Acetylation of Nrf2 by p300/CBP Augments Promoter-specific DNA Binding of Nrf2 during Antioxidant Response

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Summary

To maintain intracellular redox homeostasis, genes encoding many antioxidants and detoxification enzymes are transcriptionally upregulated upon deleterious oxidative stress through the cis- antioxidant responsive elements (AREs) in their promoter regions. Nrf2 is the critical transcription factor responsible for ARE-dependent transcription. We and others have previously demonstrated that Nrf2 is targeted for ubiquitin-mediated degradation by Keap1 in a redox sensitive manner through modifications on distinct cysteine residues of Keap1. Here we report that p300/CBP directly acetylates Nrf2 in response to arsenite-induced stress. We have identified multiple acetylated lysine residues within the Nrf2 Neh1 DNA-binding domain. Combined lysine-to-arginine mutations on the acetylation sites, with no effects on Nrf2 protein stability, compromised the DNA-binding activity of Nrf2 in a promoter-specific manner. These findings demonstrated that acetylation of Nrf2 by p300/CBP augments promoter-specific DNA binding of Nrf2 and established acetylation as a novel regulatory mechanism that functions in concert with Keap1-mediated ubiquitination in modulating the Nrf2-dependent antioxidant response.

Running title: Acetylation of Nrf2 by p300/CBP
INTRODUCTION

Oxidative stress, known as adverse effects of oxidants on physiological functions, has long been shown to play important roles in both acute toxicity induced by many environmental insults and pathogenesis of cancer, neurodegenerative disorders and other aging-related diseases (27, 31, 48, 51, 64). The cause for oxidative stress is that the production of reactive oxygen species (ROS) exceeds the capacity of body's natural antioxidant defense mechanisms. Understanding the molecular signaling events that govern these antioxidant defense mechanisms is critical for antioxidant therapeutic intervention (9).

Nrf2 is a critical transcription factor that regulates endogenous antioxidants, phase II detoxification enzymes and other cellular defensive proteins through the antioxidant responsive element (ARE) in the promoter regions of these genes (42, 43, 63). Among the most well-characterized Nrf2 downstream genes are NAD(P)H quinone oxidoreductase 1 (NQO1), heme oxygenase 1 (HO-1), thioredoxin reductase 1 (TXNRD1), glutathione-S-transferase A1 (GSTA1, also known as GST Ya in mouse), glutamate-cysteine ligase (also known as γ-glutamylcysteine synthetase, i.e. γGCS) modifier subunit (GCLM) and catalytic subunit (GCLC) (2, 37, 40, 60). A growing body of evidence has established the Nrf2-dependent antioxidant response as a pivotal protection system against detrimental effects of many environmental insults. Nrf2 knockout mice display increased sensitivity to chemical toxicants and carcinogens and are refractory to the protective actions of chemopreventive compounds (5, 46).

The activity of Nrf2 is negatively regulated by Keap1 (24). We and others have shown that under redox homeostasis conditions, Keap1 constitutively targets Nrf2 for ubiquitin-conjugation and subsequent proteasome degradation in the cytoplasm by acting as a substrate adaptor for Cul3-based E3 ubiquitin ligase complex (8, 14, 32, 68). Upon exposure of cells to oxidative stress or chemopreventive compounds, multiple cysteine residues on Keap1 are thought to be alkylated with electrophilic groups present in many Nrf2 inducers such as tert-butylhydroquinone (tBHQ) and sulforaphane (SF) (10, 12, 19, 61, 67). Such alkylations compromise the ability of Keap1 to efficiently ubiquitinate Nrf2, resulting in elevated Nrf2 protein levels and enhanced Nrf2-dependent gene expression (8, 14, 32, 57, 66, 68, 69).

Within the nucleus, Nrf2 is believed to exert its transcriptional function by forming heterodimers with small Maf (v-maf musculoaponeurotic fibrosarcoma oncogene family) proteins, binding to ARE-containing promoters, and recruiting transcription co-activators to help remodel chromatin structures and facilitate formation of basal transcription machinery (22, 29, 30, 41, 50, 74). Along with Nrf2, histone acetyltransferase (HAT) p300/CBP (CREB-binding protein) were detected in the ARE-binding complex as measured by microinjection of antibodies against p300 or CBP (74). CBP was shown to interact with Nrf2 and enhance Nrf2-dependent reporter gene activities (29). However, it is not clear whether or how p300/CBP might actively contribute to the dynamic regulation of Nrf2-dependent transcription.

p300 was cloned based on its interaction with the adenoviral-transforming protein E1A while CBP was identified by its association with the transcription factor CREB (6, 11). p300 and CBP share a high degree of homology and possess intrinsic HAT activity. p300/CBP is believed to...
serve as transcription co-activators by acetylating core histones to facilitate chromatin decondensation and recruiting basic RNA polymerase machinery (44, 47). Recent findings show that many non-histone proteins, particularly transcription factors, are substrates for p300/CBP, which greatly expands the possible mechanisms of p300/CBP in transcriptional activation (16, 65). On the other hand, an increasing list of signal-related proteins are discovered to have intrinsic acetyltransferase activity. For instance, acetylation of p53 by p300 and Tip60 at multiple lysine residues is indispensable for p53 activation (56). Acetylation of type I interferon (IFNα) receptor, IRF9 and STATs by p300/CBP is a critical regulatory event in IFNα signaling (54). Acetylation of BMAL1 by CLOCK is crucial for circadian control (18). Eco1-mediated acetylation of a cohesion subunit is required to establish the sister chromatid cohesion during S phase in cell cycle (3, 58, 72). Clearly acetylation as a general post-translational modification plays important roles in diverse signal transduction pathways other than “histone code”.

In this report, we demonstrate that p300/CBP directly binds and acetylates Nrf2 in response to arsenite-induced oxidative stress. We have identified multiple lysine residues as major acetylation sites within the Nrf2 Neh1 DNA-binding domain. Combined lysine-to-arginine (K>R) mutations of the acetylation sites, with no effects on Nrf2 protein stability, compromise the DNA-binding activity of Nrf2 in a promoter-specific manner. These findings demonstrate that acetylation of Nrf2 by p300/CBP augments promoter-specific DNA binding of Nrf2 and establish acetylation as a novel regulatory mechanism of the Nrf2-mediated antioxidant responses.

RESULTS

Nrf2 is acetylated by p300/CBP in vivo and in vitro.

To determine if endogenous Nrf2 is acetylated, HCT116 cells cultured in 150-mm dishes were directly lysed under denaturing conditions to inactivate deacetylases and to disrupt protein-protein interactions. Diluted cell lysates were subjected to immunoprecipitation by normal IgG or anti-Nrf2 antibodies, followed by immunoblot with antibodies specific for acetylated lysines. Acetylated Nrf2 was detectable under basal conditions (Fig. 1A, lane 2). To assess whether acetylation is upregulated in response to oxidative stress, cells were treated for the different time periods with 20 μM sodium arsenite [As(III)], an environmental carcinogen and strong Nrf2 inducer. There was a steady increase of Nrf2 acetylation along with the accumulation of Nrf2 proteins after As(III) treatment (Fig. 1B). Since accumulated Nrf2 protein level is always coupled with increased Nrf2 nuclear entry, it is conceivable that the observed enhancement in acetylation is due to prompt acetylation of Nrf2 by acetyltransferase(s) within the nucleus, which contribute to the quick activation of Nrf2 in response to oxidative stress.

To identify which acetyltransferase(s) acetylates Nrf2, HEK293T cells were co-transfected with vectors expressing HA-tagged Nrf2 and different histone acetyl-transferases (HATs) including p300, CBP and P/CAF. Cells were lysed under denaturing conditions to preserve the modification. Diluted cell lysates were subjected to immunoprecipitation with anti-HA antibodies, followed by immunoblot with antibodies specific for acetylated lysine. Nrf2 was acetylated only by p300 and CBP, but not P/CAF (Fig. 1C, lane 2, 4, and 5). An acetylase-deficient (DY) point mutant of p300 failed to acetylate Nrf2 (Fig. 1C, lane 3) (21). p300 was the
most potent in acetylating Nrf2, therefore was chosen for the following studies. The enhanced Nrf2 expression in the presence of p300/CBP was observed (Fig. 1C, lane 2 and 5). This is likely due to indirect effects of p300/CBP on the transcription of the Nrf2 transgene since similar observations were made on other transgenes carried in the same expression vector. This notion is further supported by that Nrf2 protein half-lives were not changed when p300 was overexpressed (Supplemental Fig. S4).

To determine if p300 is self-sufficient in acetylating Nrf2, an in vitro approach was utilized. Purified GST-tagged Nrf2 proteins were incubated with purified p300 proteins in the presence of \[^{14}C\text{-acetyl-CoA}\]. Acetylation of Nrf2 was detected by autoradiography (Fig. 1D, upper panel, lane 2). The sample with GST alone did not give any positive signals, indicating that the acetylation reactions are specific for Nrf2 (Fig. 1D, lane 1). These results indicate that Nrf2 is a bona fide substrate of p300.

In the in vitro acetylation reaction, a series of Nrf2 deletion mutants were constructed in an effort to identify the major acetylation regions. The boundary of each domain is defined in Figure 2D lower panel. Deletion of the Neh1 DNA-binding domain almost completely abolished acetylation of Nrf2, suggesting the Neh1 domain contains the major acetylation sites (Fig. 1D, lane 8). Deletion of Neh4 and Neh5, also significantly decreased the acetylation levels of Nrf2 (Fig 1D, lane 5 and 6), which is consistent with the finding that Neh5, in coordination with Neh4, mediates the binding of Nrf2 to p300 (Fig. 2E, lane 4 and 5). Deletion of other domains did not significantly alter the overall acetylation levels of Nrf2. Taken together, these results demonstrate that Nrf2 is acetylated by p300/CBP both in vivo and in vitro. The acetylation of Nrf2 is enhanced in response to arsenite exposure and the Neh1 DNA-binding domain contains the majority of acetylated lysine residues.

Nrf2 associates with p300.

Chromatin immunoprecipitation (ChIP) analysis was performed to assess specific and coordinated recruitment of p300 and Nrf2 to the ARE in response to oxidative stress. HCT116 cells were either left untreated or treated with 20 \(\mu\)M As(III) for 4 hours before cross-linking and harvest. Cell lysates were subjected to immunoprecipitation with either anti-Nrf2 or anti-p300 antibodies (with normal serum IgG as negative control). The genomic DNA fragments bound to either Nrf2 or p300 proteins were recovered and quantified by qPCR using primer pairs specific for the NQO1-ARE region or the tubulin promoter region as a negative control. The amounts of NQO1-ARE bound to p300 or Nrf2 were increased nearly ten folds in response to As(III) treatment compared to the mock-treated samples, while the amounts of tubulin promoter DNA bounded to p300 or Nrf2 remain unchanged (Fig. 2A and 2B). This demonstrates that endogenous p300, along with Nrf2, are specifically recruited to the ARE-containing promoters in response to arsenite.

Interaction between Nrf2 and p300 was tested in HEK293T cells co-transfected with expression vectors for Flag-tagged p300 and HA-tagged Nrf2. Cell lysates were immunoprecipitated with anti-Flag M2 matrix. Both the immunoprecipitates and the total cell lysates were analyzed by immunoblot with anti-HA antibodies. Nrf2 was detected in the Flag-p300 immunoprecipitates while an unrelated protein Cul4 was not detected (Fig. 2C), consistent with the previous finding on the interactions between Nrf2 and CBP (29).
Direct interactions between Nrf2 and p300, and interaction domains were assessed. p300 contains five major conserved domains: C/H1, KIX, BROMO, C/H2, and C/H3 (Fig. 2D, upper panel), whereas Nrf2 contains six major conserved domains: Neh2, Neh4, Neh5, Neh6, Neh1, and Neh3 (Fig. 2D, lower panel). The Neh2 domain is the “degron” that is bound and ubiquitinated by Keap1 (24, 68). The Neh4 and Neh5 domains are transactivation domains (29, 50). Neh1 contains a CNC (“cap’n’collar”)-type basic leucine zipper structure responsible for dimerization with Maf proteins and DNA binding (22, 23).

To identify the p300 binding domain(s) in Nrf2, a series of domain-specific deletion mutants of Nrf2 were used. GST-tagged Nrf2 proteins containing the deletions were purified. Equal amounts of GST-Nrf2 proteins were used for GST pull-down analysis with p300 proteins that were radiolabeled with [35S]-methionine. Consistent with the previous report (29), Neh4 and Neh5 domains of Nrf2, mainly the Neh5 domain, are required for interaction with p300 (Fig. 2E, lane 4 and 5). In another set of experiments, a series of truncated forms of p300 were constructed and radiolabeled with [35S]-methionine (Fig. 2F, upper panel). These [35S]-p300 proteins were subjected to GST pulled-down with GST-tagged Nrf2-WT proteins followed by SDS-PAGE and autoradiography (Fig. 2F, lower panel). p300 c-terminus 1725-2414 mediated interactions with Nrf2 (Fig. 2F, lane 7). Radiolabeled luciferase served as a negative control to show the specificity of the in vitro interaction (Fig. 2F, lane 1). Collectively, these data suggest that the Neh4 and Neh5 domains of Nrf2 directly interact with the C/H3 containing c-terminus of p300.

Identification of multiple acetylated lysines within the Neh1 DNA-binding domain of Nrf2.

Figure 1D showed that the Neh1 domain (amino acid 434-561) of Nrf2 contains major acetylation sites. There are 18 lysines within this domain in human Nrf2 (Fig. 5A). To further determine which lysine residues are major acetylation sites, GST-tagged Nrf2 with sub-domain deletions within the Neh1 domain were used for the in vitro acetylation assay. None of sub-domain deletion mutants were able to abolish Nrf2 acetylation to the same degree as the Neh1 deletion mutant, demonstrating that multiple lysine residues in Neh1 are acetylated (Fig. 3A, compare lane 4, 5, 6 to lane 3).

Next, mass spectrometry was used to identify exact acetylated lysine residues on Nrf2. HEK293T cells were transfected with expression vectors for HA-Nrf2 and p300. Cell lysates were subjected to immunoprecipitation with anti-HA antibodies followed by SDS-PAGE and Coomassie staining (Supplemental Fig. S1). The bands containing Nrf2 were isolated and analyzed by LC-MS/MS. Multiple acetylated lysines were detected (Fig. 3B) and were indicated by asterisks (Fig. 5A). Almost all acetylated lysines were within the Neh1 domain, which is consistent with the observation that deletion of Neh1 almost completely abolishes acetylation of Nrf2 in the in vitro acetylation assay (Fig. 1D and Fig. 3A).

Functional redundancy among different acetylation sites.

To elucidate the function of acetylation, lysine to arginine (K>R) substitutions were constructed on all lysine clusters within the Neh1 domain, and their effects on the overall Nrf2 acetylation levels (Fig. 4A) and Nrf2 transcriptional activity (Fig. 4B and 4C) were tested. As expected, none of the substitution constructs completely abolished acetylation of Nrf2, although a decrease of acetylation levels was visible for the K438R/K443R/K445R and K533R/K536R/K538R...
clusters (Fig. 4A, upper panel, lane 2 and lane 7). None of the cluster mutations had significant impacts on Nrf2 transcriptional activities, as measured by luciferase reporter gene (Fig. 4B). This observation was confirmed by qRT-PCR analysis of NQO1 and HO1 mRNA levels from the Nrf2<sup>−/−</sup> mouse embryonic fibroblast (MEF) cells expressing the cluster mutants of Nrf2 (Supplemental data in Fig. S2 showed one example). Since none of the K→R mutations within each lysine cluster alters the transcriptional activity of Nrf2, it is likely that there is intrinsic functional redundancy among acetylations on different sites. In other words, Nrf2 partial acetylation is sufficient for Nrf2 to reach its maximum transcriptional activity.

**Acetylation on the Neh1 domain does not regulate the stability of Nrf2 proteins.**

To resolve the redundancy issue, combined arginine substitution of all 18 lysines within the Neh1 domain was constructed and named ‘18KR’. In addition, two other combined mutations on the 6 lysines within the CNC (“cap ‘n’ collar”) homology region and the 12 lysines within the bZIP (basic leucine zipper) region were also constructed, and were named 6KR and 12KR respectively (Fig. 5A).

The effects of the combined mutations on overall Nrf2 acetylation levels were tested. The 18KR mutation almost completely abolished acetylation of Nrf2 by p300 (Fig. 5B, lane 3). The mutation of 6KR seemed to decrease acetylation levels more than 12KR (Fig. 5B, lane 4 and 5), implicating that the CNC homology region may be more heavily acetylated than the bZIP region. However an evident conclusion on the relative acetylation levels on each site is difficult to draw because the acetylation-specific antibody may have certain bias towards specific amino acid contexts of the acetylated residues, as shown in supplemental data when different acetylated lysine-specific antibodies were compared (Supplemental Fig. S3). Nevertheless the 18 lysines in the Neh1 domain were established as the major acetylation sites by both <i>in vivo</i> and <i>in vitro</i> acetylation assays. Therefore the combined 18KR mutant was chosen as for the functional study of Nrf2 acetylation.

Because Nrf2 is mainly regulated by Keap1 at the level of protein ubiquitination and degradation, it is important to know whether acetylation affects Nrf2 protein stability. To this end, the half-lives (t<sub>1/2</sub>) of Nrf2 proteins were measured under both basal and As(III)-treated conditions. MDA-MB-231 cells were chosen for this analysis because it is well established that the ubiquitination and stability of Nrf2 proteins is very sensitive to oxidative stress in this cell line (62, 67, 68). MDA-MB-231 cells expressing Nrf2-WT or Nrf2-18KR along with Keap1 and p300 were either left untreated or treated with 20µM As(III) for 3 hrs, followed by addition of 50 µM cycloheximide (CHX) to block protein synthesis. Cells were then lysed at different time points and Nrf2 protein levels were determined by immunoblot. There was no significant difference in Nrf2 half-lives between WT and 18KR under both basal and As(III)-treated conditions (Fig. 5C). In consistence with this result, overexpression of p300 did not cause increase of Nrf2 protein half-life (Supplemental Fig. S4). To assess if 18KR affects Nrf2 ubiquitination, MDA-MB-231 cells expressing HA-Nrf2 WT or 18KR mutant were co-treated with 20 µM As(III) and 10 µM MG132 for 4 hrs, and were then lysed under denaturing conditions. Diluted cell lysates were subjected to immunoprecipitation with anti-HA antibodies followed by immunoblot with anti-ubiquitin antibodies. Ubiquitin conjugation on Nrf2-WT or Nrf2-18KR was reduced to similar levels by As(III) (Fig. 5D). These results demonstrate that acetylation has no effect on Nrf2 ubiquitination or degradation.
Considering the importance of both Nrf2 nuclear import and Keap1-mediated Nrf2 nuclear export in turning on and off the Nrf2 signaling pathway (26, 52, 59), the effects of 18KR on the subcellular localization of Nrf2 and on Keap1-Nrf2 interactions were tested. MDA-MB-231 cells expressing Nrf2 WT or 18KR and p300 were subjected to indirect immunofluorescence staining. Both Nrf2 WT and 18KR localized mainly in the nucleus with p300 (Fig. 5E). Furthermore, Nrf2 WT and 18KR bound to Keap1 equally well (Fig. 5F). Collectively, these results indicate that 18KR almost completely abolish acetylation on Nrf2. Acetylation on Nrf2 Neh1 domain does not regulate Nrf2 ubiquitination or protein stability, nor does it seem to contribute to the regulation of Nrf2 subcellular localization.

Acetylation plays a positive role in the transcriptional activity of Nrf2.

The transcriptional activity of Nrf2-18KR mutant was measured in HEK293T cells co-transfected with expression vectors for either an NQO1- (Fig. 6A) or GSTA1- (Supplemental Fig. S5) ARE dependent firefly luciferase reporter gene, Nrf2 WT or 18KR, and p300. Luciferase reporter gene activities were measured. Nrf2-18KR showed a substantial decrease in its ability to drive the expression of both NQO1-ARE and GSTA1-ARE-dependent luciferase, indicating that loss of acetylation at these 18 lysine residues impaired the transcriptional activity of Nrf2 (Fig. 6A). Although not as significant as the 18KR mutation, the 6KR mutation and the 12KR mutation also marginally decreased the Nrf2-dependent transcription (Fig. 6A). An aliquot of cell lysates from the reporter gene assay was subjected to immunoblot to ensure that the Nrf2 protein levels were equivalent among the different Nrf2 mutants (Fig. 6C, upper panel). Similar results were obtained in MDA-MB-231 cells, suggesting that the observation is not specific to HEK293T cells (Supplemental Fig. S6).

Next, the differential effects of oxidative stress on cells expressing Nrf2-WT versus Nrf2-18KR were analyzed in HEK293T cells overexpressing either Nrf2-WT or Nrf2-18KR along with Keap1 and p300. Cells were treated with 20 µM As(III) for 12 hrs, and the NQO1-ARE dependent luciferase reporter gene expression were measured (Fig. 6B). Compared to cells expressing Nrf2-WT, cells expressing Nrf2-18KR had an obvious dampened ARE-dependent transcription induction in response to arsenite challenge, although Keap1-mediated control of total Nrf2 protein level was not affected (Fig. 6C, lower panel).

qRT-PCR was performed to confirm that acetylation positively regulate the transcriptional activity of Nrf2. mRNA was extracted from HEK293T cells expressing Nrf2-WT or Nrf2-18KR (Fig. 6D, left panels). mRNA was also analyzed in cells that were either left untreated or treated with 20 µM As(III) for 12 hrs (Fig 6D, right panels). Overexpression of Nrf2-WT caused a 2-3 fold increase in mRNA levels of NQO1 and TXNRD1 compared to mock-treatment control; the same fold increase of mRNA levels of these two genes were observed upon As(III) treatment compared to mock-treatment control (Fig. 6D, top two rows). This indicated that overexpression of Nrf2 mimicked the induction of endogenous Nrf2 by As(III) very well. However, As(III) caused a more dramatic induction of GCLM and HO-1, especially HO-1, compared to overexpression of Nrf2-WT (Fig. 6D, 3-4 rows). This is likely due to the fact that As(III) may also induce Nrf2-independent signal pathways that functions synergistically with Nrf2 to transactivate these genes (1). Consistent with the previous findings, As(III) did not change mRNA levels of Nrf2 (Fig. 6D, bottom row) (62). In line with the results from the luciferase
reporter gene assay, the 18KR mutant significantly compromised the induction of NQO1, TXNRD1 and GCLM (Fig. 6D, 1-3 rows, left panels), demonstrating that acetylation plays a positive role in the Nrf2-dependent transcription. Interestingly, the transcriptional activity of Nrf2-18KR on HO-1 is comparable to Nrf2-WT (Fig. 6D, 4th row, left panels), suggesting that acetylation of Nrf2 may preferentially regulate certain Nrf2 downstream genes. A parallel set of samples were subjected to immunoblot analysis to ensure that the cells were expressing equal amounts of Nrf2-WT and Nrf2-18KR (Fig. 6E).

To ensure that the observations made in HEK293T cells were not specific to this cell type, mRNA levels of NQO1, TXNRD1, HO-1, GCLM and Nrf2 were measured by qRT-PCR in Nrf2-/- MEF cells expressing the HA-Nrf2 WT or 18KR and p300. Consistent with the results obtained in HEK293T cells, acetylation of Nrf2 was found to be important in transactivating NQO1, TXNRD1 and GCLM, but not HO-1 (Fig. 6F and 6G).

The gene-specific effects of Nrf2 acetylation was further confirmed by comparing HCT116 p300-/- versus p300+/- cells, and MEF CBP-/- versus CBP+/- cells (Fig. 6H-K). Loss of either p300 or CBP HAT activity led to significant decrease of NQO1 induction in response to arsenite treatment, while induction of HO-1 remained unchanged (Fig. 6H and 6I). Nrf2 protein levels were elevated to similar extents upon arsenite exposure regardless of p300/CBP status (Fig. 6J and 6K), which is consistent with the observation that p300/CBP does not regulate the protein stability of Nrf2. These results provide independent support for the notion that acetylation of Nrf2 regulates its transcriptional activity in a gene-specific manner.

**Acetylation augments promoter-specific DNA-binding of Nrf2.**

Given the fact that acetylation mainly occurs in the DNA-binding domain, the effects of Nrf2 acetylation on ARE DNA-binding was analyzed in vitro. Biotinylated NQO1-ARE DNA was incubated with whole cell lysates from HEK293T expressing Nrf2-WT or the indicated mutant. ARE-bound Nrf2 proteins were pulled-down by streptavidin beads and detected by immunoblot with anti-HA antibodies. Nrf2-18KR, but not 6KR or 12KR, has a marked reduction in ARE-binding compared to WT (Fig. 7A, lane 2-5). An ARE fragment with mutations in its core region was included as a control for binding specificity (Fig. 7A, lane 1). These results demonstrate that acetylation of Nrf2 in the Neh1 DNA-binding domain promotes interaction between Nrf2 and the ARE.

Since the function of acetylation seems to be promoter specific, the binding of Nrf2-18KR to the ARE from GCLC and HO-1 were analyzed similarly. Consistent with the results of the qRT-PCR analysis (Fig. 6D and 6F), acetylation of Nrf2 promotes the binding of Nrf2 to the GCLC ARE, but not the HO-1 ARE in HEK293T cells (Fig. 7B). The same finding was obtained when MDA-MB-231 cells were used (Fig. 7C). Next, purified Nrf2 proteins were subjected to electrophoretic mobility shift assay (Fig. 7D and 7E). GST-tagged Nrf2-WT and Nrf2-18KR proteins were purified from bacteria and incubated with purified Flag-tagged p300 proteins in an in vitro acetylation reaction as described in Figure 1D. Immunoblot analysis confirmed efficient conjugation of the acetyl group on Nrf2 only in the presence of acetyl-CoA, while the 18KR mutation almost completely abolished Nrf2 acetylation (Fig. 7D). In the mobility shift assay with the radiolabeled NQO1-ARE probe, the acetylated Nrf2-WT protein showed enhanced ability to form the ARE-binding heterodimer with in vitro-translated MafG, compared to unacetylated
Nrf2-WT and Nrf2-18KR (Fig. 7E, left panel, lane 3-6). On the other hand, Nrf2-18KR retained the ability to bind the ARE, suggesting that the K>R mutation itself does not affect DNA recognition or dimerization between Nrf2 and small Maf proteins. When HO1-ARE was used, acetylated Nrf2-WT did not show increased DNA-binding compared to unacetylated Nrf2-WT (Fig. 7E, right panel, lane 3-4). These results demonstrate that acetylation of Nrf2 enhances its ARE-binding in a promoter-specific manner.

To verify the above findings obtained in vitro, the ARE-binding activity of 18KR was analyzed in vivo. ChIP analysis was performed in HEK293T cells either treated with 20 µM As(III), or overexpressing the indicated HA-Nrf2 WT or 18KR, along with p300. Immunoprecipitation was performed with either IgG or anti-Nrf2 antibodies. DNA fragments containing AREs from NQO1, TXNRD1, HO-1 and GCLM were amplified by PCR using specific primer sets and visualized on agarose gels (Fig. 7F). The tubulin promoter region was also amplified to serve as a negative control. Equal amounts of Nrf2 proteins and genomic DNA were used for each sample (Fig. 7G and 7H). The precipitated DNA from the ChIP assay was quantified by qPCR. The reading for the amount of DNA precipitated by anti-Nrf2 antibodies in the mock treatment group was set as 1 (Fig. 7I). As(III) induced a 7-9 fold increase in binding of endogenous Nrf2 to the NQO1-ARE, TXNRD1-ARE; a comparable fold increase was observed in cells expressing exogenous wild-type Nrf2 (Fig. 7I, left panels). This suggests that overexpression of Nrf2 mimicks As(III)-induced DNA binding of endogenous Nrf2 to some degree. Abolishing acetylation in the DNA-binding domain significantly decreased binding of Nrf2 to the AREs from NQO1, TXNRD1 and GCLM, but not the HO-1 ARE (Fig. 7I). These results demonstrate that acetylation augments promoter-specific DNA-binding of Nrf2 in vivo.

DISCUSSION

In this report we demonstrate that acetylation of Nrf2 by p300/CBP constitutes a novel regulatory mechanism for the Nrf2-dependent transcription. Our findings add Nrf2 to the increasing list of non-histone proteins, especially transcription factors, as substrates of p300/CBP (16). The Neh1 domain of Nrf2 is acetylated on multiple lysine residues. The relatively low substrate- and site-specificity of p300 can be explained by the Theorell-Chance catalysis model enlightened by the crystal structures of the p300 HAT domain (33). In this model, the substrate does not form tight and rigid complexes with p300; instead it weakly associates with the surface of p300 and the target lysine residue(s) slides through the catalytic tunnel of p300 to react with acetyl-CoA (33). In keeping with this notion, multiple acetylation sites have been previously observed on many substrates of p300 such as p53, c-Myc, FoxOs and STATs (4, 17, 54, 56, 73). In this study, the functional redundancy among the different acetylation sites was demonstrated by the observation that the Nrf2-dependent transcription was not affected by mutations of any single lysine or combined adjacent lysines (Fig. 4 and S2). However, complete abolishment of acetylation by the 18KR mutation significantly compromised Nrf2-dependent transcription as shown by luciferase reporter gene assays and qRT-PCR analysis performed in several cell lines (Fig. 6, S5 and S6). The 18KR mutation does not affect Nrf2 ubiquitination or stability under either basal or arsenite-induced conditions, demonstrating that these lysines in the Neh1 domain are not targets for ubiquitination (Fig. 5C and 5D). This is consistent with the previous findings that the seven lysine residues in the N-terminal Neh2 domain are the sites for redox-sensitive
ubiquitin conjugation, and that the Neh6 domain is the region for redox-insensitive ubiquitin conjugation (28, 38, 39).

We find that acetylation on Nrf2 directly modulates its DNA binding function (Fig. 7). The role of acetylation in DNA-binding was previously documented with several transcription factors (7, 13, 17, 20, 35, 36). In most cases acetylation promotes DNA binding of the transcription factor and enhances its transcription, which is in line with our findings and the overall positive function of p300/CBP in transcription. Acetylation on the bZIP region of MafG by CBP was shown to facilitate DNA-binding of the NF-E2 heterodimer composed of MafG and p45 (20). Considering MafG is also a common binding partner for Nrf2, it is conceivable that recruitment of p300/CBP by Nrf2 to the ARE may also facilitate the acetylation of small Mafs. Therefore, acetylation on both Nrf2 and MafG may have synergistic positive effects on their DNA-binding activity. Mechanistically, acetylation on the bZIP domains of those proteins may cause subtle allosteric or electrostatic changes that favor their heterodimerization and DNA binding.

A striking finding in this report is that transcription of HO-1 by Nrf2 is not regulated by acetylation, while other Nrf2 target genes such as NQO1, TXNRD1, GCL and GSTA1 are (Fig. 6 and 7). This suggests that acetylation of Nrf2 contributes to its fine discrimination among different ARE sequences, considering that there is considerable flexibility and variability in ARE sequences among different genes and species (43, 63). Similar mode of gene-specific regulation by acetylation was also found on several trans-acting elements, particularly transcription factors. For example, acetylation of p53 by Tip60 on its DNA-binding domain modulates the expression of BAX and PUMA, but not p21 or MDM2 (53, 55). Acetylation of FoxOs within its forkhead box DNA-binding domain enhances the expression of pro-apoptotic genes, while suppressing genes involved in stress resistance (15). Acetylation of p73 on multiple sites within a region between the DNA-binding domain and the oligomerization domain modulates the expression of a pro-apoptotic gene p53AIP1, but has no effects on p21 (7). The precise molecular mechanism underlying the reported differential gene regulation by acetylation in the DNA-binding domains of transcription factors requires further investigation. It is possible that acetyl groups on lysine residues within the DNA-binding domain directly contribute to conformational change during DNA recognition and binding. It is also possible that the acetylation of Nrf2 might recruit other cofactor(s) to the ARE and the cofactor(s) selectively modulate(s) the binding of Nrf2 to specific AREs. Nevertheless, these findings suggest that acetylation within the DNA-binding domain of a transcription factor is more likely to serve as a fine-tuning mechanism rather than an on-and-off switch in modulating the transcription factors.

An intriguing question is why HO-1, unlike other Nrf2 target genes, is highly activated by Nrf2 independently of Nrf2 acetylation? Since acetylation of transcription factors is a reversible process that fine-tunes transcription, it is conceivable that the ability of Nrf2 to tightly bind the HO-1 promoter, regardless of its acetylation status, is a strategy used by the cell to ensure efficient induction of HO-1 by Nrf2 at its full capacity in response to oxidative stress. HO-1 seems to play a specific role in stress responses. Compared to most other Nrf2 target genes that are mainly involved in ROS detoxification and redox cycling, HO-1 has been implicated in a variety of physiological defense responses including vasodilation, angiogenesis, anti-inflammation, anti-thrombosis, anti-proliferation and anti-apoptosis. HO-1 degrades heme to produce biliverdin, carbon monoxide (CO) and ferrous iron. Biliverdin is reduced to bilirubin,
accounting for the antioxidant activity of HO-1. CO on the other hand, has long been recognized as a vasodilating reagent like nitric oxide (NO), and has been shown to play protective roles in many inflammatory diseases, pulmonary injuries and cardiovascular diseases (34, 49). In accordance with its broad functions, HO-1 is rapidly induced at transcriptional level in response to a plethora of stimuli and stress conditions. Compared to most other Nrf2 target genes, HO-1 has a promoter region containing more cis- regulatory elements that ensure timely induction of HO-1 in response to diverse stress -associated stimuli by many transcription factors (1, 34). In support of this idea, we observed an huge (~60 folds or more) induction of HO-1 transcription compared to other target genes (<8 folds) in response to arsenite-induced stress in HEK293T cells (Fig. 6D), HCT116 cells (Fig. 6H), MEF cells (Fig. 6I) and UROtsa cells (62). The binding of Nrf2 to the promoter of HO-1 is also increased to a greater extent than that of other Nrf2 target genes in response to stress (~23 folds for HO-1 compared to ~10 folds for other genes, Fig. 7I). In line with our finding, selective regulation of HO-1 by Nrf2 was reported to be mediated by a SWI/SNF family chromatin-remodeling subunit BRG1 (71). BRG1 interacts with Nrf2 and enhances HO-1 induction, without affecting NQO1, GCLC or GCLM, by facilitating Z-DNA formation on the HO-1 promoter (70, 71).

We believe that p300/CBP-mediated Nrf2 acetylation functions in concert with, and downstream of, Keap1-mediated Nrf2 ubiquitination in modulating Nrf2 activity. In response to oxidative stress and environmental insults, distinct cysteine residues on Keap1 are modified, leading to compromised Nrf2 ubiquitination and enhanced Nrf2 protein levels. This allows Nrf2 accumulation in the nucleus, where it is acetylated by p300/CBP. Acetylation is essential for maximum binding of Nrf2 to specific ARE-containing promoters. As a dynamic and reversible process, acetylation of Nrf2 is determined by the relative activities of HATs and deacetylases (HDACs), both of whose activities are tightly regulated by many signaling pathways and are subject to change under diverse pathophysiological conditions. Thus, identification of Nrf2 acetylation as a novel regulatory mechanism will shed light on how redox homeostasis is maintained and fine-tuned by diverse signal transduction pathways in different cellular environments.

**EXPERIMENTAL PROCEDURES**

**Recombinant DNA molecules.**
Plasmids expressing p300 WT and DY mutant are generous gifts from T.P. Yao (21). The vector for Flag-tagged p300 was kindly provided by M.A. Ikeda (45). The Nrf2 K>R point mutants were generated by site-directed mutagenesis using the PCR and DpnI based method as previously described (52). Constructions of other plasmids are described in Supplemental data.

**Cell culture, transfection, and reporter gene assay.**
HCT116, HEK293T, MDA-MB-231 and COS-1 cells were purchased from the American Type Culture Collection (ATCC). Nrf2+/− MEF cells are a generous gift from J.Y. Chan. HCT116 p300+/− and p300+/+ cells are kindly provided by C. Caldas (25). CBP+/− and CBP+/+ MEF cells are kind gifts from D. Fang. Cells were maintained in either Dulbecco’s modified Eagle’s medium (DMEM) or Eagle’s minimal essential medium (EMEM) in the presence of 10% fetal bovine serum (FBS). Transfection and reporter gene assay are described in Supplemental data.
**Antibodies, immunoblot, immunoprecipitation and immunofluorescence analysis.**

Antibodies against HA (hemagglutinin) epitope (Covance), CBD (chitin binding domain) epitope (New England Biolab), acetylated lysines (Cell Signaling), Flag and ubiquitin (Sigma), Nrf2, p300, and α-tubulin (Santa Cruz Biotech) were purchased from commercial sources. Immunoprecipitation and immunofluorescence analysis are described in Supplemental data.

**In vivo acetylation assay, ubiquitination assay, and mass spectrometry.**

For detecting acetylation in vivo, cells were lysed directly in TBS buffer (10mM Tris-HCl pH8.0, 150mM NaCl) containing 2% SDS and 1mM DTT. Cell lysates were boiled, sonicated, and diluted 5 times with TBS buffer without SDS. The solution was then subjected to immunoprecipitation with anti-HA antibody and immunoblot with anti-acetylated lysine antibodies. For ubiquitination assay, cells were treated with 50 µM MG132 for 4 hrs to block proteasome degradation before lysis for immunoprecipitation as described above. Ubiquitination was detected by anti-ubiquitin antibodies. For mass spectrometry, immunoprecipitated proteins were visualized by Coomassie staining and recovered from the gel. LC-MS/MS analysis was performed by the Harvard Taplin mass spectrometry core facility.

**In vitro acetylation and GST pull-down.**

GST-Nrf2 proteins were expressed in *E. coli* Rosetta (DE3) LysS cells and purified with Glutathione Sepharose™ 4B matrix (Amersham Biosciences). Flag-p300 proteins were purified from COS-1 cells 48 hrs after transfection by using immunoprecipitation with anti-Flag M2 matrix, and eluted with 3×Flag peptide elution buffer (Sigma). GST-Nrf2 proteins were incubated with p300 proteins in the presence of [14C]-acetyl-CoA (55 mci/mmol, Amersham) in reaction buffer at 30°C for 1 hr. The reaction mixtures were then resolved on SDS-PAGE followed by autoradiography.

For GST pull-down assay, wild-type and truncated forms of p300 were radio-labeled with [35S]-methione using the in vitro TNT® Transcription/Translation system (Promega), and were incubated with Sepharose beads conjugated with GST-Nrf2 fusion proteins in binding buffer at room temperature for 1 hr. The Sepharose beads were then washed four times. The proteins were eluted by boiling in SDS sample buffer followed by SDS-PAGE and autoradiography. The detailed information is described in Supplemental data.

**Biotin-DNA pull down.**

The transfected cells were lysed in RIPA buffer (10 mM sodium phosphate pH7.2, 150 mM NaCl, 1% sodium deoxycholate, 2 mM EDTA, 0.1% SDS, 1% NP-40). Cell lysates were pre-cleared with protein A beads and incubated with 2 µg biotinylated 25-41 bp dsDNA probes that contains the ARE sequences in the promoter regions of Nrf2 target genes. The DNA-protein complexes were captured by streptavidin beads. After washing three times, DNA-bound proteins were eluted from beads by heating in SDS sample buffer. Proteins were resolved on SDS-PAGE and subjected to immunoblot with anti-HA antibodies to detect Nrf2. The sequences of the probes are described in Supplemental data.

**Mobility shift assay**

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The mobility shift assay was conducted as previously described (52). MafG was synthesized using the in vitro TNT® Transcription/Translation system (Promega) with non-radiolabeled methionine. GST-tagged Nrf2 proteins were purified and acetylated in vitro as described above. 2 μl MafG and 4 μl (20 ng) Nrf2 proteins were pre-incubated with poly(dI-dC) in the binding buffer (50 mM HEPES pH 7.5, 60 mM KCl, 2 mM MgCl2, 0.004% NP40, 5 mM EDTA, 10% glycerol, 100 μg/ml BSA) for 10 min in room temperature. 32P-end-labeled DNA probes were added and further incubated for 20 min before loading on 4% native gel. Gel was dried and analyzed by autoradiography. For cold probe competition, 150 times excess of cold probes were used. For supershift, 1 μg antibody was added in the pre-incubation mixture. The sequences of the probe are identical to those used in the biotin-DNA pull down assay.

**Chromatin immunoprecipitation (ChIP), qPCR and qRT-PCR.**

ChIP was previously described (52). qPCR and qRT-PCR was performed by LightCycler 480 system (Roche) for ChIP and mRNA profiling respectively. The detailed information and primer sequences are described in Supplemental data.

**Statistical test.**

Student’s t-test was used to determine the significant difference between two samples from three independent assays in reporter gene analysis and qPCR analysis.

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**REFERENCES**


FIGURE LEGENDS

Figure 1. Nrf2 is acetylated by p300/CBP in vivo and in vitro. (A) Endogenous Nrf2 is acetylated. HCT116 cells were lysed under denaturing conditions. Cell lysates were diluted and subjected to immunoprecipitation (IP) by normal IgG or anti-Nrf2 antibodies, followed by immunoblot (IB) with antibodies specific for acetylated lysine (AcK). (B) Sodium arsenite [As(III)] enhances acetylation of Nrf2. HCT116 cells were treated with 20 μM As(III) for the indicated time periods. Nrf2 acetylation levels were measured as described above. (C) Nrf2 is acetylated by p300/CBP, not P/CAF. HEK293T cells were co-transfected with vectors expressing HA-tagged Nrf2 and the indicated histone acetyl-transferases. Cells lysates were immunoprecipitated with anti-HA antibodies, followed by immunoblot with antibodies specific for acetylated lysine. (D) Nrf2 is a bona fide substrate of p300. Upper Panel: purified GST-tagged Nrf2 proteins were incubated in the presence of [14C]-acetyl-CoA with immunoprecipitated p300 proteins from COS-1 cells overexpressing Flag-p300. The reaction mixtures were resolved on SDS-PAGE followed by autoradiography. Lower Panel: the same amounts of GST-Nrf2 proteins as used in the above in vitro acetylation assay were subjected to Coomassie stain.

Figure 2. Nrf2 associates with p300. (A) Endogenous Nrf2 and p300 are coordinately recruited to the ARE in response to As(III)-induced stress. Chromatin immunoprecipitation (ChIP) analysis was performed in HCT116 cells with the indicated antibodies after 4 hr treatment of 20μM As(III). Upper Panel: the genomic DNA fragments bound to either Nrf2 or p300 proteins were recovered and quantified by qPCR using primer pairs specific for the NQO1-ARE region or the tubulin promoter region as a negative control. Lower Panel: the total DNA were amplified and visualized as above to ensure equal input between mock and As treatment groups. (B) The above precipitated DNA fragments were quantified by qPCR. (C) Nrf2 interacts with p300 in vivo. HEK293T cells expressing Flag-tagged p300 and HA-tagged Nrf2 were lysed. Cell lysates were immunoprecipitated with anti-Flag M2 matrix, followed by immunoblot with anti-HA antibodies. An unrelated protein Cul4 was included as a negative control. (D) Schematic draw of conserved domains in p300 and Nrf2 proteins. p300 contains five conserved domains: C/H1, KIX, BROMO, C/H2, and C/H3. The catalytic region, also known as the “HAT domain” is between amino acid 1195–1673. Nrf2 contains six conserved domains: Neh2, Neh4, Neh5, Neh6, Neh1, and Neh3. (E) Nrf2 transactivation domain Neh4 and Neh5, especially Neh5, directly binds p300. Upper Panel: p300 proteins were labeled with [35S]-methione and pulled-down by GST-Nrf2 with different deletion mutants. The Nrf2-bound p300 were visualized by
Figure 3. Identification of multiple acetylated lysine residues within the Neh1 DNA-binding domain of Nrf2. (A) Neh1 domain contains multiple acetylation sites. GST-tagged Nrf2 proteins with indicated deletions within the Neh1 domain were subjected to in vitro acetylation analysis as described in Figure 1D. (B) Multiple lysines in the Neh1 domain were identified as acetylation sites by LC-MS/MS. Immunoprecipitation was performed in HEK-293T cells expressing HA-Nrf2 and p300 with anti-HA antibodies. Immunoprecipitated Nrf2 proteins were visualized by Coomassie staining, isolated, and analyzed by LC-MS/MS.

Figure 4. Functional redundancy among different acetylation sites. (A) Arginine substitution on single or several adjacent lysine residue(s) does not change overall Nrf2 acetylation levels. Acetylation on Nrf2 was analyzed as described in HEK293T cells expressing the indicated HA-Nrf2 and p300. (B) HEK293T cells were co-transfected with vectors for the NQO1-ARE dependent firefly luciferase reporter gene, TK Renilla luciferase gene, the indicated HA-Nrf2 and p300. Luciferase reporter gene activities were analyzed using the Promega dual-luciferase reporter gene assay system. Relative luciferase activity and standard deviation were calculated from three independent experiments. (C) Total cell lysates from the above luciferase assay were subjected to immunoblot analysis with anti-HA antibodies.

Fig5. Acetylation on the Neh1 domain does not regulate the stability of Nrf2 proteins. (A) Distribution of lysines within the Neh1 domain of human Nrf2 protein. ** indicates acetylated lysines as identified by mass spectrometry (MS). (B) Arginine substitution of all 18 lysines in the Neh1 domain abolishes acetylation of Nrf2 by p300. Acetylations on Nrf2 were analyzed as described in HEK293T cells expressing the indicated HA-Nrf2 and p300. (C) Mutation of 18KR in Nrf2 does not affect the half-life of Nrf2 proteins under both basal and As-induced conditions. MDA-MB-231 cells expressing the indicated HA-Nrf2, Keap1 and p300 were either left untreated or treated with 20µM As(III) for 3 hrs, followed by co-treatment with 50 µM cycloheximide (CHX) for the indicated time periods. Cells lysates were analyzed by immunoblot with anti-HA antibodies. The relative intensities of the Nrf2 bands were quantified, normalized to tubulin, and plotted on a semi-log scale. The calculated half-lives of Nrf2 in each group were shown. (D) Mutation of 18KR in Nrf2 does not affect overall Nrf2 ubiquitination levels. MDA-MB-231 cells expressing the indicated HA-Nrf2, CBD-tagged Keap1 and p300 were co-treated with 20 µM As(III) and 10 µM proteasome inhibitor MG132 as indicated for 4 hrs, and then lysed under denaturing conditions. Cell lysates were diluted and subjected to immunoprecipitation with anti-HA antibodies followed by immunoblot with anti-ubiquitin (Ub) antibodies. (E) Mutation of 18KR in Nrf2 does not affect co-localization of Nrf2 with p300 in the nucleus. MDA-MB-231 cells expressing the indicated HA-Nrf2 and Flag-tagged p300 were subjected to indirect immunofluorescence analysis using anti-HA and anti-Flag antibodies. (F) Mutation of 18KR in Nrf2 does not affect the interaction between Nrf2 and Keap1. HEK293T cells expressing the indicated HA-Nrf2, Keap1-CBD and p300 were lysed. Keap1-containing
protein complexes were pulled-down with Chitin beads and immunoblotted with anti-HA antibodies. CBD: Chitin binding domain.

Fig6. Acetylation plays a positive role in the transcriptional activity of Nrf2. (A) Nrf2-18KR has decreased transcriptional activity compared to Nrf2-WT. Luciferase reporter gene analysis was performed with NQO1 ARE-reporters as described in Fig. 4B in HEK293T cells expressing the indicated HA-Nrf2 and p300. * indicates significant difference with Nrf2-WT. (B) Arsenite had decreased induction effects on Nrf2-18KR compared to Nrf2-WT. HEK293T cells expressing the indicated HA-Nrf2, Keap1 and p300 were treated with 10 µM As(III) for 12 hrs. NQO1-ARE luciferase reporter gene expression was analyzed as above. * indicates significant difference with Nrf2-WT. (C) Total cell lysates from the above two luciferase assays were subjected to immunoblot analysis with anti-HA antibodies. (D) Nrf2-18KR has decreased activity in driving the transcription of NQO1, TXNRD1 and GCLM, but not HO-1. qRT-PCR was performed in HEK293T cells either expressing the indicated HA-Nrf2 and p300, or treated with 20 µM As(III) for 12 hrs. The error bars indicate the standard deviation from three experiments. * indicates significant difference with Nrf2-WT. (E) Total cell lysates from HEK293T cells prepared in parallel with cells for the above qRT-PCR analysis were subjected to immunoblot analysis with anti-HA antibodies. (F, G) MEF Nrf2-/- cells overexpressing the indicated HA-Nrf2 and p300 were analyzed by qRT-PCR and immunoblot. (H, J) HCT116 p300-/- and p300+/- cells were treated with 20 µM As(III) for 12 hrs, followed by qRT-PCR and immunoblot analysis. * indicates significant difference between p300-/- and p300+/- cells within the same treatment group. (I, K) MEF CBP-/- and CBP+/- cells were treated with 10 µM As(III) for 12 hrs, followed by qRT-PCR and immunoblot analysis. * indicates significant difference between CBP-/- and CBP+/- cells within the same treatment group.

Fig7. Acetylation augments promoter-specific DNA-binding of Nrf2. (A) Nrf2-18KR has decreased binding affinity to NQO1 ARE compared to Nrf2-WT. HEK293T cells expressing the indicated HA-Nrf2 and p300 were lysed. Whole cell lysates were incubated with either wild-type (wt) or mutated (mu) biotinylated ARE from the NQO1 promoter region. The protein-DNA binding complexes were pulled-down by streptavidin beads and analyzed by immunoblot with anti-HA antibodies. (B) Nrf2-18KR has decreased binding affinity to GCLC ARE, but not to HO-1 ARE, compared to Nrf2 WT. HEK293T cells expressing the indicated HA-Nrf2 and p300 were subjected to DNA-binding assay with the AREs from GCLC and HO-1 as described above. (C) The same DNA-binding assay was repeated in MDA-MB-231 cells. (D) Protein input for mobility shift assay. 200ng of purified GST-tagged Nrf2-WT or Nrf2-18KR protein was incubated with purified p300 protein in the absence or presence of acetyl-CoA in an in vitro acetylation reaction of 50 µl. 1/10 of the reaction products were analyzed by immunoblot with acetylation-specific antibody and Nrf2 antibody. (E) Mobility shift assay was performed with 32P-labeled ARE probes in the presence of 2µl in vitro-translated MafG protein (Promega kit) and 4µl indicated Nrf2 proteins from the in vitro acetylation reaction described above. (F) ChIP analysis was performed on HEK293T cells either treated with 20 µM As(III), or overexpressing the indicated HA-Nrf2 and p300. ChIP analysis was as described in Figure 2A with either IgG or anti-Nrf2 antibodies. DNA fragments containing AREs of NQO1, TXNRD1, HO-1 and GCLM were amplified by PCR using specific primer sets and visualized on agarose gel. Tubulin promoter region was also amplified to serves as a negative control. (G) Total cell lysates from HEK293T cells prepared in parallel with cells for the ChIP assay were subjected to immunoblot
analysis with anti-HA antibodies. (H) Total DNA input was examined as in (F). (I) The precipitated DNA fragments from the ChIP assay were quantified by qPCR. DNA precipitated by anti-Nrf2 antibodies in the mock treatment group was set as 1. The error bars indicate the standard deviation from three experiments. The relative units of DNA amounts were plotted on the same scale for convenient comparison. ‘*’ indicate significant difference with Nrf2-WT.