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Reengineering of a fluorescent zinc sensor protein yields the first genetically encoded cadmium probe

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Supporting Information

Supporting Figures

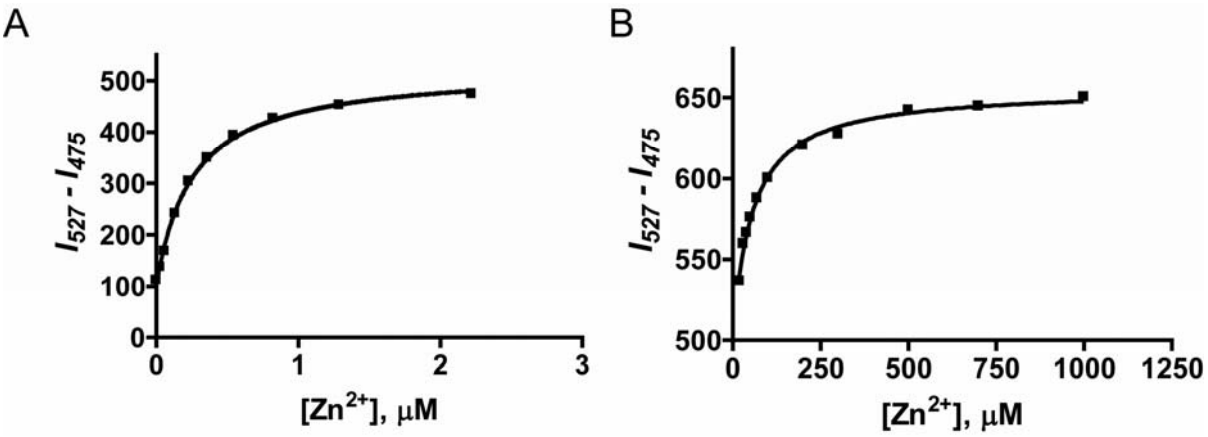

Figure S1: *Zn^{2+} binding properties of eZinCh-1. Zn^{2+} titration curves of eZinCh-1 that show the difference in fluorescence intensities at 527 and 475 nm ($I_{527} - I_{475}$) fitted using a single binding event, yielding a K_d of 258 ± 19 nM for the high affinity site (A) and a K_d of 53 ± 8 μM for the low affinity site (B). Data in (A) were obtained using Ba^{2+}-EGTA as a buffer system for Zn^{2+}. Titrations were performed in 50 mM Tris/HCl, 100 mM NaCl, 10% (v/v) glycerol, 0.05% (v/v) Tween-20, 2 μM DTT, 0.5 mM TCEP, pH 8.0 at 20 °C.*

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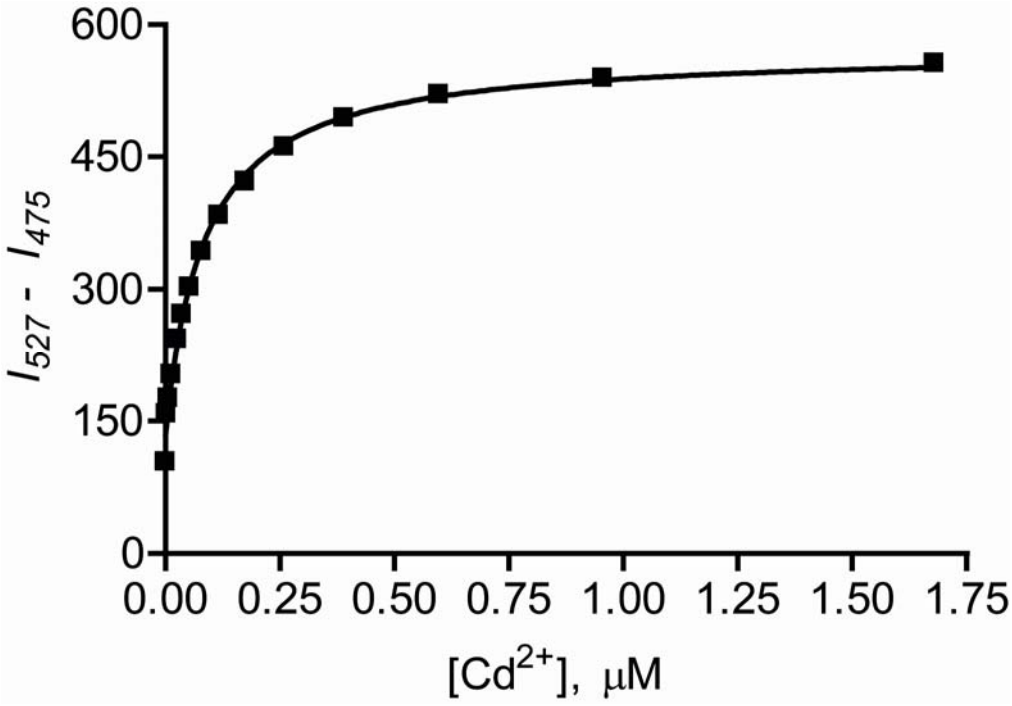

Figure S2: *Cd^{2+} binding properties of eZinCh-1. Cd^{2+} titration curve, showing the difference in fluorescence intensities at 527 and 475 nm for eZinCh-1 at increasing Cd^{2+} concentrations. The solid line represents a fit using a single binding event, yielding a K_d value of 84 nM. Titrations were performed in 50 mM Tris/HCl, 100 mM NaCl, 10% (v/v) glycerol, 0.05% (v/v) Tween-20, 2 µM DTT, 0.5 mM TCEP, pH 8.0 at 20 °C. For eZinCh-1, 0.5 mM Ca^{2+} was used together with 1.0 mM NTA as a buffer system for Cd^{2+}.*

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Hydrophobic interactions affect the emission ratio of Cd-FRET-2

The emission ratio of Cd-FRET-1 in the absence of metal (1.3) was similar to that of eZinCh-1, while substantially higher emission ratios were observed for Cd-FRET-2 (3.3) and Cd-FRET-3 (2.7) (Main article, Figure 2). The increased emission ratios for the latter two variants cannot be simply explained by the formation of interdomain disulfide bonds. Cleavage of the flexible linker by treatment of Cd-FRET-2 with proteinase K resulted in a large decrease in emission ratio, which would not be expected when the domains were connected by disulfide bonds. In addition, SDS-PAGE analysis of the proteinase K-treated Cd-FRET-2 in absence of β-mercapto-ethanol displayed only a weak band at 60 kDa and intense bands at 30 kDa (Figure S3). We therefore attribute the increased emission ratio to the increased hydrophobicity of the dimerization interface in these two mutants.

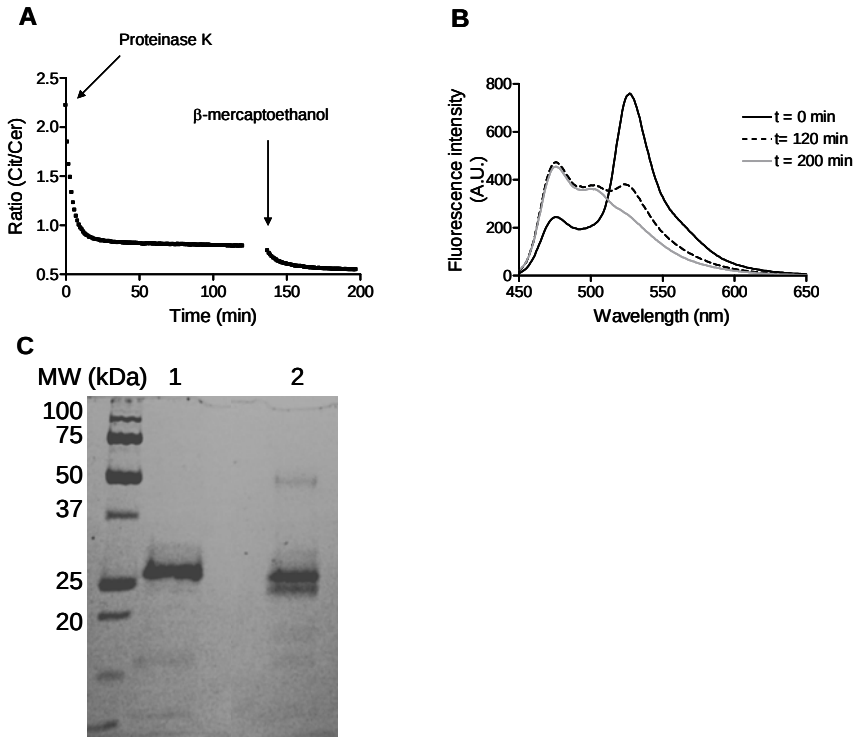

Figure S3: *(A) Emission ratio of Cd-FRET-2 monitored over time after addition of proteinase K (0.01 U) and addition of β-mercaptoethanol (5% (v/v)) at t = 135 min. (B) Fluorescence emission spectra of Cd-FRET-2 before addition of proteinase K (0 min), before addition of β-mercaptoethanol (120 min) and at the end of the experiment (200 min). The experiment was performed under anaerobic conditions. (C) SDS-PAGE analysis of Cd-FRET-2 after digestion with proteinase K. Lane 1 and 2 contain Cd-FRET-2 in the presence and absence of β-mercaptoethanol, respectively.*

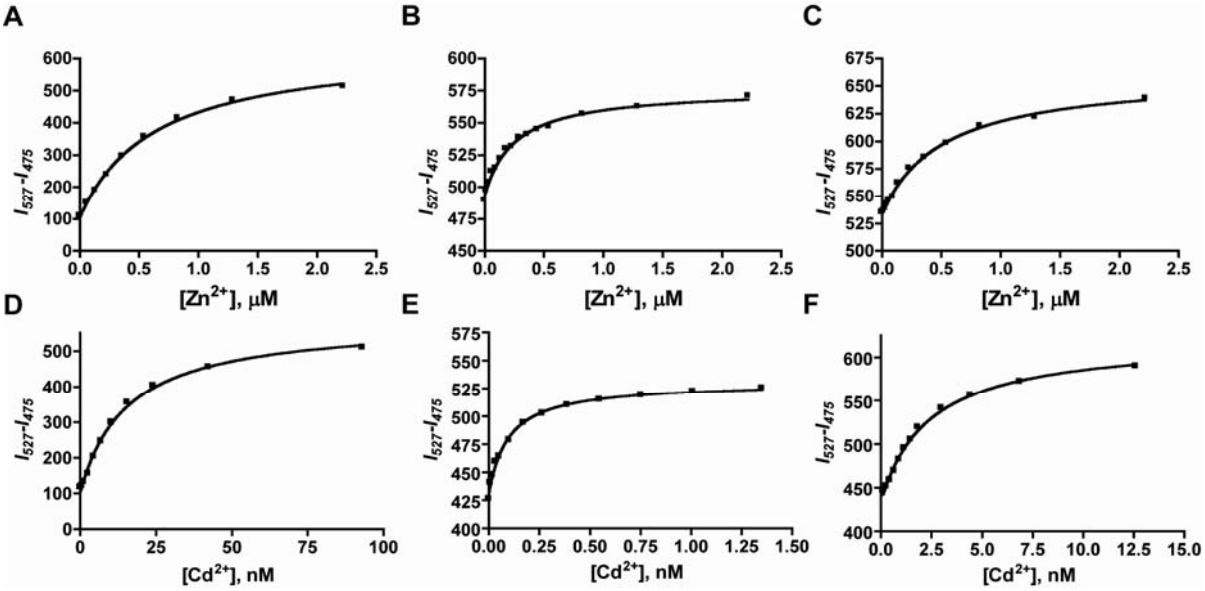

Figure S4: *Zn^{2+} and Cd^{2+} binding properties of the Cd-FRET variants.* (A-C) *Zn^{2+} titration curves, showing the difference in fluorescence intensities at 527 and 475 nm (I_{527} - I_{475}) for Cd-FRET-1 (A), Cd-FRET-2 (B) and Cd-FRET-3 (C). The solid lines display a fit of the data using a single binding event, yielding* K_d*'s of 631 nM, 253 nM and 509 nM for Cd-FRET-1, Cd-FRET-2 and Cd-FRET3, respectively. (D-F) Similar to (A-C), yet showing titration curves for Cd^{2+} instead of Zn^{2+}. The solid lines display a fit of the data using a single binding event, yielding* K_d*'s of 16 nM, 0.1 nM and 2.4 nM for Cd-FRET-1, Cd-FRET-2 and Cd-FRET3, respectively. Titrations were performed in 50 mM Tris/HCl, 100 mM NaCl, 10% (v/v) glycerol, 0.05% (v/v) Tween-20, 2 µM DTT, 0.5 mM TCEP, pH 8.0, 20 °C. In (A-C) 10 mM Ba^{2+} was used in combination with 1 mM EGTA as a buffer for Zn^{2+}. In (D) and (G), 1 mM NTA was used as a buffer for Cd^{2+}, whereas in (E), 1.1 mM Ca^{2+} was used in combination with 1 mM HEDTA.*

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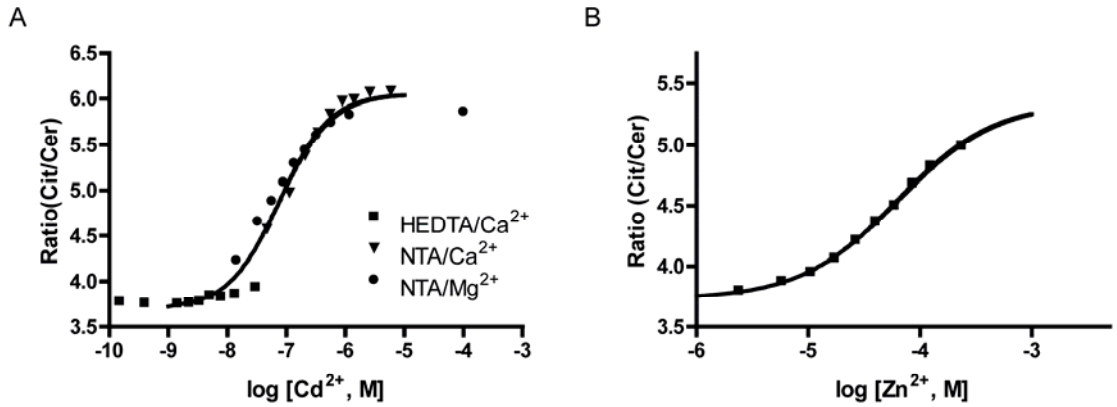

Figure S5: *Metal binding properties of Cd-FRET-2 at pH 7.1. (A and B) Emission ratio of Cd-FRET-2 as a function of Cd^{2+} and Zn^{2+} concentrations, respectively. The solid lines depict a fit using a single binding event, yielding K_d values of 42 nM for Cd^{2+} and 48 μM for Zn^{2+}, respectively. Measurements were performed in 100 mM Hepes, 100 mM NaCl, 10% (v/v) glycerol, 0.05% (v/v) Tween-20, 2 μM DTT, 0.5 mM TCEP, pH 7.1, 20 °C. The Cd^{2+} titrations were performed in buffering systems consisting of HEDTA/Ca^{2+} (squares), NTA/Ca^{2+} (triangles) and NTA/Mg^{2+} (circles). No buffer system was used in the Zn^{2+} titration.*

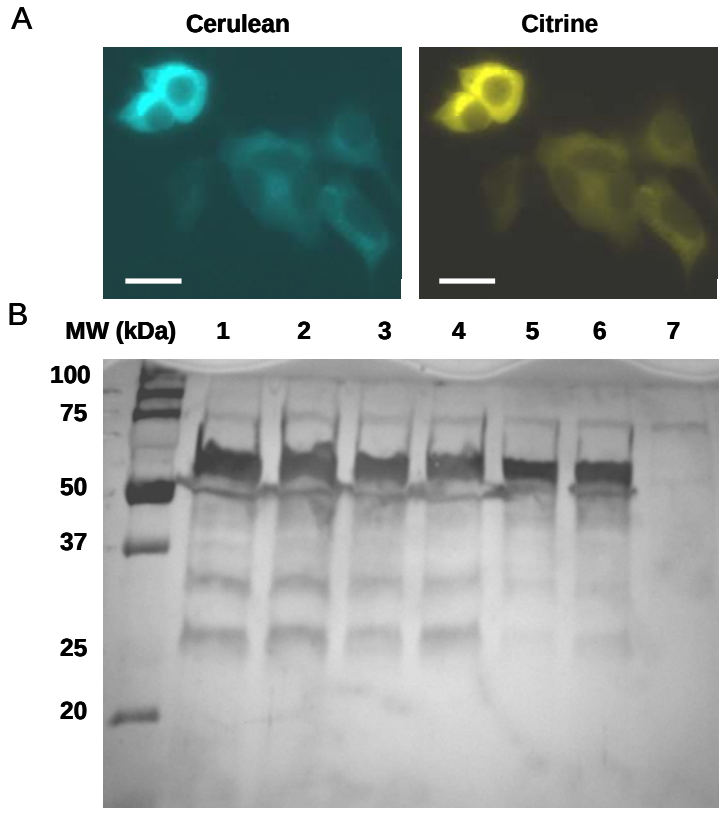

Figure S6: *Intracellular expression of Cd-FRET-2, eZinCh-1 and Cer-L9-Cit, which was used as a non-binding control,in HEK293 cells. (A) Epifluorescence images showing Cerulean (left) and Citrine (right) emission of cells expression Cd-FRET-2. The bright spots in the two cells in the top left indicate aggregation of Cd-FRET-2 and these cells also display a more rounded phenotype than the cells with a lower expression level. Scale bars indicate 20 μm* (B) *Western blot analysis of HEK293 cell lysates from cells expressing different Cd-FRET variants. Lanes 1-6 display lysates of HEK293 cells transfected with eZinCh-1 (1 and 2), Cd-FRET-2 (3 and 4) or Cer-L9-Cit (5 and 6). Lane 7 shows the lysate of non-transfected cells.*

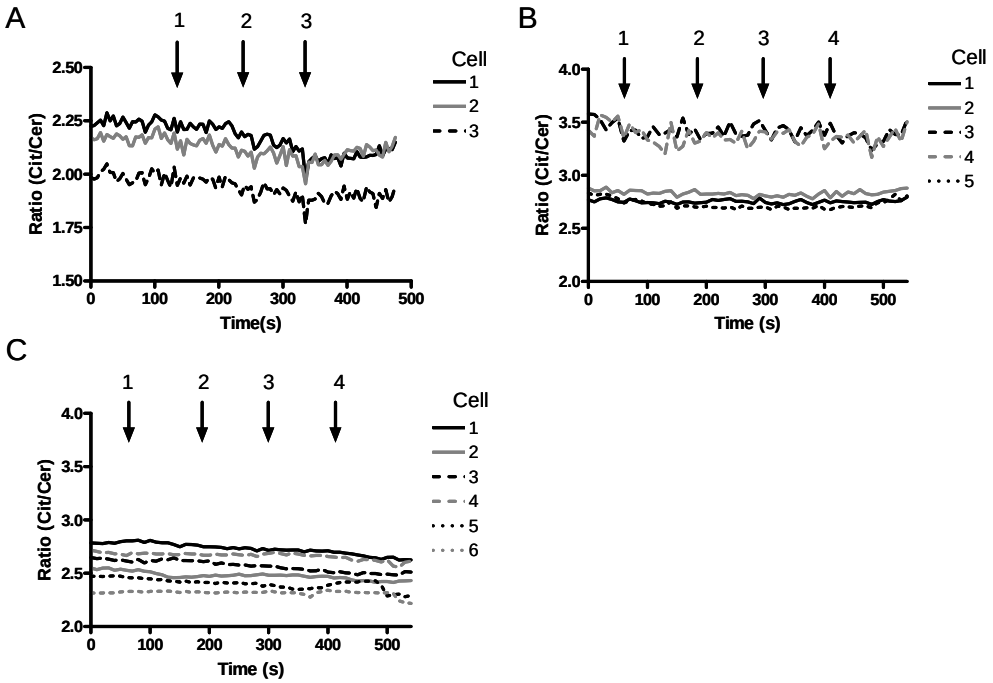

Figure S7: *Metal response of Cd-FRET-2 in HEK293 cells. (A, B, C) Ratio of Citrine over Cerulean emission for three individual HEK293 cells expressing Cd-FRET-2 in time. (A) At time point 1 cells were perifused with a krebs-bicarbonate-plus buffer (KBP) containing 50 µM Cd^{2+}/5 µM pyrithione, while 100 µM of the strong chelator TPEN was added at time point 2. At time point 3 perifusion was switched to KBP again. (B, C) Emission ratio of HEK293 cells that were permeabilized by incubation in intracellular buffer (IB) containing 12.5 µg/ml digitonin for 6 minutes. Traces show the ratios of individual cells in time upon addition of 1 µM (1), 10 µM (2), 100 µM (3) or 250 µM of either Cd^{2+} (B) or Zn^{2+} (C). KBP consisted of 140 mM NaCl, 3.6 mM KCl, 0.5 mM NaH_2PO_4, 0.5 mM $MgSO_4$, 1.5 mM $CaCl_2$, 10 mM Hepes, 25 mM $NaHCO_3$ and 3 mM glucose pH 7.4. IB comprised 140 mM KCl, 5 mM KH_2PO_4 0.7 mM $MgSO_4$, 100 µM ATP, 2 mM Na^+ succinate, 20 mM Hepes, and 5.5 mM glucose, pH 7.05.*

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Materials and methods

Generation of expression vectors for the Cd-FRET variants. A plasmid encoding for Cerulean and Citrine fused by nine GGSGGS repeats (Cer-L9-Cit), called pUC-Cer-L9-Cit was synthesized at Genscript (USA). Using *Nde*I and *Not*I digestion, the Cer-L9-Cit fragment was ligated into a pET28a-derived vector called pET28a-ZinCh-8 (ref. 1), yielding pET28a-Cer-L9-Cit. A Quikchange Multi Site-Directed mutagenesis kit (Stratagene) was then used to simultaneously introduce mutations Y39H and S208C in pET28a-Cer-L9-Cit (primers 1 and 2 for mutations Y39H and S208C respectively, Table SI), generating pET28a-eZinCh-1. Expression vectors for Cd-FRET-1, Cd-FRET-2 and Cd-FRET-3 were generated similar to eZinCh-1, but using primers 3-6 for Cd-FRET-1, primers 7 and 8 for Cd-FRET-2 and 9 and 10 for Cd-FRET-3. Sequencing results confirmed the correct open reading frames for all expression vectors.

Table SI: *Overview of primers used to generate the expression vectors for the different sensor variants.*

Primer	Sequence
1	5'-CGAGGGCGATGCCACCCACGGCAAGCTGACC-3'
2	5'-CCAGTCCGCCCTGTGCAAAGACCCCAACG-3'
3	5'-CACAAGCTGGAGTACTGCGCCATCAGCGACAAC-3'
4	5'-CTGGGGCACAAGCTTGAGTACTGCTACAACAGCCACAAC-3'
5	5'-CTGAGCACCCAGTCCTGCCTGAGCAAAGACCCCAAC-3'
6	5'-CTGAGCTACCAGTCCTGCCTGAGCAAAGACCCCAAC-3'
7	5'-CTGAGCACCCAGTCCTGCCTGTGCAAAGACCCCAACGAG-3'
8	5'-CTGAGCTACCAGTCCTGCCTGTGCAAAGACCCCAACGAG-3'
9	5'-CACTACCTGAGCACCTGCTCCTGCCTGAGCAAAGACCCCAAC-3'
10	5'-CACTACCTGAGCTACTGCTCCTGCCTGAGCAAAGACCCCAAC-3'

Protein production. eZinCh-1 and the Cd-FRET variants were expressed via the protocol reported by Evers *et al.*[1]. Briefly, proteins were expressed in *E. coli* BL21(DE3) cells and harvested after overnight culturing at 25 °C. Cells were lysed using Bugbuster reagent (Novagen), followed by Ni^{2+}-affinity chromatography. Subsequently, the protein solution was dialyzed against a buffer suitable for thrombin cleavage to remove the His-tag. Further

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purification was achieved using size exclusion chromatography on a Sephacryl S-200 HR column (GE Healthcare). Purified proteins, dissolved in 50 mM Tris and 100 mM NaCl at pH 8.0, were frozen in liquid nitrogen and stored at -80 °C.

Spectroscopy. UV-VIS spectra were recorded on a Shimadzu Multispec 1501 photodiode array spectrometer. Protein concentrations were determined using molar extinction coefficient of 77,000 M^{-1} cm^{-1} at 516 nm (ref. 2). Occasionally some protein aggregation was observed. Therefore, protein samples were incubated with 4 M urea and 50 mM DTT overnight prior to fluorescence spectroscopy measurements. Urea and DTT were subsequently removed by passing the protein solution three times through a Zeba desalt spin column (Pierce), equilibrated with 50 mM Tris/HCl, 100 mM NaCl, 10% (v/v) glycerol, 0.05% (v/v) Tween-20, 2 µM DTT, 0.5 mM TCEP, pH 8.0. At pH 7.1, a similar buffer composition was used, yet using 150 mM Hepes instead of 50 mM Tris/HCl. Fluorescence emission spectra of all proteins were recorded using ~1 µM protein concentrations on a Varian Cary Eclipse photoluminescence spectrometer using an excitation wavelength of 420 nm at 20 °C. Emission spectra were normalized using the emission intensity at 527 nm after excitation at 500 nm.

Zn^{2+} titrations. Zn^{2+} titrations were performed in 50 mM Tris/HCl, 100 mM NaCl, 10% (v/v) glycerol, 0.05% (v/v) Tween-20, 2 µM DTT, 0.5 mM TCEP, pH 8.0 by addition of 0.1 nM – 1 mM Zn^{2+} from slightly acidic $ZnCl_2$ stock solution to ~1 µM protein solutions. At pH 7.1, a similar buffer composition was used, yet using 150 mM Hepes instead of 50 mM Tris/HCl. Titrations at low Zn^{2+} concentrations were performed in a Zn^{2+} buffer system consisting of 1 mM EGTA, 10 mM Ba^{2+} and 0.1 – 0.8 mM Zn^{2+}. Free Zn^{2+} concentrations were calculated using the MaxChelator program (http://www.stanford.edu/~cpatton/maxc.html). After each addition, the emission spectrum was recorded and corrected as described above. The difference in emission intensity $I_{527} - I_{475}$ was fitted with a 1:1 binding model (equation 1). In equation 1, the difference in fluorescence intensity at 527 nm and 475 nm is used instead of the ratio, since large changes in emission ratio can introduce an error when fitting a plot showing the ratio on the y-axis. I_{527}-I_{475} is the difference in emission intensity, $[Zn^{2+}]_{free}$ is the free Zn^{2+} concentration, K_d is the dissociation constant of the zinc binding site and α and β are constants.

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$$I_{527} - I_{475} = \alpha \cdot \frac{[Zn^{2+}]_{free}}{K_d + [Zn^{2+}]_{free}} + \beta \qquad (1)$$

Cd^{2+} titrations. Cd^{2+} titrations at pH 8.0 were performed in 50 mM Tris/HCl, 100 mM NaCl, 10% (v/v) glycerol, 0.05% (v/v) Tween-20, 2 µM DTT, 0.5 mM TCEP. At pH 7.1, a similar buffer composition was used, yet using 150 mM Hepes instead of 50 mM Tris/HCl. Titrations at low Cd^{2+} concentrations were performed in Cd^{2+} buffer systems consisting of 1 mM HEDTA, 1.1 mM Ca^{2+} and 0.025 – 0.5 mM Cd^{2+}, 1 mM NTA and 0.01 – 0.9 mM Cd^{2+}, or 1 mM NTA, 0.5 mM Ca^{2+} and 0.05 – 0.9 mM Cd^{2+}. Free Cd^{2+} concentrations were calculated using the MaxChelator program (http://www.stanford.edu/~cpatton/maxc.html). After each addition, the emission spectrum was recorded and corrected as described above. The difference in emission intensity $I_{527} - I_{475}$ was used to fit the fluorescent response using a 1:1 binding model (equation 1).

Metal specificity. Metal additions were performed in 150 mM Hepes, 100 mM NaCl, 10% (v/v) glycerol, 0.05% (v/v) Tween-20, 2 µM DTT, 0.5 mM TCEP, pH 7.1 using a protein concentration of 125 nM in a final volume of 1 mL. Metals were added by 1000-fold dilution of concentrated stock solutions of 0.5 M $MgCl_2$, $CaCl_2$ and $BaCl_2$, or 20 mM of $CuSO_4$, $FeCl_3$, $CoSO_4$ and $NiSO_4$. Pb^{2+} was added from a 1000 µg mL^{-1} lead atomic standard solution (Sigma). Emission spectra after excitation at 420 nm were recorded prior to metal addition, after metal addition and after subsequent addition of 10 µM $CdCl_2$.

Cell Culture. HEK293 cells were a kind gift from Prof. Dr. G.A. Rutter (Imperial College, London, UK) and were grown in DMEM (Sigma) containing 10% (vol/vol) FBS (Life Technologies), 3 mM glucose, 2 mM glutamine, 100 units ml^{-1} penicillin and 100 µg mL^{-1} streptomycin at 37 °C and 5% CO_2 levels. Cells were plated on untreated glass coverslips (25 mm) and transiently transfected with 1.5 µg of plasmid DNA by using 5 µg polyethyleneimine (PEI). Cells were imaged 2 d after transfection.

Fluorescence microscopy. Brightfield microscopy was performed on an Axio observer D.1 (Zeiss) equipped with an Axiocam MRm monochrome digital camera (Zeiss) using Axiovision 4.7 software. Samples were excited using a HXP 120 Mercury lamp (Zeiss) and Cerulean and Citrine emission was recorded via sequentially using emission using filterset 47

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(excitation BP 436/20; emission BP 480/40) and 48 (excitation BP 436/20; emission BP 535/30) (Zeiss) in a motorized filter turret. Images were acquired using an exposure time of 200 ms and an imaging frequency of 0.1 Hz.

Intracellular FRET imaging. Intact cells were imaged in Krebs-bicarbonate plus buffer (KBP), which consisted of 140 mM NaCl, 3.6 mM KCl, 0.5 mM NaH_2PO_4, 0.5 mM $MgSO_4$, 1.5 mM $CaCl_2$, 10 mM Hepes, 25 mM $NaHCO_3$ and 3 mM glucose pH 7.4. Where stated, cells were permeabilized by adding 200 µl of 10 µg ml^{-1} digitionin (Sigma) dissolved in calibration buffer (IB) to 200 µl of HEK293 cells in IB. IB comprised 140 mM KCl, 5 mM KH_2PO_4 0.7 mM $MgSO_4$, 100 µM ATP, 2 mM Na^+ succinate, 20 mM Hepes, and 5.5 mM glucose, pH 7.05. After 300 s of incubation with digitonin, recording of images was started and followed by addition of IB containing indicated amounts of reagents to alter cytosolic Cd^{2+} or Zn^{2+} levels.

Western blot analysis. Cells were transfected as described above and cultured for 48 hours in 6-well plates. Next, cells were kept on ice, washed 3 times with phosphate buffered saline (PBS) and subsequently incubated for 30 minutes in 100 µL of PBS containing 1 mM EDTA, 1 % (v/v) Igepal® CA630 (Sigma) and 1x complete inhibitor protease cocktail (Roche) to lyse the cells. Next, gel samples (20 µL) where made and loaded on a 12% SDS-PAGE gel. The protein was transferred to a nitrocellulose membrane, followed by blocking overnight using 5 % skimmed milk in Tris-buffered saline containing Tween-20 (TBS-T) and 1 h incubation of mouse α-GFP (Ab3277, AbCam) (1:2000) in the same buffer. Horse radish peroxidase (HRP)-functionalized rabbit α-mouse immunoglobins (1:5000, Dako) was used as a secondary antibody. Proteins were visualized using a tetramethylbenzidine (TMB) solution (Sigma) according to manufacturers' instructions. PBS consisted of 137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4 and 2 mM KH_2PO_4, pH 7.4. TBS-T consisted of 20 mM Tris/HCl, 150 mM NaCl, and 0.05% (v/v) Tween-20, pH 7.4.

References

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