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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?

Swapna V. Shenvi^{1,2,§}, Eric Smith^{2,3}, and Tory M. Hagen^{2,3}

¹Molecular and Cellular Biology Program, Oregon State University, Corvallis, OR 97331

²Linus Pauling Institute, Oregon State University, Corvallis, OR 97331

³Department of Biochemistry and Biophysics, Oregon State University, Corvallis, OR 97331

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 (Suh et al., 2004), 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.

Keywords

Antioxidant Response Element (ARE); Bach1; small maf proteins; CREB-binding protein (CBP); Glutamate cysteine ligase catalytic subunit (GCLC)

Send Correspondence To: Tory M. Hagen, 571 Weniger Hall, Corvallis, OR 97330, USA, Telephone: 541-737-5083, Fax: 541-737-5077, tory.hagen@oregonstate.edu.

[§]Present Address: Nutrition & Metabolism Center, Children's Hospital Oakland Research Institute, Oakland CA 94609

Author Contributions

S.V.S. and E.S. contributed to the acquisition of data in this manuscript. S.V.S., E.S., and T.M.H. all contributed to the conception and study design as well as analysis and interpretation of the data. All three authors were responsible for the writing of the manuscript.

Introduction

A hallmark of the aging process is a decreased response to both exogenous and endogenous stresses (Finkel and Holbrook, 2000). In the liver, this inadequate stress response is reflected by a lower detoxification capacity (Hagen et al., 2002), which is exemplified by diminished levels of certain low molecular weight antioxidants like glutathione (GSH) and related phase II detoxification and antioxidant enzymes (Suh et al., 2004; Suh et al., 2004). In this context, we and others previously showed that GSH levels decline in the liver of aging Fischer 344 rats (Suh et al., 2004; Suh et al., 2004; Hall et al., 2001), 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 (Huang et al., 1993; Huang et al., 1993). 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 (Huang et al., 1993; Huang et al., 1993; Lu et al., 1999). Data from many laboratories conclusively show that hepatic activity, protein levels and gene expression of GCL are significantly lower in aging rat liver (Suh et al., 2004; Suh et al., 2004; Stio et al., 1994). 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 (Lu, 2009). 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 (Lu, 2009; Lee et al., 2006). Though GCLC is elaborately regulated at kinetic, post-translational and transcriptional levels (Torosier et al., 2006), its transcriptional regulation produces a more persistent effect and thus is more important for the maintenance of GSH homeostasis in response to oxidative stress (Yang et al., 2005; Zipper and Mulcahy, 2000; Song et al., 2005; Shenvi et al., 2009).

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) (Suh et al., 2004). 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 (Shenvi et al., 2009). 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.

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 Student's 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 (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 (Shenvi et al., 2009), this transcription factor associates with the fourth ARE ("ARE4") 3.8 kb upstream of the transcriptional start site. Nrf2 binding to ARE4 decreases significantly ($59 \pm 17\%$, $P = 0.05$) 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 (Dhakshinamoorthy et al., 2005; Reichard et al., 2007; Kaspar and Jaiswal, 2010). 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 (Suh et al., 2004).

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 (pGL4.23*Gclc*[3X]ARE4.luc2) or the ARE2 (pGL4.23*Gclc*[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 (Shenvi et al., 2009), 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 $27 \pm 10\%$ ($P = 0.05$) 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 $42 \pm 8\%$ ($P = 0.05$) relative to that seen in young rat hepatocytes (Figure 5B). Furthermore, an

examination of GCL activity showed a significant $60 \pm 1.5\%$ ($P = 0.05$) 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.

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, atherosclerosis, 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 (Ungvari et al., 2011).

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 (Suh et al., 2004) (Shenvi et al., 2008). It should be emphasized that diminished basal nuclear Nrf2 values was not accompanied by equivalent losses in cytosolic Nrf2 levels, which suggests that Nrf2 expression and translation, or its typical Keap1/cul3 ubiquitin ligase-dependent turnover is not affected 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 (Jaiswal, 2004). Thus, the mechanisms for the age-dependant and nuclear-specific loss of Nrf2 are presently unknown but points to a fundamental perturbation in Nrf2 homeostatic mechanism(s) that have the potential to be remediated. 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 (Hyun et al., 2006; Pearson et al., 2008) (Ungvari et al., 2011). 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 (Li et al., 2006; Theodore et al., 2008). 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 (Li et al., 2008; Levy et al., 2009; Venugopal and Jaiswal, 1996; Dhakshinamoorthy and Jaiswal, 2000). 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 (Dhakshinamoorthy and Jaiswal, 2000). 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 *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 (Igarashi et al., 1998). 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 (Kaspar and Jaiswal, 2010). Furthermore, Yoshida and co-workers showed that Bach1 represses transcription by complexing with small maf homodimers, thereby initiating DNA loop formation (Igarashi et al., 1998). Thus, Bach1 acts as a potent architectural chromatin-remodeling agent, which may ultimately result in the observed decline in *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 *Gclc* transcription. CBP is a histone acetyltransferase involved in chromatin opening (Shen et al., 2004; Zhu and Fahl, 2001), and its levels decline with age in the liver, kidney, and cerebral cortex of rats (Chung et al., 2002; Bandyopadhyay et al., 2002). It is normally recruited to ARE sequences via its association with the Neh4 and Neh5 domain of Nrf2 (Katoh et al., 2001; Shen et al., 2004) 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 (Sun et al., 2009; Katoh et al., 2001). 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 (Shenvi et al., 2009; 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 (Shenvi et al., 2009), 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 only 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 α promoter-switching mechanism (Iakova et al., 2003). Similarly, there are numerous reports of promoter silencing by methylation-dependent mechanisms during aging (Burzynski, 2005; Strunnikova et al., 2005; Gupta et al., 2006). Of relevance to the results obtained in this study, Barzilai and co-workers (Thompson et al., 2010) 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 (Shenvi et al., in preparation), 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.

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 (ChIP) 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, CA). 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 × 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, Pforzheim, 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, MD). Relative transcript amounts of *Gclc* and *Gclm* were determined by quantitative real-time PCR using primers, cycling conditions, and housekeeping genes as described elsewhere (Suh et al., 2004).

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 (ANOVA) was used when multiple comparisons were made, followed by Tukey's post-hoc analysis for multiple comparisons to a control.

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Figure 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 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 $59 \pm 17\%$, ($P = 0.05$) 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.

Figure 3. 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. 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 $47 \pm 14\%$ ($P = 0.05$) 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 result 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 $\pm$ SEM and are representative of reporter assays from 4 young and 4 old rats.

Figure 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 (* $P = 0.05$). (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 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.

Table 1Age-Related Alterations in Transcription Factor Binding to *Gclc* ARE4 and ARE2

	ARE 4	ARE 2
PullDown	Fold Change (From Young Control)	Fold Change (From Young Control)
Nrf2	0.41*	49.83*
mafK/G	0.86	0.92
c-jun	0.04*	0.38*
c-fos	0.12*	0.5*
CBP	0.02*	1.12
Bach1	49.48*	1.61
IgG	1.36	1.47
Fra-1	0.64	1.02

^aFigures marked with a star are calculated as statistically different by student's t-test.