Title: Age-related Loss of Nrf2, a Novel Mechanism for the Potential Attenuation of Xenobiotic Detoxification Capacity

Abstract approved:

Tory M. Hagen

Nuclear Factor, Erythroid Derived 2, Like 2 (NFE2L2 or Nrf2) is the primary transcription factor in cellular defense against oxidative and xenobiotic stresses in higher eukaryotes. This basic leucine zipper transcription factor regulates over 200 antioxidant, detoxification, and lipid metabolizing genes by binding to the Antioxidant Response Element (ARE; a cis-acting element) as a transcription enhancer. Our group, and others, have identified that the nuclear steady state levels of this highly inducible transcription factor declines with age which coincides with decreased expression of many ARE regulated genes. To elucidate the mechanism(s)
involved in lowered Nrf2 levels we used hepatocytes isolated from young and old rats as a relevant cellular model that maintains the aging phenotype with respect to Nrf2 levels. Our results show that steady state Nrf2 levels decline by ~40% with age (N=4, p<0.05), which led us to investigate the multifaceted regulation of Nrf2 homeostasis. Specifically, analysis of Nrf2 mRNA levels, and the rate of Nrf2 protein turnover and translation were investigated. Surprisingly, Keap1-mediated degradation of Nrf2, its primary negative regulator, did not account for the age-related Nrf2 decline, nor did mRNA levels change with age. Rather, Nrf2 protein synthesis declines 5.3-fold with age (N=3, p<0.05). Furthermore, we identify that rno-miR-146a, a key inflammation regulating miRNA, both inhibits Nrf2 translation and increases significantly with age. Taken together, our data suggest that the age-related loss of Nrf2 stems from the loss of its translation and may be mediated through miR regulation.

Our lab previously demonstrated in rats, that the age-related loss of Nrf2 consequentially attenuates synthesis and steady state levels of liver glutathione (GSH) synthesis and manifests as a diminished level of GSH. This antioxidant is the most abundant non-protein thiol in most cells and is pivotal for the detoxification of many xenobiotics. The rate-limiting enzyme in GSH synthesis, ɣ-glutamyl-cysteine ligase (Gcl), is composed of a catalytic (Gclc), and a modulatory (Gclm) subunit. In mice and humans, it is established that Nrf2, through the ARE, regulates Gclc expression. However, this had not been established in rats. From a bioinformatics analysis of the 5’
upstream sequence of the rat Gclc gene, we identified 3 putative AREs (ARE1, ARE3, and ARE4), and one cis-element containing the core but not the flanking nucleotides of the ARE (ARE2). Luciferase reporter plasmids containing each individual ARE were transfected into H4IIE rat hepatoma cells to test for ARE activity. Only one, designated “ARE4”, produced appreciable luciferase activity and was dependent on Nrf2 expression, suggesting that this ARE is a “true” enhancer element. Chromatin immunoprecipitation (ChIP) and qPCR identified that Nrf2 binds ARE4 but not ARE1-3. Further ChIP analysis identified that known partner transcription factors of Nrf2 (small maf, c-Jun and c-Fos) were also bound to ARE4. Taken together, our data demonstrates that Nrf2 regulates, in part, the expression of the rat Gclc gene through ARE4.

Having identified the Gclc-AREs in the rat we sought to describe the molecular consequence of the age-related decrement of Nrf2 in hepatocytes isolated from young and old rats. ChIPs and reporter assays (ARE1-4) were used to investigate known ARE-binding transcription factors and the ARE mediated enhancement of Gclc transcription. We identified a decline in Nrf2 occupancy of ARE4 (40%) and a loss of transcriptional activity (70%; P < 0.05), consistent with the loss in Gclc levels. In addition, hepatic chromatin from old rat samples demonstrated ARE4 enrichment of the transcriptional repressor Bach1, and a loss of the histone acetyltransferase CREB-Binding Protein (CBP). These results demonstrate that the active Gclc transcriptional complex is remodeled into a repressive motif in the liver of old rats.
Furthermore, ChIPs identified that Nrf2 binds ARE2 in liver lysate isolated from old rats but not young. In agreement with this finding, results from luciferase reporter assays suggest that ARE2 facilitates an Nrf2-dependent enhancement of Gclc expression in the old. Thus, a promoter switching mechanism may occur with age.

In summary, we have identified key mechanisms of how the age-related decrement in Nrf2 protein levels is controlled and also gathered preliminary evidence that a pro-inflammatory state may contribute the loss of Nrf2 synthesis via increased miR levels. Finally, we have characterized the consequences of the loss of Nrf2 to GSH synthesis as an example of age-related impairment of cellular stress response.
Age-related Loss of Nrf2, a Novel Mechanism for the Potential Attenuation of Xenobiotic Detoxification Capacity

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Dean of the Graduate School

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__________________________________________

Eric Jonathan Smith, Author
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Dr. Tory Hagen, through example, you have shown me what it is to be a scientist. In part, it is a never-ending drive to achieve excellence and it is this that I hope to emulate throughout my life. I express my deepest gratitude for the scientist that you have helped make in me.

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Age-related Loss of Nrf2, a Novel Mechanism for the Potential Attenuation of Xenobiotic Detoxification Capacity

Chapter 1. Introduction
1.1 Background and Significance

1.1.1 The Aging Process: Known Phenotypes Versus Unknown Molecular Mechanisms

Aging is arguably an inherent consequence of life. Living organisms age at a rate that is intrinsic to their species. Lifespans of different species vary widely, and range from as little as a day (mayflies) to colonial organisms, which are tens of thousands of years old. For humans, mean lifespan approaches 80 years of age in the United States, while the maximal lifespan of a person currently stands at 122 years, 5 months (Mrs. Jeanne Calment of France). Despite this enormous disparity in lifespans, the aging process shares common traits at least among multicellular biological organisms—especially at the cellular level. Invariably, as an organism ages, there is a ‘loss-of-function’ that manifests as decrements in energy transduction, metabolic declines, lack of tissue renewal (in multicellular organisms), and an increasing inability to ward off intrinsic and external stresses [1-3]. Collectively, the progressive loss of function is termed “senescence”, which ultimately ends in a failure to thrive [4-6].

Though there are commonly recognized phenotypes of aging, which for humans include wrinkled skin, gray hair, loss of height, and lack of stamina, the biochemical processes that lead to senescence are poorly understood despite a significant effort to understand them at the molecular level [7-12]. This process appears to be multifactorial, which is further
complicated by its longitudinal nature. For humans, aging occurs over decades. Thus, the underlying processes involved in senescence remain ill-defined.

The imprecise understanding of senescence has not prevented hundreds of aging theories from being proposed. A short-list of these theories includes programed senescence, endocrine theory, immunological theory, the free radical theory of aging, and rate-of-living \[13, 14\]. Some, especially the free radical theory of aging, have a significant body of experimental evidence that support their basic tenets. However, none of the aging theories have proven adequate to fully explain the enormous differences in the rate of aging seen between species.

One currently prominent aging theory is the “oxidative stress theory of aging” \[15, 16\], which is a corollary of the Free Radical Theory of Aging proposed by Denham Harmon in 1956 \[13\]. This theory posits that an imbalance between prooxidants and antioxidants leads to an accumulation of oxidative damage and results in a progressive loss in cellular function, leading to the aging phenotype \[7, 15, 16\]. In agreement with this theory, there is a measurable accumulation of oxidative damage to cellular components and an increase in the level of reactive oxygen species (ROS) observed with advanced age \[8-12, 17\]. Equally important, especially for this thesis, is a diminished level of cellular antioxidants and a shift towards the prooxidant state as organisms age. This decline in stress resistance molecules directly
contributes to increased oxidant levels and accumulation of damaged cellular components [17-20].

In a larger context, oxidative stress is just one of many insults to which organisms become more vulnerable as they age. These other stressors include enhanced toxicity from xenobiotics, heat, radiation and metabolites (e.g. high glucose). Thus, one hallmark of aging that encompasses the tenets of the oxidative stress but extends to other theories of aging is that, as one ages, the organism becomes susceptible to a variety of endogenous (oxidative stress, inflammation, increased apoptosis) and exogenous insults (drugs, environmental mutagens) [21-23]. In turn, the vulnerability to these stressors appear to be part of the etiology of many age-related diseases, such as Alzheimer's and Parkinson's diseases, congestive heart failure, kidney disease, and frailty syndromes [24-28].

The reason(s) why organisms fail to adequately respond to multiple exogenous and endogenous stresses are not understood. In fact, it is somewhat surprising given that organisms contain elaborate and overlapping defenses that are highly inducible. The following section will highlight an important endogenous antioxidant that declines with age, GSH, and the role of a transcription factor, Nrf2, which may play a key role in sensitizing aged organisms to multiple insults.

1.1.2 Glutathione as a relevant example for lost host antioxidants.
Given that cellular defense systems, especially antioxidant defenses, tend to degrade with advancing age [11, 23, 25, 29-31], one of the goals of the Hagen lab has been to identify mechanisms associated with the age-related decline in oxidative stress resistance. In this context, glutathione (GSH), a key antioxidant and detoxicant was chosen for further examination as its cellular concentration decreases with age in many species [32-37]. GSH is a tripeptide (γ-L-glutamyl-L-cysteinyl-glycine) and is the most abundant low molecular weight sulfur-containing compound in most mammalian cells, between 1-10 mM. This high concentration results from the proteolytic resistance conferred by the isopeptide bond between glutamate and cysteine. GSH must be exported from the cell for degradation to occur via γ-glutamyltransferase [38].

GSH has multiple biochemical functions. Its free thiolate moiety acts as a “soft nucleophile” under physiological conditions, which can terminate reactive oxygen species and electrophiles. GSH also binds redox active metals (e.g. iron and copper), which limits the formation of high-energy free radicals. Arguably, it’s most important role in antioxidant function is as a substrate for glutathione peroxidases and glutathione-S-transferases, which catalyze the reduction of peroxides to their corresponding alcohols, or conjugate electrophilic drugs and xenobiotics (vitamin K₃, estradiol-17β, acetaminophen, sulfobromophthalein, parathion, urethane and 1-chloro-2,4-dinitrobenzene [39]). Finally, GSH forms a potent redox couple with its disulfide form (GSSG) that regulates activities of multiple enzymes, including
electron transport chain complexes. Thus, the age-dependent loss of GSH has important consequences for making cells vulnerable to both oxidative stress and xenobiotic insults.

The rate of GSH decline in many organisms can often be tied to the intrinsic rate-of-aging of a given species. For example, GSH levels remain relatively stable throughout the first 40 years of life in humans; however, thereafter glutathione levels decline at an increasing rate until death [40-42]. Another clue that GSH is an aging factor is shown by studies examining how dietary restriction extends lifespan [43]. Animals placed under 60% caloric restriction exhibit consistent increases in GSH and GSH utilizing enzymes [32], which correlates with a high lifespan and resistance to disease. These results indicate that GSH is a longevity assurance factor, and warrants an examination into the role of its loss in the decline of stress resistance with age.

To understand why GSH levels are not maintained as one ages, one needs to appreciate the biochemical mechanisms associated with GSH homeostatic mechanisms. GSH levels are regulated by multiple mechanisms, including its rate of synthesis, export from the cell, and degradation. [38]. Our lab previously showed that while GSH levels declined by approximately 30-50% in most rodent tissues with age, this loss was not due to a lack in availability of L-cysteine, the rate-limiting substrate in its synthesis; rather, Suh et al. noted that both activity and levels of glutamate-cysteine ligase (the catalytic [GCLC] and modulatory [GCLM] subunits) and GSH synthetase, the
two enzymes in the GSH synthetic pathway, were significantly lower on an age basis. Most importantly for this dissertation, Dr. Hagen’s group identified that the age-related loss of GSH synthetic capacity is causally linked to decreased steady-state levels of the transcription factor, Nuclear factor (erythroid-derived 2)-like 2, also known as NFE2L2 or Nrf2 \[^{44}\]. The identification of Nrf2’s role in GSH loss with age may be a pivotal discovery as to why stress resistance mechanisms generally decline with age. Nrf2 not only governs GSH synthetic capacity, but is also a vital transcription factor for the expression of hundreds of antioxidant, lipid metabolizing, and detoxification genes both at basal and inducible levels. Interestingly, Nrf2 regulates many of the detoxification defenses previously identified to decline with age \[^{11, 23, 25, 29-31, 44-46}\]. Thus, a key transcription factor associated with the decline in a variety of stress resistance systems has likely been identified. In order to appreciate the role that Nrf2 may have on cellular stress resistance, a brief overview of its structure, regulation, and role in target gene transcription will now be presented.

1.1.3 Nrf2

Nrf2 is a transcription factor encoded by the NFE2L2 gene \[^{47}\]. A member of the Cap ‘n’ Collar (CNC) family, it contains a basic-leucine zipper (bZIP) DNA binding motif \[^{48, 49}\]. Nrf2 is ubiquitously expressed in human, rat and mouse organs \[^{50}\], especially tissues readily exposed to xenobiotics and exogenous ROS \[^{50}\].
The Nrf2 protein acts by translocating to the nucleus where it binds to the antioxidant/electrophile response element (ARE/EpRE: 50-TGACNNNGC-30) found in the 5'-flanking region of many antioxidant and detoxification genes, including those for GSH synthesis [48, 51], and regulates their gene expression (see below).

1.1.3.1 Regulation of Nrf2 Steady-State Levels: the Key to Regulating ARE-Mediated Gene Expression

The overall steady-state levels of Nrf2 appear to be important for regulating the expression of its target genes. Thus, significant research has been undertaken to identify the regulatory mechanisms involved in Nrf2 homeostasis under both non-stressed conditions and following oxidative insults. From these studies, a complex regulatory scheme has emerged which implicates transcription, translation and posttranslational modifications for Nrf2 homeostasis [48]. These overlapping regulatory mechanisms maintain a precise calibration of cellular Nrf2 levels with respect to stress stimuli and metabolic state of the cell (Figure 1).

Structurally, Nrf2 contains seven Nrf2-ECH homology domains (Neh) with the one closest to the N-terminus (Neh1) being integral for proper stress response [52] (Figure 2). Under normal cellular conditions, most newly translated Nrf2 rapidly associates with Kelch-like ECH-associated protein 1 (Keap1) via binding to the Neh1 domain, which initiates Nrf2 degradation. Keap1 is an adaptor protein for the Cul3 ubiquitin protein. Thus, Nrf2 that
associates with the Keap1/Cul3 apparatus is rapidly ubiquitinated via a Really Interesting New Gene (RING)-dependent mechanism, followed by degradation through the 26S proteosome [52]. Under basal (non-stressed) conditions, this interaction maintains Nrf2 at low levels and contributes to a short half-life of approximately 20 minutes [53].

Keap1 contains several redox sensitive residues; Cys151, Cys226/Cys613, Cys273/Cys288, or Cys434 [54-61]. Oxidation or electrophilic adduction to one or more of these sulfhydryls results in structural rearrangement of Keap1, which inhibits Nrf2 binding and thus limits proteolysis. Consequently, Nrf2 half-life increases and Nrf2 accumulates in the cell. Heightened Nrf2 steady-state levels are a key event in cellular stress response because Nrf2 accumulation enhances expression of antioxidant and detoxification genes. Thus, under a pro-oxidant environment, or in the presence of toxic electrophilic compounds (e.g. drugs, mutagens, environmental toxins), elevated Nrf2 levels rapidly increase the response capacity of cells in an attempt to mitigate the offending stimulus and return the cell to metabolic homeostasis.

1.1.3.2 Nrf2 and Transcriptional Regulation of ARE Genes

The mechanisms of how elevated Nrf2 levels lead to enhanced transcription of hundreds of ARE-dependent genes have been well documented [62-83]. ARE-dependent transcriptional activation requires Nrf2 translocation to the nucleus. There, it forms a complex with other proteins, which bind to AREs in
the 5'-flanking regions of detoxification genes [84]. The Nrf2 transcriptional complex recruits RNA polymerase and enhances gene expression [85] (Figure 3). Members of this complex, which are determined in part by the flanking sequences around the core ARE, can positively or negatively influence gene expression [62, 73, 86-88]. Furthermore, factors other than Nrf2 can bind the ARE, resulting in repression [89]. The interaction of these factors with the ARE is multifaceted and represent many additional means of regulating the expression of endogenous antioxidants.

Nrf2 heterodimerizes with small _musculoaponeurotic fibrosarcoma_ (Maf) proteins, MafF, MafG and MafK, via its Neh1 domain, in order to bind to a typical ARE [90]. It should be noted that although the small Maf proteins can dimerize, they lack a functional trans-activation domain and therefore when bound as a homodimer, they act as transcriptional repressors [84]. The ARE dependent enhancement, both increased and decreased, can be modulated by the molecular manipulation of the ratio between small Maf proteins and Nrf2, via overexpression and knockdown [91, 92]. In addition to small Maf proteins, Nrf2 can heterodimerize with transcription factor ATF4, bind to the ARE, and enhance expression [93].

Nrf2 also interacts with several other proteins, including coactivators, chromatin remodelers, histone acetylases, deacetylases, kinases, and methyltransferases [48, 87, 88, 94]. Several of these proteins are transcriptional repressors, such as caveolin-1, which has been shown to directly bind and inhibit Nrf2 [95]. Of special note, other proteins, which are
known competitors for Nrf2/ARE binding, may serve as ARE repressors when Nrf2 levels are low. In particular, Bach1 and Bach2 heterodimerize with small Maf proteins and bind the ARE consensus sequence, displacing Nrf2. Increased levels of Nrf2 can, in turn, displace Bach1, demonstrating the competitive nature of this repression [86, 89].

Transcriptional “coactivators” are proteins that bind to transcription factors in order to enhance expression. However, coactivators themselves lack a transactivation domain and therefore must act indirectly. The Neh4 and Neh5 domains of Nrf2 bind to the coactivator, CBP [cAMP-response-element binding protein (CREB) binding protein] [96, 97]. It is hypothesized that strong enhancement via ARE, at least in part, requires CBP association with the Nrf2 complex [97].

The consensus ARE contains an embedded AP-1 binding site and AP-1 binding proteins are shown to associate with the ARE [98]. For example, evidence indicates that the transcription factor c-jun binds the ARE but may not contribute to increased gene expression [99]. Similarly c-fos dimerizes with small Maf proteins and binds ARE, but it cannot facilitate enhanced expression [100]. Unlike Bach1, c-jun and c-fos have not been demonstrated to displace Nrf2 or inhibit ARE expression. The impact of these ARE binding factors on the expression of endogenous antioxidants remains to be fully elucidated.

It is currently unknown if any of the aforementioned factors contribute to the age-related loss of endogenous antioxidant expression as, before the
present project, there has been no characterization of age-related changes to the Nrf2/ARE transcriptional complex at any given antioxidant gene. It is interesting to note that the Bach1 null mouse exhibits increased antioxidant gene expression, but still shows an attenuation of these mRNAs with age [101], suggests that the age-related loss of endogenous antioxidants is not mediated solely through Bach1. The levels of the coactivator, CBP, decreases with age; however, the functional consequences of CBP loss on ARE-driven expression are not known [102]. Recent investigations have identified that c-jun has an attenuated expression in ventral horn of the spinal cord of old mice [103]. Conversely, the activity of c-fos is reported as increasing with age [104]. The age-related changes in the factors associated with ARE and Nrf2 are of particular interest due to their capacity to alter Nrf2-mediated transcriptional enhancement and the subsequent cellular antioxidant and detoxification capacity.

### 1.1.3.3 Emerging modes of Nrf2 regulation

Although Keap1-dependent degradation is thought to be the primary mechanism of Nrf2 protein homeostasis (proteostasis), other significant types of regulation have been identified, especially at the post-transcription level. For example, Nrf2 degradation may occur independently of Keap1 via the SCF/β-TrCP pathway, which is regulated in part by glycogen synthase kinase 3 [105]. However, the level/rate of degradation through this pathway as compared to the Keap1-dependent pathway is presently unknown.
In addition to degradation, the transcription and translation of the Nrf2 gene product are highly regulated. Reports regarding the increased expression of Nrf2 mRNA are scant, but there are reports that certain toxins cause elevated Nrf2 transcript levels [63, 106, 107]. Likewise, Nrf2 mRNA translation increases markedly in response to \( \text{H}_2\text{O}_2 \) via an internal ribosomal binding site (IRES) located on the mRNA of Nrf2 [108, 109].

Recent publications have demonstrated that microRNA (miR) inhibition of Nrf2 protein expression may also be an important regulator in Nrf2 proteostasis [110, 111]. MiRs are non-coding ribonucleic oligomers approximately 22 nucleotides long. Encoded by nuclear DNA, these molecules typically down-regulate target mRNA translation. MiRs bind mRNA and inhibit translation by attenuating ribosomal assembly or inducing message degradation [112-114]. This process modulates the expression of Nrf2 at least in cell culture. Narasimhan et al. showed that miR-144, miR-153, miR-27a, and miR-142-5p inhibited the translation of Nrf2 in neuronal, SH-SY5Y cells [115], while Stachurska et al. observed that miR-132 determines, in part, ochratoxin A-mediated toxicity in renal proximal tubular epithelial cells in an Nrf2 dependent fashion [116]. Other work has shown that miR-34-a inhibits the translation of Nrf2 and effects the expression of Nrf2 targets [110]. Although the interaction between miRs and Nrf2 expression is clear, the total contribution of this regulatory mechanism and their impact to cellular antioxidant defense is unknown, particularly with regard to aging. The regulation of Nrf2 proteostasis has proven to be more complex than originally
believed and further investigation is likely to yield interesting insights. However, changes in the activity of the protein as a transcription factor must also be considered when evaluating the role of declining levels of Nrf2 in the age-related loss of cellular anti-oxidant defenses.

In summary, Nrf2 is regulated in a multifactorial process to maintain a steady state level and cellular localization under basal conditions. Furthermore, various rheostats that act to increase protein expression in response to xenobiotics, cellular redox perturbation, or oxidative stress control Nrf2 regulation. Despite the apparent loss of Nrf2 with age, Nrf2 regulation has gone largely unstudied in this context.

1.1.3.4 Nrf2 and Longevity Assurance

Based on the general mechanisms for regulating Nrf2, one would anticipate that the age-related increase in oxidative stress exhibited by aging tissues would inhibit Nrf2 degradation, lengthen Nrf2 half-life, and increase the accumulation of Nrf2. This would be expected to increase antioxidant capacity and thus return the pro- versus anti-oxidant state back to a normal equilibrium. However, despite the increasingly oxidant rich environment of aging tissues, levels of Nrf2 decline, which suggests that Nrf2-Keap1 regulation or other regulatory contributors to Nrf2 homeostasis no longer function.
**1.2 Dissertation Hypothesis and Aims**

In the elderly, the mechanism(s) resulting in an attenuated response to oxidative stress and xenobiotics have yet to be fully elucidated. However, as outlined above, the Hagen lab recently identified Nrf2 as a link between lower stress resistance with age and molecular dysregulation of a critical pathway involved in stress resistance. Despite substantial literature precedence demonstrating that attenuated Nrf2 levels results in an increased susceptibility to a variety of xenobiotics and shortened lifespan, no significant attempt has been made to understand why Nrf2 levels decline with age and what effect this loss has on ARE-mediated gene expression. This gap in knowledge is particularly relevant as it prevents identification of therapies to limit loss of stress resistance in older adults. Therefore, identifying the underlying molecular events that lead to steady-state Nrf2 loss is necessary to design prophylactic therapies that may ultimately improve or maintain health during aging.

To fill this gap, the present work investigates the mechanism(s) involved in the age-associated attenuation of Nrf2 homeostasis, alterations in its function as a stress response transcriptional activator, and the potential remediation of its expression. My research project is predicated on two hypotheses:

i). Post-transcriptional dysregulation of Nrf2 homeostasis results in its age-associated decline. This hypothesis advances two key questions:

- Is the proteostatic regulation of Nrf2 perturbed with age? This question will be answered using hepatocytes isolated from young (4-6 months)
and old (24-26 months) F344 rats. We will use this model, which recapitulates the aging phenotype with respect to Nrf2, to identify potential lesions in Nrf2 regulation via transcription, translation and degradation.

- Does the age-related change in Nrf2 proteostasis limit Nrf2 accumulation in response to oxidative or toxicological challenges? Again, hepatocytes isolated from young and old rats will be used to answer this question. Cells will be treated with known Nrf2 inducers and the proteostatic parameters will be tested.

ii). The age-related loss of Nrf2 results in aberrant transcription factor complex formation at an ARE locus, which attenuates detoxication gene expression. Using Gclc as a quintessential ARE-mediated gene and marker for Nrf2-mediated gene expression in general, we explore this hypothesis by asking the following questions:

- Which potential ARE locus in the 5'-flanking region of Gclc is actively regulated by Nrf2? We will employ chromatin immune-precipitation (ChIP) on liver lysates isolated from young rats and construct luciferase reporter vectors to answer this question.

- Does the age-related loss of Nrf2 cause a normal or repressive transcriptional apparatus to develop with age? Nrf2 must heterodimerize with other proteins to form an active transcriptional complex. The steady-state loss of Nrf2 with age may alter transcription
factor profiles, which in turn could repress gene expression. Thus, the transcriptional apparatus for Gclc will be investigated in hepatocytes isolated from young and old rats using ChIP.
1.3 Figures

Figure 1.1. The schematic representation of the ARE response. Nrf2 mediates the antioxidant and detoxification response by binding to a genomic enhancer sequence called the antioxidant response element (ARE). In the presence of a cellular “stressor” or a benign inducer of Nrf2 the nuclear levels of Nrf2 are increased primarily by a translocation to the nucleus, an extension of the half-life, and increase translation.
Figure 1.2. Graphical representation of transcription factor nuclear factor-erythroid 2 p45-related factor 2 (Nrf2).
Nrf2 contains 7 Nrf2-ECH homology (Neh) domains (1-7) and are shown. The Neh1 CNC-bZIP domain is responsible for dimerization with small musculoaponeurotic fibrosarcoma (Maf) proteins, and is necessary for binding to antioxidant response element (ARE) sequences. The Neh2 domain negatively regulates Nrf2 through interaction with Keap1. Neh3 is the transactivation domain that enhances translation. Neh4 and Neh5 regions represent transactivation domains that recruit cAMP response element-binding protein (CREB)-binding protein (CBP). The Neh6 domain negatively regulates Nrf2 via SCFb-TrCP. The Neh7 domain mediates repression of Nrf2 by the retinoid X receptor (RXR).
Figure 1.3. Cartoon depicting the typical ARE binding transcription factors. The ARE can enhance transcription only when bound to a “permissive” complement of binding partners. In particular, Nrf2 must hetero-dimerize with other known bZIP translation factors. Other proteins, which lack DNA binding domains, such as the co-activator CBP, bind the Neh 4 and 5 domains and enhance expression. Loss the co-activators, loss of Nrf2, and the displacement of Nrf2 by Bach1 leads to a loss of transactivation, enhancement of translation, and hence represents a “repressive motif”.
Chapter 2. An Age-related Decline in Nrf2 Protein Synthesis, a Novel Mechanism for the Attenuation of Xenobiotic Detoxification Capacity

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To be submitted
2.1 Summary

Aging results in an inadequate response to a variety of environmental stressors, which increases risk for overt pathophysiologies. Our lab previously identified that the Nrf2 transcription factor, which regulates the expression of over 200 antioxidant and detoxification genes, is partly responsible for diminished stress resistance as its hepatocellular levels declines significantly with age, greater than 40% [44]. The objective of the present work was to identify the mechanism(s) responsible for this loss of Nrf2 steady-state levels. Using cultured hepatocytes from young (3 month) and old (24-26 month) rats, we now show Nrf2 mRNA levels do not significantly change with age (6.8%, p>0.05) and thus cannot account for the loss of hepatic Nrf2 protein content, or the loss of Nrf2-mediated gene expression (e.g. gclc, gclm). Overexpression of Nrf2 restores and reverses the age related loss of many representative Nrf2-dependent detoxification genes. Moreover, we identified that the half-life of Nrf2 increase significantly (5.5-fold, p<0.05). These results suggest that the aging lesion resides at the maintenance of Nrf2 protein levels, and not from dysregulation of antioxidant gene expression per se. In further exploring the mechanism(s) involved in lower Nrf2 steady-state levels, we show that Nrf2 protein synthesis is significantly diminished in hepatocytes isolated from old rats vs. those isolated from young (5.3-fold, p<0.05). A polysome profile confirmed that Nrf2 mRNA was associated with fewer
ribosomal subunits. In defining the mechanism associated with the age-related loss of Nrf2 protein synthesis, we show that Nrf2 protein loss is associated with increased levels of miRNA-146a, which increase over 2.6-fold with age. As elevated miR-146a levels result from pro-inflammatory conditions, our results suggest that the loss of Nrf2 and hence gene expression dependent on it may result from the chronic low-grade inflammation associated with aging. Taken together, our results show a novel mechanism for the age-related decline in Nrf2 protein levels and consequently, its ability to initiate an adequate response to oxidative and xenobiotic insults.
2.2 Introduction

A hallmark of aging is an increase in endogenous reactive oxygen (ROS) and nitrogen (RNS) species, and a loss of endogenous antioxidants [117-119]. This oxidant-enriched milieu has been hypothesized to contribute to a subclinical necro-inflammatory environment that goes unresolved by cellular stress response mechanisms [120]. It is also theorized that this so-called “inflamm-aging” contributes to heightened risk for age-associated pathologies and even may be part of the aging process itself. While the consequences of inflamm-aging to various organ systems are under intense study, the reasons why detoxification/antioxidant defense mechanisms fail to remediate the chronic pro-oxidant state remains largely unresolved. This limited response is a particular conundrum considering that most host defense mechanisms are highly inducible, especially following disturbances in the pro- vs. anti-oxidant balance of the cell.

In exploring this question, we previously showed that the levels of the transcription factor Nuclear Factor, Erythroid Derived 2, Like 2 (NFE2L2 or Nrf2) declines in the aging rat liver [44]. This decline is coincident with the development of a pro-inflammatory environment [121]. Nrf2 is a basic leucine zipper, cap ‘N’ collar transcription factor that is normally maintained at low steady-state levels [122]. This low level of Nrf2 is sufficient for its nuclear translocation and its subsequent binding to cis-acting DNA sequences termed the Antioxidant Response Elements (AREs), in the 5’-flanking regions of numerous antioxidant and detoxification genes. Under acute pro-oxidant
conditions or in the presence of electrophilic compounds, Nrf2 rapidly accumulates in the nucleus and enhances expression of detoxification genes. The accumulation of Nrf2 is, in part, a result of the attenuated degradation of Nrf2 via Keap1, a cul3 ubiquitin ligase adaptor protein. However, to our knowledge, the age-related loss of Nrf2 has yet to be attributed to changes in these regulatory mechanisms, and several additional regulatory mechanisms play a role in maintaining the proteostasis of Nrf2 [48].

Recently, it has been shown that several micro-RNAs (miRs) attenuate the expression of the Nrf2 mRNA and therefore contribute to the regulation of Nrf2 protein nuclear steady state levels [110, 111]. MiR expression changes under a myriad of conditions and biological processes. However, miR regulation of Nrf2 is of particular interest because of the redox sensitive nature of miR processing [111] and the pro-oxidative state that develops in the aging rat liver. It is currently unknown how the age-related change in miR expression affects Nrf2 regulation, expression of phase II detoxification constituents, or resistance to an oxidative or toxicological stress.

In this study we sought to elucidate the mechanism(s) that results in the age-related loss of Nrf2 protein levels. It has long been established that changes in proteostasis are a key factor in aging; decreased protein translation, mis-folded proteins, and altered rates of protein degradation. Here we investigate protein synthesis, degradation, and miR of Nrf2 in old and young rats. We hypothesize that Nrf2 protein synthesis declines with age resulting in the loss of basal Nrf2 protein levels and stress response.
2.3 Results

Previously, we showed that the levels of Nrf2, and its binding to a core consensus ARE sequence decline in aging rat liver [37]. Herein we confirmed these results and show that steady-state Nrf2 levels were 42 +/- 12% lower in livers of 24-month old rats than in young controls (Figure 2.1a, 2.1b, p<0.05). Moreover, this loss was coincident with a significant decline in expression of genes that are constitutively regulated by Nrf2. QPCR analysis showed mRNA content of *Gclc*, *Gclm*, and *Gst2a* exhibited age-dependent losses of 19.6% (p< 0.05), 31.4% (p< 0.05), and 40% (P< 0.05), respectively. However, there was no significant change in Nrf2 mRNA levels (Figure 2.1c). Thus, the decline in Nrf2 protein levels and only protein levels, parallels lower message levels of a number of important antioxidant genes.

To elucidate the relationship between the age-related loss of Nrf2 protein content and ARE-mediated transcription, Nrf2 was overexpressed in cultured hepatocytes taken from old and young rats by transfection of a CMV-driven pHA-Nrf2 expression vector (Figure 2.2). Transfection resulted in a greater than 30-fold increase in Nrf2 levels regardless of age (data not shown). More importantly, Nrf2 overexpression reversed the age-related decline in ARE-associated target gene expression (p<0.05). *Gclc* and *Gclm* mRNA content were at least 3-fold higher than observed in hepatocytes transfected with an empty vector. Taken together, these data suggest that the age-related loss of Nrf2 protein levels per se may be the most important factor in the loss of ARE-mediated detoxification with age.
Translation of Nrf2, but not its gene expression declines with age.

Because Nrf2 mRNA levels have been shown to vary in both tissues [123] and in response to some toxicological stressors [63, 106, 107], we hypothesized that limited expression of Nrf2 mRNA itself may be responsible for the decline in Nrf2 protein levels. However, contrary to this hypothesis, hepatic Nrf2 mRNA values exhibited no significant (p>0.14) age-dependent changes in Nrf2 transcripts (cf. Figure 2.1c), indicating additional mechanisms are responsible for Nrf2 protein levels than dysregulation of its gene expression alone.

As steady-state Nrf2 levels are regulated beyond the transcription level by a complex interplay of both protein synthesis and degradation [108, 109, 122, 124], we sought to define molecular lesions that may lead to lower hepatic Nrf2 protein homeostasis. In an initial assessment of potential differences in Nrf2 steady-state levels, we focused on factors associated with Nrf2 protein turnover.

Following its overexpression using a pHA-Nrf2 vector, the rate of Nrf2 turnover was quantified in hepatocytes from young and old rats treated with or without cycloheximide. Results showed marked differences in Nrf2 turnover rates. Using a single-phase decay model to fit the data, Nrf2 half-life increased >5-fold with age (p<0.05; Figure 2.3), with T_{1/2} estimates of 21.6 ± 3.0 and 119.8 ± 21.5 minutes in young and old hepatocytes, respectively. This pronounced diminished Nrf2 turnover in old rat hepatocytes did not
appear to stem from markedly altered Keap1 levels, which could otherwise influence its rate of degradation (Figure 2.S1). Thus, contrary to contributing to lower steady-state Nrf2 concentrations, our results suggest that the rate of Nrf2 turnover significantly declines with age, and thus may be an adaptive means to maintain Nrf2 levels, albeit at a lower steady-state level.

Because the protein turnover rate could not account for the observed differences in Nrf2 steady-state levels, we explored whether Nrf2 protein translation was responsible for the lower Nrf2 levels. A profile of ribosomal binding to mRNA is an appropriate means to assess general translational activity, so we examined the extent of ribosome binding to Nrf2 mRNA (Figure 2.4). Using hepatocellular extracts from young rats, ribosome sedimentation profiles revealed that the majority of Nrf2 mRNA and 18S rRNA associated with the denser fractions at the bottom of the gradient. These dense fractions represent polysomes and indicate that Nrf2 mRNA is being actively translated. In contrast, cellular cytoplasmic extracts from old rats displayed a significantly altered profile where the majority of Nrf2 message and 18S rRNA co-sedimented near the top of the gradient. This profile suggests that with aging, Nrf2 mRNA displays markedly attenuated ribosomal association and subsequent translation. In fact, this profile was equivalent to that seen when we incubated cytosolic extracts from young rats with EDTA (50 mM) to chemically induce polysome disintegration (data not shown).

To quantify what the aging polysome profile means to Nrf2 protein synthesis, we measured the rate of Nrf2 accumulation in rat hepatocytes
treated with bortezomib (100 nM) or anethole trithione (A3T) (50 µM), compounds that inhibit Nrf2 degradation by inhibition of the 26S proteasome and disruption of Keap1, respectively [107, 125]. Bortezomib induced a rapid hepatocellular accumulation of Nrf2. However, this rate of accumulation was 5.3 ± 4.0-fold greater in young versus old rat hepatocytes (Figure 2.5). Bortezomib co-treatment with A3T, which limits Nrf2 degradation by inhibiting its association with Keap1 [107, 126], resulted in an even greater disparity in Nrf2 protein accumulation with age (11.5 ± 3.2-fold difference) than observed with bortezomib treatment alone. These results, along with the polysome data, point to a significant lesion in the translational efficiency of Nrf2 mRNA with age.

**Age-related miR changes with age in the rat liver.**

Previous literature reports have demonstrated that miRs may play an important role in the regulation of Nrf2 expression. We therefore hypothesized that the lack of Nrf2 translational efficiency may stem from an increase in miR-dependent inhibition. A miR array analysis was performed using tissue extracts from young and old rat liver. Surprisingly, only 13 miRs met both the 2-fold and p-value cutoff, with most transcripts declining with age. These results are in keeping with recent literature reports showing that dicer-dependent miR maturation declines with age [127]. However, a small cadre of miR transcripts increased on an age-related basis (Figure 2.6a). While reports as to which genes some of these miR’s associate with are lacking, a general
observation can be made that many of the miR species which become elevated with age (34a, 146a, 28, 101a) are also known to be induced under chronic inflammatory conditions [128-131].

All miRs that increased, that have the potential to directly inhibit Nrf2 translation, in expression and have a p-value < 0.05 were examined for possible interaction with the Nrf2 mRNA. Using mirSVR sequencing analysis and available literature reports, five miRs were identified [110, 131-134] that change significantly with age and could theoretically bind to the 3’ region of Nrf2 mRNA. Thus, these miRs could potentially alter Nrf2 translation characteristics. In particular, rno-miR-146a had a high mirSVR score, suggesting the possibility that it inhibits the Nrf2 mRNA. For this reason and because it is both highly expressed and the fold change with age is large, we investigated the affect of rno-miR-146a on Nrf2. In order to confirm the array results, liver tissue from young and old rats were harvested, small RNA isolated, and RT-qPCR was performed (Figure 2.6b). This analysis revealed a 2.7 ±0.3 fold increase in rno-miR-146a, providing strong evidence this is a true age-affected miR.

**An miRNA-146 mimic significantly inhibits Nrf2 protein synthesis.**

In order investigate whether rno-miR-146a influences the expression of Nrf2, hepatocytes from young rats were transfected with a chemically modified double-stranded RNA that mimics the endogenous rno-miR-146a. Immunoblot analysis showed that the scramble control samples accumulated
approximately 2.2-fold more Nrf2 protein than miR transfected samples (Figure 2.6c). This confirmed that rno-miR-146a could attenuate the expression of Nrf2.

The mRNA level of Nrf2 and Nrf2-controlled genes were examined by qPCR after treatment with rno-miR-146a (Figure 2.6d). Results indicate no significant difference in Nrf2 mRNA levels between the control and rno-miR-146a-treated samples, despite the observed change in protein accumulation, which suggests that rno-miR-146a inhibits ribosomal association of Nrf2 mRNA but does not induce degradation. However, mRNA levels of the Nrf2 mediated genes, Gclc and Gclm, mRNA, were lowered by the mimic treatment, 32.1 ±4.5% and 13.4 ±2.6% respectively. These data reflect the loss of protein synthesis in spite of preserved mRNA levels seen in liver tissues and isolated hepatocytes from old rats.
2.4 Discussion

Steady-state Nrf2 levels result from a complex interplay between factors that influence its rate of transcription, translation, and degradation. For example, in response to pro-inflammatory stimuli, the transcription factor NF-κB translocates to the nucleus, and enhances Nrf2 transcription [135]. Additionally, altering the levels of Keap1 and/or GSK-3 can modulate the rate of Nrf2 protein turnover [105]. Our results suggest that Nrf2 translation must also be considered as an important factor that influences Nrf2 protein homeostasis and hence its function as a transcription factor in stress response and metabolism.

The present work defines decreased Nrf2 translation as the primary causative factor for lower Nrf2 steady state levels in livers of old rats. Our results and that of other groups, demonstrating that Nrf2 message levels and the Keap1/cul3 ubiquitin ligase system, which is primarily responsible for initiating Nrf2 protein turnover, remain unaffected by age, support this conclusion. In fact, our current data showing that Nrf2 half-life markedly increases in aged rat liver, along with reports that hepatocellular 20S-proteosome activity declines with age [136, 137], suggests that lower Nrf2 protein turnover may be a compensatory mechanism for the marked loss of Nrf2 protein translation. We have bolstered this argument by employing mathematical modeling to discern the impact of altered translation with respect to protein turnover with age (see supplementary information). Using experimentally determined rates of change in Nrf2 synthesis and protein
turnover, this model indicates that Nrf2 protein synthesis would decline by 5.3-fold in old versus young rats if Nrf2 protein turnover remained unabated. These results are in agreement with our experimental observations that a decline in Nrf2 translation is likely the key factor for the age-related loss of Nrf2 protein levels with age.

The loss of constitutive levels of Nrf2 is consistent with our previous studies examining the mechanisms behind the age-related decline in glutathione (GSH) synthesis, an Nrf2-mediated process [37]. We were one of the first groups to connect the loss of hepatic GSH to attenuated Nrf2-dependent expression of glutamate-cysteine ligase (Gcl) in aged animals [34, 36, 44, 138]. Through ChIP analysis and promoter mapping of active antioxidant responsive element (ARE) binding sites, we further showed that a decline in Nrf2 nuclear steady state levels resulted in lower levels of Nrf2 bound to an ARE (ARE4) in the 5’ flanking region of the catalytic subunit of Gcl (Gclc) and the association of Bach1 with this locus. As Bach1 competes with Nrf2 for ARE binding sites, these results suggest that the age-dependent decline in Nrf2 translation ultimately results in transcriptional repression of genes where Nrf2 is the prime transcription factor for basal expression. In particular, the constitutive expression of a significant number of antioxidant, anti-inflammatory, detoxification, and even general metabolic genes are dependent upon, or are influenced by, Nrf2 [73]. Thus, abrogated level of Nrf2 may have an impact beyond antioxidant defense and detoxification. Our work shows that attenuated Nrf2 protein translation results in decreased nuclear
localization, and manifests in a substantial loss of Nrf2 driven gene expression.

It must be noted that our data pertain to mainly age-dependent changes occurring to Nrf2 homeostasis on a constitutive level. The extent that the loss of Nrf2 affects response to acute xenobiotic stimuli or chronic inflammatory conditions remains to be fully elucidated. However, our results using both Nrf2 overexpression and A3T as well as our previous studies using the thiol redox modulator, lipoic acid, suggest that Nrf2-dependent response remains active, albeit at an attenuated level relative to young animals [44]. One would expect that aged animals would be more vulnerable to acute xenobiotic insults as basal antioxidant and xenobiotic defenses are limited. We have shown that old animals are more vulnerable to acute addition of model lipid hydroperoxides, which are detoxified in a glutathione- and Nrf2-mediated manner [33]. Nrf2 dependence has not been directly tested but several xenobiotics detoxified primarily or in part by glutathione have an increased toxicity in the elderly [139-141]

Additionally, it is known that the elderly, who often take numerous medications, are more vulnerable to even common over-the-counter drugs (e.g. acetaminophen) [142, 143]. A similar phenomenon is seen in young Nrf2-null mice [144, 145]. The cause of increased susceptibility of Nrf2-null mice to acetaminophen is known and appears to be in part reflected in the elderly: lack of appropriate Nrf2-mediated metabolism and drug disposition. Thus, lower Nrf2 steady-state levels in the liver may render the body
susceptible to acute xenobiotic and toxicological exposure with age. Examinations of age-related changes in detoxification capacity with age, and consequentially the therapeutic threshold of pharmacological agents in older individuals are scant. Further work will be necessary to elucidate the extent and precise nature that the loss of Nrf2 protein homeostasis plays in increased susceptibility to environmental and pharmacological insults with age.

The loss of Nrf2 levels and consequently attenuation of ARE controlled genes may also be a factor in increasing the risk for chronic pathophysiologies with age. For example, we previously demonstrated that significant age-related changes occur to gene transcripts in the aging rat liver. Most of the changes were clustered to genes associated with chronic inflammation. An ontological analysis of these changes reveals enrichment of immune response, immune cell infiltration, proinflammatory, and tissue remodeling genes. Most importantly, this change is not concomitant with an increase in the antioxidant response [146]. Therefore, the loss of Nrf2 could directly contribute to this low-grade subclinical inflammation as it directly regulates numerous genes involved in the negative feedback arm to down-regulate cytokine and NFκB-mediated inflammation [121]. Thus, loss of Nrf2 and the consequences to its downstream target genes may play a critical role for numerous pathophysiologies where chronic inflammation is part of the underlying etiology (e.g. dyslipidemia, fibrosis, cirrhosis, and cancer).
It is also interesting to note that the loss of Nrf2 translation may not only potentiate chronic inflammation, but may be perpetuated by the necro-inflammatory environment of the aging liver. Herein, we show that only miRs associated with inflammation increase with age, while miR species connected to other metabolic pathways actually decline. Of those that increase, rno-miR-34a has been reported to inhibit Nrf2 mRNA translation; however, the transcript levels of this miR were quite low and follow-up experiments failed to show a connection between miR-34a and the age-related loss of Nrf2 (data not shown). Rno-miR-146a, the most abundant inflammation-induced miR that increases with age, has a predicted binding sequence in 3'-flanking region of Nrf2. Moreover, mimicking the increase in miR-146a in hepatocytes from young animals, to levels evident in aging, recapitulates the loss of Nrf2 seen with age. While these results do not rise to the level of a cause-and-effect relationship, it does suggest the possibility that the pro-inflammatory environment of the aging rat liver chronically induces expression of miR-146a, which in turn, adversely affects Nrf2 translation.

The advantages of “cross-regulation” between inflammation and Nrf2, via miR, are presently unclear; however, it may be an example of antagonistic pleiotropy. In this scenario, elevated miR-146a may be a normal response to inflammation associated with aging, and an attempt by the cell to attenuate the chronic inflammatory response. The resulting loss in Nrf2 translation may thus be an indirect consequence of persistent upregulation of pro-inflammatory cytokines, such as IL-6, which is known to induce miR-146a.
The resulting attenuation of Nrf2 would ironically limit the means to resolve the inflammatory response, thus perpetuating it.

An alternative view to this scenario is that limiting Nrf2 during persistent inflammation may actually be beneficial under specific circumstances. For example, it has recently been shown that Nrf2 can function in a feed forward mechanism to enhance oxidative stress via kruppel-like factor 9, a mechanism that is hypothesized to induce cell death [147]. Therefore miR-146a, perhaps in conjunction with other miRs, functions to limit Nrf2 after an oxidative stress. Our lab is currently investigating the relationship between Nrf2, inflammation, and senescence.

It is interesting to consider if restoration of Nrf2 would be universally beneficial in the old. If the loss of Nrf2 is an antagonistic pleiotropy then restoration of Nrf2 protein levels in the old could be deleterious to the cell or the organism. In the case of several age-associated diseases, particularly Alzheimer’s disease, both an increase in oxidative damage and a loss of Nrf2 protein levels are observed. Known Nrf2 inducers have failed to show significant efficacy in treatment or prevention in these diseases. However, overexpression of Nrf2 in mouse and fly models of some neurodegenerative disease results in a delayed onset and longer average lifespan [148, 149]. Our data suggest that typical Nrf2 inducers, which primarily act through Keap1 to prevent degradation, would fail to produce a substantial accumulation of Nrf2 as a result of the age-related attenuation of translation. 

While further investigation of the regulation of Nrf2 is needed in age-related
diseases, we hypothesize that an increase in Nrf2 synthesis, rather than inhibition of degradation, is necessary to facilitate the protection and resistance observed in the overexpression model. We are currently investigating methods to increase Nrf2 synthesis in old rats.

In summary, we have identified for the first time, an age-related change that results in the attenuation of Nrf2 protein levels in the liver of old rats. Chiefly, Nrf2 protein synthesis declines with age. This observation can, in part, explain why Nrf2 levels decline despite the age-related increase in ROS. This results in the increased susceptibility to acute stressors that rely on basal levels of Nrf2 for detoxification and likely chronic challenges as well. Further research will be required to characterize the consequence of the loss of Nrf2 translation in regards to chronic inflammatory conditions. Finally, restoration of Nrf2 synthesis, perhaps via disruption of miR-146a, and the effects of this reversal are being investigated.
2.5 Experimental procedures

Reagents
All reagents were purchased from Sigma-Aldrich (St. Louis, MO) unless noted otherwise. Collagenase type four was purchased from Worthington Biochemical Corporation (Lakewood, NJ). Anti-Nrf2 (sc-13032) and anti-Keap1 (sc-15246) were ordered from Santa Cruz Biotechnology (Santa Cruz, CA). Immobilon-P PVDF Transfer Membrane was purchased from Millipore (Billerica, MA) Dual Luciferase Assay Kit was purchased from Promega (Madison, WI).

Animals
Fischer 344 male rats, both young (3-5 month) and old (25-29 month), were purchased from the National Institute on Aging colonies. The rats were allowed to acclimatize in the Linus Pauling Institute animal facility for a minimum of 1 week on a 12 hour light cycle (7am to 7pm) and fed standard chow ad libitum. All animal work was approved and in accordance to IACUC guidelines. The AAALAC-accredited Laboratory Animal Resources Center (LARC) provided management and veterinary care.

Hepatocyte Isolation and Cell Culture
Hepatocyte isolation was performed as described previously [150]. Briefly, the liver was perfused with Hank’s balanced salt solution, and disassociated to a single cell suspension using a collagenase solution (type 4, 3mg/ml). Cell
suspension was filtered through sterile gauze to remove connective tissue and debris. Parenchymal cells were isolated using gravity filtration and washed four times with Krebs–Henseleit solution. Cell count and viability were assessed using trypan blue exclusion.

Hepatocytes were suspended in a modified Williams’ E (5% FBS, 1 mM dexamethasone, 100 ng/ml insulin, 2 mM L-glutamine, 100 U/ml penicillin, and 100 mg/ml streptomycin), dispensed onto collagen coated cell culture plates and incubated for 16 hours at 37 °C in 5% CO₂ atmosphere before being used in experiments. Hepatocytes were seeded at 2.5x10⁵ cells/ml for cell culture. However, hepatocytes used for transfection were seeded at a concentration of 1.25x10⁵ cells/ml.

**Nuclear and Cytoplasmic Protein Extracts**
Cultured hepatocytes were washed twice with PBS, pH 7.4, and then collected by scraping. Nuclear and cytoplasmic protein extracts were collected by the use of Sigma-Aldrich CelLytic Nu-CLEAR isolation kit following the manufacturer’s protocol.

**Immunoblots**
Protein samples were denatured by addition of 2X Laemmli loading buffer and heating at 90 °C for 5 minutes. Protein separation was performed using a neutral Bis-tris acrylamide gel and following the BioRad XT Bis-Tris PAGE
XT-Mes protocol. Transfer was accomplished using the Bjerrum Schafer-Nielsen transfer buffer with 20% methanol on a semi-dry transfer apparatus. Proteins were deposited on Immobilon-P PVDF Transfer Membrane. All blots were blocked with a 5% milk (w/v) TBS-T solution over night at 5°C. Primary and secondary antibodies were each incubated on the membranes for 2 hours. Densitometry, performed with the ImageJ software package (http://imagej.nih.gov/ij/), was used for graphical representation and to perform statistical analysis.

**Quantitative PCR**

Briefly, total RNA was collected from hepatocytes with Trizol (Life Technologies, Carlsbad, CA), and reverse transcription was performed using the Retroscript Kit (Life Technologies) following the manufacturer’s protocol. The PCR was done on a StepOne plus (Life Technologies) using Taqman Universal Mastermix. All primer-probe mixtures were purchased from Life Technologies except for Nrf2. The Nrf2 primers were ordered from Eurofins MWG Operon (Huntsville, Alabama) with the following sequence and modifications:

```
TTTCCAGTGAGGGGATCGATGAG,
GTCAGCTACTCCCAGGTTGCCCA,
[6-FAM]ACCACTGTCCCCAGCCCAGAGGCCAC[BHQ1a-Q].
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Quantification was normalized to the housekeeping gene EIF2A.

**Transfections**
Transfections of hepatocytes were achieved with the jetPEI-hepatocyte transfection reagent (Polyplus-transfection SA, Illkirch, France). Hepatocytes were treated with 2 µg plasmid and the recommended amount of jetPEI after hepatocytes had been in culture for 30 hours. Transfection efficiency was assessed to be > than 60% by parallel transfection of EGFP vector and cell counting.

**ARE-gene Induction**

Hepatocytes were transfected with a human influenza hemagglutinin tagged Nrf2 (isoform BC061724) over-expression vector (pHA-Nrf2) or empty vector with jetPEI as detailed above. Cells were harvested 16 hours after transfection, total RNA isolated, and assayed for mRNA expression as stated above. Relative quantities were calculated based on ΔΔCt between cells transfected with pHA-Nrf2 and empty vector and assuming an amplification efficiency of 2.

**Nrf2 Half-life**

Hepatocytes were transfected with pHa-Nrf2 overexpression vector or empty vector with jetPEI as described. After 20 hours, hepatocytes were treated with 100 µM cycloheximide. Hepatocytes were harvested every 20 minutes and the nuclear and cytosolic proteins were isolated as described. Immunoblots were used to quantify Nrf2 levels. Statistical analysis was done by a two-way ANOVA.
Proteasome Inhibition

Hepatocytes in cell culture were treated with 100 nM of bortezomib (Millennium Pharmaceuticals, Cambridge, MA). In order to induce the Nrf2-ARE response, cells were treated with 50 µM 5-(4-methoxyphenyl)-3H-1,2-dithiole-3-thione (A3T) or both 100 nM bortezomib and 50 µM A3T. After 6 hours cells were harvested and cytosolic and nuclear proteins were collected. Nrf2 content was assessed by immunoblot.

Polysome Isolation

Polysome fractions were collected as described previously [151]. Briefly, 5.0x10^7 hepatocytes were cultured for 36 hours before use. Plates were washed twice with 100 µg/ml cycloheximide in PBS and collected by scraping. Cells were incubated in 1 ml lysis buffer (20 mM HEPES, 100 mM NaCl, 1.5 mM MgCl_2, 10 mM DTT, 150 µg/ml cycloheximide, 1 u/µl RNAsin, and 0.5% Triton X) for 5 minutes and then homogenized using a glass-teflon dounce homogenizer. Homogenate was centrifuged at 4 °C and 13,000 rcf for 10 minutes. Homogenate, 25 mg, was diluted to 0.5 mls in lysis buffer and added to 15-50% sucrose gradient. Samples were centrifuged in a Ti-41-SW for 2.38 hours at 30,000 RPM and 4C on an Optima LE-80K ultracentrifuge. Samples were fractionated into 200 µl aliquots. Thermo Scientific Solaris RNA Spike Control Kit (Thermo Scientific) was added to each fraction according to the provided protocol and total RNA extracted using Trizol. Reverse transcription
was performed with Retroscript kit and QPCR as described above. Each fraction was assayed for the RNA spike to control for extraction and reverse transcription efficiency using the following primer probe combination:

TGCAAAGCCAATTCCCGAAG, CCATTGTAGTGAACAGTAGGAC, [6-FAM]ATGCCTGAAAATAATGTCC[BHQ1a-Q].

**ARE Activity**

Hepatocytes in culture, 2x10^5 were transfected with 1.95 µg ARE-Luc, a Gclc-promoter driven PGL4 luciferase vector, and 0.05 µg of a Renilla expression vector (pRL-CMV). Cells were incubated 18 hours after transfection and then treated with either vehicle control (DMF), 100 µM Lipoic Acid (LA), or 50 µM 5-(4-methoxyphenyl)-3H-1,2-dithiole-3-thione (A3T). Cells were harvested and assayed for luciferase activity using the protocol provided with the Dual Luciferase Assay Kit (Promega).

**miRNA Transfection and Inhibition**

Hepatocytes were transfected with mirVana miRNA mimic or inhibitor (Life Technologies) using RNAiMax following the provided protocol. The miRNA levels were measured using the Taqman MicroRNA Assay (Life Technologies).

**Statistical Analysis**
All statistical analysis was performed using Excel (Microsoft, Inc.) and Prism 7 (GraphPad Software, Inc.) For comparisons between two samples, two-sided Student’s t-test was used. Differences between samples that resulted in a p-value of ≤ 0.05 were considered statistically significant. Statistical analysis between multiple endpoints was analyzed by two-way ANOVA and multiple comparisons were evaluated by Tukey’s post test method.
2.6 Figures

Figure 2.1. Nrf2 protein levels but not mRNA levels declines with age.
A) Liver tissue lysate from young (Y) and old (O) rats were assayed for Nrf2 protein content by immunoblot. Beta-actin (β-Actin) was utilized as a loading control and housekeeping gene. B) Densitometry indicates that the expression of Nrf2 declines with age and is statistically significant, N=4 p <0.05. C) QPCR was utilized to assay the relative expression of known Nrf2 regulated genes. Analysis shows some Nrf2-regulated messages decline significantly with age. (N=7 * denotes p<0.05)
Figure 2.2. Nrf2 over-expression results in similar induction of ARE regulated genes in hepatocytes isolated from young and old rats. Cultured hepatocytes from young and old rats were transfected with pHANrf2. These cells were then incubated for 24 hours and QPCR was utilized to assay the relative expression of messages regulated by Nrf2. Analysis shows that there is no significant difference between young and old samples.
Figure 2.3. Nrf2 half-life increases with age. Hepatocytes cultured from young and old rats were transfected with pHA-Nrf2. These cells were then incubated for 24 hours and then treated with 100 um cycloheximide (Time=0.) Nuclear fractions were isolated from the harvested samples and assayed for Nrf2 content by immunoblot. Samples are quantitated by densitometry and relative to the loading control and housekeeping β-actin. Results indicate that the half-life of Nrf2 is significantly increased with age (N=3, p<0.05).
Figure 2.4. Polysome profile from hepatocytes isolated from young and old rats liver lysate.
A cytoplasmic extract of hepatocytes isolated from young and old rats were sedimented on a sucrose gradient, collected as 20 fractions from the bottom, RNAs were extracted from each fraction, and the levels of 18S rRNA, β-Actin (Actb) and Nrf2 mRNAs were determined by QPCR.
Figure 2.5. Nrf2 synthesis declines with age.
Hepatocytes from young and old rats were cultured for 36 hours then treated with 50 uM A3T, 100 nM bortezomib, or both for 6 hours. Nuclear fractions were isolated from the harvested samples and assayed for Nrf2 content by immunoblot. (A) Representative immunoblot. (B) Samples are quantitated by densitometry and relative to the loading control beta actin. Although a significant increase in nuclear Nrf2 is seen in the young after treatment, no significant increase is seen in cells isolated from the old rats. Statistical analysis is done by two-way ANOVA and multiple comparisons are calculated by Tukey’s method. Both age and treatment effects are significant. (N=3 p<0.05).
Figure 2.6. Rno-miR 146a may attenuate Nrf2 protein synthesis with age.

A) Liver tissue from 6 young (6 months) and 6 old (24 months) rats were analyzed on a miRNA array. Results were limited to p value of less than 0.05 and corrected for multiple comparison. The miRNAs predicted and experimentally determined to modulated Nrf2 are shown. B) Liver tissue from 6 young and 6 old rats were analyzed by RT-qPCR for levels of rno-miR-146a. The difference between young and old is significant by student’s t-test p<0.05. C) Hepatocytes isolated from 3 young rats were transfected with rno-miR-146a miRNA mimic. The hepatocytes transfected with rno-miR-146a mimic or scrambled RNA oligomer were treated with bortezomib for 6 hours and compared to vehicle-treated hepatocytes. Nrf2 protein levels and loading control beta-actin were measured by immunoblot analysis and quantitated by densitometry. Rno-miR-146a transfection significantly attenuates the accumulation of Nrf2, p<0.05. D). mRNA levels were measured and compared to hepatocytes transfected with a scrambled RNA oligomer. Although no significant difference was seen in the RQ of the Nrf2 mRNA, both Gclc and Gclm decrease significantly, p<0.05.
Figure S2.1. Keap1 protein levels decline with age.
Liver tissue lysate from young and old rats were assayed for Keap1 protein content by immunoblot. Beta-actin (β-Actin) was utilized as a loading control and housekeeping gene. Densitometry indicates that the expression of Keap1 declines approximately 42% with age and is statistically significant, N=4 p <0.05.
Figure S2.2. Nrf2 stress response is attenuated with age. Hepatocytes isolated from young and old rats were transfected with the ARE-Luc and Renilla-PLG4 reporter vectors. After 24 hours cells were treated with 100 uM LA (Lipoic Acid) or 50 uM A3T. Cells were harvested 16 hours after treatment, lysed, and assayed for luciferase activity. Results indicate that the LA and A3T treatment significantly increase the luciferase activity in young samples. However, the increase in luciferase activity is significantly abrogated in cells from old rats with both treatments (N=3 * denotes p<0.05).
Supplemental Calculations.

Nrf2 half-life was measured from hepatocytes isolated from young and old rats. Although the steady state levels of Nrf2 in old rat liver have been shown to be about 60% of the young, the measured half-life is about 4.9 fold longer. A longer half-life, given all else is equal, would result in an increased steady state levels of Nrf2. This very simplified modeling of the steady state levels of Nrf2 serves to predict what factors must change if we assume both an increased half-life and a decrease in steady state levels of Nrf2 in old rats. Furthermore, we can compare our predictions to other experimental data to validate this model.

One major assumption is made in this exercise. The first assumption is that Nrf2 achieves a steady state level. Although this is likely not true because of circadian rhythm and an ever-changing environment experiments have been designed to minimize these factor.
Figure S2.3. A simplified model of steady state protein levels. This model includes the following components: the concentration of, the message (M), the concentration of protein (P), rates of translation ($\tau$) and of degradation ($\lambda$).

DIFFERENTIAL EQUATION MODEL OF PROTEIN EXPRESSION

$$\frac{\delta P}{\delta t} = \tau_1 M_1 - \lambda_1^P P_1$$

ASSUMPTION AT STEADY STATE LEVEL

$$\tau_1 M_1 = \lambda_1^P P_1$$
EXPERIMENTAL HALF-LIFE OBSERVATION

\[ 5\lambda_o^p P_o = \lambda_y^p P_y \]
\[ \lambda_o^p P_o = \frac{1}{5} \lambda_y^p P_y \]

SUBSTITUTIONS

\[ \tau_o M_o = \lambda_o^p P_o \]
\[ \tau_o M_o = \frac{1}{5} \lambda_y^p P_y \]
\[ 5\tau_o M_o = \lambda_y^p P_y \]
\[ 5\tau_o M_o = \tau_y M_y \]

Therefore, if the half-life of Nrf2 in hepatocytes isolated from old rats is 5-fold of young this model predicts that the rate of synthesis in the young is 5-fold of the old. This assumes both achieve a steady state level of Nrf2. Because we find that the both mRNA levels show no significant difference then we can also state that \( \tau \), the rate constant of translation, must also be 5-fold higher in young. However, when we examine the rate constant of removal there is not a 5-fold difference between young and old.

\[ \lambda_o^p P_o = \frac{1}{5} \lambda_y^p P_y , P_o = 0.6P_y \]
This model predicts that the rate constant of degradation should be 3-fold higher in the young, assuming a 40% decrement of Nrf2 protein levels with age. However, the rate of degradation was measured with Nrf2 overexpression. Hepatocytes from both young and old were transfected with an Nrf2 overexpression vector and had comparable levels of Nrf2, as seen by western blot analysis. Therefore, this 3-fold difference may not reflect the true difference in rate constant. Instead, we might simply assume an equal amount of Nrf2 protein and therefore predict a 5 fold change in rate constant. This exercise demonstrates that the observed changes in proteostasis could reasonable resolve to the attenuated levels of Nrf2 protein observed in the old.
Chapter 3. Basal Transcriptional Regulation of Rat γ-Glutamate Cysteine Ligase Catalytic Subunit Gene is Mediated through a Distal Antioxidant Response Element

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3.1 Summary

Despite it being a quintessential Phase II detoxification gene, the transcriptional regulation of the rat γ-glutamate cysteine ligase catalytic subunit (GCLC) is controversial. Computer-based sequence analysis identified three putative antioxidant response elements (AREs) at positions –889 to –865 (ARE1), -3170 to -3146 (ARE3) and –3901 to –3877 (ARE4) in the 5'-flanking region upstream of the transcriptional start site. Transfections of individual ARE-luciferase reporter gene constructs into H4IIE cells, a rat hepatoma cell line, identified ARE4 as the functional promoter. Chromatin immunoprecipitation assays using primary rat hepatocytes showed that the transcription factor Nrf2, which is known to regulate ARE-mediated genes, associated with ARE4. Co-transfection of H4IIE cells with Gclc ARE4-luciferase reporter plasmids and an Nrf2 expression plasmid resulted in a 3-fold activation of ARE4-mediated transcription relative to controls. “Loss-of-function” analysis for Nrf2 by small interfering RNA (siRNA) revealed that ARE4-mediated expression was significantly impaired. Finally, site-directed mutagenesis of the ARE4-luciferase reporter abolished Nrf2-mediated induction. Taken together, these results show that Nrf2 regulates the constitutive expression of rat GCLC through a distal ARE present in its 5'-flanking region. This is the first report showing that rat Gclc is under the transcriptional control of the Nrf2-ARE pathway on a constitutive basis.
3.2 Introduction

Glutamate cysteine ligase (GCL) catalyzes the first and rate-limiting step of the de novo synthesis of glutathione (GSH), the most abundant non-protein thiol in the cell [152]. GSH plays key roles in detoxifying peroxides, electrophiles, and maintaining the normal intracellular thiol redox status [153-156]. The de novo synthesis of GSH from its constituent amino acids involves two ATP-requiring enzymatic steps: the formation of γ-glutamylcysteine from glutamate and cysteine, and subsequent formation of GSH from γ-glutamylcysteine and glycine [157, 158]. GCL is the rate-controlling enzyme for GSH synthesis [159]. The GCL protein is a heterodimer that can be dissociated under non-denaturing conditions into a catalytic (GCLC, 73 kDa) and a modulatory (GCLM, 29 kDa) subunit, which are encoded by separate genes [160]. Although the heavy subunit contains the entire catalytic activity, association with GCLM regulates this activity [159]. Since GCL is a major determinant of the overall capacity of GSH synthesis, regulation of GCL subunits has been a topic of extensive research [158]. GCL has multiple levels of regulation, which ultimately affect either the catalytic or modifier subunits or both. In addition, the enzyme can be regulated at the kinetic, post-translational and transcriptional levels [158, 161, 162]. However, the regulation of GCL at the transcriptional level produces a more persistent effect and thus is important for the maintenance of GSH homeostasis in response to oxidative stress [163]. It has been well established that both GCLC and GCLM are inducible at the level of transcription by various agents.
such as quinones (tert-butyl hydroquinone), dithiolethiones (anetholedithione), isothiocyanates (sulforaphane), and dithiols (liopoic acid) [164].

The distal 5′-flanking region of Gclc has been fully sequenced in the human [165] and mouse [166], but not yet in the rat [167]. Several DNA cis-elements including those for NF-κB, the AP-1 binding site, and the antioxidant response element (ARE) have been implicated in GCL gene regulation [168]. In humans and mice, the ARE (core sequence: GTGACNNNGC) has been identified as the regulatory component responsible for the induction of Gclc both on a constitutive basis and in response to oxidative or electrophilic stress [165, 169-171]. Many transcription factors have been reported to bind the ARE, such as Nrf2 family members (Nrf1/2/3), small maf proteins (maf G/K/F), as well as AP-1 transcription factors [Jun (c-Jun, JunB, JunD), and Fos family members (c-Fos, FosB, Fra1, Fra2)] (reviewed by Jaiswal) [172]. Among them, ARE-dependent GCLC gene expression is largely dependent upon Nrf2, a member of the Cap'n'Collar (CNC) family of bZIP proteins [170, 173]. Nrf2 is located primarily in the cytosol but upon stimulation, accumulates in the nucleus, where it heterodimerizes with other leucine zipper proteins (e.g. c-Jun and small maf proteins), and binds the ARE to initiate gene transcription [51, 84]. Importantly, when overexpressed in cells by transfection, Nrf2 accumulates in the nucleus and activates transcription [174, 175].

The 5′-flanking region of rat Gclc has only been partially characterized
where only a 1.8 kb sequence upstream of the start site was thoroughly analyzed. In this region, several binding sites for AP-1 and NF-κB were reported [167]. Yang and co-workers [180] suggested that an AP-1 sequence in the proximal promoter region of Gclc is critical to its transcriptional upregulation in response to oxidative stress. In part, this contention is because AREs that are present in the human Gclc promoter, are not found in this 1.8 kb region of the rat Gclc promoter. However, Nrf2-ARE binding has been detected in the rat by transfection of an ARE-containing sequence derived from the human GCLC gene [181]. A 44-bp ARE sequence, which shares a 31 bp similarity with the human Gclc ARE, has been recently designated as the rat Gclc ARE, but no additional sequence information has been disclosed [182]. Thus it is possible that functional AREs in the rat GCLC gene may be present further upstream of the known 5'-flanking regions. Regardless, which cis-acting element is primarily involved in the basal gene expression of rat GCLC is still unknown and an ongoing subject of debate.

The aim of the present study was to determine whether Nrf2 controlled basal transcriptional regulation of rat Gclc through an ARE-dependent mechanism. Three putative AREs were identified in the promoter region of the rat GCLC gene (designated ARE1, 3 and 4), respectively. A ARE-like sequence, which contains only a partial consensus sequence, was also identified and designated as ARE2. Complete characterization of these elements as well as putative AP1 sequences show for the first time that
constitutive expression of rat GCLC is regulated at the ARE4 site in an Nrf2-driven manner.
3.3 Results

Identification of a functional ARE in the \textit{Gclc} promoter

We examined the rat \textit{Gclc} promoter for potential antioxidant response elements (AREs) by a computer-based analysis. Using the TRANSFAC database and the ARE consensus sequence as a probe \cite{163} revealed that there are three ARE motifs (designated ARE1, 2 and 4) within 5 kb of the \textit{Gclc} 5’-flanking region (Figure S1). When compared to the human \textit{Gclc} promoter sequence, rat ARE4 is identical to the human \textit{Gclc} ARE4 while ARE1 contains a single mismatch \cite{165, 183-185}. Identity between the rat and human sequence suggested that the ARE4 site of the rat sequence might be important in \textit{Gclc} transcription.

\textbf{Nrf2 binds to GCLC ARE4 in an endogenous chromatin configuration}

Since Nrf2 is the most potent transcriptional activator among CNC proteins \cite{186-189}, Nrf2 may enhance cytoprotective gene expression even if the other transcription factors are present and occupy the ARE. To examine whether each of the three complete AREs in the 5’-flanking region of \textit{Gclc} has the ability to bind Nrf2 \textit{in vivo}, we determined the extent of Nrf2 associated with individual ARE elements in their native chromatin environment by using the chromatin immunoprecipitation assay \cite{190}. To control for possible nonspecific interactions and DNA contamination, samples precipitated with rabbit immunoglobulin G were included. Primary rat hepatocytes were cross-
linked with formaldehyde, and chromatin was immunoprecipitated using an anti-Nrf2 antibody. PCR analysis revealed Nrf2 binding only to the region between 3.2 and 4 kb upstream of the transcriptional start site (Figures 1A and 2), which harbors the functional ARE4. The recruitment of Nrf2 to these ARE sites was specific because no signal was detected in the immunoglobulin G control samples (Figure 2). No recruitment of Nrf2 to the nonspecific intervening region located between ARE1 and ARE3 was observed. The results obtained from DNA that was PCR amplified from chromatin extracts before immunoprecipitation (input) are shown for comparison (Figure 2). Thus Nrf2 binds only to ARE4 out of the four putative AREs identified in the 5'-promoter of rat Gclc.

**ARE4 regulates the constitutive expression of rat Gclc**

To characterize the contribution of each ARE to the basal transcriptional regulation of Gclc, ARE constructs were created by synthesizing three tandem copies of each ARE element and subcloning them into the pGL4 minimal promoter vector. Following transient transfection into H4IIE cells, each ARE was then analyzed for luciferase activity (Figure 3). The results revealed that only ARE4 at nucleotide position −3901 was capable of mediating expression over that seen in the empty vector (0.014 ± 0.0012 RLA for ARE4 versus 0.014 ± 0.0012 RLA for empty vector). All other AREs failed to significantly instigate basal expression. Furthermore, an ARE4 mutant in which the core ARE sequence was altered failed to increase
luciferase activity over that seen in the empty vector (Figure 3). Based on sequence alignment, the rat Gclc ARE4 is identical with the previously reported minimal “Class III” ARE enhancer sequences: (a/g)TGA(C/T/G)nnnGC(a/g) [152]. In contrast, the ARE1 and ARE3 sequences differ from the consensus sequence in that the 3’ GC bases are replaced by AC deoxyribonucleotides. Thus, our results validate the data we obtained from the ChIP assays (Figure 2) showing that ARE4 was the only functional element among the three AREs in the rat Gclc gene.

Previous studies reported that the active ARE in the human Gclc (ARE4) also contained an adjacent TPA-responsive element (TRE) that was potentially involved in the induction of the gene [165, 183, 191]. Since the ARE consensus sequence resembles the AP-1 binding site and the rat Gclc ARE4 has an embedded AP-1 site at its 5’-end, we transfected an AP1 luciferase construct into H4IIE cells. Results revealed that the AP1 component displays 52% of the basal transcriptional activity of the ARE4 site (data not shown).

**Nrf2-dependent upregulation of Gclc ARE3 transcriptional activity**

Nrf2 has been identified as a target transcription factor essential for ARE transactivation. To determine if induction of ARE4-luciferase activity is linked to activation of Nrf2, we examined the effect of constitutively over expressing Nrf2 on ARE4 transcriptional activity. As detected by western blotting, Nrf2 nuclear levels were enhanced in Nrf2-overexpressing cells by
250% (data not shown). Transfection of an Nrf2 expression plasmid also led to robust induction of \textit{Gclc} ARE4-driven luciferase activity (0.3998 ± 0.0415 RLA for Nrf2 overexpression versus 0.014 ± 0.0012 for baseline) (Figure 5).

On the other hand, there was no induction of the embedded AP1 element over baseline, indicating that Nrf2 neither directly nor indirectly trans-activates \textit{Gclc} transcription through the AP1 component of ARE4 (Figure S2). As a further control, an ARE4 mutant (\textit{GclcARE4} mut) was co-transfected with the Nrf2 expression plasmid in H4IIE cells. This ARE3 mutant abolished the Nrf2 response in reporter gene assays (Figure 5). These results indicate that the identified ARE4 is functional and mediates the activation of \textit{Gclc} transcription by Nrf2.

**Nrf2 inducers activate Gclc gene expression through ARE3**

To assess whether the inducible regulation of \textit{Gclc} transcription is similar to its constitutive transcriptional regulation, we utilized two known inducers of Nrf2, viz. LA and ADT. We transiently transfected H4IIE cells with luciferase reporter constructs containing three copies of each of the \textit{Gclc} ARE elements and treated them with 100 \(\mu\)M LA or 30 \(\mu\)M ADT for 24 hours. Measurement of luciferase reporter activities comparing unstimulated transfected cells to those after LA (203 ± 18\% over vehicle) or ADT (191 ± 17\%) treatments show that the activity of only the ARE4 element increases under stimulation by these Nrf2 inducers (Figure 6). These results
underscore the necessity of the ARE4 element in regulating the constitutive as well as inducible transcription of rat Gclc through Nrf2.

**Effect of Nrf2 siRNA on ARE4-mediated transcription of GCLC**

To further confirm the role of Nrf2/ARE in GCLC gene expression, we used small interfering RNAs (siRNAs) to inhibit the endogenous expression of Nrf2 in H4IIE cells. The transfection of H4IIE cells with a siRNA specific for Nrf2 reduced Nrf2 message expression by about 30% (Figure S3A). By contrast, the scrambled siRNA did not affect expression of Nrf2 message or its protein levels as compared to endogenous levels in non-transfected cells (Figure S3A and data not shown). Nrf2 siRNA was also co-transfected with Gclc ARE4 5’-luc2 or GclcARE4 (TRE) 5’-luc2, respectively to determine its effect on the ARE promoter activity. Briefly, H4IIE cells were first transfected with Nrf2 siRNA. Four hours after the siRNA transfection, Gclc ARE4 or Gclc (TRE) ARE4 promoter-luciferase plasmids were transfected. Luciferase assays were performed 36 h after the second transfection. Cotransfecting the ARE–luciferase construct enabled the measurement of residual transcriptional ARE activity in Nrf2-depleted cells. The silencing of Nrf2 led to a significant abrogation in ARE4-driven luciferase activity (Fig. S3B). These data reveal that Nrf2 is directly involved in ARE4-mediated basal transcription of the rat Gclc gene.
Taken together, these studies indicate that the rat GCLC promoter contains one functional ARE that is responsible for Nrf2-dependent basal transcription.
3.4. Discussion

GSH is the most abundant non-protein thiol in cells and it plays key roles in multiple biological functions, including scavenging free radicals, conjugation and detoxification of electrophiles, and maintenance of normal cellular redox status \[192\]. It is well known that GSH levels are maintained via de novo synthesis through GCL gene expression \[159, 193, 194\]. The current work demonstrates that constitutive GCLC gene expression in the rat liver is regulated through the ARE (ARE4). We also present evidence that rat Gclc is under the direct transcriptional control of Nrf2 at least on a basal level, and potentially, by induction of Phase II enzyme inducers. These conclusions are supported by the following observations: (a) computer-based searches of the Gclc 5'-flanking region show three putative ARE elements in this gene; (b) transfection of H4IIE cells with Gclc ARE and AP1 promoter constructs indicate only one of the AREs (ARE4) and its embedded TRE element have transcriptional activity; (c) chromatin immunoprecipitation assays show that Nrf2 binds only to ARE4 in the Gclc 5'-flanking region, and (d) site-directed mutation of the ARE4 element abolishes luciferase activity. Collectively, the results of the present study establish that the transcriptional regulation of rat Gclc is governed by the Nrf2-ARE regulon. A schematic model of rat Gclc transcriptional regulation is depicted in Figure 5.

The data presented in this study are in keeping with other work in humans and mice which show that constitutive regulation of Gclc expression is mediated in an Nrf2/ARE-dependent manner. However, a direct link for the
role of Nrf2-ARE binding in basal Gclc gene regulation has never been demonstrated in rats. For this species, deciphering the cis-regulatory element responsible for the basal transcriptional regulation of Gclc, has been controversial [167, 177, 180, 195-197]. Lu and co-workers reported that basal as well as inducible transcription of rat Gclc is regulated by an AP1 element located 450 bp upstream from the transcriptional start site [167]. This study also did not identify any ARE in the rat Gclc promoter region. Subsequent work, including one from the same group, reported that the Nrf2 is essential for rat Gclc transcription [177, 180, 196]. In addition, it was reported that Nrf2 regulated the transcription of rat Gclc indirectly through the AP1 site [177]. However, there are two significant points that must be considered which may help in resolving the results of these previous studies with our findings. First, only the first 2 kb upstream of the transcriptional start site for Gclc was previously analyzed, so the potential role of distal elements was not assessed (80). In fact, the active ARE4 site lies outside this 2 kb region and therefore, has not been previously investigated. Secondly, the rat Gclc promoter lacking the distal ARE elements (i.e. ARE4) was analyzed in fibroblasts of Nrf2 null mice, hence an accurate mechanism was not obtained (135). Nevertheless, the mechanism of transcriptional regulation of rat Gclc was an unsolved problem at the beginning of this study.

In previous reports investigating transcriptional regulation of rat Gclc, DNA binding assays were performed to assess transcription factors bound to consensus sequences because the complete sequence of the promoter had
not been cloned. However, the DNA binding assay is limited by its inability to disclose the native state of competition between two reactive elements at one time. In this report, we obtained more direct evidence using chromatin immunoprecipitation, which involves immunoprecipitating chromatin bound by Nrf2 and amplifying the TRE- or ARE-containing regions individually by PCR. ChIP assays conclusively showed that only ARE4 (located between -3901 and -3877 bases from the transcriptional start site) displayed Nrf2 binding in the in vivo state.

A significant finding of the present work is that constitutive transcription of rat Gclc results directly from binding of Nrf2-ARE4 binding. The association of Nrf2 with rat ARE4 is actually consistent with Nrf2 regulation of GCLC in human cell lines [170]. The distal ARE/EpRE sites located in the human Gclc promoter region, between -3802 and -2752 bp, are required both for constitutive and induced expression of the human GCLC gene [165]. Recently, it was reported that like other AREs, the most distal ARE in the human Gclc promoter region consists of a consensus ARE sequence containing an embedded TRE element [184]. The presence of a 5’ AP1-like element is known to enhance NQO1 gene ARE-mediated gene expression [172, 198]. However, the function of these regulatory sequences and the interaction between them has not been fully elucidated. In this context, it is noteworthy that the rat Gclc ARE4 responsive to Nrf2 contains a functional binding site for the AP1 family of transcription factors similar to the functional Gclc ARE in humans. The 5’-TGAC-3’ tetranucleotide within the ARE4 core
sequence resembles the half-site recognized by members of the AP-1 family (consensus: 5'-TGACTCA-3'). This also matches with results from Yang and co-workers where blocking AP-1 with dominant negative c-Jun lowered the basal expression of Gclc [176]. Our results thus provide a molecular mechanism for transcriptional regulation of rat Gclc and provide the first direct evidence that Nrf2 targets rat Gclc as part of maintaining constitutive glutathione synthesis.

The ARE sequence regulates the constitutive as well as inducible transcription of Gclc in the human as well as the mouse. Therefore, we asked whether LA and ADT, two thiol-containing phase II inducers regulated rat Gclc transcription strictly through the ARE3. Data presented in Figure 6 show that both these inducers increase luciferase activity by driving transcription exclusively through the ARE3 element and not ARE1 or ARE2.

GSH levels can be severely limiting in cases of acute xenobiotic challenges. So, far strategies to increase GSH have focused on boosting its rate-limiting substrate cysteine (administration of N-acetylcysteine) rather than increasing GCL levels. The evidence provided in this study opens the potential for augmenting GSH levels through increased transcription of its synthetic enzymes. This will be beneficial for toxicological studies in the rat model.

In summary, we found that basal transcription of rat Gclc occurs via the Nrf2/ARE4 complex since mutation of a previously uncharacterized ARE abrogates Gclc promoter activity. Moreover, ChIP assays detected increased
Nrf2 binding to ARE4. Finally, a role for Nrf2 in the induction of Gclc promoter activity is also demonstrated by the ability of Nrf2 siRNA to abolish the activation of Gclc promoter activity via ARE4.
3.5 Experimental Procedures

Chemicals and antibodies

Restriction enzymes and T4 DNA ligase for subcloning were from New England BioLabs (Boston, MA). All PCR reactions for cloning the full-length Gclc promoter were performed using the LA Taq Kit (Takara Bio, Japan). The PCR products were cloned into the TOPO isomerase linked vector, pCR 2.1, using the TOPO TA Cloning Kit from Invitrogen (Carlsbad, CA). All transformations used in cloning the Gclc promoter employed TOP10 One Shot competent cells (Invitrogen). QIAamp DNA Micro kit was used to isolate genomic DNA from liver tissue (Qiagen, Valencia, CA). The Dual Luciferase Reporter Assay System™ and reporter plasmids, pGL4 minimal promoter vector and phRL-CMV vector were from Promega (Madison, WI). The expression vector for Nrf2 (pcDNA3.1-Nrf2) was a kind gift from Dr. Anil Jaiswal, at Baylor College of Medicine, Houston, TX [199]. Custom oligonucleotides used in PCR cloning, subcloning, and DNA sequencing were purchased from Invitrogen. Sequence service was provided by the Center for Gene Research and Biocomputing at Oregon State University. Rabbit anti-Nrf2 (H-300) antibody, Nrf2 siRNA and scrambled sequences were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). R-(α)-lipoic acid was a gift of Asta Medica (Frankfurt, Mainz, Germany). Anetholdithiolethione was a kind gift from Dr. Balz Frei (Oregon State
University). All chemicals used were the highest purity available and at least analytical grade.

**Animals**

Male Fischer 344 rats (age: 3 months) were purchased from the National Institute of Aging colonies. Rats were maintained in separate security barriers operated by Lab Animal Resources, Oregon State University according to the guidelines established in the Guide for the Care and Use of Laboratory Animals (Animal Resources Commission on Life Sciences, National Research Council) and under specific pathogen free (SPF) conditions.

**Sequencing the 5'-flanking region of the rat GCLC gene and identification of putative ARE(s)**

To identify the presence and location of putative ARE(s) in the rat Gclc promoter, the 5 kb upstream from the translation start site was downloaded from the NCBI database (www.ncbi.nlm.nih.gov/genome/guide/rat). This sequence was used to search for putative ARE(s) with the help of the MatInspector [200] and the Matrix Family Library software (version 2.4; MatInspector, Genomatix, Munich, Germany) using the ARE primary core sequence (RTGAYNNNGCR) as the probe. The location of the transcription start site for the rat GCLC gene has already been determined [167].
Chromatin immunoprecipitation assay

Chromatin immunoprecipitation analysis was conducted using control rabbit IgG, anti-Nrf2 (Santa Cruz; sc-13032) antibodies. PCR primers are described in Table 2. Briefly, proteins and DNA were cross-linked with 1% formaldehyde for 10 minutes at room temperature and cells lysed in SDS-lysis buffer containing protease inhibitors and then sheared to an average length of 500-800 bp by sonication using a Sonic Dismembrator Model F60 (Fisher Scientific, Pittsburgh, PA). Sheared chromatin was immunocleared with salmon sperm DNA/protein A-sepharose and 10% of the precleared chromatin was stored and labeled as “input DNA”. The remaining chromatin was immunoprecipitated with IgG (control) or Nrf2 antibodies (2 µg) overnight at 4°C. Immune complexes were adsorbed onto salmon sperm DNA/protein A-sepharose beads. Immunoprecipitates were washed sequentially with wash buffers to reduce background. Protein–DNA complexes were eluted from the antibody with elution buffer (1% SDS, 0.1 M NaHCO3) and formaldehyde cross-links reversed by addition of NaCl (5 M) and heating at 65 °C overnight. DNA was purified using the QIAquick purification kit (Qiagen, Valencia, CA) and PCR performed using primer pairs that spanned each of the ARE and AP1 elements. PCR products were quantified using SYBR Green Mastermix (New England Biolabs) with the Mini Opticon 2 Real-Time PCR Detection System (Bio-Rad). Specific enrichment of DNA by anti-Nrf2 antibody was calculated by subtracting the PCR value of normal IgG from that of anti-Nrf2 antibody and by normalizing that value to the PCR input. Triplicate PCR
reactions were conducted for each sample, and the experiments were repeated at least thrice. The specificity of the PCR products was confirmed by melting curve analysis and size (agarose gel electrophoresis).

**Construction of full-length Gclc promoter construct**

To validate the use of specific ARE plasmid constructs, the full-length Gclc promoter region incorporating -5868 to -1 bp from the translation start site was cloned into a pGL4 basic vector (Promega). Because of the high GC-rich regions contained in the 5′-flanking region of Gclc, primers designed with sequences containing high GC content failed to produce an amplified product. To overcome this obstacle, the following cloning strategy was empirically devised. QIAamp DNA Micro kit (Qiagen) was used to isolate genomic DNA from F344 rat liver tissue. The Gclc promoter was cloned using the following primers 5′-CAGGACTTTGCCATTTGTCC-3′ and 5′-CGGGAGGCAGATATTCACAT-3′ to amplify a region 310bp downstream and 5,869bp upstream of the translation start site of Gclc. The PCR product was cloned into the TOPO isomerase linked vector, pCR 2.1, using the TOPO TA Cloning Kit (Invitrogen). The primers to amplify the fragment (-5868 to -1 bp) were as follows: 5′-CAGGACTTTGCCATTTGTCC-3′ and 5′-GCCGCGTCCTCCTCCTCCT-3′. The Gclc promoter region was then cloned into the pCR 2.1 vector in the reverse orientation using the TOPO TA Cloning Kit and manufacturers instructions. Finally, the Gclc promoter region was directionally cloned into the pGL4 Basic vector using the Kpn1 and EcoRV
restriction sites. The entire insert was sequenced for fidelity. Throughout, PCR reactions were using the TaKaRa LA Taq Kit (TaKaRa, Shiga, Japan), and all vectors were characterized by Bgl1 endonuclease restriction digests and sequencing. All transformations used in cloning the Gclc promoter employed TOP10 One Shot competent cells.

**Site-directed mutagenesis of full-length Gclc promoter construct**

Site-directed mutagenesis of the ARE4 sequence was performed to determine the relative contribution of the ARE and/or AP1 core sequences on reporter gene activity. Specific mutations, as outlined in Table S1, in the ARE4 sequence of the full length Gclc pGL4 construct was performed using the Quickchange II XL kit (Stratagene). Mutagenesis was accomplished using manufacturer’s instructions with the following modifications: PCR amplification was performed using GCl buffer (TaKaRa) designed for amplification of GC-rich fragments. All transformations were performed using XL10-Gold Ultracompetent cells. pGL4 Gclc construct mutants were characterized by restriction endonuclease digestion and sequencing.

**Construction of synthetic ARE and AP1 luciferase reporter vectors**

The ARE- and AP1-luciferase reporter plasmids were generated using the pGL4-minimal promoter vector (Promega) containing a minimal TATA promoter upstream of the firefly luciferase gene. The sequences of the inserts used in the different plasmids are summarized in Table 1. Single-stranded
oligonucleotides were first annealed to form double-stranded oligonucleotides and then ligated into the pGL4.23[minP] vector following the manufacturer’s instruction (Promega). Each vector was engineered by inserting 3 copies of each of the ARE elements present in the rat Gclc 5’-flanking region. The three different Gclc 25-bp ARE (3X)-driven luciferase reporter constructs [i.e., pGL4.23Gclc-3xARE1-Luc2 (ARE1), pGL4.23Gclc-3xARE3-Luc2 (ARE2), and pGL4.Gclc-3xARE4Luc2 (ARE3)] as well as the ARE mutant (pGL4.23Gclc-3xAREm-Luc2) and the embedded AP1 element in ARE4 (pGL4.23Gclc-3xembeddedAP1-Luc2) were made by insertion of the appropriately hybridized complementary oligonucleotides [75 bp] with 2-bp overhangs into the XhoI/HindIII restriction sites of the pGL4-minimal promoter reporter vector. TOP10 competent cells were transformed with the recombinant DNA after ligation for amplification. After the plasmids were generated, the DNA sequence of the inserts was verified.

**Cell culture, transfections and luciferase assays**

Rat hepatoma-derived H4IIE cells, obtained from American Type Culture Collection (Rockville, MD) were grown in MEM supplemented with 10% FBS, 2 mM glutamine, 100 U/ml penicillin and 100 U/ml streptomycin and incubated at 37 °C in a 5% CO₂ atmosphere. Reporter gene assays were used to determine effects of different ARE and AP1 elements on transcriptional activity of the rat Gclc gene. The pGL4 minimal reporter vector contains a genetically engineered firefly luciferase gene containing a minimal
promoter. When promoter regions are cloned into the pGL4 vector upstream of the luciferase gene, there is strong transcriptional activation and expression of luciferase. Mutations in the individual promoter elements can influence transcriptional activity of the luciferase reporter gene that is detected fluorometrically vs. an internal control. Transient transfections were done in cells grown to ~50% confluence using Effectene Transfection reagent (Qiagen, Valencia, CA). The cells were transfected with 0.8 µg of Gclc-Luciferase plasmids. Briefly, the DNA and 4 µl of Enhancer were dissolved in EC buffer from the kit to a total volume of 100 µl. The DNA–enhancer mixture was incubated at room temperature for 5 min. After incubation, 5 µl of Effectene transfection reagent was added to the mixture, mixed, and incubated at room temperature for 10 min to allow transfection–complex formation. Media (200 µl) was added to the mixture and mixed. The solution was then immediately added to the well containing the cells and 1.5 ml of fresh medium. The total amount of plasmid DNA for transfection was adjusted by using empty expression vector (pGL4.23). The control plasmid phRL-CMV encoding Renilla luciferase (0.02 µg) was included in each assay to account for variability in transfection efficiency. Thirty-six hours after transfection, cells were harvested with passive lysis buffer (Promega), and the supernatant was collected by brief centrifugation. Transcriptional activity was determined by the expression of firefly luciferase and was normalized to the renilla luciferase levels by using a Dual Luciferase Reporter Assay Kit™ (Promega) on a Biolumat LB9505 luminometer (Berthold Detection Systems, Pfhorzeim,
Germany). The means of at least three independent experiments, each carried out in duplicate, are shown and expressed as the mean ± SEM.

Because the vector construct containing the full length Gclc promoter region was large, transfection protocols were modified vs. the smaller constructs to maximize transfection efficiency. To this end, the following modifications were performed: the transfection reagent used was JetPEI Hepatocyte (Polyplus-Transfection Inc., New York) where the transfection reagent (3.2 µg) was added to 1 µg of the pGL4 plasmid and 0.02 µg the Renilla Luciferase vector in accordance with protocols supplied by the manufacturer. Times of cell harvest following transfection were as indicated above.

**Treatments with ARE inducers**

H4IIE cells transfected with luciferase constructs containing empty vector or different ARE elements were treated with LA or ADT to assess Gclc promoter activity. Briefly, a 100 mM LA or ADT stock was prepared in 100% dimethylformamide. Cells were treated with vehicle (0.1% DMF), 50 or 100 µM LA or 30 µM ADT for 24 hours after transfection. Transcriptional activity was measured as outlined in Section 2.5.

**Statistical Analysis**

The data throughout are expressed as the mean ± SEM. Statistical analysis was performed using GraphPad Prism software version 3.03
(GraphPad Software Inc., San Diego, CA). We used a two-tailed Student’s *t* test to compare the luciferase activity of individual *GCLC* promoter constructs. A *P* value less than 0.05 was considered to be significant. One-way analysis of variance [201] followed by Tukey’s post-hoc analysis was used when multiple comparisons were made to a control.

**Nrf2 siRNA knockdown**

Nrf2 siRNA (50 pM) or scrambled control (50 pM) was transfected to H4IIE cells by Effectene™ (Qiagen, Valencia, CA, USA) according to the manufacturer’s instructions. Briefly, cells (400,000) were seeded in six-well plates and incubated overnight, then transfected with 50 pM siRNA for 12 h using 10 µl Effectene per well. The cells were cultured for another 24 h, then used for experiments. There was almost no visible damage due to the transfection procedure.

**RNA Isolation and Real-time PCR**

Briefly, total RNA was isolated using RNeasy kit (Qiagen, Valencia, CA) according to the manufacturer’s instructions, quantified spectrophotometrically (260 nm) and assayed for purity by determining the 260/280 ratio. Total RNA (0.5 mg) was reverse-transcribed with random primers and the resulting cDNA was amplified by real-time PCR using a MJ Research Opticon 2 machine (BioRad, Hercules, CA). To obtain appropriate template concentrations, samples were run concurrently with standard curves.
generated using plasmid standards. β-actin was used as a housekeeping control for RNA recovery and reverse transcription efficiency. Gene expression was normalized to β-actin mRNA levels and expressed as arbitrary units. The primers used were as follows: Nrf2-F; 5’-TCAGCT ACTCC CAGGTTGCCCA-3’, Nrf2-R; 5’-GGCAAGCG ACTCATGGTCATC TAC-3’, β-actin-F; 5’-CCTTCCTTCTGGGTATGGAATCC-3’ Beta-actin-R; 5’-GAGCAAT GATCTTGATCTTCATGGTG-3’, where ‘F’ and ‘R’ denote forward and reverse primers, respectively.
3.6 Figures

Figure 3.1. Position of ARE promoters in the 5'-flanking region of rat Gclc. Representation of ARE sequences in the 5'-flanking region of rat Gclc. Gray boxes denote potential ARE sites.
Figure 3.2. Nrf2 binds only to ARE4 in vivo. Primary rat hepatocytes were cross-linked with formaldehyde for 10 minutes, and ChIP analysis was performed using antibodies against Nrf2. Normal rabbit IgG was used as a control. Results show Nrf2 only binds to Gclc ARE4 efficiently (N=3).
Figure 3.3. ARE3 regulates constitutive expression of rat Gclc. H4IIE cells were transiently transfected with 0.8 µg of either of the Gclc-ARE elements or the ARE4 mutant (ARE4m) cloned into the pGL4 luciferase reporter with a minimal promoter together with a CMV-Renilla luciferase transcription control plasmid. Luciferase activity was determined in the cytosolic fraction 36 hr after transfection. Results are representative of 3 independent experiments and presented as relative luciferase activities normalized to the Renilla luciferase internal control. ‘a’ denotes statistically significant values compared with empty vector (p < 0.001), while ‘b’ denotes statistically significant values compared with ARE4 (p < 0.05).
Figure 3.4. An intact ARE4 sequence in the 5' flanking region is necessary for constitutive expression of Gclc.

H4IIE cells were transiently transfected with 1 µg each of the Gclc full length promoter region (pGL4.10FL), the full length promoter with the ARE4 site either mutated completely (pGL4.10ARE4-) or with a point mutation in the sequence of ARE4 (pGL4.10ARE4m) cloned into the pGL4.10 luciferase reporter together with a CMV-Renilla luciferase transcription control plasmid (0.02 µg). The empty vector (pGL4.10) was transfected as a control to check for non-specific transcriptional activity. Luciferase activity was determined in the cytosolic fraction 36 hr after transfection. Results are representative of 5 independent experiments and presented as relative luciferase activities normalized to the Renilla luciferase internal control. 'a' denotes statistically significant values compared with empty vector (p < 0.0001), while 'b' denotes statistically significant values compared with ARE4 (p < 0.0001).
Figure 3.5. Nrf2 activates rat Gclc gene promoter activity through ARE4. H4IIE cells were transfected with 0.8 μg of either of the three Gclc ARE elements or the ARE mutant, 0.06 μg of pcDNA3.1-Nrf2 (Nrf2) expression plasmid along with 0.02 μg of pHRL-CMV Renilla luciferase plasmid as a control for transfection efficiency. Nrf2 response of each reporter construct is indicated by fold change of activity versus the activity of pcDNA3.1 control plasmid that was co-transfected with it. Nrf2 over expression failed to activate the ARE4 mutant construct. Luc activity of the reporter construct alone was arbitrarily set as 100, and the mean values of relative luciferase activity from at least three independent experiments, each carried out in duplicate, are shown with the ± SEM as detailed in “Materials and Methods”. 'a', $p < 0.01$ relative to ARE4 alone (Tukey’s post-hoc analysis). Mean difference of the Nrf2 response for the ARE4 mutant group is significant from control group at $p < 0.01$. 
Figure 3.6. Induction of Gclc by ARE inducers is controlled by ARE4. H4IIE cells were transiently transfected with 0.8 µg of either of the Gclc-ARE elements or the ARE4 mutant (ARE4m) cloned into the pGL4 luciferase reporter with a minimal promoter together with a CMV-Renilla luciferase transcription control plasmid. Eighteen hours after transfection, cells were treated with either 0.01% DMF (vehicle control), 100 µM lipoic acid or 30 µM anetholdithiolethione. Luciferase activity was determined in the cytosolic fraction 24 hr after treatment. Luc activity of the empty reporter construct alone was arbitrarily set as 100, and the mean values of relative luciferase activity from at least three independent experiments, are shown with the ± SEM. Results are representative of 3 independent experiments and presented as relative luciferase activities normalized to the Renilla luciferase internal control. ‘a’ denotes statistically significant values compared with empty vector (p < 0.001), while ‘b’ denotes statistically significant values compared with ARE4 (p < 0.05).
Figure S3.1. Putative response elements in the promoter region of rat. Representation of the 5′-flanking region of Gclc, which is conserved between human, mouse and rat. The numbers shown underneath represent DNA bases in the 5′-promoter, as counted from the transcriptional start site. The putative ARE and AP1 elements conserved between the three species are shaded.
Figure S3.2. Nrf2 controls Gclc transcription through the ARE site but not the embedded AP1 component of the ARE3 element.
H4IIE cells were transfected with 0.8\( \mu \)g of the Gclc ARE4 element or the AP1 component only of the ARE4 element, 0.06 \( \mu \)g of pcDNA3.1-Nrf2 (Nrf2) expression plasmid along with 0.02 \( \mu \)g of pHRL-CMV Renilla luciferase plasmid as a control for transfection efficiency. Nrf2 response of each reporter construct is indicated by fold change of activity versus the activity of pcDNA3.1 control plasmid that was co-transfected with it. Nrf2 over expression failed to activate the construct containing only the embedded AP1 component of the ARE4 element. Luc activity of the reporter construct alone was arbitrarily set as 100, and the mean values of relative luciferase activity from at least three independent experiments was calculated. ‘a’, \( p < 0.01 \) relative to ARE4 alone (Tukey’s post-hoc analysis).
Figure S3.3. Effects of Nrf2 siRNA on ARE4-dependent Gclc transcription in H4IIE cells.

H4IIE cells were transfected with 50 pM control siRNA or 50 pM Nrf2 siRNA as described under Materials and Methods, and then transfected with pARE4-rGclc-Luc2. (A) 24 hours after siRNA transfection, Nrf2 RNA levels were detected by RT-PCR. (B) Nrf2 knockdown inhibits luciferase activity of ARE4, but not the empty vector. a, p < 0.05 versus ARE4 control. Luciferase activities after treatment with control siRNA is shown as a negative control. Results are shown as mean ± S.E.M. of three independent experiments.
Figure S3.4. Nrf2 inducers only activate the intact full length 5’ flanking region of the Gclc gene.

H4IIE cells were transiently transfected with 1 µg of either an empty vector (pGL4.10), the Gclc full length promoter region (FL), or the full length promoter with a point mutation in the sequence of ARE4 (pGL4.10ARE4m) together with a CMV-Renilla luciferase control plasmid (0.02 µg). Eighteen hours after transfection, cells were treated with either 0.01% DMF (Vehicle), 50 µM lipoic acid (R-LA) or 30 µM anetholdithiolethione (ADT). Luciferase activity was determined in the cytosolic fraction 24 hr after treatment. Results are representative of 4 independent experiments and presented as relative luciferase activities normalized to the Renilla luciferase internal control. ‘a’ denotes statistically significant values compared with vehicle treated controls (pGL4.10) (p< 0.001).
### 3.7 Tables

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Table 3.1. Sequence of Inserts in the pGL4 Minimal Promoter Vector.  

* The underlined letters represent those nucleotides that form the minimal functional ARE or AP1 element. The mutated ARE4 core sequence is in bold.
Chapter 4. Identification of age-specific Nrf2 binding to a novel ARE locus in the Gclc promoter: a compensatory means for the loss of glutathione synthetic capacity in the aging rat liver?

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4.1 Summary

NFE2-related factor 2 (Nrf2) transcriptionally governs the cellular response to harmful electrophiles, xenobiotics, and reactive oxygen species. Its nuclear levels decline with age [44, 197], which in part explains the age-related loss of phase II detoxification. However, little work has yet characterized how age affects Nrf2 DNA binding, or the role that alterations to the Nrf2 transcriptional apparatus plays in modulating Nrf2-mediated gene expression. In the present study we used immunoprecipitation assays to show that Nrf2 bound to the active antioxidant response element (ARE) of the catalytic subunit of glutamate cysteine ligase (GCLC) is significantly lower in hepatic chromatin from aged versus young rats. Moreover, the activity at this ARE locus is diminished during aging because of the presence of Bach1 and the absence of CREB-binding protein (CBP), a transcriptional repressor and co-activator, respectively. Further analysis reveals that Nrf2 occupies an alternate ARE site located -2.2 kb downstream from the normally active ARE binding site in livers of old rats, indicating an age-specific adaptation to maintain gene expression. Our results thus show that the conversion of Nrf2 binding from an active ARE to an alternative ARE element is not adequate to maintain basal expression of hepatic Gclc in old rats, which provides a potential mechanism for the age-related loss of glutathione synthetic and other phase II enzymes.
4.2 Introduction

A hallmark of the aging process is a decreased response to both exogenous and endogenous stresses [202]. In the liver, this inadequate stress response is reflected by a lower detoxification capacity [203], which is exemplified by diminished levels of certain low molecular weight antioxidants like glutathione (GSH) and related phase II detoxification and antioxidant enzymes[34, 197]. In this context, we and others previously showed that GSH levels decline in the liver of aging Fischer 344 rats [34, 197, 204], in part from a loss in GSH synthesis.

The *de novo* synthesis of GSH from its constituent amino acids involves two ATP-requiring enzymatic steps: the formation of γ-glutamylcysteine, followed by its conjugation to glycine [152, 160]. Glutamate cysteine ligase (GCL) catalyzes the first and rate-limiting step of *de novo* synthesis, making it a major determinant of overall GSH synthetic capacity [152, 160, 179]. Data from many laboratories conclusively show that hepatic activity, protein levels and gene expression of GCL are significantly lower in aging rat liver [34, 197, 205, 206]. The GCL protein is a heterodimer that can be dissociated under non-denaturing conditions into a catalytic (GCLC, 73 kDa) and a modulatory (GCLM, 29 kDa) subunit, which are encoded by separate genes [207]. Although the heavy subunit contains the active site, its association with GCLM, which is present in far less amounts than GCLC, modulates the overall activity of the enzyme [179, 208]. Though GCLC is elaborately regulated at kinetic, post-translational and transcriptional levels...
[209], its transcriptional regulation produces a more persistent effect and thus is more important for the maintenance of GSH homeostasis in response to oxidative stress [176, 177, 210-212].

Previously, we showed that the age-related loss of rat hepatic GCL is linked to lower nuclear steady-state levels of the transcription factor, NFE2-related factor 2 (Nrf2) [197]. Coincident with lower nuclear Nrf2 is diminished binding of the transcription factor to its cognate DNA sequence, the antioxidant response element (ARE), in the 5′-flanking region of Gclc. Although, lower steady-state nuclear Nrf2 levels seem likely to be causally linked to the age-associated decline in GSH, the exact consequences of Nrf2 loss on the Gclc transcriptome still remains to be elucidated.

In the present study, we used Gclc as a representative Nrf2/ARE-mediated gene to study the transcriptional mechanism of age-related deficiency in GSH synthesis. We previously showed that the 5′-flanking region of the rat Gclc gene has three ARE elements, but only one of these sequences displays Nrf2-binding and transcriptional activity[212]. Therefore, we hypothesized that in aged rats, there is lower Nrf2 binding to the Gclc, which leads to a transcriptional remodeling and contributes to a decline in gene expression.
4.3 Results

**Nuclear steady-state levels of Nrf2 decline with age**

Nuclear and cytosolic Nrf2 levels were measured to establish the extent of age-related changes in this key transcription factor. Compared with hepatocytes from young rats, those from old animals exhibited a significant $50 \pm 17\%$ decline ($P < 0.05$) in steady-state nuclear Nrf2 levels (Figure 1A & B). Although results show that there is an age-associated change in cytosolic Nrf2 compared to the nuclear fraction, the change was not found to be significant by a Students t-test. These results show that the age-related loss of Nrf2 is primarily restricted to the nuclear compartment.

**Nrf2 enrichment at ARE4 is lower in hepatocytes from old rats**

Figure 2A shows a schematic representation of the putative ARE elements present in the 5'-untranslated region of Gclc. Only one ("ARE2") of the four ARE sequences that is located 2.1 kb upstream from the transcriptional start site does not contain the all the elements that are characteristic of typically active AREs. To determine the consequences of lower nuclear Nrf2 levels on binding of this transcription factor to individual ARE promoters, chromatin immunoprecipitation [190] (ChIP) assays were performed using an Nrf2 antibody. While Nrf2 does not bind to the two sequences located 1.0 kb and 3.1 kb upstream of the transcriptional start site (designated: “ARE1” and “ARE3”, respectively) in hepatocytes isolated from both young and old rats [212], this transcription factor associates with the
fourth ARE ("ARE4") 3.8 kb upstream of the transcriptional start site. Nrf2 binding to ARE4 decreases significantly (59 ± 17 %, P <0.05) in old rat hepatocytes (Figures 2B, 2C and Table 1) relative to young controls. Thus the loss of Nrf2-ARE4 binding in old rat hepatocytes is consistent with lower nuclear Nrf2 levels.

We further asked the question whether the ARE2 element displays any Nrf2-binding activity. ChIP analysis revealed no detectable binding of Nrf2 to the ARE2 promoter in hepatocytes from young rats (Figures 2B, 2C and Table 1), indicating that ARE4 likely mediates the increase in Gclc expression by Nrf2. In contrast, Nrf2 reproducibly associated with the ARE2 sequence in chromatin isolated from old rat hepatocytes (Figures 2B, 2C and Table 1). Taken together, these results demonstrate that Nrf2-ARE4 complex formation is attenuated in the aged rat liver, but there is an age-specific recruitment of Nrf2 to an alternate ARE promoter in Gclc.

**Aging induces transcriptional remodeling of the Gclc/ARE4 complex**

While the age-related loss of nuclear Nrf2 levels may be responsible for Nrf2 binding to the ARE4 site, it cannot explain why Nrf2 is partially re-directed in aged tissue to the ARE2 element, which is neither transcriptionally active nor contains the core Nrf2 binding sequence. Thus, the simple decline in steady-state nuclear Nrf2 levels may not completely address the age-associated loss in Gclc transcriptional activity. We hypothesized that, along with lower Nrf2 associated with ARE4, a repressive-type of transcriptional
protein complex develops at that site, while Nrf2 binding at the ARE2 locus may be a compensation for this loss. Initially, this hypothesis was explored by using ChIP assays to identify Nrf2 partner proteins that bind to both the ARE4 and ARE2 sites in chromatin from young and old rat hepatocytes. Table 1 shows that in the young rat liver, along with Nrf2, small maf proteins were present at ARE4. However, because no antibodies specific to mafG, mafK or mafF are commercially available, it was not possible to identify the precise small maf protein partnering with Nrf2. In addition, c-Jun and c-Fos, which are known to bind and recognize the ARE, were also detected at this locus. Finally, the histone acetyltransferase, CREB-binding protein (CBP), is also present on ARE4 in hepatocytes from young rats (Table 1 and Figure 3).

While these ChIP assays cannot absolutely quantify a given amount of a transcription factor because of differences in antibody-binding affinities, relative quantification by qPCR analysis shows that the ARE4 in cells from young rats is transcriptionally active because only permissive binding partners with Nrf2 are present at significant levels above a non-specific IgG protein (see Table 1). Thus, transcription factors that would permit Gclc expression reside at the ARE4 locus in hepatocytes from young rats.

Using a similar strategy to map age-related changes to the ARE4 locus, we found that the ARE4 transcriptome of Gclc in hepatocytes from old rats is significantly altered relative to young rats (Table 1). First, chromatin from aged cells show the presence of Bach1, a known transcriptional repressor of ARE-mediated gene expression [86, 89, 213]. Bach1 has a DNA-
binding motif and associates with the ARE promoter when Nrf2 is limiting, but has no trans-activating domain. Thus, the presence of Bach1 at ARE4 suggests a repressive transcriptional motif develops on the Gclc promoter with age.

Also, in sharp contrast to young hepatocytes, ChIP analysis for the presence of CBP at the ARE4 locus revealed a complete lack of amplification for this key transcriptional co-activator at ARE4. These results suggest no co-activation of Nrf2 at this site (Table 1 and Figure 3). Taken together, we interpret the observed age-associated changes to the Nrf2-ARE transcriptome to mean that a repressive transcriptional environment occurs at the Gclc ARE4 site. These results correlate with our data showing an age-related loss in Nrf2 binding to the ARE and also reflect diminished Gclc expression shown previously [197].

**Nrf2 occupies ARE2 in old rats along with AP1 transcription factors**

Further ChIP experiments were undertaken to understand whether the age-specific formation of the Nrf2-ARE2 complex was accompanied by a permissive transcription factor profile. Table 1 shows that, of the transcription factors examined in young rats, the ARE2 motif binds only c-Jun and c-Fos, not Nrf2. As c-jun and c-Fos constitute the typical AP1 transcription heterodimer, these results may indicate that gene expression through the ARE2 element could be possible and partially contribute to the expression of Gclc, but in an Nrf2 independent manner. It should also be noted that
elements of a core AP1 sequence are contained in the ARE2 promoter. Consistent with the observations made in hepatocytes from young rats, c-jun and c-Fos also associated with ARE2 in old rat hepatocytes; however, AP1 binding was also accompanied by Nrf2 interacting with the ARE2 element (Table 1). No evidence of Bach1 binding to this element was observed. Thus, only co-activating transcription factors and no repressors were observed at ARE2 site with age.

“Nrf2 promoter switching” in old rats does not compensate for the “age-related” decline in ARE-mediated transcriptional activity

To examine the consequences of the age-specific changes in Nrf2 binding to both ARE4 and the ARE2 elements, we transiently transfected primary hepatocytes from young and old rats with minimal promoter-luciferase reporter constructs, each containing three copies of either ARE4 (pGL4.23Gclc[3X]ARE4.luc2) or the ARE2 (pGL4.23Gclc[3X]ARE2.luc2) locus. To verify that no reporter activity occurred through the ARE1 and ARE3 sites even in the absence of Nrf2 binding, transfection experiments were carried out with the ARE1 and ARE3 luciferase reporters as well. Additionally, the pHRL-CMV Renilla luciferase construct was co-transfected as a control for transfection efficiency. Data from these experiments show that the ARE1 and ARE3 promoters are not transcriptionally active [212], suggesting that these are pseudo-ARE sites and not involved in gene expression. However, when the ARE4-luc construct was transfected into hepatocytes, robust activity
was evident. With age, there was a significant $47 \pm 14\%$ decline in luciferase activity induced by the ARE4 promoter, indicating that lower Nrf2 binding at this motif correlates well with a decline in transcriptional activity (Figure 4). Interestingly and in concert with the ARE4 site, luciferase activity induced by the ARE2 promoter also exhibited an age-related decline to the same extent as seen in the ARE4 promoter (Figure 4). Given the age-specific occupation of the ARE2 promoter by Nrf2, these results were somewhat surprising.

Based on the aforementioned results, we hypothesized that even though Nrf2 was found on the ARE2 site in aging rat liver, the amount of Nrf2 actually bound to ARE2 was inadequate to compensate for lower age-related expression through the normal ARE4 element. To test this hypothesis, hepatocytes from old rats were co-transfected with an Nrf2 expression plasmid along with the ARE4 and ARE2 luciferase reporter constructs in order to substantially increase nuclear Nrf2 levels. Figure 4 shows the results of Nrf2 over-expression on ARE4 and ARE2 luciferase activities in old animals compared with baseline activities in the young. An examination of both the ARE4 as well as ARE2 driven luciferase activities reveals that Nrf2 over-expression compensates for the overall loss of ARE-governed transcription in aging primarily through the ARE2 element, if not exclusively. However, overexpression of Nrf2 in hepatocytes from young rats only increases ARE4-driven, but not ARE2-controlled luciferase activity. These results confirm that Nrf2 levels are limiting to transcriptional activity of the ARE2 promoter in the old rat liver. Additionally, these data also provide credibility to the hypothesis
that loss in activity of the ARE2 site is governed by a two-pronged mechanism: lower Nrf2 binding as well as formation of a repressive transcriptional motif.

The lesion in Nrf2-ARE-mediated transcription contributes to age-related decline in GCL activity and GSH levels

We verified the consequences of an age-related decline in the Nrf2-ARE regulon on hepatic Gclc mRNA expression, protein levels, GCL activity and GSH levels. Gclc mRNA levels measured using qPCR revealed a significant 27 ± 10% (P < 0.05) decline in message levels in hepatocytes isolated from old rats compared to young (Figure 5A). A western blot using total cell extracts from old rat hepatocytes shows that GCLC levels decline 42 ± 8% (P < 0.05) relative to that seen in young rat hepatocytes (Figure 5B). Furthermore, an examination of GCL activity showed a significant 60 ± 1.5% (P < 0.05) age-dependent loss in GSH synthetic capacity (Figure 5C). Lastly, we ascertained that old rats exhibited 40% lower total GSH levels, but this difference was not statistically significant (Figure 5D). These results suggest that aberrant Nrf2-ARE-mediated transcriptional regulation in old rats contributes to the attenuated hepatic GSH content with age.
4.4 Discussion

The findings in this paper mark a novel observation regarding age-related changes in the transcriptional machinery of an important stress-response gene, Gclc. To our knowledge, this is the first comprehensive report of the protein composition of the ARE transcriptome, and how the aging process modifies its composition. The Nrf2-ARE system is the master regulator of over 200 genes related to detoxication, antioxidant defense and anti-inflammatory processes. Other than the loss of nuclear Nrf2, our results demonstrate that the make-up of the permissive transcriptional machinery of the ARE is fundamentally altered with age. Since older individuals are at increased risk for cancer, artherosclerosis, and other diseases associated with aging, these observations are significant as it points to a rationale why stressors which may be effectively mitigated in younger individuals enhance risk for these pathologies in the elderly [214, 215].

The current data using a primary hepatocyte culture model are consistent with our previous studies using isolated rat liver and suggests that the culture system recapitulates the aging phenotype with respect to age-related decline in Nrf2 nuclear tenure [36, 197]. It should be emphasized that diminished basal nuclear Nrf2 values was not accompanied by equivalent losses in cytosolic Nrf2 levels, which suggests that Nrf2 regulation via translocation is altered with age. In this regard, we found no evidence for an age-associated loss of Nrf2 mRNA levels (data not shown), which is also in keeping with the consensus view that Nrf2 is constantly transcribed at high
rates and its expression does not change in response to antioxidants or electrophiles [172]. Thus, the mechanisms for the age-dependant and nuclear-specific loss of Nrf2 are a fundamental perturbation in Nrf2 homeostatic mechanism(s) that have the potential to be remediated (Chapter 2). For example, caloric restriction as well as lipoic acid treatment in aging increases Nrf2 nuclear translocation, which indicates that a greater nuclear pool of Nrf2 is available under those regimens to initiate gene transcription [214-217]. As Nrf2 has constitutively active nuclear localization signals but elaborately regulated nuclear export mechanisms, our data may point to enhanced nuclear efflux of Nrf2 with age [218, 219]. While the mechanisms associated with the age-associated disruption in Nrf2 nuclear tenure has not been fully identified, nevertheless, such a loss may profoundly influence basal profiles of phase II detoxification enzymes as well as overall xenobiotic stress response.

Nrf2 is obligated to bind to ARE sequences as a heterodimer with other bZIP proteins in order to elicit gene expression [199, 220-222]. Recent data indicates that Nrf2 binding proteins serve an overall modulatory role to fine-tune the degree of gene transcription. When Nrf2 concentrations are low, such as our data suggests happens with age, a repressive-type transcriptome may develop at the ARE and modulate gene expression. For instance, small Maf homodimers may form on ARE sites and actually limit expression of detoxification genes [199]. In keeping with this concept that age modifies an active Nrf2 transcriptional complex, it is interesting to note we observed that
Bach1 resides on the ARE4 locus of the \textit{Gclc} promoter in hepatocytes isolated from old rats. Bach1 is a cellular heme sensor that represses the NFE2 promoter, a responsive element that displays sequence homology to maf recognition elements \cite{223}. Bach1 binds to ARE sequences when either Nrf2 or small maf proteins are limiting and results in silencing of both HO-1 and NQO1 expression \cite{86}. Furthermore, Yoshida and co-workers showed that Bach1 represses transcription by complexing with small maf homodimers, thereby initiating DNA loop formation \cite{223}. Thus, Bach1 acts as a potent architectural chromatin-remodeling agent, which may ultimately result in the observed decline in \textit{Gclc} expression. We therefore interpret our results to mean that lower Nrf2 levels in aging rat hepatocytes limits Nrf2 availability for ARE4 and instead permits Bach1 association. Further experiments will be needed to assess the nature of this hypothesized Bach1-mediated chromatin modification, which is outside the scope of the present paper.

Along with the presence of Bach1, the age-related loss of CBP in the Nrf2 transcriptional complex in hepatocytes from old rats would also theoretically inhibit for \textit{Gclc} transcription. CBP is a histone acetyltransferase involved in chromatin opening \cite{34, 224}, and its levels decline with age in the liver, kidney, and cerebral cortex of rats \cite{225, 226}. It is normally recruited to ARE sequences via its association with the Neh4 and Neh5 domain of Nrf2 \cite{96, 188} and directly modulates Nrf2 association with ARE sequences through acetylation of Nrf2. Interestingly and in relation to the present study,
CBP inhibitors also lower nuclear Nrf2 localization similar to that seen in aging [94, 188]. Thus, the age-related disappearance of CBP from the ARE4 site may link lower nuclear levels of Nrf2 with the repressive transcriptional phenotype that develops at that locus with age. Additionally, loss of CBP may lead to the lack of chromatin opening at the ARE4 site, prompting Nrf2 binding to an alternate locus (i.e. ARE2) as seen in this study. We are currently assessing the role of CBP as a central mediator for the decline in Nrf2 stress response in aging.

There are multiple AREs in the Gclc promoter and, although we and others have looked at the functional significance of each of these, it was not known until this report whether aging affects Nrf2-ARE binding universally or specifically affects only certain ARE sequences [212] Erickson et al., 2002; Wasserman and Fahl, 1997) (Malhotra et al., 2010). Aside from age-related alterations to the Nrf2/ARE4 transcriptome, we observed that Nrf2 also binds to an alternative ARE (ARE2) in old rats; no Nrf2 binding was detected at this site in young rat hepatocytes. This is an intriguing result with large ramifications for transcriptional adaptation in aged animals. ARE2 contains a consensus sequence in the anti-sense strand and a partial ARE in the sense. In contrast, as we previously reported [212], ARE4 contains a consensus ARE in both the sense and anti-sense strand. Further research is necessary to determine if the observed age-related ARE2-mediated Nrf2 induced expression is exclusively sequence dependent. While the consequences of activating this unique ARE2 sequence are not yet fully known, it is interesting
that only co-activating bZip partner proteins and no repressors (e.g. Bach1) were found to bind at ARE2 in cells from aged animals. Moreover, our data from the Nrf2 overexpression experiments suggest that the ARE2 site can increase expression of Gclc. Previously, we showed that the nuclear Nrf2 as well as GCLC lost during aging was replenished by feeding old rats (R)-α-lipoic acid, a redox active dithiol compound known to induce a phase II response. This finding is very important, especially because it provides credence to our hypothesis that the age-associated transcriptional lesion in ARE4 can be remediated through Nrf2-ARE2 binding. Thus, promoter switching by Nrf2 during aging may potentially be an attempt at compensating for the loss in expression of Gclc. Taken together, these results suggest that ARE2 is comparatively more accessible to Nrf2 in the aging liver and can serve as a potential means to compensate for the decline in GCLC expression through ARE4 (Figure 6).

Partial promoter switching to maintain gene activity with age has literature precedent. For example, Iakova et al. recently that aging induces growth arrest of proliferative pathways in the liver by a C/EBP alpha promoter-switching mechanism [227]. Similarly, there are numerous reports of promoter silencing by methylation-dependent mechanisms during aging [228-230]. Of relevance to the results obtained in this study, Barzilai and co-workers [231] recently showed that the aging liver, in particular, is more susceptible to hypermethylation at promoters of genes involved in metabolic regulation and stress response. These findings are important because ARE
promoters, especially ARE4 is extremely GC-rich (89%) and is plausibly susceptible to hypermethylation.

In summary, this report identifies a novel Nrf2-mediated promoter switching mechanism that, nevertheless cannot compensate for the age-related decline in hepatic GSH synthetic capacity. Moreover, we have preliminary evidence that the expression of other ARE-containing genes (e.g. GST2A, HO-1, and NQO1) also decline with age, which indicates that the information contained in this report may not only pertain to Gclc. Experiments are ongoing as to whether alternative ARE site(s) and/or promoter switching is also responsible for their diminished expression in aged tissue. Thus, there is the potential that a common mechanism has been identified that leads to compromised phase II stress response with age.
4.5 Experimental Procedures

Reagents

Restriction enzymes and T4 DNA ligase for subcloning were from New England Bio Laboratories (Boston, MA). The Dual Luciferase Reporter Assay system and reporter plasmids, pGL4 minimal promoter vector and phRL-CMV vector were from Promega (Madison, WI). The expression vector for Nrf2 (pcDNA3.1-Nrf2) was a kind gift provided by Dr. Anil Jaiswal at University of Maryland School of Medicine, Baltimore, Maryland. Custom oligonucleotides used in PCR cloning, subcloning, and DNA sequencing were purchased from Invitrogen (Carlsbad, CA). Sequence service was provided by Center For Gene Research and Biotechnology, Oregon State University. Rabbit anti-Nrf2 (H-300), anti-lamin B1, small maf antibodies, and Nrf2 siRNA as well as scrambled oligonucleotide sequences were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Other antibodies were purchased from the following suppliers: c-Jun and Bach1 (Abcam, Cambridge, MA), c-Fos (eBioscience, San Diego, CA), CBP (Upstate Biotechnology, New York), and IgG (Calbiochem, San Diego, CA). Protein A-Sepharose was purchased from Sigma (St. Louis, MO). All chemicals used were at least analytical grade.

Animals

Rats (Fischer 344, virgin male, outbred albino), both young (2–5 months: \( n = 12 \)) and old (24–28 months, \( n = 25 \); National Institute of Aging
animal colonies), were acclimatized in the Oregon State University animal facilities for at least 1 week before experimentation. Animals were maintained on a standard chow diet, and food and water were given *ad libitum*. All animal procedures were performed in accordance with the Oregon State University guidelines for animal experimentation.

**Cell isolation and culture**

Hepatocytes were plated on collagen-coated culture dishes in William’s Medium E supplemented with 5% FBS, 1 mM dexamethasone, 100 ng/ml insulin, 2 mM L-glutamine, 100 U/ml penicillin, and 100 mg/ml streptomycin for 4 h in 5% CO2 at 37°C to allow attachment to the dishes. The medium was then replaced with fresh supplemented William’s Medium E, and the cells were cultured for an additional 48 h before chromatin immunoprecipitation or transfection with the appropriate ARE-luciferase constructs.

**Preparation of nuclear extracts and Nrf2 analysis**

Nuclear and cytosolic proteins isolated from rat hepatocytes were resolved by SDS-PAGE and Nrf2 levels determined by western blotting using Nrf2 antibody (1:1000) as described before. Anti-lamin B1 and β-actin were used as loading controls for nuclear and cytosolic proteins, respectively.

**Chromatin immunoprecipitation assay**

Chromatin immunoprecipitation analysis was conducted using control
rabbit IgG, anti-Nrf2, anti-small maf, anti-c-Jun, anti-c-Fos, anti-CBP, and anti-Bach1 antibodies. PCR primers are described in Table 2. Eighteen million hepatocytes were used for each chromatin immunoprecipitation experiment.

**Construction of luciferase reporter vectors**

The ARE- and ARE-like-luciferase reporter plasmids were generated using the pGL4-minimal promoter vector (Promega, Madison, WI) containing a minimal TATA promoter upstream of the firefly luciferase gene. The sequences of the inserts used in the different plasmids are summarized in Table 3. Single-stranded oligonucleotides were first annealed to form double-stranded oligonucleotides and then ligated into the pGL4.23[minP] vector following the manufacturer’s instructions. The vectors were engineered by inserting 3 copies of each of the ARE elements present in the rat Gclc 5’-flanking region. After the plasmids were generated, the DNA sequence of the inserts was verified.

**Hepatocyte transfection and luciferase assays**

Reporter gene assays were used to determine the transcriptional activities of individual Gclc ARE elements and the ARE-like element in primary hepatocyte cultures from young and old rats. Transient transfections were done in hepatocytes cultured on 6-well collagen-coated plates for at least 48 hours using the Effectene Transfection reagent (Qiagen, Valencia,
The cells were transfected with 1.6 μg of Gclc-luciferase plasmids. The total amount of plasmid DNA for transfection was adjusted by empty expression vector (pGL4.23). The control plasmid phRL-CMV encoding Renilla luciferase was included in each transfection (0.02 μg) to account for variability in transfection efficiency. In some cases, 0.5 μg of Nrf2 expression plasmid (pcDNA2.1 Nrf2) was co-transfected with luciferase reporter constructs. Thirty-six hours after transfection, cells were harvested with 1 x passive lysis buffer (Promega), and the supernatant was collected by brief centrifugation. Transcription activity was determined by the expression of firefly luciferase and was normalized to the renilla luciferase levels by using a dual luciferase reporter assay kit (Promega) on a Biolumat LB9505 luminometer (Berthold Detection Systems, Pfhorzeim, Germany). The means of at least three independent experiments, each carried out in duplicate, are shown with the + S.E. Statistical significance is determined by an one way ANOVA and individual comparison calculated by Bonferroni’s Multiple Comparison Test.

Real-Time PCR of GCLC and GCLM mRNA

A portion of each liver was excised and stored in RNAlater (Ambion, Austin, TX) at 80°C and homogenized using a Dounce homogenizer. Total RNA was isolated from both young and old rat livers (n=4) by using an RNeasy Midi Kit (Qiagen, Valencia, CA). cDNA was prepared from 1 μg of total RNA per group using SuperScript II (Life Technologies, Gaithersburg,
Relative transcript amounts of Gclc and Gclm were determined by quantitative real-time PCR using primers, cycling conditions, and housekeeping genes as described elsewhere [197].

Statistical Analysis

The data are expressed as the means ± S.E. Statistical analysis was performed with the GraphPad Prism software version 3.03 (GraphPad Software Inc., San Diego, CA). We used a two-tailed Student’s t test to compare the luciferase activity of individual Gclc promoter constructs. A P value less than 0.05 was considered to be significant. One-way analysis of variance [201] was used when multiple comparisons were made, followed by Tukey’s post-hoc analysis for multiple comparisons to a control.
4.6 Figures

Figure 4.1. Nuclear levels of Nrf2 decline with age. 

(A) The nuclear fraction of hepatocytes was isolated and subjected to Western blot analysis for Nrf2 levels. (B) Results show that nuclear Nrf2 levels decline significantly by 50% (* P < 0.05) with age. Blots are representative of N=3 (young) and N=4 (old) animals.
Figure 4.2. Aging induces promoter shifting of Nrf2 from ARE4 to an alternate ARE site (ARE2).

(A) Schematic representation of 5′-flanking region of Gclc showing locations of the four ARE promoters. (B) Amount of Nrf2 binding to Gclc ARE4 declines with age, while Nrf2 binds to the ARE2 promoter only in hepatocytes from old rats. ChIP assays were performed on young and old rat hepatocytes using an antibody to Nrf2 and amplifying the region spanning the ARE4 and ARE2 using specific primers. The sheared input chromatin is used as a positive control. (C) Quantification of immunoprecipitated chromatin by qRT-PCR shows that Nrf2 binding to ARE4 is 59 ± 17 %, (P <0.05) lower in liver of old rats as compared to young. Nrf2 is also more enriched at the ARE2 promoter (50 ± 3; P<0.05) compared to the ARE4 promoter with age. No Nrf2 was detected at the ARE2 promoter in young rat hepatocytes, showing that the promoter switching was age-specific. Results are representative of ChIPs performed on hepatocytes from 3 young and 3 old animals.
Aging induces loss of CBP binding to the active Gclc ARE4 transcriptome. ChIP assays performed on hepatocytes from young rats show the presence of the histone acetyltransferase, CBP at the ARE4 promoter with Nrf2. With age, CBP binding to the ARE4 drops below that of the non-specific IgG control (see Table 1), indicating the absence of this key co-activator at the ARE4 locus. Results are representative of ChIPs performed on hepatocytes from 3 young and 3 old rats.
Figure 4.4. Nrf2 promoter switching in the old rat liver does not compensate for the age-related decline in ARE transcriptional activity. Primary hepatocytes from young and old rats were transfected with a luciferase reporter construct containing three copies of either ARE4 or ARE2 and luciferase activities determined after 24 hours. A renilla luciferase construct was co-transfected to normalize for transfection. Results show that ARE4 driven luciferase activity drops 47 ± 14% (P < 0.05) with age. Nrf2 over-expression resulted in a statistical significant increased in luciferase activity driven by ARE4 (P < 0.05). In contrast, ARE4 co-transfected with Nrf2 in hepatocytes isolated from old rats was not found to be statistically different. Conversely, Nrf2 over-expression did not resulted in a statistical significant increased in luciferase activity driven by ARE2 in young but ARE2 co-transfected with Nrf2 in hepatocytes isolated from old rats was found to be statistically different. Luciferase reporter activities are calculated as Firefly/Renilla and are normalized to the activities of the empty vector in young and old rat hepatocytes respectively. Results are depicted as mean ± SEM and are representative of reporter assays from 4 young and 4 old rats.
Figure 4.5. Age-related decline in hepatic GSH synthesis. (A) Gclc mRNA levels are lower in liver tissues from old rats. Quantitative PCR analysis revealed a significant $27 \pm 10\%$ ($P < 0.05$) decline in message levels. (B) GCLC protein levels decline with age in hepatocytes. Western analysis of GCLC shows a $42 \pm 8\%$ loss in old rat hepatocytes compared to young. (C) GCL activity declines approximately $60\%$ with age in rat hepatocytes ($* P \leq 0.05$). (D) Measurement of GSH levels in young and old rat hepatocytes show a $40\%$ loss with age. Results are expressed as mean $\pm$ SEM and are representative of 4 young and 4 old rats.
Figure 4.6. Schematic representation of age-dependent alterations in the Gclc-ARE transcriptome. Nrf2 binds to the active ARE4 promoter along with the histone acetyl transferase CBP and partner proteins small maf, c-jun and c-Fos in the young rat liver. The transcriptional activity of ARE4 significantly declines with age by a two-pronged alteration: loss in Nrf2 binding accompanied by loss of CBP and binding of the negative regulator Bach1. Age-related decline in Nrf2-ARE4 binding is attempted to be compensated by Nrf2-ARE2 binding in the old liver.
### 4.7 Tables

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<tr>
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Table 4.1. Age-Related Alterations in Transcription Factor Binding to *Gclc* ARE4 and ARE2.

*Figures marked with a star are calculated as statistically different by student’s t-test.*
Chapter 5. Conclusions

Author

Eric Smith and Tory M. Hagen
Advances in the treatment of infection and traumatic injury, and a reduction in the number of people living in poverty have led to a dramatic increase in average life span. During the 20th century life expectancy nearly doubled and a ten-fold increase in the number of Americans age 65 or older has occurred in the last 100 years [24-28, 232]. A consequence of this extension in average life span is the increased prevalence of age-related disease with people 65 and older living with one or more chronic medical conditions [233]. Currently in the United States, 75% of all healthcare costs are spent on the elderly and with this age demographic projected to rapidly expand in the near future, the implications are staggering.

In typical age-associated diseases (e.g. atherosclerosis, cardiovascular disease, hypertension, cancer, arthritis, type 2 diabetes, and various neurodegenerative diseases [232]), the etiology, in part, appears causally linked to an increased susceptibility to endogenous and exogenous xenobiotic stresses [234]. These diseases are commonly treated with pharmacological interventions, with people over the age of 65 taking an average of one to four drugs on a daily basis to mitigate their symptoms [235]. However, this use of multiple pharmaceuticals in combination with the age-related loss of xenobiotic detoxification increases the likelihood of drug induced side effects, leading to the overwhelming number of adverse drug reactions in the elderly, as compared to all other age demographics [236]. Thus, there is a compelling need to understand the underlying mechanisms
that result in the age-related decline in antioxidant expression and detoxification.

Therefore, in order to identify the underlying molecular events that lead to age-related decline in antioxidant expression and detoxification, this dissertation investigated alterations in Nrf2 protein homoeostasis and elucidated the potential consequences incurred from lower Nrf2 levels evident with age. This dissertation shows:

• translation of the master regulator of endogenous antioxidant and detoxification expression, Nrf2, is inhibited with age.

• the magnitude of Nrf2 synthesis attenuation is far greater than general loss of protein synthesis.

• miR-146a inhibits Nrf2 synthesis and its expression increases with age. Thus, increased miRs (which are pro-inflammatory) may drive the loss of Nrf2 protein translation.

• the loss of Nrf2 translation directly leads to diminished nuclear Nrf2 steady state levels, which in turn causes formation of a repressive transcriptome on ARE4 via a Bach1 competition.

• Gclc expression declines significantly with age, and directly results in decreased hepatic GSH levels.

Thus, we have developed a comprehensive body of knowledge showing that translation loss of Nrf2 leads to a known cellular phenotype of aging: GSH attenuation. However, the implications of Nrf2 loss extend far beyond the decline of this tripeptide. Based on the breadth of ARE regulation lower Nrf2
synthesis should result in many of the phenotypes typically associated with senescence: increased damaged cellular components, decreased detoxification capacity, altered metabolism, and loss of endogenous antioxidants. Described below is a more detailed discourse of the findings in this dissertation.

Corresponding to this loss, a master regulator of detoxification and antioxidant defense, Nrf2, declines with age (Chapter 2). The precise consequences of the age-related loss of Nrf2 are unknown. However, in model organisms, the total ablation of Nrf2 results in the loss of endogenous antioxidants, attenuated drug detoxification, and decrease average lifespan [217]. Thus, we hypothesize that the age-related decrement of Nrf2 will manifest similar characteristics. In agreement to this hypothesis, initial data from our lab identified that the loss of Nrf2 is causal to the loss the of an important antioxidant and detoxification co-substrate, GSH. (Chapter 4). Despite this overwhelming evidence, no significant effort has been made to elucidate the mechanism(s) that lead to the loss of Nrf2 or the consequence(s) of this loss.

This dissertation investigated the age-related changes that lead to the loss of Nrf2 (Chapter 2). Chiefly, in old rats, we find that an attenuated rate of Nrf2 translation results in decreased steady state levels. This is particularly relevant to the known decreases in antioxidants and detoxification. This observation was perhaps overlooked in previous studies regarding the age-related changes in "expression" because the mRNA levels of Nrf2 do not
significantly change with age. Furthermore, Nrf2 has both a particularly short half-life, 10-20 minutes as reported in the literature [237], and low basal levels. These qualities of Nrf2 make it inherently difficult to identify and study by current proteomic techniques [238].

The age-related decrement in Nrf2 synthesis has broad implications in regard to the hallmarks of aging and diseases often associated in the elderly. In chapter 4 we show that the age related loss of Nrf2 synthesis directly results in decreased glutathione levels. However, based on the known targets of Nrf2, we hypothesize that this decrement contributes to decreased expression of many key endogenous antioxidant and detoxification genes. Therefore, particularly where a pro-oxidant cellular environment is causal, it is not unreasonable to suspect that loss of Nrf2 synthesis drives the increased risk for many age-related diseases. Thus, our work identifies Nrf2 synthesis as an important target for prophylactic or therapeutic interventions in the elderly.

Beyond the direct implications that loss of Nrf2 synthesis results in decreased expression of cellular defense genes in the elderly, this work also demonstrates that synthesis is a key regulatory mechanism of Nrf2 proteostasis. The current dogma regarding the regulation of Nrf2 is centered on Keap1-mediated degradation. In support of this, the response to a variety of xenobiotics and endogenous reactive species mediated through Keap1 are well documented. Due to these findings, one may suspect that the Keap1-Nrf2 interaction plays a role in the age related loss of Nrf2. However, we
identified that Keap1-mediated Nrf2 degradation has little impact on the age-related loss of Nrf2. In hepatocytes isolated from old rats, the half-life of Nrf2 is significantly prolonged, approximately 5-fold, concomitant with a significant loss of Keap1 protein expression (Chapter 2). The age-related change in the Nrf2-Keap1 association is another lucrative area of research. I hypothesize that this interaction is inhibited by, or mediated through, the redox-sensitive thiols in Keap1 and the increased ROS levels in the aging cellular environment. In this manner, the master stress-response regulatory mechanism correctly “senses” an increase in endogenous ROS and reduces Keap1-Nrf2 association, but ultimately fails to accumulate sufficient Nrf2 due to attenuated translation.

This underlying mechanism that results in the age-related decrement of Nrf2 outlines the potential efficacy of therapies being developed for the treatment of age-related diseases. In particular, potent inducers of Nrf2, via Keap1-Nrf2 disassociation, are being investigated in relation to various age-associated conditions [239-242]. This therapeutic strategy has two potential hurdles to overcome. Firstly, based on our findings, we hypothesize that prevention of Keap1-mediated Nrf2 degradation will not overcome the age-related attenuation of translation which will prevent substantial or sufficient accumulation of Nrf2 in the elderly. Secondly, if constitutively high levels of Nrf2 are achieved, metabolic dysregulation may develop, as seen in the mouse model of Nrf2 overexpression [243]. To this point, it has recently been demonstrated that high levels of Nrf2 function in a feed forward mechanism,
to enhance oxidative stress, leading to cell death [147]. Based on these findings, we posit that restoration of normal Nrf2 synthesis in combination with maintaining the endogenous Nrf2 regulatory mechanisms, namely Keap1-Nrf2 directed degradation, is fundamentally a better strategy to restore antioxidant stress resistance in older adults.

Introducing ancillary Nrf2 translation may be a potential means by which the onset of age-related diseases can be delayed or treated. To this end, the alternative regulatory mechanism of Nrf2, via increased translation, is a lucrative prospect for therapeutic targeting. In particular, our lab identified that lipoic acid increases the translation of Nrf2 through an IRES in addition to the disruption of Keap1-Nrf2 association [109]. Furthermore, it is increasingly apparent that miRs are important regulators of Nrf2. We have identified at least one miR that both increases with age and inhibits the translation of Nrf2 (Chapter 2). Although miR knockdowns have yet to be applied to human disease, this technique has been used in mice and non-human primates, and the application to humans is conceivable [244-246]. Taken together, this dissertation has presented research that opens the door to many future studies exploring the potential remediation of the age-related loss of Nrf2 and the molecular mechanisms of lost stress response during aging.

The second objective of this dissertation was to identify potential consequences that stem from the age-related loss of Nrf2 at the gene level. Aside from overall Nrf2 loss, our lab previously showed that the availability of nuclear Nrf2 significantly declines in the aging rat liver [44]. The results from
Chapter 4 show that low steady-state nuclear levels of Nrf2 creates conditions at the *Gclc* upstream region that lead to diminished expression. In particular, low nuclear Nrf2 levels result in lower Nrf2 binding to the active ARE. In turn, we showed that Bach 1, a transcriptional repressor, binds at ARE4 of *Gclc* and CBP is no longer present at this locus in aged tissues. We conclude a repressive transcriptional phenotype develops at ARE 4 in old rat liver, which reflects the loss of Gclc activity and GSH levels. Thus, the consequence of lower Nrf2 steady-state levels, at least for Gclc, is the decline in GSH and potential GSH-dependent detoxification. The present work only examined baseline changes to Gclc stemming from Nrf2 loss, future work should examine whether lower Gclc expression is also found under a toxicological threat or under pro-oxidative environments.

On a more speculative note, the transcription factor profile shown in Chapter 4 further suggests that this locus may be recalcitrant to Nrf2 binding. Two GC rich regions flank the ARE4 locus (approximately 80% GC) and because it is known that the GC-rich regions are prone to hypermethylation with age, particularly in the liver, this may be indicative of a chromatin state change from euchromatin to heterochromatin. Moreover, the loss of CBP, a histone acetyltransferase, may result in aberrant acetylation of histones and thus chromatin state, an event that can also result in heterochromatin formation. Hence, our data indicate that two independent events may drive the loss of Nrf2-dependent ARE activation:
• a decreased nuclear Nrf2 concentration allows the competitive inhibitor Bach1 to bind the ARE.

• chromatin state change may decrease transcription factor binding.

In support of a possible chromatin state change, we identified an alternative transcription factor binding site down stream of ARE4. Nrf2 shifts from this inactivated enhancing element to another site resembling the ARE in aging cells. This enhancer shifting by Nrf2 may be a compensatory mechanism attempting to maintain Gclc expression with age.

Mirroring the loss of GSH synthetic enzymes, we have also observed the age-related decline in a number of phase II detoxification and antioxidant enzymes; NQO1, GST2A and HO-1 (Chapter 2 and data not shown). It is an intriguing possibility that the identified shift in transcriptional control of Gclc with age may be a common mechanism involved in the repression of other phase II genes during aging or perhaps all ARE controlled genes. A provocative area of study would be the identification of other age-specific ARE-like transcription factor binding sites. To this point, advancement of sequencing methods, in combination with ChIP techniques developed in this dissertation (Chapter 3), allow for the genome-wide mapping of all AREs.

In summary this dissertation has shown that transcriptional attenuation of a quintessential ARE-mediated gene, Gclc, largely stems from the age-related decline in Nrf2 steady-state levels. In turn, loss of Nrf2 homeostasis is from inhibition of Nrf2 mRNA translation. These findings, while inherently significant, also describe a mechanism that contributes to a hallmark of aging:
the loss of Nrf2 synthesis clearly contributes to the imbalance between oxidants and the biological system's ability to readily detoxify them that develops with age. This imbalance results in the accumulation of damage and is implicated in the etiology of several common age-related diseases. Therefore, disruption of Nrf2 proteostasis may be an important risk factor in the development of these age-related diseases. Furthermore, Nrf2 is the primary cellular defense against cytotoxic effects of a variety of xenobiotics, its loss with age correlates with and may be causal to the increased prevalence of serious adverse reactions to drugs seen in the elderly. In summary, this mechanism provides new insights into the lower antioxidant and detoxification capacity observed in aging and that Nrf2 translation may serve as an age specific prophylactic or therapeutic target.
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