arrest was similar in both Brm miR and LacZ miR HaCaT cells 1h after UVR.
Furthermore, the lack of apoptotic evasion in Brm miR HaCaT cells as compared to Brm-/ MNKs in response to UVR may also explain why mouse skin shows significantly increased CPD formation much later in the time course, as it would be expected that these cells evading apoptosis would be harbouring large levels of CPDs, since the main function of apoptosis is to clear cells with irreversible damage (De Gruijl et al. 2001). This suggests cells harbouring excessive lesions were undergoing cell death, however, this same mechanism was not present in Brm-/ MNKs. Studies have also shown that the UVB-induced gene expression profile of human epidermis in vivo is different to that of cultured keratinocytes, which may also explain the varied DNA repair and apoptotic response seen in mouse skin and HaCaT cells (Enk et al. 2007).

Nevertheless, these results support that Brm plays a role in NMSC, which is likely at least partially p53-independent. Any increase in CPDs in these cells would mean an increased CPD repair response would be necessary in these cells. Considering UDS was similar in both cells, this suggests that repair was not being increasingly regulated in these cells. Misrepair of CPD lesions produce UVR signature mutations as C>T and CC>TT mutations (Pfeifer and Besaratinia, 2012). Absence of repair of these lesions and the accumulation of mutations are associated with skin cancer, and are particularly prominent in patients suffering from XP, who lack DNA repair capabilities (Yarosh, 2004). The loss of Brm and Brg1 as witnessed in BCC and SCC are thus likely UVR-induced mutations, caused by excessive DNA damage over time. The loss of Brm and Brg1 in these cells would likely lead to defective cell cycle responses, as witnessed in Brm-null HaCaT cells and MNKs, thus giving the cell a proliferative advantage. This increased proliferative potential in combination with multiple UVR doses could
lead to epidermal hyperplasia as witnessed in Brm-/- mice treated with UVR (Hassan et al. 2014), eventually leading to increased tumourigenicity, as witnessed in chronic UVR-irradiated Brm-/- mice (Halliday et al. 2012).

4.4. Conclusion

With the use of lentiviral technology to knockdown Brm in HaCaT cells, it was discovered that Brm loss in these cells in combination with UVR led to a defective proliferative response, via decreased cell cycle control of the G1 cell cycle. Further, the loss of Brm led to increased levels of UVR-induced CPD lesions formed following the initial UVR treatment, suggesting that the loss of Brm in HaCaTs leads to increased sensitivity to ROS or increased levels of ROS, leading to increased CPD lesion formation. HaCaT cells also show UVR-induced mutations of the p53 gene, indicating that the functionality of the Brm gene in NMSC is likely at least partially p53-independent. These findings further prove the importance of SWI/SNF signalling in NMSC and confirm earlier findings of Bock et al. (2011) that suggested Brm plays an important role in the development of a benign lesion into a NMSC. The loss of Brm in skin keratinocytes could thus lead to the development of epidermal hyperplasia and tumourigenicity as witnessed in chronic UV-irradiated Brm-/- mice.

CHAPTER 5: The Q203K mutation inhibits Brm function in cell growth and viability
5.1. Introduction

The final chapter of my thesis investigates the effects of a novel hotspot mutation of Brm in NMSC, previously discovered in my laboratory. As an important part of the SWI/SNF complex, Brm has been highly conserved over time, and evidence has linked it to tumour suppression in multiple tissue types. Mutations in SWI/SNF are also common in human cancers, with an average of 19% of primary cancers expressing SWI/SNF mutants. Although several studies have linked the loss of Brm with carcinogenesis, no studies of primary tumours have demonstrated mutations of Brm. However, upon screening of genes from 27 Caucasian patients using DHPLC and the sequencing of PCR products, a same single-base substitution in Brm exon 4 was found to occur in 2 of 6 BCCs and 1 in 10 SCCs. Therefore, this mutation was found to be present in 17% of all NMSC that were studied. No other mutations were discovered, nor were there any mutants detected in non-sun-exposed skin, sun-exposed skin or AK. This G:C to T:A transversion resulted in the substitution of a glutamine by lysine at codon 203 (Q203K) (Moloney et al. 2009). Analyses of exon 4 using online alignment-based algorithms predicted that the Q203K mutation would likely be poorly tolerated and affect protein function. Therefore, to test the effect of the Q203K mutant, plasmids were designed, incorporating either the full wildtype Brm sequence, Q203K or control DNA, and these plasmids were transfected in the immortalised human keratinocyte cell line, HaCaT, as used in Chapter 4. These cells were previously shown to contain wildtype exon 4 DNA and do not contain the Q203K sequence. Firstly, it will be determined whether Q203K contributes to the reduced amount of Brm seen in SCC and BCCs, by comparing parallel HaCaT cultures transfected with either wildtype or Q203K Brm. Following this, it will be
determined whether Q203K is able to alter basic cell functions in response to damaging UVR, such as cell growth, cell death, cell cycle regulation and DNA repair.

5.2. Results

5.2.1. The transfection of HaCaT cells with wildtype and Q203K Brm using the nucleofection technique

Previously in Chapter 4, cellular transductions using the lentiviral technique were utilised to transduce HaCaT cells with miRNAs. For these experiments, plasmids were designed incorporating either the entire wildtype Brm sequence, or Brm with the Q203K mutation. Each plasmid also contained an mCherry fluorescent protein fused to the C-terminus of Brm, for visualisation and selection of positive cells. Therefore, an mCherry alone plasmid was also used in all experiments to control for the fluorescent protein. Unfortunately in this case, the plasmid constructs were found to be too large to use lentiviral techniques as used previously, as the size of the constructs greatly inhibited the production of lentivirus containing the DNA. Therefore, nucleofection technology was used, utilising the Nucleofector II and an optimised nucleofection kit for HaCaT cells. Following successful transfection of each of the 3 constructs, the cells were selected with 10μg/ml blasticidin for 10 days. The cells were then removed from the flask, and mCherry positive cells were selected using a FACSaria cell-sorter (BD Biosciences) (Figs 5.1 & 5.2). The mCherry protein was directly linked to the C-terminus of the Brm wildtype or Q203K insert in the plasmid, therefore if the
cells were shown to be mCherry positive this would suggest the insert was successfully incorporated into the cells.

Figure 5.1: The transfection of HaCaT cells with Brm/Q203K plasmids containing mCherry fluorescent proteins. Post transfection, cells were selected with blasticidin, then sorted by flow cytometry to select only
mCherry positive cells, therefore leaving purely transfected cells. Objective = 20X, scale = 100μm.

Figure 5.2: The sorting of mCherry-positive transfected cells. Firstly, cells were gated for side and forward scatter in order to eliminate cellular debris (a). The cells were then separated into mCherry positive and negative and only cells from this that were high in mCherry signal were collected (purple dots) (b). If cells did not have a mCherry signal (blue dots) they were not collected. Following cell sorting, only highly pure transfected mCherry-positive cells were left.

5.2.2. Assessment of Brm protein in Brm/Q203K expressing HaCaT cells
Following the successful transfection and collection of highly pure mCherry-positive HaCaT cells, protein was collected from all cells to assess whether Brm protein was affected by either the expression of recombinant Q203K or wildtype Brm in HaCaT cells. The levels of Brm protein were analysed using a Western blot using antibody specific to Brm, as well as a housekeeping gene, GAPDH (Fig 5.3). As expected, the mCherry control cells contained endogenous levels of Brm protein from the HaCaT cells. However, both the Q203K and Brm wildtype HaCaT cells showed an extra, higher molecular weight (due to Brm being fused to mCherry) band for Brm above the endogenous Brm band. This extra band above endogenous Brm shows an extra level of Brm expression in these cells, which shows that the inserts were inserted correctly. Further, the increased level of Brm was about 50% that of endogenous Brm, similar to one Brm allele. The Q203K mutant did not affect Brm protein levels.
Figure 5.3: There is an increase in Brm protein in Q203K and Brm wildtype HaCaT cells but not mCherry. HaCaT cells were transfected with plasmids containing a mCherry control, mCherry Q203K or mCherry Brm wildtype sequence. Equal amounts of protein from these cells were loaded onto a 7.5% acrylamide gel. Gels were run at 120V and proteins were transferred to a PVDF membrane. Membranes were then stained with an anti-Brm or anti-GAPDH antibody, followed by secondary antibodies for visualisation. All blots were imaged using a Syngene G-box. The level of Brm protein was increased in Brm wildtype, as well as Brm Q203K cells, but not mCherry cells. This is seen with a higher molecular weight due to the bound mCherry, as indicated by the arrows.

5.2.3. The Q203K mutation inhibits Brm function in cell growth and viability

As investigated in the previous chapters, the effect of 4J/cm² UVR on both cell growth and death was tested on the Brm wildtype and Q203K HaCaT cells. In unirradiated cells, the growth was steady, and the cells generally reached confluence by 96h, leading to a halt in cell growth. The growth of both mCherry control and Q203K HaCaT cells were similar over the time course (P=0.864; 2-way ANOVA). Interestingly, the growth of Brm wildtype HaCaT cells was significantly increased compared to both mCherry (P=0.0132) and Q203K cells (P=0.0028; 2-way ANOVA) over the time course, with Brm cells showing increased growth over the other cell lines at both 72h and 96h. At this stage, where both Q203K and mCherry cells stopped growing due to reaching
confluence, Brm wildtype cell growth continued, suggesting these cells had a growth advantage late in culture (Fig 5.4a). This result suggests that the Q203K mutation abolishes the growth-promoting effect of overexpressed Brm, but does not have a dominant growth effect on endogenous Brm, as the mutant cells were comparable to cells with endogenous Brm alone. Irradiated cells did show a general decline in growth following UVR, however, all cell lines exhibited continued cell growth up to 96h and did not show stunted growth at 96h as unirradiated cells did. Over the time course, no cell line was found to have significantly different growth from the others following UVR (mCherry vs. Q203K, P=0.3892; mCherry vs. Brm, P=0.9883; Q203K vs. Brm, P=0.4732). This result indicated that the upregulation of the Q203K mutation or wildtype Brm in HaCaT cells did not significantly alter cell growth post UVR (Fig 5.4b).
Figure 5.4: HaCaT cells overexpressing wildtype Brm but not Q203K display a proliferative advantage that is lost with UVR. HaCaT cells containing mCherry (green circles), Q203K (red squares) or Brm (blue triangles) were (a) sham-irradiated or received (b) 4J/cm² UVR. Their cell growth was then tested at 24, 48, 72 and 96h post treatment. Sham-irradiated Brm wildtype HaCaT cells showed increased growth as compared to Q203K (P=0.0028) and mCherry (P=0.0132) HaCaT cells over the time course, however this growth advantage was lost in UVR-treated cells, which all grew similarly over time. Each time point represents mean ± SEM from of 3 independent experiments (8 independent wells per experiment; n=24). Two-way ANOVA used. Where the SEM is not obvious it is too small to be seen.

Further, when the effect of 4J/cm² UVR on each individual cell line was compared (rather than comparing the effect of Q203K in unirradiated and irradiated cells as in Fig 5.4), it was found that UVR reduced cell growth in all cell lines (Brm wildtype, P<0.0001; Q203K, P=0.0003; mCherry, P=0.0179) (2-way ANOVA) (Fig 5.5). Both irradiated Q203K Brm and mCherry HaCaT cells were found to catch up to the number of unirradiated cells by 96h, however, this was not the case for Brm cells, which showed a greater disparity between the growth of unirradiated and irradiated cells. However, these effects were likely due to cellular confluence. These results suggest that the upregulation of Brm in HaCaT cells initially has no effect on cell growth. However, upon reaching confluence, these cells continue to grow, suggesting an error in their proliferative state. However, the overexpression of the single base substitution Q203K mutant removes this proliferative advantage, similar to that seen in cells expressing
endogenous Brm levels (mCherry), suggesting that increasing wildtype Brm but not Q203K levels can lead to proliferative defects in the absence of UVR and that the Q203K mutation may reduce the function of Brm. However, upon UVR irradiation, this proliferative advantage was lost, and all cell lines grew at relatively the same rate. These results where Brm or Q203K was overexpressed in HaCaT cells differ to results obtained in the previous chapter where Brm was knocked out, indicating that the level of expression of Brm is important for cellular function.

Figure 5.5: UVR significantly decreases cell growth in mCherry, Q203K and Brm wildtype expressing HaCaT cells. HaCaT cells containing mCherry (a), Q203K (b) or Brm (c) were sham-irradiated (green) or received 4J/cm² UVR (red) and their growth was studied at 24, 48, 72 or 96h post ±4J/cm² UVR with the use of a CytoTOX-ONE assay. UVR was found to decrease cell growth in mCherry cells (P<0.0001), Q203K cells (P=0.0003) and mCherry cells (P=0.0179) over the time course. Each time point represents mean ± SEM from of 3 independent experiments (8 independent wells per experiment; n=24). Two-way ANOVA used. Where the SEM is not obvious it
is too small to be seen. Data is the same as in Figure 5.4 with different comparisons.

Following this, the effect of 4J/cm² UVR on cell viability was also tested at the time points examined above. In the untreated (No UV) cells, both Q203K and mCherry cells showed a gradual decrease in viability over time, due to increasing levels of cell death following time spent in culture. However, Brm wildtype HaCaT cells showed a decrease in viability up until 48h, which then increased at 72h and 96h. This is most likely due to the increased growth of live cells as seen in Fig 5.4a, however, it is also possible that these cells are evading cell death late in culture. The viability of unirradiated mCherry and Q203K cells were found to be similar over time (P=0.2937; 2-way ANOVA), as were mCherry and Brm cells (P=0.3782; 2-way ANOVA). However, the viability of Brm wildtype cells was significantly higher over time than Q203K cells (P=0.0086), suggesting that later in culture, when cells are at high densities, Brm wildtype cells have a survival advantage over Q203K Brm cells (Fig 5.6a). This further suggests that the Q203K mutation inhibits Brm function. Moreover, Brm wildtype cells were found to have a higher viability than Q203K cells at 72 and 96h (both P<0.0001; 2-way ANOVA), and an increased viability as compared to mCherry cells at 96h (P<0.0001; 2-way ANOVA).

In UVR-treated cells, viability decreased over time, also likely mostly due to increasing cell death following time in culture. Interestingly, Brm wildtype cells had a slight survival advantage over mCherry cells over time (P=0.024; 2-way ANOVA), which was most prominent at 48h. However, Brm cells did not show a different viability over time to Q203K cells in response to UVR (P=0.8445; 2-way ANOVA) and there was no significant difference between
mCherry and Q203K cells to UVR (P=0.1443; 2-way ANOVA)(Fig 5.6b). Further, UVR was found to have an overall affect on the viability of only mCherry cells, decreasing their viability over time (P=0.0002; 2-way ANOVA) (Fig 5.7a). However, there was no significant effect of UVR on either Q203K Brm cells (P=0.9299; 2-way ANOVA)(Fig 5.7b) or Brm wildtype cells (P=0.0645; 2-way ANOVA)(Fig 5.7c). However, in this case, the Q203K cells showed a similar curve to mCherry cells, and did not show an increasing viability with or without UVR.

Figure 5.6: The overexpression of Brm in HaCaT cells leads to increased cell viability with and without UVR. HaCaT cells containing mCherry (green circles), Q203K (red squares) or Brm (blue triangles) were (a) sham-irradiated or received (b) 4J/cm² UVR. Their cell viability was then tested at 24, 48, 72 and 96h post treatment. Interestingly, Brm wildtype cells were
found to have a survival advantage late in culture as compared to Q203K and mCherry cells. Further, upon UV-irradiation, Q203K and Brm wildtype cells had a survival advantage as compared to mCherry cells. Each time point represents mean ± SEM from 3 independent experiments (8 wells per experiment; n=24). Two-way ANOVA used. Where the SEM is not obvious it is too small to be seen.

Figure 5.7: The effect of 4J/cm² UVR on cell viability in mCherry, Q203K and Brm wildtype cells. HaCaT cells transfected with (a) mCherry, (b) Q203K or (c) Brm wildtype DNA were studied at 24, 48, 72 or 96h post ±4J/cm² UVR with the use of a CytoTOX-ONE assay to analyse cell viability. UVR led to a decrease in cell viability in mCherry cells (P=0.0002) but not Q203K cells (P=0.9299) or Brm wildtype cells (P=0.0645). Each time point represents mean ± SEM from 3 independent experiments (8 independent wells per experiment; n=24). Two-way ANOVA used. Where the SEM is not obvious it is too small to be seen. Data is the same as that shown in Figure 5.6 but with different analysis.
5.2.4. Cell cycle control is similar in Q203K mutant and Brm wildtype cells

The next experiment aimed to investigate whether the Q203K mutant in Brm played a role in cell cycle control in unirradiated cells or in response to UVR. This cell cycle assay was performed at 24h post UVR and compared Brm wildtype, Q203K mutant and mCherry control HaCaT cells. Later times were not used as HaCaT cells were found to recover quickly from UVR past this time in previous experiments (Fig 4.16, Fig 4.17), and therefore any change in cell cycle would be due to culture conditions and not UVR. This experiment used the novel methodology optimised in Chapter 3, with an EdU stain to identify S-phase cells and a phospho-histone H3 antibody to identify M-phase cells. At 24h post UVR, all 3 cell lines showed downregulation of S-phase cells when irradiated with UVR, however, there was not a corresponding decrease in M-phase cells noted (Fig 5.8). As previously, the intensity of both EdU and H3 were determined by using respective controls (Fig 5.9).
Figure 5.8: UVR reduces the number of S-phase but not M-phase cells in mCherry, Q203K and Brm wildtype HaCaT cells. Transfected HaCaT cells were irradiated with ±4J/cm² UVR and grown for 24h. Following irradiation, there was a noticeable decrease in S-phase (red) but not M-phase cells (green) in all samples. All images = 20X objective, scale = 100μm. Each image is a merger of DAPI, H3 and EdU.
**Figure 5.9: Gating of control stained cells.** Dot-plots of HaCaT cells were treated with azide but not EdU for the control of EdU (a) or a rabbit IgG isotype antibody for the control of phospho-histone H3 staining (b). These controls allowed gates to be drawn accurately for cells that were negative for EdU and H3. Thus anything with higher intensity was considered positive. Representative plots are shown of one experiment with approximately 3000 counted cells per culture.

In all 3 cell lines (mCherry, Q203K and Brm wildtype), there was a notable reduction is S-phase cells with 4J/cm² UVR (Fig 5.10). This decrease was approximately 1.5-fold in all cells, indicating that UVR reduces the number of S-phase proliferating cells 24h following irradiation. This decrease in S-phase cells with UVR was similar to that of control HaCaT cells in Chapter 4 (Fig 4.18). However, UVR did not reduce the number of M-phase cells in any of the 3 cell lines tested, but actually led to an increase in mitotic cells in all samples (Fig 5.11). Again, this was similar to results in Chapter 4 which did not show decreasing M-phase cells 24h post UVR (Fig 4.18). The increase in M-phase cells was especially prominent in mCherry cells, which showed an almost 2-fold
increase in the percentage of M-phase cells 24h post UVR, which was similar to HaCaT cells expressing lower levels of Brm in Chapter 4. However, increasing levels of Brm in both Q203K and Brm wildtype cells prevented this increase in M-phase cells, similar to cells with higher levels of Brm in Chapter 4 (Fig 4.18).
Figure 5.10: mCherry, Q203K and Brm wildtype HaCaT cells all show a decrease in S-phase cells in response to UVR. Dot-plots for EdU intensity in (a) mCherry No UV, (b) mCherry UV, (c) Q203K No UV, (d) Q203K UV, (e) Brm No UV and (f) Brm UV HaCaT cells, 24h after treatment. In all UVR treated samples, there was a respective reduction in the number of S-phase
cells as compared to unirradiated cells. Representative plots are shown of one experiment with approximately 3000 counted cells per culture.
Figure 5.11: mCherry, Q203K and Brm wildtype HaCaT cells all show an increase in M-phase cells in response to UVR. Dot-plots for histone-H3 intensity in (a) mCherry No UV, (b) mCherry UV, (c) Q203K No UV, (d) Q203K UV, (e) Brm No UV and (f) Brm UV HaCaT cells, 24h after treatment. In all UVR treated samples, there was a respective increase in the number
of M-phase cells as compared to unirradiated cells. Representative plots are shown of one experiment with approximately 3000 counted cells per culture.

To fully analyse the effect of exogenously expressed Brm and Q203K in HaCaT cells, all experiments were compiled. In total there were 3 experiments completed, each with 3 independent experimental wells (n=9). The data from each experiment was then entered into the flow cytometry program, FlowJo, and the total percentage of both EdU-positive cells (Fig 5.12) and H3-positive cells (Fig 5.13) were calculated. As completed previously, the number of cells undergoing UDS were manually counted and subtracted from the EdU-positive total, in order to analyse only S-phase cells. In the No UV cells, the level of S-phase at 24h was high and similar in all 3 cell lines (mCherry, 53.8%; Q203K, 53.8%; Brm+, 52.8%) and no sample showed a significant difference to the other (Fig 5.12). The amount of S-phase cells was lower in all UVR treated samples, however, all of the samples showed similar decreases (mCherry, 31.9%; Q203K, 32.7%; Brm+, 35.6%) with none of the UV-irradiated samples showing a statistically significant difference to another (Fig 5.12). 4J/cm² UVR was found to have a highly significant effect on all cell lines, decreasing the number of S-phase cells in each (mCherry, P=0.0006; Q203K, P<0.0001; Brm+, P=0.0009; 1-way ANOVA).
Figure 5.12: The amount of S-phase cells is reduced 24h post 4J/cm² UVR in mCherry, Q203K and Brm wildtype HaCaT cells. HaCaT cells transfected with mCherry, Q203K or Brm wildtype DNA were irradiated with or without UVR, and further incubated for 24h. S-phase cells were determined by staining with EdU/azide and counting cells with a fully stained nucleus. There was no difference in S-phase between unirradiated or irradiated samples. However, all 3 cell lines showed a significant decrease in the number of S-phase cells, indicating UVR was leading to cell cycle arrest in these cells. Each sample represents mean ± SEM from 3 independent experiments (3 wells per experiment; n=9). One-way ANOVA used.

Moreover, in unirradiated cells, the proportion of M-phase cells at 24h was similar in each sample (mCherry, 4.6%; Q203K, 4.3%; Brm+, 4.5%), with no sample showing a significant difference to the other (Fig 5.13). In UVR-treated cells, mCherry cells showed the greatest increase in mitotic cells with an increase of 4.1% as compared to unirradiated cells. Whereas Q203K cells and Brm cells showed smaller increases in M-phase cells (1.0% and 0.8% respectively). The percentage of mitotic cells in the mCherry group was significantly higher than
both Q203K and Brm wildtype HaCaT cells (P=0.0008 and P=0.0152 respectively; 1-way ANOVA). There was no significant difference between UVR-treated Q203K and Brm wildtype cells (P=0.3613; 1-way ANOVA) (Fig 5.13). Therefore, at 24h post UVR, the increasing levels of Brm or Q203K prevented a significant increase in M-phase cells, suggesting that the mCherry cells with lower levels of Brm are undergoing higher levels of cell division than the Brm overexpressing cells following UVR. This suggests that mCherry cells are exiting cell cycle arrest sooner or staying in M-phase for longer than Q203K or Brm wildtype cells following UVR. These findings are similar to results in Chapter 3 and 4, which showed that cells with lower levels of Brm undergo increased mitosis 24h post UVR.
Figure 5.13: The incorporation of Brm wildtype or Q203K DNA prevents increased M-phase numbers 24h post UVR. HaCaT cells transfected with mCherry, Q203K or Brm wildtype DNA were irradiated with or without UVR, and further incubated for 24h. M-phase cells were determined by staining with anti-histone H3 antibody and counting stained cells. There was no difference in M-phase between unirradiated cells, however, mCherry cells showed a significant increase in M-phase cells post UVR (P=0.0003), but Q203K and Brm wildtype HaCaT cells did not, indicating that the increase of Brm or Q203K prevented increased levels of M-phase cells post UVR. Each sample represents mean ± SEM from 3 independent experiments (3 wells per experiment; n=9). One-way ANOVA used.

To gather further data on the initial cell cycle patterns of these cells following UVR, the number of S-phase cells was also determined at 1h post UVR. As expected, the level of S-phase cells in unirradiated samples was similar in all cell types, with none being significantly different from the other (mCherry, 35.3%; Q203K, 32.2%; Brm+, 30.3%). The level of S-phase cells in the irradiated cells were not significantly different from each other (mCherry, 28.4%; Q203K,
There was no statistically significant difference between unirradiated and irradiated cells in any cell line (mCherry; \( P=0.4323 \); Q203K, \( P=0.229 \); Brm+, \( P=0.9113 \); 1-way ANOVA) (Fig 5.14). Therefore, these results suggest that none of these HaCaT cells are undergoing statistically significant levels of G0/G1 cell cycle arrest 1h following UVR, suggesting that cell cycle arrest in these cells may be delayed.

Figure 5.14: Neither Brm wildtype, Q203K or mCherry control HaCaT cells show significantly increased cell cycle arrest 1h post UVR. HaCaT cells transfected with mCherry, Q203K or Brm wildtype DNA were irradiated with or without UVR, and further incubated for 1h. S-phase cells were determined by staining with EdU/azide and counting cells with a fully stained nucleus. Interestingly, none of these cell lines showed a statistically significant decrease in S-phase cells 1h post UVR. Results are pooled from 3 separate experiments, 2 independent wells per experiment (n=6). Mean + SEM shown. One-way ANOVA used.
5.2.5. Increasing levels of Brm and Q203K do not influence UVR-induced DNA repair

As in previous chapters, DNA repair was also analysed in these cells using 2 different methodologies. Firstly, UDS was analysed via EdU staining and the counting of speckled or punctate stained cells. To do this, equal amounts of either mCherry, Q203K or Brm wildtype HaCaT cells were irradiated with or without 4J/cm² UVR and incubated with cell medium containing EdU, and then stained/imaged as explained in Chapter 2. A total of 2 independent experimental wells were sampled per experiment, as well as 1 control well stained with azide but unprobed with EdU, with 3 independent experiments completed (n=2 per experiment, n=6 total). Although there was a decrease in EdU intensity noted in all UV samples, there did not seem to be a major increase in the number of speckled/punctate cells indicative of UDS in the majority of samples (Fig 5.15).
Figure 5.15: The percentage of cells undergoing UDS at 1h in mCherry, Q203K and Brm wildtype cells are not drastically altered by 4J/cm\(^2\) UVR. DAPI and EdU labelled HaCaT cells visualised by fluorescent microscopy. Although there is a decrease in EdU intensity in irradiated cells, both No UV and UV samples show high levels of S-phase (fully stained cells) and limited
numbers of cells in UDS (speckled or punctate cells, green arrows). Objective = 40X, scale bar = 100μm. Each image is a merger of DAPI and EdU.

In unirradiated cells, the level of DNA repair was found to be similar in all 3 cell lines, with none of these cell lines showing a significant difference from the others (mCherry, 13.9%; Q203K, 13.4%; Brm+, 15.8%) (Fig 5.16). Similarly in UV-irradiated cells, none of these showed higher degrees of DNA repair than the others (mCherry, 17.2%, Q203K, 17.9%; Brm+, 16.3%). None of these cells indicated a statistically significant increase in UDS 1h following exposure to UVR (mCherry, P=0.1736; Q203K, P=0.2913; Brm+, P>0.9999) (Fig 5.16).
Figure 5.16: Neither Brm wildtype, Q203K or mCherry control HaCaT cells show statistically increased UDS 1h post UVR. Transfected HaCaT cells were incubated for 1h with EdU post ±4J/cm² UVR and then stained with azide to determine the percentage of punctate stained EdU-positive cells (cells undergoing UDS). No cells showed a statistically significant increase in UDS following exposure to UVR. There were no significant differences between groups in either unirradiated or irradiated samples. Results are pooled from 3 separate experiments, 2 wells per experiment (n=6). Mean + SEM shown. One-way ANOVA used.

To gather further detail of the level of DNA repair occurring in these cells, the amount of UDS at 24h was also calculated in each cell line following UVR. In unirradiated cells, the level of UDS was similar and there were no significant differences between cell lines (mCherry, 5.5%; Q203K, 6.2%; Brm+, 6.4%) (Fig 5.17). UVR was found to significantly increase the level of UDS in all cell lines (mCherry, 10.4%; Q203K, 9.4%; Brm+, 10.3%) (mCherry; P=0.0006; Q203K P=0.0173; Brm+, P=0.0131; compared to unirradiated cells using 1-way ANOVA). However there was no significant difference between the irradiated samples.
This data indicates that, UVR-induced DNA repair could not be detected using this assay until 24h after irradiation. Overexpression of Brm above control (mCherry) levels did not influence UDS at 24h and therefore it could not be determined whether or not the Q203K mutation was affecting Brm function using this assay.

**Figure 5.17:** 4J/cm² UVR leads to a significant increase in UDS in mCherry, Q203K and Brm wildtype HaCaT cells 24h post treatment. Transfected HaCaT cells were incubated for 24h post ±4J/cm² UVR and then stained with EdU/azide to determine the percentage of punctate stained EdU-positive cells (cells undergoing UDS). 24h following UVR, there was found to be a significant increase in UDS in all cell lines (mCherry; P=0.0006; Q203K P= 0.0173; Brm+, P=0.0131). The insertion of Brm or Q203K mutant DNA did not alter the rate of UDS in the cells in either unirradiated or irradiated cells. Results are indicative of 3 separate experiments, 2 wells measured per experiment (n=6). Mean + SEM shown. One-way ANOVA used.
As done previously, all cell lines were also stained for UVR-specific DNA lesions (CPDs) to analyse the possible effect that the overexpression of Q203K or Brm could have on these lesions. As seen in previous experiments, there was little to no staining apparent in any of the No UV samples, indicating these lesions were UVR-specific. There was no significant difference in CPD intensity between any of the unirradiated samples over the time course. With UVR, CPDs appeared as early as 15 min post treatment (Fig 5.18). The level of CPDs in all cell lines was similar between 15 min and 3h, indicating that little or no CPD repair was occurring during this time. There was no statistically significant decrease in CPDs from 15 min to 3h in any cell line (mCherry; P=0.9534; Q203K; P>0.9999; Brm+, P=0.7054; 2-way ANOVA). However, there were large decreases in CPDs in all cell lines from 3h to 24h (Fig 5.19). All cell lines showed a significant decrease in the level of CPDs from 3h to 24h, indicating each of these cell lines were able to repair CPDs (mCherry, P<0.0001; Q203K, P<0.0001; Brm, P=0.0004; 2-way ANOVA) (Fig 5.19). There were still levels of CPDs observed at 24h, however they were low compared to the initial levels of CPDs formed with UVR. Moreover, there was no difference in the CPD intensity between the cell lines over the time course or at any time investigated, indicating that each of these cells repaired CPD lesions at a similar rate (mCherry vs. Q203K, P=0.8568; mCherry vs. Brm, P=0.9255; Q203K vs. Brm, P=0.9866). However, as in the previous UDS assay, overexpression of Brm above control (mCherry) levels did not influence CPD accumulation and therefore it could not be determined whether or not the Q203K mutation was affecting CPDs.
Figure 5.18: 4J/cm² UVR induces CPDs in mCherry, Q203K and Brm wildtype cells that repair similarly over time. Transfected mCherry, Q203K or Brm wildtype cells were irradiated with 4J/cm² UVR or sham-irradiated and incubated in cell medium at 37°C/5% CO₂ for various times. Unirradiated (No UV) samples did not show any staining for CPDs. However, all UV treated samples showed CPD formation 15 min following treatment, with the levels at 1h being similar in all cell lines. All cell lines showed greatly decreased levels of CPDs at 24h, suggesting DNA repair. Objective=20X, scale = 100μm.
Figure 5.19: The expression of exogenous Brm or Q203K DNA in HaCaT cells does not alter CPD repair. Transfected mCherry, Q203K or Brm wildtype cells were irradiated with 4J/cm² UVR or sham-irradiated and incubated in cell medium at 37°C/5% CO₂ for various times. Unirradiated (No UV) samples did not show any staining for CPDs. All UVR-treated cells showed CPD formation as early as 15 min, which was similar up until 3h. By 24h, all samples had markedly reduced levels of CPDs. There was no significant difference between the level of CPDs or repair of CPDs between any of the cell lines studied, indicating that increased Brm or Q203K did not influence CPD repair. Results are from 3 independent experiments, with 2 independent wells measured per experiment (n=6). Mean ± SEM shown. 2-way ANOVA used. Where the SEM is not obvious it is too small to be seen.
5.2.6. Results summary

In the scope of this thesis, the Q203K mutation seems to be mostly non-functional in the presence of endogenous Brm. However, it was able to inhibit the excess cell growth effect of Brm in non-UVR exposed cells, likely due to increased viability of Brm wildtype cells. As expected, the overexpression of Brm was not comparable to the loss of Brm, and was comparable to that of control cells in Chapter 4, suggesting that endogenous Brm in these cells is enough to mediate most cell responses to UVR. Interestingly however, the upregulation of Brm in HaCaT cells led to increased cell viability, leading to increased cell numbers without UVR, which was lost when Q203K was expressed instead of wildtype Brm. There was no difference in the regulation of S-phase post UVR in any of the cell lines studied, with all cell lines showing decreases in S-phase 24h post UVR, suggesting that endogenous Brm in these samples is sufficient for this process. However, while mCherry cells led to increasing M-phase numbers at 24h post UVR, similar to cells with lower levels of Brm in Chapters 3 and 4, increasing Brm and Q203K expression prevented this mitotic cell increase, suggesting that Brm is able to protect the cells from increased mitosis following UVR. An increase in UDS was noted in all cell lines at 24h post UVR, however, increasing Brm or Q203K expression did not affect the level of DNA repair. Furthermore, there was no difference in the repair of CPDs between any of the cells studied, suggesting that endogenous Brm present in these cells is sufficient for the DNA repair response in these cells. This data indicates that Q203K is mostly non-functional, and that overexpression of normal Brm has a different functional effect to the Q203K mutant, with the exception that Q203K can compensate for some cell cycle responses post UVR.
5.3. Discussion

It has been shown in previous chapters that the loss of Brm in neonatal mouse keratinocytes and HaCaT cells led to drastic cellular changes post UVR, including defective cell cycle regulation, and increased photolesion formation. Cells with diminished Brm exhibited an increased proliferative response, decreased time in UVR-induced cell cycle arrest, as well as increased levels of CPDs following UVR, likely contributing to the increased tumourigenicity observed in UVR-treated Brm-/- mice (Halliday et al. 2012). It is likely that Brm and Brg1 play a significant role in NMSC, as both have been found to be downregulated in malignant SCC and BCC (Bock et al. 2009). Further, the loss of Brm in gastric and lung cancers also correlates with a poorer prognosis for the patient (Reisman et al. 2003; Fukuoka et al. 2004; Glaros et al. 2007; Yamamichi et al. 2007). Therefore, Brm may also play a tumour suppressive role in human skin cancer. Recently, the discovery of a novel hotspot mutation of Brm in NMSC (Q203K) (Moloney et al. 2009) was of particular importance to study. However, the functional consequences of the Q203K mutation were unknown and the aim of this work was to determine whether Q203K influences Brm function. Mutations of SWI/SNF have been thoroughly described in the literature, in both cell lines and primary tumours. Many of these mutants have been associated with cellular defects, such as the loss of Rb-mediated cell cycle arrest (Bartlett et al. 2011; Shain and Pollack, 2013). However, the mutation of Brm in the literature is unknown, as other studies have reported Brm to be epigenetically silenced rather than mutated (Glaros et al. 2007).
5.3.1. Brm protein is upregulated in Brm overexpressing wildtype and Q203K HaCaT cells

HaCaT cells were transfected with normal wildtype Brm, Q203K mutant Brm or a control (mCherry) plasmid. It was found that all 3 cell lines expressed similar levels of endogenous Brm, as expected. However, both the Brm wildtype and Q203K expressing HaCaT cells also showed an extra, higher molecular weight band for Brm, but mCherry cells did not. This result was expected as Brm is fused to mCherry, therefore, making an increased sized band approximately 30kDa higher than endogenous Brm. The single-base substitution did not affect expression of Brm protein, as there was still a band present for this sample, which was similar in intensity to the transfected wildtype Brm.

5.3.2. The effect of overexpressing wildtype Brm in HaCaT cells

Firstly, it is important to note that the design for this chapter of results was different to previous chapters in that Brm, as the Q203K mutant was overexpressed in cells already expressing endogenous Brm. Interestingly, it was found that an increase in wildtype Brm expression (Brm overexpressing) in HaCaT cells led to an increase in cell number, which was especially prominent late in culture. Cells with only endogenous Brm (mCherry) stopped growing at approximately 72h, and began plateauing, suggesting contact inhibition was present in these cells, halting their growth. However, overexpression of wildtype Brm increased growth over time, suggesting that an increase in Brm expression may have been leading to a growth defect in these cells. Further to the increased cellular growth, overexpressing Brm wildtype cells also showed increased cell
viability late in culture. In cells only expressing endogenous Brm (mCherry), the cells showed a decrease in viability over time, however, cells expressing additional Brm exhibited an increase in viability late in culture. This is most likely due to the fact that there were more living cells late in culture, however, it may also be that the Brm wildtype cells are evading cell death, leading to an increase in cell numbers. Combined, these results suggest that the increased cell number seen late in culture is due to more viable cells being present.

These results were interesting as the majority of the literature supports Brm playing a role in tumour suppression, and thus, growth suppression (Coisy-Quivy et al. 2006; Shen et al. 2008). However, it is possible that the expression of Brm is needed to be in fine balance, and that its over/under-expression may lead to cellular defects. Interestingly, the Brm overexpressing cell’s growth advantage and survival advantage were both lost with UVR. The only exception to this was that UVR-treated overexpressing Brm wildtype cells did still have a slight survival advantage over mCherry cells at 48h post UVR. These results were of interest, as the loss of Brm in HaCaT cells only led to an increase in growth when in combination with UVR. This suggests that increasing levels of Brm do not play a role in UVR-driven carcinogenesis, but may increase cell viability without UVR. Therefore, it is likely that endogenous levels of Brm expressed in HaCaT cells such as in the mCherry cells, is enough to mediate UVR-induced cellular responses. This is the first study to suggest that an increase in Brm levels above normally expressed levels could possibly lead to cellular defects. It is also therefore a possibility that an increase in Brm overexpression above normal endogenous levels could also contribute to carcinogenesis; however, this would need to be studied much more thoroughly.
Due to Brm playing a tumour suppressive role, the overexpression of Brm in cell systems and its role in cell growth has not been extensively studied. In one study however, the overexpression of Brm in melanoma cells promoted changes in cell cycle progression and apoptosis, consistent with Brm playing a tumour suppressive role in these cells (Mehrotra et al. 2014). Increasing Brm levels in those cells led to decreases in both Brg1 function as well as decreased phosphorylation of Rb. This led to suppression of proliferation of melanoma cells, as well as cell cycle arrest and apoptosis (Mehrotra et al. 2014). Moreover, the reexpression of Brm into nine Brm-deficient cell lines caused growth inhibition in all cell lines studied (Gramling et al. 2011). Therefore, these results and others in Brm-deficient cells support that Brm is important for growth inhibition. However, it should be noted that in these other studies Brm was overexpressed in cells not normally expressing Brm, and as such are not necessarily comparable to the results of this chapter. Further, it is was shown that overexpressing Brm in cells with knocked down Brm also led to increasing levels of Brg1, which may have also been responsible for changes in the cellular machinery (Mehrotra et al. 2014). However, unfortunately it is unknown if Brg1 levels were affected in these cells expressing additional Brm or Q203K. It is possible that changes in Brg1 in these cells led to altered cellular events, however Brg1 levels would have to be tested in these cells to confirm.

It is also possible that the overexpressing Brm cells lost contact inhibition near confluence, leading to increased growth, however, more experiments would need to be completed in order to analyse whether this was the case. Many immortalised cell lines do not exhibit contact inhibition; however, as exhibited by results in Chapter 4, HaCaT cells do. Cancerous cells lose this property, and
thus grow in an uncontrolled manner even when in contact with neighbouring cells (Liu and Dean, 2010). However, the change in cell growth in these cells could possibly be due to contact inhibition in these cells at a higher cell density due to a smaller cell size, or as previously mentioned, an increase in cell viability. An increase in cell growth and decrease in contact inhibition would support increasing Brm expression leading to an increase in carcinogenesis. However, it must be taken into account that these cells are expressing Brm at a level higher than normal. In a cell with normal levels of endogenous Brm, there was no evidence for Brm playing an oncogenic role, and the loss of Brm alone did not affect cell growth or cell cycle regulation. Therefore, it is possible that varying the expression of Brm in any manner can alter the way a cell behaves. Considering how many molecular events that Brm controls, it is thus not surprising that both decreasing as well as increasing its expression levels can lead to cellular changes. It may also be possible that the loss, as well as the overexpression of Brm in these cells, is leading to changes in the regulation of these cellular adhesion molecules, and thus, a change in contact inhibition. Previously, Brm and Brg1 have been shown to be required for expression of a variety of genes involved in cellular adhesion such as CD44 and E-cadherin, and Brm has been shown to be important for contact inhibition in other cells (Banine et al. 2005; Cohet et al. 2012). However, other experiments would need to be completed in order to confirm if an increase in Brm changed the contact inhibition levels of these cells, and what downstream effects this may have in the cell.

The effect of Brm overexpression was also tested on the cell cycle response on HaCaT cells. Previously, it was shown in Brm-/- MNKs and Brm-null
HaCaT cells that these cells suffered from defective G1 cell cycle responses, leading to increased proliferation following UVR. Therefore, it was of interest to see if increasing Brm levels above endogenous levels could also influence cell cycle events. All cell lines showed a decrease in S-phase 24h post UVR, indicating that these cell lines were undergoing cell cycle arrest, and exhibiting normal G1-S transition. However, mCherry control cells but not Brm wildtype overexpressing cells actually showed an increase in mitotic cells 24h post UVR. This suggests that control cells were cycling faster than cells overexpressing wildtype Brm, or that they stayed in M-phase for a longer time period. Considering that the growth of these cells were similar for the first 48h, it is thus more likely that the M-phase of these cells was extended for a longer time period and that the increased number of viable overexpressing wildtype Brm cells was due to an increase in viability rather an increase in proliferation. Interestingly, it has previously been found that knocking down Brm in MCF-10A cells reduced the percentage of cells in mitosis (Cohen et al. 2010). This was achieved not by arrest at any particular cell cycle stage, but an increase in cell cycle length of approximately 50%. Therefore, it is plausible that increasing levels of Brm in these cells in combination with UVR can also prevent excessive mitosis. However, the levels of endogenous Brm were all that was necessary for initial cell cycle responses. The results from this assay are similar to those from Chapters 3 and 4, which showed that cells with greater levels of Brm had reduced mitotic cells 24h post UVR. This suggests that Brm levels are not only important for G1-S phase transition following UVR, as seen in Brm-null HaCaT cells and Brm-/- MNKs previously, but also for prevention of increased mitosis following UVR. The endogenous Brm expressed in these cells was enough to prevent a defect in the
G1-S phase transition post UVR but not in mitosis. Regardless, even if there was a difference in cell cycle regulation between the control and overexpressing wildtype Brm cells, earlier experiments showed that there was no difference in cell numbers following UVR. This therefore shows that any cellular defect in these cells is only minor and is likely compensated for, thus unlikely to contribute to carcinogenesis.

There was also no change in unirradiated S-phase or mitotic cells between cell lines. Hence the increased cell number seen late in culture in overexpressing Brm wildtype HaCaT cells is unlikely to be due to cell cycle regulation, but instead a change in cell viability. Nonetheless, this result suggests that increased levels of Brm could be protecting cells from entering mitosis whilst still harbouring DNA damage. Although the Brm overexpressing cells showed a proliferative defect without UVR, this defect was lost with UVR. A decrease in mitosis following UVR also suggests a protective mechanism, and therefore, it is highly unlikely that overexpressing Brm above endogenous levels contributes to carcinogenesis. Considering that the reexpression of Brm in Brm-deficient lines leads to cell cycle arrest (Gramling et al. 2011; Mehortra et al. 2014), it is thus unsurprising that raising Brm levels exogenously also impacts on the cell cycle of these cells.

Lastly, the overexpression of Brm was also tested on DNA repair. Although the loss of Brm in both primary mouse keratinocytes and HaCaT cells did not lead to an overall change in DNA repair of these cells, it did cause excessive CPD accumulation in combination with UVR. However, in this case, Brm overexpressing cells did not show a significant difference to control cells in either the UDS or the CPD assays. This result suggests that the endogenous
Brm levels in these cells are sufficient to protect from increased CPD in the dark formation following UVR as in Chapters 3 and 4. This also further confirms that Brm is not necessary for total DNA repair following UVR, as increasing the levels of Brm did not influence further repair. Therefore, in keratinocytes, the overexpression of Brm on top of endogenous Brm increases the survival of viable cells in the absence of UVR, but does not affect cell responses to UVR, with the exception of preventing increased mitosis post UVR.

5.3.3. The effect of overexpressing the Brm mutant, Q203K

The effect of the Q203K mutant recently shown to occur in NMSC was also tested in HaCaT cells. Again, it is important to note that in this case, Q203K was overexpressed in cells already containing endogenous Brm. Therefore, these cells were not expressing 100% Q203K DNA, but also wildtype Brm. Therefore, in order to determine whether the Q203K had an effect in the assays, it was necessary to compare these cells to cells overexpressing wildtype Brm. Interestingly, although the overexpression of Brm wildtype led to an increase in viable cells late in culture without UVR, the growth of Q203K cells was similar to that of control cells containing only endogenous Brm. Further, although Brm overexpressing cells showed an increased viability late in culture without UVR, the Q203K expressing cells did not show a survival advantage. These results suggest that the Q203K mutation is not functional in these assays, as it did not have the effect of increased wildtype Brm. However, with UVR, the cell growth of both Q203K and wildtype Brm were similar, suggesting that the presence of endogenous Brm is all that is necessary for the cell’s response to UVR. As
observed in Chapters 3 and 4, the loss of Brm actually led to increased cell growth in combination with UVR, suggesting that functional Brm is important in the cellular response to UVR.

Interestingly however, the Brm with the Q203K mutation was not completely non-functional. In combination with UVR, the Q203K expressing cells along with overexpressing wildtype Brm cells prevented increased mitosis 24h post UVR. Only the cells expressing endogenous Brm alone showed an increase in mitosis following UVR, similar to cells expressing lower levels of Brm in Chapters 3 and 4. There was no impact on S-phase in any of the cell lines post UVR. This suggests that although the Q203K mutation reverses the proliferative phenotype of Brm overexpressing cells, when in combination with UVR, mutant Brm is still capable of maintaining this cell cycle control of the cell. However, without UVR, the Q203K mutant Brm had no observable effect. This would suggest that increasing levels of both wildtype or Q203K Brm are actually protective to the cell for UVR enhancement of M-phase cells. Brm is known to be important for entry to the G0/G1 cell cycle phase, and is also phosphorylated at the G2/M phase, suggesting that Brm accumulates at various stages of the cell cycle (Muchardt et al. 1996). Therefore, increased levels of Brm or Q203K could be protecting cells from entering mitosis whilst still harbouring DNA damage.

Lastly, it was found that similar to wildtype Brm overexpressing cells, the Q203K Brm did not have any effect on DNA repair or CPD accumulation to UVR. However, since the Brm overexpressing cells did not have any effect on DNA repair, it is not possible to determine from this assay whether the Q203K mutation affected Brm function. Thus, in order to completely negate whether Q203K had an effect on DNA repair, it would also have to be tested in cells with
knocked down endogenous Brm expression. All together, these results suggest that, if it has an effect on cell cycle or DNA repair, the Q203K mutation is most likely recessive, similar to 17.8% of other reported SWI/SNF mutations (Shain and Pollack, 2013). However, it is possible that either overexpressing Brm or Q203K in these cells is only functional in some assays in the context of other genes. Considering that Brm is a driver of SWI/SNF chromatin remodelling, and thus plays a role in the transcription and regulation of many genes, Q203K may only function in a situation where it is able to interact with some of the molecules that wildtype Brm normally interacts with. HaCaT cells are known to contain mutations in genes such as p53 and p16 (Lehman et al. 2003, Muthusamy and Piva, 2013). Thus it is possible that Q203K is only able to function in the presence of these genes, or in cooperation with other frequently mutated genes. Both Brm and Brg1 are known to cooperate with p53 to induce cell cycle responses (Park et al. 2008; Naidu et al. 2009), and therefore, its mutation may be redundant with mutant p53. This was already observed in the differences between the responses of Brm-null HaCaT cells, which exhibit p53 mutations, as compared to naïve MNKs, suggesting that Brm is at least partially dependent on normal p53 expression.

Therefore, it would also be advantageous to study the Q203K mutant in normal keratinocytes without any pre-existing mutations. This would confirm whether or not the Q203K was entirely non-functional in NMSC. The presence of UVR-induced mutations in HaCaT cells also means that other undiscovered mutations are likely present in HaCaT cells. SWI/SNF mutations are not mutually exclusive of other cancer gene mutations. For example, SWI/SNF has also been proposed to suppress tumour growth via the polycomb repressive complex 2
Mutations of Polycomb genes are also common in human cancer (Xu et al. 2015). Notably, SWI/SNF and PCR2 mutants have been discovered together in human cancers (Shain and Pollack et al. 2013). Furthermore, it was recently found that SWI/SNF mutant cancers depend on the activity of Polycomb genes for survival. This dependence on Polycomb genes was also associated with a co-occurrence of a Ras pathway mutation (Kim et al. 2015). Moreover, a study of the top 189 mutated genes in human cancer, including BRAF, PTEN and Ras revealed that the mutation of SWI/SNF subunits is not mutually exclusive to any of the other studied common mutants, suggesting that SWI/SNF mutants may cooperate with other molecular pathways in human cancer to initiate tumourigenesis (Shain and Pollack et al. 2013). Hence, it is possible that the Q203K mutation in NMSC may also need the presence of genes such as PRC2, or other commonly mutated genes in order to have its full effect.

Moreover, the mutation or overexpression of Brm may also be dependent on the expression of Brg1. Interestingly, the mutation of Brg1 in cell lines containing full Brm expression is not enough to abrogate cell cycle sensitivity; however, these cell lines become sensitive to Rb-mediated arrest when Brm is lost in these cells in combination with the mutation of Brg1 (Bartlett et al. 2011; Hoffman et al. 2014). This suggests that the mutation of Brm (Q203K) in these cells may not have been enough to alter cellular sensitivity, without the concomitant loss of Brg1. However, Brg1 levels in these cells would need to be assessed to confirm this. Furthermore, the mutation of Brg1, in combination with the loss of Brm together in other cells still leads to mutant Brg1 interacting with other SWI/SNF complex members, as well as its appearance at the promoter of target genes (Bartlett et al. 2011). Therefore, it is likely that Q203K Brm is still
interacting with other core SWI/SNF complex subunits, meaning that Brm may be partly functional in the presence of this mutation, such as seen in the mitotic response to UVR. It is likely that additional mutations would have to be present in order to cause complete loss of SWI/SNF function, and thus, a reduction in the activation of target genes. Many cancers show mutations of multiple SWI/SNF subunits (Shain and Pollack, 2013); therefore it is possible that the mutation of Brm may be partly functional in the presence of this mutation, such as seen in the mitotic response to UVR. It is likely that additional mutations would have to be present in order to cause complete loss of SWI/SNF function, and thus, a reduction in the activation of target genes. Many cancers show mutations of multiple SWI/SNF subunits (Shain and Pollack, 2013); therefore it is possible that the mutation of Brm may require the presence of other SWI/SNF mutations in order to show abrogated SWI/SNF activity. Mutations of SWI/SNF including Brm and/or Brg1 has also been associated with Coffin-Siris syndrome, a rare autosomal dominant anomaly syndrome, associated with a growth deficiency (Tsurusaki et al. 2012), showing that the mutation of SWI/SNF subunits can have immediate downstream consequences. Generally the loss of Brm in cancers is due to it being epigenetically silenced, which is reversible via HDAC inhibitors (Glaros et al. 2007). Considering that the loss of Brm in tumours correlates with decreased patient survival (Glaros et al. 2007; Yamamichi et al. 2007), and is paired with a defect in proliferation, it is likely that the epigenetic silencing of Brm is preferential to the mutation of Brm, as it gives the cell a selective advantage. However, it is unknown as to why Brm is not only lost, but also mutated in NMSC (Bock et al. 2011; Moloney et al. 2009). It is likely that UVR drives both the loss and mutation of Brm, as its loss and mutation has only been observed in UVR-driven malignant but not benign skin lesions. Although this mutation of Brm in NMSC was non-functional in the context of the majority of experiments conducted, this mutation would not be reversible, as the epigenetic loss of Brm is (Kahali et al. 2014). It is possible that this mutant could lead to further downstream mutations, as mutations in genetic stability genes such as SWI/SNF
can cause mutations in other genes (Loeb et al. 2003), eventually leading to genomic instability.

However, in order to fully analyse the effect of Q203K, other important events in carcinogenesis would have to be studied in cells expressing this mutation. It also may have been more advantageous to study Q203K in cells that did not already express endogenous Brm, however unfortunately dual transfection in this case was not possible as HaCaT cells are extremely difficult to transfect, especially with larger constructs. It is likely that endogenous Brm in these cells was able to compensate for the Q203K mutant in HaCaT cells, thus suggesting that this mutation was not dominant. If Q203K were also studied in Brm-null cells (such as SW13), this would give a better idea of its functionality. Considering that Q203K reversed the proliferative phenotype of HaCaT cells without UVR, it would be interesting to study this as well. Therefore, in the future, it would be necessary to study the Q203K mutation in other models, as well as examine its other effects before fully determining if the mutation contributes to carcinogenesis. It is also possible that Q203K may play a role in carcinogenesis in another way not explored in this thesis, such as invasion, metastasis, motility, differentiation or immune evasion. For example, the loss of Brm in C57BL/6 mice has been shown to prevent UVR-induced immunosuppression (Halliday et al. 2012).

5.4. Conclusion
As opposed to the loss of Brm in HaCaT cells, it appears that the Q203K mutation plays a mostly non-functional role, at least in the assays used in these experiments. The Q203K mutation blocked the effect of overexpressed Brm of increasing cell survival. Intriguingly, the Q203K mutation showed a lack of effect in other assays, and both wildtype and Q203K overexpressed Brm was able to prevent increased mitosis in response to UVR. Further, increasing levels of wildtype or Q203K Brm did not change the DNA repair response to UVR. However, it is possible that the activity of increased levels of Brm or Q203K is dependent on the presence of p53 or other genes.Regardless, these initial results into the function of Q203K suggest that this mutation is primarily non-functional. This may explain why Brm is preferentially silenced in many cancers, and not mutated, since it is gives the cell a selective advantage.
Chapter 6: General Discussion

6.1. Introduction

Excessive exposure to UVR is a major factor in the development of NMSCs, with between 95-99% of skin cancers being caused by exposure to UVR from sunlight (Staples et al. 2006; Ridky et al. 2007). The response of the cell to UVR-induced DNA damage is of key importance to prevent UVR-induced carcinogenesis. DNA damage provoked by UVR evokes a cellular damage response in the cell, composed of the activation of stress signalling and cell cycle checkpoint functions (Latonen and Laiho, 2005). These functions lead to the responses of cell cycle arrest, DNA damage repair mechanisms and cell death. Both UVA and UVB are known to incur DNA lesions, namely 6-4PPs and CPDs, and these photolesions are primarily repaired by NER (Palomera-Sanchez and Zurita, 2011). The success of the NER response is of utmost importance as failed repair can lead to downstream mutations and increased carcinogenesis. The NER response is also closely linked to cell cycle checkpoints and apoptosis, and any failure of these mechanisms can lead to increased cancer development (Sinha and Hader, 2002). Brm is one of the two alternative ATPase subunits of the SWI/SNF chromatin-remodelling complex; with the other being Brg1. In eukaryotes, DNA is assembled into chromatin. The access to DNA in chromatin by other transcription, repair and replication factors is driven by Brm and Brg1, which utilise the energy of ATP to mobilise DNA from nucleosomes (Halliday et al. 2009). Therefore, it is unsurprising that the loss of Brm has been associated with carcinogenesis, as its loss likely prevents or limits the access of these factors to DNA. Studies within our group have shown that Brm protein is lost in
100% of NMSC studied, but not pre-malignant AK lesions, indicating that the loss of Brm is associated with increased malignancy in NMSC (Bock et al. 2011). Further, the loss of Brm in C57BL/6 mice in combination with chronic UVR leads to increased epidermal hyperplasia, as well as increased tumourigenicity in these mice as compared to Brm wildtype littermates (Halliday et al. 2012; Hassan et al. 2014). A discovery in my laboratory also indicated that there is a novel hotspot mutation of Brm (Q203K) in 17% of NMSC, further suggesting that Brm plays a role in skin cancer (Moloney et al. 2009). Therefore, the goal of this thesis was to discover what role the loss of Brm plays in NMSC, as compared to the Q203K mutation.

6.2. Results summary

Previous results from our laboratory indicated that the loss of Brm is involved in skin carcinogenesis. The findings of this study confirm these previous results, and clarify the mechanisms for the increased tumourigenicity observed in chronic UV-irradiated Brm-null mice. The loss of Brm in MNKs altered responses to UVR. It led to several cellular defects, including an increase in proliferation, faster exit through the G1-S phase, and increased levels of UVR-specific CPD damage. Further, this DNA damage was still present in these cells while actively cycling, which would likely lead to downstream mutations and increased carcinogenesis.

The most fundamental trait of cancer cells is their ability to sustain chronic proliferation (Hanahan and Weinberg, 2011). Cell cycle checkpoints monitor the events of the cell cycle and ensure that a full cell cycle only occurs
after the completion of DNA repair, however, if a cell divides before this, the mutation or silencing of genes can occur (Vermeulen et al. 2003). The silencing as well as the mutation of SWI/SNF in the literature is highly noted, with the loss or mutation of SWI/SNF subunits linked to many cancer types (Halliday et al. 2009). The loss of Brm is noted to correlate with lower patient survival in gastric, lung and pancreatic cancer (Yamamichi et al. 2007; Glaros et al. 2007; Segedi et al. 2016). Importantly for this study, the loss as well as the mutation of Brm is also observed in NMSC (Moloney et al. 2009; Bock et al. 2011). The loss or mutation of Brm in NMSC is likely UVR-induced, in turn giving the cell a proliferative advantage as well as leading to decreased apoptosis and increased DNA damage. Brm was found to be important in the maintenance of G1 cell cycle arrest, only allowing cells to enter S-phase following DNA repair. However, with the loss of Brm, cells were prematurely exiting through G1 phase into S-phase, cycling with active CPD lesions. Persistence of CPD lesions has been linked to UVR-induced mutations, a driver of NMSC (Pfeifer and Besaratinia, 2012). UVR-induced cellular defects were especially prominent in Brm/-/- MNKs, which likely contribute to the increased epidermal hyperplasia and carcinogenesis exhibited in these mice with UVR exposure (Halliday et al. 2012; Hassan et al. 2014).

Although cellular defects were also noted in Brm-null HaCaT cells, such as increased proliferation, impaired G1 cell cycle, and increased CPD in the dark formation, these cells were less sensitive to the effects of UVR, and thus recovered from its effects more quickly. It is likely that the mutation of p53 or other molecules due to previous UVR exposures in these HaCaT cells led to a reduced dependence on p53 signalling for cell cycle and repair responses.
Brm and p53 are known to form complexes, and Brm is known to be vital for the transcription of p53 (Xu et al. 2007). Furthermore, the loss of Brm in combination with UVR led to a decreased apoptotic response in MNKs but not HaCaT cells. It is likely that an increase in live cells due to decreasing cell death would have contributed to the increased cell number noted in irradiated Brm-null cells. Evasion of cell death is also a widely accepted hallmark of cancer (Hanahan and Weinberg, 2011). Considering that the main function of apoptosis is to clear cells harbouring excess DNA damage, it is probable that these cells evading apoptosis harboured high levels of DNA damage, likely contributing to the increase in CPDs seen in Brm--/- MNKs at 24h (Jin and El-Deiry, 2005). Therefore, Brm may prevent tumourigenesis by contributing to downstream apoptotic signalling. There was no noted cell death evasion in Brm-null HaCaT cells, suggesting that the loss of Brm and its resulting cell death evasion may be p53-dependent, since HaCaT cells harbour p53 mutations. Moreover, Brg1 was also upregulated in Brm--/- MNKs as well as the majority of Brm-null HaCaT cells. Previously it was shown in Brm/Brg1 null cells that the upregulation of Brg1 in these cells led to increased apoptotic evasion (Gong et al. 2008). Therefore, it is highly likely that decreased levels of Brm are leading to increased levels of Brg1, causing the increased apoptotic evasion seen in MNKs. However, this process may be p53 dependent, hence why Brm-null cells did not show the same response. This collection of results suggests that the UVR response in HaCaT cells is likely at least partially p53-dependent. However, Brm is still able to facilitate some cellular programs in HaCaT cells in response to UVR, independent of p53.

Lastly, it was of interest to note that increasing levels of Brm on top of endogenous levels increased the number of viable cells late in culture, without
UVR. It is likely that an increase in viable cells led to the increased cell number seen in Brm overexpressing cells. Intriguingly, the Q203K mutation was able to prevent the increased viability of the cells. Interestingly however, when increasing levels of Brm expression was combined with UVR, the cells growth advantage was lost. Moreover, although the S-phase of both wildtype and Q203K Brm overexpressing cells was similar to cells only expressing endogenous Brm following UVR, both Q203K and wildtype Brm overexpressing cells showed a decreased level of mitotic cells following UVR as compared to control cells. This result, together with those from the Brm-/- MNKs and the HaCaT knockdown cells, suggest that Brm can protect from increased mitotic activation following UVR, possibly to prevent the proliferation of cells with DNA damage. However, further experiments would need to be conducted to fully understand the role of Q203K in NMSC progression.

This thesis confirms that Brm is a tumour suppressor gene in NMSC. Although the loss of Brm itself is not enough to cause tumour progression, the combination of Brm loss and UVR drives tumourigenesis. The loss of Brm is similar to the loss of other tumour suppressors such as p53 and Rb, which also lead to cell cycle and proliferative defects upon their inactivation (Vermeulen et al. 2003). Furthermore, the loss of Brm led to various cellular defects in combination with UVR. However, the Q203K mutation was not comparable to the loss of Brm in the scope of this thesis. This therefore suggests that the loss of Brm in NMSC is contributing to carcinogenesis by enhancing proliferation and impairing DNA repair, but Q203K is most likely not. However, Q203K may contribute to carcinogenesis in a manner not studied in this thesis. The results of this thesis suggest that the loss of Brm is quite damaging to the cell, which may
explain why Brm is preferentially epigenetically silenced in the majority of cancers instead of being mutated.

6.3. Clinical implications

As previously mentioned, the loss of Brm has been found in multiple tumour types, and its loss is associated with decreased survival. Recently, its loss of expression and mutation has also been observed in NMSC. Australia has the highest incidence of NMSC in the world, which is increasing over time. In 1997, there were 412,493 cases, which rose to 767,347 in 2010. The total healthcare costs of NMSC in 2010 were $93.5 million, which is expected to rise in the future (Fransen et al. 2012). Further, there were a total of 521 recorded deaths in 2012 attributed to NMSC (Cancer Council Australia, 2013). The exact molecular mechanisms underlying the inactivation/mutation of Brm in NMSC is unknown. Of importance, the majority of SWI/SNF subunits are mutated in cancer, whereas Brm is epigenetically silenced (Shain and Pollack, 2012). Brm is also silenced in 100% of studied BCC (n=5) and SCC (n=11), but not premalignant skin lesions (n=11), suggesting the loss of Brm plays a role in the progression of NMSC (Bock et al. 2011). Importantly, with the use of HDAC inhibitors, Brm expression can be restored in Brm-deficient cell lines (Glaros et al. 2007; Kahali et al. 2014). Interestingly, the human adrenal adenocarcinoma cell line, SW13 does not express Brm or Brg1, and these cells are extremely sensitive to UVR. The reexpression of Brm/Brg1 by transfection in these cells restores the apoptotic and DNA repair response in these cells (Gong et al. 2008). Moreover, the restoration of Brm/Brg1 in SW13 cells via HDAC inhibitors also reverses the
hyperproliferative response of Brm/Brg1-deficient SW13 cells (Davis et al. 2016). Therefore, it would be interesting to attempt to reexpress Brm in NMSC in addition to Brm-null MNKs and HaCaT cells and observe whether the reexpression of Brm also reverses the hyperproliferative response of these cells. Interestingly, it was recently found by Kahali and colleagues (2014) that HDAC3 and HDAC9 regulate Brm expression, whereas HDAC2 regulates Brm acetylation. Moreover, HDAC9 was clearly overexpressed in Brm-deficient cell lines, and the inhibition of HDAC9 in Brm-deficient cells also led to growth inhibition of these cells, suggesting that HDAC9 may be a potential therapeutic target in Brm-deficient cancers (Kahali et al. 2014). HDAC9 has been found to be overexpressed in a variety of cancers (Milde et al. 2010; Skov et al. 2012). It would therefore be interesting to study whether Brm can be epigenetically restored in NMSC and whether this alters the response of NMSC to UVR. Furthermore, this same study recognised that the MAPK pathway is also part of the regulatory system in Brm-deficient cells, facilitating Brm loss and thus abrogating Brm-driven growth inhibition (Kahali et al. 2014).

The targeting of kinases, such as epidermal growth factor receptor, vascular endothelial growth factor receptor and ERBB2 has been well established as a potent clinical therapy for cancer patients (Press and Lenz, 2007). However, cancer is not only driven by the acquisition of oncogenic mutations, but also activated by the loss of tumour suppressor genes. Thus it is likely that the reexpression of tumour suppressors could also be an important treatment for a variety of cancers in the future. Unfortunately however, the loss of p53 and other mutated tumour suppressors is generally not reversible, therefore limiting their therapeutic potential (Berger et al. 2011). Therefore, the
use of HDAC or MAPK inhibitors in cancer therapy in order to restore Brm expression may provide a novel opportunity in the treatment of various cancers, including NMSC. However, more research would need to be done into the use of individual HDAC inhibitors and their effect on Brm and SWI/SNF activity in different tumour types. The use of HDAC inhibitors has previously shown success in other cancers, reversing aberrant epigenetic states associated with cancer, however their ability is not fully understood as their use is generally used in combination with chemotherapy and other treatments (Ma et al. 2009; Kahali et al. 2014; Oike et al. 2014). The use of HDAC inhibitors for the treatment of NMSC and other skin cancers in the future may be a promising non-invasive alternative to other current treatments for NMSC. Further, the use of HDAC inhibitors may reduce future cancers in patients with recurrent NMSC.

6.4. Future directions

Although it was found that the loss of Brm in combination with UVR led to changes in proliferation, cell cycle control and the number of CPD lesions, there are also many future experiments that could be completed to further assess the effects of the loss of Brm in NMSC. For example, in this study, only one type of DNA damage was assessed (CPDs). There are two other types of lesions that could be studied, including 8-oxo-dG and DSBs. 8-oxo-dG lesions are produced when ROS leads to oxidative DNA damage (Fortini et al. 2003). The study of 8-oxo-dG lesions following Brm loss would be especially interesting to study following UVR, since the loss of Brm led to increased CPD in the dark formation, which is believed to result from increased ROS production and/or sensitivity
(Premi et al. 2015). Furthermore, the loss of Brm and Brg1 has been previously shown to decrease DSB repair, increasing cisplatin cytotoxicity (Kothandapani et al. 2012). DSBs are the most detrimental form of DNA damage, as they can lead to chromosomal breakage and rearrangement if not repaired, an event that may result in tumourigenesis (Dasika et al. 1999). Therefore, it would be of interest to see if the loss of Brm in combination with UVR also altered DSB formation or repair.

It would also be interesting to see if the reexpression of either Brm or Brg1 in either MNKs or HaCaTs could reverse their hyperproliferative phenotype. If so, this would suggest that the upregulation of Brm/Brg1 in cancers that are Brm/Brg1-deficient, such as SCC and BCC, via the use of HDAC inhibitors may be an alternative treatment to surgery in the future. Furthermore, it would be of importance to further understand the molecular events following the loss of Brm in combination with UVR. Brm and the SWI/SNF complex are known to form complexes with many other molecules, and can influence the transcription of many molecules, thus it would be interesting to note whether the loss of Brm could also influence the expression of other tumour suppressors such as Rb and p53. Although Brm/Brg1 is known to be important for the transcription of Rb and p53 and their target genes in other systems, it is unknown how the loss of Brm in skin keratinocytes would affect their expression (Strobeck et al. 2000; Reisman et al. 2002; Strobeck et al. 2002; Xu et al. 2007). Furthermore, it would also be of interest to assess the effect of Brm loss in MAPK pathway molecules, since these molecules have the potential to be targetted, possibly upregulating Brm expression. Lastly, it would also be advantageous to study Q203K in Brm-deficient cells, in order to gather more insight into the
functionality of this mutant, as it possible that residual Brm expression in HaCaT cells altered the results of the mutant insertion.

6.5. Conclusions

This thesis confirmed its original hypothesis, in that Brm protects from UVR-induced damage. The loss of Brm in both mice and the human keratinocyte line HaCaT, led to increased cellular sensitivity to UVR, with Brm-deficient cells exhibiting increasing cell growth via defective cell cycle responses, as well as increased CPD damage. The loss of Brm in mouse keratinocytes also led to increased apoptotic evasion, however this was not observed in the p53-mutant, Brm-null HaCaT cells. However, the Q203K mutant of Brm did not show a similar phenotype to Brm loss, suggesting that Q203K does not function in a similar way to Brm loss. The Q203K mutation was found to be mostly non-functional in the assays used, with the exception that it was able to prevent increased mitosis post-UVR, similar to overexpressing wildtype Brm. These cellular defects noted in Brm-null cells in this thesis explain why Brm-/− mice harbour increased epidermal hyperplasia, as well as increased tumourigenicity compared to Brm+/+ mice following UVR treatment. These findings support a Brm knockout phenotype that does not lead to carcinogenesis alone, but is rather dependent on a synergistic relationship with UVR in order to drive tumourigenesis via defective cellular responses. It is possible that, with the use of HDAC inhibitors, the loss of Brm expression could be reversed, thus leading to a reversal of these cellular defects, preventing the further development of tumours.
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