Exploring cellular actions and interactions of SIRT1 that may counteract ageing

Suzanne Mary Hutchinson

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Institute for Cell and Molecular Biosciences,

Newcastle University

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I dedicate this thesis to my husband Luke.
Luke has supported me throughout my PhD and
hence, has put up with a lot of stress, tears and
anti-social time course experiments, but has also
celebrated with me during the good times, so,
let the good times roll.

Declaration

I certify that this thesis is my own work, except where stated, and has not been previously submitted for a degree or any other qualification at this or any other university.

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Abstract

The NAD^+-dependent class III histone deacetylase SIRT1 appears to increase healthspan in some model organisms. Details of how SIRT1 is regulated and affects specific systems linked to ageing are limited. Here we aimed to gather a body of data to be used to generate a mathematical model of the interactions between SIRT1, resveratrol, NAD^+, Poly-ADP ribose polymerase (PARP) and chaperone mediated autophagy (CMA). There is evidence that the dietary polyphenol resveratrol can increase healthspan and is believed to do this through activating SIRT1. PARP repairs DNA single-strand breaks and is also considered to be a modifier of ageing. Both SIRT1 and PARP consume NAD^+, providing a point of interaction at the centre of cellular metabolism. Chaperone mediated autophagy (CMA) is reduced in ageing but evidence from our laboratory suggests the CMA regulator LAMP2 may be regulated by SIRT1, including by effects on DNA methylation at the *LAMP2* promoter.

Firstly, we observed that resveratrol increased SIRT1 mRNA, *SIRT1* promoter activity and NAD^+ in cultured cells (Caco-2). However, we also found that an increase in NAD^+ reduced SIRT1 mRNA dramatically. Thus, interactions between SIRT1, resveratrol and NAD^+ are complex. Secondly, SIRT1, PARP or NAD^+ were each manipulated pharmaceutically or genetically and the response of the other two variables measured. Overall, the data suggested that SIRT1 and PARP have mutually inhibitory effects, which we hypothesise is driven by competition for NAD^+. Next we developed and tested a functional assay based on a fluorescent substrate for CMA to measure activity directly. Preliminary findings indicated that reducing SIRT1 by siRNA decreased the activity of the pathway. Finally, LAMP2 mRNA expression was increased by the use of 5-aza-deoxycytidine to induce DNA hypomethylation, providing proof of principle that the gene is regulated by DNA methylation. Thus, the observed effect of SIRT1 on DNA methylation of the *LAMP2* promoter may be the basis of its stimulatory effect on CMA.

This body of work uncovers more information on the pleiotropic actions of SIRT1 relevant to modifying cellular ageing. The complexity of interactions with the other modifiers of ageing studied highlights the need for a system-level approach to data integration, and for further data to develop such a model, ultimately to identify target nodes for intervention to improve health-span.

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Abbreviations list

°C	degrees celcius
µg	micro grams
µl	micro litre
ml	milli litre
mg	milli grams
µM	micro molar
AMPK	amp-activated protein kinase α
BMI1	b lymphoma mo-mlv insertion region 1 homolog
bp	base pairs
BSA	bovine serum albumin
Caco-2	colonic adenocarcinoma
CBX	chromobox homolog 1
CFP	cyan fluorescent protein
CMA	chaperone mediated autophagy
DMEM	dulbeccos modified eagles medium
DMSO	dimethyl sulfoxide
DNMT	dna (cytosine-5)-methyltransferase 1
DR	dietary restriction
EED	embryonic ectoderm development
EZH2	enhancer of zeste homolog 2
GAPDH	glyceraldehyde 3-phosphate dehydrogenase
GFP	green fluorescent protein
h	hours
HSC70	heat shock chaperone 70
HuVEC	human umbilical vein endothelial cells
KDa	kilo daltons
KDM2B	lysine (k)-specific demethylase 2b
LAMP2	lysosomal associated membrane protein 2
m	months
MCS	multiple cloning site
min	minutes
mRNA	messenger ribonucleic acid
mTOR	mammalian target of rapamycin
NAD^+	nicotinamide adenine dinucleotide
NADH	reduced nicotinamide adenine dinucleotide
ng	nano grams
nm	nano meters
p	p-value
PARP	poly ADP-ribose polymerase
PCGT	polycomb gene target
PCR	polymerase chain reaction

PHC1	polyhomeotic-like protein 1
PRC1	polycomb pepressive complex 1
PRC2	polycomb pepressive complex 2
PRC4	polycomb pepressive complex 4
RbAP48	histone-binding protein rbbp4
RNF2	e3 ubiquitin-protein ligase ring2
RT	room temperature
RT-qPCR	reverse transcriptase-quantitative polymerase chain reaction
s	seconds
SEM	standard error of the mean
Sir2	SIRT1 homologue
siRNA	short interfering ribonucleic acid
SIRT1	Sirtuin 1
SUZ12	suz12 polycomb repressive complex 2 subunit
TMZ	temozolomide
TOP1	topoisomerase 1
α	alpha
β	beta
γ	gamma

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1 Introduction

1.1 Sirtuin1

Sirtuin1 (SIRT1) is a NAD^+-dependent deacetylase enzyme, found in the nucleus and cytoplasm that removes acetyl groups from lysine residues in proteins. In contrast to enzymes that require NAD^+ as a co factor, where it is reduced to NADH in redox reactions, SIRT1 cleaves and thus consumes NAD^+. Nicotinamide (NAM) is released from NAD^+ as the acetyl group is transferred from the protein substrate to the ADP-ribose to generate 2′-*O*-acetyl-adenosine disphosphoribose. Changes in the acetylation status of a protein can result in a functional change. For example, acetylated p53 is active but deacetylation by SIRT1 at lysine 382 attenuates p53 activity (Vaziri *et al.*, 2001).

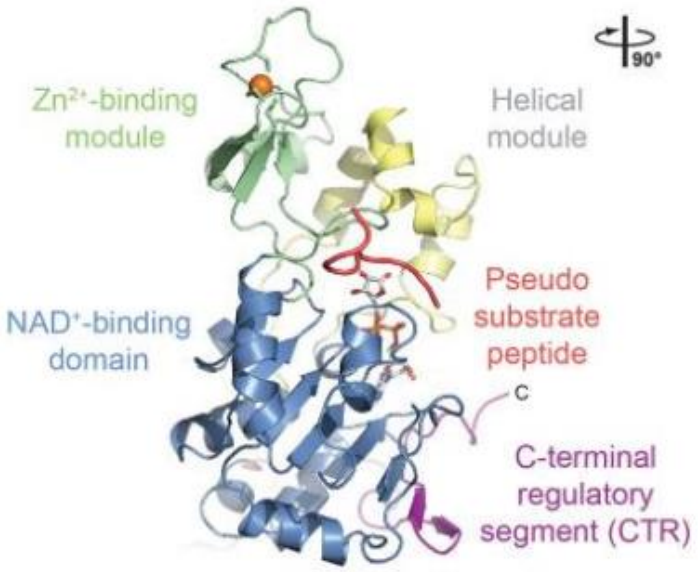


Figure 1.1.1. SIRT1 crystal structure. Red- Pseudo substrate peptide, blue- NAD^+ binding domain, green- zinc binding domain, yellow- helical module and purple- C-terminal regulatory segment Figure adapted from (Davenport *et al.*, 2014).

SIRT1 is made up of four domains: NAD^+ binding domain, zinc binding domain, helical module and the C-terminal regulatory segment (CTR) (Figure 1.1.1 (Davenport *et al.*, 2014)). The active site is located between the zinc binding domain and the helical module. Upon substrate and NAD^+ binding, the zinc binding domain and the helical module undergo a conformational change forming a hydrophobic tunnel. The CTR can

also undergo a conformational change at the C terminal end and inhibit the active site by interacting with the helical module (Davenport *et al.*, 2014).

1.2 The SIRT1 as an energy sensor in the cell

SIRT1 has a prominent role in energy sensing and metabolism in the cell, some details of which are outlined in Table 1.2.1.

Table 1.2.1. Examples of actions of SIRT1 related to energy sensing. As reviewed by (Anastasiou and Krek, 2006; Li, 2013).

SIRT1 as an energy sensor	
Adipose tissue	• SIRT1 promotes fat metabolism over fat storage in white adipocytes by repressing the activity of peroxisome proliferator-activated receptor gamma (PPAR-γ) through docking with the PPAR-γ co factors nuclear receptor co-repressor (NCoR) and silencing mediator of retinoid and thyroid hormone receptors (SMRT), causing reduced expression of PPAR-γ regulated genes. PPAR-γ promotes adipose differentiation and lipid storage (Picard *et al.*, 2004). • SIRT1 deacetylation and activation of PPAR-γ on lysine 268 and lysine 293 can promote the differentiation of white adipocytes to healthier brown adipocytes (Qiang *et al.*, 2012). • Acetyl-CoA synthetases are activated by SIRT1 deacetylation leading to the production of acetyl-CoA for fatty acid synthesis (Hallows *et al.*, 2006). • Our laboratory has shown that genistein (phytoestrogen from soya) promotes white adipocyte differentiation to brown adipocytes and increases UCP1 expression (brown adipocyte marker), the expression of which is prevented when SIRT1 is inhibited by EX-527 (Sadat Aziz personal communications).
Insulin secretion	• Uncoupling protein 2 (UCP2) is transcriptionally repressed by SIRT1 as SIRT1 directly binds to the UCP2 promoter. UCP2 reduction improves glucose stimulated insulin secretion in islet cells (Bordone *et al.*, 2006). • SIRT1 over expression using a recombinant herpes simplex virus in C2C12 myotubes caused increased glucose uptake following insulin stimulation. However, when protein-tyrosine phosphatase 1B (PTP1B), a phosphatase enzyme, which when reduced improves whole body glucose uptake after insulin stimulation in Ptp1b null mice (Klaman *et al.*, 2000), was over expressed alongside SIRT1, glucose uptake was impaired. It was suggested that SIRT1 down regulates

	PTPB1 transcriptionally through deacetylation of histone 3 located at the promoter of PTP1B (Sun *et al.*, 2007).
Liver processes	• CREB regulated transcription coactivator 2 (CRTC2) is a transcription factor that regulates genes involved in gluconeogenesis. SIRT1 inhibition using sirtinol and nicotinamide in hepatocytes increased CRTC2 activity, acetylation and gluconeogenic gene expression. SIRT1 deacetylation of CRTC2 could be a potential mechanism for SIRT1 regulation of CRTC2 and hence gluconeogenesis (Liu *et al.*, 2008b). • PGC-1α is deacetylated at several lysine residues and activated by SIRT1 in 293T cells, which causes an increase in genes related to gluconeogenesis (*PEPCK* and *G6Pase*) and a decrease in genes related to glycolysis (*Glucokinase* and *LPK*) (Nemoto *et al.*, 2005; Rodgers *et al.*, 2005). • SIRT1 deacetylates sterol regulatory element-binding protein (SREBP), which results in decreased levels of the transcription factor localising in the nucleus of cells. Inhibition of SIRT1 using sirtinol and nicotinamide in 293T and HeLa cells increased SREBP regulated gene expression (including LDLR and SCD1), such genes are involved in lipid storage, thus SIRT1 may attenuate lipid storage through repressing SREBP (Walker *et al.*, 2010).
Neuronal	• In the hypothalamus, anorexigenic POMC neurons inhibit feeding and the orexigenic agouti-related protein (AgRP) neurons promote feeding (Li, 2013). Inhibition of SIRT1 increased FOXO acetylation and reduced feeding through POMC and AgRP neurons (Cakir *et al.*, 2009; Dietrich *et al.*, 2010)

1.3 Regulation of SIRT1

The activity or expression of SIRT1 is influenced by a number of mechanisms about which current knowledge is described below.

1.3.1 Regulation of SIRT1 by NAD^+ and components of its synthetic and degradation pathways.

NAD^+ is synthesised in the cell from the dietary amino acid tryptophan or through salvage pathways from NAD^+ precursors, such as nicotinamide which, as explained, is one of the products of NAD^+ cleavage during SIRT1-mediated protein substrate (lysine) deacetylation. Nicotinamide at physiological concentrations inhibits SIRT1 activity (Bitterman *et al.*, 2002) resulting in an auto-regulatory feedback loop (Figure 1.3.1). As

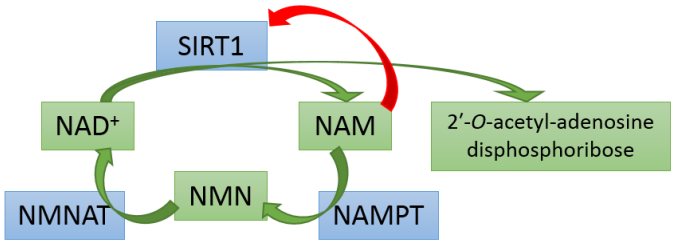


Figure 1.3.1. NAD^+ Salvage Pathway. NAD^+- Nicotinamide adenine dinucleotide, NAM- Nicotinamide, ADP- Adenosine diphosphate ribose, NAMPT- nicotinamide phosphoribosyltransferase NMN- nicotinamide mononucleotide, NMNAT- nicotinamide mononucleotide adenyltransferase. SIRT1 cleaves NAD^+ in to NAM and 2′-*O*-acetyl-adenosine disphosphoribose, NAM inhibits SIRT1 activity and is also converted to NMN by NAMPT. NMN is then converted back to NAD^+ by NMNAT.

may be expected based on the requirement for NAD^+ for SIRT1 function, Sirt1 enzymatic activity was increased in mouse fibroblasts when expression of the enzyme nicotinamide phosphoribosyltransferase (NAMPT), which is involved in NAD^+ salvage (Figure 1.3.1), was increased (Revollo *et al.*, 2004). This effect of NAMPT on SIRT1 activity may be one of the mechanisms through which reduced calorie intake activates SIRT1 since an increase in AMP-activated protein kinase (AMPK), a well-documented effect of dietary restriction (Greer and Brunet, 2009), has been shown to increase NAMPT transcription (Fulco *et al.*, 2008). Increased Nampt expression in mouse fibroblasts was seen to increase Sirt1 expression, as well as Sirt1 activity (Revollo *et al.*, 2004). The underlying mechanism here is less apparent. A direct effect of NAMPT on the SIRT1 gene is a possibility, but more likely is that the manipulation involves other feedback loops, such as, the FOXO1/SIRT1 feedback loop described below.

1.3.2 Regulatory interactions between SIRT1 and key transcription factors

Forkhead transcription factors (FOXO) control many cellular stress responses by regulating gene expression. FOXO1 has been identified as one of the targets of SIRT1 deacetylation, which results in FOXO1 activation (Brunet *et al.*, 2004). FOXO1 itself controls transcription of the SIRT1 gene, as indicated by increased expression of a reporter gene under the control of the SIRT1 promoter in HEK293 cells in which FOXO1 was overexpressed (Xiong *et al.*, 2011). Thus it appears that SIRT1 and FOXO1 interact in a positive feedback loop; SIRT1 activity increases FOXO1 activity and FOXO1 increases SIRT1 expression.

SIRT1 is also co-regulated in a feedback loop with the transcription factor c-MYC. c-MYC binds to the *SIRT1* promoter and increases SIRT1 expression. However, is itself a substrate for SIRT1-catalysed deacetylation, which has been reported both to increase and repress its activity. Lysine 323 was found to be deacetylated by SIRT1, which was linked to c-MYC instability (Yuan *et al.*, 2009). Whereas in another study SIRT1 deacetylation was linked with lysine 63-linked ubiquitin chains and the stabilization of c-MYC (Menssen *et al.*, 2012). Further research is needed to clarify the feedback loop, however, it may be the case that the feedback loop is cell line specific as both studies used different cell lines or be reliant on another stimuli.

A potential SIRT1 negative feedback loop is with PPAR-γ. In mice it was shown that Sirt1 can deacetylate Ppar-γ at lysine 268 and 293, which activated Ppar-γ and promoted brown adipocyte formation (Qiang *et al.*, 2012). PPAR-γ can also block SIRT1 activity by directly binding to SIRT1 and when PPAR-γ was reduced by siRNA SIRT1 protein expression increased in HeLa cells (Han *et al.*, 2010). However, it has also been shown that SIRT1 can repress PPAR-γ activity by binding to its cofactors NCoR and SMRT in human 3T3-L1 adipocytes (Picard *et al.*, 2004). Further data will be needed to clarify a negative feedback loop and under what circumstances SIRT1 activates or represses PPAR-γ.

1.3.3 Regulation of SIRT1 by reversible phosphorylation

Whereas changes in NAD^+ levels can take hours, regulation of SIRT1 by reversible phosphorylation provides a mechanism that causes relatively rapid changes in SIRT1 activity. Below are just a few examples of SIRT1 regulation by phosophorylation:

- Phosphorylation of SIRT1 at serine 434 located in the catalytic domain of SIRT1 by cAMP-dependent protein kinase A (PKA) amplified SIRT1 catalytic activity (Gerhart-Hines *et al.*, 2011).
- Dual specificity tyrosine phosphorylation-regulated kinase (DYRK) 1A and 3 mediated phosphorylation of SIRT1 at threonine 522 was shown to be protective against its aggregation, which may be an important heat shock response that maintains SIRT1 function at elevated temperatures (Guo *et al.*, 2012).
- Finally, AMPK can phosphorylate SIRT1 at threonine 344, which blocked Deleted in Breast Cancer 1 (DBC1) from binding to SIRT1 and inhibiting SIRT1 activity (Lau *et al.*, 2014).

1.4 Ageing

Ageing is a degenerative process occurring in the body over time. It affects all individuals but at different rates. Genetic and environmental factors, such as diet, pollution and smoking, can increase or decrease the risk of developing age-related diseases, such as dementia or cardiovascular disease. Level of exposure to these environmental factors will ultimately affect the rate of ageing and lifespan.

Genetic factors include hereditary mutations as well as mutations that occur as a result of environmental exposures. Mutations occur frequently in cells and are generally repaired through systems such as, the DNA damage response. However, excess damage occurring from toxin build-up over time can cause repair systems to become overloaded and hence mutations accumulate. These mutations may have a significant effect on systems in the cell and lead to disease. For example, a malfunctioning p53 protein can result in the cell being unable to arrest the cell cycle, which can cause uncontrolled replication and tumour formation (Richardson, 2013).

The influence of environmental factors can be positive or negative. For example, air pollution, solvents and pesticides can increase the risk of Alzheimer's disease but antioxidants and omega-3 fatty acids can decrease the risk (Migliore and Coppede, 2009). Oxidative stress due to free radicals (OH and O_2^-), is widely believed to be one of the main causes of ageing and is malleable in response to environmental factors (Migliore and Coppede, 2009).

The gradual build-up of damage to DNA, proteins and lipid through oxidative stress and other mechanisms results in malfunctioning cellular processes linked to ageing. For example, irreparable damage can cause cell death through apoptosis or replicative senescence. Cell death and/or senescence reduce tissue integrity, which ultimately leads to age-related diseases. For example, osteoporosis can result from apoptosis of osteocytes (Boskey and Coleman, 2010). Other molecular events involved in the ageing process are discussed later in this chapter.

It is well understood that smoking, drinking alcohol, air pollution and eating a diet high in saturated fats are all detrimental in ageing but it is less well understood how dietary factors or practises that reduce oxidative stress or its damaging effects affect the cell

and if such factors in the diet can reduce ageing. Such dietary modifications include dietary restriction (DR) (described below) and resveratrol, which has similar effects to DR and is a focus of the current project.

1.5 Dietary restriction extends lifespan and reduces the risk of age-related diseases.

Dietary restriction (DR) (arguably considered synonymous with calorie restriction) is a term used to describe a decrease in energy intake by 10-40 % whilst maintaining a diet complete in essential nutrients. It was first demonstrated in 1935 that DR could increase the lifespan of rodents (McCay *et al.*, 1989). Following this discovery it has been shown that the lifespan of other model organisms can be influenced by energy intake. For example, replicative lifespan of *Saccharomyces cerevisiae* was increased by a growth medium in which the glucose concentration was reduced (Lin *et al.*, 2000). It has been shown in many studies that DR can affect not only lifespan but can reduce the occurrence of age-related disease. For example, it has been shown in mice that DR can increase glucose tolerance and insulin sensitivity, reduce oxidative stress, reduce spontaneous cancers and protect neurons from environmental and intercellular stresses (Weindruch and Walford, 1982; Guo *et al.*, 2002; Masoro, 2005; Martin *et al.*, 2006; Fontana, 2008). Thus, DR has been researched extensively in the pursuit of ameliorating age-related disease in humans.

Relatively few studies have investigated the effects of DR in primates and results of those that have are conflicting. For example, a study conducted in rhesus monkeys found no effect of DR (30-40% reduction in calorie intake) on maximum lifespan and did not see risk factors of age-related diseases, including fasting serum glucose and triglycerides reduce, in animals receiving DR from a young age. However, there was a reduction in these risk factors when DR was implemented in older animals. Finally, disease incidence was measured in both young and old groups compared with controls , which indicated an improvement in diabetes and cancer rates in DR animals but not cardio vascular disease (NIA study) (Mattison .J *et al.*, 2012). A second study running in parallel reported contrasting findings. Rhesus monkeys fed a calorie restricted diet (30% reduction in calorie intake) had a longer lifespan than monkeys fed ab libitum. There was also a lower incidence of cardiovascular disease, diabetes and cancer in the

calorie restricted animals compared with controls (WNPRC study) (Ramsey *et al.*, 2000; Ricki J. Colman, 2009). Several confounding factors that may account for the differences can be identified. For example, the monkeys used in the WNPRC study were from India whereas the monkeys in the NIA study were from India and China; thus genetic variance may contribute to the discordant findings. It was demonstrated that genetic differences between strains of mice had a measurable effect on the overall outcome of a 40% DR intervention with a negative effect on lifespan in certain strains of mice being observed; thus DR may not be a universal effect (Liao *et al.*, 2010). Furthermore, the WNPRC study fed an *ad libitum* diet of purified food to control monkeys whereas the control monkeys in the NIA study where not fed *ad libitum* but received a meal twice daily following 'The National Research Council guidelines' for food quantity for the age and size of the animal, and consisted of predominantly natural ingredients (Weindruch, 1996). It is clear that more studies in primate models are needed to elucidate if DR can have an effect on lifespan in such species.

Human populations with different cultural dietary practices can give an indication of the effects of DR in humans. An associational study of the population of Okinawa, Japan, suggested that the high life expectancy is linked to a low calorie diet mainly consisting of fruit, vegetables (largely sweet potatoes) and soya, contrasting with the typical diet of the Western world, which is high in calories (Willcox *et al.*, 2009). Also cell culture models can reveal mechanistic detail relevant to likely effects of DR. One such study created conditions similar to calorie restriction by reducing glucose in cell culture medium. Human lung fibroblasts grown in the low glucose medium showed an increase in cellular lifespan, coincident with reduced expression of the *p16* gene (senescence regulator) and reduced senescence as measured by presence of β-galactosidase (a marker of senescence). In addition expression of SIRT1, which has been linked to longevity, was elevated. Consistent with the increase in SIRT1 expression being an important component of the increase in cellular lifespan (described below), SIRT1 knockdown in glucose restricted fibroblasts abolished p16 down regulation, thus allowing p16 signalling and cellular senescence but cellular lifespan was not measured under reduced glucose conditions and SIRT1 knockdown (Li and Tollefsbol, 2011).

1.5.1 SIRT1 plays a key role in the response to dietary restriction.

In 1999 it was demonstrated in *Saccharomyces cerevisiae* that the manipulation of Sirtuin 2 (Sir2, the homologue of mammalian SIRT1) had an effect on lifespan. The deletion of Sir2 shortened lifespan and the addition of an extra copy of Sir2 extended lifespan (Kaeberlein *et al.*, 1999). Many studies have now shown effects on lifespan achieved by manipulation of sirtuin expression provide evidence that sirtuin activation may be a key component of the longevity response to DR (Guarente and Picard, 2005). Mice over expressing Sirt1 from a *Sirt1* transgene showed a similar phenotype to DR mice, including reduced blood cholesterol, reduced insulin and fasting glucose (Bordone *et al.*, 2007). More recently, it was shown that overexpression of Sirt1 in the brains of mice conferred longevity, suggested to be due to increased neuronal activity caused by increased signalling through the Nk2 homeobox1 transcription factor and upregulation of Orexin type 2 g-protein coupled receptor (Satoh *et al.*, 2013). Expression of SIRT1 was also found to be increased in response to DR in rodent and human tissues (Cohen *et al.*, 2004; Civitarese *et al.*, 2007).

The view that sirtuins are an important determinant of lifespan, including under conditions of DR, remains contentious, however, for example, a recent study using strains of *Caenorhabditis elegans* and *Drosophila melanogaster* in which the *Sir2* gene was over expressed attributed extended lifespan to other confounding genetic factors (Burnett *et al.*, 2011). In contrast, however a later study that controlled for genetic background confirmed a positive influence of Sir2 over expression on lifespan (Banerjee *et al.*, 2012). Thus, the theory that SIRT1 could play a significant role in lifespan extension is still credible but requires more research to resolve these controversies.

1.5.2 The role of NAD^+ in response to DR.

A reduction in NAD^+ in response to DR has been proposed to be a key 'energy sensing' cellular mechanism that is pivoted in the longevity response (Moroz *et al.*, 2014). However, NAD^+ synthesis is also a requirement of lifespan extension by DR. For example, in *Saccharomyces cerevisiae* the Ntp1 protein (an enzyme involved in NAD^+ synthesis) was required for lifespan extension (Lin *et al.*, 2002). Similarly in

Caenorhabditis elegans Pnc-1 (a nicotinamidase found in the NAD^+ salvage pathway) was required for lifespan extension under conditions of DR (Ghislain *et al.*, 2002; Moroz *et al.*, 2014). Possible resolution of these apparently contradictory ideas and findings is that a reduction in NAD^+, signals reduced energy status and promotes longer survival, yet there is a minimum level below which, a further reduction becomes detrimental.

1.6 Resveratrol a proposed DR mimetic.

Resveratrol is a plant polyphenol found at a high concentration in the roots of plants and plant-based foods including Japanese knotweed, red grape skin and peanuts (Dolinsky and Dyck, 2011). Resveratrol has been found to mimic some of the effects of DR (Chung *et al.*, 2012), examples of what are summarised in *Table 1.6.1*.

Table 1.6.1. The effect of resveratrol on lifespan and age-related diseases

Examples of studies reporting positive effects on lifespan.	➢ In *Saccharomyces cerevisiae* fed 10 μM resveratrol there was an increase in lifespan by 70% (Howitz *et al.*, 2003). ➢ *Drosophila melanogaster* and *Caenorhabditis elegans* fed 100 μM resveratrol had up to a 20% increase in lifespan (Wood *et al.*, 2004).
Examples of studies reporting action to reduce risk factors of Cardiovascular Disease	➢ Resveratrol at 0.1 μM can potentially help reduce blood pressure through the stimulation of nitric oxide synthase in HUVEC cells (Nicholson *et al.*, 2008). ➢ In rat cardiac-myocytes 50 μM resveratrol can have an anti-hypertrophic affect through the activation of AMPK (Chan *et al.*, 2008).
Examples of studies reporting action to reduce risk factors of Diabetes	➢ Obese mice fed a diet containing 0.04 % resveratrol had improved insulin sensitivity and reduced insulin-like growth factor-1 (Baur *et al.*, 2006). ➢ Resveratrol from 10-100 μM can reversibly inhibit glucose stimulated rat pancreatic cell insulin secretion (Szkudelski, 2006).
Examples of studies reporting action to reduce risk factors of cancer	➢ Resveratrol fed in the diet at 10 ppm is an inhibitor of Nuclear factor (NF)-kappa B in female rats (Banerjee *et al.*, 2002). ➢ Resveratrol at 150 μM prevented colon cancer cell proliferation by suppressing IGF-1 (Vanamala *et al.*, 2010).

Despite the substantial body of evidence that resveratrol can have actions to extend lifespan or reduce risks of age-related disease, there are many examples of observations discordant with resveratrol having such beneficial actions. For example, a null effect

was reported in *Drosophila melanogaster* and *Caenorhabditis elegans* on lifespan extension (Bass *et al.*, 2007). Such inconsistencies in reported actions are likely to be a result of the process to supply resveratrol within specific dosing regimens (for example, nutrient medium, exposure time and concentration) for it to be efficacious.

1.6.1 Resveratrol mediates its effects through SIRT1.

It was originally believed that resveratrol was a potent activator of SIRT1 and that SIRT1 activation played a key role in the positive actions of resveratrol on healthspan in model organisms (Borra *et al.*, 2005; Zhu *et al.*, 2011; Stiaccini *et al.*, 2012). These findings, however, come under scrutiny when it was demonstrated that a fluorescent peptide substrate of SIRT1, which was used to show that resveratrol was a SIRT1 activator, bound within the enzyme active site, producing artifactual effects. When the fluorescent tag was removed resveratrol and other related compounds appeared no longer to activate SIRT1 (Beher *et al.*, 2009; Pacholec *et al.*, 2010). However, the relationship may still exist as recent findings have shown that resveratrol and other compounds do increase the activity of SIRT1 against substrates that include specific hydrophobic motifs and that glutamine 230 in the N-terminal region of SIRT1, is essential for SIRT1 activation by resveratrol (Basil P. Hubbard *et al.*, 2013). Moreover, data has shown that resveratrol can increase SIRT1 mRNA and potentially SIRT1 protein in rat ovarian cells, human prostate cells and porcine adipocytes (Bai *et al.*, 2008; Morita *et al.*, 2012; Li *et al.*, 2013).

Another proposed mechanism by which resveratrol may act is through the AMPK pathway. There is a large body of evidence to suggest that resveratrol activates AMPK (Chung *et al.*, 2012), and it is believed that resveratrol does this through the inhibition of phosphodiesterases that degrade cyclic-AMP (cAMP). cAMP activates protein kinase A, which in turn activates AMPK through liver kinase B1 (LKB1). For example, Ampk activation via phosphodiesterase inhibition by resveratrol increased NAD^+ and SIRT1 activity in mouse C2C12 myotubes (Chung *et al.*, 2012; Park *et al.*, 2012). Ampk also increases the expression of the NAD^+ salvage pathway enzyme Nampt in mouse skeletal myoblasts (Fulco *et al.*, 2008), which can decrease the SIRT1 inhibitor NAM. Sirt1 activity is also increased by Ampk activation in mouse C2C12 myotubes (Canto *et al.*,

2009). Furthermore, resveratrol has been shown to increase NAMPT activity in human primary hepatocytes (Schuster *et al.*, 2014). Thus, is likely resveratrol has an effect on SIRT1 through several pathways. Current knowledge is outlined in a schematic diagram below (Figure 1.6.1).

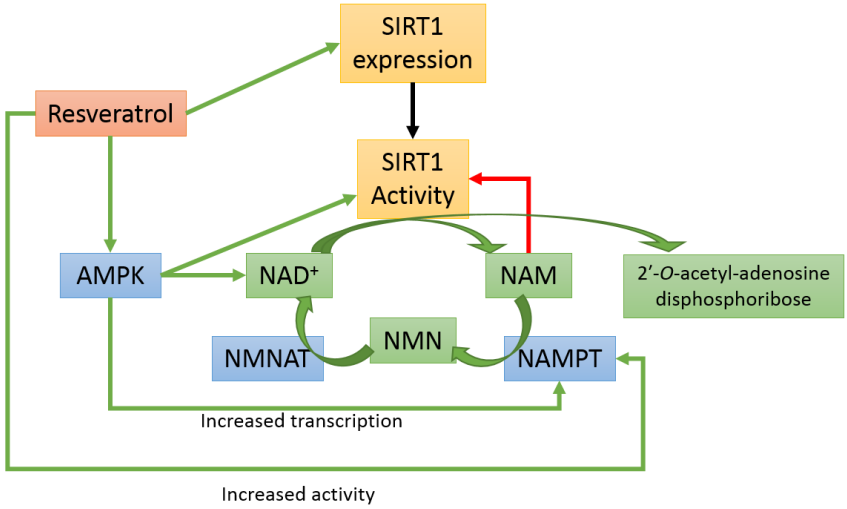


Figure 1.6.1. A schematic diagram of interactions between SIRT1 and resveratrol. Green arrows- positive effect; red arrows- negative effect. Literature used is detailed and referenced in the text.

1.7 SIRT1 and age-related diseases.

There is an extensive body of literature linking SIRT1 to age-related diseases, including diabetes, neurodegenerative diseases, multiple cancers and cardiovascular disease. SIRT1 has a central role in energy sensing and metabolism, which includes increasing insulin sensitivity (Bordone *et al.*, 2006). A mouse model of type 2 diabetes fed a high fat/high sucrose diet was used to assess the benefits of Sirt1 on insulin resistance. Following over expression of Sirt1 in the liver, blood glucose was lowered and insulin sensitivity was increased. It was also shown that Sirt1 overexpression inhibited mTOR signalling and thus the unfolded protein response, which inhibits insulin receptor signalling, a regulator of glucose homeostasis in the cell (Li *et al.*, 2011).

SIRT1 may also play a positive role in protection against neurodegenerative disorders, such as Parkinson's disease and Alzheimer's disease. Activation of SIRT1 by resveratrol in neuroblastoma cells was demonstrated to reduce the toxicity of α-synuclein found in Parkinson's disease and amyloid-β plaques found in Alzheimer's disease but on addition of sirtinol, a SIRT1 inhibitor, toxicity was restored (Albani *et al.*, 2009).

Moreover, increased SIRT1 expression from a lentivirus construct injected in to the hippocampus of p25 transgenic mice, a model known to over-produce amyloid-β plaques, was shown to have a preventative effect on neurodegeneration (Kim *et al.*, 2007). SIRT1 and other members of the sirtuin family confer protective roles against other neurodegenerative diseases and are now a focus for new therapeutic targets (Donmez and Outeiro, 2013).

While an extensive body of published work, examples of which are cited above, supports the view that SIRT1 can protect against age-related disease, an equally large body of data reports that no such actions were observed or even that such actions can be detrimental. For example, a dose-dependent effect of overexpression of Sirt1 was reported in the heart tissue of mice. It was found that mild (≤2.5 fold) over expression of Sirt1 was beneficial and involved changes including reduced oxidative stress proportionate with reducing ageing of the heart. Higher levels of Sirt1 overexpression however, (≥7.5 fold) led to cardiomyopathy (Alcendor *et al.*, 2007). Furthermore, Sirt1 expression was increased in hypertensive rats and associated with cardiac hypertrophy (Li *et al.*, 2009). Conversely, overexpression of SIRT1 by 4-5 fold using a recombinant adenovirus in neonatal cardiac myocytes, prevented hypertrophy (Planavila *et al.*, 2011).

SIRT1 also appears to play a reciprocal role in cancer. SIRT1 has been found to be elevated in many cancer cell lines including prostate cancer, skin cancer and acute myeloid leukaemia (Bradbury *et al.*, 2005; Hida *et al.*, 2007; Huffman *et al.*, 2007) but Sirt1 ectopic induction alleviated colon cancer growth in mice (Firestein *et al.*, 2008). A pertinent question is whether changes in SIRT1 expression are a consequence or cause of malignancy? (Deng, 2009). SIRT1 can deacetylate and deactivate the tumour suppressor p53 (Chen *et al.*, 2005), which would increase the risk of cancer. In contrast in mammary tumours from Brca1 mutant mice Sirt1 was reduced and survivin (negative regulator of apoptosis) increased but treatment with resveratrol increased Sirt1 and reduced survivin, which in turn reduced tumour-genesis. In normal breast cells Brca1 usually increases Sirt1 expression, and it was shown in mouse embryonic cells that Sirt1 expression reduced survivin expression (Wang *et al.*, 2008). The genetic complexity of cancer is likely to be a major factor that accounts for these differentiated

actions of SIRT1 meaning that further research that takes into account the highly heterogeneous nature of cancer, and also inter-individual differences, is essential to determine conditions where manipulating SIRT1 actions would be of potential therapeutic benefit.

The magnitude of SIRT1 expression or activation is another variable that may explain why SIRT1 in some instances has been seen to have cancer protective actions and in other sections appeared to promote cancer. As already noted, whether Sirt1 overexpression in mouse heart tissue was cardio protective or lead to cardiomyopathy was dependent of the level of expression (Alcendor *et al.*, 2007). Speculatively, higher levels of SIRT1 expression or activity could promote rather than protect against cancer if substrates for deacetylation by SIRT1 also influence cancer as there could be cancer protective acetylated agents (hence at hyper levels of SIRT1 expression their protective action would diminish) or agents that promote cancer when deacetylated.

1.8 The involvement of SIRT1 in other mechanisms of ageing

Alterations in SIRT1 function have been associated with multiple age-related diseases and there is growing evidence that SIRT1 interacts with several key mechanisms of ageing. Important processes or pathways where these relationships with SIRT1 function appear to modify activity to influence ageing or age-related disease are considered below.

1.8.1 Apoptosis

Apoptosis is a carefully managed process in the cell and is activated extrinsically by immune cell interactions or intrinsically by stressors, such as oxidative stress. Figure 1.8.1 shows the intrinsic and extrinsic pathways that trigger apoptosis in the cell. The

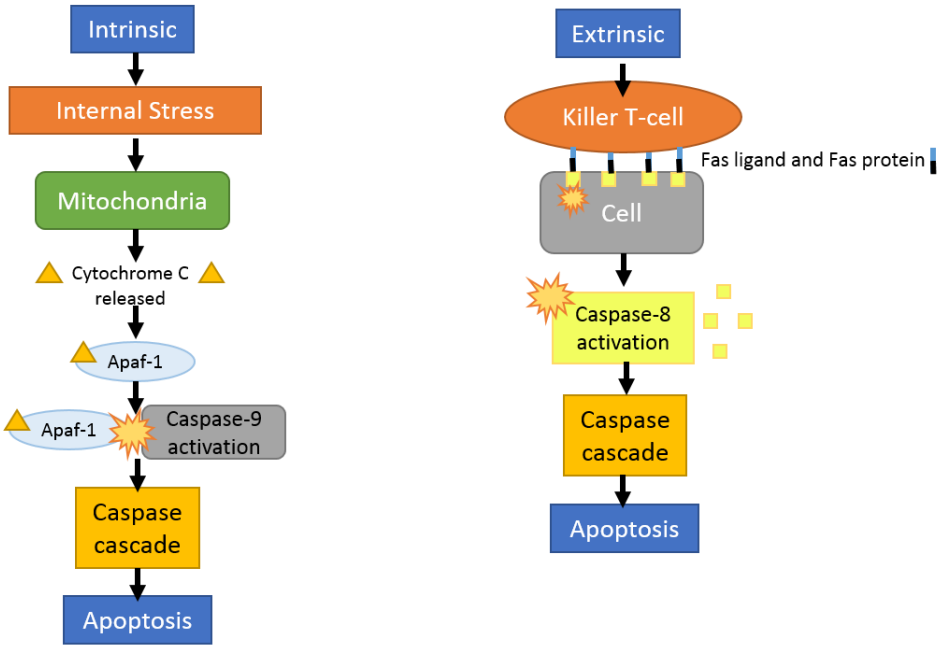


Figure 1.8.1. Schematic diagram of the intrinsic and extrinsic apoptosis pathways. A simplistic diagram of the two main apoptosis pathways and the key molecules involved in these two pathways. **Intrinsic-** stress and damage cause the release of cytochrome C from the mitochondria, which binds to Apoptotic protease activating factor 1 (Apaf-1) and in turn triggers the cleavage of caspase-9, which triggers a caspase activation cascade. **Extrinsic-** Killer T-cells bind to the cell via a Fas ligand to Fas protein on the cell surface. This triggers caspase-8 cleavage and activation triggering a caspase activation cascade**,** occasionally damaged cells will form the Fas ligand/protein themselves to trigger apoptosis. Adapted from (Bruce Alberts, 2002).

intrinsic pathway is initiated following stress by B-cell Lymphoma 2 (BCL-2) family members promoting mitochondrial release of cytochrome C, which triggers a caspase cascade. The extrinsic pathway is activated by immune cells binding to the cell via a Fas ligand/protein interaction causing the release of caspase-8 triggering a caspase cascade. Caspases contain a cysteine in their active site allowing them to cleave proteins at aspartic acid residues, cleavage of other caspases can activate them and cleavage of other proteins in the cell ultimately leads to the breakdown of the cell and cell death (Bruce Alberts, 2002). It is unclear if apoptosis is causal of ageing or occurs as a process in ageing but apoptosis markers have been observed at higher levels in ageing models, such as the Fas protein, which was increased in the livers of 24 month old Fischer 344

rats compared to 6 month old rats (Higami *et al.*, 1997), and more recently, skeletal muscle from 29 month old Fischer 344 rats compared to younger 16 month old rats had increased expression of apoptosis associated proteins Bcl-2 Associated X protein (Bax) (BCL-2 family member) and increased Apaf-1 but also increased DNA fragmentation (a marker of apoptosis) (Chung and Ng, 2006). Apoptosis has been associated with some age-related diseases, including the amyloid-β proteins found in Alzheimer's disease. Cultured neurons that were exposed to excess amyloid-β protein showed an increase in DNA fragmentation (a marker of apoptosis) compared with controls (Loo *et al.*, 1993). Also in cancer, apoptosis is often evaded in malignancy, which can be caused by the dysregulation of apoptosis proteins, such as the apoptosis initiating protein BAX. It was demonstrated in p53 mutant mice also deficient in Bax, that apoptosis was reduced by 50 % as measured by a TUNEL assay (the identification of DNA fragments using histochemistry) and brain tumour growth accelerated compared with p53 mutant mice with wild type Bax (Yin *et al.*, 1997). Thus, apoptosis can be perceived as a negative or a positive process in age-related diseases.

1.8.1.1 Mechanisms through which SIRT1 modifies apoptosis

SIRT1 can be associated with apoptosis through deacetylation of nuclear proteins including p53, NF-κB, FOXO and Ku70 (Luo *et al.*, 2001; Cohen *et al.*, 2004; Motta *et al.*, 2004; Yeung *et al.*, 2004; Takayama *et al.*, 2009). The function of these nuclear proteins to affect apoptosis and how SIRT1 may be involved in the process are summarised in *Table 1.8.1*.

Table 1.8.1. The function of nuclear proteins p53, FOXO, NF-κB and Ku70 in apoptosis and the effect of deacetylation by SIRT1

p53	p53 promotes transcription of the *p53 upregulated modulator of apoptosis/Bcl-2 binding component-3 (PUMA)* gene, which initiates apoptosis by promoting the release of cytochrome C from the mitochondria. p53 overexpression in colorectal cancer cells resulted in increased PUMA led apoptosis (Yu *et al.*, 2003). HaCaT keratinocytes were treated with a SIRT1 inhibitor caused p53 acetylation and thus activity to increase along with PARP cleavage (a marker of apoptosis), however PUMA expression was not measured in this study (Herbert *et al.*, 2014).
FOXO	FOXOs can upregulate transcription of extrinsic pro apoptotic factors including the Fas ligand and TNF-related apoptosis-inducing ligand

	(TRAIL) but has also been linked to the intrinsic apoptosis pathway through the upregulation of PUMA. FOXOs can also be inhibited by RAC-alpha serine/threonine-protein kinase (AKT) phosphorylation preventing apoptosis (Zhang *et al.*, 2011). TNF-α was used to increase apoptosis in vascular adventitial fibroblasts (VAS), and upon SIRT1 activation by resveratrol the number of cells expressing the apoptotic surface protein phosphatidylserine reduced. Knockdown of FOXO1 also reduced the number of cells expressing the apoptotic surface protein. SIRT1 and FOXO1 were found to be co-immunoprecipitated and when SIRT1 was knocked down in VAS, FOXO1 acetylation increased. It was suggested that SIRT1 activity inhibits apoptosis in TNF-α stimulated VAS through deacetylation and deactivation of FOXO1, thus attenuating FOXO1 transcription of apoptosis proteins (Wang *et al.*, 2013). However, it has also been demonstrated that SIRT1 deacetylation of FOXO1 promotes FOXO1 transcriptional activity (Daitoku *et al.*, 2004). Thus, further research is needed to clarify this relationship.
NF-κB	Nf-κB can promote the extrinsic apoptosis pathway through increased transcription of the Fas ligand (Barkett and Gilmore, 1999). SIRT1 deacetylates lysine 310 in the RelA/p65 subunit of NF-κB blocking its transcriptional activity (Yeung *et al.*, 2004) and likely its transcriptional effects on apoptosis proteins.
Ku70	Ku70 can inhibit BAX translocation to the mitochondria, thus inhibiting BAX mediated apoptosis. Rat 293T cells transfected with yellow fluorescent Bax protein used to measure Bax translocation to the mitochondria, showed reduced Bax translocation when Sirt1 activity was increased by resveratrol (Cohen *et al.*, 2004). It was also found that Sirt1 was co-immunoprecipiated with Ku70 in 293T cells and Sirt1 could deacetylate Ku70 at two key lysine residues (539 and 542), located in the critical region of Ku70 for Bax binding and was suggested to promoteKu70 binding to Bax and thus Bax inhibition (Cohen *et al.*, 2004).

Dysregulation of apoptosis to cause survival of unwanted cells or degeneration of healthy cells is clearly detrimental. Thus, if there is an increased likelihood that the process is regulated less tightly in older organisms it would be a likely major contribution to ageing and age-related disease. Indeed, there is evidence that dysregulated apoptosis by SIRT1 plays a role in specific age-related diseases osteoarthritis is one example. Chondrocytes are important in the maintenance of

cartilage; apoptotic features were observed in chondrocytes from osteoarthritis patients and it was considered that apoptosis may play a pathological role in osteoarthritis (Blanco *et al.*, 1998). Sirt1 knockout mice with induced osteoarthritis expressed increased DNA fragmentation, Parp cleavage and caspase 3 cleavage compared with wild type mice with induced osteoarthritis (Matsuzaki *et al.*, 2014). In this case SIRT1 activation may be therapeutic to prevent apoptosis of chondrocytes.

1.8.2 Senescence

Replicative senescence is the term used to describe cells that stop dividing but remain active (Hayflick and Moorhead, 1961). Senescence is known to be induced by cellular stressors including DNA damage, mitochondrial dysfunction and telomere shortening. The principle proteins that initiate senescence are p21 and p16. p21 is induced by p53 and inhibits cyclin-dependent kinase (CDK) activity, CDKs are also inhibited by p16. CDK inhibition allows the activation of pRB, which in turn inhibits E2F, which is involved in the transcription of genes that promote cell cycle progression, thus the cell cycle is halted. However, the same pathways are used to halt the cell cycle temporarily and more research is needed to distinguish how permanent cell cycle arrest occurs (Campisi and d'Adda di Fagagna, 2007).

An alteration in the proportion of senescent versus actively dividing cells may have an impact that impairs tissue function in a way observed in older organisms. Both increased and reduced cellular senescence can lead to such age-related detrimental changes in function. For example p16 (Ink4a), a biomarker of senescence, was targeted in mice to eradicate all p16 (Ink4a) positive senescent cells. Particularly in the eye, adipocyte tissue and skeletal tissue removal of senescent cells in this way improved age-related phenotypes (Baker *et al.*, 2011). On the other hand, the evasion of senescence is one of the pathological functions of cancer cells which hence divide indefinitely. Cancer cells evade senescence through the expression of oncogenes, such as c-MYC. Melanoma cells have a higher than normal expression of c-MYC and when c-MYC was reduced by siRNA in melanoma cells, senescence associated β-galactosidase increased but how c-MYC inhibits apoptosis is still not fully understood (Campisi, 1997; Zhuang *et al.*, 2008).

1.8.2.1 Mechanisms through which SIRT1 modifies senescence

Much of SIRT1s involvement in cellular senescence is through mechanisms either related to the induction or initiation of cellular senescence. For instance, SIRT1 can deactivate p53 through deacetylation and prevent cell cycle arrest, reviewed by (Yi and Luo, 2010). Furthermore, SIRT1 over expressed in embryonic lung fibroblasts lead to decreased p16 expression, increased pRB phosphorylation and lower β-galactosidase expression (a marker of senescence) (Huang *et al.*, 2008). LKB1 is a kinase, which predominantly phosphorylates AMPK, it was demonstrated in HepG2 and HEK-293T cells that LKB1 overexpression caused reduced phosphorylation of pRB and caused G1 cell cycle arrest (Liang *et al.*, 2014). Primary porcine endothelial cells undergoing senescence had increased LKB1 expression but decreased SIRT1 expression. It was shown that SIRT1 overexpression using a transiently transfected expression vector decreased LKB1 expression but increased LKB1 deacetylation and ubiquitination. However, the addition of a proteasome inhibitor (MG132) alongside SIRT1 overexpression caused LKB1 protein to increase, thus it was suggested SIRT1 was initiating LKB1 degradation through deacetylation (Zu *et al.*, 2010).

1.8.3 Telomere length

Telomeres are DNA tandem repeats (TTAAGGG) formed on the ends of chromosomes, which can loop back on themselves with the aid of shelterin protein complexes binding to the poly guanine tail to form a T-loop. These caps on the ends of the chromosomes protect the active DNA but telomere shortening and hence progressive telomere loss occurs in ageing cells (Aubert and Lansdorp, 2008; Shammas, 2011). Telomere loss is partly due to what has been described as the "end replication problem" on the lagging strand of the DNA replication fork. DNA polymerase can only travel in a 5' to 3' direction therefore, RNA primers are used to initiate replication but when these RNA primers are converted to DNA the transcription machinery are unable to bind ahead of the final RNA primer at the end of the strand (as there is no more DNA after this last primer) therefore, the RNA is degraded leaving a short DNA strand (Olovnikov, 1973; Aubert and Lansdorp, 2008). Telomerase is an enzyme that can elongate the short DNA strand following replication by adding TTAAGGG repeats (Aubert and Lansdorp, 2008). Alternatively, cells can elongate telomeres independently of telomerase by developing

alternative lengthening of telomeres (ALT), which evidence suggests is done by homologous recombination (Liau *et al.*, 2015). Many germ line cells maintain telomeres through telomerase expression, whereas adult somatic cells have lower levels of telomerase expression (Cong *et al.*, 2002). Faster telomere shortening has been linked to oxidative stress since triple guanines are particularly sensitive to oxidative damage (von Zglinicki, 2002). Telomere dysfunction/shortening can result in cell senescence or apoptosis. For example, shorter telomeres have been found in senescent human fibroblasts, and pre senescent fibroblasts were rescued from senescence by increasing telomerase expression (Martin-Ruiz *et al.*, 2004). Additionally, when Trf2 (a component of shelterin) was inhibited using a dominant-negative mutant in mouse livers, there was increased telomere shortening, apoptosis and senescence (Lechel *et al.*, 2005). Apoptosis and senescence as discussed above are associated with ageing, and are generally recognised as the outcome of excessive telomere shortening. On the contrary, telomerase or ALT gain of function have also been suggested to promote cancer progression. For example, increased TERC (RNA component of telomerase) copy number in cervical biopsies was associated with progression of uterine cervical dysplasia to invasive cancer (Hopman *et al.*, 2006). Additionally, K5-Tert mice overexpressing Tert (catalytic subunit of telomerase) in the skin had increased tumourgenesis but also had improved wound healing (Cayuela *et al.*, 2005).

1.8.3.1 Mechanisms through which SIRT1 effects telomere length.

SIRT1 can have a positive action on telomere maintenance, which may help prevent apoptosis or senescence in stem cell populations and adult somatic cells but equally may also promote malignancy in cancer cells. For example, in a mouse model overexpressing Sirt1, telomere length was increased in the embryonic fibroblasts but this lengthening of telomeres was not observed when Terc was knocked out, thus Sirt1 mediated increase in telomere length may be in part reliant upon telomerase (Palacios *et al.*, 2010). Telomere length measured by southern blot analysis in induced pluripotant stem cells decreased in the absence of SIRT1 (De Bonis *et al.*, 2014). Finally, Sirt1 overexpression using a lentivirus expression vector in rat mesenchymal stem cells caused an increase in Tert expression, telomerase activity and Ttp1 expression (a shelterin component) but in contrast to other studies did not increase telomere length

(Chen *et al.*, 2014). Further work will be required to assess if targeting SIRT1 therapeutically could be used to improve telomere maintenance/length in age-related diseases, which are in part caused by telomere loss; or reduce telomere maintenance/length by inhibiting SIRT1 in cancer cells, which have a gain of function of telomerase or ALT to evade apoptosis and senescence.

1.8.4 Oxidative stress

Oxidative stress is caused by the accumulation of reactive oxygen species (ROS) within the cell. The mitochondrial electron transport chain is the main source of these free radicals. Cytochrome p450 enzymes present in the endoplasmic reticulum also make a substantial contribution to the intracellular production of ROS, and NADPH oxidases produce ROS when the immune system is triggered. Many extrinsic environmental factors can also contribute to oxidative stress including pollution, smoking, alcohol and UV light. The superoxide (O_2^-) oxidises iron clusters to release free iron, which in turn can react with H_2O_2 in a Fenton reaction ($Fe^{2+}+H_2O_2 \rightarrow Fe^{3+}+OH+OH^-$), producing the highly reactive hydroxyl radical (OH^-). Consumption of these ROS in reactions catalysed by enzymes including superoxide dismutase (SOD) ($O_2^- + SOD \rightarrow H_2O_2$) and catalase ($2H_2O_2 + catalase \rightarrow 2\ H_2O + O_2$) reduces their accumulation within the cell. Hence these enzymes are often described as having an antioxidant protective function. The antioxidant defence mechanisms of the cell also include a wide variety of molecules derived from the diet. These dietary antioxidants include vitamin E, β-carotene, flavonoids, ubiquinol and many others. Accumulation of ROS causes damage to the cellular macromolecules notably DNA, proteins and lipids. Overloading the cells defence capability or impairment of its function results in a build-up of macromolecule oxidative damage in the cell, a characteristic feature of ageing and age-related diseases (Jezek and Hlavata, 2005; Jomova *et al.*, 2010; Romano *et al.*, 2010).

1.8.4.1 Mechanisms through which SIRT1 affects oxidative stress

Since SIRT1-catalysed reactions consume NAD^+, the source (in its reduced form) of electrons that feed into the mitochondrial electron transport chain, it is a key candidate/major modifier of cellular oxidative stress. Thus, a substantial body of research has explored how SIRT1 influences oxidative stress, with the balance of evidence towards a protective role. For example, overexpression of Sirt1 in mouse

hearts reduced oxidative stress from paraquat and increased catalase expression through FOXO1 activation (Alcendor *et al.*, 2007). Secondly, when SIRT1 was activated by the small molecule activator SRTAW04 in a transgenic mouse model with symptoms of multiple schlerosis in the optic nerve, expression of Sod was increased and oxidative stress was reduced (Khan *et al.*, 2014). As a further example, SIRT1 activates PGC-1α by deacetylation as mentioned above, which promotes mitochondrial biogenesis and the expression of antioxidant enzymes, including catalase and SOD (Nemoto *et al.*, 2005; Rodgers *et al.*, 2005; St-Pierre *et al.*, 2006).

1.8.5 Protein degradation

Protein degradation in the cell occurs principally through the activity of two systems, ubiquitin proteasome degradation and lysosomal autophagy. The ubiquitin proteasome system requires unwanted proteins to be marked for degradation by the ligation of ubiquitin molecules to the protein to form a polyubiquitin chain. This chain is subsequently recognised by the proteasome, a multicatalytic enzyme made up of several subunits of which the 20s subunit is the core enzyme involved protein degradation (Martinez-Vicente *et al.*, 2005).

Lysosomal autophagy is the process of internal degradation in the cell to remove unwanted cellular components including organelles and proteins, recycle the constituents of old cellular components, destroy pathogens and, under some conditions, activate cell death (Martinez-Vicente *et al.*, 2005; Levine *et al.*, 2011). Under conditions of DR, autophagy is up regulated to recycle the constituents of cellular components and, hence, maintain growth and development of the organism (Jia and Levine, 2007; Hansen *et al.*, 2008). There are currently three main types of lysosomal autophagy, which are described below:

Microautophagy is the process where the lysosomal membrane invaginates and/or protrudes from the plasma membrane to engulf large areas of the cytoplasm including organelles, which are then degraded inside the lysosome (Martinez-Vicente *et al.*, 2005).

Macroautophagy is when organelles and unwanted proteins are surrounded in the cytosol by a new membrane to form an autophagosome. The autophagasome then fuses with the lysosome allowing the contents to be degraded (Martinez-Vicente *et al.*, 2005).

Chaperone mediated autophagy (CMA) is a more specific mechanism where by individual proteins are chaperoned to the lysosome. A KFERQ motif in the protein sequence is recognised by heat shock chaperone 70, which binds to the protein along with other co-chaperones. The protein is transported to the lysosome membrane where the chaperones dock to the LAMP2 receptor. The protein is then fed through LAMP2 in to the lysosome for degradation (Martinez-Vicente *et al.*, 2005).

Misfolded proteins, damaged proteins and unwanted proteins build-up in the cell with age and are often stored as plaques or aggregates, which is due to overload or malfunction of degradation pathways (Cuervo and Dice, 2000b; Martinez-Vicente *et al.*, 2005). For example, it has been demonstrated in epidermal cells harvested from human females of varying ages, that the 20S proteasome component (the proteasome is a multicatalytic enzyme which degrades ubiquitin tagged proteins (Martinez-Vicente *et al.*, 2005) decreases with age (Bulteau *et al.*, 2000). LAMP2 the lysosomal membrane receptor for protein substrates chaperoned during CMA, had reduced self-recycling capability from the lysosomal lumen to the lysosomal membrane in 22 month old rats compared with 4 month old rats (Kiffin *et al.*, 2007). Also the mitochondrial unfolded protein response is a response to cellular stress that corrects misfolded proteins. Alterations in the activity of this pathway have been seen to affect lifespan in *Caenorhabditis elegans.* A transgenic strain of *Caenorhabditis elegans* carrying a deletion of the mitochondrial ribosomal protein S5 lived longer than controls, coincident with increased activity of the mitochondrial unfolded protein response pathway (Houtkooper *et al.*, 2013). Protein aggregation in mammalian cells can lead to age-related diseases particularly neurodegenerative diseases such as Alzheimer's disease, associated with amyloid-β plaques, Huntington's disease associated with huntingtin misfolded proteins and Parkinson's disease associated with protein aggregates in dopaminergic neurons (Martinez-Vicente *et al.*, 2005; Gundersen, 2010; Arrasate and Finkbeiner, 2012).

1.8.5.1 Mechanisms through which SIRT1 influences protein degradation and specifically autophagy.

SIRT1 is believed to be involved in the regulation of autophagy. Treatment of THP-1 cells with sirtinol (a SIRT1 inhibitor) impaired autophagy function and resulted in inflammation, which could be through inhibition of the mTOR pathway (Takeda-Watanabe *et al.*, 2012). However, another study demonstrated that depletion of the SIRT1 substrate NAD^+ using the drug FK866 induced autophagy, which could be interpreted to be an observation discordant with the view that SIRT1 action promotes autophagy (since SIRT1 should be less active under these conditions) or that SIRT1 promotes autophagy by consuming and depleting NAD^+ (Billington *et al.*, 2008). Effects of SIRT1 on CMA is a relatively under-investigated area, which forms the focus of some of the work in this thesis presented in Chapter 5.

1.8.6 DNA damage response

The DNA damage response comprises several pathways that act to repair single base pair mutations, double strand breaks and base additions. Figure 1.8.2 shows a simplistic diagram of the DNA damage response. DNA damage sensors recruit Ataxia telangiectasia-mutated (ATM) or ataxia telangiectasia rad3 related (ATR) kinases to the site of damage, ATM is recruited to DNA single strand breaks and ATR is recruited to damage, which occurs during replication. ATM and ATR activate DNA damage response mediators, which remain at the site of damage and initiate repair, and initiate signalling through check point kinases (CHK) to effectors such as, p53 which can halt the cell cycle until DNA is repaired (Sulli *et al.*, 2012). It has been well documented that DNA damage increases with age. For example, in normal human fibroblasts, W134 fibroblasts and prostate epithelial cells, γ-H2AX foci (a marker of DNA damage) increased with

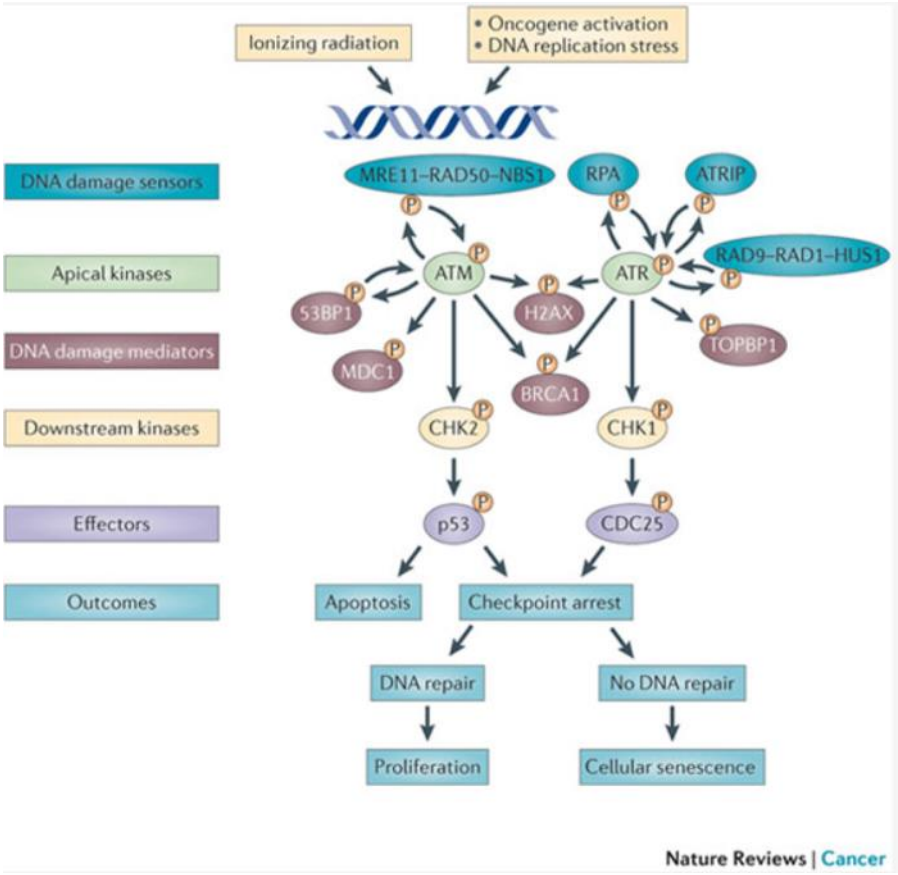


Figure 1.8.2. The DNA damage response. DNA damage occurs during DNA replication and can be caused by stressor, such as oxidative stress. Sensors detect the damage and recruit the signalling molecule ATM or ATR kinases to the site of damage. ATM and ATR phosphorylate and recruit DNA damage mediator, which can initiate repair, and cause phosphorylation and activation of CHKs, which cause cell cycle arrest through effectors like p53, to allow for DNA repair or apoptosis if the damage is unrepairable. Diagram sourced from (Sulli *et al.*, 2012)

passages (Sedelnikova *et al.*, 2004).Many studies have also shown changes in the activity of DNA repair pathways with age. For example, ATM and ATR kinases, involved in the initiation of the DNA damage response, were shown to be more active with age and following paraquat treatment in intestinal stem cells of *Drosophila melanogaster* (Park *et al.*, 2015). Another major point of interaction between ageing and the response to DNA damage arises because DNA damage accumulates progressively with age, to a point where the DNA damage response becomes overloaded, ultimately leading to cell senescence, apoptosis and in some circumstances cancer (Seviour and Lin, 2010).

1.8.6.1 Mechanisms through which SIRT1 affects DNA damage repair

SIRT1 has been associated with components of the DNA damage response, examples include:

- SIRT1 and the DNA single strand break sensing enzymes PARPs appear to cross talk. Sirt1 can deacetylate and deactivate Parp1 and Sirt1 expression was increased in Parp2-knockout mice (Luna *et al.*, 2013), discussed in more detail below.
- Interestingly, it would appear that SIRT1 is repressed when the DNA damage response is initiated. For example, the Homeodomain interacting protein kinase 2 (HIPK2) is a signaling kinase, which promotes apoptosis in response to DNA damage, also phosphorylates SIRT1 at serine 682, deactivating SIRT1 upon severe DNA damage (Conrad *et al.*, 2015). Once more, Deleted in bladder cancer protein 1 (DBC1) is phosphorylated at threonine 454 and activated by ATM or ATR, DBC1 is a potent inhibitor of SIRT1 activity, thus repressing p53 deacetylation by SIRT1 and allowing active p53 to arrest the cell cycle in the DNA damage response (Magni *et al.*, 2014) (Zannini *et al.*, 2012).

1.9 The sirtuin family

SIRT1 is the most intensively researched and thus best understood member of the sirtuin family. However, several other members of the sirtuin family have also been associated with ageing and/or the response to DR, as summarised below:

- **SIRT2-** SIRT2 similar to SIRT1 is elevated in response to DR. However, research on the effects of SIRT2 on lifespan and healthspan is currently lacking (de

Oliveira *et al.*, 2012). SIRT2 has been linked to some age-related diseases including Parkinson's disease but unlike SIRT1, SIRT2 appears to have a toxic effect. For example, SIRT2 was inhibited by siRNA in a human cell line Parkinson's disease model, which resulted in α-synuclein toxicity reducing (Outeiro *et al.*, 2007).

- **SIRT3-** SIRT3 is also elevated in DR and plays an essential role in metabolism by activating multiple mitochondria enzymes involved in the electron transport chain and fatty acid oxidation (Kincaid and Bossy-Wetzel, 2013). SIRT3 also directly deacetylates and promotes ROS scavenging in the mitochondria by SOD2 (Qiu *et al.*, 2010), thus reducing oxidative stress.
- **SIRT4-** Reduced SIRT4 activity was measured in mice undergoing DR and it was shown in mouse pancreatic β cells that SIRT4 ribosylates glutamate dehydrogenase (involved in the metabolism of glutamate to produce ATP) and thus inhibits its catalytic activity. This resulted in reduced insulin release (Haigis *et al.*, 2006).
- **SIRT5-** SIRT5 was found to be elevated alongside SIRT1 in the cerebral tissue of DR rats (Geng *et al.*, 2011). Minimal research has been undertaken on SIRT5. Although not a direct link to ageing, it was shown that SIRT5 promotes ammonia detoxification by deacetylating and activating carbamoyl phosphate synthetase 1 (urea cycle regulator) (Nakagawa *et al.*, 2009). Further research is needed to expand our knowledge of SIRT5 and determine if it has a role in ageing.
- **SIRT6-** SIRT6 has been associated with DR, for example HEK293 cells grown in reduced serum medium to mimic DR had elevated SIRT6 protein levels (Kanfi *et al.*, 2008). More recently it was also shown in male SIRT6 transgenic mice over expressing SIRT6 that lifespan increased by 14.5% compared to wild type littermates. These SIRT6 transgenic mice also showed lowered IGF1 serum levels and phosphorylation changes in the IGF signaling pathway, which has been previously linked to lifespan regulation (Kanfi *et al.*, 2012) (Zarse *et al.*, 2012).
- **SIRT7-** SIRT7 was measured in adipocyte, muscle, heart and liver tissue from DR rats but SIRT7 protein, and mRNA levels were either unaffected or

inconsistently reduced (Wronska *et al.*, 2015). SIRT7 overexpression was shown to improve aged hematopoietic stem cell regeneration ability (Mohrin *et al.*, 2015).

The examples presented above suggest the high likelihood that all members of the sirtuin family play a role in ageing. Although SIRT1 was the specific focus of the current project but other sirtuin family members may play equally important roles.

1.10 Cross talk between SIRT1 and the DNA damage response enzyme PARP.

1.10.1 PARP enzymes

Poly ADP-ribose polymerases (PARPs) are a group of enzymes that synthesise ADP-ribose polymers through the cleavage of NAD^+ to release ADP-ribose. There is evidence that PARP enzymes are involved in several cellular processes and pathways including:

- Cell death caused by extensive DNA damage resulting in PARP over activation;
    - This then either depletes cellular NAD^+ reserves causing necrosis or causes the release of Apoptosis Inducing Factor (AIF) from the mitochondria, which relocates to the nucleus to initiate apoptosis (Chiarugi and Moskowitz, 2002).
- Chromatin compaction and de-condensation through attachment of PARP to nucleosomes;
    - The mechanism for chromatin compaction has not been fully elucidated but PAR polymer synthesis on histones can destabilise the nucleosome causing de-condensation (Poirier *et al.*, 1982).
- Transcriptional regulation through chromatin remodelling or through promoter enhancement or inhibition, which is cell and gene dependent (Pavri *et al.*, 2005);
- Inflammation;
    - PARP is a coactivator of NF-κB, which initiates the inflammatory response. Inhibition of Parp in rats where cerebral bleeding (stroke) was induced, reduced the inflammatory response and improved long term neuronal survival (Kauppinen *et al.*, 2009).
- Repair of DNA damage;

- This is considered to be the primary function of PARP enzymes. PARP binds to DNA single strand breaks to stabilise the break and poly ADP-ribose (PAR) is synthesised to recruit other enzymes to the break for bass excision repair (Malanga and Althaus, 2005). It was shown that PAR synthesis triggers ATM signalling; when DNA damage was induced using MNNG in A-T fibroblasts PAR synthesis increased and phosphorylation of ATM substrates p53, SMC1, and H2AX also increased but in cells deficient in ATM, MNNG caused PAR synthesis to increase but phosphorylation of p53, SMC1, and H2AX did not change(Haince *et al.*, 2007).

The PARP family consists of multiple enzymes (listed below). Research to date has focused on PARP1 and PARP2, hence understanding of other members of the family is minimal:

- The function of PARP1 in DNA strand break repair has been studied extensively. The important contribution made by PARP1 to this process was demonstrated in *Parp1* -/- mouse embryonic fibroblasts. Whole cell lysate incubated with a DNA substrate containing a basic sites was half as efficient at repairing the DNA compared to control cell lysate (Dantzer *et al.*, 2000).
- PARP2 is highly similar to PARP1 but has been identified as a nucleolar enzyme (Ame *et al.*, 1999; Ame *et al.*, 2004). PARP2 also functions in DNA strand break repair, which is revealed through multiple published observations, including increased sensitivity of Parp2 -/- mouse embryonic fibroblasts to DNA damage causing agents (Menissier de Murcia *et al.*, 2003).
- PARP3 has been localised to the nucleus and believed to be involved in transcriptional silencing through binding with polycomb group bodies (Rouleau *et al.*, 2007). Immunofluorescence in HeLa cells has revealed that PARP3 was a component of the daughter centrosome during the cell cycle (Augustin *et al.*, 2003).
- PARP4 is the largest family member and has been shown to interact with telomerase-associated protein 1 (TEP1). However, telomere maintenance and

length appeared unaffected in Parp4 deficient mice suggesting action in this context is dispensable (Liu *et al.*, 2004).

- PARP5, also known as Tankyrase, has been found in 3T3-L1 fibroblasts to associate with the Golgi apparatus and specifically with insulin-responsive amino peptidase (IRAP) a member of the GLUT4 vesicle, leading to the suggestion the enzyme may play a role in the regulation of glucose metabolism (Chi and Lodish, 2000).
- PARP6 through to PARP16 have also been identified but currently little is known about their function.

1.10.2 PARP activity can modify the ageing process.

Like SIRT1 PARPs appear to have actions that modify cellular ageing and age-related disease. *Table 1.10.1* presents and summarises some specific actions of PARPs in this context.

Table 1.10.1. PARP involvement in ageing processes and disease.

Ageing process or disease	Relevant actions of PARPs
Telomere shortening	• PARP2 binds with a TTAAGGG repeat binding protein (TRF2) (a telomere t-loop forming protein) and inhibits its ability to bind DNA by poly ADP-ribose formation in U2OS cells (Dantzer *et al.*, 2004). However, Parp2 -/- mouse embryonic fibroblasts maintained telomerase activity and telomere length similar to wild type cells (Dantzer *et al.*, 2004).
Cancer	• Parp1 -/- mice exposed to the carcinogen N:-nitrosobis (2-hydroxypropyl) amine, experienced faster tumour progression compared with Parp1 +/+ mice (Tsutsumi *et al.*, 2001). • Inhibition of PARP1, reducing DNA single strand break repair, led to apoptosis of embryonic stem cells with mutated BRCA1 and BRCA2 (Farmer *et al.*, 2005). • PARP1 over expression in breast cancer has been associated with higher tumour grade and a poorer prognosis (Rojo *et al.*, 2012). Thus, PARP activity may increase tumour progression after cancer initiation but appears to be protective against cancer in normal cells.
Lifespan extension	• Increased PARP activity across several species was associated with longer lifespans. For example, PARP activity was 5x higher in human mononuclear leukocytes compared with rat mononuclear leukocytes, corresponding with a 20x difference in lifespan. PARP activity decreased with age in both rats and humans (Grube and Burkle, 1992). • PARP activity was higher in human centenarians compared with controls aged 20-70 years (Muiras *et al.*, 1998).
Neurodegeneration	• Studies have indicated that PARP1 activity can promote or progress neurodegenerative diseases, including, Parkinson's disease and Alzheimer's disease. PARP1-/- mice were resistant to the neurotoxin MPTP, which induces Parkinsonism. PARP1-/- mice experienced decreased microglial response to amyloid-β injected into the brain compared with controls. This microglial response was also shown to involve activation of NF-κB by PARP1 (Love *et al.*, 1999; Mandir *et al.*, 1999; Kauppinen *et al.*, 2011)

Cardiovascular disease	• Cardiac toxicity was induced using doxorubicin in Parp -/- and Parp +/+ mice. PARP -/- mice had greater left ventricle dysfunction compared with Parp +/+ mice (Pacher *et al.*, 2002). • Parp1 activity correlated positively with cardiac hypertrophy in mice and humans and PARP1 activation by a PERP expression plasmid in human cardiac myocytes led to cell death (Pillai *et al.*, 2005).
Diabetes	• The destruction of islet cells in mice using streptozocin caused Parp activation. Parp inhibition using PJ34 post streptozocin treatment improved vascular responsiveness, which is affected by hyperglycemia that reduces NO production (Garcia Soriano *et al.*, 2001). • Furthermore, Parp -/- mice were resistant to induction of diabetes by the β-cell toxin streptozocin (Burkart *et al.*, 1999).

1.10.3 PARP1 and PARP2 associations with SIRT1

Thus far some of the separate effects of SIRT1 and PARPs on cellular ageing and age-related diseases have been outlined. However, a notable commonality in their catalytic function, the cleavage of NAD^+, suggests that effects of both enzymes in this context may be inter-dependent. Indeed, published findings show that such interactions do play out both through likely competition for the cellular NAD^+ pool but also through other NAD^+-independent mechanisms. For example, PARP1 has been shown to have an indirect effect on *SIRT1* transcription through the transcription factors c-MYC and E2F1. PARP1 binds to E2F1 promoting E2F1 transcription of *c-MYC* (Simbulan-Rosenthal *et al.*, 2003). Over expression of c-MYC has been shown to increase *SIRT1* transcription, which in turn led to deacetylation at lysine 323 of c-MYC by SIRT1 and its consequent de-stabilisation (Yuan *et al.*, 2009). However, in contrast to the findings of the earlier study deacetylation at lysine 63 of c-MYC by SIRT1 appeared to increase stability of c-MYC (Menssen *et al.*, 2012). On the other hand, it appears that expression of Sirt1 can be repressed by Parp. Specifically, knockout of Parp2 in mice increased the expression and activity of Sirt1 (Bai *et al.*, 2011).

SIRT1 and PARP1 can also interact more directly, for example, SIRT1 can deacetylate and hence inactivate PARP1, as indicated by the observation that Sirt1 -/- mice showed increased acetylation of Parp1 (Rajamohan *et al.*, 2009a). Another example of a more direct interaction between SIRT1 and PARP is that transcription of the PARP1 gene

appears to be inhibited by SIRT1, as suggested by the observation that Parp1 expression was decreased in rat cardiac myocytes by overexpression of Sirt1 (Rajamohan *et al.*, 2009a). However, published data on the interactions of SIRT1 and PARP are limited and thus will be revisited in Chapter 4 of this thesis.

1.11 The involvement of epigenetics in ageing and how SIRT1 may influence epigenetics.

Epigenetic modifications can influence gene expression without changing the underlying DNA sequence; this is achieved through the organisation and structure of DNA, which is influenced by covalent chemical modifications of both the DNA and associated histone proteins. Nucleosomes are stretches of 147 bps of DNA organised around an octamer of the histone proteins: H2A, H2B, H3 and H4. The DNA is wrapped around each histone octamer twice and the nucleosomes are joined by segments of linker DNA. The N-terminal domains of histone proteins undergo epigenetic modifications that have the ability to activate or repress gene expression. Modifications of histone N-terminals include methylation, acetylation, phosphorylation and ubiquitination. The outcome of single/multiple histone modifications on gene expression is dependent upon the location and the type of modification; a large body of research is dedicated to unlocking further the histone code (Quina *et al.*, 2006; Kondo, 2009; Ford *et al.*, 2011).

Additional to histone modifications, DNA can also undergo direct chemical modification, including methylation at cytosine basics when followed by a guanine (CpG site). This modification also affects gene activation and repression. When a CpG site in or around a gene promoter region is methylated the methyl group may block the binding of the transcriptional machinery hence blocking gene transcription. It is still currently unknown how many CpG sites need to such functional effects or whether the methylation of just one critical CpG site is sufficient. Specific genes may require different patterns of CpG site methylation to alter their expression and effects at the level of tissue function are likely dependent on the proportion of cells with DNA methylation at critical sites (Quina *et al.*, 2006; Kondo, 2009; Ford *et al.*, 2011).

DNA methylation changes over time and may be linked to age-related phenotypes. The epigenome of monozygotic twins is barely distinguished as babies but in older monozygotic twins there is a discordant pattern of global DNA methylation (methylation across the genome measured at repetitive elements) between twin pairs. It is proposed that the change in global DNA methylation between monozygotic twin

pairs comes about through environmental exposures (Fraga *et al.*, 2005). More recently, an Icelandic cohort of 111 individuals and a Utah cohort of 126 individuals were tested 11 and 16 years apart respectively. Changes in global DNA methylation over time showed considerable inter-individual variation with some of the individual's genomes becoming more methylated where others lost DNA methylation over time (Bjornsson *et al.*, 2008).

The studies cited above measured age-related changes in global, DNA methylation and hence are very limited with respect to providing any insight into influences of these changes on parameters of ageing at a molecular level. Information on gene-specific alterations in DNA methylation is essential to understand how DNA methylation may affect age-related phenotypes. Studies of gene-specific effects of age on DNA methylation take one of two approaches, either gene-targeted investigations, which study candidate genes or genome wide measurement of DNA methylation. As examples of gene targeted approaches an increase in DNA methylation in genes previously linked to prostate cancer specifically *RARbeta2, RASSF1A, GSTP1, NKX2-5, ESR1* and *CLSTN1* [1] was observed in prostate tumour tissue compared to normal prostate tissue (Kwabi-Addo *et al.*, 2007). In Parkinson's disease patients compared to healthy controls, the *Neuronal PAS Domain Protein 2 (NPAS2)* clock gene was found to have significantly decreased DNA methylation (Lin *et al.*, 2012).

Although there was an observed change in DNA methylation of specific genes in these two studies, the DNA methylation changes can only be *associated* with age-related phenotypes until models are developed to test directly if these observed alterations in DNA methylation actually affect gene expression in a manner that contributes to the disease process. In this regard the most incisive test would be to recapitulate specifically the observed alteration in DNA methylation on a control background to test for the appearance of the age-related phenotype in question. Currently genome editing tools with such capability have not to our knowledge, been developed. An approach that could derive data consistent with causality would be to manipulate DNA methylation *in*

[1] Retinoic acid receptor beta2 (RARbeta2), Ras Association (RalGDS/AF-6) Domain Family Member 1 (RASSF1A), Glutathione S-Transferase Pi 1 (GSTP1), NK2 Homeobox 5 (NKX2-5), Estrogen Receptor 1 (ESR1) and Calsyntenin (CLSTN1).

vivo through (tissue specific and possibly age specific) over expression of a DNMT transgene or through reduced DNMT expression using (again targeted) gene knockout approaches.

A comprehensive map of the human epigenome has not yet been produced, but has potential to improve understanding of how changes in DNA methylation could affect ageing. Recent epigenome-wide association studies (EWAS) have begun to pave the way forward in linking epigenetic variations with complex diseases and ageing (Rakyan *et al.*, 2011). One EWAS study conducted in females aged 32 through to 80 measured changes in DNA hyper-methylation with age and identified 490 sites with increased DNA methylation with age but found that very few age-related phenotype genes had differential methylation in ageing (Bell *et al.*, 2012).

1.11.1 Epigenetic functions of SIRT1.

Both epigenetic alteration, notably but not exclusively DNA methylation and SIRT1 have well documented likely roles in modifying ageing. Evidence as described below, that SIRT1 itself has epigenetic actions links these two modifying variables.

SIRT1 is classified as a (class III) histone deacetylase (Liu *et al.*, 2009). Thus, histone deacetylation is one of the epigenetic actions of SIRT1 that is best understood. SIRT1 deacetylation of lysine residues in histones 1, 3 and 4 (lysine 26, 9 and 16 respectively) in turn promotes histone methylation. It has also been shown that SIRT1 can activate the histone methyltransferase SUV39H1 through deacetylation at lysine 266, SUV39H1 methylates histone3 at lysine 9 (Vaquero *et al.*, 2007).

There are also observations that suggest SIRT1 affects DNA methylation. SIRT1 was found localised at hypermethylated promoters of tumour suppressor gene (which were repressed) but not localised at these promoter regions when the tumour suppressor genes were expressed in other cell lines. When SIRT1 was inhibited by splitomycin or nicotinamide in cell lines with repressed tumour suppressor genes, the genes were no longer repressed. However, DNA hypermethylation of the tumour suppressor gene promoters remained after SIRT1 inhibition and it was suggested SIRT1 inhibition increased tumour suppressor gene expression through an increase in histone acetylation observed (Pruitt *et al.*, 2006). Possible interpretations of these observations

are that SIRT1 promotes DNA methylation at CpG islands (which may not be reversed by SIRT1 inhibition alone) or that SIRT1 binds preferentially to these sites once they have been methylated through other mechanisms. In our laboratory we observed that when SIRT1 was knocked down or over expressed in Caco-2 cells and HuVECs and whole genome analysis was performed to observe DNA methylation patterns in an array of genes. Significant changes in DNA methylation across the array were observed in response to SIRT1 manipulations in both cell lines (Wakeling *et al.*, 2015), suggesting SIRT1 has an effect on DNA methylation. One mechanism suggested for SIRT1 to cause DNA methylation is through DNA methytransferase 1 (DNMT1), SIRT1 deacetylates DNMT1 at lysine residues 1349 and 1415 instigating increased DNMT1 catalytic activity (Peng *et al.*, 2011).

Epigenetic modifications like methylation could be a key mechanism for SIRT1 to regulate systems in the cell through targeting gene expression. Epigenetic modifications by SIRT1 specifically DNA methylation has been hypothesised by our laboratory to be a contributing factor to ageing (Ions *et al.*, 2012). As a regulatory mechanism, genome methylation in response to SIRT1 manipulation will be investigated further in later chapters of this thesis.

1.12 Effects of ageing on stem cell function and the influence of SIRT1.

Several organs and tissues are known to be renewed through the differentiation of an adult stem cell population. Adult stem cells are vulnerable to the same mechanisms of cellular ageing that affect differentiated cells, including telomere shortening and damage from oxidative stress (Smith and Daniel, 2012). An example of this is in mouse embryonic stem cells exposed to oxidative stress via H_2O_2 treatment, which resulted in increased expression of the apoptotic surface protein phosphatidylserine, a marker of apoptosis (Guo *et al.*, 2010). Stem cells can also develop age-related diseases similar to differentiated cells. For example, in the rat colon mucosa there was as an increase in the expression of cancer biomarkers, including, cell surface markers Cd44 and Cd166 and the cytoplasmic marker alcohol dehydrogenase1, with age (Nautiyal *et al.*, 2012).

1.12.1 Polycomb group proteins regulate stem cell differentiation.

Polycomb group proteins were first discovered to be gene repressors in *Drosophila melanogaster* in the context of Hox gene inhibition (Lewis, 1978). Polycomb group proteins form two complexes, Polycomb Repressive Complex 1 (PRC1) and Polycomb Repressive Complex 2 (PRC2), which can ubiquitinate and methylate histones respectively. PRC2 is made up of the components EED, EZH2, SUZ12 and RbAP48 (defined in the list of abbreviation) and PRC1 is made up of the components BMI1, PHC1 and RNF2 (defined in the list of abbreviation). PRC1 and PRC2 silence chromatin at polycomb gene targets (PCGTs). PCGTs are typically lineage-specific genes expressed only in mature, differentiated cells, thus PRCs regulate stem cell differentiation (Di Croce and Helin, 2013).

Polycomb group protein function can influence cellular ageing. For example, premature senescence of haematopoietic stem cells was observed in a Bmi-1 knockout mouse model and further studies revealed that Bmi-1 inhibition of Arf1 and Ink4a is important in maintaining the haematopoietic stem cell population. Arf1 stabilises p53, and Ink4a is a cyclin dependent kinase inhibitor, both Arf1 and Ink4a promote cellular senescence (Park *et al.*, 2003; Molofsky *et al.*, 2005). As another example, transplanted haematopoietic cells differentiated and provided a sustained population of mature blood cells only when the Prc2 component Ezh2 was overexpressed, which concurs with an observation that overexpression of Ezh2 in mouse embryonic fibroblasts allowed by-pass of cellular senescence, which suggested to be due to stabilised chromatin structure (Kamminga *et al.*, 2006).

1.12.2 Effects of SIRT1 on stem cell differentiation.

Several studies have shown an effect of SIRT1 on stem cell differentiation. For example, Sirt1 knockout in mesenchymal stem cells in mice resulted in defects in tissue function with age, which was shown to be due to reduced deacetylation of β-catenin by Sirt1 and consequently reduction in β-catenin in the nuclei of mesenchymal stem cells, which normally promotes the transcription of genes involved in mesenchymal stem cell differentiation (Simic *et al.*, 2013). A similar function of Sirt1 to slow stem cell differentiation has also been shown in mouse embryonic stem cells. Sirt1 deacetylates retinoic acid binding protein II and hence prevents its localisation to the nucleus to

promote differentiation. When Sirt1 was deficient, mouse embryonic stem cells entered a state of hyper differentiation resulting in severe developmental abnormalities (Tang *et al.*, 2014). Furthermore, differentiation of mouse pluripotent stem cells into neural stem cells was associated with a reduction in Sirt1 expression, and Sirt1 inhibition increased differentiation into neural stem cells (Hu *et al.*, 2014). On the other hand, embryonic stem cells from Sirt1 knockout mice were unable to form substantial blast cell colonies (Ou *et al.*, 2011).

As already described, SIRT1 has been associated with DNA methylation, based on evidence that includes data on gene-specific actions from our own laboratory (Ions *et al.*, 2012). Further exploration of the action of SIRT1 on DNA methylation carried out in our laboratory using MeDIP then either hybridisation to a promoter microarray (Caco-2 cells) or sequencing (HuVECs) revealed that across the genome the effects of SIRT1 on DNA methylation clustered in particular at PCGTs. This observation formed the basis of a hypothesis investigated in work presented as Chapter 6 of this thesis that SIRT1 affects DNA methylation at PCGTs and hence may influence stem cell differentiation through actions on the polycomb proteins (Wakeling *et al.*, 2015).

In summary, it appears that SIRT1 plays varying roles in different types of stem cells, and can either promote or prevent stem cell differentiation. Further research is required to fully understand the likely pleiotropic roles of SIRT1 in stem cell renewal and differentiation. DNA methylation is a potential novel aspect of the function of SIRT1 that may be important in this context.

1.13 Aims and Objectives

1.13.1 Project Aims

The aim of the work presented in chapters 3, 4 and 5 is to acquire data ultimately to populate a model of the interrelationship between the activity of SIRT1, PARP and CMA that will enable us to generate testable predictions about the effect on the other variables in the system of modifiers of their activity in particular NAD^+ and resveratrol. Over the longer term such a model may inform how targeted manipulations to this system can counteract effects of cellular ageing.

Another level at which SIRT1 may act to influence cellular ageing is on DNA methylation. As already stated, other work in the laboratory uncovered the finding that the effects of SIRT1 on DNA methylation cluster in particular at loci that are targets of the polycomb repressive complexes. These complexes repress the expression of genes involved in stem cell differentiation through epigenetic actions. The work presented in Chapter 6 began to explore the mechanism through which SIRT1 affects DNA methylation at the target loci of the polycomb repressive complexes.

1.13.2 Specific objectives

- To investigate if resveratrol affects SIRT1 expression and/or NAD^+ concentration in Caco-2 cells.
- To determine *in vivo*, using mice, and *in vitro*, using Caco-2 cells, if there are associations between levels of expression of Sirt1, Parp activity and NAD^+ concentration.
- To determine if SIRT1 and/or resveratrol affects CMA in Caco-2 cells and to determine if DNA methylation is a likely mediating mechanism.
- To determine if SIRT1 affects the expression of any of the components of the polycomb repressive complexes and/ or if SIRT1 associates directly with the component of PRC2 EZH2.

2 Materials and Methods

2.1 Reagents

Unless otherwise stated chemicals and reagents were purchased from Sigma Aldrich.

2.2 Mouse tissue

PARP 1 -/- mice were a gift from de Murcia JM and de Murcia G. PARP -/- mice were generated by electroporation of mouse embryonic stem cells with a targeting vector consisting of a 9 kb region of the *Parp1 gene* (introns 2-7) into which the neo gene driven by the phosphoglycerate kinase promoter was inserted at exon 4. Cells resistant to G418 were selected and positive clones were injected into C57BL/6 blastocysts. The F2 generation resulting from mating of the chimeric F1 offspring were genotyped by Southern blotting to detect germ-line (de Murcia *et al.*, 1997). Intestinal tissue was harvested from the mice and immediately frozen in liquid nitrogen and stored at -80 °C until protein extraction (described below).

2.3 Cell culture

Human intestinal Caco-2 cells (ATCC® HTB-37™) sourced from a colorectal adenocarcinoma of a 72 year old caucasian male were cultured at 37 ˚C with 5 % CO_2 in air in175 ml flasks with complete cell growth medium (15 ml of DMEM containing 0.6 µl/ml gentamycin, 10% foetal bovine serum (FBS) and 1% non-essential amino acids). During routine passage (approximately every 7 days), confluent cells had the complete cell growth medium removed and were washed twice with 10 ml PBS before adding 3 ml trypsin and incubating for 10 min to detach the cells from the base of the flask. Seven millilitres of complete cell growth medium was then added to the detached cells before transferring the suspension in to a universal tube. The cells were pelleted by centrifuging (3000 g, 5 min, RT) and the supernatant fluid was discarded. The Cells were resuspended in 10 ml complete cell growth medium before counting the cells using a haemocytometer to enable reseeding at a density of 3.5 x 10^4 cells/ml. Caco-2 cells when confluent differentiate in to mature enterocytes forming a monolayer similar to that found in the intestinal epithelium, thus making them a firm choice to study the effects of diet and ageing (Sambuy *et al.*, 2005).

Human umbilical vein endothelial cells (HuVECs) (Lonza) were cultured at 37 °C with 5 % CO2 in air in 175 ml flasks with 15 ml of EBM-2 medium (Lonza) containing Bullet Kit™ (Lonza). HuVEC cells were attached to the bottom of the flask using 1 % Gelatin Attachment Factor (Life Technologies). Routine passage was as described above for Caco-2 cells, substituting the complete cell growth medium for EBM-2 medium but also using a seeding density of 3.5 x 10^4 cells/ml.

For counting, cells were suspended in 50 % complete cell culture medium (as above) and 50 % Trypan Blue, which allows visualisation of only living cells only under a light microscope. A seeding density of 3.5x10^4 cells/ml was used for experimental manipulations, which were performed in 6 well transparent plastic plates (Greiner BioOne).

For cryogenic storage cells were detached from 175 ml flasks using trypsin and collected by centrifugation (for 5 min at 3000 g). Each cell pellet was resuspended in 4 ml of freezing medium (90 % FBS, 10 % DMSO) and resuspended cells were placed in cryotubes in 1 ml aliquots. The cryotubes were sealed then placed in a Mr. Frosty™ freezing container (Thermo Scientific) containing isopropanol and incubated overnight at -80˚C. Cryotubes were stored for future use in liquid nitrogen.

2.4 Experimental manipulation of cultured cells

All experimental manipulations on Caco-2 cells and HuVECs were conducted 24 h after cells were seeded.

2.4.1 Genetic manipulation of cells

2.4.1.1 siRNA

SIRT1 knockdown was achieved by transfecting Caco-2 cells and HuVECs with Stealth RNAi (Life Technologies). Two siRNA constructs were used:

siRNA1: 5'UACAAAUCAGGCAAGAUGCUGUUGC3' sense

siRNA2: 5'UUUGUCAUACUUCAUGGCUCUAUGA3' sense

A [DT][DT] 3' overhang was added to each sequence. A Stealth RNAi™ siRNA negative control Lo GC (Life Technologies) was also used in all experiments. Cells were transfected using Lipofectamine® RNAIMAX Transfection Reagent (Life Technologies) Lipofectamine® RNAIMAX Transfection Reagent was incubated with opti-MEM medium for 5 min at RT (4 µl of reagent and 150 µl opti-MEM for 1 well) and siRNA was also incubated with opti-MEM medium for 5 min at RT (5 µl siRNA and 150 µl opti-MEM for 1 well). The two mixtures were then combined and incubated for a further 20 min at RT before adding to the cells alongside 2 ml per well complete cell growth medium. The medium was refreshed with 2 ml complete cell growth medium 24 h post transfection and cells were then harvested 72 h post transfection.

2.4.1.2 Transfection with pPS-CFP2-N

Caco-2 cells were transfected with a recombinant plasmid generated from the pPS-CFP2-N vector (Appendix C) (Evrogen) using Lipofectamine® 2000 Transfection Reagent (Life Technologies). Lipofectamine® 2000 Transfection Reagent was incubated with opti-MEM medium for 5 min at RT (9 µl of reagent and 150 µl opti-MEM for 1 well) and DNA was also incubated with opti-MEM medium for 5 min at RT (2500 ng DNA and 150 µl opti-MEM for 1 well). The two mixtures were then combined and incubated for a further 20 min at RT before adding to the cells alongside 2 ml per well complete cell growth medium The medium was refreshed with 2 ml complete cell growth medium 24 h post transfection immediately before capturing images.

2.4.1.3 Transfection with a SIRT1 promoter reporter plasmid

The *SIRT1* promoter-reporter construct generated using the vector pBlue-TOPO® (Appendix A) (Life Technologies) was transfected in to Caco-2 cells using 1.7 µg/well with Genejammer® transfection reagent (Stratagene). Four micro litres per well Genejammer® and 100 µl per well DMEM were mixed together and incubated for 5 min at RT. DNA (1700 ng per well) was then added to the Genejammer®/DMEM mixture and incubated for 15 min at RT. The mixture was then added to the cells alongside 900 µl per well complete cell growth medium. The medium was refreshed with complete cell growth medium containing either resveratrol or vehicle only (DMSO) 24 h after transfection.

2.5 Pharmocological/chemical treatment of cells

2.5.1 Resveratrol

Resveratrol was incubated for 30 min in FBS prior to addition to FBS-free cell growth medium, which was applied to the cells for 24 h at a final concentration of 60 μM from a 10 mM stock. This was repeated after 24 h incubation for experiments performed over 48 h.

2.5.2 3-aminobenzamide

The PARP inhibitor 3-aminobenzamide was dissolved and diluted in DMSO and applied in the cell culture medium at a concentration of 1 mM from a 100x (100mM) stock for 0, 4, 6, 12 and 16 h.

2.5.3 Apigenin

The CD38 NADase inhibitor Apigenin (Santa Cruz) was dissolved and diluted in DMSO and applied in the cell culture medium at a concentration of 25 μM from a 10 mM stock for 4 h.

2.5.4 FK866

The nicotinamide phosphoribosyltransferase inhibitor FK866 (Merck Chemicals LTD) was dissolved and diluted in DMSO and applied in the cell culture medium at a concentration of 0.1 μM form a 100 mM stock for 4 h.

2.5.5 Temozolomide

Temozolomide (TMZ) (Santa Cruz) was dissolved and diluted in DMSO and applied in the cell culture medium at a concentration of 30 μM from a 10 mM stock for 4 h.

2.5.6 5-azacytidine

5-azacytidine was dissolved and diluted in PBS and applied in the cell culture medium for 24 h at a concentration of 10 μM from a 10 mM stock.

2.5.7 EX-527

Ex-527 was dissolved and diluted in DMSO and applied in the cell culture medium at a concentration of 10 μM from a 10 mM stock for 4, 6, 12 and 16 h.

2.6 Routine manipulation of DNA

2.6.1 Restriction digests

DNA was digested with restriction enzymes in the manufacturer's supplied buffers (Promega). Reactions typically were performed on 1 µg DNA using 5 units of restriction enzyme and then incubated for 1 h at 37 ˚C.

2.6.2 Ligation of DNA into plasmid vectors

Ligation was carried out using the T4 DNA Ligase (Promega) in the supplied buffer. Reactions typically contained a 5x molar excess of insert over vector and was incubated at 16 ˚C over-night.

2.6.3 Polymerase Chain Reaction (PCR)

All PCR reactions were based on the following protocol. One microliter cDNA (~100 ng) was added to 2 µl forward and reverse PCR primers (10 µM), 12.5 µl Hotstart Taq Master Mix (Qiagen) and made up to 25 µl with distilled H_2O. Thermal cycling (Techne TC-5000 PCR machin) was as follows: 95 ˚C, 5 min (initial denaturation) followed by 35 cycles of 95 ˚C, 30 s (denature), 45-65 ˚C, 30 s (anneal), 72 ˚C, 1 min (elongate) then 72 ˚C, 5 min. Samples were then held in the thermal cycler at 4 ˚C and stored at -20 ˚C for future use.

2.6.4 Agarose gel electrophoresis

One gram of agarose was dissolved in 50 ml 1x Tris-EDTA buffer (Tris-HCl 10 mM, EDTA 1 mM) by heating in a microwave for 30-60 sec. Two microliters of SafeView DNA dye (NBS Biotechnologies) was mixed in to the agarose solution before pouring in to an electrophoresis tank, applying a comb to create 20 µl wells and allowing to solidify. To prepare samples, 2 µl 5x DNA loading dye (Bioline) was mixed with 8 µl PCR product. Five microlitres HyperLadder™ (Bioline) was loaded alongside samples as a size marker. Electrophoresis was in 1x Tris-EDTA buffer at 65 V for 30 min. Gels were viewed and images were captured under UV light on a gel documentation system (Quantity One® Biorad).

2.6.5 Purification of PCR products

PCR products were purified for use in downstream applications using the QIAquick PCR Purification Kit (Qiagen). PCR product was mixed with Buffer PB at a ratio of 1:5 and

added to the QIAquick spin column and centrifuged (17,000 g, 30 sec, RT). The flow through was discarded from the collection tube before the column was washed with 750 µl Buffer PE by centrifuging (17,000 g, 30 sec, RT). The flow through was again discarded and the column was dried by centrifuging (17,000 g, 1 min, RT). The QIAquick column was then placed in a new collection tube and 50 µl of distilled H_2O was added to the centre of the column and the column left to stand for 2 min. The sample was finally eluted by centrifuging (17,000 g, 1 min, RT).

2.6.6 Purification of DNA from agarose gels

Bands were excised under UV light then DNA was purified using QIAquick Gel Extraction Kit (Qiagen). The gel weighing no more than 0.2 g was added to 1 ml Buffer QG and incubated at 55 °C with occasional vortexing until the gel was dissolved. The mixture was then put in to a QIAquick gel extraction spin column and centrifuged (12,000 g, 1 min, RT) and the flow through discarded. The column was then washed by adding 750 µl Buffer PE and centrifuged (12,000 g, 1 min, RT) and the flow through discarded. Finally, the column was dried by centrifuging (12,000 g, 1 min, RT) before 30 µl Buffer EB was added to the centre of the column and incubated for 2 min at RT. The column was inserted in to a new collection tube and centrifuged (12,000 g, 1 min, RT) to elute the product.

2.6.7 Transformation of E.coli

SIG10 chemically competent cells were used in the transformation reaction. Fifty microlitres of competent cells were incubated on ice for 20 min before addition of 20 ng DNA and then incubated on ice for 30 min before heat shocked at 42 ˚C, 30 s in a water bath before placing back on to ice for 2 min. Luria Broth (LB) growth medium (250 µl) (10 g Tryptone, 10 g NaCl and 5 g yeast extract, 1 l distilled H_2O) was added to the competent cells under a Bunsen Burner flame and incubated for 1 h at 37 ˚C in a shaking incubator. Then 100 µl, 50 µl, 25 µl and 10 µl volumes of competent cells in LB were evenly spread on to room temperature LB Agar 10 cm plates (LB containing 15 g/l agar and antibiotic to select for antibiotic resistant colonies) under a Bunsen Burner flame. Plates were incubated over night at 37 ˚C and checked for colonies the next day.

2.6.8 Small scale preparation of plasmid DNA from bacterial cultures

Several bacterial colonies grown on antibiotic LB agar plates were selected and inoculated individually in to 2 ml of LB containing antibiotic under a Bunsen Burner flame and incubated for 6 h at 37 ˚C in a shaking incubator. Plasmid DNA was harvested from the bacterial starter broths using the QIAprep® Spin Miniprep Kit (Qiagen). The bacteria were pelleted by centrifuging (6000 g, 3 min, RT). The supernatant was discarded and the pellet re-suspended in 250 µl Buffer P1. Two hundred and fifty micro litres Buffer P2 was added to the re-suspension and inverted to mix then immediately 350 µl Buffer N3 was added to the mixture and inverted to mix before centrifuging (17,000 g, 13 min, RT). The supernatant was then added to a QIAprep spin column and centrifuged (17,000 g, 30 sec, RT) and flow through discarded. The column was then washed with 750 ml Buffer PE by centrifuging (17,000 g, 30 sec, RT) and the flow through discarded. The column was finally dried by centrifuging (17,000 g, 1 min, RT) before placing the column in a new collection tube and addition of 50 µl Buffer EB to the centre of the column, incubating for 1 min at RT. The plasmid was then eluted by centrifugation (17,000 g, 1 min, RT). Plasmid DNA was then sent for sequencing (Eurofins Genomics).

2.6.9 Large scale preparation of plasmid DNA from bacterial cultures

Two millilitre bacterial starter broths were prepared as described above. The bacterial starter broths were added to 250 ml LB containing antibiotic under a Bunsen Burner flame and incubated over night at 37 ˚C in a shaking incubator. Plasmid DNA was then harvested using a QIAGEN Plasmid *plus* Maxi Kit (Qiagen). The bacteria were pelleted by centrifuging (6000 g, 15 min, 4 °C). Supernatent was discarded and the bacterial pellet was re-suspended in 10 ml Buffer P1. Ten millilitres of Buffer P2 was added to the sample and inverted to mix before incubating at RT for 5 min. The QIAfilter cartridge was prepared by capping, adding 10 ml Buffer P3 and placing in a universial tube before pouring the lysate in to the QIAfilter cartridge and incubating at RT for 10 min. The QIAfilter cap was then removed and the plunger inserted to filter the lysate in to the universial tube. To the lysate 2.5 ml Buffer ER was added and inverted 10 times before incubating on ice for 30 min. The Qiagen-tip 500 column was equilibrated by allowing 10 ml Buffer QBT to flow through it by gravity. The lysate was poured in the Qiagen-tip 500 column and allowed to flow through it and then the column was washed

twice with 30 ml Buffer QC. The plasmid was eluted in to a new universal tube by adding 15 ml Buffer QN to the column. The elution was divided in to 750 µl samples and each sample was mixed with 525 µl 100 % isopropanol and centrifuged (15,000 g, 30 min, RT). The supernatant was discarded and the pellet washed with 250 µl 100 % ethanol by centrifuging (15,000 g, 10 min, RT). The supernatant was discarded and the pellet re-suspended in 75 µl Buffer TE. Plasmid DNA was then sent for sequencing (Eurofins Genomics) and stored at -20 ˚C until future use. Before use plasmid DNA concentration was checked by Nanodrop (Nanodrop® ND-1000).

2.7 RNA analysis

2.7.1 RNA extraction

Cells were washed once with ice cold PBS (1x) before the addition of TRIzol® Reagent (Life Technologies) using 500 µl/9.5 cm^2 cell monolayer. Cells were scraped in to 1.5 ml microfuge tubes and frozen for 1 h at -80 ˚C. RNA was extracted from cells by adding 100 µl phenol:chloroform (5:1) and centrifuging in a bench top centrifuge (13,000 g, 15 min, 4 ˚C), 200 µl of the aqueous layer produced was removed in to a new centrifuge tube with equal volume of 70 % ethanol. RNA was then purified from the aqueous layer plus ethanol using the Purelink® RNA mini kit (Life Technologies). Seven hundred micro litres was transferred in to a spin column and centrifuged (12,000 g, 15 sec, RT), flow through was discarded from the collection tube and the column was then washed with 700 µl wash buffer 1 by centrifuging (12,000 g, 15 sec, RT) and the flow through discarded. With a new collection tube the column was washed twice with 500 µl wash buffer 2 by centrifuging (12,000 g, 15 sec, RT). The column was then dried by centrifuging (12,000 g, 1 min, RT) before adding 50 µl H_2O to the centre of the column and centrifuging (12,000 g, 2 min, RT) to elute the RNA in to a new collection tube and stored at -80 ˚C. The concentration of RNA was determined using a Nanodrop spectrophotometer at 260 nm (NanoDrop® ND-1000).

2.7.2 DNase treatment

All RNA samples were treated with DNAse to eradicate DNA contamination. RNA (4.5 µg) was added to 2 µl DNAse buffer and 4.5 U DNAse (DNAse RQ1 kit, Promega) and distilled H_2O was added to make the final volume to 20 µl. The DNAse/RNA mix was incubated at 37 ˚C for 30 min then 4 µl of Stop Solution (DNAse RQ1 kit, Promega) was added before incubating the mixture at 65 ˚C for 10 min. Samples were then placed on ice before immediately performing reverse transcription.

2.7.3 Reverse transcription

Immediately following DNAse treatment, RNA samples were reverse transcribed to generate cDNA. Ten microlitres of each sample was added to 1 µl random primers (150 ng/µl, Promega), 0.4 µl dNTPs (25 µM, Bioline) and 0.6 µl distilled H_2O. The sample was then incubated at 65 ˚C for 5 min. Following incubation, 1 µl Superscript®III (Invitrogen), 1 µl RNasin® ribonuclease inhibitor (20-40 U/µl, Promega), 1µl DTT (0.1M, Invitrogen) and 4 µl 5x Superscript buffer (Invitrogen) was added to each sample. Samples were then incubated at 25 ˚C for 5 min, 50 ˚C for 45 min then 70 ˚C for 15 min. Samples were stored at -20 ˚C until used.

2.7.4 Real time PCR

cDNA was quantified using real time PCR. One microliter cDNA (~100 ng) was added to 2 µl forward and reverse primers (10 µM), 10 µl SYBR Green Master Mix (Roche) and made up to a volume of 17 µl with distilled H_2O. Samples were loaded in to a 96 well white plate (Starlab) and sealed. PCR was performed on a Roche Lightcycler® 480 using the following parameters: 4 min, 95 °C then 50 cycles of 95 ˚C, 10 s (denature), 52 ˚C, 10 s (anneal), 72 ˚C, 15 s (elongation). The ΔΔCt method was used to calculate quantities of specific cDNAs relative to reference genes and the control condition.

2.7.5 Primers used for PCR are listed in *Table 2.7.1*

Table 2.7.1 Primers used for real time PCR

Primer name	Sequence (note reverse primers are in reverse complement)	Annealing temperature (˚C)	Supplier
Homo sapien			
SIRT1 Forward	gaacttcagtggctggaacagtg	55	Eurofins Genomics
SIRT1 Reverse	cagagtctgaatatacctcagcgc	55	Eurofins Genomics
GAPDH Forward	tgaaggtcggagtcaacggatttg	55	Eurofins Genomics
GAPDH Reverse	catgtaaaccatgtagttgaggtc	55	Eurofins Genomics
TOP1	designed by manufacturer	55	Primer Design
Rn18s Forward	aggaattgacggaagggcaccac	58	Eurofins Genomics
Rn18s Reverse	gtgcagccccggacatctaagg	58	Eurofins Genomics
LAMP2 Forward	cctacaacactggtgataacac	55	Eurofins Genomics
LAMP2 Reverse	catttgtgctgctcactgtgc	55	Eurofins Genomics
HSC70 Forward	gcttatggtgcagctg	55	Eurofins Genomics
HSC70 Reverse	gaatggtggtattacgc	55	Eurofins Genomics
EZH2 Forward	gtcctcattggcacttactatg	58	Eurofins Genomics
EZH2 Reverse	cttggaggagtatccacatc	58	Eurofins Genomics
SUZ12 Forward	gtgatacctgcttacctctcc	58	Eurofins Genomics
SUZ12 Reverse	catgacatggagattccag	58	Eurofins Genomics
RNF2 Forward	gatgacagtgcacagacgag	58	Eurofins Genomics
RNF2 Reverse	gtatactgcttctcactggctg	58	Eurofins Genomics
KDM2B Forward	ggacatcacagatgcctc	58	Eurofins Genomics
KDM2B Reverse	cttattgcagtcagacaggttg	58	Eurofins Genomics
PHC1 Forward	cagtgctaccaccttgac	55	Eurofins Genomics

PHC1 Reverse	aggagactgagcagatgg	55	Eurofins Genomics
DNMT1 Forward	gagctgaaccttcacctagc	58	Eurofins Genomics
DNMT1 Reverse	cttccttgatggactcatcc	58	Eurofins Genomics
DNMT3B Forward	gaagactcgatcctcgtc	55	Eurofins Genomics
DNMT3B Reverse	gtagcttagcagactggacac	55	Eurofins Genomics
BMI1 Forward	gcagctcgcttcaagatg	58	Eurofins Genomics
BMI1 Reverse	gagggtacttcattgatgcc	58	Eurofins Genomics
EED Forward	ggaatatccagacggacactc	58	Eurofins Genomics
EED Reverse	gagaatgatccataccacagg	58	Eurofins Genomics
RBAP48 Forward	cagtggaagaacgagtgatc	58	Eurofins Genomics
RBAP48 Reverse	gcattca tcgacttgtcc	58	Eurofins Genomics
Mus musculus			
SIRT1 Forward	gtgctacagctctctgacgaac	58	Eurofins Genomics
SIRT1 Reverse	gtgctacagctctctgacgaac	58	Eurofins Genomics
GAPDH Forward	gtgctacagctctctgacgaac	58	Eurofins Genomics
GAPDH Reverse	gtgctacagctctctgacgaac	58	Eurofins Genomics
TOP1 Forward	gactggcagaagtatgagactgc	55	Eurofins Genomics
Top1 Reverse	gtgctacagctctctgacgaac	55	Eurofins Genomics
Bovine			
Ribo A Forward	ctcaagcttatggctctgaagtccctggt	55	Eurofins Genomics
Ribo A Reverse	gaggaattctctggttacagtagttggag	55	Eurofins Genomics

2.8 Protein analysis

2.8.1 Protein extraction from cells

Cells were washed with 1 ml 1x ice cold PBS before the addition of 1 ml 1x PBS containing 1x protease inhibitor (Roche) per 9.5 cm^2 of cell monolayer. Cells were then scraped in to 1.5 ml microfuge tubes and centrifuged (4°C, 13,000 rpm for 15 min) (Fisher Scientific AccuSpin Micro 17R). The cell pellet was resuspended in 200 µl Ripa buffer containing 1x protease inhibitor (Roche) and incubated for 30 min on ice. Samples were centrifuged (4 ˚C, 13,000 rpm for 5 min) and the supernatant fluid was stored at -80 °C until future use.

2.8.2 Protein extraction from tissue samples

Tissue samples weighing 10 mg were homogenised in 4 volumes of ice cold isotonic buffer. The homogenate was then diluted 1:5 with Ripa Buffer containing 1x protease inhibitor (Roche). Sonication was performed twice for 10 s with 1 min incubation on ice in between sonication's. The samples were then incubated at room temperature for 10 min before centrifuging (4 °C, 16,000 g, 10 min) (Fisher Scientific AccuSpin Micro 17R). The supernatant fluid was then stored at -80 °C until further use.

2.8.3 Determination of protein concentration

Protein concentration was determined using Bradford Reagent (Biorad). Samples were diluted 1:100 with distilled H_2O. Fifty microliters was then added to 200 µl of 1x Bradford Reagent in a clear flat bottomed 96 well plate and then the absorbance was measured at 595 nm (Thermo Labsystems Multiscan Ascent). Final concentrations of protein were calculated using a standard curve with standards of 0, 20, 40, 60, 80 and 100 (ug/ml) bovine serum albumin.

2.8.4 Western blotting

2.8.4.1 SDS-PAGE gels

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was used to separate protein on 10% acrylamide gels (3.5 ml bis-acrylamide (37.5:1 40%), 4.8 ml H_2O, 5.6 ml 2.5x separating buffer (1.875 M TRIS base, 0.25 % SDS, pH 8.9), 12 µl tetramethylethylenediamine (TEMED) and 130 µl 10 % ammonium persulphate) and a 5 % Acrylamide gel was used as a stacking gel (500 µl bis-acrylamide (37.5:1 40%), 2.64 ml H_2O, 800 µl 5x stacking buffer (0.3 M TRIS base, 0.5 % SDS, pH 6.7), 5 µl TEMED and 36 µl 10 % ammonium persulphate). The separating gel was poured first between two sealed glass plates (filling ¾ way up the glass plates) and left to solidify at room temperature (~ 20 min). The stacking gel was poured on top of the separating gel, a comb inserted to form wells and left to set at room temperature (~ 1 h). The glass plates were then wrapped in cling film and stored at 4 ˚C overnight prior to SDS-PAGE.

2.8.4.2 SDS-PAGE

Gels were placed in electrophoresis tanks and submerged in 1x running buffer (from a 5x stock of TRIS base 60.6 g, Glycine 144.1 g and SDS 5 g in 1 l of distilled H_2O). Protein lysate was mixed with loading buffer and 2-mercaptoethanol (1 µg/µl protein lysate, 0.25 µl of 5% 2-mercaptoethanol and 2.5 µl loading buffer (2.25 ml 1 M TRIS base, 5 ml glycerol, 0.5 g SDS, 5 mg bromophenol blue and 2.5 ml 1 M dithiottreitol). The samples were then denatured at 95 °C for 5 min. Ten microliters of each sample was loaded on the gel alongside 5 µl of 8000-220,000 Dalton Colourburst Ladder (Sigma). Electrophoresis was for 2 h at 120 V.

2.8.4.3 Transfer of protein to membrane

A wet blot system was used to blot proteins from the polyacrylamide gel on to a polyvinylidene difluride (PVDF) membrane (Roche). The membrane was prepared by immersing it in 100 % methanol (30 s) then washing in distilled H_2O (2 min) before immersing in transfer buffer (15 min) (3.86 g TRIS base, 9 g glycine, 200 ml 100 % methanol, 1800 ml distilled H_2O). The gel and membrane were sandwiched together between two pieces of Whatman® blotting paper soaked in transfer buffer (1:5 100 % methanol to running buffer). The assembly was then immersed in transfer buffer in a wet blotting tank. Transfer was for 4 h at 30 V or overnight at 20 V.

2.8.4.4 Probing with antibodies

Following transfer, the membrane was blocked overnight at 4 °C in 1x Odyssey Blocking Buffer and 1x PBS (1:1). The membrane was then incubated for 1 hour with primary antibody diluted in 1x Odyssey Blocking Buffer and 1x PBS (1:1) containing 0.1 % Tween-20. The membrane was washed for 5 min 4 times in membrane wash buffer (1x PBS and 0.1 % Tween-20) before incubating for 1 h with fluorescently-labelled secondary antibody in 1x Odyssey Blocking Buffer and 1x PBS (1:1) containing 0.1 % tween-20. The membrane was washed for 5 min 4 times in membrane wash buffer. The antibodies used are listed in *Table 2.8.1*

2.8.4.5 Antibody detection

Fluorescently-labelled secondary antibody bound to membrane was detected using the Li-Cor Odyssey Scanner. Images were captured and densitometry was performed on the signal using the Li-Cor Odyssey Scanner software.

2.8.5 Immunoprecipitation

Immunoprecipitation of protein from Caco-2 cells and HuVECs was carried out using the Pierce Classic IP Kit (Pierce Biotechnologies). The cell monolayer (175 ml flask) was washed with ice cold PBS before addition of 1.5 ml IP lysis Buffer. The cells were incubated on ice for 5 min and then transferred to a microfuge tube for centrifugation (13,000 g, 10 min, RT). Supernatant was transferred in to a new microfuge tube for analysis. Protein concentration was determined using a Bradford Reagent (see above for details). The protein lysate was pre-cleared by adding Control Agarose Resin slurry to a spin column (80 µl per 1 mg protein) and centrifuged (1000 g, 1 min, RT) to remove the storage buffer. The Control Agarose Resin was then washed by adding 100 µl sodium phosphate 0.1 M and sodium chloride 0.15M buffer and centrifuging (1000 g, 1 min, RT) the flow through was discarded. The protein lysate was then added to the column and incubated with the Control Agarose Resin for 1 hour at 4 °C with end-over-end mixing. The protein lysate was then eluted from the column by centrifuging (1000 g, 1 min, RT) and the flow through (protein lysate) was saved at this point. Ten micro litres of antibody (antibodies used are listed in *Table 2.8.1*) was added to 1000 µg protein lysate and diluted to 600 µl using IP Lysis/Wash Buffer before incubating overnight at 4 °C with end-over-end mixing. Twenty micro litres Pierce Protein A/G

Agarose resin slurry was added to a new spin column and centrifuged (1000 g, 1min, RT) to remove the storage buffer. The resin was washed twice by adding 100 µl IP Lysis/Wash Buffer and centrifuging (1000 g, 1 min, RT) then the flow through discarded. The overnight incubated protein lysate/antibody solution was then added to the spin column containing the Protein A/G Agarose resin and incubated for 1 hour at 4 °C with end-over-end mixing. The spin column was then centrifuged (1000 g, 1 min, RT) and the flow through discarded. The resin in the spin column was washed 3 times using 200 µl IP Lysis/Wash Buffer and centrifuged (1000 g, 1 min, RT) and once with 100 µl 1X Conditioning Buffer, each time the flow through was discarded. The protein was eluted from the column by adding 2X reducing sample buffer (50 µl 2X Non-reducing Lane Marker Sample buffer and 20 mM DTT) and incubating for 10 min at 100 °C before centrifuging (1000 g, 1 min, RT) to elute the sample. Finally the sample was cooled to RT before immediately loading on to a SDS-PAGE gel for further analysis.

2.8.6 Antibodies

Table 2.8.1 Antibodies used with the concentrations used, the system they were used on and where they were manufactured.

Antibody	Concentration	System	Supplier
Rabbit Anti-SIRT1 primary	1:500	Odyssey Li-Cor	Pierce
Rabbit Anti-LAMP2 primary	1:250	Odyssey Li-Cor	Abcam
Rabbit Anti-α-tubulin primary	1:1000	Odyssey Li-Cor	Abcam
Goat Anti-EZH2 primary	1 µg/ml	Immunoprecipitation and Odyssey Li-Cor	R & D systems
Rabbit Anti-SIRT1 primary	1 µg/ml	Immunoprecipitation	Abcam
Rabbit Anti-HSC70 primary	1:500	Odyssey Li-Cor	Abcam
Secondary Anti-Rabbit Fluorescent IRDye®	1:10,000	Odyssey Li-Cor	Li-Cor
Secondary Anti-Goat Fluorescent IRDye®	1:10,000	Odyssey Li-Cor	Li-Cor

2.9 SIRT1 promoter reporter assay

Caco-2 cells transfected with a SIRT1 promoter-reporter vector (Appendix A and B) were lysed using 200 µl of lysis buffer (0.25 M Tris-HCL, 2.5 mM EDTA and 0.25 % Nonident p40) per 9.5 cm^2 of cell monolayer. Cells were completely lysed by freeze thaw action. Twenty microlitres of cell lysate and 130 µl 1x chlorophenolred-ß-D-galactopyranoside diluted in buffer A (25 mM MOPs, 100 mM NaCl and 10 mM $MgCl_2$ in 100 ml distilled H_2O) were incubated in a clear flat bottomed 96 well plate at 37 °C. Absorbance was measured at 560 nm (Thermo Labsystems Multiscan Ascent) every 15 min. The protein concentration in each sample was measured using Bradford Reagent (Biorad) in a Bradford assay (described above) and data were expressed relative to total protein.

2.10 Measurement of NAD^+

NAD^+ was measured in cell lysate as the change in NADH fluorescence following addition of 100 % ethanol and alcohol dehydrogenase.

Whole cell lysate was prepared by washing cell monolayers with 1x PBS and lysed the with 200 µl 0.6 M perchloric acid. Lysed cells were then scraped in to a 1.5 ml microfuge

tubes. The samples were centrifuged (4 °C, 5000 g, 5 min) to pellet the debris. The supernatant fluid was removed into new 1.5 ml microfuge tubes and 1:5 1 M potassium phosphate was added to each sample on ice. 3 M potassium hydroxide was then added to neutralise each sample to pH 7.2-7.4. In a clear flat bottomed 96 well plate, 100 µl of sample was added to 1 µl 100 % ethanol and 100 µl diphosphate buffer (4.5 g tetrabasic sodium pyrophosphate, 0.5 g semicarbazide hydrochloride and 100 ml H_2O) and incubated for 10 min at room temperature. Absorbance was measured after incubation (340 nm excitation and 460 nm emission) on a BMG LABTECH FLVOstar Omega Plate Reader. One microlitre 300 U/ml alcohol dehydrogenase (*Saccharomyces cerevisiae*) was added to each sample before incubating for 6 min at room temperature, then taking a final absorbance reading (340 nm excitation and 460 nm emission).

NAD^+ concentration was calculated using a standard curve (Melford) with concentrations of 0, 0.02, 0.04, 0.05, 0.08, 0.1 µM. The NAD content of cells was expressed as µM/mg protein, using the protein concentration of each sample as determined using the Bradford assay (described above).

2.11 Measurement of PARP activity

PARP activity was measured in Caco-2 cells following treatment with SIRT1 siRNA or apigenin. The PARP activity assay was performed with our collaborator (Professor Nicola Curtin, Newcastle University). Caco-2 cells were harvested using trypsin (as described above) and resuspended in 1 ml PBS. The cells were centrifuged (5000 g, 5 min, 4 °C) and the supernatant fluid was discarded. The pellet was resuspended in 1 ml PBS before centrifuging (5000 g, 5 min, 4 °C) and removing the supernatant fluid. Cells were then permeabilised using 100 µl digitonin (0.15 mg/ml) and incubated for 10 min on ice. Nine hundred micro litres ice cold PBS was then added to the permeabilised cells before counting the cells using a Coulter counter. Five hundred cells were exposed to a blunt-ended oligonucleotide (5′-CGGAATTCCG-3′, Invitrogen, 200 µg/ml) and NAD^+ (7 mM) in the reaction buffer (100 mM Tris–HCl, 120 mM $MgCl_2$, pH 7.8) for 6 min at 26 °C. The reactions were then suctioned in individual wells through a membrane to capture the newly synthesized PAR polymer. The membrane was washed twice in 10 ml PBS before blocking in 10 ml milk overnight. PAR was detected using mouse monoclonal anti-PAR 10H antibody (1.5 mg/mL, a gift from Professor Alex Bürkle,

University Konstanz, Germany) with horseradish peroxidase–conjugated polyclonal goat anti-mouse IgG (DAKO) as a secondary antibody. Both antibodies were diluted 1:1000 in phosphate-buffered saline with 0.05 % Tween 20 and 5 % milk power). The membrane was incubated for 1 h with anti-PAR antibody then washed 3 times for 10 min in 10 ml wash buffer (1x PBS and 0.1 % Tween-20) and incubated for a further 1 h with the secondary antibody then washed 3 times for 10 min in 10 ml wash buffer (1x PBS and 0.1 % Tween-20). Secondary antibody was detected using ECL (Amersham) by a Fujifilm LAS 3000 imager (Raytest) (Drew *et al.*, 2011).

2.12 Fluorescence microscopy on Caco-2 cells a CMA substrate

Fluorescence microscopy was carried out on Caco-2 cells transformed with a fluorescent substrate for CMA in the Newcastle University Bioimaging suite with the help of Dr. Alex Laude. A Nikon A1R confocal microscope was used for the photoconversion (20X lense, ND1-4.42mW LED array, 8 min) of the protein product from cyan to green. Images were captured before and after photoconversion at 30 min intervals for 45 h using the Nikon A1R microscope (40X lense). Nikon Elements Viewer software was used to view the images and Volocity 3D image analysis software (Perkin Elmer) was used to measure the fluorescent intensity of individual cells.

2.13 Statistical analysis

Statistical analysis was performed using IBM SPSS Statistics 22 software. All data had a normal distribution and were analysed by ANOVA combined with Tukey's post hoc statistical test for comparison of greater than two data sets with control or Student's T-test where there were only 2 conditions.

2.14 Equipment list

Techne TC-500 PCR machin- PCR machine

Roche lightcycler® 480- Real-time PCR machine

Fisher Scientific-accuSpin MICRO 17R- Desktop centrifuge

Thermo Labsystems Multiskan Ascent- Plate reader used for Bradford reagent and promoter-reporter assay

BMG LABTECH FLVOstar Omega- Plate reader used for NAD^+ assay

Techne Dri-Block DB2A- Heat block

Li-Cor Odyssey- fluorescence scanner

NanoDrop® ND-1000- Nanodrop machine

Fujifilm LAS 3000 imager Raytest- PARP assay scanner

Nikon A1R confocal microscope- CMA assay

3 Chapter 3. Interactions between SIRT1, resveratrol and NAD^+

3.1 Introduction

A reduction in calorie intake by around 30-40 %, can extend lifespan by 40 % in mice (McCay *et al.*, 1989). Similar responses to reducing energy and/or specific nutrients available from food have been seen in other model organisms including *Saccharomyces cerevisiae, Caenorhabditis elegans and Drosophila melanogaster* (Lin *et al.*, 2000; Clancy *et al.*, 2001; Guarente and Picard, 2005; Greer and Brunet, 2009). Furthermore, the healthspan of animals maintained under such conditions of dietary restriction (DR) is markedly improved. For example, studies in Rhesus monkeys have shown that DR can decrease the risk of developing neoplasia and diabetes (Colman. R, 2009; Mattison .J *et al.*, 2012).SIRT1 is thought to play a role in mediating the effects of DR on cell and/or systemic ageing. When the SIRT1 homologue Sir2 was mutated in *Saccharomyces cerevisiae* the lifespan-extending effects of DR were lost (Lin *et al.*, 2000), whereas, increasing the copy number of Sir2 in *Caenorhabditis elegans* extended lifespan (Tissenbaum and Guarente, 2001). Thus, SIRT1 is believed to play a fundamental role in ageing. However, interactions between SIRT1 and many other molecular modulators of ageing are understood only partially.

Resveratrol can also increase the lifespan and healthspan of model organisms. For example, in obese mice fed resveratrol insulin sensitivity increased and in *Caenorhabditis elegans and Drosophila melanogaster* resveratrol extended lifespan by up to 20 % (Wood *et al.*, 2004; Baur *et al.*, 2006). Resveratrol has thus been labelled a DR mimetic. Moreover, in mice resveratrol treatment improved mitochondrial biogenesis; however, when SIRT1 was knocked down resveratrol had no effect on mitochondrial biogenesis (Price *et al.*, 2012). Such findings have led to the view that SIRT1 also plays an essential role in mediating the cellular response to resveratrol that counteracts some features of ageing.

Originally it was considered that resveratrol was a direct activator of SIRT1 but this theory has been controversial with no clear resolution. Resveratrol has also been shown to affect the expression of SIRT1. For example, resveratrol increased SIRT1 mRNA and protein levels in human ovary cells (Morita *et al.*, 2012). Moreover,

resveratrol has been shown to increase intracellular NAD^+, through the AMPK pathway, which may result in the NAD^+-dependent activation of SIRT1 (Park *et al.*, 2012). In *Saccharomyces cerevisiae* it was shown that deletion of the *Ntp1* gene (involved in NAD^+ synthesis) abolished the extension of lifespan seen under conditions of DR, suggesting that NAD^+ may be essential for DR to extend lifespan (Lin *et al.*, 2000). Together these studies link SIRT1, resveratrol and NAD^+ as key components that can extend lifespan but whose potentially complex interactions remain to be elucidated. The work presented in this chapter explores possible mechanisms through which resveratrol and/or NAD^+ affect SIRT1 expression in human Caco-2 cells and investigate if resveratrol can affect intracellular NAD^+ levels.

3.2 Resveratrol increases SIRT1 mRNA in human Caco-2 cells.

To determine if resveratrol affects SIRT1 expression, SIRT1 mRNA and protein were measured in Caco-2 cells after adding 60 μM resveratrol to the extracellular medium for 48 h. This concentration of resveratrol was used because previous work carried out in the laboratory testing a range of resveratrol concentrations from 10 μM to 100 μM showed that a minimum of 60 μM (a pharmaceutical concentration) extracellularly was required to produce level changes in gene expression and in DNA methylation (Dr. L. Wakeling personal communications). mRNA was measured by RT-qPCR and protein was measured by Western blotting.

Resveratrol significantly increased SIRT1 mRNA relative to the reference genes *GAPDH* and *TOP1* (Figure 3.2.1 A), preliminary measurements of SIRT1 protein relative to the α-tubulin loading control displayed a trend to increase by approximately 2 fold but further repeats are needed to show statistical significance (Figure 3.2.1 B and C).

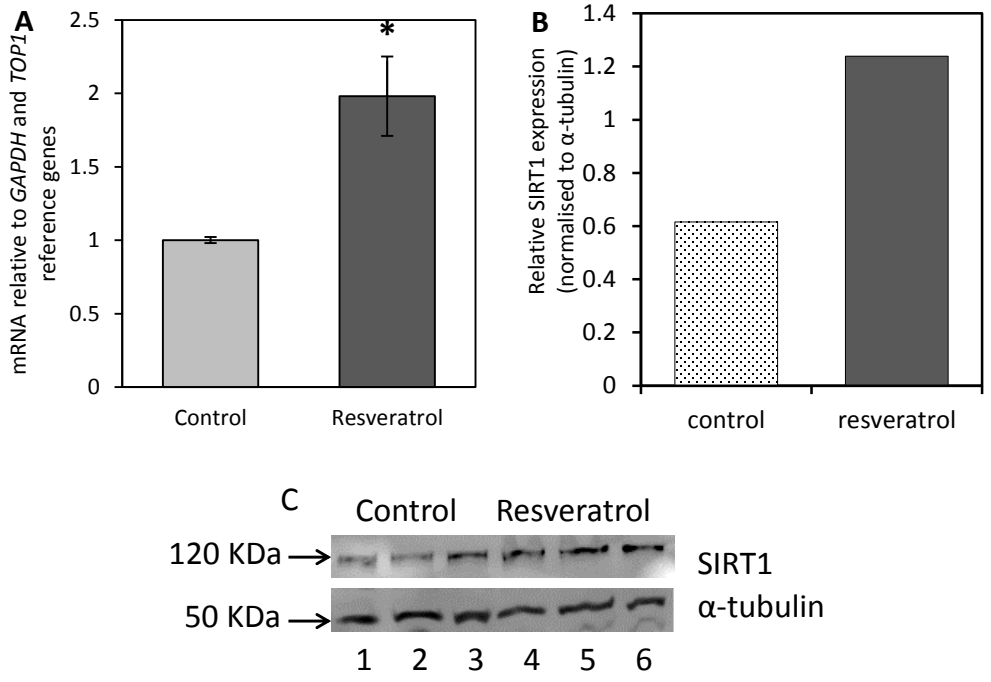


Figure 3.2.1. The effect of resveratrol on SIRT1 mRNA and protein in Caco-2 cells. Resveratrol was included in the extracellular medium at 60 μM over 48 h (replenished at 24 h). **A** SIRT1 mRNA levels measured by RT-qPCR, relative to *GAPDH* and *TOP1* n=2 (based on 6 data points comprising 2 biological replicates, each the mean of 3 wells measured separately). **B** SIRT1 protein measured by densitometry from western blot against α-tubulin loading control n=1. **C** Representative western blot raw data; lanes 1-3 control, lanes 4-6 medium containing resveratrol. SIRT1 produced a signal at 120 KDa and α-tubulin produced a signal at 50 KDa. *P<0.05, by Student's T-test. Data are shown as ± standard deviation (SD).

3.3 Resveratrol increases SIRT1 promoter activity in human Caco-2 cells.

Having observed that resveratrol increased SIRT1 mRNA we sought to determine if this response was due to an increase in transcription of the *SIRT1* gene. We thus investigated if resveratrol affected the activity of a *SIRT1* promoter-reporter construct. Caco-2 cells were transfected with the promoter-reporter construct then treated with 60 μM resveratrol over 48 h. Promoter activity was measured as activity of β-galactosidase, the product of the reporter gene, in the cell lysate.

Resveratrol significantly increased *SIRT1* promoter activity by around 20%, providing evidence that the increases in SIRT1 mRNA observed included at least a component that resulted from an increase in *SIRT1* gene transcription (Figure 3.3.1).

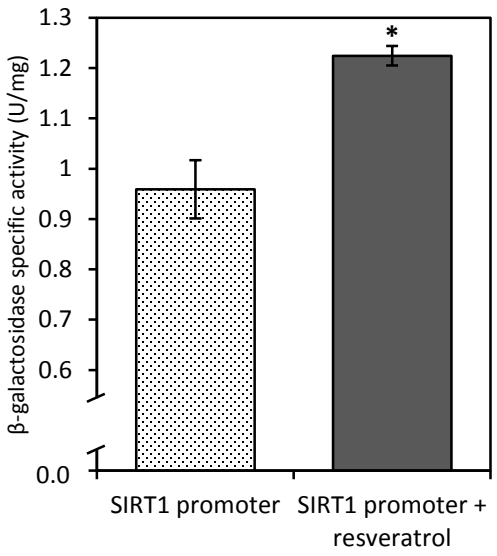


Figure 3.3.1. The effect of resveratrol on the activity of a SIRT1 promoter-reporter construct in Caco-2 cells. Caco-2 cells were transfected with a SIRT1 promoter-reporter construct and incubated with 60 μM resveratrol for 48 h extracellularly (replenished at 24 h). β-galactosidase activity was then measured by incubating the cell lysate with CPGR β-galactosidase substrate. Colour change was measured at 560 nm. *P<0.05 by Student's T-test. Data are shown as mean ± standard deviation (SD), n=2 (based on 6 data points comprising 2 biological replicates, each the mean of 3 wells measured separately).

3.4 Preventing the breakdown of NAD⁺ reduced SIRT1 mRNA in Caco-2 cells, whereas an inhibitor of de novo synthesis of NAD⁺ had no effect on SIRT1 mRNA but appears to increase SIRT1 protein.

Sir2 (SIRT1 homologue) and Ntp1 (involved in NAD^+ synthesis) are required for lifespan extension in *Saccharomyces cerevisiae* under conditions of DR (Lin *et al.*, 2000). As NAD^+ is required for SIRT1 to function as an enzyme, NAD^+ may be required for SIRT1 to extend lifespan. It has been reported previously that resveratrol can increase intracellular NAD^+ levels through the AMPK pathway (Park *et al.*, 2012) and we have shown that resveratrol can also increase SIRT1 expression. To determine if the change in expression of SIRT1 by resveratrol is linked to changes in NAD^+, we investigated if manipulation of NAD^+ can change SIRT1 mRNA and protein levels. NAD^+ was manipulated using the CD38 NADase inhibitor apigenin, which prevents the breakdown of NAD^+, and the nicotinamide phosphoribosyltransferase inhibitor FK866, which prevents NAD^+ synthesis. SIRT1 mRNA was measured by RT-qPCR relative to *GAPDH* and *TOP1* reference genes. SIRT1 protein was measured by Western blot relative to the α-tubulin loading control. Apigenin is typically used in cell culture at a concentration in the range of 0.5-100 μM (Escande *et al.*, 2013; Fale *et al.*, 2013). FK866 has been used previously in experiments reported in the literature at a range of 0.01 μM-0.4 μM but concentrations higher than 20 nM produced over a 80 % reduction in NAD^+ after 1 day incubation in Leukemia cells (Hasmann and Schemainda, 2003; Gehrke *et al.*, 2014; Schuster *et al.*, 2015). Previous work carried out by Professor Nicola Curtin's Laboratory with whom we collaborated for this work, used apigenin at a concentration of 25 μM and FK866 at a concentration of 0.1 μM over 4 h and found that these concentrations were sufficient to show effects on K564 cells after 4 h incubation. We used these same concentrations in our Caco-2 cell culture model to be consistent with our collaborators.

Addition of apigenin (25 μM, 4h), which we confirmed increased NAD^+, resulted in a 90 % reduction in SIRT1 mRNA and a slight decrease in SIRT1 protein but FK866 (0.1 μM, 4h), which we showed reduced NAD^+, had no effect on SIRT1 mRNA but showed a trend to increase SIRT1 protein (Figure 3.4.1).

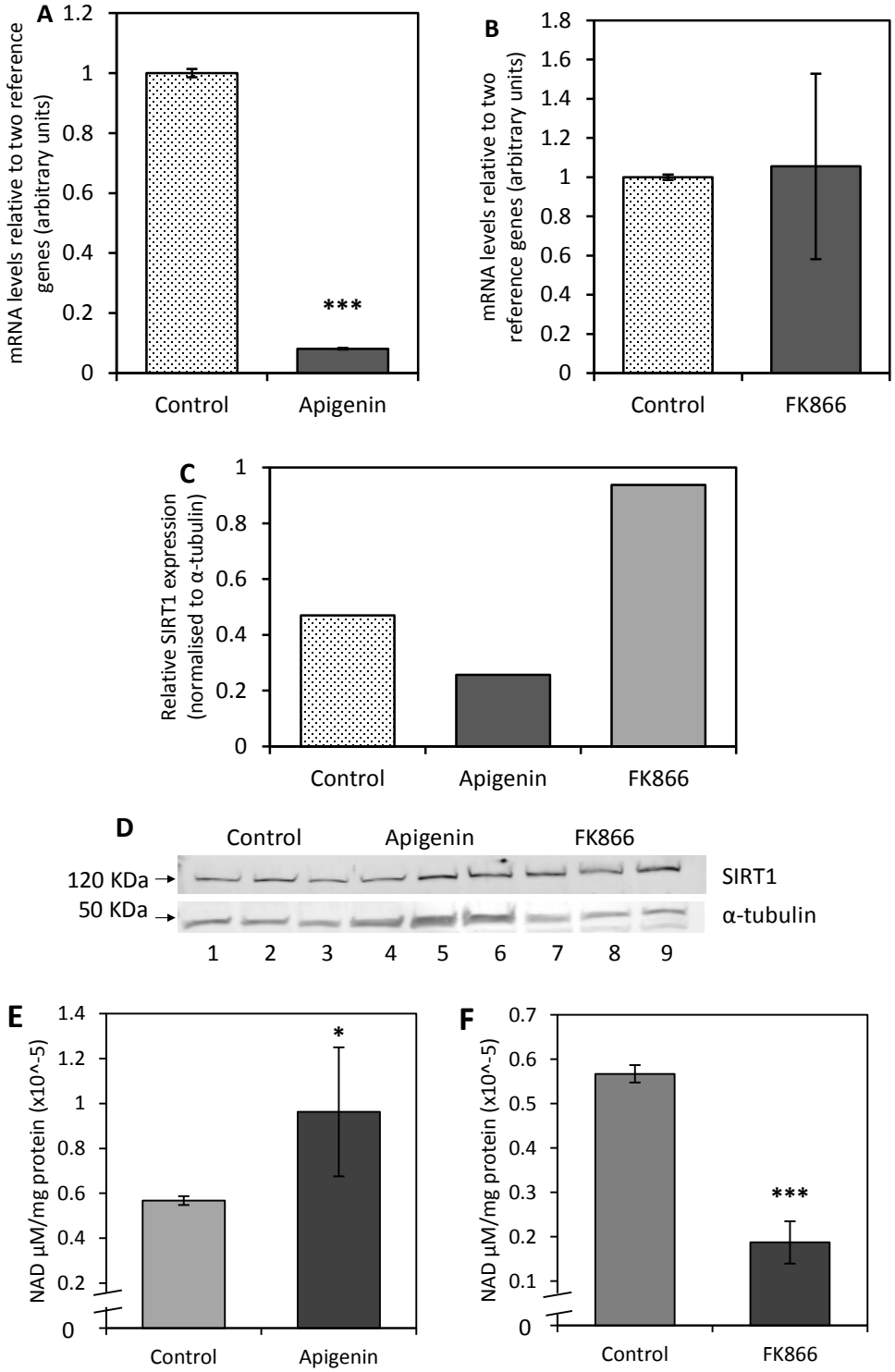


Figure 3.4.1. The effect of Apigenin and FK866 treatment on NAD⁺ and SIRT1 mRNA and protein in Caco-2 cells. Apigenin (25 µM), or FK866 (0.1 µM) were added to the medium for 4 h. SIRT1 mRNA was measured by RT-qPCR relative to *GAPDH* and *TOP1.* **A-** Effect of apigenin on SIRT1 mRNA. **B-** Effect of FK866 on SIRT1 mRNA. **C-**Quantification of SIRT1 protein by densitometry to measure the response to apigenin and FK866 n=1**. D-**Western blot raw data of SIRT1 protein in response to apigenin and FK866 (SIRT1 120 KDa and α-tubulin 50 KDa) lanes 1-3 control, lanes 4-6 apigenin, lanes 7-9 FK866**. E-** Effect of apigenin on NAD^+ levels. **F-** Effect of FK866 on NAD^+ levels. *p<0.05, ***p<0.0001 by Student's T-test. Data are shown as mean ± standard deviation (SD) n=2 (based on 6 data points comprising 2 biological replicates, each the mean of 3 wells measured separately).

3.5 The effect of resveratrol on NAD⁺ availability in Caco-2 cells.

Apigenin decreased SIRT1 mRNA and protein and FK866 increased SIRT1 protein, indicating that NAD^+ availability may have an effect on SIRT1 expression. It was reported previously that resveratrol can increase intracellular NAD^+ levels (Park *et al.*, 2012) but here we observed that when NAD^+ was increased using apigenin SIRT1 was reduced. However, resveratrol increased SIRT1 expression. We proposed a scheme to account for the observed increase in SIRT1 expression we observed in response to resveratrol and the reduction in SIRT1 expression we observed in response to apigenin whereby NAD^+ is the point of SIRT1 regulation and resveratrol reduces NAD^+ to reduce its responsive action on SIRT1 expression. Figure 3.5.1 is a schematic diagram representing this proposed scheme.

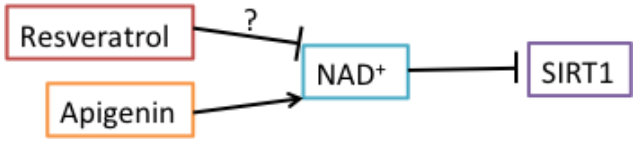


Figure 3.5.1. Schematic diagram of the proposed scheme that resveratrol reduces NAD^+ in Caco-2 cells to increase SIRT1 expression.

To test this model we investigated how NAD^+ was affected by resveratrol in Caco-2 cells. Resveratrol (60 μM) was applied to cell culture medium for 48 h. NAD^+ was then measured in the cell lysate by measuring a change in NADH fluorescence intensity before and after the addition of alcohol dehydrogenase and ethanol.

Published data demonstrate that resveratrol increased NAD^+ (Park *et al.*, 2012). Here, we saw a significant trend for resveratrol (60 μM, 48 h) to increase intracellular NAD^+ (Figure 3.5.2). Thus, NAD^+, which supresses SIRT1 expression, may not be the pivot point for the increase in SIRT1 expression observed in response to resveratrol and the reduction in SIRT1 expression we observed in response to apigenin. However, lack of statistical validity means that further experimental repeats are required to substantiate this interpretation.

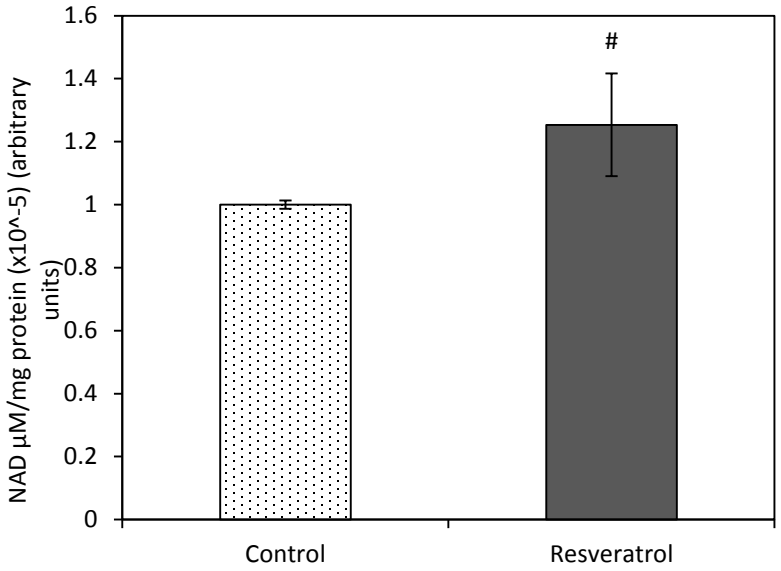


Figure 3.5.2. The effect of resveratrol treatment on NAD^+ in Caco-2 cells. NAD^+ is converted in to NADH by Alcohol dehydrogenase, the change in NADH fluorescence is measured at 360 nm excitation and 420 nm emission. Resveratrol (60 µM) was applied to the cell culture medium for 48 h (replenished at 24 h). #p=0.08 by Student's T-test. Data are shown as mean ± standard deviation (SD), n=2 (based on 6 data points comprising 2 biological replicates, each the mean of 3 wells measured separately).

3.6 Discussion

The work presented in this chapter aimed to determine if resveratrol could affect the expression of SIRT1 in Caco-2 cells and to uncover details of the mechanism through which any such effects were mediated and, in particular, to determine if NAD^+ was involved in the interactions.

SIRT1 mRNA and protein were measured by RT-qPCR and Western blot respectively. SIRT1 mRNA expression was doubled in response to the treatment of cells with resveratrol (60 μM, 48 h) but although there was a trend of SIRT1 protein increasing in response to resveratrol, further experimental repeats are required to show statistical significance. Similar effects of resveratrol (100 μM) on SIRT1 expression have also been observed in human ovary cells (Morita *et al.*, 2012). To investigate if the increase in SIRT1 mRNA by resveratrol was via increased transcription from the *SIRT1* promoter, Caco-2 cells were transfected with a *SIRT1* promoter-reporter construct and resveratrol (60 μM, 48 h) was applied in the cell medium. *SIRT1* promoter activity increased in response to resveratrol but the increase was only a modest 20 % and hence substantially smaller than the parallel increases in SIRT1 mRNA and protein. Without information on the absolute transcription rate and mRNA half-life it is not possible to determine if the increase in transcription could account in full for the increase in mRNA. However, it seems likely that other actions of resveratrol may contribute to the effect on SIRT1 mRNA.

We hypothesised that resveratrol affected the expression of SIRT1 through changing intracellular NAD^+ levels, as it was reported previously that resveratrol can increase intracellular NAD^+ through the AMPK pathway in mice (Park *et al.*, 2012). To investigate if NAD^+ can alter SIRT1 expression, SIRT1 mRNA was measured by RT-qPCR in response to NAD^+ manipulation. Apigenin (CD38 NADase inhibitor) increased NAD^+ availability and FK866 (nicotinamide phosphoribosyltransferase inhibitor) decreased NAD^+ availability. There was no change in SIRT1 mRNA in Caco-2 cells in response to FK866 but SIRT1 protein was increased. This effect could be indicative of lowered NAD^+ levels increasing the stability of SIRT1 mRNA leading to an increase in SIRT1 translation to protein. However, measurement of SIRT1 mRNA stability, for example by measuring the rate of decline after the addition of actinomycin to halt transcription, is required to

investigate this suggestion further. Furthermore, SIRT1 mRNA and protein decreased in response to increasing NAD^+ availability by treatment of Caco-2 cells with apigenin. This mode of regulation, whereby an increase in the essential co-factor NAD^+ for SIRT1-mediated substrate deacetylation leads to reduced SIRT1 transcription and mRNA, could in theory be mediated through SIRT1 deacetylating a transcription factor that acts to stimulate transcription from the *SIRT1* promoter that is deactivated by deacetylation (shown schematically in Figure 3.6.1). To our knowledge, however, examples of such auto-regulatory processes have not yet been fully uncovered. However, PPAR-γ has been shown to share a negative association with SIRT1 by inhibiting SIRT1 transcription and activity (Han *et al.*, 2010), and SIRT1 has been shown

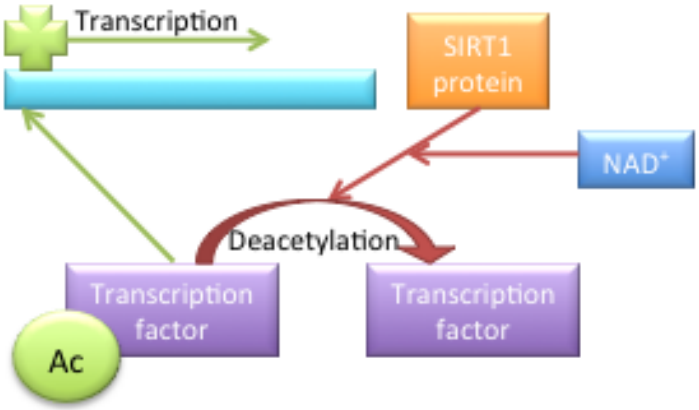


Figure 3.6.1. A proposed NAD^+-dependent auto-regulatory feedback loop acting on SIRT1 gene transcription. NAD^+ is required for SIRT1-mediated deacetylation and hence inactivation of a transcription factor that in its acetylated form increases transcription from the SIRT1 promoter. Ac=Acetyl group.

it activate PPAR-γ through deacetylation in mice (Qiang *et al.*, 2012). However, SIRT1 can also bind to PPAR-γ and inhibit its activity (Picard *et al.*, 2004), thus further work is needed to clarify a negative feedback loop.

Resveratrol was shown previously to increase NAD^+ levels through the AMPK pathway in mice (Park *et al.*, 2012) but our observations that increased NAD^+ decreases SIRT1 mRNA and protein, and resveratrol increases SIRT1 expression, are difficult to reconcile with this *in vitro* observation. Therefore, to determine the effect of resveratrol on NAD^+ in Caco-2 cells, resveratrol was applied in the cell culture medium and NAD^+ was measured in the cell lysate. Resveratrol appeared to increase NAD^+ but to draw a

firm conclusion more experimental repetition is needed and thus the apparent discordance remains.

It appears that the interrelationships between SIRT1, NAD^+ and resveratrol are highly complex. It is reasonable to assume that many other pathways interlink and intersect in regulatory feedback loops. The large number of substrates, including transcription factors that can be deacetylated by the NAD^+-dependent action of SIRT1 makes such a scenario highly likely. A full understanding of these interactions and therefore of how they could be manipulated to achieve benefits to healthspan requires further work.

4 Chapter 4. Does competition between SIRT1 and PARP1 for the cellular NAD^+ pool drive cross-talk that has implications for cell vitality?

4.1 Introduction

The work presented in Chapter 3 shows that resveratrol can increase the expression of SIRT1 and may also increase intracellular NAD^+. However, when intracellular NAD^+ was increased using the pharmaceutical agent apigenin we observed a reduction in SIRT1 expression, rather than the increase predicted if there was a simple relationship between these three variables such that resveratrol would increase NAD^+, which in turn would increase SIRT1 expression. Thus, it was important to explore how other demands on the cellular NAD^+ pool, in particular demands believed to be important in ageing, affected SIRT1 expression.

Poly ADP ribose polymerases (PARPs) consume NAD^+ and are involved in DNA single strand break repair. There is good evidence that PARPs play a role in ageing. For example, in mononuclear leukocytes from mammalian species of varying lifespans higher PARP activity was associated with longer lived species (Grube and Burkle, 1992). However, PARP has also been negatively associated with ageing, for example, when Parp2 was reduced in mouse embryonic fibroblasts telomere maintenance increased (Dantzer *et al.*, 2004) and in mice with destroyed islets, a mark of diabetes, Parp inhibition improved vascular responsiveness (Garcia Soriano *et al.*, 2001).

Crosstalk between SIRT1 and PARPs has been reported. For example, PARP1 can increase the transcription of *c-MYC* by activating the *c-MYC* transcription factor ESF1. Increased *c*-MYC can in turn increase SIRT1 expression (Simbulan-Rosenthal *et al.*, 2003; Yuan *et al.*, 2009; Marshall *et al.*, 2011). In contrast to this positive relationship between SIRT1 and PARP activity, Parp2 knockdown by siRNA caused an increase in Sirt1 mRNA in mouse C2C12 myotubes, and Sirt1 protein was also increased in Parp2 -/- mice (Bai *et al.*, 2011). Finally, PARP1 is activated by acetylation. Acetylation of Parp1 was increased in Sirt1 -/- mice compared with controls, suggesting that deacetylation by SIRT1 deactivates PARP1 (Rajamohan *et al.*, 2009a).

NAD^+ may be pivotal in the interactions between SIRT1 and PARP, because both enzymes consume NAD^+. NAD^+ appears to be a key factor in ageing. For example, the NAD^+ scavenger Pnc1 was required for lifespan extension in response to DR in *Caenorhabditis elegans* (Moroz *et al.*, 2014). Similarly in *Saccharomyces cerevisiae* mutants for Ntp1, an enzyme involved in NAD^+ synthesis, failed to live longer under conditions of DR (Lin *et al.*, 2002).

The work presented in this chapter measures some of the complex relationships between SIRT1, PARPs and NAD^+ and addresses the hypothesis that SIRT1 and PARPs compete for the same intracellular NAD^+ pool. Based on this hypothesis we predicted that PARP and SIRT1 would negatively affect the activity of each other, such that when PARP activity was high SIRT1 activity would be repressed and when SIRT1 activity was high PARP activity would be repressed. We took two main approaches. First, we used tissue available from mice of a range of different ages and also a Parp1 knockout mouse line to measure how age affected PARP activity and SIRT1 expression and if PARP1 expression had a direct effect on SIRT1 expression. Second, we manipulated each of the three variables separately in a human cell line model (Caco-2 intestinal cells) and measured the effect on each of the other two variables. We were unable to establish a robust assay to measure SIRT1 activity. Thus, in addition to manipulating SIRT1 activity through reducing expression (using siRNA) we measured effects of manipulating PARP activity on SIRT1 expression. The data will be used in a mathematical model to help understand the links between SIRT1, PARPs and NAD^+ and identify areas of intervention that may be beneficial or detrimental to cell vitality and ageing.

4.2 Sirt1 expression was negatively correlated with Parp activity in mouse liver, with a trend towards younger mice having higher levels of Sirt1 and lower Parp activity.

To begin to understand the relationship between SIRT1 and PARPs, we measured Parp activity and Sirt1 protein in adult mice of different ages. Preliminary data were collected from male and female mouse liver samples ranging from 3 to 29 months of age. PARP activity was measured in these liver extracts as synthesis of the PAR polymer, which was then detected using an anti-PAR antibody. These measurements were made by the research group of Professor Nicola Curtin, Newcastle University, with whom we collaborated with for this part of the work. SIRT1 protein levels were measured by Western blot relative to the α-tubulin loading control.

We saw no clear relationship between age, and either Parp activity or Sirt1 protein individually. However, we did observe a negative relationship between Sirt1 expression and Parp activity with a trend for younger mice to have higher Sirt1 and lower Parp that was particularly evident when the data for female and male mice were separated (Figure 4.2.1).

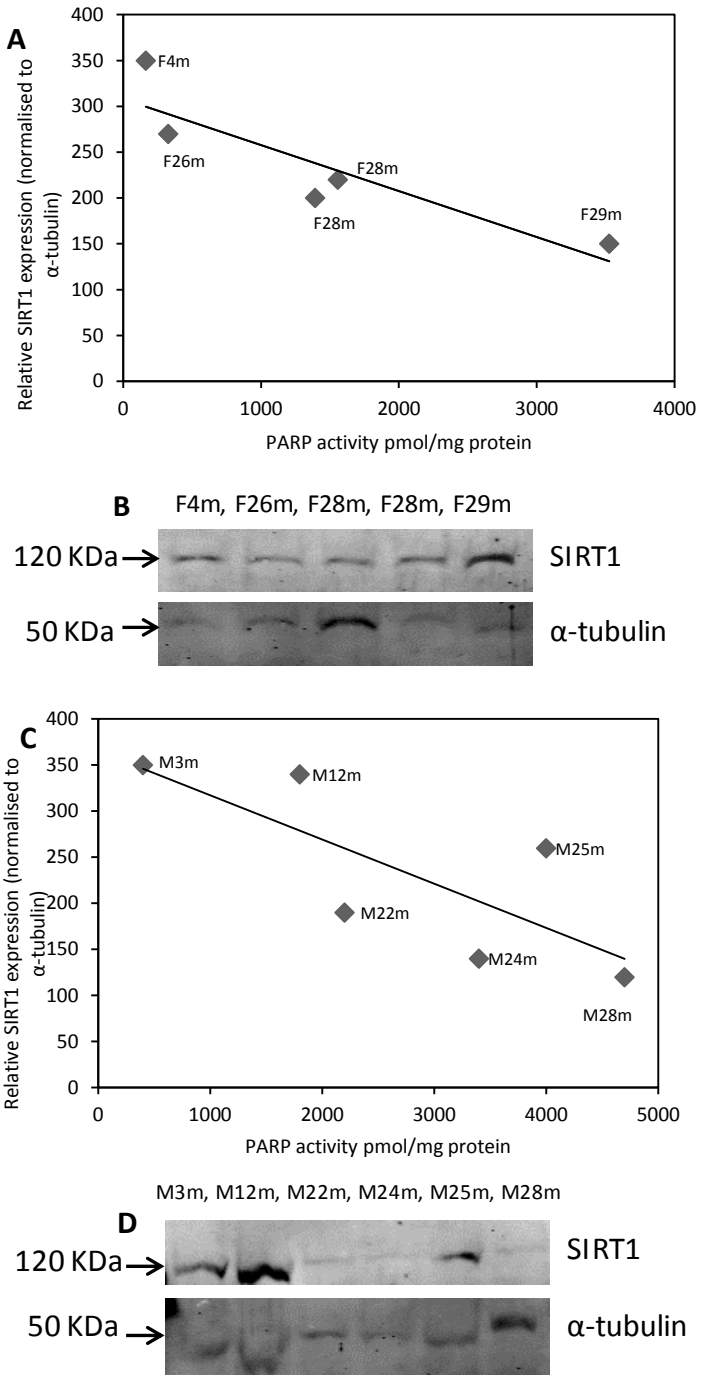


Figure 4.2.1. Female and male mouse liver samples analysed for Parp activity and Sirt1 protein levels. F=female, M=male, m=months old. Sirt1 protein was measured by western blot and is relative to α-tubulin loading control. Parp activity was measured by stimulating synthesis of the PAR polymer, which was detected by anti-PAR antibody. **A-** Sirt1 protein levels against Parp activity for female mice. **B-** Sirt1 protein measured by western blot (data as used for the plot in A) **C-** Sirt1 protein levels against Parp activity for male mice. **D-** Sirt1 protein measured by western blot (data as used for the plot in C). Measurements of Parp activity were provided by Professor Nicola Curtin, Newcastle University.

4.3 Sirt1 protein was unaffected in intestinal samples of Parp1 -/- mice.

We took advantage of the availability of samples of intestinal tissue from a Parp1 -/- mouse line, provided by our collaborator Professor Nicola Curtin, Newcastle University, to measure if these animals had different levels of expression of Sirt1 compared with controls. The samples were taken when the mice were 3 months of age. Sirt1 was measured by Western blotting and compared with α-tubulin as a loading control.

There was no difference in Sirt1 protein in the intestine between Parp1 -/- mice and Parp1 +/+ mice. Moreover, there were no apparent differences in Sirt1 protein between female and male mice (Figure 4.3.1).

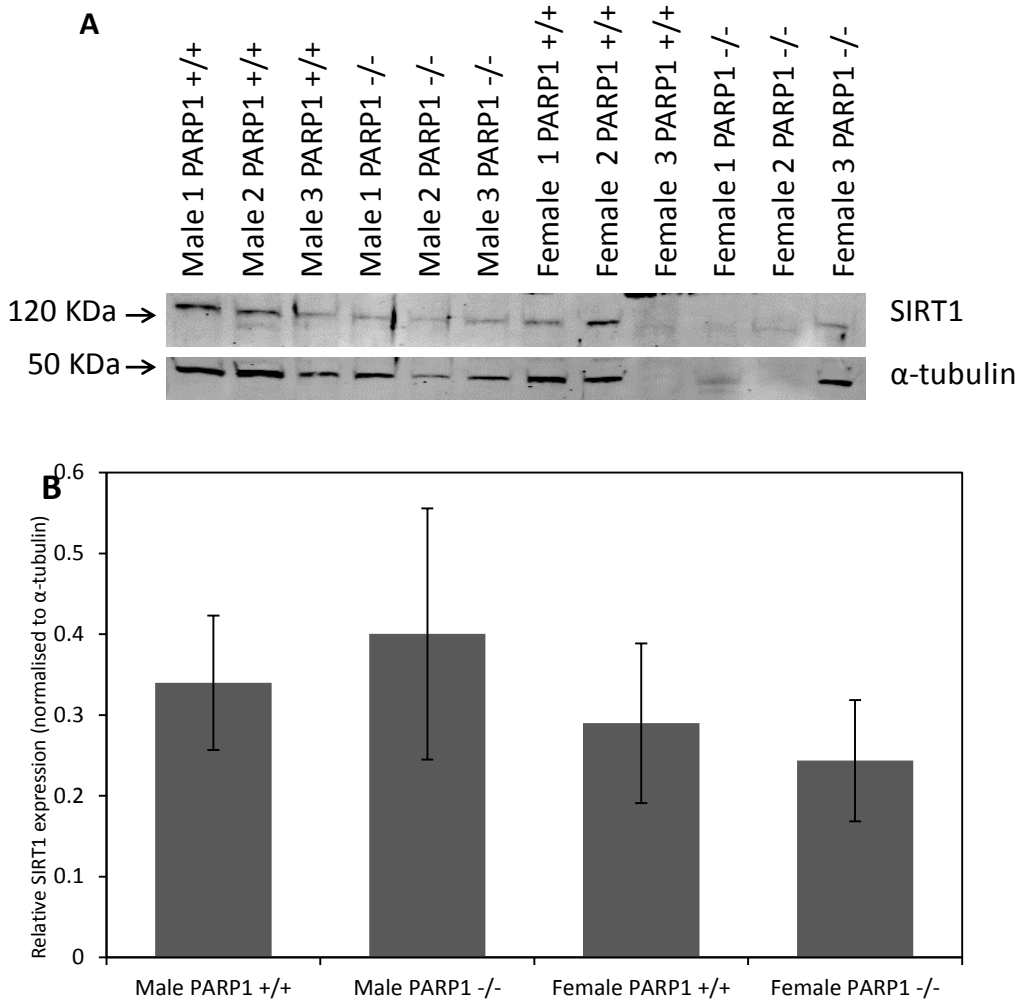


Figure 4.3.1. SIRT1 protein measured in samples of the small intestine from Parp1 +/+ and Parp1 -/- male and female mice. Representative figure showing three samples taken from each of the four groups of five mice. Mice were 3 months old. **A-** The raw data showing Sirt1 protein measured by western blot. **B-** Quantitative data derived using densitometry to measure signals on western blots. Samples were provided by Professor Nicola Curtin, Newcastle University. ANOVA and Tukey's post hoc statistical test were used. Data are shown as ± standard deviation (SD), n=3.

4.4 Inhibition of PARP led to a transient increase in SIRT1 mRNA levels in Caco-2 cells.

Thus far, preliminary data collected using mouse liver tissue had shown an apparent inverse correlation between Parp activity and Sirt1 expression in liver but no effect of Parp1 knockout on Sirt1 expression in intestine. Possible interpretations of these findings are that: i) intestine and liver differ with respect to the influence of Parp acting on Sirt1 expression; ii) the activity of Parp does not directly influence Sirt1 expression and the relationship observed in liver was the result of another factor influencing both variables (in opposite directions); iii) the small number of samples of tissue available and inter-animal variation in Sirt1 expression led to a lack of power to detect an effect of Parp1 knockout in intestine. Thus, to determine if PARP activity in intestinal cells influenced SIRT1 expression we inhibited PARP pharmaceutically in human intestinal Caco-2 cells and measured SIRT1 expression. PARP was inhibited using 3-aminobenzamide (1 mM, 0-16 h). In published work 3-aminobenzamide has been commonly used at concentrations of 1-10 mM. Concentrations of >10 mM have been reported to delay growth of cultured cells which require a source of purines in the cell culture medium (Cleaver, 1984; Zingarelli *et al.*, 1996; Karczewski *et al.*, 1999). Therefore, 1 mM 3-aminobenzamide was used. SIRT1 mRNA was measured at 0 h, 4 h, 6 h, 12 h and 16 h by RT-qPCR relative to *GAPDH* and *TOP1* reference genes.

After 6 h 3-aminobenzamide increased SIRT1 mRNA levels by 2.5 fold in Caco-2 cells. SIRT1 mRNA had returned to the control level at 12 h and at 16 h was reduced below the control level (Figure 4.4.1). Thus, we showed that PARP activity can influence SIRT1 expression in human intestinal cells but the effect may be transient and also biphasic. These time dependent features of the response are a possible explanation for the fact that we measured no effect of Parp1 knockout on Sirt1 expression in intestine *in vivo*. However, a transient response is difficult to reconcile with the observed proposed relationship between Parp activity and Sirt1 expression we measured in mouse liver.

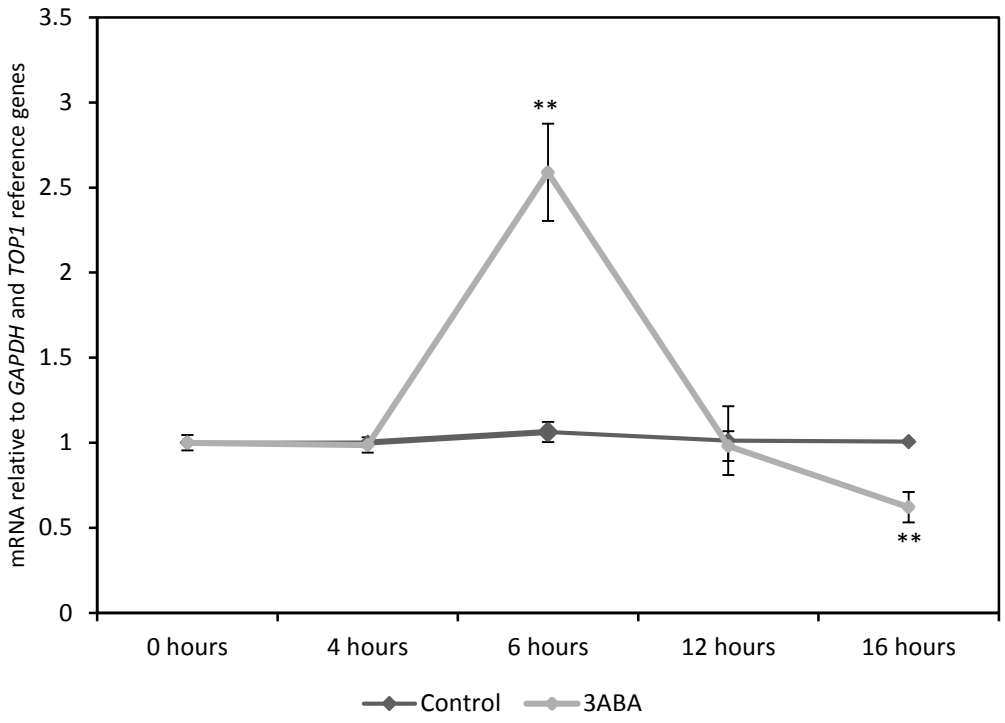


Figure 4.4.1. The effect of the PARP inhibitor 3-aminobenzamide at 4, 6, 12 and 16 h, on SIRT1 mRNA levels in Caco-2 cells. 3-aminobenzamide (3ABA) was applied in the medium at 1 mM for 0, 4, 6, 12 and 16 h. SIRT1 mRNA was measured by RT-qPCR relative to *GAPDH* and *TOP1* reference genes. **P<0.001 by Student's T-test. Data are shown as mean ± standard deviation (SD), n=2 (based on 6 data points comprising 2 biological replicates, each the mean of 3 wells measured separately).

4.5 Activating the DNA damage response, and thus PARP activity, did not change SIRT1 mRNA in Caco-2 cells.

Having observed that PARP inhibition in Caco-2 cells increased SIRT1 mRNA, demonstrating an inverse relationship similar to that seen in the livers of young and old mice, we aimed to determine if this inverse relationship can be seen when PARP activity is increased. Thus, we treated Caco-2 cells with temozolomide (TMZ) and measured SIRT1 mRNA. TMZ causes DNA damage in the cell, which activates the DNA damage response, including PARP. TMZ was applied in the cell culture medium at a concentration of 30 μM for 4 h. TMZ has been used previously in the literature at concentrations of around 25-50 μM but also as high as 1000 μM (Liu *et al.*, 2008a; Sahm *et al.*, 2013). Previous work carried out by Professor Nicola Curtin's Laboratory used TMZ at 30 μM over 4 h and found that this concentration caused sufficient damage without killing K564 cells after 4 h incubation. We thus used these same concentrations in our Caco-2 cell culture model to be consistent with our collaborators. SIRT1 mRNA was measured using RT-qPCR relative to *GAPDH* and *TOP1* reference genes.

We measured no effect of TMZ on SIRT1 mRNA under these conditions (Figure 4.5.1). Given the time-dependent nature of the response of SIRT1 to the PARP inhibitor 3ABA and the fact that the maximum response was measured after 6h (not 4h) further measurements are necessary; however, time did not permit us to pursue this part of the investigation further.

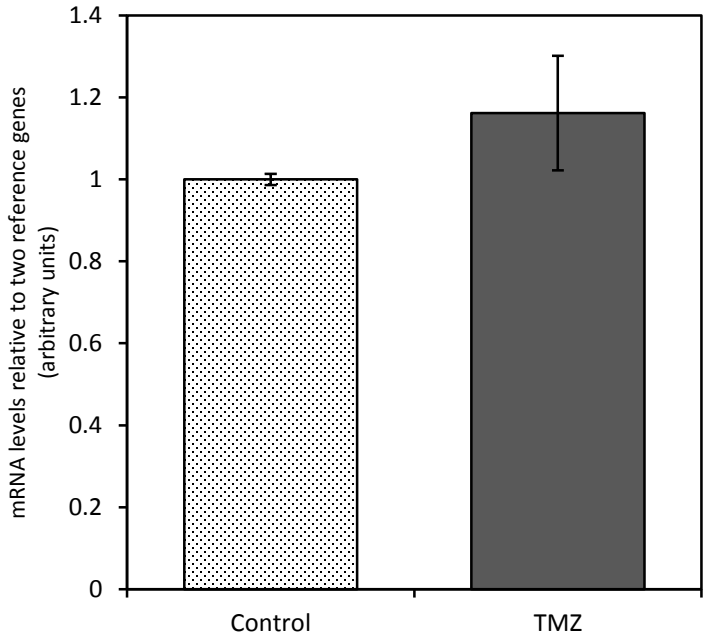


Figure 4.5.1. The effect of the PARP activator TMZ on SIRT1 mRNA in Caco-2 cells. TMZ was applied in the medium for 4 h at 30 μM. SIRT1 mRNA was measured by RT-qPCR relative to *GAPDH* and *TOP1* reference genes. Student's T-test was used. Data are shown as mean ± standard deviation (SD), n=2 (based on 6 data points comprising 2 biological replicates, each the mean of 3 wells measured separately).

4.6 SIRT1 knockdown appeared to increased PARP activity in Caco-2 cells.

Having manipulated PARP activity and measured the effect on SIRT1 expression we manipulated SIRT1 expression and measured the effect on PARP activity, to derive more data on mutual interactions between these two apparent modifiers of ageing with the aim, ultimately, of populating a detailed model of this system. Thus, we used siRNA to reduce SIRT1 expression in Caco-2 cells then measured the effect on PARP activity. Two different siRNAs targeted to SIRT1 (see methods for details) were both highly effective in multiple respects of experiments involving their use in Caco-2 cells and compared with control siRNA consistently reduced SIRT1 mRNA by approximately 90 % and reduced SIRT1 protein to levels barely detectable by Western blotting 72 h after transfection. Typical data confirming efficiency of these siRNAs are shown in Figure. 4.6.1.

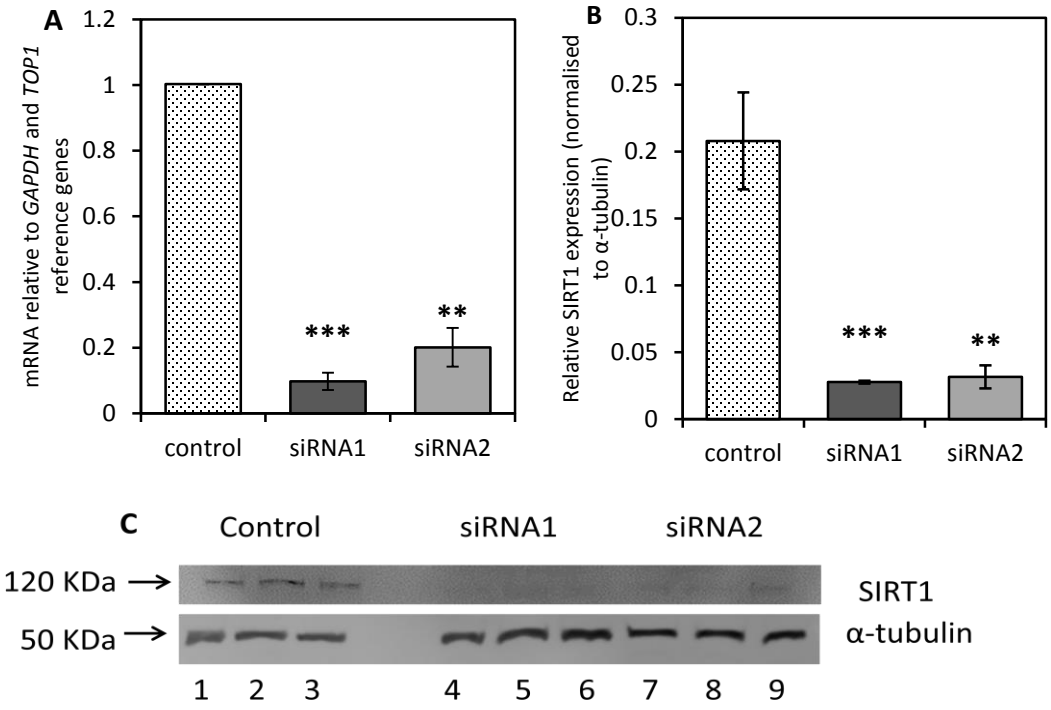


Figure. 4.6.1 SIRT1 knockdown by siRNA reduced SIRT1 mRNA and protein at 72 h. SIRT1 mRNA was reduced consistently in Caco-2 cells by two different siRNAs. mRNA was measured by RT-qPCR relative to *GAPDH* and *TOP1* reference genes 72 h after transfection. Protein was measured by western blot. **A-** The effect of SIRT1 knockdown on SIRT1 mRNA. **B-** The effect of SIRT1 knockdown on SIRT1 protein derived by measuring signals on western blots by densitometry. **C-** Representative western blot, lanes 1-3 control, lanes 4-6 siRNA1 and lanes 7-9 siRNA2, SIRT1 (120 KDa) and α-tubulin (50 KDa). **p<0.001, ***p<0.0001, by Student's T-test. Data are shown as mean ± standard deviation (SD), n=2 (based on 6 data points comprising 2 biological replicates, each the mean of 3 wells measured separately).

PARP activity was measured by stimulating synthesis of the PARP-synthesised polymer, PAR, which was detected using an anti-PAR antibody by researchers working with our collaborator Professor Nicola Curtin, Newcastle University.

Following SIRT1 knockdown by siRNA1, PARP activity appeared elevated in Caco-2 cells but was not statistically significant, which may be due to a lack in experimental repeats (Figure 4.6.2).

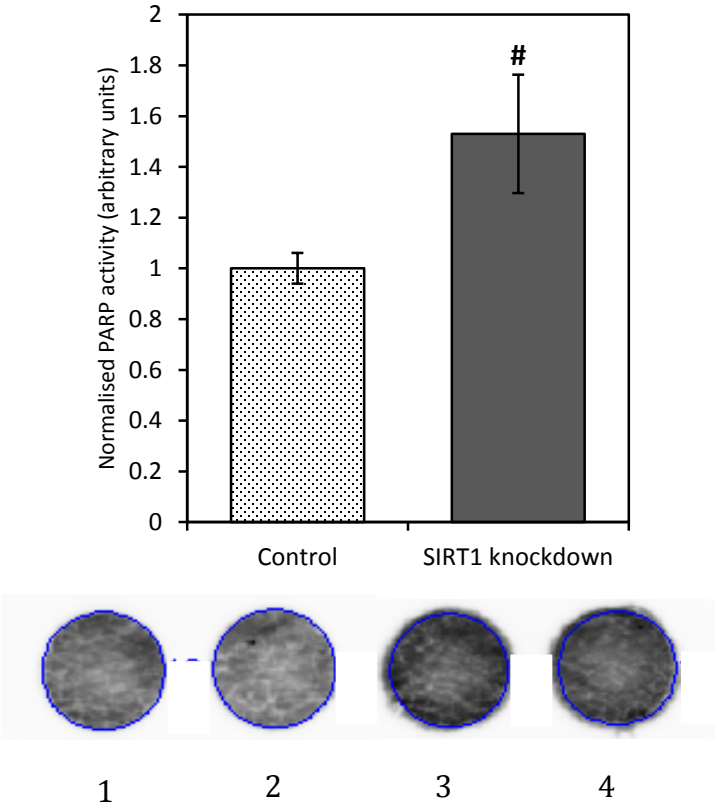


Figure 4.6.2. The effect of SIRT1 knockdown by siRNA on PARP activity in Caco-2 cells. PARP activity was measured through the generation of the PARP polymerase product PAR following stimulation in Caco-2 cell lysate with NAD^+ and a PARP substrate. siRNA1 was used to knockdown SIRT1 over 72 h. SIRT1 knockdown was verified using RT-qPCR. Below each bar is the corresponding representative raw data, 1-control, 2-control, 3- SIRT1 knockdown, 4- SIRT1 knockdown. #P=0.12 by Student's T-test. Data are shown as mean ± standard deviation (SD), n=2 (based on 6 data points comprising 2 biological replicates, each the mean of 3 wells measured separately). PARP1 activity data were provided by Professor Nicola Curtin, Newcastle University.

4.7 Increasing cellular NAD^+ by apigenin did not affect PARP activity in Caco-2 cells.

We had established previously that treatment of Caco-2 cells with apigenin was effective in increasing NAD^+ and that SIRT1 mRNA was simultaneously reduced dramatically, concomitant with a reduction in SIRT1 protein (Chapter 3.4). This reduction in SIRT1 was of similar magnitude to the reduction we achieved using siRNA, which resulted in an apparent but not significant increase in PARP activity. Thus, we predicted that apigenin would cause an increase in PARP activity and tested this prediction. Caco-2 cells were treated with apigenin (25 μM) in the cell culture medium for 4 h. PARP activity was measured in the cell lysate by stimulating synthesis by PARP of the PAR polymer, which was detected using an anti-PAR antibody. We confirmed that the treatment with apigenin invoked the same reduction in SIRT1 expression we observed previously (Figure 4.7.1). PARP1 activity data were provided by Professor Nicola Curtin, Newcastle University.

Contrary to our prediction, PARP activity was not affected in Caco-2 cells after treatment with apigenin (Figure 4.7.1). It is difficult to suggest reasons for this observation, since a likely mechanism through which a reduction in SIRT1 potentially invokes an increase in PARP activity is through reducing the drain on cellular NAD^+ reserves, which in turn allows greater PARP activity. Were this the cause then the prediction would be that apigenin (through further increasing NAD^+) should augment rather than supress this response. It is unlikely that the effect of the siRNA1 against SIRT1 on PARP activity was due to an off target action (on other genes) because we observed the same response with two different siRNAs. A possible explanation is that apigenin has other actions that influence PARP activity through other pathways.

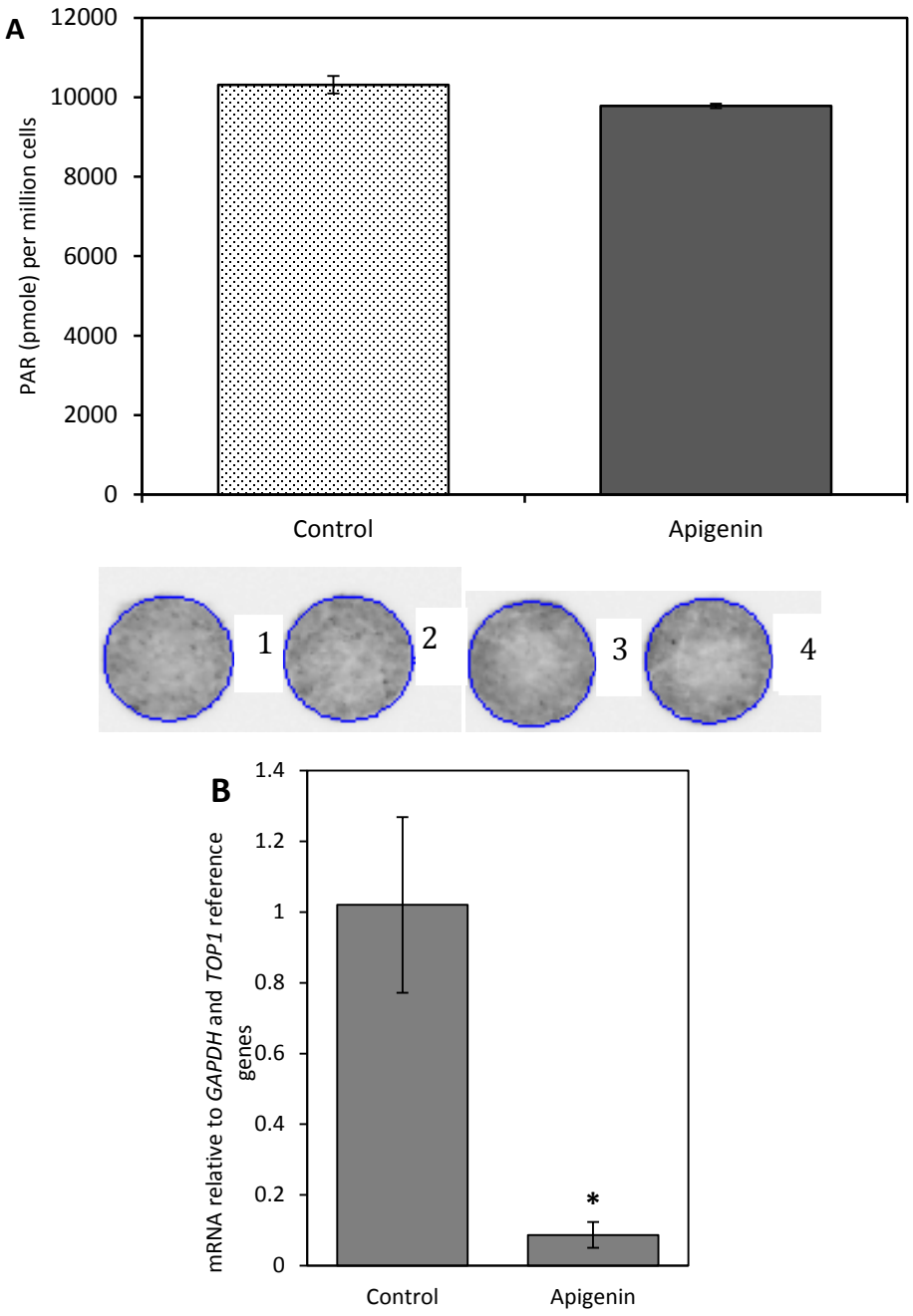


Figure 4.7.1. The effect of apigenin on PARP activity in Caco-2 cells. A- PARP activity was measured in the cell lysate after stimulating PARP synthesis of the PAR polymer. Apigenin was applied in the cell culture medium for 4 h at 25 µM. Below each bar is representative raw data, 1-control, 2-control, 3-apigenin, 4-apigenin. **B-** SIRT1 mRNA measured by RT-qPCR in response to apigenin in Caco-2 cells. **P<0.01. Data was analysed by Student's T-test. Data are shown as mean ± standard deviation (SD), n=2 (based on 6 data points comprising 2 biological replicates, each the mean of 3 wells measured separately).

4.8 At endogenous levels of SIRT1 expression, PARP inhibition and knockdown of SIRT1 appeared to have no effect on NAD^+ in Caco-2 cells.

Thus far we had observed effects of manipulating PARP on SIRT1 expression in Caco-2 cells and manipulating SIRT1 expression on PARP activity that in some instances satisfied the observed predictions for a model whereby PARP and SIRT1 compete for the same intracellular NAD^+ pool but in other instances were contrary to predictions. Given the proposed central role of NAD^+ in the proposed model we next measured the effect of inhibiting separately and together SIRT1 and PARP on intracellular NAD^+. SIRT1 was reduced using interfering siRNA and PARP was inhibited using 3-aminobenzamide (1 mM, 6 h). NAD^+ was measured in the Caco-2 cell lysate by measuring the change in NADH fluorescence before and after the addition of ethanol and alcohol dehydrogenase (causing NAD^+ to be reduced to NADH).

Following SIRT1 knockdown and/or 3-aminobenzamide treatment there were no statistically significant changes in NAD^+ (Figure 4.8.1). Due to the challenging nature of measuring small changes in intracellular NAD^+, as NAD^+ is strictly controlled in the cell, further experimental repeats are required to help elucidate if the small changes observed are true and significant representatives of the effect of SIRT1 knockdown and/or 3-aminobenzamide on NAD^+ or just small divergences.

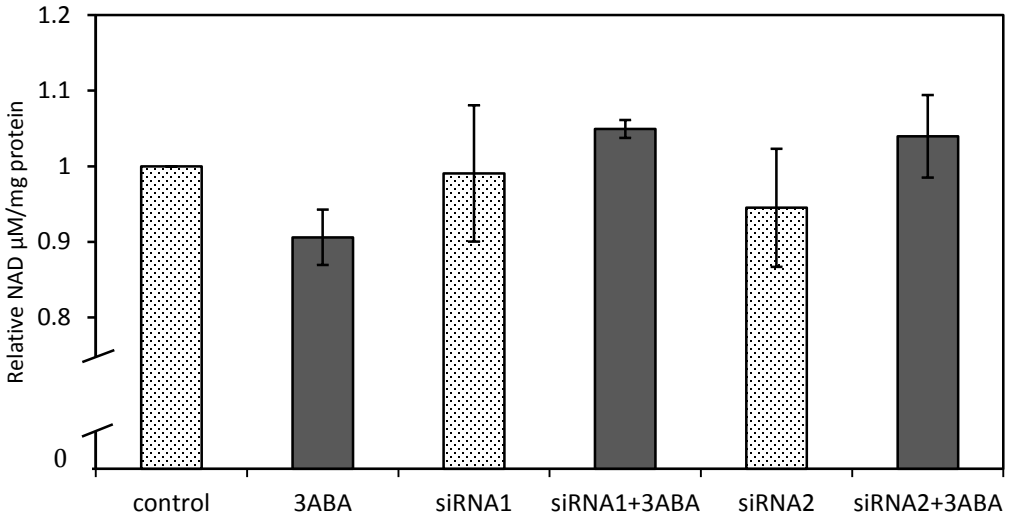


Figure 4.8.1. The effect of SIRT1 knockdown and PARP inhibition on NAD^+ in Caco-2 cells. NAD^+ was measured by the change in fluorescence of NADH following addition of alcohol dehydrogenase and ethanol to cell lysate. Fluorescence was measured at 360 nm excitation and 420 nm emission. Two siRNA's were used separately to knockdown SIRT1 over 72 h. The PARP inhibitor (3-aminobenzamide- 3ABA) was applied in the cell medium at 1 mM over 6 h. Data are normalised to the control. Statistics measured using ANOVA and Tukey's post hoc statistical test. Data are shown as mean ± standard deviation (SD), n=2 (based on 6 data points comprising 2 biological replicates, each the mean of 3 wells measured separately).

4.9 Modelling the interactions between SIRT1, PARP enzymes and NAD^+.

The aim of the work presented in this chapter was to obtain data that would provide further insight into the interactions between SIRT1, PARPs and NAD^+ and ultimately feed in to a mathematical model. Such a model would improve understanding of the interactions between SIRT1, PARPs and NAD^+ that may be beneficial or detrimental with respect to cell vitality and ageing. Due to the time constraints on the project we were unable to generate sufficient detailed data to generate a meaningful model; however, we show progress towards this goal as a schematic diagram and explanatory key of the interactions, incorporating also information from an existing model (Luna *et al.*, 2013) (Figure 4.9.1).

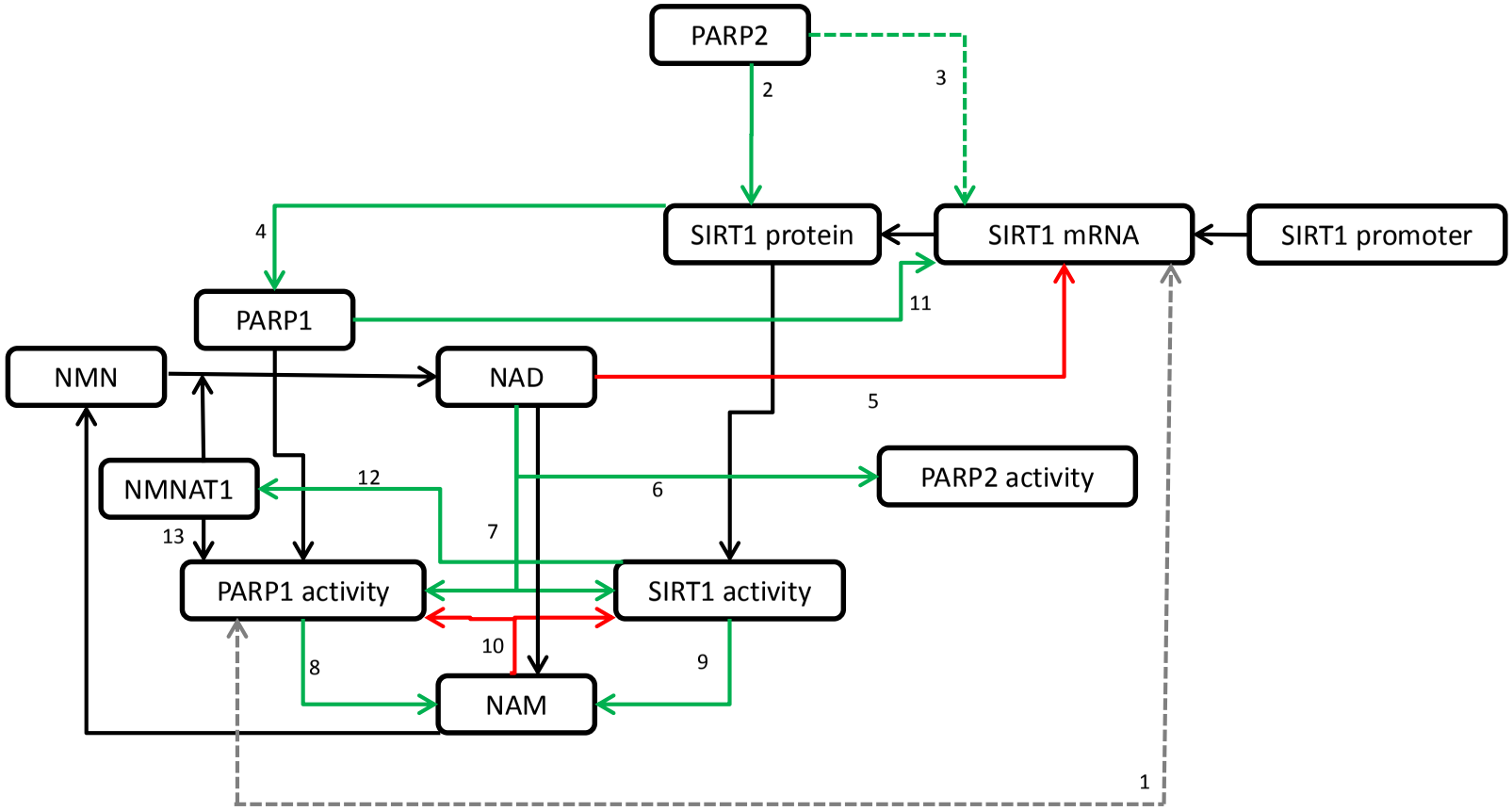


Figure 4.9.1. A schematic diagram showing the interactions between SIRT1, PARPs and NAD$^+$. Green lines indicate a positive action, red lines indicate a negative action, grey lines indicate preliminary data and black lines indicate a known direct relationship, dashed lines indicate a factor that reduced by either knockdown by siRNA or inhibited, NAM (nicotinamide), NMNAT1 (nicotinamide mononucleotide adenyltransferase 1). Numbers are represented in key with corresponding detail on the connection shown.

Key for Figure 4.9.1	
1	PARP inhibition by 3-aminobenzamide increased SIRT1 mRNA and SIRT1 knockdown by siRNA appeared to increase PARP activity in Caco-2 cells (preliminary data) (current thesis)
2	Parp2-/- mice had increased Sirt1 protein (Bai *et al.*, 2011).
3	PARP2 knockdown by siRNA in C2C12 myotubes increased SIRT1 mRNA (Bai *et al.*, 2011).
4	Parp1 is activated through acetylation; Sirt1-/- mice had higher Parp1 activity due to a decrease in deacetylation of PARP1 by SIRT1 (Rajamohan *et al.*, 2009b).
5	Apigenin increased NAD^+ and reduced SIRT1 mRNA and protein in Caco-2 cells (current thesis).
6	NAD^+ is consumed by PARP1. PARP2 and SIRT1, the Km values for NAD^+ are: PARP1~20-60 μM, PARP2~130 μM, SIRT1~150-200 μM (Houtkooper *et al.*, 2010).
7	
8	The NAD^+ precursor NAM is salvaged and recycled to NAD^+ following NAD^+ consumption by SIRT1 and PARP1 (Burgos, 2011; Luna *et al.*, 2013).
9	
10	NAM inhibits SIRT1 (Bitterman *et al.*, 2002) and PARP1 (Hageman and Stierum, 2001).
11	PARP1 can promote SIRT1 expression through promoting C-*MYC* expression, which increases SIRT1 expression (Simbulan-Rosenthal *et al.*, 2003; Yuan *et al.*, 2009; Marshall *et al.*, 2011).
12	SIRT1 recruits NMNAT1 to promoter regions to convert NMN to NAD^+ (Zhang *et al.*, 2009).
13	NMNAT1 recruits to the PARP1 polymer PAR and converts NMN to NAD^+ to be utilised by PARP1 (Berger *et al.*, 2007).

4.10 Discussion

The work presented in this chapter probes aspects of the interactions between SIRT1, PARPs and NAD^+, based on the proposal that NAD^+ is a key mediator of interactions between SIRT1 and PARP, which both can influence the process of ageing and which share NAD^+ as a substrate. A stimulus for the work was an apparently discordant effect of NAD^+ when increased by resveratrol compared with apigenin on expression of SIRT1. Resveratrol increased SIRT1 expression in Caco-2 cells and also showed a trend to increase NAD^+. However, when NAD^+ was increased using the pharmaceutical agent apigenin SIRT1 expression was reduced dramatically. Thus, we sought additional information on how other factors that influence NAD^+ affect SIRT1 expression, with an objective, ultimately, to develop a mathematical model of these seemingly complex interactions. Based on a simple model of competition between SIRT1 and PARP for a limited intracellular NAD^+ pool we predicted that PARP and SIRT1 would affect the activity of each other such that PARP represses SIRT1 and vice versa, shown schematically in Figure 4.10.1.

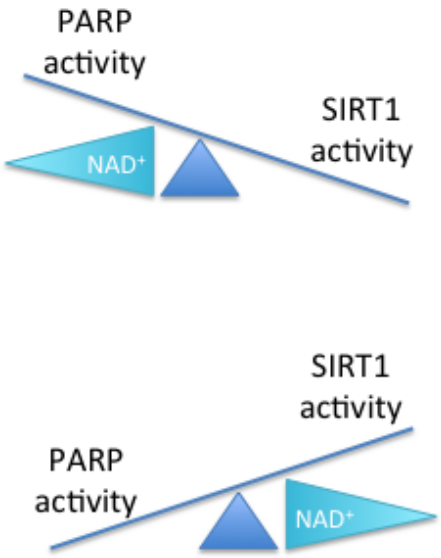


Figure 4.10.1 Schematic diagram on the predicted relationship between SIRT1, PARP and NAD^+. When PARP utilises NAD^+ SIRT1 is reduced and when SIRT1 utilises NAD^+ PARP is reduced.

Our ability to test this hypothesis rigorously was compromised by the fact that we were unable to measure SIRT1 activity. Thus, we manipulated SIRT1 activity by reducing expression using siRNA and measuring the effect on PARP activity. We also measured the effect of inhibiting and activating PARP on the expression of SIRT1. We also used liver and intestinal tissues available from mice of different ages and PARP1 knockout mice (respectively) to determine if there was evidence for a reciprocal relationship between PARP activity and SIRT1 expression.

Preliminary data collected from young and old mouse liver showed that Sirt1 protein and Parp activity were negatively correlated. There was a trend towards higher SIRT1 protein levels in younger mice than in older mice and lower Parp activity in younger than in older mice. The correlation was more prominent in female mice than in male mice. There appears to be an interaction between the female hormone oestrogen and SIRT1; signalling through ERα was increased in breast cancer cells when SIRT1 was inhibited by sirtinol (Moore and Faller, 2013). However, this relationship provides no obvious explanation for the observed tighter correlation between Sirt1 protein and Parp activity in female mice. Moreover, the number of mice analysed was small, hence the apparent tighter correlation in the female mice may be a chance feature of specific animals included in the sample. Establishing uniquely a difference between male and female mice requires the analysis of additional animals. Should such a study observe that there is a difference between sexes in the correlation between Sirt1 expression and Parp activity it would be worth determining if the same pattern is observed in humans and to investigate the reason. Such information could be incorporated in to the system level mathematical model to which this work is eventually aimed and could be important with respect to different influences on ageing in men and women.

We also gathered preliminary data on Sirt1 protein levels in the intestine of male and female Parp1 -/- mice. Sirt1 protein levels in Parp1 -/- mice did not appear to differ in either male or female animals. The data suggest that Parp1 knockout alone is not sufficient to effect Sirt1 protein levels. It is possible that other PARP enzymes, such as Parp2 (Bai *et al.*, 2011), need also to be reduced to affect Sirt1 protein levels. Another explanation for Parp1 knockout having no effect on Sirt1 expression may be that in a whole body system Sirt1 remains in homeostasis due to other regulatory feedback

mechanisms controlling expression, such as the Foxo1/Sirt1 negative feedback loop, described in detail in the introduction to this thesis (Xiong *et al.*, 2011).

The data also indicate that the reciprocal relationship between Parp activity and Sirt1 expression we observed in the liver of mice of different ages was not due to an effect of Parp1 on Sirt1 expression. However, alternative explanations include that a reciprocal relationship may be tissue specific or that the sample size of Parp1 -/- animals studied was too small to reveal an effect of the lack of Parp1 on Sirt1. Thus, further work should include measurement of Sirt1 in the livers of Parp1 -/- mice and also the analysis of a larger sample of Parp1 -/- mice.

As predicted by our model whereby SIRT1 and PARP compete for the same intracellular NAD^+ pool, SIRT1 knockdown in Caco-2 cells increased PARP activity. We did not test the effect on PARP activity of overexpressing SIRT1 in Caco-2 cells because an extensive body of work conducted in the laboratory has revealed that, although overexpression can be achieved by several fold using an expression plasmid construct, other measurements on which SIRT1 knockdown had a profound effect, such as DNA methylation (Wakeling *et al.*, 2015), are unperturbed. Thus we have concluded that Caco-2 cells express SIRT1 at endogenous levels where all of its actions are already at the maximum.

As already explained, we were unable to test the effect of PARP inhibition and activation on SIRT1 activity. The measurements of SIRT1 protein we made instead are not a direct substitute, thus the measurements cannot be linked directly with PARP in a model where both enzymes compete for NAD^+. However, we observed a profound effect of increasing NAD^+ using apigenin on SIRT1 expression, which was reduced dramatically. This finding uncovers a link between SIRT1 and the cellular NAD^+ pool, and hence between PARP activity and SIRT1 expression, and provides a focal point from which other data derived in this study can be considered in the context of the predicted model.

If the assumption is that NAD^+ has a negative influence on SIRT1 expression then other predictions could include the following:

1. Inhibition of PARP would conserve NAD^+ and lead to a suppression of SIRT1 expression.
2. Activation of PARP would consume NAD^+ and lead to increased SIRT1 expression.
3. Reducing NAD^+ pharmaceutically would increase SIRT1 expression.
4. Increasing NAD^+ using apigenin and hence reducing SIRT1 expression (as we observed) would increase PARP1 activity to mimic the response we observed when SIRT1 expression was reduced using siRNA.

The outcomes of experiments we conducted that test these predictions were as follows:

1. Inhibition of PARP using 3-aminobenzamide increased SIRT1 expression, but only transiently (6 h post application of apigenin) and showed no significant effect on NAD^+.
2. Activation of PARP using TMZ did not affect SIRT1 expression (4h post application of TMZ).
3. Reducing NAD^+ using FK866 appeared to increase SIRT1 protein, but there was no effect on SIRT1 mRNA.
4. PARP1 activity was unaffected by apigenin.

Thus, some of the predictions were met but others were not, and there remain many conflicts and limitations largely due to the fact that the current data are only a small component of all the data that must be obtained to test rigorously the predictions and also, more broadly, populate a system level mathematical model. Some of the specific limitations, relating to each of the four listed predictions and outcomes, are as follows:

1. A further time course, including more data points around the 6 h point, should be derived.
2. The effect of TMZ on SIRT1 expression should be measured over a time course, rather than a single time point.
3. The measurements of SIRT1 protein based on a small number of samples using Western blotting are unreliable and should be validated. Moreover, the effect of FK866 should be measured over a time course, rather than at a single time point, which showed no effect on SIRT1 mRNA.

4. More experimental repeats would increase the robustness of the data and also allow more confidence in interpretation particularly where data show a strong trend or consistent pattern that fails to reach statistical significance (specifically the measurement of PARP activity in response to SIRT1 knockdown and the measurement of NAD^+ in response to SIRT1 knockdown and PARP inhibition).

A further complexity with respect to interpretation of our findings comes from the start point from which the work was initiated-i.e. that resveratrol may increase NAD^+ but also increased SIRT1 expression in Caco-2 cells. An immediate priority for future work would hence be to measure the effect of resveratrol on PARP activity, with a view to eventually including information from experiments that measure the effect of resveratrol at different time points in the holistic mathematical model.

5 Chapter 5. An investigation into the effects of SIRT1 and resveratrol on chaperone mediated autophagy.

5.1 Introduction

Several observations and lines of investigation suggest that SIRT1 generally promotes protein degradation. For example, SIRT1 inhibition by sirtinol in human THP-1 cells induced inflammation and decreased the expression of LC3, a biomarker of macroautophagy. Furthermore, SIRT1 can inhibit the mammalian target of rapamycin (mTOR), which is a known inhibitor of autophagy via deactivation of autophagy initiating proteins, including, ULK family proteins (Jung *et al.*, 2010; Takeda-Watanabe *et al.*, 2012).

It has also been shown that resveratrol can increase autophagy through the AMPK pathway. Increasing AMPK activity results in the inhibition of mTOR, which is a known inhibitor of autophagy (Vingtdeux *et al.*, 2010). Moreover, the resveratrol derivative trans-3,4-dimethoxystilbene, which has a greater bioavailability than resveratrol, also inhibited mTOR through AMPK and increased autophagy (Zhang *et al.*, 2012). Another study investigated the effects of two analogues of resveratrol, RSVA314 and RSVA405, on autophagy. The analogues were shown to have the same effect on autophagy as resveratrol but with 40 times more potency (Vingtdeux *et al.*, 2011).

The observations considered above all relate to macroautophagy; here auto phagosomes form around unwanted protein then combine with lysosomes (Martinez-Vicente *et al.*, 2005). Chaperone mediated autophagy (CMA) is a different process that fulfils the same ultimate function as macroautophagy to deplete cellular components and hence release their constituents for recycling. CMA is shown in Figure 5.1.1. HSC70 binds to proteins containing the KFERQ motif and chaperones them to LAMP2 at the membrane of the lysosome. The protein is then unfolded and transported through the membrane to be degraded. LAMP2 is then either recycled or degraded (Cuervo, 2011).

Figure 5.1.1. The process of CMA. Proteins containing the KFERQ motif are recognised by HSC70 and its co-chaperones and transported to the lysosomal membrane where the motif binds to a LAMP2 dimer (stabilised by HSP90). The protein is unfolded for translocation through the LAMP2 multimer (stabilised by GFAP and lysosomal HSC70). LAMP2 is then disassembled by HSC70 and degraded by Cathepsin A unless EF1α binds to GFAP. Sourced from (Cuervo, 2011).

DNA microarray data collected previously in the laboratory indicated that *LAMP2* gene expression was reduced by SIRT1 knockdown in Caco-2 cells (L. Wakeling personal communications). This observation is concordant with the view that one of the functions of SIRT1 to counteract ageing may be maintenance or enhancement of CMA. It has been shown that LAMP2 was reduced in models of ageing. For example, in 22 month old rats a decline in CMA was linked to reduced LAMP2 expression (Cuervo and Dice, 2000a). Lower levels of LAMP2 may accelerate ageing as a result of accumulation of unwanted proteins in the cell (Martinez-Vicente *et al.*, 2005; Kiffin *et al.*, 2007).

The work presented in this chapter first aimed to confirm the microarray data showing that SIRT1 knockdown can decrease LAMP2 mRNA. We also measured HSC70 mRNA in parallel, reasoning that both proteins are involved functionally in the process of CMA and, hence, may provide a read out of the level of activity of this process. A second aim was to determine if pharmacological manipulation of SIRT1 activity and/or resveratrol treatment can affect expression of LAMP2 and HSC70. The effect of pharmacological manipulation of the cellular NAD^+ pool on the expression of LAMP2 and HSC70 was also measured. This work was aimed to build on and link with work presented in Chapter 4 of this thesis that contributes to developing a system level mathematical model of SIRT1 actions and interactions centred on the consumption of NAD^+ by SIRT1-catalysed deacetylation reactions. A forth aim was to investigate further preliminary findings that showed SIRT1 knockdown affected DNA methylation of the LAMP2 promoter, providing insight in to the mechanism through which SIRT1 affects lAMP2 expression. As stated, we measured both LAMP2 and HSC70 as likely markers of the level of activity of CMA. However, these molecules can only provide an indirect indication of CMA activity and also are not necessarily regulated in a tight relationship with CMA function. Indeed, the observations we present in this chapter show that LAMP2 and HSC70 are not themselves tightly co-regulated. Thus, to understand the influence of SIRT1 and modifiers of its activity on the process of CMA a direct functional assay is required. A final aim was, therefore, to develop such an assay. The data collected in this chapter will then be linked in to the system level mathematical model.

Caco-2 cells were used for this work because the microarray data collected following SIRT1 knockdown previously was from this cell line and the choice of Caco-2 cells for

most of the work in the laboratory on SIRT1 function is based on the fact that dietary influences on SIRT1 function are a focus and that intestinal cells are exposed directly to compounds in the diet.

5.2 SIRT1 knockdown reduced expression of LAMP2 but had no effect on the expression of HSC70.

To confirm the effect of SIRT1 knockdown on LAMP2 mRNA, indicated in earlier work by microarray analysis, we used two siRNAs to reduce SIRT1 expression in Caco-2 cells. HSC70 mRNA and protein were measured alongside LAMP2 to determine if the effects of SIRT1 knockdown extend to this second component of CMA function. mRNA was measured by RT-qPCR relative to *GAPDH* and *TOP1* reference genes and protein was measured by Western blotting relative to the α-tubulin loading control.

SIRT1 knockdown by siRNA significantly decreased LAMP2 mRNA but did not significantly affect HSC70 mRNA. Visual inspection of corresponding Western blots showed no clear effect on LAMP2 protein (but a response may have been masked by the fact the signals were weak). Attempts to derive quantitative data on protein expression by densitometry analysis of Western blots were not successful because of the weak signals (Figure 5.2.1).

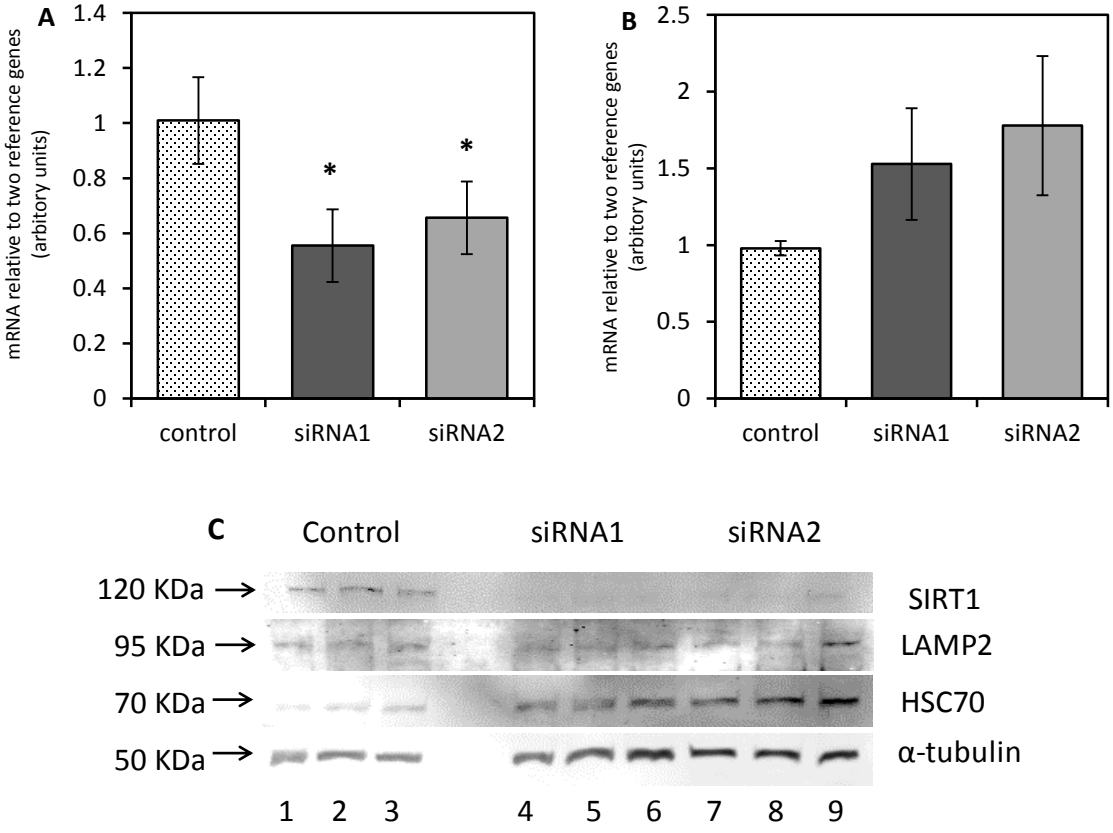


Figure 5.2.1. The effect of SIRT1 knockdown on LAMP2 and HSC70 mRNA and protein in Caco-2 cells. SIRT1 was knocked down using two different siRNAs separately. mRNA was measured by RT-qPCR relative to *GAPDH* and *TOP1* reference genes. Protein was measured by western blotting relative to α-tubulin loading control. **A-** LAMP2 mRNA in response to SIRT1 knockdown. **B-** HSC70 mRNA in response to SIRT1 knockdown. **C-** Representative western blot signals for SIRT1 (120 KDa), LAMP2 (95 KDa), HSC70 (70 KDa) and α-tubulin (50 KDa), (lanes 1-3 are controls, lanes 4-5 SIRT1 knockdown with siRNA1 and lanes 7-9 SIRT1 knockdown with siRNA2). *P<0.05, by Student's T-test. Data are shown as mean ± standard deviation (SD), n=2 (based on 6 data points comprising 2 biological replicates, each the mean of 3 wells measured separately).

5.3 Pharmaceutical inhibition of SIRT1 reduced LAMP2 mRNA but had no effect on HSC70 mRNA.

Having shown an effect of SIRT1 knockdown on LAMP2 mRNA we also investigated how LAMP2 and HSC70 were affected by pharmacological inhibition of SIRT1. Pharmacological inhibition offers the opportunity to collect more data on effects at multiple specific time points, which is particularly important to develop, ultimately, a system-level mathematical model of functional interactions of SIRT1. Caco-2 cells were exposed to the SIRT1 inhibitor EX-527 (10 μM) in the cell culture medium for up to 48 h and samples of cells for the measurement of LAMP2 and HSC70 were taken at several time points. EX-527 has been shown to decrease SIRT1 activity by 15x at a concentrations of both 10 μM and 100 μM (Solomon *et al.*, 2006). The lower concentration of 10 μM was used here in Caco-2 cells. mRNAs were measured by RT-qPCR relative to *GAPDH* and *TOP1* reference genes.

Treatment with EX-527 caused LAMP2 mRNA to be decreased transiently below the control level, which was significant only at 24 h. HSC70 mRNA also decreased transiently in response to EX-527 but was not significant. The lack of experimental repeats again limits interpretation of the data (Figure 5.3.1). Interestingly, the data does suggest that there may be a degree of reciprocity with respect to the levels of these two mRNAs; the shape of the response profiles are almost a mirror image. However, this interpretation is very speculative and further data is required to confirm such a relationship (Figure 5.3.1).

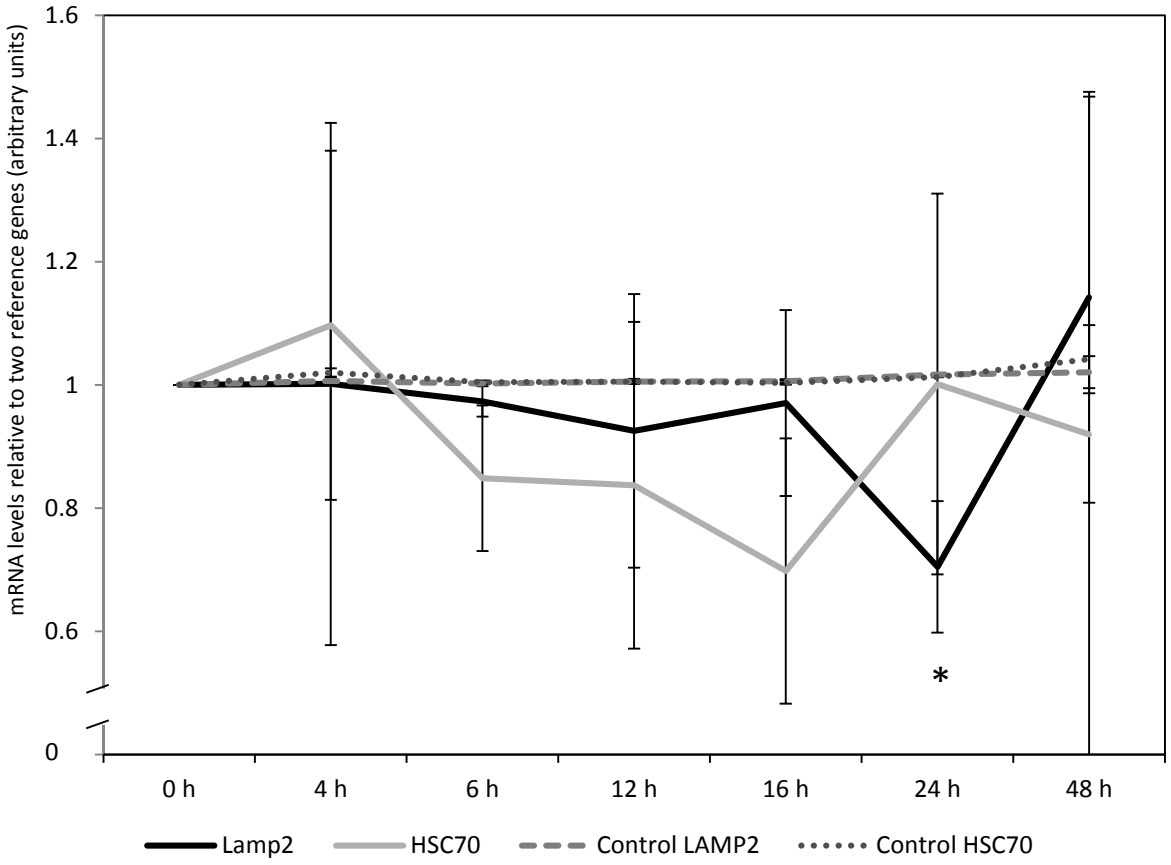


Figure 5.3.1. The effect of EX-527 on LAMP2 and HSC70 mRNA in Caco-2 cells. SIRT1 was inhibited by EX-527 (10 μM) applied in the cell culture medium for 4,6,12,16,24 and 48 h. mRNA was measured by RT-qPCR relative to *GAPDH* and *TOP1*. *P<0.05 by Student's T-test. Data are shown as mean ± standard deviation (SD), n=2 (based on 6 data points comprising 2 biological replicates, each the mean of 3 wells measured separately).

5.4 Resveratrol appears to increase LAMP2 mRNA but decreased LAMP2 protein.

Work included in this thesis shows that resveratrol increases SIRT1 expression in Caco-2 cells. We proposed that some of the reported effects of resveratrol associated with increased healthspan may be due to this increase in SIRT1 expression, which in turn increases CMA (as suggested by the positive relationship we observed between SIRT1 and LAMP2 expression). Thus, we measured the effect of resveratrol on LAMP2 and also on HSC70 in Caco-2 cells. Caco-2 cells were treated with 60 μM resveratrol for 48 h (applied to the cell culture medium and refreshed at 24 h); LAMP2 and HSC70 mRNA and protein levels were measured. mRNA was measured by RT-qPCR relative to *GAPDH* and *TOP1* reference genes and protein was measured by Western blotting relative to the α-tubulin loading control.

When resveratrol was applied to Caco-2 cells it had no effect on HSC70 mRNA or LAMP2 mRNA, however more observations will be required to confirm this (Figure 5.4.1). Visual inspection of the Western blots shows clearly a reduction in LAMP2 protein after treatment of cells with resveratrol but no clear effect on HSC70 mRNA. Analysis of blots using densitometry confirmed that resveratrol reduced LAMP2 protein and showed no effect on HSC70 protein but for statistical analysis more experimental repeats are needed.

If a discordant response of LAMP2 mRNA and LAMP2 protein to resveratrol does exist, the opposite of which was not observed with SIRT1 knockdown (as would be predicted if the action of resveratrol was purely through increasing SIRT1 expression), may reflect actions of resveratrol through additional pathways that lead to more rapid clearance of LAMP2 protein.

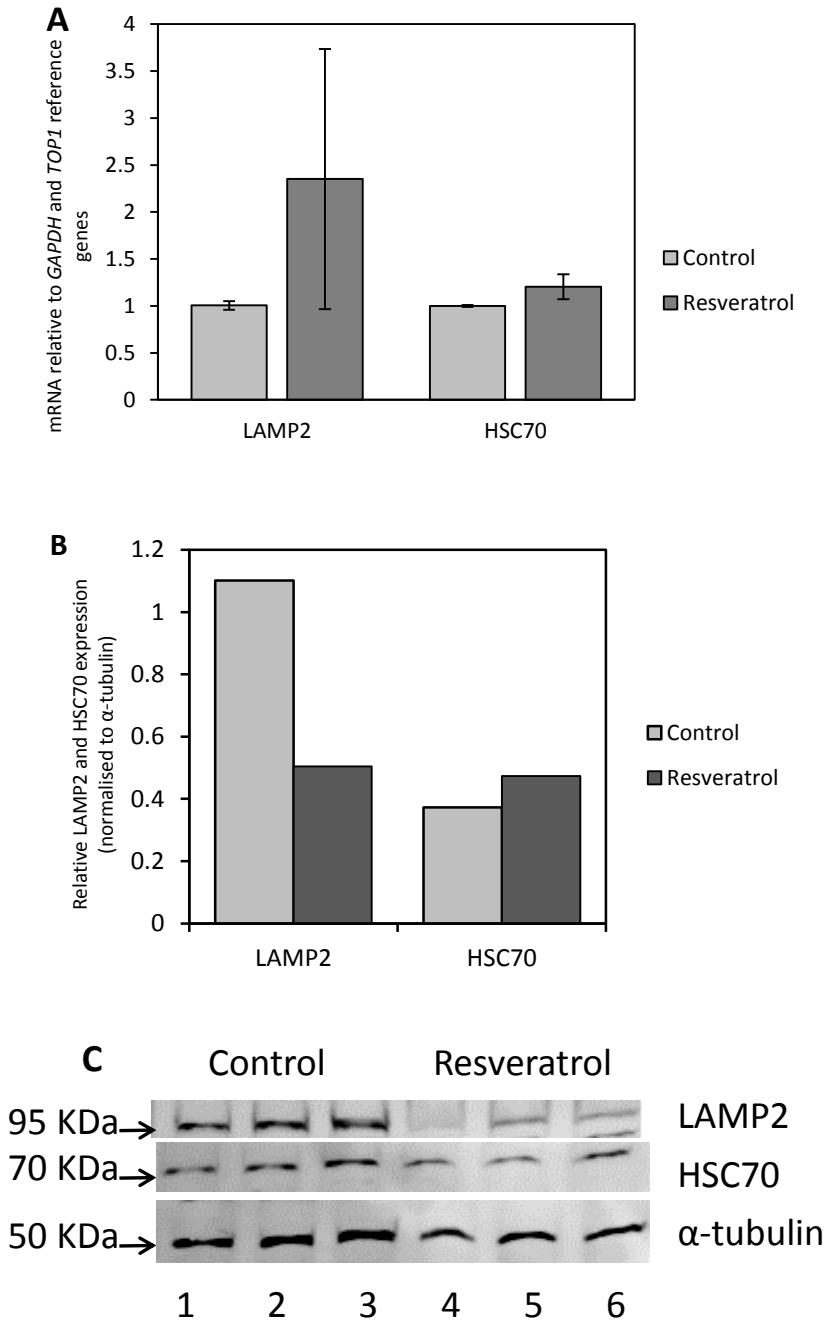


Figure 5.4.1. The effect of resveratrol on LAMP2 and HSC70 mRNA and protein in Caco-2 cells. Resveratrol was applied in the cell culture medium at a concentration of 60 μM for 48 h (replenished at 24 h). mRNA was measured by RT-qPCR relative to *GAPDH* and *TOP1* reference genes. Protein was measured by western blot relative to α-tubulin loading control. **A-** LAMP2 and HSC70 mRNA in response to resveratrol treatment n=2 (based on 6 data points comprising 2 biological replicates, each the mean of 3 wells measured separately). **B-** LAMP2 and HSC70 protein densitometry measurements n=1 **C-**Representative western blot signals for LAMP2 (95 KDa), HSC70 (70 KDa) and α-tubulin (50 KDa), lanes 1-3 are controls, lanes 4-5 resveratrol. Data are shown as mean ± standard deviation (SD) and did not differ significantly according to analysis using Student's T-test.

5.5 Increasing NAD⁺ using apigenin did not affect LAMP2 expression but reducing NAD⁺ using FK866 increased LAMP2 mRNA.

Work presented in Chapter 3-5 of this thesis are aimed towards deriving data ultimately to populate a system level mathematical model of interactions of SIRT1 with other molecules and cellular pathways that influence ageing. NAD^+, which is consumed in deacetylation reactions catalysed by SIRT1 and that we found may inhibit SIRT1 expression, provides a common node for some of these interactions, particularly for proposed interactions with PARP. Also, we found that resveratrol may increase NAD^+ in Caco-2 cells and in published findings resveratrol increases NAD^+ in C2C12 myotubes (Park *et al.*, 2012). Having thus far measured effects of manipulating SIRT1 and of resveratrol on LAMP2, a next logical step was to determine if manipulation of NAD^+ affected LAMP2 expression. NAD^+ was increased in Caco-2 cells using apigenin (CD38 NADase inhibitor, 25μM applied in the cell culture medium for 4 h) and decreased by FK866 (nicotinamide phosphoribosyltransferase, 0.1 μM applied in the cell culture medium for 4 h). LAMP2 mRNA was measured by RT-qPCR relative to *GAPDH* and *TOP1* reference genes. LAMP2 protein was measured by Western blot relative to the α-tubulin loading control.

Apigenin did not affect LAMP2 mRNA but FK866 increased LAMP2 mRNA (Figure 5.5.1 A and B). Visual inspection of Western blots did not indicate any obvious effect of either agent on LAMP2 protein (Figure 5.5.1 C). However, quality of these data were poor so we avoid any firm conclusion concerning effects on LAMP2 protein. We confirmed the expected effects of apigenin and FK866 on NAD^+ (Figure 5.5.1 D and E). The observed increase in LAMP2 mRNA driven by a reduction in NAD^+ achieved using FK866 is consistent with a model whereby NAD^+ has a negative effect on SIRT1 expression; thus SIRT1 is increased by treatment of cells with FK866 (Chapter 3) and (as we confirm in the work presented in this chapter) increases LAMP2 mRNA. Given that SIRT1 knockdown reduced LAMP2 mRNA and that our model proposes that NAD^+ reduces SIRT1, the prediction was that apigenin should also reduce LAMP2 mRNA. A possible explanation for the fact that this prediction was not met is that the response of SIRT1 to NAD^+ and the response of LAMP2 to SIRT1 could be time-dependent. Indeed, we show time dependent effects on SIRT1 in other experiments (Chapter 4) that PARP

inhibition by 3ABA over 6 h caused SIRT1 to increase but promoting PARP activity using TMZ over 4 h had no effect on SIRT1. These observations highlight the need for detailed data on these complex and multiple interactions of SIRT1 to understand its influences on cellular ageing.

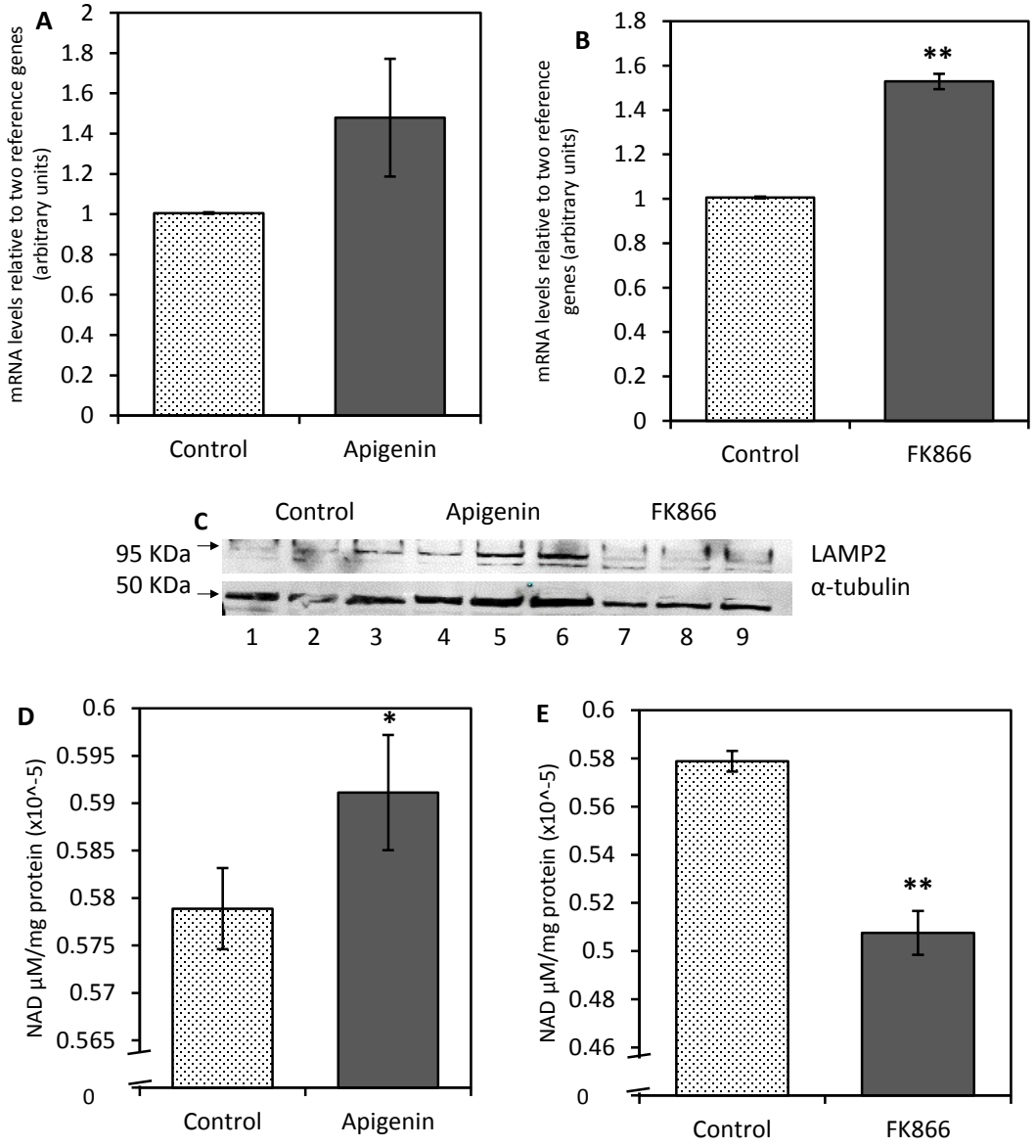


Figure 5.5.1. The effect of apigenin and FK866 treatment on NAD$^+$ and LAMP2 mRNA and protein in Caco-2 cells. Apigenin (25 µM), or FK866 (0.1 µM) was applied in the cell culture medium for 4 h. SIRT1 mRNA was measured by RT-qPCR relative to *GAPDH* and *TOP1*. **A-** Effect of apigenin on LAMP2 mRNA. **B-** Effect of FK866 on LAMP2 mRNA. **C-** Representative western blot signals for LAMP2 protein in response to apigenin and FK866, LAMP2 95 KDa and α-tubulin 50 KDa, lanes 1-3 control, lanes 4-6 apigenin and lanes 7-9 FK866 **. D-** Effect of Apigenin on NAD$^+$. **E-** Effect of FK866 on NAD$^+$. *p<0.05, **p<0.001 by Student's T-test. Data are shown as mean ± standard deviation (SD), n=2 (based on 6 data points comprising 2 biological replicates, each the mean of 3 wells measured separately).

5.6 Hypomethylation of DNA by 5-azacytidine treatment increased LAMP2 mRNA expression.

Thus far, we had accumulated a body of data all broadly consistent with a model whereby SIRT1 has a positive influence on the expression of LAMP2. A body of work conducted in our laboratory has uncovered multiple and widespread effects of SIRT1 on DNA methylation (Ions *et al.*, 2012). With respect to LAMP2 specifically, SIRT1 knockdown in Caco-2 cells and HuVECs caused an increase in DNA methylation at specific CpG sites in the *LAMP2* promoter (L. Wakeling and D. Ford personal communication). This effect of SIRT1 on DNA methylation of the *LAMP2* gene could be the mechanism through which expression is affected, but causality had not been demonstrated. Indeed it is always the case that showing a causal relationship between a change in DNA methylation and gene expression is challenging. Towards showing causality, here we determined if DNA methylation of *LAMP2* gene affects its expression. To achieve this aim, Caco-2 cells were treated with 5-azacytidine, which blocks DNA methyltranferases causing DNA hypomethylation. 5-azacytidine (10 μM) was applied in the cell culture medium for 24 h. Concentrations of around 1-20 μM 5-azacytidine have been commonly used in experiments reported in the literature to de-methylate DNA over 24-48 h (Choi *et al.*, 2004; Escher *et al.*, 2005; Kiziltepe *et al.*, 2007). For this study we used 10 μM over 24 h. mRNA was measured by RT-qPCR relative to *GAPDH* and *RN18S* reference genes.

Treatment of Caco-2 cells with 5-azacytidine caused LAMP2 mRNA to increase (Figure 5.6.1); showing that LAMP2 expression is affected by DNA methylation and hence further corroborating this as a likely mechanism through which SIRT1 affects LAMP2 expression.

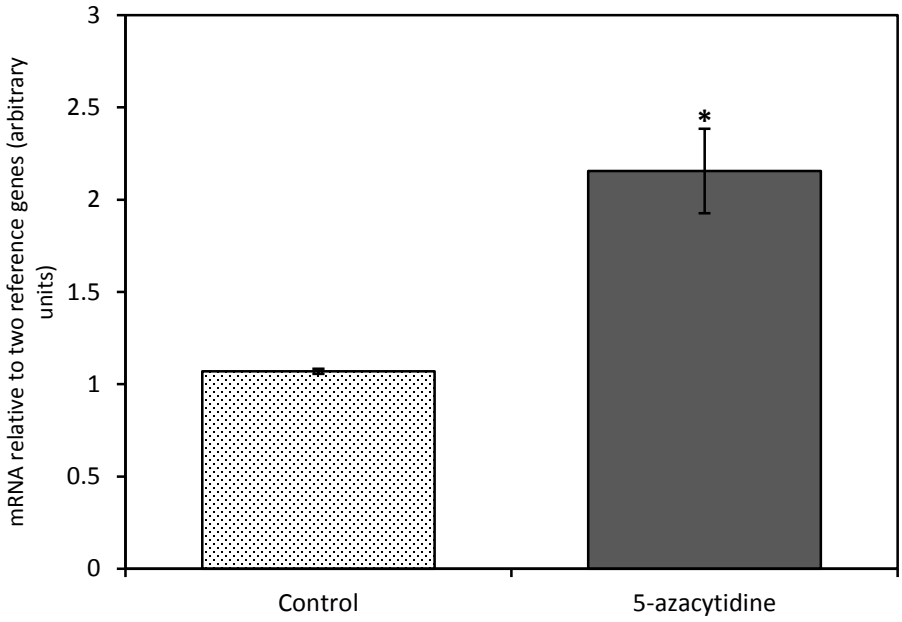


Figure 5.6.1. The effect of 5-azacytidine on LAMP2 mRNA in Caco-2 cells. 5-azacytidine (10 μM) was applied in the cell culture medium for 24 h. mRNA was measured by RT-qPCR relative to *GAPDH* and *TOP1* reference genes. *P<0.05 by Student's T-test. Data are shown as mean ± standard deviation (SD), n=2 (based on 6 data points comprising 2 biological replicates, each the mean of 3 wells measured separately).

5.7 The development of a functional assay to measure chaperone mediated autophagy.

All of the data presented in this chapter so far, which on the whole but with some caveats, support the view that SIRT1 and resveratrol promote the process of CMA, relies on indirect measures of activity of the CMA pathway (LAMP2 and HSC70 mRNA and protein). Thus, we aimed to develop a directly functional assay of CMA in Caco-2 cells to investigate these likely effects more reliably.

Our assay was based on a published procedure (Koga *et al.*, 2011), with minor adjustments. The assay measures emission from a fluorescent tag attached to the KFERQ motif. HSC70 binds to the KFERQ motif and chaperones protein bound to the fluorescent tag to lysosomes for degradation. The PS-CFP fluorescent tag used is photoconvertable, which means the fluorescence emission changes from cyan to green fluorescence upon LED array exposure. Following photoconversion and upon CMA activation the green fluorescent protein (GFP) will localise to the lysosomes and be degraded. Green fluorescence will thus decrease. If CMA is not activated the green fluorescent emission will remain relatively constant, as the protein remains in the cytosol rather than being chaperoned to lysosomes. The photoconvertable fluorescent tag allows the measurement of CMA at a snapshot in time. Fluorescent tagged protein translated post photoconversion will have cyan emission and will not increase background fluorescence that may hinder the measurement of GFP (Koga *et al.*, 2011). Figure 5.7.1 shows a schematic diagram outlining the assay principle.

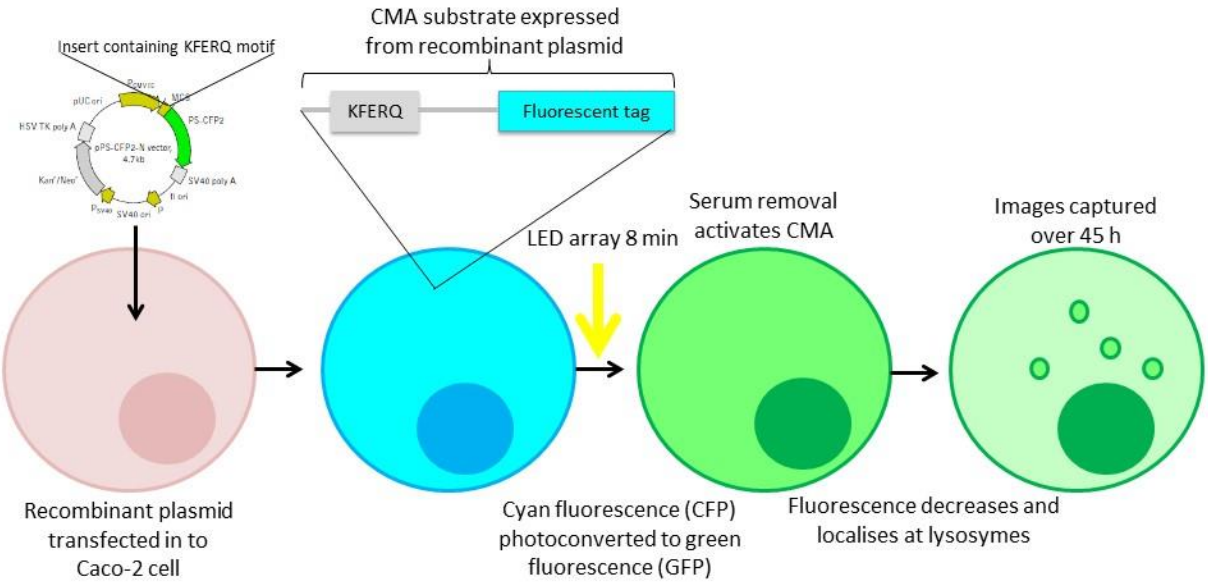


Figure 5.7.1. Schematic diagram to show the principle of the CMA assay. Caco-2 cells are transfected a plasmid expression construct for a photoconvertable CMA substrate (recognised by the sequence KFERQ). Irradiation for 8 min causes a colour change from CFP to GFP. Serum removal activates CMA causing the GFP to be chaperoned to lysosomes and degraded. Figure adapted from (Koga *et al.,* 2011).

To generate the plasmid construct for expression of the photoconvertable CMA substrate, a 287 bp region of the *bovine Ribonuclease A* gene containing the KFERQ motif was generated by PCR from *bovine* genomic DNA. PCR primers (Methods, Table 2.1) contained *HindIII* and *EcoR1* restriction sites. PCR was performed as described in Methods (2.5.3). The insert sequence, protein sequence and MCS of the destination vector pPS-CFP2-N (Evrogen) and vector map are shown in Appendix C and D. The PCR product was purified (QIAquick PCR Purification Kit, Qiagen) then restriction enzyme digestion was performed on both the PCR product and the pPS-CFP2-N vector using *EcoR1* and *HindIII* as described in Methods (2.5.1). Enzymes were then removed from the PRC product using (QIAquick PCR Purification Kit, Qiagen). The digested vector was also purified by agarose gel electrophoresis using (QIAquick Gel Extraction Kit, Qiagen) to purify the excised band as described in Methods (2.5.6). Ligation was carried out as described in Methods (2.5.2) then success of the ligation reaction was confirmed by running a sample of the product alongside a sample first digested with *EcoR1* and

HindIII (Appendix E). *E.coli* cells were transformed using the ligation mixture then plasmid was prepared from 6 individual colonies and sequenced to confirm presence and correct identity of the insert. To prepare cells for the CMA assay, Caco-2 cells were seeded at a density of 3.5 x 10^4 cells/ml in a black, glass bottomed, 24 well plate (Greiner Bioone) and incubated at 37 ˚C, 5 % CO_2 and 80 % humidity overnight. Cells were transfected with SIRT1 siRNA and incubated for a further 24 h. Cells were then transfected with the plasmid construct using Lipofectamine® 2000 (Life Technologies) following the manufacturer's instructions. The cells were incubated for a further 24 h before capturing images.

Images captured using the cyan and green channels selected at random were analysed using Volocity 3D image analysis software. Unexpectedly, there was no change in the intensity of cyan fluorescent protein (CFP) after photoconversion. However, the intensity of green fluorescent protein (GFP) increased significantly after photoconversion indicating successful photoconversion of the substrate. The fact that CFP appeared to remain the same was possibly due to continued expression of new protein during the photoconversion period (Figure 5.7.2).

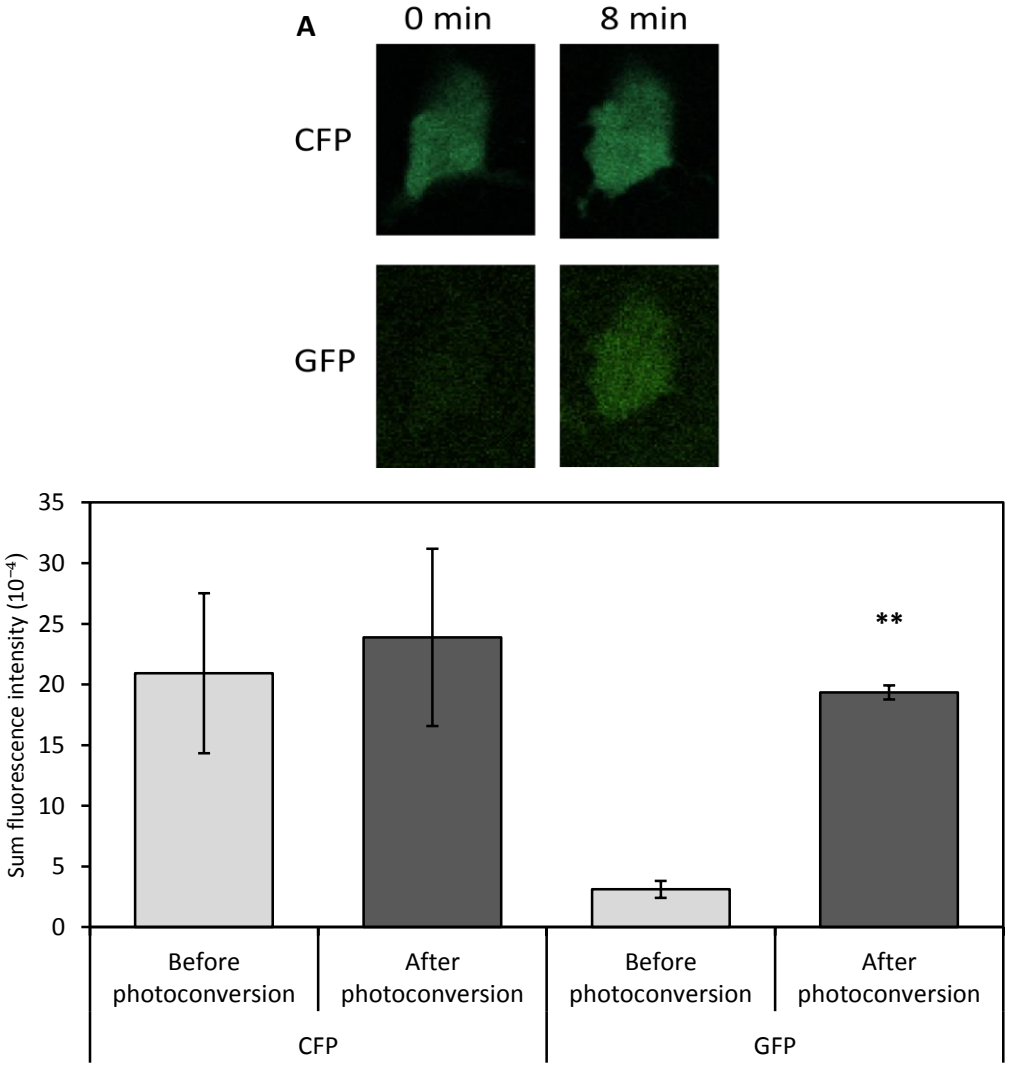


Figure 5.7.2. The effect of 8 minutes of photoconversion on CFP and GFP in Caco-2 cells expressing a photoconvertable CMA substrate. Photoconversion was for 8 m using an LED array (ND1-4.42 mW) on a Nikon A1R microscope. Nikon Elements viewer was used to view images and Volocity 3D image analysis software used to measure fluorescent intensity. **A-** Representative images of CFP and GFP in cells pre and post photoconversion **.B-**CFP and GFP intensities pre and post photoconversion**.** **p<0.001 by Student's T-test. Data are shown as mean ± standard deviation (SD), n=2.

CMA was stimulated by the removal of serum from the cell culture medium. Based on analysis of three images for control and stimulated conditions it appeared that serum removal caused GFP to decrease by approximately 30 % over 45 h. A similar decrease is reported for the original assay (Koga *et al.*, 2011). In contrast there was no apparent decrease in the GFP under control conditions. However, data for control and stimulated conditions did not differ significantly. Capture and analysis of a larger number of images is required to validate the assay (Figure 5.7.3).

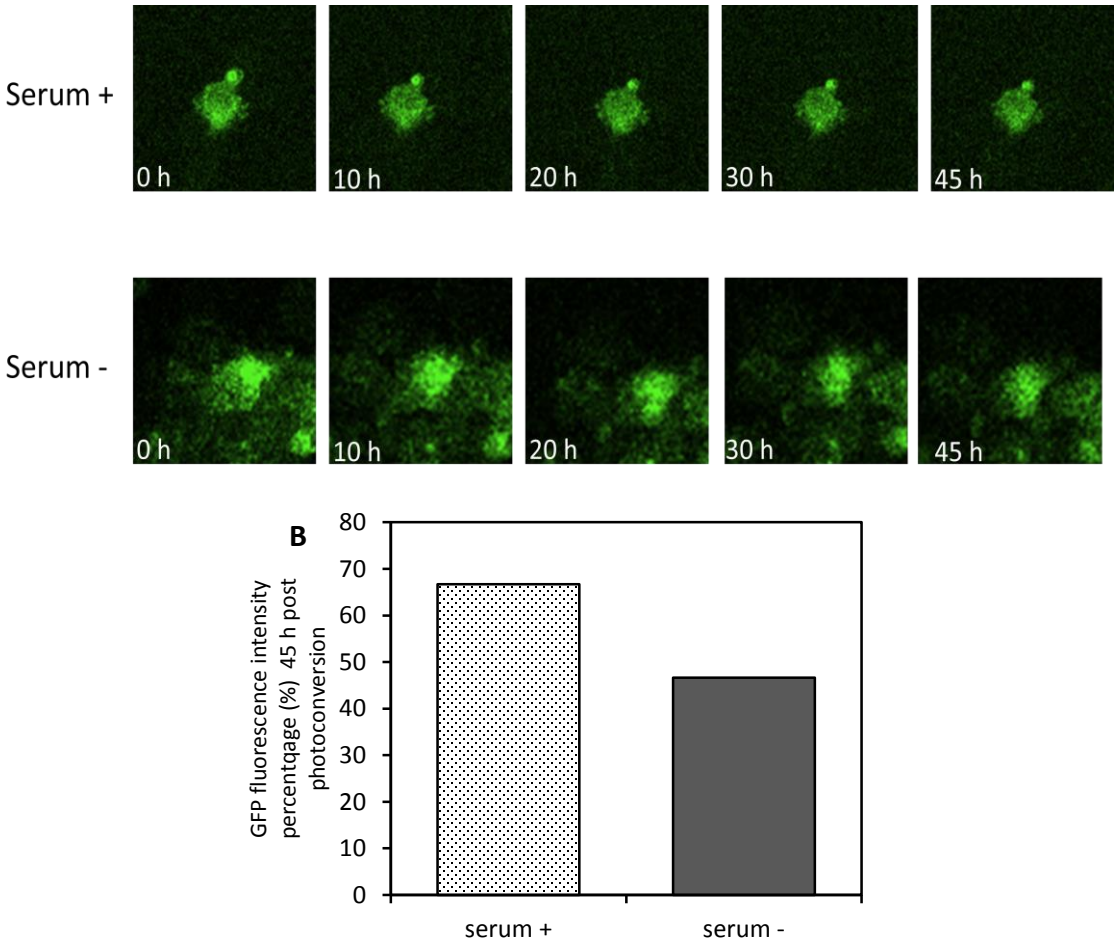


Figure 5.7.3. The effect of stimulating CMA by serum removal on the GFP signal in Caco-2 cells transfected to express the substrate for CMA. Foetal bovine serum was excluded from the cell culture medium for the serum free samples. Images were captured using a Nikon A1R microscope. **Serum +:** Representative images taken at 0, 10, 20, 30 and 45 h in serum positive medium. **Serum -:** Representative images taken at 0, 10, 20, 30 and 45 h in serum negative medium. **B:** GFP intensity at 45 h under conditions of serum+ and serum-. Photoconversion of CFP to GFP was by LED array (ND1-4.42 mW) on a Nikon A1R microscope. Nikon Elements viewer was used to view images and Volocity 3D image analysis software was used to measure fluorescent intensity. n=1.

To determine if knockdown of SIRT1 inhibited CMA, as we predicted, Caco-2 cells were transfected with siRNA targeted to SIRT1 then with the expression construct for the CMA substrate and images were captured after substrate photoconversion using the green channel over 45 h. Again, only very few useable images were captured and further optimisation and repetition is needed. However, those preliminary data indicated that CMA was active only under conditions where it was stimulated by serum removal but not when serum was removed from the cells transfected with the siRNA to reduce SIRT1 expression. Visual inspection of the typical images shown in Figure 5.7.4 reveals a clear reduction in GFP intensity over 45 h where cells were transfected with the control siRNA and serum was removed (middle panel) but no apparent change when cells were transfected with the control siRNA then serum retained (top panel) or when serum removed from cells transfected with SIRT1 siRNA (lower panel). Due to small sample number and variability in intensities image analysis revealed no statistical significance, however these first observations are consistent with SIRT1 being required for CMA to be stimulated.

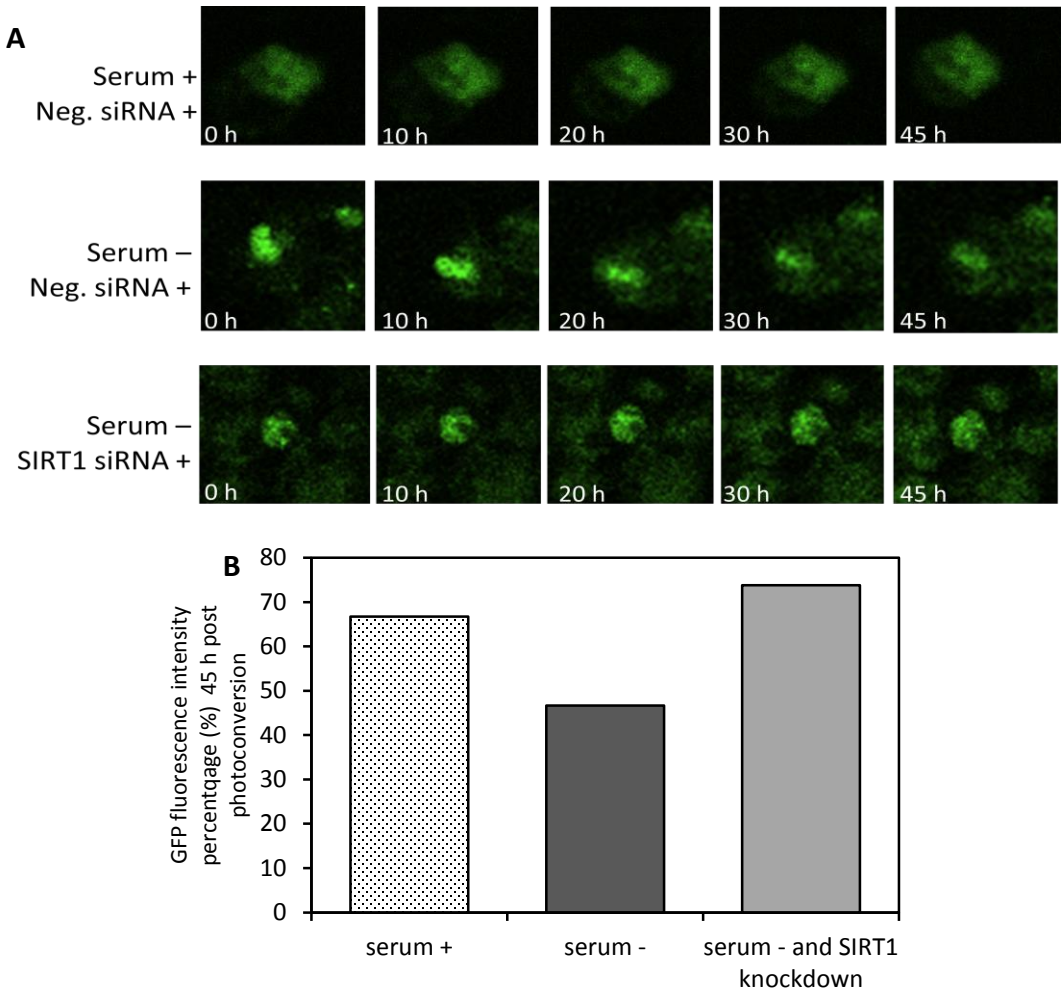


Figure 5.7.4. The effect of stimulating CMA by serum removal alongside SIRT1 knockdown on GFP intensity in Caco-2 cells. Photoconversion was done for 8 min to convert CFP to GFP using an LED array (ND1-4.42 mW) on a Nikon A1R microscope. Nikon Elements viewer was used to view images and Volocity 3D image analysis software was used to measure fluorescent intensity. **A-** Representative images from 0-45 h of GFP in cells that were retained in serum and transfected with control (Neg) siRNA, had serum removed and were transfected with control siRNA or that had serum removed and were transfected with SIRT1 siRNA**. B-** GFP intensities at 45 h for the conditions serum+, serum- and serum- plus SIRT1 siRNA n=1.

5.8 Discussion

Based on work carried out in the laboratory previously whereby microarray analysis revealed that SIRT1 knockdown in Caco-2 cells reduced LAMP2 expression (Dr. L. Wakeling personal communications), an aim of the work presented in this chapter was to explore the possibility that SIRT1 and/or resveratrol may have an influence on components of the CMA pathway as indicated by effects on the expression of LAMP2 and also HSC70, a second component of the pathway. Since we had shown in the current study that manipulation of NAD^+ affected SIRT1 expression and published data showed that resveratrol affected NAD^+ (Park *et al.*, 2012), we also sought to measure how manipulation of NAD^+ affected expression of LAMP2 and HSC70. A further aim was to develop a functional assay to measure CMA activity.

We first determined if a reduction in SIRT1 using siRNA or pharmaceutical inhibition using EX-527 affected LAMP2 and HSC70 expression in Caco-2 cells. Reducing SIRT1 using siRNA decreased LAMP2 mRNAs after 72 h in Caco-2 cells and when SIRT1 was inhibited pharmaceutically LAMP2 mRNA decreased at 24 h. SIRT1 manipulation had no significant effect on HSC70 mRNA. A limitation with respect to comparing directly the effect of SIRT1 knockdown by siRNA compared with its pharmaceutical inhibition on these mRNAs is that the response to pharmacological inhibition was very time-dependent, and it is not possible to relate directly the single time point at which the effect of the siRNA was measured (72 h after transfection) with the time course of pharmacological inhibition. This apparent transient effect of SIRT1 on these LAMP2 further highlights the need for high-resolution detailed data collected at multiple time points ultimately to populate a system-level mathematical model to understand these complex interactions. Moreover, it is essential to measure in parallel with mRNAs the corresponding proteins. Here we were able to obtain only poor quality Western blots to measure effects on LAMP2 and HSC70 proteins, which were inconclusive.

Given we had shown that resveratrol increases SIRT1 expression (Chapter 3) and, here, that SIRT1 affects LAMP2 mRNA, we proposed resveratrol would affect in particular LAMP2 expression. We also measured HSC70 expression in response to resveratrol alongside LAMP2 in Caco-2 cells. Resveratrol did not significantly affect HSC70 or LAMP2 mRNA and had no measurable effect on HSC70 protein. However, resveratrol

appeared to decrease LAMP2 protein but further observations are required to confirm this effect statistically. The anticipated effects of resveratrol on LAMP2 mRNA are concordant with the model whereby resveratrol increases SIRT1 expression which in turn activates CMA. For LAMP2 protein, the data on visual inspection appear convincing (although further repeats are necessary), we propose that actions of resveratrol additional to the effect on SIRT1 expression may underlie this response. Given that stimulation of CMA is a likely beneficial action of SIRT1 this counteracting effect of resveratrol may be a reason to seek alternative activators of SIRT1 as imitators of the response to dietary restriction to develop as dietary supplements.

As SIRT1 was affected by NAD^+ manipulation in Caco-2 cells (Chapter 3) the next logical step was to manipulate NAD^+ and measure LAMP2 mRNA and protein. Apigenin was used to increase NAD^+ and FK866 was used to reduce NAD^+ in Caco-2 cells. FK866 increased LAMP2 mRNA but apigenin had no effect on LAMP2 mRNA or protein (however the protein measurements were again of poor quality). However, the response to apigenin is inconsistent with the earlier observation that apigenin reduced SIRT1 mRNA and protein, which we would predict to reduce LAMP2 expression. As already discussed one explanation could be that the response is time-dependent. Therefore, future work should include measurement of these responses at multiple time points.

Although our work has shown repeatedly and reproducibly that SIRT1 increases LAMP2 mRNA the mechanism is not known. We proposed here that altered DNA methylation of the *LAMP2* gene by SIRT1 may be one possibility. This hypothesis arose from the earlier observation made in the laboratory whereby SIRT1 knockdown increased DNA methylation at specific CpG sites in the *LAMP2* promoter in Caco-2 cells and HuVECs (Dr. L. Wakeling personal communications). We predicted that decreased promoter DNA methylation would increase LAMP2 expression because DNA methylation would negatively affect the binding of transcription factors to the *LAMP2* gene. To determine if in principle *LAMP2* gene expression is repressed by DNA methylation 5-azacytidine was used to hypomethylate the genome in Caco-2 cells and then LAMP2 mRNA was measured. LAMP2 mRNA increased significantly following DNA demethylation, showing that LAMP2 expression is affected by DNA methylation; hence

this could be a mechanism that underlies the effect of SIRT1 on LAMP2 expression. Future work may include measuring HSC70 expression following DNA demethylation to determine if *HSC70* is also affected by DNA methylation. Given we have shown that SIRT1 has effects on DNA methylation across the genome this is an attractive mechanism through which LAMP2 and HSC70 could respond to SIRT1. Measurement of DNA methylation at the *HSC70* promoter following SIRT1 knockdown could be used to test this theory.

Measurement of LAMP2 and HSC70 provide only indirect indications of the activity of the CMA pathway. As discussed, this may account to some degree for discordance between these two measurements leading to difficulties concerning data interpretation. A direct functional assay would give a more robust measurement of the effects of SIRT1 and resveratrol on CMA. Thus, we began to optimise a published assay (Koga *et al.*, 2011) for use in our experimental model. The assay is based on expression of CFP expressed with a CMA substrate tag (KFERQ) that is photoconverted to GFP to track degradation of protein by CMA over time. The photoconversion step means that newly synthesised fluorescent protein is not "seen", allowing only the disappearance of the CMA substrate to be measured.

We observed a marked increase in GFP post photoconversion in Caco-2 cells transfected with a plasmid expression construct for this substrate, suggesting that the fluorescent tag was successfully converted from CFP to GFP. To test if the assay can be used to measure CMA in Caco-2 cells, CMA was activated by serum removal and GFP intensity was measured over 45 h. Compared to serum+ conditions there was an apparent decrease in the GFP signal after 45 h in the serum- conditions that could be seen by visual inspection of images, suggesting that CMA was active and could be measured by our assay. However, data acquisition was limited by time and resources and further experiments are required to show statistical significance. Preliminary data on the effects of SIRT1 knockdown by siRNA on CMA were also collected and appeared to show that SIRT1 knockdown prevented the reduction in GFP intensity observed over 45 h induced by the removal of serum, indicating that CMA became inactive under these conditions and hence suggesting that SIRT1 plays a role in stimulating CMA. Again

repetition of the experiment is necessary since data were very limited and did not differ significantly.

The CMA assay presented here will act as an experimental basis for future work to test if SIRT1 and/or resveratrol can affect CMA. As developed thus far, the assay is very time consuming, inflexible and (because number of images captured is limited by cell transfection efficiency) generated very limited data. It would not be practical to collect data on effects of SIRT1 inhibition/resveratrol exposure or other test treatments at different time points, for example. Thus, in its current form, the assay is only useful as a complementary approach to measuring LAMP2 and HSC70. A stable transfection of the recombinant plasmid in to Caco-2 cells would increase the number of images that can be captured in one experiment. Secondly, a concurrent stable knockdown of SIRT1 could be achieved in cell lines by genome editing using the CRISPR/CAS9 system (Roy *et al.*, 2015) and would improve assay utility.

To conclude, the work presented in this chapter has shown that resveratrol and SIRT1 may have a positive effect on CMA. Confirmation of this finding requires the collection and analysis of further measurements of LAMP2 and HSC70 expression over a range of timed exposures to resveratrol, NAD manipulation and SIRT1 manipulation and also direct measurement of CMA using a refined version of the assay developed. There is good evidence that SIRT1 affects LAMP2 expression through changes in DNA methylation.

6 Chapter 6. An investigation into the potential mechanisms through which SIRT1 has effects on DNA methylation that are clustered at the gene targets of the polycomb repressive complexes.

6.1 Introduction

It has become apparent in recent years that stem cells play a major role in ageing, which has led to 'the stem cell theory of ageing'. The theory suggests that ageing happens due to an increase in failed differentiated stem cells and/or the depletion of stem cells. Stem cell differentiation requires that particular genes are silenced through chromatin modifications that result from the action of polycomb group proteins (Orlando, 2003; Kirmizis *et al.*, 2004). Failed silencing due to changes in chromatin can lead to abnormal adult stem cell behaviour, which affects tissue regeneration and renewal (Brack and Rando, 2007; Bork *et al.*, 2010; Beerman *et al.*, 2013). However, not all tissues rely on adult stem cells for renewal but are affected by ageing; thus 'the stem cell theory of ageing' cannot be the sole cause of ageing. Nonetheless it has a likely place in the ageing process (Smith and Daniel, 2012).

Previously in the laboratory, SIRT1 was shown to affect DNA methylation at specific CpG sites in several genes that also showed altered DNA methylation patterns in ageing and were affected by DR (Ions *et al.*, 2012). A genome-wide analysis of effects of SIRT1 on DNA methylation had been carried out subsequently by SIRT1 over expression and knockdown in HuVECs and Caco-2 cells. Of the genes that had either an increase or decrease in DNA methylation in response to SIRT1 manipulation, there was a statistical over representation of polycomb group protein target genes (PCGTs) (genes which are targeted for epigenetic changes by polycomb group proteins) (Wakeling *et al.*, 2015). Hypermethylation at PCGTs has also been shown to increase with age in humans and mice (Maegawa *et al.*, 2010; Teschendorff *et al.*, 2010). We hypothesise here that SIRT1 can affect DNA methylation of PCGTs through polycomb group proteins, which may impact on ageing. This idea is based on knowledge that the polycomb proteins themselves can effect epigenetic changes at their target loci.

Two polycomb group protein complexes have been identified, polycomb repressive complex 1(PRC1) and polycomb repressive complex 2 (PRC2). PRC2 and PRC1 are each

made up of core proteins: EED, EZH2, SUZ12 and RbAP48, and: RNF2, BMI1 and PHC1, respectively (Vire *et al.*, 2006; Margueron and Reinberg, 2011). PRC1 is targeted to PCGTs by KDM2B, which recognises non-methylated DNA in CpG islands (Farcas *et al.*, 2012). PRC1 has a CBX domain that recognises and binds to methylation marks on histone 3 lysine 27. PRC1 then monoubiquitinates histone 2A resulting in compaction of the chromatin. PRC2 also contributes to chromatin compaction by recruiting histone methyletransferase1 to methylate histone 3 lysine 27.

The work presented in this chapter addressed the hypothesis that SIRT1 causes DNA methylation at PCGTs through PRCs, by determining if SIRT1 knockdown by siRNA affected expression (mRNAs) of polycomb proteins. We also measured if SIRT1 knockdown affected DNMT1 and DNMT3b mRNAs, since an effect on DNMT1 or DNMT3b expression and hence activity is a second plausible mechanism through which DNA methylation could be affected. However, should an effect be shown further work would be necessary to explore how such a non-specific action could target preferentially DNA methylation at PCGTs. Finally, we determined if SIRT1 forms a protein-protein complex with EZH2, the best candidate of the polycomb proteins for mediating any action of SIRT1 on DNA methylation of PCGTs as EZH2 associates with DNA methyltransferases and has been shown to form an interaction with SIRT1 in HeLa cells (Kuzmichev *et al.*, 2005).

6.2 SIRT1 knockdown had no effect on the mRNA of polycomb repressive complex proteins, DNA methyltransferases 1 and 3b or lysine (K)-specific demethylase 2B in human Caco-2 cells or HuVECs.

To test our hypothesis that SIRT1 affects DNA methylation at PCGTs through PRCs, we determined if SIRT1 affected the mRNA of individual components of PRC1 and PRC2 using two different siRNAs to reduce SIRT1 expression in HuVECs and Caco-2 cells. The components of PRC2 measured were: EED, EZH2, SUZ12 and RbAP48. The components of PRC1 measured were: BMI1, PHC1 and RNF2. The PRC1 recruiting protein KDM2B mRNA and the mRNAs of two DNA methyltransferase DNMT1 and DNMT3b were also measured. mRNA was measured by RT-qPCR relative to two reference genes *GAPDH* and *TOP1*.

SIRT1 knockdown by siRNA in Caco-2 cells and HuVECs did not significantly affect any of the mRNAs measured (Figure 6.2.1). In some instances we observed a difference in mRNA as a result of SIRT1 knockdown using only one siRNA. Specifically siRNA1 caused a statistically significant increase in RNF2 mRNA and siRNA2 caused a statistically significant increase in SUZ12 and PHC1 mRNAs. Since the second siRNA induced none of these effects they must be considered as "false results" and not true effects of SIRT1 knockdown. A possible explanation is that they arose from "off target" actions of the siRNA; i.e. a reduction in expression of other genes caused by siRNA that in turn influenced the affected mRNAs.

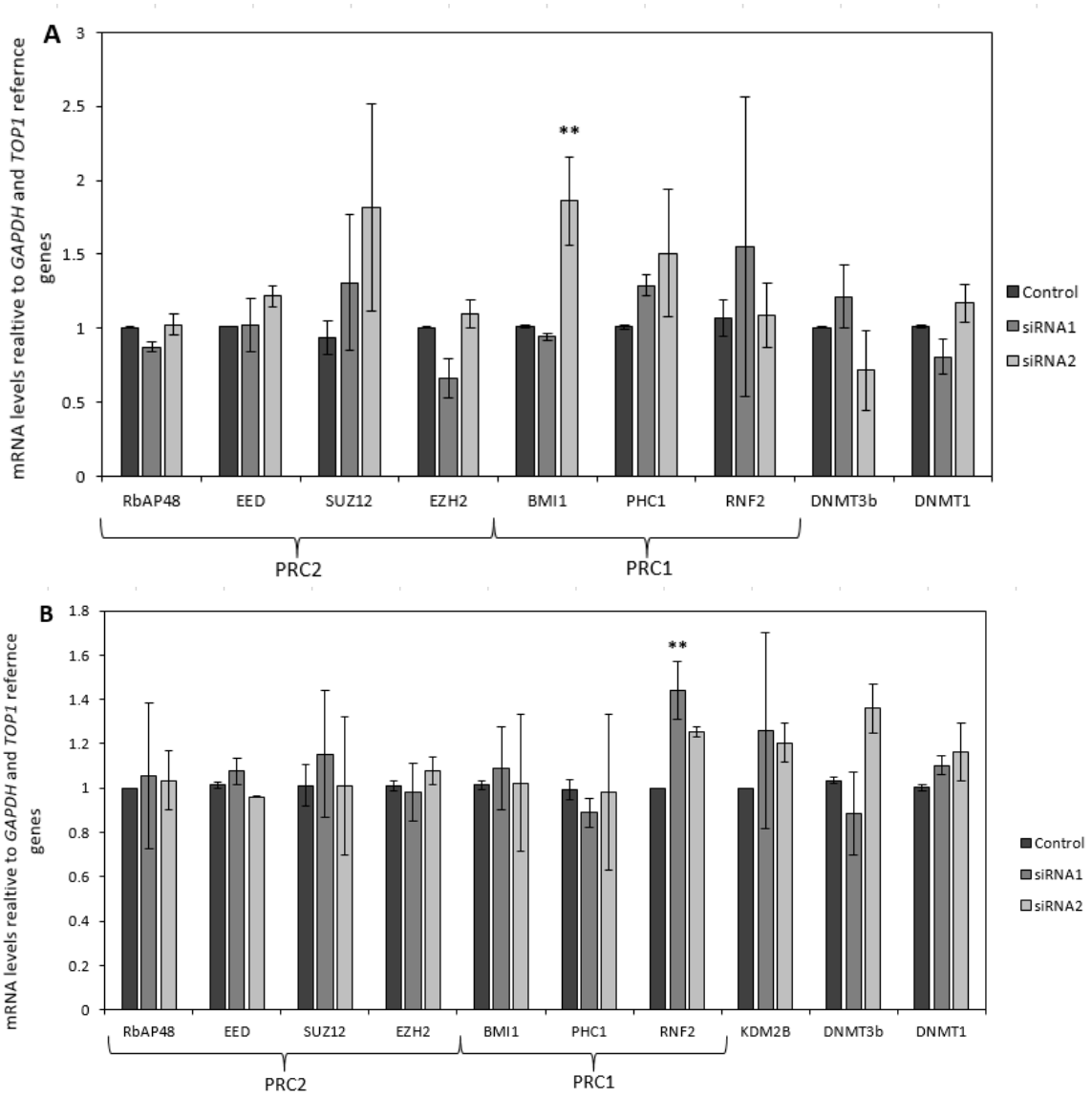


Figure 6.2.1. The effect of SIRT1 knockdown on PRC1, PRC2, DNMT1 and DNMT3b mRNA in HuVEC and Caco-2 cells and KDM2B mRNA in Caco-2 cells. A-SIRT1 knockdown by two siRNAs in HuVECs **B-**SIRT1 knockdown by two siRNAs in Caco-2 cells**. PRC2:** RbAP48, EED, SUZ12 and EZH2. **PRC1:** BMI1, PHC1 and RNF2. SIRT1 was knockdown was over 72 h using two different siRNAs separately (siRNA1 and 2). Results are relative to two reference genes *GAPDH* and *TOP1*. **p<0.005 by ANOVA and Tukey's post hoc statistical test. Data are shown as ± standard deviation (SD), n=2-6.

6.3 SIRT1 and EZH2 do not appear to form a direct inter-molecular association.

Having found no evidence that SIRT1 affects expression of components of the PRCs we next sought to determine if SIRT1 associates directly with EZH2 in a protein-protein complex that could modify EZH2 activity and hence DNA methylation at PCGTs. SIRT1 has been shown to associate with EZH2 in a PRC4 complex in HeLa cells overexpressing EZH2 (Kuzmichev *et al.*, 2005). Initially we measured EZH2 protein after SIRT1 knockdown by siRNA in Caco-2 cells. EZH2 protein was measured by Western blot relative to the α-tubulin loading control.

SIRT1 knockdown in Caco-2 cells had no effect on EZH2 protein levels (Figure 6.3.1 A and B)

To determine if SIRT1 can bind to EZH2, SIRT1 and EZH2 were immunoprecipitated separately from total lysate from HuVECs and Caco-2 cells. Input and immunoprecipitated protein was then resolved by SDS-PAGE, transferred to PVDF membrane then probed with anti-EZH2 or anti-SIRT1 antibody. Both SIRT1 and EZH2 were self-enriched in the fraction achieved after immunoprecipitation as shown by a band intensity increase over input on the Western blot but neither antibody resulted in cross-enrichment (Figure 6.3.1 C). Thus we found no evidence that SIRT1 and EZH2 associate directly under our cell culture conditions.

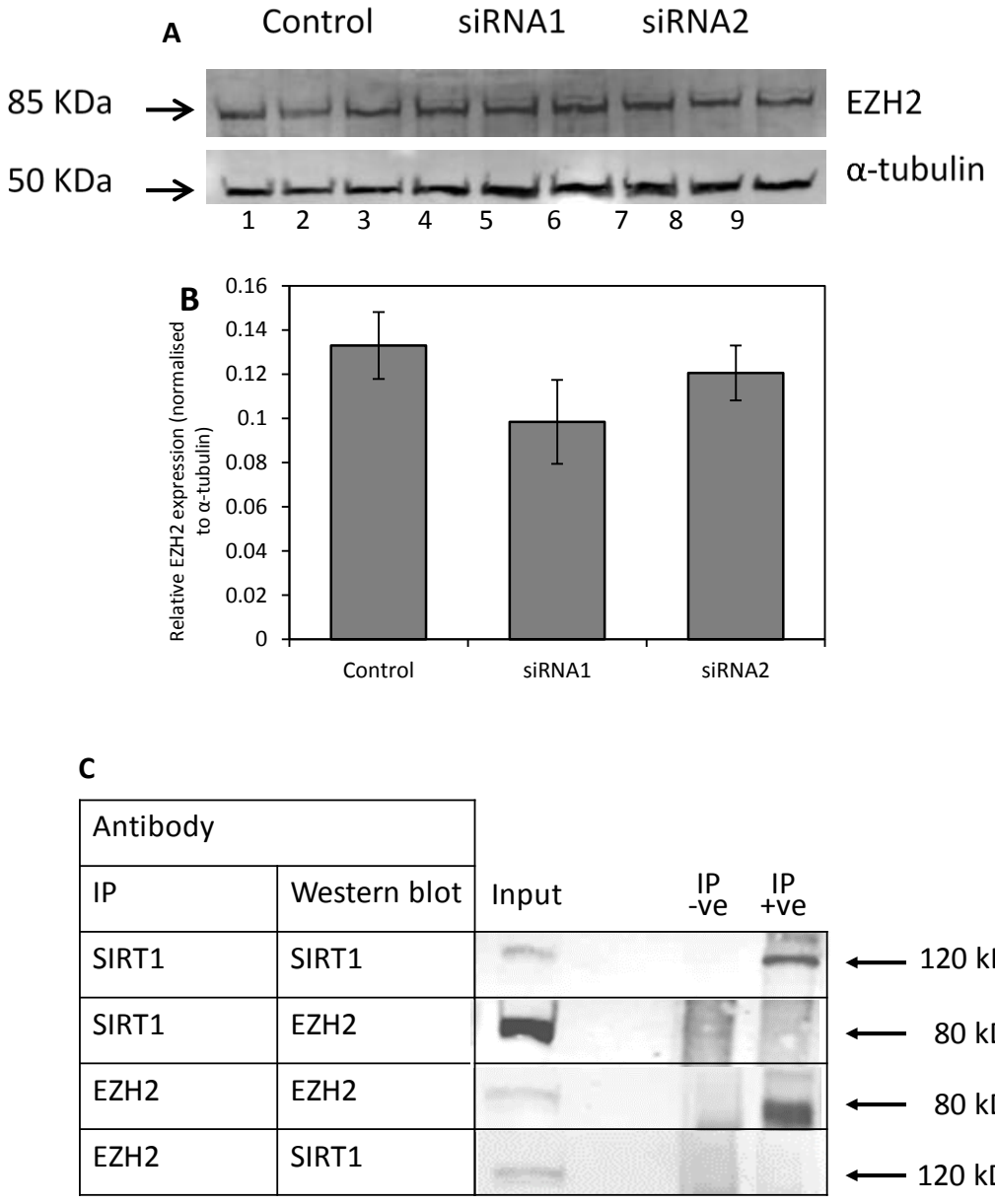


Figure 6.3.1. The effect of SIRT1 knockdown by siRNA on EZH2 protein, and immunoprecipitation of SIRT1 and EZH2 to determine if either binds to the other in HuVEC and Caco-2 cells. SIRT1 was reduced by two siRNAs separately (siRNA 1 and 2)**. A-.**Representative western blot raw data in response to SIRT1 knockdown by siRNA in Caco-2 cells. **B-** The densitometry measurement of the western blot raw data for EZH2 protein in response to SIRT1 knockdown in Caco-2 cells**. C-** Western blot raw data following on from immunoprecipitation of SIRT1 or EZH2 protein in Caco-2 cells or HuVECs**.** SIRT1 was 120 KDa, α-tubulin 50 KDa and EZH2 80 KDa. 'Input'-whole protein lysate run alongside immunoprecipitated samples and probed for the corresponding immunoprecipitated protein, 'IP–ve'- Immunoprecipitate probed for the other protein (for example, SIRT1 protein immunoprecipitate was probed with anti-EZH2 antibody). 'IP+ve'- Immunoprecipitate probed for the same protein as a control (for example, for example, SIRT1 protein immunoprecipitate was probed with anti-SIRT1 antibody) ANOVA and Tukey's post hoc statistical test were used for statistical analysis. Data is shown ± standard deviation (SD), B n=2 (based on 6 data points comprising 2 biological replicates, each the mean of 3 wells measured separately), C n=3.

6.4 Discussion

SIRT1 knockdown has been shown to change DNA methylation at PCGTs (Wakeling *et al.*, 2015), which are DNA methylation sites targeted by PRCs for gene silencing by epigenetic mechanisms including histone methylation and ubiquitination (Margueron and Reinberg, 2011). We proposed that the action of SIRT1 to change DNA methylation at PCGTs was in some way mediated through PRCs to affect DNA methylation at these sites. Specifically we proposed that this action of SIRT1 could be through affecting the expression of components of the PRCs (i.e. the individual polycomb proteins) or KDM2B. Although not a PRC component KDM2B has been shown to recruit PCR1 to CpG islands in mouse embryonic stem cells (Farcas *et al.*, 2012). Alternatively, we postulated that SIRT1 may act via components of the PRCs to alter DNA methylation at PCGTs through forming intermolecular associations with specific polycomb proteins, thereby affecting the way in which the PRC affects epigenetic modification at these target sites. A third idea was that SIRT1 may influence the expression of DNMTs. In this case, a mechanism that results in the genome wide actions of DNMTs being focused at PCGTs would also have to come into play to explain the observations. To explore these ideas the expression of SIRT1 was reduced by siRNA in Caco-2 cells and HuVECs. There were no significant changes in mRNA corresponding to any of the PRC1 or PRC2 components or in DNMT1, DNMT3b or KDM2B mRNAs. Any significant changes observed were not replicated by both siRNA1 and siRNA2 and were therefore put down to off target effects of the siRNAs.

EZH2, a component of PRC2 that methylates histone 3, is a likely candidate for affecting DNA methylation. A PRC4 complex has been described, which was observed when EZH2 was over expressed in HeLa cells and consists of EZH2, SIRT1 and isoform2 EED (Kuzmichev *et al.*, 2005). We therefore reasoned that of the multiple PRC components EZH2 was the most likely binding partner with SIRT1 to explain our observations. Before determining if EZH2 and SIRT1 appeared to form a direct intermolecular association we first confirmed that, consistent with SIRT1 having no effect on EZH2 mRNA. SIRT1 knockdown did not affect EZH2 protein expression. We then determined if SIRT1 associated directly with EZH2 protein in Caco-2 cells and HuVECs by performing immunoprecipitation on SIRT1 and EZH2 then using Western blot to probe

for the other protein. SIRT1 and EZH2 proteins were not co-immunoprecipitated, suggesting they do not bind one another in Caco-2 cells or HuVECs. Possible reasons for the apparent discordance with the published findings (Kuzmichev *et al.*, 2005) are that the association may be cell line specific or that higher levels of EZH2 (as EZH2 was overexpressed in HeLa cells) may be required for association with SIRT1.

The data presented in this chapter revealed that SIRT1 does not affect the expression of components of PRCs, KDM2B or DNMTs at the mRNA level. Further work is needed to measure the effect of SIRT1 knockdown on EED, SUZ12 and RbAP48, BMI1, PHC1, RNF2, KDM2B, DNMT1 and DNMT3b protein levels. We reasoned that EZH2 was the most likely component of the PRCs to explain the targeted action of SIRT1 on DNA methylation at PCGTs but we found no evidence for a direct association in Caco-2 cells and HuVECs. These data only begin to explore the many possible mechanisms through which SIRT1 may have effects on DNA methylation that are targeted to PCGTs and extensive further experimentation is required to shed light on the underlying mechanism.

7 Discussion

The overall aim of the work presented in this thesis was to explore interactions of SIRT1 with other cellular functions pertinent to ageing, specifically PARPs and CMA, towards developing a system-level model of which testing and refinement may ultimately reveal points of intervention to slow ageing or maintain better health during ageing. Each of the four chapters of results addressed a specific hypothesis.

- Resveratrol increases SIRT1 expression through increasing NAD^+.
- Interactions between SIRT1 and PARP are driven by competition for the same intracellular NAD^+ pool.
- SIRT1 and resveratrol increase expression of components of CMA and CMA activity. We also proposed that effects on DNA methylation of the *LAMP2* gene is one mechanism through which this effect is mediated.
- SIRT1 alters DNA methylation at polycomb gene targets through polycomb repressive complexes.

7.1 Hypothesis 1

As discussed in Chapter 1, SIRT1 plays a central role in cell metabolism and is believed to mediate some of the effects of DR and resveratrol on ageing. Current evidence sways us towards a view that resveratrol activates SIRT1 (Basil P. Hubbard *et al.*, 2013). Here we have shown that resveratrol increases SIRT1 mRNA, in agreement with previous studies (Bai *et al.*, 2008; Morita *et al.*, 2012), and increases *SIRT1* promoter activity, revealing that an increase in transcription accounts for or at least contributes to this effect. These effects of resveratrol on SIRT1 would augments each other, which given well-evidenced actions of SIRT1 on aspects of cellular function that counteract features of ageing, such as activation of FOXO1, increased catalase expression and reduced oxidative stress (Alcendor *et al.*, 2007) support a view that dietary resveratrol may be an efficacious intervention to increase healthspan. The efficacy of resveratrol to extend lifespan has been shown robustly in model organisms (Howitz *et al.*, 2003; Wood *et al.*, 2004) but it may be premature, without further human intervention studies, to recommend dietary resveratrol as a supplement to human diets.

Alongside intervention studies, further research, such as presented in this thesis, on the interactions between resveratrol and SIRT1 and other cellular components at the molecular level may be illuminating in the same context. It has been shown that resveratrol can increase NAD^+ in mouse C2C12 myotubes through activation of AMPK (Park *et al.*, 2012). Thus we proposed that an increase in NAD^+ may be a mechanism through which resveratrol increases expression of SIRT1. Thus, we investigated if NAD^+ manipulation could alter SIRT1 expression. The most striking observation was that an increase in NAD^+ induced using apigenin resulted in a 90 % reduction in SIRT1 mRNA and a reduction in SIRT1 protein levels a finding discordant with this hypothesis. Assuming that this measured increase in NAD^+ drives the reduction in SIRT1 expression (which is unproven; the effect of apigenin on SIRT1 maybe through other unknown actions) then uncovering the mechanism requires further work. A plausible scenario is that increased activity of SIRT1 that results from the increased availability of NAD^+ leads to deacetylation of a transcription factor that controls transcription of the *SIRT1* gene. The regulation of SIRT1 by PPAR-γ provides a precedent for such an auto-regulatory feedback loop. PPAR-γ can inhibit SIRT1 transcription (Han *et al.*, 2010) and is in turn deacetylated and hence activated by SIRT1 (Qiang *et al.*, 2012).

Since we did not confirm that resveratrol increased NAD^+ in our Caco-2 cell model we proposed that the effect to increase SIRT1 expression maybe due to a reduction in NAD^+, which attenuates the repressive action of NAD^+ on SIRT1 expression indicated by the action of apigenin. We thus measured NAD^+ in response to treating Caco-2 cells with resveratrol. Although, the data appeared to show an increase in NAD^+ in response to resveratrol (with a p value of 0.08), the data were inconclusive and further experimental repeats are necessary to determine if data differ with statistical significance. Assuming that resveratrol increases NAD^+, which agrees with published data (Park *et al.*, 2012), it is possible that this response may underlie an observed increase in NAMPT and SIRT1 activity (both dependent on NAD^+) in human primary hepatocytes induced by resveratrol (Schuster *et al.*, 2014). Nampt was also shown to increase Sirt1 expression and activity in mouse fibroblasts (Revollo *et al.*, 2004). A potential mechanism for this increase in SIRT1 expression in response to NAMPT is the FOXO1/SIRT1 positive feedback loop. NAMPT reduces the SIRT1 inhibitor NAM (in the

NAD^+ salvage pathway) allowing SIRT1 activity to increase, active SIRT1 deacetylates FOXO1, which promotes FOXO1 activity and thus increasing SIRT1 transcription by FOXO1 (Brunet *et al.*, 2004; Xiong *et al.*, 2011).

7.2 Hypothesis 2

Predictions of the hypothesis is that SIRT1 and PARP compete for NAD^+ in the cell are that activating one or other enzyme would reduce the activity of the other whereas inhibiting the activity of one or other enzyme would increase the activity of the other. The hypothesis also predicts that their effects would be magnified if NAD^+ was reduced in the cell but attenuated when NAD^+ is abundant. We tested these predictions as far as we were able by manipulating one of the three variables in the system and measuring the effects on the other two. Based on the observation discussed above, that NAD^+ appears to reduce SIRT1 expression, the system is predicted to be far more complex, however. Also these experiments were limited by the fact that we were unable to measure SIRT1 activity. However, we measured SIRT1 expression and observed effects on this variable. As expected, the observation revealed that interactions between SIRT1, PARP and NAD^+ are complex.

1. There appeared to be a reciprocal relationship between Parp activity and Sirt1 expression in mouse livers, with older mice having higher Sirt1 and lower Parp activity. However, this observation was made in a limited number of samples. Future work should include analysis of Sirt1protein and Parp activity in a larger sample of ageing mice to draw out any common relationships and allow statistical analysis.
2. We found no difference in Sirt1 expression in the intestine of Parp1 -/- in mice compared with controls. However, only a limited number of samples were available, hence at this point we do not draw any definitive conclusions from these data.
3. Pharmacological inhibition of PARP in Caco-2 cells increased SIRT1 mRNA at 6 h but pharmacological induction of PARP through initiation of the DNA damage response did not affect SIRT1 mRNA at 4 h.
4. SIRT1 knockdown by siRNA appeared to be increasing PARP activity.

5. An increase in NAD^+ achieved using apigenin reduced SIRT1 mRNA but had no effect on PARP activity.
6. Reduction of SIRT1 by siRNA and/or PARP inhibition did not affect NAD^+.

These findings add to other published studies that show an extensive and complex network of interactions between SIRT1 and PARP that appear relevant in the overall context of ageing and generally support the view that the activities of the two enzymes have a reciprocal relationship. For example, Sirt1 -/- mice had increased acetylated and thus activated Parp1, suggesting Sirt1 deacetylates and deactivate Parp1 (Rajamohan *et al.*, 2009a). As further examples, Parp2 -/- mice had increased Sirt1 activity (Bai *et al.*, 2011), and PARP1 appeared to increase SIRT1 transcription indirectly by promoting transcription of the SIRT1 transcription factor C-*MYC* (Simbulan-Rosenthal *et al.*, 2003). Our own data presented in this thesis reveal further interactions, we show in ageing mice that SIRT1 protein is high when PARP activity is low, and our preliminary data indicate that SIRT1 knockdown increases PARP activity and PARP inhibition increases SIRT1 mRNA.

The balance of reported effects of PARP and SIRT1 support the idea that increased activity of either enzyme is protective against effects of ageing, and thus that the consequent reduction in the actions of one enzyme that results from activating the other would limit efficacy to reduce features of ageing. However, this is not universally the case. For example, PARP1 over expression was associated with higher tumour grades in breast cancer (Rojo *et al.*, 2012) whereas Sirt1 activity was associated with lower Survivin expression resulting in increased apoptosis and thus reduced tumour progression in mice with Brca1 associated breast cancer (Wang *et al.*, 2008). Furthermore, PARP2 has been shown to inhibit TRF2 from binding to DNA in telomere T-loop formation (Dantzer *et al.*, 2004) while SIRT1 can increase Tert expression, telomerase activity and Ttp1 expression (Chen *et al.*, 2014). As a final example, PARP activity was higher in human centenarians compared to younger controls (Muiras *et al.*, 1998) but Sirt1 protein was found reduced with age in rat hippocampus neurons (Quintas *et al.*, 2012).

We hypothesised that the opposing relationship between SIRT1 and PARP is driven by competition for NAD^+. No previous studies had investigated SIRT1, PARP and NAD^+ together, however it proved challenging to measure changes in NAD^+, which is tightly regulated in the cell. However, apigenin, which only slightly increases NAD^+ availability, had a large impact on reducing SIRT1 mRNA and therefore it is plausible that small NAD^+ variations could have large implications.

Overall, the observations outlined show that a simple linear model cannot describe the co-dependency of the actions of SIRT1 and PARP and the influence thereon of NAD^+. Thus, the findings show that a system-level network is required to understand these interactions and to make further predictions. The data generated within the time constraints of the current project provide an important component of such a model but are currently inadequate to develop a robust mathematical model. However, tools are now in place to facilitate the generation of detailed data over suitable time courses and concentration ranges.

7.3 Hypothesis 3

SIRT1 appears to have a role in macroautophagy, as when SIRT1 was inhibited in THP-1 cells macroautophagy function was lost (Takeda-Watanabe *et al.*, 2012). Furthermore, there is also evidence that resveratrol can promote macroautophagy, since the resveratrol derivative trans-3,4-dimethoxystilbene inhibits mTOR, which itself inhibits autophagy (Zhang *et al.*, 2012). As discussed earlier a reduction in protein degradation can be detrimental and cause age-related diseases through mechanisms, such as contributing to amyloid-β plaques in Alzheimer's disease. Thus, if protein degradation was maintained at a sufficient level it may reduce the risk of age related diseases. Rapamycin, an mTOR inhibitor, has already been shown to reduce the build-up of amyloid-β_{42} protein and promote macroautophagy in brain tissue from PDAPP mice (Spilman *et al.*, 2010) and is thus a strong contender as a therapeutic therapy.

A second type of autophagy, CMA, also has strong links to ageing. The CMA receptor and regulator LAMP2 has been shown to be reduced in ageing rats (Cuervo and Dice, 2000b) and is likely to be a contributor to age-related diseases linked to protein build-up in

cells. However, CMA is difficult to measure and has not, until now, been the focus in autophagy studies.

Previous work in the laboratory showed that SIRT1 knockdown by siRNA reduced LAMP2 mRNA in Caco-2 cells (L. Wakeling and D. Ford, personal communication). We thus sought to investigate if SIRT1 and resveratrol can alter CMA activity. Prior to the development of a functional assay to measure CMA we measured the components of the CMA pathway LAMP2 and HSC70 under conditions where SIRT1 and resveratrol were manipulated in Caco-2 cells. SIRT1 inhibition by EX-527 decreased LAMP2 mRNA and resveratrol increased LAMP2 mRNA, in agreement with the hypothesis that resveratrol can increase SIRT1 expression which in turn increases CMA. Corresponding measurements of LAMP2 and HSC70 proteins by Western blotting were not entirely consistent with the mRNA data. However, data quality was compromised and we draw no firm conclusion at this stage. As we had shown that NAD^+ affected SIRT1 expression we manipulated NAD^+ and measured LAMP2 mRNA and protein. In agreement with the prediction that reducing NAD^+ would alleviate a repressive action on SIRT1 expression and hence activate CMA, we observed an increase in LAMP2 mRNA on treatment of Caco-2 cells with FK866, which we confirmed to be effectively increasing NAD^+. However, apigenin, which increased NAD^+ effectively, had no effect on LAMP2 mRNA, which was discordant with this overall scheme for the effect of SIRT1 on LAMP2 expression. A limitation of these data, however, are that they are constrained to being at a single time point. Moreover, given other data here discussed that show clearly a complex, non-linear effect of NAD^+, and systems (for example PARP) that draw on this cellular resource, this finding is not surprising and highlights further the need to acquire further data for input into a comprehensive system-level mathematical model.

Previous work in the laboratory suggested that SIRT1 may increase LAMP2 expression, through affecting DNA methylation of the *LAMP2* promoter. However, we were unable to attribute any causal relationship between the observation that SIRT1 knockdown in Caco-2 cells and HuVECs increased *LAMP2* promoter methylation and reduced LAMP2 mRNA. Thus, we aimed to determine proof of concept that methylation of the *LAMP2* promoter does affect expression of the gene. We did this by inducing global DNA Hypomethylation in Caco-2 cells using 5-azacytidine. In response, we observed an

increase in LAMP2 mRNA, which showed that expression of the gene is influenced by DNA methylation. These observations still fall short of showing a direct causal relationship, but add more support to the proposal that SIRT1 affects LAMP2 expression through an effect on DNA methylation of the gene promoter.

Measuring the expression of components of the CMA pathway is only a surrogate for CMA activity and may give misleading information on activity of the pathway per se. We thus set up and tested a published assay to measure CMA activity (Koga *et al.*, 2011). The assay is based on expression of a substrate for CMA with a fluorescent tag that undergoes photoconversion to allow measurement of CMA unimpeded by any background expression of new tagged substrate. Preliminary results indicated that the assay is useful and that knockdown of SIRT1 reduced CMA activity. However, use of cells transfected only transiently with the plasmid for expression of the tagged substrate meant that, due to relatively low transcription efficiency, only a small number of cells could be assayed by timed image capturing a single experiment. Improvement and optimisation of the assay for future use should include stable knockdown of SIRT1 and stable transfection of the recombinant plasmid for expression of the CMA substrate to enable capture of more images. Future work will also include measuring CMA in response to resveratrol and NAD^+ manipulation.

Along with the improvements mentioned above, this CMA assay will allow us to gather a larger body of data on the functional effects in the cell of SIRT1 and resveratrol. The preliminary data suggest that SIRT1 reduction may reduce CMA. A future experiment will be to induce SIRT1 activity (perhaps through the use of resveratrol) to see if this promotes CMA. The preservation of CMA could be used therapeutically to reduce protein build-up in age-related diseases, including Alzheimer's disease, Parkinson's disease and Huntington's disease. Furthermore, we have shown that *LAMP2* mRNA expression can be altered through DNA methylation. A second experiment would thus be to measure CMA following global DNA methylation. This would determine if CMA is also affected by DNA methylation changes. We believe DNA methylation changes found in ageing (Kwabi-Addo *et al.*, 2007; Teschendorff *et al.*, 2010; Lin *et al.*, 2012) may be a causal factor of multiple cellular function changes linked to ageing, such as a reduction in autophagy.

7.4 Hypothesis 4

The DNA methylation profile changes with ageing (Kwabi-Addo *et al.*, 2007; Christensen *et al.*, 2009; Javierre *et al.*, 2010; Koch *et al.*, 2011) and has been linked to specific age-related disorders. These include for example, prostate cancer where increased DNA methylation was observed in cancerous cells compared to normal prostate cells in prostate cancer related genes (RARbeta2 RASSF1A) (Kwabi-Addo *et al.*, 2007). Changes to DNA methylation which in theory may be caused by changes in cell function related to age could trigger changes in gene expression, resulting in age-related disease.

Stem cells are specifically affected in ageing through chromatin modifications, which hinder their ability to differentiate successfully and/or stay in a stem like state (Brack and Rando, 2007; Bork *et al.*, 2010; Beerman *et al.*, 2013). Genes involved in stem cell differentiation (PCGTs) are silenced through chromatin modifications, like DNA methylation, by PRCs (Orlando, 2003; Kirmizis *et al.*, 2004). It has also been shown that PCGT hypermethylation increases with age in mice and humans (Maegawa *et al.*, 2010; Teschendorff *et al.*, 2010). SIRT1 knockdown also caused changes in DNA methylation in Caco-2 cells and HuVECs that clustered to an extent greater than expected by chance at PCGTs (Wakeling *et al.*, 2015). To investigate if this change in DNA methylation was mediated through the PRCs, which invoke epigenetic modifications including DNA methylation at PCGTs, we began by reducing SIRT1 using siRNA to determine if this had an effect on any of the components of the PRCs and also KDM2B (a protein that recruits PRC1 to non-methylated CpGs) and the genome wide DNA methyltransferases (DNMT 1 and 3b), likely candidates for methylating CpG islands at PCGTs. SIRT1 reduction had no effect on the mRNAs of the components measured. We therefore concluded that the effect of SIRT1 on DNA methylation at PCGTs is unlikely to be through an effect of SIRT1 on expression of the PRC components. However, it remains possible that SIRT1 affects PRC component expression at the protein level. We measured EZH2 protein by Western blotting and found no effect of SIRT1 knockdown. EZH2 can associate with DNMTs and has been previously shown to interact with SIRT1 in PRC4 in HeLa cells (Kuzmichev *et al.*, 2005). Thus, of all the PRC components EZH2 in particular is a likely candidate for mediating the effects of SIRT1. However, it remains important that potential effects of

SIRT1 on the expression of other PRC components are measured at the protein level before excluding this as a mechanism through which SIRT1 effects DNA methylation at PCGTs. We next proposed that SIRT1 may have action on DNA methylation targeted to PCGTs through an intermolecular association with EZH2. However, SIRT1 and EZH2 did not co-immunoprecipitate in HuVECs or Caco-2 cells. Time constraints prevented further progress of this work, and there remains many lines of investigation to adopt to uncover the underlying mechanism.

One of the notable limitations in this project was the use of cell culture and specifically a cancer cell line (Caco-2 cells). Cancer cells are useful for cell culture as they tend to replicate more quickly than normal cells and as already mentioned Caco-2 cells differentiate in to an epithelial monolayer when they reach 100 % confluency generating a truer representation of the gut (Sambuy *et al.*, 2005). However, cancer cell lines also have a high mutation rate and have even been shown to have increasing DNA methylation with progression and often genes expressed by the original tumour cells change when the cells are grown in culture (van Staveren *et al.*, 2009). Gene expression may also alter between passage phases within cell culture and small differences in experimental technique, such as temperature change, could lead to gene expression changes. This is a limiting factor when collecting data and may account for discrepancies. For example, resveratrol reduced LAMP2 protein but had no effect on LAMP2 mRNA. This may be a true representation or simply due to these sorts of factors that can lead to variability in data. To minimise discrepancy and generate more reliable data it is important to use cells of the same passage between experimental repeats and also collect a sufficient number of experimental repeats to ensure a true representation of the gut (van Staveren *et al.*, 2009).

As explained a major objective of this whole project was to gather a body of data to be used in a system-level mathematical model of interactions between SIRT1, PARP, NAD^+ and modifiers of these variables notably resveratrol. Due to the limitations in the data we were unable to construct a system-level mathematical model but Figure 7.4.1 below shows a schematic diagram linking the observations made to previous studies.

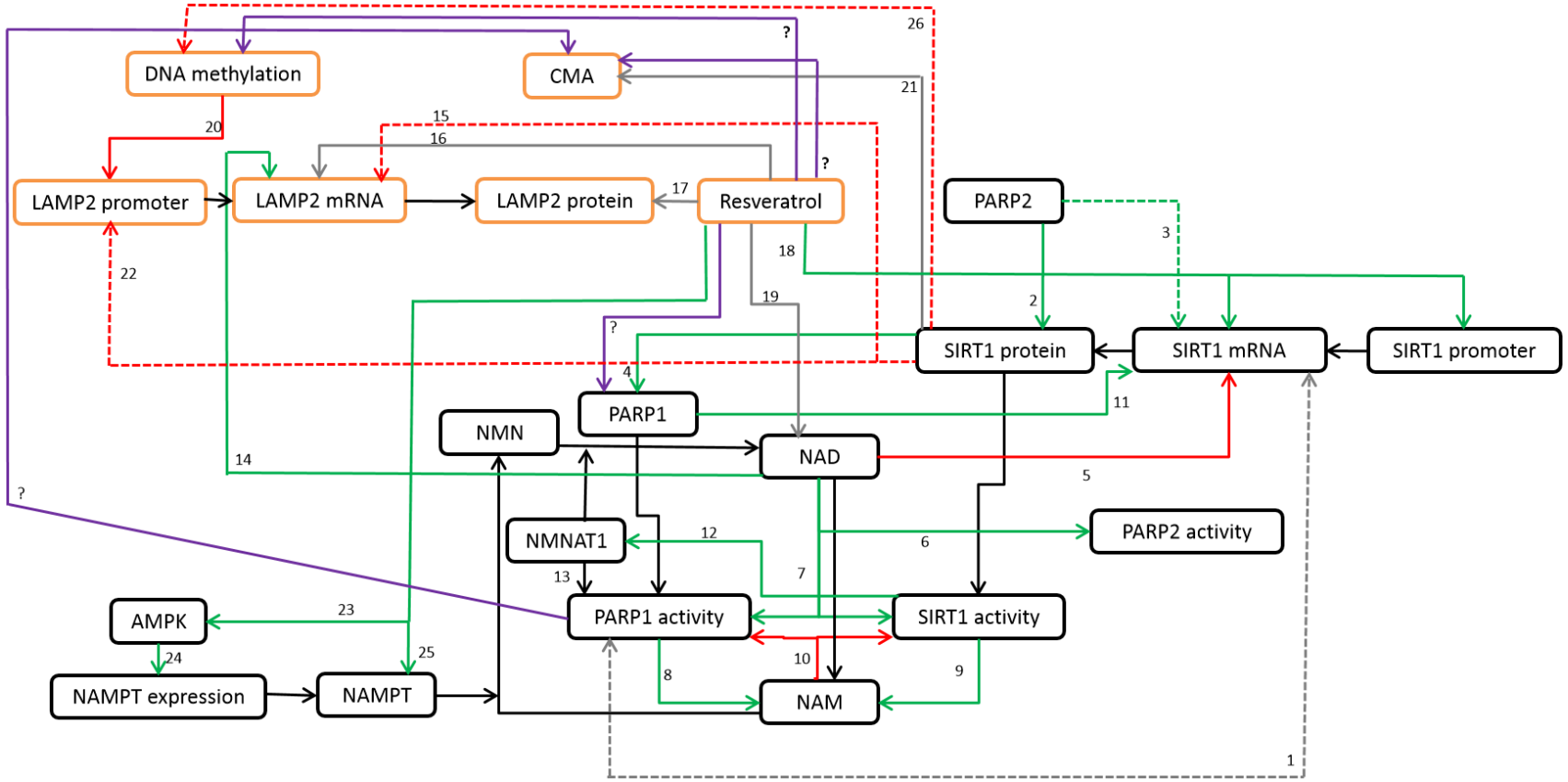


Figure 7.4.1 A schematic diagram showing the interactions between SIRT1, PARPs, resveratrol, components of CMA and NAD$^+$. Green lines indicate a positive action, red lines indicate a negative action, purple lines indicate unknown, grey lines indicate preliminary data and black lines indicate a known cellular action, dashed lines indicate a factor which is reduced by either knockdown by siRNA or inhibited, NAM (nicotinamide), NMNAT1 (nicotinamide mononucleotide adenyltransferase 1) NAMPT (nicotinamide phosphoribosyltransferase) NMN (nicotinamide mononucleotide). Numbers are represented in the key correspond with numbers on the arrow connectors.

Key for Figure 7.4.1	
1	PARP inhibition by 3-aminobenzamide increased SIRT1 mRNA and SIRT1 knockdown by siRNA increased PARP activity in Caco-2 cells (current thesis)
2	PARP2-/- mice had increased SIRT1 protein (Bai *et al.*, 2011).
3	PARP2 knockdown by siRNA in C2C12 myotubes increased SIRT1 mRNA (Bai *et al.*, 2011).
4	PARP1 is activated through acetylation; SIRT1-/- mice had higher PARP1 activity due to a decrease in deacetylation of PARP1 by SIRT1 (Rajamohan *et al.*, 2009b).
5	Apigenin increased NAD^+ and reduced SIRT1 mRNA and protein in Caco-2 cells (current thesis).
6	NAD^+ is consumed by PARP1. PARP2 and SIRT1, the Km values for NAD^+ are: PARP1 ~20-60 μM, PARP2 ~130 μM and SIRT1 ~150-200 μM (Houtkooper *et al.*, 2010).
7	
8	The NAD^+ precursor NAM is salvaged and recycled to NAD^+ following NAD^+ consumption by SIRT1 and PARP1 (Burgos, 2011; Luna *et al.*, 2013).
9	
10	NAM inhibits SIRT1 (Bitterman *et al.*, 2002) and PARP1 (Hageman and Stierum, 2001).
11	PARP1 can promote SIRT1 expression through promoting C-*MYC* expression, which increases SIRT1 expression (Simbulan-Rosenthal *et al.*, 2003; Yuan *et al.*, 2009; Marshall *et al.*, 2011).
12	SIRT1 recruits NMNAT1 to promoter regions to convert NMN to NAD^+ (Zhang *et al.*, 2009).
13	NMNAT1 is recruits to the PARP1 polymer PAR and converts NMN to NAD^+ to be utilised by PARP1 (Berger *et al.*, 2007).
14	A reduction in NAD^+ by FK866 increased LAMP2 mRNA expression in Caco-2 cells (current thesis, chapter 3).
15	SIRT1 reduction pharmaceutically and by siRNA decreased LAMP2 mRNA at 24 h and 72 h respectively in Caco-2 cells (current thesis, chapter 3).
16	Resveratrol increased LAMP2 mRNA but decreased LAMP2 protein at 48 h in Caco-2 cells (current thesis, chapter 3)
17	
18	Resveratrol increased SIRT1 expression at 48 h in Caco-2 cells (current thesis, chapter 1).

	Resveratrol increased SIRT1 mRNA expression in human ovarian, prostate and porcine adipocytes (Bai *et al.*, 2008; Morita *et al.*, 2012; Li *et al.*, 2013).
19	Resveratrol appears to increase NAD^+ at 48 h in Caco-2 cells (current thesis, chapter 1). Resveratrol increased AMPK activity and NAD^+ in C2C12 myotubes (Park *et al.*, 2012).
20	DNA hyper methylation of the genome decreased LAMP2 mRNA expression (current thesis, chapter 3).
21	Preliminary data, SIRT1 reduction by siRNA reduced CMA (data needs clarification) (current thesis, chapter 3).
22	SIRT1 knockdown by siRNA increased LAMP2 promoter DNA methylation at specific CpG sites (L. Wakeling and D. Ford personal communication).
23	Resveratrol increases AMPK activity in mouse C2C12 myotubes (Chung *et al.*, 2012; Park *et al.*, 2012)
24	AMPK increases NAMPT expression in mouse skeletal myoblast cells (Fulco *et al.*, 2008).
25	Resveratrol increased NAMPT activity in human primary hepatocytes but did not increase NAMPT mRNA expression (Schuster *et al.*, 2014).
26	SIRT1 knockdown and overexpression changed DNA methylation across the genome in Caco-2 cells and HuVECs (Wakeling *et al.*, 2015).

The work presented in Figure 7.4.1 has helped to pin point important areas for future work. In addition to the future work already mentioned, which would build on the data sets presented, other areas for investigation highlighted by this research include the following:

- The effect of resveratrol on PARP activity. Our simple hypothesis predicts that resveratrol will reduce PARP activity by increasing SIRT1 expression, which in turn depletes intracellular NAD^+.

- The effect of resveratrol on CMA. We predict that resveratrol will effect CMA through increasing SIRT1 expression.
- The effect of PARP on CMA. Our simple hypothesis that PARP and SIRT1 have reciprocal co-inhibitory actions predicts that PARP activation would reduce CMA through a reduction in SIRT1.
- An effect on *LAMP2* promoter DNA methylation, is a plausible mechanism through which SIRT1 may affect LAMP2 expression in as much as reducing SIRT1 affects DNA methylation at this site and expression of the gene increases when DNA is demethylated. However, as with all similar observations, showing a direct causal link is challenging and is not yet established for this scenario.
- We predict that resveratrol will have an effect on DNA methylation through its effects on SIRT1 expression. An informative experiment would be to carry out parallel analysis of effects genome wide as we reported here with respect to SIRT1 manipulation where we saw that changes in DNA methylation clustered at PCGTs.
- As well as probing the mechanism behind DNA methylation changes at PGCTs elucidated by SIRT1, work is also required to determine if such DNA methylation changes may alter the expression of PCGTs.

7.5 Conclusion

To conclude, this project set out to explore further interactions of SIRT1 particularly relevant in the context of the ageing cell. We have obtained a body of data that will contribute to a system-level mathematical model to help us understand the relationships between SIRT1, PARP, NAD^+, resveratrol and CMA. An underpinning hypothesis was that SIRT1and PARP compete for cellular NAD^+ and therefore show reciprocal effects on activity. The balance in this relationship could have a fundamental effect on how a cell responds to stress including by CMA. Stressors themselves, such as oxidative stress, may change the balance of activity between SIRT1 and PARP. A system-level mathematical model may highlight nodes for intervention to affect ageing and age-related diseases in predictable ways.

8 Appendix A

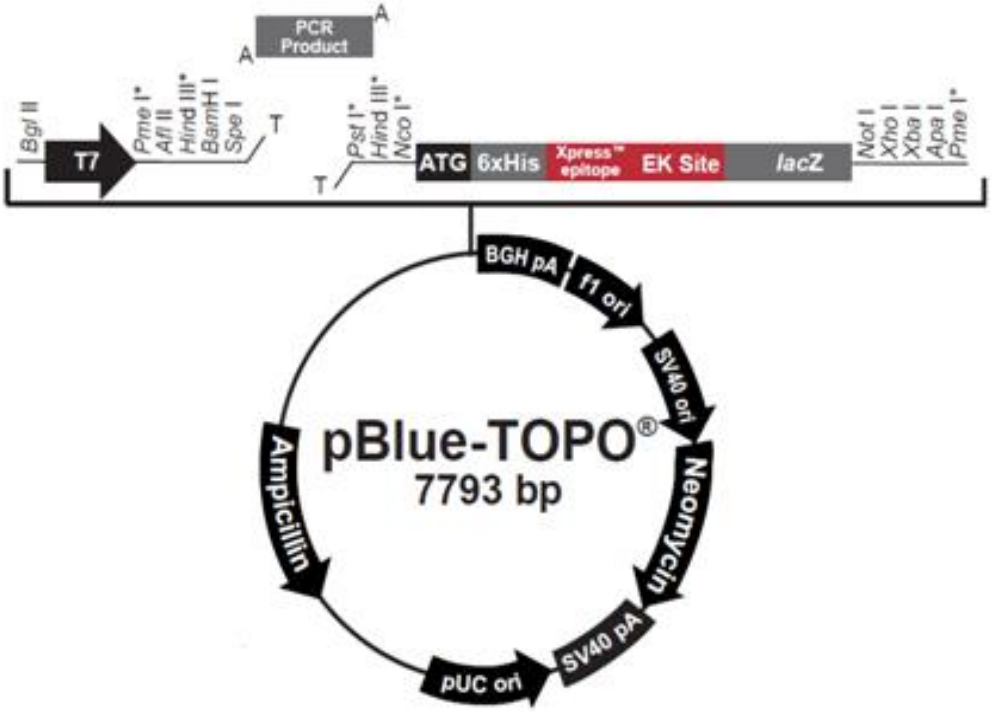


Figure 7.5.1. Appendix A. pBlue-TOPO®. The SIRT1 promoter was inserted upstream to the *LacZ* gene. Upon promoter activity this then produced the enzyme β-galactosidase. The vector contains ampicillin resistance gene (50 μg/ml). The (image sourced from https://www.lifetechnologies.com/order/catalog/product/K483101).

9 Appendix B

-1050 GAGTCACAGTGTGCCAGAATTTCAGGGAGAGAGGAAAGTGGAAGGGCTTTCCACTAAGCCTTTTGA

ACTACTAGGTACCCCTCGTTTTACATCTGGTTATCTCATTTAAATCTATGACGTTTTAAAATACTT

ATTACCATTTAAGACATGAGAAAAATTAAGTTTAGAAACGGCTAGATAGCTCACGCTAGAAAGGAA

GGACTCCAAATTTTAACCAAGGGCAGATGTGCATGGAGGCCAAGTCATTTCCTTCCCATGCTCTCA

TACTGACCCAACAAACCCATTCTGCACGTGAGAAAACTGAGGCCCGGAGGAGGGAATTCACACACG

TTTGAAGCCAAGCTGGGGCCAGAAAGTAGATCGGCTGATCTCCAAACCTCCACGTCAAAGGTCTTC

CCAGGAGGACATATGCCTTCAAGGATTTTACAATGTATACCACCCTACAAGTGATGGGAGAGAGGG

GAAAAAAGCAACCGACTAAGGAGAAAAGCAAGGAGCAGAAAAAGGAGCAAAAGAGGAGCTGTCAGA

ACGGTGTGAGGAGAGTGGGAAAGGAGCCGCCTCCTTTTGCCTCTCTTCCTACTTATTAACAAAACA

GAACGACTATCCAACGTATTTCAGGGAGCTAAGTCTTAGCCAGCTTCAGCTGTGTTTTAACCCTTA

GCTAAATATAGACAAGGCTAAGGCAGGCCAGGTGTACACTTCAGGAAGACGTGGAAATTCCCAGGG

CGGACCAAAACTTGAGCTGTTCCGGCGGTAGTGATTTGAGGTCAGTTTGAAAGAGAAGTTGAGAAA

GCGGCCGAGGGGCGAATTTGGCTGCACTACACGCTCGCCACAAAGAGGAAGGGCCGCCGGCCGCCG

GGGCCGAGTGCGCTTCCAGCCCAGGCGGAGCGGTAGACGCAACAGCCTCCGCCCGCCACGTGACCC

GTAGTGTTGTGGTCTGGCCCGCGTGGGTGGCGGGAGCGCCGAGAGGGCGGGGGCGGCGATGGGGCG

GGTCACGTGATGGGGTTTAAATCTCCCGCAGCCGGAGCCGCGGGGGCGCCAGTGCCGCGCgtcgag

cgggagcagaggaggcgagggaggagggccagagaggcagttggaagatggcggacgaggcggccc

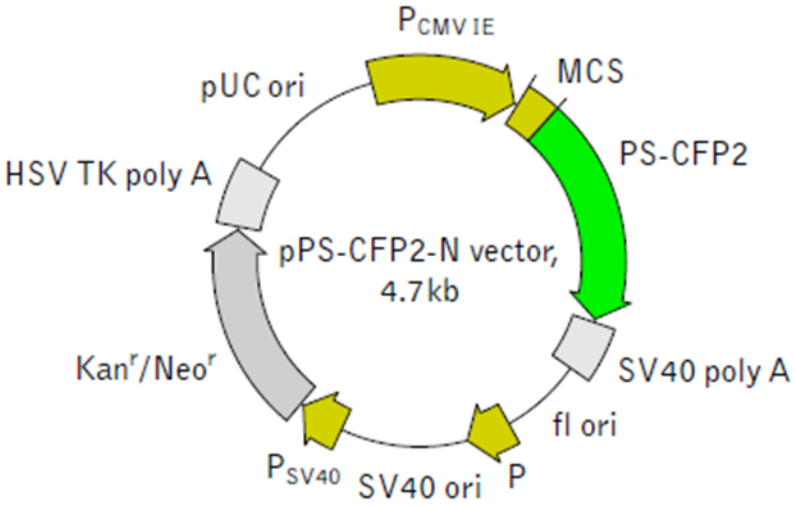
tcgcccttcagcccggcggctccccctcggcggcgggggccgacagggaggccgcgtcgtcccccg

+100 ccggggagccgc

Figure 7.5.1. Appendix B. The SIRT1 promoter region used in the pBlue-TOPO® plasmid. A PCR product was previously generated using the forward primer highlighted in green and reverse primer highlighted in yellow. This product was cloned in to a pBlue-TOPO® plasmid. The black uppercase part of the sequence is the promoter region and the red lower case section of the sequence is the first exon.

10 Appendix C

A



B Multiple cloning site

CTAGCGCTACCGGACTCAGATCTCGAGCTCAAGCTTCGAATTCTGCAGTCGACGGTACCGCGGGCCCGGGATC

Hind III *EcoR I*

Figure 7.5.1. Appendix C. pPS-CFP2-N vector and multiple cloning site. A- pPS-CFP2-N vector (Evrogen) has the PS-CFP2 fluorescent tag at the N terminal of the multiple cloning site and a kanamycin resistance gene (30 µg/ml). **B-** *HindIII* (red) and *EcoR1* (blue) were the chosen restriction enzyme sites for cloning. Vector map sourced from http://www.evrogen.com/products/vectors/pPS-CFP2-N/pPS-CFP2-N.shtml

11 Appendix D

Ribonuclease A DNA sequence:

ATGGCTCTGAAGTCCCTGGTCCTGTTGTCGCTGTTGGTCCTGGTGCTGCTGCTGGTGCGGG
TCCAGCCTTCCCTGGGCAAGGAAACTGCAGCAGCCAAGTTTGAGCGGCAGCACATGGACTC
CAGCACTTCCGCTGCCAGCAGCTCCAACTACTGTAACCAGA

Ribonuclease A protein sequence:

MALKSLVLLSLLVLVLLLVRVQPSLGKETAAAKFERQHMDSSTSAASSSNYCNQ

pPS-CFP2-N plasmid MCS containing insert sequence:

G CTA GCG CTA CCG GAC TCA GAT CTC GAG CTC AAG CTT ATG GCT CTG AAG TCC
CTG GTC CTG TTG TCG CTG TTG GTC CTG GTG CTG CTG CTG GTG CGG GTC CAG CCT
TCC CTG GGC AAG GAA ACT GCA GCA GCC AAG TTT GAG CGG CAG CAC ATG GAC TCC
AGC ACT TCC GCT GCC AGC AGC TCC AAC TAC TGT AAC CAG AGA ATT CTG CAG TCG
ACG GTA CCG CGG GCC CGG GAT CCA CCG GTC GCC ACC ATG AGC AAG

Figure 7.5.1. Appendix D. Bovine Ribonuclease A DNA sequence and protein sequence and multiple cloning site (MCS) of pPS-CFP2-N plasmid containing PCR product generated from Ribonuclease A. Green-KFERQ motif, yellow-MCS, pink-*HindIII* restriction site and blue- *EcoR1* restriction site. Ribonuclease A DNA sequence is from 136-298 bp. MCS located 591-671 bp in pPS-CFP2-N vector.

12 Appendix E

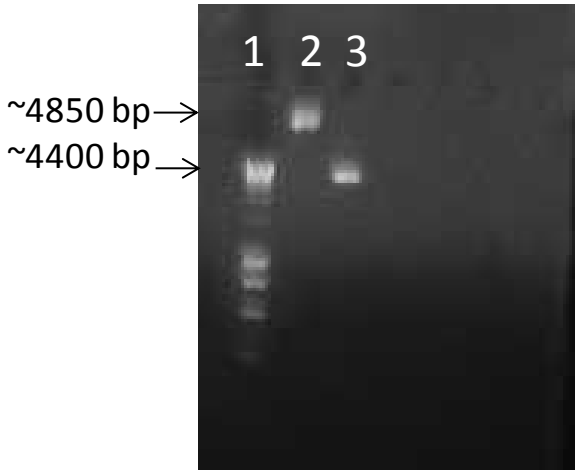


Figure 7.5.1. Appendix E. Agarose gel electrophoresis of the product generated in a ligation reaction between the vector pPS-CFP2-N and the PCR product comprising of a region of the bovine ribonuclease A gene with the CMA target sequence. Lane 1 Hyperladder™III (Bioline), lane 2 ligation reaction, lane 3 ligation reaction after digestion with *EcoR1* and *HindIII*. The smaller size of the product seen after digestion corresponds with the size of the vector only and confirms that the ligation reaction was successful.

13 Related publications.

A

Hum Genomics. 2015 Jun 24;9:14. doi: 10.1186/s40246-015-0036-0.

SIRT1 affects DNA methylation of polycomb group protein target genes, a hotspot of the epigenetic shift observed in ageing.

Wakeling LA[1], Ions LJ[2], Escolme SM[3], Cockell SJ[4], Su T[5], Dey M[6], Hampton EV[7], Jenkins G[8], Wainwright LJ[9], McKay JA[10], Ford D[11].

⊕ Author information

Abstract

BACKGROUND: SIRT1 is likely to play a role in the extension in healthspan induced by dietary restriction. Actions of SIRT1 are pleiotropic, and effects on healthspan may include effects on DNA methylation. Polycomb group protein target genes (PCGTs) are suppressed by epigenetic mechanisms in stem cells, partly through the actions of the polycomb repressive complexes (PRCs), and have been shown previously to correspond with loci particularly susceptible to age-related changes in DNA methylation. We hypothesised that SIRT1 would affect DNA methylation particularly at PCGTs. To map the sites in the genome where SIRT1 affects DNA methylation, we altered SIRT1 expression in human intestinal (Caco-2) and vascular endothelial (HuVEC) cells by transient transfection with an expression construct or with siRNA. DNA was enriched for the methylated fraction then sequenced (HuVEC) or hybridised to a human promoter microarray (Caco-2).

RESULTS: The profile of genes where SIRT1 manipulation affected DNA methylation was enriched for PCGTs in both cell lines, thus supporting our hypothesis. SIRT1 knockdown affected the mRNA for none of seven PRC components nor for DNMT1 or DNMT3b. We thus find no evidence that SIRT1 affects DNA methylation at PCGTs by affecting the expression of these gene transcripts. EZH2, a component of PRC2 that can affect DNA methylation through association with DNA methyltransferases (DNMTs), did not co-immunoprecipitate with SIRT1, and SIRT1 knockdown did not affect the expression of EZH2 protein. Thus, it is unlikely that the effects of SIRT1 on DNA methylation at PCGTs are mediated through direct intermolecular association with EZH2 or through effects in its expression.

CONCLUSIONS: SIRT1 affects DNA methylation across the genome, but particularly at PCGTs. Although the mechanism through which SIRT1 has these effects is yet to be uncovered, this action is likely to contribute to extended healthspan, for example under conditions of dietary restriction.

B

Age (Dordr). 2013 Oct;35(5):1835-49. doi: 10.1007/s11357-012-9485-8. Epub 2012 Nov 16.

Effects of Sirt1 on DNA methylation and expression of genes affected by dietary restriction.

Ions LJ[1], Wakeling LA, Bosomworth HJ, Hardyman JE, Escolme SM, Swan DC, Valentine RA, Mathers JC, Ford D.

⊕ Author information

Abstract

Changes in DNA methylation across the life course may contribute to the ageing process. We hypothesised that some effects of dietary restriction to extend lifespan and/or mitigate against features of ageing result from changes in DNA methylation, so we determined if genes that respond to dietary restriction also show age-related changes in DNA methylation. In support of our hypothesis, the intersection of lists of genes compiled from published sources that (1) were differentially expressed in response to dietary restriction and (2) showed altered methylation with increased age was greater than expected. We also hypothesised that some effects of Sirt1, which may play a pivotal role in beneficial effects of dietary restriction, are mediated through DNA methylation. We thus measured effects of Sirt1 overexpression and knockdown in a human cell line on DNA methylation and expression of a panel of eight genes that respond to dietary restriction and show altered methylation with age. Six genes were affected at the level of DNA methylation, and for six expressions were affected. In further support of our hypothesis, we observed by DNA microarray analysis that genes showing differential expression in response to Sirt1 knockdown were over-represented in the complied list of genes that respond to dietary restriction. The findings reveal that Sirt1 has effects on DNA methylation across the genome and affects, in particular, the expression of genes that respond to dietary restriction. Sirt1-mediated effects on DNA methylation and, consequently, gene expression may thus be one of the mechanisms underlying the response to dietary restriction.

Figure 7.5.1. Related publications. A- contains data gathered on SIRT1 and polycomb group proteins shown in Chapter 4 of this thesis **B-** contains data gathered prior to this thesis gathered in a masters project, which lead up to the current work **.**Please note Escolme SM was my maiden name before July 2015.

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