Electronic Supplementary Information

The Tightly Regulated Copper Window in Yeast

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Experimental details

1. Expression and purification of proteins.

The metal binding domain of Ace1(36-100), the metal binding domain of Mac1(203-295), Cup1, and Crs5 were cloned between ECFP and EYFP using *Sph*I and *Sac*I in the plasmid system described in previous reports to yield Ace1-FRET, Mac1-FRET, Cup1-FRET, and Crs5-FRET, respectively (Fig. S1 and Fig. S5, ESI).[1] The resulting plasmids were transformed into BL21star(DE3). 10 ml overnight pre-cultures grown from single colonies were each inoculated into 1 L autoclaved LB medium containing 50 mg ampicilin. The cells were grown at 37 ^{o}C, 250 rpm to OD_{600} = 0.6 and then the temperature was reduced to 16 ºC. Protein expression was induced with 0.5 mM IPTG. 30 min later, 1.4 mM $CuSO_4$ was added to the growth medium and cells were grown overnight. Cells were harvested at 4 ^{o}C by centrifugation at 6,000 rpm for 8 min. All subsequent steps were performed at 4 ^{o}C. The pellet was suspended in 20 ml buffer A, (10 mM Tris-HCl [pH 7.4], 300 mM NaCl, 4 mM DTT) and 10 mM PMSF. The cells were lysed by sonication and centrifuged at 12,000 rpm for 25 min. The supernatant was filtered through a 0.45 μm filter and applied to a Ni-NTA column. The column was washed with 5% buffer B (10 mM Tris-HCl [pH 7.4], 500 mM imidazole, 300 mM NaCl,

4 mM DTT) and eluted with a linear gradient from 5% to 100% buffer B over 40 ml. Peak fractions were pooled and kept at 4 ºC. The purity was verified by SDS-PAGE gel (Fig. S2, ESI).

To obtain apo Ace1-FRET or Mac1-FRET, 10 mM NaCN were added to the purified proteins and the proteins were run through a desalting column using Buffer A containing 8 mM DTT.

2. Fluorescence measurements.

All fluorescence measurements were carried out at room temperature on a Varian Cary Eclipse Fluorescence Spectrophotometer. The samples were excited at 433 nm, the excitation and emission slit widths were set to 5 nm and the emission spectrum was scanned from 453 nm to 650 nm at 120 nm / min. The intensity ratio at the peaks was taken as a measure of FRET change ($R = I_{527} / I_{477}$). The % FRET change in response was calculated by setting the apo proteins ratio at 0% change and the fully copper-loaded protein at 100% change in the FRET using the following formula.

% FRET Change = $(R - R_{min}) / (R_{max} - R_{min}) \times 100$

where R is the intensity ratio for a given sample, R_{min} the intensity ratio for apo protein and R_{max} the intensity ratio for copper(I) bound protein.

$H^+ + CN^- \rightleftarrows HCN \qquad \log K_a = 9.04$

$Cu^+ + 2\ CN^- \rightleftarrows Cu(CN)_2^- \qquad \log\beta_{2Cu} = 21.7$

$Cu^+ + 3\ CN^- \rightleftarrows Cu(CN)_3^{2-} \qquad \log\beta_{3Cu} = 26.8$

$Cu^+ + 4\ CN^- \rightleftarrows Cu(CN)_4^{3-} \qquad \log\beta_{4Cu} = 27.9$

$Zn^{2+} + 2\ CN^- \rightleftarrows Zn(CN)_2 \qquad \log\beta_{2Zn} = 11.07$

$Zn^{2+} + 3\ CN^- \rightleftarrows Zn(CN)_3^- \qquad \log\beta_{3Zn} = 16.05$

$Zn^{2+} + 4\ CN^- \rightleftarrows Zn(CN)_4^{2-} \qquad \log\beta_{4Zn} = 19.62.$

For the K_d measurement of Ace1-FRET, Mac1-FRET, Cup1-FRET, and Crs5-FRET with Cu^+, Cu^+/ CN^- buffers were used to obtain free Cu^+ concentrations ranging from 9.63×10^{-16} M to 5.6×10^{-23} M. (Table S2) The Cu^+_{free} concentrations in these buffers were calculated using the program HySS2006 and the Cu^+ binding constants to cyanide and the proton association constant (K_a) for CN^- given above obtained from the *NIST Critical Stability Constants of Metal Complexes*.[2, 3] Free copper concentrations were calculated for total Cu^+ concentrations in the buffer from 4.0×10^{-5} M to 1.0×10^{-3} M and CN^- concentrations from 3.0×10^{-4} M to 5.0×10^{-2} M. In the preparation of these buffers, Tris-HCl pH 7.4 (100 mM) > CN^- (0.3- 50 mM) > Cu^+ (40 μM- 1 mM) >> protein (1 μM) considering the metal buffering capacity at a certain cyanide concentration to precisely control Cu^+_{free} concretions. Protein concentrations used in this measurement is 1 μM in 100 mM Tris-HCl (pH 7.4) and 100 mM NaCl and the copper(I) loading was measured for each copper(I) buffer prepared. The Cu^+_{free} concentrations were considered unchanged in the prepared Cu^+/ CN^- buffers upon addition of the proteins because the maximum possible perturbation by the protein can be only ± 4 μM Cu^+ which were calculated to have a negligible effect on Cu^+_{free} concentrations (Table S2). The % FRET responses which are also equal to the copper occupancies of the protein versus the free copper(I) concentrations in the buffer were plotted and the binding curves were fitted for the Hill equation using Origin 8 (Fig. 1 and Fig. 3, Table S1). $[Cu]_{min}$ was calculated as the point

where 95 % of Ace1-FRET is activated and $[Cu]_{max}$ was calculated as the point where 95 % of Mac1-FRET is activated using the K_ds and errors obtained from the respective fitting curves.

For the K_d measurement of Ace1-FRET and Mac1-FRET with Zn^{2+}, similar Zn^{2+}/CN^- buffers were used using the Zn^{2+} complex formation constants with cyanide given above. Free Zn^{2+} concentrations ranging from 5×10^{-11} to 2.5×10^{-5} M were prepared by using total Zn^{2+} concentrations ranging from 5.0×10^{-5} M to 1.0×10^{-3} M and CN^- concentrations ranging from 5×10^{-4} M to 2.0×10^{-2} M (Table S3). For higher Zn^{2+} concentrations (from 2.5×10^{-5} M to 1.0×10^{-4} M), Zn^{2+} solutions with desired concentrations were used directly. The apo protein used for these measurements was at 1 μM in a buffer containing 100 mM Tris-HCl (pH 7.4), 100 mM NaCl, and 100 μM TCEP-HCl and binding curves were fitted for the Hill equation using Origin 8 (Fig. 2 e-f, Table S1). The Zn^{2+}_{free} concentrations were considered unchanged up on the addition of the proteins as the perturbation in Zn^{2+}_{free} was calculated to be negligible (Table S3).

The titration and selectivity measurements for Ace1-FRET and Mac1-FRET were performed in a buffer containing 10 mM Tris-HCl (pH 7.4), 300 mM NaCl, and 4 mM DTT. Tetrakis(acetonitrile) copper(I) hexafluorophosphate dissolved in acetonitrile at 100 μM concentration was used as the copper(I) source in all measurements. For the other metals 100 μM stock solutions were prepared from the following salts with purity > 99 %: $ZnCl_2$, $MgCl_2$, $CaCl_2$, $MnCl_2$, $Fe(NH_4)_2SO_4$, $CoCl_2$. $6H_2O$ and $NiCl_2$. $6H_2O$. For the titration curves, 0 μM to 6 μM of each metal were added to a solution of 1 μM apo

Ace1-FRET or Mac1-FRET protein (Fig. 2 a-b). For the selectivity measurement for Ace1-FRET and Mac1-FRET, 5 μM of each metal was added to 1 μM apo protein, and the response to the metal was measured. 5 μM Cu^{+} was added to the same solution and the spectrum was recorded again (Fig. 2 c-d).

3. UV-Vis Measurements.

UV-Vis absorption spectrum for apo Ace1-FRET and Mac1-FRET in 10 mM Tris-HCl (pH 7.4), 300 mM NaCl and 8 mM DTT were measured after treatment with 10 mM CN^{-} and running the desalting column. Then, these samples were buffer-exchanged into a Cu^{+}/CN^{-} buffer (100 mM Tris-HCl [pH 7.4], 100 mM NaCl, 7.5×10^{-5} M Cu^{+} and 5.0×10^{-4} M CN^{-}) with a 3.84×10^{-16} M free Cu^{+} concentration where both proteins bind to copper. Finally, the UV-Vis spectra of the protein in the Cu^{+}/CN^{-} buffer were corrected by subtracting the absorption spectrum of 0.5 mM CN^{-} in buffer (Fig. S4, ESI).

Notes and references

1 J. Zhang, Y. Ma, S. S. Taylor and R. Y. Tsien, *Proc Natl Acad Sci U S A*, 2001, **98**, 14997-15002.
2 A. E. Martell and R. M. Smith, *Standard Reference Database 46*, 2001.
3 L. Alderighi, P. Gans, A. Ienco, D. Peters, A. Sabatini and A. Vacca, *Coordination Chemistry Reviews*, 1999, **184**, 311-318.

(a) **Ace1**

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1   MVVINGVKYA CETCIRGHRA AQCTHTDGPL QMIRRKGRPS TTCGHCKELR RTKNFNPSGG
61  CMCASARRPA VGSKEDETRC RCDEGEPCKC HTKRKSSRKS KGGSCHRRAN DEAAHVNGLG
121 IADLDVLLGL NGRSSDVDMT TTLPSLKPPL QNGEIKADSI DNLDLASLDP LEQSPSISME
181 PVSINETGSA YTTTNTALND IDIPFSINEL NELYKQVSSH NSHSQ
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(b) **Mac1**

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1   MIIFNGNKYA CASCIRGHRS STCRHSHRML IKVRTRGRPS PMAIRDAILV DSTSQSTEYE
61  NGAQIEGDCC SAMNQQPILF VRASAVRKAR MINGKLHILM EEGFTAHEPK DISTFTDDGN
121 KYITETEFLR KHSPKAPATG TISPDSTKSS SSSEKKERSR LQQEPIRHFS NCCKKDKSQN
181 PASNGKTNKA PSDDIFTPYG SLESTSAFND ILQENYNSSV PGAHDSSETL TPQSTTTIAA
241 PHSSDVASKV EVLTHKGIFL STQCSCEDES CPCVNCLIHR SEEELNSYIQ QSGVPLTNIG
301 EAQITDKMMD YLDDCKCTDK ECICPPDNCT CDGCFSHSTN IIPFEKFFFY GILNARLTRK
361 TQIKFKGKLV PSKYWWDFLK LQVPLMTDAQ LELLDIHAWF QKLVSNYAPH LSDATTS
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Fig. S1 (a) Sequence of Ace1. Residues 36-100 are the copper-binding domain. (b) Sequence of Mac1. Residues 203-295 are the copper-binding domain. The copper-binding domains are highlighted in yellow and ligands are highlighted in red.

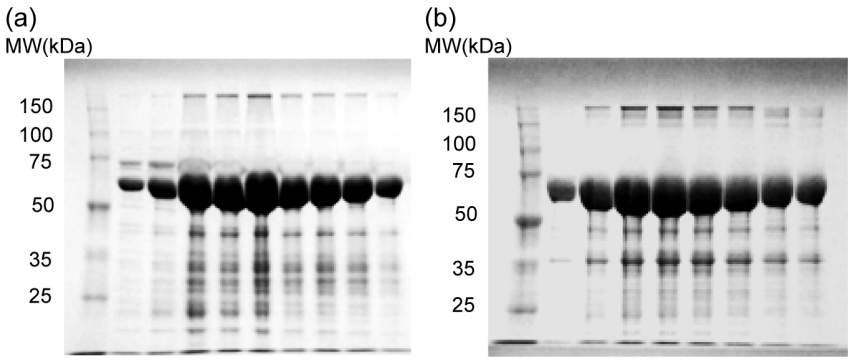

Fig. S2 SDS-protein gel of the purified (a) Ace1-FRET and (b) Mac1-FRET. The most concentrated fractions were used for experiments.

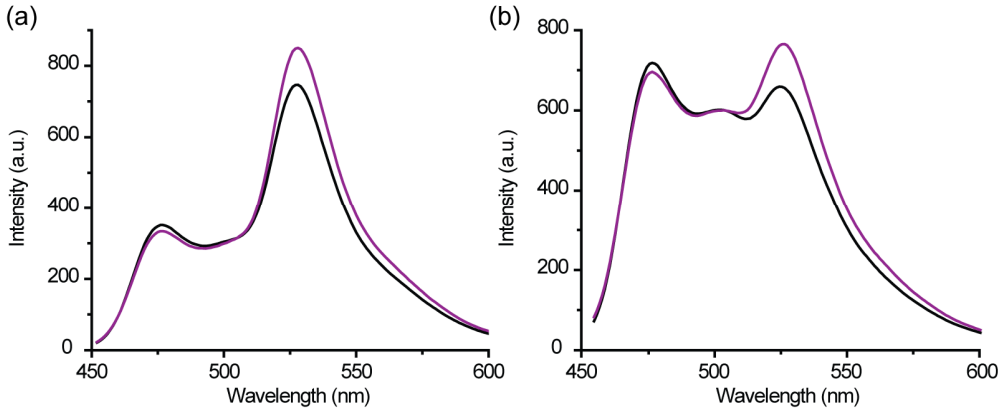

Fig. S3 Fluorescence spectra of (a) Ace1-FRET and (b) Mac1-FRET. 1 μM of protein was used for each curve. Apo protein is shown in black and copper(I) bound protein in purple.

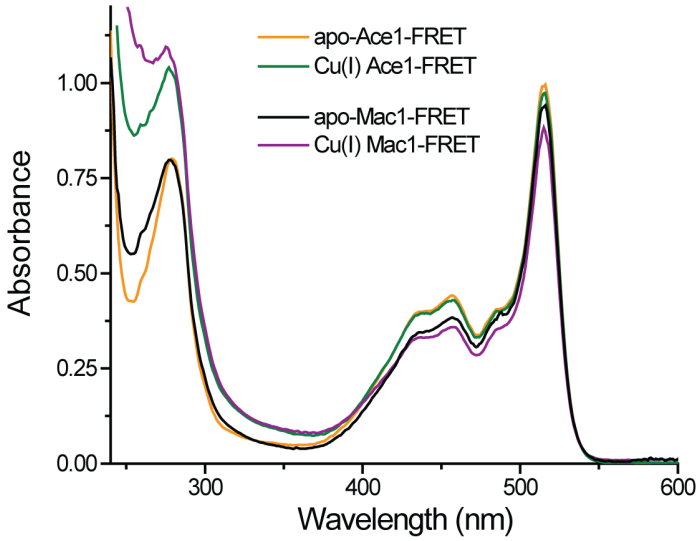

Fig. S4 UV-Vis spectra of Ace1-FRET and Mac1-FRET with and without bound copper(I). apo-Ace1-FRET shown in orange, Cu(I)-Ace1-FRET shown in green, apo-Mac1-FRET shown in black, and Cu(I)-Mac1-FRET shown in purple.

(a) Cup1

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1  MFSELINFQN EGHECQCQCG SCKNNEQCQK SCSCPTGCNS DDKCPCGNKS EETKKSCCSG
61 K
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(b) Crs5

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1  MTVKICDCEG ECCKDSCHCG STCLPSCSGG EKCKCDHSTG SPQCKSCGEK CKCETTCTCE
61 KSKCNCEKC
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Fig. S5 (a) Sequence of Cup1. (b) Sequence of Crs5. The copper-binding ligands are highlighted in red.

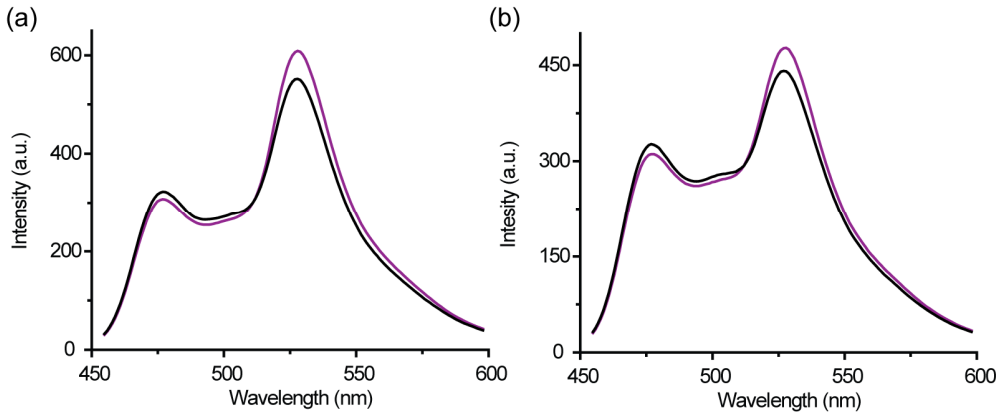

Fig. S6 Fluorescence spectra of (a) Cup1-FRET and (b) Crs5-FRET. 1 μM of protein was used for each curve. Apo protein is shown in black and copper(I) bound protein in purple.

Table S1 K_d for copper(I) and zinc(II) binding to Ace1-FRET, Mac1-FRET, Cup1-FRET and Crs5-FRET. K_ds were obtained by fitting the binding curves to the Hill equation using Origin 8. R^2 describes the quality of the fit.

Protein: Metal	**K_d**	**Standard Error**	**R^2**
Ace1-FRET: Cu^+	4.7×10^{-18}	$\pm 8.8 \times 10^{-19}$	0.996
Mac1-FRET: Cu^+	9.7×10^{-20}	$\pm 1.3 \times 10^{-20}$	0.955
Cup1-FRET: Cu^+	3.6×10^{-18}	$\pm 8.1 \times 10^{-19}$	0.998
Crs5-FRET: Cu^+	2.3×10^{-18}	$\pm 5.7 \times 10^{-19}$	0.999
Ace1-FRET: Zn^{2+}	1.1×10^{-6}	$\pm 3.1 \times 10^{-7}$	0.979
Mac1-FRET: Zn^{2+}	2.4×10^{-7}	$\pm 3.8 \times 10^{-8}$	0.980

Table S2 Cu^+/ CN^- buffers prepared for copper(I) K_d measurements. The Cu^+_{free} concentrations were calculated using the program HySS2006. The standard errors were calculated as the change in Cu^+_{free} up on addition or removal of 4 μM Cu^+ from the buffers which is the maximum possible change when 1 μM of the proteins are added. Because the possible error from this source is very small Cu^+_{free} concentrations were assumed to be unchanged when proteins were added to the Cu^+/ CN^- buffers in the calculations.

	CN^- (mM)	Cu^+ (mM)	Cu^+_{free} (M)	Standard Error
1	50	0.05	5.58E-23	± 4.5E-24
2	50	0.1	1.13E-22	± 4.5E-24
3	50	0.2	2.29E-22	± 5.0E-24
4	50	0.3	3.5E-22	± 5.0E-24
5	50	0.6	7.41E-22	± 5.5E-24
6	30	0.3	1.68E-21	± 2.5E-23
7	30	0.5	2.99E-21	± 2.5E-23
8	20	0.4	8.29E-21	± 9.5E-23
9	10	0.1	1.49E-20	± 6.5E-22
10	10	0.3	5.38E-20	± 9.5E-22
11	10	0.4	7.9E-20	± 1.1E-21
12	5	0.1	1.26E-19	± 6.0E-21
13	10	0.8	2.42E-19	± 2.5E-21
14	10	1	3.83E-19	± 3.0E-21
15	5	0.3	5.55E-19	± 1.2E-20
16	5	0.4	9.16E-19	± 1.7E-20
17	5	0.6	2.21E-18	± 1.0E-18
18	3	0.3	3.69E-18	± 1.0E-19
19	3	0.4	7.32E-18	± 1.9E-19
20	3	0.5	1.43E-17	± 4.0E-19
21	1	0.1	2.42E-17	± 1.9E-18
22	1	0.15	6.01E-17	± 5.2E-18
23	0.5	0.07	1.36E-16	± 1.7E-17
24	1	0.2	1.43E-16	± 1.0E-17
25	0.3	0.04	2.39E-16	± 4.9E-17
26	0.5	0.1	3.46E-16	± 4.3E-17
27	1	0.25	3.51E-16	± 2.6E-17
28	1	0.3	9.27E-16	± 7.8E-17

Table S3 Zn^{2+}/ CN^- buffers prepared for zinc(II) K_d measurements. The Zn^{2+}_{free} concentrations were calculated using the program HySS2006. The standard errors were calculated as the change in Zn^{2+}_{free} up on addition or removal of 2 μM Zn^{2+} from the buffers which is the maximum possible change when 1 μM of proteins are added. Because the possible error from this source is very small Zn^{2+}_{free} concentrations were assumed to be unchanged when proteins were added to the Zn^{2+}/ CN^- buffers in the calculations.

	CN^- (mM)	Zn^{2+} (mM)	Zn^{2+}_{free} (M)	Standard Error
1	0.5	0.1	1.12E-05	± 5.0E-07
2	0.25	0.05	1.50E-05	± 9.5E-07
3	1	0.2	6.06E-06	± 1.8E-07
4	1	0.1	1.16E-06	± 4.5E-08
5	5	1	6.63E-07	± 6.0E-09
6	5	0.8	2.64E-07	± 2.5E-09
7	5	0.5	6.81E-08	± 6.5E-10
8	5	0.3	2.46E-08	± 3.0E-10
9	10	0.8	9.59E-09	± 5.0E-11
10	10	0.5	3.94E-09	± 2.5E-11
11	10	0.2	1.07E-09	± 1.5E-11
12	20	0.5	2.59E-10	± 1.5E-12
13	20	0.3	1.35E-10	± 1.0E-12