KIROS HAILEMARIAM. HIPK2 is a Novel ATF1 Kinase and Regulates Transcription of the Human Ferritin H Gene Through an Antioxidant Responsive Element. (Under the direction of Dr. Yoshiaki Tsuji.)

Iron is required for normal cell growth and proliferation. However, excess iron is potentially harmful, as it can catalyze the formation of reactive oxygen species via the Fenton reaction and induce oxidative stress. Ferritin, the ubiquitous iron storage protein, is activated in response to pro-oxidants and is thought to serve a protective function in iron catalyzed oxidative damage. We previously demonstrated that oxidative stress-activated transcription of the ferritin H gene occurs through the antioxidant responsive element (ARE). We have identified Activating Transcription Factor 1 (ATF1) as one of binding proteins to the ferritin H ARE; however, whether ATF1 has a role in ferritin H regulation was unknown. The purpose of this study was to elucidate the molecular mechanisms involved in the transcriptional regulation of the human ferritin H gene. In transient transfection assay, ATF1 repressed the human ferritin H promoter reporter through the ARE. Using yeast two-hybrid method, we identified the nuclear serine-threonine protein kinase, homeodomain interaction protein kinase 2 (HIPK2), as an ATF1 binding protein. Wild type HIPK2 overrode ATF1-mediated ferritin H promoter reporter repression, while the kinase dead HIPK2 failed to do so. Moreover, HIPK2 phosphorylated ATF1 in vivo as well as in vitro. HIPK2 phosphorylated ATF1 on a residue other than Ser63 indicating that it phosphorylates ATF1 on a novel site. UVB irradiation of human keratinocytes
activated HIPK2 and up-regulated human ferritin H mRNA in a time-dependent manner. Similar results were also obtained from human breast carcinoma cells irradiated with UVB. Our results suggest that ATF1 phosphorylation by HIPK2 is important for the transcriptional activation of the ferritin H gene. Taken together, these results demonstrate that HIPK2 phosphorylates ATF1 on a novel site and modulates ATF1-mediated repression of the human ferritin H gene.
HIPK2 IS A NOVEL ATF1 KINASE AND REGULATES TRANSCRIPTION OF THE HUMAN FERRITIN H GENE THROUGH AN ANTIOXIDANT RESPONSIVE ELEMENT

by

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DEDICATION

This work is dedicated to my lovely and strong wife, Tsehay Berhe and my son Naode Kiros as well as to our beloved mother Silas Gebrekidan, to my parents, my beloved mother, Fitsumbirhan Mezgebe and my beloved father, Hailemariam Fanta.
BIOGRAPHY

Kiros Hailemariam was born on May 12, 1962 in Adua, Ethiopia, where he went through high school and college. He graduated from Addis Ababa University, Alemaya agricultural college, Ethiopia, in 1984 with a Bachelor of Science degree in plant sciences. He worked for the Ethiopian ministry of agriculture as an agronomist and extension officer from 1984-1987. However, due to the political turmoil and instability, at the time, he fled Ethiopia and took refuge in a neighboring country before getting resettled here in the U.S.A in 1990. He attended North Carolina State University as a post-baccalaureate student before joining the graduate school in Toxicology program at the North Carolina State University in 2001, and has been working toward a Ph. D under the direction of Dr. Yoshiaki Tsuji.
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GENERAL INTRODUCTION

Functions and Effects of Iron

Iron is an essential element required for normal cell growth and proliferation. It is a required constituent of numerous enzymes, including iron-sulphur and heme proteins of the respiratory chain, as well as ribonucleotide reductase, which catalyzes the rate-limiting step in DNA synthesis. Excess iron is sequestered by ferritin in a nontoxic and readily available form in cells. Iron is necessary for the transport, binding and release of oxygen; hence the ready availability for incorporation of iron to heme is essential to the survival of organisms.

However, excess iron is potentially harmful as it catalyzes the formation of the highly toxic hydroxyl radical (104) that damages DNA, lipids, and proteins, and has been implicated in the etiology of diseases such as cancer and aging (2). Redox cycling is a characteristic of transition metals such as iron. Iron donates electrons for the generation of the superoxide radical, and can participate in the generation of hydroxyl radical via the Fenton reaction (Fe (II) + H2O2 $\rightarrow$ Fe (III) + OH- + OH.) (131). Iron-catalyzed oxidative stress is believed to be the main mechanism involved in the pathogenesis of iron-induced cancer (140). Excess iron accumulation within tissues, cells and organelles, can result in toxicity and is associated with pathological disorders. In addition to the disorders such as genetic haemochromatosis and thalassaemias causing iron overloading (24), there are some diseases associated with excess iron. Iron accumulation in the brain has been associated with Parkinson’s disease, Alzheimer's disease, Huntington chorea and HIV
encephalopathy (119). Furthermore, excess mitochondrial iron accumulation in brain and cardiac tissues has been associated with Friederich ataxia (69).

**Ferritin Structure and Function**

Ferritin is a ubiquitous and highly conserved iron-sequestering protein. It is composed of two subunits, termed H (heavy) and L (light). Twenty-four ferritin subunits constitute the appoferritin shell, each of which can sequester up to 4500 iron atoms (52). The ratio of H to L subunits in ferritin can vary depending on the tissue type and physiological status of the cell, with L subunit being highly prevalent in such tissues as liver and spleen and the H subunit abundant in heart and kidney (5) (14) (20) (75). The H subunit has a molecular weight of 21 kDa and has a relatively acidic electrophoretic mobility, whereas the L subunit is a smaller protein with a molecular weight of 19 kDa (135). Since ferritin H can bind to and release iron more readily than ferritin L, it is generally believed that ferritin H plays a key role in rapid detoxification of iron and intracellular iron transport. Ferritin H functions as a ferroxidase that oxidizes Fe (II) to the Fe (III) form, whereas ferritin L is associated with iron nucleation, mineralization and long-term storage (52) (120).

Ferritin H and L subunits are encoded by separate genes (154) (23). Multiple copies of ferritin H and L genes have been discovered, but most of them turned out to be intronless pseudo genes (62) (79) (30). A human mitochondrial ferritin encoded by an intronless gene has been identified (82). Although it is believed that mitochondrial ferritin might have a role in regulating mitochondrial iron homeostasis and heme synthesis, its significance in the overall iron homeostasis is largely unknown.
Ferritin synthesis is regulated at both transcriptional and translational levels (138). While the translational regulation of ferritin by iron through the interaction of iron regulatory protein (IRP) and iron response element (IRE) in the 5’-untranslated region of both ferritin H and L has been well studied, the mechanism by which transcription of ferritin is regulated is poorly understood.

The following is an attempt to give a general overview of how ferritin synthesis is regulated under different oxidative stress conditions.

**Ferritin Regulation by Iron**

Ferritin plays an essential role in the maintenance of cellular iron homeostasis by sequestering intracellular iron in a nontoxic form. Hence, the ability of cells to quickly synthesize ferritin will prevent damage to vital macromolecules stemming from excess iron. The amount of cytoplasmic ferritin is controlled by the translation of ferritin H and L mRNAs in response to an intracellular pool of chelatable or labile iron (7). When iron is depleted, ferritin synthesis is decreased; conversely when in iron replete state, ferritin synthesis increases.

Ferritin is regulated by intracellular iron at the translational level. However, the transcriptional regulation of ferritin is less understood. The translational control of ferritin synthesis involves a unique stem-loop structure, called the iron responsive elements (IREs), in the 5’ untranslated region (UTR) of ferritin H and L mRNA. Two RNA binding proteins (IRPs) termed, iron regulatory proteins 1 and 2 (IRP 1 and 2) that recognize and bind the IRE inhibit mRNA translation. The IRP protein themselves are regulated differently. IRP1 exists as a bi-functional protein that functions either as a cytosolic aconitase when iron is abundant or as an RNA-
binding protein when iron is scarce (71). Unlike IRP1, IRP2 is regulated by proteasomal degradation. It lacks an iron-sulfur cluster and contains an additional 73 amino acid motif (49) that eventually leads to its degradation. Cellular iron homeostasis is accomplished by the coordinately regulated expression of the transferrin receptor and ferritin, which mediate iron uptake and storage respectively. Iron regulatory proteins also bind the 3’ untranslated region (UTR) of transferrin receptor (TfR) mRNA and increase the half-life of mRNA, ultimately leading to increased transferrin receptor display on the cell surface in situations of iron starvation (51).

Iron may also regulate ferritin H expression through the myc oncogene family of transcription factors. Deferoxamine was shown to decrease N-myc expression in neuroblastoma cells in an iron-specific manner (42). In a study of multiple gene expression using gene array, Alcantra O. et al demonstrated that iron chelation by deferoxamine decreased c-myc expression (1). In turn, c-myc suppresses ferritin H expression and increases IRP2 levels (155), which increases the labile iron pool. Moreover, IRP2 binds to IREs in the transferrin receptor RNA, conferring it stability and preventing its degradation resulting in iron import. This suggests that down regulation of ferritin H may be required for cell transformation by c-myc.

**Ferritin Regulation by Cytokines**

In contrast to translational mechanism of ferritin genes by iron, transcriptional regulation of ferritin is less understood. Ferritin is also regulated by cytokines both transcriptionally and posttranscriptionally during development, cellular differentiation and proliferation and inflammation. Cytokines have been shown to play an important
role in cellular response to infection and to induce the expression of ferritin genes. Tumor necrosis factor alpha and interleukin-1 alpha separately or together have been demonstrated to induce the expression of ferritin H gene at the transcriptional level in mouse TA1 adipocytes, MRC5 cells, human muscle cells and other cell types (139) (145) (152). In U937 macrophage cell line, tumor necrosis factor alpha and interferon gamma increased ferritin H mRNA (41), and in A549 cells ferritin H mRNA was up-regulated by both tumor necrosis factor alpha and interleukin 1 beta in response to iron treatments (127). The responsiveness of ferritin H induction by tumor necrosis factor- alpha is conferred by NF-kB/Rel binding site about 4.8 kb upstream of the transcriptional site (77).

Ferritin has been demonstrated to be regulated by cytokines at a posttranscriptional level. In HepG2 cells, IL-1beta, IL-6 or TNF alpha induced translation of ferritin (98) (141) and iron was needed for this regulation as this increase in ferritin was inhibited by deferoxamine (56) (116). Moreover, a study by Pinero D. J. and colleagues suggested that IL-1 beta increases ferritin accumulation posttranscriptionally in human astroma cells, hence, minimizes the labile iron pool (112).

Cytokines may also have an effect on ferritin translation through the induction of nitric oxide synthase (iNOS) and the resultant increase in nitric oxide (39) (153). Nitric oxide subsequently triggers the activation of both IRP1 and IRP2 and thereby cause the translational repression of ferritin mRNA.
Ferritin and Oxidative Stress

Molecular oxygen is essential for the survival of all aerobic organisms. Oxygen serves as a final electron acceptor in a mitochondrial electron transport chain during energy metabolism and becomes partially reduced during the electron transfer reactions. Highly reactive metabolites including superoxide anion and hydrogen peroxide are formed by one- and two- electron reduction of oxygen, respectively (46). In the presence of transition metal ions such as iron (in a Fenton reaction), the even more reactive hydroxyl radical (OH.) can be formed. These partially reduced metabolites of oxygen are referred to as reactive oxygen species (ROS) due to their higher reactivities relative to molecular oxygen. Thus, oxidative stress results from metabolic reactions that use oxygen, and represents a disturbance in the equilibrium status of pro-oxidants/antioxidants reactions in living organisms.

To protect against the potentially damaging effects of ROS, cells possess several antioxidant enzymes such as superoxide dismutase, which reduces superoxide anion radical to hydrogen peroxide (H$_2$O$_2$); catalase and glutathione peroxidase, which reduce H$_2$O$_2$ to H$_2$O. Due to its ubiquitous prevalence, glutathione acts as a homeostatic redox buffer. Ferritin molecule traps iron cations and thereby limits the deleterious effect of Fenton reaction. Therefore, ferritin is not only a part of a group of iron regulatory proteins that includes transferrin and transferrin receptor, but is also an integral part of the battery of phase II detoxification genes including glutathione S-transferase and NADPH quinine oxidoreductase-1 (NQO1). These enzymes are induced as part of a concerted response to oxidative stress. Induction of these genes, in response to diverse groups of xenobiotics and electrophilic
compounds, is regulated at a transcriptional level and is mediated by a specific enhancer element known as the antioxidant responsive element (ARE) located in the 5’ region of the promoter of the genes (53) (63) (122). The functional ARE sequence essential for the activation of these genes, which was determined through deletion and mutational analysis, is composed of a conserved core AP1-like 5’-TGACnnnGCA 3’ motif (122). Several studies have demonstrated that the basic-leucine zipper (b-Zip) transcription factors, NFE2-related factor 2 (Nrf2) and small Maf proteins (Maf K and Maf G) differentially regulate the ARE enhancer activity. Nrf2 was shown as intimately involved in activating the ARE, whereas the small Maf proteins as having a repressive effect on the ARE activity. (96) (149) (36).

Ferritin is regulated by oxidative stress through gene expression. Hydrogen peroxide (H$_2$O$_2$) and tert-butylhydroquinone (t-BHQ) were shown to transcriptionally activate ferritin H gene by directly targeting the antioxidant responsive element (ARE) that is located upstream of the transcription initiation site (143). Transcriptional activation of ferritin H and L genes was also observed in rat livers after injection with phorone, which reduces glutathione concentration and therefore limits free radical defense mechanisms (22). Ferritin expression is also translationally regulated by oxidative stress via IRPs. However, the response of both IRP1 and IRP2 to oxidative stress and their contribution to ferritin induction differ. Some studies observed that oxidative stress did increase ferritin translation by inactivating IRP1 through reversible oxidation of critical cysteine residues (21). Other studies, on the other hand, showed that H$_2$O$_2$ activated IRP1, possibly through direct disassembly of 4Fe-4S cubane cluster (103). This results in the activation of IRP1
and thus enhancing IRE-binding activity and repression of ferritin biosynthesis, potentially leaving the cells more susceptible to oxidative damage.

Ferritin H gene transcriptional control is regulated by distinct enhancer element known as antioxidant responsive element (ARE). The ARE is a highly conserved element in many phase II detoxification or antioxidant genes (113) (102). In mouse this sequence is located 4.1 Kb upstream of the ferritin H promoter region and contains an AP-1 like element. In addition, there is a second ARE in antisense orientation that contains an AP-1/NFE2 site. The basal element FER-1 contains part of the AP-1 like sequence and GC rich region dyad symmetry to which the transcription factors c-jun, c-fos and the CREB/AP1 family of b-zip transcription factors, bind (143).

A recent study on human ferritin H gene conducted by Tsuji, Y 2005 identified a regulatory element termed antioxidant responsive element 4.5 kb 5’ from the transcription start site and showed that this element was responsible for transcriptional activation of the human ferritin H gene (142). Moreover, this study revealed that the ferritin H ARE is composed of two copies of bidirectional AP1 motifs (142). The other enhancer element in the human ferritin H gene is a 60 bp 5’ from transcriptional initiation site, and is of a CCAAT motif (10) (87). This enhancer element was shown to be regulated by NF-Y transcription factors, p300/CBP and PCAF (11) (43). Furthermore, ferritin H transcription increased during monocyte to macrophage differentiation (87) as well as tissue specific expression of ferritin H (11) was attributed to the basal enhancer function of the CCAAT element.
Cytoprotective Function of Ferritin

The expression of ferritin has been implicated in the regulation of cell growth based on its potential for modulating the cellular labile iron pool (LIP). LIP is the metabolically active cellular iron, which constitutes only a fraction (<1%) of the total cellular iron (18). Recently a study has demonstrated that repression of ferritin H accelerated cell cycling via changes in the LIP (66). Pronounced overexpression of ferritin H in HeLa cells, on the other hand, attenuated normal cell growth in a manner that was dependent on ferritin H capacity to incorporate iron (66). This is attributed to the fact that the major role of iron in cell growth and proliferation is associated with the activation of ribonucleotide reductase, which is the rate-limiting enzyme for de novo DNA synthesis. These observations provide experimental support for the assumption that the transcriptional repression of ferritin H is a cellular strategy for enhancing growth stimulation by oncongenes. C-myc was shown to repress the expression of ferritin H and to stimulate the expression of the iron regulatory protein-2 (IRP-2), which increased the intracellular iron pool (155). In addition, IRP2 binds to IREs in the transferrin receptor RNA and inhibits its degradation thus enhancing the iron import. Hence, down-regulation of ferritin H might be required for cell transformation by c-myc.

Ferritin H is thought to play a role in the rapid detoxification of iron because it contains the majority of the ferroxidase activity that oxidizes Fe (II) to Fe (III) for deposition within its core. The L subunit facilitates iron nucleation, mineralization and long term iron storage (134) (120). Therefore, ferritin molecules that are rich in H subunit are involved in rapid iron uptake and release, contrasting to the long term
storage by L-subunit rich molecules (83), hence, predominant production of ferritin H under acute environmental changes appears to be a reasonable response for adaptation.

In the face of sustained oxidative insult caused by continuous generation of reactive oxygen species (ROS) within the cells, and pro-oxidant xenobiotics, the induction of the antioxidative and detoxification genes as regulated by the ARE is an important mechanism of cell defense against oxidative damage (54). Ferritin plays an important role in limiting iron availability to take part in the generation of reactive oxygen species (ROS). Several compelling experimental evidence have demonstrated the protective function of ferritin against oxidative stress. An earlier study conducted by Balla et al showed that hemin treatment of endothelial cells induced ferritin synthesis which was proportional to the reduction in the level of the cells response to cytotoxicity induced by hydrogen peroxide (8). In K562 cells, increased ferritin H levels were observed to reduce the labile iron pool, suggesting that ferritin H can sequester cellular iron and regulate the levels of labile iron pool (109).

Ferritin H has also been identified as an important factor in antioxidant defense in cultured human skin cells. The putative protective protein, ferritin, is regularly present in the basal layer of unirradiated epidermis \textit{in vivo} and that the induction of ferritin by UV was dependent on wavelength and cell type (4). Following UVA I and UVA II radiation ferritin protein was increased both in epidermal and dermal tissue (4). This demonstrates that ferritin is UV inducible in human skin as part of the putative defense mechanism. The increased ferritin in human skin
following acute UV radiation could afford protection against subsequent oxidative stress. Studies showed that corneal epithelial cells with nuclear ferritin H, which was found only in the nucleus of corneal epithelial cells and was indistinguishable from the cytoplasmic ferritin H, had less DNA breakage from UV radiation than other cell types with less ferritin (19). Moreover, corneal epithelial cells in which the expression of nuclear ferritin was inhibited by addition of the iron chelator, deferoxamine, became much more susceptible to UV-induced DNA damage.

**UVB and Ferritin Regulation**

**Role of Iron and Ultraviolet radiation in carcinogenesis**

Exposure to UV light is a significant factor in the development of skin cancers including malignant melanoma. UV appears to act as both an initiator and as a promoter of tumor development (3) (111). UV radiation is emitted by the sun and consists of UVC (200-290 nm), UVB (290-320 nm), and UVA (320-400nm). All three wavelengths of naturally occurring UV light may induce pyrimidine and thymine dimer formation, DNA strand break and DNA-protein cross-linking. UV-induced DNA damage then stimulates signal transduction pathways resulting in either repair and cell survival or cell death. It is thought that UV-induced skin cancer may largely result from such DNA damage (84).

Although UVC is a potent photocarcinogen, its biological significance may be less important since it does not reach the earth’s surface. It is generally thought that UVB irradiation and to a much less extent UVA is responsible for sun light-induced cancers (27) (33). UV irradiation can also indirectly damage cells through the formation of reactive oxygen species via the Fenton reaction (48). The resulting
oxidative damage can be mutagenic and may also be involved in carcinogenesis. UVB-dependent generation of hydroxyl radical and lipid peroxides has been demonstrated in human keratinocytes and fibroblast cultures (88) (105). The iron content in the skin of mice exposed to UVB irradiation and in the skin of sun-exposed body sites of healthy individuals was substantially elevated over the basal levels (12). Reactive oxygen species resulting from acute UVB exposures may release iron from ferritin in the cell, causing a temporary increase in free iron, which may participate in Fenton chemistry in the cell.

Elevated iron dramatically increased UV-induced damage to DNA in corneal and skin fibroblasts (19). The enhancement of UV-induced DNA damage by iron could be direct, since iron-DNA complexes increase photo absorption from the sun (6). However, it is more likely that this effect involves the iron-catalyzed damage in a Fenton reaction to DNA. Moreover, UVB generated hydroxyl radical and lipid peroxidation were responsible for the up-regulation of matrix-degrading metalloproteases (MMP-1 and MMP-3) and an increase in mRNA during invasion and metastasis in a variety of tumors including nonmelanoma skin cancer (15).

Thus, iron may contribute to the ROS formed by UVB irradiation and be involved in the pathogenesis of UVB-induced skin cancers (76). Therefore, free iron storage, the main function of ferritin H, can contribute to the regulation of iron homeostasis and the prevention of UV-induced carcinogenesis and other diseases.

**Ferritin Regulation by Ultraviolet Radiation**

Both transcriptional activation and posttranscriptional repression mechanisms have been involved in ferritin by oxidants such as H2O2 and t-BHQ. Oxidants induce
ferritin transcription by directly targeting the antioxidant responsive element of ferritin H gene (143). Other forms of oxidant stress such as ultraviolet radiation are also known to alter ferritin gene expression. UV radiation, which produces ROS and damages DNA, induce ferritin H mRNA, (118) and protein (4). In addition, in a recent study using a cDNA micro array, ferritin H was identified as one of the genes responding to UVB irradiation of HaCaT keratinocytes (80). However, the mechanisms responsible for the increased expression of the ferritin H gene after DNA damage by UV radiation are not well understood.

It is well known that by virtue of the need to maintain genetic integrity of nucleic acids, DNA damage itself can trigger cellular responses to UV light including activation of DNA repair and cell cycle enzymes, and this is an important process contributing to many of the biological effects of sunlight (45). Ultraviolet light can both decrease and increase the expression of genes in skin cells. For example, UVB light has been reported to up regulate the expression of c-jun and c-fos (17).

Many DNA damage-inducible genes have been shown to contain phorbol ester response elements, and a UVC induction of gene transcription has been localized to specific phorbol ester response element in the 5'-flanking regions of several UVC-responsive genes such as collagenase and c-fos (86). Various AREs contain two or more phorbol ester response elements in a short stretch of 40-45 nucleotides of the DNA. The phorbol ester response elements present in the promoter region of several eukaryotic genes is known to increase the transcription of these genes in response to phorbol ester (92).
Previous studies have indicated that both Jun and Fos protein family members such as Jun D and Fos B, which are known to bind ARE of the ferritin H gene along with ATF1 (147), bind to TRE (92). This suggests that Jun D and Fos B may be involved in the regulation of ferritin H gene in response to UV radiation. ATF1 is activated by UV radiation in B16 melanoma cells and strongly implicated as an important factor in the activation of the transferrin receptor gene promoter (95).

Considerable evidence has accumulated to indicate that ROS, in particular, superoxide anion, hydrogen peroxide, and hydroxyl radical, are key mediators of many solar UV light-induced biological effects in the skin (13) (94) (148). Nitric oxide readily reacts with superoxide anion to form peroxynitrite, a highly toxic intermediate (133). Increased production of superoxide anion by UV light can lead to excessive formation of peroxynitrite in nitric oxide producing tissues and stimulate a variety of signaling pathways such as mitogen-activated protein kinase (MAPK) pathways (99). It can also activate transcription factors including nuclear factor kB (NF-kB) (28) (89) and the NF-E2-related factor 2 (68). However, the mechanisms utilized by UVB and the proteins involved in the transcriptional regulation of ferritin H as mediated by the antioxidant response are still unexplored.

**Ultraviolet radiation mediated signaling and Ferritin**

The model for the role of reactive oxygen species in carcinogenesis states that iron-driven generation of hydroxyl radicals and lipid peroxides activates signal transduction pathways and modulates the activities of genes (24). Iron-dependent reactions the like Fenton reaction and lipid peroxidation are the underlying mechanism of UVB-induced JNK2 activity and c-jun transcription (15). The iron-
driven generation of hydroxyl radical and lipid peroxides were identified as early events in the downstream signaling pathway of the UVB response leading to a 15-fold increase in JNK2 activity in cultured human dermal fibroblasts (15).

Although, UV light can damage many tissue components including phospholipids, proteins, and nucleic acids, it is now recognized that many of its cellular effects are due to alterations in growth factor-and cytokine-mediated signal transduction pathways leading to aberrant gene expression. It is generally thought that ROS are mediators of some of the damage induced by UV light. Generated when UV light is absorbed by endogenous photosensitizers in the presence of molecular oxygen, ROS and their metabolites induce damage by reacting with cellular electrophiles, some of which can directly initiate cell signaling processes (55).

Some transcription factors are activated not only by growth factors, but also by ROS. Thus, while AP-1 and NF-kB are highly regulated by various growth factors/cytokine signal transduction pathways, oxidative stress is a potent activator of these transcription factors potentially via a redox-transducer mechanism (65) (35). Reactive oxygen intermediates such as hydrogen peroxide formed from UV light may act as a second messenger and modulate a variety of signal transduction pathways including ERK 1/2 and p38 signaling pathways (106) and EGF receptor phosphorylation (107) (93) (114).

The signaling pathways that transduce UVB-initiated signals to modulate gene expression involve the superfamily of the proline-directed mitogen activated protein (MAP) kinases identified as: the extracellular signal-regulated kinase (ERK),
C-jun amino terminal kinase or Stress-activated protein kinase (JNK/SAPK) and p38 subgroup of kinases. ERK, p38 and JNK MAPK activation in murine peritoneal macrophages on irradiation with UVB was demonstrated by phosho-p42/44 MAPK, p38 and JNK (129). They also observed the activation of down stream transcription factors, c-jun and c-fos in response to UVB irradiation. Their data suggest the involvement of p42/44, p38 and JNK MAPK pathways and subsequent induction of c-fos and c-jun in the signal transduction process leading to activation of macrophages exposed to UVB.

It has been reported that the action of IRP proteins can be further modulated. Activation of protein kinase C (PKC) by phorbol esters phosphorylates both IRP1 and IRP2 thereby increasing their binding to IRE, resulting in inhibition of ferritin mRNA translation (124). It appears that the UV induction of ferritin H gene expression may be mediated through signals converging on both transcriptional and posttranscriptional pathways.

**Activating Transcription Factor 1 (ATF1)**

We have identified ATF1 as one of the binding proteins to the ferritin H ARE (147). ATF1 is a member of the ATF/CREB family, which belongs to the basic leucine zipper superfamily. Members of ATF1/CREB family bind to cAMP responsive elements (CREs) with in the promoter and enhancer sequences of many genes (91). Transcriptional activation of ATF1 and CREB is enhanced by protein phosphorylation. ATF1 is phosphorylated on serine 63 (homologous to serine 133 in CREB) to activate its transcriptional activity by cAMP-dependent protein kinase (PKA), calcium-calmodulin-dependent protein kinases (CaMK), and mitogen and
stress activated kinase 1 (MSK1) (91). Phosphorylation of ATF1 or CREB is known to enhance their binding affinity for transcriptional co-activators such as CBP (CREB-binding protein) (125) (26) (78). ATF1 is overexpressed in metastatic melanoma cells (64) and in transformed lymphocytes (60) possibly enhancing tumorigenesis.

ATF1 has been demonstrated to activate genes that regulate cell proliferation (9) (74) (81) (117) (157) (158). In addition to transcriptional activation, ATF1 is known to repress genes that are involved in tumor suppression such as thrombospondin I, RB, and gelsolin (38) (100) (123). The molecular mechanism underlying ATF1 dual transcriptional function on the regulation of its target genes is presently unknown. Our studies indicate that ATF1 has a repressive effect on the transcription of the human ferritin H gene. To gain insight into how ATF1 regulates ferritin H transcription, we employed a yeast two-hybrid method and identified homeodomain interacting protein kinase 2 (HIPK2) as well as other several important proteins as interaction partners of ATF1.

**Yeast two-hybrid system for identifying protein-protein interaction**

The repressive effect of ATF1 on the transcription of the human ferritin H gene prompted us to hypothesize that some potential interacting proteins may be cooperating with ATF1 in regulating transcription of the ferritin H gene. In order to understand this mechanism, it was imperative that we identify the co-factors that are potentially involved in this regulation. To this end, we employed the yeast two-hybrid
The yeast two-hybrid assay is a system that allows the identification and analysis of protein-protein interaction.

The yeast two-hybrid assay was developed by Stanley Fields (44). The assay is based on the observation that proteins that regulate gene expression, or transcription factors, consist of two separate domains; one domain (the DNA-binding domain) mediates binding of the transcription factor to the gene promoter by sequence specific DNA recognition and the other domain (the activation domain) recruits the transcriptional apparatus to the gene. The yeast two-hybrid assay takes advantage of the fact that Gal4 function as a transcriptional activator when physically bound to an activation domain. In the yeast two-hybrid system, the protein of interest, termed bait, is fused in frame with the Gal4 DNA-binding domain, whereas the other, termed prey is fused to the Gal4 transcription activation domain. The bait and prey fusion proteins are transcriptionally inactive when expressed in the yeast strain, Saccharomyces Cervevisiae, alone. However, when an interacting bait and prey are coexpressed they associate, bringing together the DNA-binding and transcription activating domains and causing expression of a reporter gene, HIS3, downstream of the bait’s DNA-binding site. So, yeast two-hybrid system is devised to identify genes encoding proteins that are physically associated with a given protein in vivo. The yeast two-hybrid system has been used extensively to screen the cDNA library for identification of proteins that interact with a known protein. This system has some advantages over the traditional biochemical and genetic approaches, such as mass spectrometry and 2D-gel analysis: (1) it is very useful to analyze the unknown interacting protein as it can be easily identified by DNA
sequencing of the cDNA insert; (2) It is an in vivo technique that uses the yeast cell as a living test-tube; (3) Weak and transient interactions are more easily detected with the yeast two-hybrid system since the reporter gene responses often lead to significant amplification (156).

However, since this system is based on transcriptional activation and forces the expressed bait and prey proteins to localize in the nucleus, there are a large number of false-positives and false-negatives (61) (97). Therefore, to eliminate false-positives; it is very important to ascertain that these two interacting proteins exist in the same sub-cellular compartment (34). Moreover, protein interactions that are identified from yeast two-hybrid need to be confirmed by other methods such as expressing both proteins by transfection and analyzing their interaction by immunoprecipitation and Western blotting studies.

**Homeodomain Interacting Protein Kinase 2 (HIPK2)**

HIPK2 is a member of a novel family of nuclear serine/threonine kinases. HIPK2 belongs to the HIPKs (homeodomain interacting protein kinases) subfamily of dual specificity tyrosine-phosphorylated and regulated kinase (DYRK) that are distinguished by their dual ability to autophosphorylate tyrosine residues and to phosphorylate on serine and threonine residues (57) (90). HIPKs are termed homeodomain interacting protein kinases owing to their conserved protein kinase domain apart from a domain that interacts with homeoproteins (73). To date, HIPK2 is one of the well-characterized members of the HIPK family. HIPK2 was originally cloned and identified through its ability to interact with the homeoprotein transcription factor neurokinin 3 (NK-3), that has been shown to have important functions during
embryonic development and organogenesis, and was demonstrated to enhance its repressor activity (73).

HIPK2 is a component of a corepressor complex consisting of Groucho, NK-3, and a histone deacetylase complex (25) (73). HIPK2 has been shown to bind to and activate p53 by phosphorylating it at serine 46 after irradiation with UV and thereby activating p53 function and promoting apoptosis (31) (58). Moreover, HIPK2 induces apoptosis by acting on proteins different from p53 such as the p53 inhibitor, mouse double minute 2 (MDM2) (151), the scaffold protein Axin (121), and the transcriptional corepressor C-terminal binding protein (CtBP) (160). Following DNA damage, HIPK2 was shown to phosphorylate CtBP at serine 422, inducing its protease degradation and thereby abolishing its antiapoptotic function (159).

In addition to activation by UV irradiation, HIPK2 has been shown to be activated by Wnt signaling and TAK1 action (67), TGF-beta treatment (59), and antineoplastic treatments (cisplatin) (37). HIPK2 is regulated by the ubiquitin-like protein SUMO-1 and the covalent SUMO-1 modification correlates with its localization to nuclear speckles or nuclear dots (72). A very recent study has revealed that HIPK2 is differentially expressed in cells exposed to cytostatic or lethal dose of DNA damaging agents and that HIPK2 is a target of MDM2-mediated, ubiquitin-dependent degradation (115). Furthermore, another new study showed that HIPK2 was activated by ionizing radiation and specifically phosphorylated p53 at serine 46. The study also discovered that HIPK2 function is regulated by the DNA damage-kinase, ataxia telangiectasia mutated (ATM) (32).
To shed light on how ATF1 regulates the transcription of human ferritin H gene, we investigated the role HIPK2 plays in ferritin H regulation through ATF1 modulation. We hope this study will give some insights into possible function of HIPK2 in protecting cells from iron-mediated toxicity and subsequent pathophysiological disorders that are afflicting humans around the world.
HIPK2 is an ATF1 Binding Protein and Regulates Transcription of the Ferritin H Gene through an Antioxidant Responsive Element

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Running title: Ferritin H Regulation by ATF1 and HIPK2

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Abstract

Iron is required for normal cell growth and proliferation. However, excess iron is potentially harmful, as it can catalyze the formation of reactive oxygen species via the Fenton reaction and induces oxidative stress. Ferritin, the ubiquitous iron storage protein, is transcriptionally activated in response to pro-oxidants and is thought to serve a protective function from iron catalyzed oxidative damage. We previously demonstrated that oxidative stress activated transcription of ferritin H gene through the antioxidant responsive element (ARE). We have identified Activating Transcription Factor 1 (ATF1) as one of binding proteins to the ferritin H ARE; however, the mechanism by which ATF1 regulates the transcription of ferritin H gene was unexplored. The purpose of this research is to study the molecular mechanisms involved in the transcriptional regulation of the human ferritin H gene in oxidative stress. In transient transfection assay, ATF1 repressed transcription of the human ferritin H gene through the ARE. Using yeast two-hybrid method we identified the nuclear serine-threonine protein kinase, homeodomain interaction protein kinase 2 (HIPK2), as an ATF1 binding protein. HIPK2 activated the transcription of the ferritin H gene and overrode ATF1-mediated ferritin H repression. Moreover, HIPK2 phosphorylated ATF1 in vivo as well as in vitro and that HIPK2 phosphorylated ATF1 on a residue other than Ser63. UVB irradiation activated HIPK2 and increased the human ferritin H mRNA in a time-dependent manner in human keratinocytes or human breast cancer cells. Our results suggest that ATF1 phosphorylation by HIPK2 is important for the transcriptional activation of the ferritin H gene. Taken together, these results demonstrate that HIPK2 modulates ATF1-mediated repression of the
human ferritin H gene, suggesting a critical cytoprotective role for HIPK2 in response to pro-oxidants.
Introduction

Oxidative stress resulting from cellular exposure to oxidants, toxicants and heavy metals disrupts normal biological functions and may eventually contribute to pathogenesis of disease and ageing of organisms. Iron is essential for various metabolic functions, however, excess iron is potentially harmful because it catalyzes the formation of the highly toxic hydroxyl radical (40) in Fenton chemistry. Ferritin is a ubiquitous and highly conserved protein whose major function is to limit iron to participate in the generation of reactive oxygen species (ROS). Ferritin has the capacity for storing up to 4500 atoms of iron in a soluble, bioavailable and non-toxic form. Each ferritin molecule consists of 24 subunits of two types, heavy (H) and light (L) (19) which can combine in highly variable ratios depending upon the cellular requirement for iron (19).

Ferritin H is thought to play a role in the rapid detoxification of iron because it contains the majority of the ferroxidase activity that oxidizes Fe (II) to Fe (III) for deposition within its core. The L subunit facilitates iron nucleation, mineralization and long term iron storage (48) (3). Therefore, ferritin molecules that are rich in H subunit are involved in rapid iron uptake and release, contrasting to the long term storage by L-subunit rich molecules (34), hence, predominant production of ferritin H under these acute environmental changes appears to be a reasonable response for adaptation.

Ferritin synthesis is regulated at both transcriptional and translational levels (49). Translational regulation of ferritin and transferrin receptor by iron through the interaction of iron regulatory protein (IRP) and iron response element (IRE) in the 5′-
untranslated region of both ferritin H and L has been well studied (20) (21) (30). Iron-
-independent transcriptional regulation of ferritin has also been demonstrated under
conditions of oxidative stress (49), inflammation (37) and cell differentiation (6) (35).

We have identified the antioxidant responsive element (ARE) in both human
(50) and mouse (51) ferritin H promoter. ARE is a highly conserved element in many
phase II detoxification or antioxidant properties (42) (46). Induction of genes
containing ARE occurs through the activation of protein capable of binding to the
ARE consensus sequence. In the mouse ferritin H gene, this sequence is located
4.1 KB upstream of the ferritin H promoter region and contains an AP-1 like element
(51). In addition, there is a second ARE in antisense orientation that contains an AP-
1/NFE2 site, to which the b-zip transcription factors bind (51).

Both transcriptional and posttranscriptional mechanisms have been involved
in ferritin regulation by oxidants such as H2O2 and t-BHQ. Oxidants induce ferritin
transcription by directly targeting the antioxidant responsive element (ARE) region of
ferritin H gene (51). Other forms of oxidant stress such as ultraviolet radiation are
also known to alter ferritin gene expression. UV radiation, which produces ROS and
damages DNA, induce ferritin H mRNA, (44) and protein (2). Studies showed that
corneal epithelial cells with nuclear ferritin had less DNA breakage from UV radiation
than other cell types with less ferritin (9) . Moreover, in a recent study using a cDNA
microarray, ferritin H was identified as one of the genes responding to UVB
irradiation of HaCaT keratinocytes (31).

We have identified ATF1 as one of the binding proteins to the ARE of ferritin
H gene. ATF1 belongs to the CREB transcription factor family and has a basic-
leucine zipper (bZIP) transcription motif (13) (36). ATF1 is activated by UV radiation in B16 melanoma cells and strongly implicated as an important factor in the activation of the transferrin receptor gene promoter (38). Elevated expression of transferrin receptor is associated with cell proliferation and is required for an iron-dependent step in late G1 of cell cycle (33).

Using yeast-two hybrid screen, we have identified HIPK2 as the interaction partner of ATF1. HIPK2 is a nuclear serine/threonine kinase and is well conserved in various organisms (28). Recent studies have demonstrated that HIPK2 co-localizes and interacts with p53 and CREB-binding protein (CBP) within the promyelocytic leukemia (PML) bodies. HIPK2 is activated by UV radiation and selectively phosphorylates p53 at its ser 46 thereby facilitating the CBP-mediated acetylation of p53 (24) (12). The ubiquitin-like protein SUMO-1 regulates HIPK2 and the covalent modification correlates with its colocalization to nuclear speckles or nuclear dots (22). HIPK2 also regulates apoptosis independent of p53, either through directly phosphorylating CtBP (59) or through activating JNK signaling pathway (25). HIPK2 function was important for the c-ski-mediated inhibition of Smad1/4-dependent transactivation in a bone morphogenetic protein (BMP)-induced signaling pathway (18) and for p53 phosphorylation following its activation by axin through ternary complex formation (45). A recent study suggests that HIPK2 cooperates with PCAF, the histone acetylase, to induce selectively p53 transcriptional activity toward the p21Waf1 promoter while depletion of either HIPK2 or PCAF abolished this function (15). Other studies have shown that P/CAF is a component of the basal transcription apparatus of the ferritin H gene and that the relative amounts of the P/CAF in
different cell types could account for the cell specific control of the ferritin H gene transcription (8). Treatment of HeLa cells with the phosphatase inhibitor; okadaic acid, increased the transcription of ferritin H gene, suggesting that a protein kinase might be involved in the transcriptional regulation of ferritin H gene (4).

Although studies showed that ferritin H is induction by oxidative stressors confers a cytoprotective function, the molecular mechanism by which the transcription of ferritin H is regulated is largely unknown. Here we show that HIPK2 associates with ATF1 and that ATF1 has a repressive effect on the transcription of ferritin H gene. We have observed that overexpression of HIPK2 into HepG2 cells activated transcription of the ferritin H gene. Furthermore, both ATF1 and HIPK2 have targeted the ferritin H ARE and overexpression of HIPK2 reversed the ATF1 mediated transcriptional repression of ferritin H gene. HIPK2 phosphorylated ATF1 in vivo and in vitro, but Ser63 was not the phosphorylation target of HIPK2. Moreover, HIPK2 was activated by UVB in cultured cells and our northern blot analysis revealed ferritin H mRNA induction in a time-dependent manner. Our study on the effect of oxidative stress in the transcriptional regulation of ferritin H gene through its influence on the activity and functions of HIPK2 and ATF1 may shed light into the understanding of the mechanisms and pathways by which oxidative stress regulates the expression of ferritin H gene.
Materials and Methods

Cell culture: HepG2 human hepatocarcinoma cells, human embryonic kidney 293 cells, MCF-7 human breast adenocarcinoma cells were purchased from the American Type Culture Collection (ATCC). They were cultured in minimum essential medium supplemented with 2 mM L-glutamine, 1 mM sodium pyruvate, 0.1 mM nonessential amino acids and 10% fetal bovine serum (Mediatech). HaCaT cells were cultured in Dulbecco’s modified Eagle essential medium (DMEM) supplemented with 10% FBS. These cell lines were incubated at 37°C in a humidified 5% CO2 atmosphere.

Yeast two hybrid screening: A Saccharomyces strain, PJ69-4A (MATa, trp1-901, leu2-3, 112, ura3-52, his3-200, gal4 , gal80 , GAL2-ADE2, LYS2:: GAL1-HIS3, met2:: GAL7-LacZ,) was used in a yeast two-hybrid screening of a mouse B cell pGAD10 cDNA library using a full length human ATF1 as a bait. PJ69-4A was transformed with pGBDATF1 (cultured in Tryptophan-deficient medium) and the authenticity of a GAL4DBD-ATF1 expression was verified by immunoblotting using anti-ATF1 antibody. Twenty µg of B cell cDNA library was used to transform yeast cells expressing the bait protein, in a series of treatment of the yeast strain with 0.1M lithium acetate, 44% polyethylene glycol 3350 for 30 min at 30 °C and 10% DMSO for 15 minutes at 42 °C. Sixteen clones were able to grow on Tryptophan/Leucine/Histidine-deficient agar media, (a selection media on which yeast cells transformed with the cDNA library were plated), and cDNA plasmids were recovered using mechanical extraction of plasmid DNA with glass beads (425-600
microns, Sigma) and transformation of E. coli HB101 by electroporation (1300V, in 0.1 cm cuvette, BTX). HB101 were cultured on M9/Leucine-deficient agar media containing 50 µg/ml ampicillin, plasmid DNA isolated, characterized with restriction enzymes and sequenced (SeqWright Co., TX). One clone was identified as a cDNA C-terminal region of mouse HIPK2.

**DNA transfection, luciferase reporter assays and Western blotting**

Cloning of the human ferritin H 5' upstream enhancer/promoter region and construction of luciferase plasmids were reported previously (50). 0.5-1 µg of ferritin H luciferase plasmid containing ARE (-4.5kb) or lacking ARE (-4.4kb) was transiently transfected into HepG2 cells. Transient DNA transfection into HepG2 cells was carried out by the calcium phosphate precipitation method as described previously (52) except the following minor modifications: cells were plated at a density of 4 x10^5 cells/35 mm plate containing 2 ml of the culture medium, 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. After incubation for 18–24 h, the cells were fed with fresh growth medium and incubated for 24 h. 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). 293 cells were transfected with pCMVFlag-HIPK2 or pCMVFlag-HIPK2 (K221R) and/or pCMVHA-ATF1 by calcium phosphate precipitation method. To detect ATF1-HIPK2 interaction in mammalian cells whole cell lysates were immunoprecipitated with anti-HIPK2 antibody (Santa Cruz biotechnology) and subjected to Western blotting with anti-HA antibody followed by ECL detection.
**Gal4 reporter system and plasmids:** In GAL4-luciferase reporter assays, $2 \times 10^5$ 293 cells were transfected with 50 ng of five direct repeats of the yeast GAL4 binding site that controls expression of the luciferase gene, 50 ng of pFA2-ATF1 (1-221) and 0 - 50 ng of HIPK2 expression plasmid DNA by CaCl$_2$ method. After incubation for 36-48 hrs, cells were harvested and luciferase activity was measured with single luciferase assay reagent (Promega).

**UVB irradiation.** The UVB lamp (model EB 280C; Spectronics) emits wave lengths between 280 to 320 nm with a spectrum peak at 312 nm. The light intensity of the lamp was measured by the IL-1700 Research Radiometer lo equipped with an SED 240 sensor. The UVB lamp was positioned 15 cm above the cells. The medium was removed and the cells were washed with PBS and irradiated in the presence of PBS for the amount of time corresponding to the indicated UVB dose. After irradiation, PBS was removed and replaced with the specified medium.

**Northern blot analysis.** Total RNA was isolated from UVB irradiated or nonirradiated HaCaT or MCF-7 cells in culture using Tri-reagent system (Ambion). Ferritin H cDNA was labeled with [alpha-$^{32}$p]dCTP with Mega prime DNA labelling system (GE Healthcare). RNA was electrophoresed on an agarose-formaldehyde gel, transferred to Protran BA85 membrane (Whatman), and UV cross-linked. Membranes were incubated at 42 $^\circ$C in hybridization buffer overnight, washed at 52 $^\circ$C in 0.5 x SSC, 0.1 SDS solution and exposed to film at –80 $^\circ$C for autoradiography.

**Phosphatase treatment.** 800 unints of 1x lamda phosphatase (New England Biolabs) was added to 40 µg of total cell lysates, incubated at 37 $^\circ$C for 20 min. The
reaction was stopped by adding 2x SDS-PAGE sample buffer. Samples were boiled for 2-5 min., separated on SDS-PAGE and analyzed by Western blotting.

**In vitro kinase assay.** 4, 8 or 12 ng of recombinant HIPK2 (Up-state) and 2 µg of recombinant ATF1 (Santa Cruz) were incubated in a kinase buffer (10 mM HEPES, pH 7.4, 5 mM MgCl₂, 1 mM DTT) in the presence of 5 µCi [gamma-³²P] ATP and 100 µM unlabelled ATP at 30 °C for 30 minutes. Samples were then separated by 10% SDS-PAGE and visualized by autoradiography and subjected to coomassie blue staining for evaluation of protein amounts in the kinase reactions. For endogenous proteins, UVB irradiated cells were harvested in lysis buffer A (pH 7.4) containing 10 mM dibasic sodium phosphate, 150 mM NaCl, 1% Triton X 100, 0.5% sodium deoxycholate, 0.1% SDS and 0.2% sodium azide. 500 µg of cell lysate was immunoprecipitated with anti-HIPK2 antibody (Santa Cruz). Immunocomplexes were used in the kinase assay by the addition of 1 µg of MBP substrate (Sigma).
Results

ATF1 represses transcription of the human ferritin H gene

We have previously identified Activating Transcription Factor 1 (ATF1) as one of the binding proteins to the ferritin H gene (53). However, the mechanism by which ATF1 regulates transcription of the ferritin H gene was unexplored. To test the effect of ATF1 on ferritin H gene transcription, HepG2 cells were transiently transfected with expression vectors encoding ATF1 together with the -4.5 kb ferritin H reporter, containing the antioxidant responsive element (ARE), or the -4.4 kb reporter lacking ARE. As shown in Fig. 1, ATF1 decreased the luciferase expression driven by the -4.5 kb ARE(+) ferritin H reporter in a dose dependent manner. However, -4.4 kb ferritin H luciferase reporter lacking the ARE sequence showed lower basal expression with no further decrease in luciferase activity. This suggests that ATF1 represses the transcription of the human ferritin H gene and that this repression is mediated via the antioxidant responsive element (ARE).

HIPK2 is an ATF1 binding protein

To identify ATF1-interacting factors and thereby gain insight into the mechanism by which ATF1 represses the transcription of ferritin H gene, yeast two-hybrid method was employed. We screened a B cell mouse cDNA library using a full length human ATF1 as a bait and identified a cDNA encoding C-terminal 0.8 Kb region of the homeodomain interacting protein kinase-2 (HIPK2) (Fig. 2A). As HIPK2 is highly conserved in its amino acid sequence between mouse and human (96 % homology) (56), including the C-terminal region isolated from our yeast two-hybrid
screening, throughout the rest of the study we examined the function of the human HIPK2 in the regulation of human ATF1. To test for the reproducibility of the yeast two-hybrid interaction in mammalian cells between ATF1 and HIPK2, we carried out co-immunoprecipitation assay. 293 cells were co-transfected with full length Flag HIPK2 or a kinase-dead mutant, HIPK2-K221R (28) along with HA-ATF1 expression plasmids and cell lysates were subjected to immunoprecipitation with anti-HIPK2 antibody followed by Western blotting with anti-HA antibody. Under equal expression of ATF1, HA-ATF1 was coimmunoprecipitated when Flag HIPK2 or the kinase-dead mutant Flag HIPK2-K221R was expressed in the cells (Fig. 2B, top panel, lane 5 and 6). A kinase-dead HIPK2 (K221R) interaction with ATF1 (Fig. 2B, top panel, lane 6) suggests that HIPK2 binds in a manner that is independent of its kinase activity.

**HIPK2 activates transcription of the ferritin H gene through the ARE**

To test the functional role of HIPK2 in transcriptional regulation of the ferritin H gene, and to evaluate whether the ARE mediates this function, expression plasmids for HIPK2 and ferritin H luciferase reporters were transfected into HepG2 cells and luciferase assays were performed. We used human ferritin H luciferase constructs containing the ARE sequence, -4.5 kb ferritin H luciferase reporter or lacking ARE sequence, -4.4 kb ferritin H reporter. Our data show that HIPK2 increased the luciferase expression of the -4.5 kb reporter in a dose-dependent fashion while it had no effect on the luciferase expression of the -4.4 kb reporter (Fig. 3). These results suggest that HIPK2 activates the transcription of ferritin H gene and that the ARE mediates this activation.
**HIPK2-mediated activation of the ferritin H transcription depends on its kinase activity**

To begin to determine whether HIPK2 regulates ferritin H gene through a mechanism involving its kinase activity, we co-transfected increasing amounts of wild type HIPK2 or kinase-dead HIPK2 (K221A) expression plasmid DNA along with an ARE-containing ferritin H luciferase reporter into HepG2 cells. As shown in Fig. 4, wild type HIPK2 increased the activity of the luciferase reporter in a dose-dependent manner. In contrast the kinase-dead HIPK2 (K221A) failed to activate the reporter expression. This finding suggests that HIPK2 kinase activity is required for the transcriptional activation of the human ferritin H gene.

**HIPK2 overrides ATF1-mediated transcriptional repression of ferritin H gene**

We have observed that ATF1 and HIPK2 have opposing effects on the transcription of ferritin H gene, repression by ATF1 (Fig. 1) and activation by HIPK2 (Fig. 3), respectively. To examine the effect of HIPK2 on ATF1-mediated ferritin H transcriptional repression, fixed amount of ATF1 was co-transfected with increasing amounts of wild type or kinase-dead HIPK2 plasmid DNA into HepG2 cells along with -4.5 kb (ARE containing) ferritin H reporter. In the absence of HIPK2, ATF1 reproducibly repressed the luciferase activity of the ferritin H reporter (Fig 5). We observed that, under almost equal expression of transfected ATF1, wtHIPK2 increased the activity of the luciferase reporter in a dose-dependent manner. In contrast, kinase-dead HIPK2 had no effect on the reporter activity (Fig. 5). Taken
together, these results suggest that HIPK2 reversed ATF1-mediated transcriptional repression of the ferritin H gene and that HIPK2 kinase activity is required for the reversal of ATF1 repression of the ferritin H.

**HIPK2 phosphorylates ATF1 in vivo and in vitro**

Our finding suggested that HIPK2-mediated activation of the ferritin H gene is dependent upon its kinase activity (Fig. 4). Moreover, when ATF1 and wild type HIPK2 were co-expressed in 293 cells, we consistently observed a slower migrating band in immunoblottings using anti-HA antibody (Fig. 2b, middle panel lane 5). This suggested that the occurrence of the slower migrating band might be a result of ATF1 phosphorylation by HIPK2. To confirm this observation, HA-ATF1 was co-expressed in 293 cells with empty vector, kinase-dead HIPK2 (K221R) or wild type HIPK2. Total cell lysates were prepared and analyzed by Western blotting using anti-HA antibody. As shown in Figure 6A, retarded HA-ATF1 was evident where cell lysates obtained from cells co-transfected with wild type HIPK2 and HA-ATF1 were applied. To verify whether the slower migration of the extra band was caused by phosphorylation of ATF1 by HIPK2, phosphatase treatment was performed. Phosphatase treatment of cell lysates obtained from 293 cells co-transfected with HA-ATF1 and wild type HIPK2 abolished the retarded HA-ATF1 band, leaving behind a band size similar to that of HA-ATF1 and kinase-dead HIPK2 (K221R) (Fig. 6B). Taken together, this suggests that HIPK2 phosphorylates ATF1 in cultured cells albeit at as a yet unidentified site.

We next examined whether HIPK2 phosphorylates ATF1 directly. To this end, recombinant HIPK2, amino acids 165-564, was used in an *in vitro* kinase assay by
the addition of [gamma-32P] ATP and recombinant ATF1 as substrate. PKA, a known kinase for Ser63 of ATF1 and Ser133 of CREB, was used as a control. As shown in Fig. 7A, HIPK2 underwent auto-phosphorylation and phosphorylated ATF1 in a dose-dependent manner, indicating that HIPK2 phosphorylates ATF1 directly.

Our data showed that ATF1 was phosphorylated by HIPK2 in vivo and in vitro (Figs. 6 and 7A). Previous studies revealed that ATF1 was phosphorylated on Ser63 to activate its transcriptional activation in response to camp, calcium and stress (10). Hence, we asked whether HIPK2 also could phosphorylate ATF1 on Ser63. To investigate this, we performed an in vitro kinase assay. Comparable amounts of bacterially expressed and purified His-ATF1 or His-ATF1 Ser63Ala mutant fusion protein were incubated with increasing amounts of recombinant HIPK2 in the presence of [gamma-32P] ATP. As shown in Fig. 7B, HIPK2 underwent auto-phosphorylation and efficiently phosphorylated wild type ATF1 as well as ATF1 Ser63Ala mutant in a dose-dependent manner. Protein kinase A (PKA), a known kinase to phosphorylate ATF1 and CREB on Ser63 and Ser133, respectively, was used as a control, and as expected, it phosphorylated wild type ATF1, but not ATF1 Ser63Ala mutant (Fig. 7B), suggesting that HIPK2 phosphorylates ATF1 on a new site (s) besides Ser63. Moreover, this data confirms our observation shown in Fig. 7A that ATF1 was directly phosphorylated by HIPK2.

To confirm this observation, we transiently transfected 293 cells with expression plasmids for wild type ATF1 or ATF1 Ser63Ala mutant and wild type HIPK2, kinase-dead HIPK2 (K221R) or PKA. Total cell lysates were resolved by SDS-PAGE and analyzed by Western blotting with antibody recognizing phospho-
ATF1 Ser63. Over-expression of wild type ATF1 alone also caused its phosphorylation on Ser63 (Fig. 8 top lane 2). While PKA, the known ATF1 Ser63 kinase had efficiently phosphorylated ATF1 on Ser 63 as evidenced by the intense signal, there appears to be no difference in signal intensity between those produced by co-expression of the wild type or kinase-dead HIPK2 and wild type ATF1 (Fig. 8 top, lanes 2, 5 and 7). Furthermore, co-expression of wild type HIPK2 and wild type ATF1 or ATF1 Ser63Ala mutant yielded same sized slower migrating bands (Fig. 8, lanes 5 and 6, middle). We did not observe this when the mutant HIPK2 or the empty vector was co-expressed with wild type ATF1 (Fig. 8, lanes 2 and 7), suggesting that the retarded migration of the binds was the outcome of HIPK2 kinase-mediated phosphorylation of ATF1 at Ser/Thr sites other than Ser63. The manifestation of the same pattern of migration of the extra bands of the wild type ATF1 and mutant ATF1 is indicative of ATF1 phosphorylation by HIPK2 at a residue other than Ser63. Taken together, these results strongly suggest that ATF1 is the substrate of HIPK2 and that ATF1 is phosphorylated by HIPK2 at a site yet to be identified.

**HIPK2 increases transcriptional function of ATF1**

Our data have shown that ATF1 repressed transcription of the ferritin H gene and that HIPK2 reversed ATF1-mediated ferritin H repression. To examine whether ATF1 phosphorylation by HIPK2 has an activating, or inhibitory effect on the ATF1 transcription function, we used a Gal4 luciferase assay system. Gal4ATF1 or Gal4 were co-transfected with wild type or kinase-dead HIPK2 into HepG2 cells. We observed that wild type HIPK2 activated Gal4ATF1 in a dose-dependent manner,
while kinase-dead HIPK2 failed to activate the reporter. This result suggests that HIPK2 activates ATF1 (Fig. 9).

**UVB activates HIPK2 and increases ferritin H mRNA**

HIPK2 activation by UV has been reported by several studies (24) (12) (58), in which activated HIPK2 regulated the functions of p53 or CtBP through phosphorylation at Ser46 or Ser422, respectively. However, the role of HIPK2 in iron homeostasis in general and ferritin H regulation, in particular in response to DNA damage, has not been explored. To investigate this, HaCaT cells and MCF-7 cells were irradiated with single dose of 10 mj/cm^2 or 5 mj/cm^2 UVB, respectively. Whole cell lysates were subjected to immunoprecipitation with anti-HIPK2 antibody followed by in vitro kinase assay by the addition of [gamma ^{32}P] ATP and the myelin basic protein (MBP) (23) (12) substrate directly to the immunocomplexes. In MCF-7 cells, MBP phosphorylation started as early as 5 minutes after UVB irradiation and began to return to basal level at 12 hours post-UVB treatment (Fig. 10A, top). In HaCaT cells, MBP phosphorylation started at 10 minutes and returned to baseline level 60 minutes post-UVB irradiation (Fig. 10B, top). To evaluate whether or not HIPK2 activation can correlate with ferritin H transcriptional activation, we isolated total RNA from UVB-treated HaCaT or MCF-7 cells and carried out Northern blot analysis. As shown in Fig. 10A or 10B (middle panels), UVB treatment increased ferritin H mRNA in either HaCaT or MCF-7 cells in a time-dependent manner. These results demonstrate that UVB induces the ferritin H mRNA.
Discussion

Oxidative stress plays a central role in the etiology of many human diseases including cancer, neurodegenerative disease and ageing (5) (26). Cells respond to oxidative stress by inducing expression of the phase II detoxification genes that are regulated by an antioxidant responsive element (ARE). Therefore, understanding the molecular mechanisms by which these genes are regulated might lend an insight into the treatment or prevention of the aforementioned human diseases. We have previously identified ATF1 as a binding protein to the human ferritin H ARE (53). In the present study, we found that ATF1 is a repressor of the human ferritin H transcription (Fig. 1). ATF1 is known to function as either a transcriptional activator (7) (29) (32) (43) (57) or repressor (16) (39) (47) of genes involved in cell proliferation, however, the molecular mechanism underlying its dual role is not understood.

A body of evidence has shown that ferritin plays an important functional role in protecting cells against damages arising from oxidative stress and inflammatory cytokines (11) (17) (41). To gain insight into the molecular mechanisms underlying the transcriptional regulation of the human ferritin H gene by ATF1, we used yeast two-hybrid screening and identified HIPK2 as an ATF1 binding protein (Fig. 2). This finding led us to hypothesize that ATF1-mediated transcriptional regulation of the human ferritin H gene may be further regulated by HIPK2. HIPK2, a nuclear serine/threonine kinase, is involved in transcriptional regulation and apoptosis (24). HIPK2 is known to be activated by UV irradiation (24) (12), Wnt signaling and TAK1 action (27), TGF-beta treatment (25) and cisplatin (14). Therefore, we predicted that
HIPK2 might regulate the transcription of the ferritin H gene under oxidative stress condition through modification of ATF1.

We recently identified the human ferritin H ARE located -4.5 kb upstream of transcriptional initiation site (50). Promoter reporter assays in HepG2 cells showed that HIPK2 activated the ARE-containing, but not the ARE-lacking, ferritin H luciferase reporter (Fig. 3). These results indicate that HIPK2 stimulates transcriptional activation of the ferritin H gene and that the ARE mediates this activation. In this study we demonstrated that HIPK2 phosphorylates ATF1 in vivo and in vitro. We reproducibly observed a second ATF1 band with a slower migration when ATF1 was co-expressed with wild type HIPK2, but not when co-expressed with kinase-dead HIPK2 in our immunoblotting experiments (Fig. 2, lanes 5 and 6). Phosphatase treatment abolished the retarded band (Fig. 6B), suggesting that the slower migrating ATF1 band was phosphorylated ATF1. Previous studies observed that HIPK2 directly phosphorylated p53 at Ser 46 (24) (12). In addition, the co-repressor CtBP was shown to be directly phosphorylated by HIPK2 in response to UV irradiation (59). To assess whether HIPK2 directly phosphorylates ATF1, in vitro kinase assays were performed, in which we showed that ATF1 was directly phosphorylated by HIPK2 in a dose-dependent manner (Fig. 7A and 7B). Transcriptional activation of ATF1 and CREB is enhanced by protein phosphorylation (36). To-date, serine 63 in ATF1 (homologous to serine 133 in CREB) is the only phosphorylation site known to be phosphorylated by cAMP-dependent protein kinase (PKA) (36), calcium-calmodulin-dependent protein kinases (CaMK) (36), and mitogen and stress activated kinase 1 (MSK1) (36), resulting in its
enhanced transcriptional activity. Our in vitro kinase assay result showed that HIPK2 phosphorylated both wild type ATF1 and Ser63Ala mutant ATF1, while PKA phosphorylated wild type ATF1 but not Ser63Ala mutant, as predicted (Fig. 7B). In addition, co-expression of wild type HIPK2 with either wild type ATF1 or Ser63Ala mutant ATF1 resulted in the generation of the slower migrating ATF1 bands (Fig. 8). These results suggest that HIPK2 phosphorylates ATF1 at a site other than Ser63, indicating that this is a novel site. However, we cannot rule out the possibility that HIPK2 can also phosphorylate ATF1 on Ser63.

We have observed that co-expression of wild type HIPK2 and ATF1 resulted in the reversal of ATF1-mediated repression of ferritin H promoter activity whereas HIPK2 K221R mutant co-expression with ATF1 failed to do so (Fig. 5). Excess iron is potentially harmful (40) and has been implicated in the etiology of diseases such as cancer and ageing (1). The biological significance of HIPK2 can be partly explained by the observation that it reverses the ferritin H repression by ATF1, which ultimately leads to ferritin H expression and thereby helping alleviate iron-mediated toxicity and related diseases. In Gal4 reporter assays, we have found that co-expression of wild type HIPK2 but not of kinase-dead HIPK2 with Gal4-ATF1 activated the expression of luciferase reporter, suggesting that HIPK2 activates ATF1 (Fig. 9). ATF1 phosphorylation by HIPK2 may lead to the recruitment of co-factors like P300/CBP that play central role in coordinating and integrating multiple signaling events with transcriptional apparatus. Alternatively, ATF1 phosphorylation may alter its heterodimerization status with other b-zip or ATF1/CREB subfamily members giving way for other activators such as Nrf2 (54) (55) and JunD (50) to
bind ferritin H ARE. Previous study has observed that HIPK2 mediated phosphorylation of the transcriptional corepressor, CtBP, on Ser-422 marked it for degradation in response to UV irradiation (58). Although we have consistently observed that ATF1 phosphorylated by HIPK2 appears to be stable, we cannot rule out the possibility that HIPK2-mediated phosphorylation may mark ATF1 for degradation or clearance. Taken together, our results indicate that modification of ATF1 by phosphorylation as mediated by HIPK2 expression is required for the activation of ATF1 and the subsequent transcriptional activation of the human ferritin H gene. Identification of HIPK2 phosphorylation site on ATF1 and characterization of the biological significance of this site in mediating the reversal of ATF1 repressive effect on transcription of the ferritin H gene have yet to be performed.

A previous study by others showed that UV irradiation induced ferritin H mRNA (2). ATF1 has been shown to be activated in response to UV irradiation in B16 melanoma cells and its role in the activation of transferrin receptor gene promoter (38) was delineated. Elevated expression of transferrin receptor is linked with cell proliferation and is essential for an iron-dependent step in late G1 of cell cycle (33). HIPK2 has been shown to activate p53 by phosphorylating it at serine 46 after irradiation with UV and thereby activating p53 function and promoting apoptosis (12) (24). In agreement with these results, we have found that UV irradiation activated HIPK2 and induced ferritin H mRNA expression in MCF-7 or HaCaT cells in a time-dependent manner S. Nevertheless, our work falls short of providing data supporting a direct connection between HIPK2 activation and the increase in ferritin H mRNA. This can be addressed by first identifying HIPK2 phosphorylation site on
ATF1 followed by point mutation of this residue and test whether or not this mutation prevents HIPK2 and UV-induced activation and the corresponding ferritin H transcription.

In summary, we have identified HIPK2 as a novel regulator of ATF1 and inducer of ferritin H synthesis. HIPK2 appears to be at the forefront of maintaining the delicate balance between eliminating unhealthy cells through apoptosis and protecting healthy uninitiated ones, at least in part, by helping minimize iron-mediated cell death.
ACKNOWLEDGMENTS

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Figure Legends

Figure 1. ATF1 represses transcription of the human ferritin H gene
HepG2 cells were transfected with 1.0 µg of -4.5 kb luciferase (+ARE) or 1.0 µg of -4.4 kb luciferase (-ARE) plus increasing amounts of ATF1. 48-60 h after transfection, cell lysates were obtained to perform luciferase assay. DNA transfection was carried out in duplicate in each experiment with pRL-CMV as an internal transfection control. Relative luciferase expression after normalization with renilla luciferase activity from five independent experiments and standard error are shown. Relative luciferase activity in extracts from cells transfected with 1.0 µg of pcDNA3.1 (empty vector) and 1.0 µg of -4.5 kb luciferase (+ARE) was defined as 100.

Figure 2

A) Physical interaction of ATF1 and HIPK2 in yeast
PJ69-4A yeast cells were transformed with pGBD and pGAD10 (-/-), pGBDATF1 and pGAD10 (ATF1/-), pGBD and pGAD10 HIPK2 (-/HIPK2), and pGBDATF1 and pGAD10 HIPK2 (ATF1/HIPK2) and transformed colonies were tested on SD-Tryptophan, Luecine and Histidine deficient agar plate. pGBD ATF1 is a human full length ATF1, and pGAD10 HIPK2 is a mouse C-terminal region of HIPK2 isolated from pGAD10 B cell cDNA library.

B) ATF1 and HIPK2 are interaction proteins in mammalian cells
Expression plasmids for Flag HIPK2, Flag HIPK2 (K221R), or empty vector were co-transfected with empty vector or HA-ATF1 expression plasmids into 293 cells. 40 to 48 hr after transfection, cells were lysed and proteins separated by SDS-PAGE. Interaction of ATF1 and HIPK2 was analyzed by immunoprecipitation with anti-
HIPK2 antibody followed by Western blotting with anti-HA antibody (upper panel). The expression levels of HA-ATF1 (middle panel) and of HIPK2 (bottom) are analyzed using anti-HA or anti-HIPK2 antibodies, respectively.

**Figure 3. HIPK2 activates transcription of the human ferritin H through the ARE**

1.0 µg of -4.5 kb human ferritin luciferase reporter (+ARE) or -4.4 kb luciferase reporter (-ARE) was cotransfected with 0.1, 0.25 or 0.5 µg of Flag HIPK2 plasmid DNA into HepG2 cells. 40-48 h after transfection, cell lysates were obtained to perform luciferase assay. Fold induction in extracts obtained from cells transfected with 1.0 µg of -4.4 kb luciferase (-ARE) and empty vector was defined as 1.0. DNA transfection was carried out in duplicate in each experiment, and the result of five independent experiments and standard error are shown.

**Figure 4. HIPK2 kinase activity is involved in ferritin H transcriptional activation**

1.0 µg of -5.2 kb human ferritin luciferase reporter was cotransfected with 0.1, 0.25 or 0.5 µg of HIPK2, wild type or kinase defective (K221R) DNA into HepG2 cells. 40-48 h after transfection cell lysates were obtained to perform luciferase assay. Fold induction in extracts obtained from cells transfected with empty vector DNA was defined as 1.0. DNA transfection was carried out in duplicate in each experiment, and the result of five independent experiments and standard error are shown.
**Fig. 5. HIPK2 reverses ATF1-mediated repression of the ferritin H transcription**

1.0 µg of -4.5 kb human ferritin luciferase reporter (+ARE) and 0.5 µg of HAATF1 were cotransfected with 0 µg, 0.1 µg or 0.5 µg of WT/KD HIPK2 into HepG2 cells. 40-48 h after transfection, cell lysates were obtained to perform luciferase assay. Fold induction in extracts obtained from cells transfected with empty vectors (0 µg WT or KD HIPK2 + 0 µg ATF1) was defined 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. ATF1 expression levels from cell lysates obtained from coexpression of wild type HIPK2 and ATF1 were analyzed by Western blotting using anti-ATF1 antibody (bottom).

**Fig. 6. HIPK2 phosphorylates ATF1 in vivo**

**A)** 293 cells were co-transfected with 10 µg of empty vector, Flag HIPK2 wt, or Flag HIPK2 KD (K221R) mutant and 10 µg of HA-ATF1 expression plasmids. 24 hours after transfection, whole cell lysates were prepared and proteins separated on SDS-PAGE followed by analysis with Western blotting using anti-HA antibody a duplicate of same cell lysates were used for each combination (**Figure 6A, top**). The expression level of HIPK2 representing each duplicate was analyzed by Western blotting using anti-HIPK2 antibody (**Figure 6A, bottom**). **B)** A portion of the same cell lysate was used for phosphatase treatment. 800 units of lamda phosphatase was added to 40 µg of cell lysates obtained from the co-expression of wild type HIPK2 and HA-ATF1 or left untreated. Cell lysates obtained from the co-expression of the empty vector or kinase dead HIPK2 (K221R) were left untreated and were
used as control. Samples were incubated at 37 °C for 20 minutes and subjected to SDS-PAGE and migration pattern of ATF1 bands was analyzed by Western blotting using anti-HA antibody (Figure 6B).

**Figure 7. HIPK2 directly phosphorylates ATF1 besides Ser63**

A) Increasing amounts of recombinant HIPK2 was mixed with 2µg of recombinant ATF1 and then incubated in the presence of gamma [32P]-ATP at 30 °C for 20 minutes. Samples were analyzed by SDS-PAGE and visualized by autoradiography. PKA and MBP were used as controls. The bottom panel shows coomassie blue staining of the same gel.

B) Increasing amounts of recombinant HIPK2 was mixed with equal amount of bacterially expressed His-ATF1 or His-ATF1 Ser63Ala mutant and incubated in the presence of [gamma 32P]-ATP at 30 °C for 20 minutes. Samples were separated by SDS-PAGE and visualized by autoradiography. PKA was used as a control. Asterisk indicates autophosphorylation of HIPK2. Coomassie blue staining of the same gel is shown (bottom).

**Figure 8. Ser63 is not a major phosphorylation site by HIPK2**

10 µg of Wild type HIPK2 or kinase dead HIPK2 (K221R) plasmid DNA were co-transfected with 10 µg of wild type ATF1 or Ser63Ala mutant ATF1 expression plasmid into 293 cells. 24 hrs after transfection, total cell lysates were collected and 40 µg of sample separated by SDS-PAGE and then analyzed by Western blotting using anti-phospho ATF1 Ser63 antibody, anti-ATF1 antibody or anti-HIPK2 antibody. Sample obtained from co-expression of PKA and ATF1 was used as a positive control of ATF1 Ser63 phosphorylation.
Figure 9. HIPK2 activates ATF1 transcription function

50 ng of Gal4DBD-Luciferase plasmid was transfected together with 50 ng of Gal4 or Gal4ATF1 a.a. (1-228) plus 0, 25, or 50 ng of pcDNA3.1HIPK2 or pcDNA3.1kdHIPK2 by CaPO4 transfection method. Luciferase assay was performed 48 hrs after transfection. Total amount of plasmid DNA (150 ng) was adjusted with pcDNA3.1 empty vector. The value of the luciferase activity of the cell lysates obtained from no HIPK2 (50 ng of Gal4) was set as 1.0.

Figure 10. UVB activated HIPK2 and increased ferritin H mRNA

MCF-7 or HaCaT cells were irradiated with UVB at a single dose of 5 or 10 mj/cm², respectively, and harvested at various time points. Samples were immunoprecipitated with anti-HIPK2 antibody and [gamma ^32p]-ATP was added to the immunoprecipitates and incubated at 30 °C for 20 min in an in vitro kinase assay using MBP as a substrate (10A and 10B, top panels). Total RNA was isolated from UVB irradiated cells MCF-7 or HaCaT cells (5 or 10 mj/Cm2) and harvested at different time points and Northern blot analysis was conducted (middle panels 10A or 10B for MCF-7 or HaCaT cells, respectively). 18S and 28S were shown for equal loading (bottom panels, 10A or 10B).
Fig. 1  ATF1 represses transcription of the human ferritin H gene via the ARE
Fig. 2  HIPK2 is an ATF1 binding protein.
Fig. 3 HIPK2 activates transcription of the human ferritin H gene via the ARE.
Fig. 4  HIPK-2-mediated activation of the ferritin H transcription depends on its kinase activity.
Fig. 5 HIPK2 overrides ATF1-mediated transcriptional repression of the human ferritin H gene.
Fig. 6 HIPK2 phosphorylates ATF1 in the cells
Fig. 7  HIPK2 directly phosphorylates ATF1 besides Ser63.
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Fig. 8  ATF1 Ser63 is not a major phosphorylation site by HIPK2.
Fig. 9  HIPK2 activates ATF1 transcription function.
Fig. 10A  UVB increases the ferritin H mRNA
Fig. 10B UVB increases the ferritin H mRNA
General Discussion and possible future directions

The induction of phase II detoxification genes including ferritin H gene is an important protective mechanism against oxidative stress or xenobiotic metabolites-mediated cell damage. Studies have shown that antioxidant responsive element (ARE), a cis-acting element in the 5’ region of these genes (63), is responsible for their transcriptional activation (122). Ferritin, the major iron storing protein, has been shown to serve cytoprotective function against oxidative stress, or inflammation-mediated damages (29) (40) (101) (108). Several reports have implicated ATF1 in either a transcriptional activation (9) (74) (81) (117) (158) or repression (38, 100) (123) of genes involved in cell proliferation. We previously found that ATF1 is one of the binding proteins to the mouse ferritin H ARE (Iwasaki, K., et al., unpublished observation).

In this study, we have found that ATF1 repressed transcription of the human ferritin H gene. In an attempt to identify genes that encode proteins that interact with ATF1 and to evaluate their contribution to the modification of ATF1-mediated ferritin H repression, yeast two-hybrid screening was performed. Here, we found that HIPK2 is an ATF1 binding protein and an ATF1 kinase, resulting in the modulation of ATF1-mediated transcriptional repression of ferritin H. HIPK2 has been demonstrated to mediate either transcriptional activation (58) (31) (110) or transcriptional repression (25) (50). To delineate the functional role of HIPK2 and to specify the means by which it exerts this function on the ferritin H regulation, we performed transient DNA transfection and luciferase assays in which we observed that HIPK2 activated ferritin H ARE luciferase reporter but not ARE-lacking ferritin H reporter. This result
suggests that HIPK2 activates transcription of ferritin H gene and that the ARE mediates this activation. Furthermore, our data indicates, as expected, that HIPK2 kinase activity is involved in the transcriptional activation of ferritin H gene. Our study shows that HIPK2 can phosphorylate ATF1 \textit{in vivo}. We have consistently observed that overexpression of wild type HIPK2 but not of kinase-dead HIPK2 with ATF1 resulted in an additional, retarded band of ATF1 and phosphatase treatment abolished the slower migrating ATF1 band, indicating that this retarded band is phosphorylated ATF1 (Fig. 6B). Previous reports showed that HIPK2 directly phosphorylated p53 (58) (31), and the co-repressor CtBP in response to UV irradiation (160). On the other hand, HIPK2 was shown to activate JNK signaling pathway and to participate in TGF-beta-induced activation and apoptosis (59). Our \textit{in vitro} kinase assay using recombinant HIPK2 and ATF1 results suggest that HIPK2 directly phosphorylates ATF1 in a dose-dependent manner.

In addition to being implicated in the etiology of diseases such as cancer, neurodegenerative disease and aging engendered by the Fenton reaction-mediated DNA, lipids and protein damage (2) (70), excess iron can be used by cancer cells for growth and proliferation (126) (128) (136). Previous studies have suggested that transcriptional repression of ferritin H is a cellular strategy for enhancing growth stimulation by oncogenes. C-myc was shown to repress ferritin H expression and to stimulate the intracellular iron pool (155). Moreover, Tsuji and colleagues demonstrated that the viral oncoprotein, E1A, preferentially repressed ferritin H expression (144) (146). We observed that overexpression of wild type HIPK2 overrode ATF1-mediated ferritin H promoter reporter repression in a dose-
dependent fashion, while overexpression of the kinase-dead HIPK2 failed to do so. This data suggests that HIPK2 reversed ATF1-mediated transcriptional repression of the ferritin H gene. Furthermore, our Gal4 assay shows that co-expression of Gal4ATF1 with wild type HIPK2, but not with kinase-dead HIPK2 increased the Gal4 luciferase reporter expression. This result indicates that HIPK2 kinase activity is required for activation of ATF1 transcriptional functions. Taken together, we have shown that ATF1 is phosphorylated by HIPK2 and that this phosphorylation is required for the reversal of ATF1-mediated ferritin H transcriptional repression.

The biological significance of HIPK2 and ATF1 interaction can be explained in the context that HIPK2, by abolishing the repressive effect of ATF1 on the ferritin H transcription, ultimately increases ferritin H expression and thereby leading to sequestration of excess iron. The mechanism underlying ATF1 repression of ferritin H is currently poorly understood. However, in our yeast two-hybrid screening, we identified that chromatin remodeling factors such as Chromodomain helicase DNA binding protein 3 (CHD3) and MCAF1 are ATF1 binding proteins (table 1). A study reported that CHD3, in association with histone deacetylase1/2 (HDAC1/2), has an important role in gene transcriptional repression (137). A study by Zhang, Y. et al; demonstrated that MCAF1 interacted with SETDB1, a novel histone H3, lysine 9-specific methyltransferase, and caused transcriptional repression through cooperation with SETDB1 in the conversion of H3-K9 dimethyl to trimethyl (150). Investigation of the functional role of these two proteins, in association with ATF1, in the transcriptional regulation of ferritin H is underway.
Transcriptional activation of ATF1 and CREB is enhanced by protein phosphorylation. However, to-date, only serine 63 in ATF1 (homologous to serine 133 in CREB) has been demonstrated to be phosphorylated by kinases such as cAMP-dependent protein kinase (PKA), calcium-calmodulin-dependent protein kinases (CaMK), and mitogen and stress activated kinase 1 (MSK1) (91) resulting in the activation of ATF1 transcription function. In a western blotting analysis using anti-phospho Ser63 antibody, we observed signals in lanes where cell lysates obtained from co-expression of wild type ATF1 with wild type HIPK2 or kinase-dead HIPK2 were used. In an in vitro kinase assay we have shown that HIPK2 phosphorylated wild type ATF1 as well as Ser63Ala mutant ATF1. Furthermore, we observed that HIPK2 overexpression with wild type ATF1 or Ser63Ala mutant ATF1 resulted in the generation of the retarded ATF1 bands of the same size. Taken together, this data suggest that HIPK2 phosphorylates ATF1 on a site other than Ser63. UVB is known as a major cause of human non-melanoma skin cancer (47) and as acting both as a tumor initiator and tumor promoter (130) (132). Iron is thought to contribute to the UVB-mediate production of ROS, via the Fenton reaction, and to be involved in the pathogenesis of UVB-induced skin cancers (76). An earlier report by Cai and colleagues showed that elevated iron dramatically increased UV-induced damage in corneal and skin fibroblasts (19). As a major iron storage protein, ferritin H plays a key role in controlling iron homeostasis and thereby preventing UV-induced carcinogenesis and other pathogenesis (4).

Reports have shown that ferritin H mRNA was up-regulated in rat keratinocytes (118) and in HaCaT cells (80) and ferritin protein induced (4) in
response to UVB irradiation, however, the mechanism by which ferritin H mRNA induction occurs was unclear. In an effort to shed light on this mechanism, we tested HIPK2 and ferritin H response to UVB irradiation of HaCaT and MCF-7 cells. Our data show that UVB activated HIPK2 and induced ferritin H mRNA in these cell lines. We speculate that UVB-activated HIPK2 phosphorylates ATF1 on the new residue leading to activation of ferritin H transcription. An earlier study identified ATF1 as a responsible transcription factor for activation of transferrin receptor promoter after UV irradiation in B16 melanoma cells (95). Transferrin receptor mediates cellular uptake of iron by binding transferrin and carrying it into cells through endocytosis (95). The function of ATF1 appears to have a double edge sword effect on iron regulation and homeostasis. Not only does ATF1 repress transcription of the ferritin H gene, but also activates transcription of transferrin receptor gene, thus contributing to the accumulation of excess iron, which is required for an iron-dependent progression through the cell cycle in tumor cells (16) (85). Therefore, it is reasonable to assume that ATF1 modulation through phosphorylation by HIPK2 can play a dual role, protecting cells against iron-mediated damage and controlling cell proliferation and tumorigenic process.

In summary, we have presented compelling evidence that HIPK2 is an ATF1 kinase and a novel regulator of ferritin H gene.

1. ATF1 repressed transcription of the human ferritin H gene through the ARE.
2. HIPK2 and ATF1 are interaction proteins in mammalian cells.
3. HIPK2 activation of the ferritin H gene transcription is mediated by the ARE and is dependent on HIPK2 kinase activity.

4. HIPK2 phosphorylates ATF1 in vivo and in vitro.

5. HIPK2 overrode ATF1-mediated repression of the ferritin H gene.

6. Serine 63 of ATF1 is not the major phosphorylation site of HIPK2.

7. UVB irradiation of HaCaT and MCF-7 cells up-regulated ferritin H mRNA.

It is our belief that further investigation to gain better understanding and knowledge of the mechanism underlying the regulation of ferritin H can contribute toward alleviating iron-induced toxicity and oxidative damage related diseases that are afflicting humans. Therefore, an expansion and further development of this project would be a worthwhile scientific endeavor.

The following is an attempt to offer a possible future direction of this study.

1. Identification of HIPK2 phosphorylation site on ATF1. HIPK2 is a proline directed Serine/Threonine kinase and there are 6 possible phosphorylation sites in human ATF1, namely Thr99, Ser164, Ser198, Ser122, Thr184, and Ser186. While the first three sites are specific to ATF1, the last three are also conserved in CREB.

   a. Using point mutation of potential phosphorylation residues followed by in vitro kinase assay.

   b. Overexpression of wild type ATF1 or point mutants in suitable cell lines and analyzing by Western blotting using anti-HA or anti-ATF1
antibodies. An ATF1 point mutant that fails to yield slower migrating band on SDS-PAGE must contain the phosphorylation site of HIPK2.

2. Test whether or not mutation of the newly identified residue prevents HIPK2 and UV-induced ATF1 activation and the corresponding ferritin H transcription.

3. Generation of phospho-ATF1 antibody specific to the newly identified phosphorylation residue of HIPK2.

4. Test whether or not HIPK2 overexpression, UV-irradiation or treatment with oxidative stressors such as H2O2, tBHQ or hemin or with the chemotherapeutic drug, cisplatin can phosphorylate the newly identified residue on ATF1.

5. Investigate whether or not HIPK2 is involved in transcriptional activation of the ferritin H gene through phosphorylation of ATF1 in response to UV-irradiation or to treatments with other stressors, by knocking down HIPK2 using siRNA method.

6. Investigate whether or not other kinases also phosphorylate ATF1 on the newly identified site. This study may possibly open a new avenue for better understanding and characterization of ATF1 regulation by HIPK2 and other potential kinases and of its function not only in the regulation of ferritin H gene and iron-homeostasis, but also in its role as an enhancer of cell proliferation and tumorigenicity.

Our findings will open the way for better understanding of the role of HIPK2 in iron homeostasis and ferritin regulation and its potential significance in protecting
cells against oxidative stress-mediated damage and associated pathophysiological disorders.
GENERAL REFERENCES


Ferritin H Regulation by UVB

Introduction

Exposure to sunlight is a significant factor in the development of skin cancer including malignant melanoma. UV has been implicated in the initiation and promotion of tumor development (1) (10). UV can directly damage cells through pyrimidine and thymine dimmer formation, DNA strand break, and DNA-protein cross-linking to induce skin cancer (7). UV, however, can also have an indirect harmful effect on cells through the formation of reactive oxygen species via the Fenton reaction. The oxidative damage ensuing from this reaction can have mutagenic effect and may be involved in carcinogenesis. UVB-dependent generation of hydroxyl radical and lipid proxides has been observed in human keratinocytes and fibroblast cultures (8) (9). Furthermore, high iron medium was observed to drastically increase UV-induced damage to DNA in corneal and skin fibroblasts (3).

Ferritin H plays a significant role in the swift detoxification of iron under oxidative stress conditions owing to its ferroxidase activity that oxidizes (FII) to Fe (III) form for deposition within its iron core. Studies have shown an increase in ferritin H mRNA and protein in rat keratinocytes (11) and in HaCaT cells (6) (2) in response to UVB irradiation. Activation of many phase II detoxification and antioxidant genes including ferritin H is mediated by the highly conserved element termed antioxidant responsive element (ARE). We have identified ARE in both human (13) and mouse (14) ferritin H promoters. We previously showed that the transcriptional co-activator p300/CBP was a component required for mouse ferritin H ARE enhancer activity and was associated with ATF1 along with JunD and FosB (15). Moreover, we have
identified ATF1 as a binding protein to ferritin H ARE. Using yeast two-hybrid screening we isolated and identified HIPK2 as a binding protein to ATF1. HIPK2 has been demonstrated to be activated by UV irradiation (5) (4).

Although some studies showed that ferritin H expression was induced by UVB, little is known about the mechanism involved in the transcriptional regulation of the ferritin H gene in response to UVB. Therefore, based on these studies, we hypothesized that UVB radiation stimulates HIPK2 and modulates transcription of the ferritin H gene through ARE by posttranslational modification of ATF1 and the co-activator p300/CBP. The data provided here are neither sufficient nor conclusive to state that ARE mediates transcriptional activation of ferritin H gene in response to UVB irradiation.
Results and Discussion

UVB up-regulates ferritin H mRNA

To investigate UVB-mediated induction of ferritin H mRNA, HaCaT cells were irradiated with UVB at doses of 0, 5, 10, 15, 20 mj/cm², total RNA was isolated and Northern blot analysis was carried out. As shown in Figure 1, a dose-response analysis shows that UVB treatment increased ferritin H mRNA in a dose-dependent manner peaking at a dose of 10 mj/cm² and significantly declining at 20 mj/cm². This drastic decline in ferritin H mRNA suggests that 20 mj/cm² may be highly toxic resulting in excessive death of cells. This result suggests that UVB can activate transcription of human ferritin H gene.

UVB increases mouse ferritin H ARE promoter reporter activity

To begin to determine whether ferritin H transcriptional activity was mediated by the ARE, we transiently transfected a full length, 4.8 Kb 5' mouse ferritin H luciferase reporter plasmid into mouse keratinocytes, followed by UVB irradiation at doses of 0, 2, 4, 6, 8, and 10 mj/cm². The dose-response analysis in Fig. 2 shows that UVB increases ferritin H ARE reporter expression peaking at 8 mj/cm².

UVB may target two other unidentified elements besides ARE in the ferritin H promoter region

To confirm that the ferritin H ARE is indeed activated by UVB irradiation, mouse keratinocytes were transiently transfected with 0.22 kb (ARE-lacking) or 0.22 kb plus ARE ferritin H luciferase reporters. Following transfection, cells were subjected to UVB treatment at doses of 0, 10, 20, and 40 mj/cm² and luciferase expression levels measured. As shown in Figure 3, UVB increased the luciferase
activity of both the ARE-containing and ARE-lacking reporters, although the basal expression of the ARE-containing reporter is significantly higher than that of the ARE-lacking. This suggests that UVB may activate an enhancer element besides ARE in the 0.22 Kb ferritin H promoter region. To see if the combined effect of these two elements would have a synergistic effect on the luciferase expression driven by the ferritin H promoter of ferritin H promoter, we transfected mouse keratinocytes with a full length, 4.8 kb, containing the ARE, 5’ mouse ferritin H luciferase or a 0.22 kb plus ARE reporter. The cells were then irradiated with UVB at doses of 0, 10, or 20 mj/cm² and then subjected to analysis by luciferase assay. However, as shown in Figure 4, UVB activated both the full length and the 0.22 kb plus ARE constructs. However, the full-length ferritin luciferase reporter was activated at a lesser degree compared to that of the 0.22 Kb reporter. Although sufficient data is not presented, we speculate that there may be an element in the 4.8 Kb promoter region with inhibitory effect on further activation of the ARE.

To confirm these results in human keratinocytes, we transiently transfected HaCaT cells with +ARE (-4.5 kb) human ferritin H luciferase reporter. The cells were subsequently subjected to UVB irradiation at increasing doses of 0, 2.5, 5, 7.5, 10, or 15 mj/cm² (Fig. 5) or 0, 5, 10, or 15 mj/cm² (Fig. 6), and luciferase expression levels measured. We observed that, unlike with mouse keratinocytes, UVB did not activate the ferritin H ARE reporter.

The repression could probably be attributed to the possibility that the human ferritin H luciferase reporter plasmid being sensitive to UVB damage. In addition, the inevitable death of certain number of HaCaT cells from UVB-mediated damage
could contribute to the ineffectiveness of the ARE reporter plasmid DNA, resulting not only in the lack of reporter activity but also in its repression.
REFERENCES


UVB induces ferritin H mRNA in a dose-dependent manner

| 0 | 5 | 15 | 10 | 20 mj/cm² |

Figure 1. HaCaT cells were either left untreated or irradiated with UVB at doses of 5, 10, 15, or 20 mj/cm². Total RNA was isolated 44-48 hours after irradiation and northern blot analysis conducted. Ethidium bromide RNA staining is shown for RNA loading (bottom)
Fig. 2. Mouse keratinocytes (BALB-1-P-4), were transfected with full-length (-4.8 Kb) ferritin H luciferase reporter using transfast transfection method. 40-48 hours after transfection, cells were either left untreated or irradiated with UVB at doses of 2, 4, 6, 8, or 10 mj/cm². 24 hours after irradiation cells were lysed and total extracts collected to perform luciferase assay. Fold induction of the lysates obtained from unirradiated cells was set as 1.0. DNA transfection was carried out in duplicates and the result of one experiment is shown.
Fig. 3. Mouse keratinocytes (BALB 1-P-4), were transfected with (+ARE) or (-ARE) ferritin luciferase reporters using transfast transfection method. 48 hours after transfection, cells were irradiated with increasing doses of 0, 10, 20, or 40 mj/cm². 24 hours after irradiation whole-cell lysates were prepared and luciferase assay performed. Fold luciferase activity obtained from unirradiated cells was used as control. DNA transfection was carried out in duplicate, and the result of one experiment is shown.
Fig. 4. Mouse keratinocytes (BALB 1-P-4), were transfected with full length (4.8 Kb) or 0.22 Kb ferritin H luciferase reporters using transfast transfection method. 48 hours after transfection, cells were irradiated with increasing doses of 0, 10, or 20 mj/cm². 24 hours after irradiation whole-cell lysates were prepared and luciferase assay performed. Fold luciferase expression from unirradiated cells was used as control. DNA transfection was carried out in duplicate, and the result of one experiment is shown.
Fig. 5. HaCaT cells were transfected with +ARE (-4.5 kb) ferritin H luciferase reporter using transfast (tfx-20) method. 40-48 hours after transfection cells were irradiated with UVB at doses of 0, 2.5, 5, 7.5, 10, or 15 mj/cm². 24 hours after UVB irradiation total cell extracts were collected and luciferase assay performed. The luciferase activity of lysates obtained from cells that were left unirradiated was defined as 1.0. DNA transfection was carried out in duplicates and the results of three independent experiments and standard errors are displayed.
Fig. 6. HaCaT cells were transfected with +ARE (-4.5 kb) ferritin H luciferase construct using transfast transfection method. 40-48 hours after transfection cells were either irradiated with UVB at doses of 5, 10, or 15 mj/cm² or left untreated. 40-48 hours following irradiation, whole cell lysates were collected to perform luciferase assay. Fold luciferase activity of lysates obtained from cells left unirradiated was used as control. DNA transfection was conducted in duplicate and the result one experiment is shown.
Yeast Two-Hybrid Screening

We found that ATF1 repressed transcription of the human ferritin H promoter activity. However, the mechanism by which ATF1 exerts its repressive effect on transcription of ferritin H was unclear. We hypothesized that some potential interacting proteins may be cooperating with ATF1 in regulating transcription of the human ferritin H gene.

To begin to understand the mechanism by which ATF1 represses transcription of the human ferritin H gene, we set out to identify the potential interacting proteins with ATF1 using the yeast two-hybrid screening system. The known gene encoding human ATF1 was cloned into a bait vector (pGBD). In this way the gene for ATF1 was placed into a plasmid next to the gene encoding a DNA-binding domain for the yeast transcription factor, Gal4. We screened two cDNA libraries derived from mouse fibroblast and mouse B cell cloned in prey vectors pact II or pGAD 10, respectively.

The following is an overview of the steps that had been involved in our yeast two-hybrid screening.

1. Construction of a yeast two-hybrid plasmid, pGBDATF1, for screening cDNA libraries (match maker cDNA libraries) obtained from mouse fibroblast and mouse B cell to identify interacting molecules with ATF1.

   - Characterization with restriction enzyme digestion
   - Isolation of ATF1 DNA fragment
   - ATF1 DNA purification
   - Characterization, isolation and purification of the bait vector, pGBD
DNA ligation of ATF1 and pGBD

2. Library transformation and screening under –Histidine selection

The yeast strain, PJ69-4A was transformed with pAct II NIH3T3 cDNA library (prey) or pGAD 10 mouse B cell cDNA library (prey) and gave rise to 209 or 400 positive clones, respectively, on SD/A-WLH 2mM AT plates. Since HIS3 has a leaky expression in many yeast strains, we inhibited the basal expression of this gene with 3-amino-triazole (AT), a known inhibitor of the HIS3 product. Through titration we determined that 2 mM AT was optimal for our screening.

3. Master stock and 2\textsuperscript{nd} screening-Adenine selection

- All colonies that grew on SD/A-WLH 2 mM AT plates were streaked on SD/A-WL plates (master plates) and incubated at 30 \Celsius for 1-2 days.
- Colonies that grew very well on SD/A-WL plates (master plate) were streaked on SD/A-WLH 2 mM AT plates or SD-Ade-WL plates.
- 39 or 114 transformed yeast colonies from NIH3T3 cDNA library or mouse B cell cDNA library, respectively, were identified as having grown on both plates.

4. Library (prey) DNA from each of the 39 colonies from NIH3T3 and 16 colonies from the mouse B cell library was recovered using competent E.coli HB101 and alkali-lysis mini-prep method and the size of each insert was characterized with restriction enzymes.
5. Retransformation of the yeast PJ69-4A with the bait (PGBDhATF1) and the 39 pActII NIH3T3 cDNA clones and the 14 pGAD10 mouse B cell cDNA positive clones was performed as follows:

1. pGBD + pActII/pGAD10
2. pGBDATF1 + pActII/pGAD10
3. pGBD + pActII cDNA clone/pGAD10 cDNA clone
4. pGBDATF1 + pActII cDNA clone/pGAD10 cDNA clone

These combinations of DNA were plated on SD/A-WL plates for two to three days to allow yeast colony growth. Three to five independent colonies were picked and streaked on SD/A-WL plate and colonies were allowed to grow. The colonies were then streaked on SD/A-WLH 2 mM AT plate or on SD/A-Ade-WL plate. 21 clones from NIH3T3 cDNA library and 6 clones from mouse B cell cDNA library grew on both of these plates and were identified as definitely positive clones.

6. DNA was purified from each of the total 27 positive cDNA clones.

7. DNA was characterized with restriction enzyme digestion and gel electrophoresis.

8. Clones were identified by DNA sequencing.

The following is a brief description of some of the cDNAs identified as binding to ATF1 by the yeast two-hybrid screening. Also refer to the tables.

1. Homeodomain interacting protein kinase 2 (HIPK2), which is the subject of this dissertation.
2. Protein inhibitor of activated STAT3 (PIAS3). PIAS3 was originally identified as a specific inhibitor in the signaling pathway of signal transducer and activator of transcription protein 3 (STAT3). Some lines of evidence showed that the PIAS family is involved in the regulation of various cytokine/growth factor signaling pathways and transcription factors including NF-kB and SMADs through protein-protein interactions (12). The study on PIAS3 interaction with ATF1 and its role on the regulation of the human ferritin H gene through the antioxidant responsive element has been finalized and submitted for publication.

3. Chromodomain helicase DNA binding protein 3 (CHD3) is a zinc finger helicase. Although the functional role of CHD3 is not thoroughly understood, some studies revealed that in association with histone deacetylase1/2 (HDAC1/2), it does have an important role in gene transcriptional repression. Investigation of the functional role of CHD3/4 in the transcriptional regulation of the human ferritin H gene is in progress in our lab.

4. ATF7-interacting protein, also known as MCAF1 (methyl CpG-binding domain-containing chromatin associated factor 1); or ATFa-associated modulator. MCAF1 is known to interact with the novel histone H3, lysine 9-specific methyltransferase, SETDB1 and to cause transcriptional repression through cooperation with SETDB1 in the conversion of H3-K9 dimethyl to trimethyl. We propose that MCAF1 cooperation with ATF1 on histone H3 methylation leads to transcriptional repression of the human ferritin H gene.
5. Ubiquitin-conjugating enzyme E-2I is a member of the family of proteins involved in the ubiquitin-dependent protein degradation system, but its functional role in cooperation with ATF1 in ferritin H regulation is currently unknown.

6. Zinc finger protein (Miz1), a DNA-binding protein; contains at least one zinc-finger that permits it to interact with DNA. This protein is known to involve in the regulation of gene expression, but its significance in the transcriptional regulation of human ferritin H gene as related to ATF1 has not been characterized so its function in this area unknown.
Table 1. List of cDNA clones identified by yeast two-hybrid screening as binding to ATF1

<table>
<thead>
<tr>
<th>Sequence</th>
<th>Clone #</th>
<th>Insert Size (EcoRI/Xho)</th>
<th>Insert Size (Bgl II)</th>
<th>Calculated size (kb)</th>
<th>Retransformation (-His)</th>
<th>Retransformation (-Ade)</th>
<th>Alignment</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. HIPK2</td>
<td>22</td>
<td>0.80</td>
<td>1.86</td>
<td>1.86</td>
<td>(+)</td>
<td>(+)</td>
<td>In frame</td>
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<tr>
<td>2. PIAS3</td>
<td>63A</td>
<td>1.2 + 0.75 + 0.25</td>
<td>1.2 + 0.70</td>
<td>1.70</td>
<td>(+)</td>
<td>(+)</td>
<td>In frame</td>
</tr>
<tr>
<td>3. CHD3</td>
<td>88A</td>
<td>2.30</td>
<td>2.30</td>
<td>1.75</td>
<td>(+)</td>
<td>W(+)</td>
<td>In frame</td>
</tr>
<tr>
<td></td>
<td>187A</td>
<td>2.0</td>
<td>smear</td>
<td>2.0</td>
<td>(+)</td>
<td>(+)</td>
<td>In frame</td>
</tr>
<tr>
<td>4. ATF7-interacting protein</td>
<td>70B</td>
<td>1.50</td>
<td>1.50</td>
<td>1.50</td>
<td>(+)</td>
<td>(+)</td>
<td>In frame</td>
</tr>
<tr>
<td></td>
<td>175A</td>
<td>1.50</td>
<td>1.50</td>
<td>1.50</td>
<td>(+)</td>
<td>(+)</td>
<td>In frame</td>
</tr>
<tr>
<td></td>
<td>180A</td>
<td>1.50</td>
<td>1.50</td>
<td>1.50</td>
<td>(+)</td>
<td>(+)</td>
<td>In frame</td>
</tr>
<tr>
<td>5. Ubiquitin-conjugating enzyme E-21</td>
<td>171A, 19, 21, 53</td>
<td>2.80</td>
<td>1.90 + 0.5</td>
<td>1.0</td>
<td>(+)</td>
<td>W(+)</td>
<td>In frame</td>
</tr>
</tbody>
</table>
Table 1 (continued)

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<tr>
<th>Sequence</th>
<th>Clone #</th>
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<th>Insert Size (Bgl II)</th>
<th>Calculated size (kb)</th>
<th>Retransformation (-His)</th>
<th>Retransformation (-Ade)</th>
<th>Alignment</th>
</tr>
</thead>
<tbody>
<tr>
<td>6. Zinc Finger Protein</td>
<td>105B,172A,10B,52A,75A,92A,184A</td>
<td>2.50</td>
<td>2.50</td>
<td>1.98</td>
<td>(+)</td>
<td>(+)</td>
<td>In frame</td>
</tr>
<tr>
<td>7. Ranbp9 (RANBPM)</td>
<td>56A</td>
<td>0.8 +0.7</td>
<td>0.8 +0.6</td>
<td>1.90</td>
<td>(+)</td>
<td>(+)</td>
<td>In frame</td>
</tr>
<tr>
<td>8. Srebp2</td>
<td>85A,1, 14</td>
<td>2.50</td>
<td>2.50</td>
<td>1.75</td>
<td>(+)</td>
<td>(+)</td>
<td>In frame</td>
</tr>
<tr>
<td>9. Sperm associated antigen 5</td>
<td>89A</td>
<td>1.2+.75+.1</td>
<td>1.75+.35</td>
<td>2.0</td>
<td>(+)</td>
<td>(+)</td>
<td>In frame</td>
</tr>
<tr>
<td>10. Collagen alpha</td>
<td>199A</td>
<td>0.9+0.3</td>
<td>2.20</td>
<td>-</td>
<td>(+)</td>
<td>(+)</td>
<td>Out of frame</td>
</tr>
</tbody>
</table>

Identification of ATF1 interacting proteins by yeast two-hybrid method

The yeast strain, PJ69-4A was transformed with mouse fibroblast or mouse B cell cDNA library using pGBDATF1 as bait in the screening. Positive clones were screened under histidine or adenine deficient media selection process. Library DNA from yeast colonies was recovered, characterized and retransformed into yeast cells. DNA was purified from positive cDNA clones, characterized and subjected to DNA sequencing.