Age-related epigenetic changes at base excision repair genes and their modulation by dietary restriction in mice

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Abstract

Accumulation of damage in DNA is a characteristic feature of ageing which may result from a decline in DNA repair efficiency. Base excision repair (BER) is the primary mechanism used to repair small-scale DNA damage such as that caused by oxidation. I hypothesised that epigenetic events contribute to the ageing process through deregulation of BER gene expression and that these adverse effects of ageing may be modulated by dietary restriction (DR). To test these hypotheses, I quantified DNA methylation and histone post-translational modifications at BER-gene (*Ogg1* and *Apex*) promoters, together with expression of these genes, in tissues from ageing mice and from mice exposed to DR. Phenotypic changes in DNA incision activity and oxidative damage were also quantified.

Cytosine methylation was measured by pyrosequencing at the *Ogg1* promoter in brain and livers from *ad libitum* (AL) and 40% DR mice at 3, 12, 24 and 30 months of age (n=5/group). *Ogg1* promoter methylation decreased with age in the liver ($p=0.018$) and brain ($p=0.016$) and DR significantly reduced *Ogg1* promoter methylation ($p=0.014$) in the brain. Additionally, in the brain there was a 2 fold enrichment in histone 4 acetylation (H4Ac) and histone 3 lysine 27 tri-methylation (H3K27Me3) as measured by the ChIP assay at *Ogg1* and *Apex* promoters in 30 month old AL animals compared with 3 month old animals ($p<0.05$). H4Ac was 2.5-fold higher in the *Ogg1* promoter in liver in 30 month old DR versus 30 month old AL animals ($p=0.004$). Furthermore H4K27Me3 was significantly ($p=0.023$) lower at the liver *Ogg1* promoter in 30 month old compared with 3 month old animals.

Ogg1 gene expression decreased with age in the brain (n.s.) and liver ($p=0.005$). Perhaps surprisingly, *Ogg1* and *Apex* expression levels were higher in the brain ($p=0.034$) but lower in the liver ($p=0.003$) of DR compared with AL animals. I used a comet-based *in vitro* assay to quantify BER-related incision activity but did not observe any significant changes in the liver or in whole brain in response to ageing or to DR. However DNA incision activity varied considerably between different brain regions and DR enhanced incision 2 fold in the cortex ($p=0.031$) and subcortical regions ($p=0.019$). 8-oxoguanine lesions measured by HPLC-ECD decreased with age (AL and DR) ($p<0.001$) in the liver but no effect of age was detected in the brain.

This study revealed for the first time that tissue-specific epigenetic changes at BER genes occur during ageing and the data presented here suggest that epigenetic changes at BER-related gene promoters may affect BER activity in some tissues. Furthermore, I have shown that DR influences the epigenetic and transcription changes in BER-related genes observed during ageing.

Dedicated to my grandmother Halina

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Abbreviations

3’-P – 3’-phosphate

3’-PUA – 3’-phospho-α,β-unsaturated aldehyde

3AL/12AL/24AL/30AL – 3/12/24/30 month old *ad libitum* fed mice

5’-dRP – 5’-deoxyribose-5-phosphate

8-oxodG – 8-oxoguanine/8-hydroxoguanine/7,8-dihydro-8-oxoguanine

12DR/24DR/30DR – 12/24/30 month old dietary restricted mice

A- Adenine

AD – Alzheimer’s disease

ADP - Adenosine diphosphate

AL – *Ad libitum*

AKT – Protein kinase B

AMPK - 5' adenosine monophosphate-activated protein kinase

ANOVA – Analysis of variance

AP - Apurinic/apyrimidinic

AP-1 – Activator protein 1

AP2F – Activator protein 2

APEX/APE1– Apurinic/apyrimidinic endonuclease 1

ATG – Autophagy-related protein

ATP – Adenosine triphosphate

BCP - 1-bromo-3-chloropropane

BER – Base excision repair

BM – Bisulphite modification

BRCA – Breast cancer susceptible gene

C – Cytosine

CAAT – CCAAT binding factors

CDEF – Cell cycle regulators

Cdk4 – Cyclin-dependent kinase 4

CHFR – Forkhead-associated and RING finger E3 ubiquitin-protein ligase

ChIP – Chromatin immunoprecipitation

CISBAN - Centre for Integrative Systems Biology of Ageing and Nutrition

CLOX – Cut-like homeobox and CLOX homology factors

CpG – Cytosine-phosphate-guanine

CREB - cAMP-responsive element binding proteins

CS – Cockayne Syndrome

CSB – Cockayne syndrome group B protein

Daf-2 – Homolog of insulin-like growth factor receptor

DDB1 – DNA-binding protein 1

DMEM – Dulbecco's Modified Eagle Medium

DNA – Deoxyribonucleic acid

DNMT - DNA methyltrasferase

DR – Dietary restriction

DSB –Double strand break

E4FF – Ubiquitous GLI Krueppel like zinc finger

EBOX – E-box binding factors

ELISA – Enzyme-linked immunosorbent assay

ERCC – Excision repair cross-complementing protein

FCS – Foetal calf serum

FEN1 – Flap structure-specific endonuclease 1

FOXO – Forkhead box protein O

FPG – Formamidopyrimidine DNA glycosylase

Fwd - Forward

G – Guanine

GAPDH – Glyceraldehyde-3-phosphate dehydrogenase

GG-NER – Global genome nucleotide excision repair

γH2Ax – Gamma histone 2A

GLIF – FLI zinc finger family

GLM – General linear model

H4Ac – Histone 4 acetylation

H3K27Me3 – Histone 3 lysine 27 trimethylation

HAT - Histone acetyltransferase

HDAC – Histone deacetylase

HDBP – Huntington's disease gene regulatory binding protein

HIC-5 – Hydrogen peroxide-inducible clone-5

HIF1α/HIFF - Hypoxia-inducible factor 1-alpha

HMD – Histone demethylase

HMT – Histone methyltransferase

HPLC – High-performance liquid chromatography

HPSF – High purity salt free

HR – Homologous recombination

IAA - Isoamylalcohol

IGF-1R – Insulin-like growth factor receptor

IGF2 – Insulin-like growth factor 2

IgG – Immunoglobulin G

IKK – Iκ kinase

IL-2 – Interleukin 2

INSM – Insulinoma associated factors

JNK – c-Jun N-terminal kinase

K –Lysine

KO – Knock out

LKB – Serine/threonine kinase

LMP – Low melting point agarose

LUMA - Luminometric Methylation Assay

Klotho - Related to β-glucuronidase

MBD4 – Methyl CpG binding domain protein 4

MDA - Malondialdehyde

MiTF - Microphthalmia-associated transcription factor

Min - Minutes

MMR – Mismatch repair

MPG – N-methylpurine-DNA glycosylase

mRNA – Messenger ribonucleic acid

MSH – Mutator S homolog

mtDNA – Mitochondrial deoxyribonucleic acid

MTEN – Core promoter motif ten elements

mTOR – Mammalian target of rapamycin

MUTYH/MYH – Muty homolog

NER – Nucleotide excision repair

NEIL – Nei endonuclease VII-like

NFκB - Nuclear factor kappa-light-chain-enhancer of activated B cells

NFYA - Nuclear transcription factor Y subunit alpha

NHEJ – Non-homologues end joining

NKXH – NKX homeodomain factors

noRo – control cells without Ro 19-8022 and light treatment

NRF2 – Nuclear factor 2

NRSF – Neuron-restrictive silencer factor

NTH1 – Nth endonuclease III-like 1

OGG1 – 8-oxoguanine-DNA glycosylase

p53 – Tumour suppressor protein 53

PARF – PAR/bZIP family

PARP1 – Poly ADP-ribose DNA polymerase 1

PCNA – Proliferating cell nuclear antigen

PCR – Polymerase chain reaction

PDK – 3-phosphoinositide dependent protein kinase

PI3K – Phosphatidylinositide-3 kinase

PKC – Protein kinase C

PNKP – Polynucleotide kinase 3’-phophatase

qPCR – Quantitative polymerase chain reaction

PBS – Phosphate buffered saline

R - Arginine

Rev - Reverse

RNA – Ribonucleic acid

RNase – Ribonuclease

Ro – cells with R0 19-8022 and light treatment

RORA –v-ERV and RAR-related orphan receptor alpha

ROS – Reactive oxygen species

RT – Reverse transcriptase

RT – Room temperature

S6K – Ribosomal protein S6 kinase

SAM – S-adenosylmethionine

SAMM – Senescence accelerated mouse model

SD – Standard deviation

SDS – Sodium dodecyl sulphate

Sec – Seconds

SEM – Standard error of the mean

Seq – Sequencing

SIRT - Sirtuin

SMUG – Single-strand-selective monofunctional uracil DNA glycosylase

T – Thymine

Taq – DNA polymerase

TC-NER – Transcription coupled nucleotide excision repair

TDG – Thymine DNA glycosylase

TERT – Telomerase reverse transcriptase

TET – Ten-eleven translocation methylcytosine dioxygenase

TF2D – General transcription factor II

TFIIH – Transcription factor II human protein

TTD - Trichothiodystrophy

UDG/UNG – Uracil-DNA glycosylase

UV – Ultra violet

V – Vault

WBGS – Whole-genome bisulphite sequencing

WRN - Werner syndrome, RecQ helicase-like protein

WT – Wild type

XAB2 – XPA-binding protein 2

XLF – XRCC4-like factor

XP – Xeroderma pigmentosum

XP A-G – Xeroderma pigmentosum complementation group A-G

XRCC1 - X-ray repair cross-complementing protein 1

ZBDF – Zinc binding protein factors

Chapter 1: General Introduction

1.1 The ageing population

The World Health Organisation predicts that by 2050 there will be 2 billion people aged 60 and older and that the proportion of older people is increasing in both developed and developing countries. This increase highlights the economic and medical advances made over the last years which have led to decline in childhood mortality as well as better nutrition and improved living conditions. However, with age being the biggest risk factor for non-communicable diseases, an ageing population has resulted in an increase in cases of cardiovascular disease, dementias, diabetes, stroke and cancer (Dobriansky *et al.*, 2007). In light of this, there has been much interest into understanding the ageing process and in the development of interventions to enhance healthy ageing.

The elderly exhibit cognitive decline and difficulties with movement (Mitchell and Shiri-Feshki, 2009; Rosso *et al.*, 2013) highlighting that ageing of the brain and neurodegeneration are important processes to understand and sustain healthy ageing. Dementia, in particular is a debilitating condition characterised by memory loss, confusion as well as problems with speech and understanding which often lead to loss of independence for the elderly individuals (Alzheimer's Society, 2015). The Office for National Statistics reported that in 2011 in England and Wales dementias contributed 5.1% of all deaths in men and 10.3% in women (Office for National Statistics, 2012). Furthermore the costs of dementia have a profound impact on an individual as well as population level. The Alzheimer's Association states that in the United States caregivers of dementia patients, who are often the closest family, provided 17.7 billion hours of unpaid care in 2013 and it is the caregiver who bears majority of the cost for taking care of a dementia patient (Alzheimer's Association, 2014). In addition, the Alzheimer's Society states that on a national level dementia costs the United Kingdom £23 billion per year (Alzheimer's Society, 2015).

Despite the social and economic impact of ageing there is still limited understanding of the ageing process. Basic research on the cellular processes that contribute to the biology of ageing will be helpful in providing the evidence base for developing healthy ageing interventions.

1.2 Biology of ageing

To date a multitude of mechanistic theories of ageing have been proposed and these fall mainly into either of two categories: programmed or error. The programmed theories of ageing state that ageing is a result of scheduled changes in genes being switched on or off (programmed longevity), hormone levels (endocrine theory) or decline in immune system (immunological theory). On the other hand error theories such as wear and tear, metabolic rate, somatic deoxyribonucleic acid (DNA) damage and free radical theory propose that ageing occurs due to cellular damage (Jin, 2010). Furthermore, theories such as that of the 'disposable soma' bring in an evolutionary principle. This theory predicts that natural selection favours allocating cellular resources to reproduction rather than somatic cell maintenance. As such, ageing results from an accumulation of molecular damage when the cell shifts resources to fuel the limited maintenance systems (Drenos and Kirkwood, 2005). Owing to the vast changes observed with age, these theories are not necessarily mutually exclusive.

In multicellular organisms such as the worm *Caenorhabditis elegans* the rate of ageing is slowed by mutations in genes encoding *age-1* (phosphatidylinositol-3 kinase, PI3K) and *daf-2* (homologue of insulin-like growth factor receptor, IGF-1R) which extend lifespan (Friedman and Johnson, 1988; Kenyon *et al.*, 1993; Gottlieb and Ruvkun, 1994; Kimura *et al.*, 1997; Gems *et al.*, 1998). In *Drosophila melanogaster*, the common fruit fly, a mutant form of the *mth* gene (related to secretin receptor family) extends lifespan by 35% (Lin *et al.*, 1998). Similarly to the *daf-2* mutant in *C. elegans,* knockout of *Igf-1R* extends lifespan by 26% in mice (Holzenberger *et al.*, 2003). Not surprisingly mutations in some genes are also able to reduce lifespan; for example in mice a mutation in the *klotho* gene (related to β-glucuronidase) results in reduced lifespan (Kuro-o *et al.*, 1997). Such studies highlight certain cellular pathways e.g. nutrient sensing via IGF-1, as key players in the ageing process.

Furthermore, genetic variants are thought to account for approximately 25% of the variance in human lifespan (Finch and Tanzi, 1997; Cournil and Kirkwood, 2001) and the decline in physiological function which is characteristic of ageing is influenced by both environmental and genetic factors (Brink *et al.*, 2009) emphasising the complexity of cellular ageing.

1.3 Molecular damage and ageing

In the current view of ageing, the process is characterised by: genomic instability, telomere attrition, epigenetic alterations, loss of proteostasis, deregulated nutrient sensing, mitochondrial dysfunction, cellular senescence, stem cell exhaustion and altered intercellular communication (López-Otín *et al.*, 2013).

A number of the ageing hallmarks can be attributed to macromolecular damage and it is widely accepted that damage to macromolecules plays a major role in the age-related decline in physiological function (Kirkwood, 2008). Lipids, proteins and DNA within the cell can all serve as targets of damaging agents. There is a large number of damaging agents varying from extracellular ultraviolet (UV) radiation to intracellular reactive oxygen species (ROS). According to error theories of ageing, damage to macromolecules can directly or indirectly impair cellular function and hence lead to an ageing phenotype. In this section I will focus mainly on DNA damage and its importance in driving ageing, the role of protein and lipid damage will only be covered in brief.

1.3.1 *Damage to DNA*

An array of DNA damaging lesions can be found in the body and numerous studies have addressed the effect of age on DNA damage, with results being tissue and lesion specific (Table 1.1). Meta-analysis of studies shows a positive relationship between DNA damage and ageing in humans (Soares *et al.*, 2014). Furthermore the telomere shortening; a characteristic feature of ageing, can be regarded as DNA damage. The inability of cells to replicate telomeres results in loss of sequence and in diminished protection of chromosome ends (Blasco, 2007; López-Otín *et al.*, 2013).

Unrepaired DNA damage can lead to somatic mutations and an increase in mutation load with age occurs in a tissue specific manner. Somatic mutations increased in ageing mouse liver, small intestine and heart, but in the brain mutations increased during development only (1day old to 6 month old) after which there was no further increases (Dolle *et al.*, 1997; Dollé *et al.*, 2000). Mutation type is also tissue specific, with point mutations being associated with the small intestine while chromosomal rearrangements are more common in the heart (Dollé *et al.*, 2000; Busuttil *et al.*, 2007). As well as nuclear DNA,

mitochondrial DNA (mtDNA) is also susceptible to DNA damage. Age-related accumulation of mitochondrial point mutations in colonic crypts are observed (Greaves *et al.*, 2012) and these lead to dysfunction in the mitochondrial respiratory chain and apoptosis (Nooteboom *et al.*, 2010). Furthermore, the mitochondrial mutator mouse model, defective in mtDNA polymerase, accumulates mtDNA mutations and shows a reduced lifespan (Trifunovic *et al.*, 2005).

Lesion	Description	Age	Tissue	Author
Micronuclei	Small extra-nuclear bodies formed by incomplete separation of chromosomes	↑	Fibroblasts (human)	(Weirich-Schwaiger *et al.*, 1994)
		↑	Lymphocytes (human)	(Bolognesi *et al.*, 1997)
AP site	Apurinic/apyrimidinic (AP) sites generated by depurination	↑	Leukocytes (human) Liver (rat)	(Atamna *et al.*, 2000)
Oxidative lesion	Oxidative damage to DNA	↑	Muscle (rat) Heart (rat) Brain (rat) Liver (rat) Kidney (rat)	(Sohal *et al.*, 1994a)
Double strand breaks	Break in DNA at both strands	↑	Fibroblasts (human) Liver (mouse) Testes (mouse) Kidney (mouse) Lung (mouse) Brain (mouse)	(Sedelnikova *et al.*, 2004)

Table 1.1 Subset of studies quantifying damaging lesions in ageing tissues.

In 1956, Denham Harman proposed the free-radical theory of ageing where he postulated that reactive oxygen species (ROS) produced during cellular metabolism induce damage to macromolecules leading to mutations, cancer and ageing (Harman, 1956). Since then a large number of studies have looked at the role of ROS in the ageing phenotype. ROS can cause more than 100 different types of oxidative base modifications (Cooke *et al.*, 2003). A common product is 8-oxoguanine. It has been widely used as a marker in biological ageing and is thought to be important because of the specific repair mechanism to remove this lesion (Halliwell, 1999). Guanine is particularly vulnerable to oxidative damage due to its low redox potential (Steenken and Jovanovic, 1997). Oxidation of a guanine molecule involves the addition of an oxygen group on the carbon at position 8 and a hydrogen atom on the nitrogen at position 7 forming 8-oxoguanine/8-hydroxyguanine/7,8-dihydro-8-oxoguanine

(8-oxodG). This causes a stable base pair with an adenine base (David *et al.*, 2007). Since 8-oxoguanine does not block DNA replication, accumulation of 8-oxodG can lead to GC to TA transversion mutations if unrepaired (Thomas *et al.*, 1997). It has been estimated that around 180 guanines are oxidised to 8-oxoguanine per mammalian genome per day (Lindahl, 1993).

Oxidative damage may play an integral role in ageing and in the development of age-related diseases. Increased levels of 8oxodG have been reported in nuclear and mitochondrial DNA in the ageing human brain (Mecocci *et al.*, 1993) and in patients with Alzheimer's disease (AD)(Lovell and Markesbery, 2007) as well as in brain, kidney, liver and heart of rats (Kaneko *et al.*, 1997; Hamilton *et al.*, 2001) and in brain, muscle, heart, liver and kidney of mice (Sohal *et al.*, 1994a). Other DNA oxidative damage such as 8,5'-cyclopurines, thymine glycol and 8-hydroxyadenine also accumulate in ageing mice in a tissue-specific manner (Wang *et al.*, 1995; Wang *et al.*, 2012a).

1.3.2 *Protein and lipid damage*

Although not covered in much detail in this thesis it is also important to note the effect of damage on proteins and lipids and the consequences of such damage on cellular function.

Increased protein oxidation has been demonstrated in older individuals and in AD patients (Smith *et al.*, 1991) and in the brain, heart and testis of the Mongolian gerbil (Sohal *et al.*, 1995). Oxidised proteins increase in ageing mouse cartilage (Scharf *et al.*, 2013) and muscle (Li *et al.*, 2012), rat brain and liver (Tiana *et al.*, 1998) as well as human lymphocytes (Gautam *et al.*, 2010) and muscle (Mecocci *et al.*, 1999). An age-related accumulation of oxidative damage measured as protein carbonyls is tissues specific (Davies *et al.*, 2001). Furthermore oxidative damage to proteins in the brain correlates with an age-related impairment in cognitive function and motor skills (Forster *et al.*, 1996).

Protein aggregates where misfolded proteins accumulate together are characteristic of neurodegenerative diseases such as AD. An increase in insoluble proteins and protein aggregates is observed with age in *C. elegans* (David *et al.*, 2010). AD features extracellular aggregates of amyloid β-peptide and intracellular aggregates of Tau (Ross and Poirier, 2004).

Damage to lipids is also observed during ageing. Lipid peroxidation increases with age in human lymphocytes (Gautam *et al.*, 2010) and muscle (Mecocci *et al.*, 1999). Malondialdehyde (MDA), a marker of lipid peroxidation increases in ageing mouse muscle (Li *et al.*, 2012). Furthermore, MDA increases with age in human erythrocytes and this increase is more pronounced in AD patients (Casado *et al.*, 2008).

Accumulation of macromolecular damage is likely a result of down-regulation of cell homeostatic mechanisms. The proteasome, is a complex which degrades damaged and undamaged proteins and dysregulation of proteostasis is also considered a hallmark for ageing (López-Otín *et al.*, 2013). The resulting accumulation of damaged proteins may occur because of lower proteosomal activity, which is observed in aged rat liver (Agarwal and Sohal, 1994), senescent fibroblasts (Sitte *et al.*, 2000) and keratinocytes (Petropoulos *et al.*, 2000). Furthermore, autophagy also maintains the homeostatic environment of the cell by degrading and recycling damaged molecules and organelles (Pyo *et al.*, 2013b). Autophagy-related genes are down-regulated in the ageing brain (Lipinski *et al.*, 2010). However, overexpression of the gene encoding autophagy protein 5 in mice extends their lifespan emphasising autophagy as being an important player in ageing (Pyo *et al.*, 2013a).

1.4 DNA repair

The accumulation of DNA damage in the ageing cell can be modulated by a number of factors one of which is DNA repair efficiency. The cell has multiple DNA repair mechanisms to repair damage to the genome. These include base excision repair (BER) which removes small DNA alterations, nucleotide excision repair (NER) which removes bulky lesions, and mismatch repair (MMR) which repairs mispaired bases from replication errors. In addition, homologous recombination (HR) and non-homologous end joining (NHEJ) deal with double strand breaks. As a consequence of unrepaired damage, the cell exhibits inhibition of transcription, replication and chromosomal segregation which lead to apoptosis as well as mutations and chromosome aberrations which lead to cancer, disease and the ageing phenotype (Hoeijmakers, 2001).

After DNA damage induction in proliferative cells, cell cycle checkpoints are activated, leading to cell cycle arrest and providing time for the activated DNA damage repair machinery to repair the DNA damage. These checkpoints occur

at G1/S and G2/M boundaries, with an additional intra-S checkpoint. In such cells repair is important so that incorrect information is not passed onto daughter cells. Post-mitotic cells which do not go through the cell cycle also undergo DNA repair and this process is initiated directly (Iyama and Wilson, 2013) and also involves initiations of cell cycle machinery (Schwartz *et al.*, 2007). DNA repair in post-mitotic cells acts to ensure integrity of the transcribed genome (Houtgraaf *et al.*, 2006). If damage remains undetected and unrepaired, this leads to mutations and to genomic instability. Accumulation of errors in DNA may contribute to both carcinogenesis and cellular ageing (senescence).

1.4.1 *Base excision repair*

Base excision repair is required for removal of small DNA damage induced by damaging agents such as reactive oxygen species. BER includes 2 sub-pathways: short-patch repair for removal of single nucleotides and long-patch repair for removal of 2-13 nucleotides (Figure 1.1). DNA damage in the form of oxidation, alkylation or deamination of bases is removed by BER. Multiple enzymes contribute to BER including: DNA glycosylases, AP endonuclease, DNA polymerase and DNA ligase.

The core steps of the pathway are;

1. Recognition of a damaged base by DNA glycosylase which cleaves the N-glycosidic bond removing the damaged base to create an apurinic or apyrimidinic site (AP site).
2. Cleavage of the DNA backbone by AP endonuclease (APEX/Ref-1) or by the AP lyase of bifunctional DNA glycosylases. AP endonuclease activity creates a single-stranded nick in the DNA backbone which is 5’ to the AP site generating a 5’-deoxyribose-5-phosphate (5’-dRP) and 3’-hydroxyl strand break product. Meanwhile an AP lyase creates a nick in the DNA backbone at 3’ to the AP site leaving a single strand break containing a 3’-phospho-α,β-unsaturated aldehyde (3’-PUA) or 3’-phosphate (3’-P). AP endonuclease then cleaves 3’-PUA and polynucleotide kinase 3’-phosphatase (PNKP) cuts out the 3’-P residue generating a 3’-hydroxyl group.
3. A DNA polymerase β removes 5’dRP and fills in the gap with a new nucleotide.

4. Finally a DNA ligase and X-ray repair cross-complementing protein 1 (XRCC1) complex seal the nick and complete the repair process (Robertson *et al.*, 2009; Iyama and Wilson, 2013).

In long-patch repair after cleavage by APEX, DNA polymerase (β or δ), proliferating cell nuclear antigen (PCNA), flap structure-specific endonuclease 1 (FEN1) and a DNA ligase are recruited to the site to complete the strand sealing process (Robertson *et al.*, 2009).

Furthermore, repair enzymes interact with each other to allow for efficient repair and progression of the pathway. Enzymes at the start of the pathways initiate activity of enzymes down-stream in the repair process. OGG1 binds to poly ADP-ribose polymerase (PARP1) and stimulates its activity (German *et al.*, 2013). OGG1 and APEX interact with XRCC1 enhancing their incision activity (Marsin *et al.*, 2003). APEX also interacts with and enhances activity of β-polymerase, FEN1 and DNA ligase 1 (Bennett *et al.*, 1997; Tom *et al.*, 2001). Furthermore, cell signalling by OGG1 is a result of 8-oxodG activation of Ras GTPases and resultant phosphorylation of the downstream kinases (Boldogh *et al.*, 2012; German *et al.*, 2013).

Many of the genes involved in BER are highly conserved from bacteria to humans, which indicates that BER is a fundamental repair pathway in all organisms. Furthermore, BER operates throughout the cell cycle suggesting it is important in both dividing and non-dividing cells (Wilson Iii and Bohr, 2007).

1.4.1.1 *Significance of DNA glycosylase and AP endonuclease*

The initial step of BER is performed by a DNA glycosylase, which reacts specifically with DNA damage. Known glycosylases and their substrates are summarised in Table 1.2. DNA repair enzymes (e.g. OGG1 and APEX) are expressed ubiquitously in all mammalian tissues but at different intensities (Wilson *et al.*, 1996; Verjat *et al.*, 2000). Activity of DNA repair enzymes varies between tissues. OGG1 activity in mice was reported in descending order of activity in testis, kidney, muscle, liver, brain and then heart whereas UDG activity was reported in descending order in muscle, testis, kidney, heart, liver and brain. In contrast, NTH1 activity is similar in all these tissues (Karahalil *et al.*, 2002; Intano *et al.*, 2003).

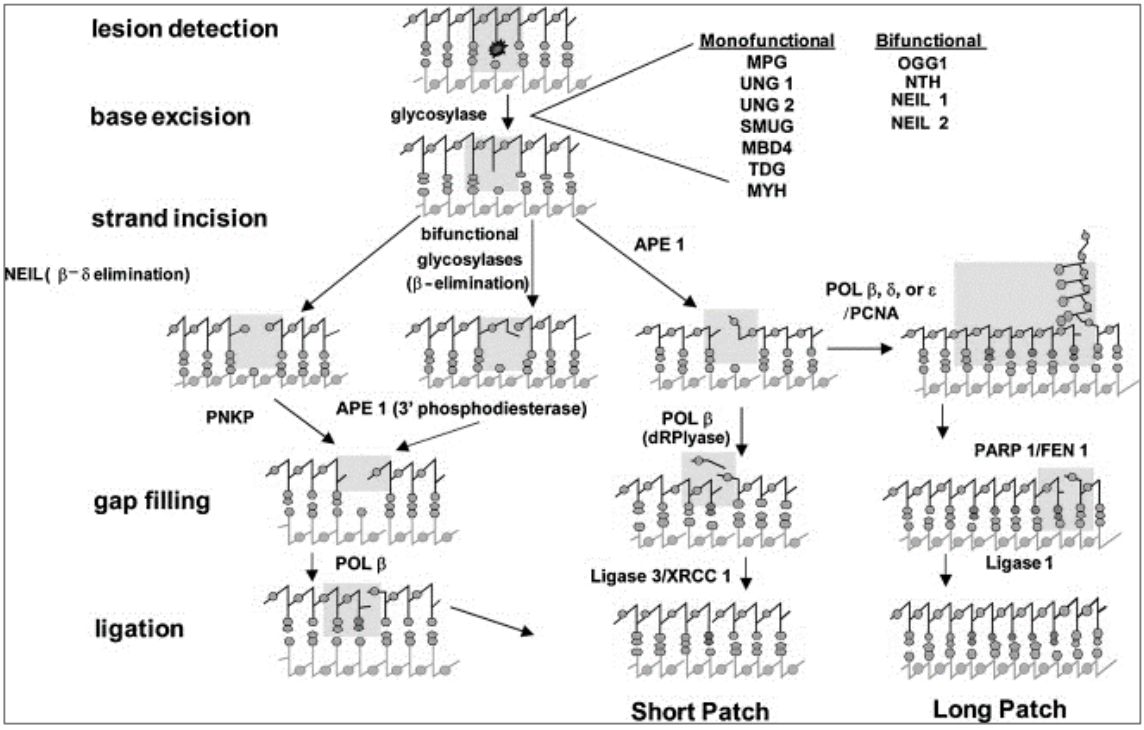


Figure 1.1 Overview of short-patch and long-patch mammalian BER pathways. Damage detection and base excision are performed by DNA glycosylases (listed in the Figure and in Table 1.2). The DNA glycosylase action dictates which gap filling and ligation route of the pathway precedes base excision. APE1, AP endonuclease; FEN1, flap structure-specific endonuclease 1; MBD4, methyl-CpG-binding domain protein 4; MPG, N-methylpurine-DNA glycosylase; MYH, muty homolog; NEIL, nei endonuclease VIII-like; NTH, nth endonuclease III-like 1; OGG1, oxoguanine DNA glycosylase; PARP1, poly (ADP-ribose) polymerase 1; PCNA, proliferating cell nuclear antigen; PNKP, polynucleotide kinase 3'-phosphotase ; POL β, δ and ε, DNA polymerase; SMUG, single-strand-selective monofunctional uracil-DNA glycosylase; TDG, thymine-DNA glycosylase; UNG1 and 2, uracil-DNA glycosylase; XRCC1, X-ray repair cross-complementing protein 1. Figure from Xu et al. (Xu et al., 2008a).

Knockout of individual DNA glycosylases does not produce lethality. The similarities in substrate specificity between the different DNA glycosylases may mean that there is inbuilt redundancy in the system and that other glycosylases can compensate for the one that was knocked out (Klungland *et al.*, 1999; Osterod *et al.*, 2001). However, mice defective in DNA glycosylase activity develop cancers and a metabolic syndrome-like phenotype. Mice deficient in uracil-DNA glycosylase develop B-cell lymphomas. Double knockout (KO) mice of *Ogg1* and *Mutyh* result in increased tumour development (Xie *et al.*, 2004). *Ogg1* inactivation leads to an increase in mutation frequency and 8-oxodG levels in the liver (Minowa *et al.*, 2000). *Neil1* knock out gives rise to mice demonstrating metabolic syndrome, with an obesogenic phenotype (Vartanian *et al.*, 2006) and increased number of mtDNA deletions compared with wild type

(WT) controls (Vartanian *et al.*, 2006). Finally, *Nth* KO mice show increased DNA lesions at telomeric loci compared with non-telomeric loci (Vallabhaneni *et al.*, 2013). On the other hand mice with KO of genes further down the repair pathway such as *Apex, β-polymerase* or *DNA ligase* do not survive embryogenesis (Barnes and Lindahl, 2004; Larsen *et al.*, 2007). However, inhibition of *Apex* by RNA interference, results in abasic DNA site accumulation, halts cell proliferation and induces apoptosis (Fung and Demple, 2005). Cells deficient in *β polymerase* have increased number of chromosomal breaks and are also prone to apoptosis (Ochs *et al.*, 1999).

Although DNA glycosylases are the primary enzymes for recognition of damage, APEX can also remove larger oxidative lesions such as 8,5-cyclopurines independently of DNA glycosylase activity (Gros *et al.*, 2004; Mazouzi *et al.*, 2013). APEX is also able to stabilise telomeric DNA (Madlener *et al.*, 2013) although it has reduced activity to single stranded DNA and DNA loops compared with double stranded DNA (Li *et al.*, 2014). In addition to APEX's repair activity it is also involved in redox regulation of transcription factors, enhancing transcription factor binding to DNA (Evans *et al.*, 2000).

In humans, single base mutations in *OGG1* which cause a single amino acid change have been detected in lung and kidney tumours (Chevillard *et al.*, 1998). Patients with adenocarcinoma of the lung showed a polymorphic allele in OGG1 exon 1 (Ishida *et al.*, 1999). Furthermore, *OGG1* localises to a chromosome region often deleted in cancers (Lu *et al.*, 1997). A mutation in the gene coding for *MUTYH* was found to be responsible for colorectal cancer development (Al-Tassan *et al.*, 2002). Therefore it is apparent that BER dysfunction plays a role in cancer development. In the neurodegenerative disorder ataxia oculomotor apraxia shows reduced activity of PARP-1, APEX and OGG1 (Harris *et al.*, 2009).

DNA glycosylase	Acronym	AP lyase activity	Damage recognised	Acronym
8-oxoguanine DNA glycosylase	OGG1	+	8-oxoguanine 2,6-diamino-4-hydroxy-5-formamidopyrimidine	8-oxodG fapyG
Uracil DNA glycosylase	UDG/UNG	-	Uracil	U
Nei endonuclease VIII-like 1	NEIL1	+	Thymine glycol 2,6-diamino-4-hydroxy-5-formamidopyrimidine Dihydrouracil 5-hydroxyuracil 5-hydroxycytosine 4,6-diamino-5-formamidopyrimidine 8-oxoguanine	Tg fapyG DHU 5-OHU 5-OHC fapyA 8oxodG
Nth endonuclease III-like 1	NTH1	+	Thymine glycol 2,6-diamino-4-hydroxy-5-formamidopyrimidine Dihydrouracil 5-hydroxyuracil 5-hydroxycytosine	Tg fapyG DHU 5-OHU 5-OHC
Muty homolog	MUTYH/MYH	+	Adenine opposite 8-oxoguanine	A opposite 8-oxodG
Thymine DNA glycosylase	TDG	-	Thymine, uracil and ethenocytosine (CpG sites)	T, U and ethenoC
Single strand selective monofunctional uracil DNA glycosylase	SMUG1	-	Uracil 5-hydroxymethyluracil	U 5-OH-meU
Methyl CpG binding domain protein	MBD4	-	Thymine and uracil opposite guanine (CpG sites)	T and U opposite G
N-methylpurine-DNA glycosylase	MPG	-	N3-methyladenine Hypoxanthine Ethenoadenine	3-meA Hyx ethenoA

Table 1.2 DNA glycosylases found in mammalian cells and the damaged bases which they recognise. Table adapted from Robertson et al. (Robertson et al., 2009).

1.4.1.2 *Initiation of Ogg1 and Apex expression*

BER expression is initiated as a response to a number of stimuli such as oxidative stress (Cabelof *et al.*, 2006), high glucose (Pang *et al.*, 2012), ionizing radiation (Yang *et al.*, 2004) and physical damage (Riis *et al.*, 2002). Transcription factors such as nuclear transcription factor Y subunit alpha (NFYA), tumour suppressor protein 53 (p53) and nuclear factor 2 (NRF2) have been shown to up-regulate *Ogg1* expression (Chatterjee *et al.*, 2005; Habib *et al.*, 2010; Singh *et al.*, 2014; von der Lippen *et al.*, 2015). Furthermore it is known that activation by NFYA involves the c-Jun N-terminal kinase (JNK), a mitogen-activated protein kinase (von der Lippen *et al.*, 2015) and/or 5' adenosine monophosphate-activated protein kinase (AMPK). In addition, *Apex* expression can also been up-regulated by transcription factors such as nuclear factor kappa-light-chain-enhancer of activated B cells (NFκB), microphthalmia-associated transcription factor and p53 in cancerous cells (Liu *et al.*, 2008; Jung *et al.*, 2013; Song *et al.*, 2013).

1.4.1.3 *Post-translational modifications of OGG1 and APEX protein*

Post-translational modifications to repair enzymes can alter their function. Acetylation of OGG1 by p300 increases its activity and about 20% of OGG1 is acetylated in cultured cells (Bhakat *et al.*, 2006). *In vitro*, phosphorylation of OGG1 by cyclin-dependant kinase 4 (Cdk4) increases its activity (Hu *et al.*, 2005) while phosphorylation by protein kinase C (PKC) enhances OGG1 binding to DNA (Dantzer *et al.*, 2002). APEX has to be in its unacetylated form to localise to the nucleus; however upon genotoxic stress it is acetylated which results in an increase in its activity (Lirussi *et al.*, 2012). Furthermore, glyceraldehyde 3-phosphate dehydrogenase (GAPDH) associates with APEX and initiates its activity (Azam *et al.*, 2008). Paradoxically, an increase in APEX activity can lead to genomic instability, as observed in cancers (Evans *et al.*, 2000), and overexpression of this enzyme prevents repair of mismatched DNA (Chang *et al.*, 2005). Therefore regulation of APEX by reducing its activity by s-glutathionylation, phosphorylation and ubiquitination is equally important to prevent error prone DNA repair (Borjigin *et al.*, 2010; Kim *et al.*, 2011; Meisenberg *et al.*, 2012). Trochostatin A-sensitive deacetylases are responsible for OGG1 deacetylation (Bhakat *et al.*, 2006); furthermore these enzymes induce APEX secretion and translocation to the cytoplasm (Choi *et al.*, 2013).

1.4.1.4 *BER in ageing*

The role of DNA glycosylase dysfunction in ageing may also be important as shown by age-related accumulation of oxidative damage in livers from *Ogg1* KO mice (Osterod *et al.*, 2001). In addition, the senescence-accelerated mouse model (SAMM) has been reported to have mutations in the *Ogg1* gene (Choi *et al.*, 1999). Whether this mutation is a cause of the SAMM phenotype is difficult to determine as mutations in multiple other genes have also been found in these mice (Tanisawa *et al.*, 2013). An age-related decline in repair has also been demonstrated in senescent cells where repair of AP sites was slower in senescent compared with young cells in culture (Atamna *et al.*, 2000). Furthermore activity of human OGG1 declines with age in lymphocytes (Chen *et al.*, 2003). Measurements of DNA repair activity in ageing tissues have mostly shown a decline in activity. OGG1, UDG and polymerase β activity declined with age in brain, liver, spleen and testes of mice (Cabelof *et al.*, 2002; Intano *et al.*, 2003). Furthermore, repair in the brain was region specific with an age-related decline in BER-related incision activity of OGG1, UDG and NTH1 in nuclear DNA in the cerebellum and brain stem and decline in mitochondrial DNA repair in the caudate nucleus, frontal cortex, hippocampus, cerebellum and brain stem (Imam *et al.*, 2006). However, some studies did not find evidence of age-related decline in DNA repair. No changes in nuclear BER activity with age were detected in mouse liver (de Souza-Pinto *et al.*, 2001), kidney and heart (Szczesny *et al.*, 2010). Mikkelsen *et al.* did not report a significant difference in messenger ribonucleic acid (mRNA) expression levels of *Ogg1* or *Neil1* in mouse liver (Mikkelsen *et al.*, 2009) furthermore, Lu *et al.* even reported OGG1 and UDG expression to be up-regulated with age in the frontal cortex (Lu *et al.*, 2004). Thus, the effect of age on BER activity requires further study.

In humans, a reduction in BER OGG1 activity is observed in ageing lymphocytes (Chen *et al.*, 2003). Polymorphisms in *OGG1* and other BER-related proteins are associated with cognitive performance and its decline during ageing (Lillenes *et al.*, 2011) and age-related macular degeneration (Synowiec *et al.*, 2012). Furthermore, in Alzheimer's disease brains there is a decrease in OGG1 activity (Mao *et al.*, 2007; Jacob *et al.*, 2013) and increase in APEX activity (Marcon *et al.*, 2009).

1.4.2 *Nucleotide excision repair and ageing*

Nucleotide excision repair is the primary pathway for removal of bulky adducts and helix-distorting lesions such as those induced by UV light or polycyclic aromatic hydrocarbons (Hoeijmakers, 2001). However, when BER is defective, NER can also remove oxidative damage (Reardon *et al.*, 1997; Sunesen *et al.*, 2002; D'Errico *et al.*, 2006). NER is divided into global genome NER (GG-NER) and transcription coupled NER (TC-NER) and schematic of the pathway and the proteins involved in the pathway are summarised in Figure 1.2.

Nucleotide excision repair has also been associated with ageing (Hart and Setlow, 1974). NER as measured by unscheduled DNA synthesis declines with age by 50% in hepatocytes and kidney cells (Weraarchakul *et al.*, 1989). The removal of thymine dimers is slower in dermal fibroblasts from old compared with new-borns and in young adult (Goukassian *et al.*, 2000). Furthermore, mRNA and protein levels of NER-related enzymes decline with age (Goukassian *et al.*, 2000).

Mutations in human NER-related genes cause xeroderma pigmentosum (XP), trichothiodystrophy (TTD), Werner syndrome and Cockayne syndrome (CS) with affected individuals showing sun sensitivity, delay in development and neurodegeneration (Kraemer *et al.*, 2007). Many of the symptoms associated with mutations in NER-genes are similar to those observed in ageing. The syndromes characterised by premature ageing are termed progerias. Mouse models of NER deficiency e.g. mutation or deletion of *Xpd*, *Ercc1* and *Xpf* also show a premature ageing phenotype and may provide a useful model for studies of ageing interventions. Such mice exhibit susceptibility to skin and other cancer, deficiency inn hematopoetic stem cells, neurological deficits and decreased growth hormone axis, (de Boer *et al.*, 2002; Niedernhofer *et al.*, 2006; Rossi *et al.*, 2007; Niedernhofer, 2008; Goss *et al.*, 2011)

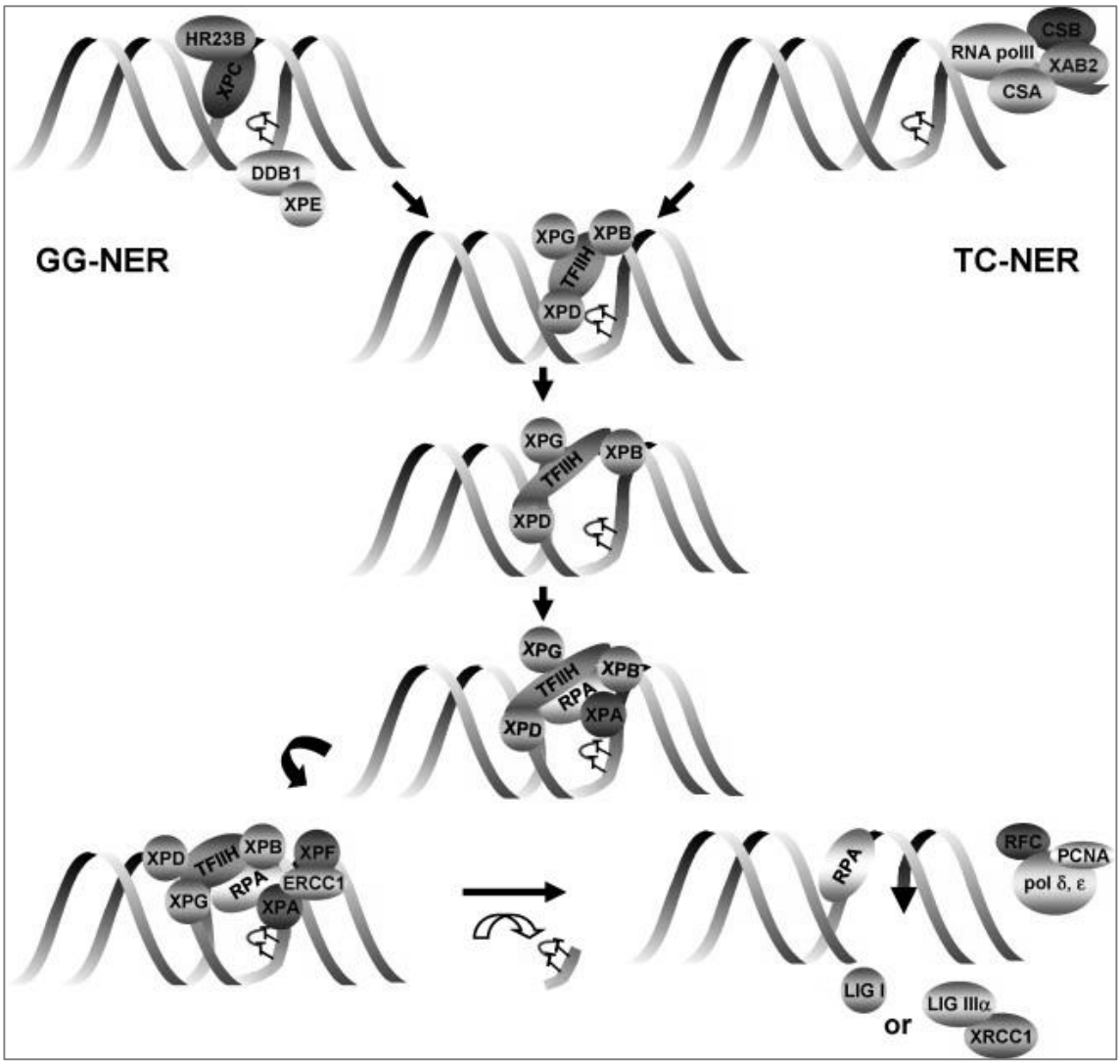


Figure 1.2 Overview of the global genome and transcription coupled NER. Lesion recognition is performed by XPC, DDB1 and XPE (GG-NER) or CSB, CSA and XAB2 (TC-NER). XPC or CSB recruit the transcription factor TFIIH complex. XPB and XPD unwind the DNA while XPA and RPA bind and stabilize the unwound lesion and recruit XPF-ERCC1. XPF-ERCC1 incises the damaged strand of DNA 5' to the lesions while XPG makes the 3' incision. The lesion is removed as a single-stranded oligonucleotide, leaving behind a gap, which is filled by polymerase δ and ε, PCNA, RPA and RFC. The DNA backbone is sealed by DNA ligase I or DNA ligase II-XRCC1. CSB, cockayne syndrome group B protein; DDB1, DNA-damage binding protein 1; ERCC1, excision repair cross-complementing protein 1; GG-NER, global genome nucleotide excision repair; PCNA, proliferating cell nuclear antigen; RPA, replication protein A; RFC, replication factor X; TC-NER, transcription coupled nucleotide excision repair; TFIIH, transcription factor II human protein; XAB2, XPA-binding protein 2; XP A-G, ;xeroderma pigmentosum complementation group A-G; XRCC1, X-ray repair cross complementing protein 1. Figure from (Niedernhofer, 2008).

1.4.3 *Other repair mechanisms and ageing*

Accumulation of DNA double strand breaks (DSB) is observed in senescent human fibroblasts and ageing mouse tissue (Sedelnikova *et al.*, 2004). Two main pathways for repair of double strand breaks (DSB) are NHEJ and HR; the pathway initiated depends on cell cycle phase. Efficiency of repair also differs between these pathways with HR giving rise to more accurate repair compared with NHEJ (van Gent *et al.*, 2001). Studies from *Drosophila melanogaster* suggest that HR repair activity increases with age while NHEJ decreases with age and it is hypothesised that unrepaired DSBs lead to apoptosis (Preston *et al.*, 2006).

Homologous recombination uses the undamaged sister chromatid as a template to give an accurate repair product; it occurs in dividing cells. This pathway is catalysed by the recombinase RAD52 family of proteins. Recombination between misaligned sequences, can result in sequence rearrangement. Perhaps the best known cause of defective HR, is the mutations in breast cancer susceptible genes (BRCA, encode proteins which recognise DSB) and their role in inherited cancer (Venkitaraman, 2002). Furthermore, Fanconi anemia is a disorder which results from a mutation in any of the FANC genes, encoding proteins for recognition of DNA crosslinks and leads to cancer and decrease in number of hematopoetic stem cells (Walden and Deans, 2014). In a population of cells from ageing pancreas (but not fibroblasts) there is an increase in number of recombinant cells (Wiktor-Brown *et al.*, 2006).

In contrast, NHEJ does not use the homologous strand but rather recognises a DSB, which may require processing by addition or removal of nucleotides, and ligates the DNA strand (van Gent *et al.*, 2001). It can occur in both dividing and non-dividing cells. The proteins involved in this pathway are: Ku70, Ku80, DNA-dependent protein kinase catalytic subunit, X-ray repair cross-complementing protein 4 (XRCC4), DNA ligase IV and XRCC4-like factor (XLF). NHEJ is important for integrity of hematopoietic stem cells during aging, as cells from mice with a mutation in DNA ligase IV show reduced NHEJ repair and a decrease in proliferation (Nijnik *et al.*, 2007). Age-related telomere shortening is associated with DSB; furthermore in senescent cells such sites are associated with a persistent DNA damage response (Hewitt *et al.*, 2012).

Mismatch repair (MMR), corrects mismatched bases made during the process of replication. It involves the proteins mutator Sα homolog (MSH), mutator Lα, exonuclease 1, RPA, PCNA, replication factor C, DNA polymerase δ and DNA ligase I (Conde-Pérezprina *et al.*, 2012). Deficiency in mismatch repair is associated with DNA microsatellite instability (Lin and Wilson, 2009) and a decline in MMR activity is reduced in senescent fibroblasts (Chang *et al.*, 2008). Furthermore, MMR may be important in telomere function specifically telomere capping as *Msh2* deficient mouse fibroblasts exhibit an increase in chromosome end fusion (Campbell *et al.*, 2005)

In summary, ample evidence supports the role of defective DNA repair mechanisms in the ageing process albeit in a tissue specific manner. Data suggest that the proliferative nature of the tissue may influence the accumulation of DNA damage, DNA repair and hence rate of ageing of the tissue.

1.5 Epigenetic regulation of gene expression

Epigenetics is defined as the study of chromatin-associated marks and molecules that can alter gene transcription but without altering the original DNA sequence. The most studied epigenetic marks are cytosine methylation and post-translational histone modifications.

These marks have chromatin altering properties, being able to shift chromatin between transcription active and repressed states. Transcription active euchromatin is loosely packaged during interphase. In contrast, heterochromatin is associated with transcription silencing and is found tightly packed during interphase. Heterochromatin can be subdivided into constitutive, found at centromeres and telomeres which is fixed, and facultative, synthesised as needed, e.g. X-linked silencing (Howard, 1996). Formation of euchromatin or heterochromatin is determined by modifications of the histones and DNA; e.g. histone acetylation is associated with euchromatin and DNA methylation with heterochromatin (Sedivy *et al.*, 2008). Together epigenetic marks give rise to an epigenetic code, which may be linked to specific functions giving rise to explicit gene expression patters (Turner, 2007).

Epigenetics has been proposed as an important player in ageing and age related diseases (López-Otín *et al.*, 2013). This is because of i) the effects that

alteration in epigenetic marks may have on genomic stability and ii) the role of epigenetics in regulation of gene transcription.

1.5.1 *DNA Methylation*

DNA methylation occurs by the addition of a methyl group by a DNA methyltransferase at the 5' position of the carbon ring in a cytosine nucleotide. The majority of DNA methylation occurs in CpG dinucleotides i.e. where a cytosine residue is followed by a guanine residue in a 5' direction (denoted by CpG for cytosine adjacent to G bound by phosphate bond and to distinguish from CG pairing). CpG sites occur rarely on their own in the genome but rather occur as clusters which are called CpG islands where the ratio of observed over expected CpG dinucleotides is more than 65%.

DNA methylation functions to silence the specific gene and keep a closed chromatin state (Gonzalo, 2010). An estimated 80% of all CpG sites in the mammalian genome are methylated. This is because the majority are found in DNA repetitive sequences made up of transposable elements which need to be silenced. However, cytosines found in CpG islands within gene promoter regions are generally unmethylated, as depicted by Figure 1.3 (Maegawa *et al.*, 2010).

DNA methylation can regulate gene expression either by inhibiting the binding of transcription factors to the promoter sequence (Medvedeva *et al.*, 2014) or by enhancing the binding of specific proteins to the methylated DNA that block other proteins from binding (Razin and Cedar, 1991). DNA methyltranseferases (DNMT) are enzymes responsible for the addition of methyl marks to cytosine; 3 enzymes have been identified in mammals; maintenance DNMT1 and *de novo* DNMT3a and DNMT3b. DNA methylation patterns are established during the early developmental period (Law and Jacobsen, 2010). Methylation patterns are maintained from cell to cell by the maintenance DNMT1 which copies the methylation pattern in the parent DNA strand to the daughter strand (Gonzalo, 2010). Recently 5-hydroxymethyl cytosine has also been shown to occur in cells although at a much lower level compared with 5-methylcytosie (Kriaucionis and Heintz, 2009). Although the biological role of this molecule is still not known, the enzymes involved in this conversion were identified as the ten-eleven translocation methylcytosine dioxygenase (TET) enzymes (Ito *et al.*, 2011).

1.5.2 *Effects of age on DNA methylation*

Although methylation patterns are developed early on in embryogenesis, studies in monozygotic twins who start off with the same methylation show divergence with age indicating that environmental factors and age can influence DNA methylation (Fraga *et al.*, 2005; Christensen *et al.*, 2009). Global DNA methylation has been shown to decrease with age in the brain, liver and small intestine mucosa in mice (Singhal *et al.*, 1987; Wilson *et al.*, 1987; Thompson *et al.*, 2010), human leukocytes (Fuke *et al.*, 2004) and cultured cells (Wilson and Jones, 1983). Furthermore, assessment of individual methylated sites showed both hypermethylation and demethylation with age; however, less than 1/3 of CpG sites studied show differential methylation with age in the mouse, and these changes were tissue specific. Hypermethylation was observed at 21%, 11%, 12% and 14% of sites studied in the small intestine, liver, brain and muscle respectively. On the other hand demethylation was observed at 13%, 12%, 4% and 3% of sites studied in the small intestine, liver, brain and muscle respectively (Maegawa *et al.*, 2010; Takasugi, 2011). It is hypothesised that epigenetic changes occur depending on mitotic activity of the tissue with the less active cells not exhibiting age-related hypermethylation (Chu *et al.*, 2007). Interestingly, the difference in methylation between the tissues exceeded that of age related changes (Thompson *et al.*, 2010). Differential methylation patterns have been reported between young and old humans. Furthermore, age-related changes in methylation are dependent on region studied with intron and intergenic regions showing >3 fold more differentially methylated regions compared with promoter and exon (Heyn *et al.*, 2012).

Changes in DNA methylation at gene promoter regions are associated with altered gene expression. Methylation of specific gene promoters has been associated with repression of transcription at those genes. One of the hypothesised modes of action for epigenetic gene silencing is by inhibiting binding of proteins involved in transcription e.g. transcription factors (TFs) at gene promoter regions (Watt and Molloy, 1988; Deaton and Bird, 2011). For example in neuroblastoma cells the N-myc transcription factor which binds to the E-box sequence cannot do so when the CpG within the binding sequence CACGTG is methylated and this prevents transcription of genes such as *APEX1, XRCC3, nibrin* and a DSB repair protein *MRE11A* (Perini *et al.*, 2005).

A third of housekeeping genes are methylated in older individuals (Heyn *et al.*, 2012), and methylation-related dysregulation of expression of housekeeping genes may be responsible for progression of ageing. In cancer, a disease of which age is a risk factor, methylation of tumour suppressor genes may be important in tumorigenesis. Age-related cytosine methylation of tumour suppressor genes occurs in a number of tissues (Ahuja *et al.*, 1998; Waki *et al.*, 2003; So *et al.*, 2006). Oestrogen receptor promoter CpG island is hypermethylated with age and in tumours, which correlates with its repression of transcription observed in tumours and cancer cell lines (Issa *et al.*, 1994). Furthermore, DNA methylation of sub-telomeric regions is associated with increased activity of telomerase in cancer cell lines (Ng *et al.*, 2009).

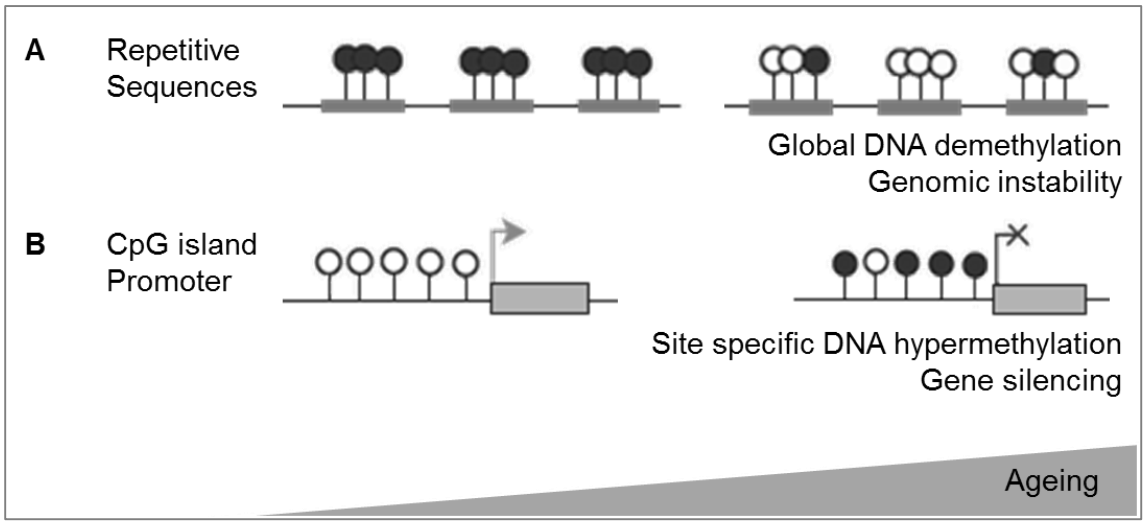


Figure 1.3 Schematic showing the effect of age on DNA methylation. Global DNA methylation as measured by cytosine methylation (filled lollipops) within repetitive sequences is demethylated during ageing (A). Site specific DNA methylation in gene promoters is usually unmethylated (clear lollipops) and results in active expression of genes, upon ageing promoters can become hypermethylated leading to gene silencing (B).

A number of other genes whose methylation may lead to deleterious effects have been studied. Hernandez *et al.* found that in the human brain age-related enrichment in methylation was found in genes coding for regulatory elements involved in activation of transcription and DNA binding (Hernandez *et al.*, 2011). *IGF2* methylation has also been shown to increase with age in cultured human fibroblasts, colon and lymphocytes (Issa *et al.*, 1996). Cytochrome C oxidase subunit VIIa polypeptide 1 promoter methylation is higher in muscle of older individuals and correlates with a decrease in its mRNA expression, and may be

important in down-regulation of oxidative phosphorylation with age (Rönn *et al.*, 2008).

Epigenetic silencing of a few DNA repair genes has been observed in cancer. Multiple cancer cell lines have hypermethylated *WRN* promoter region, while normal tissues show unmethylated regions (Agrelo *et al.*, 2006). In addition analysis of gene expression shows that these cell lines have little or no expression of *WRN* mRNA. The *BRCA1* gene promoter is hypermethylated in some breast cancer xenografts and this is associated with silencing of *BRAC1* mRNA transcript (Esteller *et al.*, 2000). An age-related change in methylation is also observed in telomerase reverse transcriptase (*TERT*), *ERCC1*, *RAD50* and *WRN* in humans (Christensen *et al.*, 2009). *MLH1* DNA repair gene is hypermethylated in colonic mucosa, which was associated with age and cancer development (Nakagawa *et al.*, 2001) and in thyroid cancer (Guan *et al.*, 2008). Furthermore, it has been suggested that differential methylation state in Werner's Syndrome and Huntington-Guilford progeria contributes to the phenotype (Heyn *et al.*, 2013). However to date no studies have addressed weather epigenetic silencing of BER-related genes could be responsible for the age-associated decline in repair activity.

1.5.3 *Histone modifications*

The nucleosome is the basic unit of chromatin consisting of an octamer of histones H2A, H2B, H3 and H4 (2 copies of each globular protein) with a 146bp of DNA wrapped around it. Together with other proteins the nucleosome is packed into a spiral arrangement of 30nm fibres containing six nucleosomes (Zhang and Reinberg, 2001). Positively charged amino acids including lysine and arginine found in histone tails can be post-translationally modified by the addition of methyl, acetyl, phosphorous or ubiquitin moieties (Figure 1.4). This chemical modification can alter the structure of chromatin. Therefore, histone modifications are important not only in regulating gene transcription but also for chromosome packaging and DNA repair.

The first experiments linking histone acetylation and methylation to gene transcription were conducted in 1964 (Allfrey *et al.*, 1964). Histone methyltransferases and demethylases can add and remove respectively methyl groups to/from lysine (K) and arginine (R) molecules. Methylation at H3K4, H3K36 and H3K79 is associated with activation of transcription, while

methylation of H3K9, H3K27 and H4K20 is associated with repression (D'Aquila *et al.*, 2013). Promoters are linked with high levels of H3K4me3 regardless of their transcription state, while gene enhancer regions are marked by H3K4me1, H3K4me1 or H3K27me3 (Zentner and Henikoff, 2013). Addition and removal of acetyl groups to lysine residues is catalysed by histone acetyltransferase (HAT) and histone deacetylase (HDAC) enzymes. It is hypothesised that acetylation of lysine neutralises its positive charge and hence weakens the bond between histones and DNA, allowing the transcription machinery access to the DNA (Bannister and Kouzarides, 2011). For example, the *CHFR* gene, coding for checkpoint forkhead-associated RING finger domain E3 ubiquitin-protein ligase, is silenced in a number of cancer cell lines and re-expression of this gene correlated with deacetylation of H3 and H4 (Toyota *et al.*, 2003). An example of histone methylation is the silencing of X chromosome (Zentner and Henikoff, 2013).

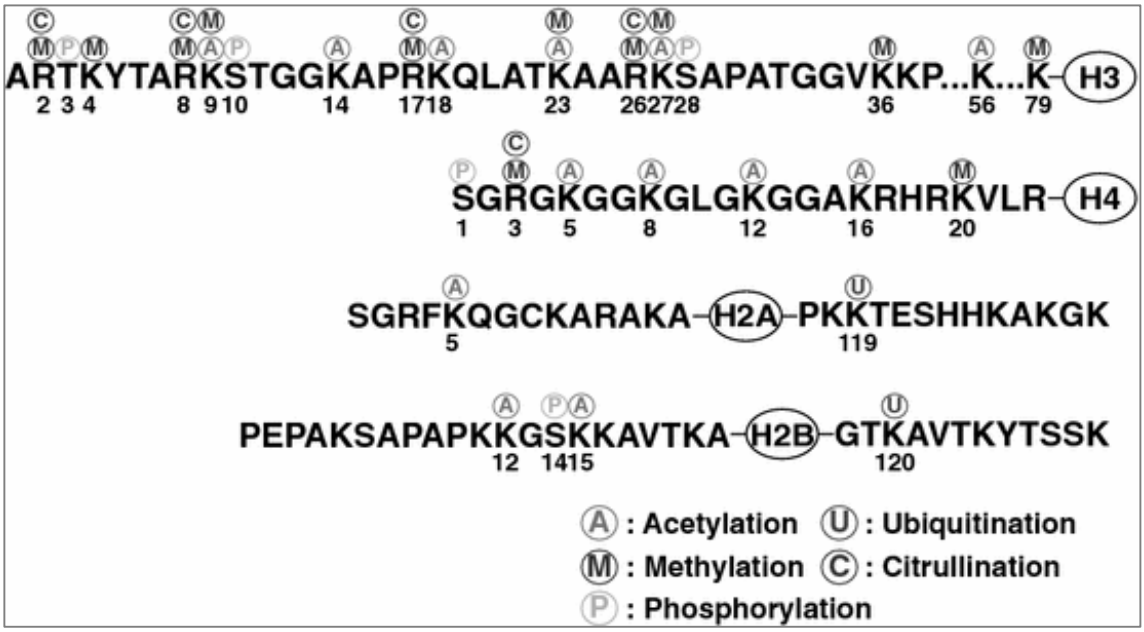


Figure 1.4 Map of histone modifications. N-terminal domains protrude from the nucleosome in H3 and H4 and N and C-terminal domains protrude in H2A and H2B. These domain can be post-translational modified by acetylation, methylation, phosphorylation, ubiquitination, citrullination, etc. and therefore alter chromatin structure, and influence transcription, replication and DNA repair. Figure from Kawakami *et al.* (Kawakami *et al.*, 2009).

1.5.4 *Effect of age on histone modifications*

A number of studies have investigated changes in histone modification with age. Increased levels of H4K16 acetylation are observed in sub-telomeric regions in old yeast cells (Dang *et al.*, 2009). Histone 4 methylation has been shown to increase in the ageing rat liver and kidney (Sarg *et al.*, 2002). Furthermore, in the ageing rat liver there is a decrease in H3K9Ac, an increase in H3S10Ph and no changes in H3K14Ac or H3K9Me1/2/3 (Kawakami *et al.*, 2009). Aged mice show deregulation in H3K12 acetylation and reduced expression of genes associated with learning memory in the hippocampus (Peleg *et al.*, 2010). In addition, an age-related DNA hypermethylation is associated with H3K4Me3, H3K27Me3 and H3K27Ac while DNA hypomethylation in associated with H3K27Ac, H3K4Me1 and H3K27Me3 (Raddatz *et al.*, 2013).

On a gene specific level in rat cortical neurones there is a decrease in caspase-3, a protease involved in cell apoptosis, expression with age and this change correlates with lower levels of H3K14 acetylation and H4 acetylation at the *caspase-3* gene promoter (Yakovlev *et al.*, 2010). Senescent cells exhibit transcription silent heterochromatin and decreased levels of H3 lysine 9 and 14 acetylation at *PCNA* and *cyclin A* promoters (Narita *et al.*, 2003). Furthermore, spermidine associated increase in longevity in yeast, flies and mice correlates with a decrease in H3 lysine 18 acetylation and increased expression of autophagy-related genes (Eisenberg *et al.*, 2009).

More studies have looked at the enzymes that are associated with addition of such marks. Overexpression of S*ir2*, a NAD-dependant histone deacetylase in yeast and worm extends lifespan (Hekimi and Guarente, 2003) while a mutation in *sir4* delays aging in yeast (Kennedy *et al.*, 1995). In mice, overexpression of *Sirt6* is also able to extend lifespan (Kanfi *et al.*, 2012). Furthermore, a deletion of the *RPD3* gene encoding a histone deacetylase is also able to increase lifespan of yeast (Kim *et al.*, 1999) and inhibition of histone demethylase utx-1 increases lifespan in nematodes (Jin *et al.*, 2011). An age-related decline in Sir2 and its mammalian homolog SIRT1 occurs in yeast and mice respectively (Sasaki *et al.*, 2006; Dang *et al.*, 2009). In rodent hippocampus and frontal cortex, expression of HDAC2 increases with age as does HDAC activity (Chouliaras *et al.*, 2013; dos Santos Sant' Anna *et al.*, 2013). Studies of other

epigenetic enzymes during ageing have shown that overexpression of EZH2, a HMT, in hematopoietic stem cells inhibits their cellular senescence (Kamminga *et al.*, 2006).

Epigenetic dysregulation has also been identified as one of the hallmarks of ageing and can contribute to the age-related genomic instability (López-Otín *et al.*, 2013). Although the hierarchy of epigenetic events is not known, it appears that together DNA methylation and histone modifications form an 'epigenetic code' which governs the transcription of genes. Furthermore, evidence points to histone deacetylation and histone methylation as important in the ageing process.

1.6 Nutritional modulation of ageing – dietary restriction as an example

Calorie/dietary restriction (CR/DR) i.e. the reduction of food intake below *ad libitum* levels without causing malnutrition is a very powerful tool in studies of ageing. The phenomenon of increased lifespan in laboratory animals fed a calorie restricted diet was reported as far back as 1930s (McCay *et al.*, 1935). Since then DR has been reported to increase lifespan in multiple animal models such as the worm *Caenorhabditis elegans*, the fruit fly *Drosophila melanogaster* and rodents such as mice and rats (Guarente and Kenyon, 2000; Swindell, 2012). Two studies have explored the effects of DR in non-human primates, the rhesus monkey. However, results were conflicting as only one study demonstrated lifespan extension (Colman *et al.*, 2009; Mattison *et al.*, 2012) which casts doubt whether DR can have life extending properties in primates. Nevertheless, DR remains a powerful tool for ageing research.

1.6.1 *Cellular effects of DR*

Multiple reports have shown DR to down-regulate signalling of various pathways e.g. growth hormone (GH) and insulin-like growth factor-1 (Sell, 2003), DR reduces levels of circulating IGF-1 and secretion of the growth hormone from the pituitary. It increases levels of glucocorticoids and reduces free radical accumulation and lipid peroxidation (Sohal *et al.*, 1995; Ramsey *et al.*, 2000). Animals fed a DR diet have a lower metabolic rate (Bartke *et al.*, 2001), higher levels of interleukin 2 (IL-2) and T-cell growth factor providing better immunological protection (Pahlavani, 2000). Furthermore, DR attenuates the age-related increase in inflammatory markers in the brain (Lee *et al.*, 2000)

and protects from apoptosis (Hiona and Leeuwenburgh, 2004). A summary of processes up- and down-regulated with DR is presented in Figure 1.5.

DR activates redox-sensitive transcription factors such as activator protein 1 (AP-1), hypoxia inducible factor 1 (HIF-1) and NFκB (Kim *et al.*, 2002). It reduces mitochondrial ROS production (Sohal *et al.*, 1994b) and retards age-associated accumulation of mutations in lymphocytes (Aidoo *et al.*, 2003) and 8-oxoguanine lesions in multiple organs in the rat (Kaneko, 1997; Hamilton, 2001). DR is able to reduce the concentration of 8-oxoguanine in the brain, heart and muscle by 40%, although it has minimal effects on 8-oxoguanine in liver or kidney, of mice (Sohal *et al.*, 1994a).

A study of the effect of DR on gene expression measured in livers of female mice at 7 and 27 months found that out of 11,000 genes measured, expression of 46 genes changed with age. Of these genes increased expression of mediators of inflammation (40%) and of stress response genes (25%) were most prominent. Of the genes whose expression decreased with age, 23% were responsible for DNA replication and the cell cycle, while others were involved in xenobiotic metabolism. Long-term DR prevented the majority of the age-related changes in gene expression and short-term DR (4 weeks) was also able to reproduce most of the changes (Cao *et al.*, 2001)

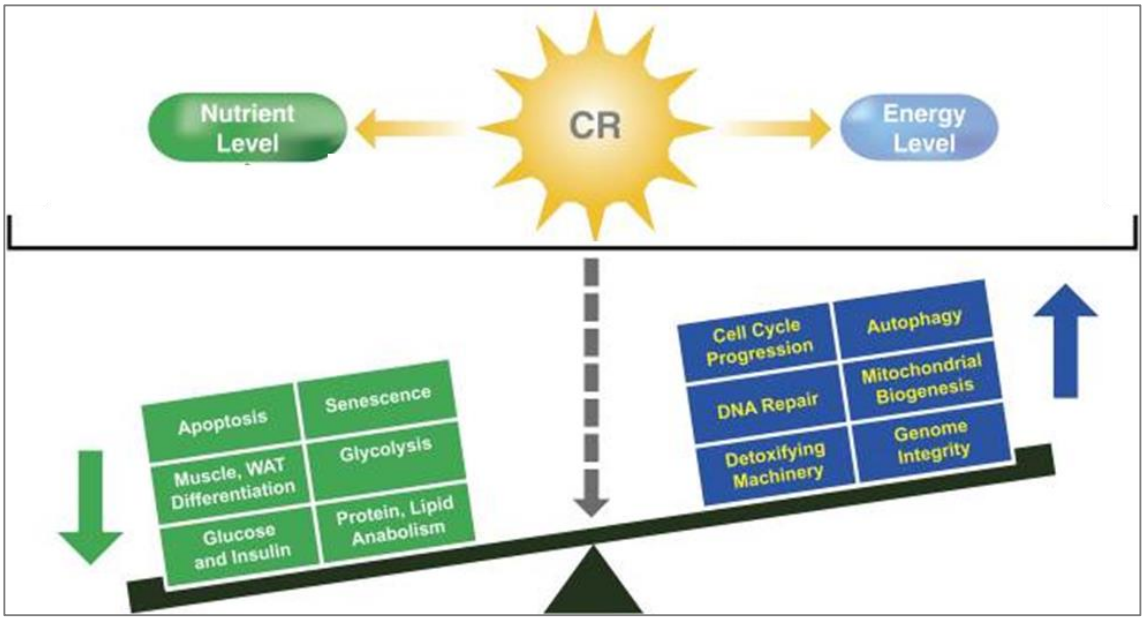


Figure 1.5 Summary of the processes activated by calorie restriction. Nutrient and energy levels signal to up-regulate stress response pathways. Figure adapted from Vaquero and Reinberg (Vaquero and Reinberg, 2009).

1.6.2 *Potential modes of action of DR*

DR has multifaceted effects on cells and organisms. It is believed to work by its action on nutrient and energy sensing pathways (Figure 1.5 and 1.6). One of these pathways is the mammalian target of rapamycin (TOR) signalling pathway (Kapahi *et al.*, 2010). Low nutrient levels down-regulate TOR kinase and its inhibition is associated with down-regulation of ribosomal biogenesis and the process of translation (Hansen *et al.*, 2007). Furthermore, deletions in the TOR pathway in yeast increase lifespan and DR is unable to further increase this effect (Kaeberlein *et al.*, 2005). DR may increase longevity by enhanced insulin sensitivity as mouse knock-out of growth hormone receptor increases lifespan in AL fed mice but not in DR fed animals (Coschigano *et al.*, 2003; Bonkowski *et al.*, 2006). The insulin/insulin-like growth factor is a hormone, which binds to its tyrosine-kinase receptor expressed on the cell membrane. Downstream it activates the protein kinase B (AKT) signalling pathway; inducing proliferation and cell growth and inhibiting apoptosis. A mutation in homologue of IGF in *C. elegans* and inhibition of the IGF pathway in *Drosophila* and mice all extend lifespan. Furthermore, in humans mutations in the receptor have been reported in cohorts of centenarians (Suh *et al.*, 2008). AMP kinase (AMPK) is also activated in low energy levels and expression of active form of protein kinase A extends lifespan via FOXO transcription factor in *C. elegans* (Greer *et al.*, 2007). Furthermore, DR is unable to extend lifespan in yeast cells not expressing Sir2 a NAD-dependant deacetylase, suggesting DR may in part act via the Sir2 pathway (Lin *et al.*, 2000). In CR mice, reduction of oxidative stress is mediated by SIRT3 activation of antioxidant SOD enzyme (Qiu *et al.*, 2010) It has been suggested that which pathway is activated likely depends on at what point and in which form DR is administered (Kenyon, 2010). Resveratrol has been termed a DR mimetic, in high doses it increases yeast lifespan through sir2 signalling (Howitz *et al.*, 2003). Resveratrol is also able to extend lifespan in nematodes, mice and fruit flies (Hector *et al.*, 2012). It prevents age-related DNA and RNA oxidation in non-human primates (Marchal *et al.*, 2013).

1.6.3 *Effects of DR on DNA repair*

DR causes tissue specific changes in DNA repair genes. Up-regulation of such DNA repair genes as is observed in mouse brain but down-regulation was seen in mouse muscle tissue (Lee *et al.*, 1999; Lee *et al.*, 2000). DR also influences

the DNA repair process. DR increases the BER-related synthesis in liver and kidney of mice (Stuart *et al.*, 2004). In rodents, DR ameliorates the age-related decline in unscheduled DNA synthesis in lymphocytes, hepatocytes, keratinocytes and kidney cells (Licastro *et al.*, 1988; Lipman *et al.*, 1989; Weraarchakul *et al.*, 1989). DR prevents the age-related decrease in G:U mismatch repair, β polymerase activity and 8-oxoguanine incision activity in the brain, liver, kidney and spleen of mice (Cabelof *et al.*, 2003; Langie *et al.*, 2011) and cyclobutane pyrimidine dimer removal in the liver of rats (Guo *et al.*, 1998). However, no effect of DR was observed on BER-related repair in lymphocytes (Gedik *et al.*, 2005) or UDG activity, AP endonuclease activity, 8-oxoguanine incision or 5-OH uracil and cytosine incision in the liver and kidney of mice (Stuart *et al.*, 2004). DR ameliorates the age-related decline in APE activity in the rat brain (Kisby *et al.*, 2010). It may enhance neuroprotection by increasing DNA repair activity; for example DR is able to prevent the age-related increase in cleaved PARP in the frontal cortex of rats (Hiona and Leeuwenburgh, 2004; Heydari *et al.*, 2007). In addition, it has been proposed that during cellular energy depletion (such as would be observed with DR), long-patch BER is the favoured pathway because it can generate ATP from poly ADP-ribosylation (Petermann *et al.*, 2003).

1.6.4 *Modulation of the epigenome by dietary restriction*

Environmental factors, including nutritional factors, can alter the epigenetic marks and molecules (Mathers *et al.*, 2010). Perhaps the best evidence of this comes from studies of epigenetic divergence observed in monozygotic twins (Fraga *et al.*, 2005). A number of studies have addressed the effect of nutritional modulation on epigenetic changes and this has been review by Mathers and Ford (Mathers and Ford, 2009). However, only a few have looked at the effect of dietary restriction on the epigenome.

A number of studies addressed gene specific changes after dietary restriction. Nutritional intervention by dietary restriction attenuates the age-related hypermethylation of the *c-myc* gene in mouse liver (Miyamura *et al.*, 1993) and *N-cadherin* gene in rat kidney (Akintola *et al.*, 2008). Furthermore, DR increases methylation and silences proto-oncogenes (Hass *et al.*, 1993). An *in vitro* model of DR by cell culture medium glucose restriction, shows that DR decreases H3 acetylation and H3K4 dimethylation (active marks) and increases H3K4

trimethylation (silencing mark) at *p16* promoter in cultured fibroblasts (Li and Tollefsbol, 2011). Furthermore, in this model DNA methylation was also observed at the *p16* promoter with glucose restriction (Li *et al.*, 2010). In humans, a low calorie diet given to obese individuals results in a decrease in DNA methylation at the *TNFα* promoter in blood mononuclear cells (Campión *et al.*, 2009). To my knowledge no study has addressed the effect of DR on global landscape of DNA methylation and histone modifications.

Although many of the effects of DR take place in the cytoplasm through phosphorylation of proteins, DR also affects chromatin (Figure 1.6). Dietary restriction may exert its longevity effects by promoting genomic stability, as it prevents the age-associated gene silencing (Jiang *et al.*, 2013). Reversal of aberrant methylation may be one of the mechanisms through which DR maintains genomic integrity. It may do so by activating enzymes responsible for generation of epigenetic marks. In fact, this hypothesis has great support from studies of Sirtuins. DR was not able to extend lifespan in yeast cells not expressing Sir2 (Lin *et al.*, 2000) suggesting that Sir2 and DR act within the same pathway. These NAD dependent histone deacetylases are known to play a role in DR function in yeast, worm, flies and mice (Guarente, 2013). Of note, the Sirtuins act as protein deacetylases and can therefore either affect protein activity directly (e.g. deacetylation of repair proteins) or indirectly via their epigenetic effect of histone deacetylation. The SIRT enzyme modulates chromatin by deacetylation of lysine residues on histone 1, 3 and 4 (Vaquero *et al.*, 2004; Vaquero *et al.*, 2006).Only a few studies have addressed the effect of DR on epigenetic enzymes other than Sirtuins. One group in particular is leading these studies. They found that DR attenuates the age-related increase of DNMT3a (Chouliaras *et al.*, 2011) and HDAC2 in the mouse hippocampus (Chouliaras *et al.*, 2013). Interestingly, changes in DNA methylation by DNMT enzymes can result from activation of this enzyme via SIRT1 as a number of genes were differentially methylated with *SIRT1* overexpression and knockdown in cultured cells (Ions *et al.*, 2012). Furthermore SIRT1 can also regulate the HAT p300 and HMT SUV39H1 (Bouras *et al.*, 2005; Vaquero *et al.*, 2007). Studies of DR mimetics have also shed some light on DR mediated chromatin effects. Rapamycin has been found to induce TET2 which binds to promoters and activates expression (Liu *et al.*, 2013). Resveratrol inhibits LSD1, a histone

demethylase, activity in cultured cells (Abdulla *et al.*, 2013) and activates SIRT1 in mouse tissues (Lagouge *et al.*, 2006). It also alters histone acetylation at the *BRCA1* promoter in cultured cells (Papoutsis *et al.*, 2010). SIRT6 deficient cells have increased susceptibility to BER-related lesions highlighting a role of SIRT6 in base excision repair (Mostoslavsky *et al.*, 2006). APEX is a target for SIRT1 deacetylation, which is important for APEX translocation (Lirussi *et al.*, 2012). In addition SIRT1 deacetylates APEX and hence resveratrol can activate SIRT1 and so promotes APEX activity (Yamamori *et al.*, 2010). Upon genotoxic stress APEX is acetylated and this is prevented by SIRT1, SIRT1 promotes APEX association with XRCC1 (Yamamori *et al.*, 2010).

In addition, DNA methylation and histone modifications are said to affect the ability of transcription factors (TFs) to bind at gene promoters. Transcription of the genes is dependent on transcription factor binding and DR may act by up-regulation of the expression or binding of transcription factors. Such an example is the FOXO family of transcription factors which are upregulated during DR and initiate a myriad of stress response genes (Nakae *et al.*, 2008). Interestingly, other transcription factors which increase with age such as NFκB, AP-1 and HIF1α are down-regulated by DR (Kim *et al.*, 2002). As such, TF-related gene expression may be either up or down-regulated via DR with the aid of epigenetic mechanisms.

Together some evidence exists for DR mediating genomic integrity in a gene-specific and global manner via several epigenetic mechanisms. However, there is still a lack of studies exploring this effect. Although DR enhances DNA repair activity, to date no studies have established the effect of DR on epigenetic regulation of BER repair genes.

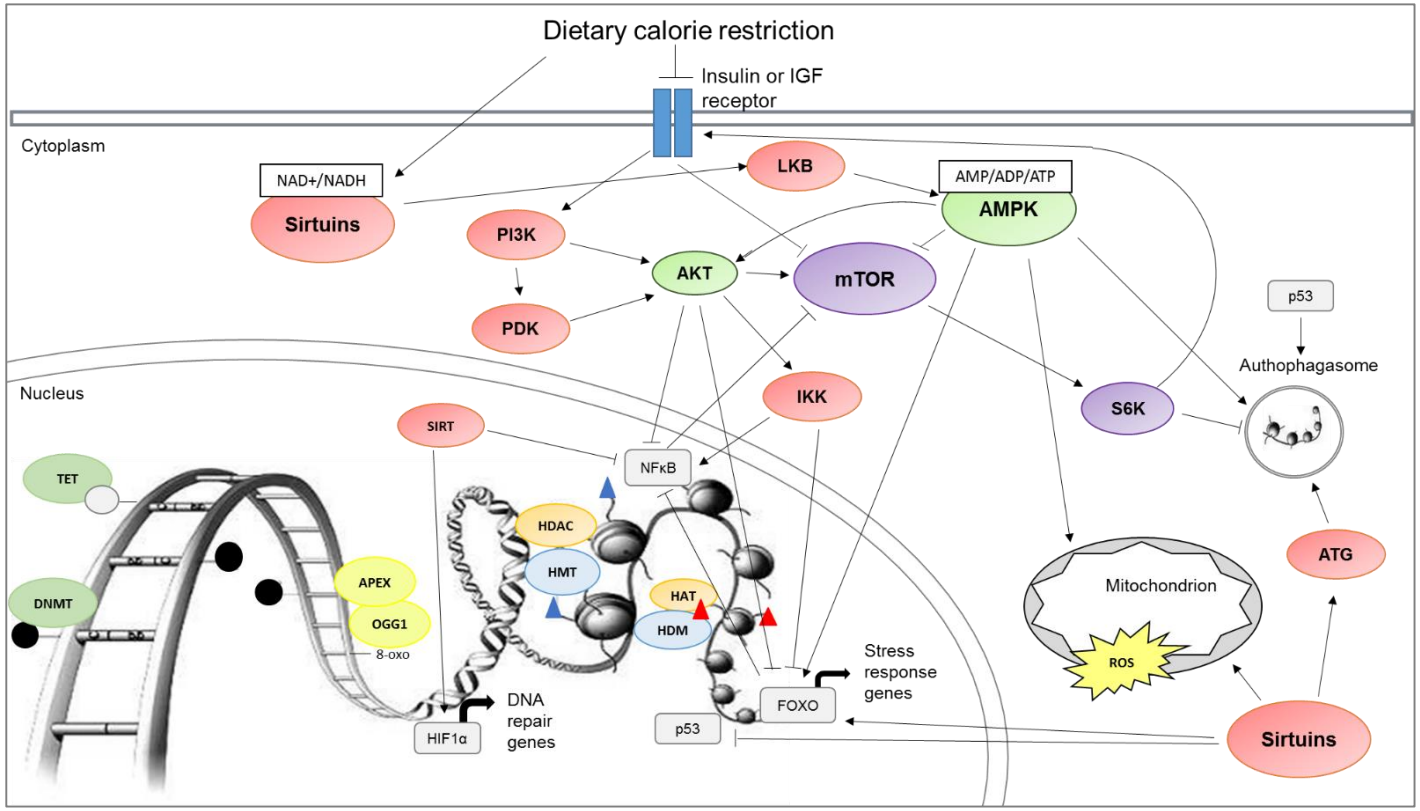


Figure 1.6 Molecular pathways influenced by dietary restriction. AKT, protein kinase B; AMPK, AMP-activated protein kinase; APEX, AP endonuclease; ATG, autophagy-related protein; DNMT, DNA methyltransferase; FOXO, forkhead box O; HAT, histone acetyltransferase; HDAC, histone deacetylase; HIF1α, hypoxia-inducible factor 1 alpha; HMD, histone demethylase; HMT, histone methyltransferase; IGF, insulin-like growth factor; IKK, Iκ kinase; LKB, serine/threonine kinase; mTOR, mammalian target of rapamycin; NFκB, nuclear factor kappa-light-chain-enhancer of activated B cells;OGG1, 8-oxoguanine DNA glycosylase; p53, tumour phosphoprotein 53; PDK, 3-phosphoinositide dependent protein kinase; PI3K, phosphatidylinositol-3-kinase; ROS, reactive oxygen species; S6K, ribosomal protein S6 kinase; SIRT, sirtuin; TET, ten-eleven translocation methylcytosine dioxygenase; red triangles, acetyl group; blue triangles, methyl group; black filled lollipop, methyl cytosine; grey-filled lollipop, hydroxymethyl cytosine. Figure adapted from (Vaquero and Reinberg, 2009; Langie et al., 2012).

1.7 Overview

Accumulation of damage to DNA is an integral feature of the ageing cell that results from exposure to damaging agents and reduced repair. Base excision repair is the primary mechanism for removal of small damaging lesions such as 8-oxoguanine. An age-related silencing of BER-genes could explain the inefficiency of DNA repair, which could have arisen due to deleterious epigenetic changes associated with BER genes. DR is a powerful tool in studies of ageing; it has been shown to affect many pathways by which it can exert its life extending properties. One such pathway is DNA repair. Although DR can evoke changes in expression of DNA repair-related genes the mechanisms responsible for these changes are poorly understood. DR causes transcription changes and some of these may be epigenetically mediated (Mathers, 2006). The purpose of this thesis was to measure the effects of age and DR on BER-gene promoter associated marks and the subsequent expression of these genes. Furthermore the effects of age and DR on functional activity of BER-related incision activity were also addressed.

1.8 Hypothesis, Aims and Objectives

For the purpose of the current thesis, I hypothesise that:

1. Base excision repair activity declines during ageing leading to accumulation of oxidation damage.
2. This age-related dysfunction is mediated through epigenetic mechanisms.
3. Dietary restriction ameliorates the decline in repair activity via effects on epigenetic regulation of BER gene expression.

The aim of this work is to test the above hypothesis and to further understanding of the role of epigenetic mechanisms in regulation of the age-related decline in DNA repair activity and its modulation by DR. This was accomplished through the following objectives:

a) To develop pyrosequencing assays to measure *Ogg1* and *Apex* promoter DNA methylation and to apply these assays to DNA from tissues from ageing and dietary restricted mice;

b) To develop ChIP based assays to measure histone acetylation and methylation changes corresponding to *Ogg1* and *Apex* promoters in tissues from ageing and dietary restricted mice;
c) To develop qPCR assays to measure mRNA expression of *Ogg1* and *Apex* in tissues from ageing and dietary restricted mice;
d) To use the comet-based *in vitro* repair assay to measure BER-related incision activity of oxidative damage in tissues from ageing and dietary restricted mice;
e) To quantify levels of 8-oxoguanine in brain and liver from ageing and DR mice;
f) To study correlations between epigenetic marks, gene expression, DNA repair activity and oxidative damage levels in tissues from ageing and DR mice;
g) To study the difference in epigenetic marks, gene expression, DNA repair activity and oxidative damage levels in tissues of different proliferative nature (brain vs. liver);
h) To determine if expression of transcription factors alters during ageing and dietary restriction;
i) To determine if epigenetic enzyme levels alter during ageing and dietary restriction;

Chapter 2: General Methods

2.1 Animal husbandry

Male and female mice were taken from a long-established colony of the inbred C57BL/6 (ICRFa) strain. This strain provides a good model of ageing as it is free from specific age-related pathologies. Mice were housed at 20±2ºC under a 12h light/12h dark photoperiod with lights on at 7am. All mice were provided with sawdust and paper bedding, standard rodent pelleted chow (CRM (P), Special Diets Services) and free access to water. Animals were subjected to either *ad libitum* (AL), 26% short-term dietary restriction (DR) or 40% long-term DR feeding and sacrificed at specific ages (details of individual animal study designs will be provided in further Chapters). Whole brain, liver, colon and lung tissues were collected, snap frozen in liquid nitrogen and stored at -80ºC until use.

2.2 Grinding of tissue samples

Tissue samples were ground under liquid nitrogen to give a homogenous mixture and to prevent tissue and macromolecule degradation. A tamper, mortar and a spatula were placed in the liquid nitrogen and allowed to cool. Pre-weighed micro-centrifuge tubes and tissue samples were placed on dry ice. The mortar was removed from the liquid nitrogen using tongs and a tissue sample was placed in the mortar. Quickly the tamper was removed from the liquid nitrogen with tongs and placed in the mortar. Using a hammer the tamper was tapped 3-4 times and then twisted while in the mortar to grind the tissue. The tamper was cleaned with a paper towel and placed back in the liquid nitrogen. Using a pre-chilled spatula the ground tissue was aliquoted into the pre-weighed micro-centrifuge tubes on dry-ice. The mortar was cleaned and placed back in the liquid nitrogen. Tubes containing ground tissue were weighed. This procedure was repeated for all tissues. Ground tissues were stored at -80˚C until further use.

2.3 Chemicals and oligonucleotides

All chemicals used for experimental procedures were purchased from Sigma Aldrich unless otherwise stated. Lyophilised oligonucleotides (primers) were purchased from Eurofins MWG Operon at 1µmol purified by HPSF or HPLC. Water used in preparation for buffers in protein assays was Millipure quality.

Nuclease-free water was used in DNA and RNA assays, furthermore buffers used in these assays were also passed through a filter prior to use.

2.4 Extraction of DNA

500µl of SET-SDS (50mM Tris-HCl, 12.5mM EDTA and 0.5% Sodium Dodecyl Sulphate) and 15µl proteinase K (20mg/ml, Thermo Scientific) were added to 30mg ground whole brain or liver tissue and incubated overnight shaking at 37˚C and 700 revolutions per minute (RPM). The following morning tissue was homogenised with a micro-centrifuge pestle and incubated for further 2 hours at 56˚C. Next, 100µl of 8M potassium acetate (KAc) was added to the tissue suspension and mixed by inversion for 2 minutes. 600µl chloroform:isoamylalcohol (IAA, 24:1) was added to the suspension and mixed by inversion for 10 min. Samples were centrifuged at 14,000RPM for 5 min and 400-450µl of the upper water phase was transferred to a new micro-centrifuge tube. 400-450µl chloroform:IAA was added and samples were mixed by inversion for 10 min. As before, samples were centrifuged at 14,000RPM for 5 min and 400-450µl of the top phase was transferred to a new tube. 20µl RNase A/T1 mix (2mg/ml RNase A and 5000U/ml T1 RNase, Thermo Scientific) was added to the sample and incubated for 30 min - 1 hour at 37˚C. 900µl of cold 100% ethanol were added to the samples and mixed by inversion for 2 min, and then incubated for 30min at -20˚C. Samples were centrifuged at 14,000RPM and the supernatant was discarded. The pellets were washed twice with 1ml 70% ethanol and samples centrifuged at 14,000RPM for 2min. The supernatant was discarded and pellets were dried on air and then eluted in approx. 30µl 2mM Tris-HCl, dissolved overnight on a shaker at 4˚C, and the following day concentrations were determined using Nanodrop 1000 (Thermo Scientific). Samples were diluted to 100ng/µl where appropriate and stored at -20˚C.

2.5 Pyrosequencing

Pyrosequencing of bisulphite converted DNA can be used to detect ratio of 5-methylcytosine to cytosine nucleotides in a genomic DNA sample. Bisulphite modification converts any non-methylated cytosine to a uracil nucleotide, whereas any methylated cytosine will remain unchanged. Uracil is complementary to adenine and will therefore be noted as thymine after polymerase chain reaction (PCR). Pyrosequencing is a sequence-by-synthesis reaction: a single nucleotide is added in succession to a reaction mixture. Upon

incorporation of a complementary nucleotide by the DNA polymerase a pyrophosphate is released. This is then converted into ATP by ATP sulfurylase using adenosine 5' phosphosulphate as a substrate. ATP drives a luciferase-based reaction which emits light. The amount of light released is proportional to the amount of base incorporated and detected by a charge-coupled device and displayed as a peak on the pyrogram. Apyrase degrades unincorporated nucleotides, so that another nucleotide can be added. The variable degrees of methylation can be analysed as C/T polymorphisms (Tost and Gut, 2007).

2.5.1 *Identification of promoter regions and CpG islands*

Sequences of the gene promoter regions under study (*Ogg1* and *Apex*) were identified using Genomatix Software. The Gene2Promoter function of this bioinformatics tool provides genomic data and only experimentally verified 5' complete transcripts were used for analysis. Sequences were downloaded in FASTA format and were then run through an online software, able to detect CpG Islands in a nucleotide sequence, to predict the site of the CpG islands within the promoter region; such a region was defined as being 200bp in length with greater than 50% GC content and 0.6 ratio of observed/expected CpG. Genomatix was used to analyse the sequences for transcription factor binding sites; TF binding sites containing a CG in the sequence and that were specific for brain and liver tissue were selected.

2.5.2 *PSQ primer design*

Bisulphite modification (BM) is used in the pyrosequencing reaction to distinguish between methylated and unmethylated cytosine nucleotides. Microsoft Office Word is used to predict the BM sequence. In a Word document, all CpG sites are converted to C/TG then, all Cs are converted to Ts and finally, all T/ sites are converted to C/ to generate *in-silico* sequences. These BM sequences were inserted into the PSQ Assay Design Software v 1.0.6 (Qiagen), to allow the software to recognise all the possible CpG sites. Primers were designed with the highest possible score to identify a PCR product <350bp and a sequencing region of 80-100bp long. The sequencing primer was placed as close to the sequence start as possible. The reverse primer was labelled with biotin on the 5' end.

2.5.3 *Bisulphite modification*

EZ DNA Methylation Gold Kit (Zymo Research) was used to bisulphite modify the DNA. As per kit instructions the CT conversion reagent was prepared by adding 900µl of nuclease-free water, 300µl of M-Dilution buffer and 50µl M-Dissolving buffer to each tube of CT conversion reagent, then shaken vigorously for 10min. 130µl of CT conversion reagent was added to 1µg DNA sample (in a 20µl volume). Samples were incubated at 98˚C for 10 min followed by 64˚C for 2.5 hours. Samples were then added to a Zymo-Spin IC column filled with 600µl of M-binding buffer and mixed by inversion. Samples were centrifuged at 13,000g for 30 seconds and the flow-through was discarded. 100µl of M-wash buffer was added to the column, centrifuged at 13,000g for 30 sec. 200µl of M-disulphonation buffer was added to each column and left to incubate for 20min at room temperature, columns were centrifuged at 13,000g for 30 sec. Columns were washed twice by adding 200µl M-wash buffer and spinning at 13,000g. After each centrifugation step the flow-through was discarded. Bisulphite modified DNA was eluted in 12µl of M-elution buffer by spinning columns at full speed for 1 min.

2.5.4 *Optimisation of pyrosequencing assays*

2.5.4.1 *PCR of promoter regions*

To optimise the pyrosequencing assays a nested-PCR is used to amplify the promoter region of a gene of interest. This product is then *in vitro* methylated and used to confirm specificity of primers designed by the PSQ software.

Primers were designed to amplify the whole promoter region, approximately 600bp. Using the nested PCR primers, whose sequences can be found in Table 2.1, for each region of interest 1µl of 100ng DNA was used in a PCR reaction with 12.5µl HotStart Taq Mastermix (Qiagen), 1µl each of forward and reverse primers at 10pmol and 9.5µl nuclease-free water. Firstly a gradient PCR was performed to determine the optimal annealing temperature for the primers with cycling conditions as shown in Table 2.2. The product was confirmed on a 1% agarose gel. Once the best annealing temperature was determined the nested PCR was performed with the optimal annealing temperature, shown in Table 2.1 and previously stated cycling conditions shown in Table 2.2.

Name	Sequence	T_a (˚C)
Ogg1 Fwd	5'-GGTTTATTTCTTGAGACAGAG	48
Ogg1 Rev	5'-TTTAATTTGAGTCCCTGTT	
Apex Fwd	5'-CCCCAGGTGTCTGACTCTTC	58
Apex Rev	5'-TTCGTTGGGAGGTCAGGTAC	

Table 2.1 Sequence and annealing temperatures of primers used for nested PCR.

Step	Temperature (˚C)	Time	Cycles
Enzyme Activation	95	15 min	
Denaturation	95	15 sec	30
Annealing	45-60	30 sec	
Extension	72	45 sec	
Elongation	72	10 min	

Table 2.2 Cycling program for nested PCR reactions.

2.5.4.2 *Generating methylated controls*

During the nested PCR reaction all methylation is wiped out creating a 0% methylated control. To create a 100% methylated product, 9µl of nested PCR product was incubated with 3µl S-adenosylmethionine (SAM, 1:8 dilution in water), 2µl NEB2 buffer, 1.5µl CpG Methyltransferase (enzymes obtained from New England BioLabs) at 30˚C for 16 hours. To check the efficiency of *in vitro* methylation a single digestion with HpaII Methyltransferase enzyme (New England BioLabs) was performed. This enzyme is methylation specific and only cleaves unmethylated CCGG sequence. 2µl NEB1 buffer, 0.5µl HpaII and 9.5µl of nuclease-free water were mixed with 8µl of either 0% or 100% DNA and incubated in a water bath at 37˚C for 2 hours. The product was run on a 1% agarose gel, 80V, 30 min and resulting gel compared with a virtual gel generated by NEB Cutter software, from New England BioLabs website, to confirm methylation.

2.5.4.3 *Bisulphite modification of methylated controls*

BM was performed as described in section 2.3 except that when adding the template 5µl of 0% control and 15µl of water or 10µl of 100% control and 10µl of water were added to the CT conversion reagent, the remaining steps of the reaction were the same.

2.5.4.4 *Pyro PCR*

A Pyro-PCR was set up: 15µl HotStart Taq Mastermix (Qiagen), 1µl each of forward and biotinylated reverse primers at 10pmol (sequences can be found in Table 2.3), 9.5µl nuclease-free water and 1µl BM DNA product. A gradient PCR

was performed with cycling conditions as shown in Table 2.4 to determine optimal annealing temperatures. The product was confirmed on a 1% agarose gel after electrophoresis at 80V for 30 minutes.

Mixes of 0% and 100% *in vitro* methylated samples were used to give a dilution series of 0%, 5%, 10%, 25%, 50%, 75%, 90%, 95% and 100% and Pyro-PCR was performed for each methylation dilution using the cycling conditions from Table 2.4 and annealing temperature as seen in Table 2.3.This made up a pre dilution series. Another dilution series was made from 0% and 100% methylated product after the pyro-PCR to make up the post pyro-PCR dilution series.

Name	Sequence	T_a (˚C)
Ogg1 Fwd1	5'-GGTTTATTTTTTGAGATAGA	42
Ogg1 Rev1	5'BIO-ACTAAAACCACATCATTA	
Ogg1 Seq1	5'-TTTAGTTAAGTTTTAAA	-
Ogg1 Fwd2	5'-GTAGGTTTTGAGATTGTAT	44
Ogg1 Rev2	5'BIO-ATTTAACCCTAAAAATAAC	
Ogg1 Seq2	5'-GAAAGTTTTGAAATGGTAGA	-
Apex1 Fwd1/2	5'-GGATAGAATAAAATTGGATAAG	53
Apex1 Rev1/2	5'BIO-TTAATCCTCCTAACACCTC	
Apex1 Seq1	5'-GAATAAAATTGGATAAGG	-
Apex1 Seq2	5'-GTTGTTTTAGAATTTTAGTA	-

Table 2.3 Sequence and annealing temperatures of primers used in Pyro-PCR.

Step	Temperature (˚C)	Time	Cycles
Enzyme Activation	95	15 min	
Denaturation	95	15 sec	50
Annealing	40-50	30 sec	
Extension	72	45 sec	
Elongation	72	10 min	

Table 2.4 Cycling program for Pyro-PCR reactions.

2.5.4.5 *Pyrosequencing of BM DNA controls*

Both the pre and post dilution series were used as products for the pyrosequencing reaction. Biotinylated PCR products were immobilised on streptavidin-coated sepharose beads (GE Healthcare) in a 96 well PCR plate, 5-10µl of pyro PCR product was added to 70µl bead solution (2µl beads, 38µl Qiagen Binding buffer and 30µl nuclease-free water) in duplicate, covered and shaken for 10 min. Pyrosequencing plate (Qiagen) was prepared by adding 0.5µl 10pmol sequencing primer and 11.5µl Annealing buffer (Qiagen) to corresponding wells in the plate. Using a prep tool, biotinylated PCR product bound to streptavidin beads was aspirated from the wells, passed through denaturing buffer (0.2M NaOH), 70% ethanol and wash buffer (Qiagen

containing 10mM Tris, pH 7.6). The resulting single stranded DNA was released into the pyrosequencing plate which was then incubated at 80˚C for 2 minutes, allowing the plate to cool permitted the sequencing primer to anneal (sequences of primers can be found in Table 2.3). The pyrosequencing reaction was performed on a PyroMark MD System (Qiagen). Pyro Q CpG software version 1.0.6 (Qiagen) was used to quantify the percentage methylation.

The resultant methylation from the dilution series was plotted on a scatter graph against the expected methylation percentages. A good assay showed a linear trend with r^2 above 0.8.

2.5.5 *Methylation analysis of DNA from brain and liver*

Once the pyrosequencing assays were optimised the methylation analysis was performed on bisulphite modified DNA samples from brain and liver. A Pyro-PCR was set up: 15µl HotStart Taq Mastermix (Qiagen), 1µl each of forward and biotinylated reverse primers at 10pmol (sequences can be found in Table 2.3), 9.5µl nuclease-free water and 1µl BM DNA product. Pyro-PCR was performed with cycling conditions as shown in Table 2.4. The product was confirmed on a 1% agarose gel. Biotynylated PCR products were analysed on the pyrosequencer as described in section 2.5.4.5.

2.6 Extraction of RNA

A 20-30mg aliquot of ground tissue was thawed on ice. 500µl cold TRIzol (Invitrogen) was added to each sample and vortexed. A micro-centrifuge pestle was used to homogenise the tissue for 1 min. Another 500µl of TRIzol was added to each sample and vortexed. Samples were then left to incubate at room temperature for 5 min and centrifuged at 12,000g for 10 min at 4˚C. The supernatant was transferred to an RNase free tube (Ambion) and 100µl 1-bromo-3-chloropropane (BCP) was added to each tube. Samples were shaken for 15 seconds, incubated for 10 min at room temperature, shaken for 15 seconds again and centrifuged at 12,000g for 10 min at 4˚C. 400µl of the top water phase was transferred to a new RNase free tube and RNA clean-up was carried out using an E.Z.N.A. Total RNA Kit (Omega, VWR) according to manufacturer's protocol. 200µl of kit TRK lysis buffer and 300µl 100% ethanol were added to the aqueous phase and mixed by inversion. The solution was loaded onto an E.Z.N.A. spin column and centrifuged at 10,000g for 1 minute

and flow-through was discarded. 350µl kit RNA Wash Buffer I was added to the column, centrifuged at 10,000g and flow-through discarded. This step was then repeated. 500µl kit RNA Wash Buffer II was added to the column, centrifuged at 10,000g and flow-through was discarded. This step was repeated and column was spun at 13,000g to remove any remaining buffer. Finally, RNA was eluted in RNA storage solution (Ambion) and quantified using Nanodrop 1000. RNA with 260/280 and 260/230 values above 1.8 were used for further experiments. RNA degradation was determined by running the RNA template on 1% agarose gel for 30 min at 80V.

2.7 Complementary DNA synthesis

To remove genomic DNA, 500ng of RNA was added to 1µl DNase I, 1µl 10X kit reaction buffer (Thermo Scientific) and made up to a final volume of 10µl with nuclease-free water and incubated at 37˚C for 30 min. Each sample was run in duplicate (one as a reverse transcriptase (RT) positive and one RT negative). 1µl 50mM EDTA was added to the reaction and incubated further for 10 min at 65˚C. Complementary DNA (cDNA) was synthesised using RivertAid H Minus First Strand cDNA Synthesis Kit (Thermo Scientific): 1µl oligo(dT)18 primer, 4µl 5X reaction buffer, 1µl RiboLock RNase Inhibitor, 2µl 10mM dNTP Mix and either 1µl RevertAid H Minus M-MulV Reverse Transcriptase (RT positive) or 1µl nuclease-free water (RT negative). Reactions were incubated at 45˚C for 60 min and 70˚C for 5 min. Confirmation of cDNA synthesis was performed by running a control PCR for the *GAPDH* gene (forward primer 5'-CAAGGTCATCCATGACAACTTTG and reverse primer 5'-GTCCACCACCCTGTTGCTGTAG). 5µl cDNA (diluted 1:200), 12.5µl HotStart Taq Master Mix (Qiagen), 0.25µl forward primer and reverse primer each at 10pmol/µl and 7µl nuclease-free water were mixed and run in a PCR reaction as shown in Table 2.5.

Step	Temperature (˚C)	Time	Cycles
Enzyme Activation	95	15 min	
Denaturation	95	15 sec	30
Annealing	58	30 sec	
Extension	72	30 sec	
Elongation	72	5 min	

Table 2.5. Cycling program for GAPDH control PCR for reverse transcription.

Presence of a strong product at 495bp on a 1% agarose gel confirmed if cDNA synthesis had worked, and no product in the RT negative samples indicated no genomic DNA contamination.

2.8 Quantitative PCR

Reverse-transcription quantitative polymerase reaction (RT-qPCR) has become the gold standard for measuring mRNA levels in molecular biology. This highly sensitive method has been used in many applications to show changes in gene expression.

2.8.1 *QPCR with SYBR Green*

Ogg1 and *Apex* gene expression was measured using qPCR with SYBR Green dye for fluorescence measurements. SYBR Green is a dye commonly used in qPCR because it emits a fluorescent signal when bound to double-stranded DNA.

2.8.1.1 *Primer design and validation*

Primers were designed using Primer3 online primer design software. Complementary DNA sequence was obtained from NCBI. Primers were designed to give a product size of 90-110bp with melting temperature of 59-61˚C. UCSC website was used to check that primers did not bind at non-specific sites and NetPrimer program was used to check that the primers did not or to a minor extend form duplexes or hairpin loops.

A standard curve for each primer assay was run with 1/3rd dilutions of sample cDNA to check for efficiency of the reaction. The reaction contained 12.5µl Maxima SYBR Green Master Mix (Thermo Scientific), 0.75µl of each forward and reverse primer at 10pmol/µl, 6µl nuclease-free water and 5µl cDNA. Primer sequences and cycling conditions can be found in Table 2.6 and Table 2.7 respectively. Assays given an efficiency 100±10% i.e. product amount doubling during each cycle, were required for an optimal PCR reaction.

Name	Primer Sequence	Product Size (bp)
Ogg1 Fwd	5'-TGGCTTCCCAAACCTCCAT	65
Ogg1 Rev	5'-GGCCCAACTTCCTCAGGTG	
Apex Fwd	5-'CCTAAGGGCTTTCGTCACAG	108
Apex Rev	5'-GCTGCCCCCTTACTCTTCTT	
Hprt Fwd	5'-AGGAGAGAAAGATGTGATTGATATT	98
Hprt Rev	5'-TCCACTGAGCAAAACCTCTT	
B2m Fwd	5'-ATGCTGAAGAACGGGAAAAAAA	93
B2m Rev	5'-CAGTGTGAGCCAGGATATAGAA	

Table 2.6 Primer Sequences for qPCR.

2.8.1.2 *Gene expression in samples of interest*

Complementary DNA was diluted 1:5 with nuclease free water. 25µl or 15µl reactions were set up in triplicate (depending which real-time instrument was used) as described in Table 2.7. Comparative delta-delta Ct program was used with cycling conditions as described in Table 2.8. Roche Light Cycler 480 instrument was used for samples run in short-term DR study (Chapter 4), while Life Technologies Step One Plus real time instrument was used for samples from the long-term DR study (Chapter 5-7).

Reaction Component	Light Cycler 480	StepOne Plus
	25µl reaction	**15µl reaction**
SYBR Green Master Mix	12.5µl	7.5µl
Forward Primer	0.75µl	0.45µl
Reverse Primer	0.75µl	0.45µl
ROX	-	0.03µl
Nuclease-free water	6µl	3.57µl
cDNA	5µl	3µl

Table 2.7. Reaction set-up for qPCR.

Step	Light Cycler 480			StepOne Plus		
	Temp (˚C)	**Time**	**Cycles**	**Temp (˚C)**	**Time**	**Cycles**
Enzyme Activation	95	15 min		95	15 min	
Denaturation	95	15 sec	40	95	15 sec	40
Annealing and Extension	60	1 min		60	1 min	
Melt curve	95 65 97	5 sec 1 min continuous		95 65+0.5 increments 95	15 sec 1 min 15 sec	

Table 2.8. Cycling conditions for qPCR reaction.

The instrument's software was used for analysis of melt curves and quality of data. Gene expression was determined using the equation outlined below,

where ΔCt represents Ct $_{\text{average reference gene}}$ – Ct $_{\text{target gene}}$. This equation was used instead of ΔΔCt method as no true control group was present in the study.

$$Expression = 2^{-\Delta Ct}$$

2.8.2 *QPCR with TaqMan assay*

Transcription factor (*Hif1α* and *Nfya*) gene expression was measured using the TaqMan assay (Life Technologies). These predesigned assays contain the forward and reverse primers as well as a probe with a fluorescent dye label. The probe is target specific which means a fluorescent signal will only be released when the target DNA is amplified.

cDNA was diluted 5X and 15µl reactions were set up as shown in Table 2.9. The list of TaqMan assays used is provided in Table 2.10. Each sample was run in duplicate with *B2m* and *Hprt* used as reference genes. As with SYBR Green qPCR reactions, gene expression was measured using the $2^{-\Delta Ct}$ method.

Reaction Component	15µl reaction
20X TaqMan Assay	0.75µl
2X TaqMan Mastermix	7.5µl
Nuclease-free water	3.75µl
cDNA	3µl

Table 2.9 qPCR reaction set up for TF TaqMan assays.

Assay	Gene	Code
Hif1α	murine hypoxia inducible factor 1, alpha subunit	Mm00468869_m1
Nfya	murine nuclear transcription factor-Y alpha	Mm00477820_m1
B2m	murine beta-2 microglobulin	Mm00437762_m1
Hprt	murine hypoxanthine guanine phosphoribosyl transferase	Mm01545399_m1

Table 2.10 Inventory of TaqMan assays.

2.9 Extraction of proteins

2.9.1 *Extraction of a crude protein extract*

A crude protein extract can be used for the comet based assay, the ELISA and enzyme activity assays. A 20-30mg aliquot of ground tissue was thawed. 75µl of buffer A (45mM Hepes, 0.4M KCl, 1mM EDTA, 0.1mM DTT and 10% Glycerol, pH 7.8) was added per 25mg of tissue, vortexed thoroughly and snap frozen in liquid nitrogen. The sample was thawed and 22.5µl buffer A1 (0.1% Triton X-100 in buffer A) was added per 25mg of tissue, vortexed thoroughly and incubated on ice for 5-10 min. Each sample was then homogenized using a

micro-centrifuge pestle, vortexed thoroughly and incubated on ice for a further 5-10 min. Samples were centrifuged at 14,000g and 4˚C for 5 min, supernatant was collected, aliquoted and stored at -80˚C until required.

2.9.2 *Protein quantification*

Bovine serum albumin (BSA) standard curve was prepared in buffer A2 (0.0023% Triton X-100 in buffer A) at concentration ranging from 0mg/ml to 4mg/ml. An aliquot of protein extract from each tissue sample was diluted 10 times in buffer A2. BIO-RAD DC Protein assay was used for the colorimetric reaction. Kit reagent A and S were used to make reagent A', to adjust for the Triton X-100 present in the buffer. Reaction was prepared by adding sample/control, buffer A' and buffer B from the kit in a ratio 1:5:40. Samples were incubated at room temperature in the dark for 15 min. Absorbance was measured at 650nm using either Lowry program on Nanodrop 1000 in duplicate or the FLUOstar Omega Microplate reader in triplicate.

2.10 Comet-based in vitro repair assay

The comet assay was firstly modified to asses base excision repair (BER) in mononuclear cells (Collins *et al.*, 2001). This assay quantifies the ability of a lymphocyte protein extract to carry out the first incision step of the BER process on a DNA substrate containing specific DNA lesions. Since then the protocol has been adapted for use with mouse tissue protein extracts to look at DNA incision of singlet oxygen-induced DNA damage (Langie *et al.*, 2011). This technique has been used in the current project and was further optimised in Chapter 3.

2.10.1 *Cell culture and preparation of substrate cells*

HeLa cells were grown in 10% Foetal calf serum Dulbecco's Modified Eagle's Medium (FCS DMEM) with 1% Pencillin/Streptamycin in 175cm^3 flasks (Greiner Bio-One) until near confluence. Media was fully removed and cells washed once with PBS. Next, 25ml of cold 1μM Ro 19-8022 (Ro cells) or PBS (noRo cells) was added to the flask and exposed to light on ice, 33cm from 500W halogen lamp for 5 min (under these conditions Ro 19-8022 induces DNA damage in form of primarily 8-oxoguanine (Will *et al.*, 1999), Ro 19-8022 obtained from Roche). PBS or Ro 19-8022 were removed and cells washed once with PBS. Cell were trypsinised for 5 min at 37˚C, trypsin was inactivated

with FCS DMEM and cells centrifuged at low speed, 400g at 4˚C for 5 min. Cells were gently re-suspended in DMEM at $6x10^6$ cell/ml. The same volume of cold freezing media (20% DMSO in DMEM) was added to give a final concentration of $3x10^6$ cell/ml (in DMEM plus 10% DMSO). Cells were slowly frozen in a thick walled polystyrene box as aliquots of 200µl and stored at -80˚C.

2.10.2 *Embedding cells in gels*

An aliquot of $3x10^6$ cell/ml noRo and Ro cells were washed with cold PBS and centrifuged at 700g, 4˚C for 5 min. Cells were re-suspended in 3ml of 0.7% low melting point agarose (LMP), 70µl gels were made by pipetting onto a glass slide (pre-coated with 1% agarose) and covered with a 22mm x 22mm cover slips (Leica Microsystems), 2 gels were set per slide (1 containing noRo cells and other containing Ro cells). Gels were left to set for 10 min at 4˚C, after which cover slips were removed. Slides were placed back to back in a Coplin jar and cells were incubated in lysis buffer (2.5M NaCl, 100mM EDTA, 10mM Tris, 250mM NaOH at pH10; prior to use 20ml DMSO and 2ml Triton X-100 was added to 180ml of lysis buffer) for at least 1 hour.

2.10.3 *Incubation with protein extract*

After lysis, only the nucleoids of the noRo and Ro gel-embedded cells remain. Slides were washed twice with cold buffer F (40mM Hepes, 0.1M KCl, 0.5mM EDTA, 0.2mg/ml BSA at pH 8). Protein extracts from tissues were diluted to the required concentration (specified in the following Chapters) in buffer A2 to a volume of 50µl, 4 volumes of buffer F and Aphidicolin at 1.5µM final concentration were added. Formamidopyrimidine DNA glycosylase (FPG, New England Biolabs) was used as a positive control at a 1/3000 dilution in buffer F. Slides were placed on a cold plate and 100µl of the prepared protein extract was added to each slide and covered with a large cover slip 24mm x 50mm (Leica Microsystems) so that extract covers both gels. Slides were incubated in a humid chamber (Boekel Slide Moat) for 25 min at 37˚C.

2.10.4 *Single cell gel electrophoresis*

Slides were placed in electrophoresis tanks (Thistle Scientific) with the frosted label edge towards the cathode (negatively charged DNA moves towards the anode). Electrophoresis buffer (300mM NaOH, 1mM EDTA at pH>12) was

added to each tank (required volume of buffer was added to reach 1V/cm over the platform during electrophoresis). Slides containing nucleoids were left to denature/unwind in the alkaline buffer for 40 min at 4˚C before being subject to electrophoresis for 30 min at 25V and 4˚C. Slides were placed in the slide holder and washed once with PBS for 10 min and once with Millipore water for 10 min.

2.10.5 *Staining and comet visualisation*

Slides were placed in a humid box and 500µl of 1X SYBR Gold (Life Technologies) diluted in pH 8 TBE buffer (Life Technologies) was added to each slide. Slides were left to stain for 40 min after which they were washed 2 times for 10 min with Millipore water. Gels were left to dry overnight. Comets were visualised using an Olympus BX51 fluorescence microscope and the Comet Assay IV software (Perceptive Instruments). 50 comets were scored per gel.

2.10.6 *Calculation of DNA incision activity*

Tail Intensity (% DNA in Tail) were used to represent the quantification of single strand breaks in substrate nucleoids. DNA repair measured as DNA incision activity was calculated using the following equation, after subtracting background levels (TI from noRo cells/buffer incubation) from all values:

$$\begin{aligned} BER-related\ incision\ activity \\ &= TI\left({}^{Ro\ nucleoids}/_{extract\ incubation}\right) \\ &- TI\left({}^{noRo\ nucleoids}/_{extract\ incubation}\right) \\ &- TI\left({}^{Ro\ nucleoids}/_{buffer\ incubation}\right) \end{aligned}$$

2.11 ELISA

To measure specific concentration of DNA repair enzymes, Enzyme-linked Immunosorbent Assay Kits for murine Oxoguanine Glycosylase 1 and APEX Nuclease 1 were obtained from Uscn Inc. (Antibodies-Online). Assays were performed as described in the manufacturer's protocol. Kit standards were prepared in doubling dilution using Standard Diluent for OGG1 (2,000pg/ml to 31.2pg/ml) and APEX (10ng/ml to 0.156ng/ml). Samples were diluted to 1mg/ml in biology-grade water. For each kit the steps were the same. 100µl of blank

(standard diluent), standard and samples were added to a corresponding well of the ELISA plate pre-coated with antibody, covered with a plate sealer and incubated for 2 hours at 37˚C. Liquid was removed from the plate and 100µl of Detection Reagent A was added to each well, the plate was covered and incubated for 1 hour at 37˚C. Liquid was removed and wells washed 3 times with 350µl of Wash solution; in between each step, the plate was snapped onto absorbent paper to remove all liquid. 100µl of Detection Reagent B was then added, plate was sealed and incubated for 30 minutes at 37˚C. Liquid was removed and the wells washed 5 times with 350µl Wash solution. 90µl of Substrate solution was added to each well, plate was covered and incubated for 25 min at 37˚C. 50µl Stop solution was added to each well and mixed by tapping the side of the plate, addition of this solution turned the reaction yellow. To quantify OGG1 and APEX protein concentration, the absorbance was read at 450-480nm using a FLUOstar Omega Microplate reader. Standard curve was constructed and protein concentrations for each sample were extrapolated from the graph.

2.12 Enzyme activity assays

2.12.1 *HAT activity assay*

Histone Acetyltransferase Activity Assay Kit (Abcam) was used to measure HAT activity as described in the manufacturer's protocol. Test samples were prepared at 2.5 mg/ml protein in 40µl reaction (diluted with water). Kit HeLa cell nuclear extract was used as a positive control at 1mg/ml in a 40µl reaction. In a 96-well flat bottomed plate (Corning), 50µl 2X HAT Assay Buffer, 5µl HAT Substrate I, 5µl HAT Substrate II and 8µl NADH Generating Enzyme were added to 40µl of each sample crude protein extract. The plate was incubated at 37˚C for 4 hours. After incubation the absorbance was read at 440nm using the FLUOstar Omega Microplate reader. Activity was calculated as OD440nm/µg protein.

2.12.2 *HDAC activity assay*

Histone Deacetylase Assay Kit (Merck Millipore) was used to measure HDAC activity as described in the manufacturer's protocol. Test samples were prepared at 10mg/ml in 20µl reaction.10µl of 2X HDAC Assay Buffer was added per well in a 96 well plate (flat bottomed). 20µl of sample protein extract or kit HeLa nuclear extract (2µg/µl) was added to the corresponding well and plate

was allowed to equilibrate to 37˚C. 10µl of 4mM HDAC Assay Substrate was then added to each well, mixed thoroughly and incubated at 37˚ for 1 hour. 20µl of Activator Solution was added to each well, mixed and incubated at room temperature for 10-20minutes. After incubation the absorbance was read at 405nm using the FLUOstar Omega Microplate reader, samples were analysed in duplicate. Activity was calculated as OD405nm/µg protein.

2.13 Chromatin Immunoprecipitation

The principle of chromatin immunoprecipitation (ChIP) is the selective enrichment of a chromatin fraction containing a specific antigen. Antibodies that recognise protein modification of interest can be used to determine the relative abundance of that antigen at one or more loci in the genome. The chromatin immunoprecipitation assay was used to study epigenetic histone modifications relative to the promoter regions of BER-related gene. ChIP was performed for brain and liver tissue from 3 month old AL, 30 month old AL and 30 month old DR fed animals.

2.13.1 *Chromatin crosslinking*

10ml of ice-cold PBS containing protease inhibitors (Sigma Aldrich) was poured into a 10cm diameter petri dish. A 100 micron cell strainer (BD biosciences) was placed at the centre of the petri dish, so that it was immersed in the solution. 50-100mg ground tissue was placed in the strainer and sieved with the help of a syringe plunger. The content of the petri dish was poured into a 15ml falcon tube and placed on ice. 37% Formaldehyde was added to the cell suspension (1% final volume), placed on a rocker and left to cross-link for 3-4 minutes. 1/10th 1.25M glycine was added to the mixture (final concentration 0.125M) to quench the formaldehyde and mixture was kept on rocker for further 1 minute. Cell suspension was then centrifuged at 1,200RPM for 4 min at 4˚C and supernatant was discarded. Cells were lysed with lysis buffer (1% SDS, 10mM EDTA, 50mM Tris-HCl pH 8.1 and protease inhibitors; 800µl for liver and 300µl for brain tissue) and incubated on ice for 20 minutes. Samples were sonicated on ice, sonicator was set to high with program 30sec ON, 20sec OFF, for 10 cycles. Samples were incubated on ice for few minutes then centrifuged at 13,000g for 10 min at 4˚C, the collected supernatant contained the cross-linked DNA.

2.13.2 *Immunoprecipitation*

Chromatin was quantified using Nanodrop (DNA quantification setting), 25µg of chromatin was used in subsequent reactions. 1 tube per sample per antibody was set up. Chromatin was diluted 1:10 in buffer (1.1% Triton X-100, 1.2mM EDTA, 16.7mM Tris-HCl pH 8.1, 167mM NaCl and protease inhibitors). 10µl blocked Staph A cell (or protein A/G mix, Merck Milipore) were added to the chromatin for pre-clearing for 30 min at 4˚C. Samples were centrifuged at 6,000RPM for 5 min and the supernatant used for the immunoprecipitation (IP) reaction. 3µg of corresponding antibody (Anti-IgG, Abcam; Anti-H4Ac acetylation on lysines 5, 8, 12 and 16, Merck Milipore; Anti-H3K27Me3, Merck Milipore) was added to the sample and incubated with rotation over night at 4˚C. The next day 50µl blocked Staph A (or protein A/G mix) were added to each sample and incubated further for 1 hour at 4˚C followed by 30 minutes at room temperature. Samples were centrifuged at 6,000RPM for 5 min at 4˚C and the supernatant was discarded. The pellet was re-suspended in 1ml cold low salt buffer (1% Triton X-100, 2mM EDTA, 20mM Tris-HCl pH 8.1, 150mM NaCl and protease inhibitors), samples were rotated on a wheel for 5 min at RT and then spun at 6,000RPM for 2 min, the supernatant was discarded. Pellets were re-suspended in 1ml of cold high salt buffer (1% Triton X-100, 2mM EDTA, 20mM Tris-HCl, pH 8.1, 500mM NaCl and protease inhibitors), samples were rotated on a wheel for 5 min at RT and then spun at 6000RPM for 2 min, and the supernatant was discarded. Pellets were washed in lithium chloride wash buffer (0.25M LiCl, 1%NP Igepal, 1mM EDTA, 10mM Tris-HCl pH 8.1 and protease inhibitors), samples were rotated on a wheel for 5 min at RT and then spun at 6000RPM for 2 min, and the supernatant was discarded. Pellets were washed twice with 1ml Tris-EDTA buffer, samples were rotated on a wheel for 5 min at RT and then spun at 6000RPM for 2 min, and the supernatant was discarded. Pellets were re-suspended in 500µl elution buffer (100mM sodium bicarbonate and 1% SDS), vortexed and incubated at room temperature with rotation for 15-20 min. Samples were centrifuged at 13,000g for 10 min and supernatant was transferred to a new tube. 20µl of 5M NaCl was added to each 500µl sample and incubated at 65˚C for 4 hours to reverse the cross-links.

2.13.3 *DNA clean-up*

10µl 0.5M EDTA, 2µl 1M Tris-HCl pH 6.5 and 2µl 10mg/ml proteinase K (Thermo Scientific) was added to each sample and incubated at 45˚C for 1 hour. DNA was extracted by adding equal volume of phenol:chloroform:isoamylalcohol to each sample, vortexing and spinning at 13,000g for 5 min. The top layer was transferred to a new tube and DNA was cleaned up using QIAQuick Gel Extraction Kit (Qiagen) according to manufacturer's protocol. 200µl isopropanol was added to each sample and incubated for 15 min at room temperature. 300µl kit solubilisation buffer and 4µl of 3M sodium acetate were added to each sample. Samples were placed in columns and centrifuged at 13,000g for 1 min, flow-through was discarded. 750µl kit Buffer PE was added to each column to wash the DNA, centrifuged and flow-through was discarded. Columns were spun at full speed for 1 min to remove residual liquid. The columns were then placed in silicone 1.5ml centrifuge tubes, 50µl buffer EB (30µl for brain sample) was added to each column and centrifuged at full speed for 1 min. The eluted DNA was used in the qPCR reaction.

2.13.4 *ChIP-qPCR*

SYBR Green technology was used in the ChIP-qPCR assay. Firstly a standard curve was set up with genomic DNA to validate the primer sets (primer sequences can be found in Table 2.11). Reaction set up is shown in Table 2.12. Once the primers were validated they were used to set up the qPCR mix with DNA from gDNA from the ChIP reaction. Amplification of enriched regions in the *Ogg1* and *Apex* promoters was performed using the StepOne Plus instrument (Applied Biosystems) with cycling conditions as described in Table 2.13. Fold enrichment was calculated compared to the 3AL group.

Name	Sequence
Ogg1 Fwd	5'-CCCAAAGTGCTGGGATTAAA
Ogg1 Rev	5'-AGTTCACTCCGTCCAAATGC
Apex Fwd	5'-GCTGCCTCGGAACACATATT
Apex Rev	5'-CTAGGTCACGTGGGTTCGTT

Table 2.11 Primers used for ChIP-qPCR.

Reaction Component	13µl reaction
SYBR Green Master Mix	6.5µl
Forward Primer	0.5µl
Reverse Primer	0.5µl
ROX	0.027µl

Nuclease-free water	3.473µl
gDNA	2µl

Table 2.12 Reaction set up for ChIP-qPCR with genomic DNA.

Step	**StepOne Plus**		
	Temperature (˚C)	**Time**	**Cycles**
Enzyme Activation	95˚C	15 min	
Denaturation	95˚C	15 sec	45
Annealing and Extension	55˚C	1 min	
Melt curve	95˚C 65˚C+0.5˚ increments 95˚C	15 sec 1 min 15 sec	

Table 2.13 qPCR cycling conditions for ChIP-qPCR.

2.14 HPLC-ECD

The base oxidation product 8-oxoguanine (8-oxodG) was detected by HPLC coupled with electrochemical detection. Genomic DNA was extracted from ~ 50mg ground frozen tissue using a standard radical-free phenol extraction (Godschalk *et al.*, 1998), to minimise artificial induction of 8-oxodG. In addition, exposure to oxygen was reduced by addition of 1mM deferoxamine mesykate and 20mM TEMPO (2,2,6,6-tetramethylpiperidine-N-oxyl), according to the European Standards Committee on Oxidative DNA damage ('Comparison of different methods of measuring 8-oxoguanine as a marker of oxidative DNA damage. ESCODD (European Standards Committee on Oxidative DNA Damage),' 2000). HPLC-ECD was performed as described by de Kok *et al.* (de Kok *et al.*, 1994). 30µg DNA was digested to deoxyribonucleosides by treatment with P1 (0.02U/µl) for 90min at 37˚C and then with alkaline phosphatase (0.014 U/µl) for 45˚min at 37˚C. The digest was injected into a isocratic pump (Gynkotek) coupled with Midas injector (Spark Holland) and connected to a 250 X 4.6mm Supelcosil LC-18S column (Supelco) and an electrochemical detector (Antec). The mobile phase consisted of 10% aqueous methanol containing 94mM KH_2PO_4, 13mM K_2HPO_4, 26mM NaCl and 0.5mM EDTA. Elution was performed at a flow rate of 1.0ml/min. Lower absolute detection limit of 40fmol of 8-oxodG or 1.5 residues/10^6 2'-deoxyguanosine (dG) was used and dG was simultaneously monitored at 260nm.

2.15 ICP-MS

Magnesium ion concentrations in protein extracts were measured using inductively coupled plasma mass spectrometry (ICP-MS). 10mg/ml of crude tissue protein extracts were diluted to 1mg/ml in 65% HNO_3 (Merck) and

incubated for 24h to decompose. Samples were then diluted 1:10 into 2.5% HNO_3 (w/v) containing 20µg/L cobalt and 20µg/L silver as internal standards (BDH) and analysed by inductively coupled plasma mass spectrometry (Thermo X-series). ^{24}Mg was measured 100 times (20ms integration time, 5 channels, 0.02AMU separation) using the peak-jump method. Triplicate measurements were taken for each sample and detector pulse counts were converted to concentration by comparison with matrix-matched metal standards. Resulting data were presented as parts per billion µg/L.

2.16 Statistical Analysis

All analyses were performed using IBM SPSS Statistics v 19 software package. Data were tested for normality using the Kolmogorov-Smirnov test. Normally distributed data were tested for analysis of variance (ANOVA), significance was tested at α less than 0.05. Non-parametric Kruskal-Wallis test was used where data were not normally distributed. Further statistical analyses are specified in results Chapters.

Chapter 3 – Optimisation of the comet *in vitro* repair assay

The data presented in this Chapter are published in the article 'Tissue difference in BER-related incision activity and non-specific nuclease activity as measured by the comet assay' Mutagenesis 2013, 28(6):673-681 (Gorniak et al., 2013), the Figures used in this Chapter are a duplication of those in the publication.

3.1 Introduction

Accumulation of damage to DNA can lead to genome instability; leading to loss of cell function and cell death (Dizdaroglu, 2012). Repair of DNA damage is one of the key pathways to maintain genomic integrity and hence phenotypic assays able to assess DNA repair capacity can provide a measure of genomic stability. Such assays can be used as biomarkers for cell survival in molecular epidemiology and biomonitoring studies of cancer (Goukassian *et al.*, 2000; Collins *et al.*, 2003; Paz-Elizur *et al.*, 2007; Collins and Azqueta, 2012). Furthermore the DNA damage response is of particular interest in the ageing process when cells have progressively greater difficulty in maintaining their genomic stability and hence measurement of DNA repair can also be used in studies of cellular function (Cabelof *et al.*, 2003; Intano *et al.*, 2003; Imam *et al.*, 2006; Kisby *et al.*, 2010).

Numerous DNA repair pathways exist including base excision repair (BER) to remove single base lesions and nucleotide excision repair (NER) to remove bulky adducts such as those produced by UV light (Hoeijmakers, 2001). A number of disease states and ageing have been linked to DNA oxidative damage induced by intracellular reactive oxygen species (ROS) (Langie *et al.*, 2012). Guanine in particular is vulnerable to oxidation and it is estimated that approximately 180 guanine bases are oxidised to 8-oxo-7,8-dihydro-2'-deoxyguanosine (8-oxodG) per mammalian cell per day (Lindahl, 1993). A specific enzyme, oxoguanine DNA glycosylase is present in the cell to recognise and incise this damaging lesion. Thus assays able to measure removal of 8-oxodG can indicate cellular repair activity of DNA oxidative damage.

To date assays developed for measurement of DNA repair are mostly suitable for use with cells in culture or with lymphocytes but are not suited for use with

tissues. However, to investigate BER *in vivo* in animal models or in humans (e.g. in biopsies), functional assays which can quantify BER activity in the tissues of interest are needed. The comet assay is a simple and sensitive assay for measurements of DNA damage. Cells are embedded in an agarose gel and lysed to leave nucleoids of supercoiled DNA. Electrophoresis of nucleoids gives structures resembling comets which can be stained using a DNA dye and visualised under the microscope. The intensity of the comet tail is relative to the number of strand breaks present in the nucleoids. The assay has also been modified to measure DNA repair (Collins *et al.*, 2001; Collins, 2004).

Recently Langie *et al.* (Langie *et al.*, 2011) developed the comet-based assay for measuring BER-related DNA incision activity in animal tissues and optimised its use for mouse liver and brain tissues. In this assay photosensitiser Ro-198022 is used to induce 8-oxodG lesions in substrate cells. These cells are then imbedded in an agarose gel and lysed as in the standard comet assay. The nucleoids are then incubated with protein extracts from tissues of interest; then electrophoresis, staining and visualisation are performed as for the standard assay. The resultant quantified strand breaks (SB) provide an indication of recognition and incision of the damage, and incision activity of BER enzymes such as oxoguanine DNA glycosylase (Figure 3.1).

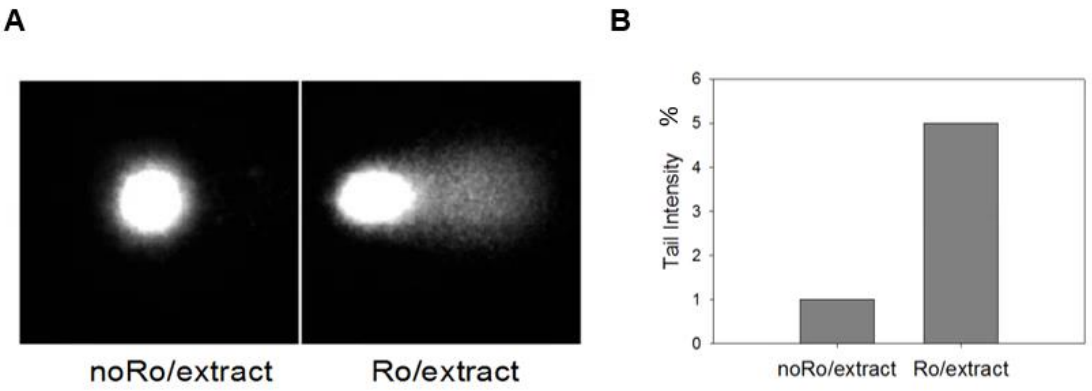


Figure 3.1 A microscope image of example comet from noRo and Ro nucleoids incubated with protein extract (A) and a plot of TI corresponding to the comet % DNA in the tail (B).

BER-related incision activity is calculated by subtracting TI in noRo nucleoids (control; without Ro 19-8022 and light treatment) incubated with protein extract and TI from Ro nucleoids (damaged, with Ro 19-8022 and light treatment) incubated with buffer from TI in Ro nucleoids incubated with protein extract (TI

in noRo nucleoids incubated with buffer i.e. the background damage, is first subtracted from all values).

In the present study, the work of Langie *et al.* was extended to optimise the *in vitro* comet based assay in other mouse tissues, specifically colon and lung, as well as brain and liver (Chapter 3) and to measure and compare BER-related incision activity in ageing mouse tissues (Chapter 4).

The aims of this study were to conduct a series of optimisation steps for use of the comet-based *in vitro* DNA repair assay with animal tissues.

The objectives of the present study were to:

a) Determine optimal tissue protein extract concentrations to use in the assay
b) Reduce non-specific nuclease activity by:
    (i) Aphidicolin block;
    (ii) performing wash steps;
    (iii) changing buffer composition.

3.2 Materials and methods

3.2.1 *Animal studies and human colon samples*

Methods are described in detail in Chapter 2. Whole brain, liver, lung and colon were collected from mice and tissues were snap frozen immediately in liquid nitrogen and stored at -80°C until processing. Colon tissue was either ‘clean’, where the tissue was flushed with PBS during collection (free of faecal matter) or ‘dirty’ where the tissue was not flushed at collection (faecal matter present).

Human mucosal biopsies came from patients taking part in the Dietary Intervention, Stem cells and Colorectal cancer (DISC) study (Clinical trial NCT01075893). Ethical approval was gained from Newcastle and North Tyneside Research Ethics Committee 2 (09/H0907/77). Patients were recruited via endoscopy. After the endoscopy procedure mucosal biopsies were taken from the rectum, 10cm from the anal verge, with 2.3mm spiked flexible endoscopy forceps, biopsies were immediately snap frozen in liquid nitrogen and further stored at -80˚C.

3.2.2 *Comet-based in vitro repair assay*

Before extraction of proteins from mouse tissue the ground tissue was washed with either 500µl PBS or buffer A. In some experiments the tissue was not washed. Extraction of protein from animal and human tissue was performed as described in Chapter 2. In case of human tissue where the weight was much smaller than the required 30mg, i.e. ~5mg, the subsequent volumes of buffers added were adjusted (1/5th of normal volume).

The comet-based BER incision assay was performed as described in Chapter 2. Protein extracts were diluted to the desired concentration in buffer A containing 0.23% Triton X-100. 50µl of this extract was further diluted in 4 volumes of buffer. Protein extracts were either left on ice before incubation with substrate nucleoids or heat inactivated for 10 minutes at 100ºC. Additional, optimisation steps were made by changing composition of the buffer protein extracts are diluted in, as shown in Table 3.1.

Data were presented as Tail Intensity (TI) which represents the percentage of DNA in the comet tail. Each extract was analysed in duplicate within one assay and in at least in 2 independent experiment where possible.

Test	Aphidicolin (µM)	Hepes (mM)	EDTA (mM)	KCl (M)	BSA (mg/ml)	Glycerol (%)	ATP (mM)	polyAT (ng)
Polymerase block	1.5-12	40	0.5	0.1	0.2	-		
B	1.5	45	0.25	-	0.3	2		
Test 1	1.5	45	0.25	0.1	0.3	2		
Test 2	1.5	40	0.5	0.1	0.2	2		
Test 3	1.5	45	0.5	0.1	0.3	2		
ATP	1.5	40	0.5	0.1	0.2	-	2.5	
polyAT	1.5	45	0.5	0.1	0.2	-		100
polyAT	1.5	45	0.5	0.1	0.3	2		100

Table 3.1 Composition of buffers used in optimisation of comet-based *in vitro* repair assay.

3.2.3 *Statistical Analysis*

Student's t-test and one-way ANOVA (with a Dunnett's or Tukey's post-hoc tests) were used to test for changes in TI and magnesium concentration.

3.3 Results

The BER-related incision is calculated by subtracting TI in noRo nucleoids from TI in Ro nucleoids. Thus, the specificity of the assay depends on minimising

non-specific nuclease activity and sensitivity relies on increasing the difference in TI between noRo and Ro treated nucleoids.

3.3.1 *Effect of extract protein concentration on assay sensitivity*

BER-related incision activity in the brain and liver was previously shown to be measurable by Langie *et al.* (Langie *et al.*, 2011). To compare DNA incision activity in other tissues the protocol described by Langie *et al.* was used on lung and colon tissue. Figure 3.2 shows the TI in substrate nucleoids treated with 5mg/ml of tissue protein extract from brain, liver, colon and lung. Substrate nucleoids incubated with protein extract from the brain showed percentage DNA in noRo and Ro nucleoids to be 2.50% and 8.20% respectively. Substrate nucleoids incubated with protein extract from the liver had 2.80% DNA in the comet tail of noRo nucleoids and 13.90% DNA in comet tail of Ro nucleoids. Substrate nucleoids treated with protein extract from colon tissue showed 95.00% and 93.70% DNA in comet tail in noRo and Ro nucleoids respectively. Substrate nucleoids incubated with protein extract from lung tissue had 63.80% DNA in comet tail of noRo and 68.70% DNA in comet tail of Ro nucleoids.

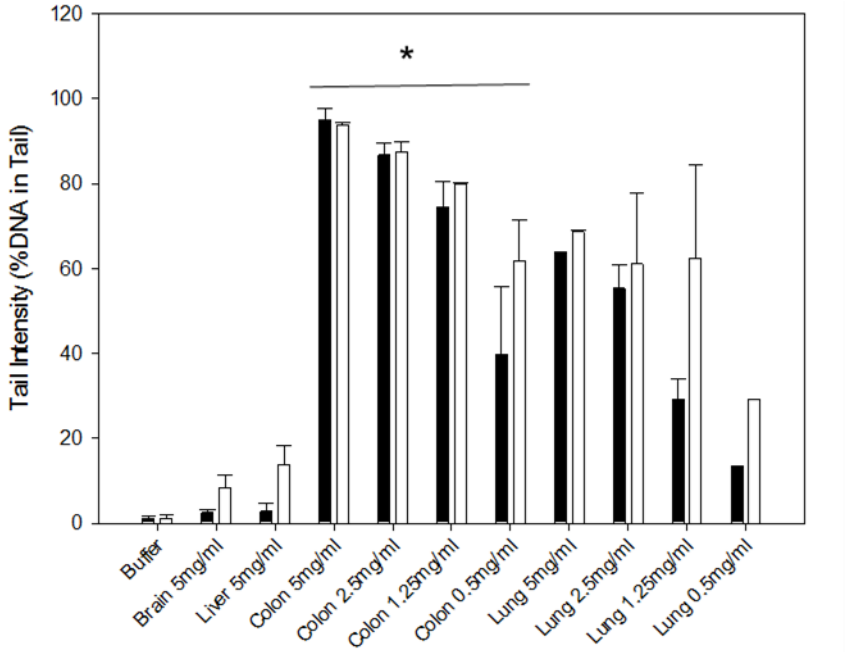


Figure 3.2 Effect of protein extract concentration on tail intensity. Tail Intensity measured in noRo (black bars) and Ro (white bars) nucleoids treated with protein extracts from brain, liver, colon and lung. Data are presented as mean of 2 replicates from a single assay with SD as the error bars. ANOVA analysis of protein extract concentration on TI in Ro nucleoids in colon (P=0.012) and lung (n.s.).

The high TI values seen in both noRo and Ro nucleoids incubated with colon and lung extracts reduce the sensitivity of the assay. Dilution curves of protein extracts were performed to determine whether TI in noRo nucleoids could thus be reduced and the difference between TI in noRo and Ro nucleoids can be maximised. Figure 3.2 shows the effect of doubling dilutions of colon and lung protein extracts on TI in noRo and Ro nucleoids. In noRo nucleoids, a tenfold dilution of colon protein extract reduced TI by 55%, while reducing lung protein extract tenfold decreased TI by 50%. As expected, TI in Ro nucleoids decreased with protein dilution of colon (P=0.012) and lung (not statistically significant) protein extracts. The sensitivity of the assay was thus improved by incubating substrate nucleoids with colon or lung extract at 0.5mg/ml and 1.25mg/ml, respectively.

3.3.2 *Non-specific nuclease activity versus cleavage activity*

To determine whether the high TI observed in substrate nucleoids incubated with colon extract was due to non-specific nuclease activity (enzymatic) or non-specific cleavage activity (non-enzymatic) the assay was performed with heat inactivated tissue extract. Figure 3.3 shows the TI in noRo and Ro substrate nucleoids treated with FPG positive control, ‘clean’ colon (PBS flushed and dissection) and ‘dirty colon (not PBS flushed at dissection). TI in noRo nucleoids incubated with heat inactivated protein extracts reduced DNA in comet tail to background levels with both ‘clean’ (P=0.01) and ‘dirty’ (P=0.001) colon. Compared with the normal assay condition, there was a decrease in TI of Ro nucleoids incubated with heat inactivated protein extracts relative to FPG (P=0.001), ‘clean’ colon (P=0.001) and ‘dirty’ colon (P=0.003). Elimination of TI in noRo nucleoids shows that enzymatic nuclease activity is responsible for the non-specific activity rather than non-enzymatic cleavage activity.

3.3.3 *Effect of Aphidicolin on non-specific nuclease activity*

Sensitivity of the assay is improved when lower concentrations of colon and lung protein extract are used; however, a somewhat high level of TI in noRo nucleoids incubated with these tissues is still observed compared to brain and liver. Aphidicolin (a polymerase inhibitor) has previously been shown to reduce non-specific nuclease activity (Speit *et al.*, 2004; Vande Loock *et al.*, 2010; Langie *et al.*, 2011). In this assay it was already used routinely at a concentration of 1.5µM. However, I tested whether increasing Aphidicolin

concentration can further reduce TI in noRo nucleoids. Table 3.2 shows that increasing Aphidicolin concentration in the assay did not alter TI in substrate nucleoids incubated with buffer, colon or lung protein extract (all P>0.05).

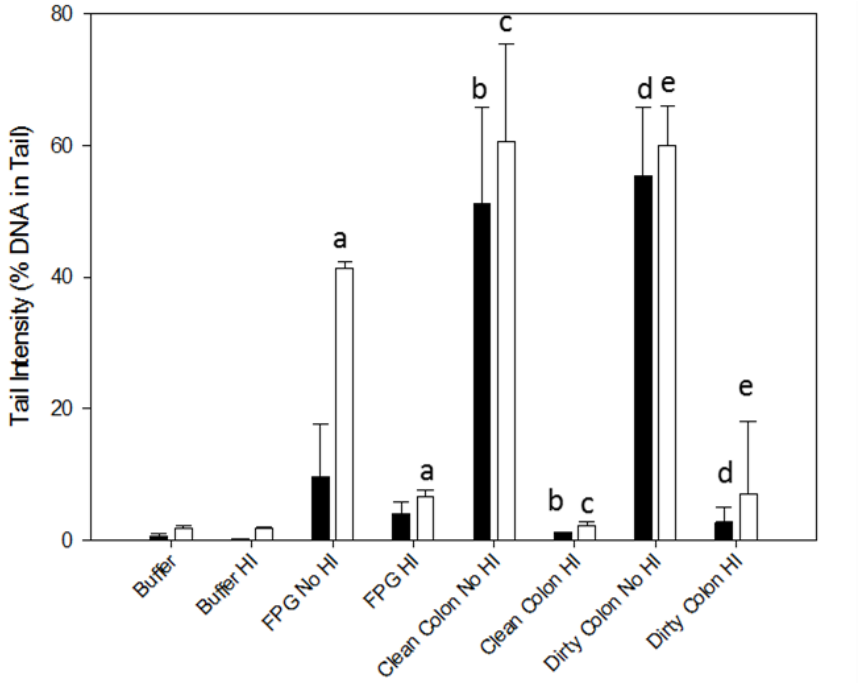


Figure 3.3 Effect of heat inactivation (HI) on comet tail intensity in cells treated with buffer control, FPG positive control and colon protein extract. Tail Intensity measured in noRo (black bars) and Ro (white bars) nucleoids. Data represent the mean values of 2 independent assays with error bars presenting pooled SEM, clean colon (n=2) and dirty colon (n=4), Student's t-test comparison of TI in HI and untreated protein extracts; FPG (P_a=0.001 Ro nucleoids), clean colon (P_b=0.01 noRo and P_c=0.001 Ro nucleoids) and dirty colon (P_d=0.001 noRo and P_e=0.003 Ro nucleoids).

Aphidicolin (µM)	**TI Buffer**		**TI Colon**		**TI Lung**	
	noRo nucleoids	Ro nucleoids	noRo nucleoids	Ro nucleoids	noRo nucleoids	Ro nucleoids
1.5	1.70 ±0.00	5.85 ±1.16	79.17 ±22.48	86.94 ±12.81	11.97 ±2.89	32.83 ±5.15
3	1.66 ±1.05	3.65 ±0.28	63.49 ±19.11	84.38 ±18.63	9.92 ±4.55	35.19 ±5.03
6	1.28 ±0.98	4.66 ±0.48	67.40 ±41.60	85.79 ±12.90	11.97 ±8.37	37.86 ±11.79
12	1.56 ±0.20	4.34 ±0.09	81.03 ±23.55	87.12 ±14.05	12.24 ±3.99	34.31 ±11.35

Table 3.2 Effect of increasing Aphidicolin concentration on Tail Intensity in nucleoids treated with buffer, colon or lung protein extract at 5mg/ml (washed with PBS during protein extraction). Data represent the mean values of 2 independent assays with error bars presenting pooled SD. Increasing [Aphidicolin] had no effect on TI in noRo or Ro nucleoids for either buffer, colon or lung treated nucleoids.

3.3.4 *Effect of washing tissue, during protein extraction, on non-specific activity and magnesium concentration*

The next step taken to reduce non-specific activity was washing the tissue during protein extraction. Often, after tissue is collected at dissection and snap frozen it still contains other materials such as blood, but by washing the tissue prior to protein extraction such residual matter can be removed. The effect of washing tissues with PBS or buffer A on non-specific nuclease activity was tested. Lung, colon and liver protein extract concentrations were tested at 5mg/ml and 2.5mg/ml for each wash step.

Figure 3.4A shows TI in substrate nucleoids treated with lung protein extract. At 5mg/ml TI in noRo nucleoids was 30.59%, 14.86% and 9.95% for non-washed, PBS washed and buffer A washed tissue respectively. TI in Ro nucleoids was 54.00% in non-washed, 41.40% in PBS washed and 43.50% in buffer A washed tissue. The reduction observed with washed tissue was not statistically significant for either noRo or Ro nucleoids. When lung protein extract was reduced to 2.5mg/ml, TI in noRo nucleoids was 10.30%, 4.99% and 3.20% in non-washed, PBS washed and buffer A washed tissue respectively. TI in Ro nucleoids was 33.90% in non-washed, 26.10% in PBS washed and 20.60% in buffer A washed tissue. The reduction observed with washes was not statistically significant for either noRo or Ro nucleoids.

Figure 3.4B shows TI in substrate nucleoids incubated with colon protein extract. At 5mg/ml TI in noRo nucleoids was 90.20%, 76.90% and 83.40% in non-washed, PBS washed and buffer A washed tissue respectively. TI in Ro nucleoids was 92.90% in non-washed, 92.80% in PBS washed and 90.90% in buffer A washed tissue. The observed reduction in TI seen with washing was not statistically significant for both noRo and Ro nucleoids. When protein extract was reduced to 2.5mg/ml, TI in noRo nucleoids was 84.30%, 75.80% and 62.20% in non-washed, PBS washed and buffer A washed tissue respectively. TI in Ro nucleoids was 90.80% in non-washed, 86.00% in PBS washed and 80.70% in buffer A washed tissue. The reduction in TI observed with wash steps was not statistically significant for both noRo and Ro nucleoids.

Figure 3.4C shows nucleoids incubated with liver protein extract. At 5mg/ml TI in noRo nucleoids was 3.20%, 7.40% and 7.50% in non-washed, PBS washed

and buffer A washed tissue respectively. TI in Ro nucleoids was 23.90% in non-washed, 32.60% in PBS washed and 21.30% in buffer A washed. The different wash steps did not significantly affect TI. When protein extract was reduced to 2.5mg/ml, TI in noRo nucleoids was 8.03%, 2.99% and 3.80% in non-washed, PBS washed and buffer A washed tissue respectively. TI in Ro nucleoids was 23.90% in non-washed, 15.30% in PBS washed and 13.50% in buffer A washed tissue. TI in Ro nucleoids was reduced when washing with PBS (P=0.016) and buffer A (P=0.01) as compared with non-washed tissue.

Magnesium concentration was measured as an additional factor that can affect nuclease activity. Many nucleases require magnesium as a cofactor, including those specific to BER as well as other non-specific ones. The crude protein extracts used in the assay contain both types of enzymes (MacLellan and Forsberg, 2001; Nishino and Morikawa, 2002; Wilson Iii, 2005; Paz-Elizur *et al.*, 2007; Gaivão *et al.*, 2009). Magnesium concentration was measured in lung, liver and colon tissue that has been either left unwashed, washed with PBS of washed with buffer A.

Highest magnesium concentration was observed in colon tissue, with lung showing 25% less and liver 50% less compared with colon (Table 3.3). Washing tissue with PBS reduced [Mg] by 32% in the lung, 15% in the colon and 28% in the liver. Washing tissue with buffer A reduced [Mg] by 42% in the lung, 13% in the colon and 18% in the liver. The changes observed in magnesium concentration with the different wash steps were not statistically significant for any of the tissues (all P>0.05).

Tissue	**Magnesium (µg/L)**		
	Non-washed	PBS washed	Buffer A washed
Lung	536±81	365±148	313±73
Colon	1089±143	925±139	947±369
Liver	808±87	578±98	660±9

Table 3.3 Effect of washing tissue before protein extraction on magnesium concentration in the extract. Magnesium concentration in 1mg/ml protein extract and after washing with PBS or buffer A before protein extraction. Data are presented as mean of 3 replicates from a single assay with SD as the error bars. Changes in magnesium concentration with wash steps were not statistically significant. Measurements of [Mg] were conducted by Dr Kevin Waldron.

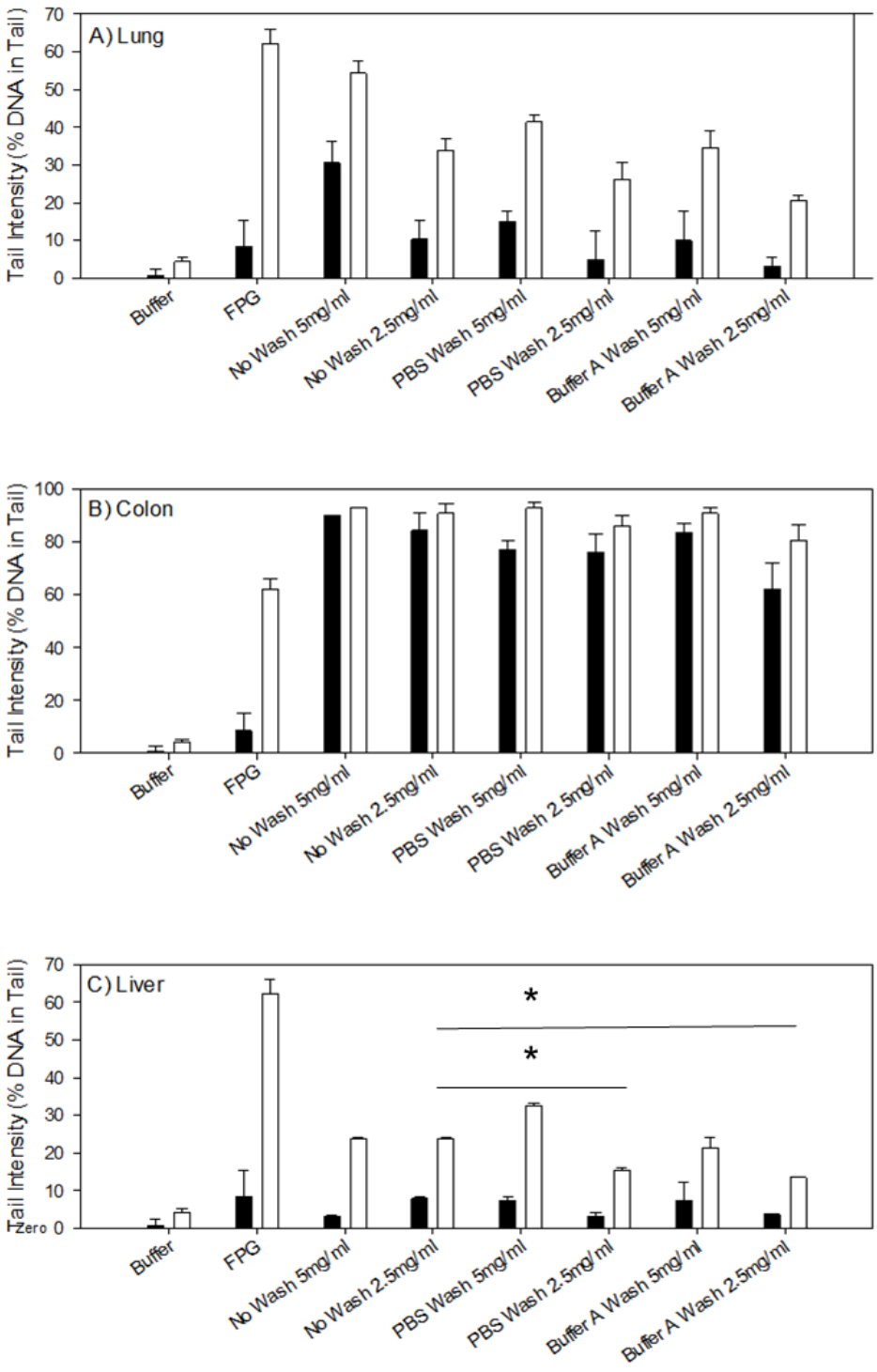


Figure 3.4 Effect of wash steps during protein extraction on comet tail intensity. Tail Intensity measured in noRo (black bars) and Ro (white bars) substrate DNA treated with protein extracts from A) mouse lung (n=3), B) mouse colon (n=3) and human colon biopsy (n=2) and C) mouse liver (n=2). Protein extracts have been not washed, washed with PBS or washed with buffer A during extraction of proteins. Data represent mean of 2 independent assays with error bars presenting pooled SEM. ANOVA comparison of TI with non-washed extracts: in 2.5mg/ml liver protein extract PBS washed (P=0.016 in Ro nucleoids) and buffer A washed (P=0.01 Ro nucleoids).

3.3.5 *Effect of washing colon tissue, during dissection, on non-specific nuclease activity*

When collecting mouse colon tissue during dissections, faecal matter is present within the intestine. To remove the faecal content the colon can be flushed with PBS leaving a ‘clean’ tissue or the content of the colon can be scraped which often still leaves the tissue ‘dirty’. To determine if washing the colon tissue at collection has an effect on non-specific nuclease activity, TI was measures in ‘clean’ and ‘dirty’ mouse colon tissue as well as human rectal mucosa which would have been thoroughly cleaned at collection. Figure 3.5 shows that when substrate nucleoids were incubated with protein extract from ‘dirty’ colon tissue, TI was 71% in noRo nucleoids and 77% in Ro nucleoids. When substrate nucleoids were incubated with protein extract from ‘clean’ colon tissue, TI was 49% in noRo nucleoids and 60% in Ro nucleoids. ‘Clean’ colon showed DNA in comet tail to be 20% lower compared with ‘dirty’ colon ($P>0.05$).

Substrate nucleoids were incubated with protein extract from human biopsies at two different concentrations; high (3mg/ml) and low (0.25mg/ml). TI in noRo nucleoids was 0.7% and 0.3% in noRo nucleoids incubated with 3mg/ml and 0.25mg/ml respectively. TI in Ro nucleoids incubated with 3mg/ml human colon biopsy was 5.6% while Ro nucleoids incubated with 0.25mg/ml protein concentration was 1.3%. Human colon biopsy showed significantly lower TI levels in substrate nucleoids than those from the mouse colon (TI in substrate nucleoids incubated with 3mg/ml protein extract from human biopsies compared with pooled clean and dirty colon $P=0.03$ in noRo nucleoids and $P=0.02$ in Ro nucleoids).

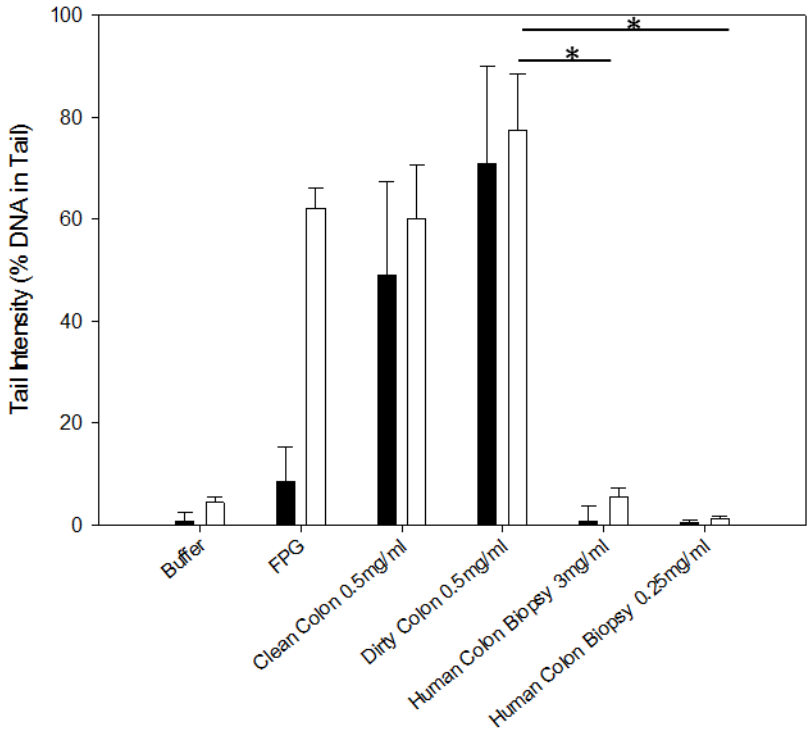


Figure 3.5 Effect of washing colon tissue at collection on comet tail intensity. Tail Intensity measured in noRo (black bars) and Ro (white bars) substrate DNA treated with protein extracts from 0.5mg/ml clean mouse colon (PBS flushed at collection, n=2), 0.5mg/ml dirty colon (not flushed at collection n=2) and human colon biopsy (flushed at collection, n=2) at 3mg/ml and 0.25mg/ml. Data represent mean of 3 independent assays (colon) and 2 assays (human biopsy) with error bars presenting pooled SEM. ANOVA comparison of TI in Ro nucleoids incubated with human colon biopsies compared to pooled mouse colon (P=0.02 at 3mg/ml and P=0.03 at 0.25mg/ml colon biopsy).

3.3.6 *Effect of buffer composition on non-specific nuclease activity*

To determine if adjusting buffer composition can reduce non-specific nuclease activity by reducing TI selectively in noRo nucleoids, changes were made to the buffer which the protein extracts are prepared. A description of the buffer composition and the resultant TI in substrate nucleoids incubated with tissue with this buffer is shown in Table 3.4. The modified buffers showed little changes to non-specific activity (all P>0.05).

One of the changes made to the buffer was addition of glycerol as seen in buffer B (used in comet based NER assay), ‘Test 1’, ‘Test 2’ and ‘Test 3’. However, addition of glycerol reduced the changes in TI induced by the FPG positive control. This effect was exacerbated when KCl was not present in the buffer as seen in buffer B. ATP is also usually added to the NER comet assay

(at 2.5mM of ATP) as a source of energy for the enzymatic reaction. Furthermore, ATP at 3.3mM can be used to inhibit bacterial and endogenous nucleases (Rosenberg, 1987). However, as seen in Table 3.4, addition of ATP had little effect on reducing non-specific nuclease activity.

Increasing concentrations of Hepes, EDTA or BSA had no further effect on reducing non-specific nuclease activity (buffer B, ‘Test 1’, ‘Test 2’ and ‘Test 3’). Buffer ‘Test 1’ and buffer ‘Test 2 showed some reduction in TI in substrate nucleoids incubated with colon protein extract. However, this decrease was accompanied by an unwanted increase in TI in noRo nucleoids incubated with buffer alone. PolyAT was added to buffer F and ‘Test 3’ to act as an alternate substrate for non-specific nucleases. This addition increased the difference in TI between noRo and Ro nucleoids incubated with extract (i.e. assay specificity) but only when 5mg/ml but not 0.5mg/ml protein extract was used. Furthermore an increase in TI in substrate nucleoids incubated with buffer alone was observed under these conditions.

Buffer	TI Buffer Incubation		TI FPG positive control		TI Colon Extract	
	noRo nucleoids	Ro nucleoids	noRo nucleoids	Ro nucleoids	noRo nucleoids	Ro nucleoids
F	0.42±0.08	0.71±0.61	5.86±2.38	43.78±0.76	53.55±8.30	73.66±4.65
B	0.95±1.03	1.11±0.09	6.77±4.29	6.42±1.19	59.28±14.37	67.29±20.15
Test 1	2.16±0.12	3.96±2.52	11.35±7.31	54.35±3.21	69.80±2.58	87.05±4.71
Test 2	0.80±0.07	4.03±0.01	14.15±1.07	44.32±1.64	78.74±24.13	79.12±0.00
Test 3	1.03±0.75	3.06±0.52	5.55±2.87	46.24±5.69	70.41±34.56	83.89±15.94
F ATP	0.55±0.34	1.72±0.41	5.47±2.15	43.16±0.63	65.58±12.51	79.05±0.62
F polyAT	0.36±0.06	2.75±2.51	2.69±2.16	43.20±22.10	50.47±54.18	81.51±16.66
Test 2 polyAT	1.08±1.10	2.86±2.08	17.63±6.08	43.97±11.57	56.05±50.77	77.69±23.41
F polyAT (0.5mg/ml colon extract)	2.85±3.60	1.59±0.35	6.81±2.13	40.29±0.64	41.27±57.55	48.23±57.04
Test 2 polyAT (0.5mg/ml colon extract)	1.33±1.30	4.40±0.43	20.50±2.68	54.28±12.44	41.62±56.70	48.21±57.70

Table 3.4 Effect of buffer composition on Tail Intensity. TI was measured in noRo and Ro nucleoids incubated with buffer control, FPG positive control and protein extracts from colon. All tests were performed with 5mg/ml protein extract concentration unless otherwise stated. Buffers F and Test 2 where at pH 8 which buffer B, Test 1 and Test 3 were at pH 7.8. Data are presented as mean of 2 replicates from a single assay with SD as the error bars, colon extract n=2 per assay. No significant differences were observed between the difference assay conditions.

3.4 Discussion

A robust and sensitive assay is needed to measure DNA repair activity. With varying repair activity between different tissue types (Cabelof *et al.*, 2003; Intano *et al.*, 2003; Mikkelsen *et al.*, 2009), a successful assay should be sensitive to measure DNA repair over a wide range. Such an assay can then be used in investigations assessing genomic stability in conditions such as ageing and cancer. Assays used to measure DNA incision are sensitive to problems of non-specific nuclease activity ((Salles *et al.*, 1995; Paz-Elizur *et al.*, 2007; Langie *et al.*, 2011) and discussions at the International Comet Assay Workshop). The comet based assay has been previously developed to quantify DNA incision activity in mouse brain and liver (Langie *et al.*, 2011). However to use the assay for measurements of DNA incision in other tissues such as lung and colon further optimisation steps presented in this chapter were performed.

3.4.1 *Tissue specific differences in non-specific incision activity*

When incubating protein extract from colon, and to a lesser extent lung tissue, a much higher TI is observed in substrate nucleoids compared to those incubated with protein extract from brain and liver tissue. When heat inactivating the protein extract the ‘saturating’ TI is abolished suggesting that non-specific activity is the cause of such high TI. This observation highlights the importance of using a control low damage nucleoids (noRo) without which it could have been mistakenly concluded that DNA repair activity is much higher in colon and lung tissue, rather than these tissues being found to have a high non-specific nuclease activity (Salles *et al.*, 1995). Non-specific nuclease activity also appears to be a problem in the comet assay with cultured cells (Azqueta *et al.*, 2009) confirming that control low damage nucleoids should be used when measuring DNA repair using the comet-based assay.

The data presented here also show that this assay may not be suitable for making direct comparisons of DNA incision activity in different tissues as different protein extract concentrations have to be used for different tissues studied.

3.4.2 *Optimisation of the assay to increase sensitivity*

The main objective of this study was to reduce non-specific nuclease activity and thereby improve the specificity of the assay. One of the most successful

methods used to decrease non-specific nuclease activity is the addition of the DNA polymerase inhibitor Aphidicolin (Speit and Schütz, 2008; Vande Loock *et al.*, 2010; Langie *et al.*, 2011). In the assay developed by Langie *et al.* (Langie *et al.*, 2011) Aphidicolin is present at a concentration of 1.5µM. In the present study, it was found that increasing Aphidicolin concentration up to 12µM did not reduce TI levels/non-specific nuclease activity any further. This may be because the original 1.5µM concentration used in the assay is sufficient to inhibit the activity of polymerases (Ikegami *et al.*, 1978) contributing to the non-specific nuclease activity.

Commercially available nuclease inhibitors are available but have not been tested here. However, they are unlikely to be suitable for use with the comet assay because of their modes of action. For example, aurintricarboxylic acid inhibits protein-DNA binding (Hallick *et al.*, 1977) while DEPC becomes unstable in Tris and Hepes containing buffers (Material Safety Data Sheet CAS# 1609-47-8). Other steps such as changing buffer composition, addition of ATP and polyAT have previously been shown to inhibit nuclease activity (Paz-Elizur *et al.*, 2007). However, they showed little effect of TI levels in the comet-based assay.

The most effective step taken to increase sensitivity was preparation of tissue beforehand. Reducing the protein extract concentration from lung tissue and washing before protein extraction and thereby reducing magnesium concentration levels and non-specific nuclease activity increased sensitivity of the assay. This may be because lung tissue contains blood, which can be removed by the PBS wash.

The most effective approach to increasing sensitivity of the assay for mouse colon was decreasing the protein concentration in the assay although washing tissues at both collection and during protein extraction, which reduced non-specific nuclease activity. The wash steps before protein extraction were not as effective in reducing magnesium concentration and non-specific nuclease activity in the colon compared with lung tissue. It may be that non-specific activity observed with colon tissue occurs due to enzymes within the autochthonous bacteria that are closely associated with the colonic mucosa (Nava and Stappenbeck, 2011) and, therefore, relatively difficult to remove.

Interestingly, human colon biopsies did not exhibit non-specific activity as observed with mouse colon tissue, and this suggests that non-specific activity is not a problem when measuring incision activity in human biopsies.

3.4.3 *Conclusions*

Measurements of DNA repair activity in mammalian tissue are difficult. The observed high levels of non-specific nuclease activity which differ between tissues require a specific and sensitive assay. Furthermore, data suggest that differences in repair activity are found between species and tissues with incision in the human colon being much lower than in the rodent extracts. This study demonstrates effective approaches used to reduce non-specific nuclease activity in the comet-based assay. Furthermore, data presented here highlight the importance of including control low-damage nucleoids (such as noRo) in the assay to prevent misinterpretation of the findings.

Despite the challenges arising in measuring DNA repair activity using the comet-based assay, with the modifications described in this Chapter, the assay proves to be suitable for use with a wide range of mammalian tissues. By selecting appropriate protein extract concentration for each tissue and undertaking wash steps to reduce non-specific nuclease activity the assay can be used to quantify BER-related incision activity. The quantified BER-related incision activity in these tissues is presented in Chapter 4.

3.5 Acknowledgments

I would like to thank Dr Kevin Waldron who performed the analysis of magnesium ion concentration in the tissue samples and the DISC study for providing me with human colon biopsies. I would also like to acknowledge CISBAN for providing the animal tissues. Lastly, I would like to thank Dr Sabine Langie for the feedback in the optimisation of the assay.

Chapter 4: The effects of ageing and short-term dietary restriction on BER in mouse tissues

4.1 Introduction

The addition of a methyl group to cytosine residues in CpG dinucleotides is responsible for regulation of gene expression during embryogenesis, foetal development and tissue differentiation (Zawia *et al.*, 2009). Recently the role of altered patterns of DNA methylation in the process of ageing and age-related diseases has also become of interest. Aberrant methylation patterns have been observed in cancer while global demethylation associated with genomic instability has been noted during ageing (Mathers, 2006; Fraga and Esteller, 2007; Calvanese *et al.*, 2009; Gonzalo, 2010; Gravina and Vijg, 2010).

Throughout the genome, most cytosine residues in CpG dinucleotides are methylated. However, the cytosine residues within CpG islands found in gene promoters are usually unmethylated in normal healthy tissues. In many cases, hypermethylation at such promoter regions is associated with a closed chromatin structure and therefore gene silencing (Richardson, 2003). The first experiments suggesting a correlation between DNA methylation of a gene and its expression were conducted by McGhee and Ginder with work on the chicken β-globin gene (McGhee and Ginder, 1979). Furthermore inhibition of DNA methylation by 5-azacytidine reverses DNA methylation associated gene silencing. Since then many studies have investigated the relationship between gene expression and DNA methylation and, often, have sought confirmation of a causal relationship by using 5-azacytidine treatment to de-methylate methylated promoter regions (Jaenisch *et al.*, 1985; Herman *et al.*, 1996; Cameron *et al.*, 1999).

Accumulation of oxidative damage to DNA and to other cellular macromolecules plays a central role in cellular ageing. Although numerous repair mechanisms exist (including BER and NER) to detect and to repair DNA lesions, increased oxidative damage to DNA is observed with age (Mecocci *et al.*, 1993; Sohal *et al.*, 1995; Gan *et al.*, 2012). This increase may result from greater oxidative stress during ageing or may be a consequence of the reported age-related decline in DNA repair capacity (Cabelof *et al.*, 2003) or a combination of both processes. The abundance of mRNA encoding BER repair enzymes as well as

their protein levels appear to fall during ageing (Cabelof *et al.*, 2002; Intano *et al.*, 2003) suggesting that lower transcript levels may be responsible for the age-related decline in repair and, consequently, increased damage accumulation. Silencing of DNA repair genes (e.g. *Ogg1* and *Apex*) by hypermethylation of the corresponding gene promoters may be one mechanism accountable for lower DNA repair capacity during ageing but, to date, there have been no published tests of this hypothesis.

DR is the most widely-used experimental approach known to extend lifespan and to reduce age-related pathologies in several animal models and has been shown to alter oxidative damage and DNA repair (Weraarchakul *et al.*, 1989; Hirano *et al.*, 1996; Schmerold and Niedermüller, 2001; Cabelof *et al.*, 2003; Swain and Subba Rao, 2011). Although the mechanism of action of DR is not yet fully elucidated, there is good evidence that it has profound effects on gene expression in several tissues (Lee *et al.*, 1999) and it has been hypothesised that these transcription changes may in part be mediated by epigenetic mechanisms (Mathers, 2006).

This Chapter describes a pilot study which was set up to test the following hypotheses:

1. During ageing *Ogg1* and *Apex* gene promoters become hypermethylated which causes silencing of the corresponding genes.
2. This gene silencing results in decreased BER activity and hence increased DNA oxidative damage accumulation.
3. DR ameliorates the adverse effects of age on methylation of *Ogg1* and *Apex,* increases the capacity for DNA repair and reduces age-related accumulation of DNA oxidative damage.

The Aims of this study were to conduct a pilot study test the above hypotheses in mice and to prepare for a larger, more definitive, later study (described in Chapter 5 and 6).

The objectives of the present study were to:

a) Optimise and validate a comet-based assay for quantification of DNA repair capacity in mouse tissues;

b) Using RT-qPCR, set up expression assays and quantify expression of *Ogg1* and *Apex* in liver and brain of mice during ageing and in response to short-term DR;
c) Set up assays to characterise and quantify methylation of specific CpG sites within the promoters of *Ogg1* and *Apex* in DNA from liver and brain of mice during ageing and in response to short-term DR;
d) Using HPLC-ECD measurements of 8oxodG, quantify oxidative damage in DNA from liver and brain of mice during ageing and in response to short-term DR.

4.2 Methods

Mice for these studies came from a colony of male C56BL6 animals. For investigation of the effects of ageing, mice were killed and tissue samples collected at 6 months, 17 months and 32 months of age (n=4-7 per age group). For investigation of the effects of DR, mice were exposed to 26% reduction in dietary energy supply for 3 months starting at age 14.2±1.2 months (n=9 per diet group). Tissues were collected and processed as described in Chapter 2. Methods for measurements of promoter methylation, gene expression, DNA incision and 8-oxoguanine are all as described in Chapter 2. One-way ANOVA was used to investigate the effects of age with the Bonferroni post-hoc test used to identify differences between ages. Student's t-test analysis was used in comparisons of diet (AL vs DR), $P<0.05$ was considered statistically significant.

4.3 Results

These sets of pilot studies were used to test the hypothesis outlined above and to critically assess the assays used in these studies. This section will briefly summarise the characteristics of animals and tissues used to assess the effects of ageing and short-term DR. It will then report on the separate types of analysis performed: DNA methylation at *Ogg1* and *Apex* promoter regions, *Ogg1* and *Apex* mRNA expression, DNA incision activity and 8-oxoguanine concentrations. The techniques and findings will then be interpreted in the discussion.

4.3.1 *Animal information*

To show the effects of ageing, mice were divided into 3 age groups: young, mid-aged and old representing distinctive ages of the adult life. Table 4.1 shows a

representation of the age and mouse weights at collection as information for all mice was not available. No information on organ weights from these mice was available.

Measurement	Young adult	Mid-age	Old-age
Age (months) at tissue collection	3-6	14-17	28-32
Body mass (g)	29.08±0.54	40.00±8.89	28.57±2.48

Table 4.1 Body mass of mice at 3 stages of lifespan: young, mid-age and old. Data represent mean ± SD of a representative set of mice from the study. Data curtesy of Dr Kerry Cameron.

In a separate study to test the effects of short-term DR mice from the C57BL6 colony were placed on either a 26% DR or AL control diet at 14 months; after 3 months mice were sacrificed. Table 4.2 shows the age, body weights and tissue weights from this study (published data (Wang *et al.*, 2010; Cameron *et al.*, 2011)).

Measurement	AL	DR
Age (months) at tissue collection	16-18	16-18
Body mass (g) (mean ± SD)	39.9±2.98	29.0±1.59
Liver weight (g) (mean ± SD)	1.17±0.228	1.18±0.253
Brain weight (g) (mean ± SD)	0.394±0.0681	0.3911±0.0548
Colon weight (g) (mean ± SD)	0.457±0.0571	0.370±0.0388
Lung weight (g) (mean ± SD)	0.230±0.0358	0.247±0.0761

Table 4.2 Body and organ masses of mice after short-term DR and their AL controls. Data represent mean ± SD for s subset of mice from the study.

4.3.2 *DNA methylation*

Oxoguanine DNA glycysylase (OGG1) and apurynic/apyrimidinic endonuclease 1 (APEX) were the chosen genes/proteins of study as representative of the first steps of the BER repair pathway. The details of the location and genomic promoter sequence of the genes encoding these murine enzymes can be found in Appendix A and B respectively. Promoter regions of both of these genes contained a CpG island each. The CpG dinucleotides in each promoter regions were counted; the *Ogg1* and *Apex* promoter regions contained 44 and 53 CpG sites respectively. A table of transcription factors known to bind at the promoter regions of these genes can also be found in Appendix A and B respectively (data generated by the Genomatix Gene2Promoter software). Pyrosequencing assays were set up to measure cytosine methylation at 13 CpG sites in the *Ogg1* promoter (of which 7 were in transcription factor binding sites) and 22 CpG sites in the *Apex* promoter region (of which 16 were in transcription factor binding sites, validation of assays can be found in Appendix A and B

respectively). Cytosine methylation was measured in brain and liver from ageing and short-term DR mice.

4.3.2.1 *Effect of age on Ogg1 promoter DNA methylation in the brain and liver*

In the brain, cytosine methylation at the *Ogg1* promoter ranged from 0% to 6.2%. Lowest methylation was observed at CpG site 14 and highest at CpG site 16. Despite overall low methylation across the promoter, methylation between individual CpG sites was not always correlated (Figure 4.1); therefore analysis for each CpG site as well as mean of all sites was performed. Mean *Ogg1* promoter methylation was 1.38% (median 1.31%, SD ±0.247%), 1.49% (median 1.43%, SD ±0.268%), and 1.27% (median 1.32%, SD ±0.254%), in young adult, mid-aged and old animals respectively; no statistically significant difference was observed in mean methylation. A difference between the age groups was found only at CpG 3 and CpG 13 (Table 4.3 and 4.4). At CpG site 3 methylation increased by 0.38% from young to mid age but then decreased again by 0.45% from mid to old age (P=0.026). At CpG site 13 methylation decreased by 0.11% from young to mid-age mice but then increased 0.95% from mid-aged to old mice (P=0.013).

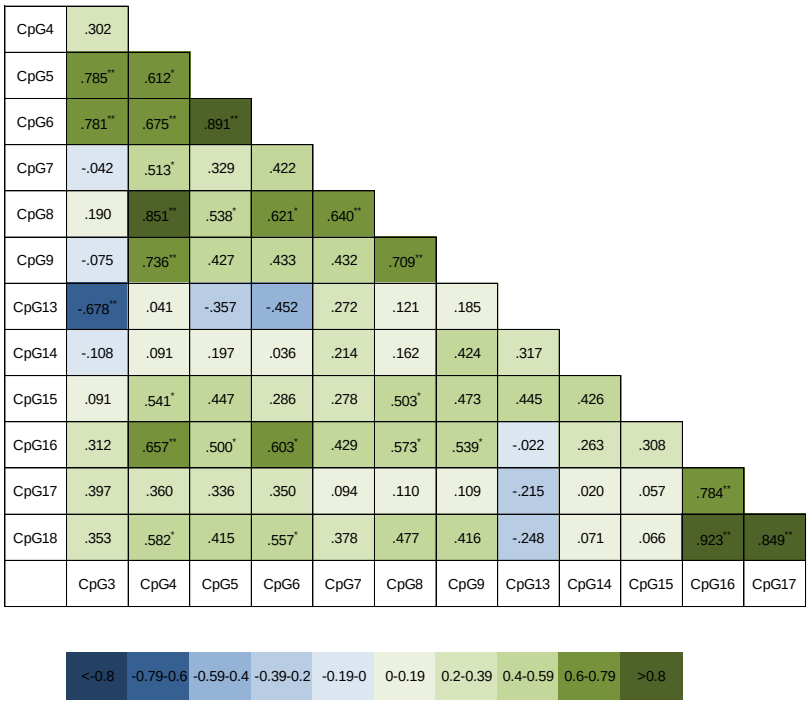
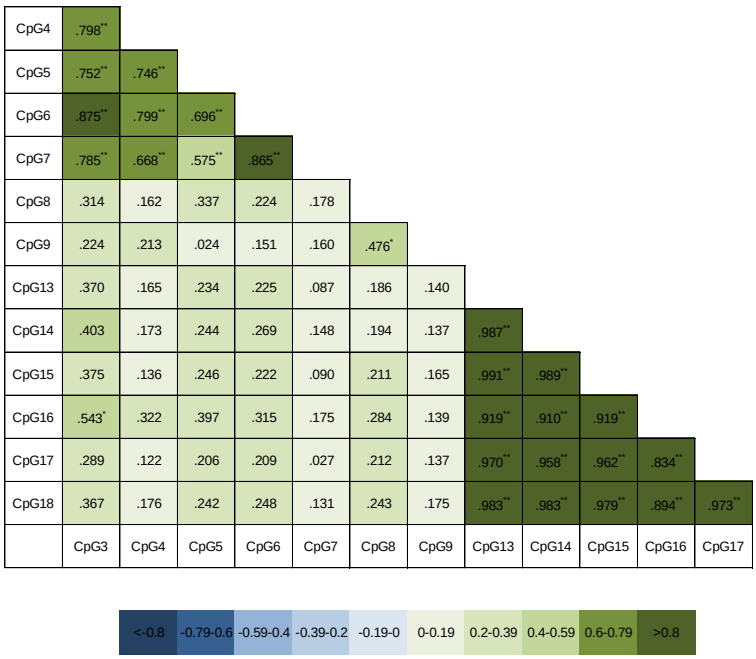


Figure 4.1 Pearson's correlation for % methylation between CpG sites within the *Ogg1* promoter from the brain. Statistically significant correlation at P<0.05 (*) and P<0.01 (**).

In the liver, cytosine methylation at the *Ogg1* promoter ranged from 0 to 8.77% with lowest methylation at CpG 3 and highest at CpG 16. Analysis was performed for each individual site as well as mean promoter methylation (Figure 4.2). A correlation analysis of percentage DNA methylation between the 13 CpG sites showed much variability in DNA methylation at individual sites studied. Mean *Ogg1* promoter methylation was 1.70% (median 1.60%, SD ±0.873%), 1.59% (median 0.96%, SD ± 0.94%) and 0.64% (median 0.44%, SD ± 0.60%) in young adult, mid age and old mice respectively. Mean *Ogg1* promoter methylation was higher in old animals compared with young adult and mid-aged however this was not statistically significant. There was a difference in methylation between the age groups at CpG sites 6 and 7 (Table 4.3 and 4.4). At CpG site 6 cytosine methylation declined by 0.52% from young to mid-aged mice (P=0.048) and by 0.58% from young to old mice (P=0.041). At CpG site 7 cytosine methylation declined by 1.02% from young to mid-aged mice



(P=0.003) and by 1.11% from young to old mice (P=0.003).

Figure 4.2 Pearson's correlation for % methylation between CpG sites within the *Ogg1* promoter from male mouse livers. Statistically significant correlation at P<0.05 (*) and P<0.01 (**).

CpG site		Brain			Liver		
		Young	Mid	Old	Young	Mid	Old
CpG 3	Mean	0.198	0.582	0.128	0.600	0.379	0.368
	Median	0.230	0.550	0.095	0.610	0.428	0.185
	SD	0.142	0.367	0.145	0.123	0.154	0.351
	P value	0.02			>0.05		
CpG 4	Mean	1.960	1.897	1.908	2.746	2.243	2.064
	Median	1.975	1.950	2.045	2.735	2.293	2.110
	SD	0.204	0.183	0.380	0.362	0.497	0.710
	P value	>0.05			>0.05		
CpG 5	Mean	0.793	0.900	0.717	1.023	0.842	0.837
	Median	0.770	0.930	0.685	1.030	0.840	0.848
	SD	0.114	0.179	0.182	0.143	0.255	0.347
	P value	>0.05			>0.05		
CpG 6	Mean	0.788	0.915	0.632	1.256	0.741	0.676
	Median	0.840	0.925	0.735	1.213	0.830	0.558
	SD	0.127	0.189	0.260	0.300	0.298	0.385
	P value	>0.05			0.028		
CpG 7	Mean	0.993	0.877	0.833	1.771	0.752	0.660
	Median	0.980	0.815	0.825	1.718	0.660	0.415
	SD	0.165	0.145	0.324	0.574	0.333	0.467
	P value	>0.05			0.001		
CpG 8	Mean	1.655	1.542	1.458	1.890	1.202	1.339
	Median	1.670	1.450	1.660	1.595	1.412	1.348
	SD	0.325	0.197	0.420	1.034	0.816	0.655
	P value	>0.05			>0.05		
CpG 9	Mean	1.368	1.177	1.265	1.695	1.586	0.638
	Median	1.335	1.170	1.320	1.600	0.963	0.440
	SD	0.124	0.098	0.254	0.873	0.936	0.596
	P value	>0.05			>0.05		

Table 4.3 Effect of age on methylation at individual CpG sites within the *Ogg1* promoter sequence 1 in DNA from brain (young n=4, mid-age n=6 and old n=6) and liver (young n=4, mid-age n=6 and old n=6). Data represent mean ± SEM for each age group. DNA methylation for individual animal tissues were measured in duplicate. ANOVA analysis of age was performed. Primer design Bartłomiej Tomaszewski and Dr Sabine Langie.

CpG site		Brain			Liver		
		Young	Mid	Old	Young	Mid	Old
CpG 13	Mean	0.893	0.783	0.128	0.600	0.379	0.368
	Median	0.720	0.610	0.095	0.610	0.428	0.185
	SD	0.401	0.622	0.145	0.123	0.154	0.351
	P value		0.009			>0.05	
CpG 14	Mean	0.075	0.047	1.908	2.746	2.243	2.064
	Median	0.000	0.000	2.045	2.735	2.293	2.110
	SD	0.150	0.114	0.380	0.362	0.497	0.710
	P value		>0.05			>0.05	
CpG 15	Mean	1.448	1.553	0.717	1.023	0.842	0.837
	Median	1.380	1.705	0.685	1.030	0.840	0.848
	SD	0.508	0.477	0.182	0.143	0.255	0.347
	P value		>0.05			>0.05	
CpG 16	Mean	4.063	4.418	0.632	1.256	0.741	0.676
	Median	3.890	4.155	0.735	1.213	0.830	0.558
	SD	0.640	1.030	0.260	0.300	0.298	0.385
	P value		>0.05			>0.05	
CpG 17	Mean	0.998	1.698	0.833	1.771	0.752	0.660
	Median	0.805	1.245	0.825	1.718	0.660	0.415
	SD	0.490	1.447	0.324	0.574	0.333	0.467
	P value		>0.05			>0.05	
CpG 18	Mean	2.715	2.918	1.458	1.890	1.202	1.339
	Median	2.750	2.485	1.660	1.595	1.412	1.348
	SD	0.444	1.009	0.420	1.034	0.816	0.655
	P value		>0.05			>0.05	

Table 4.4 Effect of age on methylation at individual CpG sites within the *Ogg1* promoter sequence 2 in DNA from brain (young n=4, mid-age n=6 and old n=6) and liver (young n=4, mid-age n=6 and old n=6). Data represent mean ± SEM for each age group. DNA methylation for individual animal tissues were measured in duplicate. ANOVA analysis of age was performed. Primer design Bartłomiej Tomaszewski and Dr Sabine Langie.

4.3.2.2 *Effect of age on Apex promoter DNA methylation in the brain and liver*

In the brain, cytosine methylation at the *Apex* promoter ranged from 0% to 42%. Lowest methylation was observed at CpG site 7 and highest at CpG site 26. A correlation analysis between individual sites showed variability hence further analysis was performed on individual CpG sites (Figure 4.3). Methylation at the *Apex* promoter was higher than at the *Ogg1* promoter. Furthermore more variability in methylation was observed between animals compared with the *Ogg1* promoter, as seen by the error bars. Mean *Apex* promoter methylation was 3.65% (median 3.34%, SD ± 1.23%), 4.16% (median 4.19%, SD ± 1.69%) and 3.79% (median, SD ± 2.17%) in young adult, mid aged and old animal respectively; no significant difference was seen in mean promoter methylation or at any individual CpG site at the different ages (Table 4.5 and 4.6).

In the liver, cytosine methylation at the *Apex* promoter ranged from 0-6.53% with lowest methylation observed at CpG site 6 and highest at CpG site 9 (Table 4.5 and 4.6). Correlation of methylation at individual sites was not constant (Figure 4.4). Mean promoter methylation was 3.33% (median 3.1%, SD ± 0.95%), 2.49% (median, 2.63%, SD ± 1.23%) and 1.04% (median 1.84%, SD ± 0.25%) in young, mid age and old animals. Mean promoter methylation decline with age (young vs old P=0.005). A ~1.67% decrease in cytosine methylation was observed in older animals compared with young at CpG sites 8 (P=0.03), 9 (P=0.015), 11 (P=0.021), 12 (P=0.033), 14 (P=0.016), 26 (P=0.002), 27 (P=0.03), 29 (P=0.013) and 30 (P=0.018).

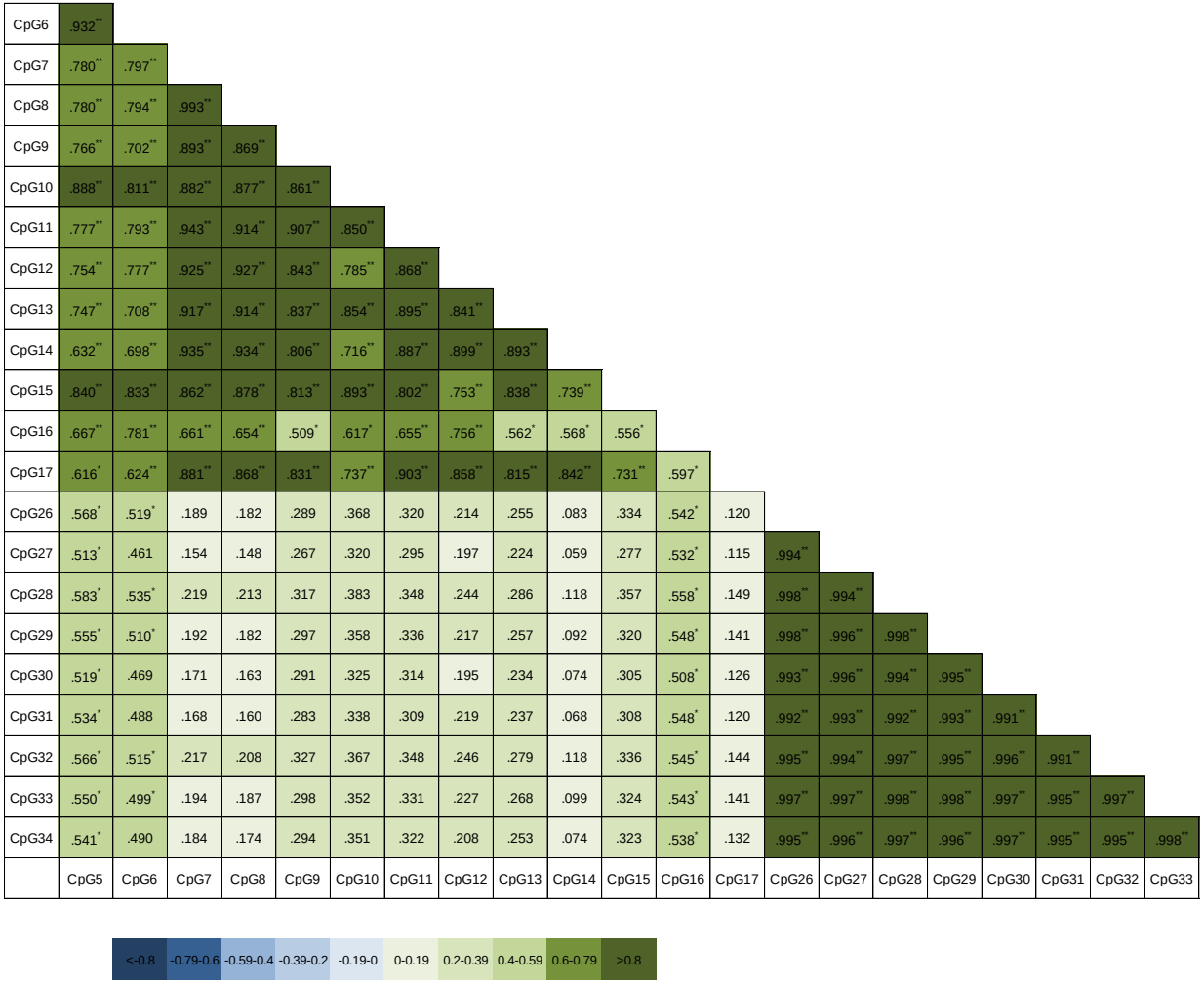


Figure 4.3 Pearson's correlation for % methylation between CpG sites within the *Apex* promoter from male mouse brains. Statistically significant correlation at P<0.05 (*) and P<0.01 (**).

	CpG5	CpG6	CpG7	CpG8	CpG9	CpG10	CpG11	CpG12	CpG13	CpG14	CpG15	CpG16	CpG17	CpG26	CpG27	CpG28	CpG29	CpG30	CpG31	CpG32	CpG33
CpG6	.894**																				
CpG7	.584*	.584*																			
CpG8	.873**	.854**	.659**																		
CpG9	.909**	.874**	.609**	.863**																	
CpG10	.830**	.748**	.692**	.806**	.816**																
CpG11	.829**	.743**	.733**	.824**	.820**	.844**															
CpG12	.905**	.847**	.745**	.856**	.905**	.899**	.926**														
CpG13	.821**	.911**	.743**	.896**	.861**	.828**	.854**	.910**													
CpG14	.747**	.801**	.749**	.866**	.781**	.774**	.920**	.847**	.930**												
CpG15	.779**	.668**	.763**	.774**	.805**	.857**	.946**	.924**	.823**	.860**											
CpG16	.689**	.723**	.696**	.730**	.694**	.721**	.885**	.795**	.840**	.887**	.798**										
CpG17	-.197	-.295	.011	-.264	-.277	-.137	.062	-.037	-.205	-.032	.108	-.072									
CpG26	.709**	.808**	.723**	.842**	.783**	.740**	.729**	.751**	.892**	.867**	.677**	.677**	-.315								
CpG27	.287	.434	.318	.338	.528*	.278	.303	.397	.400	.386	.301	.316	.013	.378							
CpG28	.799**	.828**	.659**	.799**	.812**	.779**	.710**	.797**	.870**	.788**	.729**	.648**	-.255	.892**	.336						
CpG29	.835**	.843**	.567*	.800**	.948**	.759**	.770**	.904**	.838**	.722**	.788**	.641**	-.135	.688**	.552*	.730**					
CpG30	.436	.617**	.549*	.536*	.643**	.556*	.456	.590*	.653**	.583*	.466	.462	-.133	.690**	.868**	.635**	.653**				
CpG31	.348	.517*	.446	.380	.455	.425	.246	.388	.509*	.390	.267	.406	-.311	.569*	.592*	.675**	.413	.793**			
CpG32	.596*	.742**	.585*	.539*	.619**	.658**	.490*	.646**	.727**	.579*	.490*	.497*	-.226	.662**	.439	.750**	.598*	.704**	.751**		
CpG33	.608**	.460	.379	.382	.662**	.711**	.533*	.683**	.455	.335	.662**	.387	-.026	.294	.337	.535*	.696**	.445	.431	.547*	
CpG34	.188	.056	.126	-.009	-.014	.250	.038	.104	.046	-.048	.181	.009	-.186	-.033	-.520*	.263	-.085	-.304	.067	.256	.366

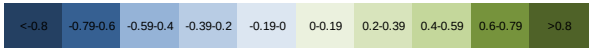


Figure 4.4 Pearson's correlation for % methylation between CpG sites within the *Apex* promoter from male mouse livers. Statistically significant correlation at P<0.05 (*) and P<0.01 (**).

CpG site		Brain			Liver		
		Young	Mid	Old	Young	Mid	Old
CpG 5	Mean	4.191	4.662	2.726	2.985	2.187	1.909
	Median	3.078	4.583	3.035	3.075	1.620	1.980
	SD	2.983	0.719	0.756	0.263	1.036	0.337
	P value	>0.05			>0.05		
CpG 6	Mean	3.873	3.928	2.382	2.316	1.559	1.228
	Median	2.905	3.755	2.485	2.316	1.455	1.275
	SD	2.565	0.524	0.823	0.393	1.093	0.434
	P value	>0.05			>0.05		
CpG 7	Mean	2.281	0.923	0.363	2.360	2.345	1.606
	Median	0.860	1.140	0.000	2.490	2.380	1.513
	SD	2.893	0.750	0.601	0.294	0.818	0.298
	P value	>0.05			>0.05		
CpG 8	Mean	2.420	0.927	0.348	2.851	2.073	1.407
	Median	1.195	1.265	0.000	2.893	2.095	1.408
	SD	2.822	0.735	0.559	0.304	0.953	0.629
	P value	>0.05			0.03		
CpG 9	Mean	4.773	4.255	3.337	5.229	4.234	3.811
	Median	3.410	4.320	3.048	5.100	3.890	3.825
	SD	3.110	0.833	1.368	0.643	0.851	0.270
	P value	>0.05			0.015		
CpG 10	Mean	3.520	3.472	2.270	3.340	2.924	2.073
	Median	2.195	3.628	2.020	3.313	2.530	2.248
	SD	3.232	0.975	0.959	0.269	1.003	0.616
	P value	>0.05			>0.05		
CpG 11	Mean	3.583	2.378	1.824	3.093	2.029	1.171
	Median	2.033	2.275	1.685	3.148	1.585	0.843
	SD	3.364	0.829	0.697	0.296	1.277	0.634
	P value	>0.05				0.021	
CpG 12	Mean	3.584	2.503	2.003	2.966	1.961	1.167
	Median	2.470	2.285	1.738	2.965	1.700	0.975
	SD	2.691	0.718	0.865	0.341	1.277	0.675
	P value	>0.05			0.033		
CpG 13	Mean	2.495	1.461	0.721	3.113	2.694	2.001
	Median	1.333	1.623	0.503	3.100	2.480	1.840
	SD	2.330	0.788	0.840	0.270	0.903	0.408
	P value	>0.05			0.048		
CpG 14	Mean	2.936	1.004	0.446	3.614	2.662	1.404
	Median	1.735	0.803	0.000	3.680	2.170	1.058
	SD	2.676	1.124	0.723	0.377	1.441	0.711
	P value	>0.05			0.016		
CpG 15	Mean	3.439	2.515	1.186	2.821	2.133	1.345
	Median	2.688	2.340	0.888	2.943	1.940	0.935
	SD	2.526	0.855	1.229	0.346	1.044	0.790
	P value	>0.05			0.049		
CpG 16	Mean	4.220	3.733	3.342	3.244	2.446	1.747
	Median	3.175	3.685	3.560	3.305	2.345	1.295
	SD	3.180	1.271	1.143	0.833	1.712	0.790
	P value	>0.05			>0.05		
CpG 17	Mean	5.149	2.601	3.080	5.190	2.528	3.200
	Median	4.035	2.610	3.023	2.305	0.000	2.075
	SD	3.233	0.637	1.092	3.034	1.567	2.338
	P value	>0.05			>0.05		

Table 4.5 Effect of age on methylation at individual CpG sites within the *Apex* promoter sequence 1 in DNA from brain (young n=5, mid n=6 and old n=6) and liver (young n=4, mid n=7 and old n=6). Data represent mean ± SEM for each age group. DNA methylation for individual animal tissues were measured in duplicate. ANOVA analysis of age was performed.

CpG site		Brain			Liver		
		Young	Mid	Old	Young	Mid	Old
CpG 26	Mean	11.488	18.511	8.219	3.513	3.059	1.739
	Median	7.198	12.910	6.025	3.508	2.870	1.755
	SD	10.134	12.696	5.510	0.342	1.003	0.129
	P value	>0.05			0.002		
CpG 27	Mean	7.448	13.695	5.492	4.074	2.280	2.383
	Median	3.295	7.765	2.960	3.690	2.365	2.298
	SD	9.692	12.647	5.890	0.857	1.231	0.795
	P value	>0.05			0.03		
CpG 28	Mean	10.906	17.176	7.562	4.065	3.741	2.932
	Median	6.433	11.718	5.188	4.070	4.050	2.955
	SD	10.510	12.293	5.416	0.343	1.057	0.141
	P value	>0.05	>0.05				
CpG 29	Mean	8.971	14.603	6.543	4.519	3.463	3.170
	Median	4.818	9.693	4.405	4.523	3.160	2.998
	SD	9.087	11.170	4.764	0.563	0.786	0.524
	P value	>0.05			0.013		
CpG 30	Mean	7.388	13.107	4.554	3.143	1.985	0.983
	Median	3.393	7.783	1.695	3.100	1.990	0.765
	SD	9.445	12.464	6.392	0.519	1.360	0.724
	P value	>0.05			0.018		
CpG 31	Mean	9.693	16.488	7.479	2.248	2.057	1.089
	Median	5.420	10.470	6.255	2.700	1.900	1.158
	SD	9.804	13.282	5.411	1.621	1.737	0.622
	P value	>0.05			>0.05		
CpG 32	Mean	9.365	16.780	5.352	3.073	3.284	1.865
	Median	3.633	10.628	2.455	3.070	3.180	2.183
	SD	12.196	14.045	7.290	1.046	1.523	1.153
	P value	>0.05			>0.05		
CpG 33	Mean	10.569	17.169	7.093	4.256	3.396	2.909
	Median	5.685	10.993	4.596	4.465	3.250	2.993
	SD	11.219	14.132	6.383	0.926	2.016	0.818
	P value	>0.05			>0.05		
CpG 34	Mean	8.921	14.305	7.043	0.000	3.700	2.444
	Median	5.043	9.948	5.033	0.000	3.460	2.405
	SD	8.614	10.916	4.861	0.000	2.481	0.843
	P value	>0.05			0.03		

Table 4.6 Effect of age on methylation at individual CpG sites within the *Apex* promoter sequence 2 in DNA from brain (young n=5, mid n=6 and old n=6) and liver (young n=4, mid n=7 and old n=6). Data represent mean ± SEM for each age group. DNA methylation for individual animal tissues were measured in duplicate. ANOVA analysis of age was performed.

4.3.2.3 *Effect of short-term DR on Ogg1 promoter DNA methylation in the brain and liver*

In the brain, cytosine methylation at the *Ogg1* promoter varied between 0 and 6.21% with lowest methylation observed at CpG site 14 and highest at CpG site 16. Mean promoter methylation was 1.65% (median 1.48%, SD ± 0.28%) and 1.57% (median 1.56%, SD ± 0.06%) in AL and DR animals respectively. No difference was observed between AL and DR mice at any of the CpG sites or with respect to mean promoter methylation (Table 4.7 and 4.8). In the liver, cytosine methylation at the *Ogg1* promoter ranged from 0% to 3.95% (Table 4.7 and 4.8). Mean promoter methylation was 1.48% (median 1.54%, SD ± 0.21%) in AL and 1.40% (median 1.42%, SD ± 0.18%) in DR animals. Methylation at CpG sites 9 and 17 was lower in DR animals compared with AL animals. It declined by 0.80% at CpG 9 (P=0.041) and by 0.47% at CpG 17 (P=0.025).

4.3.2.4 *Effect of short-term DR on Apex promoter DNA methylation in the brain and liver*

In the brain, mean Apex promoter methylation was 5.16% (median 6.17%, SD ± 2.64) and 3.40% (median 3.36%, SD ± 0.92%) in AL and DR animals respectively. Methylation varied from 0 to 41%. Methylation at CpG sites 24-26 was ~15% lower in DR animals compared with AL animals (Table 4.9 and 4.10). In the liver, cytosine methylation at the *Apex* promoter ranged from 0 to 11.8%, mean methylation was 2.18% (median 2.62%, SD ± 0.83%) and 2.97% (median 2.86%, SD ± 0.74%) in AL and DR animals respectively (Table 4.9 and 4.10). Cytosine methylation was higher in DR animals at 2 CpG sites. At CpG site 6 DR animals had 1.58% higher methylation compared with AL animals (P<0.001) and at CpG site 13 DR animals had 1.53% higher methylation compared with AL animals (P=0.034).

CpG site		Brain		Liver	
		AL	DR	AL	DR
CpG 3	Mean	0.830	1.103	0.374	0.368
	Median	0.875	1.085	0.440	0.355
	SD	0.280	0.124	0.187	0.108
	P value	>0.05		>0.05	
CpG 4	Mean	2.006	1.841	2.191	2.098
	Median	1.990	1.818	2.295	2.225
	SD	0.146	0.134	0.590	0.244
	P value	>0.05		>0.05	
CpG 5	Mean	1.040	1.229	0.789	0.877
	Median	0.970	1.185	0.840	0.890
	SD	0.162	0.183	0.265	0.130
	P value	>0.05		>0.05	
CpG 6	Mean	1.065	1.246	0.701	0.868
	Median	1.010	1.165	0.850	0.800
	SD	0.154	0.469	0.354	0.319
	P value	>0.05		>0.05	
CpG 7	Mean	0.875	0.826	0.769	0.733
	Median	0.900	0.988	0.750	0.735
	SD	0.063	0.577	0.376	0.281
	P value	>0.05		>0.05	
CpG 8	Mean	1.566	1.651	0.865	1.410
	Median	1.535	1.583	0.805	0.000
	SD	0.156	0.197	0.738	0.783
	P value	>0.05		>0.05	
CpG 9	Mean	1.204	1.325	1.624	0.598
	Median	1.195	1.352	0.760	0.000
	SD	0.113	0.114	1.029	0.265
	P value	>0.05		>0.05	

Table 4.7 Effect of short-term DR on methylation at individual CpG sites within the *Ogg1* promoter sequence 1 in DNA from brain (AL n=5 and DR n=4) and liver (AL n=7 and DR n=9). Data represent mean ± SEM for each age group. DNA methylation for individual animal tissues were measured in duplicate. Primer design Bartłomiej Tomaszewski and Dr Sabine Langie.

CpG site		Brain		Liver	
		AL	DR	AL	DR
CpG 13	Mean	0.779	0.816	1.569	1.224
	Median	0.585	0.770	1.535	1.410
	SD	0.782	0.269	0.222	0.457
	P value	>0.05		>0.05	
CpG 14	Mean	0.297	0.196	1.151	0.923
	Median	0.000	0.180	1.215	0.870
	SD	0.664	0.228	0.223	0.338
	P value	>0.05		>0.05	
CpG 15	Mean	1.835	2.089	2.015	1.719
	Median	1.880	2.088	2.000	1.790
	SD	0.479	0.298	0.101	0.367
	P value	>0.05		0.045	
CpG 16	Mean	4.430	3.766	3.305	3.248
	Median	4.130	3.713	3.340	3.135
	SD	1.104	0.369	0.409	0.350
	P value	>0.05		>0.05	
CpG 17	Mean	2.274	1.263	1.484	1.014
	Median	1.705	1.228	1.435	0.960
	SD	1.405	0.403	0.314	0.407
	P value	>0.05		0.021	
CpG 18	Mean	3.154	2.668	2.334	2.411
	Median	2.865	2.695	2.370	2.550
	SD	1.041	0.207	0.286	0.516
	P value	>0.05		>0.05	

Table 4.8 Effect of short-term DR on methylation at individual CpG sites within the *Ogg1* promoter sequence 2 in DNA from brain (AL n=5 and DR n=4) and liver (AL n=7 and DR n=9). Data represent mean ± SEM for each age group. DNA methylation for individual animal tissues were measured in duplicate. Primer design Bartłomiej Tomaszewski and Dr Sabine Langie.

CpG site		Brain		Liver	
		AL	DR	AL	DR
CpG 5	Mean	5.677	4.713	2.447	3.027
	Median	5.085	4.508	2.285	2.910
	SD	2.321	1.621	0.854	2.085
	P value	>0.05		>0.05	
CpG 6	Mean	5.066	5.135	2.124	3.705
	Median	4.105	5.595	2.090	3.735
	SD	2.391	1.220	0.625	0.599
	P value	>0.05		<0.001	
CpG 7	Mean	1.688	0.558	2.106	2.655
	Median	1.170	0.493	2.140	2.795
	SD	2.410	0.652	0.802	0.681
	P value	>0.05		>0.05	
CpG 8	Mean	1.769	0.000	1.929	2.745
	Median	1.320	0.000	2.095	2.600
	SD	2.510	0.000	1.208	1.979
	P value	>0.05		>0.05	
CpG 9	Mean	4.530	2.934	4.010	4.730
	Median	3.810	2.748	3.930	5.690
	SD	1.631	1.170	1.264	1.950
	P value	>0.05		>0.05	
CpG 10	Mean	4.091	3.408	2.566	3.015
	Median	3.485	3.405	2.975	3.695
	SD	1.939	1.443	1.610	2.351
	P value	>0.05		>0.05	
CpG 11	Mean	3.400	0.858	1.917	2.935
	Median	2.410	0.780	1.370	3.520
	SD	2.064	0.998	1.542	1.566
	P value	>0.05		>0.05	
CpG 12	Mean	3.457	2.450	1.621	2.412
	Median	2.470	2.568	1.245	2.270
	SD	2.172	0.643	1.579	1.316
	P value	>0.05		>0.05	
CpG 13	Mean	3.091	1.731	2.369	3.901
	Median	1.750	1.823	2.480	3.905
	SD	3.219	0.278	1.452	1.160
	P value	>0.05		0.043	
CpG 14	Mean	1.838	0.618	2.066	3.677
	Median	0.000	0.000	1.250	3.773
	SD	3.176	1.235	1.972	2.110
	P value	>0.05		>0.05	
CpG 15	Mean	3.439	2.060	1.539	2.843
	Median	2.400	2.038	1.285	3.128
	SD	2.370	1.740	1.474	1.508
	P value	>0.05		>0.05	
CpG 16	Mean	4.900	3.170	2.448	2.708
	Median	4.820	3.290	2.830	1.543
	SD	1.539	0.597	2.067	3.488
	P value	>0.05		>0.05	
CpG 17	Mean	4.208	1.796	1.795	5.758
	Median	2.760	2.150	1.1795	5.440
	SD	2.928	1.247	2.539	5.924
	P value	>0.05		>0.05	

Table 4.9 Effect of short-term DR on methylation at individual CpG sites within the *Apex* promoter sequence 1 in DNA from brain (AL n=5 and DR n=4) and liver (AL n=7 and DR n=9). Data represent mean ± SEM for each age group. DNA methylation for individual animal tissues were measured in duplicate.

CpG site		Brain		Liver	
		AL	DR	AL	DR
CpG 26	Mean	22.645	7.218	2.669	2.987
	Median	18.085	7.465	3.020	3.010
	SD	11.269	2.401	1.548	1.235
	P value	0.036		>0.05	
CpG 27	Mean	17.991	2.700	1.897	2.303
	Median	13.350	2.685	1.740	2.450
	SD	11.097	2.603	1.259	1.176
	P value	0.035		>0.05	
CpG 28	Mean	20.966	6.393	3.471	4.291
	Median	17.145	6.288	3.685	4.325
	SD	11.245	2.179	1.270	1.066
	P value	0.043		>0.05	
CpG 29	Mean	18.236	4.926	2.851	3.226
	Median	14.295	5.143	3.095	3.460
	SD	9.947	1.969	1.179	1.050
	P value	0.039		>0.05	
CpG 30	Mean	17.471	3.245	2.117	2.301
	Median	13.830	3.078	1.870	2.520
	SD	10.823	2.229	1.196	1.101
	P value	0.041		>0.05	
CpG 31	Mean	21.269	5.939	2.436	2.949
	Median	16.885	6.093	2.185	3.160
	SD	11.430	2.275	1.380	1.193
	P value	0.038		>0.05	
CpG 32	Mean	21.721	4.039	3.075	3.038
	Median	26.865	3.980	2.820	3.110
	SD	12.127	2.620	1.816	1.353
	P value	0.029		>0.05	
CpG 33	Mean	21.723	5.249	2.766	3.368
	Median	16.315	5.775	1.740	3.475
	SD	12.611	2.925	2.267	1.234
	P value	0.041		>0.05	
CpG 34	Mean	17.967	5.601	3.886	3.602
	Median	14.375	5.700	3.6300	3.805
	SD	9.589	1.600	1.746	0.972
	P value	0.044		>0.05	

Table 4.10 Effect of short-term DR on methylation at individual CpG sites within the *Apex* promoter sequence 2 in DNA from brain (AL n=5 and DR n=4) and liver (AL n=7 and DR n=9). Data represent mean ± SEM for each age group. DNA methylation for individual animal tissues were measured in duplicate.

4.3.3 *Expression of BER genes*

Expression of *Ogg1* and *Apex* mRNA was measured in brain and liver from ageing and short-term DR mice using RT-qPCR. Sequences of cDNA for *Ogg1* and *Apex* can be found in Appendix A and B respectively.

4.3.3.1 *Effect of age on gene expression in the brain*

An age-related decline in expression of *Ogg1* and *Apex* was seen in the brain (Figure 4.5). Expression of *Ogg1* decreased by 34% from mid aged to old animals (P=0.004) and by 43% from young adult to old animals (P=0.001). Expression of *Ogg1* in 32 month old animals was significantly lower than 6 month (P=0.001) and 17 month old (P=0.004). Expression of *Apex* decreased by 75% from young to old aged animals (P=0.001) and by 63% from mid aged to old animals (P=0.04).

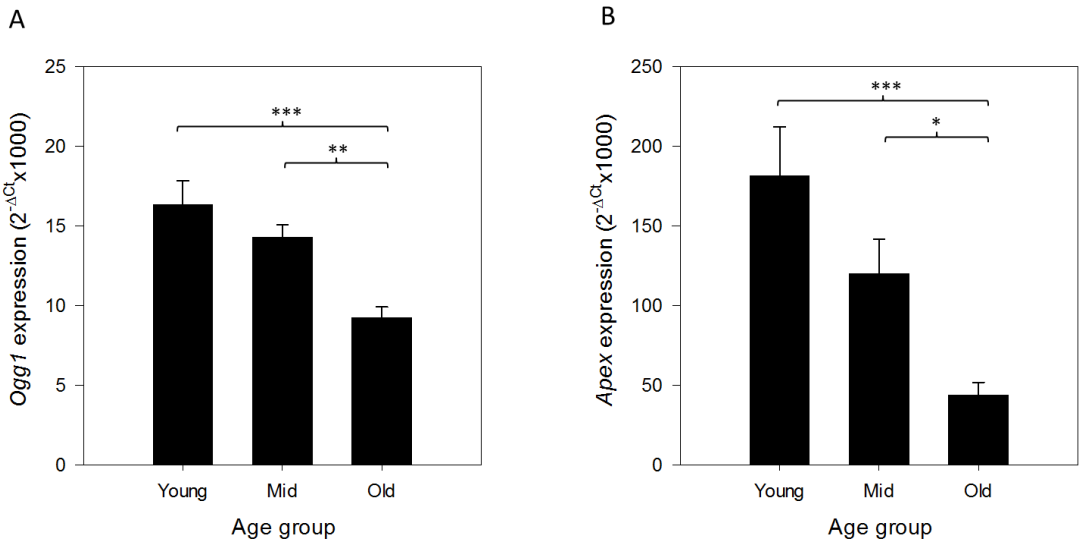


Figure 4.5 Effect of age on expression of *Ogg1* (A) and *Apex* (B) in the brain. Data represent mean ± SEM per group and have been multiplied by 1000 (young n=4, mid-age n=6, old n=6). Expression per gene was measured in duplicate for individual animal tissues. ANOVA with Bonferroni comparison for *Ogg1* P=0.001 (young vs old) and P=0.004 (mid aged vs old) and *Apex* P=0.001 (young vs old) and P=0.04 (mid aged vs old). *Ogg1 p*rimer design Dr Sabine Langie, *Apex* primer design Joanna Górniak.

4.3.3.2 *Effect of age on gene expression in the liver*

No difference in expression of *Ogg1* was observed in the liver. Expression of *Apex* was increased by 19% between young and mid aged animals and decreased by 59% from mid aged to old animals. However, these changes were not statistically significant (Figure 4.6).

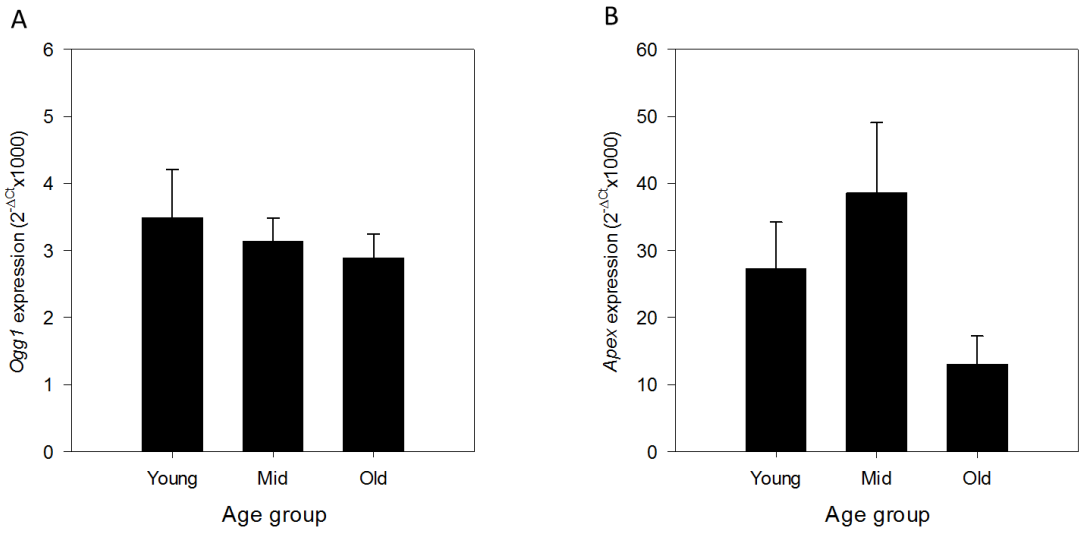


Figure 4.6 Effect of age on expression of *Ogg1* (A) and *Apex* (B) in the liver. Data represent mean ± SEM per age group (young n=4, mid-age n=4 and old n=4) and have been multiplied by 1000. Expression per gene was measured in duplicate for individual animal tissue. *Ogg1 p*rimer design Dr Sabine Langie, *Apex* primer design Joanna Górniak.

4.3.3.3 *Effect of short-term DR on gene expression in the brain and liver*

No effect of short-term DR on expression of *Ogg1* and *Apex* was found in the brain (Figure 4.7) or liver (Figure 4.8).

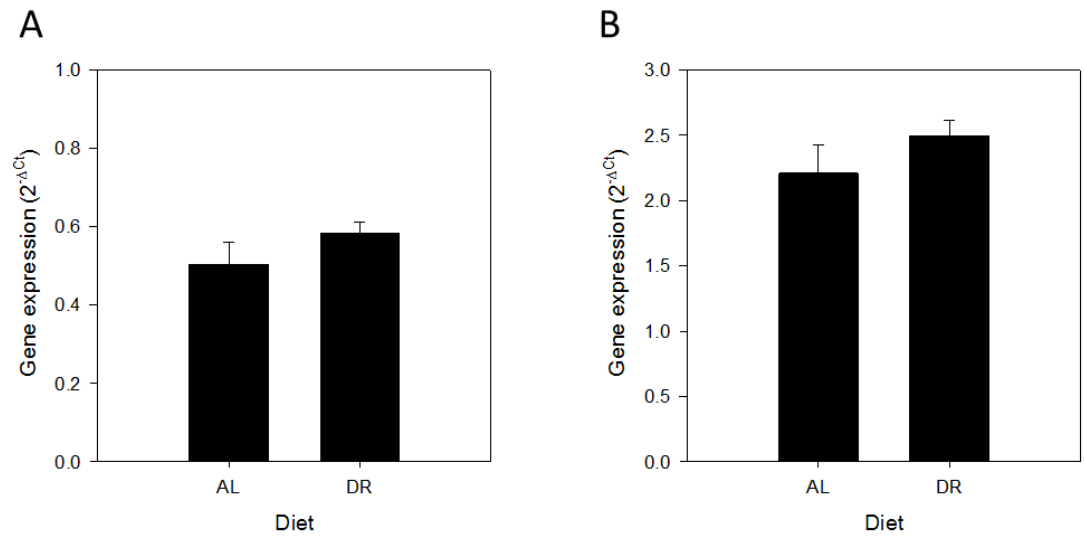


Figure 4.7 Effect of short-term DR on expression of *Ogg1* (A) and *Apex* (B) in the brain. Data represent mean ± SEM per dietary group (AL n=5 and DR n=4). Expression per gene was measured in duplicate for individual animal tissue. *Ogg1 p*rimer design Dr Sabine Langie, *Apex* primer design Joanna Górniak.

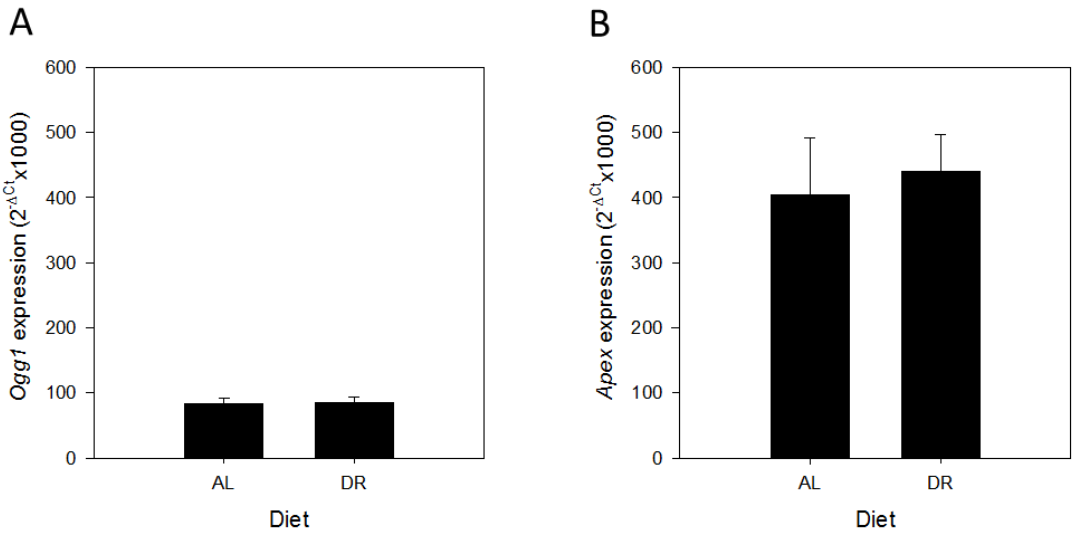


Figure 4.8 Effect of short-term DR on expression of *Ogg1* (A) and *Apex* (B) in the liver. Data represent mean ± SEM per dietary group (AL n=7 and DR n=7) and have been multiplied by 1000. Expression per gene was measured in duplicate for individual animal tissue. *Ogg1 p*rimer design Dr Sabine Langie, *Apex* primer design Joanna Górniak.

4.3.4 *DNA incision activity*

DNA repair was measured as DNA incision activity using a comet-based assay. Incision activity was measured in the brain, liver, lung and colon from ageing mice and in brain and liver from short-term DR mice.

4.3.4.1 *Effect of age on DNA incision activity in ageing tissues*

DNA incision activity varied between the tissues, with activity being highest in liver > colon > lung > brain. Biological variation was observed in each age group and this was demonstrated by the large error bars. Correcting for protein concentration and proliferative nature showed that DNA incision activity was more than 2 fold higher in proliferative tissues (colon and liver) compared with non-proliferative (brain and lung).

In the brain, BER-related incision activity was 15.4AU in young, 12.0AU in mid and 11.6AU in young animals. A 25% decrease in incision activity was observed between young and old animals; however, no significant difference was detected between the groups (Figure 4.9A). In the liver, BER-related incision activity increased significantly with age, activity was double in old animals (32 months) compared with young animals (P=0.024, Figure 4.9B). In the lung, DNA incision activity was 6.9AU in young, 8.4AU in mid and 4.5AU in old aged animals. DNA incision increased by 22% from young to mid aged animals but then it decreased by 45% from mid aged to old animals. However, these

differences were not statistically significant (Figure 4.9C). In the colon, DNA incision activity was 12.2AU, 19.6AU and 6.6AU in young, mid and old aged animals respectively (Figure 4.9D). Incision activity increased 61% between young and mid aged animals but decreased ~66% in old animals compared with mid aged ones. None of these values differed between age groups with statistical significance.

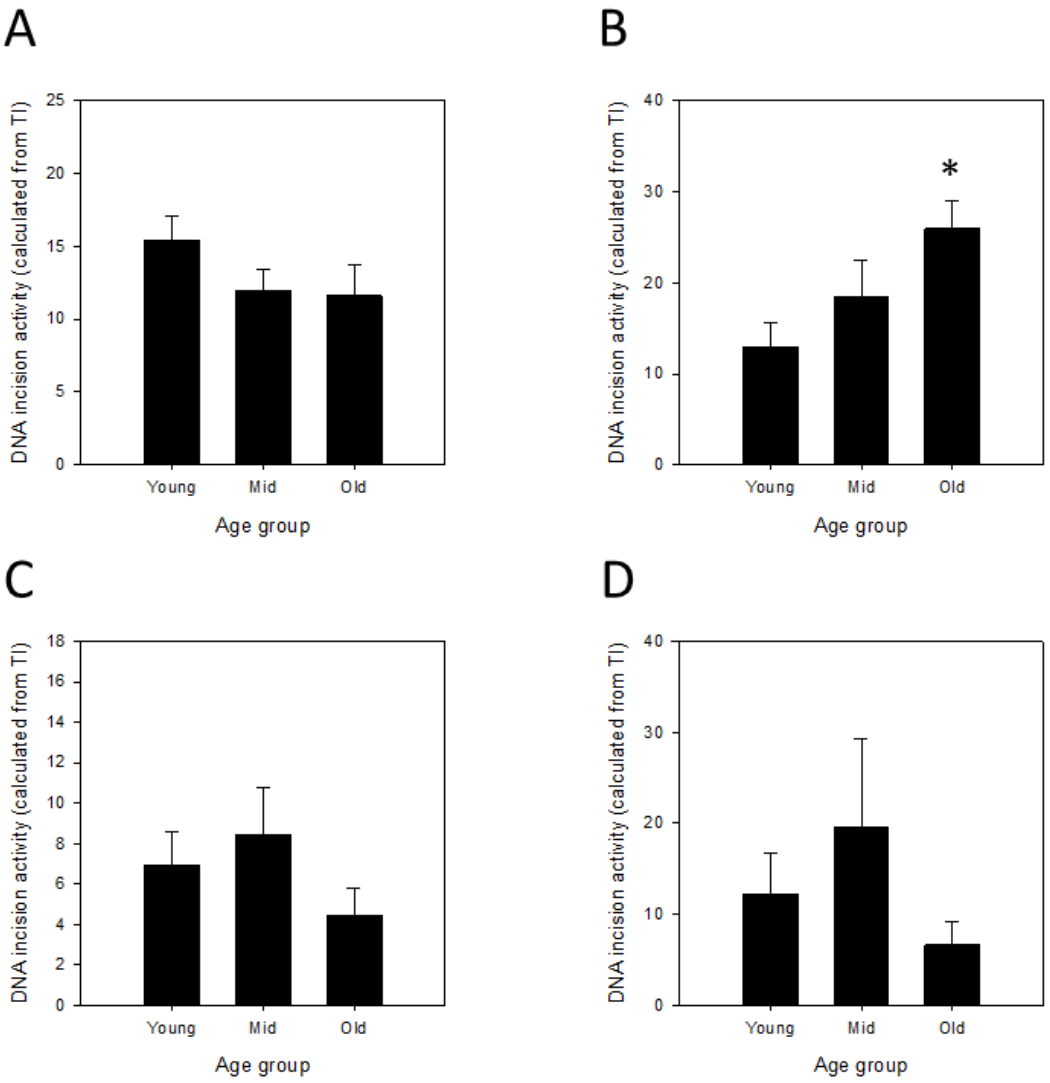


Figure 4.9 Effect of age on DNA incision activity in brain (A), liver (B), lung (C) and colon (D). Data represent mean of two individual assays ± SEM for each age group. DNA incision activity per age group n=4-6 brain, n=6 liver, n=6 lung, n=3 colon. ANOVA with Bonferroni post hoc comparison P=0.024 (young vs old). Figure published in (Gorniak *et al.*, 2013)

4.3.4.2 *Effect of short-term DR on DNA incision activity in the brain and liver*

No difference in DNA incision activity was observed in the brain. Activity was 27.7AU and 28.5AU in AL and DR animals respectively (Figure 4.10A). DNA incision activity in the liver was 15.9AU in AL animals and 23.6AU in DR animals (Figure 4.10B). DR caused a 1.5 fold increase in repair activity (P=0.032).

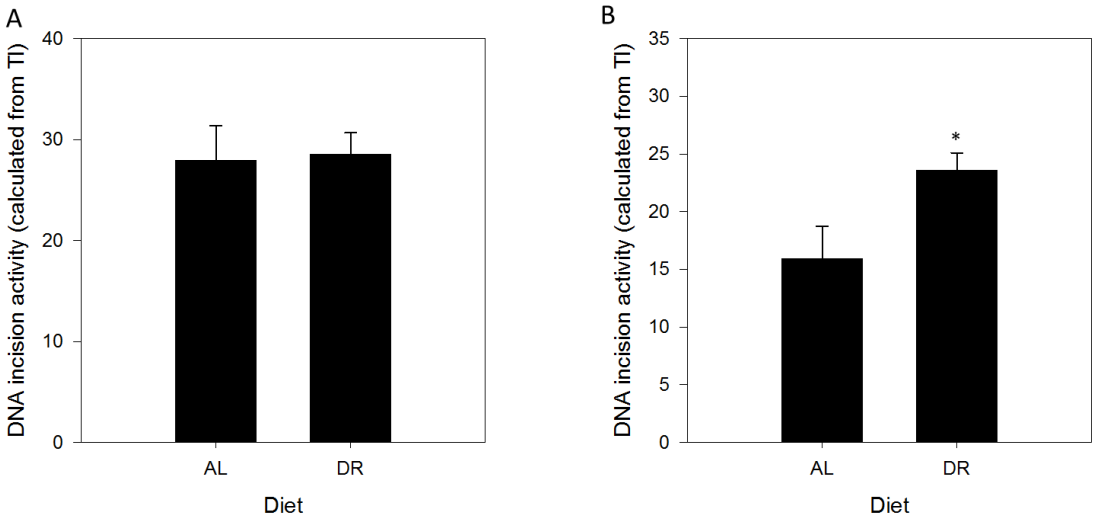


Figure 4.10 Effect of short-term DR on DNA incision activity in brain (A) and liver (B). Data represent mean of 2 individual assays ± SEM for each dietary group. DNA incision activity per group n=5-6 brain and n=7 liver. Student's t-test comparison of AL vs DR: P=0.032. Data for this Figure have been kindly provided by Sabine Langie.

4.3.5 *Oxidative damage*

Oxidative damage was measured as number of 8-oxoguanine bases using HPLC-ECD. 8-oxoguanine lesions were measured in the brain, liver, lung and colon from ageing and short-term DR mice.

4.3.5.1 *Effect of age on 8-oxoguanine concentration in mouse tissues*

Concentration of 8-oxoguanine lesions was different between the 4 organs studied (P<0.001). The highest number of lesions was found in the liver > lung > brain > colon. Oxidative damage in the brain increased with age (Figure 4.11A). In young animals 15.5 8-oxoguanines per 10^6 guanine molecules were detected. The number of oxidised guanines increased by 1.8 fold in mid-aged compared with young mice (P=0.012) and by 1.6 fold in old compared with young mice (P=0.046). The concentration of 8-oxodG in DNA from liver was similar at all ages. Mean values were 48.8, 37.8 and 43.4 8oxodG/10^6dG for

animals from young, mid-aged and old age groups respectively (Figure 4.11B). In the lung, similar levels of oxidative damage were observed in young and mid aged animals. 29.0 and 32.4 8-oxoguanines per 10^6 guanine molecules were detected in young and mid-aged animals respectively (Figure 4.11C). Interestingly, the concentration of 8-oxoguanine decreased in the oldest age group and was 40% lower compared with the mid-aged animals (P=0.006). Oxidative lesions in the colon decreased by 25.6% in the mid-aged compared to young animals (P=0.009) but increased again 1.43 fold in old animals compared to mid-aged animals (P=0.006, Figure 4.11D).

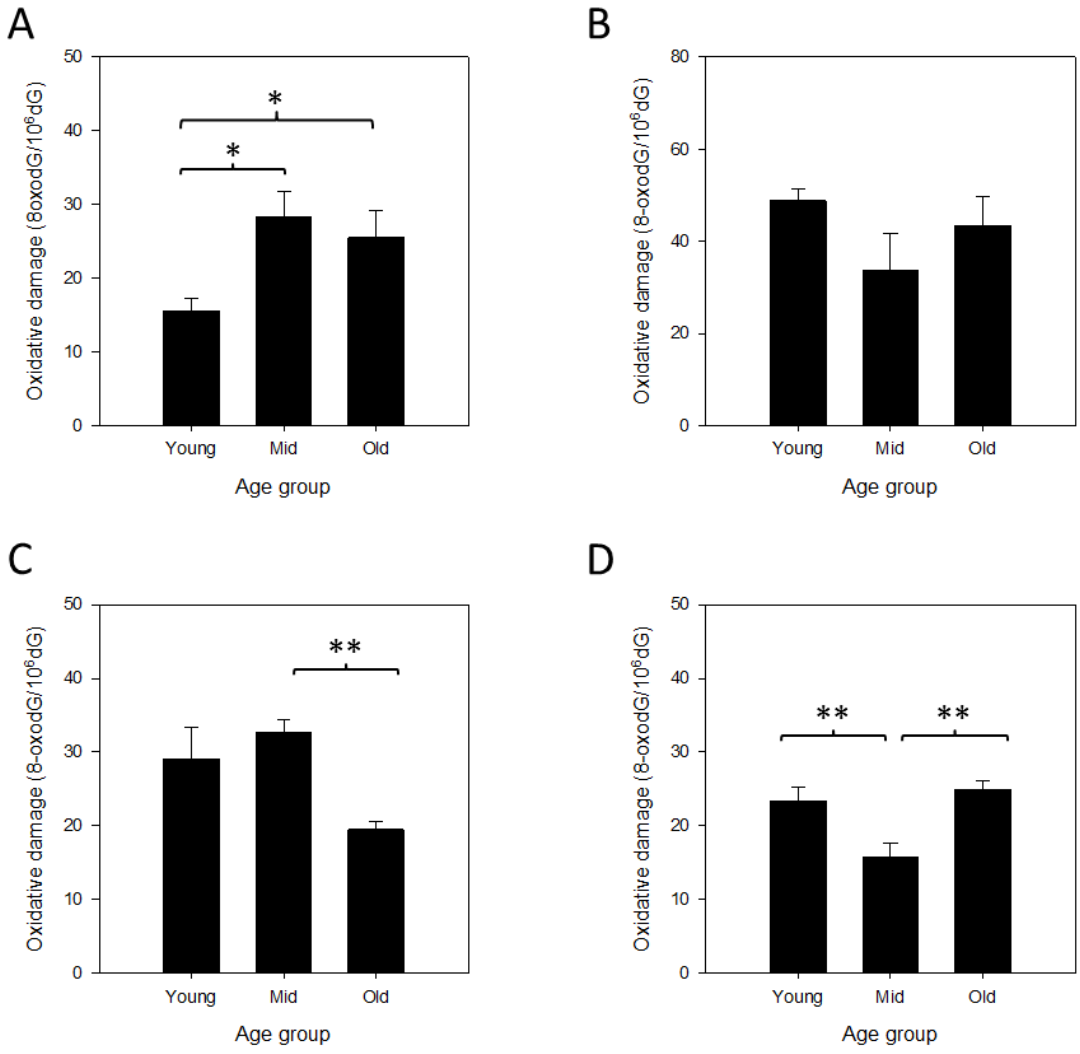


Figure 4.11 Effect of age on 8-oxoguanine concentration in brain (A), liver (B), lung (C) and colon (D). Data represent mean ± SEM for each age group, measurements were taken in duplicate. 8-oxoguanine measured per age group in brain n=4-10, liver n=3-6, lung n=6-12 and colon n=6-12. ANOVA with Bonferroni post hoc analysis in the brain P=0.012 (young vs mid) and P=0.046 (young vs old); in the lung P=0.006 (mid vs old); in the colon P=0.009 (young vs mid) and P=0.006 (mid vs old). Data collected by Maastricht University, in collaboration with Dr Roger Godschalk.

4.3.5.2 *Effect of short-term DR on 8-oxoguanine concentration in the mouse tissues*

Short-term DR had little effect on oxidative damage in the brain, liver or lung. Highest concentration of 8-oxoguanine was in the liver > lung > brain > colon. In the brain there were 21.0 and 23.4 8oxodG/10^6dG in AL and DR animals respectively (Figure 4.12A). Levels of 8-oxoguanine were ~44 and 34 per 10^6 guanines in the liver (Figure 4.12B) and lung (Figure 4.12C) respectively. In the colon DR caused a 2 fold increase in 8-oxoguanine lesions (P<0.001) compared with AL (Figure 4.12D).

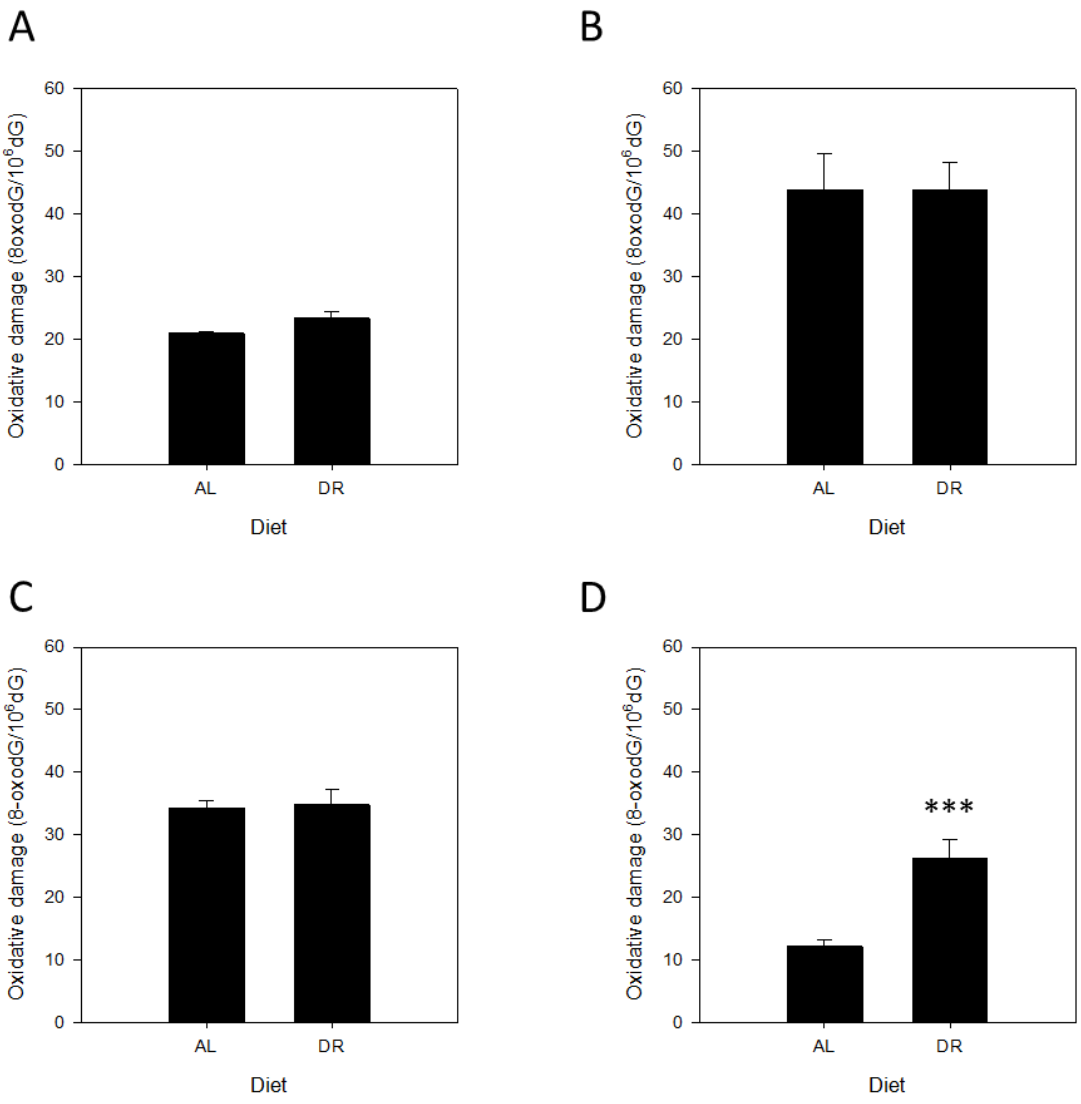


Figure 4.12 Effect of short-term DR on 8-oxogyuanine concentration in brain (A), liver (B), lung (C) and colon (D) from mice. Data represent mean ± SEM for each age group. . 8-oxoguanine measured per age group in brain n=5, liver n=9, lung n=8-9 and colon n=8-9. Student's t-test comparison of AL vs DR: P<0.001. Data collected by Maastricht University, in collaboration with Dr Roger Godschalk. Graphs in panels A and B curtesy of Sabine Langie.

4.4. Discussion

The purpose of this chapter was to optimise assays to test BER related activity and epigenetic and transcription profiling of DNA repair genes *Ogg1* and *Apex* in mouse brain and liver. Furthermore this study was set out as a pilot to test the effects of age and short-term dietary restriction in mouse tissues. In this discussion I will primarily focus on evaluation of the assays selected for this study. I will then discuss the results in light of the hypothesis.

4.4.1 *Evaluation of experimental techniques*

4.4.1.1 *Pyrosequencing*

DNA methylation as a regulator of gene expression has been of interest for some time. Recently this mechanism has been implicated as playing an important role in ageing. OGG1 and APEX were chosen as the 2 enzymes representative of the first step of BER. The pyrosequencing assay for *Ogg1* was designed and validated previously (Langie *et al.*, 2013) while the design and validation of *Apex* assay can be found in the Appendix. Both assays show strong specific amplification of the PCR product and no non-specific amplification. It is evident from the pyrosequencing assays that promoter methylation of *Ogg1* and *Apex* are low at all ages and near the lower limit of detection of the Pyrosequencer. The CpG sites covered in these assays are only a representation of the total number of sites in each of the promoters as it is not possible to look at all of them.

4.4.1.2 *RT-qPCR*

RT-qPCR is the gold standard for quantifying gene expression. The above experiments were conducted using the MIQE guidelines to ensure the best quality results (Bustin *et al.*, 2009). Stringent criterion for RNA quality ensured degradation of RNA was minimal and results were conducted on intact mRNA. Furthermore, a check assay was run after cDNA synthesis to ensure no genomic DNA contamination occurred. The results from gene expression studies were expressed using a modification of the $2^{-\Delta\Delta Ct}$ method i.e. $2^{-\Delta Ct}$, as no true control group was present. Assays gave good amplification of product with no non-specific amplification as demonstrated by the melt curves. By assessing DNA methylation as well as expression of *Ogg1* and *Apex* a relationship between methylation at CpG sites studied and their effect on expression could be explored.

4.4.1.3 *Comet in-vitro repair assay*

The comet-based *in-vitro* repair assay is a sensitive technique for measurements of repair of singlet oxygen induced DNA damage (Langie *et al.*, 2011). This assay may require multiple optimisation steps as demonstrated in Chapter 3.Optimal concentrations of protein extracts to use in the comet assay were 5mg/ml for both brain and liver, 1.25mg/ml for lung and 0.5mg/ml for colon Here I show that the comet assay is able to detected age-related changes in DNA repair as observed in the liver, and also differences in repair profiles of other tissues. Many other assays able to measure incision activity focus on repair of a single modified base in an oligonucleotide or a plasmid. Although this may give a more controlled assay it can be speculated that the comet assay gives a more realistic view of DNA incision (Langie *et al.*, 2011).

4.4.1.4 *HPLC*

Finally 8-oxodG has been used as a marker of oxidative damage. It is implicated in mutagenic GC to TA transversions. Different methods have been used to measure oxidative damage and problems arise when comparing results from each of the tests. HPLC is a sensitive method used in detection although it has been speculated that high levels are detected as a result of oxidation during the DNA extraction process. In comparison measurements using the basic comet assay show much lower estimates of oxidative damage but one may speculate where this is because of inability of enzymes to reach damage in tightly wound DNA.

4.4.2 *Effects of age*

Although no age or diet changes have been reported here, a small but significant increase in *Ogg1* promoter methylation in the aged brain has previously been shown (unpublished data by Sabine Langie). As mentioned in this Chapter, not all CpG sites in the promoter region were studied here. It may be likely that methylation at specific CpG sites is of more relevance to transcription silencing of these genes. Although a lack of change in promoter methylation was observed for both *Ogg1* and *Apex* the expression of the genes was shown to decline with age. The observed decrease in transcript levels is highly significant in the aged brain and the effects may have arisen due to other epigenetic modifications. A decline in BER gene expression has been

previously shown in ageing rat lens (Zhang *et al.*, 2010) and carcinoma (Kumar *et al.*, 2012).

DNA repair is linked with accumulation of DNA damage and genomic instability although this view is controversial as not all studies have found increased damage with age. A direct comparison of repair cannot be made between all tissues as they were tested at different concentrations. Both liver and brain were measured at 5mg/ml protein concentration and data show that liver has higher incision activity than the brain. It has been suggested that the proliferative nature of the tissue may have an influence on repair. Here liver and colon represent mitotic tissue while lung and brain represent post-mitotic tissue; however; no clear pattern is seen in either cell type, indicating that tissue-specific (rather than proliferative nature of the cell) changes in repair may occur during ageing.

Interestingly an increase in incision activity was observed in the liver in older animals and this has previously also been reported (Mikkelsen *et al.*, 2009). Furthermore although DNA repair activity has often been said to decline with age no statistically significant changes in repair was observed here in brain, liver or colon and lack of age-related effect on repair has also been reported before (Hirano *et al.*, 1996).

The data suggest that concentrations of oxidative damage as measured by 8-oxodG vary between tissues and with age. Overall (data pooled from all ages), highest levels were observed in the liver followed by lung, brain and colon. It has been previously demonstrated that oxidative damage accumulation is tissue dependant (Schmerold and Niedermüller, 2001). Increase in oxidative damage accumulation has been reported in brain and liver (Sohal *et al.*, 1994a) but this has not always been confirmed (1990).

4.4.3 *Effects of short-term DR*

Data presented here have not found an effect of short-term DR on DNA methylation or gene expression and one can speculate whether this is due to the short period of DR the mice were subjected to or more likely that the 26% DR is relatively low compared with other studies (Weraarchakul *et al.*, 1989; Cabelof *et al.*, 2003; Gedik *et al.*, 2005). Furthermore the results from the aged study suggest that the biggest change is observed in the oldest age group in

which case more informative results may have been acquired if an additional group of animals was kept alive (on DR or AL diet) until 30 months of age.

Multiple studies have reported a decline in DNA repair. PARP, a polymerase involved in DNA repair, has been shown be less active in rat frontal cortex and this was attenuated by DR (Hiona and Leeuwenburgh, 2004). Similarly unscheduled DNA synthesis as measured by incorporation of a radiolabelled cytosine nucleotide at a G:U mismatch has been shown to decline in rodent tissue with age while DR animals displayed higher repair compared with AL controls (Cabelof *et al.*, 2003; Intano *et al.*, 2003). Furthermore, DR was observed to reduce oxidative damage levels in multiple organs (Chung *et al.*, 1992; Sohal *et al.*, 1994a).

4.4.4 *Study design*

A major limitation to the 'pilot study' arises from the selection of samples that were used, which although they came from the same strain of mice bred at the university have all come from slightly different studies. Furthermore a more stringent statistical analysis could not be used as the samples tested in the brain and the liver did not come from the same animal. Thus caution should be taken when looking at the differences between the tissues. In Chapter 5 and 6; the long-term DR study this issue will be overcome as tissue samples under comparison have been taken from the same animal.

4.5 Conclusions

To conclude, CpG islands have been found in the promoters of both *Ogg1* and *Apex* genes and assays for measuring DNA methylation at sites in these CpG islands have been developed. Furthermore any changes in gene expression can be studied using the *Ogg1* and *Apex* gene expression assays. Studies of age-related accumulation of oxidative damage are still conflicting and damage accumulation will in part depend on efficiency of DNA repair hence it is important to assess both the repair capacity and damage accumulation. Finally the data presented here support further studies into ageing and long-term dietary restriction.

4.6 Acknowledgments

I would like to thank:

- *Dr Sabine Langie for permitting use of data for DNA incision activity and 8-oxoguanine levels in brain and liver from dietary restricted animals*
- *CISBAN for design of the animal study and Dr Kerry Cameron and Professor Thomas von Zglinicki for information on animal and tissue weights.*
- *Maastricht University who performed the 8-oxoguanine analysis*
- *Dr Sabine Langie and Bartłomiej Tomaszewski for design of Ogg1 pyrosequencing and qPCR primers.*
- *Dr Sabine Langie for developing the comet in vitro repair assay.*

Chapter 5: The effects of ageing and of long-term dietary energy restriction (DR) on base excision repair (BER) in the brain

5.1 Introduction

Preliminary data described in Chapter 4 suggest that age and short-term dietary restriction (DR) affect base excision repair (BER) in the brain. In this chapter I describe the changes in BER in the brain of male and female mice exposed to long-term DR.

5.1.1 *Epigenetic regulation of chromatin*

Epigenetic marks control the chromatin structure thereby regulating transcription activity of genes. While cytosine methylation is accompanied by gene silencing specific histone modifications are also associated with particular transcription states. Addition of acetyl groups to histone tails opens chromatin structure which allows transcription factors to bind to the gene promoter regions and initiate transcription (Struhl, 1998). On the other hand, methylation marks on histone tails have been implicated in both transcription activation and repression dependent on the site of methylation. Tri-methylation of the lysine residue at position 27 of histone 3 (H327Me3) in particular is associated with repression of the corresponding gene (Zhang and Reinberg, 2001). Although the mechanism for H3K27Me3 is not fully understood it is known that this histone modification associates with polycomb repressive complexes which cause repression of transcription possibly by preventing transcription elongation (Simon and Kingston, 2009).

DNA methylation and histone modifications are two independent mechanisms and although the hierarchy of epigenetic events is not known, it is evident that cross-talk occurs between these epigenetic marks. Cytosine hypermethylation often occurs together with histone deacetylation and either event can initiate gene silencing by 1) directly preventing binding of transcription factors or 2) indirectly by binding proteins, e.g. those containing a methyl binding domain, that prevent transcription initiation (Vaissière *et al.*, 2008). Furthermore, enzymes responsible for epigenetic changes form complexes, such as the association of DNA cytosine-5-methyltransferase 1 (DNMT1) with histone deacetylase 1 (HDAC1). These epigenetic enzymes can also affect numerous epigenetic marks; e.g. a mutation in *dim-5,* encoding a histone 3

methyltrasferase, in *Neurospora* abolishes DNA cytosine methylation (Fuks *et al.*, 2000; Robertson *et al.*, 2000; Tamaru and Selker, 2001).

This multiplayer regulation has led me to explore additional epigenetic marks such as histone 4 acetylation (H4Ac) and H3K27me3 as well as cytosine methylation at the *Ogg1* and *Apex* promoter regions.

5.1.2 *Enzymes regulating epigenetic marks*

These epigenetic changes are a result of the activity of enzymes such as DNA methyltrasferases (DNMT), histone acetyltransferases (HAT) and histone deacetylases (HDAC) (2009). Therefore the age-related changes in expression and function of these enzymes play a part in the observed epigenetic alterations. An age-related decline in expression of DNMT1 and DNMT3a and an increase in DNMT3b has been reported in fibroblasts (Lopatina *et al.*, 2002; Casillas *et al.*, 2003). These findings can in-part explain the changes in global and promoter specific methylation seen in ageing (D'Aquila *et al.*, 2013). Multiple classes of HAT and HDAC enzymes exist. Decreases in HATs p300 and CBP are seen with increased population doubling in human melanocytes (Bandyopadhyay *et al.*, 2002). Decline in HDAC expression has been reported in senescent stem cells compared with young cells (Jung *et al.*, 2010). Furthermore the Sirtuin family of HDACs has been linked to life extension; e.g. via Sir2 over-expression in yeast. The mammalian homologue SIRT1 is down-regulated in senescent fibroblast cells (Sasaki *et al.*, 2006). Furthermore, evidence supports HDAC activation as one of the modes of action for dietary restriction as DR was not able to extend lifespan in yeast cells not expressing Sir2 (Lin *et al.*, 2000), suggesting that Sir2 and DR act within the same pathway. In addition, DR attenuates the age-related increase in DNMT3a expression observed in mouse hippocampus (Chouliaras *et al.*, 2011).

5.1.3 *Redox-sensitive transcription factors*

It is well documented that redox-sensitive transcription factors (TF), such as forkhead box protein (FOXO) and hydrogen peroxide-inducible clone-5 (HIC-5), are up-regulated during ageing and DR (Lee *et al.*, 1999; Weindruch *et al.*, 2001; Greer and Brunet, 2008). Furthermore hypoxia-inducible factor 1-alpha (HIF1α) increases with age in rodent brain and liver (Kang *et al.*, 2005; Benderro and LaManna, 2011) while DR prevents this response (Kang *et al.*, 2005). These TFs initiate transcription of cellular response genes such as those

coding for DNA repair proteins. A number of such transcription factors have binding sequences in *Ogg1* and *Apex* promoters. A comprehensive list of these TFs can be found in Appendix A and B. Of these, nuclear transcription factor Y subunit A (NFYA) has been more thoroughly studied in the context of *Ogg1* activation. Decreased expression of NFYA was associated with decreased OGG1 protein concentration in renal cancer and tuberous sclerosis complex disease (Habib, 2009). Treatment of kidney cells with rapamycin (mTOR inhibitor) resulted in increased NFYA and OGG1 protein expression (Habib *et al.*, 2010). HIF1α and NFYA are two known TFs that bind both in the *Ogg1* and *Apex* promoter regions. Exploring the expression of these TFs during ageing and DR could provide insight into BER related expression.

5.1.4 *Aims and Objectives*

The study described in this chapter aimed to test the following hypotheses:

1. During ageing, epigenetic changes at *Ogg1* and *Apex* gene promoters cause silencing of these genes.
2. This gene silencing results in decreased BER activity and hence increased DNA oxidative damage accumulation.
3. DR ameliorates the adverse effects of age on epigenetic modifications at *Ogg1* and *Apex* promoters, by mediating changes in enzyme activity, DNA repair, expression of redox-sensitive TFs and reducing concentration of DNA oxidative damage.

The objectives of the present study were to:

a) Characterise and quantify methylation of specific CpG sites within the promoters of *Ogg1* and *Apex* in DNA from the brain of mice during ageing and in response to long-term DR;
b) Characterise and quantify histone acetylation and methylation associated with *Ogg1* and *Apex* promoters in DNA from the brain of mice during ageing and in response to long-term DR;
c) Quantify histone acetyltransferase and histone deacetylase activity in extracts from the brain of mice during ageing and in response to long-term DR;
d) Using RT-qPCR, quantify expression of the transcription factors *Hif1α* and *Nfya* which are associated with *Ogg1* and *Apex* gene activation, in the brain of mice during ageing and in response to long-term DR;

e) Using RT-qPCR, quantify expression of *Ogg1* and *Apex* in the brain of mice during ageing and in response to long-term DR;
f) Quantify BER-related incision activity in mouse brain tissue using the *in vitro* comet-based repair assay;
g) Using ELISA quantify OGG1 and APEX protein expression in extracts from the brain of mice during ageing and in response to long-term DR;
h) Using HPLC-ECD measurements of 8-oxoguanine, quantify oxidative damage in DNA from the brain of mice during ageing and in response to long-term DR.

5.2 Materials and Methods

Male and female mice for the long-term DR main study were of the C57BL6 strain. At 3 months of age, mice were allocated at random to either the ad libitum (AL) feeding regime or to DR. Dietary restricted mice were placed on 40% by weight food restriction i.e. the DR mice received 60% of the food consumed by the AL mice. Mice were killed at ages 3, 12, 24 and 30 months of age and brain and liver tissues were removed, snap frozen in liquid nitrogen immediately and stored at -80˚C. Figure 5.1 provides an overview of the design of the animal study. This chapter describes epigenetic changes observed in brain tissue and Chapter 6 will discuss epigenetic changes in liver tissue.

Figure 5.1 Schematic overview of long-term DR mouse study.

The laboratory methods have been described in detail in Chapter 2. Briefly, ground frozen tissue was used for extraction of DNA and mRNA for promoter DNA methylation and gene expression studies respectively. Protein extracts from ground tissue were also used for the *in vitro* comet-based repair assay and to quantify concentration of OGG1 and APEX proteins using ELISA kits plus

HAT and HDAC activity using colorimetric kits. Ground tissue was also used for the chromatin immunoprecipitation assay to quantify histone 4 acetylation (H4Ac) and histone 3 lysine 27 tri-methylation (H3K27Me3) relative to the *Ogg1* and *Apex* promoter regions.

ANOVA general linear model (ANOVA GLM) was used for statistical analysis testing effects of age, diet and sex and their interactions when data were normally distributed. Bonferroni post-hoc test was used for comparisons of all groups. When data were not normally distributed the non-parametric Kruskal-Wallis test was used except when analysing individual CpG site methylation in which case ANOVA GLM was performed despite no normality. Non-parametric Mann-Whitney U test, with a correction for multiple testing (significance α=0.05/number of comparisons made), was used as a post hoc test in non-normally distributed data. Where no statistically significant difference was seen between AL and DR animals at same age, the two diet groups were pooled together to increase the power for age related calculations. Similarly when no statistically significant difference was seen with age, the age groups were grouped together to increase the power for diet related calculations.

5.3 Results

5.3.1 *Animal and organ characteristics*

The long-term DR mouse study from which tissues were obtained for the present investigation was designed and managed by the Centre for Integrative Systems Biology of Ageing and Nutrition (CISBAN) to investigate the ageing process and its amelioration by DR. At 3 months of age, when mice are considered adult, the animals were randomised to *ad libitum* (AL) feeding or to the 40% dietary restricted (DR) regime. A parallel longitudinal study, designed for phenotyping purposes, run at the same time as the long-term DR study, found mean lifespan for female mice was increased by 2.3 months ($P<0.001$, mean lifespans of 26.9±0.4 and 29.2±0.4 months for AL and DR mice respectively). The increase in lifespan in DR compared with AL male mice was greater by 3 months ($P<0.001$) i.e. 26.1±0.1 and 29.1±0.4 months for AL and DR mice respectively. Overall, lifespan for male and female mice was not significantly different (unpublished data from Kerry Cameron and Thomas von Zglinicki).

Group	Male			Female		
	Body mass (g)	Brain (g)	Brain mass (%)	Body mass (g)	Brain (g)	Brain mass (%)
3AL	29.2 ±2.04	0.426 ±0.0055	1.46 ±0.049	22.9 ±1.53	0.398 ±0.0312	1.74 ±0.099
12AL	36.7 ±4.12	0.422 ±0.0154	1.16 ±0.046	25.2 ±3.12	0.423 ±0.0136	1.70 ±0.087
12DR	24.8 ±2.75	0.428 ±0.0175	1.75 ±0.086	17.1 ±1.91	0.418 ±0.0140	2.47 ±0.105
24AL	47.9 ±3.85	0.488 ±0.0426	1.02 ±0.046	35.9 ±3.20	0.483 ±0.0146	1.35 ±0.050
24DR	25.3 ±2.12	0.420 ±0.0730	1.66 ±0.088	19.2 ±0.32	0.448 ±0.0479	2.33 ±0.091
30AL	35.1 ±2.60	0.395 ±0.1085	1.12 ±0.137	28.4 ±4.38	0.467 ±0.0158	1.68 ±0.144
30DR	23.0 ±2.94	0.387 ±0.0517	1.70 ±0.150	18.1 ±1.04	0.429 ±0.0244	2.37 ±0.039

Table 5.1 Body masses and weights of brains from a subset of male and female mice in the CISBAN longitudinal phenotyping study. Specific data for the animals from which tissues were used in the present study are not available. Data represent mean ± SEM, n=5-7, 3AL – 3 month old AL fed mice, 12AL – 12 month old AL fed mice, 12DR – 12 month old DR fed mice, 24AL – 24 month old AL fed mice, 24DR – 24 month old DR fed mice, 30AL – 30 month old AL fed mice and 30DR – 30 month old DR fed mice. Data courtesy of Dr Kerry Cameron and Professor Thomas von Zglinicki.

Data for body mass and brain weights in male and female mice are shown in Table 5.1. In both male and female mice, body mass increased with age to reach a maximum at 24 months and then declined at 30 months. DR reduced body mass at all ages in both males and females ($P<0.001$). Brain weights were similar between dietary regimes and across ages although the brain weight was reduced by DR in female but not in male mice. Furthermore when measured as tissue mass as a percentage of body weight DR actually increased brain weight.

5.3.2 *Epigenetic regulation of Ogg1 and Apex promoters in the brain*

To determine the underlying mechanisms of the effects of age and of long-term DR on base excision repair, I measured epigenetic marks at the promoter regions of *Ogg1* and *Apex* genes. This included quantification of cytosine methylation, H4Ac and H3K27Me3 in the brain from mice from the CISBAN long-term DR study.

5.3.2.1 *DNA methylation of the Ogg1 promoter*

As described in Chapter 4, DNA methylation was quantified at 13 CpG sites in the *Ogg1* gene promoter and 22 CpG sites in the *Apex* gene promoter.

Measurements were made at all ages (3, 12, 24 and 30 months) and in both AL and DR animals. Cytosine methylation data are prone to non-normal distribution. Furthermore, a Box-Cox power transformation was unable to transform the data to a normal distribution. The factorial (age and diet) design of the study meant that non-parametric tests were not powerful to test for trends and interactions. As the ANOVA is a robust test, able to withstand deviations from normality (Glass *et al.*, 1972; Sawilowsky, 1990), I used the ANOVA GLM for analysis of individual CpG sites despite violation for normality of data.

Figure 5.2 and 5.3 show methylation at individual CpG sites and mean methylation across the CpG sites in the *Ogg1* promoter in male and female mice respectively. Overall, levels of methylation were quite low and ranged from 0-12.7%. In the *Ogg1* promoter, average methylation across all 7 groups was lowest in CpG 3 at 0.14% in males and 0.07% in females and highest in CpG 16 at 2.67% in males and 3.16% in females. CpG 4, 16 and 18 showed consistently higher methylation levels compared with other CpG sites measured. Supplementary Tables for mean and median cytosine methylation values per CpG site can be found in Appendix A.

Mean *Ogg1* promoter methylation in the brain was normally distributed, although methylation at most individual CpG sites was not. There were differences between the sexes ($P<0.001$), with females showing overall higher methylation compared with males and so further analyses considered data for male and female animals separately. Correlation of methylation at individual CpG sites are shown in Figure 5.4 and Figure 5.5 for male and female mice respectively. The correlation between the different sites varied from -0.122 to 0.793 in males and -0.264 to 0.753 in females. Overall the correlation between CpG sites was not high; therefore, mean methylation may not be ideal for presenting these data and analysis were also run at individual CpG sites.

In male mice cytosine methylation at 6 CpG sites decreased with age in a linear trend: CpG 3 ($P_a=0.002$), CpG 6 ($P_b=0.005$), CpG 7 ($P_d<0.001$), CpG 13 ($P_e=0.027$), CpG 14 ($P_f=0.013$) and CpG 15 ($P_h=0.026$). Cytosine methylation at 4 CpG sites was lower in DR fed animals compared with AL fed controls by: 0.16% at CpG 6 ($P_c=0.002$), 0.18% at CpG 14 ($P_g=0.031$), 0.25% at CpG 17 ($P_i=0.042$) and 0.41% at CpG 18 ($P_j=0.029$). Mean *Ogg1* promoter methylation across the 13 CpG sites decreased with age in a linear trend ($P_k=0.016$). In

addition, overall *Ogg1* promoter methylation was significantly lower in DR animals (Pl=0.014) although the absolute difference of 0.23% was quite modest.

In female mice, only cytosine methylation at CpG 8 changed with age in a quadratic trend (Pa=0.01). In addition, cytosine methylation at CpG 3 and 17 was 0.09% (Pb=0.014) and 0.33% (Pc=0.025) lower in DR compared with AL fed animals respectively. There was no evidence that mean methylation of the *Ogg1* promoter in DNA from the brains of female mice was affected by either age or diet.

5.3.2.2 *DNA methylation of the Apex promoter*

Figure 5.6 and 5.7 show methylation at individual CpG sites and mean methylation across the CpG sites in the *Apex* promoter in male and female mice respectively. Overall, levels of methylation were quite low and ranged from 0-13.9%. In the *Apex* promoter CpG 30 showed least methylation in male mice at 0.95% while CpG 27 showed least methylation in female mice at 0.26%. CpG 28 was most methylated in males at 4.57% while CpG 9 was most methylated in females at 2.73%. CpG sites 9, 17, 28, 29 and 34 showed consistently higher methylation compared with other CpG sites. A supplementary table with mean and median cytosine methylation at individual CpG sites can be found in Appendix B.

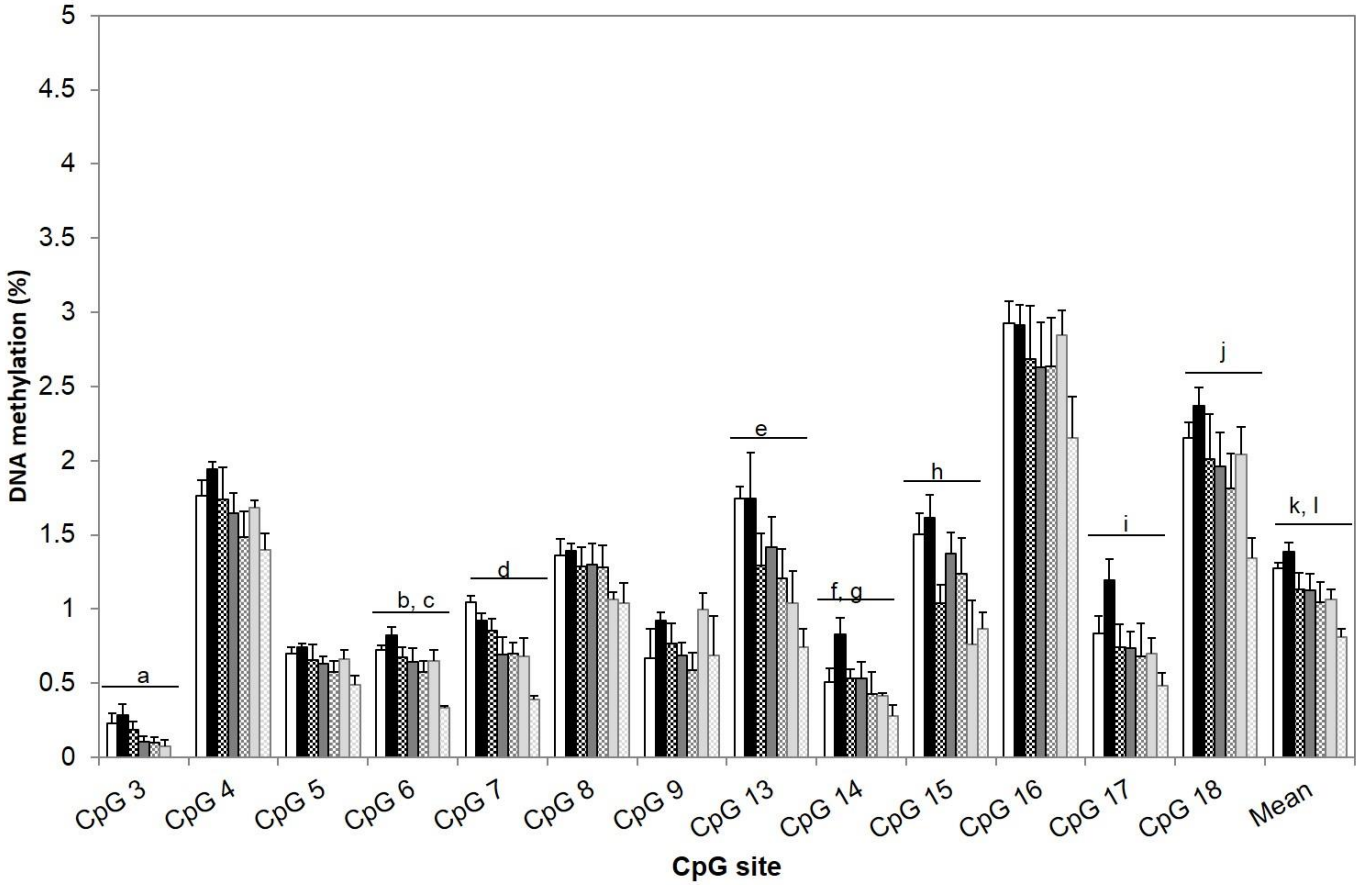


Figure 5.2 Effect of age and DR on methylation at individual CpG sites within the *Ogg1* promoter in DNA from male brain. Data represent mean ± SEM per treatment group, 3AL (white bars, n=5), 12AL (black bars, n=7), 12DR (black checker bars, n=7), 24AL (dark grey bars, n=7), 24DR (dark grey checker bars, n=7), 30AL (light grey bars, n=5) and 30DR (light grey checker bars, n=7). Methylation for individual animal tissues were measured in duplicate. Univariate ANOVA-GLM analysis for age and diet: Pa=0.002, Pb=0.005, Pc=0.002, Pd<0.001, Pe=0.027, Pf=0.013, Pg=0.031, Ph=0.026, Pi=0.042, Pj=0.029, Pk=0.016 and Pl=0.014. Primer design Bartłomiej Tomaszewski and Dr Sabine Langie. Data collected by Joanna Górniak.

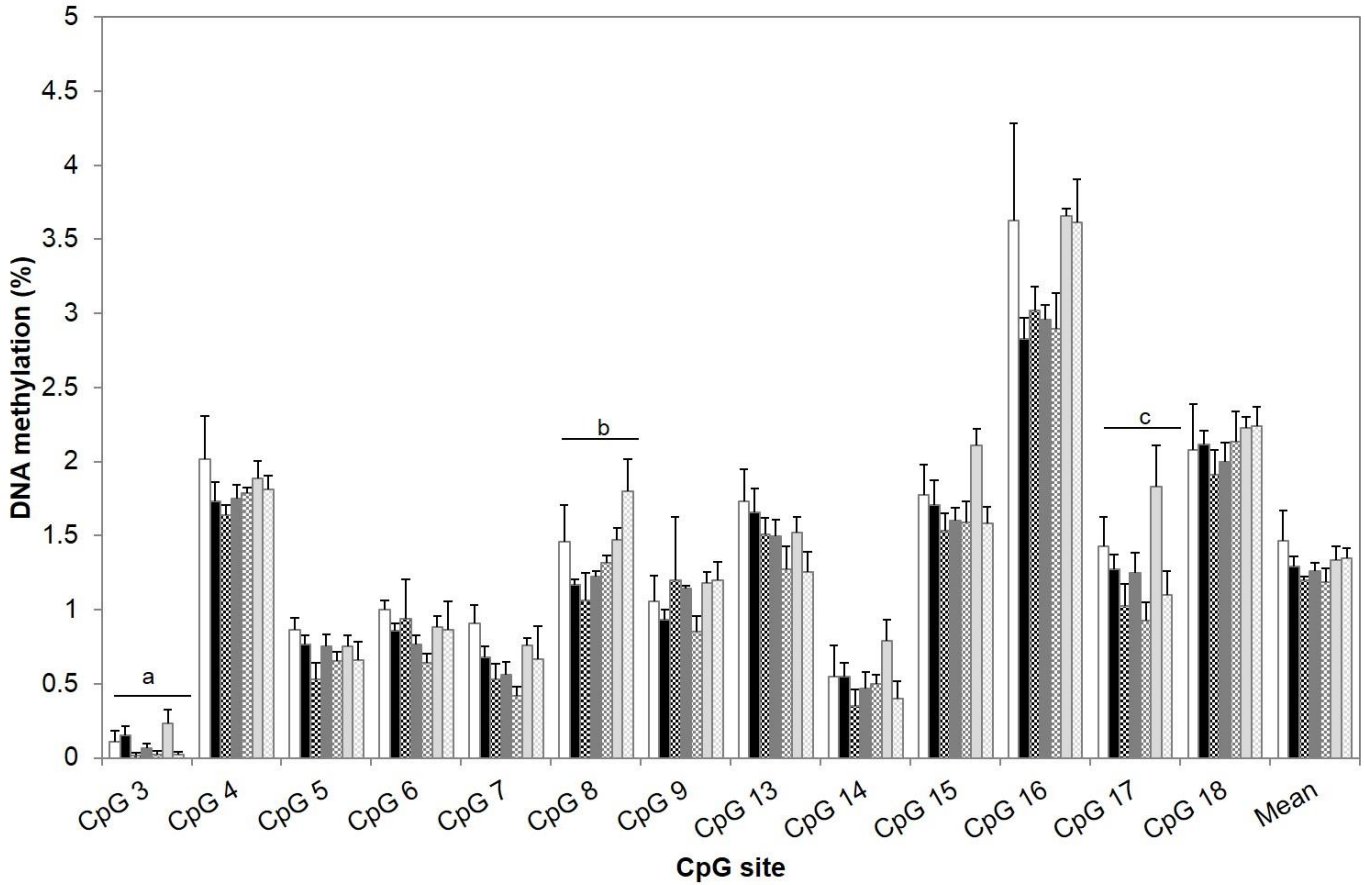


Figure 5.3 Effect of age and DR on methylation at individual CpG sites within the *Ogg1* promoter in DNA from female brain. Data represent mean ± SEM per treatment group, 3AL (white bars, n=5), 12AL (black bars, n=7), 12DR (black checker bars, n=7), 24AL (dark grey bars, n=7), 24DR (dark grey checker bars, n=7), 30AL (light grey bars, n=7) and 30DR (light grey checker bars, n=7). Methylation for individual animal tissues were measured in duplicate. Univariate ANOVA-GLM analysis for age and diet: Pa=0.014, Pb=0.010 and Pc=0.025. Primer design Bartłomiej Tomaszewski and Dr Sabine Langie. data collected by Joanna Górniak.

CpG4	.158											
CpG5	.184	.793**										
CpG6	.259	.655**	.640**									
CpG7	.380**	.580**	.536**	.773**								
CpG8	.253	.686**	.591**	.501**	.481**							
CpG9	-.122	.142	.302*	.186	.089	.049						
CpG13	.552**	.370*	.320*	.408**	.592**	.348*	.075					
CpG14	.578**	.460**	.519**	.596**	.494**	.386**	.279	.625**				
CpG15	.371*	.426**	.439**	.479**	.485**	.482**	.108	.631**	.603**			
CpG16	.132	.687**	.566**	.520**	.423**	.725**	.272	.327*	.410**	.520**		
CpG17	.353*	.614**	.624**	.510**	.446**	.520**	.038	.333*	.513**	.475**	.505**	
CpG18	.270	.698**	.669**	.612**	.562**	.629**	.245	.494**	.543**	.539**	.728**	.577**
	CpG3	CpG4	CpG5	CpG6	CpG7	CpG8	CpG9	CpG13	CpG14	CpG15	CpG16	CpG17

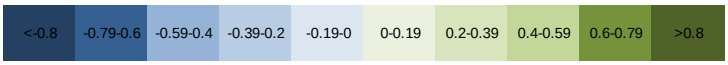


Figure 5.4 Pearson's correlation for % methylation between CpG sites within the *Ogg1* promoter from male mouse brains. Statistically significant correlation at P<0.05 (*) and P<0.01 (**).

CpG4	.359*											
CpG5	.376**	.441**										
CpG6	.152	.347*	.302*									
CpG7	.328*	.471**	.688**	.420**								
CpG8	.192	.570**	.496**	.108	.672**							
CpG9	.043	.483**	.002	.753**	.072	.053						
CpG13	.337*	.417**	.548**	.350*	.358*	.177	.236					
CpG14	.230	.209	.366*	.047	.343*	.247	-.109	.397**				
CpG15	.323*	.332*	.052	.149	.134	.100	.378**	.210	.193			
CpG16	.207	.519**	.218	-.024	.207	.468**	.186	.382**	.154	.500**		
CpG17	.523**	.538**	.496**	.094	.455**	.296*	.116	.447**	.358*	.466**	.406**	
CpG18	.274	.246	.207	-.264	.064	.320*	-.096	.301*	.191	.365*	.760**	.289*
	CpG3	CpG4	CpG5	CpG6	CpG7	CpG8	CpG9	CpG13	CpG14	CpG15	CpG16	CpG17

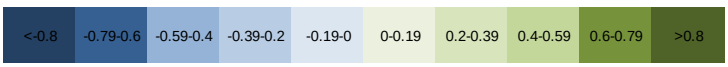


Figure 5.5 Pearson's correlation for % methylation between CpG sites within the *Ogg1* promoter from female mouse brains. Statistically significant correlation at P<0.05 (*) and P<0.01 (**).

Mean *Apex* promoter methylation in the brain was normally distributed and DNA methylation varied between the sexes (P=0.001). Correlation of methylation at individual CpG sites are shown in Figure 5.8 and Figure 5.9 in male and female mice respectively. The correlation between the different sites varies from -0.226 to 0.728 in males and 0.029 to 0.779 in females. Overall the correlation between CpG sites was not high therefore mean methylation may not be ideal for presenting these data and analysis were also run at individual CpG sites.

In male mice cytosine methylation at 3 CpG sites declined with age in a linear trend: CpG 31 (Pa=0.01), CpG 32 (Pb=0.036) and CpG 33 (Pc<0.001). In male mice no CpG sites in the *Apex* promoter were affected by diet. No difference was detected in mean methylation across the CpG sites between the seven groups, mean methylation was 1.44% ranging from 1.26% in 24DR group to 1.64% in 3AL group.

In female mice, cytosine methylation at 5 CpG sites was lower in DR fed animal compared with AL fed controls by: 0.33% at CpG 6 (Pa=0.01), 0.28% at CpG 7 (Pb=0.027), 0.43% at CpG 10 (Pe=0.009), 0.61% at CpG 17 (Ph=0.032) and 0.28% at CpG 27 (Pj=0.037). Furthermore 10 CpG sites were affected by age. Cytosine methylation decreased in a linear trend at: CpG 10 (Pd=0.007), CpG 27 (Pi=0.023), and CpG 33 (Pn=0.023). Cytosine methylation varied in a quadratic trend at: CpG 28 (Pk=0.005), CpG 29 (Pl=0.041), CpG 30 (Pm=0.007) and CpG 34 (Po=0.02). Lastly, cytosine methylation varied in a cubic trend at: CpG 9 (Pc=0.011), CpG 14 (Pf=0.005), and CpG 17 (Pg=0.024). In female mice age did not have an effect on mean *Apex* promoter methylation however, DR fed females had 0.28% lower methylation levels compared with AL fed animals (Pp=0.021).

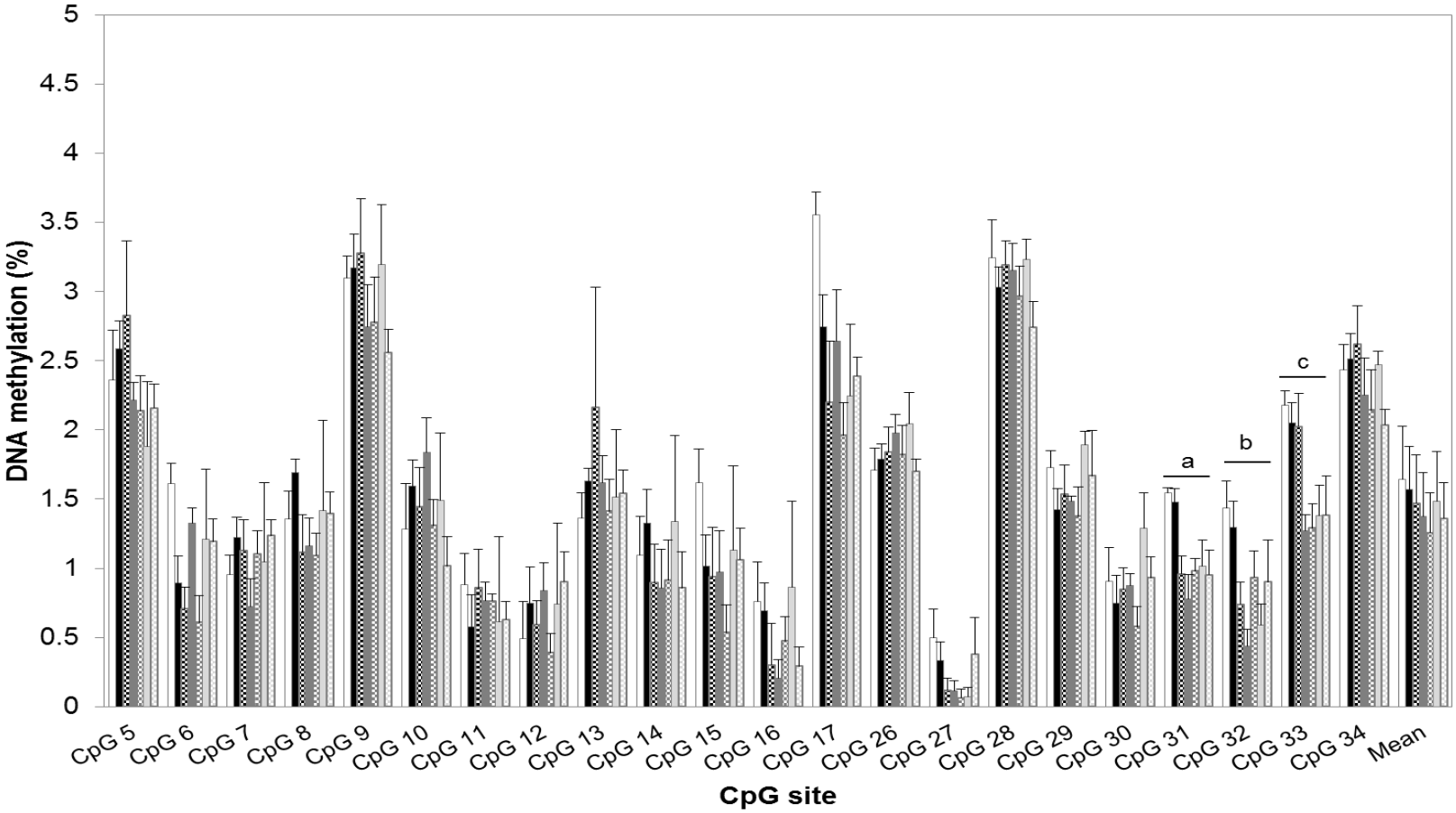


Figure 5.6 Effect of age and DR on methylation at individual CpG sites within the *Apex* promoter in DNA from male brain. Data represent mean ± SEM per treatment group, 3AL (white bars, n=5), 12AL (black bars, n=7), 12DR (black checker bars, n=7), 24AL (dark grey bars, n=7), 24DR (dark grey checker bars, n=7), 30AL (light grey bars, n=5) and 30DR (light grey checker bars, n=7). Methylation for individual animal tissues were measured in duplicate. Univariate ANOVA-GLM analysis for age and diet: Pa=0.010, Pb=0.036 and Pc<0.001.

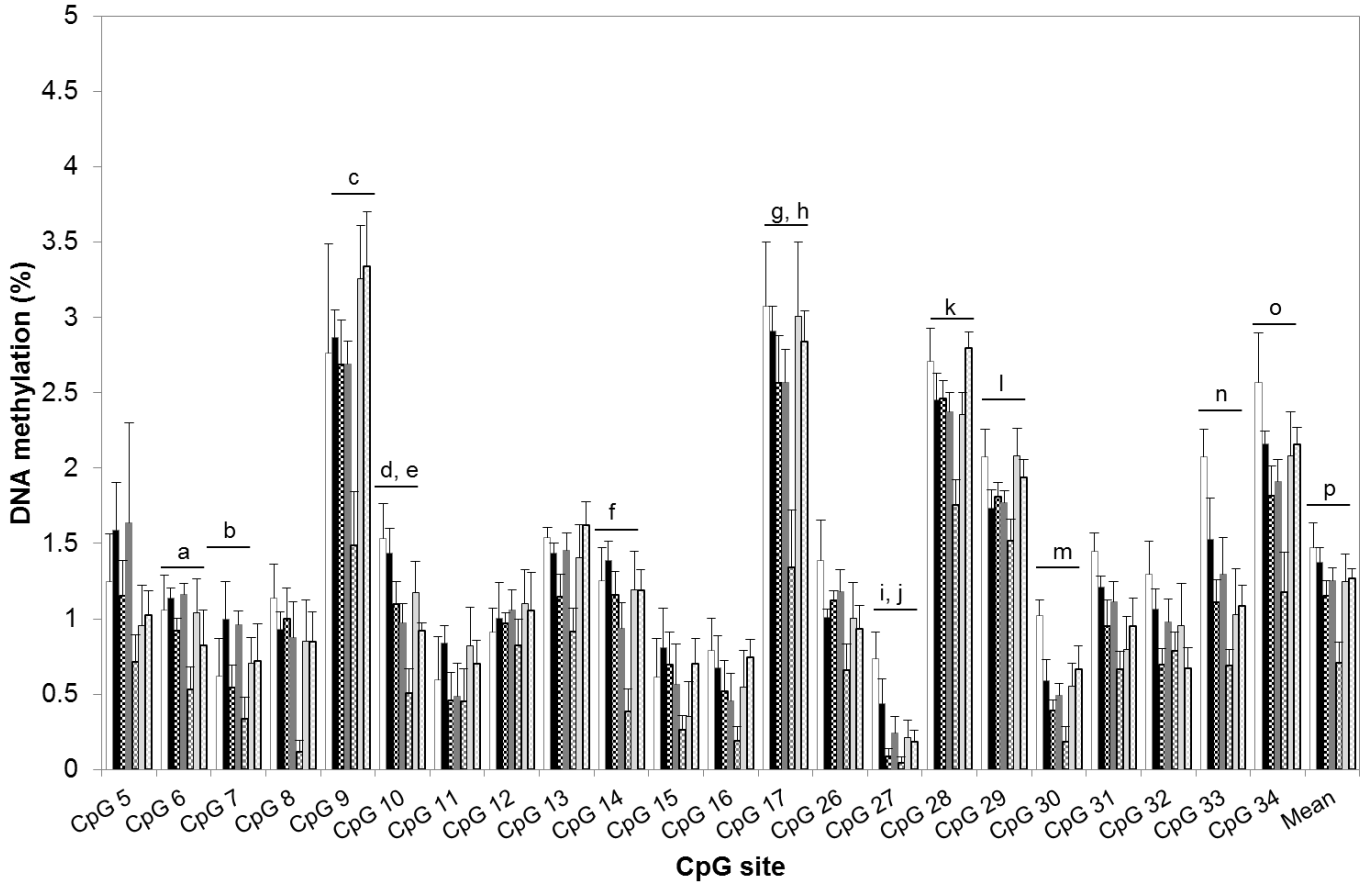


Figure 5.7 Effect of age and DR on methylation at individual CpG sites within the *Apex* promoter in DNA from female brain. Data represent mean ± SEM per treatment group, 3AL (white bars, n=5), 12AL (black bars, n=7), 12DR (black checker bars, n=7), 24AL (dark grey bars, n=7), 24DR (dark grey checker bars, n=7), 30AL (light grey bars, n=7) and 30DR (light grey checker bars, n=7). Methylation for individual animal tissues were measured in duplicate. Univariate ANOVA-GLM analysis for age and diet: Pa=0.010, Pb=0.027, Pc=0.011, Pd=0.007, Pe=0.009, Pf=0.005, Pg=0.024, Ph=0.032, Pi=0.023, Pj=0.037, Pk0.05, Pl=0.041, Pm=0.007, Pn=0.023, Po=0.020 and Pp=0.021.

CpG5																					
CpG6	.251																				
CpG7	.588**	.273																			
CpG8	.435**	.480**	.460**																		
CpG9	.614**	.203	.462**	.405**																	
CpG10	.376*	.405**	.302*	.461**	.459**																
CpG11	.388**	.471**	.452**	.498**	.452**	.539**															
CpG12	.303*	.431**	.491**	.536**	.360*	.579**	.674**														
CpG13	.305*	.192	.414**	.289	.463**	.529**	.451**	.346*													
CpG14	.322*	.265	.422**	.475**	.278	.343*	.472**	.374*	.202												
CpG15	.388**	.459**	.466**	.470**	.341*	.443**	.501**	.436**	.243	.522**											
CpG16	.404**	.547**	.437**	.618**	.334*	.324*	.659**	.433**	.206	.564**	.410**										
CpG17	.388**	.529**	.454**	.489**	.378*	.484**	.488**	.507**	.476**	.367*	.549**	.333*									
CpG26	-.046	-.006	-.134	-.112	.264	.156	-.098	.099	.044	-.167	-.197	-.021	-.152								
CpG27	-.137	.267	-.014	.123	.032	.065	.200	.201	.004	-.032	.194	.164	.124	.113							
CpG28	-.087	.010	-.113	.068	.187	.453**	.235	.122	.324*	-.011	.204	-.001	.186	.389**	.249						
CpG29	-.128	.233	-.039	.061	.210	.093	.065	.119	.072	.026	.024	-.008	.064	.255	.559**	.164					
CpG30	-.041	.011	-.162	-.029	.288	.135	-.066	.153	-.018	-.159	-.045	-.099	.016	.440**	.243	.331*	.456**				
CpG31	.054	.053	.044	.094	.140	.086	-.226	-.023	-.131	-.061	.084	-.074	.220	-.011	.477**	.144	.260	.248			
CpG32	-.060	.247	.070	.119	.160	.162	.064	.130	.025	.047	.145	.165	.163	.088	.728**	.120	.443**	.098	.649**		
CpG33	.345*	.060	.213	.114	.379*	.242	.102	.146	.266	.039	.226	.015	.316*	.081	.461**	.306*	.312*	.274	.612**	.555**	
CpG34	.352*	-.041	.154	.244	.653**	.191	.019	.095	.212	.165	-.033	-.034	.261	.343*	.046	.197	.292	.331*	.237	.144	.448**
	CpG6	CpG7	CpG8	CpG9	CpG10	CpG11	CpG12	CpG13	CpG14	CpG15	CpG16	CpG17	CpG26	CpG27	CpG28	CpG29	CpG30	CpG31	CpG32	CpG33	CpG34

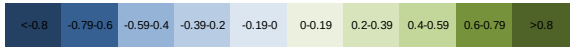


Figure 5.8 Pearson's correlation for % methylation between CpG sites within the *Apex* promoter from male mouse brains. Statistically significant correlation at P<0.05 (*) and P<0.01 (**).

	CpG6	CpG7	CpG8	CpG9	CpG10	CpG11	CpG12	CpG13	CpG14	CpG15	CpG16	CpG17	CpG26	CpG27	CpG28	CpG29	CpG30	CpG31	CpG32	CpG33	CpG34
CpG5																					
CpG6	.405**																				
CpG7	.447**	.439**																			
CpG8	.244	.545**	.449**																		
CpG9	.332*	.361*	.249	.515**																	
CpG10	.426**	.504**	.528**	.659**	.494**																
CpG11	.264	.440**	.309*	.530**	.635**	.464**															
CpG12	.250	.415**	.446**	.557**	.368*	.306*	.530**														
CpG13	.313*	.495**	.457**	.576**	.587**	.601**	.518**	.502**													
CpG14	.349*	.592**	.460**	.619**	.617**	.698**	.507**	.405**	.553**												
CpG15	.182	.440**	.573**	.531**	.235	.390**	.269	.429**	.460**	.412**											
CpG16	.319*	.418**	.409**	.484**	.277	.543**	.167	.282	.455**	.367*	.559**										
CpG17	.347*	.620**	.555**	.722**	.607**	.779**	.599**	.513**	.632**	.708**	.455**	.541**									
CpG26	.209	.161	.156	.207	.460**	.448**	.285	.260	.473**	.308*	.172	.206	.341*								
CpG27	.131	.319*	.140	.190	.181	.529**	.182	.029	.403**	.253	.063	.207	.310*	.529**							
CpG28	.158	.134	.276	.476**	.554**	.531**	.356*	.281	.562**	.527**	.318*	.360*	.536**	.630**	.344*						
CpG29	.101	.167	.058	.359*	.580**	.313*	.451**	.348*	.487**	.385**	.049	.152	.450**	.574**	.374*	.527**					
CpG30	.224	.434**	.342*	.346*	.303*	.609**	.255	.149	.499**	.539**	.192	.378**	.490**	.427**	.649**	.477**	.385**				
CpG31	.430**	.389**	.316*	.394**	.294*	.581**	.374*	.305*	.546**	.494**	.308*	.347*	.443**	.632**	.539**	.517**	.421**	.585**			
CpG32	.123	.180	.397**	.312*	.238	.497**	.246	.254	.390**	.355*	.305*	.277	.389**	.537**	.514**	.292*	.512**	.495**	.503**		
CpG33	.238	.245	.285	.444**	.224	.699**	.187	.157	.489**	.488**	.254	.331*	.419**	.570**	.715**	.505**	.417**	.646**	.685**	.707**	
CpG34	.327*	.444**	.447**	.480**	.584**	.664**	.475**	.326*	.683**	.621**	.405**	.427**	.680**	.670**	.518**	.625**	.585**	.581**	.741**	.535**	.619**

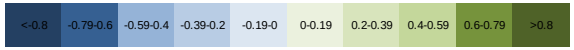


Figure 5.9 Pearson's correlation for % methylation between individual CpG sites within the *Apex* promoter from female mouse brains. Statistically significant correlation at P<0.05 (*) and P<0.01 (**).

5.3.2.3 *Histone acetylation at the Ogg1 promoter*

To assess the effect of age and of DR on chemical modifications to histone tails, histone modifications were measured in 3 groups i.e. 3 month old mice, 30 month old AL mice and 30 month old DR mice. Histone 4 acetylation (H4Ac) and histone 3 lysine 27 trimethylation (H3K27Me3) were measured as prominent examples of epigenetic marks associated with transcription activation and repression respectively.

At the *Ogg1* promoter, H4Ac in the brain was higher in female compared with male mice therefore subsequent analyses were performed for each sex separately. In male mice there was a significant difference in H4Ac between the 3 groups (P=0.02, Figure 5.10A), a Bonferroni post hoc test for age (3AL vs 30AL) and for diet (30AL vs 30DR) was performed. A 2.5 fold enrichment of H4Ac was observed in older animals compared with the young (n.s) furthermore, at age 30 months, DR animals showed a 3 fold decrease in H4Ac levels compared with AL fed controls (P=0.02). In female mice, the pattern of H4Ac was the mirror image of that seen for the males. The lowest H4Ac values were observed in the 30AL female mice and, in comparison with this group, acetylation was enriched by 1.8 fold and 2.6 fold in 30DR and 3AL mice respectively but, given the substantial inter-individual variation, these differences were not statistically significant (Figure 5.10B).

5.3.2.4 *Histone acetylation at the Apex promoter*

At the *Apex* promoter, H4Ac was higher in female compared with male mice; therefore, subsequent analyses were performed for each sex separately. No difference between the 3 groups was observed at the *Apex* promoter in males (Figure 5.11A), although a 2.4 fold enrichment of H4Ac was seen in 30AL compared with 3AL animals. Furthermore at 30 months, AL animals had 3.3 fold higher H4Ac enrichment compared with DR fed animals. No significant effect of age or diet was observed in the *Apex* promoter of female mice where H4Ac was lowest in the 30AL group and was enriched by 1.4 fold in both 3AL and 30DR animals when compared with 30AL group (Figure 5.11B).

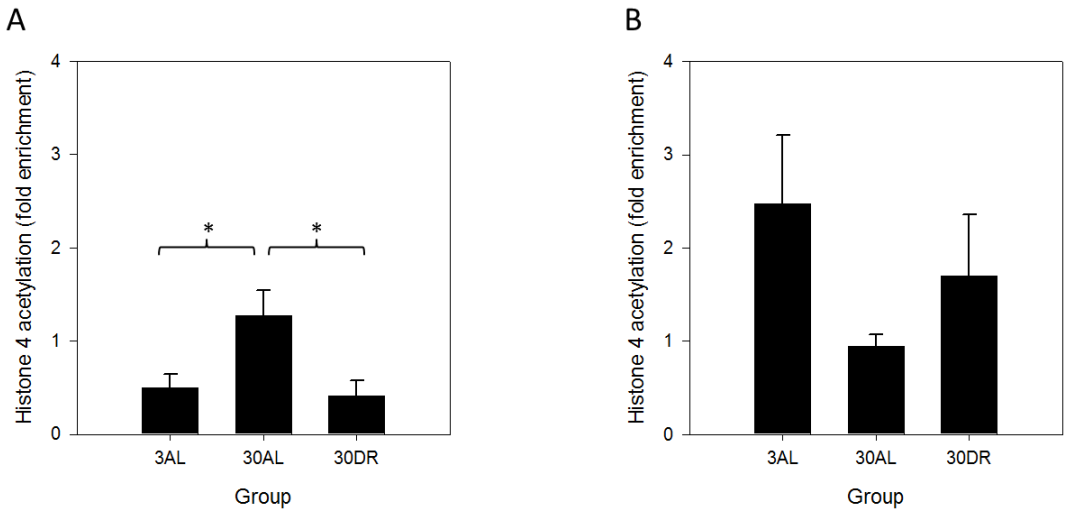


Figure 5.10 Effects of age and of DR on histone 4 acetylation at the *Ogg1* promoter in brain of male (A) and female (B) mice. Data represent mean enrichment ± SEM. ChIP-qPCR was performed in duplicate per animal, n=5 per age group. H4Ac was normalised to IgG. Univariate ANOVA-GLM analysis with Bonferroni post hoc test: 3AL vs 30AL P=0.035 and 30AL vs 30DR P=0.02. Primer design by Dr Jelena Mann, data collection Joanna Górniak.

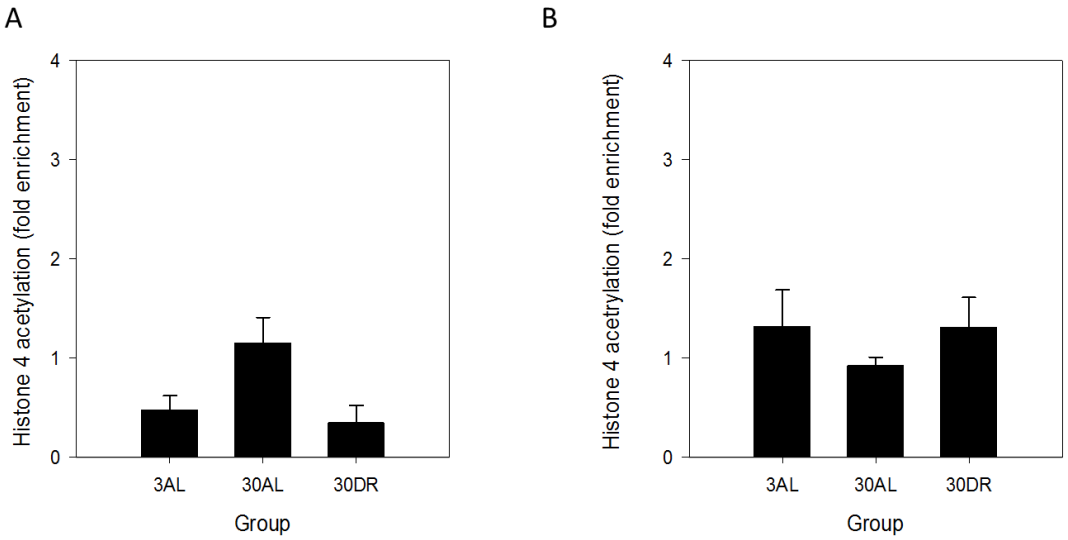


Figure 5.11 Effects of age and of DR on histone 4 acetylation at the *Apex* promoter in brain of male (A) and female (B) mice. Data represent mean enrichment ± SEM. ChIP-qPCR was performed in duplicate per animal, n=5 per age group. H4Ac was normalised to IgG. Primer design by Dr Jelena Mann, data collection Joanna Górniak.

5.3.2.5 *Histone methylation at the Ogg1 promoter*

In the brain there was no overall effect of sex on H3K27Me3 but there were interactions between sex and both age and diet therefore subsequent analyses were performed separately for each sex. No significant difference was seen between the groups in the brain of male mice. Despite this nearly a 2 fold enrichment in H3K27Me3 was observed in the 30AL group compared with both 3AL and 30DR groups (Figure 5.12A). In contrast, in females no enrichment of H4Ac was observed with age but 30DR animals did show 1.4 fold enrichment compared with 30AL animals, although this difference was not significant (Figure 5.12B).

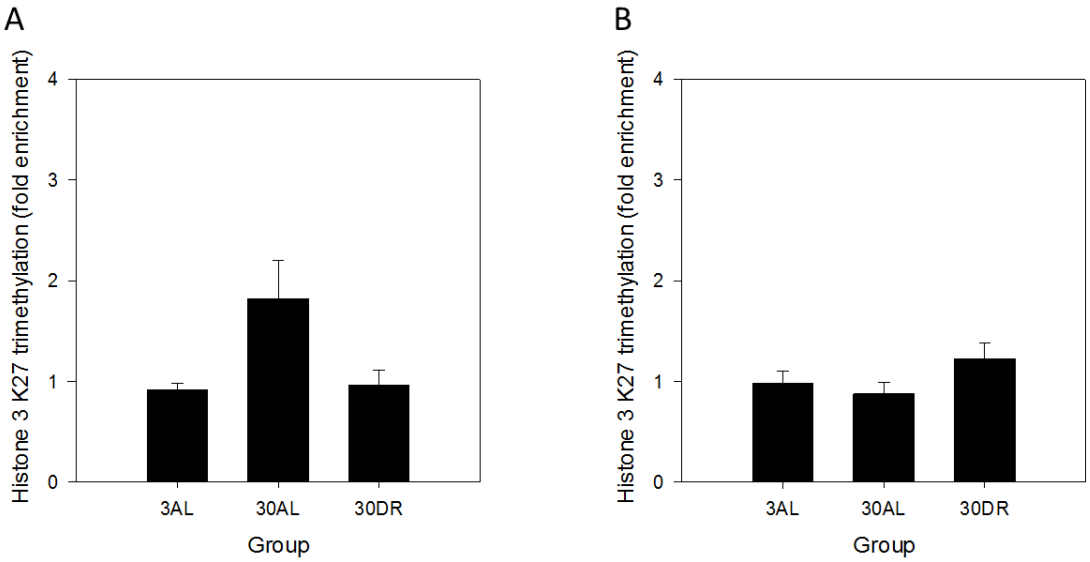


Figure 5.12 Effects of age and of DR on histone 3 lysine 27 tri-methylation at the *Ogg1* promoter in brain of male (A) and female (B) mice. Data represent mean enrichment ± SEM. ChIP-qPCR was performed in duplicate per animal, n=5 per age group. H3K27Me3 was normalised to IgG. Primer design by Dr Jelena Mann, data collection Joanna Górniak.

5.3.2.6 *Histone methylation at the Apex promoter*

A difference between sexes was not observed for methylation at the *Apex* promoter; however, an interaction of sex and diet was observed; therefore, sexes were analysed separately. No difference between the groups in either male or female mice was observed. In male mice H3K27Me3 was enriched nearly 2 fold in the 30AL group compared to the others (Figure 5.13A). In female mice H3K27Me3 was enriched 6.7 fold and 4.1 fold in 30DR group compared to 3AL and 30DR respectively (Figure 5.13B).

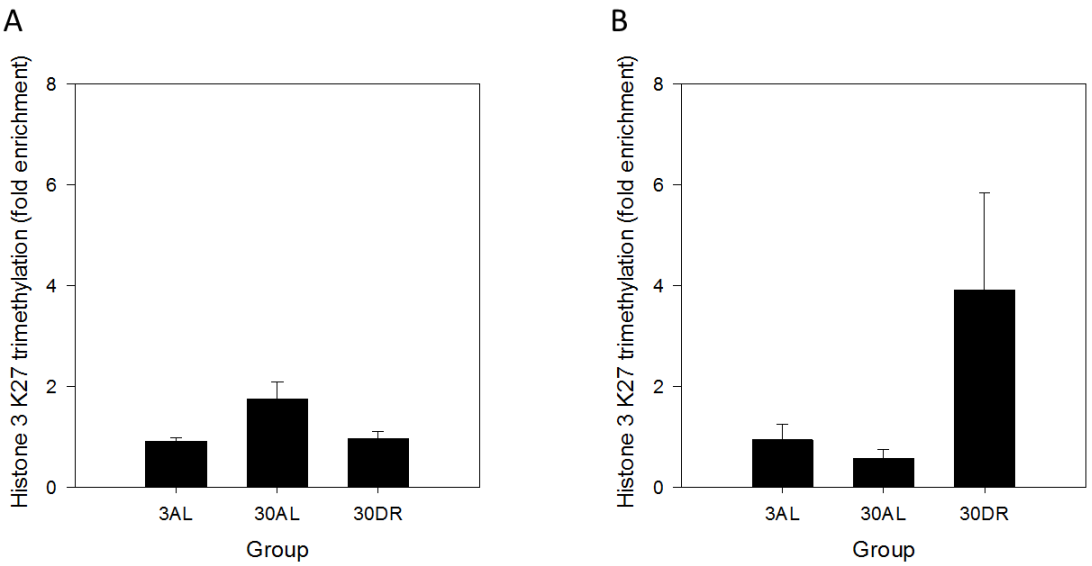


Figure 5.13 Effects of age and of DR on histone 3 lysine 27 tri-methylation at the *Apex* promoter in brain of male (A) and female (B) mice. Data represent mean enrichment ± SEM. ChIP-qPCR was performed in duplicate per animal, n=5 per age group. H3K27Me3 was normalised to IgG. Primer design by Dr Jelena Mann, data collection Joanna Górniak.

5.3.2.7 *HAT and HDAC activity*

To assess the effect of age and of DR on HAT and HDAC, enzyme activity was measured in 3 groups: 3AL, 30AL and 30DR. No difference in HAT activity was observed between the sexes. Age or diet did not have an effect on HAT activity, which was around 7AU (Figure 5.14). However, DR animals showed slightly higher levels of HAT activity (n.s.).

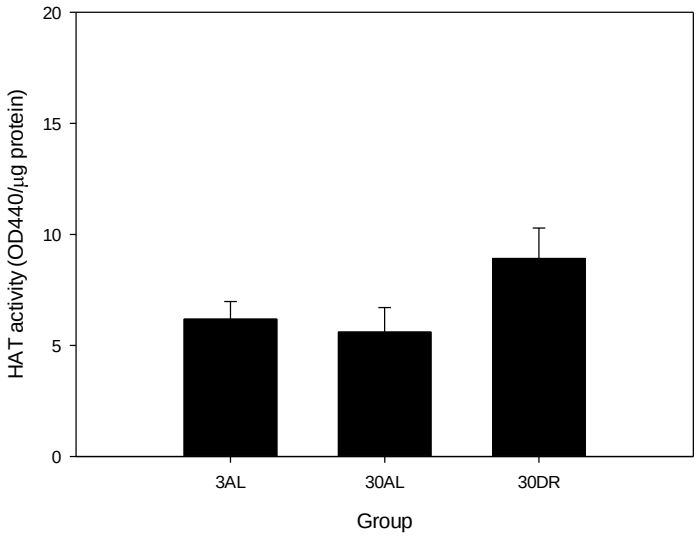


Figure 5.14 Effect of age and DR on HAT activity in the brain. Data represent mean ± SEM from a single assay, n=10 per group (pooled for male and female mice).

HDAC activity was different between the sexes with male mice showing higher activity. In male mice, HDAC activity was 7 fold higher in older animals compared with 3 month old animals (P=0.047), but no effect of diet was observed (Figure 5.15A). In female mice, age did not have an effect on HDAC activity; however, activity was 2.6 fold higher in 30DR group compared with 30AL group (P=0.049, Figure 5.15B).

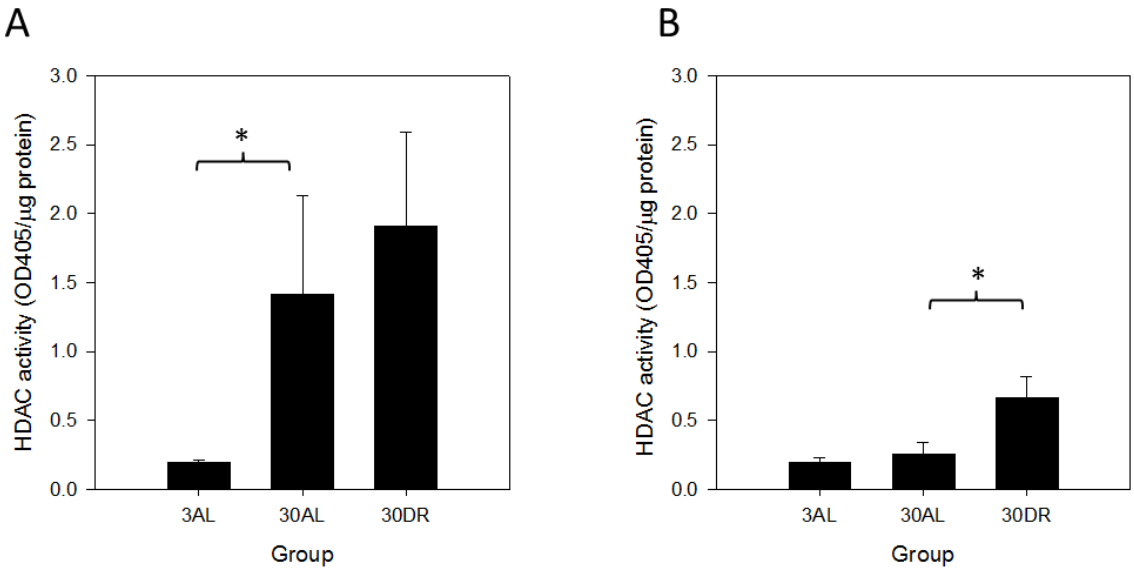


Figure 5.15 Effect of age and DR on HDAC activity in the brain of male (A) and female (B) mice. Data represent mean ± SEM absorbance from a single assay, n=5 per group. In males, Mann-Whitney U test between 3AL vs 30A: P=0.047. In females, univariate ANOVA-GLM analysis between groups with Bonferroni post hoc between 30AL vs 30DR: P_e=0.049.

5.3.2.8 *Expression of transcription factors*

To assess the effect of age and DR on the expression of *Hif1α* and *Nfya* TFs, mRNA expression was measured in 3AL, 30AL and 30DR groups. *Hif1α* expression was different between the sexes with male mice showing higher expression levels. In male mice, expression of *Hif1α* was 2 fold higher in the 30AL group compared with 3AL (P=0.012) but DR did not have an effect on expression (Figure 5.16A). In female mice, no effect of age or diet are observed although the 30AL group had ~30% lower expression compared with the other 2 groups (Figure 5.16B). No difference in *Nfya* expression was seen between the sexes; therefore, data were pooled. Although a significant difference between the groups was observed (P=0.007) a post hoc comparison did not show an effect of age or diet (Figure 5.17).

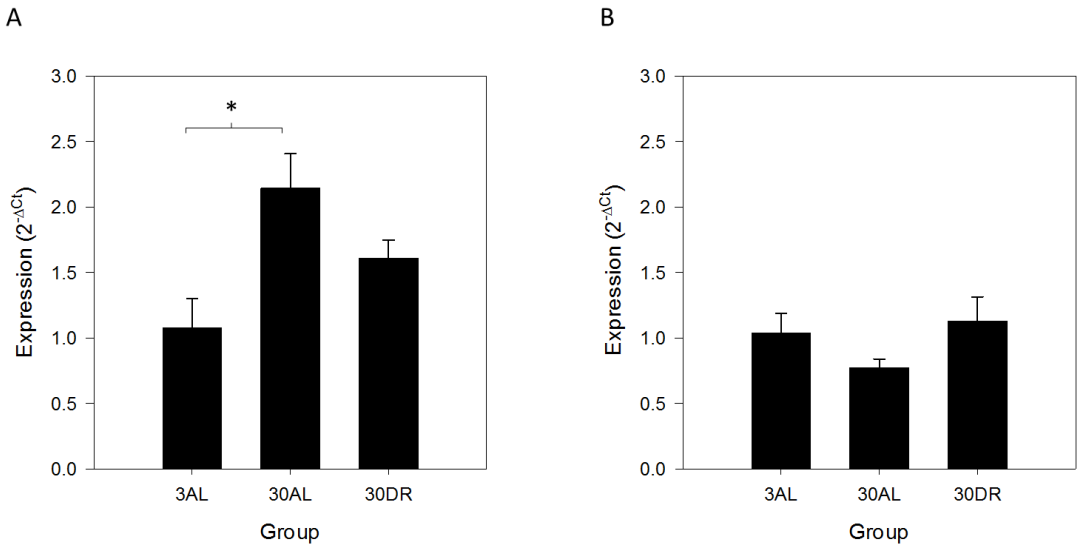


Figure 5.16 Effect of age and DR on expression of *Hif1α* in male (A) and female (B) mice in in the brain. Univariate ANOVA-GLM analysis with Bonferroni post hoc comparison 3AL vs 30AL: P=0.012.

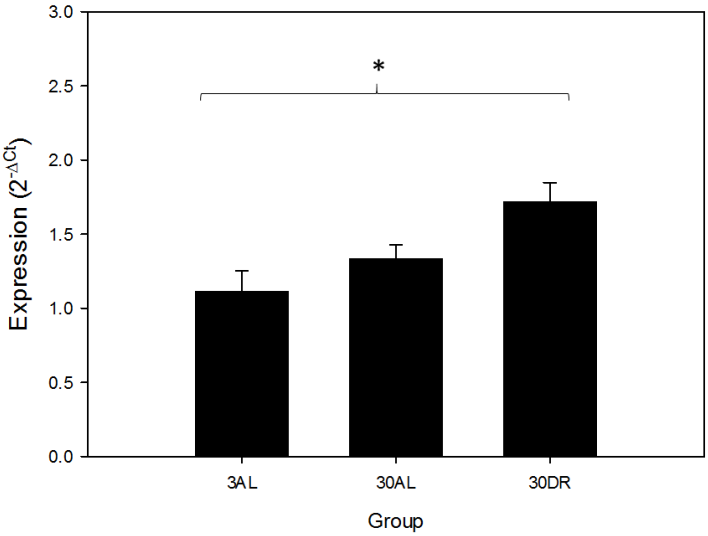


Figure 5.17 Effect of age and DR on expression of *Nfya* in in the mouse brain. Data represent mean ± SEM, n=10 per group (pooled sexes). Kruskal-Wallis analysis between groups: P=0.007.

5.3.3 *BER gene expression*

Expression of the 2 BER-related genes *Ogg1* and *Apex* was measured in mRNA extracted from the brain in 3AL, 12AL, 12DR, 24AL, 24DR, 30AL and 30DR groups. In all cases, assays were conducted on mRNA from individual animals and data are presented as $2^{-\Delta Ct}$.

5.3.3.1 *Ogg1 expression in the brain*

Overall, *Ogg1* expression was higher in males compared with females. Expression of *Ogg1* was 1.4 fold and 1.3 fold higher in the 3AL group compared with 30AL groups in male and female mice; however, this trend in decline was not statistically significant in either sex (Figure 5.18). On the other hand in both male and female mice, DR animals had significantly higher *Ogg1* expression levels compared with AL fed animals (P_{male}=0.034 and P_{female}=0.019). There were no interactions between age and diet for either sex which suggests that the effect of DR in raising *Ogg1* expression occurs across the life-course in all mice.

5.3.3.2 *Apex expression in the brain*

No difference was observed in expression between sexes, with no interactions between age, diet or sex; therefore, the data were pooled from both male and female mice. *Apex* expression in the brain is shown in Figure 5.19. An age-related decline in expression was observed (P=0.003), which followed a linear trend (R^2=0.091, P=0.004). Furthermore DR animals showed higher levels of *Apex* gene expression compared to AL controls (P=0.005). A Bonferroni post hoc analysis between the groups revealed that expression was 1.7 fold higher in 3AL (P=0.038), 1.7 fold higher in 12AL (P=0.046), 1.9 fold higher in 12DR (P=0.001), 1.4 fold higher in 24AL (n.s.), 1.7 fold higher in 24DR group (P=0.044) and 1.6 fold (n.s.) compared with 30AL group.

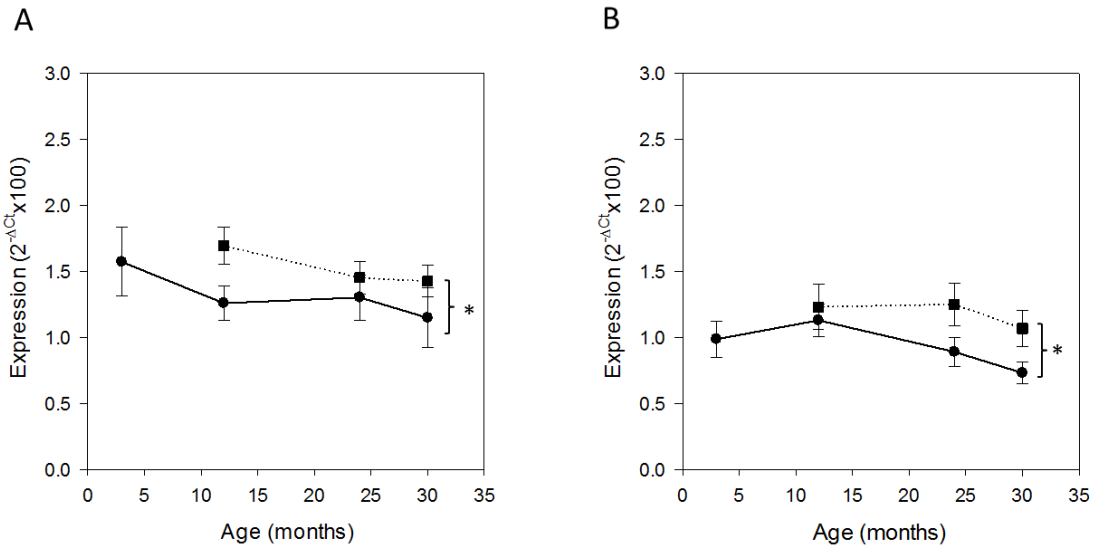


Figure 5.18 Effect of age and of DR on expression of *Ogg1* at the mRNA level in the brain of male (A) and female (B) mice. Solid line with round symbols represents AL fed animals dotted line with square symbols represents DR fed animals. Data represent mean ±SEM, n=5-7 per group. Univariate ANOVA-GLM analysis for diet: male mice P=0.034 and female mice P=0.019. Primer design Dr Sabine Langie. Data collection Joanna Górniak.

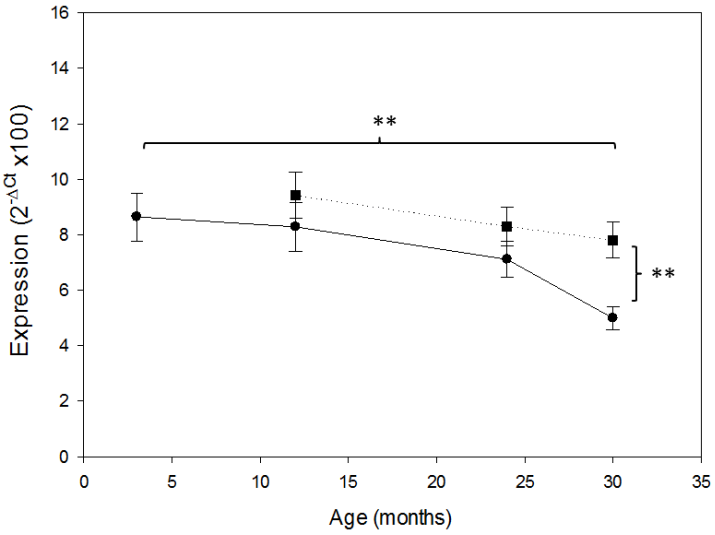


Figure 5.19 Effect of age and of DR on expression of *Apex* at the mRNA level in the brain. Solid line with round symbols represents AL fed animals and dotted line with square symbols represents DR fed animals. Data represent mean ± SEM. n=10 per group (data pooled for male and female mice. Univariate ANOVA-GLM analysis for age (P=0.003) and diet (P=0.005).

5.3.4 *Base excision repair (BER)-related DNA incision activity in the brain*

BER-related DNA repair activity was quantified as incision of DNA at sites of damage, induced by photosensitiser Ro 19-8022, using the *in vitro* comet-based repair assay, which measures the first step (incision of the strand) of the repair process. DNA incision was measured in protein extracts from brain from individual animals in all 7 groups.

DNA incision activity was highly variable between animals. For example, for the brain this ranged from 0.02 to 17.92 AU with a mean of 4.8AU. These data were skewed to the left with only a few animals showing high rates of incision activity. No difference in DNA incision activity was observed between male and female mice and so data for both sexes were pooled for subsequent analyses. No detectable effects of age or diet on DNA incision activity were observed. ANOVA-GLM show an interaction between these 2 factors (P=0.042, Figure 5.20). This is likely because DNA incision in the 24AL group was 2.28AU higher compared with 24DR but at 30 months there was a cross over and incision was 1.94AU higher in 30DR group compared with 30AL.

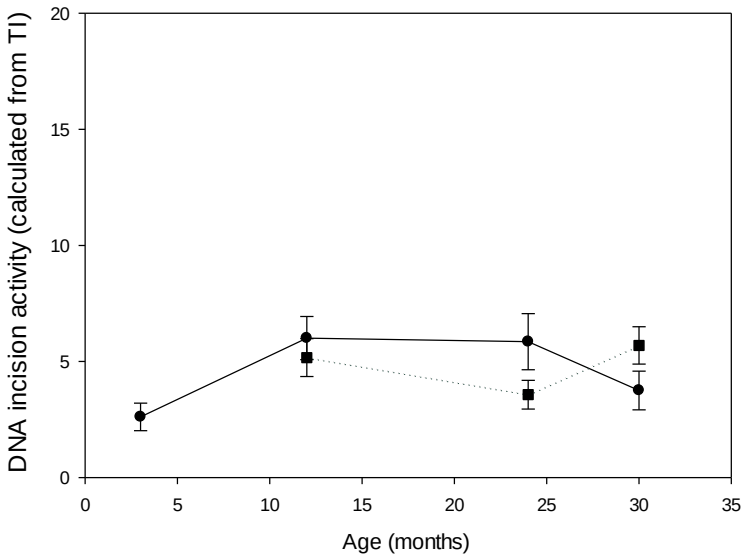


Figure 5.20 Effects of age and of DR on DNA incision activity in the brain. Solid line with round symbols represents AL fed animals. Dotted line with square symbols represents DR fed animals Data represent mean ± SEM, n=10-14 per group (pooled for male and female mice). Assay design by Dr Sabine Langie. Data collection Joanna Górniak.

5.3.5 *Expression of OGG1 and APEX at the protein level in brain*

Protein concentration of OGG1 and APEX were measured in extracts of brain from 3AL, 30AL and 30DR groups. For both proteins, concentration did not differ significantly between male and female mice, therefore the data were pooled for further analyses. OGG1 protein concentration was increased with age but no effect of diet was observed (Table 5.2). A 20% increase in protein concentration was seen in the 30AL group compared with the 3AL group (P=0.049). Furthermore OGG1 protein concentration was 1.8 fold higher in the 30DR group compared with the 3AL group. A difference in APEX concentration was seen between the 3 groups tested (P<0.001). Post hoc analysis revealed a 1.7 fold increase with age (Mann Whitney U test 3AL vs 30AL, P=0.002) and a >2 fold higher APEX concentration in DR fed animals compared with AL fed controls (Mann Whitney U test 30AL vs 30DR, P<0.001, Table 5.3).

OGG1 (ng/ml)	Male	Female	All mice
3AL	0.61±0.10	0.40±0.03	0.51±0.02
30AL	0.96±0.09	0.68±0.01	0.58±0.03*
30DR	1.00±0.02	0.82±0.02	0.90±0.03

Table 5.2 Effects of age and of DR on OGG1 protein concentrations in brain. Data represent mean ± SEM from a single assay, n=5 per group. Univariate ANOVA-GLM with Bonferroni post hoc comparison in 3AL vs 30AL: P=0.049.

APEX (ng/ml)	Male	Female	All mice
3AL	0.13±0.03	0.16±0.04	0.15±0.07
30AL	0.27±0.02	0.24±0.02	0.25±0.05**
30DR	0.72±0.14	0.42±0.07	0.55±0.26***

Table 5.3 Effect of age and DR on APEX protein concentrations in the brain. Data represent mean ± SEM from a single assay, n=5 per group. Kruskal-Wallis analysis with Mann Whitney U test comparison; 3AL vs 30AL P=0.002 and 30AL vs 30DR P<0.001.

To investigate relationships with function, protein concentration as well as mRNA levels of *Ogg1* and *Apex* were correlated with BER-related DNA incision activity. Figure 5.21 shows correlation plots for both protein vs repair and mRNA vs repair. There were positive and significant correlations between incision activity and concentrations of both OGG1 protein (R^2=0.167, P=0.028) and APEX protein (R^2=0.163, P=0.03) but no significant relationships with the corresponding mRNA abundances.

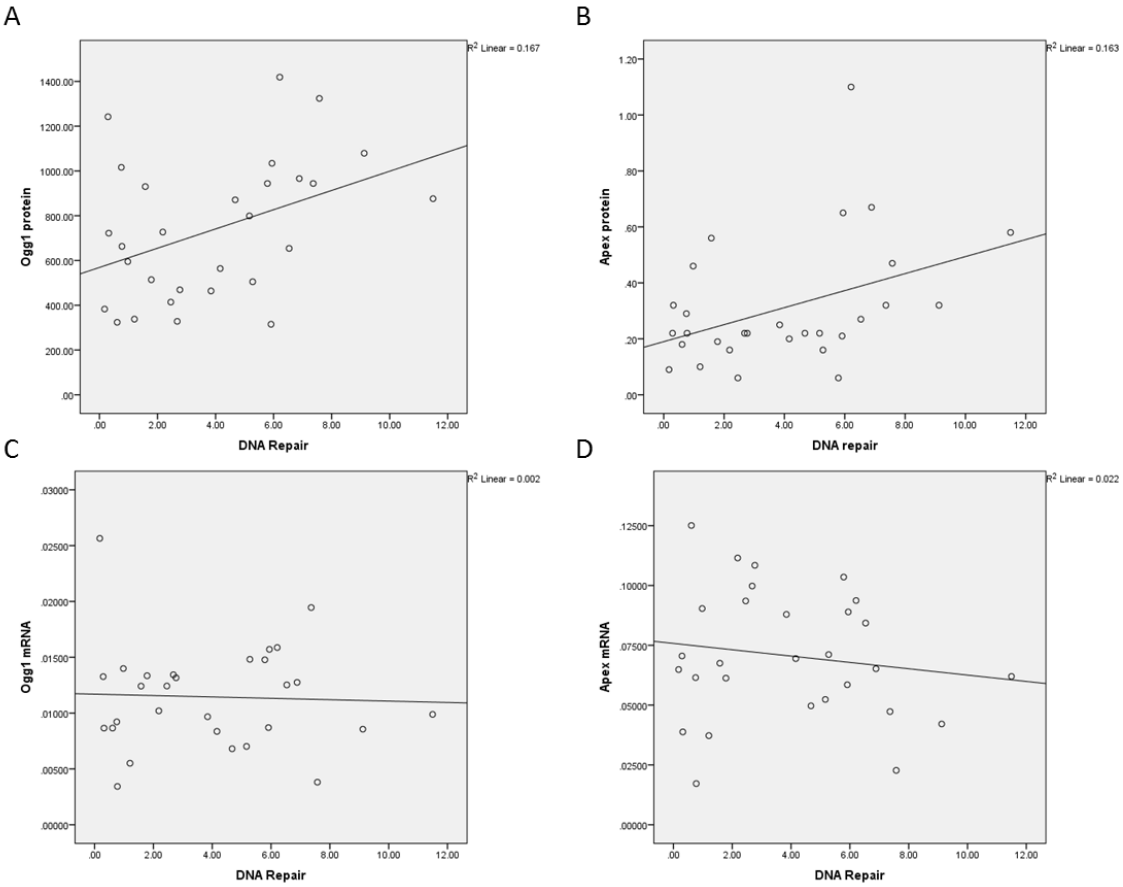


Figure 5.21. Correlations between BER-related DNA repair (incision activity) and OGG1 protein (A) APEX protein (B) *Ogg1* mRNA (C) and *Apex* mRNA (D) in the brain. Data represents mean ± SEM for all animals in the 3AL, 30AL and 30DR groups. The correlations with DNA incision activity were positive and significant for both OGG1 (P=0.028) and APEX (P=0.03) protein concentrations but there were no significant correlations between incision activity and mRNA abundance.

5.3.6 *Effects of age and DR on oxidative damage to DNA in the brain*

Oxidative damage in the brain was measured in the form of 8-oxoguanine in all 7 groups using HPLC-ECD. No difference in oxidative damage lesions were detected between males and females thus for further analyses the data from both sexes were pooled. As summarised in Table 5.4, mean values for 8-oxodG/10^6dG were lowest (11.7) in the 12 month old DR mice and highest (20.1) in the 30 month old DR animals. There were statistically significant differences in 8-oxoguanine concentration between the 7 groups (P=0.014). Multiple Mann-Whitney post hoc tests were performed between the groups, adjusting for multiple comparisons ($\alpha<0.024$). At this significance level the only difference detected was a 1.7 fold increase in 8-oxoguanine concentration in the 30DR group compared with the 12DR (P<0.001). Interestingly highest damage was observed in 3 month old animals but then fell to lowest levels in 12 month

old animals, after which there was a progressive increase. A quadratic model of age-related 8-oxoguanine concentration was fitted to the data (R^2=0.095, P=0.023) and can be seen in Figure 5.22.

Group	Age (months)	Diet	8-oxodG/10^6dG
3AL	3	AL	18.6±4.15
12AL	12	AL	15.8±2.35
12DR	12	DR	11.7±0.67
24AL	24	AL	14.9±1.04
24DR	24	DR	13.7±1.02
30AL	30	AL	16.4±2.53
30DR	30	DR	20.1±2.48***

Table 5.4 Effects of age and of DR on 8-oxoguanine concentrations in the brain. Data represent mean ± SEM, n=10-14 per group (data pooled for male and female mice). Kruskal-Wallis analysis between the groups (P=0.014) with multiple Mann-Whitney U test post hoc comparison (adjusted α<0.024): 12DR vs 30DR P<0.001. Data collected by Maastricht University, in collaboration with Dr Roger Godschalk.

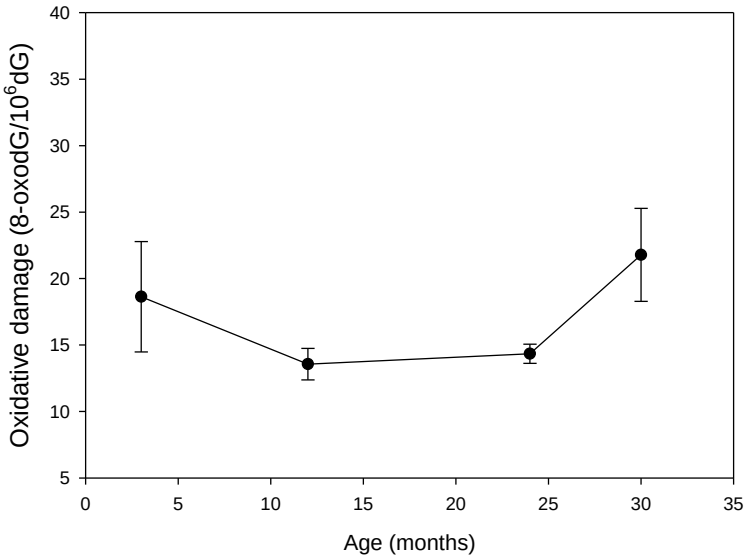


Figure 5.22 Effects of age on DNA oxidative damage in the brain. Data represent mean ± SEM, n=10-28 (pooled for both male and female and for AL and DR animals). Quadratic trend (R^2=0.095, P=0.023). Data collected by Maastricht University, in collaboration with Dr Roger Godschalk.

5.4 Discussion

In this chapter I have investigated age and DR-related epigenetic changes (DNA methylation and histone marks) at promoter regions of genes coding for the BER-related proteins: oxoguanine DNA glycosylase (OGG1) and apurinic/apyrimidinic endonuclease (APEX). I quantified the resultant changes in *Ogg1* and *Apex* transcription levels (mRNA abundance) and their repair capability (assessed as BER-related incision activity).

Two types of epigenetic marks, and their effect on BER gene expression, were measured: DNA methylation and histone post-translational modification (histone 4 acetylation and histone 3 lysine 27 tri-methylation) as it has been suggested that these may work in unison to mediate transcription changes (Vaissière *et al.*, 2008). Figure 5.23 shows the conceptual model for epigenetic regulation of BER-related genes investigated in this chapter, where during ageing BER-related genes are silenced by an increase in DNA methylation and H3K27 tri-methylation as well as a decrease in H4 acetylation and DR is able to ameliorate these changes. In addition a brief investigation of the modes of action for epigenetic changes were undertaken. The effect of age and long-term DR on activity of epigenetic enzymes HAT and HDAC as well as TF expression were studied.

The subsequent changes in BER protein, repair activity and number of 8-oxoguanine lesions were also measured to understand the effect on the epigenetic through to phenotypic profile. These observations were studied in two contrasting tissues in the mouse: the brain, which is largely post-mitotic, and the liver, which retains capacity for cell proliferation throughout much of the life-course. The decision to investigate 2 contrasting tissues was taken since age-related epigenetic changes are tissue specific (Thompson *et al.*, 2010) and so is damage and repair activity (Sohal *et al.*, 1994a; Karahalil *et al.*, 2002; Intano *et al.*, 2003). This chapter describes epigenetic changes observed in brain tissue and Chapter 6 will discuss epigenetic changes in liver tissue.

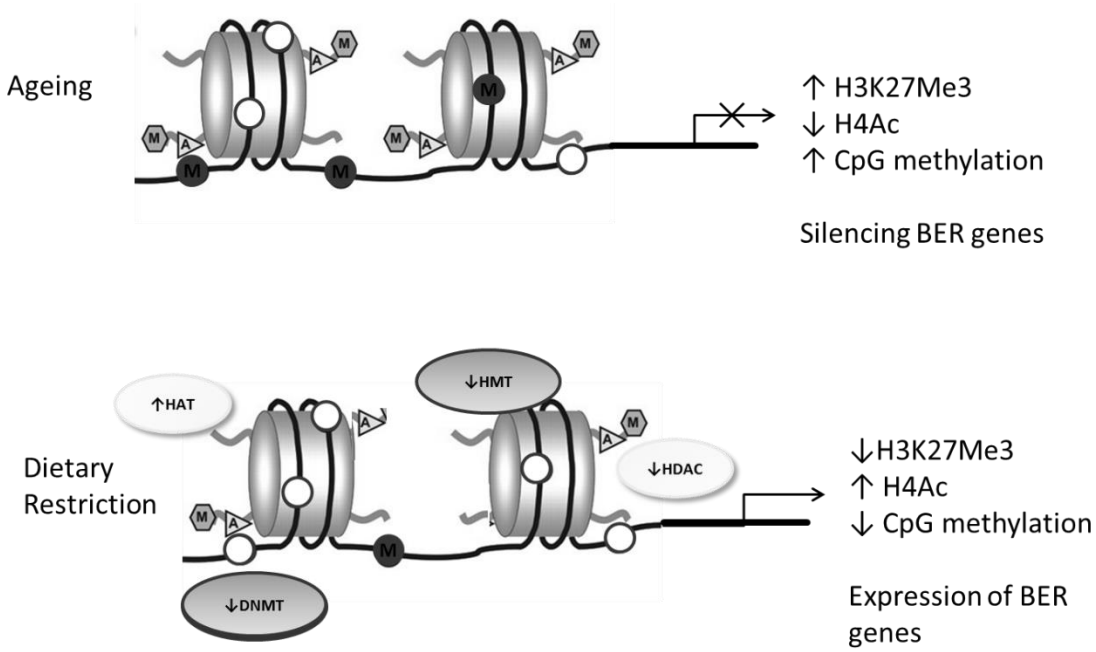


Figure 5.23 Schematic overview of proposed epigenetic mechanisms through which age and DR may influence expression of BER-related genes.

With the complexity of this study I have designed this conceptual model to simplify the data. The brain and liver tissues studied will be discussed separately in Chapters 5 and 6 respectively and comparisons between the results will be made in Chapter 6 and the general discussion in Chapter 8. I will discuss the data based on the model, identify gaps, conflicts and anomalies. However, I will do this for a selection of the data to present a story that is more streamlined. I will discuss: a) the observed epigenetic changes in reference to gene expression, b) epigenetic mechanisms that may be involved i.e. epigenetic enzyme activity and TF expression and finally, c) the phenotypic changes observed in repair activity and oxidative damage.

5.4.1 *Epigenetics and BER-related gene expression*

5.4.1.1 *Effect of age and DR on DNA methylation*

Epigenetic changes have been proposed as one of the hallmarks of ageing (López-Otín *et al.*, 2013). Decline in global DNA methylation during ageing has been well documented and evidence shows that decrease in global DNA methylation contributes to age-related genomic instability (Bell *et al.*, 2012). In addition, alterations of epigenetic marks at gene promoter regions may be responsible for the transcription changes occurring with age. Ageing is characterised by down-regulation of cell cycle and maintenance genes and up-

regulation of inflammatory and stress response genes (Lee *et al.*, 2000). The age-related decline in genomic maintenance is one of the factors that facilitate the accumulation of macromolecular damage seen during ageing (Kirkwood, 2008).

I hypothesised that there would be an increase in cytosine methylation (at BER-related gene promoters) with age, which would cause silencing of BER-related genes. DNA methylation at most CpG sites within both *Ogg1* and *Apex* promoter regions was low. In a given DNA molecule a specific cytosine residue within a CpG dinucleotide is either methylated or not and; therefore, to a first approximation, the values for methylation reported in this chapter correspond to the proportion of genomes (i.e. cells within the sample) at which the investigated CpG sites are methylated (Mathers and Ford, 2009). Overall, only a small percentage of the cells found in the brain had methylated cytosine bases in the promoter regions of *Ogg1* and *Apex*. A group of enzymes known as Ten-eleven-translocation (TET) proteins convert methylated cytosine to 5-hydroxymethylcytosine. It is hypothesised that 5-hydroxymethylcytosine prevents binding of methyl binding proteins and DNMTs. Furthermore, TET enzymes themselves may bind to unmethylated DNA and prevent DNA methylation by DNMTs (Xu *et al.*, 2011). In the mouse neurons TET1 together with a deaminase and thymine DNA glycosylase causes DNA demethylation (Cortellino *et al.*, 2011; Guo *et al.*, 2011). Although I did not quantify levels of 5-hydroxymethylcytosine there is evidence for increased levels in the ageing mouse hippocampus (Chen *et al.*, 2012) and human brain (Wen *et al.*, 2014). Such a phenomenon may explain the low levels of DNA methylation in the studied promoters.

Interestingly, contrary to my hypothesis I found that the differentially methylated cytosines within CpG dinucleotides experienced an age related demethylation and this was accompanied by decline of *Ogg1* and *Apex* expression. Investigation of cytosine methylation often centres around hypermethylation and the related gene silencing; however, demethylated genes are also observed during ageing (Takasugi, 2011). Demethylation of the *Ogg1* gene correlated with increased expression in rat liver (Tsubota *et al.*, 2008) and human breast cancer cells (Singh *et al.*, 2012). Growing evidence highlights methylation at other sites such as gene bodies and CpG island shores and shelves in

regulating transcription activity (Suzuki and Bird, 2008; Sanyal *et al.*, 2012). For example, non-promoter DNA methylation enhances transcription of genes involved in neurogenesis by inhibiting repressive polycomb proteins (Wu *et al.*, 2010). Similarly, non-promoter methylation could be responsible for the regulation of expression of *Ogg1* and *Apex* genes.

I hypothesised that DR can attenuate an-age related increase in methylation. I observed that the promoter regions of the chosen genes remain demethylated throughout chronological age and that DR animals had lower methylation levels. Furthermore at a number of CpG sites studied demethylation occurred in DR fed animals independently of age. Therefore rather than prevent age-related methylation changes I show here that DR potentiates this phenomenon. It has previously been reported that in the hippocampus, DR prevented the age-related increase in DNMT3a protein (Chouliaras *et al.*, 2011) through the Sirtuin pathway, as these deacetylases associate with DNA methyltransferases (Fuks *et al.*, 2000; Fuks *et al.*, 2001).

A possible mode of action for DNA methylation is the prevention of binding of transcription factors. I used the Genomatix software to determine the TF factors binding at the *Ogg1* and *Apex* promoters. Comparison of differentially methylated CpG sites with age at the *Ogg1* promoter (sites 15, 17 and 18) mapped to cut-like homeobox and its homolog factors (CLOX), core promoter motif ten element (MTEN), neuron-restrictive silencer factor (NRSF), v-ERV and RAR-related orphan receptor alpha (RORA), GLI zinc finger family (GLIF), zinc binding protein factors (ZBPF) and Huntington's disease gene regulatory region binding protein (HDBP) transcription factors. CpG sites showing age-related methylation at *Ogg1* promoter site 3 mapped to hypoxia inducible factor (HIFF) and 14 mapped to general transcription factor II (T2D), CCAAT binding factors (CAAT), CLOX, MTEN and NRSF. In the *Apex* promoter, sites CpG 10 and 27 were affected by both age and DR and these mapped to activator protein 2 (AP2F), insulinoma associated factors (INSM), cAMP-responsive element binding proteins (CREB) and NRSF transcription factors. The common factors between both promoters were CAAT, NRSF, HDBP. These transcription factors represent both positive and negative regulators of transcription initiated by oxidative stress and DNA damage response pathways ((Christmann and Kaina, 2013) and search of NCBI gene database). Overall DNA methylation data could

not provide implicit relationship with expression; therefore, I went on to look at histone modification marks relative to the repair genes.

5.4.1.2 *Effect of age and DR on histone post-translational modification*

The histone code provides another level of epigenetic regulation of transcription. In the present project, I investigated H4 acetylation and H3K27Me3, which have been correlated with transcription activation and repression respectively (Akhtar and Becker, 2000; Asp *et al.*, 2011). I observed both a repressive H3K27Me3 and active H4Ac modification at the promoters of BER-related genes, implying a tight regulation of transcription as is observed in development to allow rapid activation of gene expression (Bernstein *et al.*, 2006).

I found that histone modifications with age and DR were sex specific and although strong trends were observed these were not significant. Non-normality of the data and use of a non-parametric test which is not as robust as ANOVA may have masked the biological effects. The large variation within the data could have arisen because whole brain tissue rather than individual sub-regions was used in this study. It has been demonstrated that age-related cytosine methylation changes in cortical tissue correlated with the pons but not with the cerebellum (Hernandez *et al.*, 2011). Furthermore, histone methylation does not correlate between sub-regions of the hippocampus and these react differently to stress stimuli (Hunter *et al.*, 2009).

In the brain of male mice, in both the promoter regions studied H4Ac and H3K27Me3 increased with age and DR prevented this increase. Global histone modification changes occur during ageing (Feser and Tyler, 2011). However, here I focus on changes observed in relevance to *Ogg1*, and *Apex* promoters. The fact that antagonistic histone marks changed in opposite directions may be predicted to result in there being no effect on transcription activity. This prediction is supported by the observed expression of *Ogg1* which did not change with age. On the other hand expression of *Apex* declined with age despite the theoretically neutralising effect of the histone modifications measured. One explanation for this could be that the overall histone code at the *Apex* promoter displays a silencing signal; however, because only 2 of the histone marks were studied, this did not report on the overall signal (Strahl and

Allis, 2000; Dong *et al.*, 2012). While H3K27Me3 is very well correlated with repression of transcription (Barski *et al.*, 2007), H4Ac can initiate activation or repression of transcription depending on the initiator molecule (Deckert and Struhl, 2001). Furthermore, in yeast inducible genes can be marked by histone acetylation even in the inactive chromatin state (Roh *et al.*, 2004) thus highlighting the complexity of the histone code.

What is striking about the data is that DR is able to prevent these age-related changes in histone modifications and the histone modifications measured in 30DR mice were comparable to 3AL mice. This is not surprising since sirtuins (NAD dependant histone deacetylases) are one of the pathways of DR action (Lin *et al.*, 2000). Interestingly, here H4 acetylation (H4Ac) showed i) little change with age consistent with no changes in HAT activity and ii) DR decreased H4AC consistent with higher HDAC activity in the brain of DR fed animals. Changes in specific HAT and HDAC enzyme families could have occurred, but were not detected here since the overall activity was measured. However, previously no changes in CBP and p300 HAT enzyme activity were observed in the ageing brain (Li *et al.*, 2002). Furthermore the sex specific changes may be attributed to differential expression of histone demethylases, such as UTX, which is present at much higher levels in the female mice compared with male mice (Xu *et al.*, 2008b).

Based on the present results it is likely that these epigenetic mechanisms interact with each other to give rise to the specific expression pattern. Furthermore microRNAs have also been classed as an epigenetic mechanism since they act post transcription to inhibit or break down the transcript mRNA thus further complicating the transcription regulatory mechanisms. Multiple miRNAs targeting DNA repair genes have been identified. MiRNA 182-5p targets genes of the homologous recombination repair pathway (Krishnan *et al.*, 2013). Furthermore, miR 31-5p targets the *hMLH1* gene, which encodes a protein of the mismatch repair pathway, and miR overexpression is associated with MLH1 protein decrease and cell cycle arrest (Zhong *et al.*, 2013). In addition, numerous miRNAs are differentially expressed in older individuals and these changes affect expression of miRNA target genes (Noren Hooten *et al.*, 2010). *Ogg1* and *Apex* have been identified as predicted targets of multiple miRNAs in the human (Helwak *et al.*, 2013). However, there has been no

detailed study on miRNA modulation of BER genes during ageing. Such an investigation would bring important epigenetic regulatory information and could identify whether down-regulation of BER genes such as *Apex* (as identified in this study) is dependent on miRNA regulation.

On a gene specific level it seems that despite aberrant changes observed with ageing the cell attempts to counteract these changes by maintaining active expression of genes coding for repair enzymes through DNA hypomethylation and histone acetylation, in what can be explained as a survival effect. Furthermore, DR marks its own effect on the epigenome by further enhancing DNA demethylation and preventing and age-related histone changes, thus giving higher expression of BER-related proteins compared with AL fed controls.

5.4.2 *Effect of age and DR on TF expression*

I was able to detect changes in the expression of redox-sensitive transcription factors, which are involved in activation of BER-related gene expression. However, the quantified TF mRNA levels did not give an indication of the binding of these TFs to the promoter region and therefore ChIP studies might give more insight on this matter.

However, more surprisingly, no reduction in TF expression was observed in DR animals in the current study, while short-term DR showed trends to increase expression (Appendix C). DR has been proposed to reduce oxidative stress (Sohal and Weindruch, 1996) and therefore lower levels of redox-sensitive transcription factors may have been expected. On the other hand, maybe oxidative stress is lower in DR animals because they have higher levels of redox-responsive TFs which activate stress response enzymes that can maintain lower oxidative stress. This implies that DR might decrease age-related oxidative stress/damage via the activation of redox-sensitive transcription factors such as nuclear factor-kB (NFkB) and hypoxia inducible factor 1 (HIF-1) (Kim *et al.*, 2002).

Redox-sensitive transcription factors; e.g. FOXO (Greer and Brunet, 2008) and *Hic-5* (Lee *et al.*, 1999; Weindruch *et al.*, 2001) are up-regulated by ageing. Data presented here show increased *Hif1α* and *Nfya* expression in old brain. Age-related increase in HIF1α has been well documented (Kang *et al.*, 2005;

Benderro and LaManna, 2011), though, no change of human *NFYA* levels were observed (Matuoka and Chen, 2000). An increase in expression of redox-responsive genes, involved in BER activation, was not surprising in relation to the well-known implications of oxidative damage in ageing (Harman, 1956).

Data presented here show that the expression of redox-sensitive transcription factors was up-regulated with age (*Hif1α*) and DR (*Nfya*), which suggests that ageing and DR represent a state of oxidative stress, leading to the cell inducing expression of redox-sensitive transcription factors. The TFs may in turn induce expression of genes through response elements, such as in BER-related genes.

5.4.3 *Effects of age and of DR on BER-related DNA incision activity*

The observed changes in expression of BER-related genes would suggest alterations in the repair activity. I was unable to detect any changes in incision activity in the brain. I did however show an increase in OGG1 protein in cell lysates from older animals, which has been previously demonstrated (Szczesny *et al.*, 2004). Together these observations may suggest that the capacity of OGG1 to repair DNA lesions decreases with age, but the cell attempts to compensate by increasing the level of enzyme expression. A large number of protein post translational modifications regulate activity of repair enzymes, such as phosphorylation of OGG1 and acetylation of APEX. Thus any changes in post-translational modification of OGG1 and APEX will affect their function (Hu *et al.*, 2005; Almeida and Sobol, 2007; Busso *et al.*, 2010). Furthermore DR may exert its effect on DNA incision not only via epigenetic modulation but also via post-translational modification of repair enzymes by interaction with e.g. sirtuins. Search of the literature shows that the theory of age-related decline in repair has multitude of contradictory reports. Resveratrol, a DR mimetic, has been shown to increase activity of the APEX enzyme suggesting more abasic sites should be observed in DR treated animals (Yamamori *et al.*, 2010). Although I did not observe a statistical difference in incision activity in DR compared to AL animals, 30 month old AL animals showed a fall in incision activity whereas DR animals showed increased activity. These data suggest that DNA incision activity in the brain is maintained throughout mouse lifespan and only falls in the oldest mice. Furthermore, DR showed a potentially protective role by increasing incision activity in the oldest age group. It is difficult to say why this occurred but one possibility is that the 30 month group could

show ‘survival’ phenotype, having reached such a high age because of their cellular repair capacity. Cabelof *et al.* found the response of BER after exposure to oxidative stress is blunted with age (Cabelof *et al.*, 2006). Could this mean that basal repair is unaffected but any additional stressors in the older animals are not as quickly (well) removed as in the young? Again, such a report is contradictory to that of Hamilton *et al.* who found that repair after gamma irradiation did not alter in liver, brain or kidney from young or old mice (Hamilton *et al.*, 2001).

An increase in BER-related proteins was observed in response to age and DR. However others have not seen such changes (Intano *et al.*, 2003; Imam *et al.*, 2006; Kisby *et al.*, 2010; Swain and Subba Rao, 2011). Kisby *et al.* found no significant difference in APE, Pol-β or DNA ligase III (Kisby *et al.*, 2010) although they did find a decrease in activity. Imam *et al.* found no age-related change in APE and DNA ligase III in mouse cortex, hippocampus or cerebellum (Imam *et al.*, 2006) but they did find an age-related decline in OGG1 activity. Intanto *et al.* found no changes in DNA ligase I or III, XRCC-1 or APE concentration but did see a decline in Pol-β in aged mice (Intano *et al.*, 2003) while Swain *et al.* found that a decrease in DNA ligase I and III, APE and Pol-β protein expression in old rat cortical neurones (Swain and Subba Rao, 2011).The contradictory observation seen in the present study may have arisen because the protein concentration was measured in whole cell lysates, thus quantifying expression of nuclear as well as cytosolic proteins.

The comet assay was used to quantify BER-related repair activity. The damage induced in substrate cells by Ro 19-8022 is comparable to that induced by singlet oxygen, producing mainly 8-oxoguanine (Will *et al.*, 1999). The incision reaction is performed on nucleoids and therefore is much more representative of BER in a cell than incision of an oligonucleotide containing a single damaged base. On the other hand the comet-based assay, although a sensitive technique for measurements of DNA incision, suffers from non-specific nuclease activity (Chapter 3) and with high biological variability it may be difficult to obtain statistically significant changes.

Two separate groups have also shown that supplementation with one or more components of the BER pathway is required to restore DNA repair function

(Krishna *et al.*, 2005; Swain and Subba Rao, 2011), but the lack of changes in protein expression observed here suggests that function of the protein may be impaired, rather than expression affected.

Lastly there is ample evidence that NER has been shown to act as a back-up for removal of DNA oxidative damage. For example, mice lacking the *Ogg1* gene are able to minimise 8-oxodG related mutations, (Klungland *et al.*, 1999; Osterod *et al.*, 2001). This supports the notion that other repair mechanisms can be used to remove 8-oxo damage and may be another reason why no change was observed in repair but a decline in gene expression was seen with age. In the future it would be important to measure other BER as well as NER-related proteins to determine age-related changes.

5.4.4 *Effect of age and DR on oxidative damage*

The brain is a highly metabolically active tissue with a large glucose consumption, moderate ROS production and low antioxidant defences (Sohal *et al.*, 1990b). Many studies report an age-related increase in DNA oxidative damage in support of the free radical theory of ageing (Hirano *et al.*, 1996; Kaneko *et al.*, 1997; Nakae *et al.*, 2000). Similarly, evidence points to DR having a protective role in oxidative stress, with lower damage levels reported in DR fed animals (Sohal *et al.*, 1994a; Hamilton *et al.*, 2001). However, a reasonable number of studies do not report any changes in DNA oxidative damage, with changes being tissue, sex and species specific. (Hamilton *et al.*, 2001; Schmerold and Niedermüller, 2001). The present study, supports (in part) the age-related increase in DNA oxidative damage. Strikingly I observe highest 8-oxoguanine levels in the youngest mice which could be a result of an oxidative insult from birth or acquired at 3 months. If such an increase was the result of an endogenous oxidative damage source, it could be verified by measuring ROS production. However, this has to be performed on fresh tissue; therefore, is unfeasible. In mice at 3 months of age the repair capacity in the brain tissue was enough to remove the high levels of damage and by 12 months of age the number of 8-oxoguanine lesions was reduced. Furthermore from 12 months of age there is a minor increase in DNA oxidative damage with age.

The function of DNA oxidative damage during ageing remains unclear. Perhaps the problem lies in the damage lesions quantified. Although 8-oxoguanine is the

primarily damaging lesion formed, and readily used as a biomarker of DNA oxidative damage, it should be noted that other mutagenic lesions such as 5-hydroxy-2'-cytosine are formed as a result of ROS and also possess their own DNA glycosylase for repair (Kasai, 1997). Furthermore ROS can also form lesions such as 8,5'-cyclopurine-2'-deoxynucleoside, which is a bulky oxidative lesion repaired by the NER pathway (Wang *et al.*, 2012a). The comet assay could also be used to test damage in frozen tissue, but this requires steps to be taken to reduce damage induced during cryopreservation by freezing specific buffers (Hu *et al.*, 2002). Damage as measured by strand breaks using the comet assay was higher in neurones from older animal compared with young ones (Swain and Subba Rao, 2011). It should be noted that accumulation of damage depends on: nature of damaging agent, metabolic rate, presence of oxygen, efficiency of antioxidant systems, efficiency of DNA repair and rate of cell proliferation (Fraga *et al.*, 1990). Diane Cabelof suggests that mutation frequency is highest in tissues with low BER activity such as liver and lower mutation frequency is found in tissues with higher BER activity such as the brain (Cabelof *et al.*, 2002). Based on this the authors suggest that the decline seen with ageing may be attributed to the intrinsic BER activity of the tissue and not its proliferative nature. To date the role of oxidative damage and repair of such lesions has remained unresolved.

5.4.5 *Conclusions*

Key findings in the brain:

- Multiple CpG sites in *Ogg1* and *Apex* promoters were demethylated with age.
- DR fed mice showed lower cytosine methylation compared to AL controls.
- Histone modifications at *Ogg1* and *Apex* promoters were enriched in older animals.
- DR prevented the age-related enrichment in histone modifications.
- Transcription active state of chromatin is maintained in the promoter of *Ogg1* and *Apex* during ageing and DR.
- DR animals had higher mRNA levels of Ogg1 and Apex.
- There was a minor age-related decline in *Ogg1* and *Apex* mRNA.
- OGG1 and APEX protein concentration increased with age and were higher in DR fed animals compared with AL controls.

- DNA incision activity remained constant through-out chronological age.
- DNA oxidative damage increased with age and was not affected by DR.

5.5 Acknowledgments

I would like to thank:

- *Dr Jelena Mann for her help with the ChIP assay and design of primers for ChIP-qPCR.*

I would also to acknowledge:

- *CISBAN for design of the animal study and Dr Kerry Cameron and Professor Thomas von Zglinicki for information on animal and tissue weights.*
- *Maastricht University who performed the 8-oxoguanine analysis*
- *Dr Sabine Langie and Bartłomiej Tomaszewski for design of Ogg1 pyrosequencing and qPCR primers.*
- *Dr Sabine Langie for developing the comet in vitro repair assay.*

Chapter 6: The effects of ageing and of long-term dietary energy restriction (DR) on base excision repair (BER) in the liver

6.1 Introduction

Preliminary data described in Chapter 4 suggest that age and short-term dietary restriction (DR) enhance base excision repair (BER) in the liver of male mice. In this chapter I describe the changes in BER in male and female mouse liver exposed to long-term DR. I will report on outcomes from studies of liver similar to those investigated in Chapter 5 for brain tissue. In addition, I will compare the differences in epigenetic profiling at repair gene promoters and DNA incision activity in the brain and liver. The study described in this chapter aimed to test the following hypotheses:

1. During ageing, epigenetic changes at *Ogg1* and *Apex* gene promoters cause silencing of these genes.
2. This gene silencing results in decreased BER activity and hence increased accumulation of DNA oxidative damage.
3. DR ameliorates the adverse effects of age on cytosine methylation and histone modifications at *Ogg1* and *Apex* promoters, mediates changes in activity of enzymes responsible for modifications in epigenetic marks, increases the capacity for DNA repair, up-regulates expression of redox-sensitive TFs and reduces age-related accumulation of DNA oxidative damage.

The objectives of the present study were to:

a) Characterise and quantify methylation of specific CpG sites within the promoters of *Ogg1* and *Apex* in DNA from liver of mice during ageing and in response to long-term DR;
b) Characterise and quantify histone acetylation and methylation relative to *Ogg1* and *Apex* promoters in DNA from liver of mice during ageing and in response to long-term DR;
c) Quantify histone acetyltransferase and histone deacetylase activity in extracts from liver of mice during ageing and in response to long-term DR;

d) Using RT-qPCR, quantify expression of transcription factors *Hif1α* and *Nfya* associated with *Ogg1* and *Apex* gene activation in the liver of mice during ageing and in response to long-term DR,
e) Using RT-qPCR, quantify expression of *Ogg1* and *Apex* in the liver of mice during ageing and in response to long-term DR;
f) Quantify BER-related incision activity using the comet-based assay;
g) Using HPLC-ECD measurements of 8oxodG, quantify oxidative damage in DNA from the liver of mice during ageing and in response to long-term DR.

6.2 Materials and Methods

Details of the animal study are given in Chapter 5.2.

6.3 Results

6.3.1 *Animal and organ characteristics*

Data for body mass and liver weights in male and female mice are shown in Table 6.1. As expected, DR mice were lighter than AL mice at all ages and for both sexes. Proportional differences in liver weight between AL and DR animals were greater than for total body mass in male mice only (P<0.001).

Group	Male			Female		
	Body mass (g)	Liver (g)	Liver mass (%)	Body mass (g)	Liver (g)	Liver mass (%)
3AL	29.2 ±2.04	1.50 ±0.127	5.14 ±0.13	22.9 ±1.53	1.16 ±0.074	5.07 ±0.10
12AL	36.7 ±4.12	2.06 ±0.667	5.50 ±0.47	25.2 ±3.12	1.07 ±0.138	4.16 ±0.11
12DR	24.8 ±2.75	0.98 ±0.166	3.94 ±0.13	17.1 ±1.91	0.70 ±0.071	4.10 ±0.04
24AL	47.9 ±3.85	2.43 ±0.436	5.04 ±0.21	35.9 ±3.20	1.53 ±0.234	4.28 ±0.26
24DR	25.3 ±2.12	1.18 ±0.181	4.68 ±0.19	19.2 ±0.32	0.77 ±0.048	4.04 ±0.08
30AL	35.1 ±2.60	2.91 ±1.344	8.38 00.19	28.4 ±4.38	1.39 ±0.252	4.87 ±0.09
30DR	23.0 ±2.94	1.12 ±0.290	4.81 ±0.39	18.1 ±1.04	0.88 ±0.082	4.85 ±0.16

Table 6.1 Body masses and weights of livers from male and female mice in the CISBAN study. These are data from a subset of animals. Specific data for the animals used in the present study are not available. Data represent mean ± SEM, n=5-7, 3AL – 3 month old AL fed mice, 12AL – 12 month old AL fed mice, 12DR – 12 month old DR fed mice, 24AL – 24 month old AL fed mice, 24DR – 24 month old DR fed mice, 30AL – 30 month old AL fed mice and 30DR – 30 month old DR fed mice. Univariate ANOVA-GLM analysis for diet: P<0.001. Data curtesy of Dr Kerry Cameron and Professor Thomas von Zglinicki.

6.3.2 *Epigenetic regulation of Ogg1 and Apex promoter in the liver*

To determine the effects of age and of long-term dietary restriction on BER, I measured epigenetic marks at the promoter regions of *Ogg1* and *Apex* genes. This included quantification of cytosine methylation, histone 4 acetylation and histone 3 lysine 27 tri-methylation in the liver from mice from the CISBAN long-term DR study. Cytosine methylation data were not always normally distributed and Box-Cox power transformation was unable to transform the data to a normal distribution. With the assumption that the ANOVA is a robust test which is able to withstand deviations from normality (Glass *et al.*, 1972; Sawilowsky, 1990), the ANOVA GLM was used for analysis of methylation data at individual CpG sites.

6.3.2.1 *DNA methylation of the Ogg1 promoter*

DNA methylation was quantified at 13 CpG sites in the *Ogg1* gene promoter. Measurements were made at all ages (3, 12, 24 and 30 months) and in both AL and DR animals. Figures 6.1 and 6.2 show methylation at individual CpG sites and mean methylation across the CpG sites in the *Ogg1* promoter for male and female mice respectively. Overall, levels of methylation were quite low and ranged from 0-16.72%. Methylation at CpG 3 was lowest at 0.2% in male and 0.14% in female mice (average across all 7 experimental groups). Methylation at Cp16 was highest at 3.17% in male and 3.84% in female mice (average across 7 groups). In addition, in male mice CpG 4, CpG16 and CpG18 showed consistently higher methylation levels compared with other CpG sites measured while CpG 4, CpG 8, CpG 15, CpG 16 and CpG 18 showed consistently higher methylation levels in female mice.

A difference in cytosine methylation was observed between the sexes ($P<0.001$) with female mice showing overall higher methylation; therefore, further analyses were run for males and females separately. Correlations between cytosine methylation at individual CpG sites are shown in Figure 6.3 and Figure 6.4 for male and female mice respectively. The correlation between different sites varied from 0.499 to 0.829 in male mice and -0.428 to 0.629 in female mice. Overall, correlations between the sites were high in males but not in females and so effects of treatments on methylation of individual CpG sites as well as mean methylation across the sites were analysed.

In male mice methylation at CpG 8 (Pa=0.015), CpG 13 (Pb=0.007) and CpG 16 (Pc=0.007) decreased progressively with age. No effect of DR on cytosine methylation was observed in male mice at any CpG sites although CpG 7 showed an interaction between age and diet (P=0.007). Furthermore there was an age-related decline in mean methylation across the 13 CpG sites in male mice (Pd=0.018). DR had no effect on mean methylation across the *Ogg1* promoter in male mice.

In female mice cytosine methylation increased with age at CpG 9 (Pb=0.032) and CpG 16 (Pd=0.015). Furthermore, cytosine methylation was lower in DR animals compared with AL fed controls by: 0.35% at CpG 6 (Pa=0.032) and 0.40% at CpG 9 (Pc=0.023). In addition, there were significant interactions between age and diet at 4 CpG sites which appeared to be due to the higher cytosine methylation observed in 24AL group compared with all other groups (CpG 6, P=0.041; CpG 8, P=0.018; CpG 17, P=0.038 and CpG 18, P=0.049). There were no effects of age or diet on mean methylation across the 13 CpG sites in the *Ogg1* gene promoter but, there was an interaction between these variables (P=0.025).

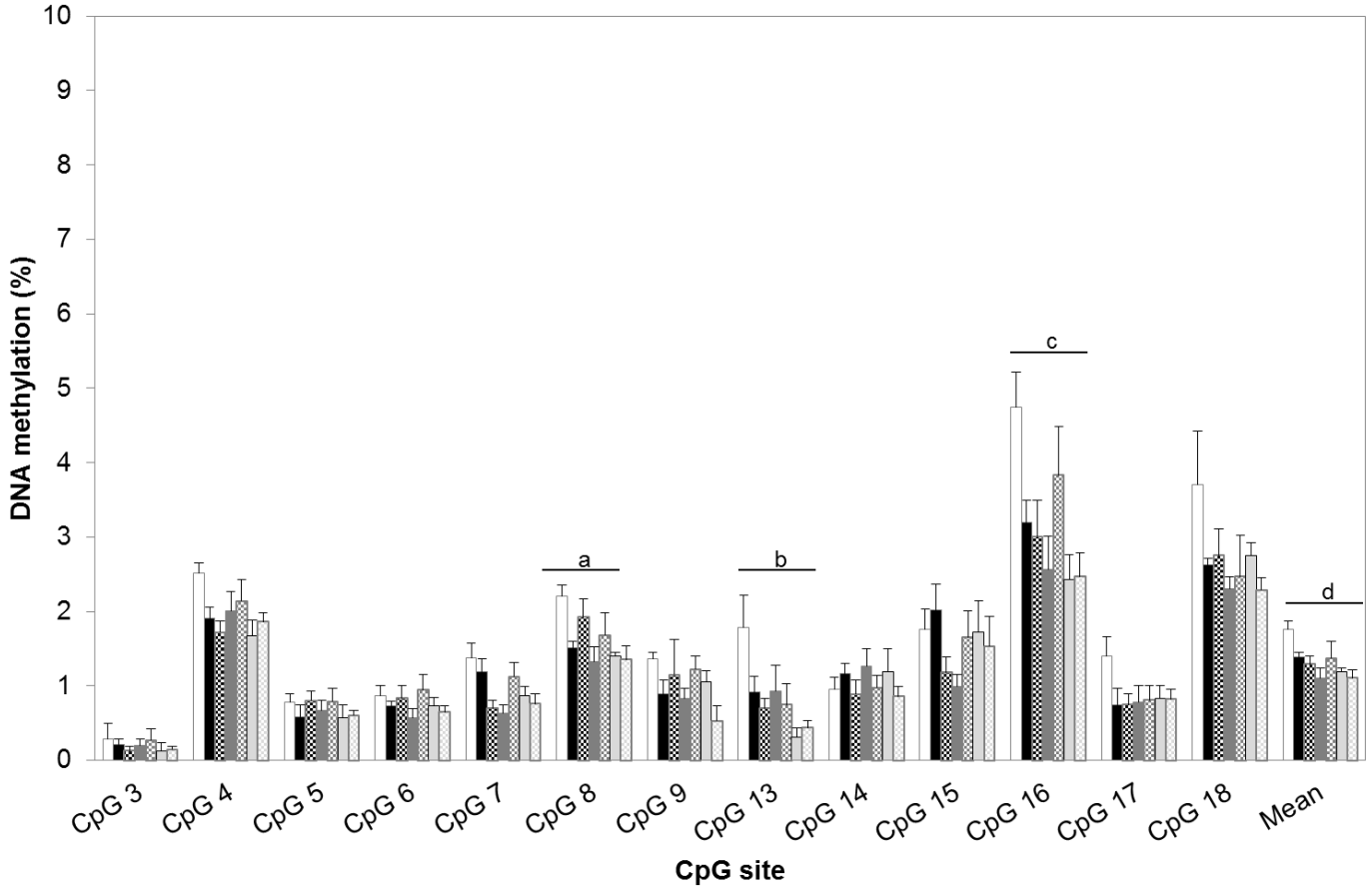


Figure 6.1 Effect of age and DR on methylation at individual CpG sites within the *Ogg1* promoter in DNA from male liver. Data represent mean ± SEM per treatment group, 3AL (white bars, n=5), 12AL (black bars, n=7), 12DR (black checker bars, n=7), 24AL (dark grey bars, n=7), 24DR (dark grey checker bars, n=7), 30AL (light grey bars, n=5) and 30DR (light grey checker bars, n=7). Methylation for individual animal tissues were measured in duplicate. Univariate ANOVA-GLM analysis for age and diet: Pa=0.015, Pb=0.007, Pc=0.007 and Pd=0.028. Primer design by Bartłomiej Tomaszewski and Dr Sabine Langie. Sample analysis and data collection Joanna Górniak.

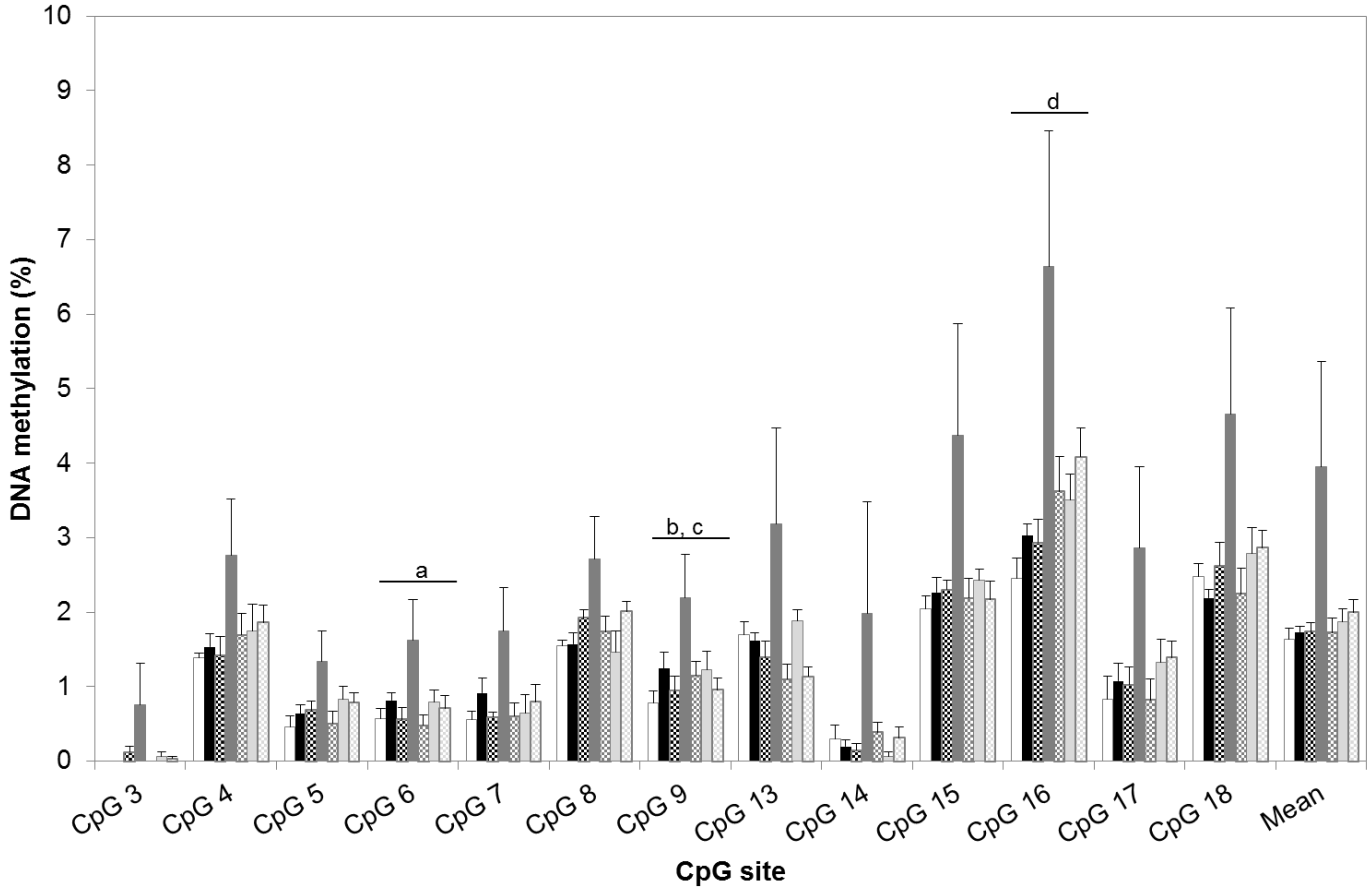


Figure 6.2 Effect of age and DR on methylation at individual CpG sites within the *Ogg1* promoter in DNA from female liver. Data represent mean ± SEM per treatment group, 3AL (white bars, n=5), 12AL (black bars, n=7), 12DR (black checker bars, n=7), 24AL (dark grey bars, n=7), 24DR (dark grey checker bars, n=7), 30AL (light grey bars, n=7) and 30DR (light grey checker bars, n=7). Methylation for individual animal tissues were measured in duplicate. Univariate ANOVA-GLM analysis for age and diet: Pa=0.032, Pb=0.032, Pc=0.023 and Pd=0.015. Primer design by Bartłomiej Tomaszewski and Dr Sabine Langie. Sample analysis and data collection Joanna Górniak.

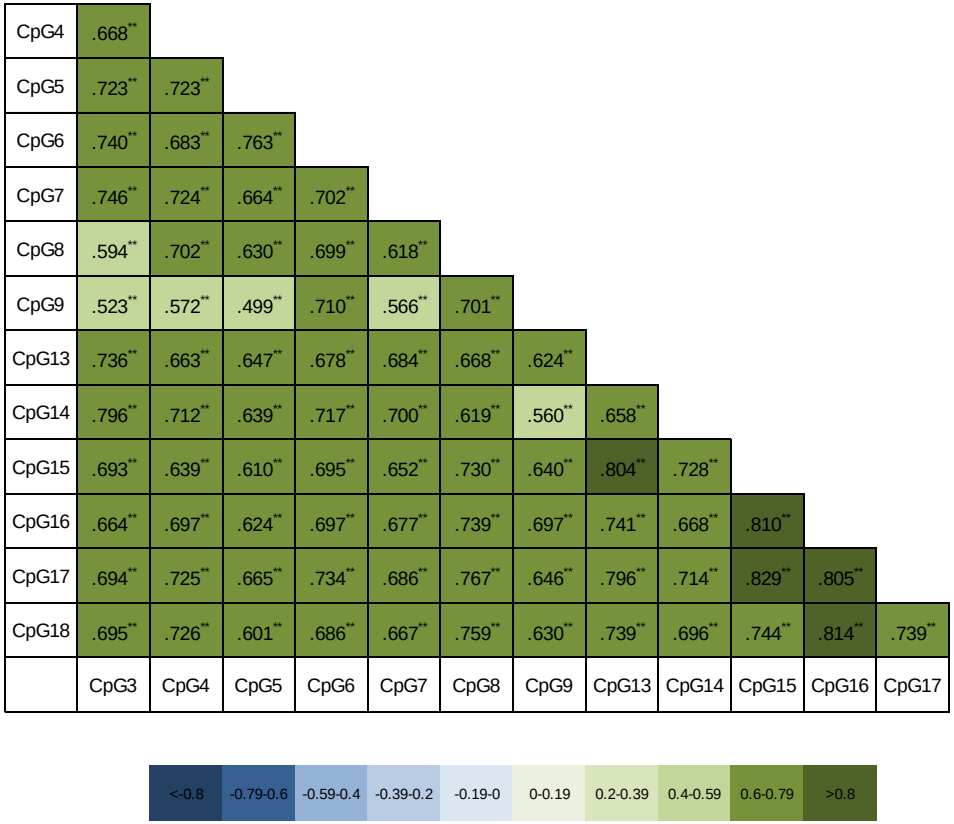


Figure 6.3 Pearson's correlation for % methylation between CpG sites within the *Ogg1* promoter from male mouse liver. Statistically significant correlation at P<0.05 (*) and P<0.01 (**).

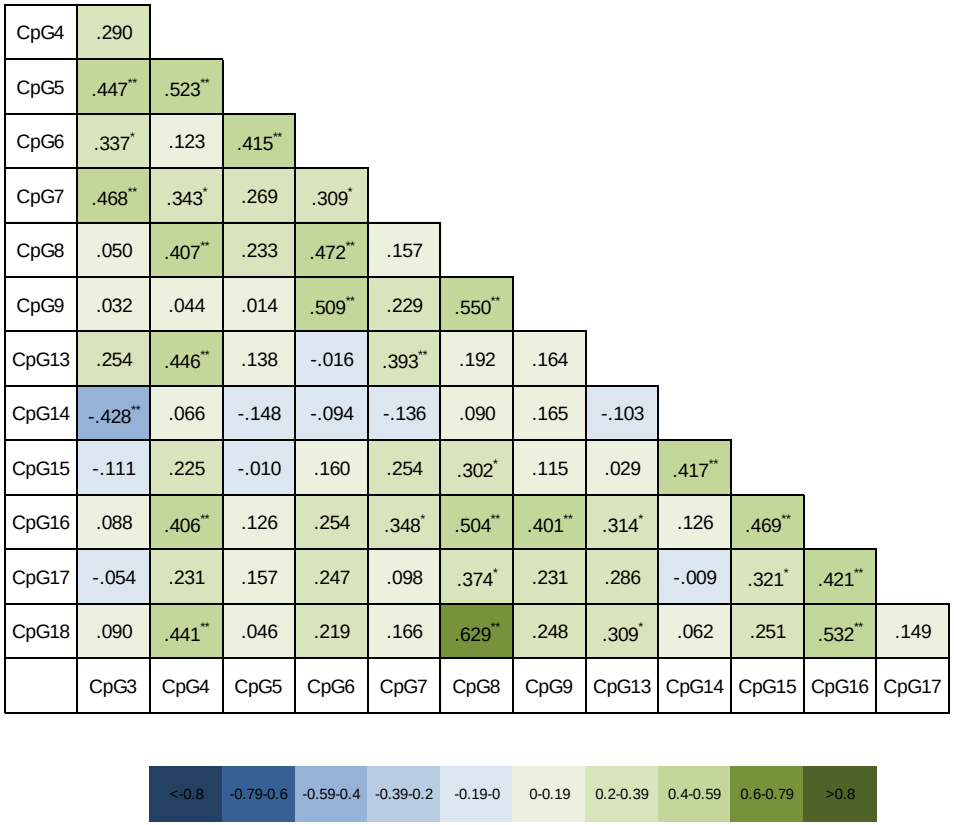


Figure 6.4 Pearson's correlation for % methylation between CpG sites within the *Ogg1* promoter from female mouse liver. Statistically significant correlation at P<0.05 (*) and P<0.01 (**).

6.3.2.2 *DNA methylation of the Apex promoter*

DNA methylation was quantified at 22 CpG sites in the *Apex* gene promoter. Measurements were made at all ages (3, 12, 24 and 30 months) and in both AL and DR animals. Patterns of methylation of the *Apex* promoter differed between sexes. For example, CpG 7 showed lowest methylation in males at 0.92% while CpG 27 showed lowest methylation in females at 0.22%. CpG 9 was most methylated in males at 3.93% while CpG 28 was most methylated in females at 3.07%. Overall, CpG sites 9, 17, 28, 29 and 34 showed consistently higher methylation compared with the other CpG sites. Cytosine methylation at individual CpG sites and overall methylation across these sites in the liver from male and female mice are shown in Figures 6.5 and 6.6 respectively. Methylation was significantly different between sexes at CpG 5 and 28 and analyses of effects of treatment on methylation at all CpG sites were performed for each sex separately. Correlations between methylation at individual CpG sites are shown in Figure 6.7 and Figure 6.8 for male and female mice respectively. The correlations between the different sites ranged from -0.035 to 0.903 in males and -0.226 to 0.910 in females. As for the *Ogg1* gene promoter, analyses of methylation data were run for individual CpG sites and for overall mean across all sites.

In the male mice methylation at individual CpG sites was low, ranging from 0% to 9.31%, and there was no significant effect of age or diet on methylation at any of the CpG sites investigated. On the other hand, in female mice age affected cytosine methylation at 11 CpG sites. Cytosine methylation decreased in linear trend at: CpG 6 (Pa=0.044), CpG 7 (Pb=0.001), CpG 8 (Pc=0.006), CpG 11 (Pf=0.006), CpG 12 (Pg=0.004) and CpG 16 (Ph=0.035). Cytosine methylation varied with age in a quadratic trend at CpG 10 (Pd=0.018), CpG 26 (Pj=0.003), CpG 27 (Pk=0.009), CpG 29 (Pl=0.006) and CpG 30 (Pm=0.011). Furthermore, cytosine methylation in DR fed animals was lower compared with AL fed animals by: 0.70% at CpG 10 (Pe=0.007) and 0.82% at CpG 17 (Pi=0.011). Mean methylation across the CpG sites declined with age in a linear trend (Pn=0.002). An interaction between age and diet was observed at CpG 16 (P=0.016) and CpG 0.019 (P=0.019).

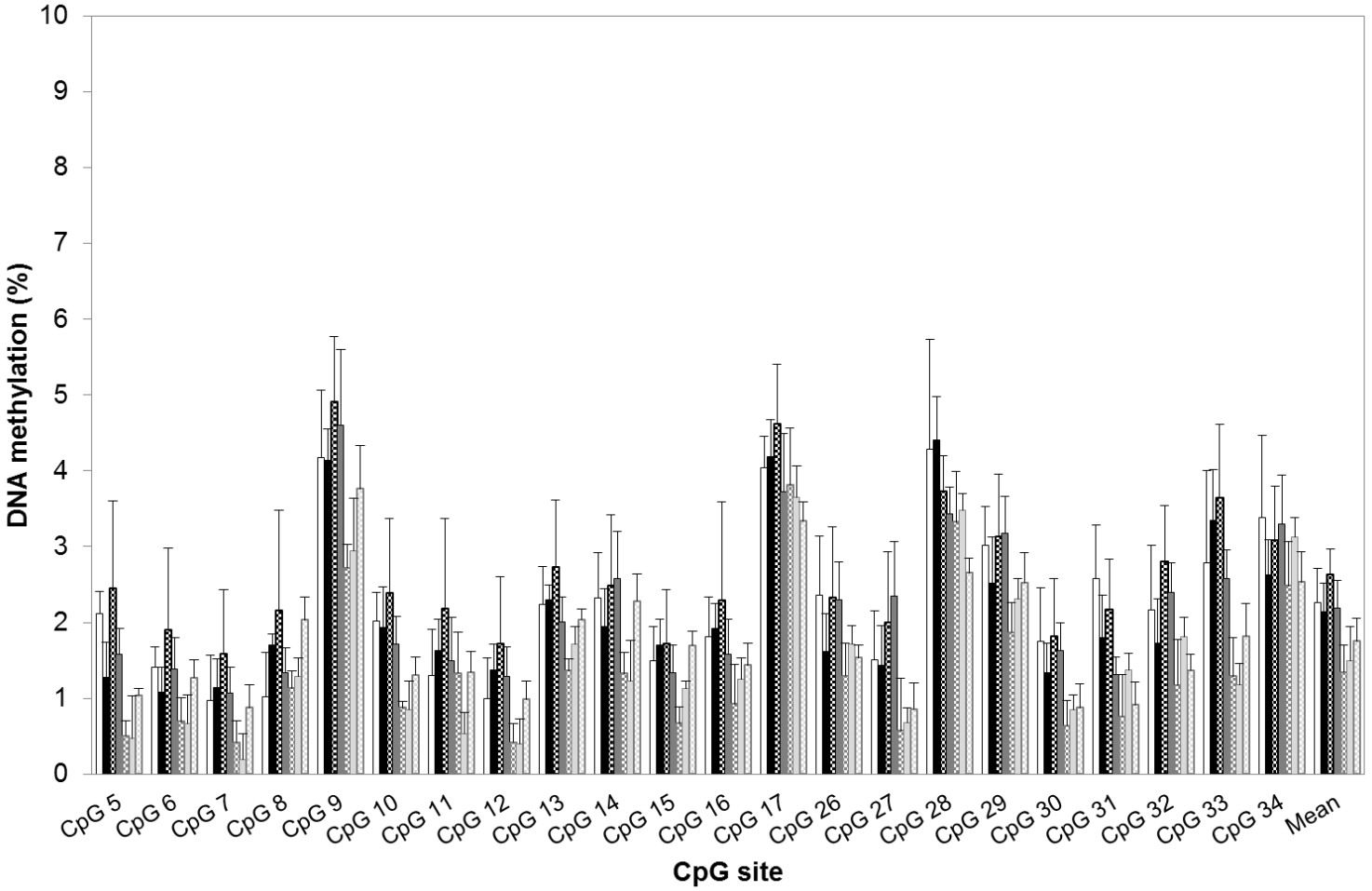


Figure 6.5 Effect of age and DR on methylation at individual CpG sites within the *Apex* promoter in DNA from male liver. Data represent mean ± SEM per treatment group, 3AL (white bars, n=5), 12AL (black bars, n=7), 12DR (black checker bars, n=7), 24AL (dark grey bars, n=7), 24DR (dark grey checker bars, n=7), 30AL (light grey bars, n=5) and 30DR (light grey checker bars, n=7). Methylation for individual animal tissues were measured in duplicate.

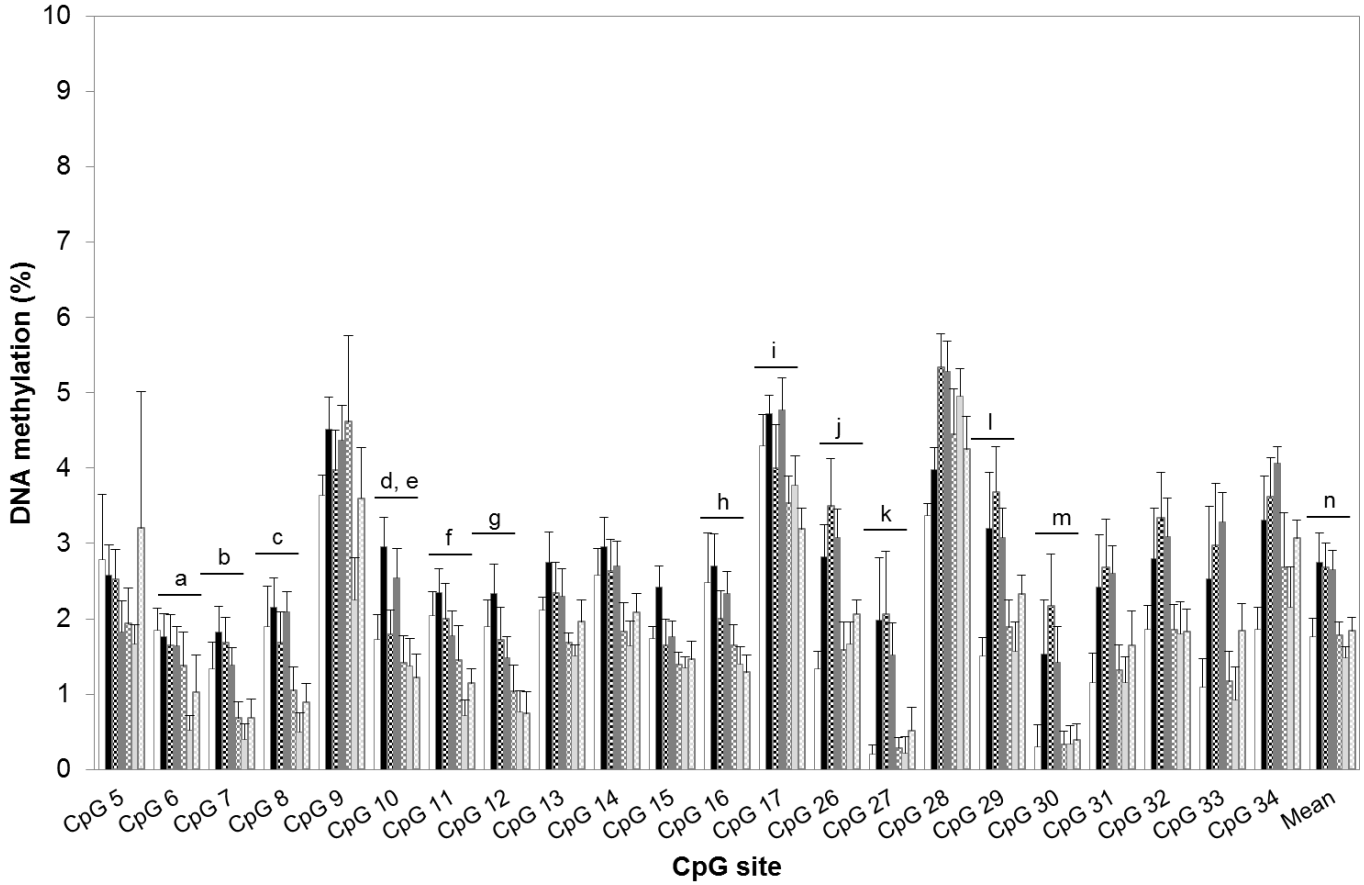


Figure 6.6 Effect of age and DR on methylation at individual CpG sites within the *Apex* promoter in DNA from female liver. Data represent mean ± SEM per treatment group, 3AL (white bars, n=5), 12AL (black bars, n=7), 12DR (black checker bars, n=7), 24AL (dark grey bars, n=7), 24DR (dark grey checker bars, n=7), 30AL (light grey bars, n=5) and 30DR (light grey checker bars, n=7). Methylation for individual animal tissues were measured in duplicate. Univariate ANOVA-GLM analysis for age and diet: Pa=0.044, Pb=0.001, Pc=0.006, Pd=0.018, Pe=0.007, Pf=0.006, Pg=0.004, Ph=0.035, Pi=0.011, Pj=0.003, Pk=0.009, Pl=0.006, Pm=0.011 and Pn=0.002.

CpG6	.876**																				
CpG7	.805**	.859**																			
CpG8	.735**	.836**	.799**																		
CpG9	.544**	.628**	.651**	.623**																	
CpG10	.840**	.848**	.769**	.794**	.730**																
CpG11	.767**	.813**	.803**	.764**	.622**	.786**															
CpG12	.773**	.833**	.818**	.806**	.703**	.873**	.849**														
CpG13	.823**	.875**	.875**	.822**	.703**	.845**	.796**	.879**													
CpG14	.757**	.720**	.743**	.737**	.625**	.823**	.726**	.740**	.710**												
CpG15	.770**	.787**	.752**	.718**	.614**	.789**	.821**	.850**	.841**	.758**											
CpG16	.838**	.858**	.775**	.797**	.650**	.834**	.846**	.812**	.832**	.724**	.855**										
CpG17	.634**	.730**	.654**	.605**	.563**	.672**	.769**	.656**	.625**	.593**	.703**	.758**									
CpG26	.375*	.484**	.327*	.448**	.381**	.525**	.438**	.493**	.376*	.398**	.394**	.465**	.447**								
CpG27	.412**	.471**	.345*	.390**	.315*	.550**	.369*	.510**	.416**	.398**	.397**	.396**	.292	.822**							
CpG28	.218	.194	-.035	.137	.065	.338*	.297*	.273	.193	.157	.297*	.209	.185	.499**	.577**						
CpG29	.420**	.410**	.280	.358*	.237	.524**	.342*	.472**	.337*	.391**	.397**	.372*	.272	.771**	.818**	.571**					
CpG30	.484**	.549**	.388**	.489**	.403**	.682**	.496**	.612**	.522**	.457**	.476**	.506**	.400**	.811**	.903**	.654**	.853**				
CpG31	.462**	.489**	.343*	.400**	.382**	.645**	.435**	.593**	.491**	.433**	.515**	.497**	.404**	.757**	.794**	.602**	.767**	.851**			
CpG32	.466**	.534**	.356*	.426**	.314*	.613**	.451**	.580**	.469**	.440**	.476**	.417**	.368*	.783**	.892**	.671**	.800**	.904**	.852**		
CpG33	.376*	.433**	.403**	.406**	.300*	.502**	.291	.428**	.416**	.267	.218	.260	.175	.565**	.726**	.481**	.662**	.788**	.635**	.715**	
CpG34	.226	.359*	.150	.350*	.366*	.459**	.302*	.374*	.296*	.292	.276	.266	.273	.758**	.742**	.684**	.687**	.733**	.694**	.767**	.570**
	CpG6	CpG7	CpG8	CpG9	CpG10	CpG11	CpG12	CpG13	CpG14	CpG15	CpG16	CpG17	CpG26	CpG27	CpG28	CpG29	CpG30	CpG31	CpG32	CpG33	CpG34

<-0.8	-0.79-0.6	-0.59-0.4	-0.39-0.2	-0.19-0	0-0.19	0.2-0.39	0.4-0.59	0.6-0.79	>0.8

Figure 6.7 Pearson's correlation for % methylation between CpG sites within the *Apex* promoter from male mouse livers. Statistically significant correlation at P<0.05 (*) and P<0.01 (**).

	CpG6	CpG7	CpG8	CpG9	CpG10	CpG11	CpG12	CpG13	CpG14	CpG15	CpG16	CpG17	CpG26	CpG27	CpG28	CpG29	CpG30	CpG31	CpG32	CpG33	CpG34
CpG6	.604**																				
CpG7	.424**	.781**																			
CpG8	.376**	.775**	.789**																		
CpG9	.423**	.685**	.526**	.591**																	
CpG10	.194	.600**	.687**	.696**	.532**																
CpG11	.260	.679**	.683**	.704**	.491**	.692**															
CpG12	.439**	.760**	.786**	.778**	.531**	.723**	.750**														
CpG13	.393**	.678**	.814**	.710**	.435**	.625**	.741**	.775**													
CpG14	.256	.681**	.727**	.755**	.519**	.692**	.781**	.734**	.835**												
CpG15	.322*	.522**	.686**	.652**	.307*	.576**	.640**	.703**	.837**	.726**											
CpG16	.360*	.643**	.761**	.784**	.447**	.649**	.640**	.648**	.684**	.626**	.700**										
CpG17	.137	.461**	.575**	.632**	.386**	.583**	.489**	.479**	.443**	.527**	.490**	.751**									
CpG26	.064	.134	.275	.285	.082	.349*	.325*	.284	.356*	.250	.196	.211	.083								
CpG27	.185	.265	.387**	.304*	.191	.392**	.301*	.381**	.448**	.322*	.285	.279	.099	.844**							
CpG28	-.164	-.170	-.055	-.088	-.189	.043	.062	-.079	-.005	-.070	-.226	-.118	-.098	.658**	.456**						
CpG29	-.061	.124	.299*	.266	.096	.300*	.314*	.321*	.369*	.275	.193	.125	-.013	.834**	.837**	.550**					
CpG30	.013	.245	.371*	.354*	.171	.372**	.362*	.414**	.453**	.346*	.248	.229	.091	.847**	.910**	.534**	.909**				
CpG31	-.034	.157	.238	.297*	.063	.282	.308*	.243	.412**	.312*	.195	.189	.036	.785**	.771**	.494**	.813**	.817**			
CpG32	.031	.147	.353*	.342*	.119	.343*	.241	.248	.314*	.251	.136	.272	.236	.785**	.761**	.661**	.740**	.804**	.656**		
CpG33	.153	.303*	.307*	.353*	.270	.376**	.315*	.344*	.361*	.272	.172	.264	.082	.832**	.843**	.479**	.807**	.845**	.802**	.730**	
CpG34	-.029	.080	.192	.301*	.135	.319*	.358*	.269	.344*	.282	.208	.134	.069	.766**	.662**	.543**	.751**	.698**	.642**	.625**	.742**

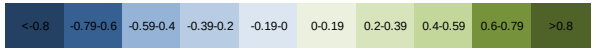


Figure 6.8 Pearson's correlation for % methylation between CpG sites within the *Apex* promoter from female mouse livers. Statistically significant correlation at P<0.05 (*) and P<0.01 (**).

6.3.2.3 *Histone acetylation at the Ogg1 promoter*

To assess the effect of age and of DR on chemical modifications to histone tails measurements were taken in 3 groups i.e. 3 month old mice, 30 month old AL mice and 30 month old DR mice. Histone 4 acetylation (H4Ac) and histone 3 lysine 27 trimethylation (H3K27Me3) were measured as prominent examples of epigenetic marks associated with transcription activation and repression respectively.

Overall, there was no effect of sex on acetylation of histone 4 at the *Ogg1* promoter. However both diet and age interacted with sex suggesting that males and females were differentially affected by these factors. In male mice, H4Ac enrichment was statistically different between the 3 groups (P=0.025). H4Ac enrichment was similar in the young (3AL) and old (30AL) groups but was enriched 3.5 fold in DR fed animals compared with age matched AL fed animals (P=0.021. Figure 6.9A). In female mice, H4Ac was also significantly different between the 3 groups (P=0.021). However, H4Ac was enriched 5.2 fold in older (30AL) compared with younger (3AL) animals (P=0.021) and H4Ac was 4 fold lower in DR fed animals compared with age matched AL fed animals, although this difference was not statistically significant (Figure 6.9B).

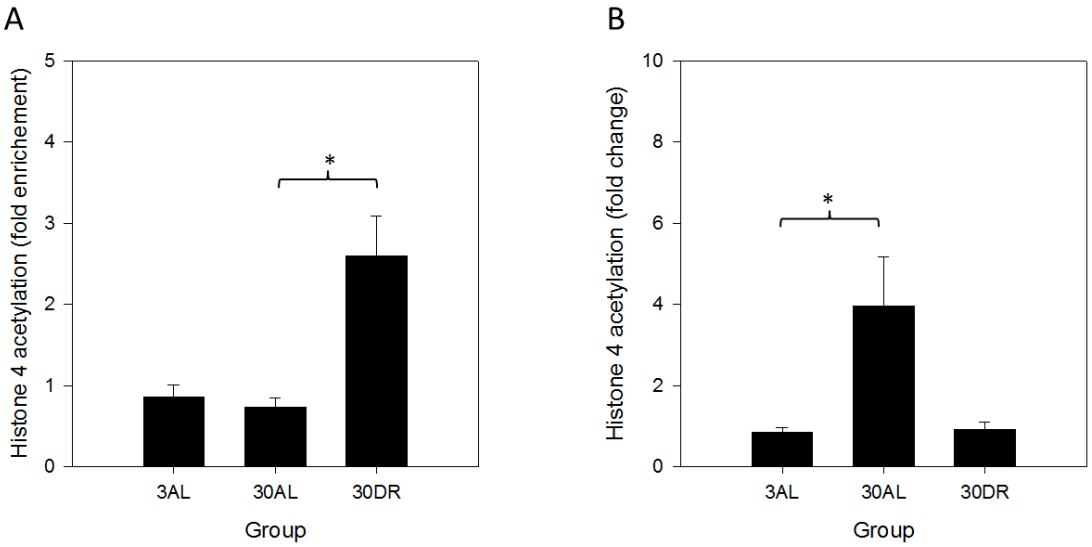


Figure 6.9 Effects of age and of DR on histone 4 acetylation at the *Ogg1* promoter in liver of male (A) and female (B) mice. Data represent mean enrichment ± SEM. ChIP-qPCR was performed in duplicate per animal, n=5 per age group. H4Ac was normalised to IgG. Kruskal-Wallis analysis with post hoc Mann-Whitney U comparison: in male mice 30AL vs 30DR P=0.021 and in female mice 3AL vs 30AL P=0.021. Primer design by Dr Jelena Mann, data collection Joanna Górniak.

6.3.2.4 *Histone acetylation at the Apex promoter*

No effect of sex was observed on acetylation of histone 4 at the *Apex* promoter. However both diet and age were shown to interact with sex suggesting that males and females were differentially affected by these factors; therefore, analysis was performed separately for each sex.

In male mice, neither age nor diet affected H4Ac (Figure 6.10A). Lowest H4Ac levels were observed in the 30DR group, there was a ~2.5 fold H4Ac enrichment in both AL groups compared with 30DR. In female mice, a difference between the 3 groups was observed (P=0.021). H4Ac was 7.9 fold higher older animals (P=0.021) and 5.8 fold higher in the 30AL group compared with the 30DR group (P=0.022, Figure 6.10B).

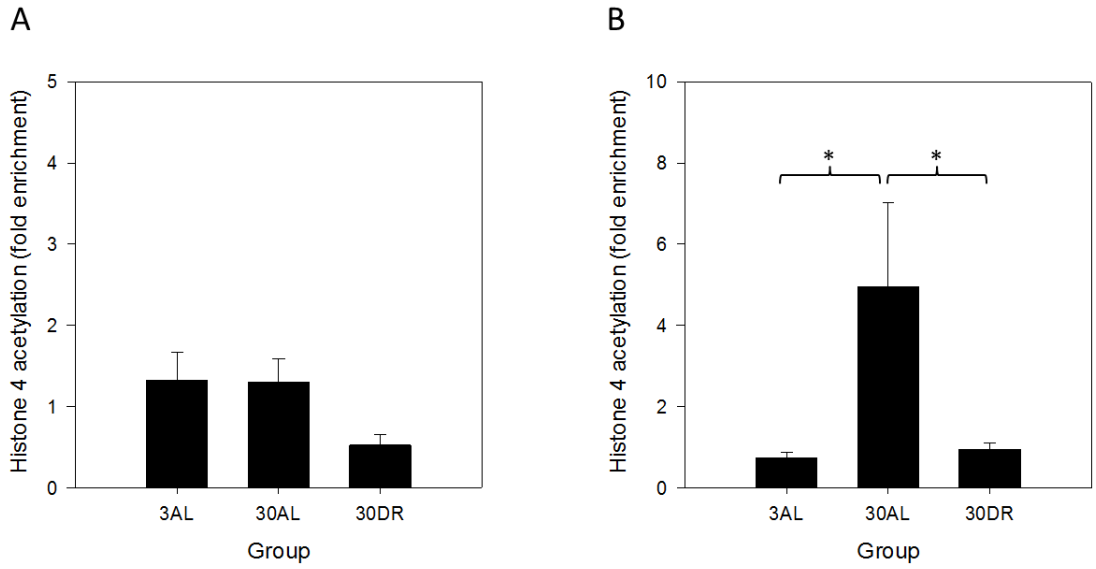


Figure 6.10 Effects of age and of DR on histone 4 acetylation at the *Apex* promoter in brain of male (A) and female (B) mice. Data represent mean enrichment ± SEM. ChIP-qPCR was performed in duplicate per animal, n=5 per age group. H4Ac was normalised to IgG. Kruskal-Wallis analysis between groups (P=0.021) with Mann-Whintey U comparison for 3AL vs 30AL P=0.021 and 30AL vs 30DR P=0.022. Primer design by Dr Jelena Mann, data collection Joanna Górniak.

6.3.2.5 *Histone methylation at the Ogg1 promoter*

No effect of sex was observed on H3K27Me3 at the *Ogg1* promoter in the liver. However, there were interactions of sex with age and sex with diet therefore data were analysed separately. In male mice, H3K27Me3 was 25% lower in older mice compared with young (P=0.037, Figure 6.11A) but no difference was observed with diet. In female mice, age did not have an effect on H3K27Me3

however, histone methylation was 20% lower in DR fed animals compared with age matched AL fed animals (P=0.037 Figure 6.11B).

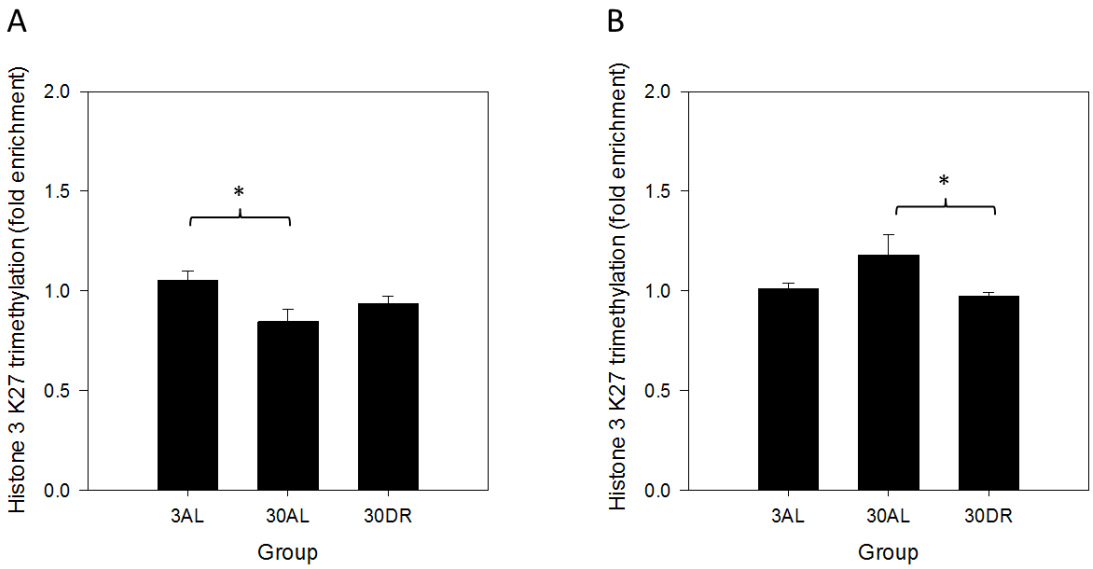


Figure 6.11 Effects of age and of DR on histone 3 lysine 27 tri-methylation at the *Ogg1* promoter in liver of male (A) and female (B) mice. Data represent mean enrichment ± SEM. ChIP-qPCR was performed in duplicate per animal, n=5 per age group. H3K27Me3 was normalised to IgG. Univariate ANOVA-GLM analysis with Bonferroni post hoc comparison: 3AL vs 30AL P=0.037 in male mice. Kruskal-Wallis analysis with Mann-Whitney U test comparison: 30AL vs 30DR P=0.037 in female mice. Primer design by Dr Jelena Mann, data collection Joanna Górniak.

6.3.2.6 *Histone methylation at the Apex promoter*

At the *Apex* promoter in the liver no difference in H3K27Me3 was observed between the sexes but, sex interacted with age; therefore, the analyses were run separately for males and females. In male mice H3K27Me3 decreased with age, with 3 month old animals showing 10.7 fold higher levels compared with 30 month old AL fed animals although this difference was not statistically significant. Diet did not have an effect on H3K27Me3 levels (Figure 6.12A). In female mice, no effect of age or diet was observed. H3K27Me3 was 13% higher in 3AL compared to 30DR group and 30AL was 2.3 fold higher than 30DR group (Figure 6.12B).

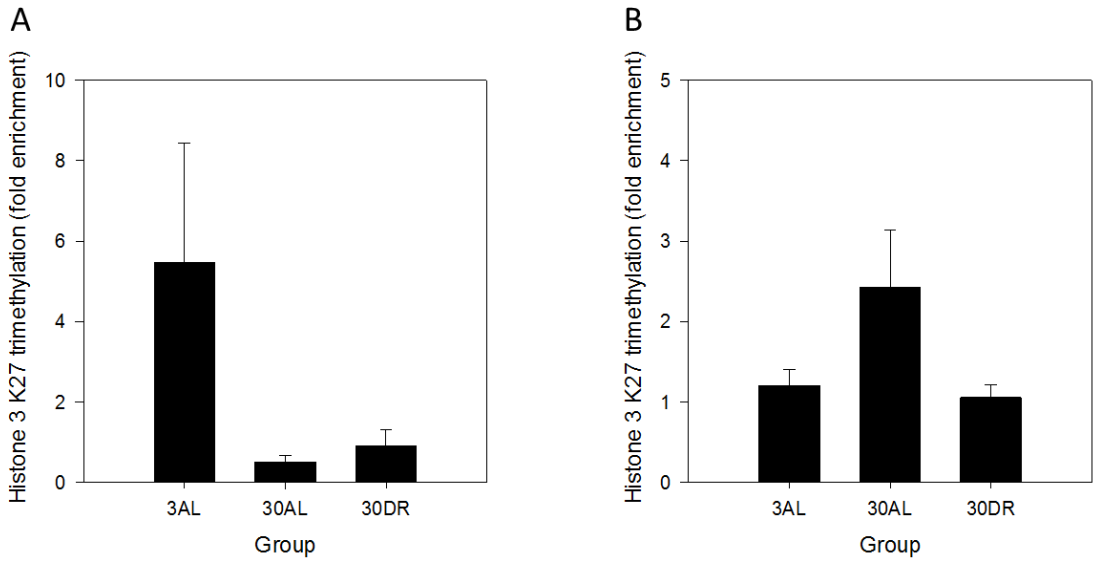


Figure 6.12 Effects of age and of DR on histone 3 lysine 27 tri-methylation at the *Apex* promoter in brain of male (A) and female (B) mice. Data represent mean enrichment ± SEM. ChIP-qPCR was performed in duplicate per animal, n=5 per age group. H3K27Me3 was normalised to IgG. Primer design by Dr Jelena Mann, data collection Joanna Górniak.

6.3.2.7 *HAT and HDAC activity in the liver*

To assess the effect of age and of DR on HAT and HDAC, enzyme activity was measured in 3 groups; 3AL, 30AL and 30DR. No difference in HAT or HDAC activity was observed between males and females; therefore, data were pooled for further analysis. No difference was observed in HAT activity between the 3 different groups; activity was 6.46AU in 3AL, 6.07AU in 30AL and 5.29AU in 30DR group (Figure 6.13A). A significant difference in HDAC activity between the 3 groups was observed (P=0.034). No change in HDAC activity was observed with age; however, 30 month old DR fed mice had a 60% higher activity compared with 30 month old AL fed mice (P=0.017, Figure 6.13B).

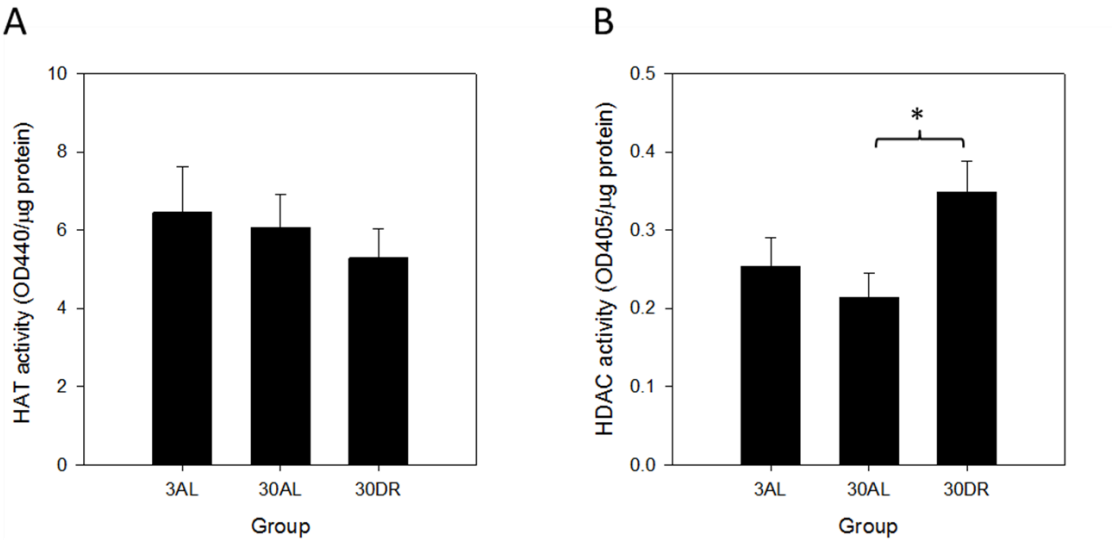


Figure 6.13 Effect of age and DR on HAT (A) and HDAC (B) activity in the liver. Data represent mean ± SEM absorbance from a single assay, n=10 per group (pooled for male and female mice). Kruskal-Wallis analysis between the groups (P=0.034) with Mann-Whitney post hoc comparison between 30AL and 30DR: P=0.017.

6.3.2.8 *Expression of transcription factors in the liver*

The next step was to determine the effect of age and DR on the expression of the redox-sensitive transcription factors *Hif1α* and *Nfya* in the liver. Expression was measured at the mRNA level in 3AL, 30AL and 30DR groups. Expression of both TFs was affected by sex, with female mice having on average higher expression of *Hif1α* and *Nfya* compared with males. In male mice, expression of *Hif1α* did not change with age; however, 30DR animals had 1.75 fold higher expression compared with 30AL animals although this was not significant (Figure 6.14A). In female mice, there was a difference between the 3 groups tested. *Hif1α* expression was 2.4 fold higher in the 30AL compared to 3AL group and 1.6 fold higher in the 30DR compared to 30AL group; however, neither of these were statistically significant when post hoc comparisons were made (Figure 6.14B). In male mice neither age nor DR had an effect on *Nfya* expression (Figure 6.15A). In female mice, expression in 30 month old AL fed mice was 2.8 fold higher compared with 3 month old mice (P=0.011) and 1.6 fold higher compared with 30 month old DR fed mice (n.s., Figure 6.15B).

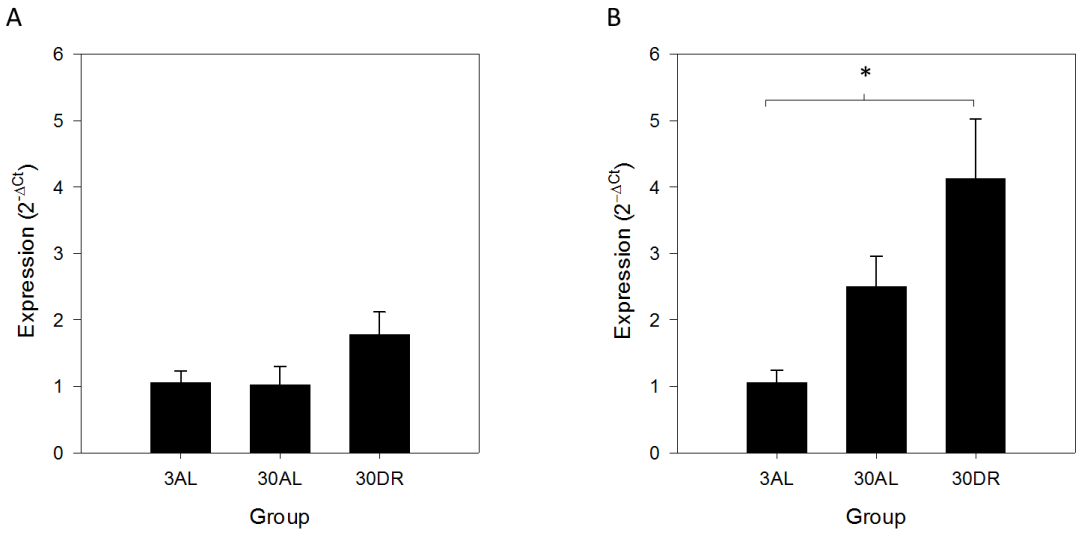


Figure 6.14 Effect of age and DR on expression of *Hif1α* in male (A) and female (B) mice in in the liver. Data represent mean ± SEM, n=5 per group. Univariate ANOVA-GLM analysis between groups: P=0.011.

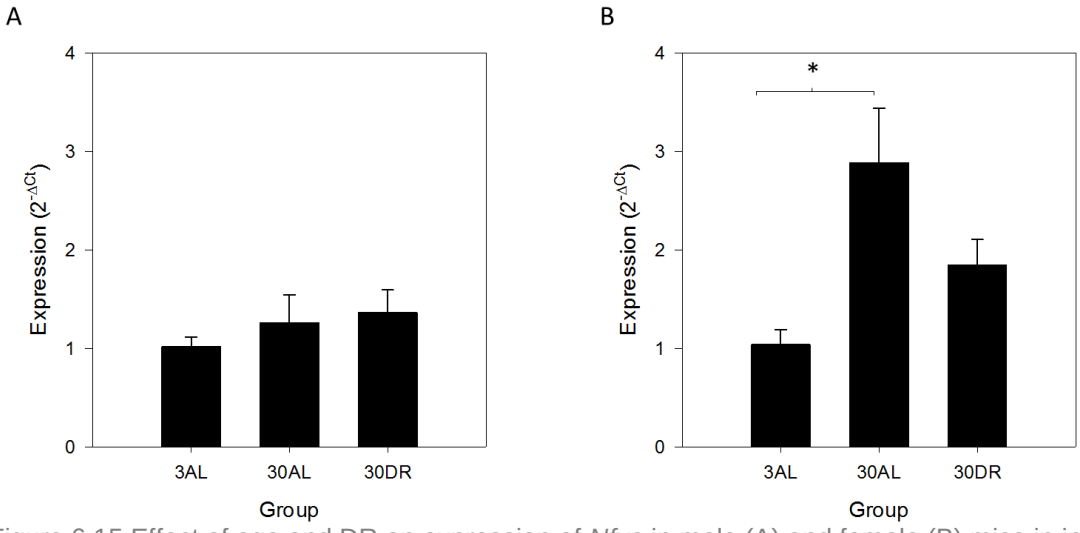


Figure 6.15 Effect of age and DR on expression of *Nfya* in male (A) and female (B) mice in in the liver. Data represent mean ± SEM, n=5 per group. Univariate ANOVA-GLM analysis with Bonferroni comparison: 3AL vs 30AL P=0.011.

6.3.3 *Ogg1 and Apex gene expression*

Expression of the 2 BER-related genes *Ogg1* and *Apex* was measured in mRNA extracted from liver in all 7 groups. In all cases, assays were conducted on mRNA from individual animals and data are presented as $2^{-\Delta Ct}$.

6.3.3.1 *Ogg1 expression in the liver*

Ogg1 was differently expressed between the sexes with expression in the female being higher compared with males (P<0.001). No effect of age was observed but DR animals showed lower expression compared with AL animals (P=0.001). In addition, there was an interaction between age and diet, which

may have appeared because in the oldest mice there was a divergent effect between the different diets. 30AL animals showed an increase in expression while 30DR animals showed a decrease in expression of *Ogg1* (Figure 6.16A). No differences between the different age and diet groups were found in female mice, although DR mice appeared to have lower *Ogg1* expression compared with AL mice especially at older age (Figure 6.16B).

6.3.3.2 *Apex expression in the liver*

Expression of *Apex* in the liver was significantly different between the sexes with female mice showing higher expression levels (P=0.02). In male mice, there was no effect of age on *Apex* expression but DR animals had on average 22% lower levels of *Apex* mRNA compared to AL animals although this was not statistically significant (Figure 6.17A). In female mice, a difference between the seven groups was observed (P=0.019, Figure 6.17B). Post hoc Mann-Whitney U comparisons with adjusted α<0.024 did not reveal a significant difference. It is likely that this change arose because expression of *Apex* in the DR animals appears to be lower (especially at the oldest age group) compared to AL fed animals.

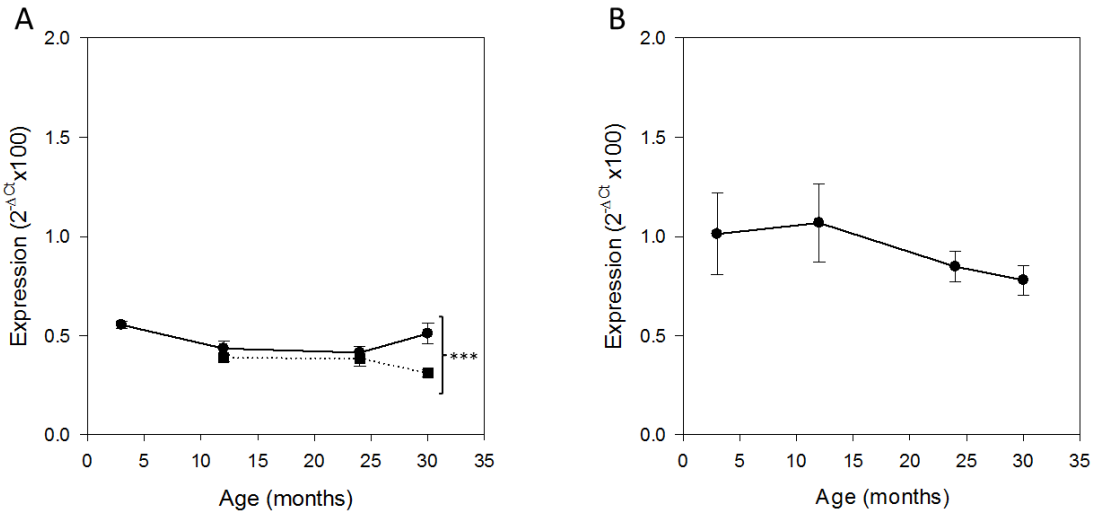


Figure 6.16 Effect of age and of DR on expression of *Ogg1* at the mRNA level in the liver of male mice (A). Solid line with round symbols represents AL fed animals dotted line with square symbols represents DR fed animals. Data represent mean ± SEM, n=5-7 per group. Univariate ANOVA-GLM analysis for diet P=0.001. Effect of age and of DR on expression of *Ogg1* at the mRNA level in the liver of female mice (B). Solid line with round symbols represents data pooled for AL and DR fed animals. Data represent mean ± SEM, n=5-14 per group. Primer design Dr Sabine Langie, data collection Joanna Górniak.

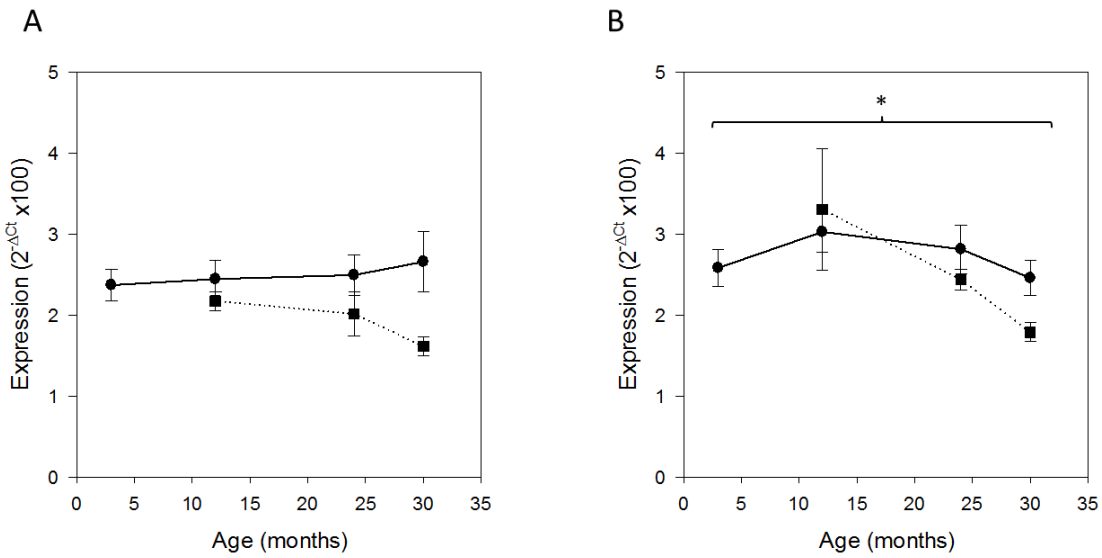


Figure 6.17 Effect of age and of DR on expression of *Apex* at the mRNA level in the liver of male (A) and female (B) mice. Solid line with round symbols represents AL fed animals; dotted line with square symbols represents DR fed animals. Data represent mean ± SEM, n=5-7 per group. Kruskal-Wallis analysis between the groups P=0.019.

6.3.4 *Base excision repair (BER)-related DNA incision activity in the liver*

BER-related DNA repair activity was quantified as the DNA incision of damage; induced by photosensitiser Ro 19-8022, using the comet-based assay which measures the first step (incision of the strand) of the repair process. DNA incision was measured in protein extracts from liver from individual animals in all 7 groups. There was a statistically significant difference between male and female mice, with male mice showing 2-fold higher activity compared with female mice (P<0.001). No effect of age or diet was observed in the males (Figure 6.18A) or females (Figure 6.18B). In males the mean incision activity was 14.2AU (range 4.7-24.5AU). Average incision activity of ~15AU was similar between 3 months, 24 months and 30 months old animals but was slightly lower in 12 months old animals (~12AU). In females mean incision activity was 7.1AU (range 0.4-13.8AU) where incision was slightly higher in 12 months old group (7.8AU) compared to the other ages (6.6-7.0AU).

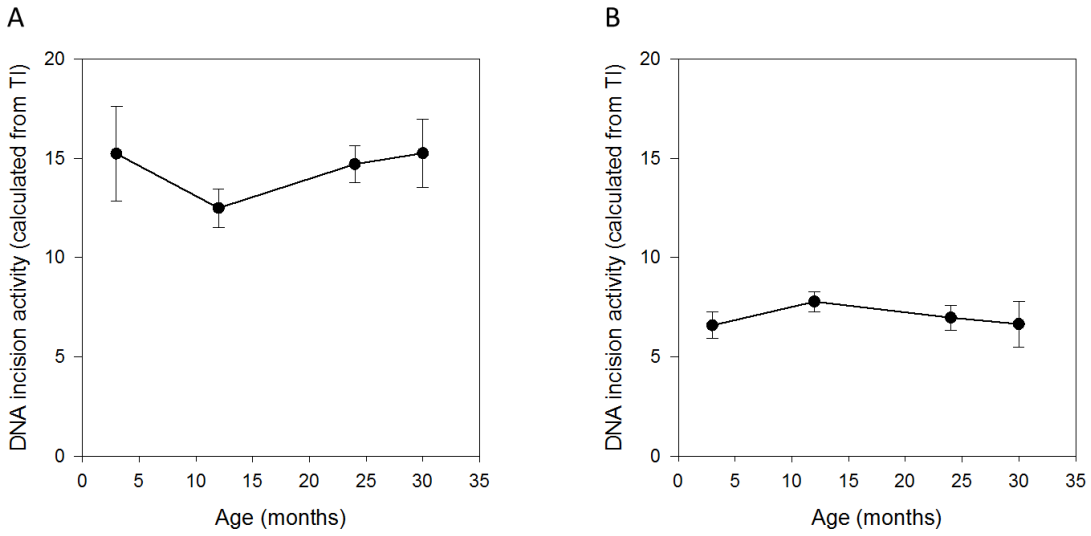


Figure 6.18 Effect of age and DR on DNA incision activity in the liver of male (A) and female (B) mice. Data represents mean ± SEM, n=5-14 per group (pooled from AL and DR animals at each age group). Assay design Dr Sabine Langie, data collected by Kirstin Halley-Hogg (male liver) and Joanna Górniak (female liver).

6.3.5 *Expression of OGG1 and APEX at the protein level in the liver*

Protein concentration of OGG1 and APEX were measured in extracts of liver from 3AL, 30AL and 30DR groups. Differences between groups were determined using ANOVA GLM and Bonferroni post hoc test for the predefined comparisons of 3 AL *v.* 30 AL and 30 AL *v.* 30 DR groups. The most dramatic finding was that protein concentrations of OGG1 and APEX were ~20 fold higher in the liver compared with brain tissue ($P<0.001$ for both enzymes), data for brain are shown in Chapter 5 in Table 5.2 and 5.3. In the liver there was significantly higher concentration of OGG1 ($P<0.001$) and APEX ($P<0.001$) in males compared with females. No effect of age or DR on OGG1 or APEX protein concentration was observed between the three different groups in males or females (Table 6.2 and 6.3 respectively). Average OGG1 concentration in males was 16.4ng/ml (range 15.0-18.9ng/ml) while in females average concentration was 10.2ng/ml (range 5.5-13.7ng/ml). Average APEX concentration in males was 10.6ng/ml (range 7.5-13.4ng/ml) while in females average concentration was 5.5ng/ml (range 2.0-9.2ng/ml).

OGG1 (ng/ml)	Male	Female	All mice
3AL	16.75±0.61	8.65±0.13	12.70±0.48
30AL	16.59±0.41	9.96±0.93	13.28±0.38
30DR	15.90±0.38	12.03±0.61	13.96±0.23

Table 6.2 Effect of age and DR on OGG1 protein concentrations in the liver. Data represent mean ± SEM from a single assay, n=5 per group.

APEX (ng/ml)	Male	Female	All mice
3AL	10.60±0.85	6.915±1.00	8.759±2.76
30AL	10.54±0.75	4.116±0.73	7.326±3.73
30DR	10.56±1.18	5.479±0.16	8.022±3.22

Table 6.3 Effect of age and DR on APEX protein concentrations in the liver. Data represent mean ± SEM from a single assay, n=5 per group.

Similarly to the brain, in the liver protein concentration but not mRNA levels of *Ogg1* and *Apex* were correlated with repair. Figure 6.19 shows correlation plots for protein vs repair and mRNA vs repair. Positive correlation was observed for both OGG1 protein and repair (R^2=0.370, P<0.001) and APEX protein and repair (R^2=0.440, P<0.001) and negative correlation was observed for *Ogg1* mRNA expression and repair (R^2=0.0145, P=0.041).

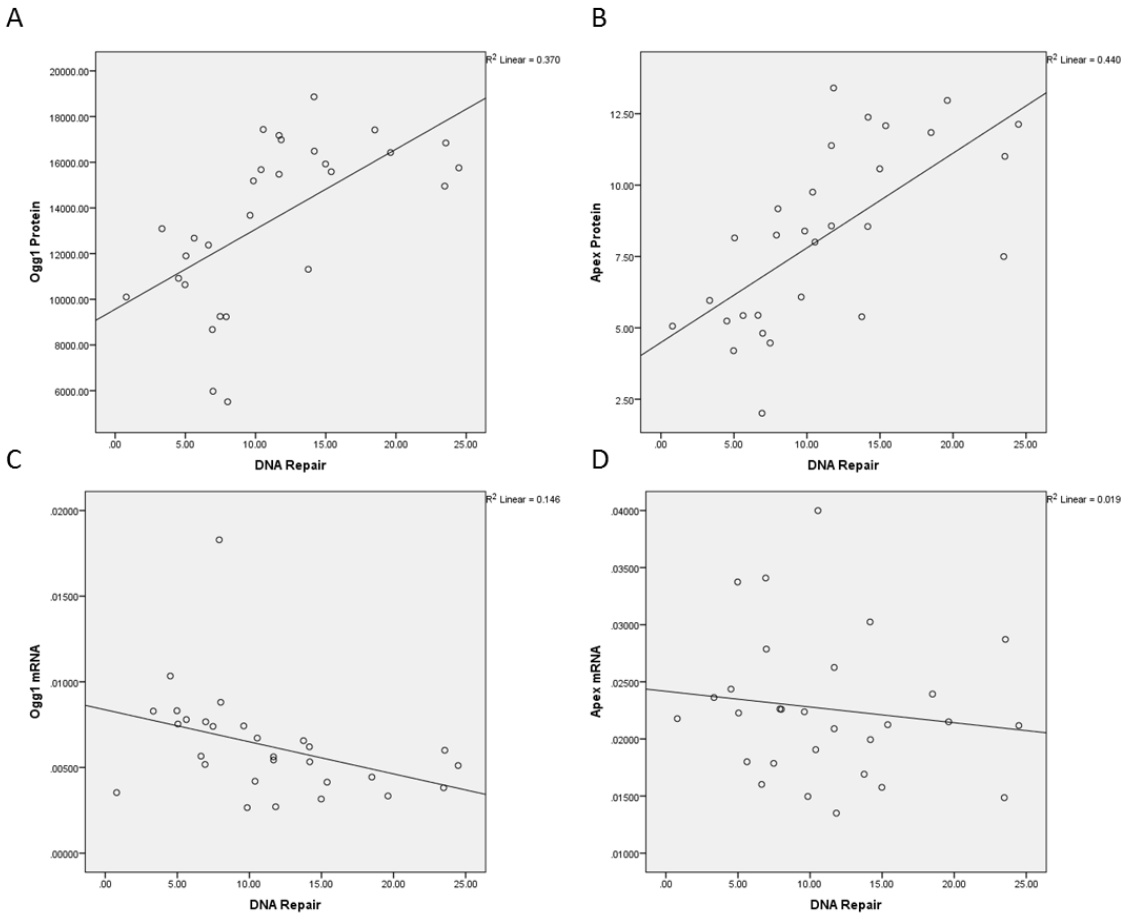


Figure 6.19 Correlation between DNA repair and OGG1 protein (A) APEX protein (B) *Ogg1* mRNA (C) and *Apex* mRNA (D) in the liver. Data represents mean ± SEM, pooled for male and female, data from 3AL, 30AL and 30DR groups. Correlation $P_{OGG1\ protein*DNA\ repair} < 0.001$, $P_{APEX\ protein*DNA\ repair} < 0.001$ and $P_{Ogg1\ mRNA*DNA\ repair} = 0.041$.

6.3.6 *Effects of age and DR on oxidative damage to DNA in the liver*

Oxidative damage in the brain was measured in the form of 8-oxoguanine using HPLC-ECD in all 7 groups. A statistically significant difference between the sexes was observed with females showing higher 8-oxoguanine levels (P<0.001). In male mice, there was a statistically significant effect of age (P=0.007) and DR (P=0.002) on 8-oxoguanine (Figure 6.20A); furthermore, there was an interaction between age and diet (P<0.001). A Bonferroni post hoc analysis between groups showed significant differences between 3AL and 12AL (P=0.005), 3AL vs 30AL (P=0.035), 12AL vs 12DR (P<0.001), 12AL vs 24AL (P<0.001), 12AL vs 30DR (P=0.008), 12DR vs 30AL (P=0.001) and 24AL vs 30AL (P<0.001, P=0.049). In female mice, there was a significant decrease in 8-oxoguanine concentration with age (P<0.001) but no effect of diet (Figure 6.20B). 30 month old animals showed a 38% decrease compared with 3 month old and 40% decrease compared with 12 month old animals (P<0.001). There

was a significant linear reduction in 8-oxoguanine concentration with age (R^2=0.375, P<0.001).

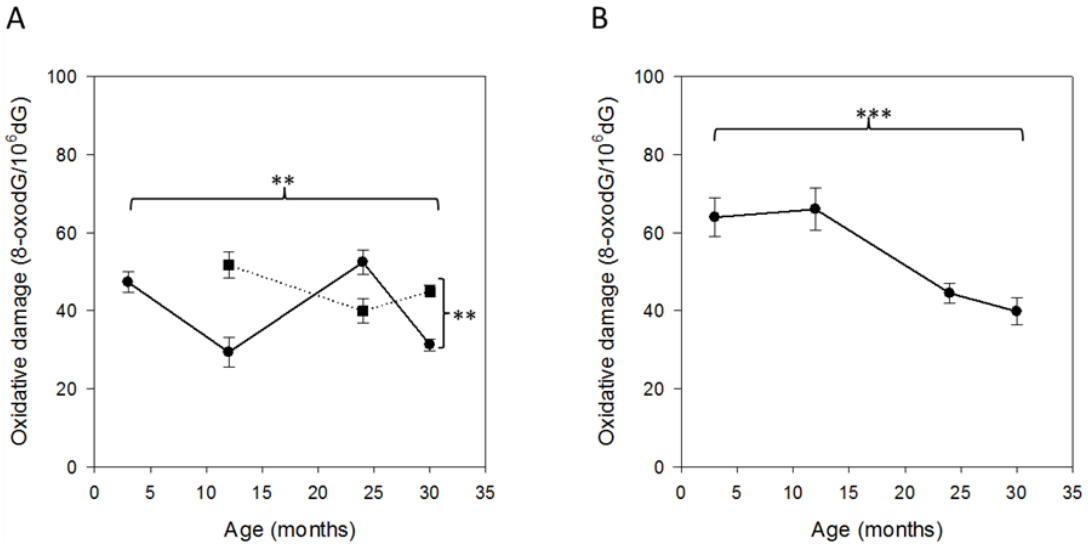


Figure 6.20 Effect of age and of DR on 8-oxoguanine concentration in the liver of male mice, (A). Solid line with round symbols represents AL fed animals dotted line with square symbols represents DR fed animals. Data represent mean ± SEM, n=5-7 per group. Univariate ANOVA-GLM analysis for age P=0.007 and diet P=0.002. Effect of age and of DR on 8-oxoguanine concentration in the liver of female mice (B). Solid line with round symbols represents data pooled for AL and DR fed animals. Data represent mean ± SEM, n=5-14 per group. Univariate ANOVA-GLM analysis for age P<0.001. Data collected by Maastricht University, in collaboration with Dr Roger Godschalk.

6.4 Discussion

In this Chapter I have investigated age and DR-related epigenetic changes (DNA methylation and histone marks) at promoter regions of the BER-related proteins: oxoguanine DNA glycosylase (OGG1) and Apurinic/apyrimidinic endonuclease (APEX) in the liver. In addition, I quantified the associated changes in *Ogg1* and *Apex* transcript levels (mRNA abundance) and the DNA repair capability (assessed as BER-related incision activity). A conceptual model for epigenetic regulation of the BER-related genes investigated in this chapter has been presented in Chapter 5.4, Figure 5.23. I will discuss: a) the observed epigenetic changes with reference to gene expression, b) the epigenetic mechanisms that may be involved in modulation of transcription i.e. epigenetic enzyme activity and TF expression c) the phenotypic changes observed in repair activity and oxidative damage and finally d) compare results from brain and liver tissue.

6.4.1 *Epigenetics and BER-related gene expression*

6.4.1.1 *Effect of age and DR on epigenetic regulation of transcription*

Tissue specific changes in DNA methylation are observed during ageing (Thompson *et al.*, 2010). These, or other, alterations of epigenetic marks at gene promoter regions may be responsible for the transcription changes occurring with age, in particular the age-related down-regulation of cell cycle and maintenance genes (Lee *et al.*, 2000).

I hypothesised that there would be an increase in cytosine methylation (at BER-related gene promoters) with age, which would cause silencing of BER-related genes. I found that similarly to the brain, in the liver DNA methylation within the CpG sites measured was low at both *Ogg1* and *Apex* promoter regions suggesting that these genes are constitutively expressed in both tissues. In fact the qPCR data showed a consistent presence of *Ogg1* and *Apex* mRNA in both brain and liver. Furthermore, the lowly methylated promoter regions imply that DNA methylation is involved in maintenance of transcription rather than in switching these genes on (Feng *et al.*, 2006). Different methylation of specific CpG sites was observed with age and DR in both genes but the expression of the corresponding genes remained constant throughout lifespan. Application of the demethylating agents 5-aza-2-deoxycitidine and zebularine to cancer cell lines increases *Ogg1* and *Apex* expression respectively, confirming that DNA methylation plays a part in regulation of transcript expression of DNA repair genes (Peng *et al.*, 2006; Singh *et al.*, 2012).

It is likely that not all CpG sites within the promoter region are important in epigenetic regulation of gene transcription. Of more interest with respect to promoter methylation are those CpG sites which are found within transcription factor consensus binding sequences. Based on the information from the bioinformatics tool Genomatix, at the *Ogg1* promoter only CpG 16, which was affected by both age and DR, occurs in the binding sequence of a transcription factor; neuron-restrictive silencer factor (NRSF). On the other hand in the *Apex* promoter, multiple transcription factors bind at sequences containing differentially methylated CpG sites. CpG site 10, which was affected by both age and DR, is within the binding sequence for activator protein 2 (AP2F), insulinoma associated factors (INSM) and cAMP-responsive element binding protein (CREB) transcription factors. Furthermore, the 6 CpG sites differentially

methylated with age only, lay within binding sequence for 9 different transcription factors. Figure 6.21 shows the transcription factors that bind to sequences containing differentially methylated regions in *Ogg1* and *Apex* promoters in brain and liver tissue. Furthermore, the Venn diagram highlights the TFs that are shared between the tissues for each of the target genes. Unsurprisingly, many of the TFs found to bind BER gene promoters are redox-responsive (Lavrovsky *et al.*, 2000; Haddad, 2002). Furthermore some have been independently shown to regulate BER-genes e.g. NFYA transcription factor belongs to the CAAT binding factors and upregulates *Ogg1* in lymphocytes and kidney cells. (Habib *et al.*, 2010; von der Lippen *et al.*, 2015). Thus the low levels of promoter methylation observed in all animals in the present study may act to maintain expression of the corresponding DNA repair genes by permitting binding of these specific transcription factors.

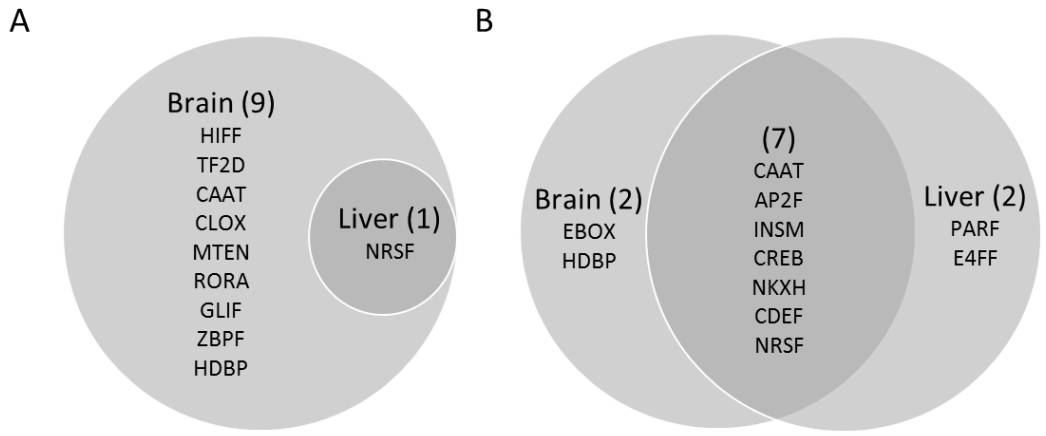


Figure 6.21 Venn diagram showing transcription factors binding at differentially methylated regions in the *Ogg1* (A) and *Apex* (B) promoters in brain and liver tissue. AP2F, activator protein 2; CAAT, CCAAT binding factors; CDEF, cell cycle regulators; CLOX, Cut-like homeobox and CLOX homology factors; CREB, cAMP-responsive element binding proteins; E4FF, ubiquitous GLI Kureppel like zinc finger; EBOX, E-box binding factors; GLIF, GLI zinc finger family; HDBP, Huntington's disease gene regulatory region binding protein; HIFF, hypoxia inducible factor; INSM, insulinoma associated factors; MTEN, core promoter motif ten elements; NKXH, NKX homeodomain factors; NRSF, neuron-restrictive silencer factor; PARF, PAR/bZIP family; RORA, v-ERV and RAR-related orphan receptor alpha; TF2D, general transcription factor II D; ZBDF, zinc binding protein factors.

In addition to cytosine methylation, I also investigated H4Ac and H3K27Me3 as a mechanism for regulation of transcription of the DNA repair genes. These two histone marks are associated with transcription activation and repression respectively (Akhtar and Becker, 2000; Asp *et al.*, 2011). In male mice

H3K27Me3 was lower in older animals at both promoters suggesting that activation of transcription during ageing occurs via demethylation of H3 lysine 27. In *C. elegans* H3K27Me3 declines with age and knockdown of UTX, a histone demethylase, increased lifespan in this worm (Maures *et al.*, 2011). Thus factors such as DR, which affect age-related methylation of promoters and hence expression of repair genes, may modulate the ageing process. Furthermore in female mice DR was able to reduce H3K27Me3. The effect of DR on histone methylation is not as well explored as that of histone acetylation, although it is possible that histone demethylases are targets for one of the downstream enzymes of DR. This is unlikely to be SIRT1 as it has been found to be readily associated with increase in chromatin repressive marks (Vaquero and Reinberg, 2009). Interestingly the DR-related histone demethylation was seen only in females suggesting that female hormones may be at play. In fact, oestrogen is able to reduce levels of H3K27Me3 via the PI3/AKT pathway (Bredfeldt *et al.*, 2010); thus, perhaps during ageing in conditions of dietary restriction oestrogen is also able to exert this role. Not surprisingly, DR fed animals showed lower histone acetylation levels for both genes. This is consistent with the role of Sirtuin family of HDACs which have been demonstrated as one of the modes of action of dietary restriction (Lin *et al.*, 2000; Cohen *et al.*, 2004; Kenyon, 2010). Deacetylation could explain the lower levels of repair gene expression in DR animals, although this was not always significant. In contrast, acetylation of the *Ogg1* promoter was enhanced in male DR fed mice, but this was not accompanied by the expected higher levels of *Ogg1* mRNA. Despite the observed changes in histone marks with age, expression of *Ogg1* and *Apex* in the liver did not change with age suggesting that other (epigenetic) regulators may counter-balance these changes in histone marks. This observation, also made in the brain, highlights the importance of studying a larger epigenetic landscape as epigenetic marks may work together to create an open/closed chromatin state (Vaquero and Reinberg, 2009). In addition, expression of *Ogg1* and *Apex* was lower in DR fed animals which, is opposite to results observed in the brain (Chapter 5) emphasising the importance of considering tissue-specific changes occurring with age and DR. It is not surprising that tissue specific changes have been found at the epigenetic level. Much work has already shown differential patterns of expression and DNA repair in different tissues (Karahalil *et al.*, 2002; Intano *et al.*, 2003).

Overall it appears that in the liver expression of BER-related genes remains constant throughout age and that this may occur due to the maintenance of an open chromatin structure induced by a consortium of epigenetic enzymes and other binding proteins which together maintain the euchromatin state.

6.4.1.2 *Effect of age and DR on epigenetic enzymes*

Only one previous study was found to assess HAT activity in ageing mouse tissues. The study reported an age-related decline in p300/CBP HAT activity in the liver, muscle and testes and no effect of age on p300/CBP HAT activity in kidney, heart, brain, lung and spleen. Furthermore when measuring overall HAT activity no difference was observed (Li *et al.*, 2002). In support of this, Chapters 5 and 6 demonstrate no age or DR related changes in HAT activity. Although the role of HATs in ageing is not as clearly understood as that of HDACs, it is not redundant as RNAi inhibition of *cpb-1* in *C. elegans* accelerates ageing and attenuates lifespan enhancing effects of DR (Zhang *et al.*, 2009).

A decline in HDAC expression has been reported in adult stem cells; however, here I show no effect of age on HDAC activity in mouse brain or liver. On the other hand, I have shown that DR enhanced HDAC activity in both tissues. This is not surprising since Sirtuins (NAD dependant histone deacetylases) have been previously linked to DR (Lin *et al.*, 2000). Furthermore, the increase in HDAC activity in DR animals corresponds with the observed decline in histone 4 acetylation. More difficult to interpret are the age-related changes observed in acetylation as neither HAT or HDAC activity was altered with age. It is possible that changes in specific HAT and HDAC enzyme families could have occurred, but were not detected here since the overall activity was measured. Such a phenomenon was observed by Li *et al.* (Li *et al.*, 2002).

These results provide a partial view of the function of enzymes responsible for epigenetic marks and focus only on the aspect of histone acetylation. Further work could explore the enzymes involved in methylation of DNA and associated histones including: DNMT, TET, histone metyltransferases and demethylases. Such a study of multiple epigenetic enzymes could be particularly valuable because epigenetic enzymes work together to give rise to the epigenetic code (Ikegami *et al.*, 1978; Kondo, 2009). Moreover, the above results suggest that specific enzyme activity rather than overall activity should be studied as the latter is too general.

6.4.1.3 *Effect of age and DR on TF expression*

I detected changes in the expression of redox-sensitive transcription factors, which are involved in activation of BER-related gene expression. Whilst neither age nor diet had an effect on *Hif1α* or *Nfya* expression in male mice, in female mice both transcription factors were up-regulated with age. No effect of age or DR on *Hif1α* in male rats has also been previously reported. Despite the lack of effect on mRNA transcripts lower HIF1α activity in DR compared with AL animals at all ages has been reported (Kang *et al.*, 2005). This observation suggests that with some TFs, DR enhances activity of this TF rather than causing up-regulation of expression. Redox-sensitive transcription factors e.g. FOXO (Greer and Brunet, 2008) and *Hic-5* (Lee *et al.*, 1999; Weindruch *et al.*, 2001) are up-regulated by ageing. I also observed *Hif1α* and *Nfya* up-regulation with age in female mice (Harman, 1956). However, more surprisingly I did not observe changes in DR animals. DR reduces age-related oxidative stress (Sohal and Weindruch, 1996) possibly via the activation of redox-sensitive transcription factors such as nuclear factor-kB (NFkB) and hypoxia inducible factor 1 (HIF-1)(Kim *et al.*, 2002).

The data presented here show that, at least in female mice, expression of redox-sensitive TFs is up-regulated with age. In future it would be helpful to study both expression and function of TFs in response to ageing and DR since there may be changes in activity in the absence of change in transcription.

6.4.2 *Effects of age and of DR on BER-related DNA incision activity*

As expected from the lack of changes in *Ogg1* and *Apex* expression, no changes in incision activity nor OGG1 and APEX protein levels were observed with age or DR. Other repair proteins such as DNA ligase I and II, XRCC1, β-Polymerase and REF-1 also do not alter in the ageing liver (Intano *et al.*, 2003). Interestingly the liver has much higher repair protein concentration than the brain and this is in line with liver also having a higher incision rate compared with the brain. Furthermore, activity of repair enzymes was also high in testis and kidney and low in muscle and heart (Karahalil *et al.*, 2002). On a cellular level repair activity was higher in proliferative cells compared with non-proliferating cells (Akbari *et al.*, 2009). In addition, in the brain OGG1 and APEX proteins seem to accumulate with age where as in the liver this does not occur. In fact, reports show greater protein degradation in the liver in comparison with

the brain (Dasuri *et al.*, 2009) and it has been argued that the proliferative nature of the tissue is responsible for this protective mechanism (Stroikin *et al.*, 2005). Although it is suggested that DR enhances genomic stability by increasing repair activity I did not observe such effects here. Previous reports show higher incision in the liver of DR fed animals (Cabelof *et al.*, 2003), but not always at a statistically significant level (Stuart *et al.*, 2004). Furthermore, the results presented show that DR does not have an effect on mRNA expression of BER genes. This is a tissue-specific effect as in the brain DR up-regulated expression of *Ogg1* and *Apex* mRNA (Chapter 5). Nevertheless, in this animal model DNA incision activity in the ageing liver is at least as good as in young animals and DR does not provide any further benefit.

Therefore the liver maintains its DNA incision activity and is able to recycle the repair proteins unlike the brain which without any proliferative activity, accumulates repair proteins. The brain may maintain vital oxidative damage removal via other repair pathways such as NER.

6.4.3 *Effect of age and DR on oxidative damage*

The free radical theory of ageing is supported by many studies reporting age-related increase in oxidative damage and by, numerous studies showing that DR attenuates this increase (Hamilton *et al.*, 2001). However, the accumulation of DNA oxidative damage with age has not always been reported (Chung *et al.*, 1992; Hirano *et al.*, 1996; Anson *et al.*, 1999) leading to controversy about the role of oxidative damage during ageing. A summary of studies reporting 8-oxoguanine concentration is shown in Table 6.9. By and large, these studies show increases in oxidative damage in the brain but not always in the liver. It can be speculated that this tissue difference is a result of the proliferative nature of the liver, where during cell division additional detection and removal of oxidative damage in DNA may occur.

I have shown that the concentration of 8-oxoguanine is higher in the liver tissue than the brain and this has also been reported by others (Schmerold and Niedermüller, 2001). Other damaging lesions such as abasic sites were also more abundant in the liver compared with the brain (Atamna *et al.*, 2000). This may be counter intuitive as it is often suggested that the brain is an organ with high oxygen consumption and little proliferative activity. Furthermore, higher damage accumulation is observed in the brain with age compared with other

tissues such as liver, kidney, spleen, lung and small intestine (Hirano *et al.*, 1996). However, it should be noted that accumulation of damage depends on: nature of damaging agent, metabolic rate, presence of oxygen, efficiency of antioxidant systems, efficiency of DNA repair and rate of cell proliferation (Fraga *et al.*, 1990). Studies of the antioxidant defence system of tissue found that superoxide dismutase activity was higher in the liver compared with the brain (Sohal *et al.*, 1990a). The high SOD activity together with efficient incision activity can in part explain the age-related decrease in oxidative lesions in the liver of female mice observed in this study. In male mice an ambiguous pattern in oxidative damage was observed; it could be speculated that the 24 month old mice experienced oxidant insult that resulted in higher 8-oxoguanine lesions. Furthermore DR did not have a protective effect in either male or female mouse livers. This is the first study to report a decline in 8-oxoguanine during ageing. This observation may highlight that this strain of mice is able to resist oxidative damage. However, somatic DNA mutations accumulate in the ageing liver (Dolle *et al.*, 1997) thus suggesting that the liver may be prone to damaging lesions other than 8-oxoguanine.

Author	Age	Species and Sex	Methods	Age Effect	DR
Gorniak *et al.* (Thesis)	3, 12, 24 and 30 months	Male and female C57Bl6 mice	HPLC-ECD Phenol DNA extraction	(↑) Brain (12 to 30mo, both male and female) (Δ) Liver (male) (↓) Liver (female)	(-) Brain (male and female) (Δ) Liver (male) (-) Liver (female)
(Schmerold and Niedermüller, 2001).	5 and 30 months	Male Sprague-Dawley rats	HPLC-ECD	(-) Brain (↑) Liver	NA
(Nakae *et al.*, 2000)	0 days to 104 weeks	Male F344 rats	Immunohistochemistry and HPLC-ECD	(↑) Brain (-) Liver	NA
(Hirano *et al.*, 1996)	3 weeks, 5 months and 30 months	Male Sprague-Dawley rats	HPLC-ECD	(↑) Brain (-) Liver	NA
(Kaneko *et al.*, 1997)	2, 6, 12, 24, 27, 30 and 33 months	Male F344 rats	HPLC-ECD Ultrapure Phenol DNA extraction	(↑) Brain (↑) Liver	(↓) Brian (only at 30mo) (↓) Liver (only at 30mo)
(Hamilton *et al.*, 2001)	6, 18 and 24 months (rats) 6 and 25/26 months (mice)	Male B6D2F1 mice Male F344 rats Female C57Bl6 mice Male C57Bl6 mice Male F344 rats	HLPC-ECD NaI DNA extraction	(↑) Brain (all species) (↑) Liver (all species)	(↓) Brain (rat) (↓) Brian (mouse) (↓) Liver (mouse) (-) Liver (rat)

Table 6.4 Comparison of studies reporting 8-oxoguanine during ageing and DR in the brain and liver.

To conclude the liver is resistant to accumulation of oxidative lesions during ageing likely due to its proliferative state, which provides an extra level for DNA repair. This is contrary to the brain which shows accumulation of DNA oxidative damage during ageing. Overall, oxidative damage appears to play a larger role in the ageing of the brain than of the liver while the liver may be vulnerable to other forms of damage.

6.4.4 *Conclusions*

Key findings:

- Transcription active state of chromatin is maintained in the promoter of *Ogg1* and *Apex* during ageing and DR.
- Neither age nor DR had an effect on BER gene or protein expression in the liver.
- DNA repair in the liver remained constant throughout ageing.
- DNA oxidative damage in the liver declined with age and was not affected by DR.
- Tissue specific changes occurred at the epigenetic, transcription and phenotypic level.

6.5 Acknowledgments

I would like to thank:

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- *Maastricht University who performed the 8-oxoguanine analysis*
- *Dr Sabine Langie and Bartłomiej Tomaszewski for design of Ogg1 pyrosequencing and qPCR primers.*
- *Dr Sabine Langie for developing the comet in vitro repair assay*
- *Miss Kirstin Halley-Hogg an undergraduate student who performed the comet-based DNA repair assay on male liver samples.*

Chapter 7. Base excision repair in specific brain regions

7.1 Introduction

Ageing is associated with decline in brain functions such as cognition and with increased risk of neurodegenerative disease. As in other tissues, ageing in the brain is characterised by the accumulation of unrepaired molecular damage. Although relatively little is understood about the age-associated molecular changes occurring in the nervous system (Bohr *et al.*, 2007; Weissman *et al.*, 2007a; Rolseth *et al.*, 2008). Due to its low antioxidant status, high metabolic activity and thus high oxygen demand, the brain appears to be particularly vulnerable to oxidative damage (Halliwell, 1992; Driver *et al.*, 2000).

Until recently, the brain had been assumed to be largely a post-mitotic tissue that is unable to regenerate by cellular proliferation (Sohal *et al.*, 1990a). However, we now know that neurogenesis occurs in brain regions such as the hippocampus (Lee *et al.*, 2012). Furthermore, sub-regions of the brain have defined roles and most have been reported to alter with age. The magnitude of such change in size or function may be region specific. Region specific differences in age-related neuronal loss have been observed, with the cerebellum being more prone to loss compared with the hippocampus (Kennard *et al.*, 2013). The hippocampus plays a role in memory formation. During ageing the volume of the hippocampus decreases and this is accompanied by an underperformance in hippocampus-related tasks. Furthermore, the hippocampus shows an increase in oxidative damage with age (Driscoll *et al.*, 2003; Navarro *et al.*, 2008; Woodruff-Pak *et al.*, 2010). The cortex is important for spatial memory formation as well as in thought and language, while the cerebellum plays a role in motor control and learning. Both these regions in mice have been reported to accumulate oxidative damage in form of protein carbonyls, which is associated with decreased performance in cognitive and motor skill related tasks (Forster *et al.*, 1996; Woodruff-Pak *et al.*, 2010). Subcortical regions (containing the amygdala, caudate nucleus, pallidus, putamen, thalamus and others), which serve as connections and are also associated with motor skills, memory and learning, show a decline in volume with age that correlates with a decrease in function (Walhovd *et al.*, 2005).

In rodents, an age-related reduction in coordination and balance correlates with increased 8-oxoguanine (8-oxodG) levels in the cerebellum and caudate putamen (Cardozo-Pelaez *et al.*, 1999). In addition, age-related accumulation of DNA damage has been reported in neurones and in astrocytes (Swain and Subba Rao, 2011). Although all brain regions exhibit age-related increases in DNA damage, its magnitude was region-specific with cortex exhibiting highest increase followed by hippocampus, striatum, hypothalamus and lastly cerebellum (Juliet *et al.*, 2005). The central nervous system contains numerous types of neurones (Weissman *et al.*, 2007a) and these may be affected differentially by ageing due to the differences in cell structure and composition. Among the different cell types found in the brain, Purkinje neurones are characteristic of the cerebellum, pyramidal neurones are found in the cortex, hippocampus and amygdala, while non-neuronal cell such as astrocytes can be found through-out the brain (Byrne and Roberts, 2009).

OGG1, the primary protein for removal of oxidised guanine bases, is expressed ubiquitously throughout the brain (Verjat *et al.*, 2000). However, differing repair activity was detected in brain sub-regions. Furthermore an age-related decline in DNA repair is observed in both cortical neurones and in astrocytes (Verjat *et al.*, 2000; Rolseth *et al.*, 2008; Swain and Subba Rao, 2011).

To date only one study has investigated the effect of dietary restriction (DR) on DNA repair in different brain regions and did so by quantifying AP endonuclease (APE) activity (Kisby *et al.*, 2010). These authors demonstrated that APE activity in the cortex, hypothalamus, midbrain, brain stem and cerebellum declined with age in rats fed ad libitum. However, whilst APE activity also declined with age in DR animals, this measure of repair capacity remained higher than that in *ad libitum*-fed rats, and this apparently protective effect of DR was observed in all brain regions investigated except the hypothalamus. Furthermore 8-oxodG adducts in the cortex were lower in the DR group at all ages up to 25 months. Apart from this study, little is understood about the effects of age and of DR on expression of BER-related genes and on capacity for DNA repair in different brain regions.

I hypothesise that the BER repair pathway in specific sub-regions of the brain is differentially affected by age and by DR. To test this hypothesis, I quantified

BER-related DNA incision activity as well as *Ogg1* and *Apex* mRNA levels in the hippocampus, cortex, cerebellum and subcortical tissues of young (3 months old) and old (24 months old) mice which had been fed either ad libitum (AL) or exposed to 40% reduction in dietary energy intake from age 3 months (DR).

7.2 Materials and Methods

Male and female C57BL6 mice aged 3 months and 24 months were obtained from the long-term DR main Study (described in Chapter 5), which included mice fed AL throughout their lives and those exposed to 40% DR from age 3 months. When the mice were killed, the brain was removed and initially dissected into left and right hemispheres, followed by further dissection of each hemisphere into the cerebellum, hippocampus, cortex and subcortical tissue (rest of brain). The anatomical location of these regions is illustrated in Figure 7.1. Tissues were snap frozen in liquid nitrogen and stored at -80˚C. Tissue from one hemisphere was used for mRNA extraction and from the other hemisphere for protein extraction.

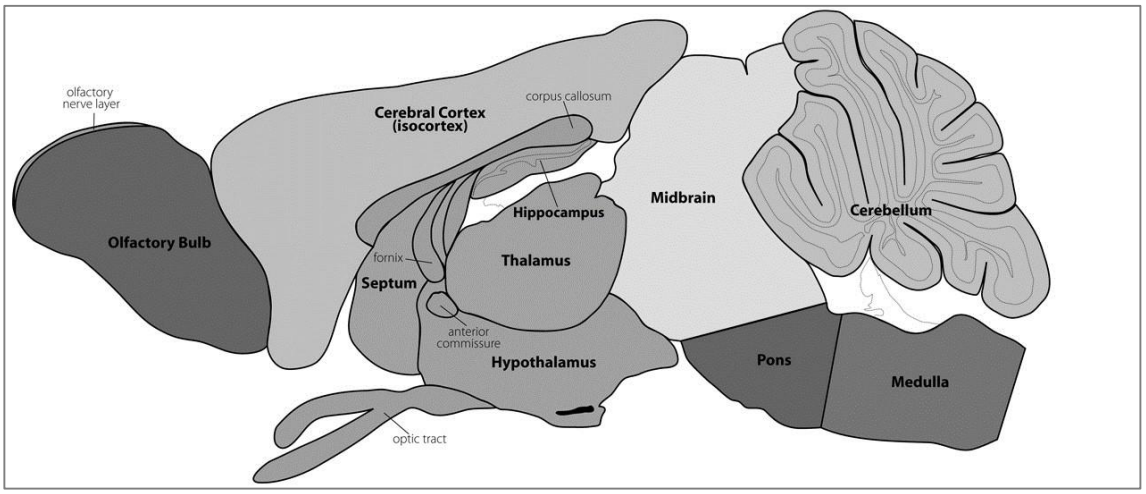

Figure 7.1 Diagram of the mouse brain showing the location of sub-brain regions. For the purpose of this study, the brain was dissected into cortex, cerebellum, hippocampus and subcortical regions (rest of brain). Image from Brain Atlas (Heintz, 2004) last accessed 3rd December 2014 at http://www.gensat.org/imagenavigator.jsp?imageID=33832.

Protein extraction from brain tissues was performed as described in Chapter 2, except for cortex and hippocampus which were too small to permit grinding and sub-sampling. Instead, for these brain regions, the whole tissue from 1 hemisphere was used. When extracting protein from tissue 75µl of buffer A and 22.5µl of buffer A1 was added to all tissues, except the hippocampus. Since the

mouse hippocampus weighs less than 15mg (Cameron *et al.*, 2011), the volumes of buffer A and buffer A1 added were reduced proportionally to 25µl and 10µl, respectively. The comet-based BER repair assay was performed as described in Chapter 2 with protein extracts for all brain sub-regions tested at 5mg/ml. RNA extraction for quantification of BER gene expression by qPCR was performed as described in Chapter 2.

ANOVA GLM was used to investigate the primary effects of age (3 month old *v.* 24 month old) and of feeding regime (24AL *v.* 24DR) and to test for possible interactions between age and feeding regime. Bonferroni post hoc comparison was used to test for differences in brain regions. $P<0.05$ was considered statistically significant.

7.3 Results

The results summarised in Chapter 5 did not reveal any significant age-related changes in DNA incision activity when measured on whole brain tissue. Since such measurements on whole brain might mask region-specific changes in DNA repair, in the present study BER-related DNA incision activity as well as *Ogg1* and *Apex* gene expression were measured in the following brain areas: hippocampus, cortex, cerebellum and subcortical regions. Measurements were made on tissues from 3 groups of animals i.e. 3 month old animals fed AL (3AL), 24 months old animals fed AL (24AL) and 24 months old which had been diet restricted from age 3 months (24DR).

7.3.1 *BER-related DNA incision activity in specific brain regions*

DNA incision activity differed between sexes ($P<0.001$) and between the different brain regions ($P<0.001$). DNA incision activity (pooled across all 3 groups: 3AL, 24AL and 24DR) in the cortex tissue was 2.5-fold higher in male compared with female animals ($P=0.003$). Incision activity in the hippocampus and cerebellum were similar for the two sexes, while DNA incision activity in subcortical tissue was 1.9-fold higher in male compared with female mice ($P=0.013$). Because of these effects of sex further analysis was performed on each sex separately.

In male mice, overall BER-related DNA incision activity was lowest in subcortical tissue and increased in the order hippocampus < cerebellum < cortex. DNA incision activity was more than 2 fold higher in the cortex than in all

other brain regions (P<0.001), but differences between the hippocampus, cerebellum and subcortical regions were not statistically significant. In female mice, overall BER-related DNA incision activity was lowest in the subcortical tissues, as was reported above for male mice, and increased in the order cerebellum < cortex < hippocampus. However, these differences in DNA incision activity between the different brain regions were not statistically significant for females.

7.3.1.1 *Effect of age*

There were no detectable effects of age on DNA incision activity in brain regions of male mice. However, DNA incision activity varied quite widely between male mice from 0.38-9.97AU in the hippocampus, 5.02-28.99AU in the cortex, 1.45-7.08AU in the cerebellum and 1.02-12.95AU in subcortical tissue (Figure 7.2). Similarly to male mice, no effect of age was observed in the hippocampus of female mice and individual mice varied considerably in DNA incision activity from 0-14.2AU across all 3 groups. In contrast, DNA incision activity in the cortex declined by 55% in older animals compared with young ones (P=0.028). In the cerebellum DNA incision activity was 2.6-fold higher in 24AL group compared with the 3AL group, but this difference was not statistically significant. DNA incision activity in subcortical tissue from 24 month old female mice was nearly double compared with 3 month old mice but this difference was not statistically significant (Figure 7.3).

7.3.1.2 *Effect of DR*

There were no effects of DR on DNA incision activity in male hippocampus or cerebellum. In contrast, DR fed male mice showed a 2-fold increase in DNA incision activity in cortex (P=0.031) and subcortical tissue (P=0.019) compared with AL animals (Figure 7.2). In female mice, an effect of DR was observed only in the cerebellum where there was a significant 2-fold increase in DNA incision activity in 24DR compared with 24AL animals (P=0.021). No effect of DR was observed in the hippocampus, cortex or subcortical tissue (Figure 7.3).

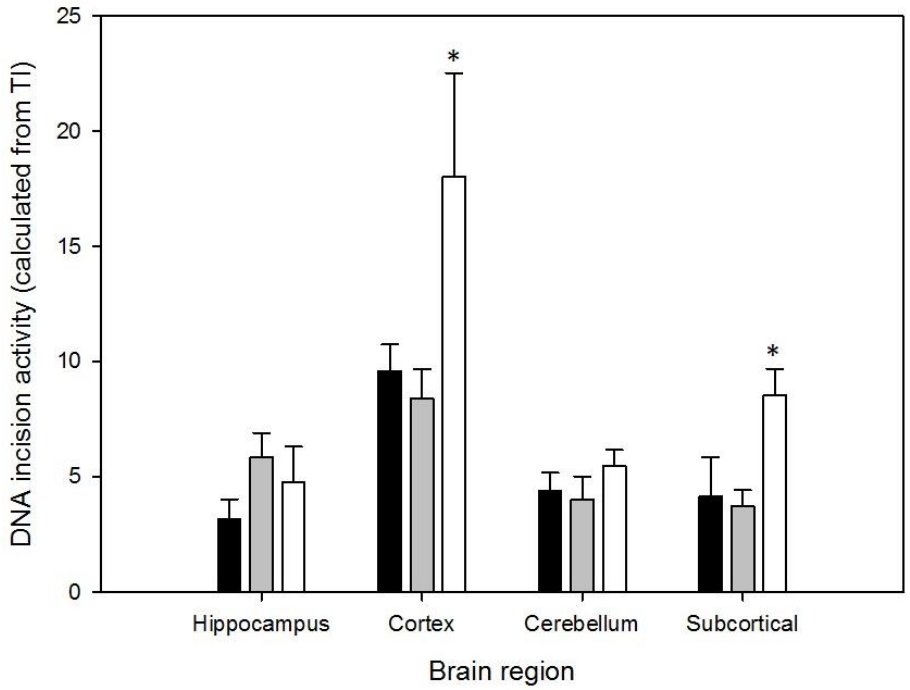

Figure 7.2 DNA incision activities in specific brain regions of male mice. Bars represent mean ± SEM, groups 3AL (black bars), 24AL (grey bars), 24 DR (white bars), n=5/group. Univariate ANOVA-GLM analysis of AL vs DR in the cortex P=0.031 and in subcortical tissue P=0.019. Data collected by Joanna Górniak (hippocampus and cortex) and Manon Lecardonnel (cerebellum and subcortical regions).

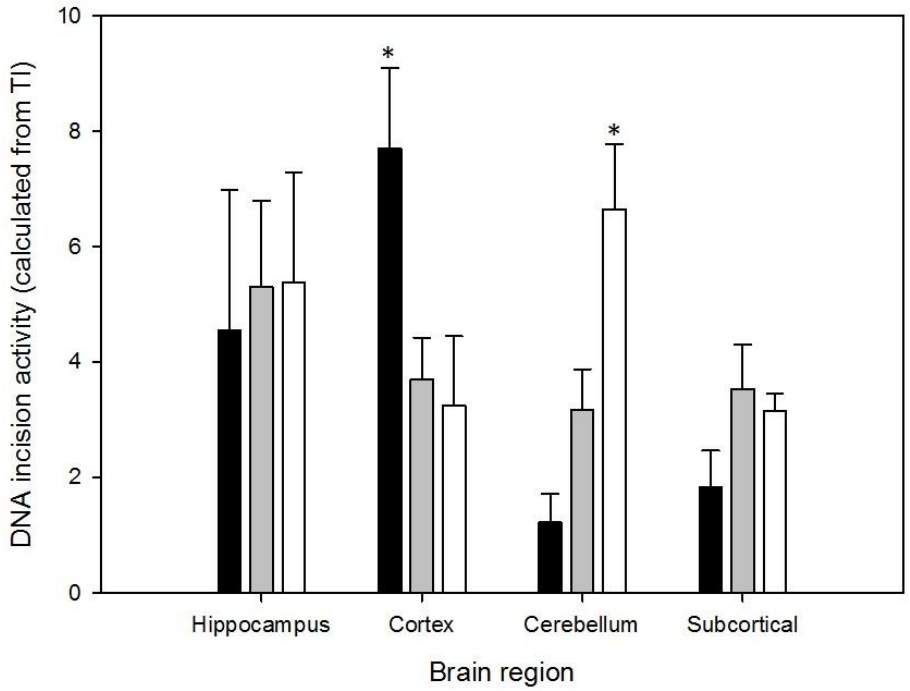

Figure 7.3 DNA incision activities in specific brain regions of female mice. Bars represent mean ±SEM, groups 3AL (black bars), 24AL (grey bars), 24 DR (white bars), n=5/group. Univariate ANOVA-GLM analysis in the cortex 3 month vs 24 month P=0.028 and in the cerebellum AL vs DR P=0.021. Data collected by Joanna Górniak (hippocampus and cortex) and Manon Lecardonnel (cerebellum and subcortical regions).

7.3.2 *BER-related gene expression in specific brain regions*

Expression of *Ogg1* and *Apex* differed significantly between brain regions (P<0.001) and sexes (P<0.001). In the cortex expression of *Ogg1* was 10 fold higher in female mice compared with male mice (P<0.001) while in the subcortical regions expression of *Ogg1* was 1.8 fold higher in female compared with male mice (P=0.002). *Ogg1* mRNA transcript levels in the hippocampus were similar for both sexes. On the other hand, in the cerebellum expression of *Ogg1* was 1.5 fold higher in male mice compared to female mice (P=0.006). Similarly to *Ogg1*, expression of *Apex* in the cortex was 9 fold higher in females compared with males (P<0.001) and in the subcortical region *Apex* mRNA was 1.4 fold higher in females compared with males (P=0.001). *Apex* mRNA levels were similar in hippocampi from both sexes. Lastly, expression of *Apex* in the cerebellum was 1.4 fold higher in males compared with females (P=0.01).

7.3.2.1 *Effect of age on Ogg1 expression in specific brain regions*

In male mice, overall expression of *Ogg1* was lowest in the cortex and increased in the order hippocampus < cerebellum < subcortical. Compared with the cortex expression of *Ogg1* was 8.8 fold higher in the hippocampus (P=0.003), 16 fold higher in the cerebellum (P<0.001) and 19 fold higher in the subcortical regions (P<0.001). In addition as compared with the hippocampus, expression of *Ogg1* was 1.8 fold higher in the cerebellum (P=0.008) and 2.2 fold higher in the subcortical regions (P<0.001). *Ogg1* transcript levels were similar in the cerebellum and subcortical region. There was an age-related decline in *Ogg1* expression in the hippocampus, cortex and cerebellum. In the hippocampus, expression of *Ogg1 was* 36% lower in 24 month old compared with 3 month old animals, although this was not statistically significant. Expression of *Ogg1* in the cortex decreased by 17% (P=0.011) and in the cerebellum by 45% (P=0.05) in 24 month old animals compared with 3 month old animals (Figure 7.4). In female mice (Figure 7.5), overall expression of *Ogg1* mRNA was highest in subcortical regions and was approximately 3.7 fold higher compared with the three other tissues (P<0.001). There was no effect of age on *Ogg1* expression in the cortex but *Ogg1* expression declined with age in the cerebellum and subcortical tissues. A 23% decline in expression is observed in 24AL animal compared to 3AL animal in the subcortical tissue

although this is not statistically significant. In the cerebellum there was a 38% decline in expression in 24AL animal compared with 3AL (P=0.001).

7.3.2.2 *Effect of DR on Ogg1 expression in specific brain regions*

24DR male mice had only 7% lower expression of *Ogg1* in the cerebellum compared with 3AL animals, suggesting that DR can attenuate the age-related decline in expression. No effect of DR was observed in any other regions in male mouse brain (Figure 7.4). In the hippocampus, cerebellum and the subcortical region of female mice, DR was able to up-regulated expression (Figure 7.5). *Ogg1* mRNA levels increased by 57% in the hippocampus (P=0.032), 10% in the cerebellum (P=0.008) and 65% in subcortical tissue (P=0.031) in DR animals compared with AL animals. No effect of DR was observed on the cortex of female mice.

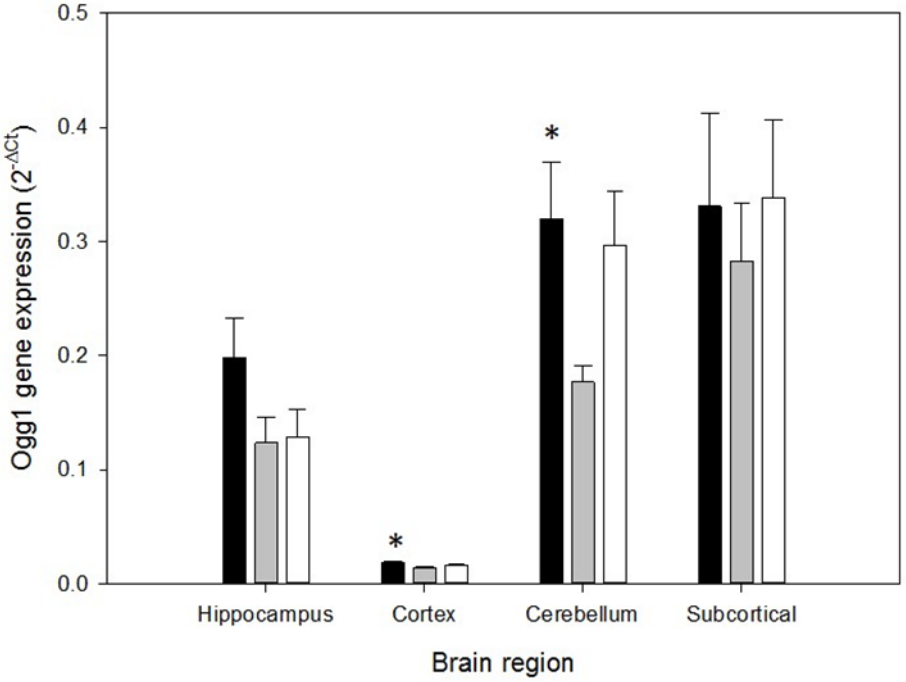

Figure 7.4 Expression of *Ogg1* in specific brain regions in male mice. Bars represent mean ± SEM, groups 3AL (black bars), 24AL (grey bars), 24 DR (white bars), n=5/group. Univariate ANOVA-GLM analysis for 3 month vs 24 month in the cortex P=0.011 and in the cerebellum P=0.05. Data collected by Joanna Górniak (hippocampus and cortex) and Manon Lecardonnel (cerebellum and subcortical regions).

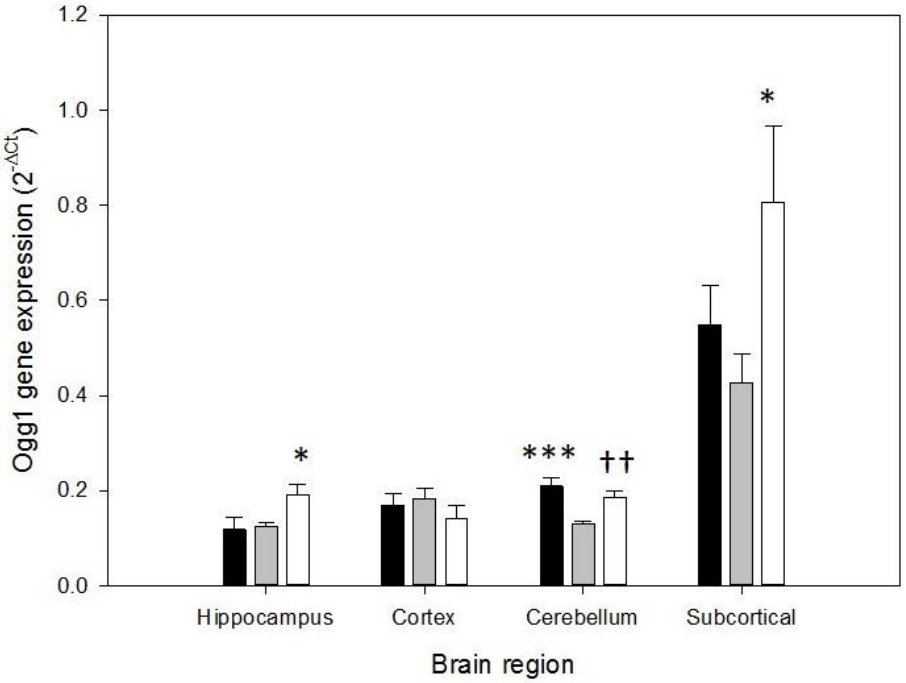

Figure 7.5 Expression of *Ogg1* in specific brain regions in female mice. Bars represent mean ± SEM, groups 3AL (black bars), 24AL (grey bars), 24 DR (white bars), n=5/group. Univariate ANOVA-GLM in the hippocampus AL vs DR P=0.032, in the cerebellum 3 month vs 24 month P=0.001 and AL vs DR P=0.008 and in the subcortical tissue AL vs DR P=0.031. Data collected by Joanna Górniak (hippocampus and cortex) and Manon Lecardonnel (cerebellum and subcortical regions).

7.3.2.3 *Effect of age on Apex expression in specific brain regions*

In male mice, expression of *Apex* was lowest in the cortex and increased in the order cerebellum < subcortical < hippocampus. Expression of *Apex* was 2.8 fold higher in the hippocampus (P<0.0001) and 2.5 fold higher in subcortical tissue (P=0.009) compared with the cortex. Furthermore expression in the hippocampus was also 1.6 fold higher compared with the cerebellum (P=0.01). An age-related decline in *Apex* expression was observed in the hippocampus, cortex and cerebellum. Expression of *Apex* declined by 50% in the hippocampus (P=0.047), 16% in the cortex (P=0.049) and 30% in the cerebellum (P=0.009) from 24 month old animals compared with 3 month old animals. No difference in expression was observed between the 3 groups in subcortical tissue (Figure 7.6). In female mice, expression of *Apex* was lowest in the cerebellum and increased in the order hippocampus < subcortical < cortex (Figure 7.7). Expression in the cortex was 2.9-fold higher compared with the hippocampus (P<0.001), 7.3 fold higher compared with the cerebellum (P<0.001) and 2.5 fold higher compared with subcortical regions (P<0.001). No difference in *Apex* mRNA levels was observed between the hippocampus or subcortical regions. However, expression of *Apex* was 2.5 fold higher in the hippocampus (P=0.003) and 2.9 fold higher in the subcortical regions (P<0.001) compared with the cerebellum. No effects of age on *Apex* expression were observed in the hippocampus, cortex or the subcortical tissue in these females. However, in the cerebellum *Apex* expression decreased by 36% in 24 month old compared to 3 month old animals (P<0.001).

7.3.2.4 *Effect of DR on Apex expression in specific brain regions*

In both male and female mice DR had no effect on *Apex* expression in any of the brain regions studied (Figure 7.6 and 7.7 respectively) except in the cerebellum of female mice where expression was higher in DR fed animals (P=0.004). Similarly to whole brain extracts there was no correlation between expression of *Ogg1* or *Apex* and DNA incision activity for any of the brain regions studied for males or females.

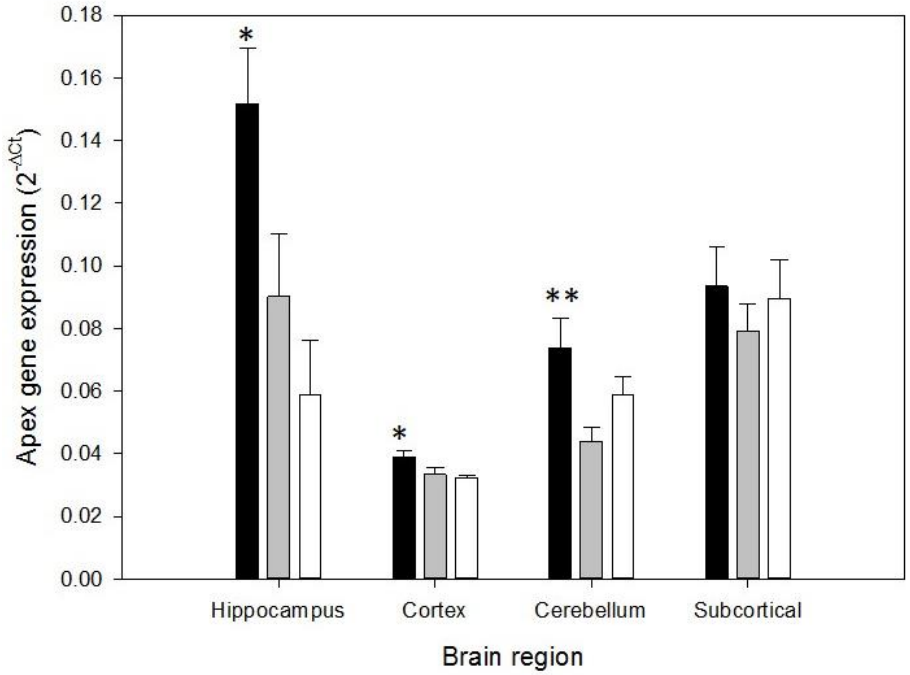

Figure 7.6 Expression of *Apex* in specific brain regions in male mice. Bars represent mean ± SEM, groups 3AL (black bars), 24AL (grey bars), 24 DR (white bars), n=5/group. Univariate ANOVA-GLM for 3 month vs 24 month in the hippocampus P=0.047, cortex P=0.049 and cerebellum P=0.009. Data collected by Joanna Górniak (hippocampus and cortex) and Manon Lecardonnel (cerebellum and subcortical regions).

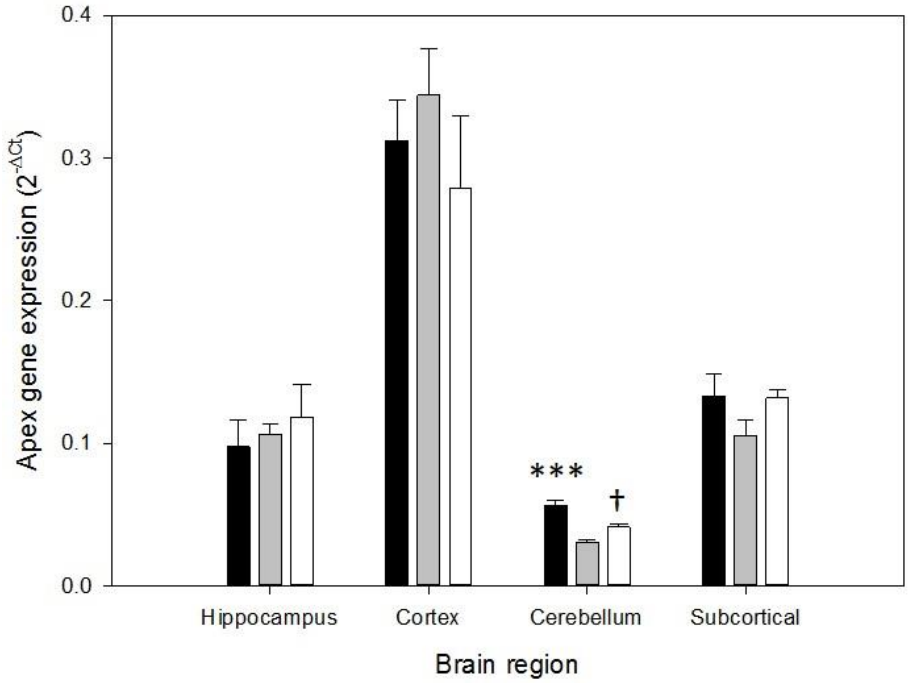

Figure 7.7 Expression of *Apex* in specific brain regions in female mice. Bars represent mean ± SEM, groups 3AL (black bars), 24AL (grey bars), 24 DR (white bars), n=5/group. Univariate ANOAV-GLM in the cerebellum for 3 month vs 24 month P<0.001 and AL vs DR P=0.004 P. Data collected by Joanna Górniak (hippocampus and cortex) and Manon Lecardonnel (cerebellum and subcortical regions).

7.4 Discussion

Repair of DNA damage is a critically important cellular defence mechanism for all tissues including the brain. Although it is clear that accumulation of DNA damage contributes to brain ageing and to the risk of developing age-related neurological disease (Martin, 2008), little is known about age-related changes in the capacity for DNA repair in different brain regions. Given the distinct functional roles of each brain region, understanding changes in DNA repair capacity with age in each brain region may help to provide a molecular basis for clinical observations. In addition, it will be important to discover whether interventions such as DR can affect the capacity for DNA repair in different brain regions. Until the present study was undertaken, there had been only one previous relevant study (Kisby *et al.*, 2010) that quantified the effect of DR on DNA repair in different brain regions using an assay to study APE activity. The present study extended that work considerably by quantifying i) BER-related incision activity and ii) expression of 2 BER-related genes *Ogg1* and *Apex* in young (3 months) and old (24 months) mice and also investigated the effect of long-term DR on expression of these genes.

7.4.1 *Effect of sex on DNA incision activity and gene expression*

In the present study, I show that capacity for DNA repair incision differs between brain regions with cortex showing highest incision activity, which likely results because of the high oxidative damage and low antioxidant levels reported in this tissue (Cardozo-Pelaez *et al.*, 2000). I observed that DNA incision activity in the brain was lower in female mice compared with male mice. This is the first direct comparison of DNA repair activity between the sexes in the mammalian brain. Interestingly even though female mice exhibit lower behavioural deficits compared with males (Dumont *et al.*, 2011) male C57BL6 mice show longer lifespan than female mice from this strain (Kunstyr and Leuenberger, 1975; Rowlatt *et al.*, 1976). Nevertheless, in the present study there was no difference between the sexes in lifespan (unpublished data Kerry Cameron, Chapter 5). Sex hormones have been linked to oxidative stress and neurodegeneration, with oestrogen showing a protective role and testosterone showing no protection (Razmara *et al.*, 2007; Lewis and Dluzen, 2008). The protective role of oestrogens has been well documented. The antioxidant properties of oestrogens are one of the modes for neuroprotection by this

hormone (Green and Simpkins, 2000). Furthermore oestrogens activate the MAPK pathway, which is in part involved in up-regulation of *Ogg1* (Green and Simpkins, 2000; von der Lippen *et al.*, 2015); in fact, in this study *Ogg1* and *Apex* mRNA levels are higher in female cortex compared with male. In addition, oestrogen can translocate OGG1 to subcellular compartments (Araneda *et al.*, 2005). It is then possible that oestrogens provide a protective role by up-regulating *Ogg1* and other oxidative damage response genes, provide antioxidant properties and compartmentalise repair proteins. Furthermore, the higher incision activity observed in male mice could result from a need for enhanced DNA repair in response to higher oxidative stress/damage in males.

7.4.2 *Effect of age on DNA incision activity and gene expression*

The effects of age differed between specific brain sub-regions studied. Although an age-related decline in DNA repair activity has previously been reported (Kisby *et al.*, 2010), here I have not observed any such changes except in cortex of female mice suggesting that differences in repair capacity are species specific. In fact, no other age related effects on incision activity were observed. It could be speculated that the assay was not sensitive enough to detect such change, but this is unlikely to be the case as the assay shows as high as 2 fold increases in DNA incision of cortex and subcortical tissue of males and cerebellum of females. Therefore, it is more likely that the incision step of the repair pathway is not affected by age. No age-related changes in incision of 8-oxoguanine were also reported in caudate nucleus, frontal cortex and hippocampus (Imam *et al.*, 2006). It has been previously speculated that enzymatic activity downstream in the BER pathway declines with age (Cabelof *et al.*, 2003). In further studies it would be interesting to observe also activity and/or expression of β-polymerase and DNA ligase. Interestingly, contrary to the incision data, mRNA expression of BER-related proteins declined with age in all regions studied. It is not the first time that DNA repair activity and gene expression levels do not correlate (Mikkelsen *et al.*, 2009). It can be speculated that basal levels of the repair proteins may be relatively stable and hence a small decline in their expression would not have major effects on the repair activity. In support of this hypothesis, no age-related changes in protein expression of AP endonuclease or DNA ligase III were seen in hippocampus, frontal cortex or cerebellum in C57BL6 mice (Imam *et al.*, 2006). Epigenetic

changes which result in gene silencing can be the reason for the observed age-related decline in BER-related gene expression. Region and dietary specific changes in cytosine methylation are observed in young mice (Langie *et al.*, 2013). Furthermore, epigenetic regulation of BER-gene expression has been explored in whole brain tissue in Chapter 5 and provided evidence in support of the above hypothesis. Unfortunately, due to the large amount of tissue required for the ChIP assay, epigenetic marks have not been explored here.

Overall, it can be said that despite an age-related decline in mRNA expression of BER-related proteins, the incision activity in brain sub-regions is unaffected by age because repair protein expression remains stable.

7.4.3 *Effect of DR on DNA incision activity and gene expression*

Interestingly, DR increased incision activity in cortex, subcortical tissue and the cerebellum. No such DR-related changes were seen when looking at the whole brain (Chapter 5), thus highlighting importance of studying sub-regions of the brain. Furthermore, the results reported here support the previously demonstrated DR-related increase in APE incision activity in multiple brain regions (Kisby *et al.*, 2010). In addition, DR up-regulated expression of *Ogg1* in the hippocampus, cerebellum and subcortical tissue although it did not have similar effects on *Apex* expression. Together this suggests that DR may affect mRNA as well as protein levels. DR is thought to act via nutrient sensing pathways. With multiple down-stream effectors it is possible that these pathways may control DNA repair at multiple levels. DR mimetics resveratrol and rapamycin up-regulate *Ogg1* via NRF2 transcription factor and AMPK activation respectively. In addition, OGG1 activity is increased upon phosphorylation by Cdk4 (Hu *et al.*, 2005) and this post-translation modification of the enzyme is required for binding to chromatin (Dantzer *et al.*, 2002). Therefore, it is plausible that DR enhances DNA repair on a transcription level via redox-responsive transcription factors such as NRF2 and on a protein level by phosphorylation of BER-related proteins which enhances their activity. Furthermore, oestrogen provides additional protective effect in females via up-regulation and compartmentalisation of OGG1.

7.4.4 *Conclusions*

To conclude, the present data suggest the importance of studying the brain specific regions in the context of ageing. Data presented here show that in the

mouse, ageing has no effect on the incision step of DNA repair process. More interestingly DR enhanced DNA repair by upregulated repair enzymes at mRNA transcript level and by enhancing DNA repair related incision, supporting the hypothesis that one of the beneficial effects of DR on the ageing brain is enhanced DNA repair.

7.5 Acknowledgments

I would like to thank:

- *CISBAN for design of the study and permission for use of brain tissues*
- *Miss Manon Lecardonnel who performed the repair and expression assay on cerebellum and subcortical tissue.*
- *Dr Sabine Langie for design of Ogg1 qPCR primers.*

Chapter 8: General Discussion

8.1 Overview

The accumulation of molecular damage is one of the characteristic features of ageing and contributes to the cellular dysfunction which results in the development of age-related frailty, disability and disease. In particular, oxidative damage to DNA may be deleterious because of its effect on genomic stability and on patterns of gene expression. This accumulation of oxidative damage to DNA is due to numerous factors such as altered antioxidant status and decline in DNA repair capacity with age (Freitas and de Magalhães, 2011). Base excision repair (BER) is the primary mechanism involved in recognition and repair of small oxidative lesions in DNA. Defective BER has been implicated in ageing (Cabelof *et al.*, 2006; Swain and Rao, 2012) as well as in the aetiology of age-related disease such as Alzheimer's disease (Weissman *et al.*, 2007b). Epigenetic marks at promoter regions in genes can regulate expression of the corresponding gene. However, there is accumulating evidence for aberrant changes in epigenetic marks in candidate genes and genome-wide epigenetic changes in ageing (Rakyan *et al.*, 2010; Tapp *et al.*, 2013). I hypothesised that such age-related changes in epigenetic marks could be responsible for age-related decline in BER-gene expression. Furthermore epigenetic events occur in a tissue-dependent manner as shown with the repair *BRCA1* gene which is only silenced by DNA hypermethylation in ovarian and breast tumours and remains unmethylated in other tumour types (Esteller *et al.*, 2000). Finally, the plastic nature of the epigenome suggests that dietary interventions may exert their beneficial effects on ageing via the epigenetic code (Mathers *et al.*, 2010).

The purpose of this project was to discern the epigenetic changes at base excision repair-related genes that occur with age and in response to dietary energy restriction. Furthermore, I investigated the effects of such epigenetic changes on transcription of BER-related genes and on functional activity (BER-related incision capacity). In addition, the net effects of these processes on the quantity of oxidative damage in DNA was also addressed. Since ageing may affect individual tissues to different extents, I tested my hypothesis in two tissues: brain and liver.

8.2 Evaluation of the Hypothesis

BER was chosen as the DNA repair pathway for study because of the evidence of 8-oxoguanine accumulating with age and contributing to the ageing process (Sohal and Weindruch, 1996; Nie *et al.*, 2013). There are multiple enzymes in the BER pathway and two were studied here: oxoguanine DNA glycosylase (OGG1) and AP endonuclease (APEX). These enzymes were chosen for study because they catalyse the initial steps of BER removal of oxidative damage and it is argued that changes in the genes encoding these enzymes would be likely to have consequences for the overall BER pathway. OGG1 recognises and removes an oxidised guanine base after which APEX excises the DNA backbone. OGG1 is the principle enzyme for removal of 8-oxoguanine lesions although other DNA glycosylases such as NEIL are also able to remove DNA oxidative damage. Furthermore, other DNA repair mechanisms such as NER can act as back-up for BER. The redundancy illustrated by these multiple pathways shows that cells are well equipped for removal of oxidative damage to DNA and BER alone may not be critical in determining the rate of ageing. However, evidence from multiple studies supports the hypothesis that DNA repair capacity falls with age and that this may contribute to the ageing process. In addition, a recent study using a data-mining approach attempted to classify DNA repair genes into those which were age-related and those which were not age-related and predicted that in humans the DNA repair genes which are age–associated include: *APEX1, ERCC5, RPA1, XRCC5 (Ku80), XRCC6, NEIL1, MUTYH* and *WRN* (Freitas *et al.*, 2011). Whilst the latter study postulated that DNA oxidative damage is central to ageing, there is significant evidence suggesting that such oxidative damage is not the key factor in determining rate of ageing. Several studies have shown that DNA oxidative damage does not accumulate with age in either nuclear or mitochondrial DNA (Chung *et al.*, 1992; Hirano *et al.*, 1996; Nakae *et al.*, 2000; Kennedy *et al.*, 2013). Nonetheless genomic instability plays a part in age-related cellular degeneration and therefore studies of DNA repair pathways such as BER are likely to be important in explaining the biology of ageing.

Epigenetic alterations have also been implicated as a hallmark of ageing (López-Otín *et al.*, 2013). Thus epigenetic changes which control the transcription state of chromatin may play a role in aberrant gene expression

during ageing. Changes in epigenetic marks at sites controlling transcription activity of the genes encoding BER enzymes may alter their expression and hence explain any age-related decline in repair activity that is observed. In this study I focussed on promoter regions as these tend to be CpG rich implying DNA methylation control and also because this is where binding of transcription factors that initiate the transcription process occurs. By looking at promoter regions we omit the gene body and other regions that affect transcription activation and therefore could have missed more complex epigenetic changes. Looking at all epigenetic marks would not be feasible and with the strong support for promoter related changes I opted to look at this region and to measure multiple epigenetic marks: cytosine methylation, histone acetylation and histone methylation. To summarise, although oxidative damage and its repair have been well documented in ageing research there is still much disagreement concerning its role in ageing. Furthermore, this is the first study to explore epigenetic mechanisms as a regulator of BER-gene expression during ageing and the effects of DR on epigenetic marks at these genes.

8.3 Evaluation of the animal model and techniques

As a mammalian system, mice belong to short-lived species (surviving up to 3 years) compared with humans who can live past 100 years. Furthermore telomere attrition is one of the hallmarks of ageing but mice have relatively long telomeres with high telomerase activity. Thus these factors among many others suggest that differences in ageing mechanisms between mice and humans are inevitable. However, animal models are essential for our understanding of ageing. The animal model chosen here was the C57BL6 mouse. This animal has often been used for studies of ageing as it is relatively free of age-related pathologies such as cancer (Swindell, 2012); it is a well-established mouse model of ageing at Newcastle University. Furthermore, manipulations of lifespan by dietary energy restrictions can provide further insight into the mechanisms of ageing and DR was able to extend lifespan in mice used in this study.

To determine an appropriate sample size for the main study a prior power calculation performed from DNA incision activity in young and old livers suggested n=6 per treatment group. This would suggest that sample groups with 5 to 7 animals should have given enough power to this study. The statistical programme often informed of low power in the test performed.

Furthermore, the main study contained 7 treatment groups; therefore, error could occur from multiple testing and care has to be taken when analysing results with a p value close to 0.05.

A strength of this study is that I was able to make measurements and comparisons in two tissue types: brain and liver and also in sub-regions of the brain. I was able to recognize the effects of age and diet at different levels based on measurements of epigenetic, transcription and phenotypic changes. In addition the study was of a longitudinal nature allowing comparison at 4 separate stages of mouse lifespan and in both *ad libitum* and dietary restricted mice.

There are limitations to techniques used to in this study which have been covered in Chapter 4. As discussed previously, the pyrosequencing assays are limited to looking at < 50% of CpG sites found in *Ogg1* and *Apex* promoters. I cannot be certain that the CpG sites studied here are essential to control transcription activation. However, pyrosequencing permits precise estimation of measurements of methylation at individual CpG level and with much higher accuracy than other techniques such as high resolution melting or methylation specific PCR. Similarly to number of CpG sites there are also a large number of histone modifications that exist; two were studied here as they are associated with transcription silencing and/or activation. The comet-based assay which measures the initial incision step of a repair process may demonstrate well the activity of OGG1 and APEX but is not ideal in terms of measuring overall BER capacity. Finally, HPLC-ECD was used to measure 8-oxoguanine concentration in these tissues; however; with this technique being prone to oxidation of DNA during extraction absolute 8-oxoguanie levels may not be directly representative of those found in the cell. While limitations to techniques exist I can be certain that those used here are appropriate for the quantifications made in this study. Overall, this study and the techniques used here present a good model for robust studies of ageing and its modulation by dietary restriction.

8.4 Principal findings

I have demonstrated that BER-related epigenetic changes are observed with age in a tissue-specific manner. The difference observed between these organs was not surprising as tissue specific age-related changes have previously been demonstrated on a functional and transcription level (Lee *et al.*, 2000). The

primary hypothesis explored the changes in DNA methylation at BER-related gene promoters in response to age (Chapter 4). This is the first study exploring epigenetic changes in promoter regions of *Ogg1* and *Apex* during age. Using the Genomatix software, the promoter regions of genes encoding *Ogg1* and *Apex* were identified; the promoter regions were both confirmed to contain CpG islands. Therefore, cytosine methylation at these promoters could theoretically be involved in their transcription activation. Chapters 4-6 showed that DNA methylation levels of *Ogg1* and *Apex* promoters were very low; this was not surprising as promoters of transcription active genes are generally unmethylated (Maegawa *et al.*, 2010). Lack of age-related changes in the methylome suggested other epigenetic mechanism may be involved. Histone modifications, in particular histone 4 acetylation and histone 3 lysine 27 tri-methylation, were quantified as this explored both chromatin active and silencing marks respectively (Chapter 5 and 6). Because increased DNA methylation is associated with gene silencing I hypothesised that during ageing there is an increase in cytosine methylation at *Ogg1* and *Apex* promoters which caused an age-related decline in their transcription. Interestingly, I observed a decrease in DNA methylation with age suggesting that the cell maintains expression of BER genes required in the DNA damage response. Furthermore, changes in contrasting histone marks were also observed with age. Since during ageing aberrant changes in the epigenome are observed on a global level (Maegawa *et al.*, 2010; Peleg *et al.*, 2010; Han and Brunet, 2012), gene specific changes in epigenetic marks must occur to preserve an open chromatin state and maintain expression of BER genes (Figure 8.1 proposed mechanism). Interestingly, the liver was more resistant to epigenetic changes compared with the brain. Furthermore DR, which prevents genomic instability, further potentiates the epigenetic code to give euchromatin. Such changes are a result of activity of epigenetic enzymes such as HDAC, HAT, HMT, DNMT, TET and histone demethylase; in fact, changes in activity of these enzymes are observed with ageing and HDAC is a known element of the DR mechanism (McMahon *et al.*, 2007; Chouliaras *et al.*, 2011; Jin *et al.*, 2011; Zhang *et al.*, 2013).

Interestingly gene expression is maintained throughout age in the liver but a decline was seen in the brain. Two possible mechanisms can explain this unexpected observation. Firstly, it is now also known that DNA methylation elsewhere such as enhancer regions or gene body can also affect transcription

activity and methylation at these sites has not been explored here. Secondly, miRNAs have been found that degrade BER-relate mRNA transcripts.

Although data suggest an open chromatin structure at the BER-promoters these studies would not be able to directly show whether transcription occurs as other factors such as binding of proteins that would inhibit transcription or binding of transcription factors have not been addressed. Transcription factor expression has been reported to correlate well with the occupancy of its binding sites ('An integrated encyclopedia of DNA elements in the human genome,' 2012). I measured expression of two redox-sensitive transcription factors: *Hif1α* and *Nfya* and found that their expression increased with age suggesting that these TF are up-regulated in response to oxidative stress and could initiate BER-related gene transcription activation. Up-regulation of TFs was observed in old DR fed animals, thus suggesting DR mediates its effects via reduction of oxidative stress by initiating oxidative stress responses. In Chapter 5 I have also shown that DR may exert one of its neuro-protective roles by maintaining levels of repair enzyme transcripts at higher levels compared with AL animals. Furthermore, *Ogg1* and *Apex* genes are also up-regulated via NFYA, NRF2, p53, NF-κB, MiTF, heat shock protein 70 (Kenny *et al.*, 2001; Mendez *et al.*, 2003; Chatterjee *et al.*, 2005; Liu *et al.*, 2008; Habib *et al.*, 2010; Jung *et al.*, 2013; Song *et al.*, 2013; Singh *et al.*, 2014; von der Lippen *et al.*, 2015).

Upon assessing the expression of *Ogg1* and Apex, I wanted to confirm the effects on functional activity of the repair proteins. Chapter 3 showed that the comet-based assay can be used to measure BER-related DNA incision activity in a number of mouse tissues. I have confirmed that DNA incision activity is tissue-specific, likely to be due to the proliferative nature of the tissue and is higher in liver compared with brain. In Chapter 5 and 6 I have shown that in the brain and liver, no significant effect of age or diet was observed on DNA incision activity. Therefore these proteins maintain their activity throughout age. Independently of their gene up-regulation, it has been shown that acetylation and phosphorylation of these proteins can enhance their activity (Dantzer *et al.*, 2002; Hu *et al.*, 2005; Bhakat *et al.*, 2006; Bhattaracharyya *et al.*, 2009; Lirussi *et al.*, 2012). A wealth of data on DNA repair activity are found in the literature but these reports are contradictory. A main finding from this study is that BER-related DNA incision activity did not change with age or diet; however, there

was an increase in protein concentration with age in the brain but not liver. One suggestion is that in the brain there is an accumulation of damaged protein resulting from lack of cell division related autophagy. I have not measured the other repair enzymes/processes that are also able to remove oxidative damage. An important hypothesis for ageing research postulates that ROS and the DNA oxidative damage induced by these molecules are a major contributor to genomic instability associated with ageing; however, here 8-oxoguanine a marker of oxidative damage did not support this statement. Currently a large number of studies provide no support for oxidative damage during ageing (most recently (Kennard *et al.*, 2013)). Thus if damage accumulation is a key player in the ageing process other repair enzymes such as those that are involved in repair of larger lesions or double strand breaks and that maintenance of genomic stability may be more noteworthy.

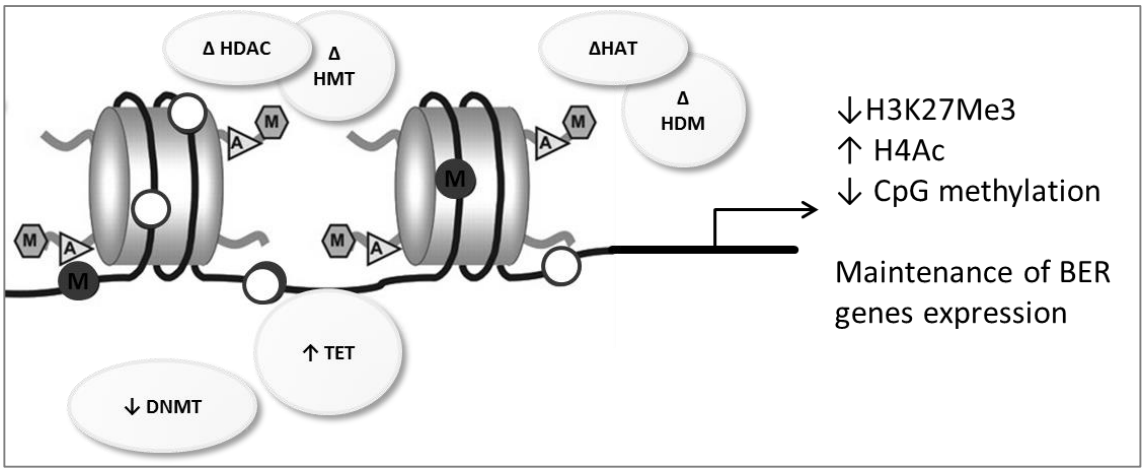

Figure 8.1 Proposed mechanism for epigenetic regulation of BER gene expression during ageing. During ageing the cell attempts to maintain BER gene expression by altering epigenetic marks to maintain an open chromatin structure. BER, base excision repair; DNMT, DNA methyltransferase; H3K27Me3, histone 3 lysine 27 trimethylation; H4Ac, histone 4 acetylation; HAT, histone acetyltransferase; HDAC, histone deacetylate; HMT, histone methyltransferase; TET, Ten-eleven translocation methylcytosine dioxygenase.

An important conclusion that can be drawn from these studies is that phenotypic changes should be measured as well as epigenetic and transcription changes as it is evident that a clear relationship is not always seen. Studies often report only the epigenetic changes occurring with a treatment. However, they fail to back up the data with effects on the transcription profile (gene expression) of the genes or further on the actual functional effect of the protein encoded by the gene. I have demonstrated that changes occur at the epigenetic and

transcription level; however, little was seen in DNA repair activity. Furthermore, although protein expression and incision activity in the liver are higher than the brain, an accumulation of BER-related proteins is observed in the brain but not liver. This suggests that in this study age and diet do not affect DNA repair capacity and highlights that epigenetic changes do not necessarily translate to functional effects on the cell and care should be taken not to over interpret epigenetic data.

8.5 Future studies

Genomic instability is one of the hallmarks of ageing (López-Otín *et al.*, 2013) and accumulation of damage often observed with age is in part a result in deficient DNA repair activity (Lombard *et al.*, 2005). No changes in DNA incision activity were observed in this study; however, this is just the representation of the first step of the repair pathway. Evidence points to a deficiency of the gap filling step with age (Cabelof *et al.*, 2002). It would be informative to confirm this observation using a gap filling assay/β polymerase activity (Cabelof *et al.*, 2003). A modification of the comet assay can also be used. Collins *et al.* previously measured the repair process including gap filling using the comet assay and supplementation of nucleotides (Collins *et al.*, 1995) although this would have to be optimised for use with animal tissues. Furthermore, numerous studies implicate NER in removal of oxidative damage (Wang *et al.*, 2012a; Melis *et al.*, 2013). Modifications of the *in vitro* comet repair assay for quantifying NER are available (Langie *et al.*, 2006). Thus in future studies it would be interesting to quantify the activity of other repair enzymes e.g. β-polymerase, XRCC1, DNA Ligase IV, PCNA, GADD45, ERCC1, XP A-G as well as to test epigenetic changes at promoter regions of these genes in response to age and DR.

This study shows a potential role for epigenetic maintenance of BER gene expression but it would benefit from a more detailed exploration of the mechanism. Numerous approaches can be taken to explore this. Firstly it would be useful to confirm using a promoter and binding assay in a vector system that DNA methylation changes can affect transcription of BER genes (Klug and Rehli, 2006). However, because both genes studied displayed low DNA methylation, mapping of the epigenome could be more informative, such as has been shown for epigenetic changes associated with X chromosome inactivation

(Marks *et al.*, 2009). Such an analysis of the epigenetic landscape can be used for correlative studies with transcription and could clarify the ambiguous epigenetic changes observed in this study. No work has looked at whether DR affects global DNA methylation. For example, can it promote genomic stability via silencing transposable elements? This could be explored by analysis of DNA methylation at Alu or LINE1 transposable elements (Lisanti *et al.*, 2013). Multiple groups, and the data presented here, show DR induced changes in DNA methylation at promoter regions confirming that DR can at least contribute to genomic stability on a loci specific level. Furthermore, Ten-eleven-translocation (TET) proteins convert methylated cytosine to 5-hydroxymethylcytosine (5-hmC). As I observe an age related decrease in DNA methylation at both promoters it would be interesting to confirm whether this was a result of an increase in 5-hmC. An age-related increase in 5-hmC has already been reported in the brain (Chen *et al.*, 2012; Wen *et al.*, 2014). Global 5-hmC can be measured using HPLC or enzymatic assay (Szwagierczak *et al.*, 2010). More interestingly, the bisulphite sequencing method has recently been optimised to measure 5-hmC and it can do so at individual CpG sites (Booth *et al.*, 2012).

Finally, only a small part of this study looked at the potential mechanisms of epigenetic changes and their effect on gene expression. I have quantified activity of HAT and HDAC enzymes and measured expression of TFs involved in up-regulation of BER-related gene. To develop on this, activity of other enzymes should also be studied e.g. DNMT (Chouliaras *et al.*, 2011), TET (Zhang *et al.*, 2013), HMT (McMahon *et al.*, 2007) and histone demethylases (Sun *et al.*, 2010; Jin *et al.*, 2011). However, these studies should be specific to individual enzymes. In addition, the TF studies should be improved by analysing TF binding to promoters. ChIP-sequencing can be used for global mapping of transcription factor binding sites and has been used so in the ENCODE project (Wang *et al.*, 2012b). The ChIP assay can also identify TF binding at promoters using antibodies specific to TF of interest (Gade and Kalvakolanu, 2012).

8.6 Conclusions

This study confirms that ageing is associated with aberrant epigenetic changes that can alter expression of BER-related genes. I have shown that promoter regions of BER-related genes *Ogg1* and *Apex* are dynamic and undergo

changes with age and diet. Changes in multiple and contrasting epigenetic marks were observed suggesting that regulation of transcription is controlled by multiple mechanisms. I have demonstrated that epigenetic changes do occur at BER-related genes in a tissue and sex specific fashion however no clear correlation with the gene expression was observed highlighting that epigenetic regulation is only one aspect of the complex regulation of transcription of genes. Furthermore this study fails to show a decline in BER-related DNA incision activity with age suggesting that at least in these mice DNA repair activity is sustained throughout life and hence there is maintenance of DNA oxidative damage levels during ageing.

Appendix A – Oxoguanine DNA glycosylase (OGG1)

A.1 Ogg1 promoter DNA Sequence with numbered CpG sites

Ogg1 (Chromosome 6, Locus ID GXL 88306, Gene ID 18294) *Mus musculus*:

ATGAGATTCTTT[1]**CG**GTTTATTTCTTGAGACAGAGTCT[2]**CG**CCATGAAGCCCAGCCTAGTTAA
GCTTCAAA[3]**CG**TGCCTCC[4]**CG**CCTCTGCCTAC[5]**CG**AGTTCTGGGA[6]**CG**AC[7]**CG**GTGTGTACC
ACTA[8]**CG**CCC[9]**CG**CAATGATGTGGCTCCAGTG[10]**CG**GGCCAGCA[11]**CG**AGAAGTTGCAGGCA
GGCCCTGAGATTGCAC[12]**CG**AAAGCCCTGAAATGGCAGAGC[13]**CG**GGTCTCTGGCAGCCAA
T[14]**CG**CCAAGCAG[15]**CG**AGGCCAGCAGGCCAAC[16]**CG**CTCCCACCTCACAGGCC[17]**CG**CCCAC
TC[18]**CG**GCACTCC[19]**CG**AGTTCTCATTGGTGCTT[20]**CG**TACTTTCTTCT[21]**CG**GATTGGCTACCT
CTAGGGTCAAATAAG[22]**CG**GGAGCAAGGATCTACTTC[23]**CG**GTGGGAGTAAACTGGGACC[24]**C**
GGAAGAACCATACTGG[25]**CG**[26]**CG**CCTTTCCAAACCT[27]**CG**AGGGCTG[28]**CG**CAGGAGGAGGA
GG[29]**CG**[30]**CG**[31]**CG**C[32]**CG**GGAGGTCCTGGGGG[33]**CG**GGGCCA[34]**CG**TC[35]**CG**GAGGAAGAGG[36]
CGGGG[37]**CG**TGCTGCA[38]**CG**GGAGAG[39]**CG**GAGTGGGG[40]**CG**TTTAGGGGTTTGGTGGTGTG[4]
[1]**CG**AGAGGCTTTCCTA[42]**CG**AGGAGAGGGAAATTCATTCAACAGGGACTCAAATTAAAATTA
CAC[43]**CG**GACCCATAAGCTAGGACCCA[44]**CG**GTGCTA

A.2 Transcription factor binding at the Ogg1 promoter

Genomatix generated transcription factor consensus sequence binding on the positive strand of the *Ogg1* promoter.

TF	Description	Spans Assay CpG
DEAF	Homolog to deformed epidermal autoregulatory factor-1 from D. melanogaster	-
HIFF	Hypoxia inducible factor, bHLH/PAS protein family	3
HESF	Vertebrate homologues of enhancer of split complex	-
HEAT	Heat shock factors	-
TF2D	General transcription factor IID, GTF2D	14
CAAT	CCAAT binding factors	14
CLOX	CLOX and CLOX homology (CDP) factors	14, 15
MTEN	Core promoter motif ten elements	14, 15
NRSF	Neuron-restrictive silencer factor	14, 15, 16
RORA	v-ERV and RAR-related orphan receptor alpha	15
GLIF	GLI zinc finger family	17
ZBPF	Zinc binding protein factors	17
HDBP	Huntington's disease gene regulatory region binding proteins	18
YBXF	Y-box binding transcription factors	-
XCPE	Activator-, mediator- and TBP-dependent core promoter element for RNA polymerase II transcription from TATA-less promoters	-
E2FF	E2F-myc activator/cell cycle regulator	-
ZF35	Zinc finger protein ZNF35	-
MOKF	Mouse Krueppel like factor	-
EGRF	EGR/nerve growth factor induced protein C & related factors	-
ZF5F	ZF5 POZ domain zinc finger	-
EBOX	E-box binding factors	-
SP1F	GC-Box factors SP1/GC	-
CHRE	Carbohydrate response elements	-
KLFS	Krueppel like transcription factors	-
PDRM	PRDI-BF1 and RIZ homologous (PR) domain proteins (PRDM)	-

Table A.1. TFs binding at the *Ogg1* promoter, expressed in brain, liver or ubiquitously. Highlighted TFs are ones present in the *Ogg1* pyrosequencing assay.

A.3 Pyrosequencing Assay Validation

Ogg1 pyrosequencing assay optimisation has been performed previously by Bartłomiej Tomaszewski and Dr Sabine Langie. No data on nested PCR product representing *Ogg1* promoter, synthesis of an in vitro 100% methylated promoter or pre and post methylation dilution series are available. Figure A.1 shows example gel from pyro PCR of Ogg1 sequences 1 and 2.

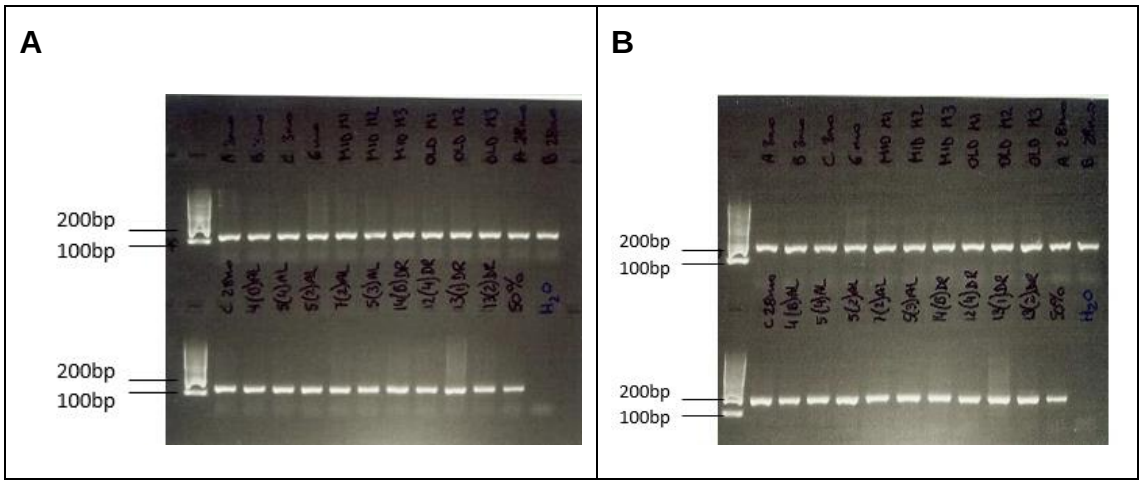

Figure A.1 Example of agarose gel from pyro PCR of *Ogg1* sequence 1: 134bp (A) and sequence 2: 184bp (B) performed with BM DNA from the brain.

A.4 Pyrosequencing Pyrograms

Example of Pyrogram read outs from pyrosequencing reactions of *Ogg1* sequence 1 and 2.

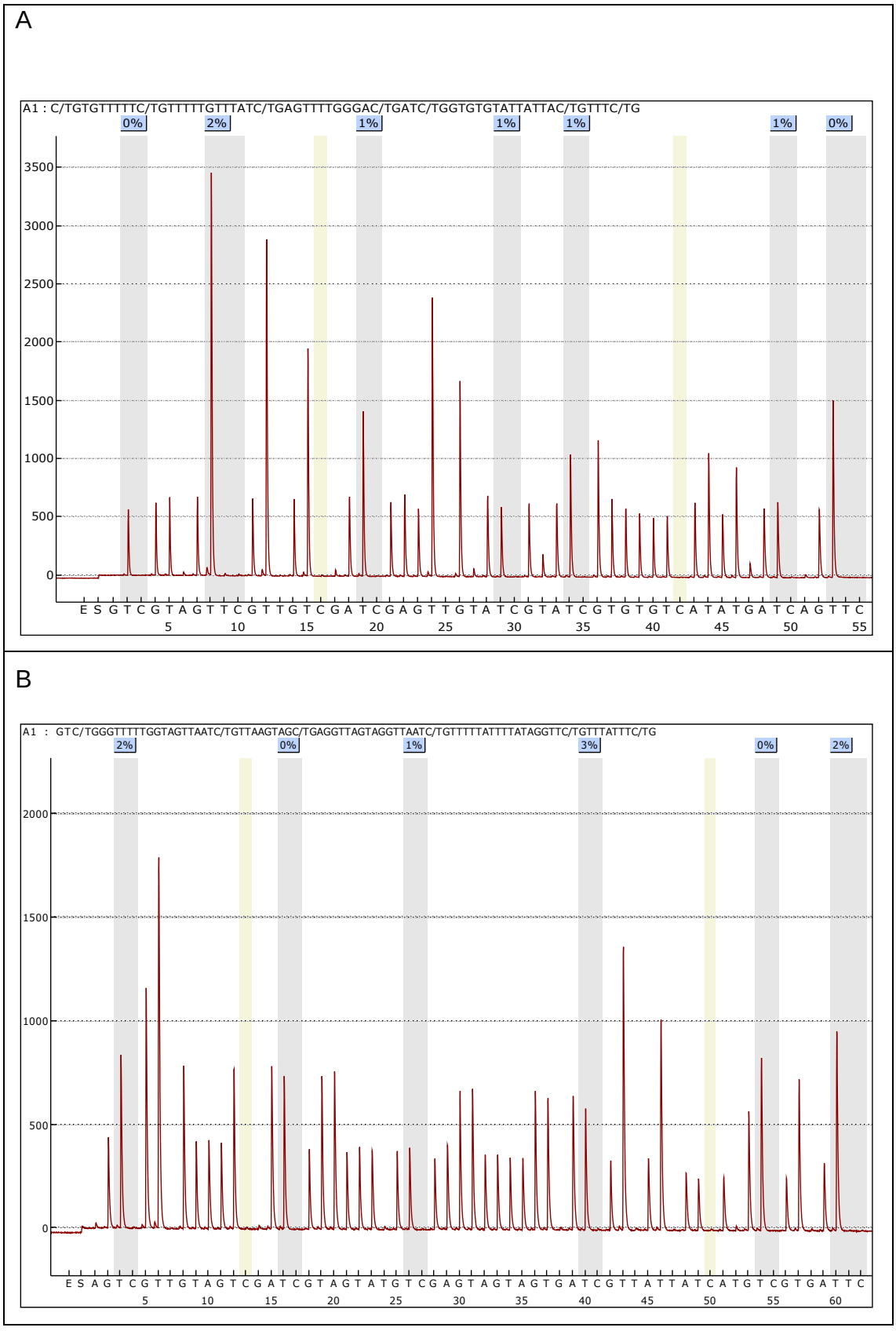

Figure A.2 Example of a pyrogram reading for *Ogg1* sequence 1 (A) and sequence 2 (B).

A.5 Cytosine methylation at individual CpG sites in the Ogg1 promoter

Mean and median cytosine methylation from animals in the long term-term DR study (Chapter 5 and 6) as pleasured by pyrosequencing. Methylation in the brain of male mice (Table A.2), brain of female mice (Table A.3), liver of male mice (Table A.4) and liver of female mice (Table A.5).

CpG site		Cytosine methylation (%)						
		3AL	12AL	12DR	24AL	24DR	30AL	30DR
3	Mean	0.2280	0.2843	0.1864	0.1050	0.0986	0.0740	0.0000
	Median	0.2650	0.3800	0.2050	0.1550	0.1500	0.0000	0.0000
	SD	0.1609	0.1974	0.1438	0.1010	0.0946	0.1019	0.0000
	P value	All > 0.05						
4	Mean	1.7640	1.9400	1.7400	1.6457	1.4857	1.6850	1.4007
	Median	1.8050	1.9600	1.7900	1.6950	1.4750	1.7050	1.4050
	SD	0.2283	0.1413	0.5614	0.3615	0.4583	0.1125	0.2941
	P value	All > 0.05						
5	Mean	0.6980	0.7443	0.6536	0.6293	0.5743	0.6630	0.4907
	Median	0.6600	0.7350	0.7250	0.6750	0.6250	0.6700	0.5550
	SD	0.1000	0.0596	0.2811	0.1439	0.2025	0.1370	0.1548
	P value	All > 0.05						
6	Mean	0.7250	0.8214	0.6736	0.6414	0.5786	0.6500	0.3321
	Median	0.7250	0.7950	0.7600	0.7250	0.6000	0.7000	0.3150
	SD	0.0639	0.1453	0.1761	0.2534	0.1954	0.1724	0.0449
	P value	1.000	>0.05	>0.05	>0.05	>0.05	>0.05	0.006
7	Mean	1.0440	0.9250	0.8536	0.6943	0.7021	0.6820	0.3914
	Median	1.0300	0.9300	0.8800	0.4950	0.5850	0.7650	0.3750
	SD	0.1069	0.1187	0.2191	0.3132	0.2458	0.2799	0.0634
	P value	1.000	>0.05	>0.05	>0.05	>0.05	>0.05	<0.001
8	Mean	1.3630	1.3929	1.2864	1.2993	1.2829	1.0620	1.0371
	Median	1.4450	1.4150	1.3900	1.3650	1.2000	1.1400	1.1650
	SD	0.2475	0.1313	0.3535	0.3706	0.4021	0.1227	0.3627
	P value	All > 0.05						
9	Mean	0.6670	0.7921	0.7671	0.6843	0.5864	0.9940	0.6886
	Median	0.7100	0.9250	0.7950	0.7800	0.5950	1.0450	0.6650
	SD	0.4519	0.3709	0.3551	0.2315	0.3222	0.2502	0.7066
	P value	All > 0.05						
13	Mean	1.7470	1.7443	1.2957	1.4150	1.2093	1.0420	0.7457
	Median	1.6450	1.9950	1.4550	1.5400	1.1400	1.3050	0.6350
	SD	0.1804	0.8232	0.5734	0.5394	0.4777	0.4742	0.3218
	P value	All > 0.05						
14	Mean	0.5090	0.8300	0.5321	0.5336	0.4300	0.4130	0.2786
	Median	0.4250	0.9200	0.4600	0.5050	0.4250	0.4250	0.3750
	SD	0.2096	0.2942	0.1654	0.2958	0.3569	0.0404	0.1942
	P value	All > 0.05						
15	Mean	1.5030	1.6136	1.0386	1.3757	1.2364	0.7600	0.8664
	Median	1.5650	1.7100	1.0350	1.4800	0.8900	0.5450	0.8200
	SD	0.3222	0.4092	0.3269	0.3784	0.7178	0.6649	0.2998
	P value	All > 0.05						
16	Mean	2.9250	2.9114	2.6857	2.6279	2.6336	2.8480	2.1550
	Median	2.8550	2.7900	2.6850	2.7950	2.8950	2.6900	2.3200
	SD	0.3386	0.3734	0.9441	0.8005	1.0613	0.3728	0.7355
	P value	All > 0.05						
17	Mean	0.8340	1.1914	0.7421	0.7379	0.6814	0.7020	0.4814
	Median	0.7950	1.3000	0.6250	0.6350	0.5550	0.6300	0.5600
	SD	0.2625	0.3860	0.4118	0.2878	0.5302	0.2349	0.2373
	P value	All > 0.05						
18	Mean	2.1520	2.3671	2.0114	1.9607	1.8136	2.0440	1.3457
	Median	2.0850	2.1600	2.1500	2.1050	1.9300	2.0650	1.5550
	SD	0.2401	0.3402	0.8061	0.6079	0.7194	0.4107	0.3604
	P value	All > 0.05						
Overall	Mean	1.2765	1.3861	1.1332	1.1282	1.0450	1.0665	0.8093
	Median	1.3075	1.4025	1.225	1.2025	0.9500	1.1075	0.8650
	SD	0.0833	0.1693	0.2916	0.2905	0.3600	0.1457	0.1490
	P value	1.000	>0.05	>0.05	>0.05	>0.05	>0.05	0.043

Table A.2 Percentage cytosine methylation in the brain from male mice from long-term DR study. Comparison to 3AL (1.000), significance level <0.05.

CpG site		Cytosine methylation (%)						
		3AL	12AL	12DR	24AL	24DR	30AL	30DR
3	Mean	0.1100	0.1521	0.0164	0.0650	0.0243	0.1286	0.0200
	Median	0.0000	0.1350	0.0000	0.0000	0.0000	0.0000	0.0000
	SD	0.1597	0.1702	0.0435	0.0812	0.0643	0.1866	0.0529
	P value	All > 0.05						
4	Mean	2.0160	1.7321	1.6364	1.7486	1.5300	1.8629	1.8129
	Median	1.6900	1.5650	1.6950	1.7400	1.7100	1.7500	1.8200
	SD	0.6525	0.3443	0.1865	0.2433	0.7015	0.3195	0.2445
	P value	All > 0.05						
5	Mean	0.8670	0.7657	0.5307	0.7550	0.5621	0.5336	0.6579
	Median	0.7750	0.8000	0.6200	0.7900	0.6250	0.3800	0.6800
	SD	0.1795	0.1661	0.2971	0.2058	0.2906	0.2205	0.3384
	P value	All > 0.05						
6	Mean	1.0010	0.8571	0.9393	0.7650	0.5479	0.6007	0.8621
	Median	1.0450	0.7800	0.6900	0.8450	0.5700	0.4650	0.7550
	SD	0.1419	0.1303	0.7108	0.1654	0.2944	0.1854	0.5161
	P values	All > 0.05						
7	Mean	0.9060	0.6757	0.5293	0.5600	0.3564	0.6271	0.6650
	Median	0.8950	0.6800	0.6700	0.4850	0.3900	0.5350	0.4850
	SD	0.2800	0.2086	0.2739	0.2279	0.1624	0.2164	0.6000
	P value	All > 0.05						
8	Mean	1.4560	1.1650	1.0621	1.2229	1.1293	1.4321	1.7986
	Median	1.3200	1.1950	1.1850	1.2700	1.2150	1.4850	1.5350
	SD	0.5570	0.1010	0.4956	0.1032	0.5528	0.2178	0.5762
	P value	All > 0.05						
9	Mean	1.0590	0.9336	1.1971	1.1400	0.7329	1.1586	1.1971
	Median	0.9150	0.9100	0.8850	1.1200	0.8500	1.1750	1.2150
	SD	0.3867	0.1787	1.1367	0.0623	0.4322	0.2149	0.3312
	P value	All > 0.05						
13	Mean	1.7300	1.6571	1.5064	1.4971	1.2750	1.1064	1.2564
	Median	1.6050	1.6900	1.5950	1.4650	1.0550	1.1850	1.2800
	SD	0.4927	0.4190	0.3055	0.2869	0.5976	0.5042	0.3531
	P value	All > 0.05						
14	Mean	0.5480	0.5514	0.3493	0.4679	0.4971	0.5593	0.3979
	Median	0.3600	0.6200	0.3550	0.4550	0.4250	0.4100	0.3500
	SD	0.4661	0.2315	0.2960	0.2932	0.1608	0.3863	0.3175
	P value	All > 0.05						
15	Mean	1.7760	1.7093	1.5321	1.6007	1.5871	1.9429	1.5807
	Median	1.6650	1.7800	1.5300	1.6100	1.7350	1.9800	1.5450
	SD	0.4585	0.4288	0.3167	0.2248	0.3910	0.3532	0.2958
	P value	All > 0.05						
16	Mean	3.6280	2.8279	3.0186	2.9607	2.8943	3.3179	3.6129
	Median	3.6050	2.8000	3.2400	2.9200	2.9000	3.4500	3.5600
	SD	1.4633	0.3742	0.4242	0.2488	0.9081	0.4095	0.7727
	P value	All > 0.05						
17	Mean	1.4290	1.2736	1.0250	1.2479	0.9286	1.4693	1.0993
	Median	1.2900	1.2400	1.0900	1.2450	0.8250	1.4100	1.0950
	SD	0.4409	0.2544	0.3909	0.3672	0.3300	0.7009	0.4199
	P value	All > 0.05						
18	Mean	2.0810	2.1150	1.9107	2.0000	2.1329	2.1936	2.2371
	Median	1.9700	2.0850	1.9850	2.0600	2.4000	2.3250	2.3250
	SD	0.6888	0.2398	0.4385	0.3450	0.6722	0.2365	0.3456
	P value	All > 0.05						
Overall	Mean	1.4630	1.2936	1.2014	1.2629	1.1843	1.3361	1.3496
	Median	1.3350	1.3700	1.2025	1.2000	1.1875	1.4250	1.3250
	SD	0.4664	0.1709	0.0653	0.1336	0.2561	0.2416	0.1813
	P value	All> 0.05						

Table A.3 Percentage cytosine methylation in the brain from female mice from long-term DR study. Comparison to 3AL (1.000), significance level <0.05.

CpG site		Cytosine methylation (%)						
		3AL	12AL	12DR	24AL	24DR	30AL	30DR
3	Mean	0.2910	0.2100	0.1264	0.2250	0.2729	0.1200	0.1467
	Median	0.0000	0.2000	0.0000	0.2175	0.0000	0.0000	0.1875
	SD	0.4574	0.1855	0.1748	0.2330	0.4077	0.2683	0.1211
	P value	All > 0.05						
4	Mean	2.5110	1.9057	1.7164	2.2525	2.1379	1.6740	1.8625
	Median	2.4600	1.7850	1.8200	2.2650	2.1850	1.5350	1.8500
	SD	0.3249	0.3909	0.4104	0.2370	0.7683	0.4643	0.3305
	P value	All > 0.05						
5	Mean	0.7800	0.5836	0.8007	0.7792	0.7893	0.5720	0.6075
	Median	0.6400	0.7200	0.8350	0.8100	0.7700	0.7150	0.6075
	SD	0.2493	0.4272	0.3406	0.2104	0.4556	0.3787	0.1564
	P value	All > 0.05						
6	Mean	0.8730	0.7336	0.8350	0.5867	0.9436	0.7260	0.6475
	Median	0.8450	0.8000	0.7450	0.7025	0.9400	0.7700	0.6725
	SD	0.2849	0.1669	0.4502	0.3641	0.5400	0.2679	0.2363
	P value	All > 0.05						
7	Mean	1.3760	1.1921	0.6986	0.6642	1.1236	0.8730	0.7600
	Median	1.2000	1.1650	0.7050	0.4950	1.0400	0.7750	0.8500
	SD	0.4373	0.4652	0.2982	0.3395	0.5087	0.2650	0.3412
	P value	1.000	>0.05	>0.05	0.48	>0.05	>0.05	>0.05
8	Mean	2.2100	1.5107	1.9257	1.4958	1.6829	1.4020	1.3550
	Median	2.1550	1.6100	1.6450	1.4625	1.6800	1.3700	1.2900
	SD	0.3276	0.2359	0.6566	0.2757	0.7833	0.1053	0.4656
	P value	All > 0.05						
9	Mean	1.3630	0.8943	1.1521	0.8783	1.2257	1.0540	0.5317
	Median	1.3850	1.1100	0.9050	0.9075	1.1850	0.9250	0.4400
	SD	0.1947	0.4794	1.2352	0.3769	0.4536	0.3241	0.5302
	P value	All > 0.05						
13	Mean	1.7790	0.9221	0.6964	0.9333	0.7486	0.3100	0.4392
	Median	1.3800	1.0700	0.6550	1.0350	0.4950	0.4350	0.5125
	SD	0.9706	0.5579	0.3453	0.9039	0.7374	0.2875	0.2501
	P values	1.000	>0.05	>0.05	>0.05	>0.05	0.016	0.026
14	Mean	0.9580	1.1664	0.8886	1.2667	0.9786	1.1880	0.8583
	Median	0.9200	1.1550	0.8050	1.1250	0.8850	1.3150	0.9275
	SD	0.3405	0.3590	0.5033	0.6103	0.4265	0.6880	0.3403
	P value	All > 0.05						
15	Mean	1.7650	2.0164	1.1800	0.9858	1.6521	1.7230	1.5350
	Median	1.5000	2.3700	1.3650	0.9925	1.8100	1.8650	1.1325
	SD	0.5945	0.9140	0.5378	0.4356	0.9459	0.9296	1.0669
	P value	All > 0.05						
16	Mean	4.7450	3.1921	3.0029	2.5667	3.8329	2.4260	2.4717
	Median	4.3900	3.5300	3.3650	2.7775	3.7400	2.7100	2.2925
	SD	1.0502	0.7898	1.2869	1.1755	1.7320	0.7608	0.8435
	P value	All > 0.05						
17	Mean	1.3950	0.7457	0.7457	0.7817	0.8107	0.8270	0.8183
	Median	1.6000	1.0900	0.8600	0.7125	0.7600	0.6700	0.6725
	SD	0.6059	0.5923	0.3841	0.5908	0.5270	0.3980	0.3454
	P value	All > 0.05						
18	Mean	3.7080	2.6207	2.7550	2.3025	2.4707	2.7520	2.2900
	Median	2.7200	2.6150	2.6450	2.2275	2.5550	2.6050	2.2850
	SD	1.5989	0.2439	0.9465	0.4212	1.4715	0.3892	0.4420
	P value	All > 0.05						
Overall	Mean	1.7630	1.3914	1.2914	1.2279	1.3704	1.1905	1.1067
	Median	1.7325	1.4425	1.2525	1.2275	1.0925	1.1725	1.0088
	SD	0.2414	0.1428	0.2940	0.1135	0.6087	0.1012	0.2738
	P value	1.000	>0.05	>0.05	0.041	>0.05	>0.05	>0.05

Table A.4 Percentage cytosine methylation in the liver from male mice from long-term DR study. Comparison to 3AL (1.000), significance level <0.05.

CpG site		Cytosine methylation (%)						
		3AL	12AL	12DR	24AL	24DR	30AL	30DR
3	Mean	0.0000	0.0000	0.1200	0.2033	0.0000	0.0321	0.0336
	Median	0.0000	0.0000	0.0000	0.2775	0.0000	0.0000	0.0000
	SD	0.0000	0.0000	0.2137	0.1617	0.0000	0.0850	0.0888
	P value	All > 0.05						
4	Mean	1.3900	1.5329	1.4129	2.0433	1.6943	1.8550	1.8600
	Median	1.4650	1.5500	1.2150	1.7625	1.3700	2.0650	1.7400
	SD	0.1303	0.4631	0.7011	0.6089	0.8777	0.5866	0.6215
	P value	All > 0.05						
5	Mean	0.4530	0.6329	0.6829	0.9258	0.5071	0.7586	0.7879
	Median	0.4600	0.7400	0.6600	0.9175	0.4900	0.8550	0.8350
	SD	0.3401	0.3358	0.3115	0.1220	0.4057	0.3295	0.3519
	P value	All > 0.05						
6	Mean	0.5730	0.8136	0.5593	1.0983	0.4829	0.6271	0.7129
	Median	0.4150	0.9150	0.4050	0.9650	0.5100	0.5000	1.0000
	SD	0.2977	0.2704	0.4318	0.2776	0.3333	0.2961	0.4304
	P value	All > 0.05						
7	Mean	0.5630	0.9136	0.5943	1.1717	0.6050	0.5807	0.7971
	Median	0.4100	1.0450	0.5200	1.0975	0.4450	0.5000	0.5450
	SD	0.2362	0.5482	0.1669	0.2926	0.4180	0.5388	0.6054
	P value	All > 0.05						
8	Mean	1.5520	1.5686	1.9229	2.1633	1.7400	1.6129	2.0121
	Median	1.5150	1.6250	1.8500	2.1750	1.9050	1.6350	1.9100
	SD	0.1568	0.3908	0.2747	0.4406	0.5372	0.4996	0.3495
	P value	>0.05						
9	Mean	0.7760	1.2414	0.9421	1.6683	1.1486	1.2686	0.9557
	Median	0.7450	1.2250	0.6150	1.5825	1.1250	1.2200	0.9200
	SD	0.3726	0.5722	0.5321	0.6837	0.6115	0.4739	0.4300
	P value	>0.05						
13	Mean	1.7020	1.6200	1.3936	1.9317	1.0993	1.2664	1.1343
	Median	1.8600	1.5850	1.2500	1.9250	0.9800	1.1750	1.1150
	SD	0.3837	0.2636	0.5686	0.7765	0.3736	0.4239	0.3320
	P value	>0.05						
14	Mean	0.2960	0.1943	0.1436	0.4750	0.3864	0.0360	0.3164
	Median	0.0000	0.0000	0.0000	0.6075	0.4050	0.0000	0.3250
	SD	0.4127	0.2462	0.2459	0.3911	0.3175	0.1682	0.3784
	P value	>0.05						
15	Mean	2.0450	2.2657	2.2993	2.8733	2.1921	2.2307	2.1707
	Median	2.0450	2.3950	2.2400	2.7700	1.9050	2.2450	2.2500
	SD	0.3901	0.5312	0.3430	0.4185	0.6204	0.4393	0.6328
	P value	>0.05						
16	Mean	2.4570	3.0279	2.9329	4.9542	3.6229	3.7207	4.0871
	Median	2.5000	3.1200	3.1800	4.0550	3.8250	3.8500	4.1950
	SD	0.6137	0.4081	0.8304	2.0457	1.1507	0.8759	1.0170
	P value	1.000	>0.05	>0.05	0.023	>0.05	>0.05	>0.05
17	Mean	0.8320	1.0743	1.0279	1.8100	0.8229	1.0500	1,3914
	Median	0.7900	1.3250	0.6850	1.7600	0.5700	0.6950	1.4900
	SD	0.6852	0.6488	0.6164	0.7825	0.6732	0.6918	0.5940
	P value	>0.05						
18	Mean	2.4840	2.1871	2.6236	3.3158	2.2443	2.8650	2.8679
	Median	2.4000	2.1600	2.8700	2.7725	2.4000	3.2250	3.0750
	SD	0.3645	0.3230	0.8403	1.2858	0.9147	0.8807	0.6049
	P value	>0.05						
Overall	Mean	1.6370	1.7293	1.7386	2.5600	1.7286	1.8671	1.9950
	Median	1.5750	1.7150	1.7450	2.2175	1.5700	1.7850	2.0400
	SD	0.3303	0.2271	0.3123	0.8060	0.5207	0.4612	0.4467
	P value	>0.05						

Table A.5 Percentage cytosine methylation in the liver from female mice from long-term DR study. Comparison to 3AL (1.000), significance level <0.05.

A.6 Validation of primers for ChIP-qPCR

Primers for amplification of *Ogg1* promoter region were validated with liver genomic DNA from the ChIP input reaction. A qPCR reaction shows good amplification for a dilution series of DNA and the primers. Primers were designed by Dr Jelena Mann.

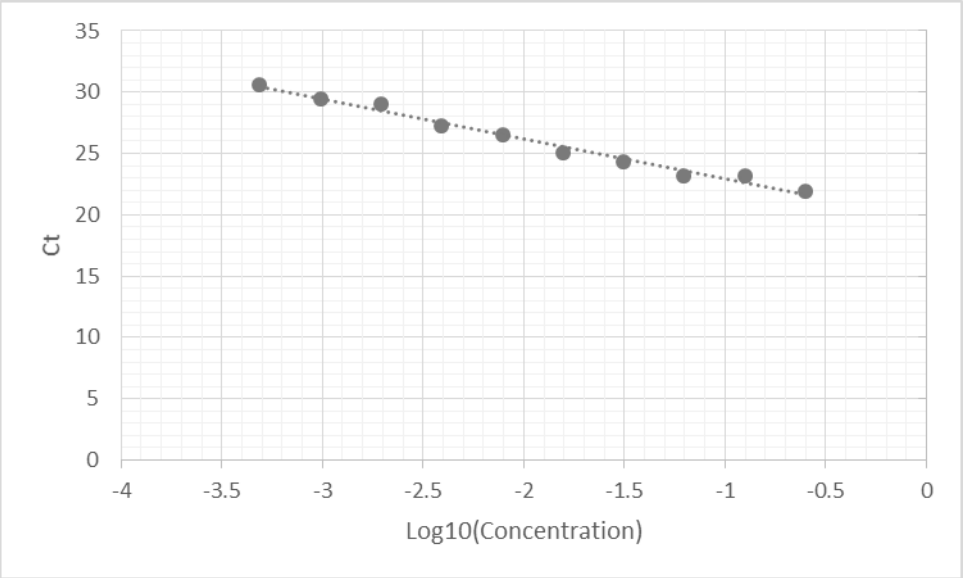

Figure A.3 Validation of assay used for ChIP-qPCR. Efficiency 102.3, slope -3.27.

A.7 Ogg1 cDNA Sequence acquired from Genomatix software

GAGGCGGGGCGTGCTGCACGGGAGAGCGGAGTGGGGCGTTTAGGGGTTTGGTGGTGTG
CGAGAGGCTTTCCTACGAGGAGAGGGAAATTCATTCAACAGGGACTCAAATTAAAATTACA
CCGGACCCATAAGCTAGGACCCACGGTGCTACACTCCCGGTGTGTGTGGTCACTTGTGTG
CGAGGCTTTAGGACTTGGGAAAGTCGAGTTCTGATTGGACAGTGCCGTAATGGGCTGGGG
CTGCTAGCCGAATGTTATTCCGTTCCTGGCTGCCTAGCAGCATGAGACATCGCACCCTAAG
CTCCAGCCCGGCCCTCTGGGCCTCTATCCCGTGTCCACGCTCTGAGCTGCGTCTGGACTT
GGTTTTAGCTTCTGGACAGTCCTTCCGGTGGAAGGAGCAGAGCCCTGCTCACTGGAGTGG
CGTGCTGGCAGATCAAGTATGGACACTGACTCAGACGGAGGACCAGCTCTATTGCACTGT
GTACCGAGGAGACGACAGCCAGGTCAGCAGGCCCACCCTAGAGGAGCTGGAAACCCTAC
ACAAGTACTTTCAGCTAGATGTCAGCCTGGCACAGCTGTATAGCCACTGGGCTTCTGTAGA
CTCCCACTTCCAAAGAGTGGCCCAGAAATTCCAAGGTGTGAGACTGCTGAGACAAGACCC
CACTGAGTGCCTTTTCTCTTTCATCTGTTCCTCCAACAACAACATTGCTCGCATTACTGGCA
TGGTGGAACGGCTCTGCCAGGCCTTTGGACCTCGACTCATTCAGCTTGATGATGTCACTTA
TCATGGCTTCCCAAACCTCCATGCCCTGGCTGGTCCAGAAGCAGAGACTCACCTGAGGAA
GTTGGGCCTGGGGTACCGTGCCCGCTATGTACGTGCCAGTGCCAAAGCCATCCTCGAAGA
GCAAGGTGGGCCAGCTTGGCTGCAGCAGCTTCGAGTGGCCCCTTATGAAGAGGCCCACA
AGGCCCTCTGCACCCTGCCCGGGGTGGGCGCCAAGGTGGCTGACTGCATCTGCTTAATG
GCCCTTGACAAACCCCAGGCTGTGCCCGTGGATGTCCATGTATGGCAGATTGCCCATCGT
GACTACGGCTGGCATCCTAAGACATCCCAGGCTAAGGGCCCGAGCCCTTTGGCCAACAAA
GAACTGGGAAACTTTTTCCGGAATCTGTGGGGACCTTATGCTGGCTGGGCCCAAGCAGTG
CTGTTCAGTGCTGACCTTCGCCAACCAAGCCTGTCTCGGGAGCCACCAGCAAAGCGCAAA
AAGGGATCTAAGAGGCCAGAGGGCTAGGTAGGGCTCAGGGGCAAAAGGTCTTTTTTCCTT
TGTTCTCCATTTCTTTCCCTTGTCTCTCCCCCCCCCCCAACCCCCGACCCCTAAGTCTCAA
GTCAGAAAGACTTAACACTCCCACCTCAGAGGCCAGAACCCCAAATCAAAGTCAGTTTTTA
CAATAGGGAAAAAGGAAGCTATTTGAGCAAACGGAACTGTCTTGTTTTATCTTCCCTTTATT
ACAAGAAGGAACAATAAAATAGAAACATTTGTATGGAAAA

A.8 Ogg1 expression qPCR primer validation

Primer validation for *Ogg1* expression with cDNA dilution series. Primers designed by Dr Sabine Langie.

Assay	Efficiency factor	Efficiency (%)	Slope
Ogg1 (Target)	1.94	93.80	-3.480
Hprt (Reference)	1.96	96.03	-3.421
B2m (Reference)	1.92	92.49	-3.516

Table A.6 qPCR assay efficiency as tested with cDNA from liver.

A.9 OGG1 ELISA assay

Protein extract concentration at 5mg/ml and 1mg/ml were tested in the OGG1 ELISA assay to optimise extract concentration to be used. Kit standard was used to set up a standard curve.

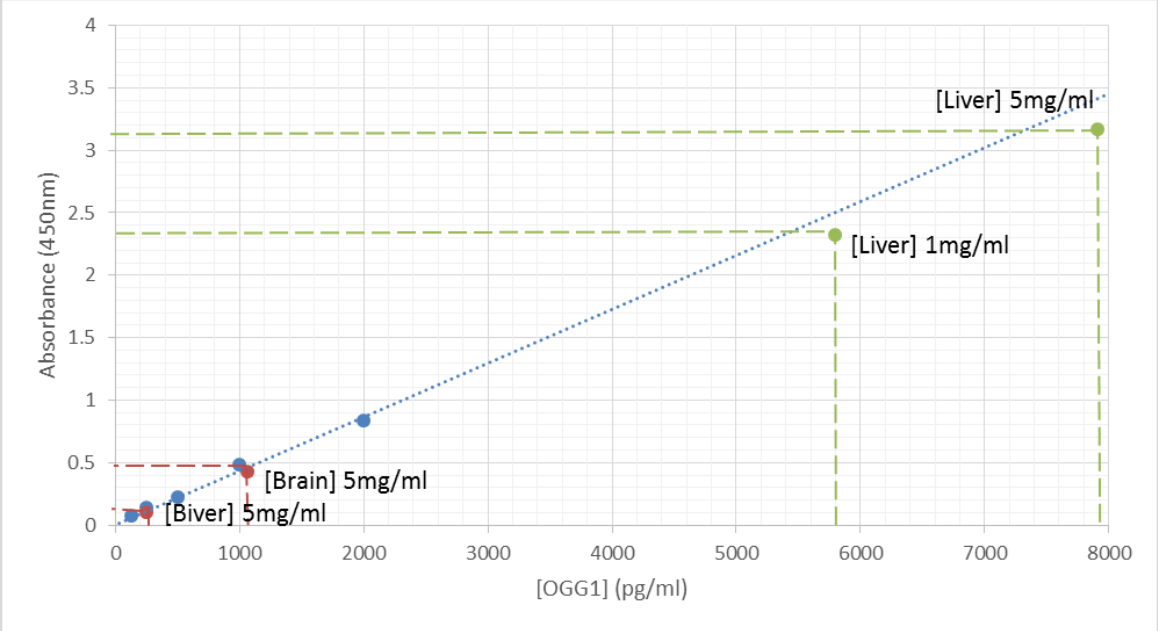

Figure A.4 Optimisation of assay OGG1 ELISA, protein extracts from brain (red) and liver (green) at 5mg/ml and 1 mg/ml were tested versus assay standard (blue) to determine concentration of extract to use in the assay.

Appendix B – Apurinic/apyrimidinic endonuclease 1 (APEX)

B.1 Apex promoter DNA Sequence with numbered CpG sites

Apex (Chromosome 14, Locus ID GLX 98783, Gene ID 11792) *Mus musculus*:

AAAACAAAACCTGAA[1]**CG**AACCCA[2]**CG**TGACCTAGCCCCAGGTGTCTGACTCTTCTAGTCAA
AAGTTACCAACCAAA[3]**CG**CACAATCAGGGGAGTCTGGAGTGGGGAAGCCTGTAAAATCTGA
CATAGGCAACAGTGTGGAATAT[4]**CG**GACAGAACAAAACTGGACAAGGAGC[5]**CG**GGAAG[6]**CG**[7]
CGGC[8]**CG**CAGCCACTCAC[9]**CG**GTGCC[10]**CG**GGGC[11]**CG**TGA[12]**CG**TAAGTG[13]**CG**C[14]**CG**[15]**CG**G
GTT[16]**CG**CCAGCAC[17]**CG**TGC[18]**CG**T[19]**CG**[20]**CG**GACCA[21]**CG**CCCA[22]**CG**C[23]**CG**ATCTTGTTGG[24]
CGCTGCCTT[25]**CG**AACCCCAGCAC[26]**CG**[27]**CG**GGCATGGTGCCTCA[28]**CG**GCTCC[29]**CG**GT[30]**CG**
TGAGGC[31]**CG**AGTGA[32]**CG**AGCTGTAAC[33]**CG**GCACTACCA[34]**CG**AACAACCCAGAACCAAG[35]
CGAAGGGACTTT[36]**CG**GG[37]**CG**CCAGAAGTTATGTCA[38]**CG**AGGTGCCAGGAGGACCAATCAT
[39]**CG**[40]**CG**CTCCAGAACCA[41]**CG**TGGGGG[42]**CG**GAGCCCT[43]**CG**AATCA[44]**CG**TGACCACACCAA
T[45]**CG**CCCA[46]**CG**TGGGAACAGGCAGACTCCATTCTTTGTGC[47]**CG**TGAGGGTCTCTGGCTT[48]
CGTTGGGAGGTCAGGTACCTTGTTCTAG[49]**CG**[50]**CG**TTT[51]**CG**[52]**CG**ACCCTGCT[53]**CG**

B.2 Transcription factor binding at the Apex promoter

Genomatix generated transcription factor consensus sequence binding on the positive strand of the *Apex* promoter.

TF	Description	Spans Assay CpG
EBOX	E-box binding factors	9
HIFF	Hypoxia inducible factor, bHLH/PAS protein famiy	-
HESF	Vertebrate homologues of enhancer of split complex	-
MTEN	Core promoter motif ten elements	-
CAAT	CCAAT binding factors	8, 9
AP2F	Activator protein 2	10, 11
INSM	Insulinoma associated factors	10, 11
CREB	cAMP-responsive element binding proteins	10, 11, 12, 13, 31, 32
PARF	PAR/bZIP family	11, 12, 13
E4FF	Ubiquitous GLI - Krueppel like zinc finger involved in cell cycle regulation	12, 13
NKXH	NKX homeodomain factors	12, 13, 14, 15
CDEF	Cell cycle regulators: Cell cycle dependent element	13, 14, 15, 16
NRSF	Neuron-restrictive silencer factor	26, 27, 28
HDBP	Huntington's disease gene regulatory region binding proteins	33, 34
E2FF	E2F-myc activator/cell cycle regulator	-
ZF01	C2H2 zinc finger transcription factors 1	-
BRN5	Brn-5 POU domain factors	-
RORA	v-ERB and RAR-related orphan receptor alpha	-
NF1F	Nuclear factor 1	-
CLOX	CLOX and CLOX homology (CDP) factors	-
EGRF	EGR/nerve growth factor induced protein C & related factors	-
KLFS	Krueppel like transcription factors	-
SP1F	GC-Box factors SP1/GC	-
XCPE	Activator-, mediator- and TBP-dependent core promoter element for RNA polymerase II transcription from TATA-less promoters	-
RXRF	RXR heterodimer binding sites	-

Table B.1 TFs binding at the *Apex* promoter, expressed in brain, liver or ubiquitously. Highlighted TFs are ones that are contained within the *Apex* pyrosequencing assay.

B.3 In vitro methylation of nested PCR product (Apex promoter)

Nested PCR was used to generate a product as close to full length of *Apex* promoter region as possible (Figure B.1A). Synthesis of an in vitro 100% methylated *Apex* promoter was confirmed using a methylation specific restriction enzyme digest. HpaII enzymes was used, the predicted sites of cleavage are shown in Figure B.1B. A predicted and actual gel of *Apex* promoter digest are shown in Figure B.1C and B.1D respectively.

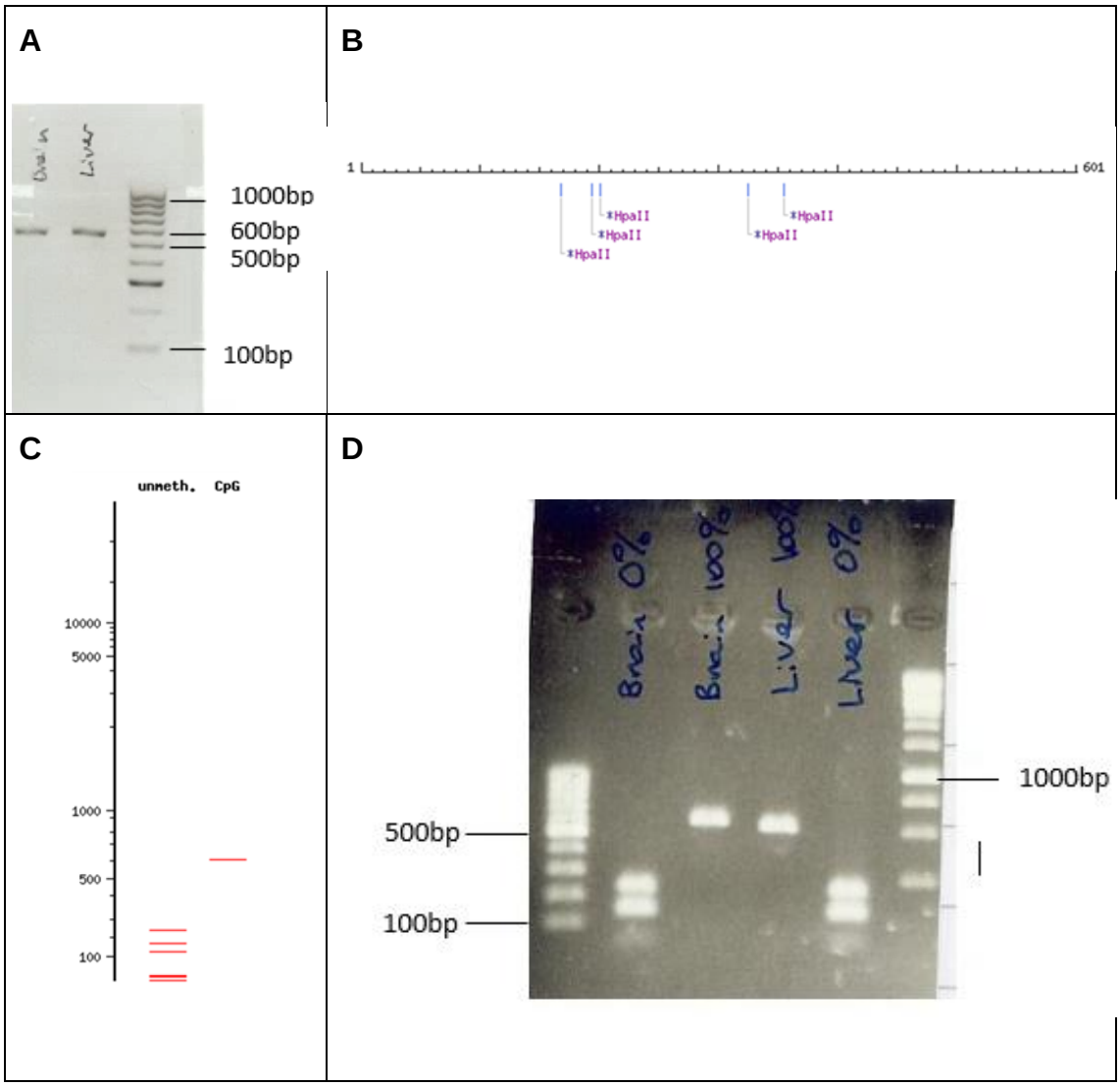

Figure B.1 *Apex* promoter, example of nested PCR gel (A), restriction sites of HpaII as generated by NEB Cutter Tool (B), virtual gel of predicted HpaII digest as generated by the NED Cutter Tool (C) and actual gel of HpaII digest (D).

B.4 Pyrosequencing Assay Validation

One forward and reverse primer were used to generate a product of 296bp and 2 separate sequencing primers were used to give the 2 *Apex* pyrosequencing

assays. Information on the primers can be found in Chapter 2. Figure B.2 shows example gel from pyro PCR of *Apex* sequences 1 and 2.

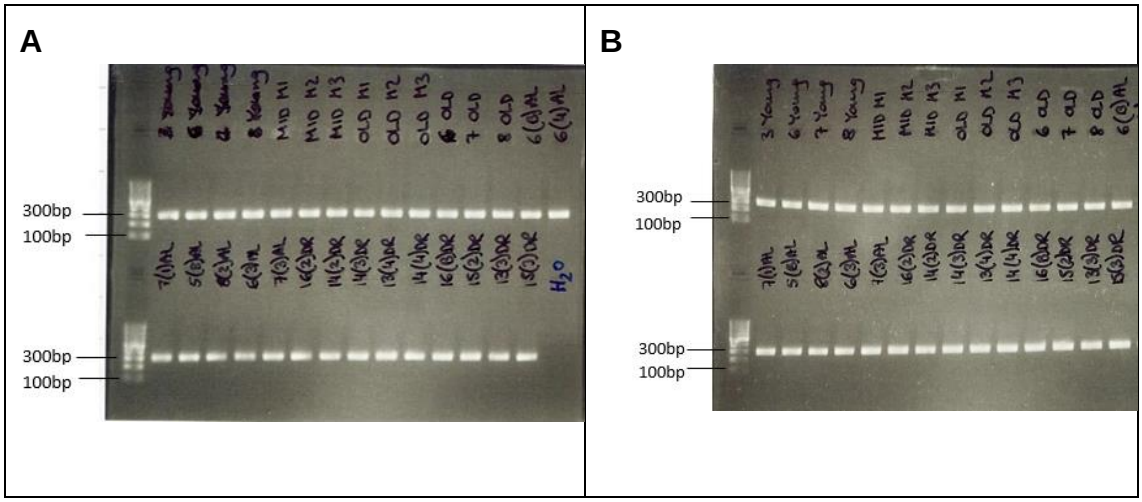

Figure B.2 Example of agarose gel from pyro PCR of *Apex* sequence 1: 296bp (A) and sequence 2: 296bp (B) with BM DNA from the liver.

Pre and post pyro-PCR dilution series, required to validate the assays, plotting observed versus expected methylation should yield an R^2 >0.9 to confirm specificity and sensitivity (Figure B.3).

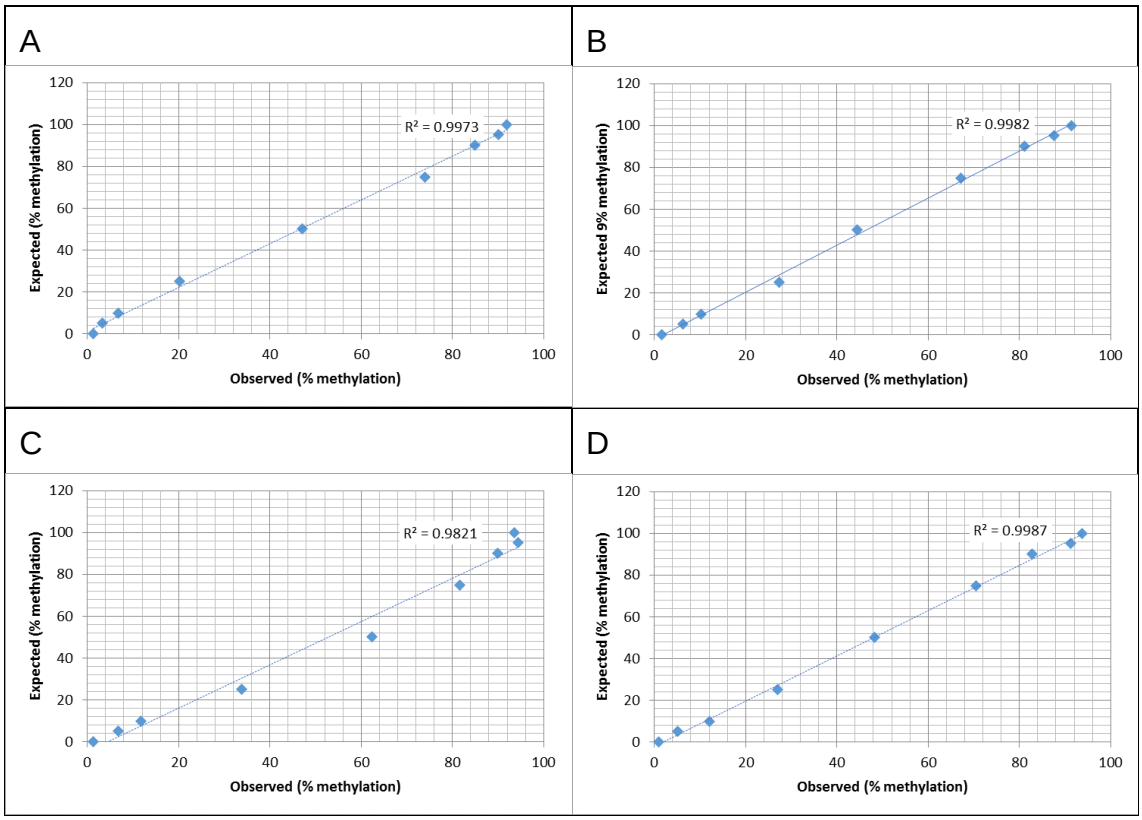

Figure B.3 *Apex* assay sequence 1 pre-PCR dilution series (A), sequence 1 post-PCR dilution series (B), sequence 2 pre-PCR dilution series (C) and sequence 2 post-PCR dilution series (D).

B.5 Pyrosequencing pyrograms

Example of pyrograms from pyrosequencing of *Apex* sequence 1 and 2 (Figure B.4).

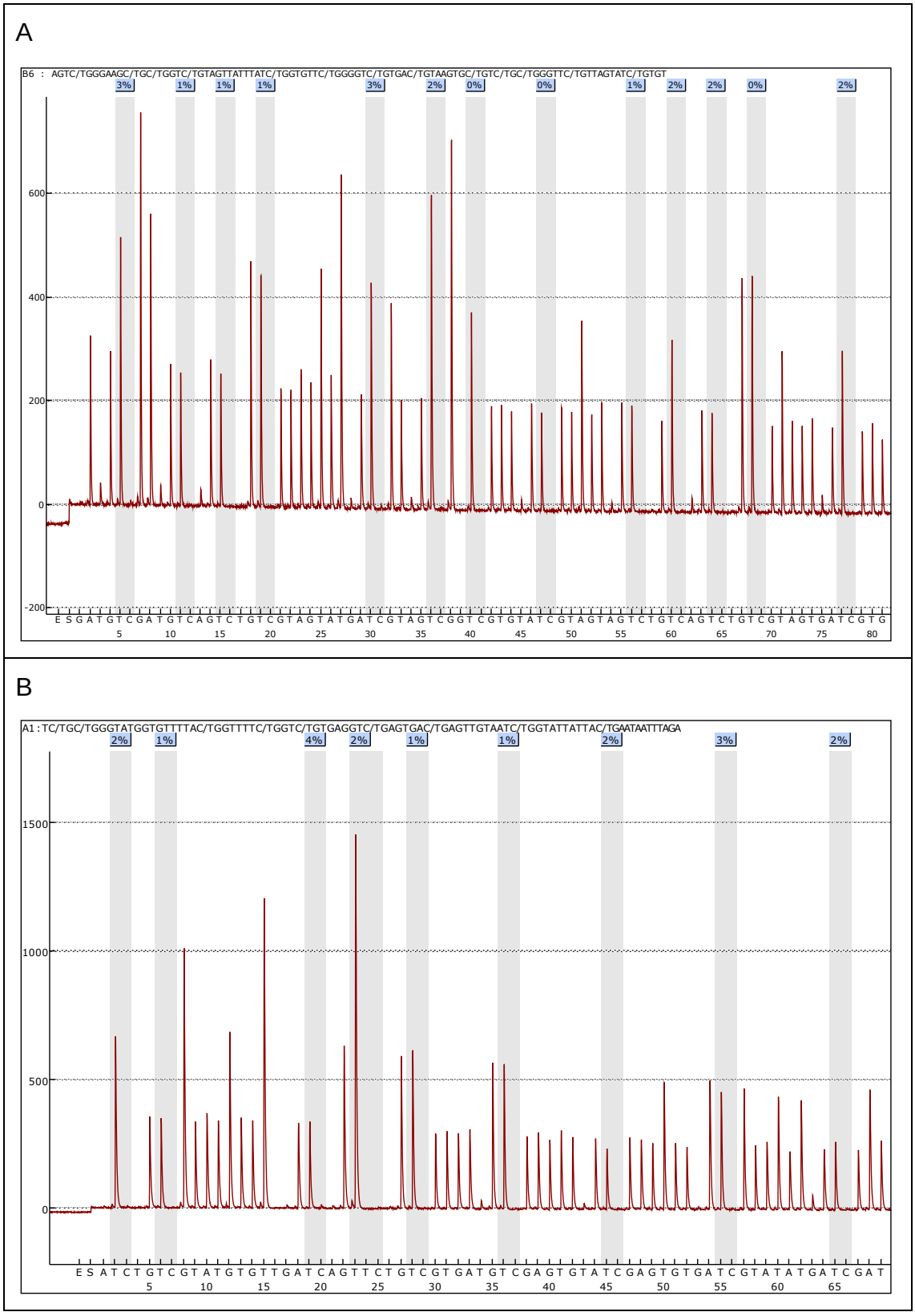

Figure B.4. Example of a pyrogram reading for *Apex* sequence 1 (A) and sequence 2 (B).

B.6 Cytosine methylation at individual CpG sites

Mean and median cytosine methylation from animals in the long term-term DR study (Chapter 5 and 6) as pleasured by pyrosequencing. Methylation in the brain of male mice (Table B.2), brain of female mice (Table B.3), liver of male mice (Table B.4) and liver of female mice (Table B.5).

CpG site		Cytosine methylation (%)						
		3AL	12AL	12DR	24AL	24DR	30AL	30DR
5	Mean	2.3630	2.5886	2.8264	2.2171	2.1371	1.8790	2.1579
	Median	2.2350	2.3850	2.9750	2.3550	2.2000	1.8200	2.1700
	SD	0.9429	0.5208	1.4253	0.3302	0.6715	1.0541	0.4637
	P value	All > 0.05						
6	Mean	1.6150	0.8964	0.7057	1.3243	0.6100	1.2120	1.1929
	Median	1.6000	1.0200	0.7750	1.3050	0.6150	0.7850	1.2750
	SD	0.3723	0.5066	0.4195	0.2919	0.5090	1.1243	0.4317
	P value	All > 0.05						
7	Mean	0.9530	1.2250	1.1307	0.7250	1.1064	1.0460	1.2364
	Median	1.1950	1.2100	1.2100	0.6150	1.2250	0.7250	1.2900
	SD	0.3793	0.3812	0.5810	0.5275	0.4295	1.2786	0.2981
	P value	All > 0.05						
8	Mean	1.3570	1.6914	1.1186	1.1600	1.0929	1.4190	1.3964
	Median	1.1700	1.6550	1.2100	1.0800	1.0650	0.9200	1.4350
	SD	0.5359	0.2636	0.7081	0.5393	0.4295	1.4523	0.4177
	P value	All > 0.05						
9	Mean	3.0970	3.1721	3.2750	2.7429	2.7786	3.1980	2.5614
	Median	2.8450	3.0800	3.0950	2.6800	2.6500	3.1500	2.6600
	SD	0.4191	0.6498	1.0532	0.8142	0.8578	0.9674	0.4371
	P value	All > 0.05						
10	Mean	1.2830	1.5964	1.4429	1.8407	1.3107	1.4920	1.0164
	Median	1.3600	1.7100	1.6050	1.6700	1.1050	0.9550	0.7700
	SD	0.8701	0.5014	0.7568	0.6520	0.4896	1.0856	0.5553
	P value	All > 0.05						
11	Mean	0.8810	0.5793	0.8586	0.7657	0.7657	0.6130	0.6307
	Median	0.9200	0.5900	0.7350	0.9200	0.7050	0.0000	0.6850
	SD	0.6001	0.5997	0.7310	0.3556	0.1217	1.3707	0.3452
	P value	All > 0.05						
12	Mean	0.4890	0.7500	0.5943	0.8364	0.3907	0.7390	0.9057
	Median	0.0000	0.7550	0.7250	0.8250	0.6100	0.0000	0.6550
	SD	0.7208	0.6889	0.4466	0.5382	0.3673	1.3195	0.5642
	P value	All > 0.05						
13	Mean	1.3650	1.6307	2.1650	1.6164	1.4121	1.5120	1.5450
	Median	1.3150	1.5250	1.6200	1.6550	1.5950	1.0600	1.8250
	SD	0.4711	0.2390	2.2845	0.5284	0.6090	1.1000	0.4308
	P value	All > 0.05						
14	Mean	1.0960	1.3257	0.8950	0.8600	0.9143	1.3390	0.8593
	Median	1.0250	1.6750	0.8350	0.8200	0.9050	0.6700	0.8150
	SD	0.7414	0.6440	0.7407	0.7415	0.7675	1.3833	0.6883
	P value	All > 0.05						
15	Mean	1.6160	1.0150	0.9393	0.9757	0.5350	1.1320	1.0636
	Median	1.6250	0.9450	1.1050	0.6850	0.6250	0.8400	1.2700
	SD	0.6469	0.6043	0.9479	0.7853	0.5369	1.3564	0.5954
	P value	All > 0.05						
16	Mean	0.7580	0.6907	0.3021	0.2064	0.4764	0.8660	0.2929
	Median	0.8800	0.6950	0.0000	0.0000	0.6750	0.0000	0.0000
	SD	0.7561	0.5378	0.7994	0.3526	0.4531	1.3898	0.3656
	P value	All > 0.05						
17	Mean	3.5550	2.7443	2.1971	2.6393	1.9614	2.2430	2.3907

	Median	3.5400	2.6650	2.4750	2.8700	1.7750	2.2100	2.3450
	SD	0.4342	0.6167	1.1807	0.9856	0.6262	1.1663	0.3639
	P value	1.000	>0.05	>0.05	>0.050	0.044	>0.05	>0.05
26	**Mean**	1.7090	1.7871	1.8386	1.9786	1.8207	2.0420	1.7007
	Median	1.7000	1.6650	1.6600	2.0400	1.9700	2.1850	1.6250
	SD	0.4172	0.2984	0.4761	0.3471	0.5621	0.5072	0.2389
	P value	All > 0.05						
27	**Mean**	0.4990	0.3343	0.1186	0.1129	0.0636	0.0700	0.3793
	Median	0.3350	0.3050	0.0000	0.0000	0.0000	0.0000	0.0000
	SD	0.5506	0.3448	0.2238	0.1928	0.1682	0.1565	0.7040
	P value	All > 0.05						
28	**Mean**	3.2460	3.0321	3.1936	3.1543	2.9693	3.2340	2.7421
	Median	3.1850	3.2150	3.0700	3.1200	2.9600	3.1950	2.6050
	SD	0.7180	0.3860	0.4531	0.5102	0.5600	0.3205	0.4867
	P value	All > 0.05						
29	**Mean**	1.7290	1.4229	1.5357	1.4829	1.3757	1.8930	1.6679
	Median	1.6950	1.4850	1.4700	1.5400	1.3600	1.9000	1.5900
	SD	0.3201	0.3992	0.5629	0.0969	0.5548	0.2234	0.8667
	P value	All > 0.05						
30	**Mean**	0.9050	0.7450	0.8464	0.8736	0.5793	1.2890	0.9307
	Median	0.8050	0.8650	0.7250	0.9100	0.4950	1.5200	0.8550
	SD	0.6406	0.5451	0.4114	0.2272	0.3853	0.5749	0.3981
	P value	All > 0.05						
31	**Mean**	1.5480	1.4793	0.9607	0.7764	0.9814	1.0180	0.9536
	Median	1.5600	1.4800	1.0850	0.7800	0.9600	1.0200	1.0150
	SD	0.0948	0.2553	0.3306	0.4651	0.2329	0.4187	0.4775
	P value	1.000	>0.005	0.014	>0.05	>0.05	>0.05	>0.05
32	**Mean**	1.4330	1.2979	0.7386	0.4386	0.9350	0.5910	0.9014
	Median	1.2500	1.5050	0.7200	0.5700	1.0450	0.7350	0.5800
	SD	0.5238	0.4953	0.4295	0.3149	0.5084	0.3332	0.8089
	P value	1.000	>0.05	>0.05	0.047	>0.05	>0.05	>0.05
33	**Mean**	2.1760	2.0529	2.0243	1.2743	1.2907	1.3800	1.3843
	Median	2.0350	1.9700	2.1900	1.3600	1.2600	1.2900	1.2350
	SD	0.2848	0.3781	0.6378	0.2912	0.4652	0.4982	0.7564
	P value	All > 0.05						
34	**Mean**	2.4350	2.5150	2.6193	2.2536	2.1429	2.4730	2.0371
	Median	2.4600	2.5150	2.9400	2.2150	2.0900	2.4750	2.1200
	SD	0.4902	0.4761	0.7279	0.7073	0.7774	0.2114	0.2879
	P value	All > 0.05						
Overall	**Mean**	1.6413	1.5715	1.4694	1.3752	1.2568	1.4855	1.3612
	Median	1.5245	1.5452	1.4564	1.3777	1.3970	1.2255	1.3225
	SD	0.3154	0.2396	0.4952	0.2304	0.2642	0.5775	0.2411
	P value	All > 0.05						

Table B.2 Percentage cytosine methylation in the brain from male mice from long-term DR study. Comparison to 3AL group (1.000), statistically significant difference <0.05.

CpG site		**Cytosine methylation (%)**						
		3AL	**12AL**	**12DR**	**24AL**	**24DR**	**30AL**	**30DR**
5	**Mean**	1.2450	1.5857	1.1529	1.8383	0.7121	0.9550	1.0264
	Median	1.2750	1.2700	0.9450	1.2150	0.4600	1.1200	0.8800
	SD	0.7126	0.8387	0.6171	1.8267	0.4813	0.7096	0.4288
	P value	All > 0.05						
6	**Mean**	1.0600	1.1350	0.9207	1.0967	0.5293	1.0393	0.8250
	Median	1.2750	1.1100	0.9250	1.1275	0.4450	0.7600	0.8350
	SD	0.5112	0.1794	0.2152	0.1329	0.3968	0.6002	0.6090
	P value	All > 0.05						
7	**Mean**	0.6170	0.9993	0.5450	0.8942	0.3379	0.7021	0.7193
	Median	0.9250	0.8350	0.4200	0.8650	0.3050	0.5650	0.6700
	SD	0.5700	0.6577	0.3927	0.1921	0.3830	0.4542	0.6520

	P value	All > 0.05						
8	**Mean**	1.1390	0.9329	1.0029	0.6683	0.1164	0.8521	0.8507
	Median	1.2600	0.9700	0.9450	0.5325	0.0000	0.4450	0.7150
	SD	0.2552	0.0879	0.2781	0.1038	0.0430	0.5272	0.2730
	P value	1.000	>0.05	>0.05	>0.05	0.033	>0.05	>0.05
9	**Mean**	0.5052	0.2965	0.5273	0.3221	0.2075	0.7261	0.5225
	Median	2.7610	2.8671	2.6864	2.7092	1.4886	3.2543	3.3400
	SD	2.3400	2.7250	2.9350	2.6000	1.4550	3.2550	3.1100
	P value	All > 0.05						
10	**Mean**	1.6302	0.4781	0.7917	0.4438	0.9471	0.9428	0.9580
	Median	1.5320	1.4350	1.0950	0.9650	0.5064	1.1750	0.9200
	SD	1.6900	1.4150	1.1000	1.0200	0.5350	1.3400	0.9300
	P value	1.000	>0.005	>0.05	>0.05	0.003	>0.05	>0.05
11	**Mean**	0.5202	0.4441	0.4047	0.3769	0.4291	0.5427	0.1341
	Median	0.5950	0.8400	0.4564	0.3242	0.4507	0.8221	0.7021
	SD	0.5600	0.9350	0.4450	0.2125	0.0000	1.0650	0.7600
	P value	All > 0.05						
12	**Mean**	0.6396	0.2986	0.4990	0.4139	0.5684	0.6804	0.4067
	Median	0.9120	1.0050	0.9686	0.9692	0.8250	1.0979	1.0571
	SD	0.7550	1.0200	0.9800	1.0650	0.5700	1.0400	1.1050
	P value	All > 0.05						
13	**Mean**	0.3570	0.6233	0.1923	0.2856	0.4624	0.6014	0.6599
	Median	1.5400	1.4350	1.1443	1.3883	0.9179	1.4057	1.6200
	SD	1.5300	1.5050	1.2700	1.3625	1.0650	1.2550	1.4600
	P value	All > 0.05						
14	**Mean**	0.1437	0.1855	0.3979	0.2710	0.4060	0.5736	0.4222
	Median	1.2520	1.3871	1.1614	0.9508	0.3871	1.1900	1.1893
	SD	1.5800	1.4850	1.3200	0.7125	0.5300	0.7450	1.4000
	P value	All > 0.05						
15	**Mean**	0.4926	0.3337	0.3978	0.4996	0.3888	0.6837	0.3565
	Median	0.6150	0.8100	0.6957	0.3350	0.2621	0.3529	0.7036
	SD	0.9550	0.5650	0.6300	0.2175	0.3950	0.0000	0.5950
	P value	All > 0.05						
16	**Mean**	0.5659	0.6878	0.5782	0.4307	0.2477	0.6120	0.4394
	Median	0.7900	0.6750	0.5200	0.5325	0.1914	0.5464	0.7464
	SD	0.9100	0.5850	0.5550	0.5800	0.0000	0.4900	0.6150
	P value	All > 0.05						
17	**Mean**	0.4782	0.5558	0.5397	0.4802	0.2417	0.6421	0.3067
	Median	3.0730	2.9107	2.5664	2.4392	1.3421	3.0079	2.8379
	SD	2.7450	2.7550	2.8650	2.4650	1.0950	2.7600	2.6850
	P value	1.000	>0.05	>0.05	>0.05	0.026	>0.05	>0.05
26	**Mean**	0.9528	0.4251	0.8271	0.4979	1.0101	1.2984	0.5514
	Median	1.3870	1.0079	1.1193	1.1808	0.6607	1.0014	0.9321
	SD	1.2800	1.0100	1.0700	1.1425	0.4400	0.9250	0.8400
	P value	All > 0.05						
27	**Mean**	0.6005	0.1458	0.1823	0.3788	0.4549	0.6397	0.4073
	Median	0.7360	0.4357	0.0850	0.2408	0.0429	0.2136	0.1864
	SD	0.5900	0.4550	0.0000	0.1350	0.0000	0.0000	0.2300
	P value	1.000	>0.05	0.007	>0.05	0.003	>0.05	0.043
28	**Mean**	0.3887	0.4406	0.1464	0.2934	0.1134	0.2958	0.1881
	Median	2.7100	2.4543	2.4600	2.3733	1.7579	2.3543	2.7943
	SD	2.6050	2.4300	2.5000	2.3775	1.6950	2.3250	2.7100
	P value	1.000	>0.05	>0.05	>0.05	0.003	>0.05	>0.05
29	**Mean**	0.4890	0.4644	0.3170	0.3347	0.4313	0.3942	0.2821
	Median	2.0760	1.7371	1.8114	1.7733	1.5186	2.0800	1.9386
	SD	2.2100	1.7100	1.8350	1.8325	1.5350	2.0600	1.8450
	P value	All > 0.05						
30	**Mean**	0.4038	0.3191	0.2413	0.1993	0.3725	0.4905	0.3084
	Median	1.0220	0.5871	0.3936	0.4925	0.1850	0.5529	0.6679
	SD	1.0100	0.5050	0.4550	0.4475	0.0000	0.5550	0.6050

	P value	1.000	>0.05	0.031	>0.05	0.001	>0.05	>0.05
31	**Mean**	0.2360	0.3762	0.1857	0.2132	0.2587	0.3983	0.4049
	Median	1.4450	1.2114	0.9529	1.1125	0.6650	0.7943	0.9529
	SD	1.5250	1.0950	0.7750	1.2100	0.5300	0.6150	0.9450
	P value	1.000	>0.05	>0.05	>0.05	0.048	>0.05	>0.05
32	**Mean**	0.2739	0.1898	0.4527	0.3561	0.3172	0.5927	0.4849
	Median	1.2930	1.0629	0.6929	0.9783	0.7893	0.9536	0.6743
	SD	1.2250	0.9900	0.5100	1.0700	0.6250	0.6050	0.7550
	P value	All > 0.05						
33	**Mean**	0.4918	0.3649	0.2856	0.4075	0.3250	0.7521	0.3594
	Median	2.0760	1.5250	1.1079	1.2933	0.6900	1.0264	1.0886
	SD	2.0850	1.6150	1.0850	1.3150	0.6150	0.7400	1.1750
	P value	1.000	>0.05	>0.05	>0.05	0.003	>0.05	>0.05
34	**Mean**	0.4065	0.7387	0.4003	0.6445	0.2842	0.8039	0.3533
	Median	2.5660	2.1629	1.8136	1.9092	1.1800	2.0800	2.1593
	SD	2.4750	2.2750	1.9950	1.8775	1.3700	1.9250	2.1000
	P value	1.000	>0.05	>0.05	>0.05	0.003	>0.05	>0.05
Overall	**Mean**	0.7452	0.2258	0.5392	0.3880	0.6856	0.7767	0.2972
	Median	1.4748	1.3730	1.1523	1.2030	0.7073	1.2479	1.2696
	SD	1.3450	1.2890	1.2590	1.2780	0.6320	1.2000	1.2180
	P value	1.000	>0.05	>0.05	>0.05	0.003	>0.05	>0.05

Table B.3 Percentage cytosine methylation in the brain from female mice from long-term DR study. Comparison to 3AL (1.00), statistically significant difference <0.05.

CpG site		**Cytosine methylation (%)**						
		3AL	**12AL**	**12DR**	**24AL**	**24DR**	**30AL**	**30DR**
5	**Mean**	2.1190	1.2750	2.4471	1.5864	0.5071	0.4770	1.0429
	Median	2.2550	1.3400	1.0000	1.5950	0.3800	0.3600	0.4700
	SD	1.1838	1.2493	2.9621	0.8913	0.4817	0.5712	1.3303
	P value	All > 0.05						
6	**Mean**	1.4060	1.0850	1.9079	1.3907	0.6936	0.6640	1.2750
	Median	1.5550	0.8550	1.3750	1.4350	0.3300	0.3600	1.0250
	SD	0.8236	0.8277	2.8127	0.9500	0.8768	0.6263	0.9875
	P value	All > 0.05						
7	**Mean**	0.9690	1.1479	1.5836	1.0729	0.4221	0.1930	0.8793
	Median	1.4850	1.1250	1.3400	1.0400	0.0000	0.0000	0.0000
	SD	0.8915	1.2663	2.2412	0.9714	0.7229	0.2669	1.2092
	P value	All > 0.05						
8	**Mean**	1.0170	1.6964	2.1564	1.3329	1.1350	1.2850	2.0329
	Median	1.4700	1.5750	0.9300	1.5600	1.1400	1.3800	1.9600
	SD	0.9474	0.9934	3.2031	0.8652	0.6826	0.2557	1.6205
	P value	All > 0.05						
9	**Mean**	4.1760	4.1386	4.9171	4.6043	2.7236	2.9440	3.7614
	Median	3.4350	4.1400	4.3700	4.6950	2.4500	3.1300	3.4250
	SD	1.5088	1.6514	2.1065	1.9585	1.1557	0.8839	1.9217
	P value	All > 0.05						
10	**Mean**	2.0130	1.9321	2.3900	1.7200	0.8771	0.8500	1.3121
	Median	2.2300	1.6450	1.6150	1.6350	1.1200	0.9400	0.8850
	SD	1.1106	1.3805	2.4573	0.9890	0.5453	0.6172	1.1909
	P value	All > 0.05						
11	**Mean**	1.3060	1.6257	2.1893	1.5000	1.3364	0.5300	1.3443
	Median	0.5250	2.1050	1.4950	1.9300	1.4100	0.5650	1.2300
	SD	1.9862	1.5744	3.0198	1.3949	1.2733	0.5491	1.3624
	P value	All > 0.05						
12	**Mean**	0.9950	1.3707	1.7200	1.2864	0.4221	0.3930	0.9879
	Median	0.6650	1.4400	1.3850	1.6050	0.4450	0.0000	1.1150
	SD	1.5036	0.9698	2.2635	0.9228	0.4243	0.6468	0.8727
	P value	All > 0.05						
13	**Mean**	2.2350	2.3043	2.7364	2.0100	1.3621	1.7110	2.0414

	Median	2.1000	2.2400	2.3600	2.1250	1.3100	1.7750	1.5650
	SD	0.8333	0.7865	2.2372	0.8564	0.4696	0.2755	0.9189
	P value	All > 0.05						
14	**Mean**	2.3200	1.9479	2.4893	2.5743	1.3271	1.2290	2.2843
	Median	2.4500	1.7850	1.9400	3.0650	1.4150	0.8200	1.8350
	SD	1.2441	1.4403	2.5088	1.2665	0.9860	1.2557	2.2232
	P value	All > 0.05						
15	**Mean**	1.5000	1.7057	1.7229	1.3364	0.6671	1.1240	1.6979
	Median	1.3250	1.9350	1.7600	1.3750	0.7100	1.1550	1.4400
	SD	1.0221	1.1018	1.8661	0.7899	0.5564	0.3197	1.1246
	P value	All > 0.05						
16	**Mean**	1.8100	1.9214	2.2950	1.5829	0.9243	1.2550	1.4357
	Median	1.5950	2.1700	1.7050	1.8300	0.5750	0.9400	1.0550
	SD	0.9574	1.3552	3.1368	1.1066	1.2714	0.7739	1.2077
	P value	All > 0.05						
17	**Mean**	4.0360	4.1929	4.6171	3.7214	3.8143	3.6540	3.3386
	Median	4.0000	4.4100	3.7000	3.5700	3.5150	3.3750	2.7900
	SD	0.7125	1.6569	2.3332	1.7058	2.5103	0.6036	1.3338
	P value	All > 0.05						
26	**Mean**	2.3570	1.6136	2.3321	2.3021	1.3000	1.7120	1.5379
	Median	3.6150	1.5050	1.6800	1.9950	0.3450	1.8450	1.4300
	SD	1.9958	1.2242	2.1285	1.5800	1.4432	1.2247	0.6790
	P value	All > 0.05						
27	**Mean**	1.5080	1.4364	2.0036	2.3486	0.5721	0.6820	0.8571
	Median	0.0000	1.2600	1.6900	0.9900	0.0000	0.0000	0.7950
	SD	2.0777	1.2050	2.4187	2.1851	1.5137	1.3635	0.6857
	P value	All > 0.05						
28	**Mean**	4.2820	4.4050	3.7314	3.4271	3.3229	3.4860	2.6571
	Median	2.5850	3.9900	3.5400	2.9450	2.9900	3.4150	2.8350
	SD	3.6271	2.6664	0.9942	1.4500	1.5890	0.9555	0.4639
	P value	All > 0.05						
29	**Mean**	3.0190	2.5129	3.1343	3.1757	1.8679	2.3110	2.5286
	Median	3.7900	2.4700	2.3800	1.9250	1.4950	1.9100	2.6300
	SD	1.6050	1.0908	2.1930	2.0552	0.9671	1.0261	0.7747
	P value	All > 0.05						
30	**Mean**	1.7550	1.3364	1.8150	1.6329	0.6400	0.8540	0.8800
	Median	0.4650	1.1400	1.5450	0.9550	0.4500	0.4750	0.8900
	SD	2.0168	1.0397	1.8866	1.4217	0.7433	1.0149	0.6350
	P value	All > 0.05						
31	**Mean**	2.5790	1.8029	2.1764	1.3136	0.7643	1.3760	0.9164
	Median	2.4400	1.3000	1.6450	1.0250	0.5600	0.8850	0.6750
	SD	1.7725	1.4253	1.6928	1.1247	0.9880	1.4687	0.6871
	P value	All > 0.05						
32	**Mean**	2.1620	1.7271	2.8029	2.3929	1.1750	1.8140	1.3693
	Median	0.5750	1.1950	2.1400	1.7600	0.7850	0.9300	1.7400
	SD	2.4639	1.5138	1.9731	1.3757	1.2461	1.5300	0.6666
	P value	All > 0.05						
33	**Mean**	2.7890	3.3421	3.6421	2.5793	1.2914	1.1830	1.8229
	Median	1.7000	3.0250	2.3900	1.3500	1.0100	0.7400	2.2300
	SD	3.1851	2.0547	2.3443	2.5577	1.2297	1.2139	0.8552
	P value	All > 0.05						
34	**Mean**	3.3830	2.6236	3.0893	3.2950	2.4871	3.1310	2.5329
	Median	3.8200	2.3600	3.0800	2.6850	2.4450	2.9600	2.7100
	SD	3.3758	1.1518	1.5823	1.6359	1.8932	0.8594	1.0864
	P value	All > 0.05						
Overall	**Mean**	2.2600	2.0629	2.6200	2.1829	1.3471	1.4940	1.7529
	Median	1.4300	2.1200	1.9700	1.8100	1.1400	1.3000	1.6200
	SD	1.2704	0.6689	2.1362	0.8851	0.5744	0.5310	0.8692
	P value	All > 0.05						

Table B.4 Percentage cytosine methylation in the liver from male mice from long-term DR study. Comparison to 3AL (1.000), statistically significant difference <0.05.

CpG site		Cytosine methylation (%)						
		3AL	12AL	12DR	24AL	24DR	30AL	30DR
5	Mean	2.7810	2.5807	2.5257	1.8257	1.9343	1.6686	3.2057
	Median	2.4250	2.4700	2.0900	1.4050	1.4200	1.3700	1.4600
	SD	1.9353	1.0530	1.0362	1.0836	1.2623	0.6750	4.7983
	P value	All > 0.05						
6	Mean	1.8430	1.7686	1.6436	1.6414	1.3736	0.5157	1.0314
	Median	1.8550	2.2450	1.4900	1.5350	1.0450	0.4450	0.6550
	SD	0.6630	0.8035	1.0991	0.6865	1.1966	0.5305	1.2774
	P value	All > 0.05						
7	Mean	1.3410	1.8193	1.6879	1.3864	0.6850	0.3971	0.6886
	Median	1.6050	1.8850	1.3350	1.2350	0.5350	0.3000	0.7200
	SD	0.7785	0.9289	0.8605	0.6001	0.5488	0.5465	0.6425
	P value	All > 0.05						
8	Mean	1.8990	2.1557	1.6814	2.0943	1.0450	0.4943	0.8979
	Median	1.8400	2.2650	1.4500	2.1500	0.9550	0.0000	0.7150
	SD	1.2063	1.0149	1.0947	0.7188	0.8361	0.6885	0.6478
	P value	All > 0.05						
9	Mean	3.6450	4.5150	3.9686	4.3679	4.6143	2.2500	3.6007
	Median	3.9400	3.9450	3.9700	4.0900	4.6550	2.3250	4.0700
	SD	0.5735	1.1309	1.4091	1.2386	3.0159	1.4695	1.7677
	P value	All > 0.05						
10	Mean	1.7280	2.9529	1.7957	2.5414	1.4193	1.3714	1.2193
	Median	2.0350	3.0100	1.8100	2.2550	0.9550	1.7250	1.0550
	SD	0.7370	1.0272	0.8373	1.0479	0.9409	0.9617	0.8199
	P value	All > 0.05						
11	Mean	2.0470	2.3529	1.9971	1.7736	1.4471	0.7207	1.1479
	Median	2.0450	2.4850	1.7650	1.5700	1.3750	0.5500	0.9800
	SD	0.6953	0.8388	1.2426	0.8699	1.2198	0.5317	0.5086
	P value	All > 0.05						
12	Mean	1.9010	2.3321	1.7257	1.4886	1.0364	0.7614	0.7436
	Median	2.1150	2.4850	1.2450	1.4600	0.5700	0.5600	0.7750
	SD	0.7927	1.0348	1.1217	0.7365	0.9090	0.7625	0.7691
	P value	All > 0.05						
13	Mean	2.1130	2.7493	2.3364	2.3021	1.6807	1.5093	1.9607
	Median	2.0600	2.3500	1.9500	1.9450	1.7450	1.3300	1.7800
	SD	0.4010	1.0494	1.0791	0.9427	0.3580	0.3743	0.7733
	P value	All > 0.05						
14	Mean	2.5800	2.9514	2.6357	2.7014	1.8357	1.6457	2.0900
	Median	2.5250	2.5650	2.4500	2.5750	1.4450	1.6650	1.9200
	SD	0.7762	1.0296	1.1221	0.8851	1.0090	0.8588	0.6473
	P value	All > 0.05						
15	Mean	1.7330	2.4186	1.6421	1.7614	1.3964	1.3500	1.4621
	Median	1.8200	2.3500	1.4100	1.5700	1.4750	1.3750	1.4950
	SD	0.3625	0.7428	0.9474	0.5412	0.4106	0.3931	0.6410
	P value	All > 0.05						
16	Mean	2.4820	2.7057	1.9971	2.3407	1.6521	1.3943	1.2886
	Median	2.0100	2.7350	2.0650	2.0750	1.4900	1.3500	1.4450
	SD	1.4646	1.1090	0.9988	0.7674	0.7096	0.6056	0.6075
	P value	All > 0.05						
17	Mean	4.2960	4.7207	3.9971	4.7671	3.5336	3.7779	3.1921
	Median	4.1150	4.7550	3.2050	4.8300	3.3650	3.1800	3.2800
	SD	0.9193	0.6598	1.5275	1.1395	0.9525	1.0174	0.7238
	P value	All > 0.05						
26	Mean	1.3330	2.8236	3.4929	3.0814	1.5807	1.6600	2.0621
	Median	1.5150	2.3600	2.7150	2.9050	1.7050	1.3550	2.0500
	SD	0.5173	1.1269	1.6646	0.9922	1.0126	0.7954	0.4855

	P value	1.000	>0.05	0.02	>0.05	>0.05	>0.05	>0.05
27	**Mean**	0.2000	1.9786	2.0629	1.5200	0.2886	0.2200	0.5171
	Median	0.0000	1.3500	1.8900	0.7000	0.0000	0.0000	0.0000
	SD	0.2857	2.2103	2.2154	1.1354	0.3664	0.5821	0.8183
	P value	All > 0.05						
28	**Mean**	3.3700	3.9800	5.3421	5.2800	4.4429	4.9500	4.2507
	Median	3.4950	3.8200	5.5700	4.7250	4.2850	5.3150	4.3400
	SD	0.3437	0.7645	1.1716	1.0600	1.6242	0.9930	1.1387
	P value	All > 0.05						
29	**Mean**	1.5040	3.2064	3.6857	3.0836	1.8857	1.5686	2.3279
	Median	1.3100	2.6000	3.3500	2.7750	2.2250	1.3900	2.1850
	SD	0.5499	1.9464	1.5705	1.0283	0.9592	1.0169	0.6609
	P value	All > 0.05						
30	**Mean**	0.2990	1.5271	2.1729	1.4271	0.3336	0.3364	0.3871
	Median	0.0000	0.7500	1.9650	0.8700	0.0000	0.0000	0.0000
	SD	0.6686	1.9091	1.8108	1.2298	0.4526	0.6373	0.5705
	P value	All > 0.05						
31	**Mean**	1.1540	2.4243	2.6864	2.5993	1.3243	1.1593	1.6479
	Median	1.3050	1.7200	2.7250	2.5900	1.1300	1.0150	1.5950
	SD	0.8823	1.8218	1.6967	0.9781	0.8675	0.8875	1.1985
	P value	All > 0.05						
32	**Mean**	1.8570	2.8007	3.3379	3.0871	1.8593	1.8057	1.8329
	Median	1.9400	2.7000	3.1200	2.7150	1.8650	1.5000	1.6800
	SD	0.7116	1.7684	1.5940	1.3549	0.8669	1.1088	0.7755
	P value	All > 0.05						
33	**Mean**	1.0880	2.5300	2.9736	3.2814	1.1664	0.9264	1.8471
	Median	1.0050	2.2250	2.4050	3.7000	1.2600	0.0000	1.5200
	SD	0.8459	2.5406	2.1872	1.0423	1.0521	1.1584	0.9513
	P value	All > 0.05						
34	**Mean**	1.8620	3.3079	3.6221	4.0650	2.6850	2.1586	3.0714
	Median	2.0150	3.4550	3.2500	4.0800	1.8100	1.9750	2.9250
	SD	0.6405	1.5402	1.3619	0.5849	1.9135	1.4182	0.6191
	P value	All > 0.05						
Overall	**Mean**	1.9572	2.7544	2.6823	2.6554	1.7829	1.4837	1.8397
	Median	2.0130	2.7650	2.5450	2.5100	2.0300	1.3630	1.5770
	SD	0.5451	1.0194	0.8588	0.6662	0.4593	0.3755	0.4895
	P value	All > 0.05						

Table B.5 Percentage cytosine methylation in the liver from female mice from long-term DR study. Comparison to 3AL (1.00), statistically significant difference <0.05.

B.7 Validation of primers for ChIP-qPCR

Primers for amplification of *Apex* promoter region were validated with liver genomic DNA from the ChIP input reaction. A qPCR reaction shows good amplification for a dilution series of DNA and the primers. Primers were designed by Dr Jelena Mann.

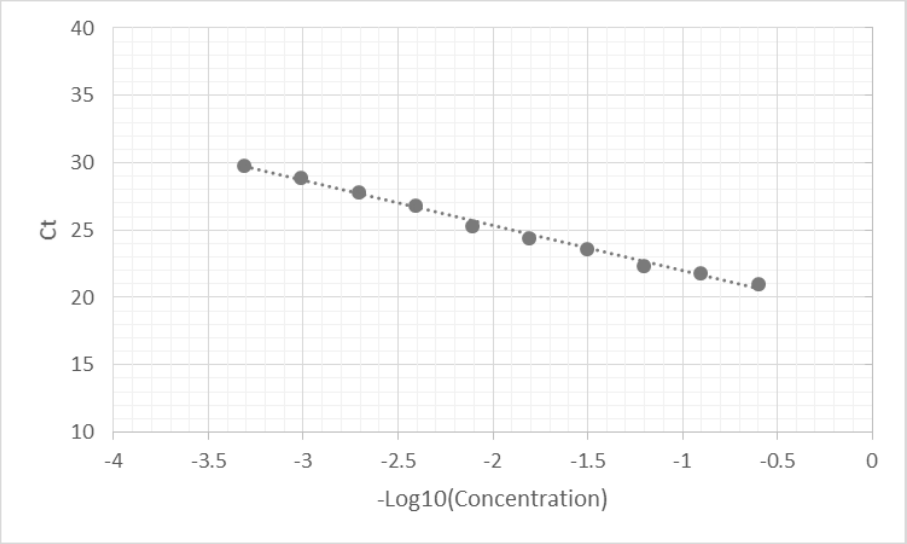

Figure B.5 Validation of assay used for ChIP-qPCR. Efficiency 98.2, slope -3.37

B.8 Apex cDNA Sequence acquired from Genomatix software

CACGTGACCACACCAATCGCCCACGTGGGAACAGGCAGACTCCATTCTTTGTGCCGTGAG
GGTCTCTGGCTTCGTTGGGAGGCAGCGCAGTAAACACTGCTTCGGTGCTCCAGGAGCCTA
AGGGCTTTCGTCACAGCGATGCCAAAGCGGGGAAAGAAAGCGGCGGCCGACGACGGGGA
AGAACCCAAGTCGGAGCCAGAGACCAAGAAGAGTAAGGGGGCAGCAAAGAAAACCGAGA
AGGAGGCCGCGGGAGAGGGCCCTGTCCTGTACGAGGACCCTCCAGATCAGAAAACCTCA
CCCAGTGGCAAATCTGCCACACTCAAGATATGCTCCTGGAATGTGGATGGGCTTCGAGCC
TGGATTAAAAAGAAAGGTTTGGATTGGGTAAAGGAAGAAGCACCAGATATCTTGTGCCTCC
AAGAGACCAAGTGCTCGGAGAACAAACTCCCGGCTGAACTGCAAGAGCTGCCTGGACTCA
CCCATCAGTACTGGTCAGCTCCGTCAGACAAAGAAGGATACAGTGGTGTGGGCCTACTTT
CCCGCCAGTGCCCGCTAAAAGTCTCTTATGGCATTGGCGAGGAAGAACATGATCAAGAAG
GCCGGGTGATTGTGGCTGAATTTGAGTCCTTTGTCCTGGTAACAGCCTATGTTCCCAATGC
AGGCAGGGGTCTGGTAAGACTGGAATACCGACAGCGTTGGGATGAAGCCTTCCGAAAGTT
TCTAAAGGACTTGGCTTCCAGAAAGCCTCTTGTGCTATGTGGGGATCTCAATGTGGCTCAT
GAAGAAATTGACCTCCGTAACCCCAAAGGAAACAAAAAGAATGCTGGCTTTACTCCCCAGG
AGCGCCAAGGTTTTGGGGAACTGCTACAAGCTGTACCATTGGCTGACAGCTTCCGGCATC
TCTACCCCAACACTGCTTACGCTTACACTTTCTGGACTTACATGATGAATGCCCGCTCTAAG
AATGTTGGTTGGCGCCTTGATTACTTTTTGCTTTCCCACTCTCTTTTACCTGCATTGTGTGA
CAGCAAGATCCGGTCCAAGGCTCTTGGCAGTGACCACTGTCCCATCACCCTTTACCTAGCA
CTGTGATACCCTCCTGCAGTAGCTTCCTGCCTGGGAGATGGCTCTCTCTGCAGAAGTCTG
GTGTTTTAGCCTTCAGGTGTTTGGTTTTGTATGTGCTCCCTCATTTTAAACATTAAACCAAAC
TTCTGGTTTCCTTTAGACAATCCAAGAGAAATAAAAGGCCCTACTTTCAGCTTGGCTTTCTT
TGTCCCTTTGTGAAAACTACATATTCCCTTTTTCAT

B.9 Apex expression qPCR primer validation

Validation of primers for qPCR assay. Primer design Joanna Górniak (Apex) and Dr Sabine Langie (*Hprt* and *B2m*).

Assay	Efficiency factor	Efficiency (%)	Slope
Apex (Target)	2.01	101.44	-3.855
Hprt (Reference)	1.96	96.37	-3.412
B2m (Reference)	1.90	90.22	-3.581

Table B.6 Primer test and assay efficiency as tested with cDNA from liver.

B.10 APEX ELISA assay

Protein extract concentration at 5mg/ml and 1mg/ml were tested in the APEX ELISA assay to optimise extract concentration to be used (Figure B.6). Kit standard was used to set up a standard curve.

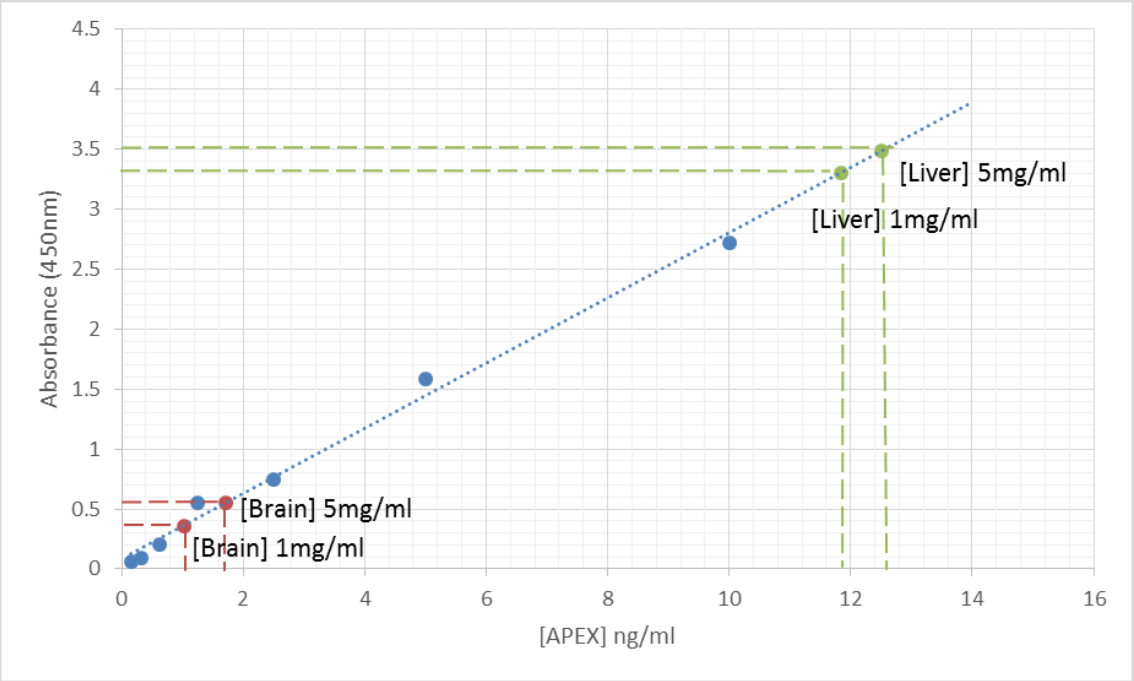

Figure B.6 Optimisation of assay APEX ELISA, protein extracts from brain (red) and liver (green) at 5mg/ml and 1 mg/ml were tested versus assay standard (blue) to determine concentration of extract to use in the assay.

Appendix C RNA quality and TF expression validation

C.1 RNA check

RNA quality was tested for purity using the Nanodrop with values for 260/280 and 230/260 of >1.8. In addition, RNA was tested for degradation by agarose gel electrophoresis. 2 clear bands or rRNA indicated intact RNA.

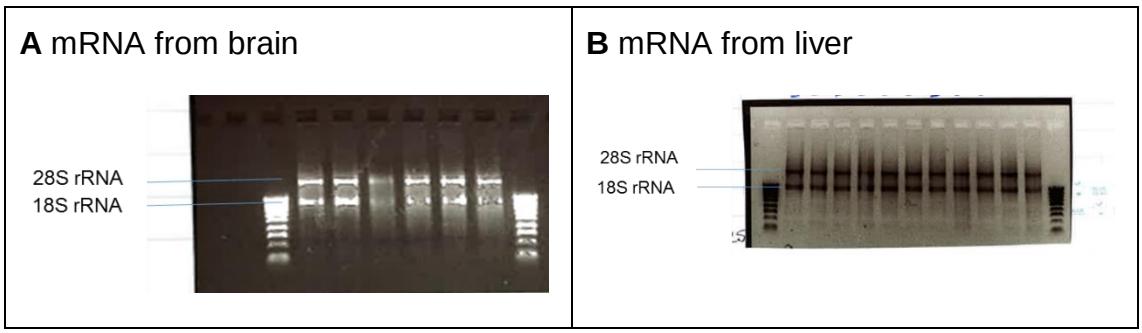

Figure C.1 Example of RNA extracted from brain (A) and liver (B). Two clear strong bands for ribosomal RNA confirm intact RNA.

C.2 cDNA check (Gapdh primers, +RT and –RT)

A PCR for *Gapdh* gene amplification was used to test for genomic contamination after cDNA synthesis (2 reactions were set up: reverse transcriptase positive and RT negative). Product in RT positive reaction indicated successful cDNA synthesise while no product in RT negative reaction indicated no genomic DNA contamination.

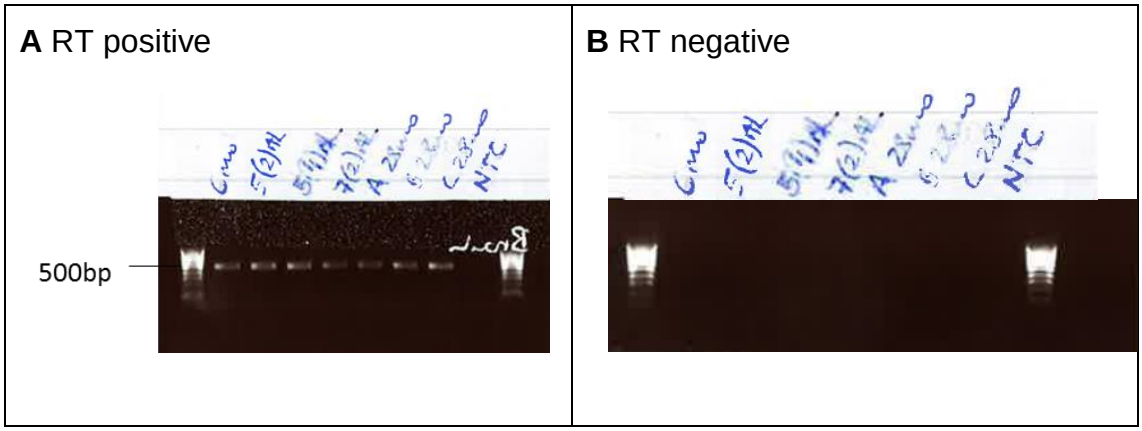

Figure C.2 Example of cDNA synthesis confirmation PCR, GAPDH amplification (495bp product) with reverse transcriptase (A) and without reverse transcriptase (B) to confirm synthesis of cDNA and no genomic DNA contamination.

C.3 TF mRNA expression assay validation (TaqMan)

Expression of 2 transcription factors was quantified as these are bind to and initiate up-regulation of *Ogg1* and *Apex* expression.

Figure C.3 A scheme showing the binding of Nfya (green) and Hif1α (purple) transcription factors at *Ogg1* gene promoter region. Schemes generated via Genomatix software.

Figure C.4 A scheme showing the binding of Nfya (green) and Hif1α (purple) transcription factors at *Apex* gene promoter region. Schemes generated via Genomatix software.

Study of transcription factor expression, HIf1α and Nfya expression were quantified using B2m and Hprt as reference genes. Expression was quantified with TaqMan Probe assay, inventoried in the table.

Assay	Gene	Code
Hif1α	murine hypoxia inducible factor 1, alpha subunit	Mm00468869_m1
Nfya	nuclear transcription factor-Y alpha	Mm00477820_m1
B2m	beta-2 microglobulin	Mm00437762_m1
Hprt	hypoxanthine guanine phosphoribosyl transferase	Mm01545399_m1

Table C.1 Name and code of *Mus musculus* TaqMan assays used.

A standard curve with cDNA was set up for each TaqMan probe to test the efficiency of the reaction.

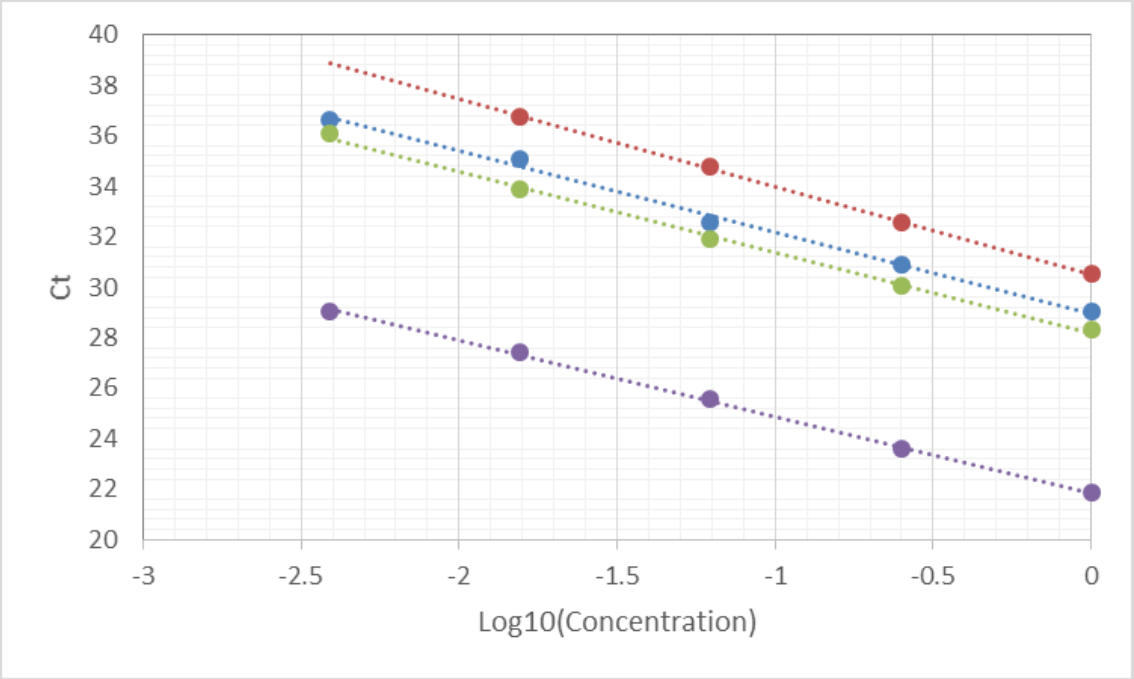

Figure C.5 Validation of assays used for qPCR. Hlf1α (dotted blue line), efficiency 104.2, slope -3.22; Nfya (dotted red line), efficiency 93.8, slope -3.48; B2m (dotted purple line), efficiency 114.6, slope -3.02; Hprt (dotted green line), efficiency 105.3, slope -3.20.

C.4 TF mRNA expression in brain and liver of short-term DR

Expression of TF was measured in tissues from long-term DR mice (Chapter 5 and 6) and also in tissues from short-term DR mice, figure below.

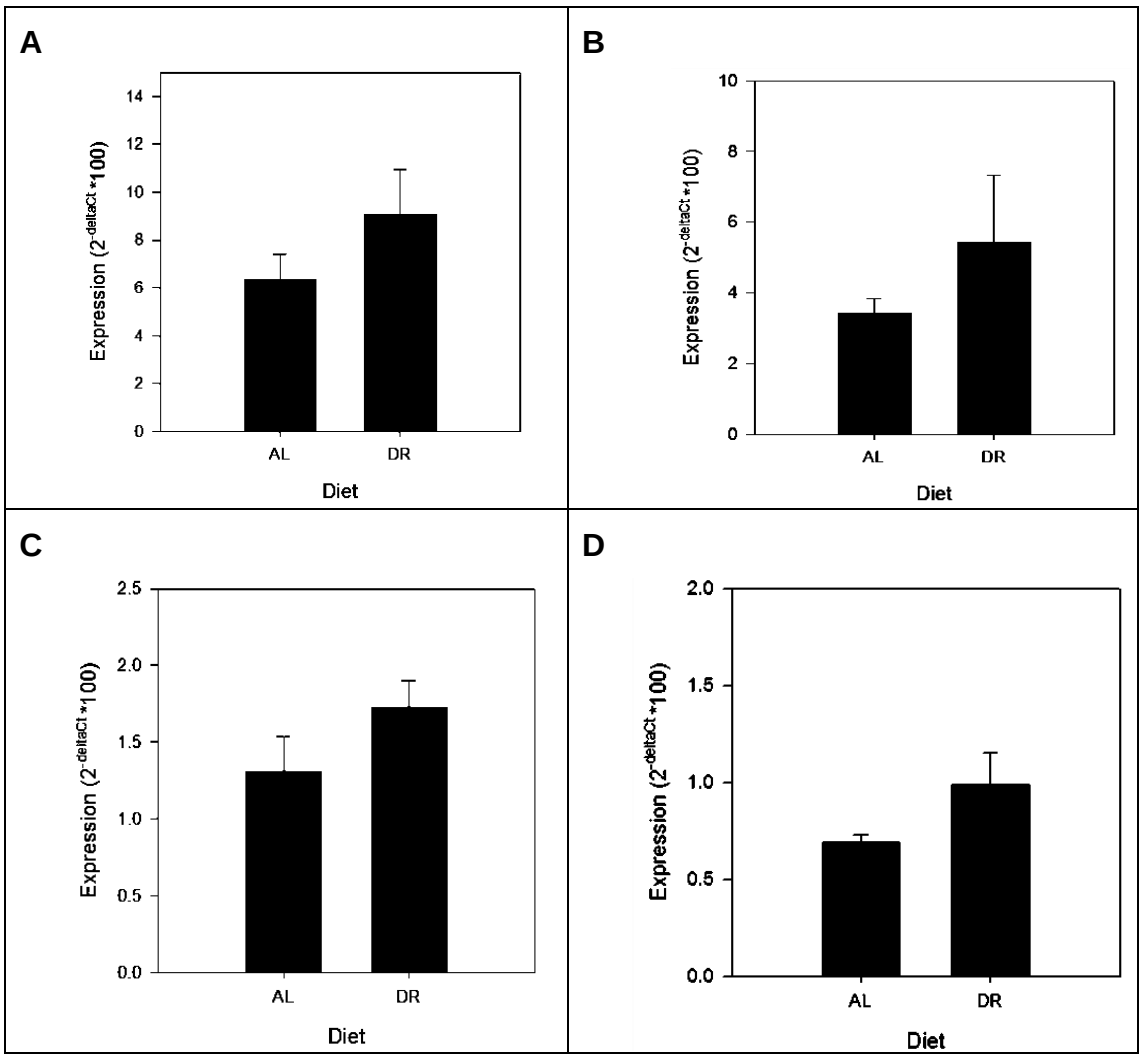

Figure C.6 The effect of short-term DR on expression of *Hif1α* in the brain (A), *Nfya* in the brain (B),*Hif1α* in the liver (C) and *Nfya* in the liver (D).

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