Supporting Information

for

Arsenic Trioxide Preferentially Binds to Ring Finger Protein PML: Understanding Target Selection of the Drug

Experimental details

Protein Expression and Purification

The gene sequence for the expression of the ring finger domain of PML was amplified by PCR from pET32M-PML (49-104) plasmid and was subcloned into the pET28a-His_6-SUMO expression vector. The pET28a-His_6-SUMO-PML expression vector was transformed into BL21 (DE3) cells. The ^{15}N isotopic-labeled protein was obtained by the growth of *E. coli* in the medium containing $^{15}NH_4Cl$ as the sole nitrogen source. The protein was first purified using Ni-NTA affinity chromatography. The SUMO-tag was removed by small ubiquitin-like modifier protease. The protein was further purified through gel filtration followed by high-performance liquid chromatography. The protein concentration was determined through UV absorption. The NCp7 and ERα proteins were expressed and purified as previously described.[1] The stock solution of proteins was typically prepared in 1.0 mM in phosphate buffer at pH 5.8, and arsenite was prepared in 40 mM at pH 10.4.

Electrospray Ionization Mass Spectrometry

All mass spectrometry experiments were conducted on an Exactive Plus (Thermo Fisher Scientific, CA, U.S.A.) mass spectrometer. For the arsenite reactions, 50 μM proteins (PML, NCp7 or ERα), were incubated with different amount of arsenite in 50 mM ammonium acetate buffer at 37 °C for 2 hours. All samples were diluted to a final solution with 10 μM protein before injection. The positive ion mode was used in the ESI-MS experiments. Data were processed using XCalibur software (version 2.0, Thermo Finnigan).

Fluorescence Measurements

The fluorescence measurements were performed on a RF-5301PC spectrofluorometer (Shimadzu) using a quartz cuvette with a path length of 5 mm. The excitation wavelength was set at 280 nm, and the emission fluorescence spectra were recorded from 300 to 500 nm. The relative intensity of the fluorescence was calculated using the formula $(F - F_S)/ (F_0 - F_S)$, where F_0 is the initial fluorescence, F_S is the final fluorescence of in titration, and F is the fluorescence at the given concentration of metal ions. The data were fitted by the equation $Y = 1-(P_0 + X + K_d)/2P_0 + ((P_0 + X + K_d)^2 – 4P_0X)^{1/2}/2P_0$ using a nonlinear least-squares fitting, where P_0 is the concentration of protein.[2] The apparent dissociation constant K_d was obtained from the data fitting.

For the kinetic study, the fluorescence spectra were recorded on the time scan mode at 37 °C, with excitation at 280 nm and emission at 350 nm was recorded. Typically, 10 μM proteins were prepared in 20 mM phosphate buffer (pH 5.8). Arsenite was prepared in the same buffer in the concentration of 250 μM. Arsenite (200 μL) was added to the 50 μL protein in the cuvette, and fluorescence intensity was recorded immediately. The control experiment was recorded by adding 200 μL of buffer to the protein solution.

NMR Spectroscopy

^{1}H-^{15}N heteronuclear single quantum coherence (HSQC) spectra were collected on a Bruker 600 MHz NMR spectrometer at 25 °C. NMR samples were prepared on 0.2 mM ^{15}N-labeled proteins in 50 mM phosphate buffer (pH 5.8) containing 100 mM NaCl and 10 % D_2O (v/v). The data were processed and analyzed using TopSpin software.

References

1. L. Zhao, S. Chen, L. Jia, S. Shu, P. Zhu and Y. Liu, *Metallomics*, 2012, **4**, 988-994.
2. A. Urvoas, M. Moutiez, C. Estienne, J. Couprie, E. Mintz and L. Le Clainche, *Eur. J. Biochem.*, 2004, **271**, 993-1003.

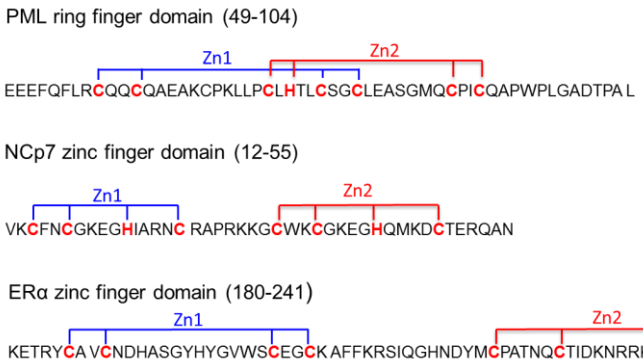

Figure S1. Protein sequences of ring finger domain of PML protein (aa 49 - 104), zinc finger domain of NCp7 protein (aa 12 - 55) and zinc finger domain of ERα protein (aa 180 - 241). The coordination residues to two zinc atoms are highlighted in red.

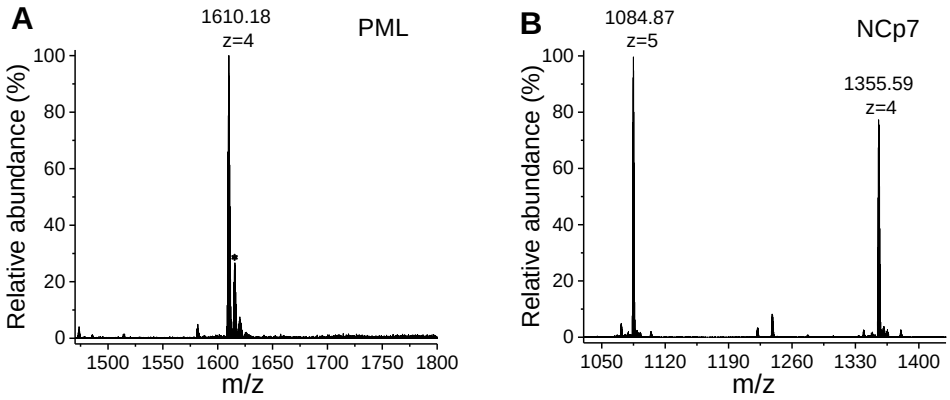

Figure S2. ESI-MS characterization of proteins. **(A)** PML, **(B)** NCp7. The m/z values and charges are labelled in spectra. Asterisks denote the addition of Na^+ on the protein.

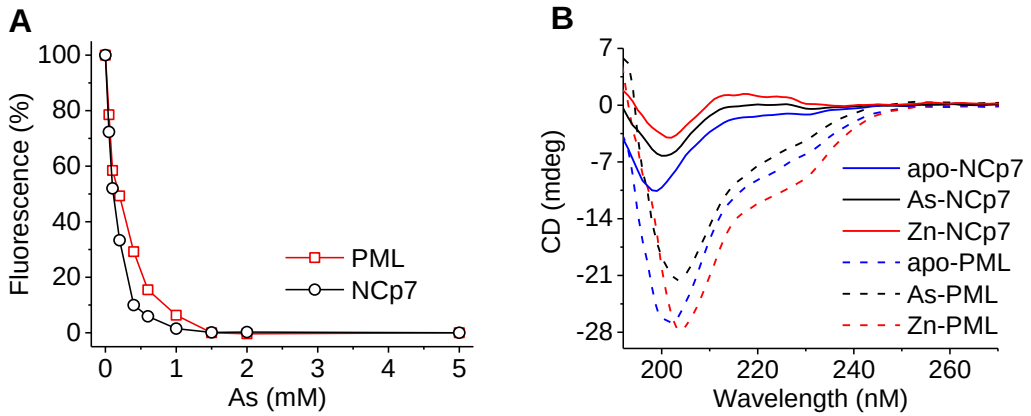

Figure S3. (**A**) Fluorescence of PML and NCp7 proteins quenched by arsenite. The reactions were performed on 20 µM protein with different ratios of arsenite in 20 mM phosphate buffer at 37°C for 2 h. **(B)** Far-UV CD spectra of PML (dash line), NCp7 (solid line). Colors denote apo- (blue), Zn-bound (red) and As-bound (black) proteins. The arsenite reactions were performed in 20 mM phosphate buffer at 37°C for 2 h.

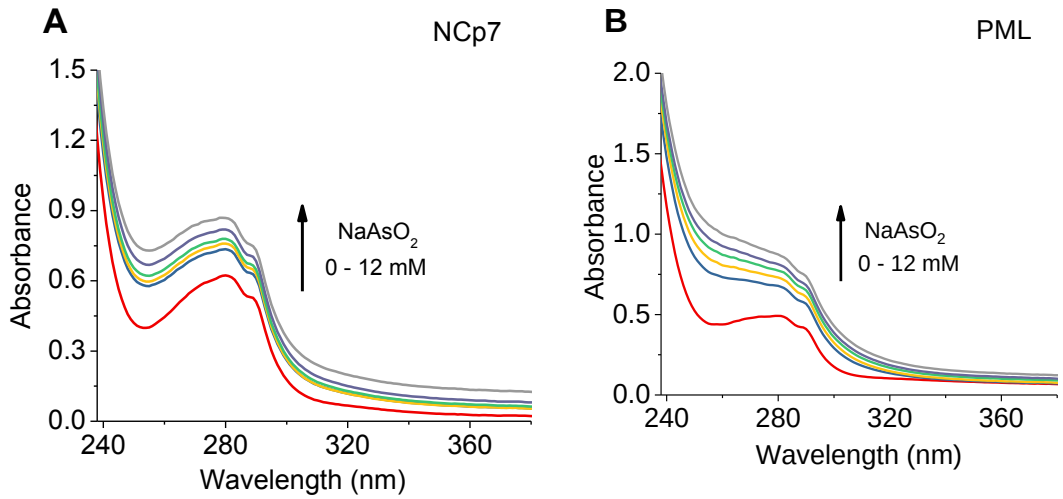

Figure S4. UV absorption spectra of the 100 µM NCp7 **(A)** and 100 µM PML **(B)** after the reaction with different amounts of $NaAsO_2$. The reactions were performed in 20 mM phosphate buffer at 37 °C for 2 h.

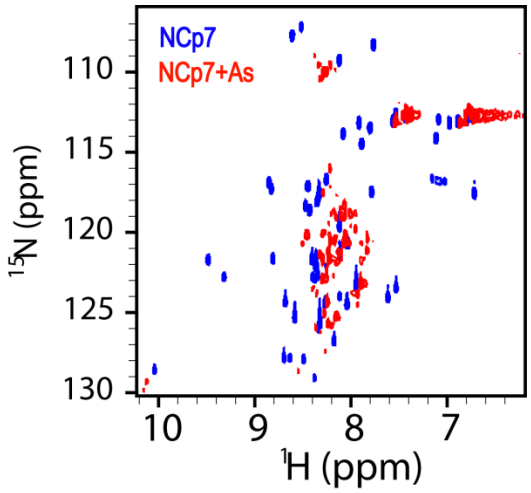

Figure S5. ^{1}H-^{15}N HSQC NMR spectra of NCp7 before (blue) and after (red) reaction with 10-molar equivalents of arsenite for 2 hour. The spectra were recorded on 0.2 mM ^{15}N-labeled protein.

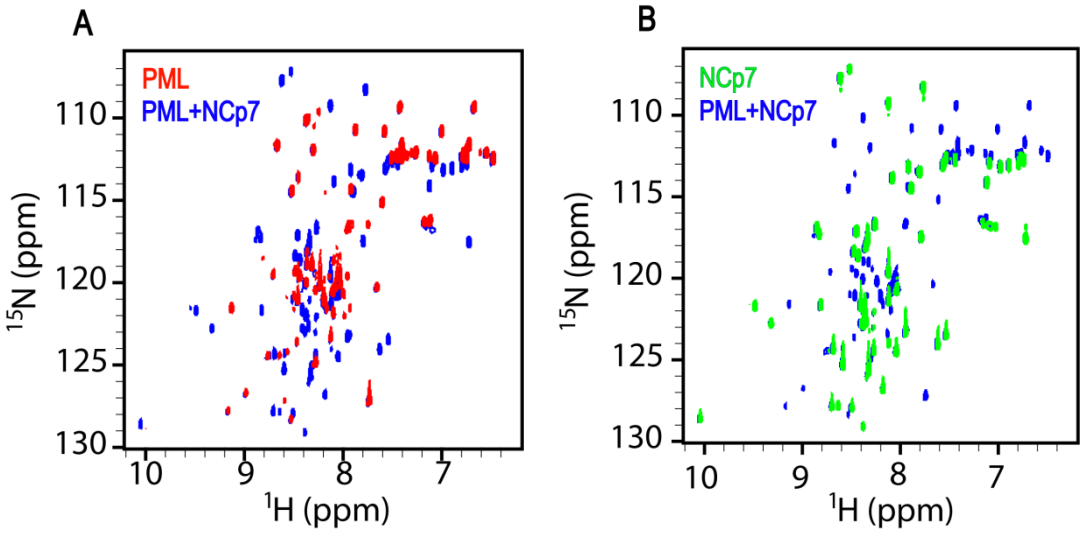

Figure S6. The superposition of ^{1}H-^{15}N HSQC NMR spectra of single protein and protein mixture of PML and NCp7. **(A)** PML (red) and protein mixture (blue). **(B)** NCp7 (green) and protein mixture (blue). All spectra were recorded at 298 K on 0.2 mM proteins in 20 mM phosphate buffer (pH 5.8) containing 100 mM NaCl.

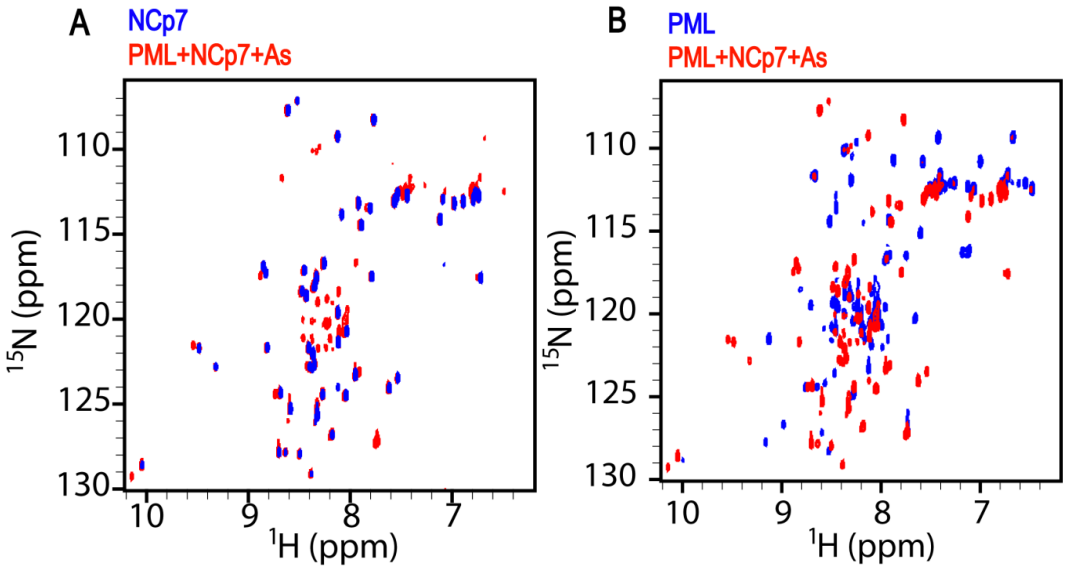

Figure S7. The superposition of ^{1}H-^{15}N HSQC NMR spectra of single protein (in the absence of arsenite) and the proteins mixture after arsenite reaction. **(A)** NCp7 without reaction (blue) and protein mixture after arsenite reaction (red). **(B)** PML without reaction (blue) and protein mixture after arsenite reaction (red). The reaction was performed on the protein mixture consisted of equimolar PML and NCp7 (0.2 mM). All spectra were recorded at 298 K in 20 mM phosphate buffer (pH 5.8) containing 100 mM NaCl.

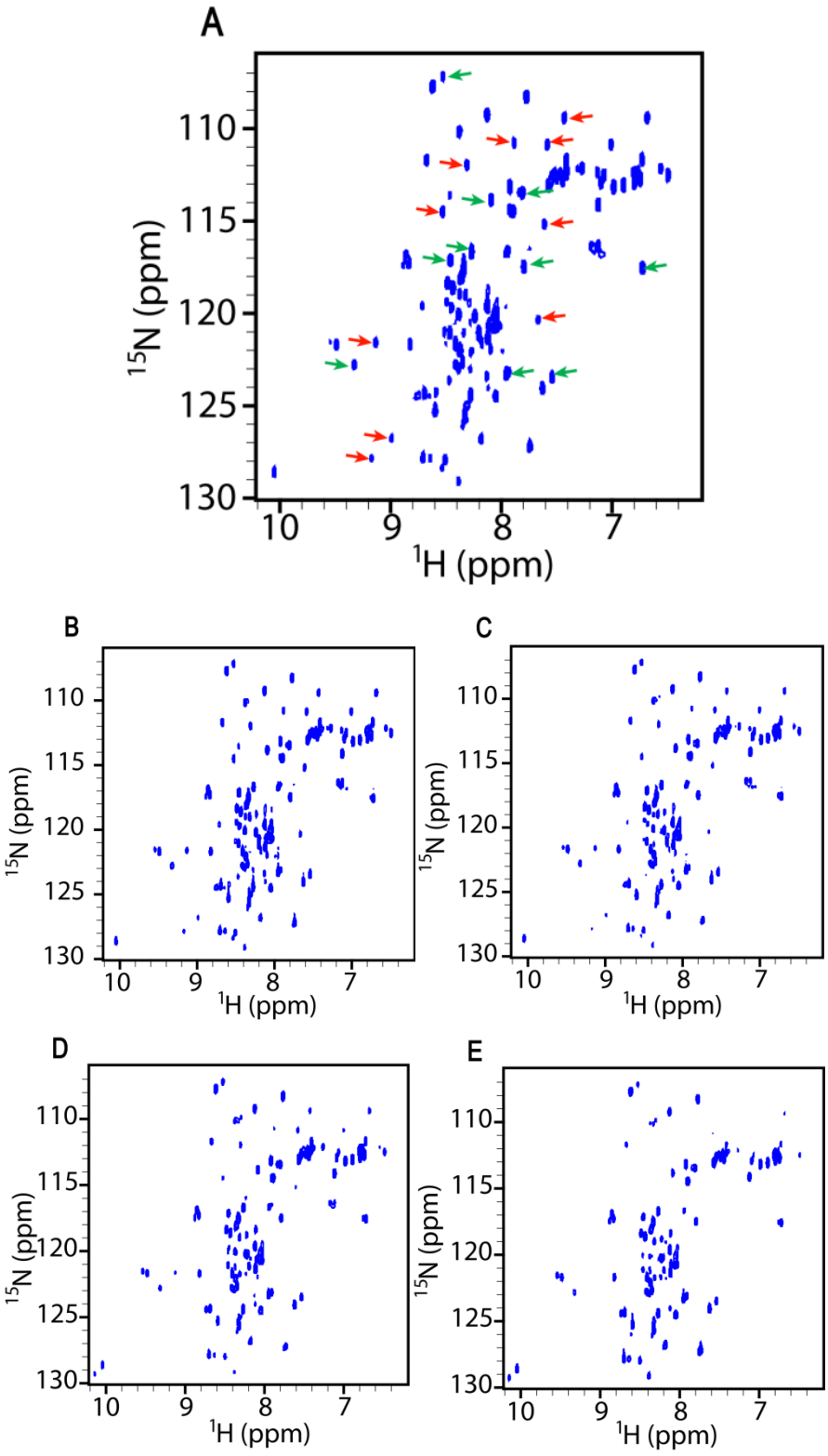

Figure S8. ^{1}H-^{15}N HSQC NMR spectra of protein mixture (PML and NCp7) in the reaction of arsenite. Arrows indicate the peaks of PML (red) and NCp7 (green) taken for the intensity analyses during the reaction. Different molar ratios of arsenite to proteins were used in the reaction: **(A)** 0; **(B)** 0.5; **(C)** 1.0; **(D)** 3.0; **(E)** 5.0. All spectra were recorded at 298 K on 0.2 mM proteins in 20 mM phosphate buffer (pH 5.8) containing 100 mM NaCl.

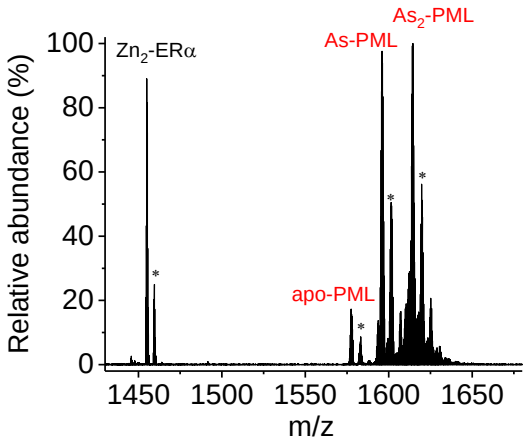

Figure S9. ESI-MS analysis of arsenite reactions with PML and ERα together. The reactions were performed on the mixture of 10 µM PML and 10 µM ERα with 15 µM arsenite at 37 °C for 2 hours. The +5 charged peaks of ERα and the +4 charged peaks of PML are shown on the spectrum. Asterisks denote the addition of Na^+ on the protein.

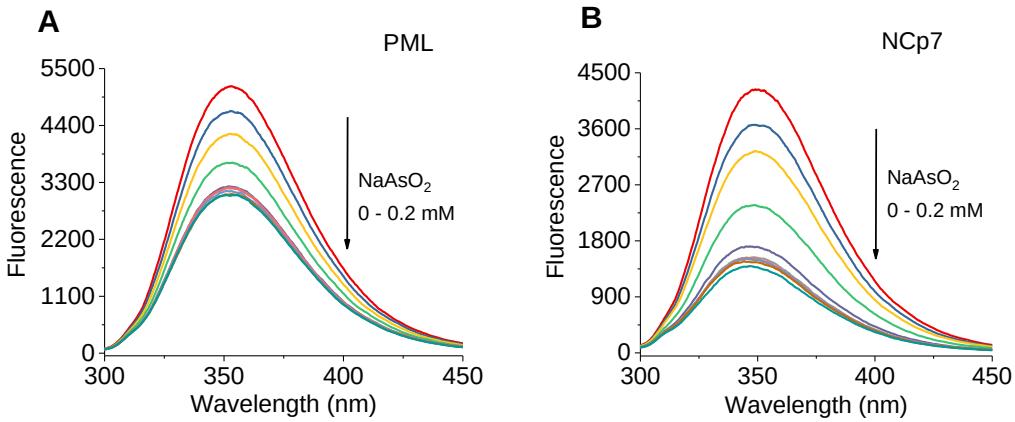

Figure S10. Fluorescence of proteins quenched by arsenite. **(A)** PML; **(B)** NCp7. 20 µM proteins were incubated with arsenite at 37 °C for 30 min in the presence of 20 mM phosphate buffer, pH 5.8.

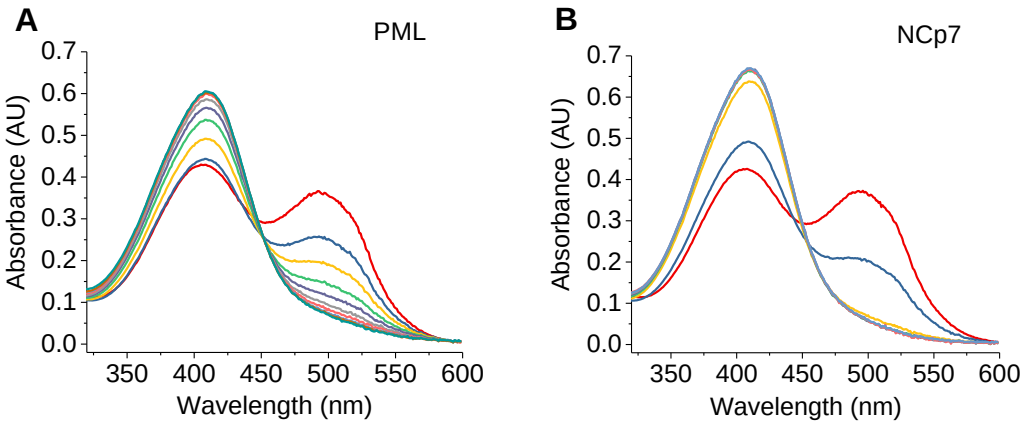

Figure S11. UV spectra of $Zn(PAR)_2$ in the reaction with PML **(A)** and NCp7 **(B)**. Different amount of proteins were titrated to 15 μM $Zn(PAR)_2$ in 20 mM phosphate buffer. 20 μM additional PAR was present in order to eliminate free Zn^{2+}. All reactions were performed at 37 °C.

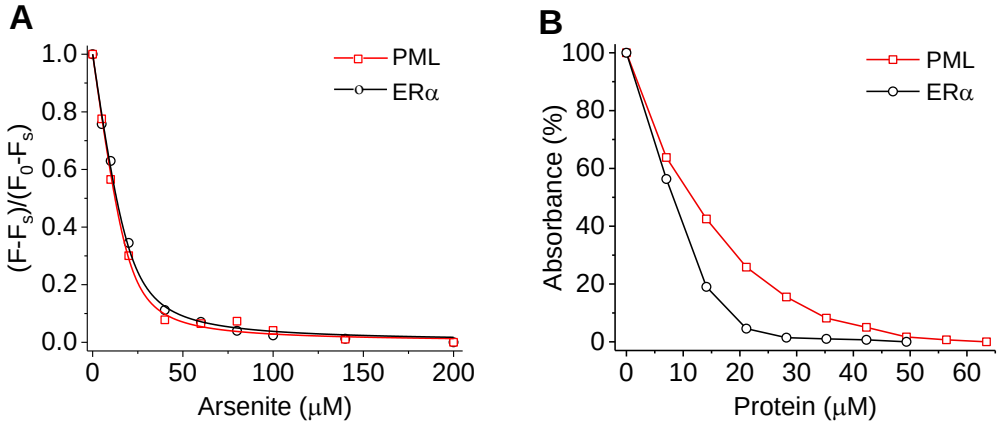

Figure S12. **(A)** Fluorescence of PML and ERα quenched by arsenite. 20 μM proteins were incubated with arsenite at 37 °C for 30 min in the presence of 20 mM phosphate buffer, pH 5.8. **(B)** UV absorbance of $Zn(PAR)_2$ at 500 nm in the reaction with PML and ERα. Proteins were titrated to 15 μM $Zn(PAR)_2$ in 20 mM phosphate buffer in the presence of 20 μM additional PAR to eliminate free Zn^{2+}. All reactions were performed at 37 °C. The color denotes the protein PML (red) or ERα (black).

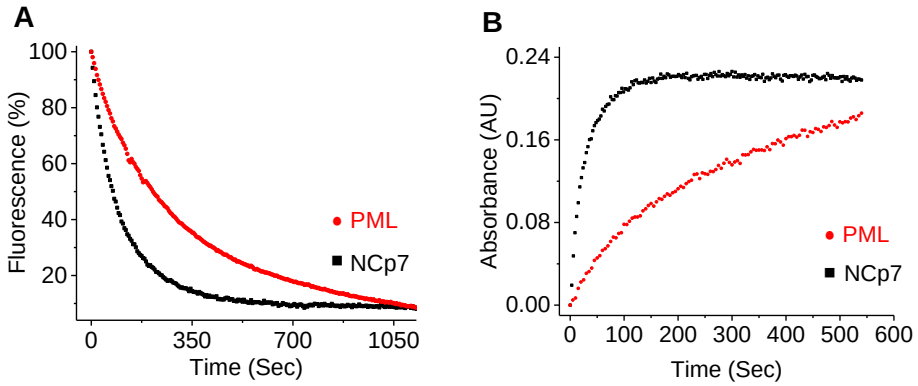

Figure S13. Kinetic study on the reaction of arsenite with PML and NCp7. **(A)** Reactions of arsenite with PML (red) and NCp7 (black) monitored by fluorescence. The reaction was performed on the 50 µM protein with 10-molar equivalents of arsenite in 20 mM phosphate buffer at 37 °C. **(B)** Time-dependent zinc-release assay on 50 µM PML (red) or 50 µM NCp7 (black). Reactions were performed in 50 mM phosphate buffer at 37 °C with protein/As(III) ratio of 1:8, respectively.

Table S1. The analysis of ESI-MS peaks detected in Figure S2

Protein	Composition	Formula	m/z (charge)	MW: obsd./cald.	Mass error (ppm)
NCp7	Zn_2-NCp7	$C_{217}H_{357}N_{77}O_{64}S_7Zn_2$	1084.87 (+5) 1355.84 (+4)	5419.35/5419.25	18.45
PML	Zn_2-PML	$C_{270}H_{425}N_{73}O_{83}S_9Zn_2$	1610.18 (+4)	6436.72/6437.11	-60.59

Table S2. The analysis of the products from the reaction of arsenite with PML and NCp7

Protein	Composition	Formula	m/z (charge)	MW: obsd./cald.	Mass error (ppm)
NCp7	apo-NCp7	$C_{217}H_{354}N_{77}O_{64}S_7$	1058.70 (+5)	5288.50/5288.27	43.49
	Zn-NCp7	$C_{217}H_{357}N_{77}O_{64}S_7Zn$	1071.69 (+5)	5353.45/5353.86	-76.58
	As-NCp7	$C_{217}H_{354}N_{77}O_{64}S_7As$	1073.47 (+5)	5362.35/5362.39	-7.46
	Zn-As-NCp7	$C_{217}H_{354}N_{77}O_{64}S_7ZnAs$	1086.46 (+5)	5427.30/5427.45	-27.64
	Zn_2-NCp7	$C_{217}H_{357}N_{77}O_{64}S_7Zn_2$	1084.87 (+5)	5419.35/5419.26	16.61
	As_2-NCp7	$C_{217}H_{351}N_{77}O_{64}S_7As_2$	1088.26 (+5)	5436.30/5436.31	-1.84
PML	apo-PML	$C_{270}H_{418}N_{73}O_{83}S_9$	1576.71 (+4)	6302.84/6303.30	-72.98
	As-PML	$C_{270}H_{418}N_{73}O_{83}S_9As$	1595.44 (+4)	6377.76/6378.25	-76.82
	As_2-PML	$C_{270}H_{418}N_{73}O_{83}S_9As_2$	1614.17 (+4)	6452.68/6453.10	-65.08