Supporting Information

Tuning the metal binding site specificity of a fluorescent sensor protein: from copper to zinc and back

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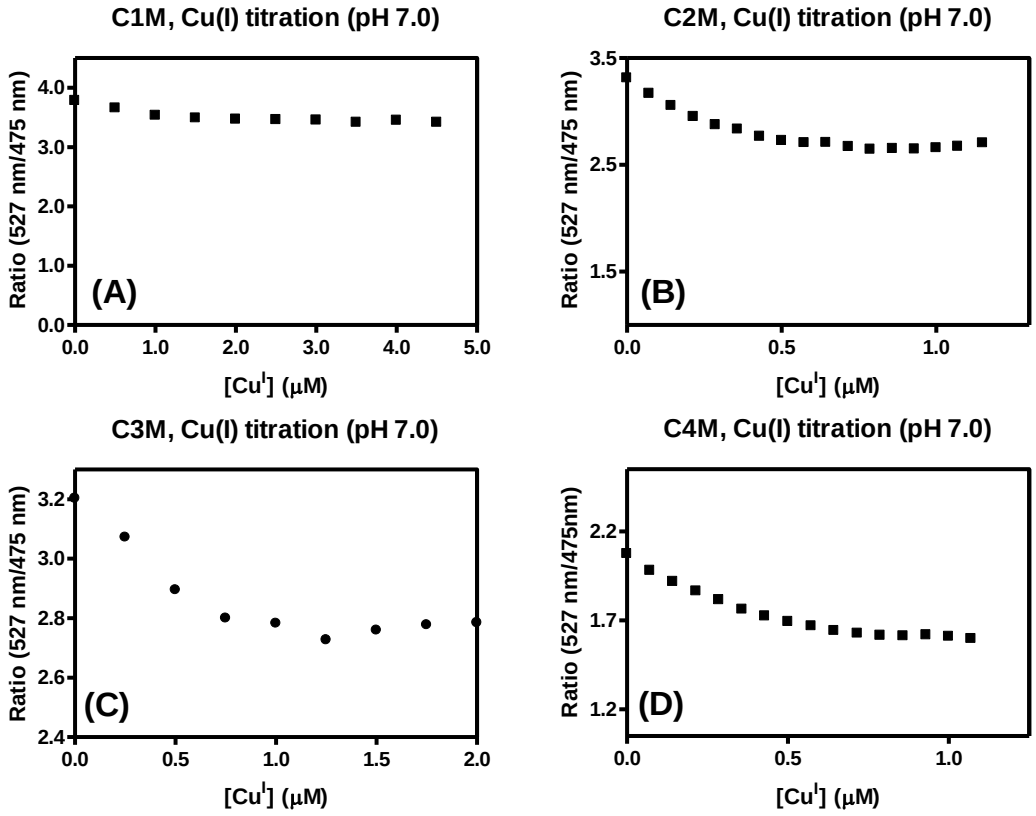

Figure S1. FRET titration plots of the single Cys → Met mutants studied in this work. Copper titration curves for single Cys → Met mutants **(A)** eCALWY-C1M; **(B)** eCALWY-C2M; **(C)** eCALWY-C3M; **(D)** eCALWY-C4M. Copper titrations were performed in 100 mM MOPS, 100 mM NaCl and 10% (vol/vol) glycerol (pH 7.0) under anaerobic conditions.

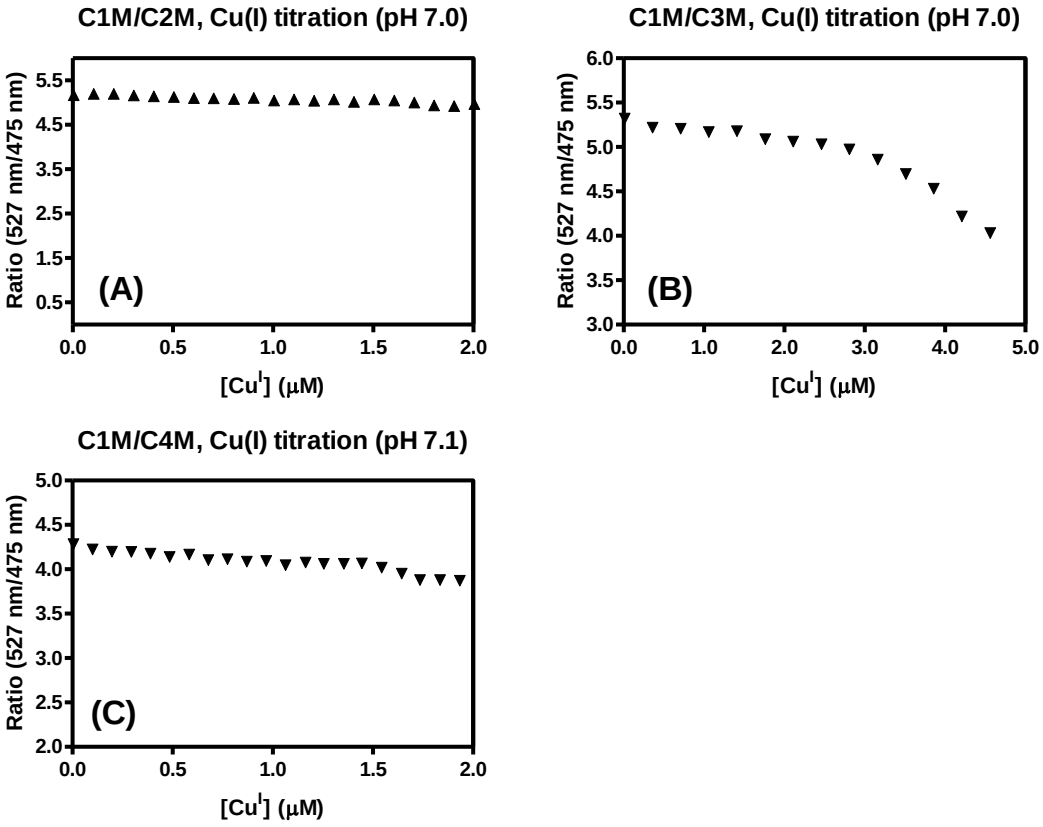

Figure S2. FRET titration plots of the double Cys → Met mutants studied in this work. Copper titration curves for double Cys → Met mutants **(A)** eCALWY-C1M/C2M; **(B)** eCALWY-C1M/C3M; **(C)** eCALWY-C1M/C4M. All copper titrations were performed in 100 mM MOPS, 100 mM NaCl and 10% (vol/vol) glycerol (pH 7.0) under anaerobic conditions.

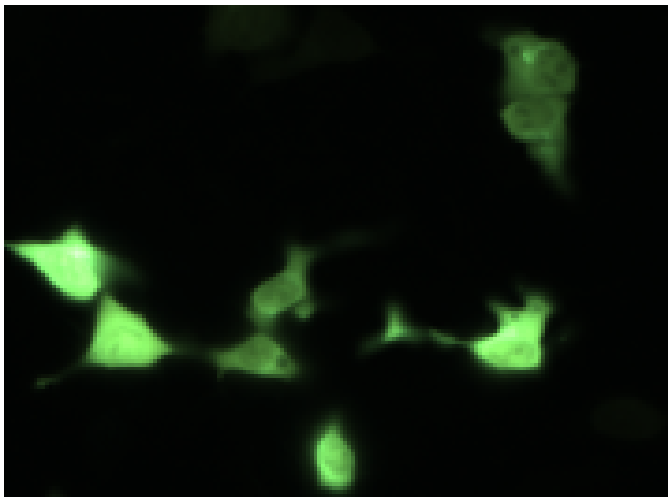

Figure S3: Intracellular imaging of eCALWY-C2M/C3M. Fluorescence imaging of HEK293 cells transfected with the copper sensor showed homogeneous expression.

Site-directed mutagenesis of pET28a-eCALWY-1

For both single and double mutants, QuikChange site-directed mutagenesis kits (Stratagene) were used. The pET28a-eCALWY-1 described by Vinkenborg *et al*[1] was used as template DNA. 100 ng of template DNA was used with the appropriate DNA primers to create a series of single and double (Cys → Met) mutants. Although several attempts were made to generate all variations of Cys → Met double mutants, double mutations C2M/C4M and C3M/C4M failed repeatedly. Based on the mutants reported here, the remaining two mutants were not pursued further. DpnI was used to digest the parental template DNA and the mixture was then transformed into XL-1 Blue competent cells (Novagen) according to supplier's instructions. Mutagenesis was confirmed by DNA sequencing (MWG Eurofins, Germany). Single colonies were selected, amplified and transformed into *Escherichia coli* BL21 (DE3) cells for protein expression. Protein expression and purification of each mutant variant was performed according to procedures reported previously.[1] Briefly, *E. coli* cells encoding for eCALWY-1 mutants were grown at 37°C in the presence of ampicillin, to an optical density (O.D.) of approximately 0.6. Protein expression was induced upon addition of 0.1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) for 4 hours at 15°C. The cells were harvested by centrifugation and lysed with BugBuster reagent (Novagen) according to standard procedures. The soluble protein supernatant was purified by nickel-affinity chromatography and the crude protein was incubated with thrombin to cleave the His-tag before a second round of nickel-affinity chromatography. Finally, size-exclusion chromatography (S200 Sephacryl column, GE) in 50 mM Tris, 100 mM NaCl, 1 mM DTT (pH 7.9) was used to obtain purified protein in good yield (approx. 7 mg/L).

Metal ion titration experiments

All zinc titrations were performed under buffered conditions in 150 mM HEPES, 100 mM NaCl, 1 mM DTT and 10 % (v/v) glycerol (pH 7.1), as described previously.[1] Dithiothreitol (DTT) was used to prevent oxidation of the cysteines in the metal binding motif. All zinc titrations were performed by mixing 0.1- 0.9 mM of Zn^{2+} from a slightly acidic stock solution of $ZnCl_2$ 99.99% (Acros) with buffering systems consisting of 1 mM EGTA, 1 mM HEDTA, 1 mM EDTA, 1 mM NTA or 1 mM 1,3-diamino-2- hydroxypropane-N,N,N',N'-tetraacetic acid (DHPTA) (purchased from Sigma). The free zinc concentrations were calculated using the program MaxChelator using the stability constants present within program. All free zinc concentrations were calculated using T = 20 °C and I = 0.1 M. Fluorescence spectra and emission anisotropy were recorded on a Varian Cary Eclipse spectrometer. Protein concentrations were determined by measuring the citrine absorbance at 527 nm using an extinction coefficient of 77,000 $M^{-1}cm^{-1}$. The citrine/cerulean (Cit/Cer) ratio was calculated by dividing the emissions at 527 and 475 nm, respectively. Dissociation

constants were calculated using the same equations and fitting parameters as described previously.[1]

Copper binding experiments were performed as described previously.[1] Stock solutions of $[Cu^{+}(CH_3CN)_4][PF_6]$ were freshly prepared in acetonitrile. Copper titrations were performed in 100 mM MOPS, 100 mM NaCl, 1 mM EDTA and 10% (vol/vol) glycerol (pH 7.0). All manipulations and sample preparation were performed under an inert atmosphere and the protein samples were sealed in a cuvette with a rubber septum for fluorescence measurements. Copper aliquots (0.1–3.0 μM Cu^{+}) were added to the protein sample via a gas-tight Hamilton syringe. All samples were pretreated with excess DTT and EDTA for 1 h to reduce all cysteine residues and to chelate any Zn^{2+} bound, respectively. The protein samples were then passed through a PD10 desalting column (GE Healthcare), pre-equilibrated with anaerobic MOPS buffer to remove all DTT. The protein was eluted and 1 mM EDTA added to the protein sample to inhibit Zn^{2+} binding. The Citrine/Cerulean ratio was calculated by dividing the emission intensities at 527 and 475 nm, respectively.

For both eCALWY-C3M and eCALWY-C2M/C3M, several attempts were made to determine the exact copper binding affinities using various copper chelators, including bathocuproïnedisulfonic acid (BCS), thiacrown ether (14-ane-S4), disulfiram and dithiothreitol (DTT). However, in all cases, full recovery of the original FRET ratio (in the absence of metal binding) failed. Even in the presence of excess bathocuproïnedisulfonic acid (BCS), with a FRET ratios could only be partially restored.

Metal ion specificity

To test the metal ion specificity, all titrations were performed in 150 mM HEPES, 100 mM NaCl, 1 mM DTT and 10 % (v/v) glycerol (pH 7.1). Stock solutions were made in water from $CoSO_4$, $NiSO_4$, $CuSO_4$. Metal titrations were performed by adding 0.1 - 0.9 mM of the appropriate metal solution to a final concentration of 20 μM. The fluorescence emission spectrum was recorded, and the Cit/Cer ratio was calculated.

Intracellular studies in HEK293 cells of eCALWY-C2M/C3M

To create the mammalian expression vector for eCALWY-C2M/C3M, the peCALWY-NB construct reported by Vinkenborg et al was used.[1] Initially, both peCALWY-NB and pET28a-eCALWY-C2M/C3M were doubly digested with AgeI and NotI restriction enzymes, according to standard procedures. The digestion products were purified by agarose gel electrophoresis and the appropriate bands were excised and the DNA extracted using a QIAGEN gel purification kit. This was then followed by ligation of the eCALWY-C2M/C3M

fragment into the *AgeI*- and *NotI*-digested peCALWY-NB vector to obtain the mammalian expression vector peCALWY-C2M/C3M. HEK293 cells 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 the same temperature and CO_2 levels. Cells were plated on poly(L-lysine)-coated glass coverslips and transiently transfected with 0.5–1.0 μg of plasmid DNA by using Lipofectamine 2000 (Invitrogen) according to the manufacturers' instructions. Cells were imaged 2 days after transfection.

Intracellular FRET imaging.

HEK293 cells were imaged in modified Krebs-HEPES-bicarbonate buffer (KHB), consisting 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, 2 mM $NaHCO_3$ and 3 mM glucose, which was pre-equilibrated with 95:5 O_2:CO_2 (pH 7.4). TPEN and pyrithione were prepared fresh on the day of use in 25 mM and 1 mM stock solutions in DMSO, respectively. All sensors were homogeneously expressed throughout the cytosol of the cells.

Copper response experiments were performed using disulfiram and copper sulfate. Solutions were prepared fresh on the day of experiments in 100 mM stock solutions in Milli-Q water. Addition of copper had little effect on the FRET ratio, suggesting that the eCALWY-C2M/C3M sensor may be fully-occupied. However, subsequent addition of the copper chelator, neocuproine, also failed to show any changes in FRET. This may suggest that eCALY-C2M/C3M is not in the correct binding regime to compete for intracellular copper.

References

1. Vinkenborg, J.L., et al., *Genetically encoded FRET sensors to monitor intracellular Zn^{2+} homeostasis.* Nature Methods, 2009. **6**(10): p. 737-740.