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Arsenite Targets the RING Finger Domain of Rbx1 E3 Ubiquitin Ligase to Inhibit Proteasome-Mediated Degradation of Nrf2

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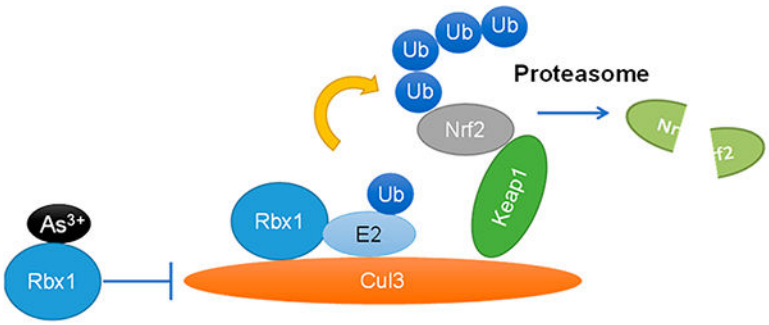
Abstract

Activation of the nuclear factor erythroid 2–related factor 2 (Nrf2) antioxidant response signaling pathway is a major mechanism for the cellular defense against oxidative stress. Arsenite, a widespread contaminant in drinking water, is known to induce oxidative stress and activate the Nrf2-dependent signaling pathway through the stabilization of the Nrf2 protein by inhibiting its ubiquitination via the Cul3–Rbx1–Keap1 (cullin 3, RING-box 1, and Kelch-like ECH-associated protein 1) E3 ubiquitin ligase, and its degradation by the 26S proteasome, though the underlying mechanism, remains elusive. In the present study, we demonstrated that arsenite could bind to the RING finger domain of Rbx1 in vitro and in cells, which led to the suppression of Cul3–Rbx1 E3 ubiquitin ligase activity, thereby impairing the Nrf2 ubiquitination and activating the Nrf2-induced antioxidant signaling pathway. Our finding provided novel insight into arsenic toxicity by uncovering a distinct mechanism accounting for arsenite-induced Nrf2 activation.

Abstract



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INTRODUCTION

Arsenic is one of the most ubiquitous toxic substances in the environment, and more than 150 million people in >70 countries are chronically exposed to excessive amounts of arsenic species in drinking water, leading to high incidences of skin, lung, kidney, liver, and bladder cancers as well as neurological disorders, cardiovascular diseases, and diabetes.[1,2] Numerous studies have been conducted to explore the molecular mechanisms underlying the toxic and carcinogenic effects of arsenic species, where DNA hypomethylation, perturbation of signal transduction, repression of DNA repair, alteration of gene expression, and induction of oxidative stress are thought to be important.[3,4]

Previous studies have revealed that arsenite could bind to side chain sulfhydryl groups of vicinal cysteine residues in the zinc finger domains of proteins, which may contribute to arsenic toxicity and carcinogenicity.[5] In this respect, arsenite was found to bind to the Cys3His (C3H)- or Cys4 (C4)-type zinc fingers much more strongly than those of the Cys2His2 (C2H2)-type.[6] Since most RING finger domains harbor a C3H- and a C4-type zinc finger,[7–9] our recently published work demonstrated the interactions between arsenite and the RING finger domains of proteins, including several E3 ligases, such as FANCL and RNF20-RNF40, which ultimately led to the inhibition of the repair of DNA interstrand cross-link lesions and double strand breaks, respectively.[10,11] Our previous work also elucidated the interaction between arsenite and the zinc finger motifs of Tip60 histone acetyltransferase and ten-eleven translocation (Tet) family proteins, which perturbs histone and DNA epigenetic marks.[12,13]

Oxidative stress has also been proposed to contribute to arsenic toxicity.[14] Under normal physiological conditions, reactive oxygen species (ROS), such as hydrogen peroxide, hydroxyl radical, and superoxide anion, are regulated, which serve as signaling molecules to modulate multiple biological processes, including inflammation, cell division, autophagy, and stress response.[15–19] Nonetheless, ROS are excessively produced in cells exposed to arsenite, which results in oxidative stress, thereby impairing cellular functions and inducing cell cycle arrest, apoptosis, and even cancer development.[20–22]

Activation of the Nrf2–Keap1 pathway is the main cellular defense mechanism to protect cells from oxidative stress.[23] Under unstressed conditions, nuclear factor erythroid 2–related factor 2 (Nrf2) is negatively regulated by Kelch-like ECH-associated protein 1 (Keap1), which functions as an adaptor of Nrf2 to facilitate its ubiquitination and degradation by cullin 3-RING box 1 (Cul3-Rbx1) E3 ubiquitin ligase and the 26S proteasome, respectively.[24] However, under stress conditions, Nrf2 is stabilized due to the deactivation of Keap1, which activates transcription of downstream antioxidant genes, such as NAD(P)H:quinone oxidoreductase (NQO1) and heme oxygenase-1 (HO-1).[25] Keap1 is a highly susceptible target for thiol-reactive chemical species because it contains a large number of cysteine residues.[26] Indeed, cysteine residues at positions 151, 257, 288, and 297 were previously identified as the sites for attack by electrophilic species and thought to be crucial for Keap1's function.[27] However, these cysteine residues are not part of C3H- or C4-type zinc fingers that are susceptible to arsenite binding.

Since arsenite is known to induce oxidative stress, previous studies have demonstrated that the Nrf2–Keap1 pathway can also be activated by arsenite.[28] However, the arsenite-induced activation of Nrf2 has been shown to be independent of cysteine residues in Keap1, suggesting a distinct mechanism contributing to the arsenite-induced activation of the Nrf2–Keap1 pathway.[29] It remains insufficiently understood about the exact mechanism underlying this pathway, though it was found to be p62-dependent.[30]

On the grounds of the previous findings that arsenite could bind to the RING finger domains of proteins, we hypothesized that arsenite is capable of binding to the RING finger domain of Rbx1, the E3 ubiquitin ligase component of the Cul3-Rbx1 complex, which may inhibit Nrf2 ubiquitination and activate the antioxidant response through stabilizing the Nrf2 protein in cells.

EXPERIMENTAL PROCEDURES

Plasmid Construction and Site-Directed Mutagenesis.

Primers 5′-AAACTCGAGGCGGCAGCGATGGATGTGGATA-3′ and 5′-AAAGATCCCTAGTGCCCATACTTTTGGAATTCCC-3′ were designed to construct the pEGFP-C3-Rbx1 plasmid. Total RNA was extracted from the HEK293T human embryonic kidney epithelial cells using the E.Z.N.A. Total RNA Kit I (Omega, Norcross, GA) and reverse-transcribed into a cDNA library, which served as a template to clone the coding sequence of Rbx1 into the BamHI/Xhol sites of the pEGFP-C3 vector by PCR.

The pCDNA3.1-Nrf2-FLAG and HA-ubiquitin plasmids were purchased from Addgene, and the pCDNA3.1-Rbx1-HA plasmid was kindly provided by Dr. Pengbo Zhou at Weill Cornell Medical College. In this vein, the amino acid sequence for the FLAG tag is DYKDDDDK, and the HA tag, with the amino acid sequence of YPYDVPDYA, was derived from residues 98–106 of human influenza hemagglutinin. Primers 5′-GTAACCATGCTTTTTGCTTCCACTG-3′, 5′-GTGGAAGCAAAAAGCATGGTTACAG-3′ or 5′-GCTTTTCACTTCCACCACATCTCTC-3′, 5′-GCGAGAGATGTGGTGGAAGTGAAAA-3′ were designed and used to construct plasmids of Rbx1-HA and Rbx1-GFP harboring the H80C and C83H mutations, respectively, by employing the GeneArt Site-directed Mutagenesis Kit (Thermo Fisher Scientific, Waltham, MA).

Cell Culture and Transfections.

HEK293T cells were maintained in a humidified atmosphere containing 5% CO_2 at 37 °C and cultured in Dulbecco's Modified Eagle Medium (DMEM, ATCC, Manassas, VA) containing 10% fetal bovine serum (FBS, Invitrogen, Waltham, MA) and 100 U/mL of penicillin and streptomycin. Typically, 1 μg of plasmid or 100 pmol siRNA was transfected into cells with 5 μL of Lipofectamine 2000 (Thermo Fisher Scientific, Waltham, MA).

In Vitro Arsenite Binding Assay.

The procedures for monitoring the interaction between the RING finger peptide of Rbx1 and As(III) were previously described.[12] Briefly, the RING-finger peptide derived from Rbx1 (VDNCAICRNAFHFHCISR) was synthesized by Genemed Synthesis Inc. (San Antonio, TX), dissolved in 50% acetonitrile, and used for the in vitro binding assays. Aliquots of 100 μM peptide were mixed with 200 μM $NaAsO_2$ and 100 μM dithiothreitol (DTT) and incubated on ice for 1 h. The solution was subsequently mixed with an equal volume of 2,5-dihydroxybenzoic acid matrix solution and spotted onto a sample plate. The samples were analyzed on a MALDI TOF/TOF 5800 system (Applied Biosystems, Foster City, CA) in the positive-ion, reflectron mode. Competition experiments were conducted by incubating the peptide with $NaAsO_2$ and $ZnCl_2$ at varying molar ratios. The peptide was also titrated with an increasing amount of $NaAsO_2$ and subjected to UV absorption measurements on a Varian Cary 50 UV–visible spectrophotometer (Palo Alto, CA). The UV absorbance was monitored in the wavelength range of 230–400 nm.

Streptavidin Agarose Affinity Assay and Western Blot.

HEK293T cells were seeded in 6-well plates at a density of 1×10^6 cells per well and transfected with wild-type or mutant pCDNA3.1-Rbx1-HA plasmids for 24 h. The cells were then mock-treated or treated with 5 μM $ZnCl_2$, $NaAsO_2$, or p-aminophenyl arsenoxide (PAPAO) for 1 h and subsequently incubated with 5 μM biotin-As probe for 1 h, as described previously.[11] The cells were then harvested and lysed in CelLytic M lysis buffer containing 1% protease inhibitor cocktail (Sigma-Aldrich, St. Louis, MO). Streptavidin agarose beads (Thermo Fisher, Waltham, MA) were incubated with the whole cell lysate at 4 °C overnight, washed with 1 × PBS three times, resuspended in SDS-PAGE loading buffer, and subjected to SDS-PAGE analysis.

After SDS-PAGE separation, proteins were transferred to a nitrocellulose membrane at 40 V for 2 h in a buffer containing 10 mM $NaHCO_3$, 3 mM Na_2CO_3, and 20% methanol. The membrane was then pretreated with phosphate buffered saline with Tween-20 (PBS-T) containing 5% nonfat milk at room temperature for 1 h and incubated with anti-HA primary antibody (Abcam, Cambridge, MA) at 4 °C overnight. The membrane was subsequently incubated with anti-rabbit secondary antibody at room temperature for 1 h and thoroughly washed with PBS-T. The protein bands were detected by the ECL Advanced Western Blotting Detection Kit (GE Healthcare, Chicago, IL) and visualized with Hyblot CL autoradiography film (Denville Scientific, Inc., Metuchen, NJ). The membranes were also probed for input Rbx1 and β-actin using anti-HA and anti-actin antibodies (Abcam, Cambridge, MA) to confirm equal protein loading.

Fluorescence Microscopy.

Wild-type and mutant pEGFP-C3-Rbx1 plasmids (0.5 μg each) were transfected individually into 1×10^5 HEK293T cells seeded on cover glasses. After 24 h, the transfected cells were mock treated or treated with 10 μM $ZnCl_2$, $NaAsO_2$, or PAPAO and then incubated with 5 μM ReAsH-EDT_2 (Invitrogen, Waltham, MA) in Opti-MEM medium at 37 °C for 1 h. They were washed with BAL buffer 3 times, fixed with 4% paraformaldehyde for 20 min, and stained with 4′,6-diamidino-2-phenylindole (DAPI). The sample slides were subjected to a

Leica TCS SP5 confocal microscope (Leica Microsystems, Buffalo Grove, IL) for imaging in the wavelength range of 425–450, 500–550, and 580–650 nm for DAPI, GFP, and ReAsH, respectively.

Nrf2 Ubiquitination Assay.

HEK293T cells were seeded in 6-well plates at a density of 2×10^5 cells per well. A 100 pmol of siRbx1 targeting the 3′-UTR of Rbx1 mRNA (5′-GACUUUCCCUGCUGUUACCUAAdTdT-3′) was transfected into each well to knock down the endogenous Rbx1 protein. After 24 h, 1 μg of siRNA-resistant plasmid encoding wild-type or mutant Rbx1-HA, 1 μg of Nrf2-FLAG, and 0.5 μg of ubiquitin-HA plasmids together with Lipofectamine 2000 were added into each well. After 1 day, the cells were treated with 5 μM $NaAsO_2$ for 24 h and subsequently incubated with 10 μM MG132 for 2 h. In this vein, MG132 was added to inhibit protein degradation mediated by the 26S proteasome. The resulting whole cell lysate was incubated with anti-FLAG M2 magnetic beads (Sigma, St. Louis, MO) at 4 °C overnight, and the beads were then resuspended in the SDS-PAGE loading buffer. After SDS-PAGE separation, Western blot analysis was performed to detect Nrf2 ubiquitination using the anti-HA antibody. The input Nrf2 and β-actin were detected using the anit-Nrf2 (Santa Cruz Biotechnology, Dallas, Texas) and anti-Actin antibody, respectively.

Statistical Analysis.

All data were presented as the mean and standard deviation of the results obtained from multiple replicates, where the numbers of replicates for the specific experiments are indicated in the figure legends. The P values were calculated using a two-tailed, unpaired Student's t test: *, $0.01 < P < 0.05$; **, $< P < 0.01$; ***, $P < 0.001$.

RESULTS

Arsenite Binds to the RING Finger Domain of Rbx1 in Vitro.

To explore if Rbx1 plays a role in arsenite-induced Nrf2 activation, we first assessed the interaction between arsenite and the RING finger domain of Rbx1. To this end, we performed a matrix-assisted laser desorption/ionization-time-of-flight (MALDI-TOF MS)-based in vitro binding assay with the use of a synthetic peptide derived from the RING finger domain of the human Rbx1 protein, which contains a $Cys_3HisCys_2HisAsp$ (C3HC2HD)-type zinc finger[31] (Figure 1a). The $[M + H]^+$ ion of the apopeptide was detected at m/z 2106 in MALDI-MS, while a mass increase of 72 Da was found upon incubation of the synthetic peptide with As^{3+} at a molar ratio of 1:2. This mass increase reflects the coordination of one As^{3+} with the peptide along with the releases of three protons from the sulfhydryl groups of cysteine residues in the RING finger domain (Figure 1b).[6] We also added increasing concentrations of Zn^{2+} to the As^{3+}-peptide mixture, and found that, even in the presence of the excessive amount of Zn^{2+} relative to As^{3+}, we could still observe the interaction between As^{3+} and the synthetic peptide in MALDI-MS, while the corresponding peptide-Zn^{2+} complex was not detectable, suggesting that As^{3+} binds to the peptide much more strongly than Zn^{2+} (Figure 1b).

The alteration in charge-transfer electronic transitions of the peptide forged by coordination of As^{3+} with the sulfhydryl group of cysteine residues in the RING finger domain can be monitored by UV absorption in the wavelength range of 230– 400 nm.[32] As expected, a heightened absorbance of the peptide was detected upon titrating the peptide with increasing amounts of As^{3+} from 0 to 2 equiv, indicating a gradual increase in binding of As^{3+} to the RING finger peptide (Figure 1c). Together, the above results demonstrated that As^{3+} could bind to the RING finger domain of Rbx1 in vitro.

Arsenite Interacts with Rbx1 in Cells.

Having established the interaction between As^{3+} and Rbx1 in vitro, we next performed a streptavidin agarose affinity pull-down assay to examine if this interaction could also occur in mammalian cells. Toward this end, we ectopically expressed the HA-tagged Rbx1 protein in HEK293T cells and incubated the transfected cells with a synthetic biotin-As probe, which is a conjugate between biotin and p-aminophenyl arsenoxide (PAPAO, Figure 2a).[33] We subsequently precipitated biotin-As-binding proteins with streptavidin beads[34] and probed for HA-Rbx1 by using Western blot. The results showed that the biotin-As probe could facilitate the pull-down of HA-Rbx1 from HEK293T cells with the use of streptavidin beads, manifesting that arsenite could indeed bind to Rbx1 in cells (Figure 2b). Next we asked if As^{3+} could compete with Zn^{2+} to bind to the Rbx1 protein. In this vein, we pretreated cells with Zn^{2+}, As^{3+}, or PAPAO before we incubated cells with the biotin-As probe. Our results showed that the interaction between biotin-As and Rbx1 was substantially compromised upon pretreatment with As^{3+} or PAPAO, though a slight suppression was also detected with Zn^{2+} pretreatment, demonstrating a stronger binding of Rbx1 toward As^{3+} than Zn^{2+} (Figure 2c,d).

Arsenite Interacts with the RING Finger Domain of Rbx1 in Cells.

To explore further the interaction between arsenite and the RING finger domain of Rbx1, we employed a fluorescence-microscopy-based assay with the use of the ReAsH-EDT_2 dye (Figure 3b), which contains two As^{3+} and displays red fluorescence when cysteine residues in proteins bind to its arsenic moieties.[35] We observed substantial co- localization of the ectopically expressed Rbx1 and ReAsH, suggesting the interaction between the As^{3+} and Rbx1 protein (Figure 3a,c).

To test if cysteine residues in the RING finger domain of Rbx1 are crucial for this interaction, we also expressed mutant Rbx1 proteins, i.e., Rbx1-H80C and Rbx1-C83H, in HEK293T cells and measured their co-localization with ReAsH. Our results revealed that, relative to wild-type GFP-Rbx1, GFPRbx1-H80C exhibited a slightly elevated co-localization with ReAsH, whereas this co-localization was nearly abolished for GFP-Rbx1-C83H (Figure 3a,b). In this vein, the stronger interaction between ReAsH and GFP-Rbx1-H80C than the wild-type Rbx1 is attributed to that fact that the two As^{3+} in ReAsH could bind all four cysteine residues in the Cys4-type of zinc finger in the mutant protein. Together, our results revealed that arsenite could co-localize with ectopically expressed Rbx1 in mammalian cells, and the co-localization requires the cysteine residues in the RING finger domain of Rbx1. In addition, we pretreated transfected cells with Zn^{2+}, As^{3+}, or PAPAO prior to incubating cells with the ReAsH-EDT_2 dye. As expected, we observed a

reduced co-localization between GFPRbx1 and ReAsH upon pretreatment with As^{3+} or PAPAO, but not Zn^{2+}, supporting the competitive binding of As^{3+} to cysteine residues in the RING finger motif of Rbx1 (Figure 4a,b).

Interaction between Arsenite and Rbx1 Is Required for Arsenite-Induced Stabilization of Nrf2.

Exposure of cells to arsenite has been known to activate the Nrf2-induced antioxidant pathway, though the detailed mechanism remains incompletely understood.[28] Having demonstrated the interaction between arsenite and Rbx1 both in vitro and in cells, we next asked if this interaction could affect the activation of Nrf2. We first exposed HEK293T cells with increasing concentrations of As^{3+} from 0 to 5 μM. Not surprisingly, the levels of endogenous Nrf2 were increased in a dose-dependent manner with arsenite exposure, indicating that arsenite could stabilize Nrf2 protein in cells (Figure 5a,b).

It is well established that Nrf2 is ubiquitinated by the Keap1–Cul3–Rbx1 E3 ligase complex.[36] When endogenous Rbx1 was depleted using siRNA, the ubiquitination level of Nrf2 was dramatically decreased (Figure 5c, lanes 1–2). Nevertheless, if we complemented cells with siRNA-resistant constructs for the expression of wild-type or mutant Rbx1 proteins after knocking down endogenous Rbx1, the ubiquiti-nation of Nrf2 was rescued to a similar level as to the control (Figure 5c, lanes 1 and 3–5). These results indicated that Rbx1 is essential for Nrf2 ubiquitination.

Previous studies have revealed that arsenite could perturb ubiquitination of proteins by targeting E3 ubiquitin ligases.[11] As expected, we observed a significant reduction of Nrf2 ubiquitination in cells upon arsenite treatment (Figure 5c, lanes 1 and 6). This result suggested that arsenite stabilized the Nrf2 protein through inhibiting its ubiquitination and subsequent proteasomal degradation.

To further demonstrate that the binding of arsenite with the RING finger domain of Rbx1 accounts for the perturbation of Nrf2 ubiquitination, we complemented HEK293T cells with plasmids for the ectopic expression of wild-type or mutant Rbx1 proteins, followed by arsenite treatment. The results showed that Nrf2 ubiquitination was rescued in cells reconstituted with Rbx1-C83H to a much greater extent those with wild-type Rbx1 or Rbx1-H80C (Figure 5c, lanes 8–10). This finding is in keeping with the fact that arsenite could not target Rbx1-C83H as strongly as wild-type Rbx1 or Rbx1-H80C, as shown from the aforementioned fluorescence microscopy experiments. Thus, the above results support that the binding of arsenite to the RING finger domain of Rbx1 is crucial for Nrf2 ubiquitination. However, arsenite could still suppress Nrf2 ubiquitination even in the presence of Rbx1-C83H (Figure 5c, lanes 5 and 10), though the suppression was not as pronounced as in the presence of wild-type Rbx1 or Rbx1-H80C (Figure 5c, lanes 8–10). The latter might be attributed to the presence of other mechanisms contributing to the arsenite-induced perturbation of Nrf2 ubiquitination.[29,30,37]

Taken together, our finding suggested that arsenite targets the RING finger domain of Rbx1 E3 ubiquitin ligase, which is responsible for stabilizing the Nrf2 protein by inhibiting its ubiquitination and proteasome-mediated degradation.

DISCUSSION

A global genome-wide analysis of Nrf2 targets has identified a number of antioxidant-element-containing genes, such as HO-1, glutathione S-transferase (GST), and NQO1, revealing the major role of Nrf2 to activate the antioxidant response and for the detoxification of harmful substances.[38] Along this line, Nrf2-knockout mice displayed higher sensitivity toward a wide range of oxidative stress inducers, including, but not limited to, tertbutylhydroquinone (tBHQ), sulforaphane (SF), and arsenite.[39–43] The activity of Nrf2 is suppressed under basal conditions. The Cul3-Rbx1 guides the polyubiquitination and rapid degradation of Nrf2 by the 26S proteasome with the aid of Keap1, which acts as a substrate adaptor to localize Nrf2 to the Cul3-Rbx1 E3 ligase, leading to a low constitutive level of the Nrf2 protein with a turnover rate of ~20 min under normal conditions.[44,45] Hence, the constant assembly/disassembly cycle of Keap1 to Cul3-Rbx1 E3 ligase is required for Keap1-mediated Nrf2 ubiquitination and degradation, whereas both enhanced and diminished associations of Keap1 with Cul3-Rbx1 E3 ligase could lead to Nrf2 stabilization.[46] Nrf2 inducers, such as tBHQ and SF, were found to activate Nrf2 by negatively modulating the interaction between Keap1 and Cul3.[47,48] Several distinct reactive cysteine residues in Keap1 serve as sensors for inducers to modify and impede Nrf2 ubiquitination. In this vein, C273 and C288 were shown to be crucial for suppression of Nrf2 under basal conditions, whereas C151 was required for the activation of Nrf2 by inducers like tBHQ and SF.[27] It was also found that different inducers might target different cysteine residues. For example, C288 is a sensor for alkenals, whereas C226/C613 serves as a sensor for Zn^{2+}.[49] The preferences of disparate cysteine residues for different inducers suggest the presence of “cysteine codes” for Nrf2 activation by various chemicals.

The “cysteine codes” of Keap1 are not always sufficient for understanding the activation of Nrf2 since some inducers require additional protein factors. For instance, unlike tBHQ and SF, arsenite was found to activate Nrf2 by enhancing the interaction between Keap1 and Cul3 in a Keap1 C151-independent manner,[29] promoting us to explore distinct mechanisms of arsenite-induced activation of Nrf2. Since the interaction between arsenite and vicinal thiols in proteins has been proposed to account for the carcinogenic and cytotoxic effects of arsenic species,[5] cysteine residues in Nrf2, which is an evolutionarily conserved protein, have been found to be arsenite sensors to facilitate Nrf2 activation. Studies in mouse cells have revealed an interaction between arsenite and wild-type Nrf2, whereas this interaction was compromised in Nrf2 C311/C316, C235, C414, and C506 mutants. Moreover, arsenite activated wild-type Nrf2 but not the C191A, C235A, C311A/C316A, C414A, or C506A mutants in mouse cells.[37] Another protein factor that is important for arsenite-induced activation of Nrf2 is p62, which is a selective substrate adaptor for the degradation of specific proteins through autophagy.[50] Arsenite deregulates autophagy and causes an overexpression of p62, which directly binds to Keap1 to block Keap1-mediated Nrf2 ubiquitination. Interestingly, this p62-dependent pathway is only responsible for arsenite-induced activation of Nrf2, but not for the activation of Nrf2 by tBHQ or SF.[30] The Bcl-XL-interacting protein PGAM5 and p53-regulated p21 were also identified as disruptors for the binding of Keap1 with Nrf2 to activate Nrf2.[51,52]

Rbx1 is a major regulator of the Nrf2 antioxidant pathway since it serves as the E3 ubiquitin ligase for Nrf2 ubiquitination. On the basis of the fact that it contains a cysteine-rich RING finger domain, it prompted us to investigate if cysteine residues in Rbx1 could also serve as sensors for arsenite-induced activation of Nrf2. In this study, we demonstrated the interaction between arsenite and the RING finger domain peptide of Rbx1 by MALDI-TOF MS and UV absorption spectrophotometry analyses. We also revealed, for the first time, the competitive binding of arsenite to wild-type Rbx1 in human cells by the streptavidin agarose affnity pull-down assay and ReAsH-EDT_2 fluorescent microscopy assay, whereas the Rbx1-C83H mutant failed to co-localize with As^{3+}-containing ReAsH, indicating the critical role of the cysteine residue in the RING finger domain for arsenite binding to Rbx1. Furthermore, we demonstrated the significance of this interaction in arsenite-mediated impairment of Nrf2 ubiquitination, where the Rbx1-C83H mutant compromised the arsenite-induced suppression of Nrf2 ubiquitination. To summarize, our present study provides a distinct mechanism underlying the arsenite-mediated activation of the Nrf2 antioxidant pathway.

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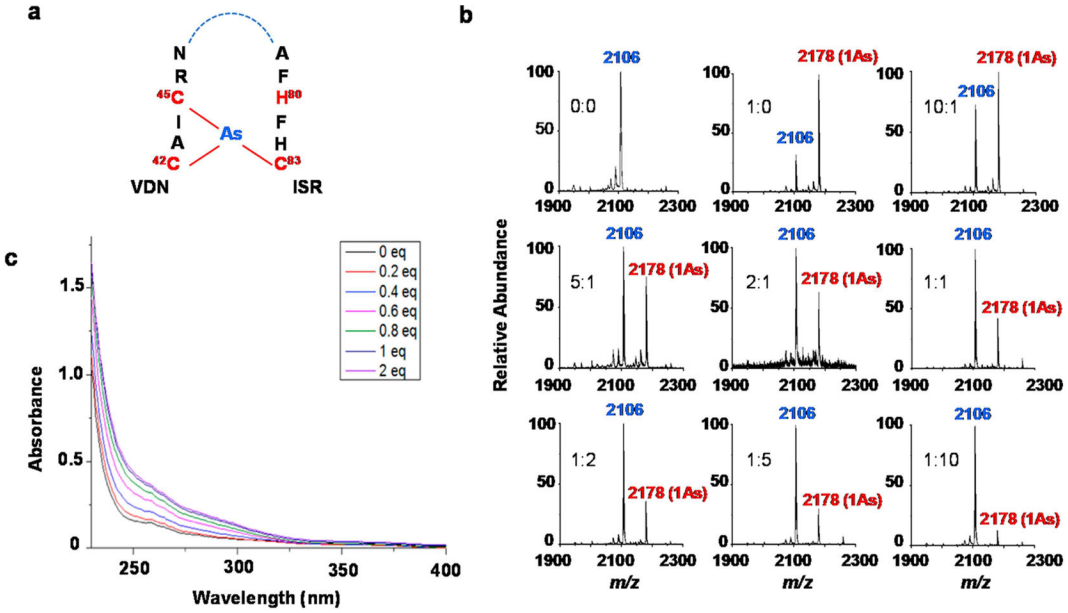

Figure 1.

Arsenite binds to the RING finger domain of Rbx1 in vitro. (a) The interaction between As^{3+} and a peptide derived from the RING finger domain of RBX1 (a.a 39–47 and 78–84). (b) MALDI-TOF mass spectrum showing the interaction between arsenite and the RING-finger peptide of Rbx1. The synthetic peptide was incubated with different molar ratios of As^{3+}/Zn^{2+} as indicated in each spectrum. The results showed that the apopeptide could bind to one As^{3+}. (c) UV absorption spectrum of the RING finger peptide of Rbx1. The synthetic peptide was sequentially titrated with increasing concentrations of $NaAsO_2$, and its UV absorbance was monitored in the wavelength range of 230–400 nm.

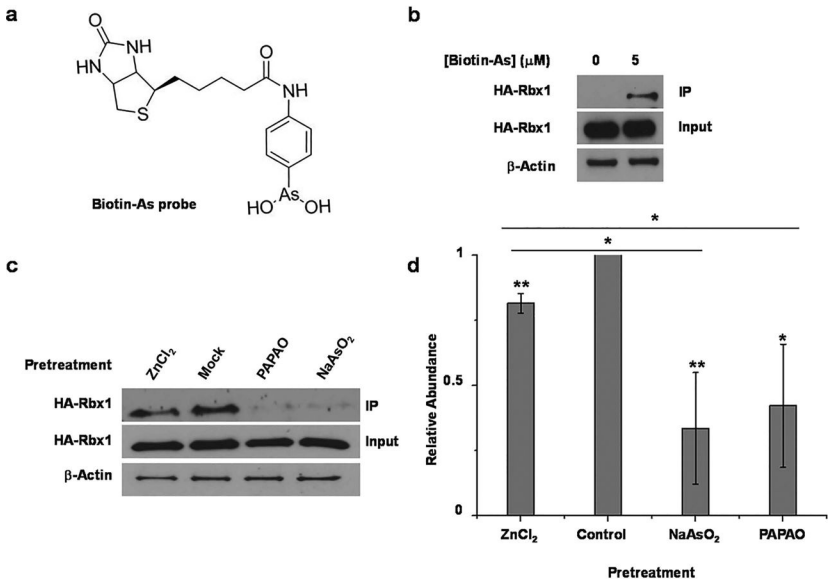

Figure 2.

Arsenite binds to the Rbx1 protein in cells. (a) The chemical structure of the biotin-As probe. (b) Streptavidin agarose affinity pull-down assay indicating the interaction between As^{3+} and Rbx1 in cells. The biotin-As probe was used to pull down ectopically expressed HA-Rbx1 in HEK293T cells. The HA-Rbx1 signal was detected using the anti-HA antibody, and the input HA-Rbx1 and actin were also detected. (c,d) The interaction between the biotin-As probe and Rbx1 was substantially diminished upon pretreatment with 10 μM $NaAsO_2$, PAPAO, and to a lesser extent, 10 μM Zn^{2+}. The Western blot images are shown in (c), and the quantification results are displayed in (d). The data represent the means and standard deviations of results obtained from 3 biological replicates. The P values (for comparisons with the control, unless otherwise indicated) were calculated using an unpaired two-tailed student's t-test (*, $0.01 < P < 0.05$; **, $0.001 < P < 0.01$).

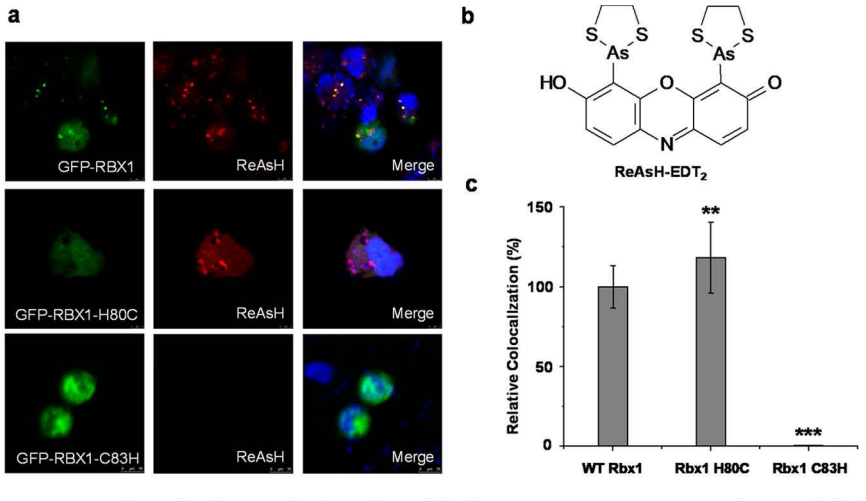

Figure 3.

As^{3+}-bearing dye ReAsH-EDT_2 co-localizes with GFP-Rbx1. (a) Fluorescence microscopy results revealed the co-localization between ReAsH-EDT_2 and ectopically expressed GFP-Rbx1. The mutation of histidine to cysteine in the RING finger domain of Rbx1 enhanced the co-localization, whereas the mutation of cysteine to histidine diminished the co-localization. (b) The chemical structure of ReAsH-EDT_2. (c) Statistical analysis of the extents of co-localization between ReAsH-EDT_2 and wild-type Rbx1, Rbx1-H80C, and Rbx1-C83H. The signal intensities of each fluorescence channel were measured using ImageJ. The ratios were calculated by dividing the signal intensity of ReAsH-EDT_2 with that of GFP. The data represented the mean and standard deviation of the ratios obtained from 30 individual cells. The P values (for comparisons with the ectopic expression of wild-type (WT) Rbx1) were calculated using an unpaired two-tailed student's t-test (**, $0.001 < P < 0.01$; ***, $P < 0.001$).

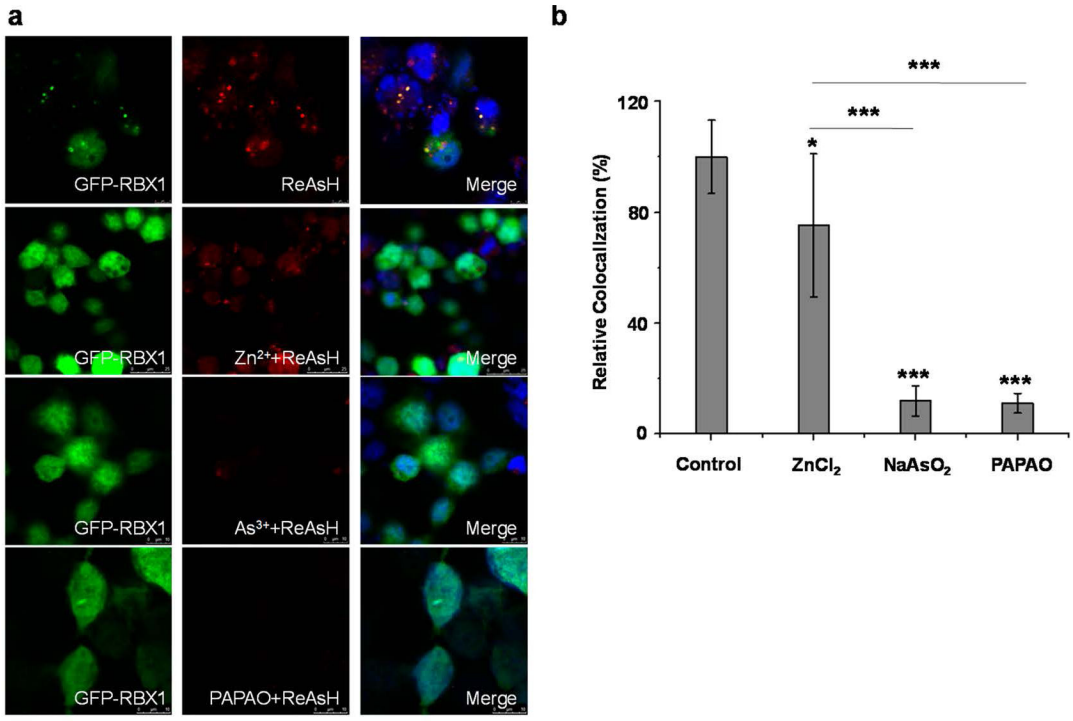

Figure 4.

Competitive binding of As^{3+} to the RING-finger domain of Rbx1. (a) Fluorescence microscopy results revealed the co-localization between ReAsH-EDT_2 and ectopically expressed GFP-Rbx1. The co-localization was significantly diminished in cells pretreated with 10 μM $NaAsO_2$ or PAPAO but not Zn^{2+}. (b) Quantitative analysis of the frequencies of co-localization between ReAsH-EDT_2 and Rbx1. The data represent the mean and standard deviation of results obtained from images of 30 different cells. The P values (for comparisons with the control) were calculated using a two-tailed, unpaired Student's t test (*, $0.01 < P < 0.05$; ***, $P < 0.001$).

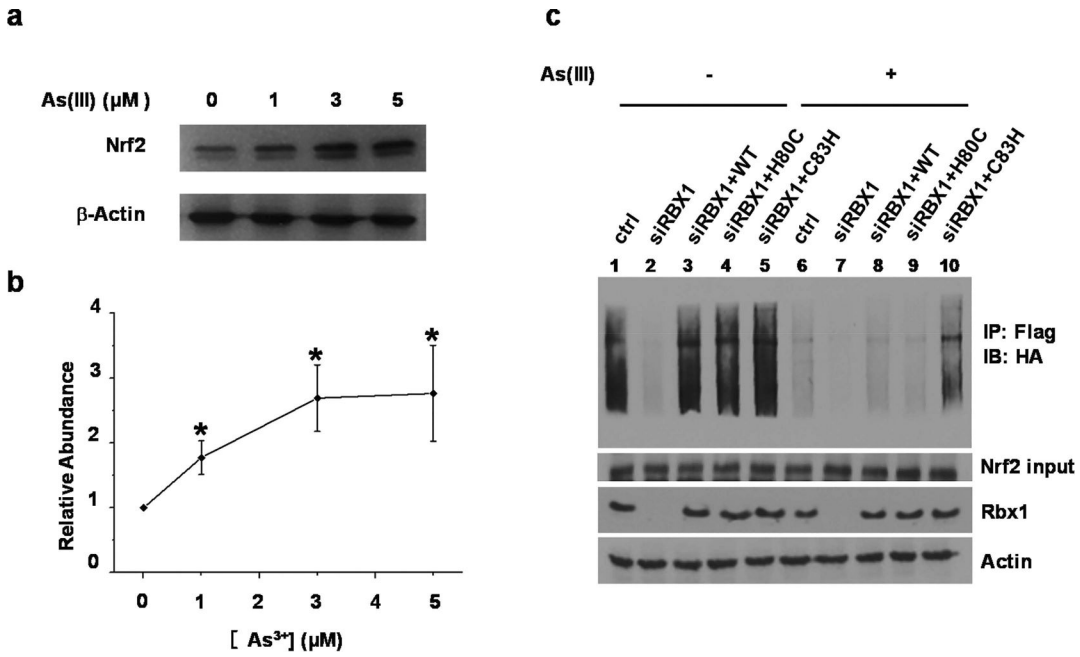

Figure 5.

Interaction between arsenite and Rbx1 is required for the stabilization of Nrf2 by arsenite. (a) Arsenite exposure stabilized the Nrf2 protein in cells. HEK293T cells were transfected with FLAG-Nrf2 and subsequently treated with increasing concentrations of As^{3+}. After MG132 treatment, the levels of Nrf2 and β;-actin were detected by using the anti-Nrf2 and anti-β-actin primary antibody. (b) Relative protein levels of Nrf2 in cells exposed to arsenite. The data represent the means and standard deviations of results obtained from 3 biological replicates. The P values were calculated using an unpaired two-tailed student's t-test (*, $0.01 < P < 0.05$). (c) HEK293T cells were either transfected with control, nontargeting siRNA (ctrl, lanes 1 and 6) or siRbx1 (lanes 2–5 and 7–10), complemented with wild-type Rbx1 (lanes 3 and 8), Rbx1-H80C (lanes 4 and 9), or Rbx1-C83H (lanes 5 and 10), and transfected with FLAG-Nrf2 and ubiquitin-HA, followed by treatment with 5 μM As^{3+} (lanes 6–10) and 5 μM MG132 (lanes 1–10). Anti-FLAG M2 beads were used to immunoprecipitate the FLAG-Nrf2 protein, and the ubiquitination of Nrf2 was detected by Western blot using anti-HA antibody.