Supporting Information for

"Arsenite Binds to the RING Finger Domains of RNF20-RNF40 Histone E3 Ubiquitin Ligase and Inhibits DNA Double-strand Break Repair"

by

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Experimental Procedures

Cell culture

HEK293T, HeLa and IMR90 cells were obtained from ATCC (Manassas, VA). U2OS cells harboring a chromosomally integrated copy of DR-GFP or EJ5-GFP reporter were provided by Prof. Jeremy M. Stark,[1] and GM00637 cells were obtained from Prof. Gerd P. Pfeifer. GM00637, HeLa and HEK293T cells were cultured in Dulbecco's modified Eagle's medium (DMEM, ATCC). IMR90 cells were cultured in Eagle's minimum essential medium (EMEM, ATCC). U2OS cells were grown in DMEM with high glucose and L-glutamine, but without sodium pyruvate (Invitrogen). All culture media except those used for transfection were supplemented with 10% fetal bovine serum (FBS, Invitrogen, Carlsbad, CA) and 100 IU/mL penicillin. Cells were maintained in a humidified atmosphere with 5% CO_2 at 37ºC, with medium renewal of 2-3 times a week depending on cell density. For plasmid transfection, cells were switched to the same media as described above except that no penicillin was added.

In vitro arsenite binding assay

The RING-finger peptides of RNF20 (with amino acid residues 921-961, i.e., TCPCCNMRKK DAVLTKCFHV FCFECVKTRY DTRQRKCPKC N) and RNF40 (with residues 941-990, i.e., EYKARLTCPC CNTRKKDAVL TKCFHVFCFE CVRGRYEARQ RKCPKCNAAF) were obtained from Genemed Synthesis (San Antonio, TX), purified by HPLC and used for *in vitro* binding assays. Arsenite binding to the RING finger peptides was monitored by MALDI-TOF mass spectrometry on a Voyager DE STR instrument (Applied Biosystems, Framingham, MA) in linear, positive-ion mode. Peptides were dissolved at a concentration of 1 mM in a buffer containing 20 mM Tris-HCl (pH 6.8) and 1 mM dithiothreitol. Aliquots of 100 μM peptides were incubated with 200 μM $NaAsO_2$ at room temperature for 1 hr. The resultant solution was diluted by 100 fold and mixed with an equal volume of 2,5-dihydroxybenzoic acid matrix solution before spotting onto a sample plate.[2] The mass spectrometer was equipped with a pulsed nitrogen laser operating at 337 nm with a pulse duration of 3 ns. The acceleration voltage, grid voltage, and delayed extraction time were set at 20 kV, 65%, and 190 ns, respectively. Each mass spectrum was acquired from an average of signal from 100 laser shots.

The UV absorption spectra were recorded in the wavelength range of 240-400 nm on a Varian Cary 50 UV-visible spectrophotometer (Palo Alto, CA), where the RING finger peptides (100 μM) in 20 mM Tris-HCl (pH 6.8) were titrated with increasing amounts of $NaAsO_2$.

Refolding of RING finger domain of RNF20 in the presence of As(III)

The cDNA encoding amino acid residues 869-974 of RNF20 was inserted into a modified pRSF-duet vector (Novagen), separated from a His6-SUMO tag by a ubiquitin-like protease (ULP1) cleavage site. The plasmid was transformed into BL21 (DE3) RIL cell strain and protein expression was induced by 0.4 mM isopropyl β-D-1-thiogalactopyranoside. After the induction, the cells continued to grow overnight. The fusion protein was first purified through a Ni-NTA column, followed by removal of the His6-SUMO tag by ULP1 cleavage and a second run of Ni-NTA column. Finally, the protein was concentrated to ~20 mg/mL for storage.

To examine the effect of As(III) on the folding behavior of RNF20, we performed denaturation and refolding experiments for the RING finger domain of RNF20 (with residues 869-974) *in vitro* in the presence of zinc chloride or sodium arsenite. Briefly, 0.3 mM RNF20 (869-947) was

dissolved in 250 μL of 6 M guanidine hydrochloride, 20 mM DTT and incubated for 45 min. Subsequently, the denatured protein sample was rapidly diluted into 4 mL of refolding solution containing 5% glycerol, 1.0 M NaCl, 0.5 mM zinc chloride or sodium arsenite, and 20 mM Tris-HCl (pH 7.5). The refolded protein sample was then concentrated and subjected to analysis with size exclusion chromatography (SEC) on a Superdex 200 HR 10/30 column, with a buffer containing 20 mM Tris-HCl (pH 7.5), 150 mM NaCl and 1 mM DTT.

Extraction and enzymatic digestion of core histones

Core histones were isolated from cultured human cells following previously reported procedures.[3] Briefly, GM00637, HEK293T and IMR90 cells, either untreated or after a 24-hr treatment with 5 μM arsenite, were harvested by centrifugation at 500g. The cell pellets were subsequently washed with a 5-mL lysis buffer containing 0.25 M sucrose, 10 mM $MgCl_2$, 0.5 mM PMSF, 50 mM Tris (pH 7.4) and 0.5% Triton X-100. The pellets were then resuspended in 5 mL of the same buffer and kept at 4ºC overnight. The histones were extracted from the cell lysis mixture with 0.4 M sulfuric acid by incubating at 4ºC for at least 4 hr with continuous vortexing, precipitated with cold acetone, centrifuged, dried and redissolved in water.

Core histone mixtures were digested with trypsin (Roche Applied Science, Indianapolis, IN) at a protein/enzyme ratio of 20:1 (w/w) in 100 mM NH_4HCO_3 (pH 8.0) at 37ºC overnight. The peptide mixtures were subjected to LC-MS/MS analysis.

LC-MS/MS for the identification and relative quantification of histone H2B K120 ubiquitination

On-line LC-MS/MS analysis was performed on an LTQ-Orbitrap Velos mass spectrometer coupled with an EASY n-LCII HPLC system and a nanoelectrospray ionization source (Thermo, San Jose, CA) for peptide sequencing and ubiquitination identification. The sample injection, enrichment, desalting, and HPLC separation were carried out automatically on a homemade trapping column (150 μm×50 mm) and a separation column (75 μm×120 mm, packed with ReproSil-Pur C18-AQ resin, 5 μm in particle size and 300 Å in pore size, Dr. Maisch HPLC GmbH, Germany). The peptide mixture was first loaded onto the trapping column with a solvent mixture of 0.1% formic acid in CH_3CN/H_2O (2:98, v/v) at a flow rate of 3.0 μL/min. The peptides were then separated using a 120-min linear gradient of 2-40% acetonitrile in 0.1% formic acid and at a flow rate of 300 nL/min.

The LTQ-Orbitrap Velos mass spectrometer was operated in the positive-ion mode with a spray voltage of 1.8 kV. All MS/MS data were acquired in a data-dependent scan mode where one full MS scan was followed with twenty MS/MS scans. To obtain high-quality MS/MS, the mass spectrometer was also set up in selected-ion monitoring (SIM) mode where the fragmentations of the $[M+2H]^{2+}$ ion of the H2B peptide containing diglycine-linked K120, which represents the ubiquitin remnant after tryptic digestion, was monitored. The quantification of ubiquitination level was conducted in SIM mode by comparing the relative abundances of ions corresponding to the target ubiquitinated peptide ($AVTK_{GG}VTSSK$) with respect to a reference H2B peptide EIQTAVR, which is not known to carry any post-translational modification, following previously published procedures.[4]

Plasmid construction

The expression plasmids for GFP-RNF20 and Myc-RNF40 were kindly provided by Dr. Yossi Shiloh,[5] and the expression plasmids for GFP-RNF8 and GFP-RNF168 were generously offered

by Dr. Jiri Lukas.[6,7] GFP-RNF40 vector was constructed by subcloning the RNF40 coding sequence from the Myc-RNF40 into the unique XhoI and BamH1 sites of pEGFP-C3 vector (provided by Dr. Alan R. Lehmann). The expression plasmids of GFP-RNF20 carrying the C922,924A or C957,960A mutations, GFP-RNF40 harboring the C948,950A or C983,986A mutations, GFP-RNF8 containing C403,406A or C437,440A mutations, and GFP-RNF168 with C16,19A or C51,54A mutations were obtained by site-directed mutagenesis, and the sequences for the mutated plasmids were verified by sequencing.

Streptavidin agarose affinity assay and Western blot

Biotin-As was synthesized by conjugating *p*-aminophenylarsine oxide (PAPAO) to activated PFP-biotin,[8,9] and used for streptavidin agarose affinity assay following previously published procedures.[10] Briefly, HEK293T cells were transfected with wild-type or mutant form of GFP-RNF20, GFP-RNF40, GFP-RNF8, or GFP-RNF168. At 24 hr after the transfection, cells were treated with 5 μM biotin-As for 2 hr and lysed in CelLytic™ M lysis buffer supplemented with a protease inhibitor cocktail (Sigma-Aldrich). The cell lysates were incubated with streptavidin agarose at 4ºC for overnight. Streptavidin agarose beads were subsequently washed with 1×PBS and resuspended in SDS-PAGE loading buffer.

After SDS-PAGE separation, proteins were transferred to a nitrocellulose membrane using a solution containing 10 mM $NaHCO_3$, 3 mM Na_2CO_3, and 20% methanol. The membranes were blocked with 5% non-fat milk in PBS buffer containing 0.1% (v/v) Tween-20 (pH 7.5) for 7 hr and incubated overnight at 4°C with rabbit anti-GFP antibody (1:20000 dilution, Sigma-Aldrich). The membranes were washed with fresh PBS-T at room temperature for five times (10 min each). After washing, the membranes were incubated with HRP-conjugated secondary antibody at room temperature for 1 hr. The membranes were subsequently washed with PBS-T for five times. The secondary antibody was detected by using ECL Advance Western Blotting Detection Kit (GE Healthcare) and visualized with Hyblot CL autoradiography film (Denville Scientific, Inc., Metuchen, NJ). Similar experiments were also conducted by pretreating cells with 10 μM $NaAsO_2$, PAPAO, or Zn^{2+} for 1 hr prior to the biotin-As treatment.

Fluorescence microscopy for monitoring the interaction between As(III) and RING-finger proteins

HEK293T cells, seeded in 6-well plates at a density of ~3×10^5 cells per well, were transfected with 1.5 μg wild-type or mutant form of GFP-RNF20, GFP-RNF40, GFP-RNF8, or GFP-RNF168 using Lipofectamine 2000 (Invitrogen). Cells were incubated at 37ºC for another 18-48 hr, transferred to cover glasses, and incubated with 5 μM ReAsH-EDT_2 (Invitrogen, Carlsbad, CA) at 37ºC for 1 hr in serum-free Opti-MEM (Invitrogen). After thoroughly washing with BAL buffer, cells were fixed with 4% paraformaldehyde and imaged using a Leica TCS SP5 confocal microscope (Leica Microsystems, Buffalo Grove, IL). Cells were also pretreated with 10 μM $NaAsO_2$ or PAPAO for 1 hr prior to transfection with wild-type GFP-RNF20, GFP-RNF40, GFP-RNF8, or GFP-RNF168 and treatment with ReAsH-EDT_2.

Fluorescence microscopy for monitoring the recruitment of DNA repair proteins to laser-localized DNA DSBs

HeLa cells were seeded in a 35-mm glass bottom culture dish (MatTek) and incubated with 0, 5 or 20 μM sodium arsenite at 37°C for 8 hr prior to laser damage. Localized irradiation was performed using a Nikon Eclipse TE2000 confocal microscope equipped with an SRS NL100

nitrogen laser-pumped dye laser (Photonics Instruments, St. Charles, IL) that fires 3-ns pulses with a repetition rate of 10 Hz at 365 nm, with a power of 0.7 nW measured at the back aperture of the 60× objective. The laser was directed to a specified rectangular region of interest within the nucleus of a cell visualized with a Plan Fluor 60×/NA 1.25 oil objective. The laser beam was oriented by galvanometer-driven beam displacers and fired randomly throughout the region of interest until the entire region was exposed. Throughout an experiment, cells were maintained at 37°C, 5% CO_2, and 80% humidity using a Live Cell^{TM} environmental chamber. After the laser treatment, cells were incubated at 37°C for 10 min and fixed immediately with 4% formaldehyde in PBS at room temperature (RT) for 10 min or with 0.5% Triton X in PBS on ice for 10 min followed by 2% formaldehyde at RT for 10 min. Fixed cells were permeabilized with 0.5% Triton X-100, 1% BSA, 100 mM glycine and 0.2 mg/mL EDTA in PBS on ice for 10 min. The cells were subsequently digested with RNase A at 37ºC. For immunofluorescence staining, cells were incubated at 37ºC for 1.0 hr with primary antibodies for γ-H2AX (Millipore), BRCA1 (Abcam) and RAD51 (Santa Cruz). Cells were subsequently incubated with corresponding secondary antibodies (Alexa Fluor goat anti-mouse or Alexa Fluor goat anti-rabbit, Molecular Probes, Invitrogen) and mounted with ProLong Gold Antifade Reagent with DAPI (Molecular Probes, Invitrogen). Immunostained cells were visualized and imaged using Hamamatsu EM-CCD digital camera attached to the Nikon Eclipse TE2000 confocal microscope.

RNA extraction and RT-PCR

Real-time quantitative RT-PCR for evaluating the expression levels of select DNA repair genes was performed by using the iQ SYBR Green Supermix kit (Bio-Rad) and gene-specific primers for *RAD51*, *BRCA1, XRCC1, KU70, KU80* or the control gene *GAPDH* as listed in Table S1.

GFP reporter assays for monitoring DNA DSB repair in cells

U2OS cells with a chromosomally integrated copy of DR-GFP or EJ5-GFP plasmid, either untreated or after a 24 hr treatment with $NaAsO_2$, were transfected with the I-SceI expression vector pCBASce.[1] The U2OS-DR-GFP and U2OS-EJ5-GFP cells without I-SceI transfection were used as negative control. Three days after the transfection, cells were washed with PBS and stored in a sorting buffer (1×PBS, 1 mM EDTA, 25 mM HEPES, 1% FBS, pH 7.0) for flow cytometry analysis (BD FACS Aria I, Franklin Lakes, NJ). The U2OS-DR-GFP cells, with or without $NaAsO_2$ treatment, were also transfected with pCAGGS-GFP to assess whether $NaAsO_2$ exposure affected the transfection efficiency.[1]

Colony survival assay

HEK293T cells, either untreated or after a 24-hr treatment with 5 μM arsenite, were plated in 6-well plates in triplicate at densities of 150-8000 cells per well. The cells were subsequently exposed to various doses of neocarzinostatin (NCS) and the cells were immediately attached to the plates. A 'split-dose' protocol was applied,[11] where each NCS dose was split into 15 ng/mL portions applied at 24 h intervals for up to 5 days. Cell colonies grown for 10-14 days were then fixed with 6% (v/v) glutaraldehyde and stained with 0.5% (w/v) crystal violet. Colonies containing at least 50 cells were subsequently counted under a microscope.

References:

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Table S1. Primers for Real-time quantitative RT-PCR

Sequence Name: *RAD51*-S	5'-CGATGTTTGCTGCTGATCC-3'
Sequence Name: *RAD51*-AS	5'-TTCTGGTTTCCCCTCTTCC-3'
Sequence Name: *BRCA1*-S	5'-AATGGCTTCCATGCAATTG-3'
Sequence Name: *BRCA1*-AS	5'-TCTGGGGTATCAGGTAGGTGT-3'
Sequence Name: *XRCC1*-S	5'-CTGACCGAGATCCAGTCTATGA-3'
Sequence Name: *XRCC1*-AS	5'-CAGCTGAAGCCAACCCA-3'
Sequence Name: *KU70*-S	5'-GACTGGGCTCCTTGGTGG-3'
Sequence Name: *KU70*-AS	5'-CCTTGGGCCTTTTGCTTC-3'
Sequence Name: *KU80*-S	5'-GTGATGTGGACGATTTATTGG-3'
Sequence Name: *KU80*-AS	5'-ATTTTGGCTCCCCTTGAA-3'
Sequence Name: *GAPDH*-S	5'-TTTGTCAAGCTCATTTCCTGGTATG-3'
Sequence Name: *GAPDH*-AS	5'-TCTCTTCCTCTTGTGCTCTTGCTG-3'

Figure S1. (a) Streptavidin agarose affinity pull-down assay without or with biotin-As probe to examine the binding between As(III) and RNF20 or RNF40 in cells. (b) Pretreatment of cells with 10 μM Zn^{2+} for 1 hr does not affect the binding between As(III) and RNF20 or RNF40 in cells. (c) Western blot results showing the expression levels of wild-type and mutant GFP-RNF20 and GFP-RNF40 in HEK293T cells. (d-e) Western blot results showing the expression levels of ectopically expressed and endogenous RNF20 and RNF40 in the presence or absence of 5 μM $NaAsO_2$. β-actin was used the as loading control in (c-e).

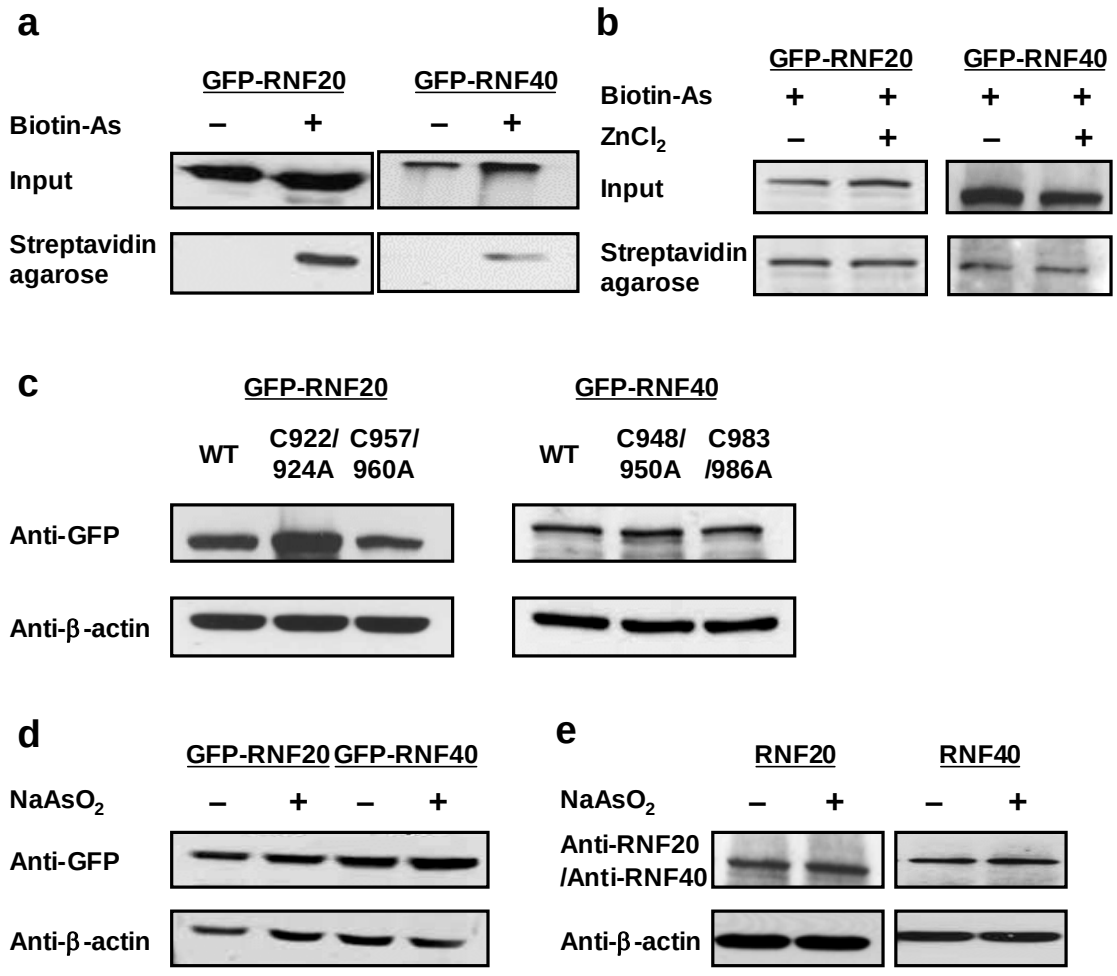

Figure S2. Fluorescence microscopy results for cells transfected with GFP-RNF20 (top) or GFP-RNF40 (bottom), but without ReAsH treatment; no red fluorescence could be detected in these cells.

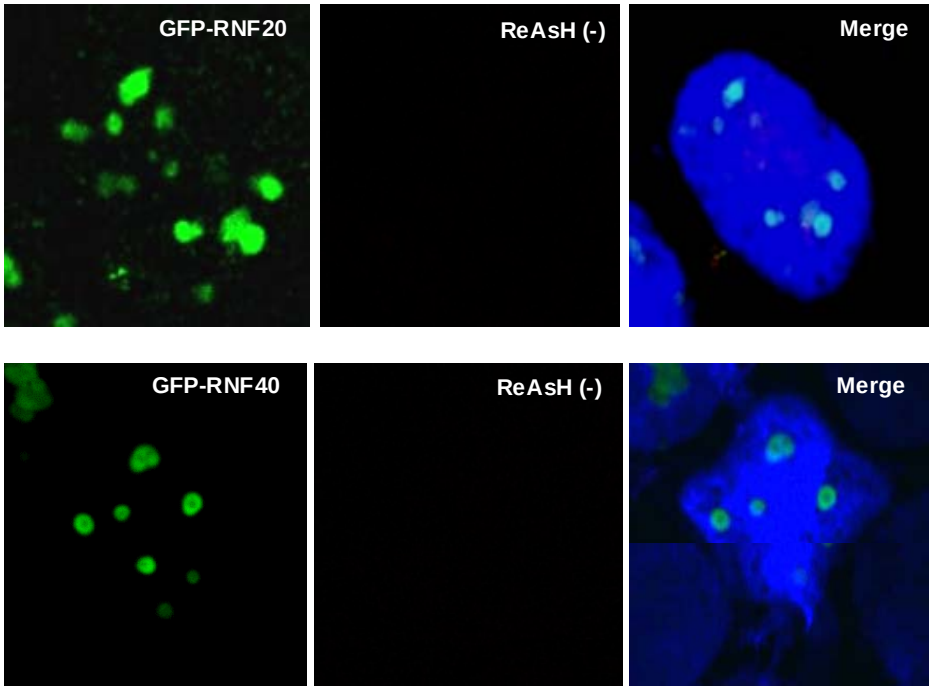

Figure S3. LC-MS and MS/MS for the quantification of the relative levels of ubiquitination of lysine 120 in histone H2B. (a) Selected-ion chromatogram for monitoring the $[M+2H]^{2+}$ ion (*m/z* 549.8) of a tryptic peptide derived from histone H2B ubiquitinated on lysine 120, $AVTK_{GG}VTSSK$, with K120 being modified with a diglycine remnant (K_{GG}). (b) Selected-ion chromatogram for monitoring the $[M+2H]^{2+}$ ion (*m/z* 408.7) of an unmodified reference tryptic peptide from histone H2B, EIQTAVR. Shown in the insets are the MS displaying the $[M+2H]^{2+}$ ions of the two peptides. (c) Product-ion spectrum (MS/MS) of the $[M+2H]^{2+}$ ion of H2B reference peptide EIQTAVR.

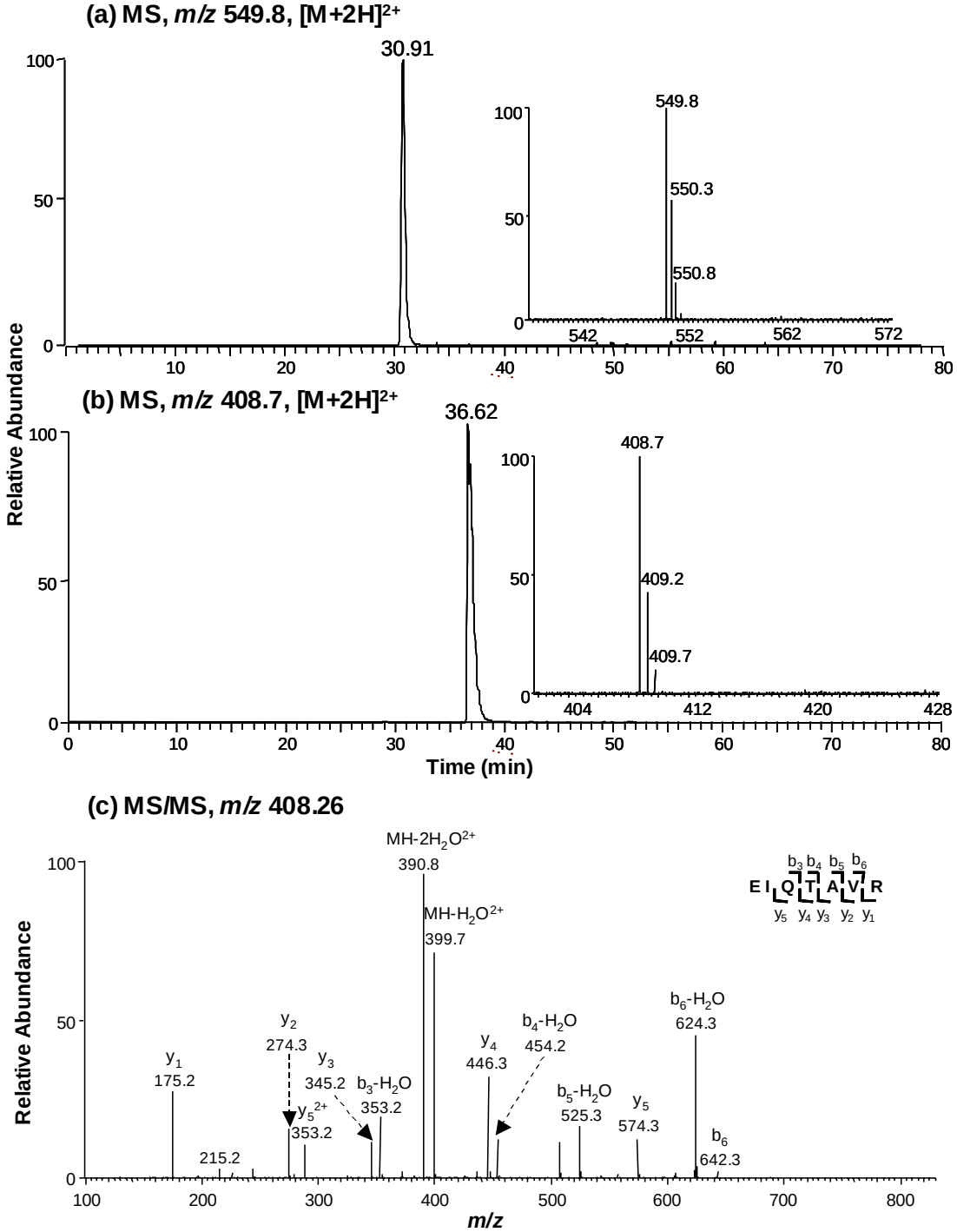

Figure S4. As(III) promotes the aggregation of the RING finger domain of RNF20. (a) Size exclusion chromatography (SEC) profile of RNF20 (869-974) refolded in the buffer containing 0.5 mM zinc chloride, with the elution volumes for proteins with different molecular weights marked. (b) SEC profile of RNF20 (869-974) refolded in the buffer containing 0.5 mM sodium arsenite.

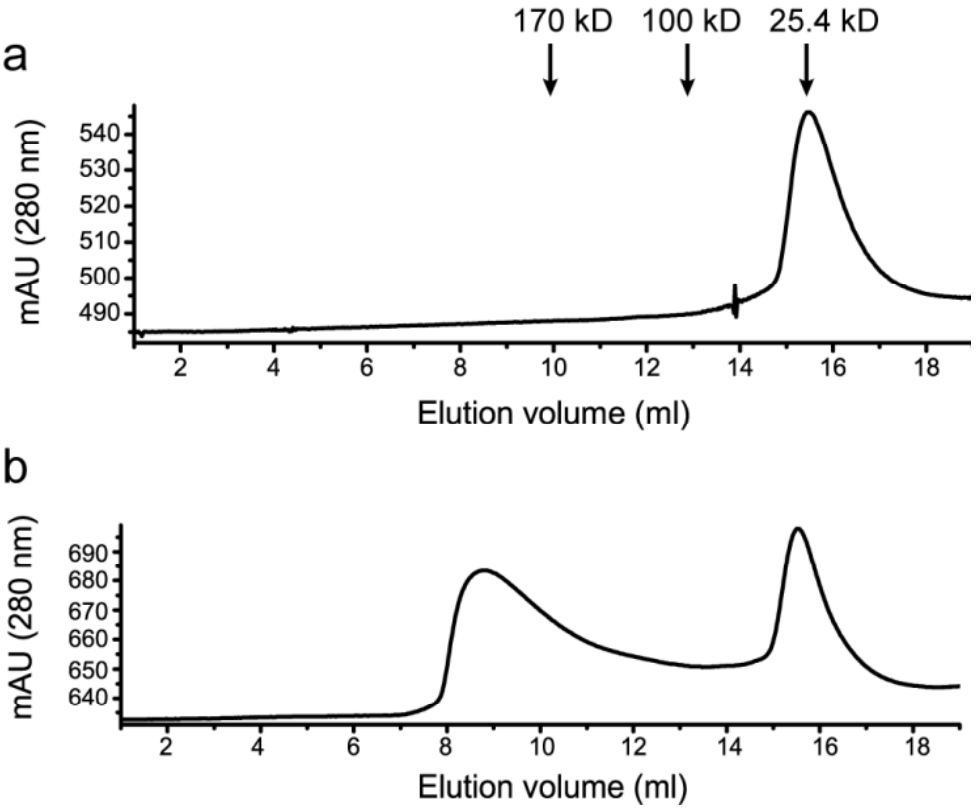

Figure S5. Quantitative real-time PCR results showing that the mRNA expression levels of five genes involved in DNA DSB repair are not reduced in HEK293T cells upon a 24-hr treatment with 5 μM $NaAsO_2$. The mRNA expression levels of these genes were normalized to that of *GAPDH*. The *p* values were calculated by using unpaired two-tailed *t*-test. "N.S" indicates that the difference was not significant ($p > 0.05$).

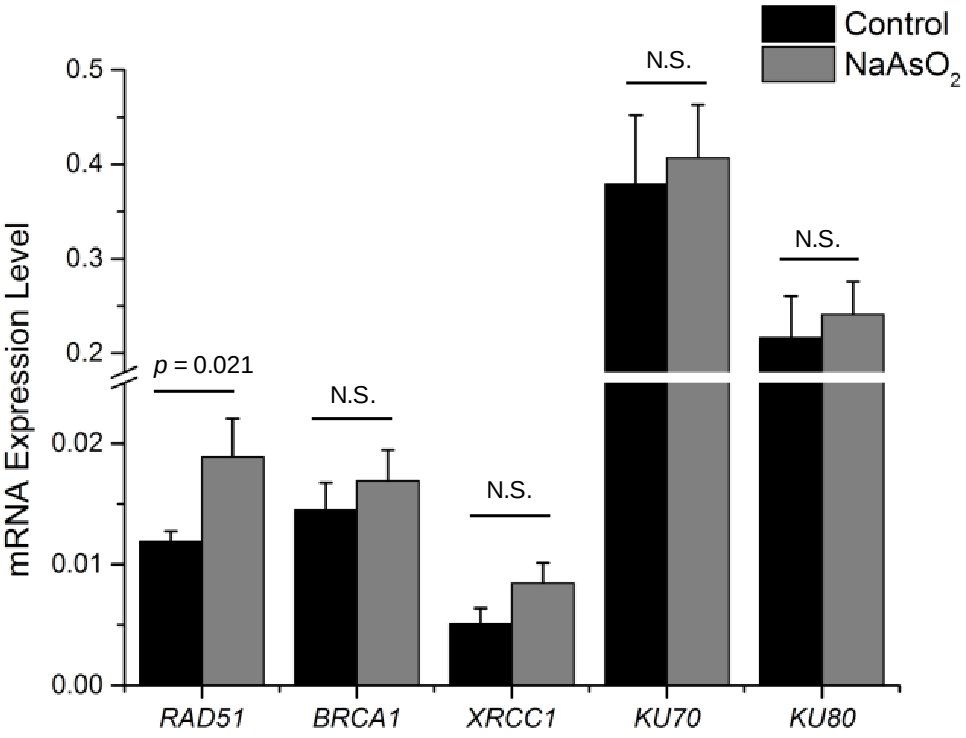

Figure S6. Flow cytometry results showing the percentage of GFP-positive cells in (a) U2OS-DRGFP and (b) U2OS-EJ5GFP cells transfected with I-SceI alone (I-Sce I) or together with a 24-hr treatment of 5 μM $NaAsO_2$ (I-Sce I + $NaAsO_2$).

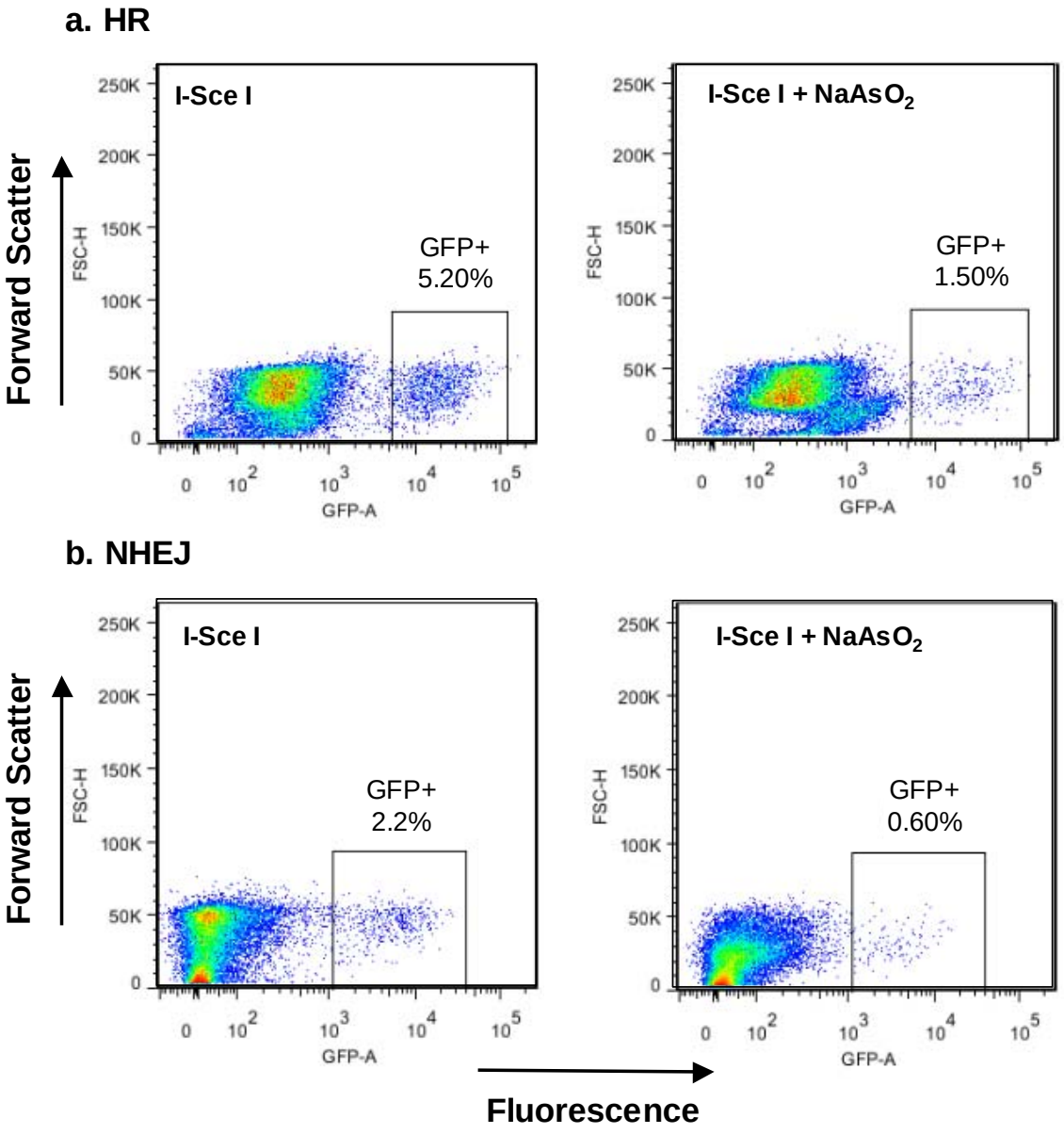

Figure S7. (a) Effect of $NaAsO_2$ on DSB repair. Quantitative results showing that $NaAsO_2$ treatment led to reduced DNA DSB repair via the HR and NHEJ pathways. "Control" refers to cells with neither I-SceI transfection nor $NaAsO_2$ treatment. The values represent the mean ± S.D. of results obtained from three independent experiments. The *p* values were calculated by using unpaired two-tailed *t*-test. (b) Quantitative results showed that the treatment with 5 μM $NaAsO_2$ does not affect the transfection efficiency in U2OS cells. "CK" and "As" represent control and $NaAsO_2$-treated cells, respectively.

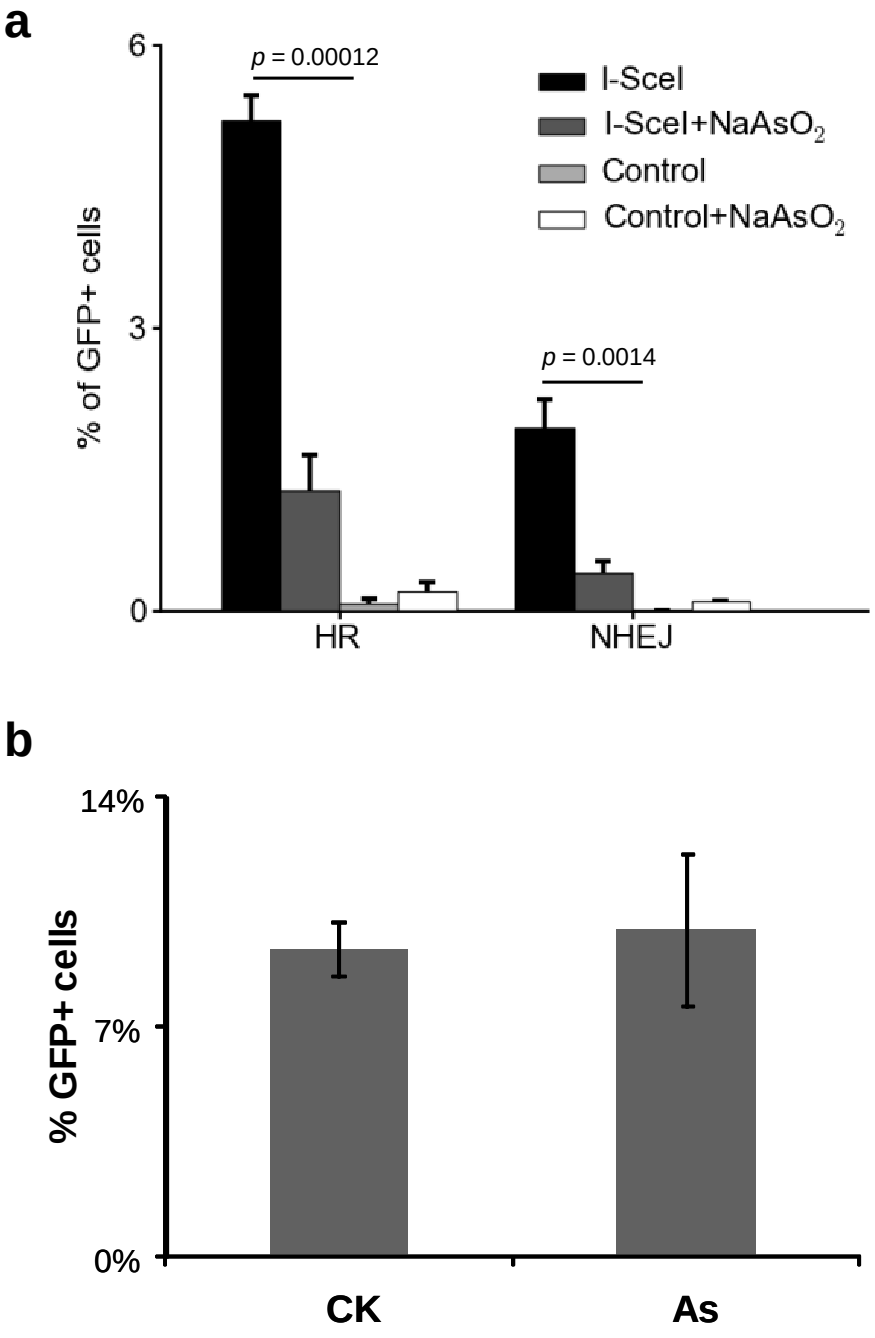

Figure S8. Clonogenic survival data showed that a 24-hr pretreatment with 5 μM $NaAsO_2$ sensitized HEK293T cells the radiomimetic drug neocarzinostatin (NCS). The values represent the mean ± S.D. of results obtained from three independent experiments. The *p* values were calculated by using unpaired two-tailed *t*-test.

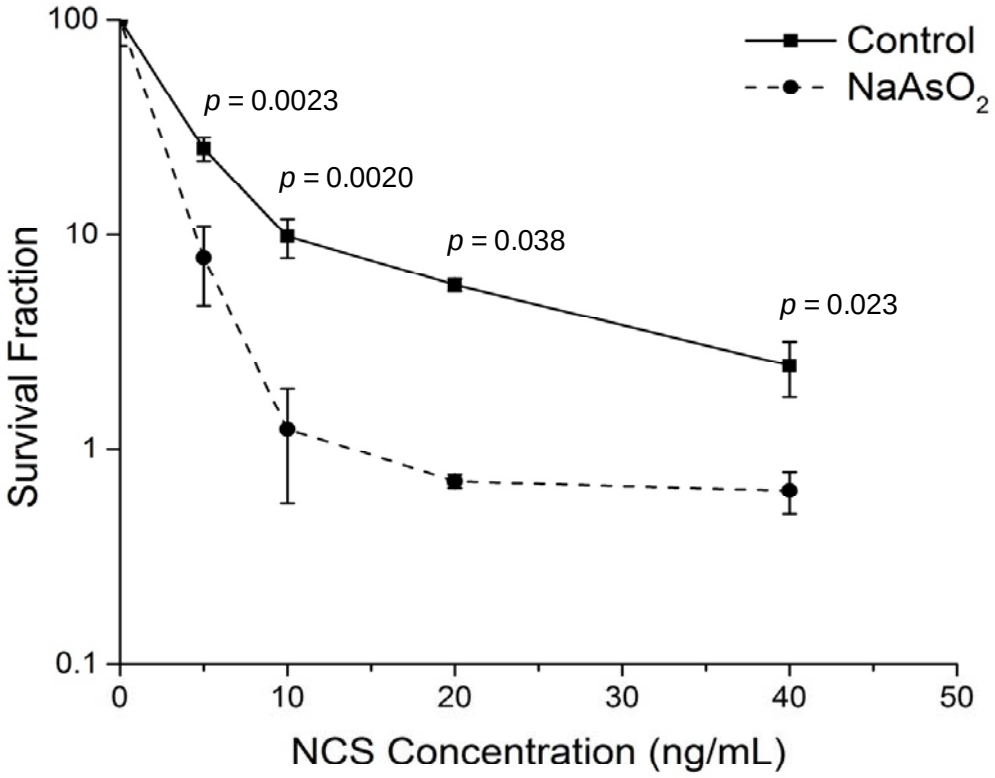

Figure S9. Streptavidin agarose affinity pull-down assay revealed the interaction between As(III) and RNF8 in cells. (a) Biotin-As probe allowed for the pull-down of GFP-RNF8 from lysate of HEK293T cells, and pretreatment of cells with 10 μM $NaAsO_2$ or PAPAO for 1 hr attenuated the binding. (b) Control experiment showed that the exclusion of Biotin-As probe led to failure in pulling down GFP-RNF8. (c) Control experiment showed that pre-treatment of cells with 10 μM $ZnCl_2$ for 1 hr did not affect the binding between As(III) and GFP-RNF8. (d) Mutation of RING finger cysteines led to diminished pull-down of GFP-RNF8 with the Biotin-As probe. (e) Western blot results showed that arsenite treatment did not alter the expression of GFP-RNF8 in HEK293T cells, where β-actin was employed as the loading control.

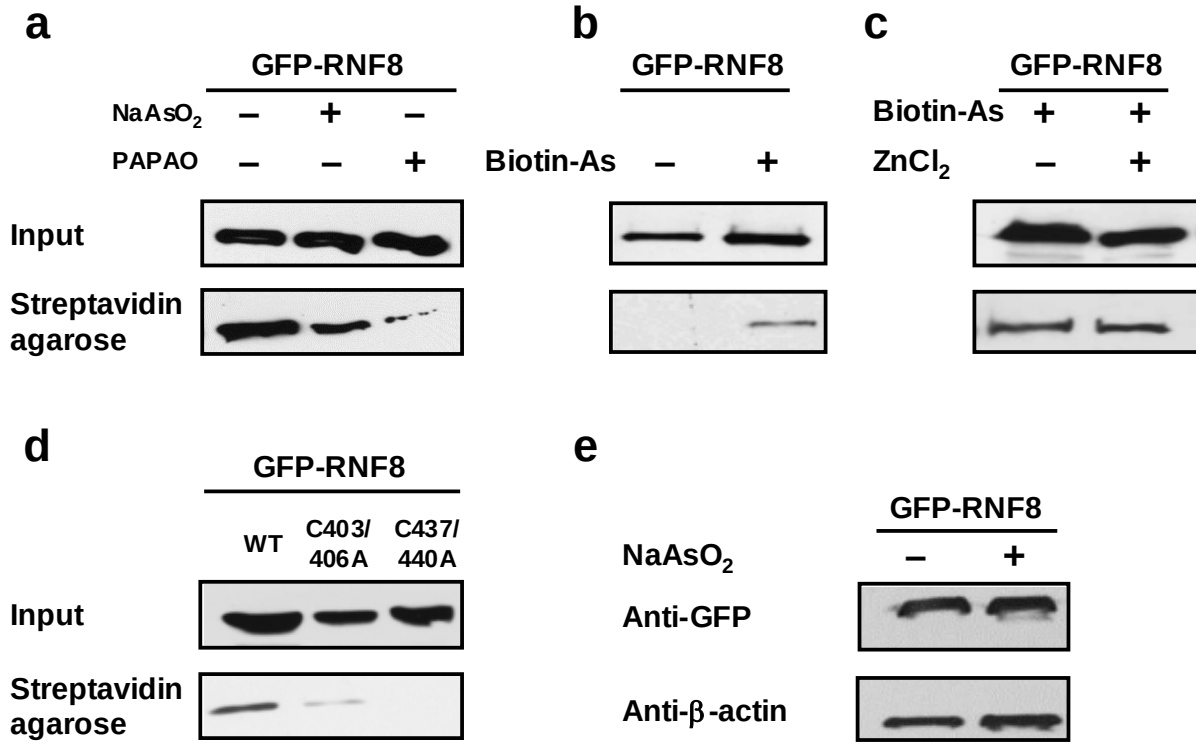

Figure S10. Streptavidin agarose affinity pull-down assay revealed the interaction between As(III) and RNF168 in cells. (a) Biotin-As probe allowed for the pull-down of GFP-RNF168 from lysate of HEK293T cells, and pretreatment of cells with 10 μM $NaAsO_2$ or PAPAO for 1 hr attenuated the binding. (b) Control experiment showed that the exclusion of Biotin-As probe led to failure in pulling down GFP-RNF168. (c) Control experiment showed that pre-treatment of cells with 10 μM $ZnCl_2$ for 1 hr did not affect the binding between As(III) and GFP-RNF168. (d) Mutation of RING finger cysteines led to diminished pull-down of GFP-RNF168 with the Biotin-As probe. (e) Western blot results showed that arsenite treatment did not alter the expression of GFP-RNF168 in HEK293T cells, where β-actin was employed as the loading control.

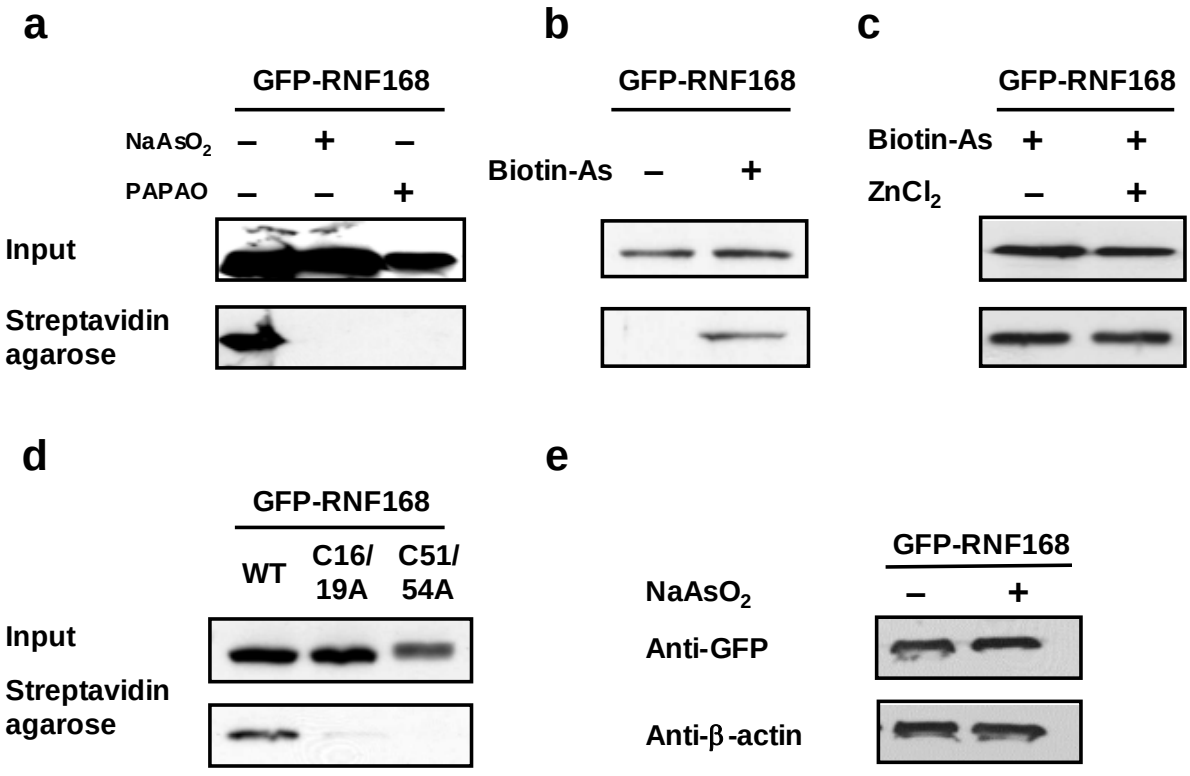

Figure S11. Immunofluorescence microscopy results demonstrated the interaction between As(III) and the RING finger domains of RNF8 and RNF168 in cells. (a-b) Colocalization of As(III)-bearing ReAsH with GFP-RNF8 and GFP-RNF168 in HEK293T cells, and such colocalization is lost in cells pretreated with 10 μM $NaAsO_2$ or PAPAO, but not Zn^{2+}. In addition, mutations of RING finger cysteines to alanines abolished the colocalization of RNF8 or RNF168 with ReAsH. (c) Fluorescence microscopy results for cells transfected with GFP-RNF8 or GFP-RNF168, but without ReAsH treatment; no red fluorescence could be detected in these cells.