Supporting Information

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SI Materials and Methods

Expression and Purification of S-Met CusF and S-Met CusB-NT. *Escherichia coli* BL21 (λDE3) containing the *cusF*$_{(6\text{-}88)}$ *trx-his*$_6$*-tev* plasmid were grown from a freshly streaked plate into LB media containing 100 μg/mL ampicillin at 37 °C until they reached an OD_{600} of 0.8, at which point protein production was induced with 0.4 M isopropyl β-D-1-thiogalactopyranoside. Growth was allowed to continue at 37 °C for 4 h, whereupon the cells were harvested by centrifugation and pelleted. The cells were resuspended, lysed using the French pressure method, and centrifuged to remove cell debris. The filtered supernatant was poured over a Ni-NTA resin column, rinsed with buffer, and eluted using a 250 mM imidazole buffer rinse. To remove the His_6-Trx tag, tobacco etch virus (TeV) protease and 5 mM β-mercaptoethanol were added to the protein solution, and the mixture was incubated at 20 °C overnight. After dialysis, the protein solution was once again poured over a Ni-NTA resin column to yield cleaved, pure apo CusF. SDS/PAGE analysis of the purified protein showed the final product to be in a single band at the appropriate molecular weight (~10 kDa), with no visible impurities.

E. coli BL21 (λDE3) containing the *cusB NT trx-his*$_6$*-tev* plasmid was prepared in exactly the same manner as for CusF. The SDS/PAGE analysis of the purified CusB-NT showed the final product to be in a single band at the appropriate molecular weight (~7 kDa), with no visible impurities.

Both purified proteins were then dialyzed and stored in Hepes buffer (50 mM, pH 7.5) for the subsequent metallation steps.

Expression and Purification of Se-Met CusF and Se-Met CusB NT. The selenomethionine-labeled variants of each of the aforementioned constructs were prepared as described previously (1). The Se-Met variants of CusF and CusB-NT were purified and stored as described above, and their Se-to-protein content was verified by inductively coupled plasma optical emission spectrometry (ICP-OES) and the bicinchoninic acid assay (BCA).

Expression and Purification of CusA. *E. coli* BL21 (pLYS) containing the *pASK cusA* vector were grown in LB media containing 100 μg/mL ampicillin at 37 °C until they reached an OD_{600} of 0.8, at which point protein production was induced with 200 μg/L anhydrotetracycline. Growth was allowed to continue at 30 °C for 5 h, whereupon the cells were harvested by centrifugation and pelleted. The cells were then resuspended and lysed using the French pressure method, and the cell debris was pelleted by centrifugation. The supernatant was then ultracentrifuged, at which point the membrane-containing pellet was collected. The pellet was resuspended and solubilized by homogenization, followed by dropwise addition of Cymal-6 detergent with stirring over ice. The remaining membrane was removed by ultracentrifugation, the supernatant poured over a streptavidin agarose affinity column, and the column washed before eluting the clean, tagged CusA protein using a solution of desthiobiotin. The protein was then dialyzed into transfer buffer and frozen for future use. SDS/PAGE of the final, dialyzed protein showed a single band at ~116 kDa with no visible impurities. The Bradford assay was used for protein quantification, and ICP-OES was used to analyze the purified CusA for transition metal (Cu, Zn) content to ensure the protein was in apo form.

Metal Reconstitution of Cus Proteins. ***Cu(I) reconstitution of CusF, CusB NT, and CusA.*** To produce the Cu(I) forms of CusF, CusB-NT, and CusA, the same protocol was carried out in all cases. The protein of interest was quantified by the BCA (for CusF and CusB-NT) or Bradford assay (for CusA), and then prepared and kept chilled overnight in a Coy anaerobic chamber to give anaerobic protein. Tetrakis(acetonitrile)copper(I) hexafluorophosphate was dissolved in pure acetonitrile [Cu(I)-ACN hereafter] and the amount of Cu(I)-ACN to add to the protein calculated such that the ACN concentration was limited to 10% of the total protein solution by volume. The Cu(I)-ACN was added to the apo protein anaerobically by syringe pump (1 μL/min rate), with stirring, at a ratio of 1.5:1 metal to protein for CusF and CusB or 0.9:1 for CusA. The mixture was then allowed to incubate 1 additional hour with stirring, over ice. The metallated protein was concentrated to the desired volume using a microconcentrator, and three cycles of desalting were accomplished using spin columns that had been equilibrated with transfer buffer containing 10%, 5%, and 0% acetonitrile, which removed excess metal and salt from the proteins. At this point, the proteins were either used immediately in anaerobic transfer experiments, or flash-frozen and stored anaerobically in liquid nitrogen for a future single anaerobic thaw and use. Metal-to-protein concentrations were verified by ICP-OES and the BCA or Bradford assay.

Ag(I) reconstitution of CusF and CusB NT. Protein concentrations were first determined by the BCA. Silver nitrate stock solutions (10 mM) were always prepared fresh and kept covered in foil and on ice until ready for use. The Ag(I) solution was added to the cold apo protein in the absence of any competing salts in the dark by syringe pump (1 μL/min rate), with stirring, at a ratio of 1.5:1 metal to protein. The mixture was then allowed to incubate 1 additional hour with stirring, over ice, in the dark, and then desalted of excess metal by three cycles of equilibrated spin columns. The protein was kept covered in foil and either used immediately in anaerobic transfer experiments or flash-frozen and stored in liquid nitrogen for a future single thaw and use. Metal-to-protein concentrations were verified by ICP-OES and the BCA.

Metal Transfer Experiments. For a typical two-protein metal transfer experiment, freshly metallated CusF and CusB were buffer exchanged from Hepes into Cus transfer buffer [50 mM Hepes, pH 7.5, 0.1% 6-cyclohexyl-1-hexyl-β-D-maltoside (Cymal-6) detergent, 50 mM NaCl], the components of which were needed specifically to maintain the solubility and structure of the membrane protein CusA. Protein concentration was kept to either 75 μM for di-protein experiments involving CusA, or 150 μM for di-protein experiments involving only CusF and CusB, and for the purposes of this work, the protein stoichiometry was kept at 1:1:1. In experiments that tested the metal transfer from CusF or CusB to apo CusA, an aliquot of metallated protein was added to the apo CusA with slow pipetting up and down to ensure adequate mixing, and then allowed to incubate for 10 min at room temperature before adding ethylene glycol to 20% and flash freezing in an XAS cuvette to produce a liquid glass for future EXAFS analysis. In experiments that tested metal transfer from metal ion-loaded CusF and CusB, metallated CusF was added in a small aliquot to a vial of metallated CusB and mixed thoroughly, whereupon the reaction was allowed to incubate for 10 min before adding glassing agent and freezing as previously described. An aliquot from these mixtures was then tested by ICP-OES to confirm that the metal ion concentration was as calculated.

For a typical three-protein metal transfer experiment, concentration was maintained at 150 μM for each protein. Metallated CusB was always added first to a vial of apo CusA, mixed well, and incubated for 10 min to allow CusB to interact with CusA.

Before adding any metallated CusF, a control sample of CusB and CusA was then prepared and frozen using an aliquot of this mixture. At this point, an aliquot of metallated CusF was added to the vial, mixed again, and allowed to incubate for an additional 5 min before adding glassing agent and flash freezing as previously described.

EXAFS Data Collection. Samples were measured as aqueous glasses in 20% (vol/vol) ethylene glycol at 10 K. Cu K-edge (8.9 keV), Se K-edge (12.5 keV), and Ag K-edge (25.5 keV) extended X-ray absorption fine structure (EXAFS) and X-ray absorption near-edge data were collected at the Stanford Synchrotron Radiation Lightsource operating at 3 GeV, with currents near 500 mA maintained by continuous top off.

All edges were measured on beamline 9-3, using a Si[220] monochromator. All data were collected in fluorescence mode using a high-count rate Canberra 100-element Ge array detector with maximal count rates per array element of <120 kHz. For each edge, four to six scans of buffer blank were averaged and subtracted for all protein samples to remove the Z-1 filter Kβ fluorescence and produce a flat preedge baseline. At each metal edge, a Cu, Se, or Ag foil, respectively, was placed between the first and second ionization chamber to provide energy calibration.

Cu K-edges were collected using an Rh-coated mirror upstream with a 12.5-keV energy cutoff to reject harmonics, and a nickel oxide filter and Soller slit inserted in front of the detector to reduce elastic scattering relative to the Cu Kα fluorescence. Se K-edges were collected using an Rh-coated mirror upstream with a 15-keV energy cutoff to reject harmonics, and an arsenic oxide filter and Soller slit inserted in front of the detector to reduce elastic scattering relative to the Se Kα fluorescence. Ag K-edge data were measured using ion chambers operating at 1.5 kV, with the energy cutoff mirror removed to achieve the high energy. The amount of harmonic contamination from this setup was not expected to be significant. A Pd filter and Soller slit were inserted in front of the detector to reduce elastic scattering relative to the Ag Kα fluorescence.

EXAFS Simulations. Data averaging, background subtraction, and normalization to the smoothly varying background atomic absorption were performed using EXAFSPAK (2). For the experimental energy threshold energy ($k = 0$), 8,995 eV was selected for Cu K-edge, 12,663 eV was used for the Se K-edge, and 25,515 eV for Ag K-edge. Spectral simulation was carried by least-squares curve fitting, using full curved wave calculations as formulated by the SRS library program EXCURVE 9.2 as previously described (3–5). We refined the parameters of the fit as follows: E_0, the photoelectron energy threshold; R_i, the distance from the central metal atom (Cu, Se, or Ag) to atom i; and $2\sigma^2_i$, the Debye–Waller (DW) term for atom i. We fixed the coordination numbers to those previously established from crystal structures whenever possible, or by other previously published values as in the case of CusB NT. Determination of residual metal bound to Se in transfer experiments was performed by refining the Se-Cu/Ag shell occupancy, using a DW factor determined from simulation of the EXAFS data from a fully metallated sample. The quality of the fits was determined using the least-squares fitting parameter, F, which is defined as follows:

$$F^2 = (1/N)\sum_i k^6 \left(\chi_i^{\text{theory}} - \chi_i^{\text{experiment}}\right)^2,$$

and is hereafter referred to as the fit index (FI).

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2. George GN (1995) EXAFSPAK (Stanford Synchrotron Radiation Laboratory, Menlo Park, CA).
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5. Gurman SJ, Binsted N, Ross I (1986) A rapid, exact, curved-wave theory for EXAFS calculations. II. The multiple-scattering contributions. *J Phys C Solid State Phys* 19: 1845–1861.

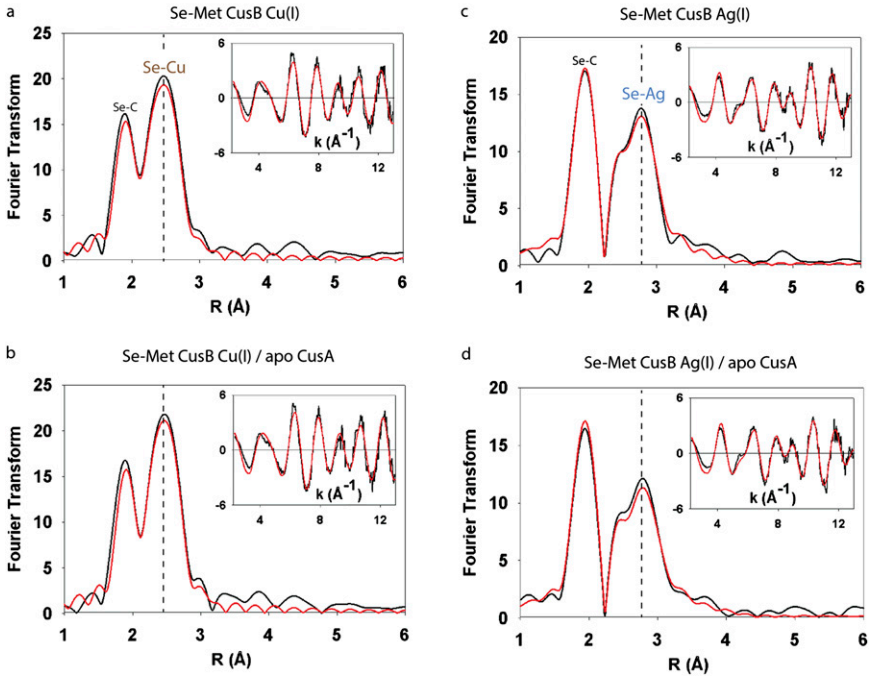

Fig. S1. CusB alone does not transfer metal ions to CusA. (*A*) Se K-edge spectra of Se-Met Cu(I) CusB. (*B*) Se K-edge spectra of Se-Met Cu(I) CusB incubated with apo CusA. No change in the Se-Cu signal of Se-Met Cu(I) CusB could be detected, indicating that no transfer occurs from CusB to CusA. (*C*) Se K-edge spectra of Se-Met Ag(I) CusB. (*D*) Se K-edge spectra of Se-Met Ag(I) CusB-incubated apo CusA. Although the Se-C and Se-Ag intensity both decreased upon the addition of apo CusA, both decreased by the same amount, indicating that no Ag(I) was removed from the Se-Met CusB metallosite.

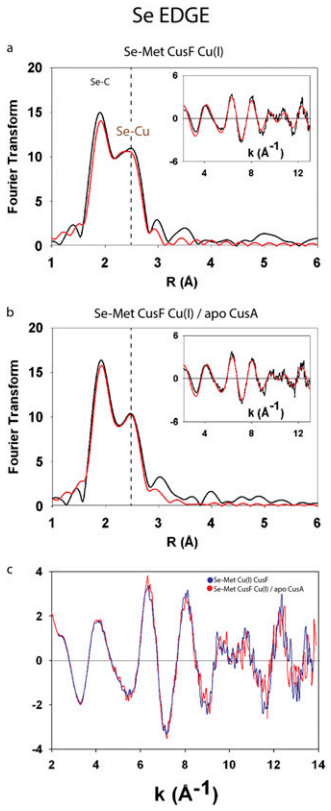

Fig. S2. CusF alone does not transfer metal ions to CusA. (*A*) Se K-edge spectra of Se-Met Cu(I) CusF. (*B*) Se K-edge spectra of Se-Met Cu(I) CusF incubated with apo CusA. No change in the Se-Cu signal of Se-Met Cu(I) CusF could be detected, indicating that no transfer occurred from CusF to CusA while not in the presence of CusB. (*C*) Overlay of the experimentally obtained EXAFS from *A* and *B* for clarity.

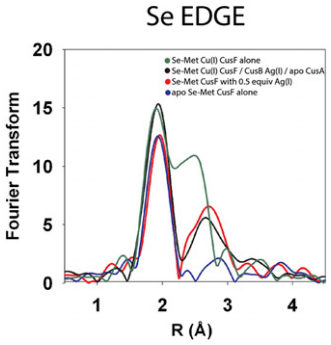

Fig. S3. CusF accepts Ag(I) from CusB upon activation of CusA, confirming CusF/CusB interaction. Green spectrum , Se K-edge FT of Se-Met–labeled Cu(I)-loaded CusF, which exhibits a Se-Cu peak at 2.40Å. Black spectrum, Se K-edge FT of the Se-Met Cu(I) CusF/Ag(I) CusB/apo CusA mixture, in which the Se-Cu peak has disappeared and is replaced by a Se-Ag signal at 2.60 Å. Red spectrum, Se-Met Ag(I)-loaded CusF. Blue spectrum, unreacted apo Se-Met CusF. The Ag(I)-loaded and apo CusF samples were from a separate preparation than the other Se-Met CusF protein used in the main work, and the intensity of the Se-C peak was lower than expected. This was likely due to detector oversaturation from excess Se k-α fluorescence.

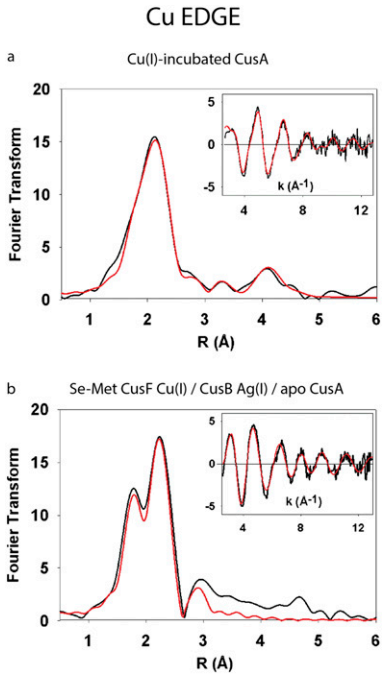

Fig. S4. The "active" CusA pump binds Cu(I) in a motif not observed previously. (*A*) Cu K-edge of apo CusA incubated with tetrakis(acetonitrile)-copper(I). (*B*) Cu K-edge FT of putative Cu(I)-CusA, which appeared upon incubation of Se-Met Cu(I) CusF, Ag(I) CusB, and apo CusA proteins. The spectra produced in each of these cases differ considerably. The spectrum in *A* has a multiple scattering contribution from a single histidine and two short sulfur bonds and is thought to be a Cu(I)-binding site on the outer surface of CusA rather than a site in the interior of the pump. In contrast, the spectrum in *B* has no imidazole ring-induced multiple scattering and is dominated by signals arising from two methionines and one oxygen/nitrogen ligand.

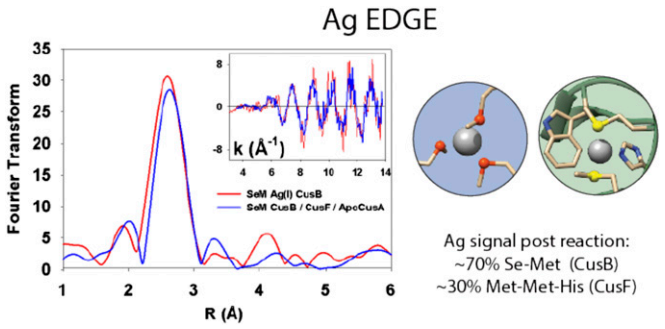

Fig. S5. Silver K-edge XAS supports the observed Se edge backtransfer of Ag(I) from Se-Met Ag(I) CusB to apo CusF. Analysis of the Ag K-edge of the Se-Met–labeled CusB triprotein incubation shows a mixed environment for Ag that is best fit to ~80% Se-Met Ag(I) CusB (3 Se) and ~20% CusF (2 Met, 1 His). CusF may only accept Ag(I) upon releasing its Cu(I) cargo, yet the Se-Met CusB spectra at the Se edge does not show a corresponding Se-Cu signal (Fig. 2*B*, main text). This one-way transfer of silver from Se-Met CusB Ag(I) to CusF indicates that, in the timescale of our reactions, CusB does not act as a metal relay toward CusA and instead operates as an on–off switch that activates the CusA pump for metal ion delivery from CusF.

Table S1. Cus protein fit parameters (via curve fitting in the EXCURV 9.2 program): Selenium K-edge

		Se-C			Se-Cu			Se-Ag			Se-Se			
Sample/fit	*F**	No[†]	R, Å[‡]	DW, Å^2	No	R, Å	DW, Å^2	No	R, Å	DW, Å^2	No	R, Å	DW, Å^2	Eo
Se-Met Cu(I) CusB	0.51	2	1.96	0.006	1	2.42	0.006				0.2	2.82	0.017	−5.58
Se-Met Cu(I) CusB/Apo CusA	0.49	2	1.96	0.006	1	2.42	0.006				0.2	2.84	0.014	−5.53
Se-Met Ag(I) CusB	0.39	2	1.96	0.003				1	2.64	0.009				−6.11
Se-Met Ag(I) CusB/apo CusA	0.5	2	1.96	0.004				1	2.64	0.010				−5.60
Se-Met Ag(I) CusB/Cu(I) CusF	0.42	2	1.96	0.006				1	2.64	0.010				−5.26
Se-Met Ag(I) CusB/Cu(I) CusF/apo CusA	0.6	2	1.96	0.008	0.1	2.37	0.009	0.9	2.64	0.009				−5.71
Se-Met Cu(I) CusF	0.74	2	1.96	0.007	0.5	2.38	0.005				0.5	2.85	0.032	−6.04
Se-Met Cu(I) CusF/apo CusA	0.62	2	1.96	0.006	0.5	2.38	0.007				0.5	2.83	0.027	−5.82
Se-Met Cu(I) CusF/Ag(I) CusB/apo CusA	0.66	2	1.96	0.004	~0.05	2.38	0.005	0.19	2.60	0.005	0.5	2.80	0.02	−5.50
Se-Met Ag(I) CusF	0.92	1.5	1.96	0.003				0.45	2.62	0.008				−6.29
Se-Met apo CusF	0.93	1.5	1.96	0.003							0.5	2.79	0.025	−5.40

**F* is a least-squares fitting parameter defined as follows:

$$F^2 = \frac{1}{N}\sum_{i=1}^{N} k^6(\text{Data} - \text{Model})^2.$$

[†]Coordination numbers are generally considered accurate to ±25%.
[‡]In any one fit, the statistical error in bond lengths is ±0.005 Å. However, when errors due to imperfect background subtraction, phase-shift calculations, and noise in the data are compounded, the actual error is closer to ±0.02 Å.

Table S2. Cus protein fit parameters (via curve fitting in the EXCURV 9.2 program): Copper K-edge

		Cu-N (His)*			Cu-S			Cu-Se			Cu-O/N[†] or Cu-C			
Sample/fit	F	No	R, Å	DW, Å^2	No	R, Å	DW, Å^2	No	R, Å	DW, Å^2	No	R, Å	DW, Å^2	Eo
Se-Met Cu(I) CusF k = 15	0.46	2	1.93	0.006				2	2.38	0.021				−1.96
Se-Met Cu(I) CusF/Ag(I) CusB/apoA	0.56				2	2.24	0.018				1 O/N 2 C	1.85 3.12	0.009 0.017	0.28
Se-Met Cu(I) CusB k = 16	1.2							3	2.41	0.007				−3.50
S-Met Cu(I) CusB	0.39				3	2.30	0.007				1 C	2.06	0.003	−3.63
Cu(I) prepared CusA	0.59	1	1.891	0.012	1.5	2.18	0.016							−0.48

*Fits modeled histidine coordination by an imidazole ring, which included single and multiple scattering contributions from the second shell (C2/C5) and third shell (C3/N4) atoms, respectively. The Cu-N-C_x angles were as follows: Cu-N-C2, 126°; Cu-N-C3, −126°; Cu-N-N4, 163°; Cu-N-C5, −163°.
[†]Distances of the Cu-N(His) and Cu-N/O (non-His) shells were constrained to be equal in fits to the oxidized proteins.

Table S3. Cus protein fit parameters (via curve fitting in the EXCURV 9.2 program): Silver K-edge

		Ag-Se			Ag-S			Ag-N (His)			
Sample/fit	F	No	R, Å	DW, Å^2	No	R, Å	DW, Å^2	No	R, Å	DW, Å^2	Eo
Se-Met Ag(I) CusB	2.8	3	2.63	0.005							6.23
Se-Met Ag(I) CusB/Cu(I) CusF/apo CusA	1.8	1.9	2.61	0.008	0.7	2.61	0.008	0.4	2.21	0.005	11.8