Identification of Labile Zn-Sites in Drug-Target Proteins

Yu-Ming Lee,[†] Yi-Ting Wang,[§] Yulander Duh,[§] Hanna S. Yuan[*,§] and Carmay Lim[*,†,‡]

[†]Institute of Biomedical Sciences, Academia Sinica, Taipei 115, Taiwan

[§]Institute of Molecular Biology, Academia Sinica, Taipei 115, Taiwan

[‡]Department of Chemistry, National Tsing Hua University, Hsinchu 300, Taiwan

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Supplementary Table S1. Predicted "druggable" labile zinc fingers

PDB:chain	Protein name	Zf core
PHD/FYVE zinc finger type		
2PUY:B	Phd finger protein 21a (BHC80)	Zn·(Cys_3His)
3LQI:C	Mll 1 phd3-bromo	Zn·(Cys_3His)
2PNX:C	Inhibitor of growth protein 4 (ING4)	Zn·(Cys_3His)
2PV0:C	DNA methyltransferase 3 like protein (dnmt3l)	Zn·(Cys_4)
1HYJ:A	Early endosome antigen 1(EEA1)	Zn·(Cys_4)
2VPD:A	Pygopus 1 (PYGO1)	Zn·(Cys_4)
RING zinc finger type		
2HYE:D	ring-box 1(RBX1)	Zn·(Cys_3His)
2JRJ:A	Pirh2 (p53-induced RING-H2 domain protein)(RCHY1)	Zn·(Cys_3His)
3Q1D:A	Tripartite motif-containing protein 54 (TRIM54 or MURF-1)	Zn·(Cys_3His)
1C9Q:A	BIR-2 DOMAIN OF XIAP	Zn·(Cys_3His)
3EYL:A	BIR-3 domain of XIAP	Zn·(Cys_3His)
3HCU:A	Tumor necrosis factor receptor associated factor 6 (TRAF6)	Zn·(Cys_4)
2H0D:B	Ubiquitin ligase protein RING2 (RING1B)	Zn·(Cys_4)
3FL2:A	E3 ubiquitin-protein ligase UHRF1 (ICBP90)	Zn·(Cys_4)
3EB5:A	ciap2 ring domain (cIAP2)(BIRC3)	Zn·(Cys_4)
A20 *zinc finger type*		
3OJ4:C	Ubiquitin-conjugating enzyme e2 d1	Zn·(Cys_4)
2C7M:A	Rab guanine nucleotide exchange factor 1	Zn·(Cys_4)
TAZ *zinc finger type*		
1L3E:B	Hypoxia inducible factor-1 alpha subunit (p300)	Zn·(Cys_3His)
3IO2:A	Histone acetyltransferase p300	Zn·(Cys_3His)
Undefined *zinc finger type*		
3C5K:A	Histone deacetylase 6 (HDAC 6)	Zn·(Cys3His)
2VRW:B	Proto-oncogene vav (an oncogene)	Zn·(Cys3His)
1F3H:A	Survivin	Zn·(Cys3His)

Supplementary Materials and Methods

Protein expression and purification

The cDNA of Pirh2, TRAF6, BHC80, UHRF1 was amplified respectively by RT-PCR using the HeLa cell extracts by the RevertAid First Strand cDNA Synthesis Kit (Fermentas GmbH, Germany). The cDNA of TRAF6, BHC80 and UHRF1 were inserted respectively into the pET28a vector (Novagen, USA) whereas the cDNA of Pirh2 was inserted into the pGEX-4T-1 vector (GE Healthcare, USA). Plasmids were then transformed into *Escherichia coli* BL21-CodonPlus (DE3)-RIPL strain (Stratagene, USA) and cultured in LB medium at 37ºC for 4 hours. Protein expression was induced by adding 1 mM IPTG at 18ºC for 22 hours. Cell extracts containing the His-tagged proteins were applied to a Ni-NTA affinity column (Qiagen, USA) equilibrated with 50 mM phosphate buffer and 100 mM NaCl at pH 7.5 and eluted with gradient imidazole. The GST-fused Pirh2 was purified by glutathione-sepharose beads (GE Healthcare, USA) with the same buffer system and eluted with a linear gradient of Glutathiol.

The cDNA of human DNMT3L was purchased from TRANSOMIC (USA) and the PCR amplified gene was cloned into NdeI/XhoI sites of the expression vector pET-28a (Novagen, USA). The plasmid was transformed into *E. coli* BL21-RIPL strain (Stratagene, USA) cultured in LB medium supplemented with 50 μg/ml kanamycin. Cells were grown to an optical density of 0.4–0.6 measured at a wavelength of 600 nm and induced by 0.8 mM IPTG at 18°C for 20 h. The harvested cells were disrupted by a microfluidizer in 50 mM phosphate buffer containing 100 mM NaCl, and the cell extracts were passed through a Ni-NTA affinity column (Qiagen, USA) equilibrated with 50 mM phosphate buffer and 100 mM NaCl at pH 7.5 and eluted with gradient imidazole.

For the control proteins, RNase T, CRN4 and TZD were expressed and purified as previously described[25,26,28]. Carbonic Anhydrase I from human erythrocytes and GAL4 from *Saccharomyces cerevisiae* were purchased from Sigma-Aldrich (USA).

Zinc ejection Assays

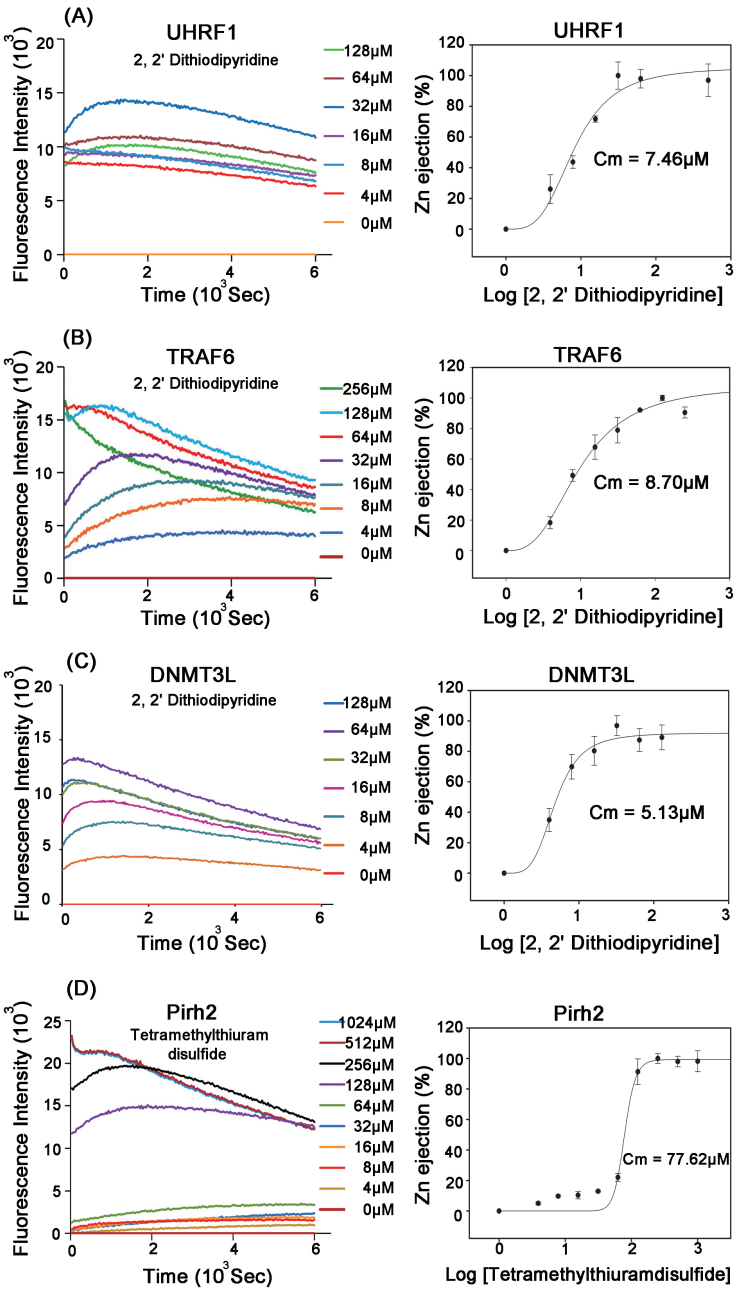
The Zn-ejecting agents, Tetramethylthiuram disulfide, 2,2'-Dithiodipyridine, Phenyl disulfide, Cystamine dihydrochloride, 3-Nitrosobenzamide(NOBA) and 2,2'-dithiobis and 5,5'-Dithiobis, were purchased from Sigma-Aldrich (USA) or Merck (Germany). Release of zinc ions from protein samples were monitored by the fluorescence emission from the zinc-specific fluorophore FluoZinTM-3 (Invitrogen/Life Technologies, USA). The Zn-ejecting agents were dissolved in DMSO to a stock solution of 100 mM and then diluted in 50 mM phosphate buffer, pH 7.5. The protein sample (5 μM) was mixed with the Zn-ejecting agent (5 μM) and 1 μM FluoZin-3 in a total reaction volume of 200 μl at room temperature. Fluorescence emission was monitored by EnSpire Multilabel Plate Reader (Perkin Elmer, USA) at an excitation wavelength of 494 nm and emission wavelength of 516 nm for 600 sec.

For the measurement of Cm (the concentration of rejecting agent corresponding to 50% Zn ions ejection from the target protein), 20 μM of UHRF1, BHC80, TRAF6 and DNMT3L were mixed respectively with 5 μM FluoZin^{TM}-3 and compound 2,2'-Dithiodipyridine, whereas Pirh2 (20 μM) was mixed with 5 μM FluoZin^{TM}-3 and Tetramethylthiuram disulfide in a total reaction volume of 50 μl at room temperature. The reaction started immediately before the plate was inserted into the spectrophotometer. Readings were taken for 200 cycles at a rate of 30 seconds per cycle and each reaction was repeated for three times. Fluorescence emission was monitored by EnSpire Multilabel Plate Reader (Perkin Elmer, USA) at an excitation wavelength of 494 nm and emission wavelength of 516 nm for 6000 seconds.

MASS Spectrometry

The zinc protein (1 mg/ml) was mixed with 2,2'-Dithiodipyridine (10 mg/ml) and incubated for 15 min. Protein samples were then denatured by DTT and passing through a

ZipTip$_{C18}$ desalting column (Millipore, USA) before analyzed by Bruker Daltonics- Autoflex III TOF/TOF mass spectrometer (Bremen, Germany). The measured MW of BHC80 was 8,890 Dalton, close to the calculated MW of 8891.30 of the expressed protein with the N-terminal Met residue deleted. The amino acid sequence for the expressed BHC80 is: MGSSHHHHHHSSGLVPRGSHMIHEDFCSVCRKSGQ
LLMCDTCSRVYHLDCLDPPLKTIPKGMWICPRCQDQMLKKEEAI.



Supplementary Figure S1. The efficiency of the Zn-ejecting compounds was estimated for cancer targets (A) UHRF1, (B) TRAF6, (C) DNMT3L and (D) Pirh2. The zinc ions released from the target protein (20 μM) by adding the Zn-ejecting agents 2,2′-dithiodipyridine or tetramethylthiuramdisulfide were monitored by the increased fluorescence signal of the zinc-specific fluorophore, FluoZinTM-3 (5 μM), using an excitation wavelength at 494 nm and emission wavelength at 516 nm for detection. For a given concentration *i* of the Zn-ejector, the highest fluorescence intensity of the curve (I_i) is proportional to the released Zn^{2+} concentration. At the maximum fluorescence intensity (I_{max}) of all the curves for a given protein, we assumed that all the Zn^{2+} was ejected from the target protein. The percentage of Zn ejected from each target protein by a given Zn-ejector concentration *i* was estimated by I_i/I_{max} and the concentration (Cm) corresponding to 50% Zn ejection was determined and shown in the right panel.