Supporting Information for

Reduction of Human Defensin 5 Affords a High-Affinity Zinc-Chelating Peptide

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This Supporting Information Includes:

Supporting Experimental Section

Supporting Materials, Tables, and Figures

Supporting Experimental Section

Materials and General Methods. The sub-sections listed below delineate the sources and general preparations of materials, and general methods employed in this work.

Commercial Materials. All reagents, and chemicals, and organic solvents were purchased from commercial suppliers in the highest available purity and used as received: ULTROL-grade HEPES (Calbiochem), TraceSELECT sodium chloride (Fluka/Sigma-Aldrich), metal-free 30% aqueous sodium hydroxide (Fluka/Sigma-Aldrich), OmniPur dithiothreitol (DTT_{red}, EMD), *trans*-4,5-dihydroxy-1,2-dithiane (DTT_{ox}, Sigma-Aldrich) sodium phosphate (BDH), *tris*(2-carboxy)ethylphosphine (TCEP, Alfa Aesar), EDTA (Malinckrodt Chemicals), NADPH tetrasodium salt (Calbiochem), high-purity metal salts (Sigma-Aldrich, *vide infra*), Zincon (Alfa Aesar), FluoZin-1 (Invitrogen), MagFura-2 (Invitrogen), Zinpyr-4 (Strem Chemicals, Inc.), HPLC-grade trifluoroacetic acid (TFA, Alfa Aesar), LC-MS-grade formic acid (Fluka/Sigma-Aldrich), and acetonitrile (EMD for HPLC-grade; Sigma for LC-MS grade). Enzymes were purchased from commercial suppliers, stored according to manufacturer protocols, and used as received: human thioredoxin (Sigma-Aldrich), rat liver thioredoxin reductase (Sigma-Aldrich), TPCK-treated trypsin (Affymetrix), and proteinase K (Worthington). Chelex resin was purchased from Biorad. Argon (Ar) and nitrogen (N_2) were obtained from Airgas.

Water Purification. Milli-Q water (18.2 MΩ) filtered with a 0.22-μm millipax filter was employed to prepare all aqueous solutions, buffers, and peptides stock solutions.

Preparation and Handling of HD5. Purified $HD5_{red}$ and $HD5_{ox}$ were obtained following overexpression of His_6-Met-HD5 in *Escherichia coli* and tag cleavage as described previously.(*S1*) Representative analytical HPLC traces of the purified peptides employed in this work are given in Figure S10. The purified peptide samples were stored at -20 ^{o}C as lyophilized powders or as aqueous solutions. Peptide stock solutions were prepared in water ($HD5_{ox}$), 0.01 M HCl ($HD5_{red}$), or 0.01 M trifluoroacetic acid ($HD5_{red}$). The stock solutions were typically partitioned into 100-μL aliquots, and immediately stored at -20 ^{o}C. The stock solutions are

stable to freeze thawing for a number of cycles. The peptide concentrations of the stock solutions were routinely quantified by optical absorption spectroscopy by using a BioTek Synergy HT plate reader outfitted with a calibrated Take3 Micro-Volume plate using the calculated extinction coefficients of ε_{278} = 2 800 $M^{-1}cm^{-1}$ for $HD5_{red}$ and ε_{278} = 3 181 $M^{-1}cm^{-1}$ for $HD5_{ox}$.(*S1*) The $HD5_{red}$ extinction coefficient was also used for quantifying solutions of the iodoacetamide-modified derivative HD5-TE (*vide infra*).

Stock solutions of $HD5_{red}$ were prepared in dilute acid (*vide supra*) to prevent oxidation of $HD5_{red}$ to $HD5_{ox}$. Acidic solutions of $HD5_{red}$ are stable under aerobic conditions. At neutral pH, $HD5_{red}$ readily folds to $HD5_{ox}$ in the presence of oxygen. With the exception of an oxidative folding experiment described below, all experiments with $HD5_{red}$ were conducted in Chelex-treated and Ar-purged buffers, and either in an anaerobic glovebox (VAC Atmospheres, N_2 atmosphere) or on the bench top in the presence of the reducing agent TCEP. When prepared in Ar-purged buffer and in the presence of excess TCEP, $HD5_{red}$ remains in its reduced form for at least 3 h at room temperature (HPLC analysis; data not shown).

Preparation of Buffers for Metal-Binding and Redox Studies. All precautions were taken to minimize metal-ion contaminations. Metal-free ULTROL grade HEPES (free-acid) was obtained from Calbiochem and used as received. Only Teflon-coated spatulas were employed to transfer all reagents. Plasticware was rinsed with Chelex-treated Milli-Q water before use. Glassware was soaked in dilute nitric acid and rinsed with Chelex-treated water before use. Chelex-treated water and freshly-prepared solutions of HEPES (20 mM or 75 mM) were treated with 10 g/L Chelex resin by stirring in a polypropylene container for at least 1 h. Prior to use, the Chelex resin was removed by centrifugation (50-mL polypropylene tube), and the pH was adjusted to the appropriate pH value by addition of metal-free 30% (w/v) NaOH (aq) (Fluka/Sigma-Aldrich). In some instances, HEPES buffer was prepared using Chelex-treated Mill-Q water and the pH subsequently adjusted using metal-free 30% (w/v) NaOH (aq). For experiments requiring anaerobic conditions, the buffers were degassed for at least 1 h by Ar bubbling. Prior to this Ar purge, the buffer was placed in a sterile 50-mL polypropylene tube

outfitted with a Teflon-coated stir bar, and 0.5 g of Chelex resin was added. The tube was sealed with a rubber septum, and a piece of Tygon microbore tubing was employed to deliver Ar to the buffer and thereby avoid contact of the buffer with a metal needle. The Ar-purged buffers were subsequently transferred and stored in the glovebox. Immediately prior to use, the buffer was passed through a 0.45-μm syringe filter to remove the Chelex resin.

Preparation of Zn(II), Cd(II), and Co(II) Stock Solutions. For experiments performed under aerobic conditions, stock solutions of Zn(II) (100 mM) and Cd(II) (10 mM) were prepared from 99.999% anhydrous $ZnCl_2$ (Sigma-Aldrich, used immediately upon first opening) and 99.999% anhydrous $CdCl_2$ (Sigma-Aldrich). The solutions were prepared using Milli-Q water and acid-washed volumetric glassware, and immediately transferred to 50-mL polypropylene tubes for storage. For experiments performed in the glovebox, the stock solutions of Zn(II) and Co(II) were prepared in the glovebox using degassed Milli-Q water. These stock solutions were stored in 50-mL polypropylene tubes.

Liquid Chromatography. Analytical and semipreparative high-performance liquid chromatography (HPLC) were performed on an Agilent 1200 instrument equipped with a thermostated autosampler set at 4 °C. A 5-μm pore Clipeus C18 column (4.6 x 250 mm, Higgins, Inc.) was employed for all analytical HPLC experiments. Solvent A was 0.1% TFA/H_2O and solvent B was 0.1% TFA/MeCN. A gradient of 10-60% B over 30 min at a flow rate of 1 mL/min was used. Absorption was monitored at 220 and 280 nm.

LC-MS analyses were performed on an Agilent 1290 Infinity LC / 6230 TOF-ESI system equipped with a thermostated autosampler set at 4 °C. A reverse-phase Poroshell C18 column (75 x 2.1 cm, 5-μm particle size) was employed. Solvent A was 0.1% formic acid/H_2O and solvent B was 0.1% formic acid/MeCN. A gradient of 10–60% B over 30 min at a flow rate of 0.4 mL/min was used and absorption was monitored at 220 and 280 nm. A sample volume of 5 μL was injected. Data was acquired by using the Agilent MassHunter Workstation Data Acquisition software and processed with the Agilent MassHunter Qualitative Analysis program. The ESI-MS spectra were acquired in positive ion mode. In the trap, the parameters were: gas temperature,

325 °C; nebulizer pressure, 20 psi; drying gas flow, 10 L/min; capillary voltage, 4000 V; sheath gas temperature, 400 °C; sheath gas flow, 12 L/min.

Midpoint Potential Determination. The midpoint potential (E_m) of HD5 was determined by incubating either $HD5_{red}$ or $HD5_{ox}$ in buffers poised at defined redox potentials. These experiments were conducted in an anaerobic glovebox. To prepare buffers with specific redox potentials, 100 mM solutions of both the reduced (DTT_{red}) and oxidized (DTT_{ox}) forms of dithiothreitol (DTT) were prepared in the glovebox at room temperature (75 mM HEPES, pH 7.0, Ar-purged). The ratio of DTT_{red} / DTT_{ox} required for the desired redox potential of each buffer (E_{Buffer}) was calculated using the Nernst equation (eq. 1)

$$E_{Buffer} = E^{o}_{m\ (DTT_{red}/DTT_{ox})} - \frac{RT}{nF} \ln\left(\frac{[DTT_{red}]}{[DTT_{ox}]}\right) \quad \text{(eq. 1)}$$

where $E^{o}_{m\ (DTT_{red}/DTT_{ox})}$ is the midpoint potential of the DTT_{ox} / DTT_{red} couple, R is the universal gas constant (8.314 $JK^{-1}mol^{-1}$), T is the temperature (295.15 K), *n* is the number of moles of electrons transferred (2 electrons per disulfide bond; *n* = 2 for DTT and *n* = 6 for HD5), and F is the Faraday constant (9.6485 x 10^4 $Cmol^{-1}$).

$E^{o}_{m\ (DTT_{red}/DTT_{ox})}$ is –330 mV when [DTT_{red}] = [DTT_{ox}] at pH = 7.0.(*S2*) Therefore, E_{Buffer} can be defined according to eq. 2.

$$E_{Buffer} = -330\ mV - \left(\frac{59.76}{2}\right) \log\left(\frac{[DTT_{red}]}{[DTT_{ox}]}\right) \quad \text{(eq. 2)}$$

Buffers with redox potentials spanning the -230 to -290 mV range were prepared in 5-mV increments. A 100-μL solution of 25 μM $HD5_{red}$ (or $HD5_{ox}$) was prepared in each redox buffer and the samples were incubated in the glovebox at room temperature (ca. 22 °C) for 20 h in order for equilibrium to be reached. At t = 20 h, the samples were quenched with 10 μL of 6% aqueous TFA, removed from the glovebox, centrifuged (13,000 rpm x 10 min, 4 °C), and the resulting supernatants were analyzed by HPLC. The percentages of $HD5_{red}$ and $HD5_{ox}$ at equilibrium were determined by integrating the HPLC peak areas ($HD5_{red}$, 19.9 min retention

time; $HD5_{ox}$, 14.4 min retention time). Each experiment was performed in triplicate. The E_m of $HD5_{red}$ / $HD5_{ox}$ was calculated by fitting the data to the Nernst equation (eq. 3).

$$E^{o}_{m\,(HD5_{red}/HD5_{ox})} - \left(\frac{59.76}{6}\right)\log\left(\frac{[HD5_{red}]}{[HD5_{ox}]}\right) = -330\ \text{mV} - \left(\frac{59.76}{2}\right)\log\left(\frac{[DTT_{red}]}{[DTT_{ox}]}\right) \qquad \text{(eq. 3)}$$

Thioredoxin Activity Assays. Four 100-μL solutions containing 20 μM $HD5_{ox}$, 15 μM human thioredoxin (Trx), 0.5 μM rat liver thioredoxin reductase (TrxR), 1 mM NADPH, and 2 mM EDTA (100 mM potassium phosphate buffer, pH 7.0) were prepared and the conversion of $HD5_{ox}$ to $HD5_{red}$ monitored at different time points. The activity assay was initiated by adding Trx to the reaction last. The mixtures were incubated at room temperature, and each reaction was quenched at a different time point (t = 0, 10, 20, 30 sec) by addition of 10 μL of 6% aqueous TFA. The acidified solutions were immediately vortexed and centrifuged (13,000 rpm x 10 min, 4 ^{o}C), and the resulting supernatants were immediately analyzed by HPLC. Control experiments were performed by omitting one of the components (Trx, TrxR, EDTA, NADPH) from the assay. The control assays were quenched at t = 10 sec. Following optimization, each experiment was performed in duplicate and representative HPLC traces are shown.

HPLC Assays for Zinc and Cadmium Coordination. Solutions (100 μL) containing 50 μM $HD5_{red}$ and either 1.0 equiv of Zn(II) or 1.0 equiv of Cd(II), or no added metal, were prepared at pH 7.4 (75 mM HEPES, 100 mM NaCl, not Ar-purged) under aerobic conditions. The resulting solutions were incubated overnight on the bench top (capped 1.7-mL microcentrifuge tubes) at room temperature and subsequently acidified with 10 μL of 6% aqueous TFA. The acidified solutions were vortexed, centrifuged (13,000 rpm x 10 min, 4 ^{o}C), and the supernatants analyzed by HPLC.

MALDI-TOF Mass Spectrometry. For Zn(II):$HD5_{red}$, a solution containing 36 μM $HD5_{red}$, 72 μM Zn(II), and 180.5 μM TCEP was prepared in 75 mM HEPES, 100 mM NaCl, pH 7.4. For Cd(II):$HD5_{red}$, a solution containing 36 μM $HD5_{red}$, 90 μM Cd(II), and 180.5 μM TCEP was prepared in 75 mM HEPES, 100 mM NaCl, pH 7.4. The MALDI matrix was 2',4',6'-

trihydroxyacetophenone (THAP). The samples and matrix were submitted to the MIT Biopolymers Laboratory for same-day MALDI-TOF analysis (reflector mode). Only poor resolution was obtained for the +Zn(II) samples and the resulting data are therefore not reported.

Optical Absorption Spectroscopy. Optical absorption spectra were collected on a Beckman Coulter DU 800 spectrophotometer thermostated at 25 ^{o}C with a Peltier Temperature Controller or an Agilent 8453 diode-array spectrophotometer controlled with manufacturer-supplied software and thermostated at 25 °C by a circulating water bath. Quartz cuvettes (Starna, 1-cm path length) were employed for all optical absorption measurements.

In a typical optical absorption titration of $HD5_{red}$ with Zn(II) or Cd(II), a 200-μL solution of 20 μM $HD5_{red}$ (20 mM HEPES, 100 μM TCEP, pH 7.4, Ar-purged) was prepared in a quartz cuvette. Aliquots of a concentrated Zn(II) or Cd(II) stock solution were added. The cuvette was capped and mixed gently after each M(II) addition, and the optical absorption spectrum was recorded. The total change in volume during each titration was less than 2%. A rising baseline and decrease in absorption intensity at ca. 230 nm, suggesting precipitate formation, was routinely observed after addition of ≥2 equiv of Zn(II) to ≥20 μM $HD5_{red}$ under these conditions.

For a typical Co(II)-binding titration, a 200-μL solution of 50 – 150 μM $HD5_{red}$ (75 mM HEPES, pH 7.4) was prepared in an anaerobic quartz cuvette in the glovebox and the optical absorption spectrum recorded. Aliquots from a concentrated Co(II) stock solution were added in the glovebox. After each Co(II) addition, the cuvette was sealed and inverted gently for mixing before recording the optical absorption spectrum. The total change in volume during the titration was less than 2%. No precipitation was observed with Co(II) addition under these experimental conditions.

Fluorescence Spectroscopy. Fluorescence spectra were collected on a Photon Technologies International QuantaMaster 40 fluorimeter outfitted with a continuous xenon source for excitation, autocalibrated QuadraScopic monochromators, a multimode PMT detector, and a circulating water bath maintained at 25 °C. This instrument was controlled by the

FelixGX software package. FelixGX was employed to integrate the emission spectra. Preliminary optical absorption and fluorescence spectroscopic measurements were performed at least twice, and all titrations were performed at least in triplicate. Quartz cuvettes (Starna, 1-cm path length) were employed for all optical absorption measurements.

Zinc Competition Experiments with $HD5_{red}$ and Zn(II) Sensors. All Zn(II) competition experiments were performed under anaerobic conditions in the glovebox under a nitrogen atmosphere. MagFura-2 (MF2) and FluoZin-1 (FZ1) were purchased from Invitrogen, Zinpyr-4 (ZP4) was obtained from Strem Chemicals, Inc., and Zincon monosodium salt was received from Alfa Aesar. Stock solutions of ~1 mM ZP4 in DMSO (anhydrous, Sigma-Aldrich) were prepared, partitioned into 50-μL aliquots, stored at -20 ^{o}C, and thawed immediately before use. Stock solutions of ~200 μM FZ1 and ~170 μM MF2 were prepared in Milli-Q water, partitioned into aliquots, and stored at -20 ^{o}C. Stock solutions of ~1 mM Zincon in DMSO were prepared immediately before use. Solutions of these metal- and light-sensitive chromophores were covered in aluminum foil and handled in the dark. Reported Zn(II) K_d values for these probes are: ZP4, 0.65 nM;(*S3*) MF2, 20 nM*;*(*S4*) Zincon, 12.6 μM;(*S5*) FZ1, 7.8 μM.(*S6*)

Competition with MF2. For Zn(II) competition titrations between MF2 and $HD5_{red}$, a 15.6-μL aliquot of a $HD5_{red}$ stock solution (640 μM in 0.01 M TFA) was added to 984.4 μL of buffer (75 mM HEPES, pH 7.4, with or without 100 mM NaCl) to provide a final $HD5_{red}$ concentration of 10 μM. For the titration in the presence of 100 mM NaCl, this solution was diluted with the buffer to a final concentration of 5 μM. The optical absorption spectrum of this solution was recorded. An aliquot of a freshly-prepared MF2 stock solution in water was then added to provide a final MF2 concentration of either approximately 5 μM (no NaCl) or 2.5 μM (with NaCl). The final concentration of MF2 was confirmed from its absorbance at 366 nm using the reported extinction coefficient of 22,000 $M^{-1}cm^{-1}$.(*S4*) This solution was titrated with Zn(II) by step-wise addition of 5-μL aliquots from either a 400 μM (no NaCl) or 200 μM (with NaCl) Zn(II) stock solution. Each Zn(II) addition was performed in the glovebox and, after each Zn(II) addition, the cuvette was capped and the solution was mixed gently prior to recording the optical absorption

spectrum. The absorption change at 324 nm was recorded and corrected for dilution, and the resulting values were employed for data fitting. The results from three independent titrations were fit by using DynaFit and a Zn(II) K_d value of 20 nM for MF2.(*S4*) A control titration (15.6 μL of 0.01 M TFA added without $HD5_{red}$ added to the buffer) was also performed and analyzed for comparison.

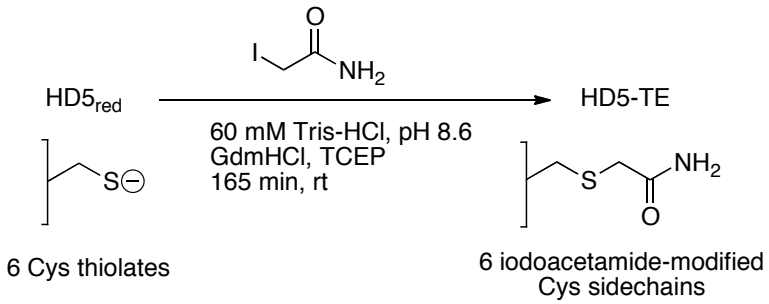
Competition with Zincon. For Zn(II) competition titrations between Zincon and $HD5_{red}$, a 15.6-μL aliquot of a $HD5_{red}$ stock solution (640 μM in 0.01 M TFA) was added to 984.4 μL of buffer (75mM HEPES buffer, pH 7.4) to provide a final concentration of 10 μM. Freshly-prepared Zincon was then added to afford a final Zincon concentration of approximately 10 μM. The actual concentration of Zincon was calculated from its absorbance at 488 nm using the reported extinction coefficient of 26,900 $M^{-1}cm^{-1}$.(*S5*) This solution was titrated with Zn(II) as described above for the MF2 titrations. The absorbance change at 367 nm was recorded and corrected for dilution. A control titration (15.6 μL of 0.01 M TFA added without $HD5_{red}$ added to the buffer) was also performed and analyzed for a comparison.

Competition with ZP4. For Zn(II) competition titrations between ZP4 and $HD5_{red}$, a 14.3-μL aliquot of a $HD5_{red}$ stock solution (700 μM in 0.01 M TFA) was added to 985.7 μL of buffer (75mM HEPES buffer, pH 7.4) to provide a final $HD5_{red}$ concentration of 10 μM. The optical absorption and fluorescence spectra of this solution were collected. An aliquot of a freshly-prepared ZP4 stock solution (~1 mM in DMSO) was then added to afford a final ZP4 concentration of approximately 1 μM. The final ZP4 concentration of ZP4 was verified from its absorbance at 504 nm using the extinction coefficient of 61,000 $M^{-1}cm^{-1}$.(*S3*) This solution was titrated with Zn(II) as described above for the MF2 titrations. The fluorescence emission spectrum was recorded after each Zn(II) addition (λ_{ex} = 495 nm). The resulting emission spectra were corrected for dilution and integrated from 500 nm to 600 nm, and the integrated emission was plotted against Zn(II) concentration. The results from three independent titrations were fit by using DynaFit and a Zn(II) K_d value of 0.65 nM for ZP4.(*S3*) Control titrations without $HD5_{red}$ added were performed and analyzed for comparison.

Competition with FZ1. For Zn(II) competition titrations between FZ1 and $HD5_{red}$, a 3.1-μL aliquot of a $HD5_{red}$ stock solution (640 μM in 0.01M TFA) was added to 996.9 μL of buffer (75mM HEPES, pH 7.4),to provide a final $HD5_{red}$ concentration of 2 μM. The optical absorption and fluorescence spectra of this solution were collected. Freshly prepared FZ1 from a ~200 μM stock solution was then added to afford a final FZ1 concentration of approximately 2 μM. Aliquots (5 μL) of an 80-μM Zn(II) stock solution were added. After each Zn(II) addition, the cuvette was capped and mixed gently, and the emission spectrum subsequently recorded (λ_{ex} = 495 nm). The resulting emission spectra were corrected for dilution and integrated from 500 nm to 600 nm, and the integrated emission was plotted against Zn(II) concentration. A titration of FZ1 with Zn(II) in the absence of $HD5_{red}$ was performed in parallel.

Data Analysis with DynaFit. The data obtained from the MF2 and ZP4 titrations were analyzed by using DynaFit. Each titration was fit to models that consider one- and two-binding sites by using an iterative approach. These models assume monomeric species. The K_{d1} values (Ka in DynaFit script) obtained from the one-site model were inputted as initial estimates for Ka in the two-site model. Examples scripts are provided below in this Supporting Information.

Preparation of HD5-TE. $HD5_{red}$ (2.4 mg, 0.67 μmol) was dissolved in 1.0 mL of 60 mM Tris-HCl buffer adjusted to pH 8.6 containing 6 M guanidinium hydrochloride. A 100-μL aliquot of 100 mM TCEP was added and the solution was vortexed briefly and incubated at room temperature for 25 min. A 100-μL aliquot of freshly-prepared 500 mM iodoacetamide in water was added and the reaction was covered in aluminum foil and incubated at 37 ^{o}C for 165 min. The reaction was centrifuged (13,000 rpm x 10 min) to pellet any insoluble material, and the product was purified from the supernatant by semi-preparative HPLC using a 5-μm pore ZORBAX C18 column (9.6 x 250 mm, Agilent Technologies) with a gradient of 10–40% B over 15 min at a flow rate of 5 mL/min. The isolated product was lyophilized to dryness, which afforded a white fluffy powder (2.0 mg, 76%). Analytical HPLC retention time, 16.8 min (10–60% B in 30 min). ESI-MS: calcd $[M+H]^+$, 3928.80; found, 3928.83.



Protease Susceptibility Assays. To determine whether Zn(II) modulates the susceptibility of $HD5_{red}$ to proteolytic degradation, a solution containing 50 μM $HD5_{red}$ in the presence of 1.2 equiv of Zn(II) was prepared and incubated at room temperature (100 mM Tris-HCl, 20 mM $CaCl_2$, 0.001% Triton-X 100, 5 mM TCEP, pH 8.2). The protease of interest was added to initiate the reaction (final concentration: trypsin, 0.25 μg/mL; chymotrypsin, 0.25 μg/mL; proteinase K, 1 μg/mL). Aliquots (50 μL) were removed at t = 1, 2, 5, 15, 30, and 60 min and immediately quenched with 5 μL of 6% TFA, vortexed, and stored at -20 ^{o}C prior to HPLC analysis. In parallel, assays in which Zn(II) was omitted from the reaction were also performed. In addition, control assays using HD5-TE, which cannot bind Zn(II), instead of $HD5_{red}$ were conducted for each protease to confirm that Zn(II) addition did not perturb protease activity.

DynaFit script for competition of $HD5_{red}$ and ZP4 for Zn(II) (one binding site)

```
[task]
        data = equilibria
        task = fit

[mechanism]

        E + M   <==> E.M                        : Ka      dissoc
        L + M <==> LM                           : Kc      dissoc


[constants]
        Ka = 0.000210?,  Kc =0.00065

[concentrations]
        E =  10.00
        L =  1.00

[responses]
        L = 0, LM= 1



[data]
        plot titration


[equilibria]

  directory    ./examples/
  extension    txt
  variable          M
  file              1uMZP4-10uMHD5-data  |  concentration E = 10.00
  response L = 0, LM= 1



[output]

  directory    ./examples/constants
```

DynaFit script for competition of $HD5_{red}$ and ZP4 for Zn(II) (two binding sites)

```
[task]
        data = equilibria
        task = fit

[mechanism]

        E + M <==> E.M                          : Ka dissoc
        E.M + M <==> E.M.M                      : Kb dissoc
        L + M <==> LM                           : Kc dissoc


[constants]
        Ka = 0.000247?, Kb = 0.020?, Kc = 0.00065

[concentrations]
        E =  10.00
        L =  1.00

[responses]
        L = 0, LM= 1



[data]
        plot titration


[equilibria]

  directory    ./examples/
  extension    txt
  variable          M
  file              1uMZP4-10uMHD5-data  |  concentration E = 10.00
  response L = 0, LM= 1



[output]

  directory    ./examples/constants
```

DynaFit script for competition of $HD5_{red}$ and MF2 for Zn(II) (one binding site)

```
[task]
        data = equilibria
        task = fit

[mechanism]

        E + M   <==> E.M                        : Ka      dissoc
        L + M <==> LM                           : Kc      dissoc


[constants]
        Ka = 0.0003308?,            Kc =0.02

[concentrations]
        E =  10.00
        L =  5.2

[responses]
        L = 0, LM= 1



[data]
        plot titration


[equilibria]

  directory    ./examples/
  extension    txt
  variable          M
  file              5uMMF2-10uMHD5-data  |  concentration E = 10.00
  response L = 0, LM= 1



[output]

  directory    ./examples/constants
```

DynaFit script for competition of $HD5_{red}$ and MF2 for Zn(II) (two binding sites)

```
[task]
        data = equilibria
        task = fit

[mechanism]

        E + M <==> E.M                          : Ka dissoc
        E.M + M <==> E.M.M                      : Kb dissoc
        L + M <==> LM                           : Kc dissoc


[constants]
        Ka = 0.000509? , Kb = 0.280?, Kc = 0.02

[concentrations]
        E =  10.00
        L =  5.2

[responses]
        L = 0, LM= 1



[data]
        plot titration


[equilibria]

  directory    ./examples/
  extension    txt
  variable          M
  file              5uMMF2-10uMHD5-data  |  concentration E = 10.00
  response L = 0, LM= 1



[output]

  directory    ./examples/constants
```

Table S1. Reported midpoint potentials for a variety of cysteine-containing biomolecules.

Biomolecule	E_m (mV)	Experimental Conditions	Reference
Yap1 redox domain (Yap1-RD)	E_{m1} = -155 ± 3 E_{m2} = -330 ± 2	100 mM HEPES (pH 7.0) [a]	S7
Protein disulfide-isomerase (PDI)	-175 ±15	100 mM potassium phosphate, 1 mM EDTA (pH 7.0) [a]	S8
Penicillamine	-186	D_2O, 0.15 M NaCl [a]	S9
Cysteine	-188 ± 1	D_2O, 0.15 M NaCl (pD 4.18 ~ 7.08) [a]	S9
E. coli Grx 3	-198	100 mM potassium phosphate, 1 mM EDTA (pH 7.0) [a]	S10
Coenzyme A	-199 ± 2	D_2O, 0.15 M NaCl (pD 6.4 ~ 7.4) [a]	S9
Cysteamine	-203 ± 1	D_2O, 0.15 M NaCl [a]	S9
N,N-dimethylcysteamine	-214	D_2O, 0.15 M NaCl [a]	S9
E. coli Grx 1	-233	100 mM potassium phosphate, 1 mM EDTA (pH 7.0) [a]	S10
Glutatione	-240	100 mM potassium phosphate, 1 mM EDTA (pH 7.0) [a]	S10
S. cerevisiae Trx2	-265 ± 10	100 mM HEPES (pH 7.0) [a]	S7
S. cerevisiae Trx1	-275 ± 1	100 mM HEPES (pH 7.0) [a]	S7
E. coli Trx	-285 ± 10	100 mM MOPS (pH 7.0) [b]	S11
Metallothionein	< -366	0.2 M Tris (pH 7.3) [c]	S12

[a] Midpoint potential determined by using GSH/GSSG redox buffers. [b] Midpoint potential determined by using DTT_{red}/DTT_{ox} redox buffers. [c] Midpoint potential determined by using an Ag/AgCl electrode.

Table S2. Observed *m/z* values of the HPLC peaks attributed to partially-oxidized $HD5_{red}$.[a]

Retention Time (min)	$[M+H]^+$ obs *m/z*	Assignment ($[M+H]^+$ calc *m/z*)
15.6	3580.68	$HD5_{ox}$ (3580.63)
17.5	3584.73	Partially-oxidized product (3584.66)[b]
17.8	3584.79	Partially-oxidized product (3584.66)[b]
18.3	3584.79	Partially-oxidized product (3584.66)[b]
18.8	3584.65	Partially-oxidized product (3584.66)[b]
20.1	3586.83	$HD5_{red}$ (3586.67)

[a] These data correspond to the HPLC trace presented in Figure 3 of the main text. [b] The partially-oxidized product corresponds to HD5 with one S—S bond and four free Cys-SH.

Table S3. Observed and calculated *m/z* for MALDI-TOF MS of the Cd(II):$HD5_{red}$ complexes.

Cd(II):$HD5_{red}$	Species	calc *m/z*	obs *m/z*
0:1	**$[M + H]^+$**	3586.83	n.o.[a]
1:1	**$[M - H + Cd(II)]^+$**	3698.56	3698.61
2:1	**$[M - 3H + 2Cd(II)]^+$**	3810.45	3810.50

[a] n.o. = not observed.

MALDI-TOF spectrum for Cd(II):$HD5_{red}$

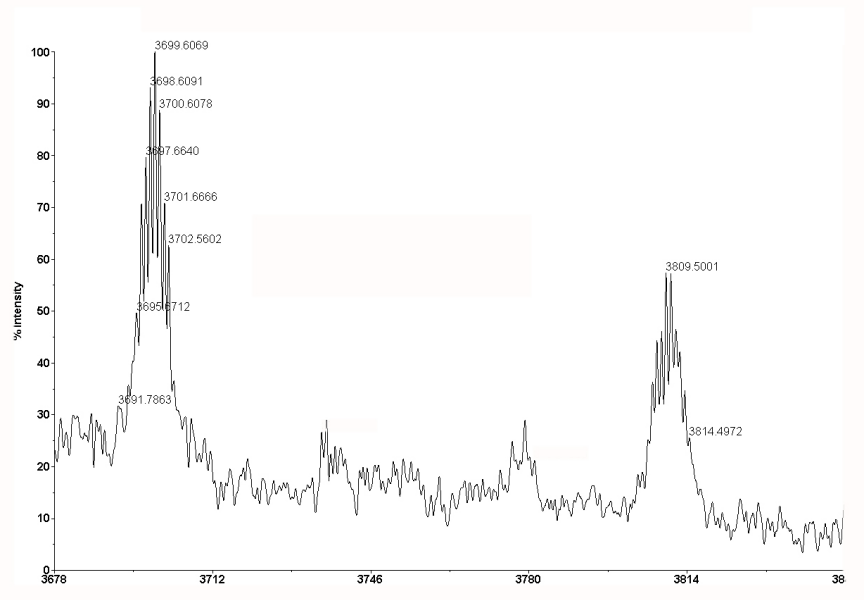


Table S4. Dissociation constants ($\log K_d$) for Zn(II) binding to cysteine-rich sites in biomolecules.

Biomolecule	$\log K_d$	Experimental Conditions	Method	Reference
ZntR	-14.8	20 mM Tris-Bis, 100 mM NaCl (pH 7.0)	Fluorescence (TPEN titration)	S13
	-14.9	20 mM Tris-Bis, 100 mM NaCl (pH 8.0)		
Dipeptidyl peptidase III	-12.3	50 mM NaPB (pH 7.4)	Enzymatic activity	S14
Aspartate transcarbamoylase	-12.1	40 mM HEPES (pH 7.0)	UV (indo-1, quin-2 titrations)	S15
Metallothionein	-7.7 to -11.8 [a]	50 mM HEPES (pH 7.4)	Fluorescence (FluoZin-3 & RhodZin-3 titrations)	S16
Human carbonic anhydrase II	-11.4	10 mM Tris (pH 7.0)	Equilibrium dialysis & UV (dithizone assays)	S17
Glyoxalase I	-10.6	0.1 M Tris, 0.1 M NaCl (pH 8.5)	Metal buffer technique & enzymatic activity	S18
Carboxypeptidase A, bovine	-10.5	50 mM Tris, 1 M NaCl (pH 8.0)	Equilibrium dialysis & isotopic/microchemical analyses	S19
Human sonic hedgehog	≤-10	100 mM HEPES, 150 mM NaCl (pH 7.5)	Fluorescence (Magnesium green)	S20
Angiotensin converting enzyme	-8.2	50 mM HEPES, 300 mM NaCl (pH 7.5)	Equilibrium dialysis & atomic absorption spectroscopy	S21

[a] Different stability constants ($\log K$) were measured for each of the seven Zn(II) ions that are bound by metallothionein: sites 1-4 ($\log K = 11.8$), site 5 ($\log K = 10.45$), site 6 ($\log K = 9.95$) and site 7 ($\log K = 7.7$).

Table S5. Reported electronic transitions for Co(II)-thiolate complexes.

Molecule	λ / nm (ε / $M^{-1}cm^{-1}$)	Reference
Zinc ribbon protein CH_3-L36 (S_3N complex)	323 (1750), 360 (1150), 609 (180), 653 (300), 689 (400)	S22
Zinc ribbon protein C_4-L36 (S_4 complex)	305 (4060), 350 (1800), 614 (230), 688 (370), 732 (300)	S22
Metallothionein (S_4 complex, clusters)	320 (2845), 400 (2170), 600 (245), 690 (420), 743 (435)	S23
DNA repair protein XPAzf (S_4 complex)	307 (7800), 355 (5100), 640 (840), 695 (1080), 742 (1290)	S24
Rubredoxin (S_4 complex; Co-substituted Fe-S cluster)	350 (9405), 470 (3010), 620 (1128), 685 (1232), 748 (1034)	S25
HSP33 peptide (S_4 complex)	300 (2470), 347 (3200), 635 (369), 686 (769), 722 (697)	S26
T-cell protein CD8α (S_4 complex)	610, 680, 760 (<300)	S27
T-cell protein Lck (S_4 complex)	695 and shoulders at ~ 660 & 720 (>400)	S27
$(Me_4N)_2Co(SPh)_4$	416 (4380), 625 (640), 693 (985), 734 (840) [a]	S28
$(Ph_4P)_2Co(SPh)_4$	625 (590), 680 (820), 725 (675)[a]	S29

[a] These values were measured in acetonitrile.

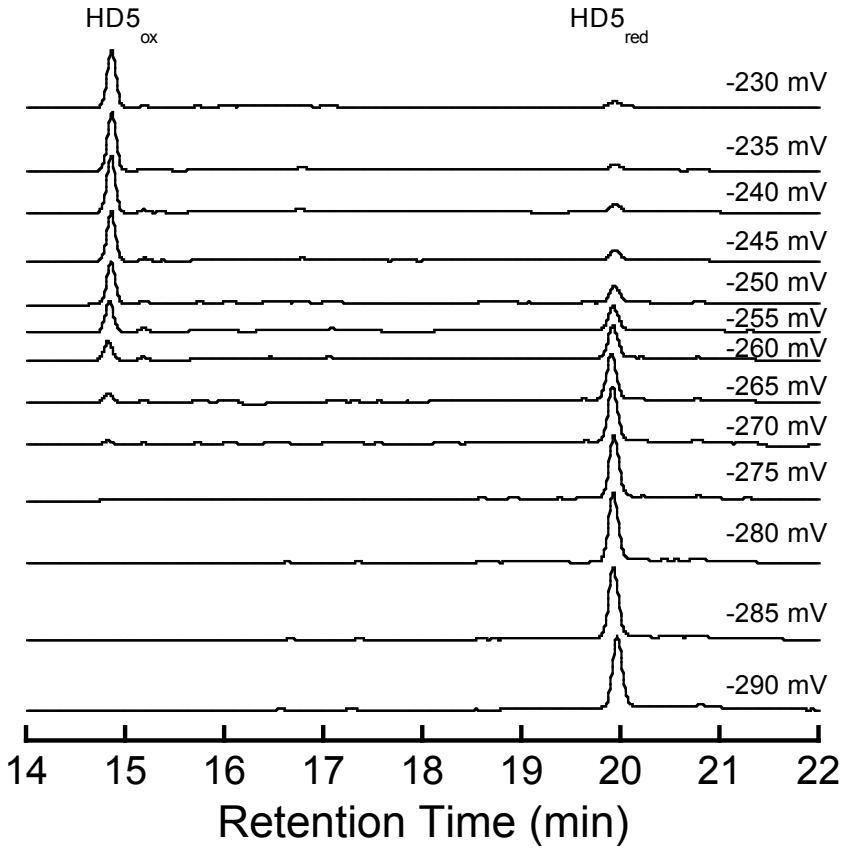


Figure S1. Representative analytical HPLC traces (220 nm) for the HD5 midpoint potential determination starting from 25 μM $HD5_{red}$ (75 mM HEPES, pH 7.0). Small peaks in the baseline with retention times between 14.9 and 20 min were not considered in the E_m determination and fell within the noise. A solution with a $HD5_{red}$ concentration greater than 25 μM, which would provide enhanced resolution of any small peaks indicative of partially-oxidized products or DTT adducts, could not be employed in these assays as a result of $HD5_{red}$ having six SH equivalents and the solubility of the redox buffer components, the latter of which must be in sufficient excess.

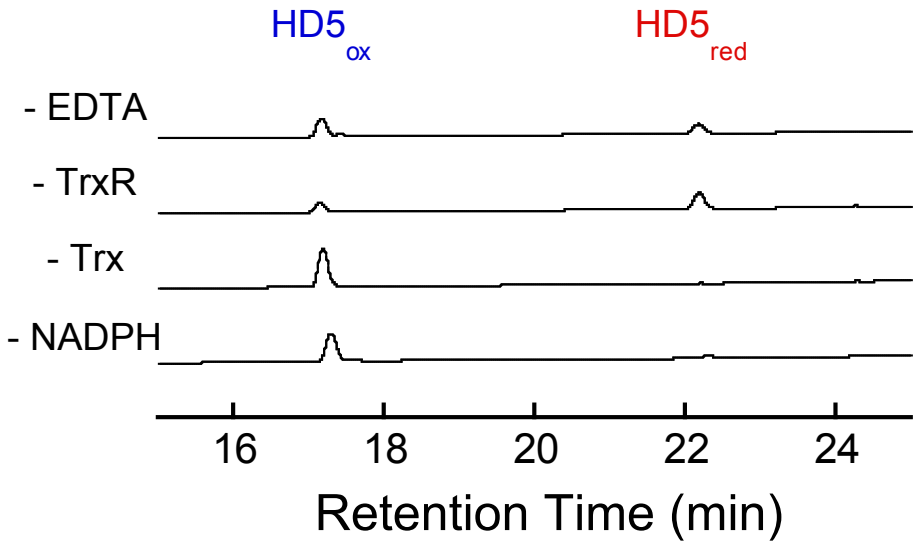


Figure S2. Analytical HPLC traces (220 nm) for the Trx/TrxR assay controls (100 mM potassium phosphate, pH 7.0, t = 10 sec). These controls correspond to the data presented in Figure 2b of the main text. No $HD5_{red}$ formation is observed when either Trx or NADPH is omitted from the assay. In contrast, Trx will reduce some $HD5_{ox}$ in the absence of its reductase, indicating that the Trx employed in this assay is partially reduced. EDTA is not essential for $HD5_{ox}$ reduction.

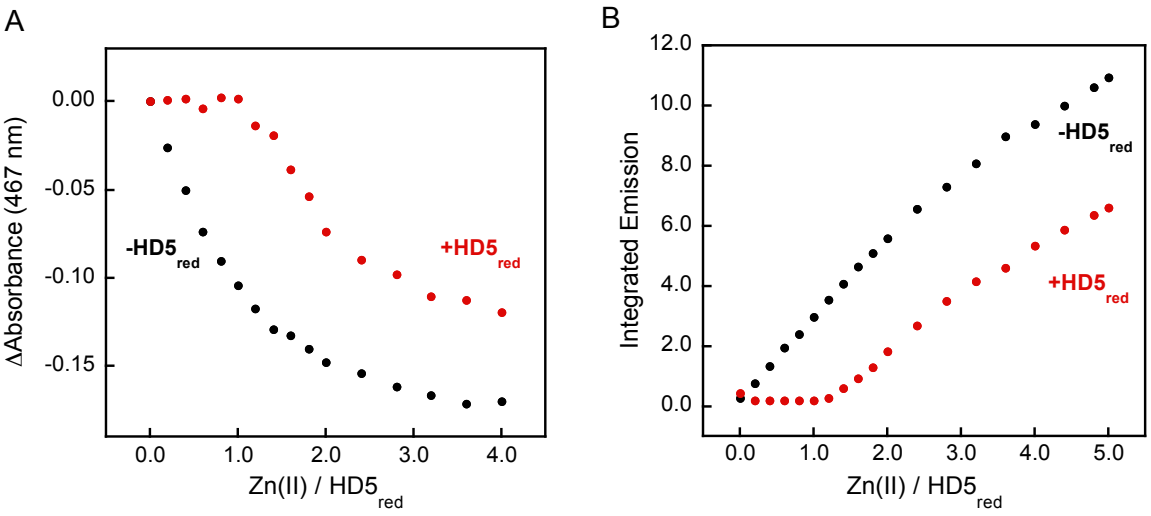
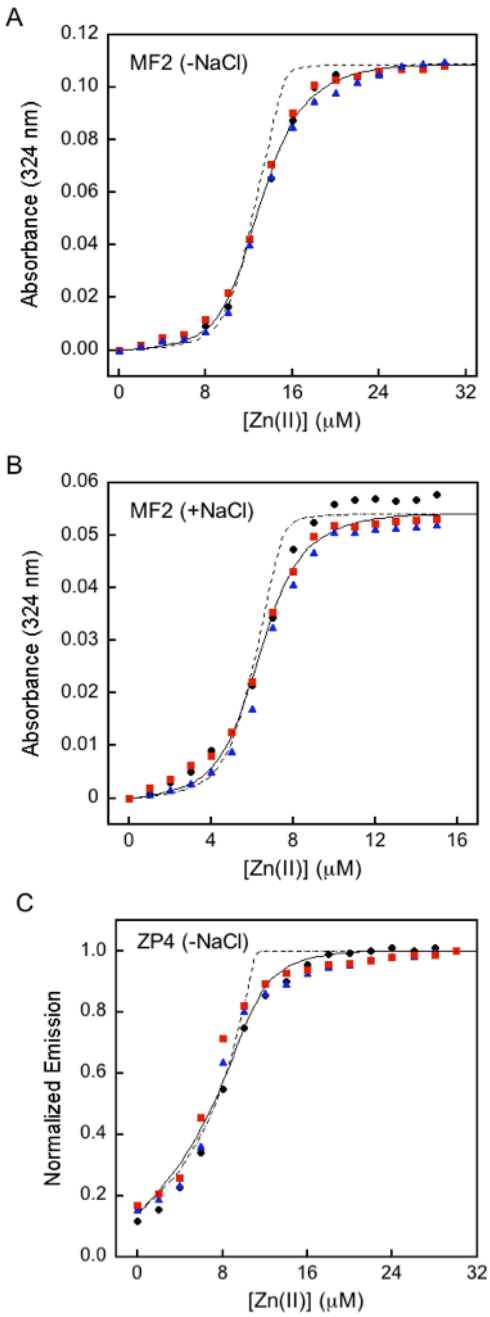


Figure S3. Zn(II)-induced response of Zincon and FZ1 in the presence of $HD5_{red}$ at pH 7.4 (75 mM HEPES) and 25 ^{o}C. (A) Anaerobic titration of a mixture containing 10 μM Zincon and 10 μM $HD5_{red}$ with Zn(II). The absorption values at 467 nm indicate that Zincon only responds to Zn(II) after $HD5_{red}$ coordinates one equivalent of Zn(II). (B) Anaerobic titration of a mixture containing 2 μM FZ1 and 2 μM $HD5_{red}$ with Zn(II). The fluorescence response of FZ1 to Zn(II) occurs only after $HD5_{red}$ coordinates one equivalent of Zn(II). In both panels, the black circles correspond to a Zn(II)-binding titration performed in the absence of $HD5_{red}$, and the red circles indicate a titration performed in the presence of $HD5_{red}$.



From fit with 1:1 only
K_{d1} = 331 ± 142 pM

From fit with 1:1 and 2:1
K_{d1} = 509 ± 54 pM
K_{d2} = 353 ± 25 nM

From fit with 1:1 only
K_{d1} = 574 ± 206 pM

From fit with 1:1 and 2:1
K_{d1} = 841 ± 154 pM
K_{d2} = 344 ± 56 nM

From fit with 1:1 only
K_{d1} = 210 ± 23 pM

From fit with 1:1 and 2:1
K_{d1} = 247 ± 23 pM
K_{d2} = 20 ± 5 nM

Figure S4. Anaerobic Zn(II) competition titrations for dissociation constant determination. (A) Zn(II)-induced response of 5 μM MF2 in the presence of 10 μM $HD5_{red}$ at pH 7.4 (75 mM HEPES) and 25 °C. (B) Zn(II)-induced response of 2.5 μM MF2 in the presence of 5 μM $HD5_{red}$ at pH 7.4 and in the presence of sodium chloride (75 mM HEPES, 100 mM NaCl) and 25 °C. (C) Zn(II)-induced response of 1 μM ZP4 in the presence of 10 μM $HD5_{red}$ at pH 7.4 (75 mM HEPES) and 25 °C. Excitation was provided at 495 nm and the emission spectra were integrated from 500–600 nm. The integrated emission was normalized to the maximum response. In each panel, the black circles, red squares, and blue triangles correspond to three independent titrations. The lines represent the global fits. The dashed line is the fit to a 1:1 binding model. The solid line is the fit to a 1:1 and 2:1 binding model. The calculated apparent K_d values corresponding to the fits are listed to the right of each plot.

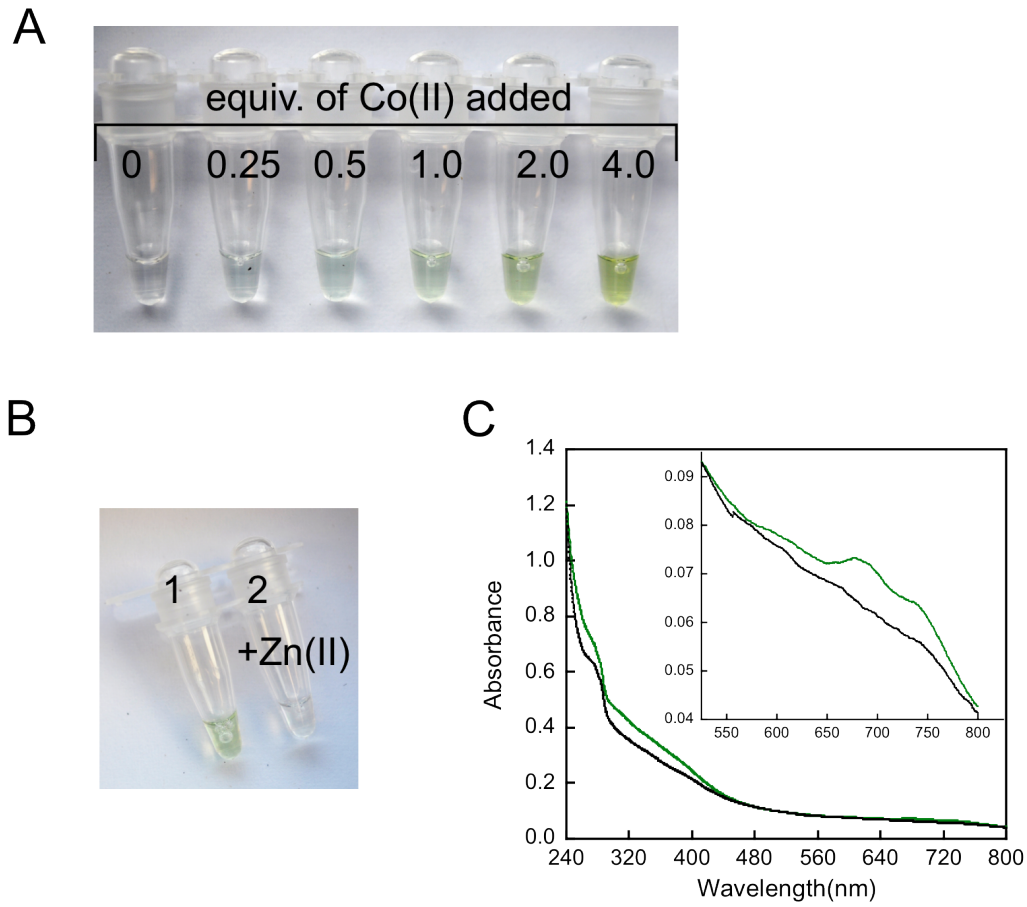


Figure S5. Coordination of Co(II) by $HD5_{red}$. (A) Colors observed following Co(II) complexation to $HD5_{red}$. Addition of 0–4 equiv of Co(II) to a solution of 1.0 mM $HD5_{red}$ at pH 7.4 (75 mM HEPES). The color changes from light blue to green with increasing Co(II) equivalents. (B) Addition of 1.0 equiv of Zn(II) to a mixture of 1.0 mM $HD5_{red}$ and 2.0 equiv of Co(II) at pH 7.4 (75 mM HEPES). (C) Optical absorption spectra of 50 μM $HD5_{red}$ and excess Co(II) before (green) and after addition of 1.0 equiv of Zn(II) (black). Loss of the LMCT and *d*—*d* transitions are observed. Exposure of Co(II):$HD5_{red}$ to oxygen results in formation of a yellow solution (data not shown).

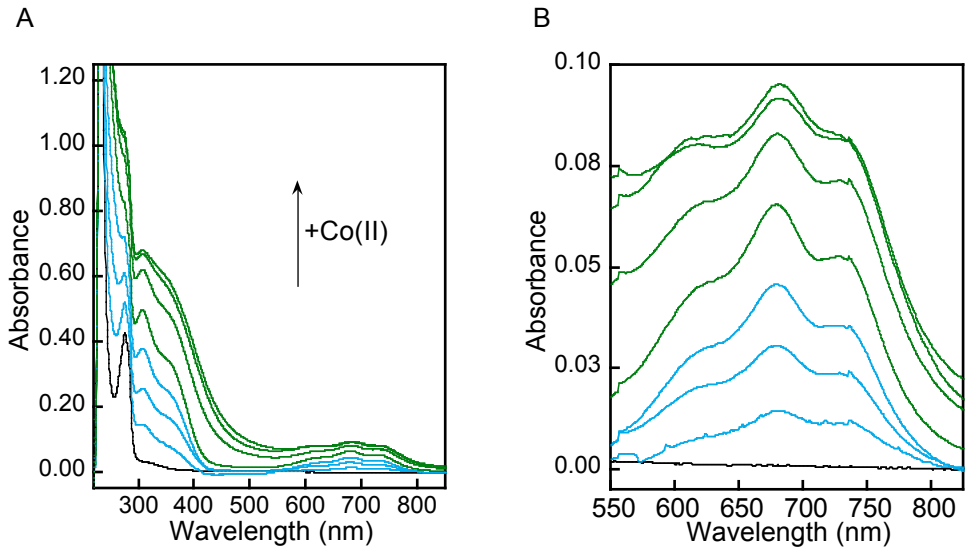


Figure S6. Anaerobic titration of 150 μM $HD5_{red}$ with Co(II) at pH 7.4 (75 mM HEPES) and 25 ^{o}C monitored by optical absorption spectroscopy. $HD5_{red}$ (black trace) was titrated with 0.25, 0.5, 0.75, 1.0, 1.5, 2.0 and 4.0 equiv of Co(II). The traces for 0.25, 0.5 and 0.75 equiv of Co(II) are in blue, and the traces for 1.0, 1.5, 2.0 and 4.0 equiv of Co(II) are in green. The green and blue represent the color of each solution as observed by eye. (A) Complete spectra. (B) Expansion of the *d*—*d* transition range.

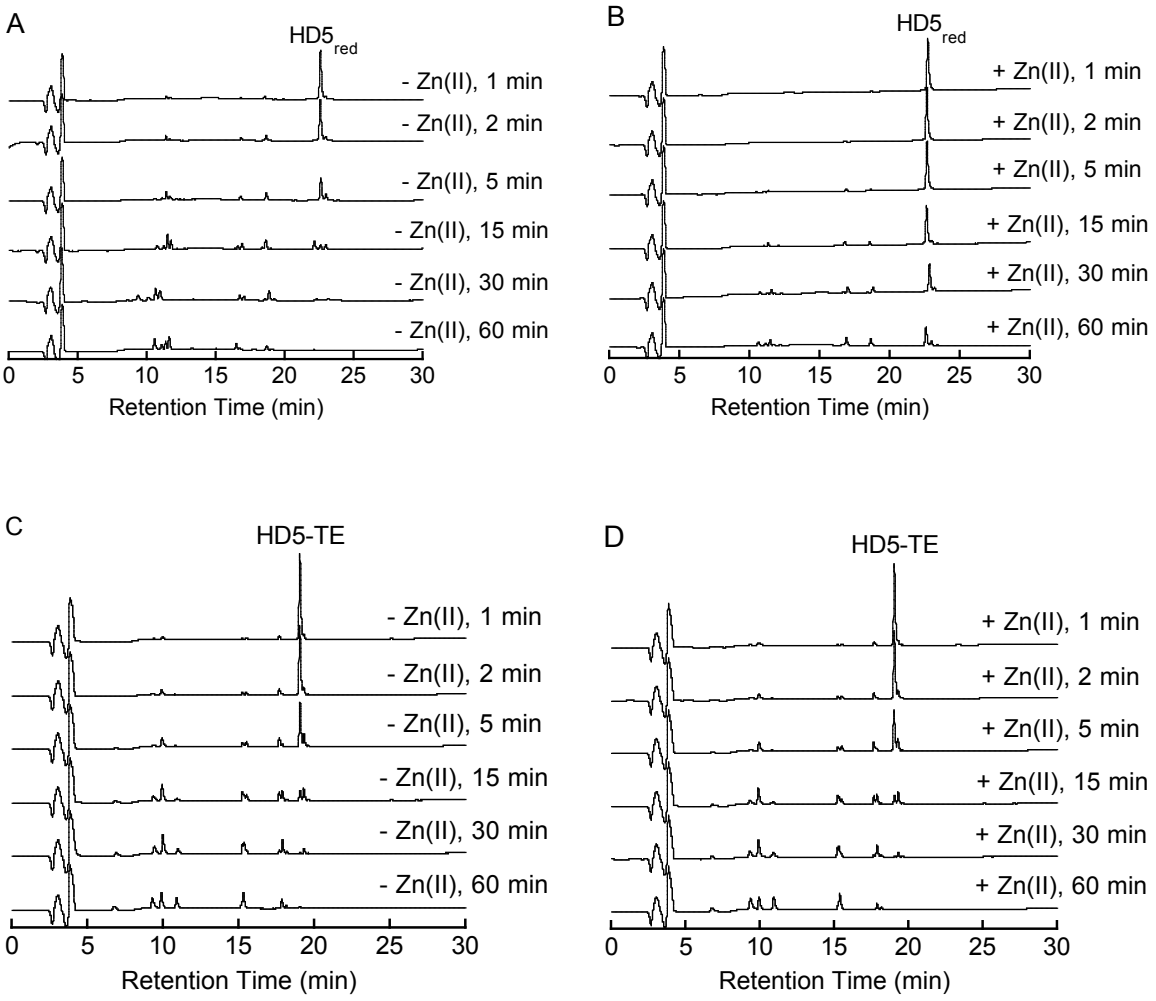


Figure S7. Analytical HPLC traces (220 nm) for trypsin susceptibility assays (100 mM Tris-HCl, 20 mM $CaCl_2$, 0.001% Triton-X 100, 5 mM TCEP, pH 8.2). The concentration of trypsin was 0.25 μg/mL. (A) Full chromatograms for the trypsin-catalyzed degradation of 50 μM $HD5_{red}$. (B) Full chromatographs for the trypsin-catalyzed degradation of 50 μM $HD5_{red}$ in the presence of 1.2 equiv of Zn(II). Panels A and B correspond to Figure 5A of the main text. (C) Full chromatograms of trypsin-catalyzed degradation of 50 μM HD5-TE in the absence of Zn(II). (D) Full chromatograms of the trypsin-catalyzed degradation of 50 μM HD5-TE in the presence of 1.2 equiv of Zn(II).

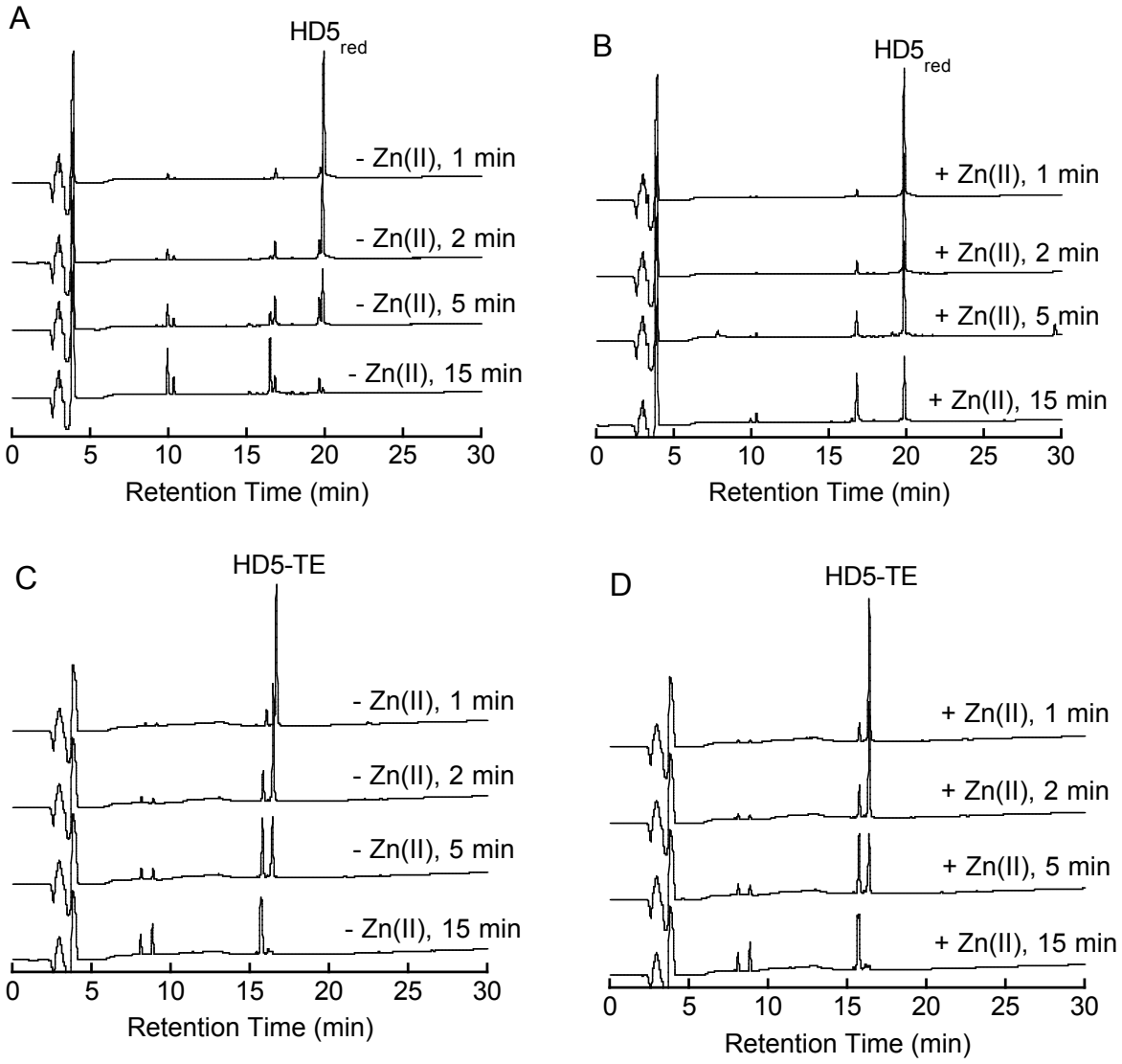


Figure S8. Analytical HPLC traces (220 nm) for chymotrypsin susceptibility assays (100 mM Tris-HCl, 20 mM $CaCl_2$, 0.001% Triton-X 100, 5 mM TCEP, pH 8.2). The concentration of chymotrypsin was 0.25 μg/mL. (A) Full chromatograms for the chymotrypsin-catalyzed degradation of 50 μM $HD5_{red}$. (B) Full chromatographs for the chymotrypsin-catalyzed degradation of 50 μM $HD5_{red}$ in the presence of 1.2 equiv of Zn(II). (C) Full chromatograms of chymotrypsin-catalyzed degradation of 50 μM HD5-TE in the absence of Zn(II). (D) Full chromatograms of the chymotrypsin-catalyzed degradation of 50 μM HD5-TE in the presence of 1.2 equiv of Zn(II).

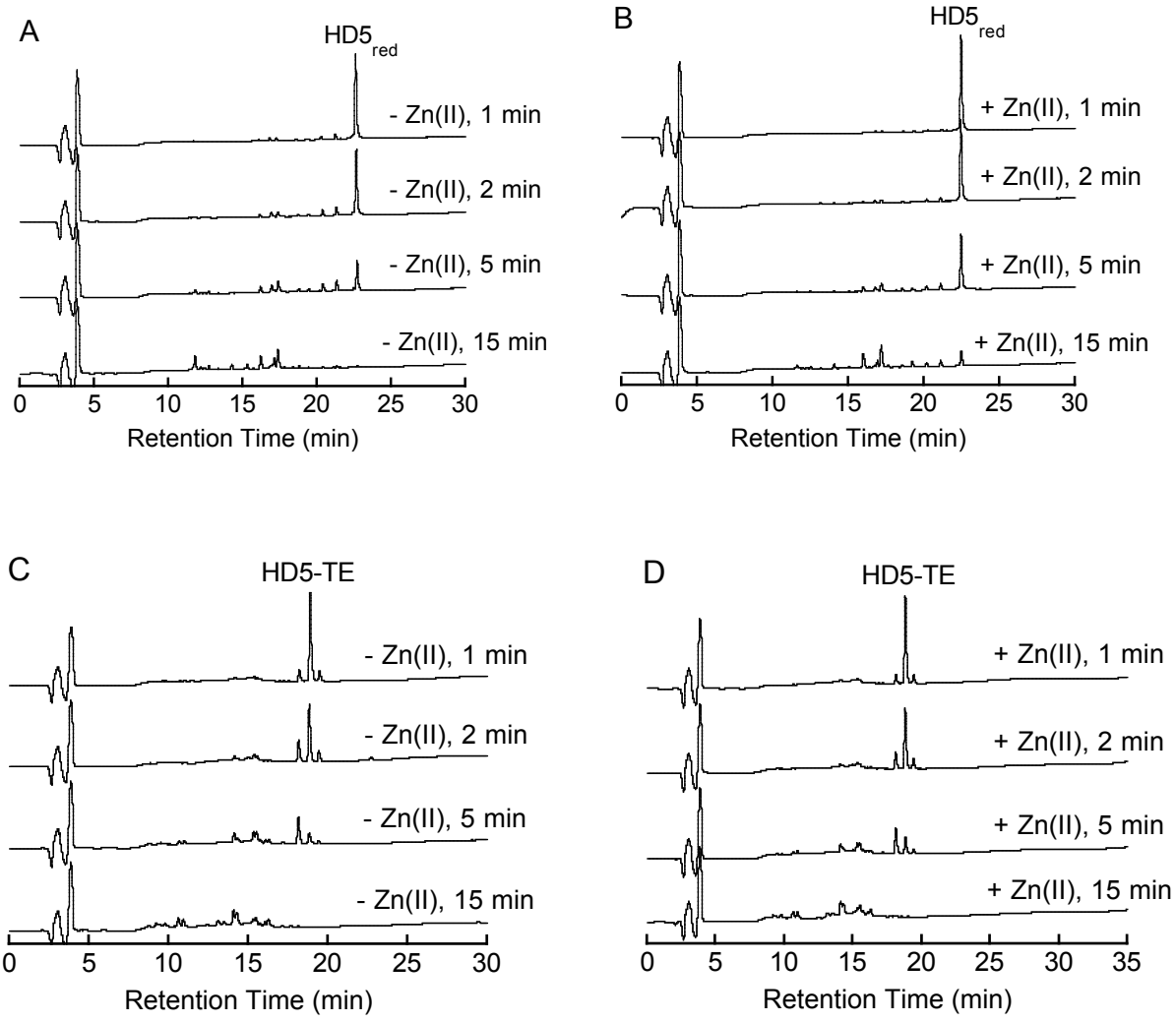


Figure S9. Analytical HPLC traces (220 nm) for proteinase K (PK) susceptibility assays (100 mM Tris-HCl, 20 mM $CaCl_2$, 0.001% Triton-X 100, 5 mM TCEP, pH 8.2). The concentration of PK was 1.0 μg/mL. (A) Full chromatograms for the PK-catalyzed degradation of 50 μM $HD5_{red}$. (B) Full chromatographs for the PK-catalyzed degradation of 50 μM $HD5_{red}$ in the presence of 1.2 equiv of Zn(II). (C) Full chromatograms of PK-catalyzed degradation of 50 μM HD5-TE in the absence of Zn(II). (D) Full chromatograms of the PK-catalyzed degradation of 50 μM HD5-TE in the presence of 1.2 equiv of Zn(II).

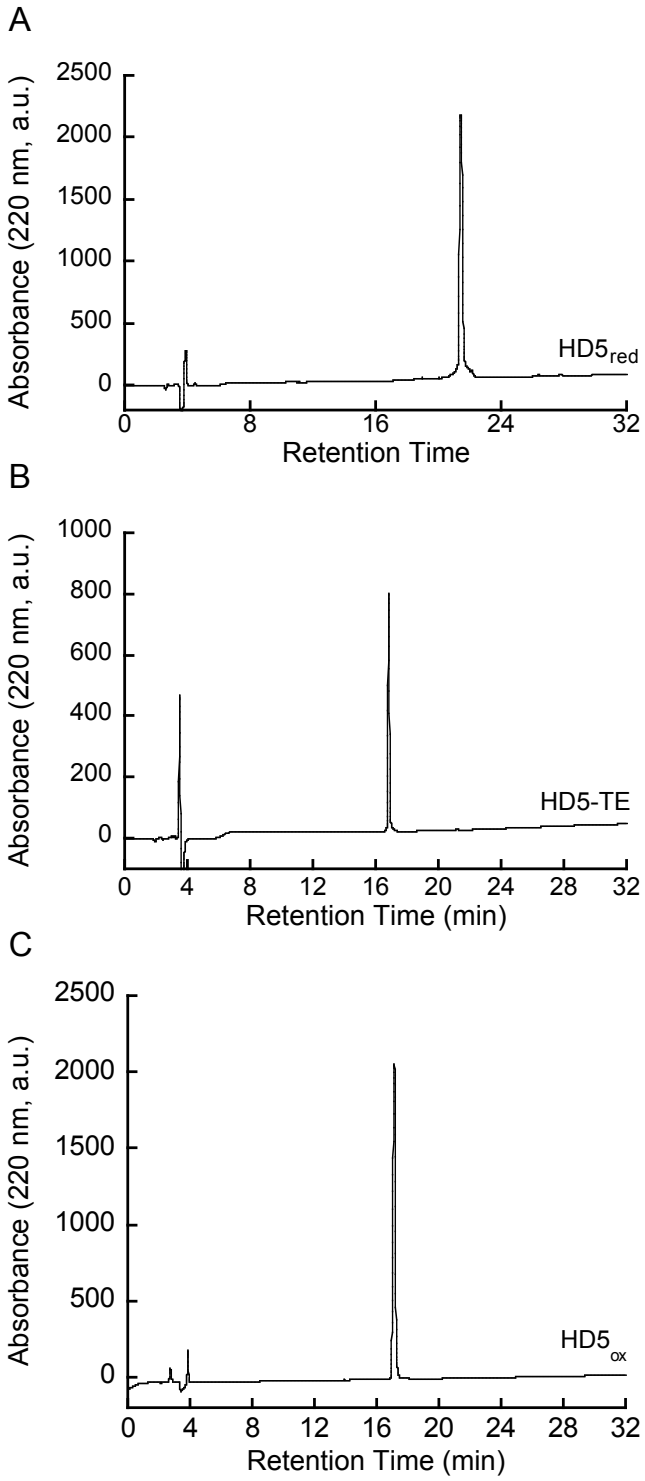
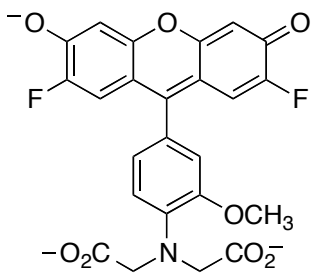
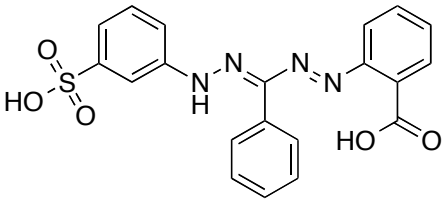


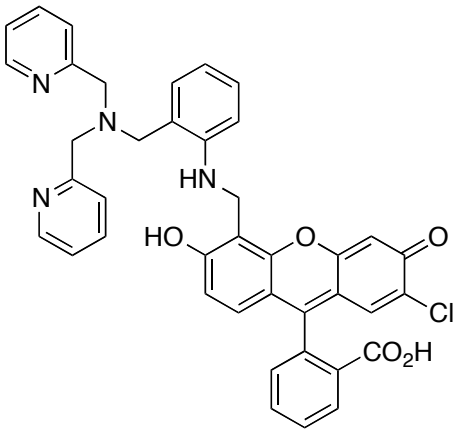
Figure S10. Representative analytical HPLC traces of the peptides prepared for and employed in this work (10–60% B over 30 min, 220 nm absorption). (A) $HD5_{red}$. (B) HD5-TE. (C) $HD5_{ox}$.



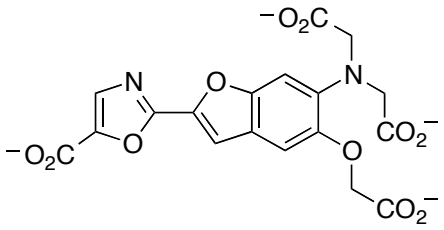
FluoZin-1 (FZ1)



Zincon



Zinpyr-4 (ZP4)



MagFura-2 (MF2)

Figure S11. Structures of small-molecule Zn(II) sensors employed in this work.

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