

ABSTRACT

HUANG, BO-WEN. Transcriptional Regulation of Ferritin by Arsenic and Hypoxia. (Under the direction of Dr. Yoshiaki Tsuji).

Antioxidant detoxification genes such as ferritin and NAD(P)H quinone oxidoreductase-1 (NQO1) are transcriptionally activated under oxidative stress conditions via a highly conserved enhancer, termed anti-oxidant responsive element (ARE), to which nuclear factor-E2-related factor 2 (Nrf2) binds and activates transcription. Histone modifications play a cooperative and essential role in transcriptional regulation; however, particular histone modifications associated with antioxidant gene transcription remain elusive. Here we found that arsenic exposure activates transcription of ferritin H gene via the ARE concomitant with increased methylation of histones H4 Arg3 (H4R3) and H3 Arg17 (H3R17). We hypothesized that methylation of histone H4R3 and H3R17 are involved in transcriptional regulation of ferritin H and some other ARE-regulated antioxidant genes. To test this hypothesis, two protein arginine methyltransferases (PRMTs) were further investigated; PRMT1 and PRMT4 (CARM1 (coactivator-associated arginine methyltransferase 1) that catalyze methylation of H4R3 and H3R17, respectively. After arsenic exposure to human HaCaT or K562 cells, we found that 1) nuclear accumulation of PRMT1 and CARM1 was induced, 2) methylation of histone H4R3 and H3R17 were induced proximal to the AREs of ferritin and NQO1 genes, 3) knocking down PRMT1 or CARM1 did not block Nrf2 nuclear accumulation but inhibited Nrf2 binding to the AREs, thus diminishing ferritin and NQO1 gene transcription, and 4) PRMT1 and CARM1 knockdown enhanced cellular susceptibility to arsenic toxicity as evidenced by caspase 3 activation. Collectively, these results suggest that PRMT1- and CARM1-mediated methylation of histone H4R3 and H3R17 regulate the ARE and cellular antioxidant response to arsenic.

In addition to transcriptional level, ferritin expression is regulated by iron at the translational level through the interaction of iron-responsive element (IRE) in the 5'-untranslated region (UTR) of ferritin mRNA with iron regulatory protein (IRP). Ferritin was also shown to be upregulated by hypoxia at the translational level, similar to excess iron, through decreased IRP binding to the IRE. Cobalt chloride has been used as a hypoxia mimetic that stabilizes a key transcription factor, hypoxia inducible factor-1 α (HIF1- α), and

activates transcription of various genes involved in oxygen homeostasis through binding to a hypoxia responsive element (HRE). Here we observed that hypoxia and hypoxia mimetic cobalt chloride induced ferritin expression by two different mechanisms. In K562 cells under hypoxia, ferritin was upregulated at the transcriptional level through HIF1 during an early phase of hypoxia. In contrast, cobalt chloride initially activated ferritin transcription through increased Nrf2 binding to the antioxidant responsive element (ARE) of the ferritin gene, followed by the translational block of ferritin synthesis through increased IRP binding to the ferritin IRE. We also identified a functional HRE in the human ferritin H gene. Collectively, these results suggest that ferritin is a novel HIF1 α -dependent gene during hypoxia and the hypoxia-mimetic cobalt chloride regulates ferritin in a HIF1 α -independent manner.

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Transcriptional Regulation of Ferritin by Arsenic and Hypoxia.

by
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DEDICATION

*To my parents, who are always supportive with their unconditional love,
and to all graduate students, who foresee that the road of research is bumpy but still persist
because of curiosity of the unforeseen.*

BIOGRAPHY

Bo-Wen Huang was born on July 24, 1979 in Kaohsiung, Taiwan. He grew up in Taiwan, a small but interesting island with hilarious things happening everyday. He graduated with the Class of 1997 from Saint Dominic High School in Kaohsiung. He attended National Changhua University of Education in Changhua, Taiwan where he received his Bachelor degree of Science in Chemistry in 2001. He began his graduate studies under the direction of Dr. Chiun-Jye Yuan in the Department of Biological Science and Technology at the National Chiao Tung University in Hsinchu, Taiwan and received a Master degree of Science. His research included constructing a drug screening system for the potential anti-inflammation compounds in Chinese herbs and also working as a research assistant and a teaching assistant in the courses of the General Chemistry Lab and the Cell Biology Lab. Upon graduation in 2003, he served in the Republic of China Military Police. He earned the rank of Second Lieutenant in 2004 and subsequently served as the Company Chief Counselor in Kaohsiung and Changhua. After discharge, he came to the United States and started pursuing his Ph.D. in the Department of Environmental and Molecular Toxicology at North Carolina State University, Raleigh, North Carolina, in 2006. In 2007, he joined the laboratory of Dr. Yoshiaki Tsuji and started his Ph.D. research in the regulation of ferritin gene under the direction of Dr. Yoshiaki Tsuji.

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GENERAL INTRODUCTION

Iron Regulation

Iron is a transition element crucial for cellular homeostasis. Iron regulates essential cellular processes such as proliferation and metabolism by being involved in a variety of biological processes including oxygen transport, oxidative phosphorylation, and DNA synthesis (1,2). As a transition metal, iron can undergo oxidation or reduction between its ferrous (Fe^{2+}) or ferric (Fe^{3+}) states and works as an important cofactor in a variety of proteins and enzymes such as hemoglobin, cytochromes, ribonucleotide reductases and dehydrogenases, all involved in the previously mentioned cellular processes. However, oxidation of ferrous iron may also result in the production of deleterious hydroxyl radicals through the Fenton reaction ($\text{Fe}^{2+} + \text{H}_2\text{O}_2 \rightarrow \text{Fe}^{3+} + \text{OH}^- + \bullet\text{OH}$), resulting in damage to macromolecules such as lipids, nucleic acids, and proteins (3). Therefore, iron homeostasis should be tightly regulated to ensure its availability for biosynthesis but preventing excess level of iron that have potentially detrimental effects.

The regulation of cellular labile iron levels is an elaborate process involving different proteins that control iron uptake, storage, and export. For example, Fe^{3+} bound to transferrin (Tf) is transported through blood and imported into cells by the plasma membrane-bound transferrin receptor (TfR) through endocytosis; on the other hand, Fe^{2+} enters cells through the divalent metal transporter 1 (DMT-1) spanning plasma membrane (4). After entering cells, the iron in the Tf-TfR complex endosome is released from transferrin due to acidification and then exported into the cytosol also through DMT-1 spanning endosomal membrane. The labile iron in cytosol is then used in the biosynthesis of proteins such as hemoglobin and enzymes. Since free iron is available for deleterious Fenton reactions, the excess cellular iron should be immediately exported by an iron exporter such as ferroportin (5), or stored by the iron storage protein ferritin (6).

The Ferritin Gene

Ferritin is the major intracellular iron storage protein, which exists in blood serum as well (1). Ferritin can sequester excess free iron by converting reactive Fe^{2+} to Fe^{3+} through ferroxidase activity, ultimately storing Fe^{3+} within its hollow shell; by removing excess intracellular iron, ferritin prevents iron-mediated production of hydroxyl radicals, thus preventing lipid, DNA, and protein damage (7). As an iron reservoir, ferritin regulates intracellular iron homeostasis, achieving the balance between iron bioavailability and prevention of iron-induced toxicity.

1. Structure, distribution and function of ferritin

Because of its important role in iron storage, ferritin is ubiquitous in the cytoplasm, nucleus, and mitochondria of cells, as well as in the serum of blood (1). Cytoplasmic ferritin is composed of 24 subunits of heavy (H) and light (L) chains (8). The H chain subunit has ferroxidase activity to convert Fe^{2+} to Fe^{3+} for storage inside the shell (9); whereas the L chain stabilizes the overall ferritin structure and aids in cage-like structure formation (10,11). The ratio of heavy and light chain depends upon tissue and cell type. For example, the ratio of ferritin H/L expression in heart is 40/60 but in liver, L chain ratio reaches 90% (12). In the brain, the ratio of ferritin H increases from microglia, oligodendrocyte to neuron cells (13-15). The ferritin subunits form a spherical, hollow shell. Regardless of subunit composition, each ferritin apoprotein can store up to 4500 iron atoms (11).

Nuclear ferritin is composed mainly of ferritin H produced from the same mRNA as cytosolic ferritin (16). Stressors affect the distribution of nuclear ferritin. For example, ferric ammonium citrate, cytokines, and hydrogen peroxide (H_2O_2) affect ferritin localization between the cytosol and the nucleus in SW1088 human astrocytoma cells; upon stimulation, ferritin translocates into nucleus, binds to DNA and may be responsible for protection of iron-mediated oxidative damage of DNA (17).

Ferritin is also found in the serum. Serum ferritin is also identical to cytoplasmic ferritin, but unlike nuclear ferritin, the primary component of serum ferritin is the ferritin L subunit (18). The serum ferritin level is usually correlated with the total amount of iron stored in the body

and is used as a biomarker; for instance, the level of serum ferritin is usually low in iron deficiency anemia patients (19) but high in patients with acute and chronic liver disease (20). Mitochondrial ferritin is composed of ferritin H-like proteins, which share 80% homology with ferritin H and also demonstrate ferroxidase activity (21,22). In human cells, the expression level of mitochondrial ferritin is very specific, mainly observed in testis but very little in other tissues (21,23).

As mentioned previously, ferritin also plays an important role in cellular antioxidant defense. The sequestration of excess intracellular labile iron prevents the production of the reactive oxygen species (ROS) hydroxyl radical and potential oxidative damage. Transgenic expression of ferritin H into mice rescued them from Parkinson's-inducing agent 1-methyl-4-phenyl-1,2,3,6-tetrapyrindine (MPTP) by preventing ROS production (24); inducible overexpression of ferritin H and L in HeLa stable cells also decreased H₂O₂-induced oxidative stress (25). On the other hand, transient knockdown of ferritin H exacerbates H₂O₂-induced cell death (26) and we have demonstrated that knockdown of ferritin H also exacerbates ROS-induced apoptosis in response to rotenone (27).

2. Regulation of ferritin expression

Ferritin is mediated by both iron-dependent post-transcriptional regulation and iron-independent transcriptional regulation (1). The former has been well investigated over the past two decades (1,28) but the latter has not been fully elucidated.

A) Iron-dependent post-transcriptional regulation

The post-transcriptional regulation of ferritin genes is controlled translationally in response to intracellular labile iron (29,30). When iron levels increase, ferritin protein is upregulated in order to store the excess free iron, while ferritin protein levels decrease in response to lowered iron levels (31). This regulation is dependent upon the interaction between the iron responsive element (IRE) located in the 5'UTR of ferritin mRNA, and the iron regulatory proteins (IRP) 1 and 2 (32); binding of IRPs to the IRE stem loop structure of ferritin mRNA inhibits ferritin protein synthesis (31). Even though both IRP1 and 2 bind to the ferritin IRE, they are regulated differently; the aconitase activity of IRP-1 relies on the formation of a 4Fe-4S iron sulfur cluster (33). Under iron-rich conditions, IRP1 forms 4Fe-

4S iron sulfur cluster and serves as a cytosolic aconitase but also loses its ability to bind to the ferritin IRE and inhibit ferritin translation (6). On the other hand, IRP2 doesn't form an iron sulfur cluster but is regulated by proteasome-dependent degradation through iron-mediated oxidation and ubiquitination (34); therefore, IRP2 is accumulated to inhibit ferritin translation while iron is scarce. Furthermore, IRP1 and IRP2 distributions differ in different tissues; IRP1 is more abundant in liver, kidney, intestine, and brain, while IRP2 is rich in pituitary and a pro-B-lymphocytic cell line (35).

B) Iron-independent transcriptional regulation.

Diverse stimuli such as hormones, cytokines, and oxidative stress have been demonstrated to regulate ferritin transcription (28). Thyroid hormone induces ferritin H transcription, probably through a cAMP pathway (28,36). The cAMP-responsive region (the B-box) of the human ferritin H gene was identified as being regulated by the B-box binding factors (Bbf) complex, consisting of the transcription factor NFY, the coactivator p300, and the histone acetylase p300/CBP associated factor (PCAF) (37,38). The adenovirus E1A oncogene can repress ferritin H expression by suppressing Bbf complex formation (39). Ferritin is also regulated by cytokines such as tumor necrosis factor alpha (TNF- α) and interleukin 1 alpha (IL-1 α). Alterations in ferritin H expression have been observed in TNF- α -related chronic inflammation (40,41). Ferritin H gene expression is upregulated by IL-1 α (42). Located 4.8 kb upstream from the transcription start site of the mouse ferritin H gene are multiple copies of the nuclear factor κ B (NF- κ B) consensus sequence, which is responsible for TNF- α -mediated ferritin H transcription (43). In the case of NF- κ B inhibition of TNF- α -induced apoptosis, NF- κ B-mediated ferritin H induction may play an antioxidant role by sequestering iron to prevent ROS production, thus inhibiting the ROS-mediated proapoptotic c-Jun N-terminal kinase (JNK) pathway (44).

Oxidative Stress and the Antioxidant Response Element (ARE)

The incomplete reduction of O_2 forms oxygen radicals, known as reactive oxygen species (ROS). In order to defend pro-oxidant xenobiotics, dietary components, chemotherapeutic drugs, cigarette smoke, and ozone cells developed defensive systems to prevent ROS toxicity (28,45,46). The cellular antioxidant defense system is a complex system through which the cell maintains redox homeostasis. In addition to controlling endogenous production of ROS, the antioxidant defense system monitors the cellular redox state and transforms ROS into less toxic molecules to prevent the generation of ROS (47). Antioxidant enzymes play a major role in the antioxidant defense system; NAD(P)H quinone oxidoreductase 1 (NQO1), glutathione-S-transferase (GST), manganese superoxide dismutase (MnSOD), catalase, and heme oxygenase 1 (HO-1) are examples (47). MnSOD catalyzes superoxide anion (O_2^-) into H_2O_2 ; H_2O_2 is then converted into H_2O by catalase (48). Other antioxidant enzymes such as ferritin, NQO1, and HO-1 reduce ROS levels in ways other than direct reactions with ROS. As previously described, ferritin prevents the generation of the highly toxic hydroxyl radical (OH^\bullet) through the ability of ferritin to sequester iron. Indeed, previous research has already shown that upregulation of ferritin expression protects cells from apoptosis by its antioxidant property (26,44,49).

While ROS can act as signaling molecules (31), at high levels they can damage cellular constituents such as nucleic acids, proteins, and lipids (1). When ROS levels overwhelm the antioxidant defense system no matter through a rapid increase from exogenous sources, or through impairment of the antioxidant defense system, it will result in a toxic state known as oxidative stress. Oxidative stress has been implicated in several disease states, including neurodegeneration, cancer, and aging (50). Therefore, an effective antioxidant defense system is essential in preventing cellular damage, and oxidative stress-related disease states. In order to maintain an effective antioxidant defense, the cell must sustain tight control over levels of antioxidant enzymes. As named “antioxidant”, these genes share similar transcriptional regulation in response to oxidative stress through a cis-element called antioxidant-responsive element (ARE) (51) with the conserved core sequence of

TGA(C/T)nnnGCA. Therefore, it is not surprising that oxidative stress could mediate an ARE-dependent transcriptional regulation of the ferritin gene. Ferritin AREs have been identified in both of human and mouse cells (7,52,53). The ferritin H ARE was first characterized in mouse NIH-3T3 fibroblast cells, and is located 4.1kb upstream of transcription start site (TSS) of the mouse ferritin H gene, containing an activator protein 1-like (AP1-like), an specificity protein 1-like (SP1-like), and an activator protein 1/ nuclear factor erythroid-derived 2 (AP1/NF-E2) sequence (53). The human ferritin H ARE, located 4.5kb upstream of the TSS region of human ferritin H gene, consists of bidirectional 55-bp ARE motifs with an AP-1-like and an AP-1/NF-E2 sequence (7). The NQO1 gene is regulated by a proximal ARE -520bp upstream of the transcription start site (54). The AREs can regulate basal expression of antioxidant enzymes as well as inducible expression activated by a wide variety of stimuli, including ROS, heavy metals, lipid aldehydes, antioxidant phenols, and hemin (1).

1. Nuclear factor erythroid-derived 2-related factor 2 (Nrf2)

The primary transcription factor involved in the activation of the ARE is nuclear factor-E2-related factor 2 (Nrf2). Nrf2 is the key mediator in transcriptional activation of ARE-containing antioxidant genes in response to oxidative stress. Nrf2 regulates expression of ferritin, NQO1, HO-1, glutathione-S transferase (GST), and glutamate cysteine ligase (GCL) genes (55). Nrf2 not only regulate inducible expression of ARE-genes, but also basal expression as well (55). Nrf2 is a member of the Cap' n' collar (Cnc) transcription factor family that share a conserved basic region-leucine zipper structure (bZip) region (56,57). Nrf2 is comprised of six Nrf2-erythroid Cnc homology (ECH) (Neh) domains (58). The bZip region, which facilitates dimerization with other bZip family members as well as DNA binding, is located in the Neh1 region. The Neh3, 4, and 5 regions are transactivation domains (58), and it has been demonstrated that Neh4 and 5 are responsible for the interaction of Nrf2 with transcriptional coactivators such as CREB (c-AMP-response-element-binding-protein)-binding protein (CBP) (59) and receptor-associated coactivator 3 (RSC3) (60). The Neh6 and Neh2 domains mediate Nrf2 degradation; Neh6 is required for Nrf2 proteasomal degradation under oxidative stress conditions, while degradation under

homeostatic conditions is mediated by the Neh2 domain (61). The rapid turnover rate of Nrf2 under quiescent conditions ($t_{1/2} < 10$ min) result from interaction of the Neh2 domain with Kelch-like erythroid-derived CNC homology (ECH)-associated protein 1 (Keap1) (62). Keap1 is a cytosolic protein that sequesters Nrf2 in the cytoplasm by binding both Nrf2 and the actin cytoskeleton (55). Keap1 also binds the cullin-3 E-3 ubiquitin ligase (Cul3) (63) and thereby promotes the association of Nrf2 with Cul3, resulting in the ubiquitination and proteolytic degradation of Nrf2 under homeostatic conditions (64). However, in response to oxidative stress, Nrf2 is released from the Keap1/Cul3 inhibitory complex resulting in stabilization of Nrf2 levels. Keap1 contains several redox-sensitive cysteine residues (Cys-151, 273, and 288) that are oxidized by ROS (65,66). Oxidation of these residues causes the release of Nrf2 from Keap1, most likely through conformational changes in Keap1 (67). Nrf2 then translocates into the nucleus; this step is also regulated by ROS through oxidation of Cys-183 in the Neh5 domain (68). Nuclear Nrf2 forms heterodimers with members of the small Maf protein family (Maf-F, G, and K) and binds to the ARE (69,70).

2. Transcriptional regulation of the ferritin AREs

While ARE activation is mediated primarily by Nrf2-Maf heterodimers, repression of the ARE is facilitated by various repressors; we demonstrated that the ferritin H gene is repressed by activating transcription factor 1 (ATF1) (71). Ferritin H and other ARE-genes such as heme oxygenase -1 (HO-1) are also constitutively repressed by the bZip family member BTB and Cnc homology 1 (BACH1) protein (72,73). BACH1 release from the ARE is regulated by oxidative stress as well; oxidation of BACH1 Cys-574 by ROS results in the release of BACH1 and translocation into the cytoplasm (74). After the release of BACH1, the Nrf2-Maf heterodimer binds and activates the ARE, leading to transcription (72,75).

Nrf2 binds and activates the human ferritin H ARE after *tert*-butylhydroquinone (tBHQ), hemin, and rotenone treatment (8,27,73). Other transcription factors are involved in ferritin H ARE as well; in response to H₂O₂ and tBHQ, JunD is activated by phosphorylation at Serine-100 and binds to the human ferritin H ARE (7). Not only restricted to transcription factors, coactivators have been shown to regulate the ferritin H ARE as well. The mouse ferritin H ARE was originally identified because of the effect of the adenovirus E1A oncogene, which

negatively regulated ferritin H gene expression through a FER-1 enhancer element (mouse ferritin H ARE) (53). The transcriptional coactivator p300/CBP is recruited to the ferritin H ARE after tBHQ treatment (73) and has been demonstrated to counteract the repression of E1A and activate ferritin H ARE through histone acetyltransferase activity (76)

Transcription factors can also serve as repressors of the ferritin H ARE. As mentioned previously, we demonstrated that activating transcription factor 1 (ATF1) repressed ferritin H gene expression through the ARE; overexpression of ATF1 decreased ferritin H transcription whereas knockdown of ATF1 conversely increased its expression (71). We have recently demonstrated that protein inhibitor of activated STAT3 (PIAS3) interacts with ATF1, antagonizing the repressive function of ATF1 partly by blocking ATF1-ARE interaction (71). Homeodomain-interacting protein kinase 2 (HIPK2) has been recently shown to activate the human ferritin H ARE and reverse the repressor role of ATF1, possibly by phosphorylation at its Serine-198 site (77). Belonging to the CREB/ATF subfamily (78), overexpression of CREB inhibits the ferritin H ARE (unpublished data), suggesting that CREB may also act as a human ferritin H ARE repressor. We also demonstrated that tBHQ robustly induced ferritin H transcription in tumor suppressor phosphatase and tensin homologue deleted on chromosome 10 (PTEN)-deficient human leukemia Jurkat cells, and restoration of PTEN reversed this induction (73), suggesting that PTEN negatively regulates the ferritin H ARE.

The human ferritin L ARE contains only one consensus ARE sequence located 1350 bp upstream from the transcription start site (52) and can be activated by tBHQ, sulforaphane, hemin, and high levels of iron; to date, transcription factors and coregulators responsible for ferritin L ARE activation have not been characterized. We have found that arsenite treatment increases Nrf2 binding to both of the human ferritin L and H AREs, suggesting that ARE-dependent transcription of ferritin H and L may be regulated in a similar manner (Figure 3, Page 46, Chapter 1).

Hypoxia and Hypoxia Inducible Factors (HIFs)

While oxidative stress is an abundance of oxygen radicals, hypoxic conditions, which can result in oxidative stress by producing ROS (79-81), may also regulate antioxidant genes such as ferritin and HO1. Indeed, hypoxia has been shown to regulate ferritin at the translational level (82-84), and activate transcription of HO1 (85).

In mammals, oxygen delivery is in the covalent diatom form of two oxygen atoms (O₂). Red blood cells are responsible for O₂ transport via travelling inside of the blood arteries to ensure each cell in any location of tissues can access O₂ without shortage; however, if O₂ supply decreases, resulting from malfunctions such as anemia, hemorrhage, heart failure or stroke (86), the arterial blood O₂ pressure reduces, resulting in hypoxemia, and eventually cause decreasing tissue O₂ pressure below physiological levels; it is named, hypoxia (87) (88). Hypoxia can cause diseases; for example, acute renal injury (89), hypoxic-ischemic brain injury (90) and several neurodegeneration disease, such as Alzheimer's, Parkinson's and Huntington's diseases (91) have been shown to be associated with hypoxia. In cells, hypoxia will force cells go to an anaerobic metabolic pathway, switching from aerobic electron transport chain to the anaerobic fermentation, resulting in the reduce of cellular ATP production (92). Furthermore, short of O₂ supply will diminish mitochondrial activity, terminate the electron transport chain and even release electron flow to produce reactive oxygen species (ROS) including superoxide and hydroxyl radicals ;and reactive nitrogen species (RNS) such as peroxynitrite (79-81). All of these radicals can directly damage lipids, nucleic acids, proteins and carbohydrates when overwhelming antioxidant protein system (81).

1. Hypoxia inducible factors (HIFs)

Since oxygen homeostasis needs tight regulation to ensure appropriate oxygen supply, a sensitive oxygen responding system is necessary to cope with the change of oxygen concentration immediately; for example, cells need ATP to maintain almost all kind of biological process and therefore have to quickly switch to the anaerobic fermentation to ensure ATP production under hypoxia insults. Hypoxia inducible factors (HIFs) are a group

of transcription factors that have been shown to quickly respond to hypoxia and regulate different variety of physiological processes involved in angiogenesis, glucose metabolism, iron homeostasis, cell proliferation and differentiation (93-95). HIF family contains three members, HIF-1, -2 and -3. Each member forms a heterodimer composed of one α - and one β - subunit, both belonging to the basic helix-loop-helix/Per-ARNT-Sim (bHLH/PAS) protein family and therefore can dimerize with each other through their bHLH/PAS domains (96). β -subunits, originally identified as the aryl hydrocarbon nuclear translocator (ARNT) and associated with aryl hydrocarbon receptor (AhR), are constitutively expressed regardless of oxygen concentration no matter under normoxia or hypoxia (97). Different from the β -subunits, α - subunit under normoxia is almost completely undetectable because of its rapid turnover rate under normoxia ($t_{1/2} < 5$ min) (98,99), resulting from an oxygen-sensitive proteasome degradation system (100). The unique oxygen-dependent degradation domain (ODDD) in α - subunit is responsible for regulating protein stability of α subunit; hydroxylation of two consensus proline residues in ODDD domains, Pro -402 and -564 of HIF-1 α ; Pro -405 and -530 of HIF-2 α (101–103), by prolyl hydroxylases (PHD) is prerequisite for polyubiquitination and results in final proteasome-dependent degradation of α subunit (93). However, PHD can only catalyze hydroxylation in the presence of oxygen and iron (104), therefore, it will be inactivated under hypoxic condition; in another words, hypoxia can stabilize α -subunit from oxygen-dependent degradation (105-108) and let α - and β - subunits be activated by forming a heterodimer. After being stabilized and activated, HIFs can bind the consensus hypoxia responsive element (HRE) and regulate HIF-targeted genes (109).

Interestingly, cobalt chloride has been reported to induce hypoxia-like response in cells (110), probably resulting from stabilization of HIF- α subunit by inactivation of PHD (111). Furthermore, cobalt chloride was reported to induce HIF-1 binding activity in a similar level as hypoxia induced (112) and it can induce a variety of HIF-dependent genes such as erythropoietin and VEGF (113,114). Therefore, cobalt chloride is named the hypoxia-mimetic.

2. Hypoxia in ferritin gene expression

Since HIFs can regulate multifaceted biological processes including iron homeostasis, it is not surprising that HIFs are involved in transcriptional regulation of proteins responsible for iron homeostasis through binding to HREs. Indeed, the functional HREs in promoter regions of transferrin receptor, transferrin, and HO-1 have already been identified (85,115,116). On the other hand, HIFs can cope with hypoxia-mediated ROS production by inducing antioxidant genes such as heme oxygenase-1 and glutathione peroxidase (85,117). Harboring dual characteristics of iron-regulation and anti-oxidation, ferritin could be a possible novel HIF-dependent gene, in which it regulates iron homeostasis and alleviates oxidative stress, responding to the exposure of hypoxia. We studied ferritin H promoter region and identified a putative HRE located 4.3kb upstream of the transcription start site, supporting the idea that HIF may regulate ferritin transcription. Previous research has shown that hypoxia can induce ferritin expression through reducing IRP-mediated translational repression (82-84). However, whether hypoxia induces ferritin through transcriptional regulation or how it regulates ferritin transcription has not been studied yet.

The regulation of the Nrf2-ARE axis by oxidative stress presents a tightly controlled system by which antioxidant genes are activated in a timely and efficient manner. ROS control all stages of the Nrf2-ARE axis, from Nrf2 stabilization and nuclear translocation to release of transcriptional repressors from the ARE. While Nrf2 activation and subsequent ARE binding leading to transcription of the ferritin H gene has been studied, several questions still remain unanswered. For example, what are the transcriptional mechanisms initiated before and after the binding of Nrf2 to the ferritin H ARE? Does ferritin H ARE activation induce RNA Polymerase II (RNAPolII) recruitment to the ferritin H transcription start site? Or does the ferritin H ARE facilitate the phosphorylation of RNAPolII that is necessary for the initiation and elongation phases of transcription? While studies exploring the recruitment of transcription factors and co-activators to the ferritin H ARE are providing a clearer picture of ferritin H ARE activation and transcriptional activation, little research has been conducted as to the role of the chromatin environment in ferritin H ARE activation and

ferritin H transcription, which may provide a clearer picture of ferritin H transcriptional regulation.

Chromatin Remodeling and Histone Modification

The chromatin environment plays a major role in gene regulation. Chromatin is the nucleic acid-protein complex by which DNA is packaged and condensed in the nucleus. DNA is wrapped around an octamer of core histone proteins, H2A, H2B, H3, and H4, as the nucleosome (118). Nucleosomal units are compacted and undergo further condensation and folding to form chromosomes. Condensed chromatin presents a significant barrier to transcription. In non-dividing cells the areas of condensed chromatin are called heterochromatin and the more open and accessible regions are called euchromatin (119). Euchromatin is formed by alterations in the chromatin environment through nucleosome shifting or removal, and provides accessible regions for transcriptional complex; ATP-dependent chromatin remodelers carry out this process by removing nucleosomes or moving them along the DNA strand through the use of ATP-hydrolysis (120). In addition to ATP-dependent chromatin remodelers, the histones themselves control the chromatin environment. Histones have N-terminal tails that can be post-translationally modified (121). The acetylation of histone N-terminal tail lysines can relax chromatin by disrupting the attraction between the negatively charged DNA phosphate backbone and the positive charge of lysine residues; methylation can increase the bulk and hydrophobicity to either lysine or arginine residue and disrupt intra- or intermolecular hydrogen-bond interactions to affect protein binding partners (122); or increases the positive charge of the lysine, thus increasing attraction between the histone tail and DNA, resulting in more condensed chromatin (123,124). Through altering the chromatin environment by ATP-dependent chromatin remodeling or histone modification, DNA regions such as enhancers or transcription start sites become accessible to transcription factors and the RNAPolII complex. However, the chromatin environment also plays a major role in transcription by recruiting non-histone proteins that are transcriptional components themselves, or induce additional chromatin

remodeling; this occurs through histone modification (125,126). In addition to the methylation and acetylation of lysines, other post-translational modifications occur as well. Phosphorylation (127), sumoylation (128), ubiquitination (129), proline isomerization (130), and arginine methylation (131) of histones have all been demonstrated to regulate transcription. Histone modifications may recruit ATP-dependent chromatin remodelers, or additional histone modifiers, to alter the chromatin environment or recruit transcriptional components such as elongation factors (127). Histone modifications therefore can serve as binding platforms by which non-histone proteins are recruited and bind through specialized binding domains, thus regulating transcription. One modification of interest is the methylation of histone N-terminal tail arginines.

Protein Arginine Methyltransferases and Histone Arginine Methylation

Histone arginine methylation has been reported to be involved in the regulation of chromatin structure and gene expression (132). They alter chromatin structure, regulating the accessibility of non-histone proteins that regulate transcription or chromatin insulator activity (133).

In eukaryotes, arginine methylation occurs in different kinds of proteins such as transcription factors and histones, which are implicated in a variety of cellular processes such as RNA processing, ribosomal biosynthesis, DNA repair, signal transduction, and transcriptional regulation (134). Arginine methylation is catalyzed by a group of enzymes known as protein arginine N-methyltransferases (PRMTs) (132). PRMTs are ubiquitously expressed in a wide variety of organisms, such as plants, fungi, yeast, *C. elegans*, *Drosophila*, vertebrate animals, and mammals (135). PRMTs are evolutionarily conserved between organisms, but differ in the number of family members; for example, four enzymes (*Rmt1/Hmt1*, *Rmt2*, *Rmt3*, *Hsl7/Skb1*) have been found in yeast (*S. cerevisiae*), nine (DART1–9) in *Drosophila* (136), and seven (*Zf1–7*) in the zebrafish (*Danio rerio*) (137). To date, 11 PRMT proteins in the human PRMT family have been identified, and except for PRMT2, PRMT10, and PRMT11, all PRMT family members possess catalytic arginine

methylation activity (138) and can be classified into two different groups based upon methylation status: the type-I PRMTs (PRMT1, 3, 4, 6 and 8) catalyze the formation of asymmetric dimethylarginine, while the type-II PRMTs (PRMT5, 7 and 9) can form symmetric dimethylarginine (138).

All PRMT members share a common catalytic methyltransferase domain including a highly conserved core region and subdomains important for binding the methyl donor S-Adenosyl-L-methionine (SAM) and their substrates (139). PRMTs have a non-conserved N-terminal domain with distinctive motifs and functions; for example, the Src homology 3 domain (SH3) of PRMT2 can be utilized to interact with the N-terminal domain of PRMT8 (140). PRMT3 harbors an N-terminal zinc-finger domain which is responsible for its substrate specificity (141). PRMT4 is also known as CARM1 (coactivator associated arginine methyltransferase1), which has a unique C-terminal domain that serves as an autonomous activation domain important for CARM1 coactivator function (142); recently, this domain was shown to be auto-methylated by CARM1 and is important for CARM1-mediated transcriptional regulation and pre-mRNA splicing (143).

1. PRMT1 and asymmetric histone H4 arginine 3 di-methylation (H4R3me2a)

PRMT1 was the first identified PRMT family enzyme (144). Homozygous mutation of *prmt1* in mice results in embryonic lethality and the loss of 85% of the whole arginine methyltransferase activity in the *prmt1*^{-/-} embryonic stem cells, demonstrating its important role in physiological functions (145,146). PRMT1 activity and subcellular localization are dependent upon association with other proteins; for example, the pregnane X receptor (PXR) can interact with PRMT1, resulting in PRMT1 accumulation in the nucleus (147). B-cell translocation gene (BTG) 1 and 2 were demonstrated to regulate PRMT1 through direct binding, resulting in increased PRMT1-mediated methylation (144). PRMT1 predominantly methylates glycine and arginine rich (GAR) motifs (134) but its substrates are diverse. PRMT1 has already been shown to mediate transcriptional regulation by controlling the activity of transcription factors such as estrogen receptor α (ER α) (148), p53 (149) and forkhead box protein O1 (FOXO1) (150); as well as by regulating histone arginine methylation (121,151).

Asymmetric histone H4 arginine 3 dimethylation (H4R3me2a) is the major histone arginine methylation of PRMT1 and highly correlated with transcriptional activation (152). Indeed, it has been shown that the induction of H4R3me2a is localized at the promoters of pS2 (153), CITED2 (121), CYP3A4 (147), and the β -globin locus (154) when transcription occurs. In an example of histone modification “crosstalk,” PRMT1 recruitment and H4R3me2a potentiate the subsequent histone H3 and H4 acetylation, contributing to the maintenance of active euchromatin structures (152,154,155). Indeed, a more recent paper has shown that H4R3me2a provides a binding surface for p300/CBP-associated factor (PCAF) and directly enhances histone H3 acetylation leading to the recruitment of transcriptional pre-initiation complexes (PIC) to active promoters (156). However, acetylation of H4 can inhibit subsequent arginine 3 methylation by PRMT1 (155,157), suggesting an order existing in H4R3me2a and histone lysine acetylation. Intriguingly, histone H4R3 can be also dimethylated symmetrically; opposite to H4R3me2a, symmetric histone H4R3 dimethylation (H4R3me2s) is correlated with transcriptional repression or gene silencing (158-160). PRMT5 and PRMT7 have been shown to mediate H4R3me2s (161).

2. *CARM1 and asymmetric Histone H3 arginine 17 di-methylation (H3R17me2a)*

CARM1 (PRMT4) belongs to the type-I PRMT enzymes and was the first PRMT identified as a transcriptional regulator and a binding partner for the p160 transcriptional co-activator, glucocorticoid receptor-interacting protein 1 (GRIP1) (162). CARM1 is ubiquitously expressed in all tissues but with an increasing level in heart, kidney, and testis (162). Because of the importance of CARM1 in development, *Carm1*-knockout mice are smaller than wild type mice and die soon perinatally (163). Similar to PRMT1, CARM1 is also involved in transcriptional regulation through interaction with transcription factors; for example, CARM1 can enhance the nuclear receptor function (162,164,165) or synergistically cooperate with p300/CBP and PCAF (166). Interestingly, CARM1 has also been showed to methylate p300/CBP and regulate its acetyltransferase activity (167-169). Posttranslational phosphorylation has been reported to regulate CARM1 activity; for example, CARM1 methyltransferase activity is negatively regulated through phosphorylation at Serine-228 in its substrate binding domain and the phosphorylation diminishes its binding activity to

methyl donor, SAM (170); on the other hand, phosphorylation at serine-217 of CARM1 not only blocks SAM accessibility but also causes CARM1 cytoplasmic localization (171). CARM1 has been shown to cooperate with PRMT1 in STAT5, NF- κ B and p53 mediated transcriptional regulation (121,151).

H3R17me2a is generated by CARM1 (172) and has been shown to be induced in the transcription of several gene promoters such as pS2, CCNE1, Oct 4, E2F1, Sox2 and CITED2 (121,173-177). In the estrogen-responsive *pS2* promoter, CARM1 is recruited and correlated with the induction of H3R17me2a (178); at the same time, CBP/p300 is also recruited and mediated H3K18 acetylation, which is important for CARM1 to increase its methyltransferase activity rate and conduct H3R17me2a more efficiently (166,179). Also, H3R17me2a was shown to be correlated with the histone H3S10 phosphorylation during mitosis (180).

Besides H3R17me2a, CARM1 can also target H3R26me2a, which has not been extensively studied, but is known to be correlated with stem cell development in the mouse embryo (181), and also induced at the CCNE1 promoter (176); however, the role of H3R26me2a is still not clear.

CHAPTER 1: Transcriptional Regulation of the Human Ferritin Gene by Protein Arginine Methyltransferases PRMT1 and CARM1

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Running title: Histone arginine methylation and Nrf2-ARE regulation

Key words: ferritin, arsenic, histone methylation, Nrf2, oxidative stress

Background: The chromatin environment plays a cooperative and essential role in gene transcription; however, particular histone modifications associated with antioxidant gene regulation remain uncharacterized.

Results: Methylation of histone H4R3 and H3R17 was involved in transcriptional regulation of ARE-regulated antioxidant genes such as ferritin and NQO1.

Conclusion: Arginine methyltransferases PRMT1- and CARM1-mediated histone H4R3 and H3R17 methylation regulate the ARE and cellular antioxidant response to arsenic.

Significance: Our finding that specific histone modifications cooperate with Nrf2 for ARE-dependent transcription may lead to better understanding of antioxidant gene expression and oxidative stress related diseases.

ABSTRACT

Antioxidant detoxification genes such as ferritin and NAD(P)H quinone oxidoreductase-1 (NQO1) are transcriptionally activated under oxidative stress conditions via a highly conserved enhancer, termed anti-oxidant responsive element (ARE), to which nuclear factor-E2-related factor 2 (Nrf2) binds and activates transcription. Histone modifications play a cooperative and essential role in transcriptional regulation; however, particular histone modifications associated with antioxidant gene transcription remain elusive. Here we found that arsenic exposure activates transcription of ferritin H gene via the ARE concomitant with increased methylation of histones H4 Arg3 (H4R3) and H3 Arg17 (H3R17). We hypothesized that methylation of histone H4R3 and H3R17 are involved in transcriptional regulation of ferritin H and some other ARE-regulated antioxidant genes. To test this hypothesis, two protein arginine methyltransferases (PRMTs) were further investigated; PRMT1 and PRMT4 (CARM1 (coactivator-associated arginine methyltransferase 1) that catalyze methylation of H4R3 and H3R17, respectively. After arsenic exposure to human HaCaT or K562 cells, we found that 1) nuclear accumulation of PRMT1 and CARM1 was induced, 2) methylation of histone H4R3 and H3R17 were induced proximal to the AREs of ferritin and NQO1 genes, 3) knocking down PRMT1 or CARM1 did not block Nrf2 nuclear accumulation but inhibited Nrf2 binding to the AREs, thus diminishing ferritin and NQO1 gene transcription, and 4) PRMT1 and CARM1 knockdown enhanced cellular susceptibility to arsenic toxicity as evidenced by caspase 3 activation. Collectively, these results suggest that PRMT1- and CARM1-mediated methylation of histone H4R3 and H3R17 regulate the ARE and cellular antioxidant response to arsenic.

INTRODUCTION

Cells are constantly exposed to harmful xenobiotics from the environment as well as endobiotics and reactive oxygen species (ROS) produced during various metabolic activities. To combat and detoxify these harmful chemicals and oxidants, cells evolved antioxidant systems and activate several key signaling pathways to biotransform them to less toxic benign molecules (1). In cells exposed to these chemicals, a battery of antioxidant detoxification genes including hemeoxygenase-1 (HO-1), NAD(P)H quinone oxidoreductase-1 (NQO1), and glutathione S-transferases (GST) are induced at the transcriptional level (2). In addition to these metabolic enzymes, we previously reported that ferritin, the major iron storage multimeric protein composed of H and L subunits, is transcriptionally and post-transcriptionally upregulated under oxidative stress conditions (3-6). The H and L subunits of ferritin play key roles in iron storage through the H subunit's ferroxidase activity and L subunit's structural stabilization involved in the oxidation and efficient incorporation of Fe^{2+} into a multimeric ferritin shell (7). Iron is an essential element for a wide variety of cellular activities including metabolism, proliferation, and differentiation; however, excess free iron is toxic to cells because it catalyzes production of highly reactive hydroxyl radical through the Fenton reaction, causing various damages to macromolecules including DNA, proteins, and lipids (7,8). Therefore, transcriptional upregulation of ferritin under oxidative stress is an important cellular defense mechanism; by chelating excess intracellular free iron thereby minimizing the hydroxyl radical formation. It should be noted that, when iron is in excess, ferritin is upregulated at the translational level by iron via the well-characterized IRE-IRP system, while in cells under oxidative stress, ferritin is upregulated at the transcriptional level in an iron-independent manner (8-10). Transcriptional activation of ferritin and other antioxidant detoxification genes is regulated via a conserved enhancer element, termed the antioxidant responsive element (ARE) (11). The core ARE sequence is an AP1-like TGACnnnGCA motif (11,12), to which nuclear factor-E2-related factor 2 (Nrf2) and small Maf proteins are recruited and activates transcription of antioxidant genes (2).

Since DNA is wrapped around core histones (an octamer of H2A, H2B, H3, and H4) and tightly packed as nucleosomes, dynamic and reversible changes in chromatin structure and conformation through posttranslational modifications of core histones are necessary to allow transcription factors access to their specific cis-acting elements and exhibit their functions properly (13). One well-studied coactivator that collaborates with various transcription factors is histone acetyltransferase (HAT). In the activation of an ARE enhancer, we and others reported that HATs such as p300, CBP (CREB binding protein) (14-16), and MOZ (monocytic leukemia zinc-finger protein)(17) are involved in transcriptional activation of ferritin H and GST genes under oxidative stress. N-terminal tails of core histones have multiple Lysine (Lys), Arginine (Arg), and Serine/Threonine (Ser/Thr) residues that are subject to reversible posttranslational modifications such as acetylation, methylation, and phosphorylation (13,18). Indeed, acetylation of histone H3 Lys9 and Lys18 (H3K9 and H3K18) in conjunction with recruitment of p300 and CBP were associated with t-BHQ (tert-butylhydroquinone)-induced ARE activation (14). These HATs may play a role in acetylation of H3K9 and H3K18, as well as direct acetylation of Nrf2 that was shown to activate Nrf2 transcription function (19,20). In addition, BRG1 (Brahma-related gene 1), a catalytic subunit of Swi2/Snf2-like ATPase involved in chromatin remodeling, was shown to interact with Nrf2 and specifically regulates the HO-1 ARE but not NQO1 ARE (21). Accumulating evidence indicates that posttranslational modifications of histones play a crucial role in transcriptional regulation; however, particular histone modifications and enzymes involved in antioxidant gene regulation under oxidative stress remain largely uncharacterized.

The protein methyltransferases (PMTs), composed of lysine PMTs (PKMTs) and arginine PMTs (PRMTs), have been characterized as important regulators of gene transcription by facilitating the transfer of methyl groups to specific Lys and Arg residues, respectively, in both histones and non-histone proteins (22). N-terminal histone tails contain Lys residues that may be mono-, di, or tri-methylated, or Arg residues which are mono- or di- (either symmetric or asymmetric) methylated, thus providing a platform for interaction with methyl-

Lys or methyl-Arg binding proteins. These proteins contain such binding motifs as Chromo domain (binding to methyl-Lys) or Tudor domain (binding to methyl-Arg)(23), allowing new protein-histone interactions that either activate or repress gene transcription in a context-dependent manner (24). For instance, methylation at Lys 4 and Lys 9 on histone H3 by several PKMTs were characterized as marks of transcriptional activation and repression, respectively (13). Similarly, Arg-methylation by PRMTs on histones causes either transcriptional activation or repression (25). The mammalian PRMT family comprises 11 members (PRMT1- PRMT11), in which type I enzymes such as PRMTs 1, 3, 4 (CARM1), 6, and 8 catalyze monomethylation and asymmetric dimethylation of Arg, whereas the type II enzymes such as PRMT5 catalyze monomethylation and symmetric dimethylation of Arg (25-28). Among them, PRMT1 is the major type I enzyme that catalyzes methylation of histone H4 Arg 3 (H4R3) (29) that is a mark of transcription activation (30). PRMT4 (CARM1; coactivator associated arginine methyltransferase 1) is another PRMT involved in transcription-activation that was originally shown to bind steroid receptor coactivators (SRC) (31) and was subsequently characterized as an enzyme involved in methylation of histone H3 Arg 17 (H3R17) (32).

Arsenic exposure is associated with toxicity in various cell types and incidence of several cancers including skin, bladder, and lung. The potential mechanism underlying arsenic-induced toxicity and carcinogenicity is mediated by oxidative stress that damages macromolecules including nucleic acids and proteins (33,34). Oxidative stress is implicated in various diseases such as cancer and neurodegeneration; therefore, gaining insight into the molecular mechanism through which antioxidant detoxifying genes are regulated is important to understand these diseases. In this study we report that transcription of the human ferritin genes were induced by arsenic treatment via the AREs in several human cell types. During arsenic treatment, we observed that PRMT1 and CARM1 proteins transiently accumulated in nucleus, and CHIP assays revealed that methylation of H4R3 and H3R17 was enriched in the ARE enhancer regions. Knocking down PRMT1 or CARM1 inhibited arsenic-induced Nrf2

recruitment to the ARE and ferritin mRNA expression, suggesting the importance of PRMT1 and CARM1 in arsenic-induced ferritin transcription through the ARE.

EXPERIMENTAL PROCEDURES

Cell Culture and chemical reagents- HaCaT human keratinocyte cells (35) were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS) (Mediatech). K562 human erythroleukemia cells (American Type Culture Collection), were cultured in RPMI1640 medium supplemented with 25 mM Hepes, 0.3g/liter L-glutamine, and 10% FBS. They were incubated at 37°C in a humidified 95% air, 5% carbon dioxide incubator. Sodium Arsenite (NaAsO₃, Thermo Fisher) and Adenosine dialdehyde (AdOx, Sigma-Aldrich) were dissolved in distilled water and DMSD, respectively.

Plasmids and antibodies- The -4.5kb ARE(+) and -4.4kb ARE(-) human ferritin H 5' upstream enhancer and promoter fused to a firefly luciferase reporter gene were described previously (6). Antibodies used in this work were purchased from the following companies: anti-ferritin H (sc-25617), anti-NQO1 (sc-32793), anti-hemeoxygenase-1 (sc-7695), anti-Nrf2 (sc-13032x), anti-rabbit IgG (sc-2027), anti-histone H4 (sc-25260), and anti-RNA polymerase II (sc-899x), all from Santa Cruz Biotechnology; anti-laminB₁ (Ab-1, Oncogene); anti-lactate dehydrogenase (LDH) (AB1222, Chemicon); anti-CARM1 (3379S), anti-caspase 3 (8G10), and anti-histone H3 (9715) from Cell Signaling Technology; anti-β-actin (A5441, Sigma); anti-dimethyl histone H4R3 antibody for ChIP assays (39706, Active Motif) and Western blotting (07-213, Millipore); anti-dimethyl histone H3R17 for ChIP assays (07-214, Millipore) and for Western blots (ab8284, Abcam).

DNA transfection and luciferase reporter assay- HaCaT cells were transiently transfected by electroporation (Bio-rad Gene Pluser X-Cell) with -4.5kb ARE(+) or -4.4kb ARE(-) human ferritin H luciferase reporter plasmids. 10 ng pRL-null (Promega) was co-transfected as an internal control and incubated for 48 hours. Cells were then treated with sodium arsenite for 20-24 hours and subjected to luciferase assays using Dual Luciferase reagents (Promega); Renilla luciferase activity was used to normalize firefly luciferase expression driven by the ferritin H promoter.

Histone Extraction – Histone extraction was carried out according to the published method with minor modifications (36). Briefly, HaCaT cells were suspended and washed with buffer C (20 mM HEPES (pH 7.9), 0.1% Triton X-100, 1.5 mM MgCl₂, 1 mM PMSF and 1 mM DTT), followed by resuspension in buffer D (10mM HEPES (pH 7.9), 10 mM KCl, 1.5 mM MgCl₂, 1 mM PMSF and 1 mM DTT). Then H₂SO₄ was added to a final concentration 0.4N and rocked for 30 minutes. Extracted histone proteins were precipitated with TCA, washed once with 0.1% HCl/acetone, then with acetone, and dissolved in 2M urea.

Western blotting- Whole cell extracts (WCE) or nuclear and cytosolic fractions were loaded on 7.5-15% sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (Mini-Protean 3, Bio-rad), transferred to an polyvinylidene difluoride (PVDF) membrane (Pierce), incubated with a primary antibody at 4°C overnight, followed by a secondary antibody-conjugated with horseradish peroxidase at room temperature for 1.5 hours. Proteins were visualized using a HyGLO detection reagent (Denville Scientific), ECL prime (GE Healthcare), or Femtomax (Rockland). Cell fractionation was carried out with a nuclear isolation kit (Active Motif).

Northern blotting - Total RNA was isolated by using TRI Reagent RT (Molecular Research Center). 2-10µg of total RNA was separated on 0.7% agarose gel with 5% formaldehyde in 3-(N-morpholino)-propanesulfonic acid buffer, followed by capillary transfer to an Immobilon-NC nitrocellulose membranes (Millipore). ³²p-labeled human ferritin H, ferritin L, NQO1, or HO-1 cDNA probe was hybridized with membranes at 42°C overnight and washed with washing buffer (0.1% SDS in 0.5X SSC) at 52°C. The dried membranes were subjected to autoradiography. Staining RNA with ethidium bromide was used for evaluation of equal RNA loading. Quantitation of autoradiography was performed by the software, *Multi Gauge* (Fujifilm).

Small interfering RNA (siRNA) transfection- 90% confluent HaCaT cells in 100mm culture dishes were electroporated with 100 pmol of non-targeting siRNA (siCon) (D-001210-01),

siPRMT1 (J-010102-07), or siCARM1 (J-004130-05) (Supplemental Table I for siRNA sequences) by Gene Pluser Xcell in 100 μ l of siRNA transfection media (sc-36868, Santa Cruz Biotechnology). After electroporation, cells are incubated at room temperature for 10 minutes, transferred into cell culture dishes with growth media, and incubated at 37°C for 48 hours. Cells were then subjected to sodium arsenite treatment and Northern blotting or ChIP assay.

Quantitative real time polymerase chain reaction (qPCR) – RNA was reverse transcribed to cDNA with iScript™ cDNA Synthesis Kit (Bio-rad). cDNA were subjected to SYBR Green qPCR with iQ™ SYBR® Green Supermix (Bio-rad) by using the primer pairs specific for detection of target genes. The relative efficiency of each primer set was determined from RNA standard curve by sequential 10 fold dilutions. Expression of mRNA level of each gene was normalized to β microglobulin (ΔC_t) and defined as the change of C_t in treated samples relative to untreated control or siCon ($\Delta\Delta C_t$). Exponential $\Delta\Delta C_t$ values were converted to linear values ($2^{-(\Delta\Delta C_t)}$) for fold induction.

Chromatin immunoprecipitation (ChIP) Assay- ChIP assays were carried out according to the fast ChIP method (37). Briefly, HaCaT cells were fixed with 1.42% formaldehyde for chromatin cross-linking and quenched with 125mM glycine. Cell lysates are sonicated to shear chromatin DNA. Chromatin immunoprecipitation (ChIP) by immunoglobulin G (IgG) or specific antibodies against interested proteins were performed at 4°C in sonication bath (Branson 2510, 40mHz) for 15 minutes and incubated with protein A agarose/ssDNA bead slurry (16-157, Millipore). After washing and decrosslinking, the genomic DNA were subjected to SYBR Green qPCR with iQ™ SYBR® Green Supermix (Bio-rad) by using the primer pairs flanking ferritin H, NQO1, and HO-1 EN2 (8) AREs or transcription start sites (sequences in Supplemental Table I). The relative efficiency of each primer set was determined using input genomic DNA. The DNA in each immunoprecipitated sample was normalized to input (ΔC_t) and defined as the change of C_t in treated samples relative to

control ($\Delta\Delta C_t$). Exponential $\Delta\Delta C_t$ values were converted to linear values ($2^{-\Delta\Delta C_t}$) for fold induction.

RESULTS

Arsenic induced transcription of the human ferritin H gene

It has been shown that arsenic causes oxidative stress (33) and induces several antioxidant detoxification genes such as HO-1 and NQO1 (38). As ferritin encapsulates excess iron and protect cells from oxidative stress (4,14,39,40), we tested whether arsenic induces ferritin expression in three human cell types. HaCaT human keratinocytes were treated with 1-10 uM sodium arsenite for 24 hr and mRNA and protein expression for ferritin H and L were measured. Northern and Western blots showed that arsenic induced ferritin H and L mRNA and protein in a dose-dependent manner (Fig. 1A). Arsenic also induced NQO1 and HO-1 as reported previously (38). Similar results were observed in K562 erythroleukemia (Fig. 1B) and Jurkat T cell-leukemia cells (supplemental Fig. 1S). Since the cellular response to arsenic on ferritin expression remains largely uncharacterized, the rest of our study focused on regulation of ferritin H and L expression following arsenic exposure. The human ferritin H gene contains an ARE, a key oxidative stress responsive enhancer comprised of one AP1-like and one AP1/NFE2 site located 4.5 kb upstream from the transcription start site (6). We therefore tested whether the ARE is responsible for the transcriptional activation of ferritin H following arsenic exposure. HaCaT cells were transiently transfected with luciferase reporters containing or lacking the ARE (4.5kb ARE (+) or 4.4kb ARE (-)) and treated with 0.5 or 1 uM arsenic for 24 hr for luciferase assays. Arsenic induced luciferase expression driven by the 4.5kb ARE (+) but not by the 4.4kb ARE (-) (Fig. 1C), suggesting that arsenic induces transcription of the ferritin H gene through the ARE. To confirm the transcriptional activation of the ferritin H gene by arsenic treatment, we used chromatin immunoprecipitation (ChIP) assay to measure the recruitment of RNA polymerase II to the transcription start site (TSS) of the ferritin H gene. Indeed, RNA polymerase II was recruited to the ferritin H TSS 4 hr after arsenic treatment in HaCaT cells (Fig. 1D). Collectively, these results indicate that arsenic activates transcription of the human ferritin H gene through the ARE.

Arsenic induced histone methylation along with nuclear accumulation of PRMT1 and CARM1

To assess the role of methyltransferases in ferritin H gene transcription, we pretreated HaCaT cells with 50 μ M adenosine periodate (AdOx), a general inhibitor of various methyltransferases, and treated them with arsenic to examine ferritin H mRNA expression. AdOx indeed inhibited the effect of arsenic on ferritin H mRNA expression (Supplemental Fig. 2). Among 11 PRMT members, PRMT1 and CARM1 (PRMT4) have been characterized as transcriptionally activating PRMTs by catalyzing histone methylation on H4R3 and H3R17, respectively (29,31,41,42). In fact, our dimethyl-H4R3 or -H3R17 Western blots showed that arsenic treatment increased dimethylation of H4R3 and H3R17 in HaCaT cells (Fig. 2A). Furthermore, we observed that PRMT1 and CARM1, localized in both the cytoplasm and nucleus in HaCaT cells, transiently accumulated in nucleus by 1 hr after arsenic treatment (Fig. 2B). These results suggest that PRMT1 and CARM1 are recruited to the nucleus to induce methylation of nuclear proteins in response to arsenic.

Methylation of histone H4R3 and H3R17 preceded or occurred concomitantly with Nrf2 recruitment to the ferritin AREs following arsenic treatment

We then asked whether methylated histone H4R3 and H3R17 are enriched in the proximity of the ferritin H ARE, and if so at what time relative to the recruitment of Nrf2 to the ARE after arsenic treatment. To address these issues, HaCaT cells were treated with 10 μ M arsenic for 15-120 min and CHIP assays for ferritin H ARE were carried out using anti-Nrf2, anti-dimethyl H4R3, or anti-dimethyl H3R17 antibody. We observed the recruitment of Nrf2 to the ferritin H ARE in 0.5-1 hr after arsenic treatment and continued to increase for 2 hr in HaCaT cells (Fig. 3). The enrichment of dimethylated H4R3 and H3R17 was seen by 15 min and 1 hr, respectively, after arsenic treatment, but it was transient and returned to basal level by 2 hr (Fig. 3). We observed a similar profile of Nrf2 recruitment and dimethylation of H4R3 in human ferritin L ARE (Fig. 3). These results suggest that methylation of histone H4R3 and/or H3R17 precedes or occurs concomitantly with Nrf2 recruitment to the ferritin H and L AREs following arsenic treatment.

Nrf2 binding to the ferritin H ARE, but not Nrf2 nuclear accumulation, was inhibited after PRMT1 or CARM1 knockdown.

These results suggest that there may be a causal association between Nrf2 activation and methylation of histone H4R3 and/or H3R17. To explore further, we transfected siRNA to knock down PRMT1 or CARM1 in HaCaT cells, then treated with arsenic for 2 hr and measured Nrf2 nuclear translocation and the recruitment of Nrf2 to the ferritin H and L AREs. Knocking down PRMT1 or CARM1 showed only marginal effects on arsenic-induced nuclear accumulation of Nrf2 (Fig. 4A); however, ChIP assay showed that it inhibited Nrf2 binding to the ferritin H and L AREs (Fig. 4B). NQO1 ARE also showed decreased Nrf2 binding under PRMT1 or CARM1 knockdown condition (Fig. 4B). In contrast, we observed only a marginal effect on Nrf2 recruitment to the HO-1 ARE under the same condition (Supplemental Fig. 3). We then measured expression of ferritin under PRMT1- or CARM1-deficient condition. Indeed, induction of mRNA and protein expression of ferritin H and L by arsenic treatment was inhibited by either PRMT1 or CARM1 knockdown in HaCaT (Fig. 5A) as well as K562 cells (Fig. 5B). Collectively, these results suggest that PRMT1 and CARM1 are important in Nrf2 binding to the ferritin H and L AREs that increases these mRNA levels in cells exposed to arsenic.

PRMT1 and CARM1 knockdown enhanced cellular susceptibility to arsenic toxicity

Ferritin as well as other antioxidant genes regulated by Nrf2 were demonstrated to be cytoprotective against oxidative stress. Arsenic-mediated oxidative stress induces apoptosis (34). To understand the role of PRMT1 and CARM1 in cellular defense against oxidative stress, we knocked down PRMT1 or CARM1 in HaCaT cells and assessed cellular susceptibility to arsenic toxicity by examining caspase 3 cleavage, a hallmark of apoptosis. Under significant knockdown of PRMT1 and CARM1 in HaCaT cells (Fig. 6), we observed increased cleavage of caspase 3 following arsenic treatment for 12 hr (Fig. 6). These results indicate that PRMT1 or CARM1 deficiency sensitizes cells to arsenic-mediated apoptosis, suggesting these histone methyltransferases playing a cytoprotective role.

DISCUSSION

Arsenic is a widely contaminated human carcinogen that causes oxidative cell damage. To understand the cellular defense program elicited by arsenic exposure, we characterized the molecular mechanism through which antioxidant genes are coordinately regulated by the Nrf2 transcription factor along with histone modifications proximal to an ARE enhancer element of antioxidant genes. In this study we found that arsenic is a potent inducer of ferritin, the major intracellular iron-storage protein, in various cell types. The induction of ferritin H by arsenic was regulated at the transcriptional level via activation of the ARE (Fig. 1), which we previously identified as serving both a basal and an oxidative stress-responsive enhancer of the mouse (3,43) and human ferritin H gene (6). We also observed ferritin L induction by arsenic exposure (Fig. 1), which may be regulated in the same manner as ferritin H because the mouse and human ferritin L gene was also shown to be transcriptionally regulated through an ARE (12,44). Iron plays an essential role in cell proliferation and transformation (45). Therefore, upregulation of ferritin in arsenic-treated cells appears to be an adaptive response to alleviate arsenic toxicity by chelating more intracellular free iron into ferritin, thereby decreasing the risk of both iron-catalyzed hydroxyl radical formation as well as cell proliferation and transformation. However, it was shown that human osteogenic sarcoma transformed after long-term exposure of arsenic exhibited higher total iron levels than the normal counterpart, in which ferritin expression in arsenic-transformed cells was not increased but decreased (46). This observation suggests that iron homeostasis appears to be adjusted during long-term exposure of arsenic in transformed cells through alteration of gene expression of ferritin and some others involved in iron metabolism. We also observed that expression of HO-1 and NQO1 was induced by arsenic in HaCaT cells and two other human cells K562 and Jurkat (Fig. 1 and supplemental Fig. 1), consistent with our understanding of these well-characterized antioxidant genes known to be induced by arsenic (38,47,48). Since arsenic was shown to activate an ARE (38), our finding characterizes ferritin as another new antioxidant gene transcriptionally induced by arsenic.

Arsenic is known to activate Nrf2 (38), the major transcription factor involved in ARE-regulated gene transcription. However, in spite of accumulating evidence for the importance of the chromatin environment in gene transcription, how Nrf2 and histone modifications proximal to an ARE regulate each other remains elusive. Our results showing that AdOx, a broad histone methyltransferase inhibitor, blocked arsenic-induced ferritin H expression (Supplemental Fig. 2) led us to investigate the role of PRMTs in particular PRMT1 and CARM1 that are transactivation-associated PRMTs (25) in ARE regulation by arsenic. In our experiments, PRMT1 and CARM1 proteins were localized in both the cytoplasm and nucleus in HaCaT cells, and nuclear PRMT1 and CARM1 transiently accumulated following arsenic exposure (Fig. 2). To our knowledge, this is the first report showing increased levels of these PRMTs in the nucleus when cells were placed under a particular stress condition. However, it should be noted that pregnane X-receptor (PXR) was shown to interact with PRMT1 and their interaction determines PRMT1 subcellular compartmentalization (49). In addition, phosphorylation of CARM1 was shown to cause CARM1 cytoplasmic localization (50). Further investigation will be necessary to better understand the molecular mechanism by which nuclear accumulation of PRMT1 or CARM1 is regulated under arsenic exposure.

In conjunction with nuclear accumulation of PRMT1 and CARM1 following arsenic treatment, we observed increased methylation of H4R3 or -H3R17 proximal to the ARE in the ferritin H and L gene (Fig. 3). However, this change does not appear to be specific to the ARE region because core promoter of the human ferritin H gene also showed higher dimethyl-H4R3 or -H3R17 (unpublished observation). Gene-wide distribution of histone modifications was previously observed in many other genes (51-53). Since we did not observe the recruitment of PRMT1 or CARM1 to the ferritin ARE after arsenic treatment (unpublished observation), these results suggest that global increases in dimethyl-H4R3 or -H3R17 may be induced by arsenic exposure. The broad methylation distribution pattern of histone H4R3 or H3R17 by PRMT1 or CARM1 appears to enable Nrf2 to bind more efficiently to the ARE and activate transcription of the ferritin gene. This is supported by the results showing increased methylation in histone H4R3 and H3R17 preceding or occurring

concomitantly with Nrf2 recruitment to the ARE following arsenic treatment (Fig. 3), and more importantly, PRMT1 or CARM1 deficiency by knocking down with siRNA did not affect Nrf2 nuclear accumulation induced by arsenic but blocked the binding of Nrf2 to the ferritin H and L AREs (Fig. 4). We also observed a similar effect on the NQO1 ARE (Fig. 4). In contrast, we did not obtain evidence of the contribution of PRMT1 or CARM1 in activation of the HO-1 ARE following arsenic treatment (Supplemental Fig. 3). Although the ARE sequences are highly conserved and its regulatory mechanism appears to be largely shared (12), nucleosome occupancy of each ARE may differ, and as a result, will significantly affect the transcriptional process. Indeed, there are several cases representing a particular regulatory mechanism applied only to a specific ARE (20,21,54,55).

Our results suggest that PRMT1- and CARM1-mediated histone methylation is likely to be involved in facilitating Nrf2 binding to the ARE. However, another possibility cannot be ruled out; that is, PRMT1- or CARM1-mediated protein Arg methylation of non-histone proteins. It was demonstrated that PRMT1 methylates and activates STAT1 (signal transducers and activators of transcription) transcriptional activity by altering its interaction with an inhibitor protein PIAS1 (56). PRMT1 also methylates FOXO1 within an Akt phosphorylation motif, resulting in inhibition of FOXO1 phosphorylation by Akt thereby blocking FOXO1 degradation (57). In addition, the activity of a transcriptional coactivator PGC1 α (peroxisome proliferator-activated receptor γ coactivator 1 α) was increased by PRMT1-mediated multiple Arg methylation in the C-terminal PGC1 α (58). In the context of an ARE regulation, several transcription factors are involved, including Nrf2 and other b-zip transcription factors such as small Maf proteins (MafF, MafK, MafG), Bach1, and AP1 family members (2). In addition, several transcriptional coactivators were shown to be involved in the regulation of an ARE enhancer activity, including p300/CBP (14-16), MOZ (17), and BRG1 (21). Therefore, in regard to the possibility of involvement of non-histone protein methylation, these ARE-interacting transcription factors and/or coactivators might also be regulated by PRMT1 or CARM1 in response to arsenic exposure.

Arsenic is metabolized by oxidative methylation along with glutathione conjugation (59). The methylation step catalyzed by arsenic methyltransferase utilize S-adenosylmethionine (SAM) as a methyl donor, resulting in decreased SAM pools. Thus arsenic causes global hypomethylation in genomic DNA (60); however, it is known that arsenic induces gene-specific DNA hypermethylation by unknown mechanism that is thought to contribute to gene expression changes (61). We believe that arsenic-induced histone Arg methylation may be a situation similar to specific DNA hypermethylation, in which cells exposed to arsenic recruit PRMT1 and CARM1 to nucleus and increase histone H4R3 and H3R17 methylation even under decreased SAM pools, which appears to be a cellular antioxidant response to arsenic toxicity by allowing Nrf2 to upregulate specific ARE-regulated antioxidant genes such as ferritin and NQO1.

In summary, this work demonstrated that 1) ferritin is a new arsenic-responsive gene, transcriptionally regulated through activation of the ARE, 2) arsenic induces histone H4R3 and H3R17 methylation along with nuclear accumulation of PRMT1 and CARM1, 3) methylation of histone H4R3 and H3R17 precedes or accompanies Nrf2 recruitment to the ARE following arsenic treatment, and 4) PRMT1 or CARM1 deficiency inhibits Nrf2 recruitment to the ferritin H and L AREs and their transcriptional activation by arsenic treatment, and 5) PRMT1 or CARM1 knockdown increased cellular susceptibility to arsenic-mediated apoptosis. Collectively, these results suggest that PRMT1 and/or CARM1 is important for arsenic-mediated ARE activation and the antioxidant cellular defense program.

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FIGURE LEGENDS

Figure 1: Arsenic induced transcription of the human ferritin H and L genes.

Total RNA and whole cell lysate (WCL) were isolated from **A)** HaCaT or **B)** K562 cells treated with 0, 1, 3 or 10 μM sodium arsenite for 24 hr. RNA was subjected to Northern blotting (left panels) and hybridized with ^{32}P -labeled human ferritin H, ferritin L, NQO1, or HO-1 cDNA probe. An equal amount of RNA loading was confirmed by staining RNA with ethidium bromide. WCL was subjected to Western blotting (right panels) using anti-ferritin H, ferritin L, NQO1, HO1 or β -actin antibodies. **C)** HaCaT cells were transfected with luciferase reporter plasmids containing 4.5kb ARE(+) or 4.4kb ARE(-) 5'- promoter region of the human ferritin H gene, and treated with sodium arsenite (0.5 or 1 μM) or t-BHQ (1 or 5 μM) for 24 hr. pRL-null renilla luciferase reporter was cotransfected and used as an internal transfection efficiency control. The firefly luciferase activity normalized with renilla luciferase activity from cells with no treatment (0) was defined as 1. Means \pm standard errors were shown from 3 independent experiments. **D)** HaCaT cells were treated with 10 μM sodium arsenite for 4 or 8 hr, and ChIP assays for RNA polymerase II (RNAPII) binding to the ferritin H transcription start site (TSS) were performed. The level of RNAP II (gray bar) bound to ferritin H TSS with no treatment was defined as 1 (control). Control IgG in the same ChIP assays were shown in white bar. Means from two independent experiments were shown.

Figure 2: Arsenic induces histone methylation along with nuclear accumulation of PRMT1 and CARM1.

A) HaCaT cells were treated with 10 μM sodium arsenite for 0, 0.5, 1, 2 and 4 hr and extracted histone was analyzed for asymmetric di-methylation of histone H4R3 (H4R3me2a) and H3R17 (H4R17me2a) by Western blotting. Histone H4 and H3 Western blots are shown for loading control. **B)** HaCaT cells were treated with 10 μM sodium arsenite for 0, 0.25, 0.5, 1, 2 and 6 hr and cytosolic and nuclear fractions were analyzed for PRMT1 and CARM1

expression by Western blotting. Lamin B₁ and lactate dehydrogenase (LDH) were used for a nuclear and cytoplasmic marker, respectively, and for loading control.

Figure 3: Methylation of histone H4R3 and H3R17 precedes or occurs concomitantly with Nrf2 recruitment to the ferritin AREs following arsenic treatment.

HaCaT cells treated with 10 μ M sodium arsenite for indicated times were analyzed for protein binding to ferritin H ARE (FH ARE) or ferritin L ARE (FL ARE) by ChIP assays using rabbit IgG, Nrf2, dimethylated histone H4R3 (H4R3me2a) or H3R17 antibodies (H3R17me2a). Protein binding to FH ARE or FL ARE in untreated cells with each specific antibody was set to 1. ARE binding of dimethylated histone H4R3, H3R17, or Nrf2 were shown in gray columns and control IgG in white columns. Means \pm standard errors of triplicate samples from three independent experiments are shown.

Figure 4: Knocking down PRMT1 or CARM1 does not affect Nrf2 nuclear accumulation but inhibits Nrf2 binding to the ferritin and NQO1 AREs.

Non-target siRNA (no siPRMT1, no siCARM1), siPRMT1, or siCARM1 was transfected into HaCaT cells. **A)** Cells were then treated with 10 μ M sodium arsenite for 12 hr. Nuclear fraction was analyzed by Western blotting with anti-Nrf2, anti-lamin B₁, anti-PRMT1, anti-CARM1, or anti- β -actin antibody. **B)** cells were then treated with 10 μ M sodium arsenite for 2 hr, and Nrf2 binding to ARE in ferritin H (FH ARE), ferritin L (FL ARE) or NQO1 ARE was analyzed by ChIP assay. Binding of Nrf2 to each ARE in arsenite-treated (gray columns) or untreated cells (white columns) is shown as non-target siRNA (siCon) and arsenic treated cells as 100%. Means \pm standard errors of were shown from three independent experiments. * P <0.05, as determined by a Student's t -test.

Figure 5: Knocking down PRMT1 or CARM1 inhibits arsenic-induced ferritin expression.

Non-target siRNA (no siPRMT1, no siCARM1), siPRMT1, or siCARM1 was transfected into **A)** HaCaT cells or **B)** K562 cells followed by treatment with 10 μ M sodium arsenite for

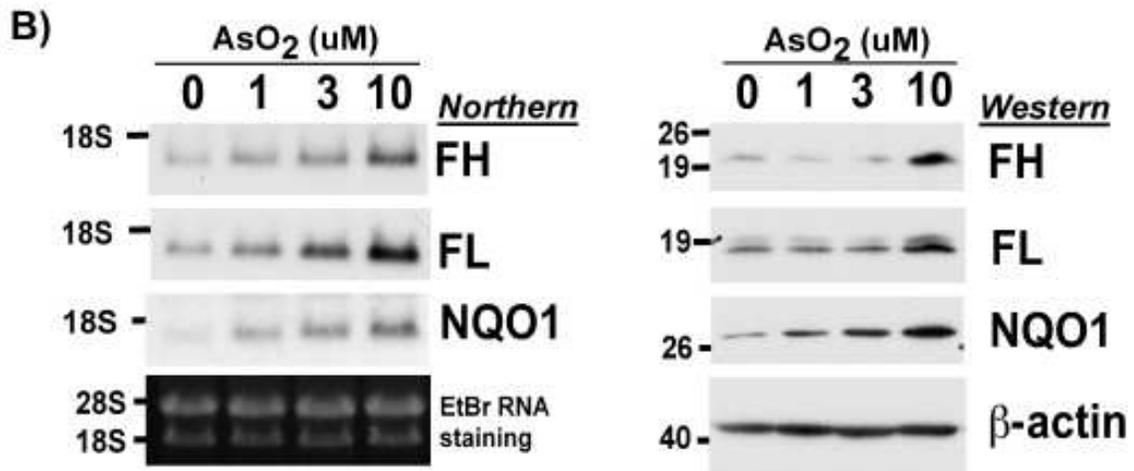
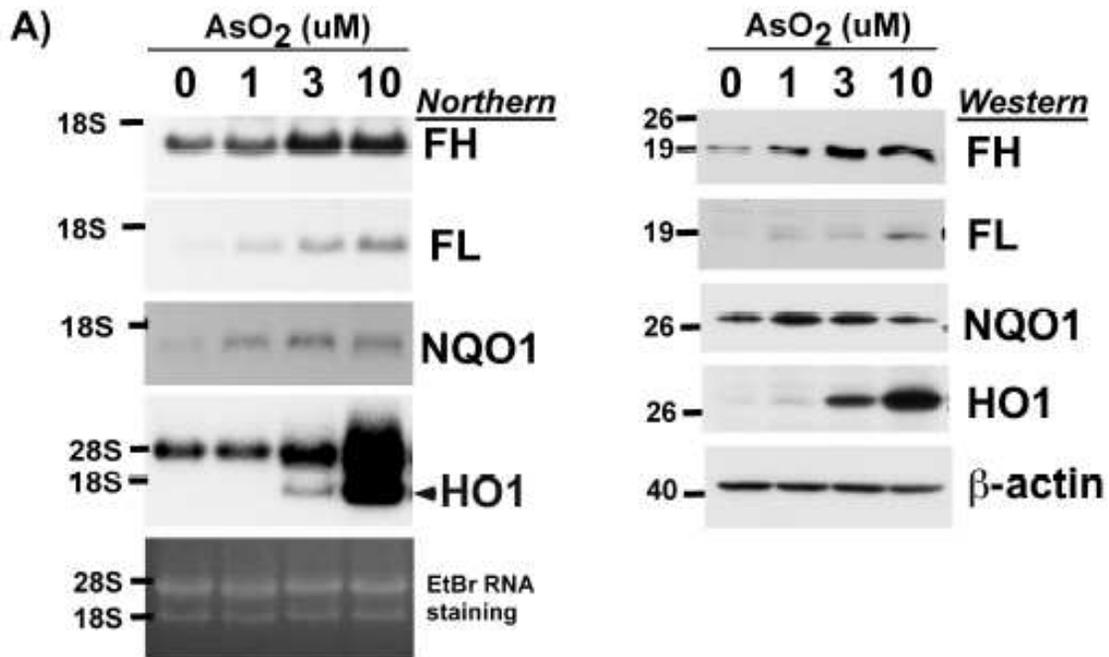
12 hr. Protein and mRNA expression of ferritin H (FH) and ferritin L (FL) were measured by Western blotting (top) or Northern blotting (middle). Knockdown of PRMT1 and CARM1 were validated in Western blotting with anti-PRMT1 or anti-CARM1 antibody. β -actin and staining RNA with ethidium bromide were used for loading control. Quantitative results of Northern blots from three independent experiments were shown in the graphics (bottom). Ferritin mRNA levels in cells transfected with SiCon (= no siPRMT1, no siCARM1) treated with 10 μ M arsenite was defined as 100%. Gray columns in cells treated with arsenic and black columns with no treatment. * P <0.05, as determined by a Student's t -test.

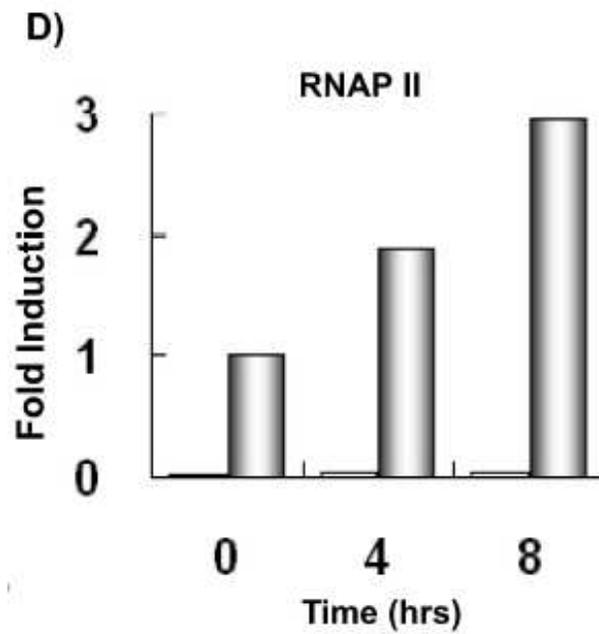
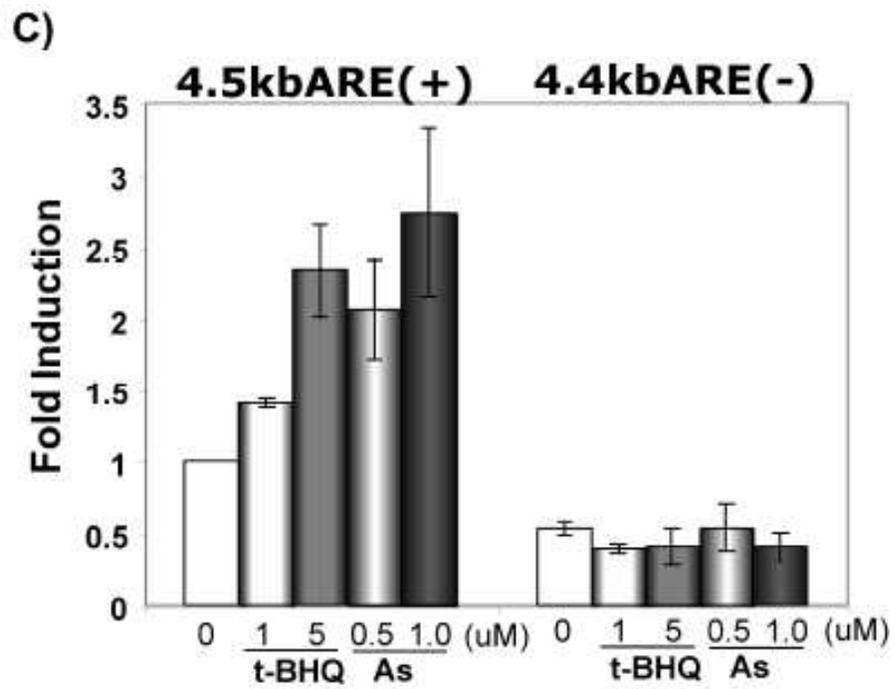
Figure 6: PRMT1 and CARM1 knockdown enhanced cellular susceptibility to arsenic toxicity

HaCaT cells transfected with non-target siRNA (no siPRMT1, no siCARM1), siPRMT1, or siCARM1 were treated with 10 μ M sodium arsenite for 12 hr and whole cell lysates were isolated and analyzed by Western blotting with anti-Caspase 3 antibody. Knockdown of PRMT1 and CARM1 were verified by Western blots with anti-PRMT1 and anti-CARM1 antibodies. β -actin as a loading control

FIGURES

Fig. 1 Arsenic induced transcription of the human ferritin H and L genes





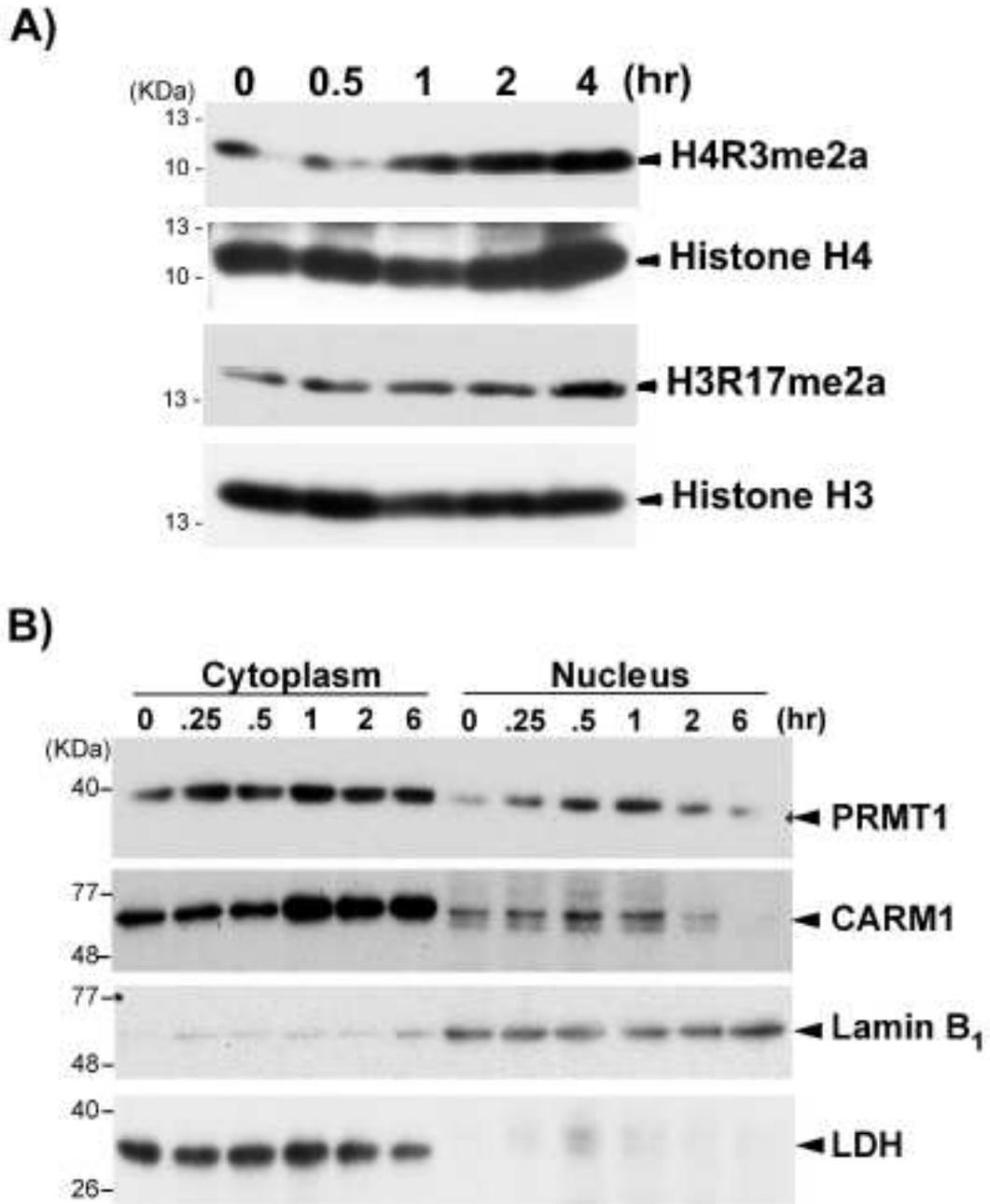


Fig. 2 Arsenic induces global histone methylation along with nuclear accumulation of PRMT1 and CARM1.

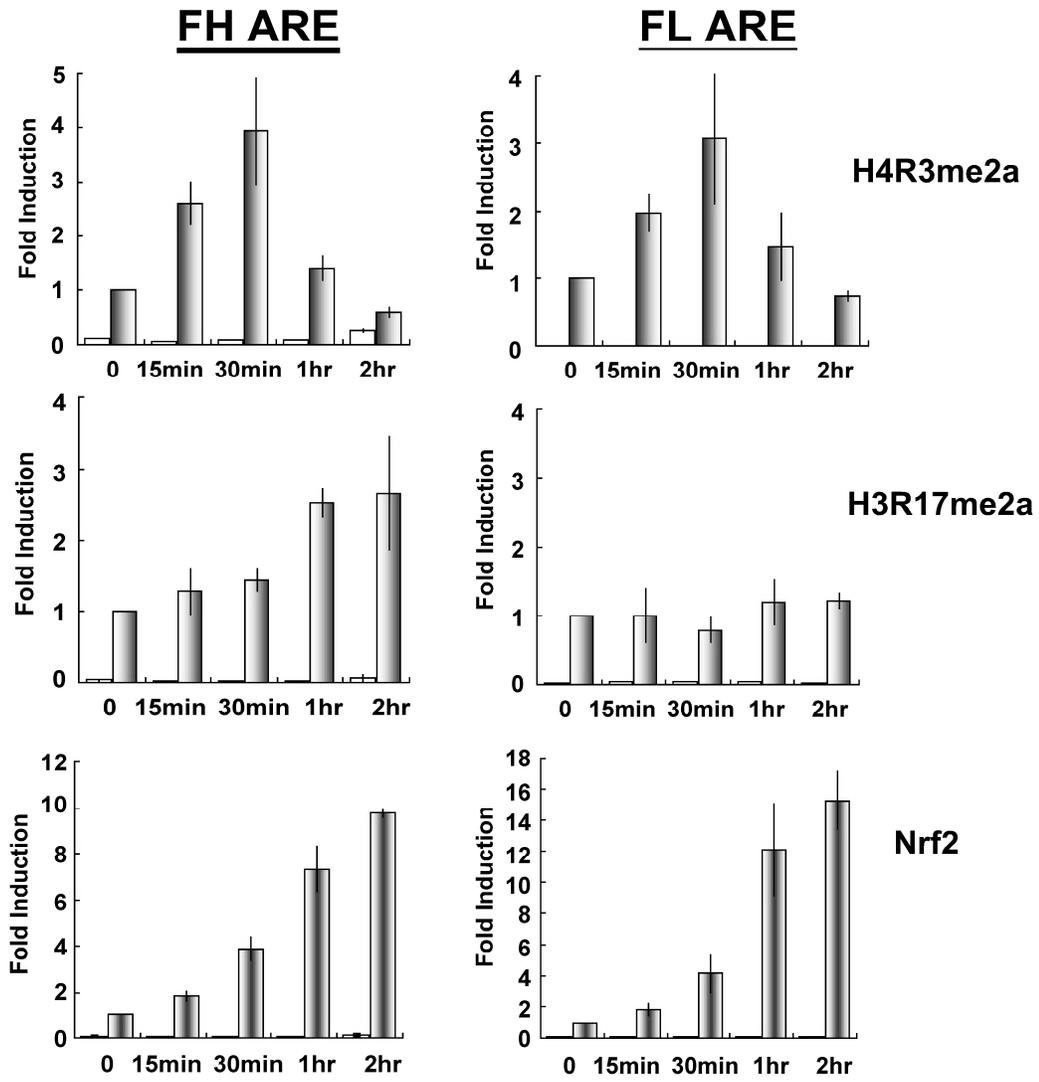


Fig. 3 Methylation of histone H4R3 and H3R17 precedes or occurs concomitantly with Nrf2 recruitment to the ferritin AREs following arsenic treatment.

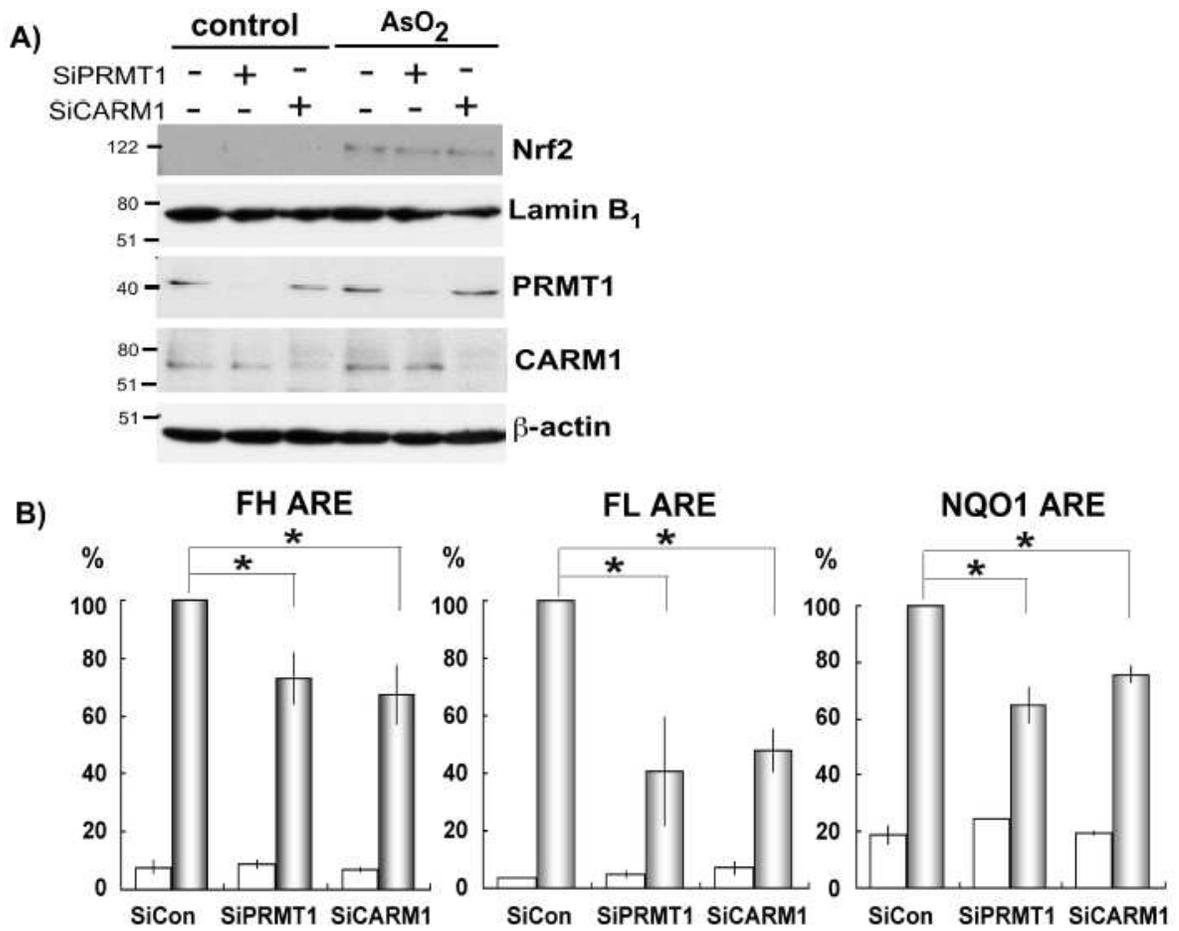


Fig. 4 Knocking down PRMT1 or CARM1 does not affect Nrf2 nuclear accumulation but inhibits Nrf2 binding to the ferritin and NQO1 AREs.

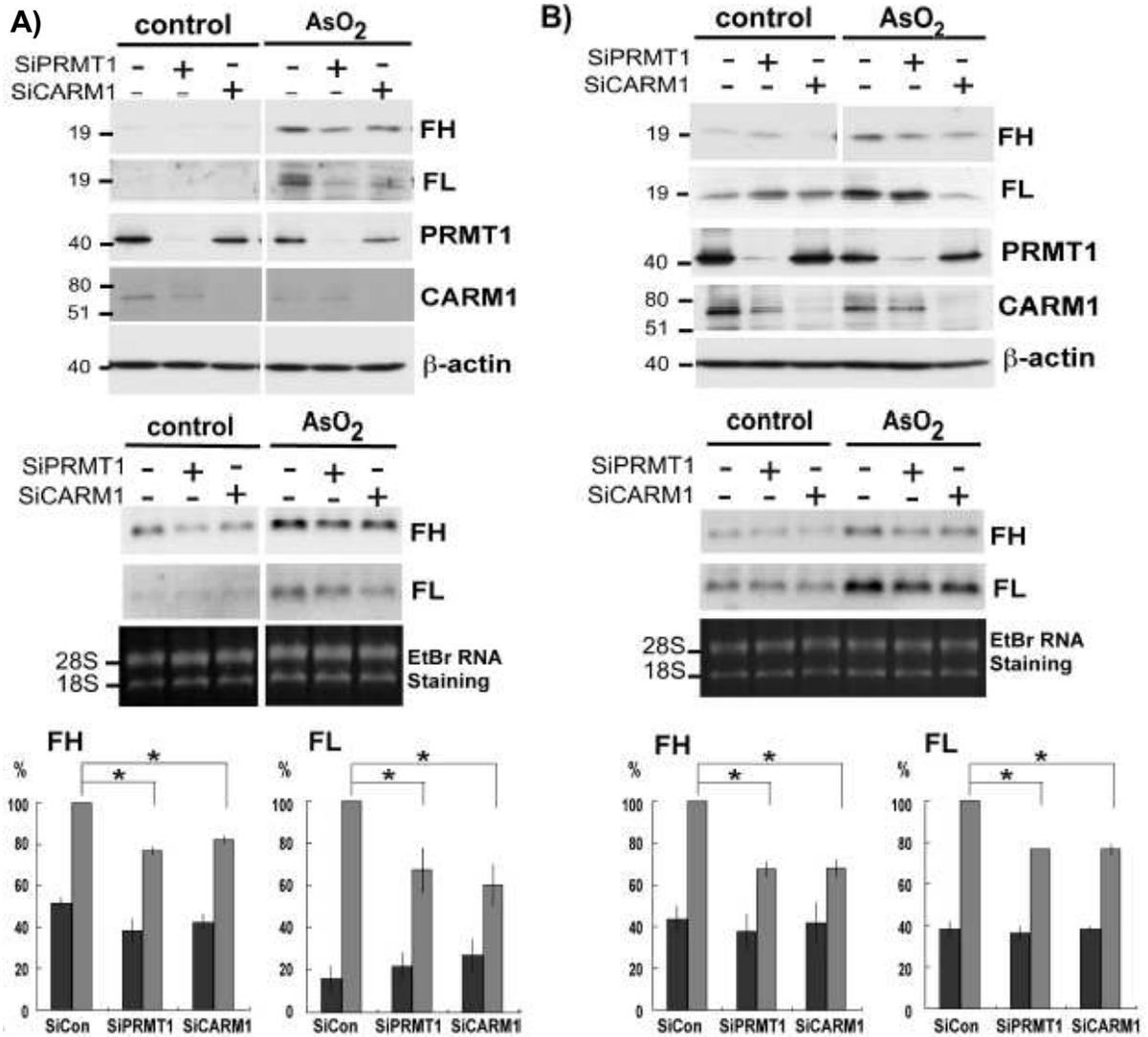


Fig. 5 Knocking down PRMT1 or CARM1 inhibits arsenic-induced ferritin expression.

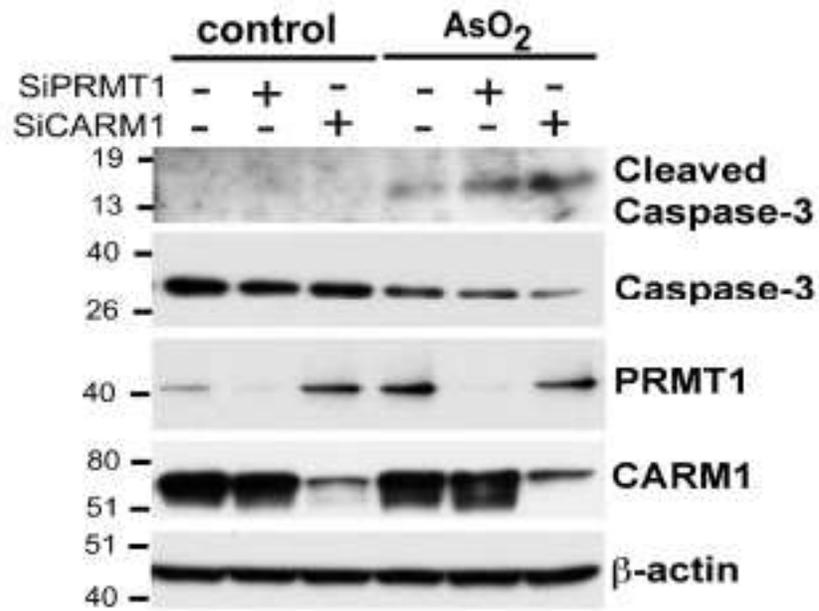


Fig. 6 PRMT1 and CARM1 knockdown enhanced cellular susceptibility to arsenic toxicity

SUPPLEMENTAL FIGURE LEGENDS

Supplemental Figure 1: Arsenic induces expression of the antioxidant genes in Jurkat T cells.

Total RNA and whole cell lysate (WCL) were isolated from Jurkat cells treated with 0, 0.3, 1, 3 or 10 μ M sodium arsenite for 24 hr. RNA was subjected to Northern blotting (left panels) and hybridized with 32 P-labeled human ferritin H, ferritin L or NQO1 cDNA probe. An equal amount of RNA loading was confirmed by staining RNA with ethidium bromide. WCL was subjected to Western blotting (right panels) using anti-ferritin H, NQO1, or β -actin antibody.

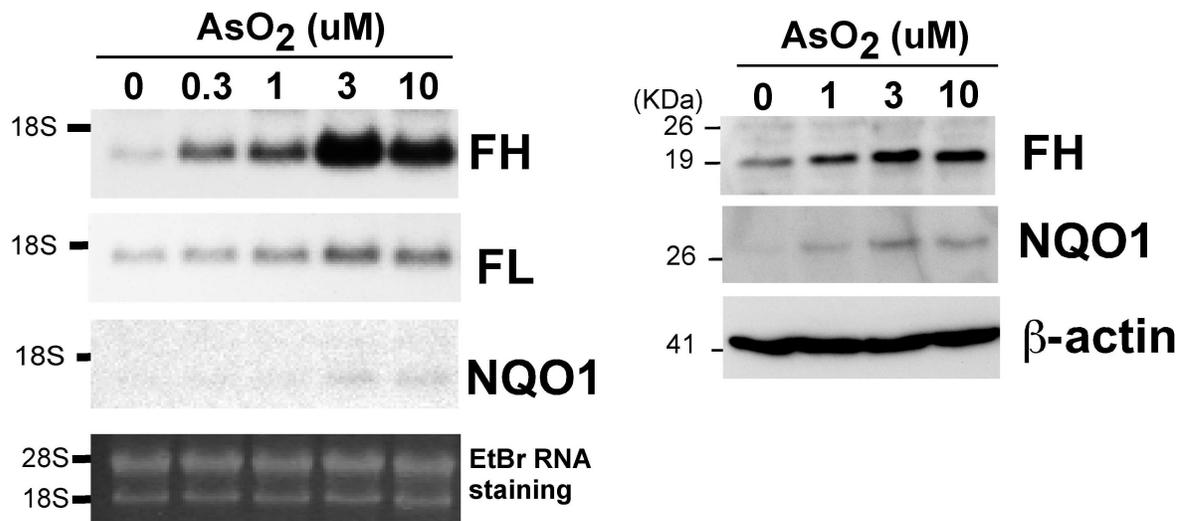
Supplemental Figure 2: Adenosine dialdehyde blocks arsenic-induced ferritin H gene expression.

Total RNA was isolated from HaCaT cells pretreated with a methyltransferase inhibitor, adenosine dialdehyde (AdOx), for 1 hr followed by 10 μ M sodium arsenite treatment for 14 hr. RNA was subjected to Northern blotting and hybridized with 32 P-labeled human ferritin H probe. An equal amount of RNA loading was confirmed by staining RNA with ethidium bromide.

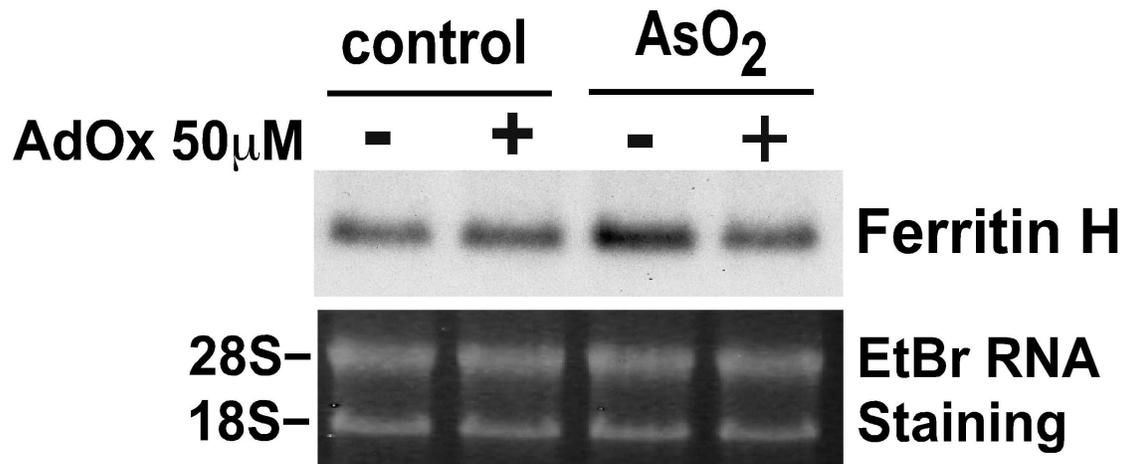
Supplemental Figure 3: Knocking down PRMT1 or CARM1 does not inhibit Nrf2 binding to the HO1 ARE.

Non-target siRNA (siCon), siPRMT1, or siCARM1 was transfected into HaCaT cells and treated with 10 μ M sodium arsenite for 2 hr. Nrf2 binding to HO1 ARE was analyzed by ChIP assay. Binding of Nrf2 to HO1 ARE in arsenite-treated (gray columns) or untreated cells (white columns) is shown as non-target siRNA (siCon) and arsenic treated cells as 100%. Means \pm standard errors of were shown from three independent experiments.

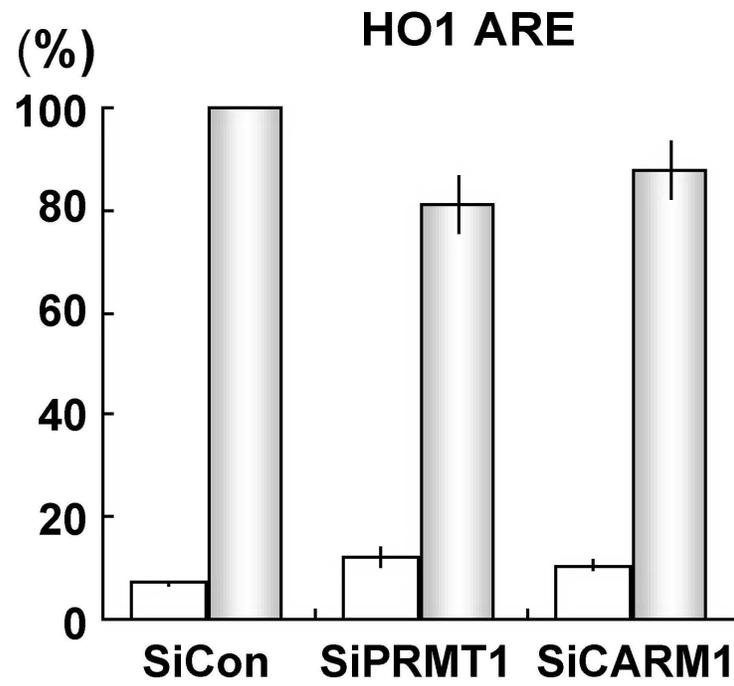
SUPPLEMENTAL FIGURES



Supplemental Fig. 1 Arsenic induces expression of the antioxidant genes in Jurkat T cells.



Supplemental Fig. 2 Adenosine dialdehyde blocks arsenic-induced ferritin H gene expression.



Supplemental Fig. 3 Knocking down PRMT1 or CARM1 does not inhibit Nrf2 binding to the HO1 ARE.

SUPPLEMENTAL TABLES

Supplemental Table I Sequence of Primers and SiRNAs

Oligo Name	Sequence 5' to 3'
FH ARE Forward	TCCAGGTCTTATGACTGCTC
FH ARE Reverse	GATGAGAGAAGAGCCAAGC
FL ARE Forward	TAGTTCTGAGGGTCCCACCA
FL ARE Reverse	GGTATCTGGGGTCCTTGTTG
NQO1 ARE Forward	CCCAGGGAAGTGTGTTGTAT
NQO1 ARE Reverse	CAAGAGAGTCCAGGGATCAA
HO1 ARE Forward	TCCTTTCCCGAGCCACGTG
HO1 ARE Reverse	TCCGGACTTTGCCCCAGG
FH TSS Forward	CCGACGCGGCTATAAGAGAC
FH TSS Reverse	GGGTTCCGTCCAAGCACT

SiRNA Name	Sequence 5' to 3'
SiControl	UAGCGACUAAACACAUCAUU
SiPRMT1	GUUCCAGUAUCUCUGAUUUAUU
SiCARM1	GGACAUGUCUGCUUAUUGCUU

**CHAPTER 2: Transcriptional Regulation of the Human Ferritin Gene by Hypoxia
Inducible Factor-1**

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ABSTRACT

Oxygen and iron are vital elements that maintain a variety of physiological functions. Ferritin is the major iron storage protein that copes with excess iron and ultimately determines intracellular iron availability. Ferritin expression is regulated by iron at the translational level through the interaction of iron-responsive element (IRE) in the 5'-untranslated region of ferritin mRNA with iron regulatory protein (IRP). Ferritin was also shown to be upregulated by hypoxia at the translational level, similar to excess iron, through decreased IRP binding to the IRE. Cobalt chloride has been used as a hypoxia mimetic that stabilizes a key transcription factor, hypoxia inducible factor-1 α (HIF1- α), and activates transcription of various genes involved in oxygen homeostasis through binding to a hypoxia responsive element (HRE). Here we observed that hypoxia and hypoxia mimetic cobalt chloride induced ferritin expression by two different mechanisms. In K562 cells under hypoxia, ferritin was upregulated at the transcriptional level through HIF1 α stabilization during an early phase of hypoxia. In contrast, cobalt chloride initially activated ferritin transcription through increased Nrf2 binding to the antioxidant responsive element (ARE) of the ferritin gene, followed by the translational block of ferritin synthesis through increased IRP binding to the ferritin IRE. We also identified a functional HRE in the human ferritin H gene. Collectively, these results suggest that ferritin is a novel HIF1 α -dependent gene during hypoxia and the hypoxia-mimetic cobalt chloride regulates ferritin in a HIF1 α -independent manner.

INTRODUCTION

All organisms from bacteria to human beings need oxygen for survival. In mammals, oxygen is gathered from the cardiovascular, hematopoietic, and respiratory systems and delivered to cells as a substrate in oxidative phosphorylation, which synthesizes ATP (1). Therefore, maintaining oxygen homeostasis is crucial; failure to tightly regulate oxygen levels results in insufficient generation of ATP and the production of harmful reactive oxygen species (ROS), which damage proteins, lipids, and DNA (2). Therefore, cells employ various mechanisms to maintain oxygen homeostasis in which hypoxia-inducible factors (HIFs) serve as the master regulators of oxygen homeostasis. HIFs sense oxygen change and mediate developmental and physiological pathways that either deliver O₂ to cells or allow cells to survive O₂ deprivation (3). The HIF family contains 3 members, HIF-1, 2, and 3; each HIF member consists of an α - and β -subunit (4). The β -subunit is constitutively expressed but the α -subunit is regulated in an oxygen-dependent manner (5). In normoxia, the α -subunit is degraded through a degradation system that recognizes hydroxyl prolines located in the oxygen-dependent degradation domain (ODD); under hypoxic conditions, prolyl hydroxylation is inhibited and this allows the α -subunit to dimerize with the β -subunit through a basic helix-loop-helix (bHLH) and regulate HIF-targeted genes through binding to the hypoxia-responsive element (HRE) (3). Not restricted to the pathways involved in regulation of oxygen homeostasis, HIFs are multifaceted transcription factors can mediate a diverse range of physiological regulations such as proliferation, differentiation, cell survival, angiogenesis, energy metabolism, and iron homeostasis (6).

In iron homeostasis regulation, the major iron storage protein ferritin plays a crucial role as an iron reservoir to store excess labile iron and release it later when needed. Ferritin is a cage-like protein composed of 24 subunits of heavy (H) and light (L) chains, in which ferritin H harbors ferroxidase activity to transfer Fe²⁺ to Fe³⁺ for storage, while ferritin L supports the overall ferritin structure (7). Ferritin gene expression is regulated by both translational and transcriptional mechanisms. Translational regulation is through interaction between iron regulatory proteins (IRPs) and the 5' ferritin iron-responsive element (IRE), by which blocks

recruitment of the translational machinery and repress ferritin translation (8). Translational control of ferritin expression is well studied as compared to transcriptional regulation (7). NF- κ B was shown to regulate ferritin transcription after TNF- α treatment (9), and an antioxidant-responsive element (ARE) has been identified 4.4 to 4.5kb upstream of the ferritin H transcription start site (10). The ferritin H ARE is an important enhancer element regulated by transcription factors such as c-Jun, JunD, ATF1, and Nrf2 in response to different types of stressors (10-13); however, the molecular mechanisms of transcriptional regulation of the ferritin gene is still not completely understood.

Previous research has demonstrated that hypoxia can induce ferritin expression mainly through translational regulation; in human oligodendrogloma cells, hypoxia induces the synthesis of ferritin and the translation inhibitor cycloheximide blocks ferritin synthesis, but not transcription inhibitors such as actinomycin D, suggesting that hypoxia induces ferritin protein expression by translational regulation (14). In mouse peritoneal macrophages, the ferritin protein expression was increased by diminishing the IRE binding activity of IRP-1 during hypoxia (15). However, whether hypoxia induces ferritin through transcriptional regulation or how it regulates ferritin transcription has not been studied yet. Here we show that ferritin H may be a novel HIF-1-targeted gene during hypoxia. In our research, we found a putative HRE sequence located in 4.3kb upstream of the ferritin H transcription start site. The hypoxia mimetic cobalt chloride transcriptionally upregulated ferritin gene expression by activating the ARE, but not the HRE. Nuclear translocation of the major ARE transcription factor Nrf2 was induced after cobalt chloride treatment. Surprisingly, IRP-mediated translational repression decreased ferritin H protein expression after cobalt chloride treatment, counteracting ferritin H transcriptional upregulation. Protein expression of other non IRE-regulating antioxidant genes, NQO1, thioredoxin, and glutathione-S-transferase- π were upregulated. On the other hand, hypoxia induced ferritin H gene expression in both K562 and SH-SY5Y cells and concomitantly stabilized HIF-1 α ; furthermore, expression of HIF-1 α activated the ferritin H promoter through the HRE, suggesting HIF-1 α is involved in transcription of the ferritin H gene through a HRE-dependent pathway

RESULTS

Cobalt chloride transcriptionally regulates the human ferritin H gene through the antioxidant responsive element

It has been shown that cobalt chloride can serve as a hypoxia-mimetic by inducing HIF-1 binding activity similarly to hypoxia (16), producing hypoxia-like responses in cells (17). We used cobalt chloride to mimic hypoxia to test whether it induces ferritin expression. K562 cells were treated with 20-200 μ M cobalt chloride for 20 hours; mRNA and protein expression of ferritin H were measured and HIF-1 α was used to validate our model of cobalt-mimicked hypoxia. We observed that ferritin H mRNA was induced in a dose-dependent manner by cobalt chloride treatment (Fig. 1A), and HIF-1 α accumulated in the nucleus (Fig. 1A), suggesting that cobalt chloride successfully produces a hypoxia-like response and upregulates ferritin gene expression. Since HIF-1 usually regulates gene transcription through binding to HRE, we searched the ferritin H promoter and found a putative hypoxia responsive element (HRE) located 4.3 kb upstream of the 5' promoter region of the ferritin H gene. To address whether cobalt chloride induces ferritin H mRNA expression by transcriptional regulation and if the putative HRE is a functional HRE during cobalt chloride treatment, K562 cells were transiently transfected with luciferase reporters constructed with different lengths of the ferritin H promoter, containing the ARE and/or putative HRE (4.5kb ARE(+)/HRE(+), 4.4kb ARE(-)/HRE(+), 4.0kb ARE(-)/HRE(-), and TATA box), followed with cobalt chloride treatment (10, 100 and 250 μ M) for luciferase assays. Cobalt chloride dose-dependently induces luciferase expression driven by the 4.5kb ARE(+)/HRE(+) but not 4.4kb ARE(-)/HRE(+), 4.0kb ARE(-)/HRE(-) or TATA box (Fig. 1B), suggesting that cobalt chloride induces transcription of the ferritin H gene mainly through the ferritin H ARE. To confirm whether cobalt chloride regulates ferritin H transcription through the ARE, we tested if Nrf2, the major transcription factor of AREs, is activated by cobalt chloride. In figure 1C, western blotting showed that Nrf2 accumulates in the nucleus after cobalt chloride treatment, suggesting that Nrf2 is activated and accumulates in the nucleus, and is involved in ferritin H ARE activation.

Cobalt chloride specifically represses ferritin H protein expression through IRP-mediated translational repression

To address if cobalt chloride induces other antioxidant genes or only specifically on ferritin H, K562 cells were treated with cobalt chloride (20, 100 and 200 μM) and protein expression of the antioxidant genes ferritin H, NQO1, thioredoxin, and glutathione-S-transferase- π , were measured. Western blotting showed that cobalt chloride dose-dependently upregulates protein expression of NQO1 and thioredoxin at 12 and 24 hours; glutathione-S-transferase- π in 12 hours, but surprisingly cobalt chloride downregulated the expression level of ferritin H in opposition to other antioxidant genes (Fig. 2A). Indeed, we observed that cobalt chloride induced ferritin transcription in figure 1A; however, ferritin gene expression can be also mediated through IRP-dependent translational regulation. To assess whether cobalt chloride increases IRP-IRE interaction resulting in translational repression, K562 cells were transfected with a luciferase reporter containing the 5'UTR of the ferritin H gene, which contains the IRE of mRNA, then treated with cobalt chloride (20, 100 and 250 μM) and harvested for luciferase assays. Cobalt chloride repressed ferritin H IRE-driven luciferase expression in a dose dependent manner similar to the iron chelator deferoxamine (DFO). (Fig. 2B), suggesting that cobalt chloride induces IRP-IRE interaction, resulting in translational repression of the ferritin H gene and thus counteracting transcriptional activation.

Hypoxia induces ferritin H mRNA expression through a HIF-1/HRE-dependent pathway

Since cobalt chloride may mediate ferritin H gene transcription through a HRE-independent pathway, to address whether hypoxia may regulate ferritin H transcription through the HRE, the human neuroblastoma cell line, SH-SY5Y, and the human erythroleukemia cell line, K562, were incubated under hypoxic conditions for the indicated times (0.5-12 hr) and ferritin H gene expression was measured. In SH-SY5Y cells, Northern and Western blotting revealed that ferritin H mRNA and protein were induced between 2 to 6 hours and HIF-1 α was accumulated concomitantly, and both of them gradually returned to basal levels at 12

hours in respectively (Fig. 3A). Similar results were observed in K562 cells, in which the protein level of ferritin H was upregulated in a time dependent manner, increasing from 2 to 8 hours, and mRNA level was slightly induced starting at 30 minutes and increasing with maximum induction at 2 hours (Fig. 3B). These results suggest that hypoxia may activate HIF-1, resulting in ferritin H transcription. To further confirm the role of HIF-1 in ferritin H transcription, K562 cells were cotransfected with HIF-1 α expression vector and luciferase reporters containing different ferritin H promoters with or without the putative HRE (4.5kb ARE(+)/HRE(+), 4.0kb ARE(-)/HRE(-), and TATA box)) were subjected to luciferase assays. Expression of HIF-1 α showed dose-dependent induction in luciferase expression driven by the 4.5kb ARE(+)/HRE(+), but marginally in 4.0kb ARE(-)/HRE(-) and no effect in TATA box (Fig. 4A), suggesting that HIF-1 can activate the ferritin H gene promoter and the ARE and/or HRE plays an important role in this regulation. To exclude the involvement of the ARE and confirm a role of the HRE in HIF-1-mediated ferritin H promoter activation, K562 cells were cotransfected with HIF-1 α expression vector and luciferase reporters containing the ferritin H promoter without ARE (4.4kb ARE(-)/HRE (+)) or with mutant ARE (4.5kb mARE/HRE(+)), in which ARE function was abolished. Luciferase assays showed that HIF-1 can activate both ferritin H promoters, which contains no ARE (4.4kb ARE(-)/HRE(+)) or mutant ARE (4.5kb mARE/HRE(+)) (Fig. 4B), suggesting that the ferritin ARE is not necessary for hypoxia-mediated ferritin H transcription. These results suggest that HIF-1 can activate the ferritin H gene promoter through the ferritin HRE but not the ARE enhancer element.

DISCUSSION

Living organisms including human beings require both oxygen and iron in a variety of processes such as oxidative phosphorylation, synthesis of enzymes and cofactors, wound healing and posttranslational modifications to maintain growth (18,19). Aberrant oxygen and iron homeostasis will diminish ATP production and also cause oxidative stress, which is involved in harming proteins, lipids, and DNA by oxidation(5). Hypoxia-inducible factors (HIFs) play important roles in the regulation of oxygen homeostasis and also in a variety of processes such as proliferation, differentiation, and iron homeostasis (6). Previous research has shown that hypoxia induces ferritin gene expression through translational regulation (14,15). Here we reported that ferritin H is a new HIF-dependent gene in response to hypoxia, and that the hypoxia mimetic, cobalt chloride, regulates ferritin H gene expression through a combination of transcriptional and translational regulation.

Originally we attempted to use cobalt chloride to mimic hypoxic conditions to answer if ferritin H transcription is induced by hypoxia. Indeed, we observed upregulation of ferritin H mRNA and HIF-1 α accumulation after cobalt chloride treatment (Fig. 1A), suggesting cobalt chloride successfully induces “chemical hypoxia” and transcription of the ferritin H gene; however, the results of luciferase assays demonstrated that deletion of the ferritin H ARE (ARE(-)/HRE(+)) abolished activation of the ferritin H promoter induced by cobalt chloride treatment (Fig. 1B), suggesting that cobalt chloride-mediated ferritin H transcription is mainly regulated by the ARE but not the HRE; also, the major transcription factor of the ARE, Nrf2, accumulated in nucleus after cobalt chloride treatment (Fig. 1C), suggesting Nrf2 is involved in the activation of ferritin H ARE after cobalt chloride treatment. However, we can not exclude the possibility that both the HRE and ARE are involved in cobalt chloride-mediated transcription; to further study the role of ARE and HRE in cobalt chloride mediated transcription, a luciferase reporter containing the ferritin H promoter with a mutant HRE (ARE(+)/mHRE) region should be constructed to address if the HRE is important for cobalt chloride-mediated ferritin H transcription. In addition, knockdown of Nrf2 or HIF-1 α , followed by cobalt chloride treatment can answer which transcription factor(s) is involved in

the cobalt chloride-mediated pathway. Furthermore, ChIP assays employing anti-Nrf2 and anti-HIF-1 α antibodies and RT-PCR with primers flanking the ferritin H ARE and HRE can be utilized to answer if these two transcription factors bind to the ARE or HRE and regulate ferritin H transcription in response to cobalt chloride treatment.

To confirm the role of ARE in cobalt chloride-mediated regulation, we analyzed gene expression of other antioxidant genes such as NQO1, thioredoxin, and glutathione-S-transferase- π along with ferritin H and found that protein expression of NQO1, thioredoxin, and glutathione-S-transferase were upregulated by cobalt chloride treatment while ferritin H was repressed in a cobalt chloride dose-dependent manner (Fig. 2A). This is paradoxical, considering our observation that cobalt chloride induced ferritin H transcription (Fig. 1). Since ferritin H is also regulated by IRP-mediated translational regulation, we asked if cobalt chloride differentially regulates transcription and translation of the ferritin H gene. Luciferase assays supported this idea and showed that cobalt chloride repressed ferritin H IRE-driven luciferase activity (Fig. 2B), suggesting that cobalt chloride increases IRP-IRE interaction and results in translational repression of ferritin H protein expression. To directly demonstrate this, IRP-IRE binding assays should be conducted to demonstrate that cobalt chloride increases IRP-IRE interaction and represses ferritin H protein expression. Knockdown of IRP-1 or IRP-2 followed with cobalt chloride treatment will help to clarify which particular IRP protein is involved in the translational repression of ferritin H protein expression.

We demonstrated that hypoxia induces ferritin H gene expression, upregulating both ferritin H mRNA and protein expression, and concomitantly stabilizes HIF-1 α in SH-SY5Y and K562 cells (Fig. 3). Furthermore, luciferase assay results showed that expression of HIF-1 α activates the ferritin H promoter via an ARE and/or HRE- dependent pathway (Fig. 4A), but ARE deletion constructs had no effect on activation, excluding the role of ARE in HIF-1 α -mediated ferritin H promoter activation (Fig. 4B). These results suggest that hypoxia may stabilize HIF-1 α , and induce ferritin H transcription through a HRE-dependent pathway. The role of HIF-1 in hypoxia-mediated ferritin H transcription needs to be further clarified by knocking down HIF-1 during hypoxia since expression of HIF-1 α is physiologically artificial.

Hypoxia is a complicated physiological stressor and has been shown to be involved in the activation of a large battery of transcription factors such as NF- κ B, AP-1, and ATF1 (20-22) as well as HIF-1 stabilization. AP-1 and ATF-1 have been shown to regulate the ferritin H ARE but the role of ferritin H ARE in hypoxia is still elusive and it is unknown whether Nrf2 is involved in hypoxia-mediated pathway. CHIP assays using anti -HIF-1 α , -Nrf2, and -ATF1 antibodies with primers flanking the ferritin H ARE and HRE can be performed to delineate the network of these transcription factors in ARE and HRE activation in hypoxia-mediated ferritin H transcription.

MATERIALS AND METHODS

Cell Culture, Culture condition and Chemical reagents- K562 human erythroleukemia cells (American Type Culture Collection) were cultured in RPMI1640 medium (Mediatech) supplemented with 25 mM Hepes, 0.3g/liter L-glutamine, and 10% FBS (Mediatech). SH-SY5Y human neuroblastoma cells (American Type Culture Collection) were cultured a 1:1 mixture of Eagle's minimum essential medium and F12 medium (Mediatech) supplemented with nonessential amino acids and 10% FBS. These cells were incubated at 37°C in a humidified 5% carbon dioxide atmosphere. For hypoxic stimulation, the culture plates were incubated in a modular incubator chamber (Billups-Rothenburg, Del Mar, CA) and pumped with a gas mixture (Airgas National Welders) containing 1% O₂, 5% CO₂, and 94% nitrogen b as indicated time. Cobalt chloride (CoCl₂) was purchased from Mallinckrodt Baker and dissolved in water.

Plasmids and Antibodies- The 4.5kb ARE(+)/HRE(+), 4.4kb ARE(-)/HRE(+), 4.0kb ARE(-)/HRE(-), 4.5kb mARE/HRE(+) and TATA box human ferritin H 5' upstream enhancer and promoters fused to a firefly luciferase reporter gene were described previously (10). 5'UTR sequence containing the IRE of ferritin H mRNA was described (23) and constructed into ferritin H TATA box luciferase reporter. Antibodies utilized in Western blotting were purchased from the following companies: anti-HIF-1 α (NB100-479), Novus Biologicals; anti-ferritin H (SC-25617), anti-NQO1 (sc-32793), anti-Nrf2 (SC-13032) and anti-thioredoxin (FL-105), Santa Cruz Biotechnology; anti-glutathione-S-transferase- π (610718), BD Biosciences; and anti- β -actin (A5441), Sigma-aldrich.

Cell extracts and Western blotting- Whole cell lysates (WCL) were prepared by washing cells with 1X cold PBS and lysed with cell lysis Buffer A (150mM NaCl, 10mM Na₂HPO₄, 1% Triton-X, 0.5% Deoxycholic Acid, 0.1% SDS, 0.2% Sodium Azide; pH 7.4). Nuclear extracts were prepared using a nuclear extraction kit (Active Motif) by following the manufacturer's direction. Cell lysates were electrophoretically separated on sodium dodecyl

sulfate (SDS)-polyacrylamide gels then transferred to polyvinylidene difluoride (PVDF) membranes (Thermo Fisher Scientific) and probed with primary antibodies. After overnight incubation, membranes were washed and incubated at room temperature with horseradish peroxidase conjugated secondary antibody (Calbiochem) and subsequently incubated in chemiluminescent HyGlo reagent (Denville Scientific) and visualized by exposure to x-ray film (Denville Scientific).

Northern blotting- Total RNA was isolated with TRI Reagent RT (Molecular Research Center) according to manufacturer's protocol. 5-10 μ g of total RNA was separated on 1.1% agarose gel with 5% formaldehyde in 3-(N-morpholino)-propanesulfonic (MOPS) acid buffer, followed by overnight capillary transfer to a Protran® BA85 nitrocellulose transfer membrane (Whatman). ³²P-labeled human ferritin H cDNA probe prepared with MegaPrime DNA Labeling Kit (GE Healthcare) was hybridized with the membrane at 42°C overnight and washed with buffer (0.1% SDS in 0.5X SSC buffer) at 52°C. The dry membranes were subjected to autoradiography and visualized on x-ray film (Denville Scientific). Staining RNA with ethidium bromide was used for equal RNA loading.

DNA transfection and luciferase reporter assay- K562 cells were transiently transfected by electroporation (Bio-rad Gene Pluser X-Cell) with indicated human ferritin H luciferase reporter or HIF-1 α expression plasmids. Cells were then treated with cobalt chloride for 24 hours after overnight incubation and subjected to luciferase assays using Luciferase assay system (Promega), or directly incubated for 36 hours in HIF-1 α coexpression experiment.

Statistics- All statistics were conducted with SPSS Statistics19 (IBM).

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FIGURE LEGENDS

Figure 1: Cobalt chloride regulates the human ferritin H antioxidant responsive element

Total RNA and nuclear protein extract were isolated from K562 cells treated with 0, 20, 100 or 200 μM of cobalt chloride for 24 hours. A) RNA was subjected to Northern blotting (top panels) and hybridized with ^{32}P -labeled human ferritin H probe. Equal amount of RNA loading was confirmed by staining RNA with ethidium bromide. Nuclear extracts were subjected to Western blotting (bottom panels) using anti-HIF-1 α antibody. β -actin blots were used as loading controls. B) K562 cells were transfected with luciferase reporter plasmids containing 4.5kb ARE(+)/ HRE(+), 4.4kb ARE(-)/ HRE(+), 4.0kb ARE(-)/ HRE(-), or TATA box 5'- promoter region of the human ferritin H gene, and treated with cobalt chloride (0, 10, 100, or 250 μM) for 24 hr. The firefly luciferase activity from untreated cells transfected with 4.5kb ARE(+)/ HRE(+) 5'- promoter reporter was defined as 1. Mean \pm standard errors were shown from 3 independent experiments. One-way ANOVA test indicated significance in 4.5kb ARE(+)/ HRE(+) (p-value < 0.001); * indicated p-value < 0.001 in post-hoc test when compared with no cobalt chloride treatment. C) Nuclear protein extract isolated from K562 cells treated with 0, 20, 100 or 200 μM of cobalt chloride for 24 hours was subjected to Western blotting using anti-Nrf2 antibody. β -actin blots were used as loading control.

Figure 2: Protein expression of ferritin H is inhibited by IRP-mediated translational repression.

A) K562 cells were treated with 0, 20, 100, or 250 μM of cobalt chloride for 12 or 24 hours and whole cell lysates were analyzed for ferritin H, NQO1, thioredoxin and glutathione-S-transferase- π by Western blotting. β -actin Western blot was used for loading control. B) K562 cells were transfected with luciferase reporter driven by the 5'UTR of ferritin H gene containing the IRE, treated with 20, 100, or 250 μM of cobalt chloride or deferoxamine (DFO) for 24 hr. The firefly luciferase activity from untreated cells was defined as 100%.

Mean \pm standard errors were shown from 3 independent experiments. One-way ANOVA test indicated significance in both of groups treated with CoCl₂ and DFO (p-value < 0.001); * indicated p-value < 0.001 in post-hoc test when compared with either no CoCl₂ or DFO treatment.

Figure 3: Hypoxia induces ferritin H gene expression and HIF-1 α accumulation concomitantly.

Total RNA, nuclear extract and whole cell lysates from A) SH-SH5Y and B) K562 cells treated with hypoxic condition for the indicated time (30 minutes to 12 hours) were isolated. RNA was subjected to Northern blotting (top panels of both A and B) and hybridized with ³²P-labeled human ferritin H probe. An equal amount of RNA loading was confirmed by staining RNA with ethidium bromide. Nuclear extracts were subjected to Western blotting (bottom panels of A) using anti-HIF-1 α antibody. Lamin B₁ blotting was shown as loading control. Whole cell lysates were analyzed with anti- ferritin H antibody (middle panels of A and bottom panels of B), and β -actin blotting shown as loading control.

Figure 4: HIF-1 activates the ferritin H promoter by regulating the HRE but not the ARE

K562 cells were cotransfected with HIF-1 α expression vector (0, 200, or 400 ng) A) plus luciferase reporters containing 4.5kb ARE(+)/HRE(+), 4.0kb ARE(-)/HRE(-), or TATA box 5'-promoter region of the human ferritin H gene for 36 hr. The firefly luciferase activity from cells transfected with 4.5kb ARE(+)/HRE(+) reporter with no HIF-1 α expression vector (0) was defined as 1; or B) cotransfected plus luciferase reporter plasmids containing 4.4kb ARE(-)/ HRE(+) and 4.5kb mARE/ HRE(+) promoter region of the human ferritin H gene. The firefly luciferase activity from cells with no HIF-1 α expression vector (0) was defined as 1 individually. Mean \pm standard errors were shown from 3 independent experiments. One-way ANOVA test indicated significance in 4.5kb ARE(+)/ HRE(+) (p-value < 0.05); * indicated p-value < 0.01 in post-hoc test when compared with no HIF-1 α expression vector.

FIGURES

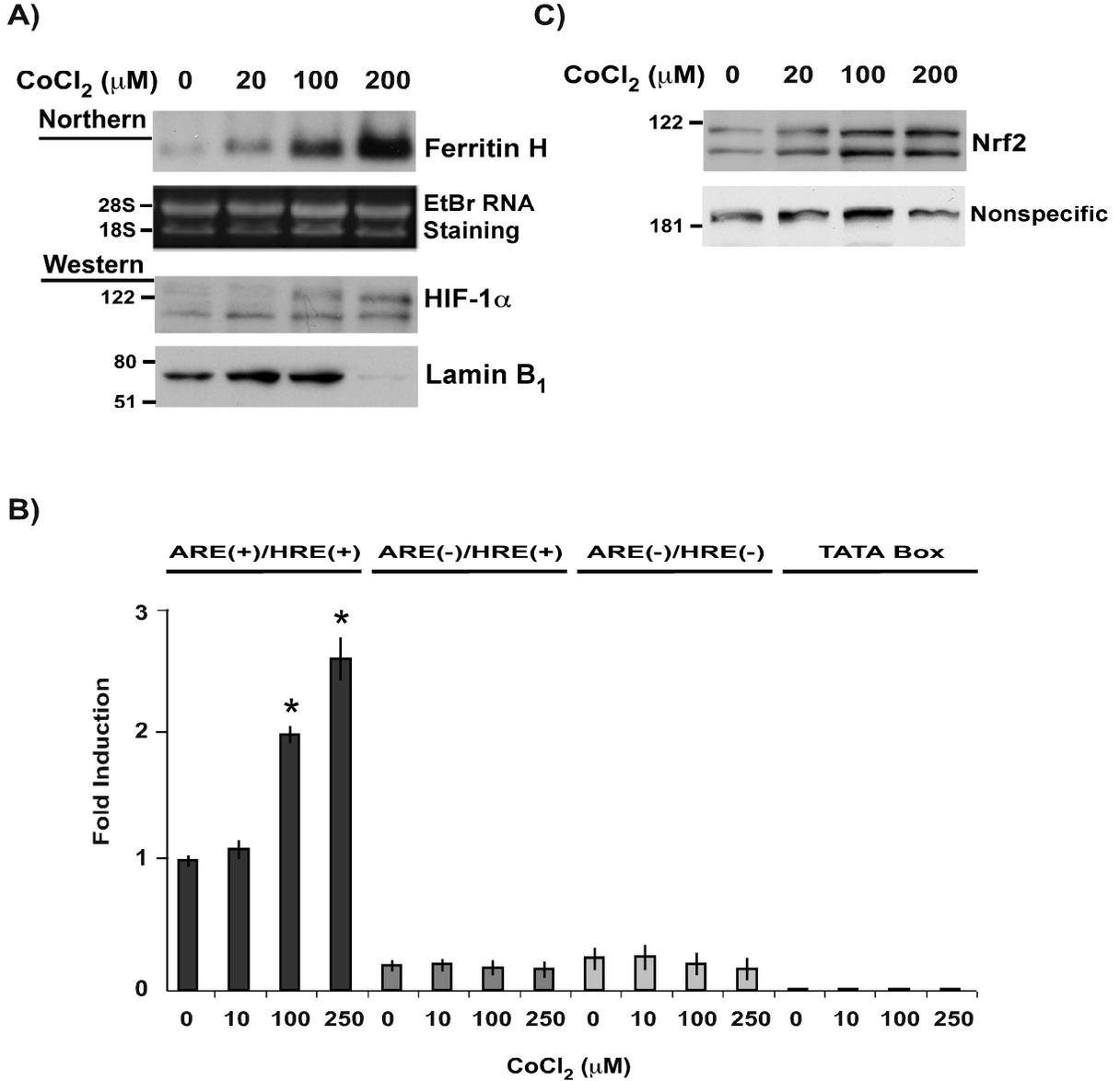


Fig. 1: Cobalt chloride regulates the human ferritin H antioxidant responsive element

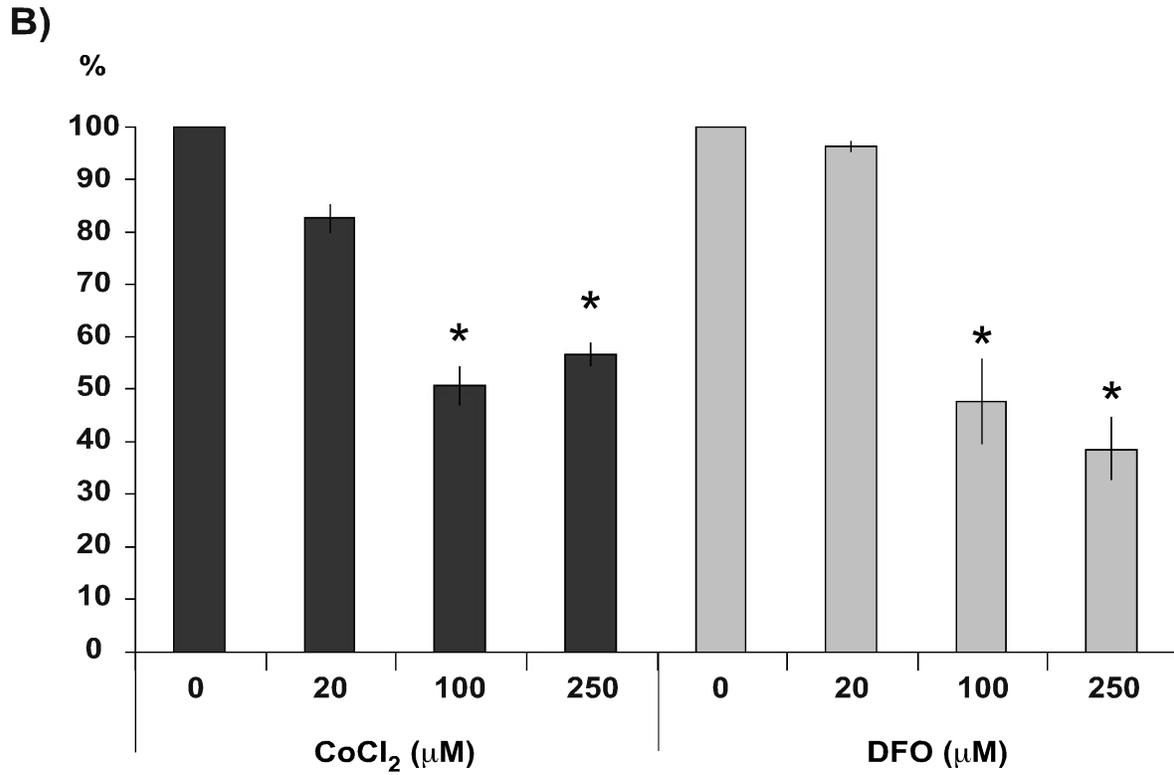
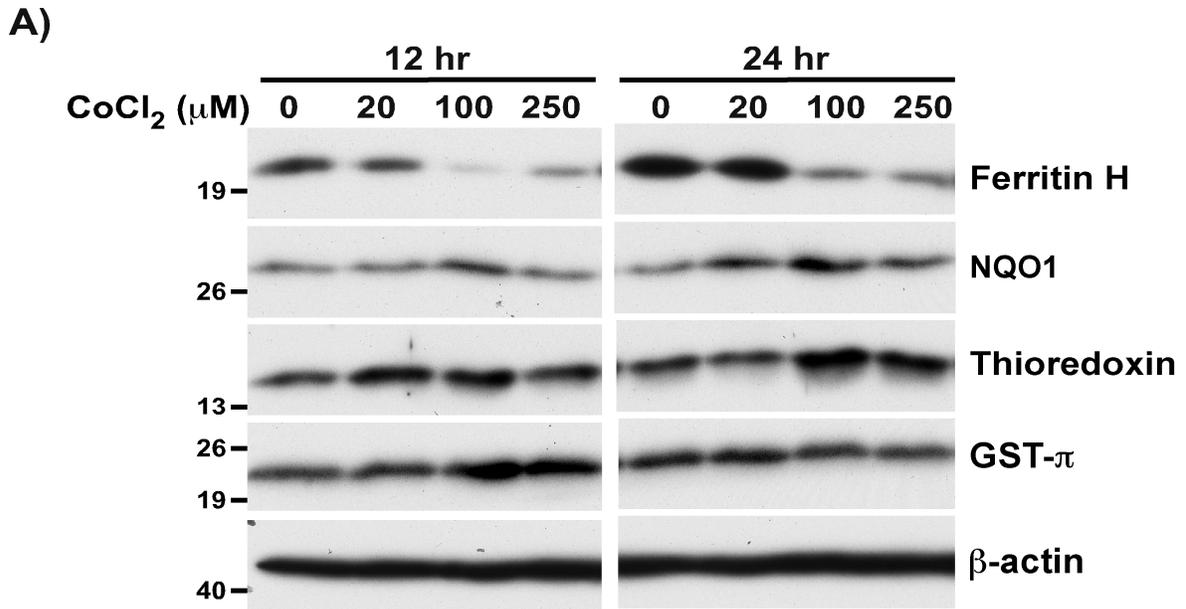
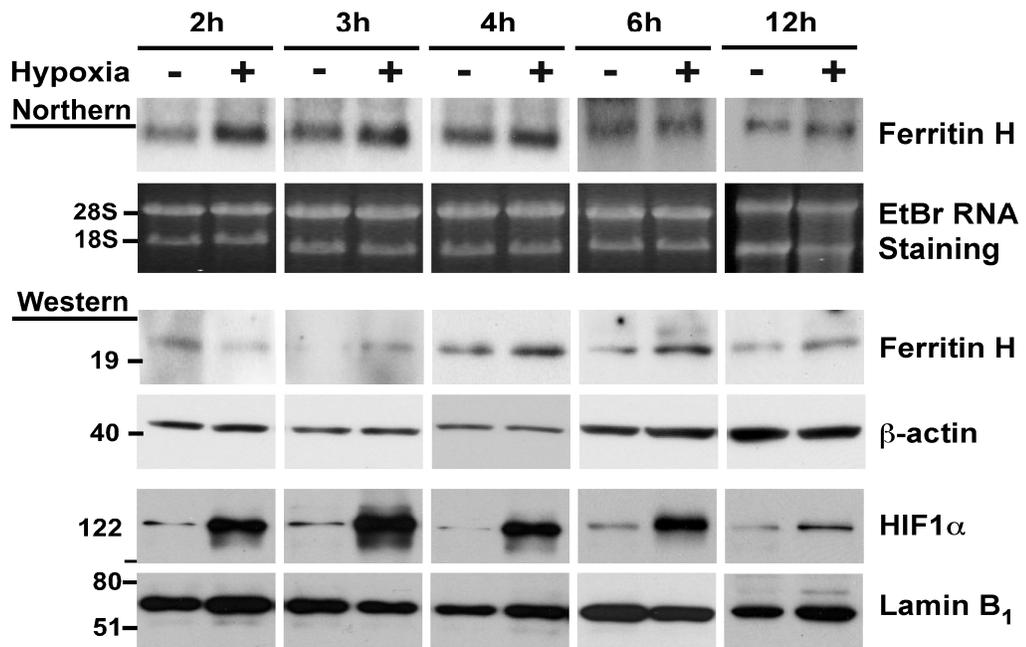


Fig. 2: Protein expression of ferritin H is inhibited by IRP-mediated translational repression.

A)



B)

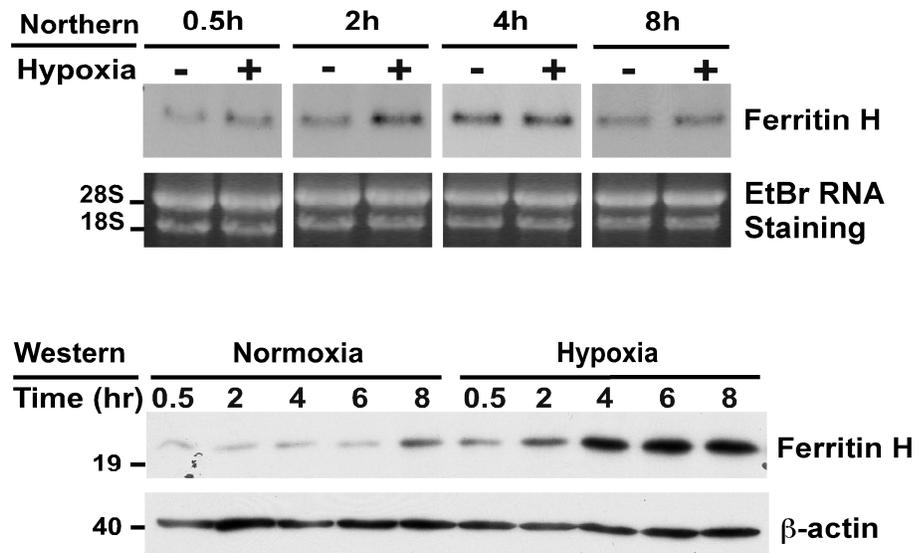


Fig. 3: Hypoxia induces ferritin H gene expression and HIF-1 α accumulation concomitantly.

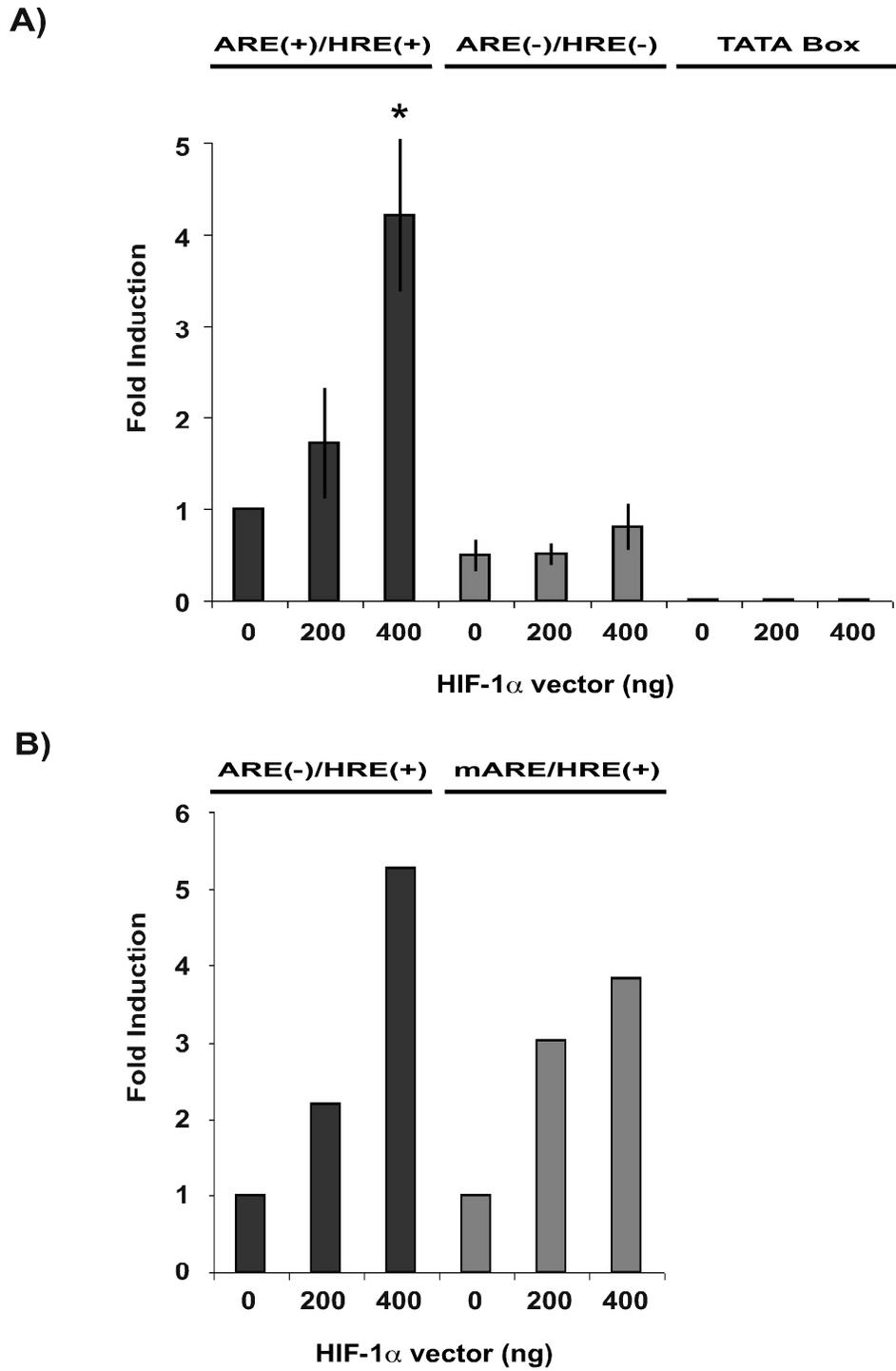


Fig. 4: HIF-1 activates the ferritin H promoter by regulating the HRE but not the ARE

GENERAL DISCUSSION

Chapter 1: Transcriptional Regulation of the Human Ferritin Gene by Protein Arginine Methyltransferases PRMT1 and CARM1

Several years ago, we started this project from the research of how histone modifications and chromatin remodeling regulates the antioxidant responsive element enhancer (ARE) activity. Very limited information was found in the research field of ARE regulation. Until now, typing key words “antioxidant responsive element” with “histone” in PubMed links only 16 publications; with “chromatin”, 40 publications are found but most of them only mention histone acetylation briefly; however, with “transcription factors”, 1493 publications are found. We thought the research will be exciting and also improve the knowledge how ARE is regulated during transcription by the dynamic chromatin structure and post-translational modifications of histones such as acetylation, phosphorylation and methylation.

Our laboratory has been interested in transcriptional regulation of the ferritin gene and found hemin-mediated ferritin H transcriptional regulation during erythroid cell differentiation (8). At the same time, we found that hemin induces several histone modifications including histone H4R3 asymmetric dimethylation (H4R3me2a), global histone H4 acetylation, histone H3K9 and K14 acetylation in the ferritin H ARE. Compared with the role of histone acetylation, which is relatively well studied and involved in gene activation with almost no doubt (50,128-131), the research of histone arginine methylations in transcriptional regulation was just attracting attention and the mechanism was still not completely understood. Therefore, we thought it will be a good field to explore. Indeed, H4R3me2a has already been shown involved in transcriptional activation (152,156,182) so its induction in the ferritin H ARE may play a role on ferritin H transcription after hemin treatment; therefore, we asked if it regulates ferritin transcription during hemin treatment. Pretreated with the PRMT family inhibitor, AMI-1, the hemin-mediated induction of ferritin H mRNA and ferritin H ARE luciferase promoter activity was attenuated in a dose dependent manner; suggesting PRMTs are involved in the transcriptional regulation of ferritin H gene.

Expression of PRMT1, the only methyltransferase found to mediate H4R3me2a, induced ARE-dependent ferritin H promoter activity, narrowing down the importance of PRMT1 in ferritin H transcription after hemin treatment. However, knockdown of PRMT1 did not clearly inhibit hemin-mediated ferritin H transcription. Meanwhile, we found previous research showed that PRMT1 can cooperate with another PRMT member, CARM1, and synergistically regulate gene transcription (121,151); and also, oxidative stress were shown to upregulate PRMTs and increase asymmetric dimethylarginine (183-185). Therefore, we wonder if PRMT1 and CARM1 are both involved in ferritin H gene transcription and a potent oxidative stressor may give us a clearer answer to whether histone arginine methylations are involved in ferritin H transcriptional regulation.

Arsenic is a potent oxidative stressor, involved in carcinogenesis (186-189) and has been shown to regulate antioxidant genes such as NQO1 and HO-1 through activating Nrf2, the major transcription factor involved in ARE-regulated gene transcription (186,190) but has not been shown to regulate the ferritin gene. We first tested if arsenic upregulate the ferritin gene transcription and if the regulation is mediated through PRMT1 and CARM1. We found arsenic upregulated transcription of the ferritin genes (Fig. 1) and knockdown of PRMT1 or CARM1 attenuated arsenic-mediated ferritin transcription showing in both of mRNA and protein expression at least in part (Fig. 5). Therefore, we would like to elucidate the molecular mechanism in which PRMT1 and CARM1 are involved in ferritin transcriptional regulation.

Since we have already confirmed that PRMT1 and CARM1 are involved in ferritin transcription after arsenic treatment, the first question we would like to ask how arsenic mediates PRMT1 and CARM1 in the pathway. We thought arsenic may activate PRMT1 and CARM1 result in 1) increasing their methyltransferase activity to induce their substrate methylation; 2) nuclear translocation of PRMT1 and CARM1 to mediate their substrates such as histones and transcription factors. Indeed, we observed the inductions of both global (Fig. 2A) and the ARE-associated (Fig. 3) histone H4R3 and H3R17 methylations after arsenic treatment, suggesting these two methyltransferase are activated and methylate their substrates, histone H3 and H4. Also, *in vitro* methylation assays by immunoprecipitation of endogenous

PRMT1 and CARM1 from HaCaT cells treated with 10 μ M arsenite showed that CARM1 is activated and able to induce methylation of its substrate, histone H3 (unpublished data); however, we did not observe activation of PRMT1 in the same assay. We further studied the molecular mechanism how arsenic activates PRMT1 and CARM1; CARM1 protein was upregulated 1 to 6 hours (Fig. 2B) after arsenic treatment, suggesting the increasing level of protein may also increase CARM1 methyltransferase activity, which is consistent with our *in vitro* methylation assay and previous research that oxidative stress upregulates PRMTs expression (183,184). Also, arsenite started to induce nuclear accumulation of PRMT1 and CARM1 in 15-30 minutes (Fig.2B), which precedes or occurs concomitantly with the methylation of ferritin ARE H4R3 (starting from 15 minutes) and H3R17 (starting in about 30-1hr), suggesting arsenite-induced nuclear accumulation of PRMT1 and CARM1 may result in methylation of histone H4R3 and H3R17 in the ferritin H ARE. The above results implied that arsenite may upregulate protein expression level and mediate nuclear accumulation of PRMT1 and CARM1, activate them and result in downstream histone arginine methylations.

Previous research showed that distribution of PRMT1 is dynamic and can shuttle between the cytosol and nucleus. Inhibition of methylation by general methyltransferase inhibitor, adenosine dialdehyde (AdOx) induces nuclear translocation of PRMT1, and let PRMT1 bind its unmethylated substrates such as core histones until the methylation reaction is complete again, suggesting nuclear translocation of PRMT1 may be critical for PRMT1 to access and methylate its nuclear substrates including histones (191). Also, cellular distribution of PRMT1 and CARM1 is cell type specific; for example, PRMT1 and CARM1 are predominantly cytoplasmic in HEK293 and UOS2, but nuclear in HeLa and MCF7 cell lines (192). Pregnane X-receptor (PXR) was shown to interact with PRMT1 and their interaction direct PRMT1 nuclear localization (147). In addition, phosphorylation of CARM1 was shown to cause CARM1 cytoplasmic localization (171). These reports suggest distribution of PRMT1 and CARM1 may be varied in different types of cells and tissues; furthermore, protein-protein interaction and post-translational modifications of PRMT1 and CARM1 may effect their localization. Further investigation will be necessary to better

understand the molecular mechanism of arsenic-mediated nuclear accumulation of PRMT1 or CARM1.

Even though the evidence of the importance of the chromatin environment in gene transcription has been accumulated, how Nrf2 and histone modifications in ARE regulate each other still remain unexplored. Similar timing of the induction of H4R3 and H3R17 methylation and Nrf2 binding to the ferritin ARE (Fig. 3) let us ask if they regulate each other during arsenic treatment. The methylation of histone H4R3 or H3R17 by PRMT1 or CARM1 probably enables Nrf2 to bind to the ARE and activate transcription of the ferritin gene. This idea is supported by the results that knockdown of PRMT1 or CARM1 did not affect Nrf2 nuclear accumulation induced by arsenic but blocked the binding of Nrf2 to the ferritin H and L AREs (Fig. 4). Previous research showed that H4R3 methylation provides binding surface for PCAF binding to chromatin, supporting the idea that histone arginine H4R3 and H3R17 methylations may facilitate Nrf2 binding after arsenic treatment. To further research the possibility, peptide binding assay by using synthetic methyl- or non-methyl histone H3 and H4 peptides to study the affinity of Nrf2 with these peptides should be performed to address the question.

However, another possibility cannot be ruled out: PRMT1- or CARM1-mediated arginine methylation of other non-histone proteins such as Nrf2, p300/CBP and other coactivator which may regulate Nrf2 binding activity. It has been shown that PRMT1 can methylate and regulate the activity of other transcription factors such as STAT1 (signal transducers and activators of transcription), FOXO1 and PGC1 α (peroxisome proliferator-activated receptor γ coactivator 1 α) (150,193,194). The idea that PRMT1 and CARM1 may methylate Nrf2 directly and increase its binding activity can be supported by the histone acetyltransferase p300/CBP, which was shown to acetylate Nrf2 and increase its binding activity to the NQO1 ARE (195). Also, CARM1 has been showed to methylate p300/CBP and regulate its activity (167-169); therefore, it is also possible that PRMT1 and CARM1 indirectly regulate Nrf2 binding activity through p300/CBP. Besides Nrf2, several transcription factors including small Maf proteins (MafF, MafK, MafG), Bach1, and AP1 family members (196) and transcriptional coactivators such as p300/CBP (59,73,197), MOZ

(198), and BRG1 (199) are involved in ARE regulation. Therefore, these ARE-interacting transcription factors and coactivators might also be regulated by PRMT1 or CARM1 in response to arsenic exposure.

Ferritin and other antioxidant genes were demonstrated to be cytoprotective against oxidative stress (1). Arsenic-mediated oxidative stress has been shown to induce caspase-3-dependent apoptosis (200). Knockdown of PRMT1 or CARM1 enhanced arsenic-mediated caspase-3 cleavage (Fig. 6), suggesting the cytoprotective role of PRMT1 and CARM1 probably through regulating antioxidant genes. However, it is also possible that PRMT1 and CARM1 are involved in the regulation of apoptosis directly without mediating antioxidant genes.

PRMT1 and CARM1 have been shown to be either apoptotic or anti-apoptotic by methylating their substrates. For example, knockdown of CARM1 can induce apoptosis in prostate cancer cells (201) and PRMT1 negatively regulates apoptosis signal-regulating kinase 1 (ASK1) by methylation two arginine residue-78 and 80 and suppresses paclitaxel-induced apoptosis in breast cancer cells (202), suggesting the anti-apoptotic role of PRMT1 and CARM1. On the other hand, PRMT1 can be also proapoptotic mainly through antagonize PI3K-Akt survival pathway based on the research of *Fukamizu's* group. First, PRMT1-mediated methylation of BCL-2 antagonist of cell death (BAD) blocks the following phosphorylation by Akt, induces BAD accumulated in mitochondrial and bound to the antiapoptotic protein, BCL-X(L), resulting in apoptosis (203). Second, methylated FOXO1 by PRMT1 inhibits FOXO1 cytosolic localization and degradation, resulting from PI3K-Akt-dependent phosphorylation and induces the downstream proapoptotic protein, Bcl-2-interacting mediator (Bim), resulting in apoptosis (150).

PRMT1 and CARM1 are known to be necessary coactivators of p53 in the regulation of its target gene, GADD45 (149), which is a stress sensor regulates cell cycle arrest, DNA repair, cell survival or apoptosis, suggesting the possible role of PRMT1 and CARM1 involved in p53-dependent apoptosis. It should be addressed that HaCaT keratinocytes have p53 point mutations in codon-179 of exon 5 and CC to TT mutations in codons-281 and -282 of exon 8 (204). However, silencing the mutated p53 can still attenuate apoptosis in HaCaT

cells in response to UV exposure, suggesting the mutant p53 in HaCaT cells is still functional in mediating apoptosis at least in part (205). Furthermore, arsenite still induces apoptosis in both of p53^{+/+} MEFs and p53^{-/-} MEFs in different pathways: in the p53^{+/+} MEFs as a p53-dependent apoptosis pathway, but in the p53^{-/-} MEFs through a p53-independent but Noxa-dependent pathway (206), arguing the possibility that there is still p53 independent apoptosis pathway if p53 in HaCaT cells completely lose its function.

Regarding histone modifications by arsenic, crosstalk between different histone modifications have been discovered and gradually understood by accumulative research. Since histone phosphorylation and their protein kinases activated by arsenic exposure were shown to be associated with gene transcription (207-210) it will be interesting to ask whether phospho histone H3 Serine (H3S10P), the major histone H3 phosphorylation, regulates transcription of antioxidant genes during arsenic exposure. My colleague, *Paul D. Ray*, recently showed that arsenic induces H3S10P at the HO-1 promoter concomitantly and upregulates HO-1 gene expression; furthermore, Nrf2 may mediate H3S10P through an oxidative stress-JNK dependent pathway (unpublished manuscript). Integrating both of our research will give us a more intact picture of how histone modifications interplay with Nrf2 in ARE-mediated transcriptional regulation after arsenic treatment. Our results suggest that after arsenic exposure, nuclear translocation of PRMT1 and CARM mediates H4R3 and H3R17 methylation, result in Nrf2 binding to the ARE; binding of Nrf2 may serve as an anchor to recruit protein kinases such as JNK and finally induce histone H3S10 phosphorylation.

Chapter 2: Transcriptional Regulation of the Human Ferritin Gene in Response to Hypoxia

We started this project while I was doing my oral preliminary examination. In the beginning of the research, we found one interesting observation that some proteins involved in iron homeostasis such as transferrin, transferrin receptor, divalent metal transporter 1 and heme oxygenase-1 harbors hypoxia responsive element (HRE) (85,115,116,211), and on the other hand, hypoxia inducible factor-2 α has 5' iron regulatory element (IRE) (212). By researching the ferritin H promoter sequence, we found that the ferritin H promoter has an HRE at 4.3kb upstream of the transcription start site. Furthermore, an iron chelator, deferoxamine (DFO), could stabilize HIF-1 α by the inhibition of PHD enzymes in SHSY5Y cells (213) and PMA-mediated ferritin expression may be involved in stabilization of HIF-1 α in THP cells (214,215), suggesting that sequestration of iron by ferritin could protect HIF-1 from degradation. These observations brought us an idea that if iron and oxygen homeostasis crosstalk to each other by the reciprocal regulation of HIF-1 and ferritin, where HIF-1 upregulates ferritin expression through binding to the ferritin HRE and ferritin upregulation in turn maintain HIF-1 stabilization.

First, we tried to use cobalt chloride to mimic hypoxic conditions to answer if ferritin H transcription is induced by hypoxia. Cobalt chloride successfully induces “chemical hypoxia” and transcription of the ferritin H gene (Fig. 1A) but mainly via the ferritin H ARE but not the HRE (Fig. 1B), possibly through Nrf2 activation (Fig. 1C). Further researching protein expression of different antioxidant genes, we found ferritin H behaved different from other antioxidant genes including NQO1, thioredoxin, glutathione-S-transferase- π , and was repressed because of IRP-IRE mediated translational repression (Fig.2). Previous research showed that low serum ferritin is associated with increasing level of cobalt concentration in women with anemia (216); (Co²⁺)transferrin reduced ferritin protein expression in HuH7 human hepatoma cells (217) and cobalt chloride can prevent IRP2 degradation (84,218) and increase IRP-1 binding resulting in repression of ferritin expression (219), suggesting that cobalt chloride may downregulate ferritin protein expression through IRP-dependent

translational repression in K562 cells. No similar research has been shown that cobalt chloride mediates both of transcriptional and translational regulation of the ferritin gene; however, in the regulation of another iron mediator protein, transferrin receptor; cobalt chloride decreased IRP-IRE interaction in the 3'UTR of transferrin receptor to destabilize transferrin receptor mRNA, but at the same time, it also induce HIF-dependent transcription, resulting in final increase of transferrin receptor mRNA in Hep3B cells (220).

We demonstrated that hypoxia induces ferritin H gene expression, upregulates both of ferritin H mRNA and protein expression; and stabilize HIF-1 α in both SH-SY5Y and K562 cells (Fig. 3). Furthermore, the luciferase assay suggested that hypoxia may stabilize HIF-1 α , and induce ferritin H transcription through an HRE-dependent pathway (Fig.4). Besides HIF-1, hypoxia has been shown to regulate a lot of transcription factors such as NF- κ B, AP-1, and ATF1 (221-223). It is possible that hypoxia regulates the ferritin H ARE through activating AP-1 and ATF-1, which are involved in regulation of the ARE (7,71). Our laboratory identified ATF1 as a ferritin H ARE repressor (71); however, ATF1 in most of situations is a transcriptional activator (224-228). We thought the possibility if ATF1 can be turned into an activator in the ferritin H ARE regulation after hypoxia treatment. Recently, ATF1 has been shown as a hypoxia-mediated transcription factor and activate gene transcription probably through a p38MAPK-dependent Ser-63 phosphorylation pathway in skeletal muscle cells (223); therefore, ATF1 is possible to be activated in response to hypoxia. Also, ATF1 can form a heterodimer with HIF-1 α (229) and has been shown to be able to recognize HRE sequence (230). These bring another possibility that both HIF-1 α and ATF1 may be activated by hypoxia and cooperate with each other in the regulation of ferritin H ARE and/or HRE.

In terms of hypoxia-mediated ferritin gene expression, IRP-mediated post-transcriptional regulation should be also taken into consideration besides transcriptional regulation. Hypoxia has already been shown to induce protein expression of ferritin through post-transcriptional regulation (82). Furthermore, protein expression of ferritin was induced by diminishing the IRE binding activity of IRP-1 during hypoxia in both mouse peritoneal macrophages and human kidney epithelial 293 cells (83,84) and also hypoxia can either downregulate (231) or upregulate (84,232) IRP-1 expression; however, protein level of IRP-1

does not necessarily represent its binding activity since it can incorporate with iron-sulfur cluster to produce aconitase; therefore, cobalt may upregulate protein expression of IRP1 but also induce IRP1 assembled with iron-sulfur cluster, eventually resulting in decrease of IRP-1 binding activity (33). Unlike IPR1, IPR2 protein level is usually consistent with its binding activity; hypoxia has been shown to induce IPR2 protein level and binding activity in different cells (84,232-235). In response to hypoxia, IRP-1 and IRP-2 seem to conflict to each other; IRP1 and IRP2 can preferentially bind different IRE structure (236,237). In addition, IRP1 can bind IRE in a more extensive manner than IRP2, including the IREs of the ferritin, transferrin receptor, and erythroid-aminolevulinate synthase mRNAs but IRP2 is limited to ferritin IREs (238). Further research will be needed to characterize how IRP-mediated post-translational regulation interplay with transcriptional regulation in ferritin gene expression responding to hypoxia.

Cobalt chloride is a well-known hypoxic-mimetic (111); however, it regulated the expression of the ferritin H gene different from hypoxia treatment in our research. We observe that cobalt chloride-mediated ferritin H transcription is involved with the ARE regulation (Fig. 1) but that hypoxia-mediated transcription with the HRE (Fig. 4). Similar observation in which cobalt chloride and hypoxia regulate mRNA induction differently has been shown in another antioxidant gene, heme oxygenase-1 (HO1), which possesses both of functional ARE and HRE regulations. In HIF activity-deficient and wild type CHO cells, cobalt chloride induces HO1 transcription through Nrf2-ARE pathway but hypoxia induces mRNA accumulation not through transcriptional regulation in wild type CHO cells (239). This result is consistent with our observation that as a hypoxia mimetic, cobalt chloride may still regulate gene expression differently from hypoxic condition.

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APPENDICES

Transcriptional regulation of ferritin and antioxidant genes by HIPK2 under genotoxic stress

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Summary

ATF1 (activating transcription factor 1), a stimulus-induced CREB family transcription factor, plays important roles in cell survival and proliferation. Phosphorylation of ATF1 at Ser63 by PKA (cAMP-dependent protein kinase) and related kinases was the only known post-translational regulatory mechanism of ATF1. Here, we found that HIPK2 (homeodomain-interacting protein kinase 2), a DNA-damage-responsive nuclear kinase, is a new ATF1 kinase that phosphorylates Ser198 but not Ser63. ATF1 phosphorylation by HIPK2 activated ATF1 transcription function in the GAL4-reporter system. ATF1 is a transcriptional repressor of ferritin H, the major intracellular iron storage gene, through an ARE (antioxidant-responsive element). HIPK2 overrode the ATF1-mediated ARE repression in a kinase-activity-dependent manner in HepG2 cells. Furthermore, DNA-damage-inducing agents doxorubicin, etoposide and sodium arsenite induced ferritin H mRNA expression in HIPK2^{+/+} MEF cells, whereas it was significantly impaired in HIPK2^{-/-} MEF cells. Induction of other ARE-regulated detoxification genes such as *NQO1* (NADPH quinone oxidoreductase 1), *GST* (glutathione S-transferase) and *HO1* (heme oxygenase 1) by genotoxic stress was also decreased in HIPK2-deficient cells. Taken together, these results suggest that HIPK2 is a new ATF1 kinase involved in the regulation of ferritin H and other antioxidant detoxification genes in genotoxic stress conditions.

Key words: HIPK2, ATF1, Phosphorylation, Ferritin, Genotoxic stress, Transcription

Introduction

ATF1 (activating transcription factor 1) belongs to the CREB transcription factor family that contains a basic-leucine zipper (b-zip) motif for DNA binding and dimerization (Rehfuess et al., 1991). ATF1 and CREB control transcription of many target genes through homo- or heterodimerization within the family or with other b-zip transcription factors on the sequences related to an AP1 or cAMP-response element (Mayr and Montminy, 2001). ATF1 plays pivotal roles in cell survival and proliferation. Increased ATF1 expression in transformed lymphocytes and metastatic melanoma cells appears to enhance the growth potential of these tumor cells (Hsueh and Lai, 1995; Jean et al., 2000). In addition, a gene fusion between Ewing's sarcoma gene *EWS* and *ATF1* by t(12;22) chromosomal translocation was found in clear-cell sarcoma (Zucman et al., 1993), and the EWS-ATF1 chimeric transcription factor plays a vital role in maintaining viability, tumorigenicity and metastatic potential of these cells (Bosilevac et al., 1999; Jean et al., 2000). Stimulus-coupled activation of ATF1 is induced by growth factors as well as stress-inducing agents, in which ATF1 is phosphorylated at Ser63 located in the kinase-inducible (KID) domain by PKA (cAMP-dependent protein kinase) and several other serine-threonine (Ser-Thr) kinases (Mayr and Montminy, 2001).

ATF1 regulates cell survival and proliferation by acting either as a transcriptional activator (Atlas et al., 2001; Belmonte et al., 2001; Kingsley-Kallesen et al., 1999; Lee and Pedersen, 2003; Lu and Sack, 2008; Rolli et al., 1999; Zaman et al., 1999; Zhang et al., 2004) or a repressor (Dong et al., 2002; Okuyama et al., 1996; Salnikow et al., 1997). We recently found that ATF1 is a

transcriptional repressor of the ferritin H gene (Iwasaki et al., 2007). Ferritin is the major intracellular iron storage protein comprising 24 subunits of H and L forms (Theil, 2003). Ferritin H contains the ferroxidase activity that oxidizes ferrous iron to ferric iron for iron storage, whereas ferritin L stabilizes the multimeric ferritin shell (Arosio and Levi, 2002). Ferritin synthesis is subject to both transcriptional and translational regulation (MacKenzie et al., 2008a). Iron-mediated translational regulation via interactions of iron regulatory proteins with iron-responsive elements of ferritin H and L mRNA was extensively and elegantly characterized (Hentze et al., 2004; Rouault, 2006). Iron-independent regulation of ferritin H and L genes was observed under such conditions as oxidative stress, inflammation and cell differentiation (MacKenzie et al., 2008a; MacKenzie and Tsuji, 2008). We demonstrated that both mouse and human ferritin H genes are transcriptionally activated under oxidative stress via a well-conserved far-upstream enhancer element, antioxidant-responsive element (ARE) (Iwasaki et al., 2006; Tsuji, 2005; Tsuji et al., 1995; Tsuji et al., 2000). The induction of ferritin during oxidative stress is an important cell defence mechanism against oxidative cell damage (Kaur et al., 2003; MacKenzie et al., 2008b; Pham et al., 2004; Sakamoto et al., 2009).

The molecular mechanism through which ATF1 transcriptional function is regulated in oxidative and genotoxic stress conditions remains incompletely understood. To better understand the ATF1-mediated ARE regulation, in this study we attempted to find ATF1-interacting proteins by yeast two-hybrid screening and have identified homeodomain-interacting protein kinase 2 (HIPK2) as an ATF1 binding protein. HIPK2 belongs to an emerging family of

nuclear Ser-Thr kinases that share significant homologies with the DYRK (MNB) dual-specificity kinases (Hofmann et al., 2000; Kim et al., 1998). These protein kinases play important roles in gene regulation, cell growth, differentiation and survival (Calzado et al., 2007; Rinaldo et al., 2007); however, molecular mechanisms behind their functions and diversities remain largely unknown. Among these kinases, HIPK2 has received much attention to its key regulatory role in DNA damage response. HIPK2 was initially identified as a binding protein and pivotal regulator of a homeodomain transcription factor, NKX1 (Kim et al., 1998). HIPK2 is involved in both cell survival and apoptosis (Sombroek and Hofmann, 2009). HIPK2 was shown to play a pivotal role in TGF- β -dependent cell survival of midbrain dopamine neurons via Smad interaction and subsequent activation of TGF- β target genes (Zhang et al., 2007). HIPK2 was also shown to be proapoptotic in response to ultraviolet (UV) exposure through p53 phosphorylation at Ser46, which inhibits MDM2-mediated degradation of p53 and ultimately activates p53-dependent apoptosis (D'Orazi et al., 2002; Hofmann et al., 2002). Furthermore, HIPK2 was shown to induce phosphorylation of anti-apoptotic transcriptional co-repressor CtBP (C-terminal binding protein) at Ser422, which in turn triggers CtBP ubiquitination and degradation (Zhang et al., 2005; Zhang et al., 2003).

In this study, we demonstrate that HIPK2 is a novel ATF1 kinase that phosphorylates a new phosphorylation site, Ser198, but not Ser63, and that HIPK2 appears to counteract the repressor function of ATF1 on the ferritin H ARE, resulting in induction of ferritin H and several other ARE-regulated antioxidant detoxification genes. These results suggest that HIPK2 is an important nuclear Ser-Thr kinase involved in cellular response to genotoxic and oxidative stress through post-translational regulation of the ATF1 transcription factor and expression of the ferritin H and other ARE-regulated antioxidant genes.

Results

HIPK2 phosphorylates ATF1

ATF1 serves as either a transcriptional activator or a repressor, including transcriptional repression of the ferritin H gene we observed (Iwasaki et al., 2007). To understand the regulatory mechanism of the ATF1 transcription factor and its effect on the ferritin H gene, yeast two-hybrid screening was employed to identify potential ATF1 binding proteins. A GAL4 DNA-binding domain fused to the human full-length ATF1 cDNA was used as bait for screening a murine B cell cDNA library, in which we identified HIPK2 as an ATF1 binding protein that allowed a transformed yeast clone to grow on His (-) dropout agar plates (Fig. 1A). To verify ATF1 and HIPK2 interaction in mammalian cells, HA-ATF1 and Flag-HIPK2 [either wild-type (wt) or kinase-dead (kd) HIPK2] were transiently transfected into HEK293 cells and cell lysates were subjected to immunoprecipitation with an anti-HIPK2 antibody followed by western blotting with anti-HA antibody. Only when HA-ATF1 and Flag-HIPK2 (either wt or kd) were co-expressed in the cells, did the HIPK2 antibody co-precipitate HA-ATF1 (Fig. 1B, top, lanes 5 and 6). In this experiment, we reproducibly detected a slower migrated ATF1 band in western blotting when co-expressed with wild-type HIPK2 (Fig. 1B, middle, lane 5) but not with kinase-dead HIPK2 (lane 6). Similar results of HIPK2-induced ATF1 retardation were observed in K562 and SH-SY5Y cells (B.-W. Huang and K. Sakamoto, unpublished observation). The fact that the retarded ATF1 migration was induced in a HIPK2 kinase-dependent manner suggests that

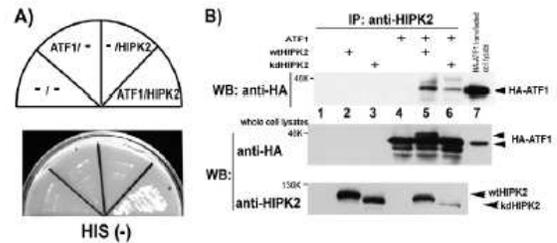


Fig. 1. HIPK2 is an ATF1 binding protein. (A) PJ69-4A yeast clones transformed with indicated combinations of bait and prey (pGBDATF1 and pAct2HIPK2) were tested for their growth on a histidine-deficient agar plate. (B) Indicated combinations of pHA-ATF1 and pFlagHIPK2 [wild type (wt) or kinase dead (kd)] were transfected into HEK293 cells, and cell lysates were immunoprecipitated with anti-HIPK2 followed by western blotting with anti-HA antibody (top). The right-end lane was loaded with 10 μ g of HEK293 whole-cell lysate transiently transfected with pHA-ATF1. The same set of cell lysates were subjected to western blotting with anti-HA (middle) or anti-HIPK2 (bottom). Of note is that wt HIPK2 (but not kd HIPK2) and HA-ATF1-transfected cell lysate exhibited a slower migrated ATF1 band (middle, lanes 5 and 6).

ATF1 might be phosphorylated either directly or indirectly by HIPK2.

To further characterize the retarded ATF1, we expressed HA-ATF1 and HIPK2 (wt or kd) in HEK293 cells and whole-cell lysates prepared from duplicated transfection plates were independently analyzed by western blotting for detection of HA-ATF1. The results in Fig. 2A reproducibly showed that the retarded HA-ATF1 band was induced only when wild-type HIPK2 was co-expressed. To characterize whether the retarded ATF1 is phosphorylated, the whole-cell lysates were treated with phosphatase prior to the western blotting with anti-HA antibody. Indeed, the phosphatase treatment abolished the retarded band (Fig. 2B), indicating that the retarded ATF1 band is phosphorylated. The possibility of phosphorylation on the HA-tag is ruled out

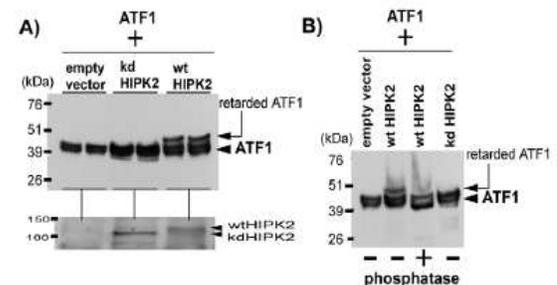


Fig. 2. HIPK2 phosphorylates ATF1. (A) Forty micrograms of HEK293 cell lysates transfected in duplicate with pCMVHAATF1 along with pCMVFlag (empty vector) or pCMVHIPK2-K221R kd or pCMVHIPK2 wt were independently separated on SDS-PAGE and analyzed by western blotting with anti-HA antibody. The HAATF1 and retarded HAATF1 bands were indicated by an arrowhead and arrow, respectively (top). The expression level of HIPK2 representing each duplicate was analyzed by western blotting with anti-HIPK2 antibody (bottom). (B) Forty micrograms of one of duplicated cell lysates in A were untreated or treated with λ -phosphatase for 37°C for 20 minutes prior to SDS-PAGE and western blotting with anti-HA antibody.

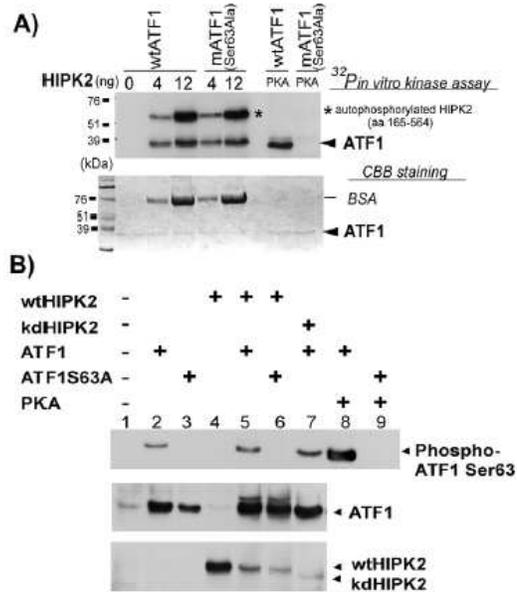


Fig. 3. HIPK2 directly phosphorylates a novel ATF1 site, not Ser63. (A) Increasing amounts of recombinant *HIPK2* (amino acids 165-564) was mixed with 2 μ g of bacterially expressed His-ATF1 or His-ATF1 Ser63Ala mutant and incubated in the presence of γ -³²P-ATP at 30°C for 20 minutes. Samples were separated on SDS-PAGE and visualized by autoradiography. PKA was used as a control. Asterisk indicates autophosphorylated HIPK2. Coomassie Blue staining of the same gel is shown (bottom). The stained band below the 76 kDa marker is BSA added to recombinant HIPK2 solution as a carrier protein. The molecular weight of the recombinant HIPK2 (a.a.165-564) is approximately 55 kDa. (B) Wt *HIPK2* or kd *HIPK2* (K221R) plasmid DNA were co-expressed with wt *ATF1* or Ser63Ala-mutant *ATF1* in HEK293 cells. Twenty-four hours after transfection, total cell lysates were collected and 40 μ g of samples were separated on SDS-PAGE and then analyzed by western blotting using anti-phospho ATF1 Ser63 antibody (top), anti-ATF1 antibody (middle) or anti-HIPK2 antibody (bottom). Cell lysate with co-expression of PKA and ATF1 was used as a positive control of ATF1 Ser63 phosphorylation (lane 8).

because there is no Ser-Thr in the tag sequence (Tyr-Pro-Tyr-Asp-Val-Pro-Asp-Tyr-Ala); a non-tagged ATF1 also showed the HIPK2-induced retardation (see below in Fig. 5).

To test whether HIPK2 directly phosphorylates ATF1, we performed an in vitro kinase assay by incubating recombinant ATF1 and HIPK2 in the presence of γ -³²P-ATP followed by SDS-PAGE and autoradiography. Recombinant PKA was used as a positive control for ATF1 phosphorylation at Ser63. ATF1 was ³²P-labeled after incubation with 4 or 12 ng of HIPK2 in vitro in a dose-dependent manner (Fig. 3A). To our knowledge, Ser63 of ATF1 is the only phosphorylation site induced by PKA and related kinases (Rehfuß et al., 1991). Interestingly, HIPK2 phosphorylated wild-type ATF1 as well as Ser63Ala-mutant ATF1 (Fig. 3A). As expected, PKA, an ATF1 Ser63 kinase (Rehfuß et al., 1991), phosphorylated wild-type ATF1 but not Ser63Ala-mutant ATF1 (Fig. 3A). Thus, we concluded that HIPK2 directly phosphorylates ATF1 at new Ser-Thr site(s).

These results do not exclude the possibility that HIPK2 also phosphorylates ATF1 at Ser63 in addition to the novel phosphorylation site. To test this possibility, Flag-HIPK2 (wt or kd) or PKA along with HA-ATF1 (wt or Ser63Ala) plasmids were expressed in HEK293 cells and the status of phospho-Ser63 ATF1 was determined by western blotting using an anti-phospho Ser63 ATF1 antibody. Expression of ATF1 alone showed a detectable amount of spontaneously phosphorylated ATF1 at Ser 63 (Fig. 3B, top, lane 2). By contrast, no phospho-Ser63 ATF1 band was detected when ATF1 Ser63Ala was expressed alone (lane 3). When wild-type HIPK2 was co-expressed with wild-type ATF1, no increase in ATF1 Ser63 phosphorylation was observed (Fig. 3B, top, lane 5, compare with lane 2). However, the anti-ATF1 western blot of the same sample (Fig. 3B, middle, lane 5) showed a clear retarded ATF1 band, suggesting that HIPK2 phosphorylated ATF1. Importantly, when wild-type HIPK2 was co-expressed with Ser63Ala-mutant ATF1, phospho-Ser63 ATF1 was undetectable (Fig. 3B, top, lane 6) but the retarded ATF1 band was similarly detected (middle, lane 6). Conversely, when wild-type ATF1 was co-expressed with kinase-dead HIPK2, the spontaneous basal level of phospho-Ser63 ATF1 was detected (top, lane 7) but no retarded ATF1 band was observed (middle, lane 7). When the catalytic subunit of PKA was co-expressed with wild-type ATF1, an increase in phosphorylation of Ser63 ATF1 was detected (Fig. 3B, top, lane 8), indicating that induced phosphorylation of ATF1 at Ser63 is detectable in our assays. Collectively, these results suggest that HIPK2 does not phosphorylate Ser63 but phosphorylates new Ser-Thr site(s) other than Ser63 of ATF1.

Phosphorylation at Ser198 of ATF1 and activation by HIPK2

To our knowledge, Ser63 in ATF1 is the only phosphorylation site so far identified by PKA (Rehfuß et al., 1991), MSK1 (mitogen and stress activated kinase 1) (Wiggin et al., 2002) and several other kinases (Mayr and Montminy, 2001), leading to activation of the transcription function of ATF1. To examine the effect of HIPK2 on ATF1 transcription function, we employed GAL4-luciferase reporter assays by co-transfection of ATF1-GAL4-DNA binding-domain-fused plasmid (pFA-ATF1) and a GAL4-luciferase reporter along with HIPK2 into HEK293 cells. PKA was used as a positive control for ATF1 activation via Ser63 phosphorylation. In this assay, HIPK2 enhanced ATF1-dependent luciferase expression as efficiently as PKA (Fig. 4), suggesting that HIPK2 activates ATF1. When both HIPK2 and PKA were co-expressed, ATF1 was further activated, suggesting that the pathways of ATF1 activation by HIPK2 and PKA are independent.

Ser63 in ATF1 is flanked by Pro62. HIPK2 preferentially phosphorylates Ser-Thr sites adjacent to proline residues [such as Pro421-Ser422-Pro423 in CtBP (Zhang et al., 2003), Pro296-Ser297 in Grocho (Choi et al., 2005), Ser46-Pro47 in p53 (D'Orazi et al., 2002; Hofmann et al., 2002)]. Therefore, we predicted that Ser63 in ATF1 is one of potential HIPK2 phosphorylation sites. However, HIPK2 did not increase ATF1 Ser63 phosphorylation (Fig. 3B) and Ser63Ala-mutant ATF1 was equivalently phosphorylated as wild-type ATF1 by HIPK2 in our in vitro kinase assay (Fig. 3A), suggesting that HIPK2 is not an ATF1 Ser63 kinase but phosphorylates new Ser-Thr site(s). There are at least six other potential HIPK2 phosphorylation sites in human ATF1 at Pro98-Thr99-Pro100, Pro163-Ser164, Ser198-Pro199 (these are conserved in CREB), Ser122-Pro123 and Thr184-Pro185-Ser186 (these are not conserved in CREB). To determine new ATF1

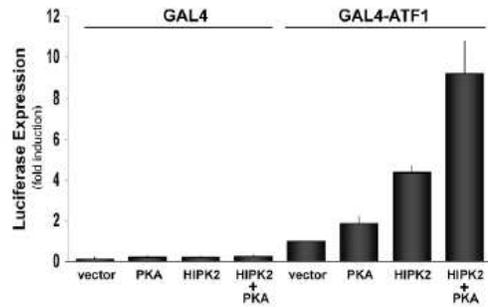


Fig. 4. HIPK2 activates ATF1 Transcription function. GAL4-DBD-luciferase plasmid was transfected into HEK293 cells together with GAL4 or GAL4-ATF1 (1–228) plus *HIPK2* or *PKA* expression plasmid. The luciferase assay was carried out 48 hours after transfection. GAL4-ATF1 plus empty-vector-transfected sample was set as 1.0. DNA transfection was carried out in duplicate in each experiment and the result of four independent experiments and standard errors are shown.

phosphorylation site(s) by HIPK2, non-tagged ATF1 plasmids mutated at the conserved Ser-Thr between ATF1 and CREB proteins (Ser63, Thr99, Ser164 and Ser198 of ATF1) to Ala were co-expressed with HIPK2 and cell lysates were subjected to western blotting with anti-ATF1 antibody and examined for whether a particular ATF1 mutation abolishes the retarded band. As shown in Fig. 5A, only the Ser198Ala ATF1 mutant failed to show the retardation under the similar expression levels of transfected HIPK2. To verify that Ser198 is the ATF1 phosphorylation site by HIPK2, we conducted an *in vitro* kinase assay using recombinant wild-type, Ser198Ala and Ser63Ala ATF1 proteins. Indeed, HIPK2 phosphorylated wild-type and Ser63Ala ATF1 proteins but failed to phosphorylate Ser198Ala ATF1 (Fig. 5B). By contrast, PKA phosphorylated wild-type and Ser198Ala ATF1 proteins but failed to phosphorylate Ser63Ala ATF1 as expected (Fig. 5B). Collectively, we concluded that HIPK2 directly phosphorylates ATF1 at Ser198.

Transcriptional regulation of the human ferritin H gene by ATF1 and HIPK2

Ferritin and a battery of antioxidant genes are transcriptionally regulated via an ARE under oxidative and chemical stress conditions (MacKenzie et al., 2008a). We recently demonstrated by gel retardation and ChIP assays that ATF1 binds to the ferritin H ARE and represses ferritin H transcription in K562 human erythroleukemia cells (Iwasaki et al., 2007). ATF1 also repressed the ferritin H ARE-dependent transcription in HepG2 cells, in which the luciferase construct containing the ferritin H ARE (–4.5 kb) was repressed by ATF1, whereas the one lacking the ARE (–4.4 kb) showed lower basal luciferase expression and no further transcriptional repression by ATF1 (Fig. 6A). To assess the role of HIPK2 in the transcriptional regulation of the human ferritin H gene, –4.5 kb ARE (+) or –4.4 kb ARE (–) luciferase was co-transfected with HIPK2 expression plasmid into HepG2 cells. As shown in Fig. 6B, HIPK2 induced expression of luciferase driven by the ARE (+) reporter, whereas there was no effect on the ARE (–) ferritin-H-luciferase expression. Then, we asked if HIPK2-mediated activation of the ferritin H transcription is dependent on the kinase activity. To address this question, wt or kd HIPK2 were

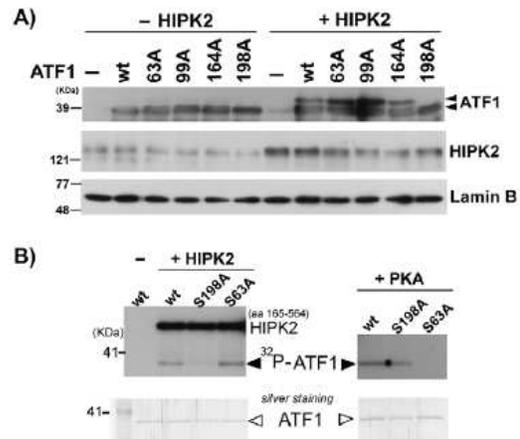


Fig. 5. HIPK2 phosphorylates ATF1 at Ser198. (A) Wild-type Flag-HIPK2 and various ATF1 mutants (Ser63, Thr99, Ser164, Ser198 to Ala) were co-transfected into HEK293 cells and nuclear fractions were subjected to western blotting with anti-ATF1 antibody, anti-HIPK2 antibody or anti-lamin B antibody. ATF1 and retarded ATF1 are indicated with arrowheads. (B) Ten nanograms of His-tagged recombinant ATF1 wt, Ser198Ala (198A) or Ser63Ala (63A) was incubated with 4 ng recombinant HIPK2 (kinase domain aa 165–564) or PKA in the presence of 5 μ Ci γ -³²P-ATP at 30°C for 20 minutes. Samples were loaded on 10% SDS-PAGE and phosphorylated bands were detected by autoradiograph. Comparable loading of the proteins was verified by gel Silver staining (bottom).

expressed with the –4.5 kb ARE (+) ferritin-H-luciferase reporter into HepG2 cells and luciferase activity was measured. Varied amounts of wt HIPK2 transfection showed increased expression of luciferase, whereas kd HIPK2 had no effect on ferritin-H-luciferase expression (Fig. 7A).

We next examined whether HIPK2 affects the ATF1-mediated repression of ferritin H. To this end, ATF1 and HIPK2 plasmids were co-expressed in HepG2 cells along with –4.5 kb ARE (+) ferritin-H-luciferase and luciferase expression was measured. ATF1 reproducibly repressed ferritin-H-luciferase expression, and wt HIPK2 but not kd HIPK2 overrode the ATF1-mediated ferritin H repression (Fig. 7B). These results suggest that the reversal effect of HIPK2 on ATF1-mediated ARE repression is dependent on the HIPK2 kinase activity and that HIPK2-mediated phosphorylation of ATF1 might play a role in the reversal effect of HIPK2 on the ATF1-mediated ferritin H repression.

The role of endogenous HIPK2 in expression of ferritin and other antioxidant genes

To elucidate the role of endogenous HIPK2 in expression of ferritin in genotoxic stress, HIPK2^{+/+} and HIPK2^{–/–} mouse embryonic fibroblasts (MEF) were treated with doxorubicin, etoposide and sodium arsenite for 20–24 hours, and ferritin H and L mRNA expression was measured. These genotoxic agents, known to activate HIPK2 (Hofmann et al., 2002; Rinaldo et al., 2007), induced ferritin H mRNA expression in HIPK2^{+/+} cells but not in HIPK2^{–/–} MEF cells (Fig. 8A). The mouse ferritin L gene was shown to be regulated by an ARE (Wasserman and Fahl, 1997);

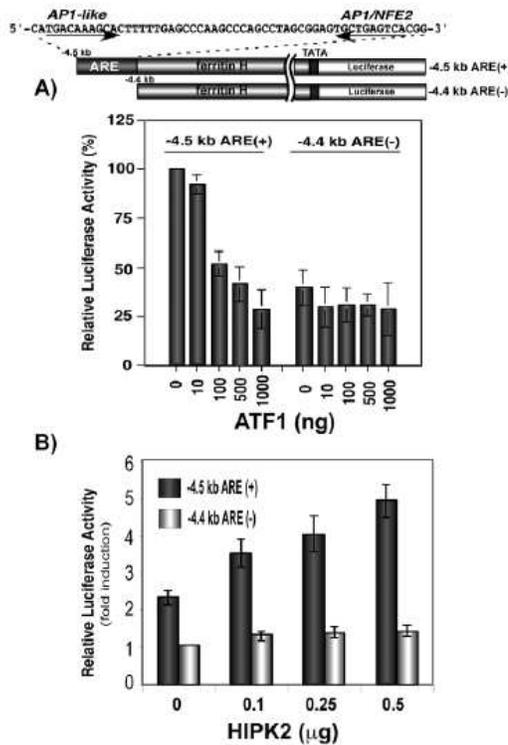


Fig. 6. Transcriptional regulation of the human ferritin H gene by ATF1 and HIPK2 via the ARE. (A) One microgram of -4.5 kb ferritin H luciferase [ARE (+)] or -4.4 kb ferritin H luciferase [ARE (-)] was co-transfected with indicated amounts of pCDNA3.1:ATF1 into HepG2 cells. Total input of plasmid DNA was adjusted to $2 \mu\text{g}$ with pCDNA3.1 empty vector. Cells were harvested for luciferase assays 48–60 hours after DNA transfection (in duplicate with pRL-CMV as an internal transfection control). Luciferase expression after normalization with pRL-CMV from five independent experiments is shown with standard errors. The luciferase activity in cell lysates co-transfected with -4.5 kb ARE (+) and empty vector was defined as 100%. (B) One microgram of -4.5 kb ARE (+)- or -4.4 kb ARE (-)-luciferase was co-transfected with indicated amounts of pFlagHIPK2 into HepG2 cells. Forty to forty-eight hours after transfection, cell lysates were prepared to run on the luciferase assay. Fold-induction in cell lysate obtained from cells transfected with -4.4 kb ARE (-) with empty vector was defined as 1.0. DNA transfection was carried out in duplicate in each experiment and the results of five independent experiments and standard errors are shown.

however, these genotoxic agents showed only marginal effects on ferritin L mRNA expression even in $HIPK2^{+/+}$ MEF cells (Fig. 8A). To examine whether the status of HIPK2 expression affects other well-characterized ARE-regulated antioxidant genes, $50 \mu\text{M}$ etoposide-treated MEF cells were analyzed in mRNA expression of *NQO1*, *GST α* and *HO1*. Etoposide induced mRNA expression of *NQO1* and *GST α* in $HIPK2^{+/+}$ MEF cells; however, the HIPK2 deficiency blunted the mRNA induction (Fig. 8B). To exclude the possibility of adaptation effects in MEF cells, similar experiments were performed in SH-SY5Y cells by knocking down *HIPK2*. As shown in Fig. 8C, etoposide treatment induced ferritin H and

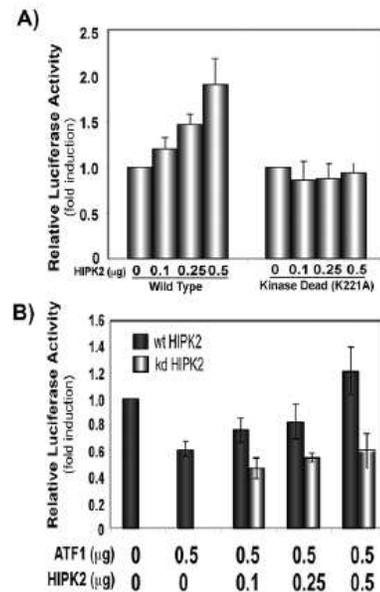


Fig. 7. HIPK2 activates ferritin H transcription. (A) One microgram of -4.5 kb human ferritin-H-luciferase reporter was co-transfected with indicated amounts of Flag-wtHIPK2 (wild type) or Flag-K221A-HIPK2 (kinase dead) into HepG2 cells. Fold-induction in cell lysate obtained from cells transfected with Flag empty vector was defined as 1.0. DNA transfection was carried out in duplicate in each experiment and the results of five independent experiments and standard errors are shown. (B) One microgram of -4.5 kb human ferritin-H-luciferase reporter was co-transfected with indicated amounts of pCMVATF1 and pFlagHIPK2 (wt or kd) into HepG2 cells. Fold-induction in cell lysate obtained from cells transfected with empty vectors was defined as 1.0. DNA transfection was carried out in duplicate in each experiment and the results of four independent experiments and standard errors are shown.

NQO1 expression and that was impaired by *HIPK2* knockdown. These results suggest that HIPK2 plays an important role in upregulation of ferritin H and other ARE-regulated antioxidant genes in response to genotoxic stress.

Discussion

Receptor-mediated activation of the CREB family has been extensively studied, in which PKA and several other Ser-Thr kinases activate transactivation function of ATF1/CREB through phosphorylation at Ser63 in ATF1 and Ser133 in CREB that are located in the KID domain (Mayr and Montminy, 2001; Shaywitz and Greenberg, 1999). In this study, we demonstrate that HIPK2 phosphorylates ATF1 at Ser198, but not Ser63, and activates ATF1 transcription function. CREB and CREM (cAMP response element modulator) are closely related to ATF1 in their amino acid sequences and functional domains (Mayr and Montminy, 2001). In fact, the human ATF1 amino acid sequences containing the HIPK2 phosphorylation site are highly conserved in CREB and CREM transcription factors: ATF1, TVVMT-S⁽¹⁹⁸⁾PV; CREB, GVVMASS⁽²⁷¹⁾PA; CREM, GVVMAAS⁽²²⁶⁾PG. Therefore, this HIPK2-mediated ATF1 phosphorylation might be applicable to the

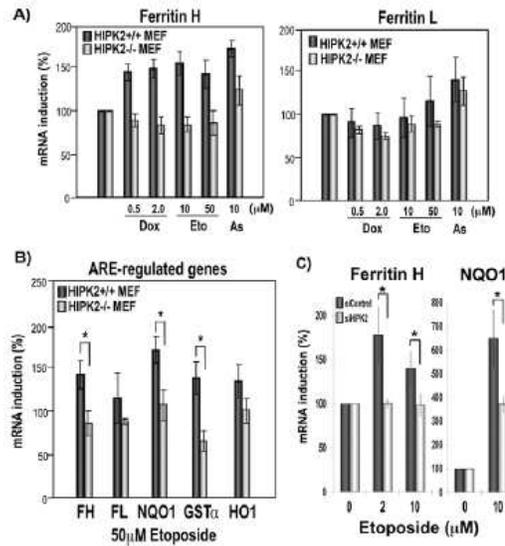


Fig. 8. HIPK2 deficiency affects expression of ferritin H and other ARE-regulated genes in genotoxic stress. (A) HIPK2^{+/+} or HIPK2^{-/-} MEF cells were treated with 0.5 or 2.0 $\mu\text{g/ml}$ doxorubicin (Dox), 10 or 50 μM etoposide (Eto) or 10 μM sodium arsenite (As). Twenty hours after the treatment, total RNA was isolated and subjected to real-time PCR for expression of ferritin H and L. (B) HIPK2^{+/+} or HIPK2^{-/-} MEF cells were treated with 50 μM etoposide for 20 hours and expression of ferritin H (FH), ferritin L (FL), NAD(P)H quinone oxidoreductase-1 (*NQO1*), glutathione S-transferase- α (*GST* α), or heme oxygenase-1 (*HO1*) mRNA was measured by real-time PCR. (C) SH-SY5Y cells were transfected with non-targeting (siControl) or HIPK2-targeting (siHIPK2) siRNA and incubated for 24 hours. Cells were then treated with 2 μM or 10 μM Etoposide for 48 hours and ferritin H and *NQO1* mRNA expression was measured by real-time PCR. In B and C, mean and standard errors from three independent experiments were shown. * $P < 0.05$, as determined by a Student's *t*-test.

entire CREB family. Indeed, we recently observed that HIPK2 phosphorylates CREB at Ser271, which activates CREB transcription function (Sakamoto et al., 2010).

CREB phosphorylation at Ser133 (equivalent to ATF1 Ser63) was shown to enhance subsequent recruitment of CREB-associated histone acetyltransferase CBP (CREB binding protein) (Arias et al., 1994; Chrivia et al., 1993; Kwok et al., 1994) via the KID, resulting in transcriptional activation of target genes. The HIPK2 phosphorylation site of ATF1 Ser198 is not in the KID but is localized adjacent to the second glutamine-rich region followed by the basic region of ATF1. We recently observed in ChIP assays that phosphorylation of CREB at Ser271 by HIPK2 showed no significant increase in DNA binding but increased recruitment of CBP on a BDNF promoter (Sakamoto et al., 2010). This suggests that HIPK2-mediated Ser198 phosphorylation might also stabilize the ATF1 and CBP interaction or facilitate the recruitment of CBP through phosphorylated Ser63. In addition, this new ATF1/CREB phosphorylation site by HIPK2 might recruit yet-unidentified coactivators to the vicinity of the binding site for transcriptional activation of target genes. The retarded migration of ATF1 with Ser198 phosphorylation but not Ser63 phosphorylation (Fig. 3B)

implies that the impact of phosphorylation at these two sites on protein structure and activation mechanism might be different. Further investigation will be necessary for understanding the new regulatory mechanism of ATF1 and CREB family members by HIPK2.

HIPK2 has been characterized as a DNA-damage-responsive Ser-Thr kinase that functions either as a transcriptional co-repressor or co-activator (Rinaldo et al., 2007) and participates in cell death (Calzado et al., 2007) or survival (Zhang et al., 2007). HIPK2 protein levels are regulated by E3 ubiquitin ligases such as WSB1 (Choi et al., 2008) and SIAH1 (Winter et al., 2008). In addition, HIPK2 has recently been shown to regulate cellular hypoxic response, in which hypoxic conditions induce the interaction of HIPK2 and another ubiquitin E3 ligase SIAH2 that in turn facilitates HIPK2 polyubiquitination and proteasomal degradation (Calzado et al., 2009a). This HIPK2 degradation allows SIAH2 to induce degradation of prolyl hydroxylases, resulting in the stabilization of HIF1 α (Calzado et al., 2009b). In accordance with the negative regulatory role of HIPK2 in HIF1 α expression, HIPK2 was also shown to repress transcription of the *HIF1a* gene that causes sensitization of cells to doxorubicin-induced apoptosis under a hypoxia-mimicking condition (Nardinocchi et al., 2009). Thus, both at transcriptional and post-translational levels, HIPK2 appears to be a negative regulator of *HIF1a* expression and hypoxia-inducible gene expression. Conversely, ATF1 and CREB were reported to bind to the hypoxia response element together with HIF1 α constitutively or an inducible manner under a hypoxia-mimicking condition (iron chelator or cobalt chloride treatment) (Ebert and Bunn, 1998; Kvietikova et al., 1995; Zaman et al., 1999) that upregulates hypoxia-responsive genes and protects cells from oxidative cell damage. Furthermore, ATF1 was identified as a hypoxia-responsive transcription factor, in which phosphorylation of ATF1 at Ser63 via a p38 MAP kinase pathway was involved in hypoxia-induced transcriptional activation of the uncoupling protein 3 gene in myotubes (Lu and Sack, 2008). The degradation of HIPK2 during hypoxia might cause the ratio to change towards more phospho-Ser63 ATF1, less phospho-Ser198 ATF1 along with HIF1 α accumulation via the aforementioned mechanism. Our results suggest that phosphorylation of Ser63 (by PKA) and Ser198 (by HIPK2) activates ATF1 transcription function additively or independently (Fig. 4); however, the hypoxia response element might have the preference of phospho-Ser63 ATF1 in cooperation with HIF1 α for expression of hypoxia-inducible genes. It remains to be determined in the future whether there is a positive or negative crosstalk between Ser63 and Ser198 phosphorylation of ATF1 in expression of its target genes.

Transcriptional repression of the ferritin H gene appears to be a strategy of oncogenes for enhancing cell proliferation because lower ferritin H expression levels ultimately increase the intracellular iron pool for cell proliferation and metabolism (Kakhlon et al., 2001). We previously demonstrated that the adenovirus E1A oncogene transcriptionally represses ferritin H in mouse NIH3T3 fibroblasts (Tsuiji et al., 1993a), later it turned out to be regulated through the ARE (Tsuiji et al., 1995; Tsuiji et al., 2000). c-myc was shown to repress transcription of ferritin H along with an increased intracellular iron pool that was required for c-myc-induced cell transformation (Wu et al., 1999). Our results show that ATF1 is another transcriptional repressor of ferritin H in non-stress conditions (Fig. 6). Expression of ATF1 is upregulated in several cancer cells such as lymphomas (Hsueh and Lai, 1995) and metastatic melanoma cells (Jean et al., 2000). In addition, the

EWS-ATF1-fused gene via t(12;22) chromosomal translocation is involved in proliferation of clear-cell sarcoma (Zucman et al., 1993). Thus, *ATF1* could be another gene product that enhances cell proliferation through transcriptional repression of ferritin H and a subsequent increase in intracellular iron levels.

Iron is an essential element for various metabolic pathways and cell proliferation; however, excess iron is potentially detrimental because it catalyzes formation of a highly toxic hydroxyl radical in Fenton chemistry (Papanikolaou and Pantopoulos, 2005). Therefore, under oxidative and some chemical stress conditions, ferritin transcription is activated through the ARE and cells limit the availability of intracellular labile iron. In this study, under exposure to such genotoxic agents as doxorubicin and etoposide that are known to activate HIPK2 (Rinaldo et al., 2007), mRNA expression for ferritin H and other ARE-regulated antioxidant genes was upregulated in HIPK2^{+/+} cells but not HIPK2^{-/-} or knockdown cells (Fig. 8). Thus, HIPK2 appears to be involved in ARE-regulated antioxidant gene transcription in these stress conditions. We observed that HIPK2^{-/-} MEF cells proliferate much faster than HIPK2^{+/+} MEF cells as previously reported (Wei et al., 2007), and of note is that they are more susceptible to oxidative and genotoxic agents such as etoposide, doxorubicin and cisplatin (Sakamoto et al., 2010). The increased susceptibility to these agents could be, at least in part, due to the lack of induction of ARE-regulated ferritin H and other antioxidant genes in HIPK2-deficient cells.

Basic-leucine zipper transcription factors such as NRF2 (NFE2-related factor 2) and small MAF proteins (MAFK and MAFG) have been demonstrated to regulate various phase II detoxification genes via the ARE enhancer (Motohashi and Yamamoto, 2004). NRF2 and JUND are involved in the transcriptional activation of the ferritin H gene via the ARE during oxidative stress through their post-translational modifications such as redox and phosphorylation (Iwasaki et al., 2006; Tsuji, 2005). We were not able to detect endogenous HIPK2 protein by western blotting in HepG2 and other cell types, even after our trials with almost all commercially available anti-HIPK2 antibodies, perhaps because of generally low expression levels of HIPK2 as reported (Wang et al., 2001), and as noted by others that currently no commercially available antibody can efficiently detect endogenous HIPK2 protein (Boucher et al., 2009). Further investigation will be necessary to elucidate the HIPK2-induced ARE activation mechanism, specifically as to whether HIPK2 primarily activates, through phosphorylation, ATF1 and/or NRF2 or other ARE-binding proteins including coactivators such as p300 and CBP (Aikawa et al., 2006) and core histones.

Materials and Methods

Cell culture

The HepG2 human hepatocarcinoma cells and HEK293 human embryonic kidney cells (ATCC) were cultured in minimum essential medium (MEM) supplemented with 2 mM L-glutamine, 1 mM sodium pyruvate, 0.1 mM nonessential amino acids and 10% fetal bovine serum (FBS; Mediatech). SH-SY5Y cells were cultured in a 1:1 mixture of MEM and HAM's F12 medium supplemented with 0.1 mM non-essential amino acids and 10% FBS. HIPK2^{+/+} and HIPK2^{-/-} mouse embryonic fibroblasts (MEF), kindly provided by Eric J. Huang at University of California San Francisco (Wei et al., 2007; Wiggins et al., 2004), were cultured in DMEM with 2 mM L-glutamine and 10% FBS. Cells were maintained and incubated during experiments at 37°C in a humidified 5% CO₂ atmosphere.

Plasmids and antibodies

Cloning of the human ferritin H 5' upstream enhancer and promoter region and construction of luciferase reporter plasmids were previously described (Tsuji, 2005). pFlagHIPK2 wild-type and K221A or K221R kinase-deficient HIPK2 were kindly provided by M. Lienhard Schmitz and Jun Ninomiya-Tsuji. ATF1 point mutations

(63A, 99A, 164A and 198A) were constructed with the QuickChange Site-Directed Mutagenesis Kit (Stratagene). Mouse *PKA* cDNA (Clontech) was subcloned into pCMV vector. Antibodies used in this study were: anti-HA (HA11, Covance), anti-Flag (M2, SIGMA), anti-HIPK2 (C15, Santa Cruz Biotechnology) and anti-ATF1 (C41-5.1, Santa Cruz Biotechnology).

DNA transfection and luciferase reporter assays

Transient DNA transfection into HepG2 cells was carried out by the calcium phosphate precipitation method as described previously (Tsuji et al., 1993b). Briefly, cells were plated at a density of 4 × 10⁵ cells per 35 mm plate containing 2 ml of the culture medium (in duplicate per transfection) and a total of 0.2 ml of calcium phosphate solution containing 0.5–1 μg of each reporter plasmid DNA was added to the cells and incubated for 40–48 hours. The GAL4 reporter assays were performed as described previously (Iwasaki et al., 2006). To monitor and normalize the differences in transfection efficiency in each plate, 0.1 μg of pRL-CMV (Promega) or pRL-EF (elongation factor promoter) was simultaneously cotransfected. Preparation of cell extracts and luciferase assays were performed using Dual Luciferase Assay Reagents (Promega) and the luciferase activity was measured with Luminometer (Model 20E, Turner Designs). Luciferase expression in each transfected sample was normalized by Renilla luciferase activity.

Yeast two-hybrid assay

Yeast two-hybrid screening of a mouse B-cell pGAD cDNA library was performed using a human full-length *ATF1* (pGBDATF1) as bait and the procedure was described previously (Iwasaki et al., 2007). Colonies of yeast P69-4A retransformed with pGADHIPK2 and pGBDATF1 (grown on tryptophan- and leucine-deficient synthetic dropout agar plates) were tested for growth on tryptophan-, leucine- and histidine-deficient agar media containing 2 mM 3-aminotriazole.

Immunoprecipitation and western blotting

HEK293 cells were transfected with 10 μg of pCMVFlagHIPK2 [wild-type (wt) or kinase-dead (kd) K221R] and 10 μg of pCMVHAATF1 plasmids by calcium phosphate transfection method. Whole-cell lysates were prepared at 40–48 hours after transfection and protein concentration of each cell lysate was measured with Bio-Rad protein assay reagent. Three hundred micrograms of the whole-cell lysates was immunoprecipitated with anti-HIPK2 antibody and then subjected to western blotting with anti-HA antibody. Expression levels of transfected Flag-HIPK2 and HAATF1 in the cell lysates (40 μg) were analyzed by western blotting with anti-HIPK2 and anti-HA antibodies, respectively. In the experiments of phosphatase treatment, 40 μg of HEK293 whole-cell lysates transfected with HAATF1 and Flag-HIPK2 (wt or kd K221R) were incubated with 800 units of λ-phosphatase (New England Biolabs) for 20 minutes at 37°C prior to western blotting with anti-HA antibody.

HIPK2 knockdown and real-time PCR

SH-SY5Y cells (1 × 10⁷) were transfected with 100 pmol non-targeting siRNA (5'-UAGCGACUAAACACAUCAAUUU-3'; D-001210-01; Dharmacon) or siHIPK2 (5'-GAGAAUCACUCCAAUCGAA-3'; J-003266-10; Dharmacon) using Gene Pulser X-Cell in 100 μl serum- and antibiotics-free media. After treatment with genotoxic chemicals, RNA was isolated with TRIzol reagent (Invitrogen) and real-time PCR was carried out to measure ferritin H, ferritin L, *NQO1*, *GSTα*, *HO1*, *HIPK2* or *GAPDH* mRNA with SYBR Green PCR Master Mix (Applied Biosystems) in the presence of primer set for ferritin H (human, Qiagen QT00072681; mouse, 5'-GCCTCTACGTCTATCTGTCTATGTC-3' and 5'-TGGTGGAGAAAG-TATTGGC-3'), mouse ferritin L (5'-AATCAGGCCCTCTGGATC-3' and 5'-GATAGTGGCTTCCAGGAAGTC-3'), *NQO1* (human, Qiagen QT00050281; mouse, 5'-CATTCTGAAAGGCTGGTTGA-3' and 5'-CTAGCTTGTATCTG-GTTGTGAC-3'), mouse *GSTα* (5'-GCCAAGTACCCTTGGTTGAA-3' and 5'-AATCTGACCACCTCAACA-3'), mouse *HO1* (5'-CCCACCAAGTTCA-AACAGCTC-3' and 5'-AGGAAGCGGCTTAGCCTC-3'), *HIPK2* (QT00051485, Qiagen) or *GAPDH* (QT00079247, Qiagen). The value of each mRNA expression was normalized by *GAPDH* mRNA expression. Approximately 70% *HIPK2* knockdown in SH-SY5Y cells was achieved by our electroporation procedure (Sakamoto et al., 2010).

In vitro kinase assay

Recombinant HIPK2 (amino acids 165–564, Upstate/Millipore), PKA (human full-length catalytic subunit, Upstate/Millipore), and ATF1 (amino acids 39–271, Santa Cruz Biotechnology) proteins were used (Fig. 3A). His-tagged human *ATF1* plasmids (pQE31ATF1 wt, 63A, and 198A) were constructed and recombinant full-length ATF1 proteins were purified as described (Sakamoto et al., 2010) with minor modifications. Indicated amounts of recombinant proteins were incubated at 30°C for 20–30 minutes in kinase buffer (10 mM HEPES, pH 7.4, 5 mM MgCl₂, 1 mM DTT) in the presence of 5 uCi γ-³²P-ATP and 100 μM unlabelled ATP. Samples were then separated on 10% SDS-PAGE and subjected to Coomassie Blue or Silver staining (SilverQuest, Invitrogen) and autoradiography.

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Regulation of Genotoxic Stress Response by Homeodomain-interacting Protein Kinase 2 through Phosphorylation of Cyclic AMP Response Element-binding Protein at Serine 271

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CREB (cyclic AMP response element-binding protein) is a stimulus-induced transcription factor that plays pivotal roles in cell survival and proliferation. The transactivation function of CREB is primarily regulated through Ser-133 phosphorylation by cAMP-dependent protein kinase A (PKA) and related kinases. Here we found that homeodomain-interacting protein kinase 2 (HIPK2), a DNA-damage responsive nuclear kinase, is a new CREB kinase for phosphorylation at Ser-271 but not Ser-133, and activates CREB transactivation function including brain-derived neurotrophic factor (BDNF) mRNA expression. Ser-271 to Glu-271 substitution potentiated the CREB transactivation function. ChIP assays in SH-SY5Y neuroblastoma cells demonstrated that CREB Ser-271 phosphorylation by HIPK2 increased recruitment of a transcriptional coactivator CBP (CREB binding protein) without modulation of CREB binding to the BDNF CRE sequence. HIPK2^{-/-} MEF cells were more susceptible to apoptosis induced by etoposide, a DNA-damaging agent, than HIPK2^{+/+} cells. Etoposide activated CRE-dependent transcription in HIPK2^{+/+} MEF cells but not in HIPK2^{-/-} cells. HIPK2 knockdown in SH-SY5Y cells decreased etoposide-induced BDNF mRNA expression. These results demonstrate that HIPK2 is a new CREB kinase that regulates CREB-dependent transcription in genotoxic stress.

INTRODUCTION

CREB (cAMP response element-binding protein) belongs to the b-zip transcription factor family that contains a basic region for DNA binding and a leucine zipper domain involving dimerization within the same family members (Shaywitz and Greenberg, 1999; Mayr and Montminy, 2001). ATF1 (activating transcription factor 1; Hai *et al.*, 1989) and CREM (cAMP response element modulator; Foulkes *et al.*, 1991) are closely related to CREB in their amino acid sequences and functional domains (Mayr and Montminy, 2001; Lonze and Ginty, 2002). Besides a constitutive transactivator function of CREB through interaction with TORCs (transducers of regulated CREB; Conkright *et al.*, 2003),

CREB is a stimulus-coupled transcription factor that plays a crucial role in a wide range of signaling pathways such as those triggered by G-protein-coupled receptors, neurotrophin receptors, growth factor receptors, and calcium channels (Lonze and Ginty, 2002). CREB is also involved in neuroprotection under hypoxia and oxidative stress conditions (Lonze and Ginty, 2002). Stimulus-coupled activation of CREB has been extensively studied, in which PKA (protein kinase A; cAMP-dependent protein kinase) and several other serine/threonine kinases activate transactivation function of CREB through phosphorylation at Serine 133 located in the kinase-inducible domain (KID domain; Mayr and Montminy, 2001). The activation of CREB-mediated gene transcription is facilitated by subsequent recruitment of CREB-associated coactivator proteins such as CBP (CREB-binding protein; Chrivia *et al.*, 1993) via the KID interaction (KIX) domain, whereas RGS13 (regulator of G-protein signaling) serves as a nuclear inhibitor of CREB transactivation function by interacting with Serine 133-phosphorylated CREB and CBP/p300 and decreasing both CREB binding to DNA and its interaction with CBP/p300 (Xie *et al.*, 2008).

Besides CREB phosphorylation at Serine 133, calcium influx into neuronal cells was shown to induce CREB phosphorylation at two additional sites at Serines 142 and 143, in which the involvement of calmodulin kinase or casein kinase II was suggested by the effects of pharmacological inhibitors (Kornhauser *et al.*, 2002). Phosphorylation of Serines 142 and 143 along with Serine 133 phosphorylation

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Abbreviations used: ATF1, activating transcription factor-1; BDNF, brain-derived neurotrophic factor; CBP, CREB-binding protein; ChIP, chromatin immunoprecipitation; CRE, cyclic AMP-response element; CREB, cyclic AMP response element-binding protein; CREM, cyclic AMP response element modulator; HIPK2, homeodomain-interacting protein kinase 2; KID, kinase-inducible domain; KIX, KID-interacting domain; PKA, protein kinase A (cyclic AMP-dependent protein kinase).

was required for efficient CREB-dependent transcription induced by calcium influx despite a decreased interaction between the CREB KID domain and the CBP KIX domain (Kornhauser *et al.*, 2002). BDNF (brain-derived neurotrophic factor) is one of the major CREB-target genes regulated by calcium and plays a vital role in cell survival and adaptive neuronal responses (Shieh *et al.*, 1998; Tao *et al.*, 1998). CREB is also involved in genotoxic stress-coupled gene transcription during oxidative stress and DNA damage, such as ionizing radiation, UV light, and hydrogen peroxide. These stressors were shown to induce multiple and sequential phosphorylation of CREB leading to Serine 121 phosphorylation by the DNA damage-response kinase ATM (ataxia-telangiectasia-mutated) and casein kinases (Shanware *et al.*, 2007). However, these phosphorylation events on CREB induced by DNA damage inhibited CREB-CBP interaction (Shanware *et al.*, 2007), resulting in inhibition of CREB transcription activity (Shi *et al.*, 2004).

Genotoxic stress activates a variety of protein kinases that relay signals to either cell cycle arrest or apoptosis. HIPK2 (homeodomain-interacting protein kinase 2; Kim *et al.*, 1998), within a family of nuclear serine-threonine kinases that share significant homologies with the Dyrk dual specificity kinase family (Hofmann *et al.*, 2000) has recently been characterized as a DNA-damage responsive kinase that plays a key regulatory role in both cell survival and apoptosis (Rinaldo *et al.*, 2007). For instance, HIPK2 was shown to be proapoptotic in response to UV light exposure through direct phosphorylation of p53 at Serine 46, preventing MDM2-mediated p53 degradation and ultimately leading to activation of p53-dependent apoptotic pathways (D'Orazi *et al.*, 2002; Hofmann *et al.*, 2002). Accumulating evidence has shown that broader genotoxic stress agents including ionizing radiation (Dauth *et al.*, 2007), DNA-damaging chemotherapeutic agents (such as cisplatin and doxorubicin; Di Stefano *et al.*, 2004; Calzado *et al.*, 2007; Rinaldo *et al.*, 2007) and a cyclin-dependent kinase 2 inhibitor roscovitine (Weierska-Gadek *et al.*, 2007) activate HIPK2, which in turn activates p53. In addition to p53-dependent proapoptotic role of HIPK2, the transcriptional corepressor and antiapoptotic protein CtBP was shown to be phosphorylated at Serine 422 by HIPK2, resulting in CtBP degradation by the proteasome and promoting UV-induced apoptosis (Zhang *et al.*, 2003). HIPK2 also plays an antiapoptotic role; for instance, HIPK2 is crucial for TGF- β -mediated survival of mid-brain dopamine neurons through its interaction with Smads for activation of TGF- β target genes (Zhang *et al.*, 2007). Interestingly, it was demonstrated that loss of HIPK2 caused increased proliferative potential in association with suppression of β -catenin-mediated activation of cyclin D1, and in the same study the loss of HIPK2 increased susceptibility to the two-stage carcinogenesis protocol (Wei *et al.*, 2007), suggesting that HIPK2 has a tumor suppressor function.

To date, only a few HIPK2 protein substrates, their phosphorylation sites, and the biofunctional impact of phosphorylation have been identified and characterized (D'Orazi *et al.*, 2002; Hofmann *et al.*, 2002; Zhang *et al.*, 2003; Aikawa *et al.*, 2006; Kim *et al.*, 2006; Wee *et al.*, 2008), even though dozens of cellular proteins have been reported to be regulated by HIPK2 (Rinaldo *et al.*, 2007). In this study we found that HIPK2 is a new CREB kinase that phosphorylates Serine 271 but not Serine 133 and regulates CREB-dependent gene transcription including the BDNF gene and genotoxic response.

MATERIALS AND METHODS

Cell Culture and Reagents

SH-SY5Y human neuroblastoma, K562 human erythroleukemia, and 293 human embryonal kidney cells transformed with adenovirus 5 DNA were purchased from American Type Culture Collection (ATCC, Manassas, VA). SH-SY5Y cells were cultured in a 1:1 mixture of Eagle's minimum essential medium (MEM) and HAM's F12 medium supplemented with nonessential amino acids and 10% fetal bovine serum (FBS, Mediatech, Manassas, VA). K562 were cultured in RPMI 1640 medium supplemented with 25 mM HEPES, 0.3g/l L-glutamine, and 10% FBS. 293 cells were cultured in MEM with 2 mM L-glutamine, Eagle's BBS, 0.1 mM nonessential amino acids, 1 mM sodium pyruvate, and 10% FBS. HIPK2^{+/+} and ^{-/-} mouse embryonic fibroblasts (MEFs), kindly provided by Dr. Huang at University of California San Francisco (Wiggins *et al.*, 2004; Wei *et al.*, 2007), were cultured in DMEM with 2 mM L-glutamine and 10% FBS. These cells were cultured at 37°C and 5% CO₂ in a humidified atmosphere. Etoposide was purchased from BioMol (Plymouth Meeting, PA) and dissolved in methanol. Cisplatin was purchased from Calbiochem (La Jolla, CA) and dissolved in water at 1 mg/ml.

Plasmids, DNA transfection, and Luciferase Reporter Assay

Human CREB α 341 cDNA was purchased from Invitrogen and subcloned into pCMV or pQE vector. CREB point mutations (133A, 271A, 133E, 271E, and double mutations) were constructed with the QuickChange site-directed mutagenesis kit (Stratagene, La Jolla, CA). Mouse PKA cDNA was purchased from Clontech (Palo Alto, CA) and subcloned into cytomegalovirus (pCMV) vector. cDNAs for mouse CBP and human p300, kind gifts from Drs. R. Goodman (Vollum Institute, Oregon Health Science University) (Chrivia *et al.*, 1993) and R. Eckner (New Jersey Medical School) (Eckner *et al.*, 1994), were described previously (Tsuji *et al.*, 1999). pCMVFlagHIPK2 and kinase dead (kd) mutant (K221R) plasmids (Kaneji-Ishii *et al.*, 2004) were used throughout the experiments along with similar effects observed with pDNA3FlagHIPK2wt and K221A kindly provided by Dr. Schmitz (Justus-Liebig University, Germany; Hofmann *et al.*, 2000, 2002). pLucMCS-luciferase reporter plasmid was purchased from Stratagene. CRE4-luciferase was constructed by insertion of four copies of an annealed double-strand oligonucleotide (5'-AGCTGACGTCAGAG-3') into pLucMCS-luciferase plasmid. Rat BDNFpIII-luciferase plasmid was generated by insertion of a 41-base pair annealed double-strand oligonucleotides (5'-AGCT-CACGTCAAGGCAGCGTGGAGCCCTCTCGTGGACTCC-3') into pLucMCS-luciferase. Transient DNA transfection into MEFs was carried out by electroporation (X-Cell, Bio-Rad, Hercules, CA) with an optimized preset condition by Bio-Rad for NIH3T3 cells (exponential decay, 1000 μ F, 155 V). Cells were suspended in 100 μ l of media with plasmid DNA in a cuvette with a 0.2-cm gap. Transient DNA transfection into K562 cells was carried out with jetPEI (Polyplus, New York, NY) according to manufacturer's instructions. As a transfection internal control, 10 ng of pRL-null (Promega, Madison, WI) was simultaneously cotransfected. Luciferase assays were performed using Dual Luciferase Assay Reagents (Promega). Firefly luciferase expression was normalized by Renilla luciferase activity.

Immunoprecipitation and Western Blotting

One hundred to 500 μ g of 293 whole cell lysates were used for immunoprecipitation with 1 μ g of anti-HIPK2 (sc-10294, Santa Cruz Biotechnology, Santa Cruz, CA) and protein G-plus agarose (Calbiochem, IP04). Proteins separated on 7.5, 10, or 12.5% SDS-PAGE were transferred to Immobilon-P polyvinylidene difluoride membrane (Millipore, Bedford, MA) and incubated at 4°C overnight with primary antibodies for HA (HA11, Covance, Princeton, NJ, or SC-805, Santa Cruz Biotechnology), Flag (F1804, Sigma, St. Louis, MO), CREB (sc-240x, sc-186x, Santa Cruz Biotechnology), HIPK2, or CBP (sc-369x, Santa Cruz Biotechnology). Protein phosphatase treatment was carried out by incubating 45 μ g of whole cell lysate from CREB or CREB plus HIPK2 transfected K562 cells with 20 U of lambda phosphatase (New England Biolabs, Ipswich, MA) for 30 min at 37°C before Western blotting with anti-CREB antibody. Nuclear fractionation was carried out using a nuclear extraction kit (Active Motif, Carlsbad, CA). Apoptosis was monitored by Caspase 3 cleavage in MEF cells treated with 2, 10, or 50 μ M etoposide for 12–48 h. Total cell lysates were subjected to Western blotting using anti-Caspase 3 antibody (9665, Cell Signaling, Danvers, MA). After incubation with secondary antibodies conjugated with horseradish peroxidase, proteins were visualized using an ECL detection kit (HyGLO, Denville Scientific, Metuchen, NJ) or advanced ECL kit (GE Healthcare, Piscataway, NJ).

In Vitro Kinase Assay

His-tagged wild-type (wt) or mutant CREB proteins were expressed and purified with pQE vector system (Qiagen, Valencia, CA). They were incubated with 9 ng of recombinant HIPK2 (aa 165–564, Millipore) or 0.1 ng of PKA (Millipore) for 30 min at 30°C in the kinase buffer (10 mM HEPES, pH 7.4, 5 mM MgCl₂, 1 mM DTT) containing 5 μ Ci [γ -³²P]ATP and 100 μ M ATP. Samples were then separated on 10% SDS-PAGE and subjected to silver staining (Sigma) and autoradiography. For measuring HIPK2 activity in cells, Flag-HIPK2-transfected K562 cells were treated with 50 μ M etoposide or 0.5

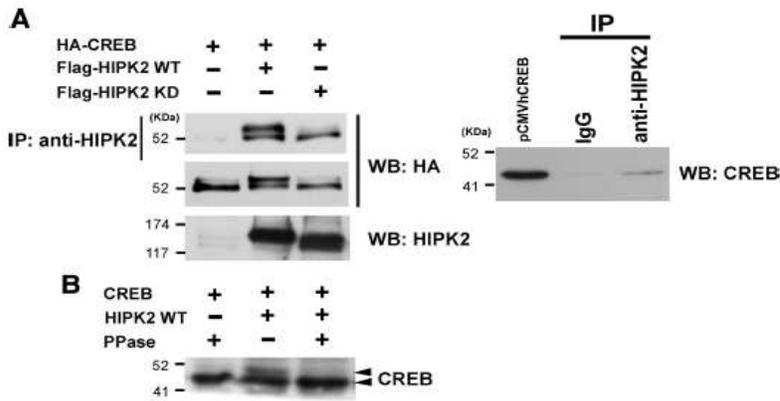


Figure 1. HIPK2 interacts with CREB and induces CREB retardation on SDS-PAGE. (A) Left, 293 cells were transfected with HA-CREB alone, HA-CREB plus Flag-HIPK2 wild type (WT), or HA-CREB plus Flag-HIPK2 kinase dead (KD). Whole cell lysates prepared after 48-h incubation were immunoprecipitated with anti-HIPK2 antibody, and the immunoprecipitates were subjected to Western blotting with anti-HA antibody (top). The whole cell lysates were also analyzed by Western blotting for expression of transfected HA-CREB (middle) and HIPK2 (bottom). Right, whole cell lysate of nontransfected 293 cells was immunoprecipitated with goat IgG or anti-HIPK2 antibody, followed by Western blotting with anti-CREB antibody. pCMVhCREB-transfected cell lysate was loaded as a CREB positive control. (B) K562 cells were transfected with nontagged CREB, or CREB plus Flag-HIPK2-WT, and whole cell lysates were treated with protein phosphatase (PPase +) before SDS-PAGE and Western blotting with anti-CREB antibody.

$\mu\text{g/ml}$ cisplatin for 6 h and harvested in the lysis buffer containing 20 mM HEPES, pH 7.4, 150 mM NaCl, 12.5 mM β -glycerophosphate, 1.5 mM MgCl_2 , 2 mM EGTA, 10 mM NaF, 2 mM DTT, 1 mM Na_2VO_4 , 0.5% Triton X-100, and 20 μM aprotinin. Cell lysates, 100 μg , were immunoprecipitated with anti-Flag antibody or control rabbit IgG in 1 \times kinase buffer containing 10 mM HEPES, pH 7.4, 5 mM MgCl_2 , 1 mM DTT, and 100 μM ATP. The immunoprecipitates were used for *in vitro* kinase assay using 1 μg of myelin basic protein (MBP, Sigma) as a substrate. After incubation at 30°C for 30 min or 22°C for 4 min, samples were separated on 12.5% SDS-PAGE, stained with Coomassie Brilliant Blue (CBB), and subjected to autoradiography.

Small Interfering RNA Transfection and real-time PCR

SH-SY5Y cells, 1×10^7 , were electroporated with 100 pmol of nontargeting small interfering RNA (siRNA; 5'-UAGCGACUAAAACACAUCAAUUU-3', D-001210-01; Dharmacon, Lafayette, CO) or siHIPK2 (target sequence: 5'-GAGAAUCACUCCAAUCGAA-3', J-003266-10; Dharmacon) using Gene Pulser X-Cell (square wave, 25 ms, 110 V) in 100 μl FBS- and antibiotic-free media. After incubation of electroporated SH-SY5Y cells in the electroporation cuvette for 10 min at room temperature, the cells were suspended in 10 ml of growth media and plated with 2 ml cell suspension per well in six-well plates and incubated for 24 h. Cells were then treated with 2 or 10 μM etoposide, or methanol as a control for 48 h, and RNA was isolated with TRIzol reagent (Invitrogen). Real-time PCR was carried out to measure HIPK2, BDNF, or GAPDH mRNA with SYBR Green PCR master mix (Applied Biosystems, Foster City, CA) in the presence of primer set for HIPK2 (QT00051485, Qiagen), BDNF (5'-CGCCATGCAATTTCCACTATCAATA-ATTTAAC-3' and 5'-ACTTTTCAGTCACTACTTGTCAAAGTAACC-3'), or GAPDH (QT00079247, Qiagen).

Chromatin Immunoprecipitation Assay

Chromatin immunoprecipitation (ChIP) assays were carried out as described previously (Sakamoto *et al.*, 2009) using a ChIP assay kit (Millipore) with minor modifications. Briefly, 1×10^7 SH-SY5Y cells were transfected with 20 μg of plasmid DNA as indicated in figure legends and plated into two dishes of 35-mm plate. After incubation for 40 h, cells were subjected to chromatin cross-linking and preparation of cell lysates. Approximately one-tenth aliquots of cell lysate containing sheared DNA by sonication (Iwasaki *et al.*, 2007) were immunoprecipitated with 2–4 μg each of antibodies for HA, Flag, CREB, CBP, or control goat IgG. PCR was performed in 50 μl reactions containing [^{32}P]dCTP, Advantage 2 PCR polymerase mix (Clontech), and a pair of primers (5'-CGCCTGAATTTTGTCTGTAAT-3', 5'-AATGG-GAAAGTGGGTGGGAG-3') to amplify 0.1-kb region of the human BDNF exon III promoter containing the CREB-binding site. The PCR samples were loaded and separated on an 8% acrylamide gel and subjected to autoradiography.

RESULTS

HIPK2 Phosphorylates CREB at Serine 271 But Not Serine 133

Our yeast-two hybrid screening for ATF1-interacting proteins cloned HIPK2 (unpublished observation). On the basis of the fact of high homology between CREB and ATF1 (Mayr and Montminy, 2001; Lonze and Ginty, 2002), we hypothe-

sized that HIPK2 may also interact with CREB and regulate CREB function. To test this hypothesis, HIPK2 and HA-CREB expression plasmids were cotransfected, and their protein-protein interaction was examined by immunoprecipitation/Western blot. Immunoprecipitation of wtHIPK2 or kdHIPK2 with an anti-HIPK2 antibody coprecipitated HA-CREB (Figure 1A), suggesting that HIPK2, regardless of its kinase status, interacts with CREB. Immunoprecipitation of nontransfected 293 cells with the anti-HIPK2 antibody also coprecipitated endogenous CREB protein (Figure 1A). Of note is that cotransfection of HA-CREB along with wtHIPK2, but not kdHIPK2, induced a retarded form of HA-CREB on SDS-PAGE (Figure 1A). To test whether this retarded migration of CREB is due to phosphorylation of CREB, CREB- and wtHIPK2-transfected cell lysate was treated with protein phosphatase before loading on SDS-PAGE. Indeed, the protein phosphatase treatment abolished the retarded CREB band (Figure 1B), suggesting that HIPK2 expression caused CREB phosphorylation.

The major regulatory mechanism of CREB is phosphorylation of Serine 133 by PKA, or several other kinases such as MSK1 (mitogen- and stress-activated kinase 1) and calcium-calmodulin kinases, leading to activation of transcription function of CREB (Mayr and Montminy, 2001). HIPK2 preferentially phosphorylates Ser/Thr sites adjacent to Proline residues (see *Discussion*). Serine 133 in CREB is flanked by Proline 132; therefore, we anticipated that HIPK2 might be another Serine 133 kinase. To test this possibility, Serine 133 to Alanine mutant CREB (133A) was coexpressed with HIPK2 in K562 cells, and cell lysates were subjected to Western blotting for the retarded migration of CREB. wtCREB reproducibly showed the retarded CREB band when wtHIPK2 was coexpressed (Figure 2A). Under this condition, the CREB 133A mutant gave rise to the same retarded band induced by HIPK2 (Figure 2A), suggesting that the CREB retardation, which was sensitive to phosphatase treatment (Figure 1B), was not due to Serine 133 phosphorylation.

There are eight potential phosphorylation sites in the human CREB protein by HIPK2, in which Serine or Threonine is adjacent to Proline (Ser-76, Ser-80, Ser-133, Thr-172, Ser-237, Thr-259, Ser-271, and Thr-276). Among these, amino acid sequences containing Ser-133, Thr-172, Ser-237, and Ser-271 of CREB are highly conserved in human ATF1 Ser-63, Thr-99, Ser-164, and Ser-198, respectively. Our preliminary experiments suggested that HIPK2 appears to phos-

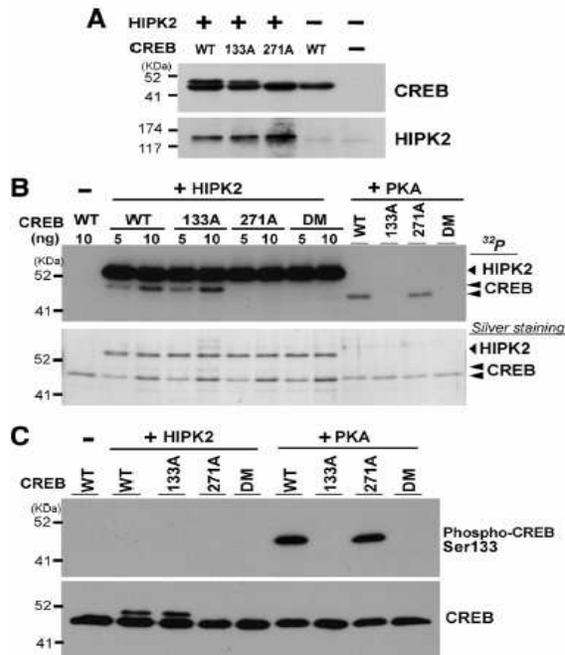


Figure 2. HIPK2 phosphorylates CREB at Serine 271 but not Serine 133. (A) CREB-WT, CREB 133A, or CREB 271A mutant was cotransfected with Flag-HIPK2-WT in K562 cells. Whole cell lysates were prepared after 48 h and subjected to SDS-PAGE and Western blotting with anti-CREB or anti-HIPK2 antibody. (B) Five or 10 ng of His-tagged recombinant CREB-WT, 133A, 271A, or 133A/271A double mutant (DM) was incubated with 9 ng recombinant HIPK2 (kinase domain aa. 165–564) in the presence of [γ - 32 P]ATP for 30 min at 30°C. Five nanograms of these recombinant CREB proteins was also incubated with recombinant PKA. Samples were loaded on SDS-PAGE, and phosphorylated bands were detected by autoradiograph. Comparable loading of the samples was verified by silver staining of the gel (bottom). (C) The same set of recombinant CREB proteins was incubated with recombinant HIPK2 or PKA in the presence of cold ATP, followed by SDS-PAGE and Western blotting with anti-phospho Serine 133 CREB (top) or CREB antibody (bottom).

phorylate ATF1 at Serine 198 (unpublished observation), suggesting that the corresponding CREB Serine 271 may be the HIPK2-mediated phosphorylation site. To test this possibility, we introduced Serine 271-to-Alanine mutation in CREB (271A) and performed coexpression with wtHIPK2 and CREB271A followed by Western blotting to detect the retarded migration. As shown in Figure 2A, the 271A mutation completely abolished the retarded migration of CREB, suggesting that HIPK2 phosphorylates CREB at Serine 271 and induces its slower migration in SDS-PAGE.

These results do not exclude the possibility of additional CREB phosphorylation sites by HIPK2, including Serine 133 that may not elicit the retarded migration. To test this possibility and to address whether HIPK2 directly phosphorylates CREB at Serine 271, we performed *in vitro* kinase assays by incubating recombinant wt or mutant CREB protein and HIPK2 in the presence of [γ - 32 P]ATP. As shown in Figure 2B, recombinant HIPK2 phosphorylated both wt-CREB and 133A mutant CREB but failed to phosphorylate

271A or 133A/271A double mutated CREB (DM). These results indicate that HIPK2 directly phosphorylates CREB at Serine 271 and that Serine 271 is the sole phosphorylation site of CREB by HIPK2. As a control experiment, we incubated recombinant PKA with these recombinant CREB proteins to verify that our 133A CREB mutants are devoid of PKA-mediated phosphorylation. Indeed, PKA phosphorylated wt and 271A CREB proteins but failed to phosphorylate 133A and 133A/271A mutant CREB proteins (Figure 2B). Furthermore, we observed in Figure 2B that the phosphorylated recombinant CREB by HIPK2 (phospho-Serine 271 CREB) showed slower migration than the phosphorylated CREB by PKA (phospho-Serine 133 CREB), which is consistent with the CREB retardation seen in the cells (Figures 1 and 2A). To further rule out the possibility of CREB Serine 133 phosphorylation by HIPK2, recombinant CREB proteins incubated with HIPK2 or PKA *in vitro* were subjected to Western blotting with a phospho-Serine 133 CREB antibody (Figure 2C, top) or CREB antibody (Figure 2C, bottom). PKA-phosphorylated CREB proteins (wt and 271A) were detected by the phospho-Serine 133 CREB antibody but HIPK2-phosphorylated CREB proteins (wt and 133A) giving rise to the retarded CREB, Figure 2C, bottom) were not detected by the phospho-Serine 133 antibody. Taken all together, we concluded that HIPK2 phosphorylates CREB only at Serine 271.

Serine 271 Phosphorylation by HIPK2 Enhances a Transactivation Function of CREB

To explore the role of HIPK2 in CREB-dependent transcription, we performed CRE-luciferase reporter assays in cells transiently transfected with CREB and HIPK2. CREB alone activated tandem four copies of CRE-dependent luciferase (CRE4) transcription by approximately 10-fold in K562 cells (Figure 3A). Cotransfection of wt HIPK2 with CREB showed further activation, whereas the kdHIPK2 showed no effect (Figure 3A). Transfection of wtHIPK2 alone also slightly activated the CRE-dependent luciferase expression (Figure 3A), probably by the effect of HIPK2 on endogenous CREB-WT. We then asked whether a CRE enhancer derived from a CREB-target gene is also regulated by HIPK2. The exon III of BDNF gene has a functional CRE-enhancer, and we tested a BDNF-luciferase reporter. As shown in Figure 3B, the BDNF CRE enhancer was activated by CREB, and it was further activated by wtHIPK2 but not by kdHIPK2. These results suggest that HIPK2 activates CREB-dependent transcription in a kinase activity-dependent manner.

To further elucidate the role of HIPK2 in CREB phosphorylation at Serine 271, we generated the CREB Serine 271-to-glutamic acid mutant (271E) to mimic Serine 271 phosphorylated CREB. As a control, we generated the Serine 133 to glutamic acid mutant CREB (133E). First, we expressed these CREB mutants in K562 cells and tested whether the replacement of Serine with glutamic acid causes the retardation in SDS-PAGE. As shown in Figure 4A, 271E CREB showed the retarded migration that was similar to one induced by co-expression of wtCREB and HIPK2, whereas the 133E CREB mutant did not show the retarded migration. The retardation of 271E CREB was resistant to phosphatase treatment (Figure 4B), excluding the possibility of other CREB phosphorylation sites for the retardation. Then, we tested the effect of these glutamic acid CREB mutants on CRE-dependent transcription by a luciferase reporter assay. Compared with the CRE-dependent transcription activated by wtCREB or 133E CREB, 271E CREB activated it 1.8–2.0-fold higher than wt or 133E CREB (Figure 4C). No difference in the CREB activity between wt and 133E in this assay appears to

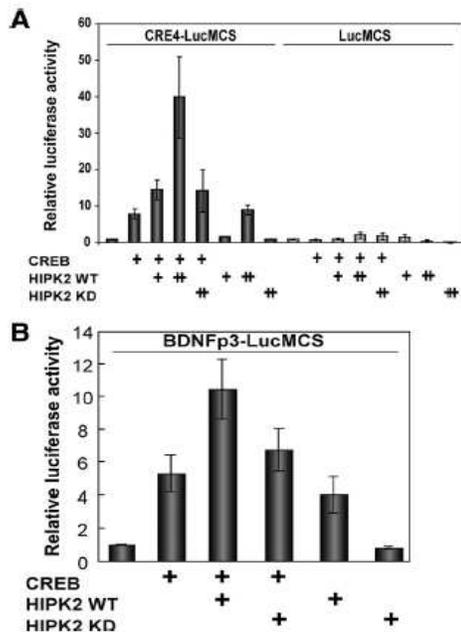


Figure 3. HIPK2 activates CREB-dependent transcription in a kinase-dependent manner. (A) CREB, 0.1 μ g, along with 0.1 μ g (+) or 0.3 μ g (++) HIPK2-WT or HIPK2-KD plasmids were cotransfected with pCRE 4 copies (CRE4)-luciferase or pTATA-luciferase reporter into K562 cells. (B) CREB, 0.1 μ g, along with 0.3 μ g HIPK2-WT or HIPK2kd plasmids were cotransfected with rat BDNF promoter III-luciferase into K562 cells. In both A and B, cells were harvested after 24 h to measure luciferase expression. Relative luciferase activity is shown by setting each reporter transfection alone as 1.0. Average of four experiments are shown with SEs.

be consistent with a previous report showing no significant impact of Serine 133 to glutamic acid replacement on CREB and coactivator interaction (Solt *et al.*, 2006). Furthermore, cotransfection of wtCREB or Serine 271-to-Alanine mutant CREB (271A) along with HIPK2 in CRE4-luciferase assays demonstrated that the mutation of Serine 271 to Alanine diminished HIPK2-mediated activation (Figure 5A). Collectively, these results suggest that the phosphorylation of CREB at Serine 271 by HIPK2 enhanced the transactivation function of CREB.

Phosphorylation of CREB by HIPK2 Facilitates the Recruitment of the Coactivator CBP

To define the mechanism through which Serine 271 phosphorylation by HIPK2 enhances the transactivation function of CREB, we asked whether Serine 271 phosphorylation affects the DNA-binding ability of CREB. To this end, we expressed HA-tagged wt or 271A CREB together with HIPK2 in SH-SY5Y human neuroblastoma cells and performed CREB ChIP assays for the BDNF exon III CRE site. Under similar expression levels of wtCREB (with or without HIPK2 expression) and 271A CREB (Figure 5B, Western blot with nuclear extracts), binding of these CREB proteins to the BDNF promoter III was correlated with their CREB expression levels (Figure 5B). We also tested nuclear extracts iso-

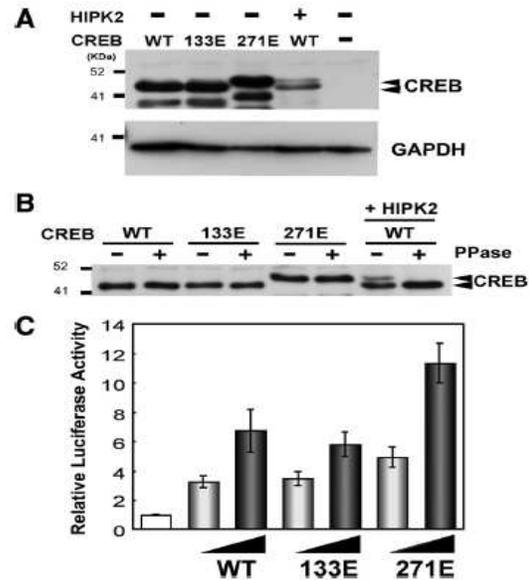
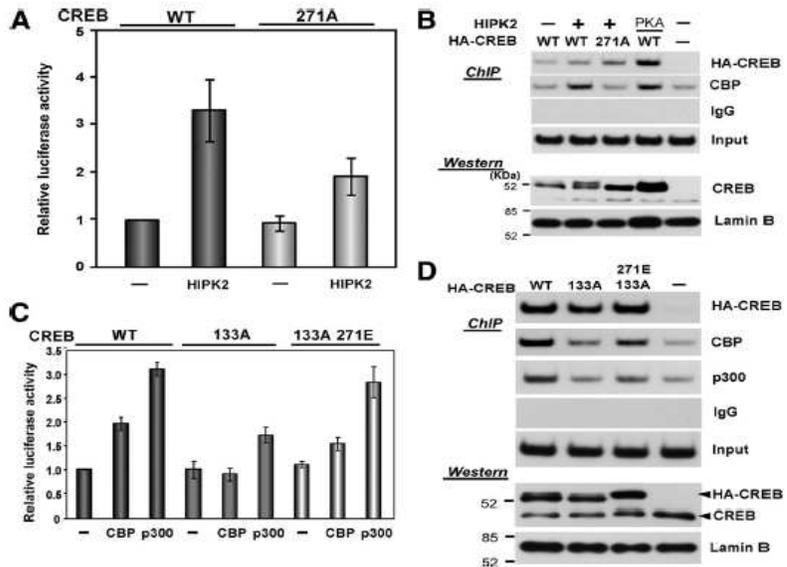


Figure 4. Replacement of CREB Serine 271 with glutamic acid caused the retarded migration in SDS-PAGE and enhanced CREB transactivation function. (A) CREB-WT, 133E, 271E mutant alone or CREB-WT plus Flag-HIPK2 was transfected into K562 cells. Whole cell lysates were prepared after 48 h and subjected to SDS-PAGE and Western blotting with anti-CREB antibody. Intact CREB proteins (retarded and nonretarded) are indicated with arrowheads. The smaller CREB proteins appear to be produced by unknown proteolytic cleavage. GAPDH Western blotting is shown as a loading control. (B) K562 cells were transfected with CREB-WT, CREB 133E, CREB 271E, or CREB-WT plus HIPK2. Whole cell lysates were treated with protein phosphatase (PPase+) before SDS-PAGE and Western blotting with anti-CREB antibody. (C) 0.1 or 0.3 μ g of CREB-WT, Serine-to-glutamic acid mutant 133E or 271E expression plasmid was transfected with CRE4-luciferase reporter into K562 cells. Cells were harvested after 24 h incubation, and luciferase expression was measured. Luciferase expression from the reporter alone was set to 1.0. Average from eight independent experiments are shown with SEs.

lated from these cells for their binding to a canonical CRE DNA sequence by gel retardation assays, in which no difference was observed in the ability of DNA binding between wtCREB and HIPK2-phosphorylated wtCREB or 271E CREB (Supplemental Figure S1). These results suggest that Serine 271 phosphorylation of CREB by HIPK2 did not alter the binding of CREB to the CRE site.

Because the CREB-dependent transcription is regulated by cooperation of CREB and CREB-associated proteins, we next examined whether the recruitment of CBP, a histone acetyltransferase serving as a coactivator of CREB, is affected by HIPK2-induced CREB Serine 271 phosphorylation. After expression of wtCREB alone or coexpression of wtCREB or 271A CREB mutant together with HIPK2 in SH-SY5Y cells, ChIP assays were performed to measure interaction of endogenous CBP with the CRE site in the BDNF promoter III. Coexpression of wtCREB along with PKA was used as a positive control of enhanced CBP recruitment to CREB via Serine 133 phosphorylation. Under equivalent expression levels of transfected CREB proteins, CBP recruitment to the

Figure 5. CREB phosphorylation at Serine 271 by HIPK2 increased CBP recruitment to the CRE site in the BDNF promoter. (A) CREB-WT or CREB 271A was transfected with HIPK2 and a CRE4-luciferase reporter into K562 cells. After 24 h, cells were harvested to measure luciferase expression. Relative luciferase activity (fold) is shown by setting CREB-WT alone as 1.0. Average of seven experiments with SEs are shown. (B) pCMVHA-CREBwt or 271A mutant was electroporated into 1×10^7 SH-SY5Y cells along with pCMVflagHIPK2 or pCMVHPKA and plated into two dishes. Forty-eight hours after electroporation, one group was used for ChIP assays for the CRE site in the BDNF promoter immunoprecipitated with control IgG, anti-CREB, or anti-CBP antibody (top). Another set of cells was used for isolation of nuclear proteins and 30 μ g each was loaded on SDS-PAGE and analyzed by Western blotting with anti-CREB, or anti-lamin B (bottom). (C) pCMVCREBwt, 133A, or 133A271E was co-transfected with CRE4-luciferase reporter into 293 cells along with pCMV-CBP or pCMV-p300. Twenty-four hours after transfection, cells were harvested for luciferase assay. Luciferase expression from CRE4-luciferase with wtCREB was set to 1.0. Average from four independent experiments with SEs are shown. (D) 1×10^7 SH-SY5Y cells were electroporated with HA-CREBWT, 133A, 133A/271E, or empty vector and divided into two plates each. One set was subjected to ChIP assays for the CRE site in the BDNF promoter immunoprecipitated with anti-HA, anti-CBP, anti-p300, or control IgG (top). Another set of cells was used for isolation of nuclear proteins and 30 μ g each was analyzed by Western blotting with anti-CREB or anti-lamin B (bottom).



BDNF promoter III was increased when wtCREB was co-expressed with HIPK2 or PKA (Figure 5B). Consistently, co-expression of 271A CREB mutant along with HIPK2 showed no increase in CBP recruitment (Figure 5B). Furthermore, in CRE4-luciferase reporter assays, cotransfection of wt or Serine 133A, or 133A271E double-mutated CREB with p300 or CBP demonstrated that CBP and p300 activated wtCREB but not 133A CREB; however, 133A271E regained the CBP/p300-mediated activation (Figure 5C). Consistently, ChIP assays in Figure 5D showed that decreased recruitment of CBP or p300 to 133A CREB compared with wtCREB was recovered by introduction of 271E phosphomimetic mutation in 133A CREB (133A/271E). Taken together, these results suggest that CREB phosphorylation at Serine 271 by HIPK2 increased the CBP/p300 recruitment or stabilized the recruited CBP/p300 in the CREB-binding complex.

Retarded Migration of Endogenous CREB by Etoposide via HIPK2

HIPK2 was shown to be activated by several genotoxic stress agents and plays a key regulatory role in both cell survival and apoptosis (Rinaldo *et al.*, 2007). We next asked whether endogenous CREB protein is phosphorylated when HIPK2 is activated. To address this question, 293 cells were treated with etoposide for 6 h and examined the retardation of endogenous CREB protein on SDS-PAGE. As shown in Figure 6A, treatment with 50 μ M etoposide showed increased expression of endogenous CREB along with retarded migration. To elucidate the involvement of HIPK2 in this retardation, wtHIPK2 (HIPK2+/+) MEFs or HIPK2 knockout MEF (HIPK2-/-) cells were treated with 10 μ M etoposide, and nuclear extracts were separated on SDS-PAGE for retardation of endogenous CREB. As shown in Figure 6B, etoposide treatment significantly induced retarded migration of CREB in HIPK2+/+

MEF cells compared with HIPK2-/- cells. Collectively, these results suggest that endogenous CREB is phosphorylated by etoposide treatment via HIPK2.

Etoposide Induces CRE-dependent Transcription and BDNF mRNA via HIPK2

Our *in vitro* kinase assays with immunoprecipitates of HIPK2 also demonstrated that treatment of cells with eto-

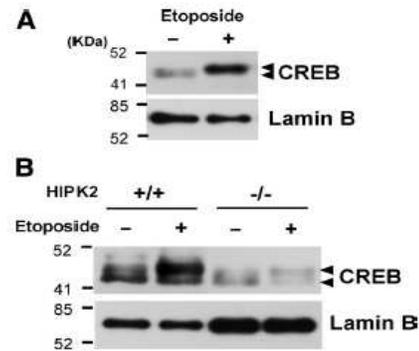


Figure 6. Retarded migration of endogenous CREB by etoposide via HIPK2. (A) 293 cells were treated with methanol (-) or 50 μ M etoposide (+) for 6 h. Nuclear extracts were subjected to SDS-PAGE and Western blotting with anti-CREB antibody. Western blotting with anti-lamin B antibody is shown as a loading control. (B) HIPK2+/+ or -/- MEF cells were treated with 10 μ M etoposide for 2 h. Nuclear extracts were subjected to SDS-PAGE and Western blotting with anti-CREB or anti-lamin B antibody.

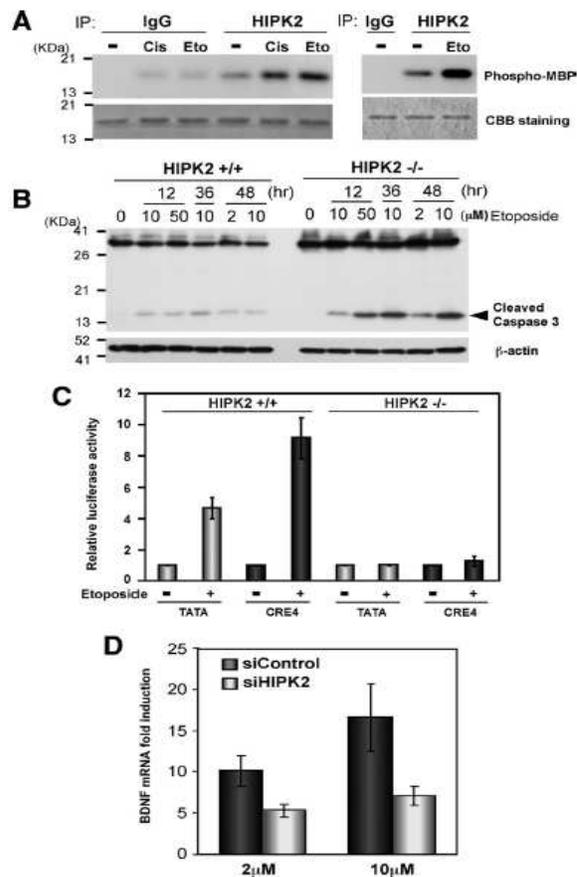


Figure 7. Etoposide induces CRE-dependent transcription and BDNF mRNA via HIPK2. (A) FlagHIPK2-transfected K562 cells were treated with 0.5 $\mu\text{g/ml}$ cisplatin or 50 μM etoposide for 6 h and harvested for immunoprecipitation with anti-HIPK2 antibody or control IgG. The immunoprecipitates from two independent experiments were incubated with recombinant MBP at 30°C for 30 min (left) or 22°C for 4 min (right) in the presence of [γ - ^{32}P]ATP and loaded on SDS-PAGE. Phosphorylated MBP was detected by autoradiography (top). Comparable protein loading was verified by Coomassie Brilliant Blue (CBB) staining (bottom). (B) Twenty-four hours after plating 5×10^6 HIPK2 $^{+/+}$ or $^{--}$ MEF cells, they were treated with indicated concentrations of etoposide for 12, 36, or 48 h. Whole cell lysates were analyzed by Western blotting with anti-Caspase 3 antibody. β -actin Western blot is shown as a loading control. (C) HIPK2 $^{+/+}$ or $^{--}$ MEF cells were transfected with TATA-luciferase or CRE4-luciferase and incubated for 24 h. Then cells were treated with 10 μM etoposide for 48 h and harvested for luciferase assay. Luciferase expression with each reporter without etoposide treatment was set to 1.0, and mean values from five independent experiments are shown; error bars, SEs. (D) 1×10^7 SH-SY5Y cells were transfected with nontargeting (siControl) or HIPK2-targeting (siHIPK2) siRNA and incubated for 24 h. Cells were then treated with 2 or 10 μM etoposide for 48 h for RNA isolation. BDNF exon III mRNA expression was analyzed with quantitative real-time PCR. BDNF mRNA expression in nontreated cells was set to 1.0; mean values from five independent experiments are shown; error bars, SEs.

poside as well as cisplatin activated HIPK2 (Figure 7A). We observed that HIPK2 $^{--}$ MEFs are more susceptible to etoposide-induced Caspase 3 activation (Figure 7B) and cytotoxicity observed by increased number of cells detaching from culture plates. To understand a physiological role of HIPK2 in genotoxic stress and CREB-dependent gene regulation, we asked whether a DNA-damaging agent activates CRE-dependent transcription and whether it is regulated by HIPK2. To address these questions, HIPK2 $^{+/+}$ or HIPK2 $^{--}$ cells (HIPK2 mRNA levels in Supplemental Figure S2) were transfected with CRE- or control- (TATA-) luciferase reporter, treated with 10 μM etoposide, and subjected to luciferase assay. In HIPK2 $^{+/+}$ MEF cells, etoposide activated CRE-dependent luciferase expression (9-fold), higher than the activation of the TATA-luciferase (4.5-fold). In contrast, HIPK2 $^{--}$ MEF cells showed much lower luciferase expression and no difference in CRE- or TATA-luciferase expression even after etoposide treatment (Figure 7C). These results suggest that etoposide treatment activated the CRE-dependent transcription through HIPK2. CREB and CBP bind the promoter of BDNF exon III and it was regulated by HIPK2 in SH-SY5Y cells (Figure 5). We then tried to verify in SH-SY5Y cells whether etoposide induces BDNF mRNA in a HIPK2-dependent manner by knocking down HIPK2 with siRNA. SH-SY5Y cells transfected with nontargeting siRNA (siControl) or HIPK2 siRNA (siHIPK2) were treated with 2 or 10 μM etoposide and BDNF mRNA levels were measured. Given $\sim 70\%$ knockdown of HIPK2 mRNA measured by RT-PCR (Supplemental Figure S2), etoposide-induced BDNF mRNA expression in siControl SH-SY5Y cells, but it was diminished in HIPK2-knockdown cells (Figure 7D). Collectively, these results suggest that HIPK2 regulates etoposide-induced CRE-dependent transcription, such as BDNF, that may be involved in cell survival against DNA damaging agents.

DISCUSSION

In this study we have demonstrated that HIPK2 is a CREB Serine 271 kinase and regulates CREB-dependent transcription in response to a DNA-damaging agent, etoposide. HIPK2 preferentially phosphorylates Serine/Threonine sites adjacent to Proline: Ser46-Pro47 in p53 (D'Orazi *et al.*, 2002; Hofmann *et al.*, 2002), Pro421-Ser422-Pro423 in CtBP (Zhang *et al.*, 2003), Pro296-Ser297 in Groucho (Choi *et al.*, 2005), Ser249-Pro250 and Ser276-Pro277 in AML1 (Aikawa *et al.*, 2006), and Thr281-Pro282, Thr304-Pro305, and Thr373-Pro374 in Pax6 (Kim *et al.*, 2006). The amino acid sequences containing CREB Serine 271 are Val-Val-Met-Ala-Ser-Ser271-Pro, which is highly conserved in ATF1 as Val-Val-Met-Thr-Ser198-Pro. Indeed, our preliminary results suggested that ATF1 at Serine 198 appears to be phosphorylated by HIPK2 (Hailemariam and Tsuji, unpublished observation). We observed retarded migration of phosphorylated CREB at Serine 271 by HIPK2 (Figures 1 and 2), similar to the retarded migration of Groucho, AML1, and Pax 6 phosphorylated by HIPK2 (Choi *et al.*, 2005; Aikawa *et al.*, 2006; Kim *et al.*, 2006), but not CREB phosphorylation at Serine 133 by PKA (Figure 2).

Protein phosphorylation has been shown to alter the stability of various transcription factors and coregulators including enhanced degradation of CtBP via Serine 422 phosphorylation by HIPK2 (Zhang *et al.*, 2003); however, CREB Serine 271 phosphorylation does not appear to affect the protein stability because CREB expression levels after HIPK2 overexpression looked unchanged, along with the results showing no difference in protein expression of wt-CREB and Ser271Ala mutant CREB when HIPK2 was coex-

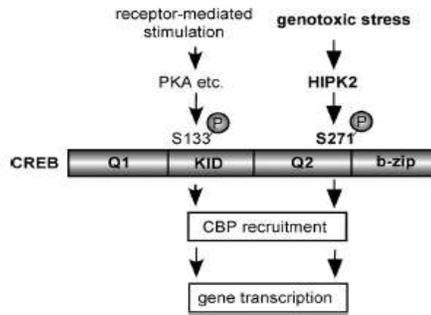


Figure 8. Regulation of CREB by HIPK2 in genotoxic stress. CREB is activated through phosphorylation of Serine 133 in the KID domain by PKA and related kinases in response to various receptor-mediated stimuli (Mayr and Montminy, 2001). This study demonstrated that genotoxic stress, such as etoposide, activates HIPK2, which in turn activates CREB through phosphorylation of Serine 271 adjacent to the Q2 domain and the b-zip domain of CREB. HIPK2 does not phosphorylate CREB at Serine 133. Phosphorylation of CREB at Serine 271 by HIPK2 increases CBP recruitment. CREB phosphorylation besides these two sites is discussed in the text and reviewed in Johannessen *et al.* (2004).

pressed for 24–48 h (Figure 2A). Phosphorylation affects protein localization such as the cases of mitogen-activated protein (MAP) kinases. We tested whether phosphorylation of CREB by HIPK2 facilitates the nuclear localization of endogenous and transfected CREB by Western blotting of cytoplasmic and nuclear fractions after HIPK2 overexpression. Our results showed that endogenous and transfected CREB proteins in K562, SH-SY5Y, and 293 cells almost exclusively localized in nucleus regardless of HIPK2 overexpression or the Serine 271 mutated CREB (Supplemental Figure S3). Phosphorylation of CREB at Serine 133 was reported to increase DNA binding to some CRE sites despite more contradictory results that indicate no impact on the DNA binding ability of CREB upon Serine 133 phosphorylation (reviewed in Shaywitz and Greenberg, 1999). We tested this possibility by ChIP assays for the CRE sequence in the exon III of the BDNF promoter or by gel retardation assays for binding to the consensus CREB sequence (TGACGCA; Figure 5, and Supplemental Figure S1), both of which suggested that CREB Serine 271 phosphorylation by HIPK2 does not modulate its DNA binding.

The transactivation function of CREB is regulated by interactions between CREB and its binding proteins. The interaction of CREB and CBP is primarily regulated by phosphorylation of CREB by PKA and other Serine 133 kinases, in which both the KID domain of CREB where Serine 133 is located and the KIX domain of CBP are involved in the interaction (Chrivia *et al.*, 1993; Figure 8). Our ChIP assay showed that phosphorylated CREB by HIPK2 increased or stabilized CBP recruitment to the CRE site in the BDNF exon III promoter (Figure 5). The CREB 271A mutant showed decreased association with CBP compared with wtCREB despite the equivalent binding to the CRE site (Figure 5). Serine 271 in CREB is located adjacent to the second glutamine-rich region (Q2/CAD) and the basic region (Figure 8), involved in constitutive activation of CREB and DNA binding, respectively (Shaywitz and Greenberg, 1999). It was demonstrated that phosphorylation of Serine 142 and 143 in the CREB KID domain during calcium influx into neurons decreased the binding of CREB to the KIX domain of CBP,

even though the CREB-dependent transcription was ultimately activated by the cooperative phosphorylation of CREB at Serine 133 by calcium signaling (Kornhauser *et al.*, 2002). Similarly, ionizing radiation/ATM-induced phosphorylation of CREB at Serine 121 in the KID domain decreased interaction between CREB and CBP, resulting in decreased CREB transactivation function (Shi *et al.*, 2004). These results suggest that additional phosphorylation events on CREB have the potential to alter interactions between CREB and CREB-binding proteins. In genotoxic stress and other yet uncharacterized conditions that activate HIPK2, cells may recruit the new mechanism of CREB activation and induction of CREB-target genes, that is through HIPK2-mediated phosphorylation of Serine 271 and facilitated interaction with CBP as demonstrated in this study (Figure 8). Furthermore, HIPK2 forms a complex with the coactivator p300 and AML1, phosphorylates p300 at multiple Serine/Threonine sites and activates p300 HAT activity and coactivator function (Aikawa *et al.*, 2006). It is therefore likely that HIPK2 activates transcription of CREB target genes in genotoxic stress conditions not only through the increased interaction of CREB-CBP but also activation CBP/p300 via direct phosphorylation. Further investigation will be necessary for understanding additional roles of HIPK2 in CREB-dependent gene transcription.

HIPK2 is expressed in various cell types, but its expression levels are relatively very low (Wang *et al.*, 2001). Despite our trials of almost all commercially available anti-HIPK2 antibodies, we failed to detect expression of endogenous HIPK2 protein by Western or immunoprecipitation/Western approach. Our results using HIPK2^{+/+} and ^{-/-} MEF cells showed that etoposide activated CRE-dependent transcription only in HIPK2^{+/+} cells (Figure 7C). Furthermore, HIPK2-deficient MEF cells were highly susceptible to etoposide toxicity (Figure 7B). We speculated that the higher susceptibility to etoposide toxicity in HIPK2-deficient cells was, at least in part, due to the lack of expression of CRE-dependent genes serving as cell survival. Particularly in neuronal cells, CREB is a crucial transcription factor for cell survival and adaptive response (Lonze and Ginty, 2002), and BDNF is a CREB-regulated gene that plays a vital role in neuronal cell survival, differentiation, and adaptive responses (Shieh *et al.*, 1998; Tao *et al.*, 1998). We measured mRNA expression of several CREB-target genes in SH-SY5Y cells after etoposide treatment, in which BDNF mRNA expression showed highest induction with 10 μ M etoposide within 24 h. The induction of BDNF mRNA by etoposide was 50% blocked by HIPK2 knockdown in SH-SY5Y cells (Figure 7D), suggesting that HIPK2 is involved in etoposide-induced BDNF mRNA expression. HIPK2 was demonstrated to be proapoptotic through p53 activation (D'Orazi *et al.*, 2002; Hofmann *et al.*, 2002) or CtBP degradation (Zhang *et al.*, 2003) while also serving as a prosurvival factor through Smads-regulated TGF- β -dependent gene expression in midbrain dopamine neurons (Zhang *et al.*, 2007). Cells may integrate these two opposing events or select either proapoptotic or prosurvival function of HIPK2, depending on the type of stress/environmental cues, magnitude of cell damage, and the type of cells and tissues.

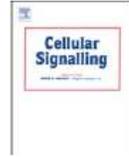
In summary, this work has demonstrated that genotoxic stress activates another pathway of CREB regulation, in which HIPK2 phosphorylates CREB Ser-271 (but not Ser-133) and activates CREB-dependent transcription such as the BDNF gene through increased interaction with the coactivator CBP (Figure 8). These results shed light on a new regulatory mechanism of CREB in genotoxic stress.

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Review

Reactive oxygen species (ROS) homeostasis and redox regulation in cellular signaling

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ABSTRACT

Reactive oxygen species (ROS) are generated during mitochondrial oxidative metabolism as well as in cellular response to xenobiotics, cytokines, and bacterial invasion. Oxidative stress refers to the imbalance due to excess ROS or oxidants over the capability of the cell to mount an effective antioxidant response. Oxidative stress results in macromolecular damage and is implicated in various disease states such as atherosclerosis, diabetes, cancer, neurodegeneration, and aging. Paradoxically, accumulating evidence indicates that ROS also serve as critical signaling molecules in cell proliferation and survival. While there is a large body of research demonstrating the general effect of oxidative stress on signaling pathways, less is known about the initial and direct regulation of signaling molecules by ROS, or what we term the "oxidative interface." Cellular ROS sensing and metabolism are tightly regulated by a variety of proteins involved in the redox (reduction/oxidation) mechanism. This review focuses on the molecular mechanisms through which ROS directly interact with critical signaling molecules to initiate signaling in a broad variety of cellular processes, such as proliferation and survival (MAP kinases, PI3 kinase, PTEN, and protein tyrosine phosphatases), ROS homeostasis and antioxidant gene regulation (thioredoxin, peroxiredoxin, Ref-1, and Nrf-2), mitochondrial oxidative stress, apoptosis, and aging (p66Shc), iron homeostasis through iron-sulfur cluster proteins (IRE-IRP), and ATM-regulated DNA damage response.

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1. Introduction

Reactive oxygen species (ROS), such as superoxide anion (O_2^-), hydrogen peroxide (H_2O_2), and hydroxyl radical (HO^\bullet), consist of radical and non-radical oxygen species formed by the partial reduction of oxygen. Cellular ROS are generated endogenously as in the process of mitochondrial oxidative phosphorylation, or they may arise from interactions with exogenous sources such as xenobiotic compounds.

When ROS overwhelm the cellular antioxidant defense system, whether through an increase in ROS levels or a decrease in the cellular antioxidant capacity, oxidative stress occurs. Oxidative stress results in direct or indirect ROS-mediated damage of nucleic acids, proteins, and lipids, and has been implicated in carcinogenesis [1], neurodegeneration [2,3], atherosclerosis, diabetes [4], and aging [5]. However, ROS involvement in the pathogenesis of disease states is not confined to macromolecular damage. There is increasing evidence that ROS signaling contributes to disease. For example, ROS have been shown to promote tumor metastasis through gene activation [6]. While there exists ample evidence demonstrating the role of ROS in

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regulating cellular signaling pathways, the question that is raised is exactly how do ROS initiate cellular signaling? The “oxidative interface” is that boundary between ROS and the signaling molecules they activate; that is, the figurative region that describes how ROS directly activate oxidative stress-responsive pathways. This review seeks to explore the oxidative interface between ROS and a functionally broad selection of cellular signaling pathways regulating a variety of cellular processes (Fig. 1).

In order to understand ROS regulation of signaling pathways, the mechanism of how ROS alters protein function should be briefly addressed. The oxidative interface consists mainly of the redox regulation of redox-reactive cysteine (Cys) residues on proteins by ROS. Oxidation of these residues forms reactive sulfenic acid ($-SOH$) that can form disulfide bonds with nearby cysteines ($-S-S-$) or undergo further oxidation to sulfinic ($-SO_2H$) or sulfonic ($-SO_3H$) acid; if nearby nitrogen is available sulfenic acid may also form a sulfenamide. These oxidative modifications result in changes in structure and/or function of the protein. With the exception of sulfonic acid, and to a lesser degree sulfinic acid, these redox modifications are reversible by reducing systems such as thioredoxin and peroxiredoxin [7] which are necessary given that these modifications function in redox sensing and signaling. For a more detailed overview of redox chemistry refer to Winterbourn [8] and Janssen-Heininger [9].

2. Regulation of MAPK signaling pathways by ROS

The mitogen-activated protein kinase (MAPK) cascades consist of four major MAPKs; the extracellular signal-related kinases (Erk1/2), the c-Jun N-terminal kinases (JNK), the p38 kinase (p38), and the big MAP kinase 1 (BMK1/Erk5). These kinases are evolutionarily conserved in eukaryotes and play pivotal roles in cellular responses to a wide variety of signals elicited by growth factors, hormones, and cytokines, in addition to genotoxic and oxidative stressors. The function and regulation of the MAPK cascades have been comprehensively covered [10–13]; therefore, this review article focuses solely on those protein kinases and phosphatases in the MAPK cascades that are directly regulated by ROS. MAPK pathways are composed of a three-ring kinase tier; MAPK kinase kinases (MAPKKK) phosphorylate and activate MAPK kinases (MAPKK), which in turn phosphorylate and activate MAPKs. Among the members of the MAPK cascades,

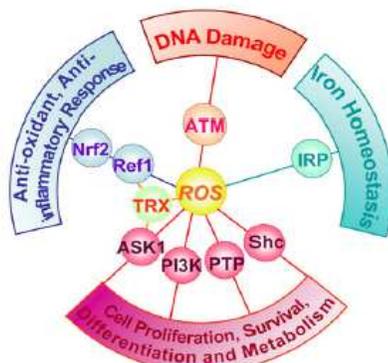


Fig. 1. Cellular signaling pathways regulated by ROS. Reactive oxygen species (ROS) regulate several signaling pathways through interaction with critical signaling molecules, affecting a variety of cellular processes, such as proliferation, metabolism, differentiation, and survival (apoptosis signal-regulated kinase 1 (ASK1), PI3 kinase (PI3K), protein tyrosine phosphatase (PTP), and Src homology 2 domain-containing (Shc)); antioxidant and anti-inflammatory response (thioredoxin (TRX), redox-factor 1 (Ref-1), and NFE2-like 2 (Nrf-2)); iron homeostasis (iron regulatory protein (IRP)); and DNA damage response (ataxia-telangiectasia mutated (ATM)).

apoptosis signal-regulated kinase 1 (ASK1) is an upstream MAPKKK that regulates the JNK and p38 MAPK pathways leading to apoptosis through phosphorylation of MKK4, MKK3, and MKK6 MAPKKs [14]. ASK1 is activated under various stress conditions including oxidative stress [15]. ASK1 is homo-oligomerized by both C- and N-terminal coiled-coil domain interaction and activation occurs through phosphorylation of a conserved threonine (Human: Thr-838, Mouse: Thr-845) residue in the activation loop of the human ASK1 kinase domain (Fig. 2A). There are many ASK1-associated proteins so far identified, among which the redox protein thioredoxin was shown to constitutively interact with ASK1 and directly inhibit its kinase activity [16]. Only the reduced form of thioredoxin interacts with ASK1; by blocking N-terminal, but not basal C-terminal interaction, thioredoxin inhibits complete ASK1 oligomerization and subsequent activation (Fig. 2A) [16,17]. ASK1 is activated when oxidants or ROS oxidize two cysteine residues in the redox center of thioredoxin, inducing formation of an intramolecular disulfide bond between Cys-32 and Cys-35 that results in the dissociation of thioredoxin from ASK1, allowing for the subsequent N-terminal homophilic interaction and complete oligomerization of ASK1 (Fig. 2A), which is enhanced by the binding of tumor necrosis factor- α receptor associated factors (TRAF) [16–18]. The ASK1 oligomer subsequently undergoes autophosphorylation of a conserved threonine residue (Human: Thr-838, Mouse: Thr-845) located in the activation loop of ASK1 (Fig. 2A) [19], which is inactivated by protein phosphatase 5 (PP5) [20]. In addition to homo-oligomerization of ASK1, ASK1 hetero-oligomerizes with ASK2, another ASK family serine/threonine MAPKKK (Fig. 2B). ASK2 binds to the C-terminal domain of ASK1, and this interaction stabilizes ASK2, resulting in autophosphorylation of ASK2 at the conserved threonine (Human: Thr-806, Mouse: Thr-807) in the activation loop. ASK1 is then phosphorylated at Thr-838 by ASK2, resulting in activation of the hetero-oligomer (Fig. 2B) [21]. ASK1-deficient mouse embryonic fibroblasts were shown to be less susceptible to TNF- or H_2O_2 -induced cytotoxicity along with decreased JNK and p38 MAPK activation, suggesting that ASK1 plays a pivotal role in promoting cell death under oxidative stress [15]; however, ROS activated ASK1 mediates p38 signaling leading to non-apoptotic outcomes also, such as differentiation [22] and immune signaling [23], thus reinforcing the role of ROS signaling in cellular homeostasis.

Evidence suggests that cGMP-dependent protein kinase (PKG) regulates MAPK activation [24,25]. It was demonstrated that PKG1 α is a redox sensor activated by ROS; oxidation of Cys-42 by H_2O_2 resulted in the formation of an active PKG1 α homodimer through intermolecular disulfide bond formation [26], though it is unknown whether ROS-activated PKG1 α regulates MAPK pathways. Similarly, protein kinase A (PKA) was shown to be activated by ROS through formation of intramolecular disulfide bonds [27], and protein kinase C (PKC) activity is also regulated through redox mechanisms [28]; both kinases have been implicated in MAPK signaling.

MAPK pathways are also activated by the direct inhibition of MAPK phosphatases by ROS. ROS produced by NADPH oxidases or in mitochondria have been shown to inhibit JNK-inactivating phosphatases [29] through reversible oxidation of a catalytic-site cysteine to sulfenic acid, thus sustaining JNK activation. Inhibition of phosphatases by ROS has also been shown to regulate p38 signaling [30,31], and it was recently demonstrated that ROS generated by commensal bacteria inactivated dual-specific phosphatase 3 (DUSP3) by oxidation of Cys-124, resulting in ERK activation [32]. DUSP is a protein tyrosine phosphatase (PTP); inactivation of classical PTPs such as protein tyrosine phosphatase 1B (PTP1B) or SH2-domain containing PTP (SHP2) by ROS in a similar cysteine redox mechanism (I/V-H-C-X-X-G-X-X-R-S/T) [33–35] ultimately potentiates MAPK and growth factor signaling pathways initiated from RTK, cytokines, and stressors [33,35–41]. Oxidation of the catalytic site cysteine in PTPs to sulfenic acid ($-SOH$) is reversible, as are disulfide bonds and sulfenamides, but further oxidation to the generally irreversible sulfinic acid

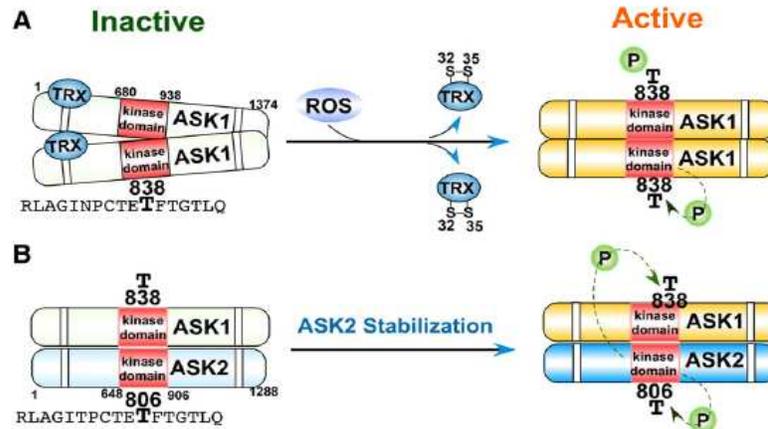


Fig. 2. Activation of ASK kinases in response to oxidative stress. A) Oxidation of thioredoxin (TRX) results in disulfide bond formation between Cys-32 and Cys-35 and subsequent dissociation from ASK1. ASK1 undergoes complete homo-oligomerization and subsequent autophosphorylation at Thr-838 located in the kinase domain. B) Hetero-oligomerization of ASK1 and ASK2 stabilizes ASK2, resulting in 1) the autophosphorylation of ASK2 at Thr-806, and 2) the subsequent phosphorylation of ASK1 at Thr-838 by ASK2.

($-SO_2H$) or sulfonic acid ($-SO_3H$) can also occur (Fig. 3). Thioredoxin or glutathione appears to be involved in reducing sulfenic acid residues and reversing the oxidative inactivation of PTPs [34]. The classical PTP family includes the ligand binding transmembrane receptor-like PTPs (RPTPs) such as RPTP α . Ligand binding to RPTP induces RPTP dimerization, which results in the catalytic-inactive conformation of RPTP [42]. ROS have been shown to inhibit human RPTP α tyrosine phosphatase activity through preferential oxidation of Cys-723 in the second catalytic domain of RPTP α rather than oxidation of Cys-433 in the first catalytic domain [43], leading to the formation of intermolecular Cys–Cys disulfide bonds as well as a reversible cyclic sulfenamide [44] and subsequent stabilization of the inactive RPTP α dimer [45]. Growth factor signaling events are frequently associated with production of ROS that are known to be important signaling molecules [46]. Oxidation and inhibition of PTPs by ROS appear to be one of the molecular mechanisms through

which growth factor-induced ROS production is essential for transducing and sustaining growth factor signals.

3. Regulation of PI3K signaling pathways by ROS

Another signaling pathway that plays a key role in cell proliferation and survival in response to growth factor, hormone, and cytokine stimulation is the phosphoinositide 3-kinase (PI3K) pathway. The PI3K, consisting of the p110 catalytic subunit and the p85 regulatory subunit, is tightly coupled with RTKs activated by various growth factors such as Epidermal Growth Factor (EGF), Platelet-Derived Growth Factor (PDGF), Nerve Growth Factor (NGF), insulin, and Vascular Endothelial Growth Factor (VEGF). PI3K is recruited to activate tyrosine-phosphorylated RTK dimers through a SH2 domain in the PI3K p85 regulatory subunit. PI3K catalyzes the synthesis of the second messenger phosphatidylinositol 3,4,5 triphosphate (PIP3) from

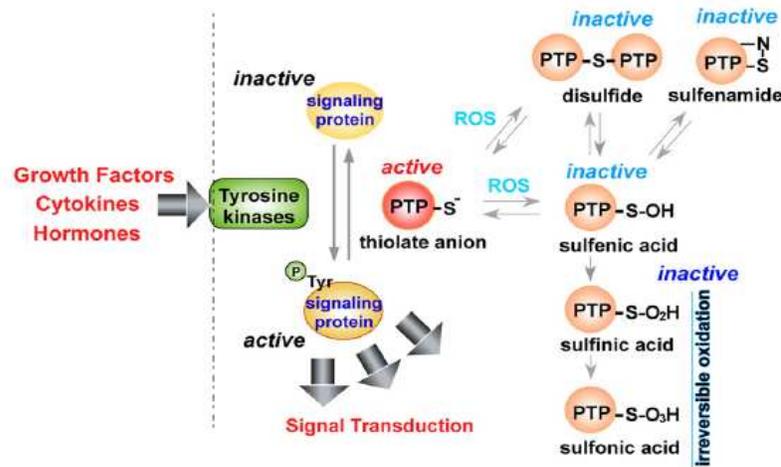


Fig. 3. Mechanism of ROS-mediated protein tyrosine phosphatase inactivation. Tyrosine kinases, activated by growth factors, cytokines, and hormones, phosphorylate target proteins. Phosphorylation can be reversed by protein tyrosine phosphatases (PTP); ROS inactivates PTP by oxidation of catalytic cysteine residues resulting in the formation of the sulfenic acid ($-SOH$) intermediate that can form disulfide bonds or sulfenamide residues. Further oxidation of sulfenic acid results in formation of sulfinic ($-SO_2H$) or sulfonic acid ($-SO_3H$), which are relatively irreversible.

phosphatidylinositol 4,5 bisphosphate (PIP₂), wherein the membrane bound PIP₃ serves as a signaling molecule to recruit proteins containing the pleckstrin homology (PH) domain. These PH domain proteins, such as the phosphoinositide-dependent protein kinase (PKD) and protein kinase B (AKT) serine/threonine kinases, are thus activated and mediate further downstream signaling events [47]. The synthesis of PIP₃ is negatively regulated primarily by the phosphatase and tensin homology (PTEN) phosphatase, which dephosphorylates PIP₃ back to PIP₂ [48]. Through PTEN, the PI3K pathway is subject to reversible redox regulation by ROS generated by growth factor stimulation. H₂O₂ was shown to oxidize and inactivate human PTEN through disulfide bond formation between the catalytic domain Cys-124 and Cys-71 residues [49,50]. It was also demonstrated that endogenously generated ROS following treatment with peptide growth factors such as insulin, EGF, or PDGF causes oxidation of PTEN leading to the activation of the PI3K pathway [51]. PTEN oxidation is reversed by peroxiredoxin II, a cytoplasmic peroxiredoxin isoform that eliminates H₂O₂ generated in response to growth factors [49]. Thus the PI3K pathway is regulated by ROS in a similar manner as the MAPK pathways; at the oxidative interface, protein phosphatases are directly oxidized by ROS resulting in sustained activation of the signaling pathways. It is noteworthy that various oxidants and ROS-producing chemicals activate transcription of a battery of antioxidant genes through a PI3K-NFE2-like 2 (Nrf2)-antioxidant response element (ARE) mechanism (Section 4), where PTEN knockdown enhances transcription of ARE-regulated antioxidant genes [52]; however, it is not known whether these oxidants induce PTEN oxidation and inhibition of phosphatase activity leading to gene activation.

4. Nrf2 and Ref-1-mediated redox cellular signaling

In order to prevent oxidative stress, the cell must respond to ROS by mounting an antioxidant defense system. Antioxidant enzymes play a major role in reducing ROS levels; therefore, redox regulation of transcription factors is significant in determining gene expression profile and cellular response to oxidative stress. Redox factor-1 (Ref-1) (Fig. 4A), identified as a 37-kDa protein that facilitates Fos-Jun DNA binding activity [53], was shown to be identical to an apurinic/aprimidinic (AP)-endonuclease named APE (AP endonuclease) [54] or human AP endonuclease 1 (HAP1) [55]. Thus Ref-1 is a multifunctional protein that not only regulates transcription factor activity, but also mediates base excision repair. The transcriptional regulatory function of Ref-1 is mediated through its redox activity on several transcription factors such as activator protein 1 (AP-1), p53, nuclear factor kappa B (NFκB), and hypoxia inducible factor 1 (HIF-1α) [56]. The N-terminus region of Ref-1 is responsible for redox activity while the AP-endonuclease activity domain is located at the C-terminal region (Fig. 4A) [57]. Cys-65 of Ref-1 appears to be a major redox active site (along with Cys-93) that is required for the reduction and increased DNA binding of targeted transcription factors [58]. Ref-1 activated the AP-1 transcription factor, Fos-Jun, through redox regulation of cysteine residues in the Fos-Jun DNA binding domains [53,59,60]. As shown in Fig. 4B, this cysteine is highly conserved in various human b-zip transcription factors, and except for CAATT enhancer binding protein (C/EBP) transcription factors, all may be regulated in a redox dependent manner by Ref-1, resulting in increased DNA binding and transcriptional activation of target genes. Indeed, it was demonstrated that this conserved cysteine in Nrf2 and cAMP response element binding (CREB) protein is subject to redox regulation. Site mutagenesis of Cys-506 interfered with mouse Nrf2-antioxidant response element (ARE) binding [61], in contrast to mutation of Cys-300/310 of CREB which increased DNA binding activity [61,62], demonstrating the importance of these redox regulated cysteine residues in transcriptional activity. Furthermore, Ref-1 was shown to be involved in the transcriptional activation of Nrf2-target genes under oxidative stress [63]. Oxidation and inactivation

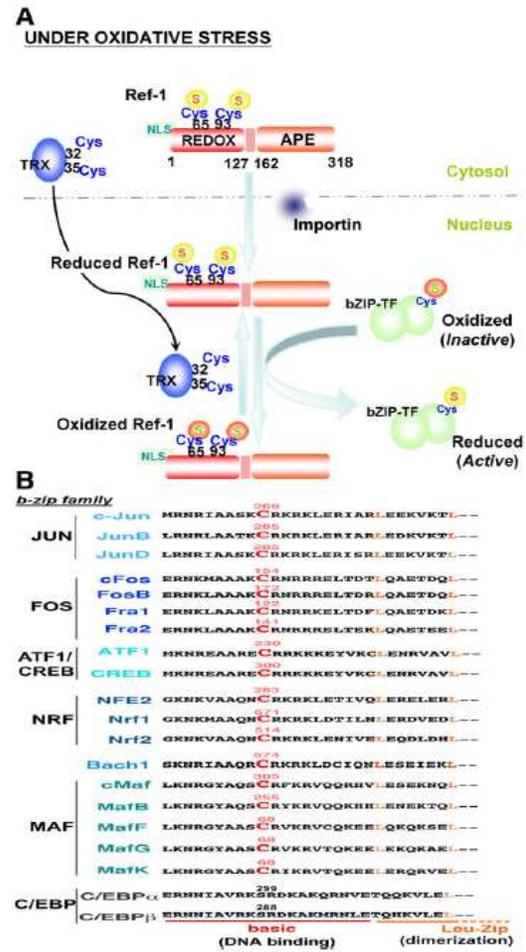


Fig. 4. Ref-1-mediated redox cell signaling. **A)** The human Ref-1N-terminal region consists of the redox domain (REDOX; residues 1–127) and a 20 amino acid nuclear localization sequence (NLS); the C-terminal region contains the apurinic/aprimidinic endonuclease domain (APE; residues 162–318). Cysteine-65 and -93 are major redox active sites. Under oxidative stress, Ref-1 translocates into the nucleus by NLS-importin-dependent or -independent pathways where Ref-1 regulates the activity of b-zip transcription factors (bZIP-TF) by redox mechanisms. Oxidized Ref-1 is subject to redox regulation by nuclear translocated thioredoxin (TRX) through the TRX catalytic center (Cys-32 and -35). **B)** The conserved redox-active cysteine of various human b-zip transcription factors is indicated in red color and labeled with the residue number. In addition to the b-zip family, other transcription factors such as p53, NFκB, and HIF-1α are also regulated by Ref-1 [56].

of b-zip transcription factors is not due to formation of intra- or intermolecular disulfide bonds, but probably the result of reversible oxidation of cysteine to sulfenic (–SOH) or sulfinic (–SO₂H) acids [59], though the possibility of an intermolecular disulfide bond between Cys-300 and Cys-310 in CREB cannot be entirely discounted [62]. It is generally accepted that reduction of Ref-1-targeted transcription factors results in oxidation of Ref-1 at Cys-65, possibly along with Cys-93 (Fig. 4A), although it has not been completely determined whether this oxidation event results in the formation of an intramolecular disulfide bond or the conversion to sulfenic or sulfinic acid [64].

The reduction of Ref-1 appears to be regulated by thioredoxin (Fig. 4A). In response to phorbol myristate acetate or ionizing

radiation, thioredoxin was shown to translocate into the nucleus and interact with Ref-1, resulting in the activation of AP-1 transcriptional activity under reducing conditions [65,66]. The interaction of thioredoxin with Ref-1 and the subsequent activation of Ref-1 target proteins appear to be regulated by the redox active Cys-32 and Cys-35 residues of thioredoxin [65–67] which are responsible for its reducing activity [68] (Fig. 4A). Ref-1 is localized in the nucleus as well as in the cytoplasm, depending on the cell type and physiological conditions. Cytoplasmic Ref-1 translocates to the nucleus under oxidative stress conditions such as H₂O₂ [69] and hypochlorous acid (HOCl) [70]. It was demonstrated that nuclear importins interact with the N-terminal 20 amino acid region of Ref-1, mediating nuclear translocation (Fig. 4A) [71]. It remains unknown how oxidants or ROS trigger nuclear localization of Ref-1, though it has been shown that nitric oxide induces nuclear export of Ref-1 by S-nitrosation of the redox sensitive Cys-93 residue [72]; p38 may also play a role in the nuclear translocation of Ref-1 via phosphorylation of Ser-54 [73]. These reports suggest that Ref-1 nuclear translocation by oxidants or oxidative stress responsive signaling pathways may occur through modifications of regions outside the accepted nuclear localization sequence of Ref-1.

Increasing the cellular antioxidant capacity by upregulation of antioxidant detoxification genes is critical in cellular adaptation to oxidative stress and protection from oxidative damage. Ref-1 was shown to be upregulated by genotoxic agents and oxidants, such as bleomycin and H₂O₂, and so protected cells from DNA and oxidative damage [70]. Ref-1 upregulation seems to be a reasonable adaptive response since Ref-1 mediates both DNA repair and the redox activation of key transcription factors involved in cellular defense, such as AP-1 and NFκB. However, under oxidative stress conditions, a group of antioxidant detoxification genes such as glutathione S-transferase (GST) [74], NADPH quinone oxidoreductase-1 (NQO1) [75], heme oxygenase-1 (HO1) [76,77], and ferritin H (FH) [78,79] are transcriptionally activated in an AP-1/NFκB-independent manner. These antioxidant genes are regulated by a highly homologous enhancer termed the antioxidant responsive element (ARE), or electrophile response element (EpRE), located ~0.5 kb–10 Kb upstream from transcription initiation sites of these genes [74–76,78–80]. The consensus core ARE sequence is TGA(C/T)nnnGCA [81] and the presence of two or more copies of the ARE in close proximity to each other often serves as a bona fide ARE [80]. Various endogenous and exogenous ROS-generating and electrophilic chemicals (such as H₂O₂ [82], lipid aldehydes [83], arsenic [84], tert-butylhydroquinone (t-BHQ) [82], hemin [63,82,85], and resveratrol [86]) activate transcription of these antioxidant genes via the ARE.

The primary transcription factor involved in ARE activation under oxidative stress conditions is NFE2-like 2 (Nrf2), a cap 'n' collar (CNC)-b-zip transcription factor (Fig. 5) [87]. Under non-stressed conditions, the majority of Nrf2 resides in the cytoplasm (though there is controversy as to the exact subcellular localization of Nrf2 [88]) and associates with a dimeric inhibitory protein, Kelch-like ECH-associated protein-1 (Keap1) [87]; Keap1 interacts with the cullin-3 E3-ubiquitin ligase (Cul3) and serves as a platform for the ubiquitination and resultant proteasomal degradation of Nrf2 (Fig. 5) [89,90]. Reactive mouse Keap1 cysteines (Cys-151, -273, and -288) [91,92] are redox sensors, and upon oxidation by ROS, results in the dissociation of Nrf2 from Keap1/Cul3 which allows Nrf2 stabilization and translocation into the nucleus. In the nucleus Nrf2 dimerizes with members of another b-zip family, the small Maf proteins (Maf-F, Maf-G, and Maf-K). The Nrf2–small Maf heterodimer binds the ARE enhancer and activates ARE-dependent transcription of target genes which serve as antioxidants and in processes such as electrophile detoxification, glutathione synthesis, and ROS homeostasis (Fig. 5) [80,93]. As shown in Fig. 4B, Nrf2 contains a conserved cysteine located in the DNA binding domain (Cys-514) which is the conserved site of Ref-1-mediated redox regulation. Indeed, mutation of mouse Nrf2 at Cys-506 (equivalent to Cys-514 of the human Nrf2) to Ser-506 affected binding to the ARE enhancer and decreased

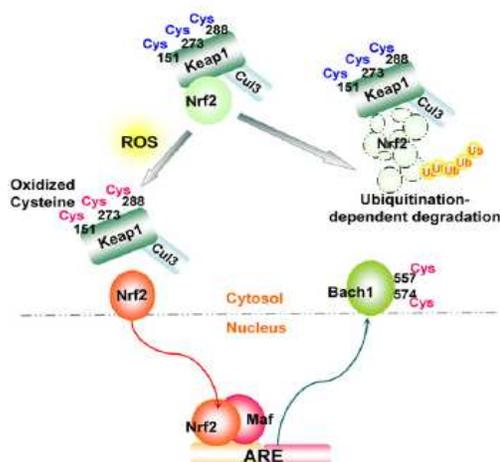


Fig. 5. Redox regulation of the Nrf2–ARE pathway. ROS oxidation of cysteines (Cys-151, -273 and -288) in the mouse Kelch-like ECH-associated protein-1 (Keap1) results in the release of Nrf2 from the Keap1/cullin-3 E3-ubiquitin ligase (Cul3) complex, preventing Nrf2 degradation. Nrf2 subsequently undergoes nuclear translocation. In the nucleus, a heterodimer of Nrf2 and small Maf members (Maf-F, Maf-G, and Maf-K) binds the antioxidant-responsive element (ARE); oxidation of a b-zip transcriptional repressor of ARE, the human BTB and CNC homolog 1 (Bach1) at Cys-557 and -574 results in cytoplasmic translocation of Bach1, both leading to activation of the ARE.

NQO1 expression [61]. It has also been demonstrated that Ref-1 nuclear localization and transcriptional activation of the ARE in the human ferritin H gene were increased following t-BHQ or hemin treatment [63], suggesting the possibility of Nrf2 redox regulation by Ref-1. Another example of possible redox regulation of Nrf2 was shown when mutation of the Cys-119 residue located in the transactivation domain of Nrf2 evidenced decreased binding to the NQO1 ARE [94].

Interestingly, the BTB and CNC homolog 1 (Bach1), a b-zip transcriptional repressor of the ARE [95], also features the Ref-1 associated conserved cysteine (Cys-574) (Fig. 4B) that is subject to redox regulation [96]. In this study, the sulfhydryl oxidizing agent diamide reversed Bach1-repressed ARE enhancer activity via Cys-574 oxidation (probably Cys-557 as well) leading to cytoplasmic translocation of Bach1 (Fig. 5) [96]. Nuclear export of Bach1 during ARE-dependent transcriptional activation of the NQO1 gene after t-BHQ treatment is also facilitated through phosphorylation of the mouse Bach1 at Tyr-486 (Tyr-483 in the human Bach1) by an undetermined tyrosine kinase [97]. Collectively, these results suggest that at least two sequential redox events, 1) the oxidation or adduct formation of Keap 1 in the cytoplasm and subsequent release and nuclear translocation of Nrf2, and 2) the redox regulation of Nrf2 and Bach1 in the nucleus, appear to be critical for maximum transcriptional activation of ARE-dependent antioxidant genes via the Nrf2 signaling pathway. Thus, through upstream redox regulators such as Ref-1, transcription factor and repressor activity is modulated indirectly through ROS, while the examples of Nrf2 and Bach1 demonstrate the direct regulation of transcription factors and repressors by ROS. Both direct and indirect control of transcriptional regulators illuminate the oxidative interface between ROS and ARE gene transcription.

5. Regulation of p66Shc, mitochondrial oxidative stress, and aging

ROS have been implicated in the process of aging. Given that the majority of endogenous ROS are generated in mitochondria [98], there has been much interest in the role that mitochondrial ROS may play in aging. Of note is the Shc adaptor protein family, encoded by the *shcA* locus in mammalian cells, consisting of the p66Shc,

p52Shc, and p46Shc isoforms (Fig. 6) [99–101]. Expression of p66Shc and p52/p46Shc isoforms is regulated by two different promoters [102] along with alternative translation initiation or splicing [99]. All isoforms share a phosphotyrosine binding domain (PTB), and a proline-rich collagen homology domain-1 (CH1) followed by a C-terminal Src homology 2 domain (SH2) (Fig. 6). p52Shc and p66Shc also share a cytochrome c binding domain (CB), while a second CH domain (CH2) is unique to pShc66 (Fig. 6). The homozygous *shcA* gene knockout mice lacking *shcA* exons 2 and 3 are embryonically lethal by E11.5 due to congestion of blood in the heart and cardiac outflow tracts [103]. The predominant expression of Shc proteins in the developing cardiovascular system indicates the importance of Shc proteins in the development of the heart and angiogenesis [103]. Among the three Shc isoforms, p52Shc and p46Shc are adaptor proteins involved in RTK signaling pathways through recruitment of the SH2 domain to phospho-tyrosines in the cytoplasmic domain of RTKs upon growth factor stimulation [99,103]. In contrast, p66Shc was revealed to play more predominant roles in mitochondrial ROS metabolism and oxidative stress response rather than serving as an RTK adaptor protein. Pelicci and colleagues demonstrated that p66Shc-deficient mice are not only more resistant to apoptosis under oxidative stress, but also have increased life span [104]. p66Shc-deficient mouse fibroblasts also showed decreased toxicity in response to oxidative stress compared with normal p66Shc fibroblasts and *in vitro* results suggest that phosphorylation of p66Shc at Ser36 is critical for stress-induced apoptosis [104]. Subsequently, a fraction of p66Shc was shown to localize in mitochondria as a high molecular mass protein complex containing heat shock protein 70 (HSP70), and a modest increase in mitochondrial p66Shc along with its dissociation from this large protein complex were observed after UVC or H₂O₂ exposure [105]. p66Shc mitochondrial translocation or proapoptotic activity may be regulated by posttranslational modifications such as phosphorylation of p66Shc at Ser-36 in the CH2 domain (Fig. 6) by PKC or JNK following exposure to UV or H₂O₂, and/or interaction with TOM-TIM protein import complexes [105–109], although it was previously noted that p66Shc in mitochondria is not serine phosphorylated [110].

Our current understanding is that p66Shc is a proapoptotic protein involved in ROS production in mitochondria leading to mitochondrial damage and apoptosis under oxidative or genotoxic stress conditions such as H₂O₂ or UV exposure. What is the initial and direct impact of oxidative or genotoxic stress on p66Shc and how is p66Shc activated? First, p66Shc protein levels in cytoplasm as well as in mitochondria appear to be increased under certain stress conditions [111,112] in addition to increased serine and threonine phosphorylation of p66Shc [100,101]. Although the molecular mechanism by which p66Shc expression is increased in response to stress signals remains largely uncharacterized, the Rac1 GTPase, which generates

ROS through activation of NADPH oxidase [113], was shown to block p66Shc ubiquitination and degradation through phosphorylation of p66Shc on Ser-54 and Thr-386 in a p38 dependent manner [114]. Furthermore, oxidative stress-activated PKC- β induces phosphorylation of p66Shc at Ser-36, which in turn triggers the interaction of the prolyl isomerase Pin1 with p66Shc, possibly inducing the isomerization of a p66Shc phospho-Ser36-Pro37 bond, resulting in the subsequent translocation of p66Shc into mitochondria [115]. Secondly, p66Shc was shown to be pro-apoptotic in mitochondria upon redox-dependent reversible tetramerization through formation of two disulfide bonds via Cys-59 in the N-terminus CH2 domain of p66Shc (Fig. 6), leading to copper-dependent ROS generation and initiation of apoptosis [106]. The active oxidized form of p66Shc is reversibly reduced by glutathione or thioredoxin leading to inactivation [106]. The molecular mechanism through which stress-activated p66Shc induces apoptosis has not been fully elucidated; however, p66Shc was shown to serve as a redox protein that produces H₂O₂ in mitochondria through interaction and electron transfer between p66Shc and cytochrome c [110], in which mutations in the redox center of p66Shc (E132–E133 to Q132–Q133 in the CB domain, Fig. 6) impaired opening of the mitochondrial permeability transition pore and thus negating the proapoptotic function of p66Shc [99]. It appears that p66Shc induces apoptosis through generation of ROS but also may be activated by ROS. Aging is associated with a decrease in mitochondrial function such as impaired oxidative phosphorylation that results in increased generation of ROS [116]. It is interesting to speculate whether increased ROS in this context would activate p66Shc to produce further ROS, resulting in apoptosis and maintaining the steady progression of aging. Regardless, p66Shc remains an interesting link between ROS and aging.

6. Regulation of the IRE-IRP system and iron homeostasis by ROS

Iron is an essential element that plays crucial roles in cell proliferation and metabolism by serving as a functional constituent of various enzymes including ribonucleotide reductase and cytochrome P450. However, when present in excess, free iron generates ROS via the Fenton reaction [117–119], placing cells under deleterious oxidative stress. Therefore, tight regulation of iron homeostasis is crucial not only to maintain normal cellular function, but also to prevent iron-mediated oxidative stress. The expression of many genes involved in iron transport and storage is regulated by iron itself at the post-transcriptional level in which iron regulatory protein-1 and -2 (IRP1 and IRP2) interact with an iron-responsive element (IRE) in the 5'- or 3'-untranslated region (UTR) of mRNAs such as, for example, ferritin (serving as intracellular iron storage) and transferrin receptor-1 (serving as iron transport into cells), respectively (Fig. 7). The IREs consist of a stem-loop structure composed of approximately 30 nucleotides with a 5'-CAGUG-3' loop to which cytoplasmic IRP1 and/or IRP2 binds and determines the fate of mRNAs. In general, IRP binding to IRE in the 5'-UTR of mRNA (e.g. ferritin) results in mRNA translational block and decreased protein expression while IRP binding to the 3'-UTR of mRNA (e.g. transferrin receptor-1) increases the stability of mRNA, increasing protein expression [117,120]. The amino acid sequence of IRP1 is highly homologous to mitochondrial aconitase, and IRP exhibits aconitase activity when forming a 4Fe-4S iron-sulfur cluster [121,122] and it is through this cluster that cellular iron levels control IRP1-IRE interaction. Under iron-rich conditions IRP1 retains the 4Fe-4S cluster (inactive IRP1) and therefore cannot bind to the IRE (Fig. 7). IRP2, on the other hand, is highly homologous to IRP1 but lacks an iron-sulfur cluster and has no aconitase activity. Furthermore, under iron rich conditions IRP2 is degraded via the proteasome pathway (Fig. 7). Thus, iron-rich conditions induce dissociation of IRPs from IREs, resulting in release of the translational block of ferritin mRNA and the destabilization of transferrin receptor 1 mRNA as well. As a result, iron-rich cells synthesize more ferritin for iron

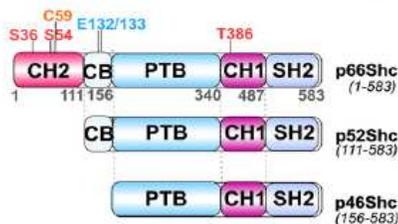


Fig. 6. Isoforms of p66Shc, p52Shc and p46Shc. Schematic representation of three isoforms produced from the human *ShcA* gene (CH1 and 2, proline-rich collagen homology domain-1 and 2; CB, cytochrome c binding domain; PTB, phosphotyrosine binding domain; and SH2, Src homology 2 domain); numbers define the regions of different domains according to human p66Shc amino acid sequence; Met-111 and -156 of p66Shc are equivalent to the first methionines of p52Shc and p42Shc. ROS-mediated phosphorylation sites are at Ser-36 and -54; Thr-386; pro-apoptotic residues are Cys-59 and Glu-132/133.

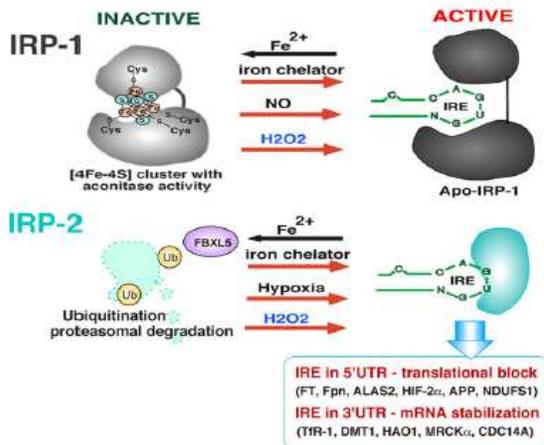


Fig. 7. Regulation of IRP-IRE interactions. Under iron rich conditions, IRP1 contains a [4Fe-4S] cluster and is unable to bind to the IRE, though loss of iron from the cluster (destabilization) under iron deficient conditions allows IRP1 to bind to the IRE. IRP2 does not contain a [4Fe-4S] cluster and is degraded by F-box/LRR-repeat protein 5 (FBXL5)-dependent ubiquitination. Iron chelators, nitric oxide (NO), hypoxia, and hydrogen peroxide (H₂O₂) increase IRP/IRE interaction. H₂O₂ destabilizes the [4Fe-4S] cluster of IRP1 and also stabilizes IRP2 protein by preventing FBXL5-dependent ubiquitination. Increasing IRP-IRE interaction in 5'UTR results in translational block of ferritin (Ft), ferroportin (Fpn), aminolevulinic acid synthase-2 (ALAS2), hypoxia inducible factor-2 α (HIF-2 α), amyloid precursor protein (APP), and NADH dehydrogenase (ubiquinone) Fe-S protein 1 (NDUFS1) genes, but in the 3'UTR it results in mRNA stabilization of transferrin receptor (TfR), divalent metal transporter 1 (DMT1), hydroxyacid oxidase 1 (HAO-1), myotonic dystrophy kinase-related Cdc42-binding kinase alpha (MRCK α), and CDC14 cell division cycle 14 homolog A (CDC14A).

storage/detoxification and less transferrin receptor-1 to halt iron transport into the cells, ultimately reducing excess intracellular iron. In contrast, iron-deficient conditions facilitate the disassembly of

the 4Fe-4S cluster in IRP1 while stabilizing IRP2, allowing IRP1 and IRP2 to bind IREs in 5'-UTR ferritin mRNA (translational block) and 3'-UTR transferrin receptor-1 mRNAs (mRNA stabilization). As a result, iron-deficient cells produce less ferritin (decreasing iron storage capacity) and more transferrin receptor-1 (increasing iron transport) to maintain iron homeostasis [117,120]. In the last decade several new iron transport and metabolism genes such as Divalent Metal Transporter 1 (DMT1) and ferroportin (Fpn) [117] were discovered. The identification of IREs present in the 5'- or 3'-UTR of Fpn and DMT1 mRNAs strengthens the view of the IRE-IRP regulatory system as the primary post-transcriptional mechanism of the majority of iron metabolism genes tightly regulated by iron (Fig. 7). In addition, other genes are regulated by the IRE-IRP system, such as NADH dehydrogenase (ubiquinone) Fe-S protein 1 (NDUFS1) [123], Alzheimer's amyloid precursor protein (APP) [124], hydroxyacid oxidase 1 (HAO-1) [125], myotonic dystrophy kinase-related Cdc42-binding kinase alpha (MRCK α) [126], cell division cycle 14 homolog A (CDC14A) [127], delta-aminolevulinic synthase 2 (ALAS2) [128], and hypoxia inducible factor-2 alpha (HIF-2 α) [129] (Fig. 7).

The fact that IRP1 contains a 4Fe-4S cluster implies that it may be subject to redox regulation. Indeed, H₂O₂ was shown to convert, or destabilize, the 4Fe-4S cluster of IRP1 (inactive) to a 3Fe-4S cluster (active) (Fig. 7) through loss of a single iron [122], and ferritin protein expression was transiently downregulated after H₂O₂ exposure [78,130] through increased IRP1 binding to the IRE, though followed by upregulation of ferritin by transcriptional activation of the ferritin gene via the ARE [78]. The question that arises is whether increased IRP1-IRE binding is the direct effect of H₂O₂ on the 4Fe-4S cluster. When IRP1 was directly incubated with H₂O₂ in vitro, there was no increase in IRP1 binding to IRE [122,130], suggesting that destabilization of the 4Fe-4S cluster is not sufficient for IRP1 binding to IREs in response to H₂O₂, implying that H₂O₂ activates an alternate signaling pathway leading to additional posttranslational modifications of IRP1 for increased IRE binding. Nitric oxide (NO) was also found to increase IRP1 binding to the IRE through destabilization of the 4Fe-4S cluster [131,132] (Fig. 7). The redox-regulated PKC was shown to

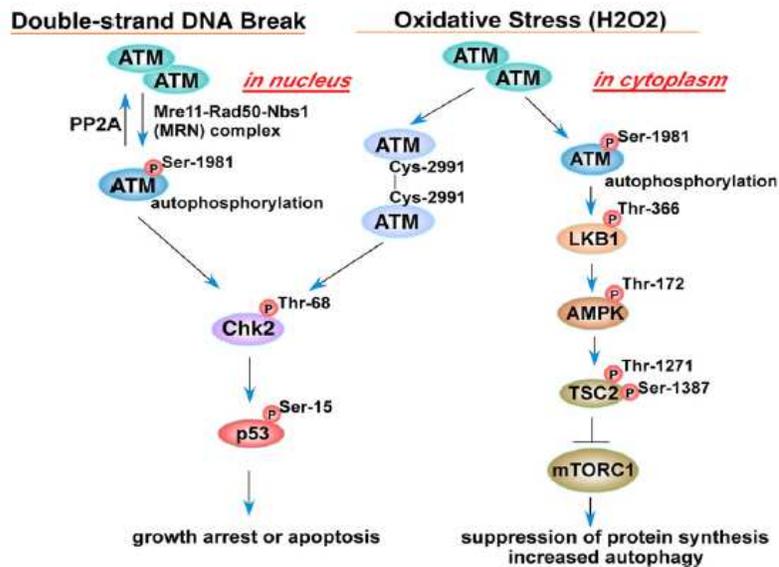


Fig. 8. Schematic of ATM signaling upon oxidative stress and double-strand DNA breaks. Double-strand DNA breaks mediate the phospho-ATM-Chk2 pathway; however, oxidative stress elicits both the phospho-ATM-LKB1 and the ATM-homodimer pathways (ataxia-telangiectasia mutated (ATM); liver kinase B1 (LKB1); AMP activated protein kinase (AMPK); tuberous sclerosis complex 2 (TSC2); mammalian target of rapamycin complex1 (mTORC1)).

phosphorylate IRP1 at Ser-138 [133], and it was later demonstrated that phosphorylation of Ser-138 results in destabilization of the 4Fe–4S cluster and increases IRP1 binding to IRE [134]. Unlike IRP1, IRP2 does not contain a Fe–S cluster and its binding to IREs is primarily decreased in iron-rich cells through iron-dependent proteasomal degradation mediated by F-box/LRR repeat protein 5 (FBXL5) [135] (Fig. 7), resulting in downregulation of iron transporting proteins (destabilization of such mRNAs as TfR1 and DMT1) and upregulation of iron storage and export proteins (release of translational block of such mRNAs as ferritin and ferroportin) [136]. It has recently been demonstrated that IRP2 is subject to redox regulation, in which oxidative stress caused by glucose deprivation in HEK293 cells induced oxidation of Cys-512 and Cys-516 in IRP2 that in turn decreased IRP2 binding to IREs [137], and decreased IRE binding ability of IRP2 was correlated with decreased transferrin receptor-1 expression that may allow cells to limit iron transport and hindering subsequent iron-mediated ROS production. However, in contrast, a recent report demonstrated that ROS increased IRP2–IRE binding [138] in addition to protecting IRP2 from iron-mediated degradation, an effect similar to that shown under hypoxic conditions [139] (Fig. 7).

Taken together, the IRE–IRP regulatory system is not only regulated by cellular iron status but also regulated by ROS, in which cells elicit a defense mechanism against iron toxicity and iron-catalyzed oxidative stress.

7. ROS and DNA-damage response

Ataxia-telangiectasia mutated (ATM) and Ataxia-telangiectasia and Rad3-related (ATR) are PI3K-like serine/threonine protein kinases activated under genotoxic stress conditions and phosphorylate various proteins involved in cell proliferation, cell death and survival, and DNA repair [140,141]. These two signaling proteins were initially thought to be activated by a particular type of DNA damage therefore serving in parallel signaling pathways; however, accumulating evidence suggests that the ATM- and ATR-pathways communicate and cooperate in response to DNA damage [141]. ATM, preferentially activated by DNA double strand breaks, has been shown to serve as a sensor of oxidative stress in which ATM-deficient cells were more susceptible to oxidative stress-inducing agents as well as DNA damaging agents [142]. However, it has recently been demonstrated that the molecular mechanisms of the activation of ATM by DNA damage and oxidative stress are different. Upon double strand DNA break induction by agents such as bleomycin, cells recruit the Mre11–Rad50–Nbs1 (MRN) complex to damaged sites together with ATM, which in turn triggers autophosphorylation of ATM at Ser-1981 and activates ATM protein kinase activity leading to phosphorylation of downstream signaling proteins such as checkpoint kinase 2 (Chk2) at Thr-68 and p53 at Ser-15 (Fig. 8). Phosphorylation of ATM at Ser-1981 and its kinase activity are reversibly regulated by protein phosphatase 2A (PP2A) [143]. Cells exposed to H₂O₂ also feature ATM activated via Ser-1981 phosphorylation [144–146], although Guo et al. showed that H₂O₂ activates ATM in an MRN/Ser-1981 autophosphorylation-independent manner (Fig. 8) based on their results that 1) ATM was activated by H₂O₂ equivalently in both wild type and mutant Mre11 cells, and 2) H₂O₂ activated both wild type and Ser-1981 to alanine mutant purified dimeric ATM in vitro [144]. Noncovalently associated dimeric (non-active) ATM is known to be dissociated into active monomers in response to DNA damage; however, Guo et al. showed that purified ATM protein incubated with H₂O₂ in vitro migrated slower in SDS-PAGE due to formation of covalent dimers that were sensitive to reducing agents, and given the fact that N-acetyl-cysteine (NAC) blocked ATM activation induced by H₂O₂ in vitro [144], these results suggest that H₂O₂ activates ATM through formation of active ATM dimers via intermolecular disulfide bond(s). Further characterization demonstrated that Cys-2991, located near the kinase domain of human ATM, is primarily involved in the disulfide bond formation and oxidative activation

of ATM (Fig. 8) [144]. It is noteworthy that a C2991A ATM mutant was fully activated by the MRN–DNA complex but not by H₂O₂ in vitro [144]. Thus H₂O₂ and possibly other ROS, elicit ATM activation not through the DNA damage and MRN mediated pathway, but directly by ATM dimer formation via Cys-2991 oxidation and intermolecular disulfide bridge formation (Fig. 8).

The fact that ATM deficient cells accumulate ROS and are sensitive to oxidative damage [142] suggests that ATM is crucial to a cellular oxidative stress defense program. What downstream signaling events are regulated by activated ATM in response to ROS? One clue to address this question has recently been presented [145] in which cytoplasmic ATM autophosphorylated at Ser-1981 in response to oxidative stress activates a liver kinase B1 (LKB1)–AMP activated protein kinase (AMPK) cascade (Fig. 8). Autophosphorylated cytoplasmic ATM activates LKB1 via phosphorylation of Thr-366, which activates AMPK through Thr-172 phosphorylation. Activated AMPK in turn activates the tuberous sclerosis complex 2 (TSC2) tumor suppressor protein via phosphorylation at Thr-1271 and Ser-1387, leading to inhibition of mammalian target of rapamycin complex 1 (mTORC1), thereby suppressing protein synthesis and inducing autophagy under oxidative stress (Fig. 8) [145]. The activation of autophagy through this pathway may be a cellular defense mechanism in response to ROS.

8. Conclusions

The disease states in which ROS signaling and toxicity have been implicated are areas of intensive research in regards to prevention and therapy. Unveiling the molecular mechanisms of disease pathogenesis and progression is therefore essential in providing relevant targets in order to develop innovative therapeutic strategies. In this context it is worthwhile not only to investigate ROS signaling in disease, but also to reveal how ROS instigate cellular signaling under homeostatic conditions. Having a clear understanding of how ROS directly regulate signaling pathways that are found to play a key role in the pathogenesis and progression of disease will allow us to understand how ROS may cause or contribute to disease and uncover new therapeutic targets. For example, ROS regulates proliferative and apoptotic pathways, and aberrant regulation of proliferation and apoptosis is essential in tumorigenesis; therapeutic strategies exploiting the role of ROS in those pathways are being developed [1,147]. While therapeutic development has primarily been concerned with reducing ROS levels to prevent toxicity, as in neurodegeneration [148], atherosclerosis, and diabetes [149], it remains to be seen whether targeting the redox sensitive molecules and signaling pathways activated by ROS will produce viable therapeutics in the prevention and alleviation of ROS-mediated disease states.

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