**Identification of Novel Acetylation Sites in Nuclear Factor
(Erythroid-Derived 2)-like 1(Nrf1) and their Regulation by
Sirtuin 1 (SIRT1)**

By

ANDREW J. SEAL

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Ottawa-Carleton Institute of Biology

Carleton University

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Abstract:

For cells to survive the damages of reactive oxygen species (ROS) they must defend themselves. Cellular defense is accomplished by means of the antioxidant response element (ARE). One of the main factors involved in regulating the ARE is the nuclear factor (erythroid-derived 2)-like1 protein (Nrf1), this protein has not been thoroughly characterized, so the manner in which it is regulated is unknown. The deacetylase Sirtuin 1 (SIRT1) has been shown to regulate a relative of Nrf1, Nrf2. When SIRT1 was chemically inhibited, the acetylation of Nrf1 increased, indicating SIRT1 to be a regulator of Nrf1. When Nrf1 was deacetylated by SIRT1, a significant decrease in the activity of Nrf1 was observed. Under hypoxic conditions, it was observed that SIRT1 was a stronger regulator of Nrf1 activity than hypoxia. Two acetylation sites were identified in Nrf1; lysines 205 and 629. The deacetylation of Nrf1 resulted in a significant decrease in cellular senescence.

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Table of Contents:

Abstract:	ii
Acknowledgments:	iii
Table of Contents:	iv
List of Figures:	vii
List of Tables:	ix
List of Abbreviations:	x
Chapter 1: General Introduction:	1
1.1 Reactive oxygen species and hypoxia	1
1.2 Antioxidant response element	2
1.3 Nuclear factor (erythroid-derived-2) like 1 protein (Nrf1)	3
1.4 The truncated p65 isoform of Nrf1	4
1.5 The role of acetylation and Sirtuin 1	6
1.6 Cellular senescence	10
1.7 Cell lines	11
1.8 Hypothesis and objectives	13
Chapter 2: Materials and Methods	13
2.1 Cells	13
2.2 Expression constructs	14

2.3 Detection of Nrf1 acetylation	14
2.4 Luciferase reporter assay	15
2.5 RNA Extraction, reverse transcriptase-PCR and quantitative RT-PCR	15
2.6 Site directed mutagenesis	16
2.7 Senescence associated β -galactosidase activity	16
2.8 Statistical analysis	17
Chapter 3: Results.....	19
3.1 Nrf1 is acetylated in mammalian cells	19
3.2 Inhibition of SIRT1 results in increased Nrf1 acetylation	21
3.3 Overexpression of SIRT1 affects metallothioneine 1 mRNA transcription	26
3.4 Regulation of Nrf1 activity by nicotinamide.....	29
3.5 Regulation of Nrf1 activity by resveratrol	31
3.6 SIRT1 inhibits full length Nrf1 but not p65-Nrf1	33
3.7 Hypoxia results in a change in Nrf1 activity independent of SIRT1	38
3.8 Location of acetylation sites on Nrf1	39
3.9 Nrf1 plays a role in cellular senescence	46
Chapter 4: Discussion	50
4.1 Nrf1 acetylation.....	50
4.2 The deacetylation of Nrf1 by SIRT1	50
4.3 SIRT1 inhibits Nrf1 activity.....	51

4.4 Chemical regulation of SIRT1	52
4.5 The interaction of SIRT1, Nrf1 and the truncated p65-Nrf1	54
4.6 The effect of SIRT1 on the regulation of Nrf1 by hypoxia.....	55
4.7 The identification of the acetylated lysine residues on Nrf1	56
4.8 The role of Nrf1 in cellular senescence.....	58
4.9 Proposed novel interactions of SIRT1 and Nrf1	60
Chapter 5: Conclusions and Future directions	61
Chapter 6: References	64

List of Figures:

Figure 1.1: CNC-bZIP transcription factors regulate antioxidant-response element (ARE)/electrophile-response element (EpRE)-driven gene expression	5
Figure 1.2: The regulation of SIRT1 under oxidative stress conditions	9
Figure 1.3: Proposed model relating mitochondrial function and lifespan in sod-2 mutant worms.	12
Figure 3.1: Nrf1 is acetylated in HepG2 and HEK293 cells under normal conditions. ...	20
Figure 3.2: Co-expression of SIRT1 results in a decrease and 10mM NAM results in increase of acetylation on Nrf1.....	23
Figure 3.3: Resveratrol 50µM does not alter Nrf1 acetylation while 10mM NAM increases it in Nrf1.....	25
Figure 3.4: Co-expression of Nrf1 and SIRT1 results in decreased MT1 gene expression in HEK293 cells.	27
Figure 3.5: Co-expression of Nrf1 and SIRT1 results in decreased MT1 gene expression in HepG2 cells.	28
Figure 3.6: NAM causes a slight increase in Nrf1 activity in HepG2 and a decrease in HEK293 cells.....	30
Figure 3.7: Resveratrol has no significant effect on Nrf1 activity in HepG2 and HEK293 cells.....	32
Figure 3.8: SIRT1 inhibits Nrf1 activity in HepG2 cells, and increases the inhibitory effect of p65-Nrf1.....	35
Figure 3.9: SIRT1 inhibits Nrf1 activity in HEK293 cells, but has little effect on the inhibitory effects of p65-Nrf1.	37

Figure 3.10: Hypoxia decreases Nrf1 activity in HepG2 cells, while it has little effect in the presence of SIRT1.	40
Figure 3.11: Hypoxia increases Nrf1 activity in HEK293 cells, while it has little effect in the presence of SIRT1.	41
Figure 3.12: Mutants K205R and K629R Nrf1 showed a significant decrease in Nrf1 activity relative to the wild type in HepG2 cells.	43
Figure 3.13: Mutant K205R Nrf1 showed a significant decrease in Nrf1 activity relative to the wild type in HEK293 cells.	45
Figure 3.14: Inhibition of Nrf1 by p65-Nrf and both mutant K205R and K629R showed similar reduction of cellular senescence relative to SIRT1.	49
Figure 4.1: Schematic diagram showing the positions of the novel acetylation sites on full-length Nrf1 and the truncated p65-Nrf1 (Chepelev et al. 2011; Zhang et al. 2006).	59
Figure 4.2: Proposed new pathway diagram for Nrf1 and SIRT1 interaction.	62

List of Tables:

Table 2.1: Oligonucleotide primers used for site directed mutagenesis.	18

List of Abbreviations:

α MEM— Alpha minimal essential media

ATCC— American Type Culture Collection

CPRG— chlorophenol red- β -D-galactopyranoside

EpRE— Electrophile response element

ER— Endoplasmic reticulum

FBS— Fetal bovine serum

GCLC— Glutamate cysteine ligase catalytic subunit

HepG2— Hepatocellular carcinoma cell line

HP-F— Human hepatocytes isolated from whole livers

IP— Immunoprecipitation

MT— Metallothionein

NAM— Nicotinamide

Nrf1— Nuclear factor erythroid 2-related factor-1

PVDF—Polyvinylidene fluoride

Res— Resveratrol

RT-PCR— Real-time polymerase chain reaction

SA- β -Gal—Senescence associated beta galactosidase

SIRT1— Sirtuin1

SDS-PAGE—Sodium dodecyl sulfate-poly acrylamide gel electrophoresis

Chapter 1: General Introduction:

1.1 Reactive oxygen species and hypoxia

Cellular responses to reactive oxygen species (ROS) and low oxygen (hypoxia) are closely linked. Under hypoxic conditions, the lack of oxygen as a final electron acceptor causes electrons to build up along the mitochondrial electron transport chain (ETC) and the cytochrome components of the ETC become highly reduced. If any oxygen is present at all (hypoxic, but not anoxic, conditions) then it can pick up readily available electrons from the electron transport chain, and becomes the first ROS produced by the mitochondria, superoxide. Under hypoxic conditions, superoxide is primarily produced from Complex III. This can then lead to production of hydrogen peroxide and, eventually, the very damaging hydroxyl radical (Murphy 2009; Guzy et al. 2005; Klimova and Chandel 2008). Thus, as oxygen decreases, there may be a critical oxygen tension at which superoxide production from the mitochondria increases and this ROS production can be utilized in cellular signalling (Murphy 2009; Guzy et al. 2005; Klimova and Chandel 2008).

One such signalling factor that is induced by hypoxia and can be affected by ROS is the Hypoxia-Inducible Factor 1, or HIF-1. HIF-1 is a transcription factor involved in the mediation of the cellular response to decreased oxygen through the induction of hypoxia-inducible genes (Murphy 2009; Guzy et al. 2005; Klimova and Chandel 2008). Under normal oxygen conditions (21% oxygen or normoxia), HIF-1 α is hydroxylated by prolyl hydroxylases (PHDs), which leads to the ubiquitination of HIF-1 α and its subsequent proteolytic degradation. Under hypoxic conditions, ROS production from

Complex III can inhibit the PHD enzymes and thus further stabilize HIF-1 α (Guzy et al. 2005; Murphy 2009; Klimova and Chandel 2008; Chepelev et al. 2011). When stabilized by hypoxia, HIF-1 activates the genes responsible for the cell's hypoxic response, which causes an anomalous increase in the ROS production of the mitochondria; the cause of this paradoxical increase is currently unknown (Guzy et al. 2005; Murphy 2009; Klimova and Chandel 2008). ROS are responsible for causing several impairments on the normal cell function including DNA mutation, DNA repair, cell growth and differentiation, and cell death (Murphy 2009).

1.2 Antioxidant response element

Due to the damaging effects of ROS on normal cell function the cell must have a defence mechanism against ROS. The antioxidant response element (ARE) is responsible for expressing the genes that encode for among others, heme oxygenase-1, ferritin (light and heavy chain), metallothioneines, NAD(P)H quinone (oxido) reductase, glutathione S-transferase, and β -globin; which combat the damaging effects of ROS, and induce phase II detoxification enzymes (Chepelev et al. 2011; Jung and Kwak 2010). Factors that bind to the ARE consensus sequence (TGACNNNGC) in the cell are the Cap'N'Collar-basic Leucine Zipper (CNC-bZIP) transcription factors which are known as nuclear factor (erythroid-derived 2)-like 1, 2 and 3 proteins (Nrf) (Chepelev et al. 2011; Ohtsuji et al. 2008; Vomhof-Dekrey and Picklo 2012). Nrfs are usually active when they form heterodimers with the Maf (musculo-aponeurotic fibrosarcoma oncogene) family of proteins (Chepelev et al. 2011; Johnsen et al. 1996). Other members of the CNC-bZIP family include, Bach1 and Bach2; these proteins also form heterodimers with Maf

proteins, but unlike the Maf-Nrf heterodimers, when Maf-Bach dimers bind to the ARE they act as dominant repressors of the ARE genes (Chepelev et al. 2011).

1.3 Nuclear factor (erythroid-derived-2) like 1 protein (Nrf1)

Nrf1 (NFE2L1) is a transcription factor responsible for regulating the cell's response to pro-oxidants. The full length Nrf1 (p120) is found in the endoplasmic reticulum (ER) where it is proteolytically cleaved to form the active p95 form, which translocates into the nucleus to bind to the electrophile response element (EpRE; aka the antioxidant response element or ARE) promoting phase (II) detoxification and antioxidant enzyme production. Nrf1 is unique from Nrf2 because it has an elongated N-terminal and acidic domain that targets Nrf1 to be translocated into the ER (Yiguo Zhang et al. 2006).

Compared to its closely-related protein Nrf2 (NFE2L2), little is known about Nrf1. Nrf2 has been shown to be inhibited by Kelch-like ECH-associated protein 1 (Keap1), which associates with the protein in the cytosol and must be released from it in order to be activated by cAMP response element-binding protein (CREB)-binding protein (CBP) (Kawai et al. 2011). CBP acetylates two lysines on Nrf2, which releases it from Keap1 and allows it to travel to the nucleus (Kawai et al. 2011; Cosgrove et al. 2006; Yiguo Zhang et al. 2006). The NAD-dependant deacetylase Sirtuin 1 (SIRT1) regulates the activity of Nrf2 by removing the acetyl groups that CREB-binding protein had attached to the protein, thereby inhibiting the binding activity of Nrf2 (Kawai et al. 2011; Chepelev et al. 2011; Yiguo Zhang et al. 2006). While Nrf1 has been shown to act independently from Keap1, little is known about SIRT1's effect on Nrf1 (Kawai et al. 2011; Chepelev et al. 2011; Yiguo Zhang et al. 2006).

1.4 The truncated p65 isoform of Nrf1

The mechanism which is responsible for the generation of p65-Nrf1 is currently unknown, most likely it results from either proteolytic cleavage of p95 or internal start sites in the Nrf1 gene. While p65-Nrf1 contains the DNA binding domain and the Maf binding domain, it does not activate the ARE region because it does not have the transactivation domain; which makes p65-Nrf1 a competitive inhibitor of the full length Nrf1, 2 and 3 proteins, as it binds to Maf proteins but does not activate AREs (Figure 1.1) (Chepelev et al. 2011; Wang et al. 2007). In previous studies, p65-Nrf1 has been shown to be repressed under hypoxic conditions (Chepelev et al. 2011). It has been speculated that the role of p65-Nrf1 is to modulate the expression of antioxidant genes by ensuring proper deactivation of their transcription when they are no longer required under low stress conditions (Wang et al. 2007). This could play an important role in the generation of precancerous cells, since it has been shown that the uncontrolled expression of antioxidant genes results in increased proliferation and survival of precancerous cells, so the inhibition of p65-Nrf1 could result in these deleterious symptoms (Motohashi and Yamamoto 2007; Padmanabhan et al. 2006).

Figure 1.1: CNC-bZIP transcription factors regulate antioxidant-response element (ARE)/electrophile-response element (EpRE)-driven gene expression. CNC-bZIP factors include activators Nrf1, Nrf2, Nrf3, and NE-F2 p45 as well as dominant-negative repressors Bach1 and Bach2 and bind to ARE (EpRE) as heterodimers with small Maf proteins. Hypoxia induces heme synthesis and heme dislodges Bach1 from its interaction with Maf-occupied AREs, favoring the binding of activators (Nrfs 1, 2, and 3) to the ARE and activating the expression of ferritin, HO-1, and β -globin genes. We hypothesize that hypoxia activates Nrf1 and Nrf2 transactivation through ROS pathways. Diagram modified from (Chepelev and Willmore 2011).

1.5 The role of Acetylation and Sirtuin 1

Nrf1 has been shown to be regulated by posttranslational modifications in the past such as, phosphorylation and glycosylation, but the modification that has not been examined more closely is acetylation (Chepelev et al. 2011; Zhang et al. 2009; Zhang et al. 2007). The acetylation of lysine residues is a posttranslational modification responsible for regulating the activity of a large number of proteins (Lin 2000; Hwang et al. 2013; Bordone and Guarente 2005). These acetyl groups can be added to lysines by several proteins such as, p300, CREB-binding protein (CBP) and the p300/CBP-associated factor (Hwang et al. 2013; Lin 2000; Bordone and Guarente 2005; Kawai et al. 2011). The acetylation of proteins is a reversible process which allows the effects of this modification to be regulated by its removal (by deacetylases such as Sirtuin1) (Hwang et al. 2013; Lin 2000; Bordone and Guarente 2005; Kawai et al. 2011).

Sirtuins are a family of NAD^+ dependant deacetylases that share homology with the silent information regulator 2 (Sir2) protein from *Saccharomyces cerevisiae*. They are responsible for the regulation of histones and transcription factors, by altering their acetylation status (Houtkooper et al. 2012; Hwang et al. 2013). Sirtuin1 (SIRT1) is the most studied of the mammalian Sirtuins because it is responsible for regulating many factors in cells, such as, inflammation, cellular senescence, mitochondrial biogenesis, apoptosis and circadian rhythms (Hwang et al. 2013; Houtkooper et al. 2012). Since SIRT1 requires NAD^+ for a co-activator of its deacetylation activity, it is thought that this aspect of the enzyme could lead to its regulation by environmental factors such as oxidative stress; since NADH/NAD^+ levels in cells have been shown to be regulated by stressors such as hypoxia (Garofalo et al. 1988). The NAD^+ is hydrolysed in the

deacetylation reaction to produce nicotinamide (Houtkooper et al. 2012; Hwang et al. 2013; Lin 2000; Bordone and Guarente 2005). Nicotinamide acts as a feedback inhibitor of SIRT1 (Hwang et al. 2013; Braidy et al. 2011; Peled et al. 2012; Bai et al. 2008). It and the NAD^+ are part of a salvage pathway that is regulated by the redox status of the cell, which would be one means by which the activity of SIRT1 could be regulated by oxidative stress, although this interaction is not fully characterized (Hwang et al. 2013; Braidy et al. 2011; Peled et al. 2012; Bai et al. 2008). Another means by which SIRT1 has been shown to be regulated by oxidative stress is through the activation of p53. Under oxidative stress, p53 inhibits the transcription of SIRT1 (Hwang et al. 2013; Nemoto et al. 2004).

The promotion of SIRT1 transcription, as well as its activity, are both increased under hypoxic conditions in a HIF-dependant manner (Hwang et al. 2013; R. Chen et al. 2011). In addition to the expression of SIRT1 being regulated by both HIF-1 α and HIF-2 α , it also is responsible for regulating the acetylation status of both HIFs. When HIF-1 α is deacetylated by SIRT1, its transactivation is suppressed, while the deacetylation of HIF-2 α leads to its transcriptional activation. This is one of several instances where SIRT1 has been shown to be partially responsible for its own regulation (Hwang et al. 2013; Dioum et al. 2009; Lim et al. 2010).

One of the more interesting factors that SIRT1 is responsible for regulating is cellular senescence, which it has been shown to decrease in the past (Wang et al. 2011; Ota et al. 2006; Hwang et al. 2013; Bai et al. 2008; Braidy et al. 2011; Zschoernig and Mahlknecht 2008; Solomon et al. 2006; Oppenheimer et al. 2012; Pillarisetti 2008; Kim et al. 2012). SIRT1 has been shown to decrease cellular senescence by several different

means; it has been shown to be partially responsible for the maintenance of telomeres in conjunction with the DNA repair enzyme XRCC6 (Hwang et al. 2013; Kim et al. 2012). Telomere shortening is believed to be one of the factors involved in aging; maintaining the length of these telomeres is critical to maintaining longevity of cells, and SIRT1 has been shown to increase cell longevity by maintaining these telomeres (Kim et al. 2012). An alternate method with which SIRT1 has been shown to decrease cellular senescence is by regulating the cellular response to oxidative stress, through the regulation of proteins induced by the ARE, such as , p53 and FOXO3 (Figure 1.2) (Hwang et al. 2013; Kawai et al. 2011; Yao and Rahman 2012; Furukawa et al. 2007; Tsuji et al. 2004; Tsuji et al. 2006).

Figure 1.2: The regulation of SIRT1 under oxidative stress conditions, and its regulation of transcription factors and histones which lead to increased cellular senescence. Diagram taken from (Hekimi et al. 2011).

1.6 Cellular senescence

One of the earliest theories used to explain why cells and multicellular organisms undergo aging, is the oxidative stress theory of aging (Harman 1956; Harman 1972). This theory proposes that ROS would cause damage to mitochondrial DNA, resulting in the increased release of ROS from the mitochondria and creating a cycle of ROS damage and production. This, in turn, would result in lipid, protein and DNA damage which would lead to cellular senescence. While this feedback cycle of mitochondrial ROS damage and production has been extensively studied, in recent years it has been proposed that ROS are not the cause of senescence, but rather a symptom that act as stress signalling molecules in response to the damages of aging (Hekimi et al. 2011; Lagouge and Larsson 2013). As signaling molecules, ROS activate pathways which modulate the activity of transcription factors such as NF- κ B, FOXO, p53, HIF-1 α and Nrf2 (Hekimi et al. 2011; Hamanaka and Chandel 2010; Trachootham et al. 2008).

It has been shown that ROS are not just damaging to cells, but in some situations an increased amount of ROS has been shown to be beneficial to the prolonging of life in *Caenorhabditis elegans*, and *Heterocephalus glaber* (Hekimi et al. 2011; Feng et al. 2001; Yang and Hekimi 2010; Andziak et al. 2006; Andziak and Buffenstein 2006). The effects of ROS seem to be very inconsistent between species that have different antioxidant genes knocked out. For example, in mice and flies, the knockout of superoxide dismutase 2 (SOD-2) was lethal while in *C. elegans* it dramatically prolonged life (Hekimi et al. 2011; Elchuri et al. 2005; Li et al. 1995; Doonan et al. 2008; Van Raamsdonk and Hekimi 2009). This lead to the proposal that the damaging effects of

ROS would increase lifespan to a point where it reaches a threshold that leads to a dramatic decrease in lifespan (Figure 1.3) (Van Raamsdonk and Hekimi 2009).

1.7 Cell lines

The interactions of Nrf1 and SIRT1 were investigated in both a cancerous liver cell line (human hepatocellular carcinoma (HepG2) cells) and a non-cancerous liver cell line (primary human hepatocyte (HF-P) cells) for their effects on cell senescence, as well as human embryonic kidney (HEK293) cells. HEK293 cells were chosen for this study because they are a model cell line in which the presence of Nrf1 has already been observed, as well as its excellent ability to accept external DNA by means of transfection (Chepelev et al. 2011; Thomas and Smart 2013). HepG2 and HF-P cells were chosen for a cancer versus non-cancer comparison as well as their endogenous ARE activity (Prasad et al. 2013). Liver cells were also chosen as well as their easily detectable senescence with respect to SIRT1 activity (Yu et al. 2013).

Figure 1.3: Proposed model relating mitochondrial function and lifespan in *sod-2* mutant worms. *N2* and *clk-1* worms, mitochondrial function is decreased leading to compensatory changes which increase lifespan. Deleting *sod-2* in *clk-1* worms further decreases mitochondrial function towards that of *isp-1* worms with a coincident increase in lifespan. Deleting *sod-2* in *isp-1* worms decreases mitochondrial function past a threshold after which the organism is no longer able to compensate for the degree of mitochondrial impairment and lifespan decreases. Diagram taken from Van Raamsdonk and Hekimi 2009.

1.8 Hypothesis and objectives

The acetylation of Nrfl may be a primary factor in its regulation. Likewise, the removal of that acetylation by SIRT1 may be a primary regulator. It is the objective of this study to 1) examine if there is any acetylation sites on Nrfl, 2) determine if SIRT1 is responsible for the deacetylation of Nrfl, 3) determine what effect acetylation/deacetylation has on the activity of Nrfl, 4) determine what effects hypoxia has on the activity of Nrfl when it is deacetylated 5) identify the lysine residues that are targeted for acetylation and 6) examine the effects that the acetylation/deacetylation of Nrfl has on cellular senescence. Our hypothesis is that Nrfl is acetylated and its activity is regulated through its deacetylation by SIRT1.

Chapter 2: Materials and methods

2.1 Cells

Two hepatocyte cell lines were used; a hepatocellular carcinoma cell line (HepG2) (ATCC, Manassas, VA), and human hepatocytes isolated from whole livers (HP-F) (Zen-Bio Inc., Raleigh, NC). HepG2 cells were cultured in alpha Minimal Essential Media (α MEM) supplemented with 10% fetal bovine serum (FBS) (Gibco, Grand Island, NY). HP-F cells were plated with Hepatocyte Plating Media (Cat.# HM-1, Zen-Bio Inc.) and were maintained with Hepatocyte Maintenance Medium (Cat.# HM-2, Zen-Bio Inc.).

2.2 Expression constructs

The human Nrf1 gene was inserted into the CMV-5a-FLAG expression vector (Sigma-Aldrich, St. Louis, MO) to give an N-terminally FLAG tagged protein and has been described previously (Chepelev et al. 2011). FLAG-SIRT1 inserted into pECE expression vector was obtained from Addgene (Cat.# 1791, Cambridge, MA). The truncated p65 form of Nrf1 inserted into the pEF1-V5-His expression vector, a luciferase reporter plasmid containing the EpRE of the GCLC gene and a β -galactosidase expression vector were acquired from Dr. Henry Jay Forman (University of California, Davis School of Medicine). Expression vectors were transfected into the cells using Lipofectamine 2000 (Life Technologies Inc., Burlington ON) according to the protocols provided.

2.3 Detection of Nrf1 acetylation

HepG2 cells were plated at a density of approximately 2.5×10^6 cells per 100mm plate, grown for 24 hours then harvested and lysed in 1ml of lysis buffer (50 mM Tris-HCl, pH 7.4, with 150 mM NaCl, 1 mM EDTA, and 1% Triton^{TM} X-100). An immunoprecipitation was done on 500 μ g of total protein lysate using anti-Nrf1 (H4) mouse IgG (sc-28379 Santa Cruz Biotechnology, Santa Cruz, CA) and protein A/G PLUS-Agarose Immunoprecipitation Reagent (sc-2003 Santa Cruz Biotech, Santa Cruz, CA) following the manufacturer's protocol. The immunoprecipitation product was run on a 10% SDS-PAGE gel and transferred to PVDF membrane (Millipore, Billerica, MA). The membrane was probed for acetyl-lysine, using 1:5,000 anti-acetyl-lysine rabbit IgG (9441, Cell Signalling, Danvers, MA) and 1:4,000 goat anti-rabbit IgG-HRP (sc-2768, Santa Cruz Biotechnology, Santa Cruz, CA) diluted in 5% blocking solution (50mM Tris,

150mM NaCl and 0.05% Tween-20, 5% Carnation milk). The membrane was stripped following the Abcam (Cambridge, MA) stripping protocol, then probed for Nrfl, using 1:500 anti-Nrfl (H4) mouse IgG and 1:5,000 goat anti-mouse IgG-HRP (Dako, Burlington, ON).

2.4 Luciferase reporter assay

Approximately 8.0×10^5 HepG2 or HEK293 cells were plated 24 hours prior to transfection with Lipofectamine 2000 (Life Technologies Inc., Burlington ON), in each well of a 6 well plate. In each well 2 μ g of GCLC luciferase reporter plasmid and 0.4 μ g of β -galactosidase vector were transfected, with the addition of one or a combination of the following: 12 μ g pCR3.1, 4 μ g N-FLAG-Nrfl, 4 μ g FLAG-SIRT1 and 4 μ g V5-p65-Nrfl. Varying amount of pCR3.1 vector were added to the wells to make each well contain 14.4 μ g of total DNA. After 24 hours transfection media was removed and replaced with fresh media. After another 24 hours, the cells were harvested and the assay was performed as previously described (Chepelev et al. 2011) with the following alterations: for the β -galactosidase assay 50 μ l of each luciferase lysate was added to 85 μ l of β -galactosidase assay buffer (0.2mg/ml CPRG, 60mM Na₂HPO₄, (pH 8.0), 10mM KCl, 1mM MgCl₂, and 1mM DTT) for 1-2 hours and measured at 580nm. Each transfection treatment had 3 sub-replicates and was repeated 5 times, values presented are means $\pm$ SEM.

2.5 RNA Extraction, reverse transcriptase-PCR and quantitative RT-PCR

Total RNA was extracted from approximately 2.0×10^6 HepG2 cells transfected with 6 μ g pCR3.1, 4 μ g FLAG-SIRT1 and/or 2 μ g N-FLAG-Nrfl for 48 hours, using RNeasy® Mini Kit and QIAshredder (QIAGEN, Toronto, ON), and cDNA was

synthesised from 1µg of total RNA from each treatment using iScript cDNA Synthesis Kit (Bio-Rad, Mississauga, ON). PCR primers are as follows: MT1-forward (5'-CAC TGG CTC CTG CAA ATG-3'), MT1-reverse (5'-ACT TCT CTG ATG CCC CTT TG-3'), 18sRNA-forward (5'-TCA ACT TTC GAT GGT AGT CGC CGT-3') and 18sRNA-reverse (5'-TCC TTG GAT GTG GTA GCG GTT TCT-3'). Quantitative real-time PCR was performed using iQ SYBR Green Supermix (Bio-Rad) and 2µl of cDNA.

2.6 Site directed mutagenesis

Lysine acetylation targets were identified using post-translational modification identification software (PhosphoSitePlus). Targeted lysine residues were mutated into arginine with the primers found in Table 2.1. These mutations were made using the QuikChange II Site-Directed Mutagenesis Kit, from Agilent (Mississauga, ON).

2.7 Senescence associated β -galactosidase activity

To assess the effects that the deacetylation of Nrf1 has on cellular senescence, primary human hepatocytes (HP-F) were transfected with 0.5µg of pCR3.1, Nrf1, SIRT1, p65-Nrf1 or the mutants, K205R and K629R, which showed decreased Nrf1 activity. Cellular senescence was observed by allowing the cells to grow for 144 hours, before an assay was performed. Senescence associated β -galactosidase activity was detected using the X-Gal Chromogenic Assay (Debacq-Chainiaux et al. 2009). Cells were rinsed twice with PBS for 30 seconds, fixed in 2% formaldehyde (vol/vol) and 0.2% glutaraldehyde (vol/vol) for 5 minutes, the fixation solution was removed and the cells were washed twice with phosphate buffered saline (PBS) for 30 seconds. The cells were then stained overnight at 37°C with X-Gal staining solution (40 mM citric acid/Na phosphate buffer, 5 mM $K_4[Fe(CN)_6] \cdot 3H_2O$, 5 mM $K_3[Fe(CN)_6]$, 150 mM sodium chloride, 2 mM

magnesium chloride and 1 mg/ml X-gal in distilled water), the cells were again washed twice with PBS for 30 seconds and once with methanol, and the cells were left to air dry. Cells were counted and photographed using phase contrast; every viable cell was counted for each treatment, the percent of cells expressing SA- β -galactosidase activity was determined by dividing the number of SA- β -galactosidase cells by the total number of cells.

2.8 Statistical analysis

All values displayed represent the mean $\pm$ the standard error of the mean (SEM) for three to five independent experiments. All statistical comparisons were made relative to the controls using one-way ANOVA followed by the post-hoc Tukey's test, for multiple comparisons. Values were considered to be significantly different from one another if $p < 0.05$.

Table 2.1: Oligonucleotide primers used for site directed mutagenesis.

Primer Name	Sequence 5'-3'
FWD K70R	TATGGTATCCACCCCAGGAGCATAGACCTGGAC
RVS K70R	GTCCAGGTCTATGCTCCTGGGGTGGATACCATA
FWD K205R	GAGCAGGATGTGGAGAGGGAGCTGCGAGATGGA
RVS K205R	TCCATCTCGCAGCTCCCTCTCCACATCCTGCTC
FWD K622R	CGAGCCCGAGCCATGAGGATCCCTTTCACCAAT
RVS K622R	ATTGGTGAAAGGGATCCTCATGGCTCGGGCTCG
FWD K629R	CCCTTTCACCAATGACAGAATCATCAACCTGCCTG
RVS K629R	CAGGCAGGTTGATGATTCTGTCATTGGTGAAAGGG
FWD K644R	CAATGAACTGCTGTCCAGATACCAGTTGAGTGAAGC
RVS K644R	CGTTCACTCAACTGGTATCTGGACAGCAGTTCATTG
FWD K675R	AACTGCCGCAAGCGCAGGCTGGACACCATCCTG
RVS K675R	CAGGATGGTGTCCAGCCTGCGCTTGCGGCAGTT
FWD K713R	CGACAGATGAAGCAGAGGGTCCAGAGCCTGTAC
RVS K713R	GTACAGGCTCTGGACCCTCTGCTTCATCTGTCG

Chapter 3: Results

3.1 Nrf1 is acetylated in mammalian cells

To determine the absence or presence of acetyl groups on Nrf1, both HEK293 and HepG2 cells were harvested in the conditions described (Figures 3.1, 3.2 and 3.3).

Acetylation was observed endogenously in HepG2 and to a lesser extent in HEK293. To increase the acetylation signal from Nrf1, both cell lines were incubated for 24 hours with 10mM nicotinamide (NAM) to inhibit SIRT1, (Figures 3.2 and 3.3). Whole cell lysates were first immunoprecipitated with a Nrf1 antibody to isolate Nrf1, and then an acetyl-lysine antibody was used to probe for acetylated Nrf1 in a Western blot. The membrane was stripped of all antibodies and reprobed for Nrf1 to confirm which acetyl-lysine band belonged to Nrf1.

Figure 3.1: Nrf1 is acetylated in HepG2 and HEK293 cells under normal conditions. HepG2 (A.) and HEK293 (B.) cells harvested from a 10 cm plate at approximately 90% confluency were lysed using lysis buffer (50 mM Tris-HCl, pH 7.4, with 150 mM NaCl, 1 mM EDTA, and 1% Triton^{TM} X-100). An immunoprecipitation was performed overnight using whole cell lysates (500µg), proteins were precipitated with anti-Nrf1 IgG antibody (H4) (2µg, sc-28379, Santa Cruz Biotechnology Inc.) and protein A/G PLUS-Agarose Immunoprecipitation Reagent (sc-2003, Santa Cruz Biotechnology Inc.) as described in Materials and Methods. A Western blot with the IP samples was performed, the blot was first probed with anti-acetyl-lysine antibody (1:5,000 in 5% blocking solution, 9441, Cell Signaling Tech. Inc.). The blot was stripped and reprobed with anti-Nrf1 antibody (1:500) to confirm the location and identity of the acetylated band.

3.2 Inhibition of SIRT1 results in increased Nrf1 acetylation

The effects of overexpressing SIRT1 on the acetylation of Nrf1 were observed by co-expressing SIRT1 and Nrf1 with and without 10mM NAM, to observe changes in Nrf1 acetylation. Endogenous and over expressed Nrf1 in HEK293 cells showed very weak acetylation signal. When SIRT activity was inhibited with 10mM NAM an increase in acetylation of Nrf1 was observed. The increase of Nrf1 acetylation (Figures 3.2 and 3.3) indicates, quite strongly, that SIRT1 is an enzyme responsible for regulating the deacetylation of Nrf1. A possible reason for the weak acetylation signal in endogenous Nrf1 in HEK293 cells is a likely indication that SIRT1 is actively deacetylating Nrf1 under normal conditions. When activating SIRT1 with 50 μ M Res or overexpressing SIRT1 in HEK293 cells (Figures 3.2 and 3.3) there was minimal decrease in Nrf1 acetylation from the control cells, a fact attributed to the high endogenous activity of SIRT1 under normal conditions in HEK293 cells. When these same treatments were applied to the HepG2 cells an increase in the acetylation of Nrf1 was observed in both NAM treatments, however neither treatment resulted in a statistically significant change in the acetylation of Nrf1 from the controls (Figures 3.2 and 3.3).

Figure 3.2: Co-expression of SIRT1 results in a decrease and 10mM NAM results in increase of acetylation on Nrf1. HEK293 (top) and HepG2 (bottom) cells overexpressing Nrf1, Nrf1 with 10mM NAM, Nrf1 and SIRT1, and Nrf1 SIRT1 with 10mM NAM were harvested from 6cm plates 48hours after transfection. Cell pellets were lysed using lysis buffer (50 mM Tris-HCl, pH 7.4, with 150 mM NaCl, 1 mM EDTA, and 1% Triton^{TM} X-100). An immunoprecipitation was performed overnight using whole cell lysates (500µg), proteins were precipitated with anti-Nrf1 IgG antibody (H4) (2µg, sc-28379, Santa Cruz Biotechnology Inc.) and protein A/G PLUS-Agarose Immunoprecipitation Reagent (sc-2003, Santa Cruz Biotechnology Inc.) as described in Materials and Methods. A Western blot with the immunoprecipitated samples was performed. The blot was first probed with anti-acetyl-lysine antibody (1:5,000 in 5% blocking solution, 9441, Cell Signaling Tech. Inc.) and then stripped and reprobed with anti-Nrf1 antibody (1:500) to be used as a loading control, also to confirm the location and identity of the acetylated band. Blots shown are representative of three separate experiments. The integrated density values of the acetylated bands were normalized to their respective Nrf1 bands to acquire a relative signal. Values shown are the means normalized to the control $\pm$ SEM. N=3, (*) indicates significance ($p < 0.05$).

Figure 3.3: Resveratrol 50 μ M does not alter Nrf1 acetylation while 10mM NAM increases it in Nrf1. HEK293 (top) and HepG2 (bottom) cells treated with DMSO, 50 μ M Res and 10mM NAM, were harvested from 6cm plates 48hours after transfection. The pellets were lysed using lysis buffer (50 mM Tris-HCl, pH 7.4, with 150 mM NaCl, 1 mM EDTA, and 1% Triton^{TM} X-100). An immunoprecipitation was performed overnight using whole cell lysates (500 μ g), proteins were precipitated with anti-Nrf1 IgG antibody (H4) (2 μ g, sc-28379, Santa Cruz Biotechnology Inc.) and protein A/G PLUS-Agarose Immunoprecipitation reagent (sc-2003, Santa Cruz Biotechnology Inc.) as described in Materials and Methods. A Western blot with the immunoprecipitated samples was performed, the blot was first probed with anti-acetyl-lysine antibody (1:5,000 in 5% blocking solution, 9441, Cell Signaling Technology Inc.). The blot was stripped and reprobed with anti-Nrf1 antibody (1:500) to be used as a loading control, also to confirm the location and identity of the acetylated band. Blots shown are representative of three separate experiments. The integrated density values of the acetylated bands were normalized to their respective Nrf1 bands to acquire a relative signal. Values shown are the means normalized to the control $\pm$ SEM. N=3, (*) indicates significance (p<0.05).

3.3 Overexpression of SIRT1 affects metallothioneine 1 mRNA transcription

In previous studies it was shown that Nrf1 is the exclusive ARE promoter of metallothioneine 1 (MT1) (Ohtsuji et al. 2008). We therefore decided to take advantage this fact by using MT1 mRNA expression as a marker of Nrf1 activity. HEK293 and HepG2 cells were transfected with Nrf1 and SIRT1, harvested and total RNA was extracted to create cDNA which was used as a template for MT1 real-time PCR. Overexpression of Nrf1 alone results in a 2-fold increase in MT1 gene expression over the control HEK293 cells (Figure 3.4), and a 2.5-fold increase in HepG2 cells (Figure 3.5), indicating the validity of MT1 as a marker of Nrf1 activity in mammalian cells. To examine the regulatory effects of SIRT1 on Nrf1, we co-expressed SIRT1 and Nrf1, and found that MT1 gene expression decreased to 0.6 and 0.56 in HEK293 and HepG2 cells respectively relative to their respective controls. This 75 and 77% decrease respectively in MT1 expression from Nrf1 alone, indicated that the deacetylation of Nrf1 inhibits its transcriptional promoter activity significantly, and that SIRT1 is one of the main regulators of Nrf1's acetylation.

Figure 3.4: Co-expression of Nrf1 and SIRT1 results in decreased MT1 gene expression in HEK293 cells. Total RNA was extracted from HEK293 cells transfected with pCR3.1, Nrf1 or Nrf1 and SIRT1, in 6 well plates, as described in Materials and Methods. Complementary DNA was produced using the iScript^{TM} cDNA Synthesis Kit (Bio-Rad Laboratories Inc.) from 2 μ g of total RNA. Real time-PCR using the MT1 and 18S primers described in Materials and Methods was performed using the IQ^{TM} SYBR® Green 2X Supermix Kit (Bio-rad Laboratories Inc.) with 2 μ l of the cDNA used as the template DNA for the MT1 genes and 2 μ l of 1 in 10,000 diluted cDNA for the 18S genes (in order to achieve a critical threshold value between 10 and 30 cycles). Values shown are the means normalized to the control $\pm$ SEM. N=3, (*) indicates significance (p<0.05).

Figure 3.5: Co-expression of Nrf1 and SIRT1 results in decreased MT1 gene expression in HepG2 cells. Total RNA was extracted from HepG2 cells transfected with pCR3.1, Nrf1 or Nrf1 and SIRT1, in 6 well plates, as described in Materials and Methods. Complementary DNA was produced using the iScript^{TM} cDNA Synthesis Kit (Bio-Rad Laboratories Inc.) from 2µg of total RNA. Real time-PCR using the MT1 and 18S primers described in Materials and Methods was performed using the IQ^{TM} SYBR® Green 2X Supermix Kit (Bio-rad Laboratories Inc.) with 2µl of the cDNA used as the template DNA for the MT1 genes and 2µl of 1 in 10,000 diluted cDNA for the 18S genes (in order to achieve a critical threshold value between 10 and 30 cycles). Values shown are the means normalized to the control $\pm$ SEM. N=3, (*) indicates significance ($p < 0.05$).

3.4 Regulation of Nrf1 activity by nicotinamide

Since we showed that Nrf1 is acetylated and that NAM treatment increases Nrf1 acetylation, we decided to examine what effect this acetylation has on Nrf1's activity. HEK293 and HepG2 cells were treated with increasing concentrations of NAM and Nrf1's activity was observed via the luciferase reporter assay. The cells were harvested and lysed after incubating for 24 hours with NAM, then 2mM ATP and 200 μ M luciferin was added to the whole cell lysates and the luminescence was measured. Contrary to our hypothesis, when SIRT1 was inhibited with high concentrations of NAM the activity of Nrf1 decreased in HEK293 cells (Figure 3.6). This decrease in activity was unexpected since other experiments showed SIRT1 having an inhibitory effect on Nrf1, one would have expected this inhibition to disappear once SIRT1 was inhibited by NAM. Instead the opposite was observed. These results (Figure 3.6) could indicate a previously unobserved effect that NAM has on the activity of Nrf1, which is independent of SIRT1 regulation. When the same concentrations of NAM were applied to HepG2 cells the activity of Nrf1 did not change until the 25mM NAM was added to the cells, which is when we saw a decrease in the activity of Nrf1. The fact that we see such little change in the activity of Nrf1 in HepG2 cells does not necessarily indicate that SIRT1 does not inhibit Nrf1; instead it lends more credence to the theory that presented above, that Nrf1 and NAM interact independently of SIRT1. An alternate theory could be that there are redundancies built into the cells to keep the activity of Nrf1 low even when SIRT1 is not active.

Figure 3.6: NAM causes a slight increase in Nrf1 activity in HepG2 and a decrease in HEK293 cells. HepG2 and HEK293 cells transfected with 4 μ g of Nrf1, 2 μ g GCLC luciferase promoter and 0.4 μ g β -galactosidase in 6 well plates were treated with increasing concentrations of NAM 24 hours post transfection, for an additional 24 hours, then luciferase and β -galactosidase assays were performed, as described in Materials and Methods. The values shown are the averages of the normalized luciferases values divided by the β -galactosidase assay values, providing the luciferase activity relative to the cell transfected with only Nrf1. The error bars are the $\pm$ SEM. N=3, (*) indicates significance ($p < 0.05$).

3.5 Regulation of Nrf1 activity by resveratrol

When activating SIRT1 using Res, little change was observed in Nrf1 acetylation in HEK293 and HepG2 cells (Figure 3.3). Since little change was observed when SIRT1 was activated with Res (Figure 3.3) we wished to examine the effects that varying concentrations of Res would have on Nrf1 activity in HEK293 and HepG2 cells. Cells were treated with increasing concentrations of Res. A luciferase assay was performed on whole cell lysates of both cell lines, after 24 hours incubation with Res, Nrf1 activity was measured. Based on our hypothesis Res was expected to lower the activity of Nrf1 in both cell lines by activating SIRT1. Surprisingly there was no decrease in the activity of Nrf1 even through the highest concentrations of Res (Figure 3.7). This was observed in both cell lines and slightly opposes our hypothesis, in that Res did not lower the activity of Nrf1. However the lack of a significant change in Nrf1 activity could be attributed to the high basal deacetylation of Nrf1 by SIRT1, so increasing SIRT1's activity with Res did not seem to lead to an increased inhibitory effect, which supports the observations of Figure 3.3. The reason that SIRT1 is most likely actively inhibiting Nrf1 in the cells endogenously would be due to the fact that the treated cells were not exposed to any additional oxidative stress which would require high Nrf1 activity, and this has been shown to be the case that under high oxidative stress conditions the activity of SIRT1 is reduced (Hwang et al. 2013; Houtkooper et al. 2012).

Figure 3.7: Resveratrol has no significant effect on Nrf1 activity in HepG2 and HEK293 cells. HepG2 and HEK293 cells transfected with 4 μg of Nrf1, 2 μg GCLC luciferase promoter and 0.4 μg β -galactosidase in 6 well plates were treated with increasing concentrations of Res 24 hours post transfection, for an additional 24 hours, then luciferase and β -galactosidase assays were performed, as described in Materials and Methods. The values shown are the averages of the normalized luciferases values divided by the β -galactosidase assay values, providing the relative luciferase activity relative to the cell transfected with only Nrf1. The error bars are the $\pm$ SEM. N=3, (*) indicates significance ($p < 0.05$).

3.6 SIRT1 inhibits full length Nrf1 but not p65-Nrf1

The real time RT-PCR results (Figures 3.4 and 3.5) showed the inhibition of Nrf1 by SIRT1 through the inhibition of MT1 gene expression. To reconfirm and extrapolate on these results, luciferase assays were performed using the GCLC promoter region to activate luciferase expression, which has been shown previously to be regulated by Nrf1 (Yang et al. 2005; Ohtsuji et al. 2008; L. Chen et al. 2003; Wang et al. 2007). Both HEK293 and HepG2 cells transfected with Nrf1 alone showed an increase in luciferase activity by approximately 2.5-fold over the negative control, indicating the assays validity. When a co-expression of Nrf1 and SIRT1 was performed, a significant decrease was seen in both cell lines. This decrease confirms what the MT1 gene expression originally showed; that SIRT1 is responsible for the inhibition of Nrf1 activity by means of deacetylation. The short form of Nrf1, p65-Nrf1, has been shown in previous papers to be a competitive inhibitor of Nrf1 (Wanget al. 2007). We hypothesised that in addition to deacetylating full length Nrf1 SIRT1 might also deacetylate p65-Nrf1 resulting in a greater inactivation of Nrf1. While this effect was seen in the HepG2 cells in a small amount, in HEK293 no compounding inhibitory effect was seen when combining both SIRT1 and p65-Nrf1, which most likely indicates that SIRT1 and p65-Nrf1 act independently of one another to inhibit full length Nrf1's activity in HEK293 cells but act together in HepG2 cells. Further investigation will be required to confirm this postulation.

Figure 3.8: SIRT1 inhibits Nrf1 activity in HepG2 cells, and increases the inhibitory effect of p65-Nrf1. The co-transfection of Nrf1, SIRT1 and p65 resulted in a significant decrease in Nrf1 activity relative to the SIRT1, Nrf1 and p65, Nrf1 co-expressions, indicating that SIRT1 regulates the competitive inhibition of Nrfs through the deacetylation of p65-Nrf1 in HepG2 cells. HepG2 cells were transfected with 2 μ g GCLC luciferase promoter and 0.4 μ g β -galactosidase, with a pCR3.1 empty vector, 4 μ g Nrf1, 4 μ g SIRT1 or 4 μ g p65-Nrf1 in different combinations in 6 well plates, the total amount of DNA transfected into each well was 14.4 μ g, the difference was made up with pCR3.1, 48 hours post-transfection luciferase and β -galactosidase assays were performed, as described in Materials and Methods. The values shown are the averages of the normalized luciferases values divided by the β -galactosidase assay values, providing the relative luciferase activity relative to the cell transfected with pCR3.1 alone. Plotted are means and $\pm$ SEM. N=5. Treatments not grouped together with the same letter are considered statistically significant from one another ($p < 0.01$).

Figure 3.9: SIRT1 inhibits Nrf1 activity in HEK293 cells, but has little effect on the inhibitory effects of p65-Nrf1. The co-transfection of Nrf1, SIRT1 and p65 resulted in no significant change in Nrf1 activity relative to the SIRT1, Nrf1 and p65, Nrf1 co-expressions, indicating that SIRT1 does not seem to regulate the acetylation status of p65-Nrf1 in HEK293 cells. HEK293 cells were transfected with 2µg GCLC luciferase promoter and 0.4µg β-galactosidase, with a combination of one or some of pCR3.1 empty vector, 4µg Nrf1, 4µg SIRT1 or 4µg p65-Nrf1 in 6 well plates, the total amount of DNA transfected into each well was 14.4µg, the difference was made up with pCR3.1, 48 hours post-transfection luciferase and β-galactosidase assays were performed, as described in Materials and Methods. The values shown are the averages of the normalized luciferases values divided by the β-galactosidase assay values, providing the relative luciferase activity relative to the cell transfected with only pCR3.1. Shown are means and ± SEM. N=5. Treatments not grouped together with the same letter are considered statistically significant from one another (p<0.01).

3.7 Hypoxia results in a change in Nrf1 activity independent of SIRT1

Previous research done by our lab has shown that under hypoxic conditions in HEK293 and COS7 cells, the activity of Nrf1 significantly increases (Chepelev et al. 2011). The effect of hypoxia on Nrf1 was never examined with a cancerous cell line such as HepG2, also the effect that hypoxia has on SIRT1's regulation of Nrf1 was never examined closely. Examining the effects of hypoxia on Nrf1 in HepG2 cells, as well as SIRT1's regulation of Nrf1 required the use of a luciferase assay to examine what changes occur under these conditions. Interestingly unlike the transformed cell lines of HEK293 and COS7 the activity of Nrf1 in HepG2 cells decreased dramatically in both the empty vector and the overexpressed Nrf1 transfected cells (Figure 3.10). This reversal of the trend, seen in HEK293 cells, could be a tissue specific response or could be due to the cancerous nature of the HepG2 cells. Whether this inhibition of Nrf1 under hypoxia is a defense measure or a down regulation of a gene that is not required under hypoxia in HepG2 cells is still unclear and requires further research to examine this phenomenon.

When SIRT1 was co-expressed with Nrf1 an interesting trend appeared. The SIRT1 seems to negate the effects of the hypoxia on Nrf1's activity both in HepG2 and in HEK293 cells (Figures 3.10 and 3.11). In HepG2 cells we saw a decrease in Nrf1's activity under hypoxia but with SIRT1 co-expressed no significant change is observed between hypoxia and normoxia (Figure 3.10). This could indicate that the cause of the decreased Nrf1 activity under hypoxia without SIRT1 is caused by the deacetylation of Nrf1, explaining the lack of change when SIRT1 is co-expressed (Figure 3.10). An alternative cause for the lack of change could be that SIRT1's inhibition of Nrf1 is a stronger regulator of Nrf1 activity than hypoxia so with the addition of hypoxia little

change is observed. In HEK293 cells we see a dramatic increase in Nrf1 activity under hypoxia, but when SIRT1 is co-expressed with Nrf1 that increase no longer becomes significant. Once again this lack of change in hypoxic conditions could indicate that SIRT1 is a stronger regulator of Nrf1 activity than hypoxia.

3.8 Location of acetylation sites on Nrf1

To more closely examine the effects that acetylation has on Nrf1's activity, the specific lysine residues that are targeted for acetylation on Nrf1 needed to be identified. Since we had already shown that the deacetylation of Nrf1 by SIRT1 resulted in a decrease in Nrf1 activity, mutating the possible lysine targets to arginine, a similarly charged and sized amino acid, should yield a decrease in Nrf1 activity relative to the wild type sequence in the same expression vector. Of the seven mutants created two showed a significant decrease in Nrf1 activity such that their activity was indiscernible from the empty vector control, indicating these mutants to be biologically inactive and the most likely sites for Nrf1 acetylation. In HEK293 cells, only mutant lysine-205-arginine was seen to be inactivated (Figure 3.13), while in HepG2 cells both lysine-205-arginine and lysine-629-arginine mutants were seen to be inactivated (Figure 3.12). The additional acetylation site in HepG2 cells could indicate one of two things. The regulation of Nrf1 activity via acetylation varies from tissue to tissue since HEK293 originated from embryonic kidney tissue while HepG2 originated from hepatocellular carcinoma tissue. Alternatively, this additional site could only be acetylated in cancerous cells, which could be a contributing factor to the proliferation or survival of cancer cells. Further investigation into these possibilities will be required.

Figure 3.10: Hypoxia decreases Nrf1 activity in HepG2 cells, while it has little effect in the presence of SIRT1. HepG2 cells were transfected with 2 μ g GCLC luciferase promoter and 0.4 μ g β -galactosidase, with a combination of one or some of pCR3.1 empty vector, 4 μ g Nrf1, or 4 μ g SIRT1 and Nrf1 in 35mm plates, the total amount of DNA transfected into each well was 10.4 μ g, the difference was made up with pCR3.1, twenty-four hours post transfection the cells were subjected to either 21 or 1% O₂ for twenty-four hours then luciferase and β -galactosidase assays were performed, as described in Materials and Methods. The values shown are the averages of the normalized luciferase values divided by the β -galactosidase assay values, providing the relative luciferase activity relative to the cell transfected with only pCR3.1 under normoxic conditions. The bars are means $\pm$ SEM. N=3. (*) indicates significance (p<0.05).

Figure 3.11: Hypoxia increases Nrf1 activity in HEK293 cells, while it has little effect in the presence of SIRT1. HEK293 cells were transfected with 2 μ g GCLC luciferase promoter and 0.4 μ g β -galactosidase, with a combination of one or some of pCR3.1 empty vector, 4 μ g Nrf1, or 4 μ g SIRT1 and Nrf1 in 35mm plates, the total amount of DNA transfected into each well was 10.4 μ g, the difference was made up with pCR3.1, twenty-four hours post transfection the cells were subjected to either 21 or 1% O₂ for twenty-four hours then luciferase and β -galactosidase assays were performed, as described in the experimental procedure. The values shown are the averages of the normalized luciferases values divided by the β -galactosidase assay values, providing the relative luciferase activity relative to the cell transfected with only pCR3.1 under normoxic conditions. The bars are means $\pm$ SEM. N=3. (*) indicates significance (p<0.05).

Figure 3.12: Mutants K205R and K629R Nrf1 showed a significant decrease in Nrf1 activity relative to the wild type in HepG2 cells. HepG2 cells were transfected with 2µg GCLC luciferase promoter and 0.4µg β-galactosidase, with either 4µg of pCR3.1, wild type Nrf1, K70R, K205R, K622R, K629R, K644R, K675R or K713R. Each transfection was done with and without 4µg of SIRT1, in 6 well plates, the total amount of DNA transfected into each well was 10.4µg. The difference was made up with pCR3.1, 48 hours post transfection the cells were harvested then luciferase and β-galactosidase assays were performed, as described in the experimental procedure. The values shown are the averages of the normalized luciferases values divided by the β-galactosidase assay values, providing the relative luciferase activity relative to the cell transfected with only pCR3.1 under normoxic conditions. The bars are means ± SEM. N=3. (*) indicates significance (p<0.05) relative to wild type Nrf1.

Figure 3.13: Mutant K205R Nrf1 showed a significant decrease in Nrf1 activity relative to the wild type in HEK293 cells. HEK293 cells were transfected with 2 μ g GCLC luciferase promoter and 0.4 μ g β -galactosidase, with either 4 μ g of pCR3.1, wild type Nrf1, K70R, K205R, K622R, K629R, K644R, K675R or K713R. Each transfection was done with and without 4 μ g of SIRT1, in 6 well plates, the total amount of DNA transfected into each well was 10.4 μ g. The difference was made up with pCR3.1, 48 hours post transfection the cells were harvested then luciferase and β -galactosidase assays were performed, as described in the experimental procedure. The values shown are the averages of the normalized luciferases values divided by the β -galactosidase assay values, providing the relative luciferase activity relative to the cell transfected with only pCR3.1 under normoxic conditions. The bars are means $\pm$ SEM. N=3. (*) indicates significance ($p < 0.05$) relative to wild type Nrf1.

3.9 Nrf1 plays a role in cellular senescence

Previous papers have shown that SIRT1 plays a role in decreasing cellular senescence (Wang et al. 2011; Ota et al. 2006; Hwang et al. 2013; Bai et al. 2008; Braidy et al. 2011; Zschoernig and Mahlknecht 2008; Solomon et al. 2006; Oppenheimer et al. 2012; Pillarisetti 2008), we wished to observe the role Nrf1 plays in cellular senescence, specifically by examining the effects of the deacetylation of Nrf1 by SIRT1. Examining these effects required the use of primary human hepatocyte cells transfected with either, pCR3.1, Nrf1, Nrf1 and SIRT1, SIRT1, K205R, K629R, p65-Nrf1, or nothing. Cells were treated for seven days, and then stained with X-Gal overnight. Both the endogenous and the pCR3.1 control cells showed roughly the same amount of cells undergoing senescence, with 34.3 and 32.8% of cells expressing senescence associated β -galactosidase (SA- β -galactosidase) activity respectively (Figure 3.14). When the cells were transfected with Nrf1 alone there was a slight decrease in SA- β -galactosidase activity to 27.5% positive cells. However this decrease was not statistically significant, indicating that biologically active Nrf1 does not play a role in senescence. Cells transfected with SIRT1 alone or co-expressed with Nrf1, showed a significant decrease in SA- β -galactosidase activity dropping the percentage of positive cells to approximately 19%, this decrease was expected based on previous literature (Wang, et al. 2011; Ota et al. 2006; Hwang et al. 2013; Bai et al. 2008; Braidy et al. 2011; Zschoernig and Mahlknecht 2008; Solomon et al. 2006; Oppenheimer et al. 2012; Pillarisetti 2008). If the deacetylation of Nrf1 plays a role in the decreased cellular senescence seen by SIRT1, then we would expect the transfection of the cells with the mutant forms of Nrf1 to show a similar percentage of cells expressing SA- β -galactosidase activity. The cells transfected

with either mutant K205R or K629R showed no statistical difference from the cells transfected with SIRT1, indicating that the deacetylation of Nrf1 at these precise sites by SIRT1 is one of the factors contributing to the decrease in cellular senescence seen when SIRT1 is activated in cells. Since previous papers have shown that Nrf1 is inhibited by its truncated p65-Nrf1 form, we used this to examine if p65 also plays a role in cellular senescence by inhibiting all Nrfs (Wang et al. 2007; Johnsen et al. 1996; Chepelev et al. 2011). After the cells were transfected with p65-Nrf1 we saw a significant decrease in SA- β -galactosidase activity from the pCR3.1 control, indicating that p65-Nrf1 does play a role in decreasing senescence, most likely by inhibiting Nrf1.

Figure 3.14: Inhibition of Nrf1 by p65-Nrf and both mutant K205R and K629R showed similar reduction of cellular senescence relative to SIRT1. Primary human hepatocyte cells isolated from liver were plated in a 96 well plate, after 24 hours they were transfected using 4.5 μ l of GenJet^{TM} ver. II (SignaGen Laboratories) and 0.5 μ g of pCR3.1, Nrf1, SIRT1, K205R, K629R or p65-Nrf1. The cells were grown for 144 hours to allow cellular senescence to occur, a SA- β -galactosidase activity assay was then performed as described in Materials and Methods. Every cell expressing SA- β -galactosidase activity was counted and divided by the total number of cells for each treatment to obtain the percentage of senescent cells. The values shown are the mean percentage of >350 cells/treatment $\pm$ SEM. (*) indicates significance ($p < 0.05$), (**) indicates significance ($p < 0.01$) relative to endogenous and pCR3.1 controls.

Chapter 4: Discussion

4.1 Nrf1 acetylation

Since it has been shown that SIRT1 regulates transcription factors, such as FOXO3, NF- κ B and Nrf2, by means of deacetylation, it was hypothesised that SIRT1 would also regulate Nrf1 in a similar manner (Hwang et al. 2013; Kawai et al. 2011). The reason Nrf1 was considered to be an exemplary target for deacetylation by SIRT1, was that it is a transcription factor critical for cell survival and it is in the same Cap-N-Collar family of transcription factors that Nrf2 is a member of (Kawai et al. 2011; L. Chen et al. 2003; Chan et al. 1998; Kobayashi et al. 2011).

For Nrf1 to be a target of direct SIRT1 regulation it has to be acetylated, so the first course of action was to determine if Nrf1 was acetylated. An IP to pull down endogenous Nrf1 was used in a Western blot to detect the acetylated band of Nrf1 (Figure 3.1). The fact that Nrf1 is acetylated can clearly be seen in both non-cancerous cells (HEK293) and cancerous cells (HepG2), indicating that it would be possible that SIRT1 could remove the acetyl groups from Nrf1 (Figure 3.1).

4.2 The deacetylation of Nrf1 by SIRT1

Now that we had a clear indication that Nrf1 is indeed acetylated it was necessary to determine if SIRT1 was responsible for the removal of the acetyl groups. Cells were transfected with and without SIRT1 to see if the acetylated band of Nrf1 would diminish in the presence of overexpressed SIRT1 (Figure 3.2). The band did not significantly decrease in the presence of an abundance of SIRT1. However, this did not necessarily rule out the possibility that SIRT1 was responsible for deacetylating Nrf1, but instead it

more likely indicates that SIRT1 is very active in the cells endogenously since the control Nrf1 band was already fairly weak.

Previous papers have examined the activity of SIRT1 using chemical regulators, such as the inhibitor NAM and the activator Res (Bai et al. 2008; Kawai et al. 2011; Wang et al. 2011; Zschoernig and Mahlknecht 2008; Hwang et al. 2013; Pillarisetti 2008; Peled et al. 2012; Lim et al. 2010). To examine the interaction of Nrf1 and SIRT1 from a different perspective, we inhibited SIRT1 with 10mM NAM. When the overexpressed SIRT1 was inhibited, a significant increase in the acetylation of Nrf1 was observed (Figure 3.2). This was the first strong indicator that SIRT1 was the primary protein responsible for the deacetylation of Nrf1. This trend was observed in both the HEK293 and the HepG2 cells (Figure 3.2). When these experiments were repeated with just chemical regulation of SIRT1, a similar trend was observed (Figure 3.3). The activator Res had little effect on the Nrf1 acetylation band, since it was already a fairly weak signal in the DMSO control cells it was not that surprising that activating SIRT1 would not result in a greater decrease in signal (Figure 3.3). This further supported our earlier hypothesis that SIRT1 is actively deacetylating Nrf1 endogenously, and once again a significant increase in Nrf1 acetylation was observed when SIRT1 was inhibited with NAM (Figure 3.3).

4.3 SIRT1 inhibits Nrf1 activity

Having a clear picture that Nrf1 was acetylated and that SIRT1 was responsible for its deacetylation lead to the examination of what effect this deacetylation had on the activity of Nrf1. Some of the most common ways of observing Nrf1 activity are to use the promoter region of different genes responsible for the antioxidant response as a target

for the DNA binding activity of Nrf1, in various assays (Yang et al. 2005; Ohtsuji et al. 2008; Chen et al. 2003; Wang et al. 2007; Kawai et al. 2011; Johnsen et al. 1998; Chepelev et al. 2011). These genes work well to measure the activity of Nrf1 however the majority of them are also regulated by Nrf2 which has already been shown to be inhibited by SIRT1, meaning that the change in Nrf1 activity seen in these assays when treating with SIRT1 could be a result of it inhibiting Nrf2 (Yang et al. 2005; Ohtsuji et al. 2008; Chen et al. 2003; Wang et al. 2007). Because of this, a novel method of detecting Nrf1 activity alone would be needed to initially determine what effect SIRT1 would have on its activity, then once its effect was known the traditional assays could be used to further examine their relationship.

A recent publication showed that unlike most ARE genes metallothionein 1 was solely regulated by Nrf1, and not Nrf2. Real-time RT-PCR primers were designed to detect the changes in MT1 gene expression, allowing us to look exclusively at the regulation of Nrf1 activity (Ohtsuji et al. 2008). When both HEK293 and HepG2 cells were co-transfected with SIRT1 and Nrf1 a significant decrease in Nrf1 activity was observed in the gene expression of MT1 relative to just the Nrf1 transfected cells (Figures 3.4 and 3.5). This decrease in MT1 gene expression indicated that the deacetylation of Nrf1 by SIRT1 inhibited the ability of Nrf1 to bind to the ARE and promote the gene expression of MT1, meaning that SIRT1 has a negative regulatory effect on the activity of Nrf1.

4.4 Chemical regulation of SIRT1

Knowing now that SIRT1 inhibits the activity of Nrf1, chemically regulating SIRT1 could be a novel means of altering the activity of Nrf1 *in vivo*. To examine how

effective chemical regulation of SIRT1 would be on the activity of Nrf1, a luciferase vector using the promoter for GCLC to promote luciferase production was used to measure Nrf1 activity, when cells were treated with increasing concentrations of chemical regulators of SIRT1 (Bai et al. 2008; Kawai et al. 2011).

In an interesting trend it was seen that the inhibition of SIRT1 with NAM resulted in no significant change in Nrf1 activity in HepG2 cells, while a decrease of between 40 and 50% was seen in HEK293 cells at concentrations at or above 10mM NAM (Figure 6). The fact that neither cells line experienced a significant increase in Nrf1 activity was contradictory to what was observed with the MT1 gene expression experiments (Figures 3.4, 3.5 and 3.6). This could indicate that there is ancillary mechanism to maintain the maximum activity of Nrf1 when cells are not undergoing oxidative stress, and their acetylation groups are removed. The cause of the decrease in the HEK293 cells could indicate that either this supplementary mechanism could be overcompensating for acetylation of Nrf1, leading to a decrease in activity. Alternatively this decrease could be a previously unobserved effect that NAM has on the activity of Nrf1, such as increasing levels of p65-Nrf1. Regardless of the correct scenario further examination will be required to more completely understand the effect that NAM has on the activity of Nrf1.

The effect that chemically activating SIRT1 had on the activity of Nrf1 was examined when the cells were treated with increasing concentrations of Res. No significant change in Nrf1 activity was observed with increasing concentrations of Res (Figure 3.7). The lack of change that was observed was somewhat expected after the results of the acetylation Western were obtained (Figure 3.3), since the acetylation band was so weak even at basal levels it indicated that the endogenous deacetylation of Nrf1

was already at very high levels, so further activating an already very active protein results in little further change. Recently the validity of Res as an activator of SIRT1 has been brought into question so the lack of change in the acetylation of Nrf1 when treated with Res could be a result of it not directly activating SIRT1 (Beher et al. 2009).

4.5 The interaction of SIRT1, Nrf1 and the truncated p65-Nrf1

The truncated form of Nrf1, p65-Nrf1, has been shown to be a competitive inhibitor of all three Nrf proteins, examining the role that SIRT1 plays in the regulation of this competitive inhibitor is an important area to examine (Wang et al. 2007). Examining this relationship required another GCLC luciferase assay, each of the three genes, SIRT1, Nrf1 and p65-Nrf1, were transfected separately and co-transfected to try and narrow down what role each of them played with regards to the activity of Nrf1. When either SIRT1 or p65-Nrf1 was co-transfected with Nrf1, they both showed an inhibition of Nrf activity to (statistically) the same extent. This was seen in both HEK293 and HepG2 cells (Figures 3.8 and 3.9). However when all three were co-transfected together, we saw a significant decrease in Nrf activity in HepG2 cells from either one of them individually co-transfected with Nrf1, but this decrease was not seen in HEK293 cells (Figures 3.8 and 3.9). This decrease in Nrf1 activity seems to indicate that SIRT1 and p65-Nrf1 work synergistically to inhibit the activity of Nrf1 by two different means 1) through the deacetylation of Nrf1 and 2) through the competitive inhibition of Nrf1 by removing available small Maf proteins. Also this competitive inhibition seems to be regulated by the deacetylation of p65-Nrf1. A similar trend was observed when SIRT1 and p65-Nrf1 were transfected together and when they were transfected on their own, in HepG2 cells the combination of the two resulted in a significant decrease from the p65-

Nrf1 alone, but in HEK293 cells no change was observed when the two were co-expressed (Figures 3.8 and 3.9). This could indicate that SIRT1 a) interacts differently with p65-Nrf1 in cancerous cells than in non-cancerous cells or b) it could be a tissue specific response. Further investigation will be needed to determine which scenario is the correct one.

4.6 The effect of SIRT1 on the regulation of Nrf1 by hypoxia

Previous research has shown that when HEK293 cells are exposed to 1% O₂ hypoxia for 24 hours, the activity of Nrf1 increases (Chepelev et al. 2011). The role that SIRT1 plays in this effect is unknown. Examining the interaction of Nrf1 and SIRT1 in hypoxia was a logical area to focus attention on. Since Nrf1 was shown to increase when HEK293 cells were treated with hypoxia, it was expected that it would behave similarly in HepG2 cells. However, in HepG2 cells, a significant decrease in Nrf1 activity was observed when the cells were subjected to hypoxia (Figures 3.10 and 3.11). This decrease in Nrf1 activity was an interesting and unexpected result, this decrease could be caused by SIRT1 and the different ways it acts on Nrf1 in different cell lines or it could be due to another regulator outside of the realm of this research, further research will need to examine the cause of this more thoroughly.

In both cell lines which were co-transfected with SIRT1 and Nrf1, there was no significant change between the cells which were treated with 24 hours of hypoxia and those which were not (Figures 3.10 and 3.11). What this indicates is that the regulatory effects that SIRT1 has on Nrf1 is a more dominant regulator of the activity of Nrf1 than that of hypoxia, this could indicate the acetylation status of Nrf1 is one of the key regulators of its activity.

4.7 The identification of the acetylated lysine residues on Nrf1

Now that it has been shown that Nrf1 is acetylated, that SIRT1 deacetylates Nrf1, and that this deacetylation causes an inhibition of Nrf1, it is important to locate the lysine residues which are acetylated, so that they can be targeted for future studies. Originally the identification of the acetylated lysine residues was going to be accomplished by purifying Nrf1 from mammalian cells and running it on an SDS-PAGE gel which would then be stained and the Nrf1 band would be excised and digested, to run on mass spectrometry. This proved to be extremely difficult because, no matter how much Nrf1 was extracted from the cells; it never seemed to be enough to obtain a signal on the mass spectrometry. A different approach was taken which proved to be more fruitful.

The Nrf1 sequence was analysed using UniProt and the PhosphoSite software to determine every possible lysine which could be acetylated (The UniProt Consortium 2013). With this list of seven possible targeted acetylated lysines, a series of seven lysine to arginine mutations were made to each one of the lysine on the list (Table 2.1). The mutations were made from lysine to arginine because arginine has a similar size and charge to lysine so it should make the mutant behave the same as the wild type, with the exception that they would lack acetyl groups. The theory was that the mutation(s) which were targeted by SIRT1 for deacetylation would result in a decrease in the luciferase signal, as they would behave as if they had already been deacetylated.

When HepG2 cells were transfected with each mutant individually, it was observed that the lysine 205 and lysine 629 resulted in a significant decrease in Nrf1 activity from the wild type Nrf1 and no significant change from the empty vector (pCR3.1) control was seen (Figure 3.12). This indicates that in HepG2 cells, there are two

lysines which are acetylated and targeted by SIRT1 for deacetylation. Again the same results were not observed in the non-cancerous HEK293 cells, which only showed the lysine 205 mutant as having reduced luciferase activity relative to the wild type (Figure 3.13). The fact that the HEK293 cells are missing the acetyl group on lysine 629 could be the cause of all of the differences observed between the HepG2 and HEK293 cells.

The lysine 205 residue is located on the first acidic domain of only the full length Nrf1 and not on the truncated p65-Nrf1 (Figure 4.1). The role of the first acidic domain in Nrf1 is responsible for directing the full length Nrf1 to the endoplasmic reticulum (ER) (Zhang et al. 2006). This translocation to the ER results in Nrf1 no longer being able to bind to the ARE, thus acting as an inhibitor to the activity of Nrf1 (Zhang et al. 2006). The location of an acetyl group in this domain could indicate that the deacetylation of the lysine residue 205 could result in the translocation of Nrf1 into the ER thereby inhibiting its activity. Further investigation is required to confirm this hypothesis.

In the HepG2 cells, when both SIRT1 and p65-Nrf1 were co-transfected with Nrf1, a significant decrease in Nrf1 activity was observed compared to either of them co-transfected with Nrf1 alone; a result that was not observed in HEK293 cells (Figures 3.8 and 3.9). The location of the second acetylated lysine residue 629 could explain the difference in the inhibition of Nrf1 in each cell line (Figure 4.1). The lysine 629 residue is located on the basic leucine zipper (bZIP) domain in both the full length Nrf1 and truncated p65-Nrf1 (Figure 4.1). The bZIP domain is responsible for binding to DNA, and acetylated lysine residues have been shown to interact with DNA in the past (Arbely et al. 2011; Johnsen et al. 1998; Chan et al. 1998). This could indicate that when the acetyl group attached to the lysine 629 residue is removed, the DNA binding activity or

transactivation from the ARE, of both full-length Nrf1 and the truncated p65-Nrf1, are inhibited. This would also provide an explanation as to why a greater decrease in Nrf1 activity was observed in the HepG2 cells when both SIRT1 and p65-Nrf1 were co-transfected, since Nrf1 contains an additional lysine residue in HepG2 cells which can be regulated on the truncated p65-Nrf1 (Figures 3.8 and 4.1). Further investigation will be required to more closely examine the role the acetylated lysine 629 plays in the activity of Nrf1 and p65-Nrf1.

4.8 The role of Nrf1 in cellular senescence

For many years now it has been shown that SIRT1 is a regulator of cellular senescence, and that when it is activated it leads to a decrease in cellular senescence (Wang et al. 2011; Ota et al. 2006; Hwang et al. 2013; Bai et al. 2008; Braidy et al. 2011; Zschoernig and Mahlknecht 2008; Solomon et al. 2006; Oppenheimer et al. 2012; Pillarisetti 2008). Since we have shown that SIRT1 inhibits the activity of Nrf1 by deacetylating it, Nrf1 becomes an excellent possible factor in the regulation of cellular senescence. Examining what role Nrf1 plays in cellular senescence required the use of primary human hepatocyte cells. These cells were maintained for one week to allow natural cellular senescence to occur. These cells were transfected with Nrf1, SIRT1 p65-Nrf1 and the two mutants Lys205Arg and Lys629Arg, individually on the second day of maintenance, to observe what impact they would have on cellular senescence (Figure 3.14).

Figure 4.1: Schematic diagram showing the positions of the novel acetylation sites on full-length Nrf1 and the truncated p65-Nrf1 (Chepelev et al. 2011; Zhang et al. 2006).

When cells were transfected with either SIRT1 on its own, or co-transfected with Nrf1, a significant decrease in cellular senescence was observed, which was expected based on the known reduction of senescence when SIRT1 is active (Wang et al. 2011; Ota et al. 2006; Hwang et al. 2013; Bai et al. 2008; Braidy et al. 2011; Zschoernig and Mahlknecht 2008; Solomon et al. 2006; Oppenheimer et al. 2012; Pillarisetti 2008). Examination of the role that the deacetylation of Nrf1 has on cellular senescence required the overexpression of the two mutant Nrf1 proteins K205R and K629R. Interestingly when both lysine 205 and lysine 629 mutants were overexpressed in the primary cells a decrease in senescence was observed to the same extent that the overexpression of SIRT1 showed (Figure 3.14). This indicates that the deacetylation of Nrf1 is a major component to the decreasing of cellular senescence seen when SIRT1 is activated. When the cells were transfected with the truncated p65-Nrf1, this also resulted in a significant decrease in cellular senescence, but not to the same extent that SIRT1 provided. This indicated that the general inhibition of any of the Nrf proteins resulted in a decrease in cellular senescence (Figure 3.14).

4.9 Proposed novel interactions of SIRT1 and Nrf1

From the observations made during the thesis study, we propose a new interaction pathway for SIRT1 and Nrf1. If cells are exposed to oxidative stress, SIRT1 is inhibited and this has been shown previously (Houtkooper et al. 2012; Hwang et al. 2013; Lin 2000; Bordone and Guarente 2005). This inhibition of SIRT1 results in the acetylation groups on Nrf1 not to be removed (Figure 4.2). These acetylation groups could potentially allow the full length Nrf1 to travel into the nucleus and form a heterodimer with the small Maf protein, allowing it to bind to the ARE and promote the production of

antioxidant response genes (Johnsen et al. 1996; Wang et al. 2007; Yang et al. 2005; Ohtsuji et al. 2008; Zhang et al. 2006; Chan et al. 1998) (Figure 16). The products of these genes fight against oxidative stress, decreasing it to non-lethal levels. This, in turn, would remove the stress that was inhibiting SIRT1, allowing it to become activated. This activation would then lead to the deacetylation of Nrf1 and inhibit it, either by a) translocating it into the ER, b) removing an acetyl group in the bZIP domain, causing it to lose its DNA binding activity or c) activating its inhibitor, p65-Nrf1 (Arbely et al. 2011; Zhang et al. 2006; Zhang et al. 2007) (Figure 4.2). This inhibition of Nrf1, in any of these cases, results in a decrease in cellular senescence (Figure 4.2).

Chapter 5: Conclusions and future directions

We were able to demonstrate that Nrf1 is indeed acetylated and that SIRT1 is responsible for the deacetylation of Nrf1, as well we were able to identify the sites at which Nrf1 is acetylated in both cancerous (lysine 205 and 629) and non-cancerous cells (lysine 205). The deacetylation of Nrf1 by SIRT1 resulted in a significant decrease in both MT1 transcription and GCLC transcription. We were not able to fully characterize the role of SIRT1 in regulating the truncated p65-Nrf1. We were able to demonstrate that SIRT1 is a stronger regulator of the activity of Nrf1 than that of hypoxia. Finally we were able to observe the effects of deacetylating Nrf1 on cellular senescence, which was shown to decrease it similarly to SIRT1.

Figure 4.2: Proposed new pathway diagram for Nrf1 and SIRT1 interaction. Novel pathway elements are the red arrows.

In future studies, the relationship between SIRT1, Nrf1 and p65-Nrf1 will need to be more closely analysed, in both cancerous and non-cancerous cells, to determine if Nrf1 and p65-Nrf1 are acetylated at lysine 629 in cancerous cells and if SIRT1 deacetylates that residue. The effects of SIRT1 on the production of p65-Nrf1 should also be analysed. The effects of the acetyl groups on the full length Nrf1 will also need to be analysed, it would be interesting to characterize the translocation of Nrf1 into the ER and what role the acetylation in acidic domain 1, would have on this translocation. The effects of NAM on Nrf1 should also be more thoroughly characterized, to determine by what pathway it is inhibiting the activity of Nrf1. The effects of hypoxia on the acetylation status of Nrf1 should also be characterized. The enzymes responsible for acetylating Nrf2, CREB-binding protein and p300, should also be examined for their ability to acetylate Nrf1. The differences in activity between HepG2 and HEK293 should be more closely examined to determine if these changes are due to the cells originating from different tissues or if it is due to the cells being cancerous and non-cancerous respectively.

Chapter 6: References

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