Supplementary Information for

Human Calprotectin Is an Iron-Sequestering Host-Defense Protein

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Supplementary Results

This Supplementary Information includes:

Supplementary Table 1. Human Calprotectin Mutant Protein Nomenclature

Protein	S100A8 Mutation(s)	S100A9 Mutation(s)
CP	n/a	n/a
CP-Ser	C42S	C3S
CP-Ser ΔHis_3Asp	C42S, H83A, H87A	C3S, H20A, D30A
CP-Ser ΔHis_4	C42S, H17A, H27A	C3S, H91A, H95A
CP-Ser $\Delta\Delta$	C42S, H17A, H27A, H83A, H87A	C3S, H20A, D30A, H91A, H95A
CP-Ser(H103A)	C42S	C3S, H103A
CP-Ser(H104A)	C42S	C3S, H104A
CP-Ser(H105A)	C42S	C3S, H105A
CP-Ser-AHA	C42S	C3S, H103A, H105A
CP-Ser-AAA	C42S	C3S, H103A, H104A, H105A

Supplementary Table 2. Metal analysis of untreated medium (+BME)

-Ca(II) Trial		Mg	Ca	Mn	Fe	Co	Ni	Cu	Zn
1	ppm	3.75	3.99	0.0126	0.192	0.0074	0.0188	0.0125	0.365
	μM	154	99.6	0.229	3.44	0.130	0.320	0.197	5.58
2	ppm	3.90	4.01	0.0065	0.167	0	0.0062	0.0095	0.341
	μM	160	100	0.118	2.99	0	0.106	0.149	5.22
3	ppm	3.68	3.78	0.009	0.172	0.006	0.013	0.018	0.338
	μM	151	94.3	0.164	3.080	0.105	0.222	0.283	5.17
4	ppm	3.69	3.92	0.006	0.201	0.002	0.010	0.007	0.384
	μM	152	97.8	0.109	3.60	0.035	0.170	0.110	5.87
5	ppm	3.52	4.46	0.010	0.210	0.004	0.018	0.013	0.394
	μM	145	111	0.180	3.76	0.067	0.310	0.203	6.03
6	ppm	3.54	3.62	0.011	0.122	0.0004	0.014	0.012	0.222
	μM	146	90.3	0.191	2.19	0.007	0.239	0.192	3.40
7	ppm	4.10	3.99	0.013	0.162	0	0.023	0.012	0.308
	μM	169	99.6	0.237	2.90	0	0.392	0.189	4.71
+Ca(II) Trial		Mg	Ca	Mn	Fe	Co	Ni	Cu	Zn
1	ppm	3.78	81.14	0.0123	0.158	0.0076	0.018	0.0099	0.36
	μM	156	2020	0.224	2.83	0.133	0.307	0.156	5.51
2	ppm	3.99	77.7	0.0066	0.154	0	0.0066	0.0073	0.341
	μM	164	1940	0.120	2.76	0	0.112	0.115	5.215
3	ppm	3.66	73.9	0.009	0.173	0.008	0.012	0.016	0.343
	μM	151	1840	0.164	3.10	0.141	0.204	0.252	5.25
4	ppm	3.47	83.8	0.006	0.173	0.002	0.008	0.008	0.338
	μM	143	2090	0.109	3.10	0.035	0.136	0.126	5.17
5	ppm	3.54	77.4	0.010	0.196	0.004	0.015	0.011	0.360
	μM	146	1930	0.177	3.51	0.061	0.250	0.168	5.51
6	ppm	3.58	82.9	0.010	0.083	0.0004	0.012	0.009	0.212
	μM	147	2070	0.177	1.49	0.007	0.204	0.146	3.25
7	ppm	3.62	70.4	0.013	0.119	0	0.041	0.010	0.316
	μM	149	1760	0.237	2.12	0	0.702	0.164	4.84

Supplementary Table 3. Metal analysis of medium (+BME) treated with 125 μg/mL CP-Ser

-Ca(II) Trial		Mg	Ca	Mn	Fe	Co	Ni	Cu	Zn
1	ppm	3.67	3.65	0.0086	0.17	0.0077	0.0184	0.011	0.0941
	μM	151	91.1	0.157	3.04	0.135	0.314	0.173	1.44
2	ppm	3.89	3.79	0.004	0.171	0	0.005	0.0101	0.133
	μM	160	94.6	0.073	3.062	0	0.085	0.159	2.034
3	ppm	3.60	3.17	0.001	0.080	0.006	0.007	0.015	0.006
	μM	148	79.1	0.018	1.43	0.105	0.119	0.236	0.092
4	ppm	3.66	3.35	0.006	0.170	0.002	0.006	0.007	0.099
	μM	151	83.6	0.109	3.04	0.035	0.102	0.110	1.514
+Ca(II) Trial		Mg	Ca	Mn	Fe	Co	Ni	Cu	Zn
1	ppm	3.77	80.6	0.005	0.124	0.0071	0.0182	0.0097	0.0233
	μM	155	2010	0.091	2.23	0.125	0.310	0.153	0.356
2	ppm	3.97	76.2	0	0.148	0	0.0054	0.0079	0.0183
	μM	163	1900	0	2.65	0	0.092	0.124	0.280
3	ppm	3.51	71.0	0.001	0.083	0.006	0.0005	0.014	0.005
	μM	144	1770	0.018	1.49	0.105	0.009	0.220	0.076
4	ppm	3.45	83.3	0.005	0.097	0	0.007	0.008	0.005
	μM	142	2080	0.091	1.74	0	0.119	0.126	0.076

Supplementary Table 4. Metal analysis of medium (+BME) treated with 250 μg/mL CP-Ser

-Ca(II) Trial		Mg	Ca	Mn	Fe	Co	Ni	Cu	Zn
1	ppm	3.64	3.18	0.0045	0.0207	0.008	0.0125	0.0089	0.0159
	μM	150	79.3	0.082	0.371	0.141	0.213	0.140	0.243
2	ppm	3.82	3.11	0	0.0011	0	0	0.0027	0.0018
	μM	157	77.6	0	0.020	0	0	0.042	0.028
3	ppm	3.60	2.93	0.001	0.009	0.005	0.007	0.010	0.006
	μM	148	73.1	0.018	0.161	0.088	0.119	0.157	0.092
4	ppm	3.48	2.92	0.005	0.171	0.001	0.008	0.002	0.004
	μM	143	72.8	0.091	3.06	0.018	0.136	0.031	0.061
5*	ppm	3.66	3.14	0	0.067	0	0.010	0.004	0.008
	μM	151	78.3	0	1.19	0	0.170	0.058	0.128
+Ca(II) Trial		Mg	Ca	Mn	Fe	Co	Ni	Cu	Zn
1	ppm	3.75	79.4	0.0044	0.019	0.0069	0.0121	0.0083	0.0125
	μM	154	1980	0.080	0.340	0.121	0.206	0.131	0.191
2	ppm	3.85	75.1	0	0	0	0	0.0003	0.0005
	μM	158	1870	0	0	0	0	0.005	0.008
3	ppm	3.57	72.0	0.001	0.007	0.006	0.004	0.008	0.004
	μM	147	1800	0.018	0.125	0.105	0.068	0.126	0.061
4	ppm	3.48	82.7	0	0	0	0.003	0.001	0.005
	μM	143	2060	0	0	0	0.051	0.016	0.076
5*	ppm	3.87	77.5	0	0.038	0.001	0.011	0.001	0.007
	μM	159	1930	0	0.682	0.009	0.187	0.022	0.104

*Sample 5 was incubated at 4ºC for t = 20 h.

Supplementary Table 5. Metal analysis of medium (-BME) treated with 250 μg/mL CP-Ser

-Ca(II) Trial		Mg	Ca	Mn	Fe	Co	Ni	Cu	Zn
1	ppm	3.29	2.98	0.002	0.082	0.003	0.007	0.008	0.007
	μM	135	74.4	0.029	1.46	0.053	0.114	0.120	0.109
2	ppm	3.76	3.22	0.004	0.107	0.001	0.010	0.013	0.006
	μM	155	80.3	0.066	1.91	0.011	0.162	0.198	0.096
3	ppm	3.80	2.77	0	0.026	0	0.018	0.017	0.058
	μM	156	69.1	0	0.469	0	0.303	0.260	0.275
+Ca(II) Trial		Mg	Ca	Mn	Fe	Co	Ni	Cu	Zn
1	ppm	3.20	73.5	0.002	0.0215	0.0031	0.007	0.0052	0.007
	μM	132	1830	0.031	0.385	0.054	0.116	0.082	0.106
2	ppm	3.71	95.1	0.004	0.059	0.002	0.008	0.011	0.007
	μM	153	2370	0.064	1.06	0.035	0.133	0.173	0.102
3	ppm	3.76	73.9	0.0003	0.036	0	0.008	0.0002	0.001
	μM	155	1840	0.005	0.639	0	0.128	0.003	0.017

Table S6. Metal analysis of medium (+BME) treated with 250 μg/mL CP-Ser ΔHis$_3$Asp

-Ca(II) Trial		Mg	Ca	Mn	Fe	Co	Ni	Cu	Zn
1	ppm	3.55	2.81	0.001	0.050	0.005	0.007	0.015	0.006
	μM	146	70.1	0.018	0.895	0.088	0.119	0.236	0.092
2	ppm	3.47	2.82	0.002	0.043	0.004	0.008	0.012	0.008
	μM	143	70.4	0.031	0.763	0.063	0.133	0.187	0.128
3	ppm	3.48	2.66	0.004	0.024	0	0.010	0.011	0.005
	μM	143	66.4	0.066	0.421	0	0.170	0.173	0.069
+Ca(II) Trial		Mg	Ca	Mn	Fe	Co	Ni	Cu	Zn
1	ppm	3.67	73.0	0.001	0.009	0.006	0.006	0.009	0.006
	μM	151	1820	0.018	0.161	0.105	0.102	0.142	0.092
2	ppm	3.72	80.1	0.002	0.0004	0.004	0.011	0.004	0.008
	μM	153	2000	0.033	0.007	0.063	0.189	0.069	0.119
3	ppm	3.92	90.2	0.004	0.007	0.002	0.008	0.008	0.006
	μM	161	2250	0.064	0.131	0.030	0.143	0.127	0.092

Supplementary Table 7. Metal analysis of medium (-BME) treated with 250 μg/mL CP-Ser ΔHis$_3$Asp

-Ca(II) Trial		Mg	Ca	Mn	Fe	Co	Ni	Cu	Zn
1	ppm	3.29	2.87	0.002	0.139	0.0032	0.006	0.0106	0.008
	μM	135	71.6	0.029	2.49	0.056	0.095	0.167	0.124
2	ppm	3.87	3.18	0.004	0.151	0.001	0.009	0.016	0.006
	μM	159	79.3	0.066	2.70	0.023	0.152	0.257	0.090
3	ppm	3.91	3.36	0.0001	0.097	0.001	0.011	0.004	0.005
	μM	161	83.8	0.002	1.73	0.012	0.184	0.065	0.081
+Ca(II) Trial		Mg	Ca	Mn	Fe	Co	Ni	Cu	Zn
1	ppm	3.44	72.1	0.002	0.132	0.004	0.006	0.008	0.009
	μM	142	1800	0.040	2.37	0.061	0.104	0.120	0.132
2	ppm	4.02	86.0	0.004	0.0531	0.0022	0.008	0.0125	0.012
	μM	165	2140	0.067	0.951	0.039	0.135	0.197	0.184
3	ppm	3.90	75.4	0	0.085	0.001	0.011	0.003	0.005
	μM	160	1880	0	1.51	0.012	0.187	0.042	0.078

Supplementary Table 8. Metal analysis of medium (+BME) treated with 250 μg/mL CP-Ser ΔHis$_4$

-Ca(II) Trial		Mg	Ca	Mn	Fe	Co	Ni	Cu	Zn
1	ppm	3.52	3.02	0.005	0.173	0.001	0.010	0.006	0.008
	μM	145	75.3	0.091	3.10	0.018	0.170	0.094	0.122
2	ppm	3.39	2.89	0.009	0.171	0.003	0.014	0.012	0.006
	μM	139	72.1	0.158	3.06	0.049	0.245	0.181	0.098
3	ppm	3.73	3.09	0.011	0.192	0.001	0.017	0.017	0.007
	μM	153	77.1	0.195	3.43	0.014	0.296	0.269	0.102
+Ca(II) Trial		Mg	Ca	Mn	Fe	Co	Ni	Cu	Zn
1	ppm	3.43	82.8	0.005	0.158	0.001	0.010	0.007	0.006
	μM	141	2070	0.091	2.83	0.018	0.170	0.110	0.092
2	ppm	2.93	76.6	0.014	0.148	0.001	0.019	0.013	0.014
	μM	121	1910	0.253	2.65	0.018	0.319	0.211	0.216
3	ppm	3.89	92.5	0.010	0.167	0.002	0.018	0.015	0.008
	μM	160	2310	0.175	2.98	0.026	0.312	0.242	0.115

Supplementary Table 9. Metal analysis of medium (+BME) treated with 250 μg/mL CP-Ser-AAA

-Ca(II) Trial		Mg	Ca	Mn	Fe	Co	Ni	Cu	Zn
1	ppm	3.46	3.12	0.005	0.168	0.002	0.007	0.016	0.011
	μM	142	77.8	0.091	3.01	0.035	0.119	0.252	0.168
2	ppm	3.69	3.23	0.007	0.176	0.002	0.013	0.016	0.013
	μM	152	80.6	0.129	3.15	0.028	0.225	0.252	0.194
3	ppm	3.90	2.80	0.010	0.124	0	0.005	0.018	0.010
	μM	160	69.9	0.182	2.23	0	0.083	0.275	0.157
+Ca(II) Trial		Mg	Ca	Mn	Fe	Co	Ni	Cu	Zn
1	ppm	3.37	82.7	0.002	0.164	0.001	0.004	0.006	0.005
	μM	139	2060	0.036	2.94	0.018	0.068	0.094	0.076
2	ppm	3.81	90.3	0.009	0.162	0.001	0.010	0.015	0.007
	μM	157	2250	0.160	2.89	0.025	0.172	0.234	0.104
3	ppm	3.50	69.6	0	0.124	0	0.012	0.012	0.013
	μM	144	1740	0	2.22	0	0.196	0.186	0.202

Supplementary Table 10. Metal analysis of medium (+BME) treated with 250 μg/mL CP-Ser ΔΔ

-Ca(II) Trial		Mg	Ca	Mn	Fe	Co	Ni	Cu	Zn
1	ppm	3.53	3.51	0.009	0.176	0.006	0.012	0.017	0.294
	μM	145	87.6	0.164	3.15	0.105	0.204	0.268	4.50
2	ppm	3.51	3.70	0.006	0.181	0.002	0.010	0.005	0.313
	μM	144	92.3	0.109	3.24	0.035	0.170	0.079	4.79
3	ppm	3.46	3.56	0.010	0.188	0.004	0.018	0.012	0.312
	μM	142	88.8	0.173	3.37	0.061	0.300	0.181	4.77
+Ca(II) Trial		Mg	Ca	Mn	Fe	Co	Ni	Cu	Zn
1	ppm	3.53	72.0	0.009	0.163	0.006	0.011	0.016	0.227
	μM	145	1800	0.164	2.92	0.105	0.187	0.252	3.47
2	ppm	3.40	83.4	0.006	0.171	0.002	0.009	0.008	0.286
	μM	140	2080	0.109	3.06	0.035	0.153	0.126	4.37
3	ppm	3.45	74.3	0.009	0.177	0.004	0.016	0.010	0.245
	μM	142	1850	0.167	3.16	0.074	0.274	0.153	3.75

Supplementary Table 11. Metal analysis of untreated MRS-modified medium (+BME)

-Ca(II) Trial		Mg	Ca	Mn	Fe	Co	Ni	Cu	Zn
1	ppm	13.2	4.76	5.47	0.286	0.069	0.004	0.011	0.725
	μM	543	119	99.6	5.12	1.17	0.068	0.173	11.1
2	ppm	13.1	4.74	5.31	0.280	0.013	0.011	0.010	0.675
	μM	538	118	96.7	5.01	0.221	0.187	0.157	10.3
3	ppm	12.1	4.15	4.91	0.224	0.010	0.010	0.010	0.634
	μM	499	104	89.4	4.01	0.170	0.170	0.157	9.70
+Ca(II) Trial		Mg	Ca	Mn	Fe	Co	Ni	Cu	Zn
1	ppm	13.8	79.1	5.63	0.298	0.008	0.002	0.010	0.766
	μM	567	1970	102	5.34	0.136	0.034	0.157	11.7
2	ppm	13.2	78.2	5.22	0.263	0.014	0.013	0.008	0.668
	μM	544	1950	95.0	4.71	0.238	0.222	0.126	10.2
3	ppm	12.3	70.9	4.92	0.225	0.010	0.009	0.009	0.637
	μM	507	1770	89.6	4.03	0.170	0.153	0.142	9.74

Supplementary Table 12: Metal analysis of MRS-modified medium (+BME) treated with 250 μg/mL CP-Ser

-Ca(II) Trial		Mg	Ca	Mn	Fe	Co	Ni	Cu	Zn
1	ppm	13.3	3.75	4.96	0.241	0.008	0.013	0.007	0.024
	μM	545	93.6	90.3	4.32	0.136	0.222	0.110	0.367
2	ppm	10.7	3.78	3.94	0.213	0.008	0.003	0.010	0.027
	μM	441	94.3	71.7	3.81	0.136	0.051	0.157	0.413
3	ppm	11.5	3.05	4.20	0.180	0.008	0.006	0.007	0.021
	μM	473	76.1	76.4	3.22	0.136	0.102	0.110	0.321
+Ca(II) Trial		Mg	Ca	Mn	Fe	Co	Ni	Cu	Zn
1	ppm	15.3	87.7	5.70	0.278	0.008	0.013	0	0.076
	μM	627	2190	104	4.98	0.136	0.222	0	1.16
2	ppm	12.8	76.3	4.64	0.262	0.013	0.011	0.010	0.052
	μM	528	1900	84.5	4.69	0.221	0.187	0.157	0.795
3	ppm	12.3	66.8	4.49	0.219	0.009	0.010	0.009	0.030
	μM	504	1670	81.7	3.92	0.153	0.170	0.142	0.459

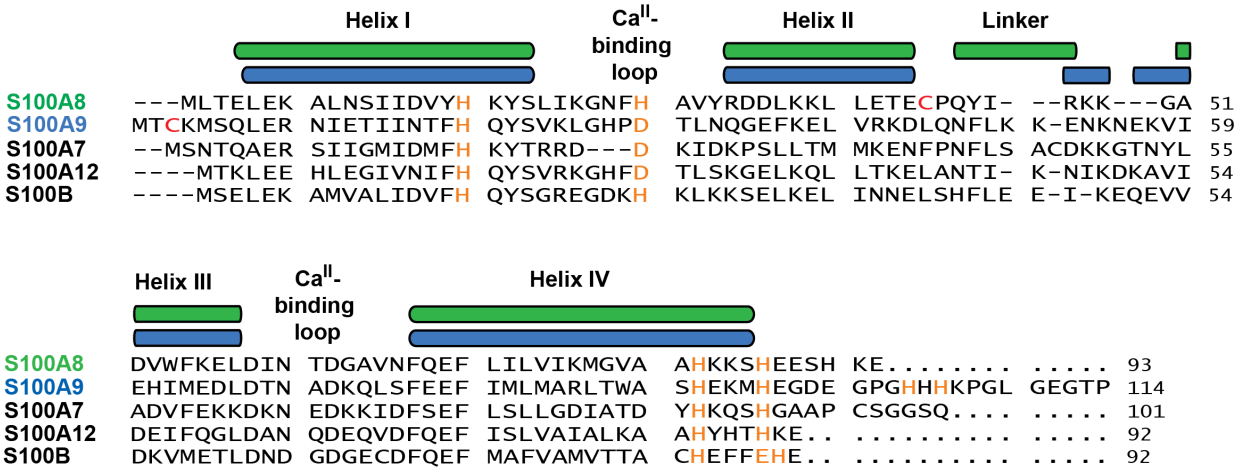
Supplementary Table 13: Metal analysis of MRS-modified medium (-BME) treated with 250 μg/mL CP-Ser

-Ca(II) Trial		Mg	Ca	Mn	Fe	Co	Ni	Cu	Zn
1	ppm	13.1	3.70	4.94	0.235	0.008	0.011	0.009	0.030
	μM	538	92.3	89.9	4.21	0.136	0.187	0.142	0.459
2	ppm	11.4	3.96	4.20	0.221	0.008	0.005	0.013	0.023
	μM	468	98.8	76.4	3.96	0.136	0.085	0.205	0.352
3	ppm	11.6	3.24	4.20	0.179	0.009	0.006	0.010	0.023
	μM	478	80.8	76.4	3.21	0.153	0.102	0.157	0.352
+Ca(II) Trial		Mg	Ca	Mn	Fe	Co	Ni	Cu	Zn
1	ppm	13.1	74.2	5.10	0.238	0.006	0.006	0.001	0.194
	μM	537	1850	92.8	4.26	0.102	0.102	0.016	2.97
2	ppm	13.0	74.9	4.67	0.259	0.013	0.012	0.015	0.047
	μM	533	1870	85.0	4.64	0.221	0.204	0.236	0.719
3	ppm	11.8	67.0	4.32	0.179	0.011	0.007	0.009	0.090
	μM	485	1670	78.6	3.21	0.187	0.119	0.142	1.38

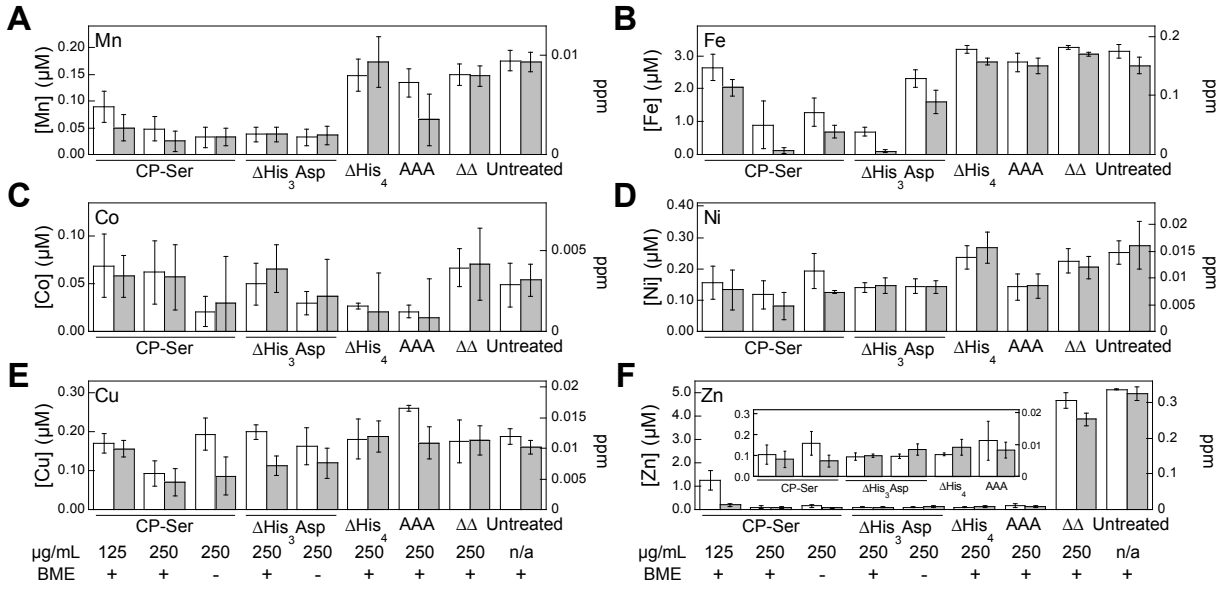
Supplementary Table 14. Fe(II) buffering conditions of Zinpyr-1 (ZP1) $K_{d,Fe(II)}$ determination

Ligand	$K_{d,Fe(II)}$ (M)	Reference	[Ligand] (M)	$[Fe(II)]_{total}$ (M)	$[Fe(II)]_{free}$ (M)
EDTA[a]	4.68×10^{-15}	1	1.0×10^{-3}	1.0×10^{-4}	4.68×10^{-16}
				9.0×10^{-4}	4.21×10^{-15}
NTA[b]	1.45×10^{-9}	2	1.0×10^{-3}	1.0×10^{-8}	1.45×10^{-14}
				1.0×10^{-7}	1.45×10^{-13}
				1.0×10^{-6}	1.45×10^{-12}
				1.0×10^{-5}	1.45×10^{-11}
				1.0×10^{-4}	1.45×10^{-10}
				9.9×10^{-4}	1.44×10^{-9}
TPA[c]	2.24×10^{-9}	3	1.0×10^{-3}	1.0×10^{-9}	2.24×10^{-15}
				1.0×10^{-8}	2.24×10^{-14}
				5.0×10^{-8}	1.12×10^{-13}
				1.0×10^{-7}	2.24×10^{-13}
				2.0×10^{-7}	4.48×10^{-13}
				5.0×10^{-7}	1.12×10^{-12}
				8.0×10^{-7}	1.79×10^{-12}
				1.0×10^{-6}	2.24×10^{-12}
				2.0×10^{-6}	4.48×10^{-12}
				5.0×10^{-6}	1.12×10^{-11}
				8.0×10^{-6}	1.79×10^{-11}
				1.0×10^{-5}	2.24×10^{-11}
				1.0×10^{-4}	4.48×10^{-11}
				2.0×10^{-4}	1.12×10^{-10}
				4.0×10^{-4}	1.79×10^{-10}
				6.0×10^{-4}	2.24×10^{-10}
				8.0×10^{-4}	4.48×10^{-10}
				9.0×10^{-4}	8.96×10^{-10}
				9.9×10^{-4}	1.34×10^{-9}
Citrate	3.33×10^{-8}	4	1.0×10^{-3}	9.0×10^{-4}	3.00×10^{-8}

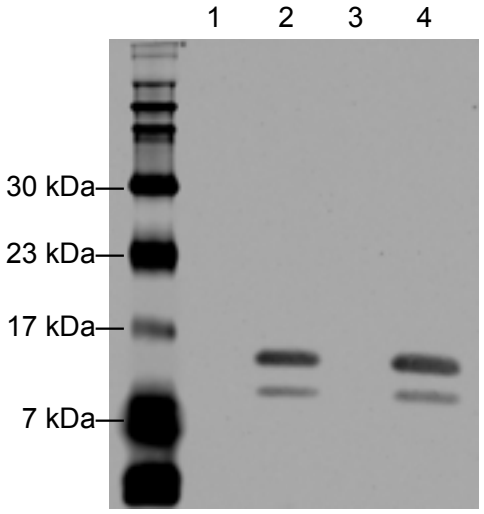
[a] Ethylenediaminetetraacetic acid. [b] Nitrilotriacetic acid. [c] Tris(2-pyridylmethyl)amine.



Supplementary Figure 1. Amino acid sequence alignment of human S100A8, S100A9, S100A7, S100A12, and S100B. The secondary structural elements of S100A8 (green) and S100A9 (blue) are presented above the sequences. The cysteine residues, (A8)Cys42 and (A9)Cys3, mutated to serine are red. The transition metal-binding residues are orange.

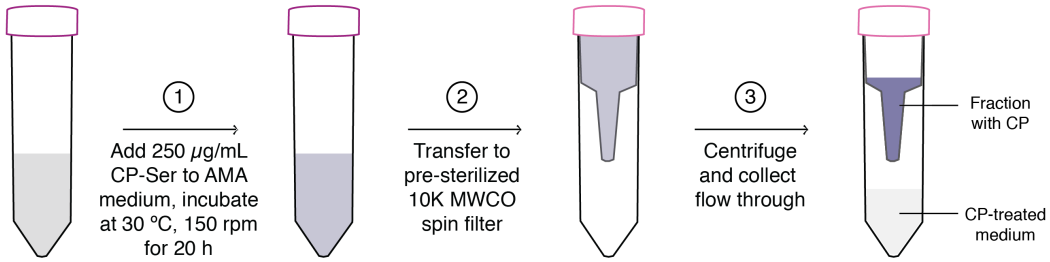


Supplementary Figure 2 Metal analyses of CP-treated medium for Mn (**A**), Fe (**B**), Co (**C**), Ni (**D**), Cu (**E**), and Zn (**F**). Medium treated with 125 or 250 μM /mL CP in the absence (white bars) and presence (gray bars) of 2 mM Ca(II). The concentration of protein and presence (+) or absence (-) of 3.1 mM BME in the media are indicated below. Concentration values in μM (left axes) and ppm (right axes) are reported (mean ± SEM, $n \geq 3$). The plots for Mn, Fe, and Zn appear in the main text and are reproduced here for comparison.

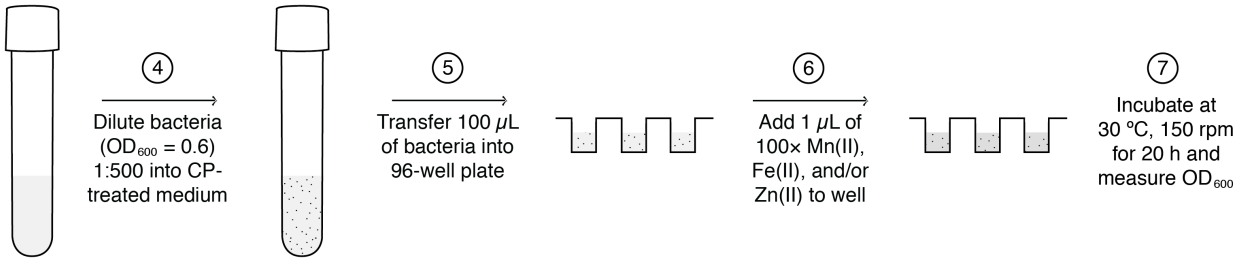


Supplementary Figure 3. Western blot analysis of CP-treated medium and flow through after filtration. Medium treated with 250 μg/mL CP-Ser, (lane 1) flow through -Ca(II), (lane 2) before filtration -Ca(II), (lane 3) flow through +Ca(II), and (lane 4) before filtration +Ca(II).

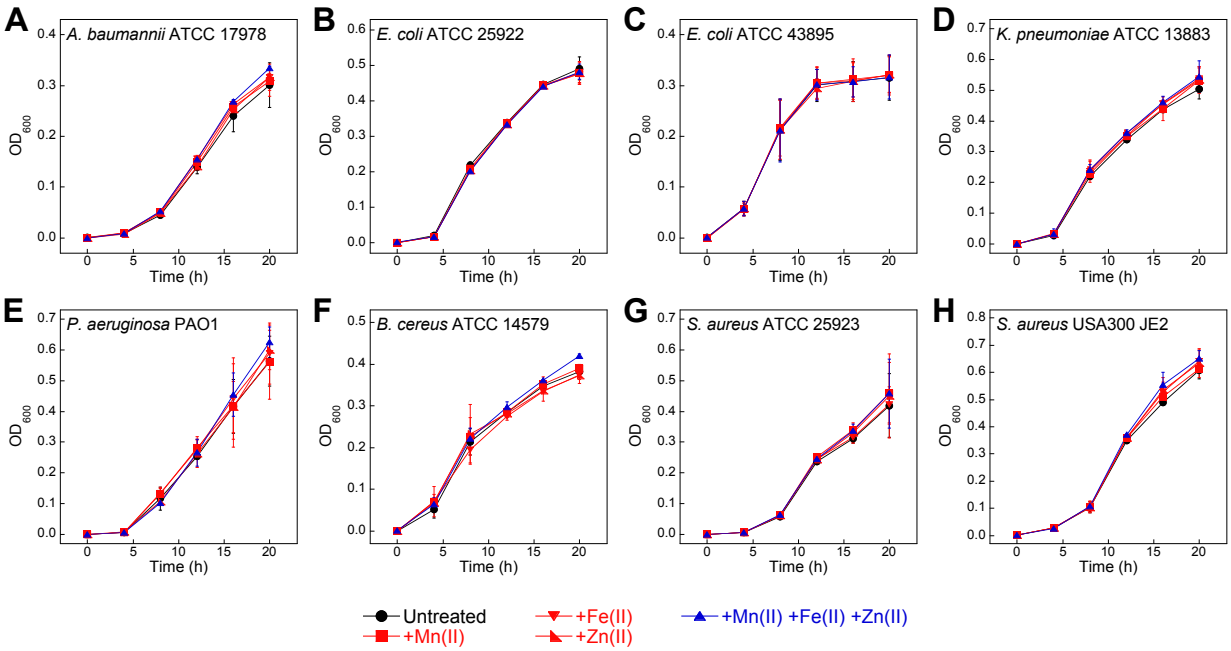
A. Preparation of CP-treated medium



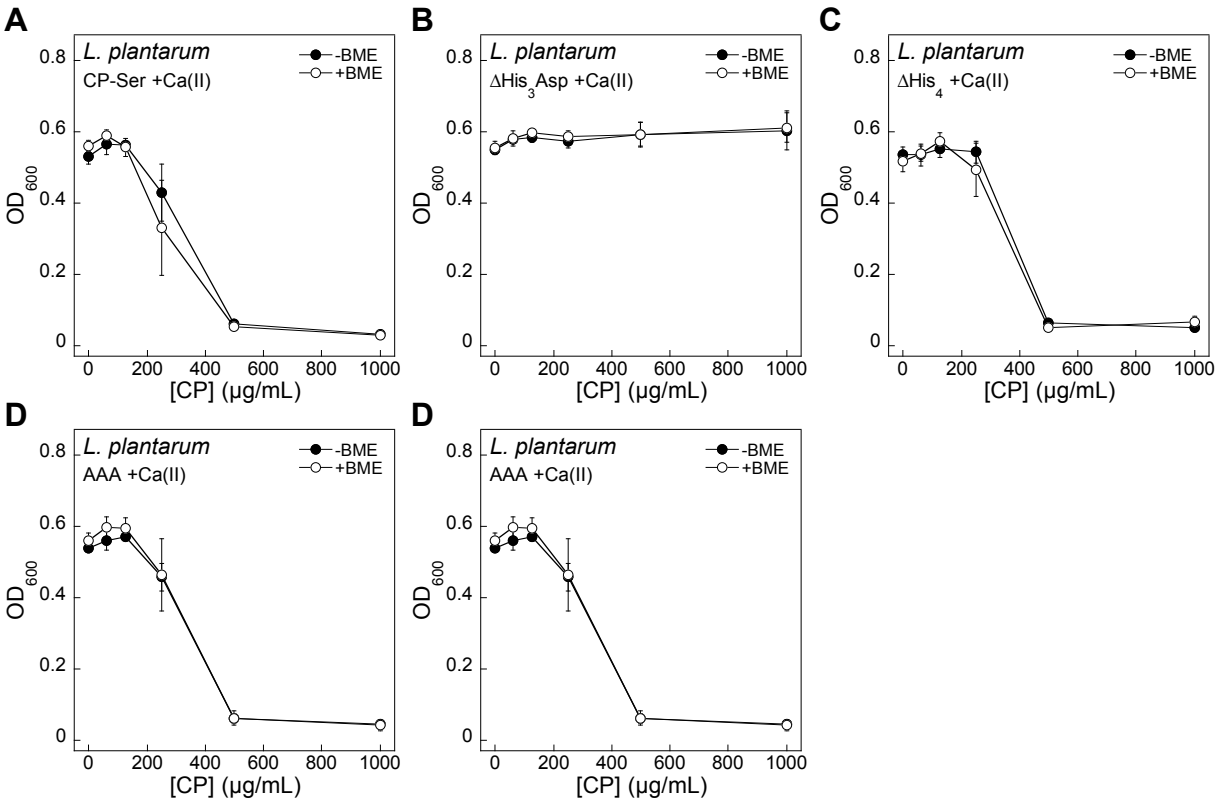
B. Bacterial growth in metal-substituted medium



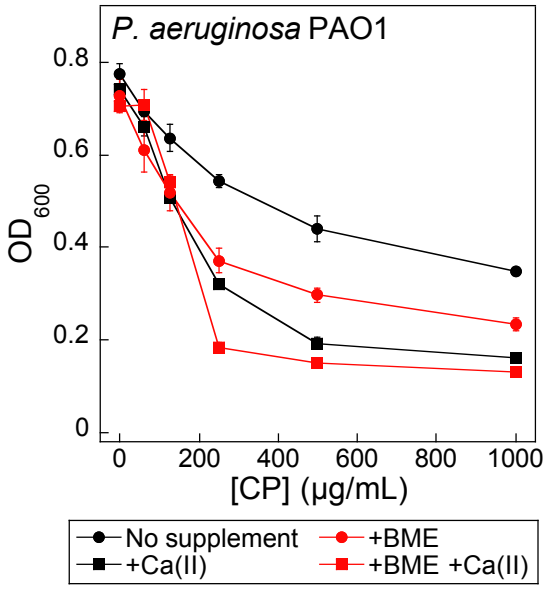
Supplementary Figure 4 Schematic cartoon of metal-substitution bacterial growth assay procedure.



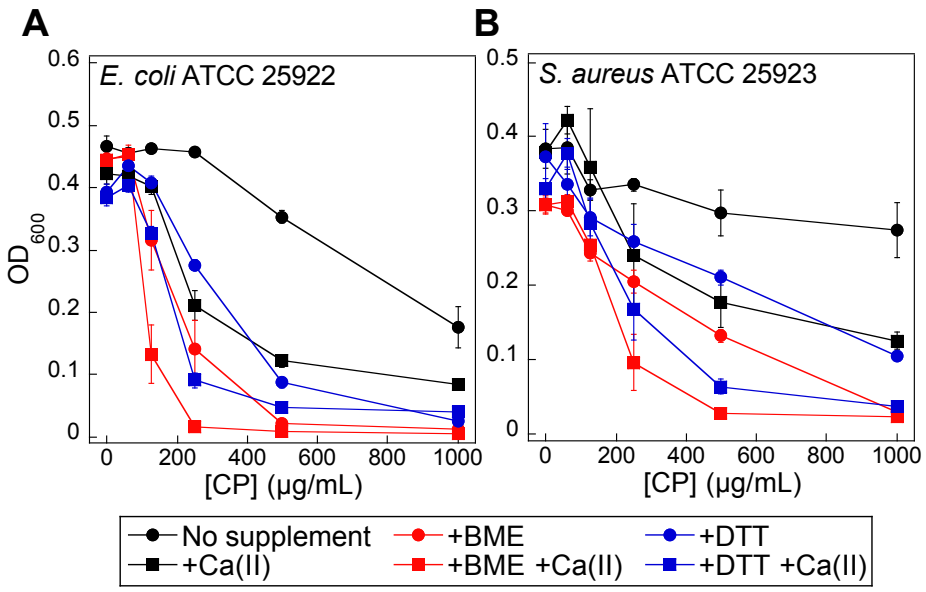
Supplementary Figure 5. Growth curves of Gram-negative and Gram-positive bacteria cultured in medium (+Ca(II), +BME) that was not treated with CP and was supplemented with 0.15 μM Mn(II), 3 μM Fe(II), and/or 5 μM Zn(II) (mean ± SDM, *n* = 2).



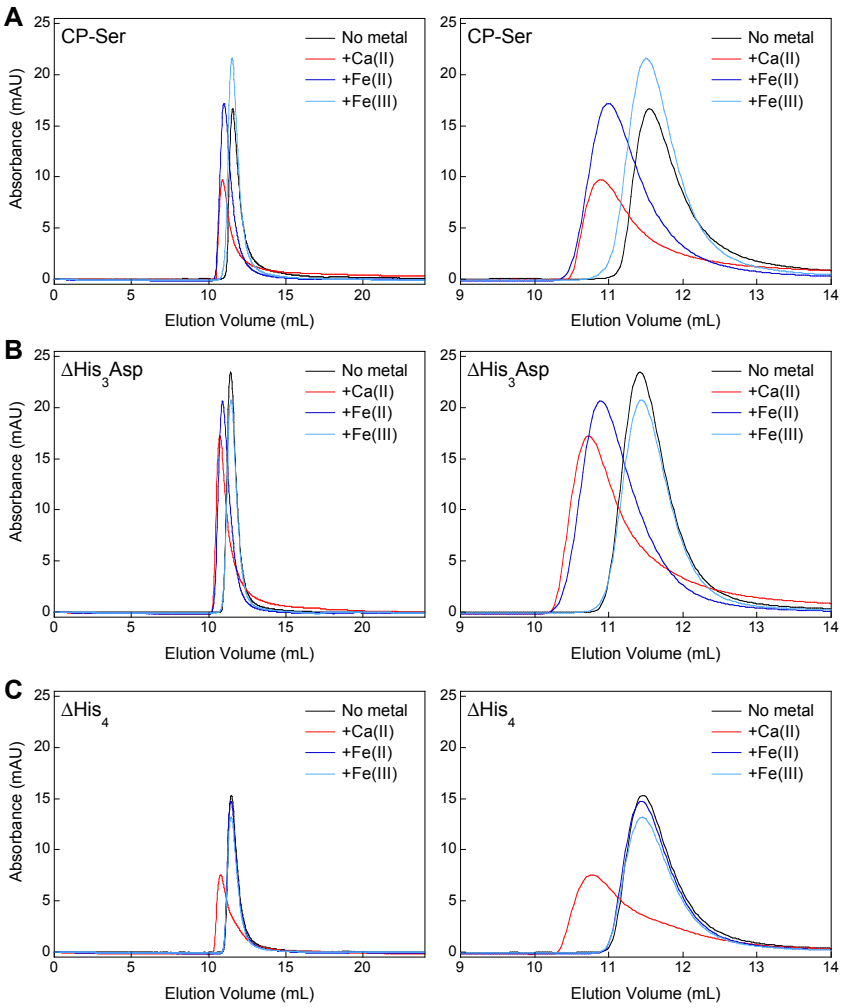
Supplementary Figure 6. Antimicrobial activity assays of CP against *L. plantarum*. Bacterial cultures were incubated with CP-Ser (**A**), CP-Ser ΔHis$_3$Asp (**B**), CP-Ser ΔHis$_4$ (**C**), CP-Ser-AAA (**D**), and CP-Ser ΔΔ (**E**) in the absence (solid circles) and presence (open circles) of 3.1 mM BME in the medium. The OD_{600} values were recorded at t = 20 h (mean ± SEM, *n* = 3).



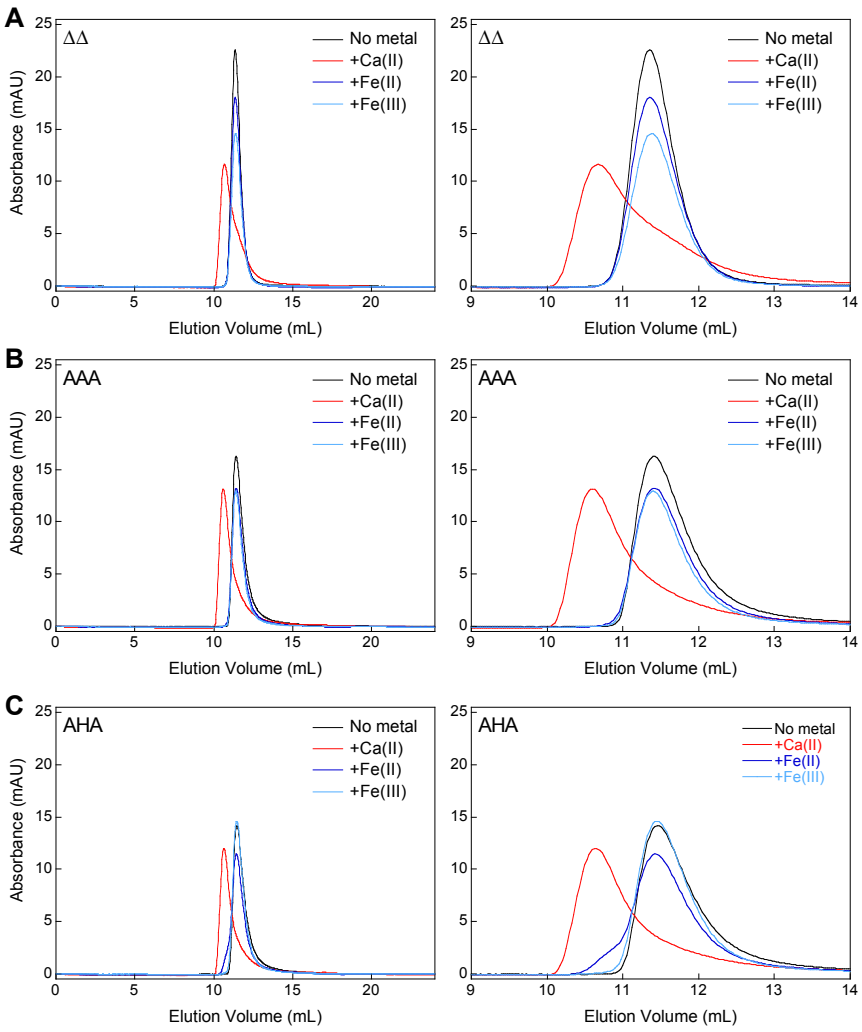
Supplementary Figure 7. Antimicrobial activity of CP against *P. aeruginosa* PAO1 in the absence and presence of ~3 mM BME and/or 2 mM Ca(II). The OD_{600} values were recorded at t = 20 h (mean ± SEM, *n* = 3).



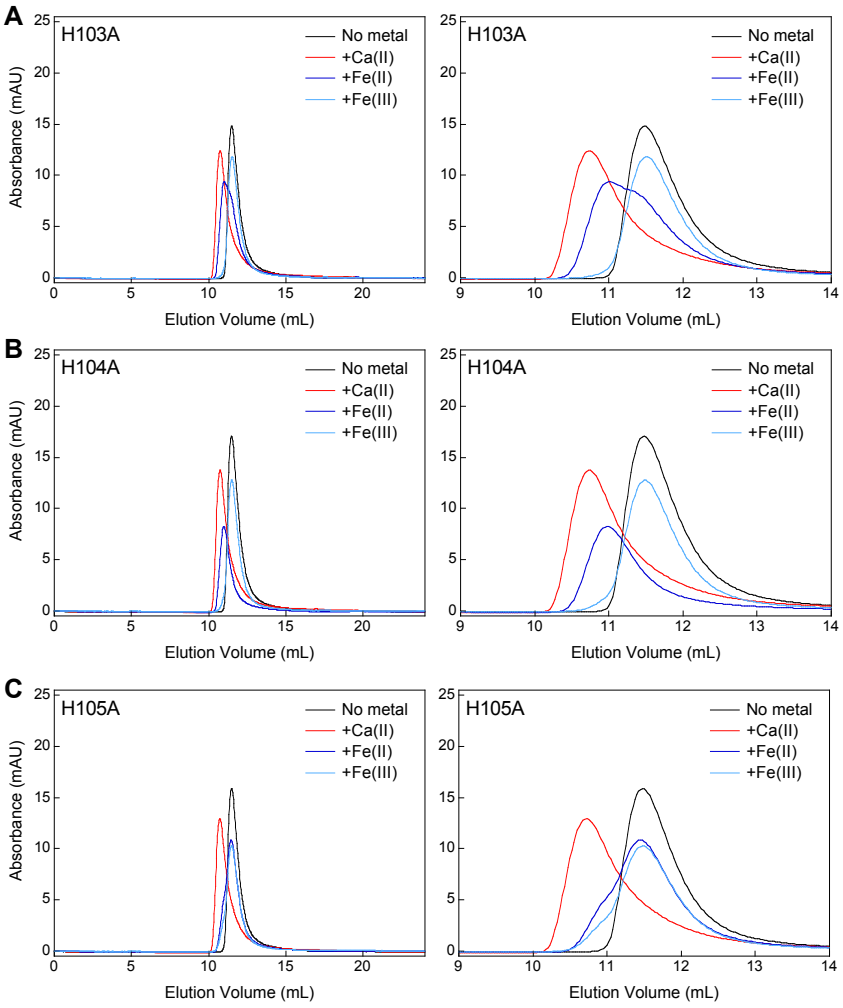
Supplementary Figure 8. Antimicrobial activity assays against *E. coli* ATCC 25922 (**A**) and *S. aureus* ATCC 25923 (**B**) with BME or DTT in the growth medium. The medium was supplemented with either no reducing agent (black), 3.1 mM BME (red), or 1.6 mM DTT (blue), in the absence (circles) and presence (squares) of a 2-mM Ca(II) supplement. The OD_{600} values were recorded at t = 20 h (mean ± SEM, *n* = 3).



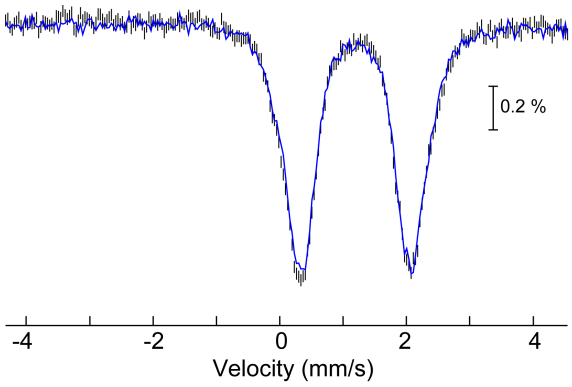
Supplementary Figure 9. Analytical size-exclusion chromatography (SEC) of CP-Ser (**A**), CP-Ser ΔHis_3Asp (**B**), and CP-Ser ΔHis_4 (**C**) preincubated with 5 equiv of Fe(II) (blue line) or Fe(III) (light blue line) in 75 mM HEPES, 100 mM NaCl, pH 7.0. The chromatograms with no metal addition (black line) and with 2 mM Ca(II) in the running buffer (red line) are shown. Protein concentration was 20 μM, and sample volume was 100 μL.



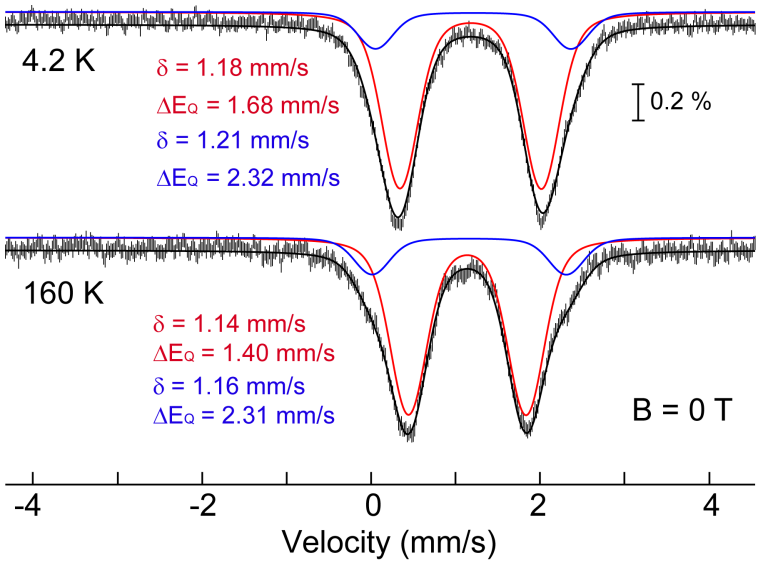
Supplementary Figure 10. Analytical SEC of CP-Ser ΔΔ (**A**), CP-Ser-AAA (**B**), and CP-Ser-AHA (**C**) pre-incubated with 5 equiv of Fe(II) (blue line) or Fe(III) (light blue line) in 75 mM HEPES, 100 mM NaCl, pH 7.0. The chromatograms with no metal addition (black line) and with 2 mM Ca(II) in the running buffer (red line) are shown. Protein concentration was 20 μM, and sample volume was 100 μL.



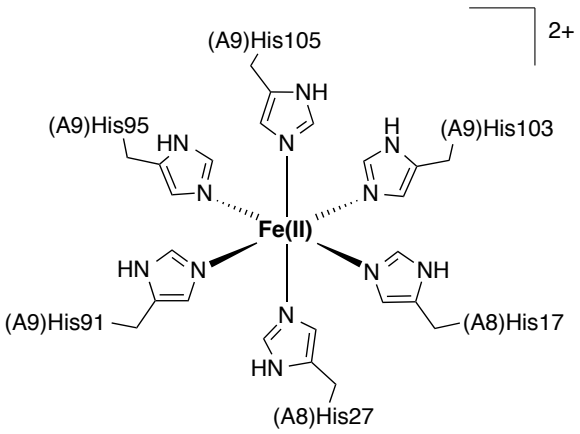
Supplementary Figure 11. Analytical SEC of CP-Ser(H103A) (**A**), CP-Ser(H104A) (**B**), and CP-Ser(H105A) (**C**) preincubated with 5 equiv of Fe(II) (blue line) or Fe(III) (light blue line) in 75 mM HEPES, 100 mM NaCl, pH 7.0. The chromatograms with no metal addition (black line) and with 2 mM Ca(II) in the running buffer (red line) are shown. Protein concentration was 20 μM, and sample volume was 100 μL.



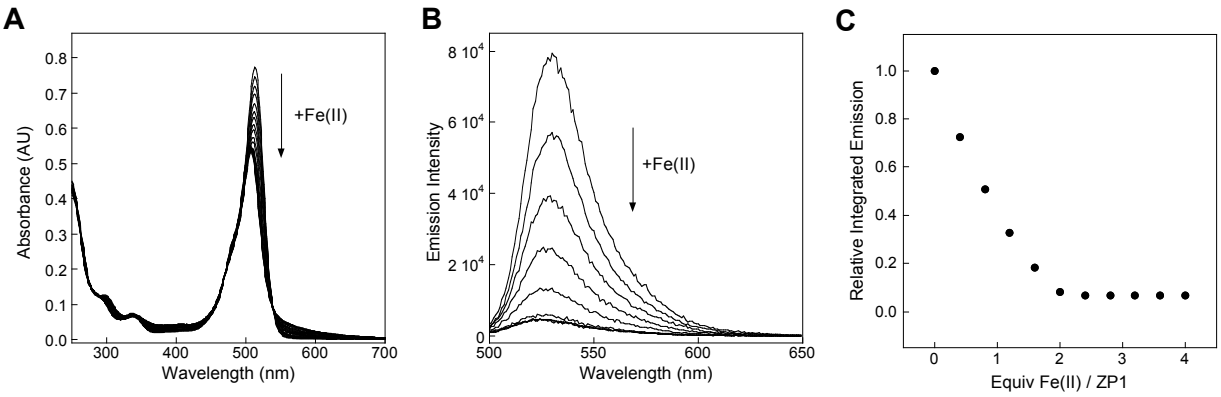
Supplementary Figure 12. Comparison of 4.2-K/53-mT Mössbauer spectra of ^{57}Fe-bound CP-Ser (black hashed marks) and the ΔHis_3Asp variant (blue solid line) in a solution of 90% (v/v) 75 mM HEPES, 100 mM NaCl, pH 7.0 and 10% (v/v) glycerol.



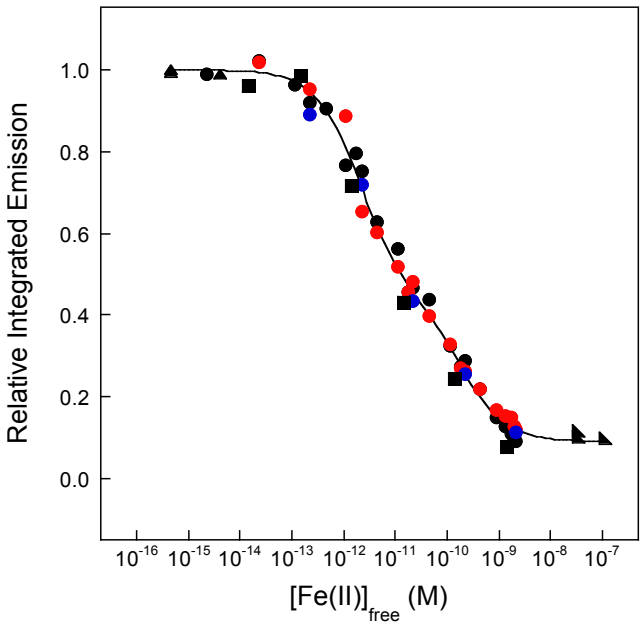
Supplementary Figure 13. Mössbauer spectra of ^{55}Fe-bound CP-Ser in a solution of 90% (v/v) 75 mM HEPES, 100 mM NaCl, pH 7.0 and 10% (v/v) glycerol collected at 4.2 K (top, vertical bars) and at 160 K (bottom, vertical bars) without an externally applied magnetic field. The spectra can be simulated with two quadrupole doublets. The solid red and blue lines are simulations of the individual subspectra of the major (83% of total intensity) and minor (17% of total intensity) component, respectively, while the solid black line is the summation of the subspectra. The simulation parameters are shown color-coded on the spectra.



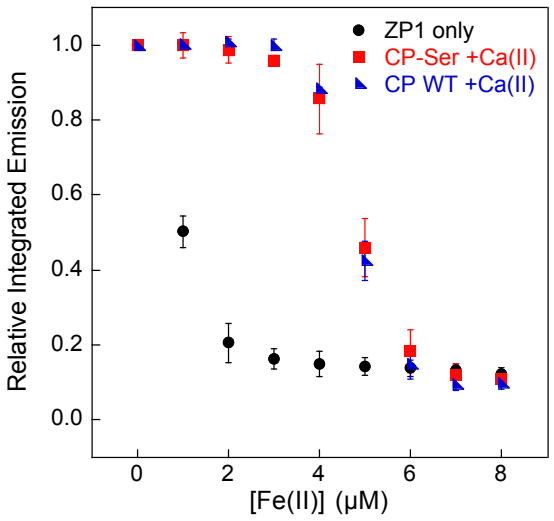
Supplementary Figure 14. Proposed Fe(II) coordination site of CP.



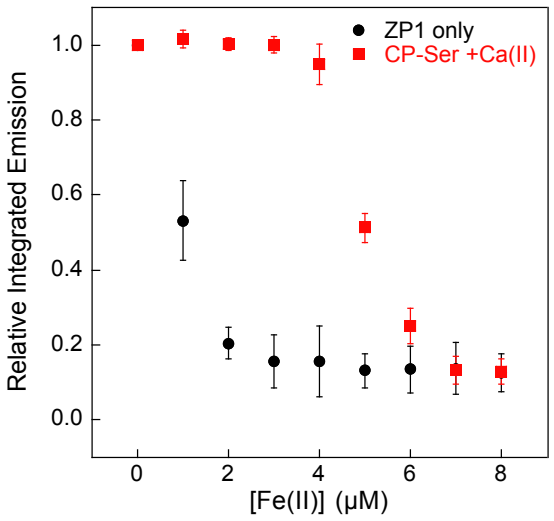
Supplementary Figure 15. Titration of ZP1 with 0 to 4.0 equiv Fe(II) monitored by optical absorption and emission spectroscopies (75 mM HEPES, 100 mM NaCl, pH 7.0). (**A**) The absorption spectra of 10 μM ZP1 with the addition of Fe(II). The λ_{max} shifts from 513 nm (ε = 77,000 M^{-1} cm^{-1}) to 506 nm (ε = 54,000 M^{-1} cm^{-1}). (**B**) The emission spectra of 1 μM ZP1 with the addition of Fe(II). There is a ~12-fold turn off response in the presence of >2 eq Fe(II). There is a slight blue shift in λ_{em} from 530 nm to 525 nm with Fe(II) addition. (**C**) Relative integrated emission of 1 μM ZP1 with the addition of Fe(II).



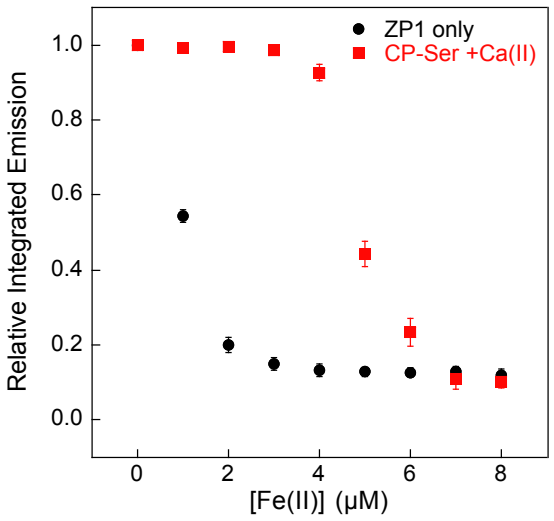
Supplementary Figure 16. ZP1 $K_{d,Fe(II)}$ determination. The relative integrated emission of ZP1 was measured in Fe(II) solutions buffered with TPA (circles), NTA (squares), EDTA (up triangles), and citrate (right triangles) in 75 mM HEPES, 100 mM NaCl, pH 7.0. The different colors represent independent trials. The black line represents the fit ($K_{d1,Fe(II)}$ = 2.15 ± 0.25 pM, $K_{d2,Fe(II)}$ = 190 ± 41 pM).



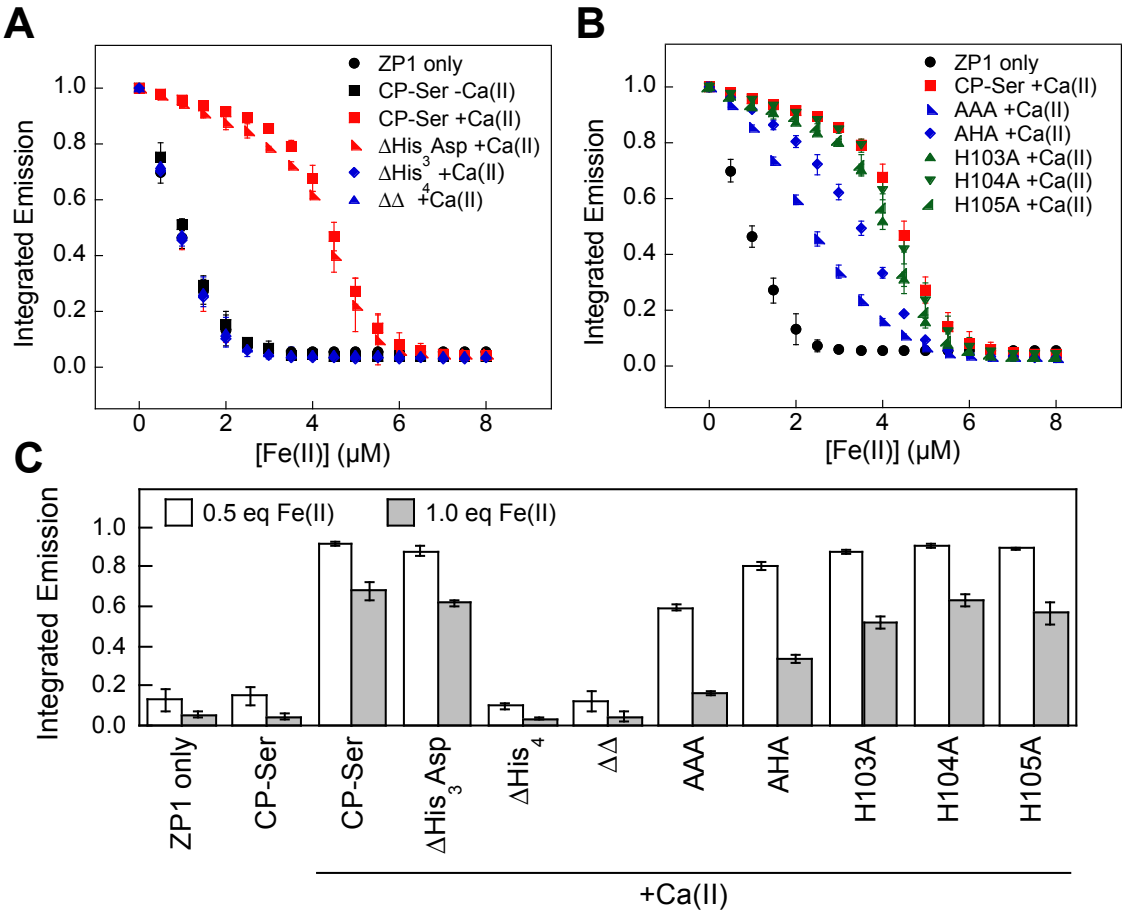
Supplementary Figure 17. Emission of ZP1 (1 μM) in the presence of 4 μM CP-Ser or native CP and 200 μM Ca(II) (75 mM HEPES, 100 mM NaCl, pH 7.0) after >12 h of incubation. The integrated emission values were normalized to that of the Fe(II)-free sample (mean ± SDM, *n* = 3).



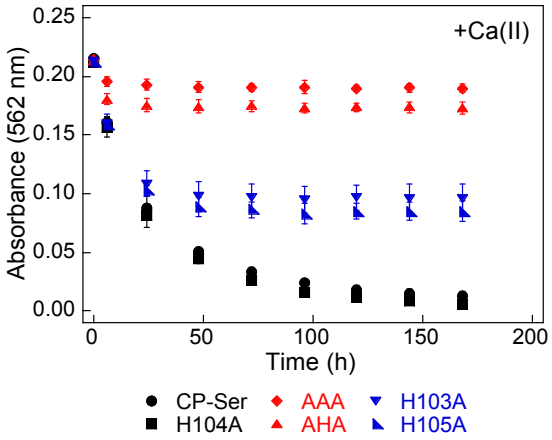
Supplementary Figure 18. Emission of ZP1 (1 μM) in the presence of 4 μM CP-Ser and 200 μM Ca(II) with 3 mM BME in the buffer (75 mM HEPES, 100 mM NaCl, pH 7.0) after >12 h of incubation. The integrated emission values were normalized to that of the Fe(II)-free sample (mean ± SDM, *n* = 3).



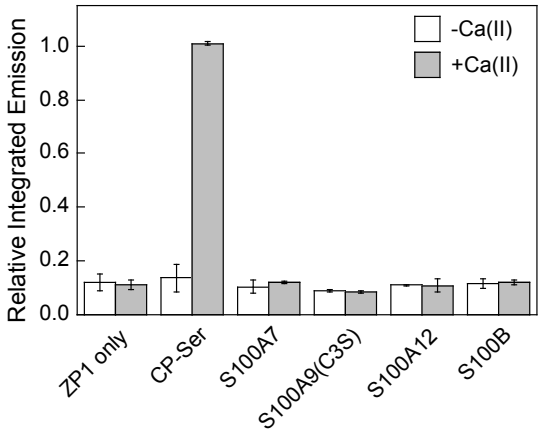
Supplementary Figure 19. Emission of ZP1 (1 μM) in the presence of 4 μM CP-Ser and 200 μM Ca(II) using $FeCl_2$ as the Fe(II) salt (75 mM HEPES, 100 mM NaCl, pH 7.0) after >12 h of incubation. The integrated emission values were normalized to that of the Fe(II)-free sample (mean ± SDM, *n* = 3).



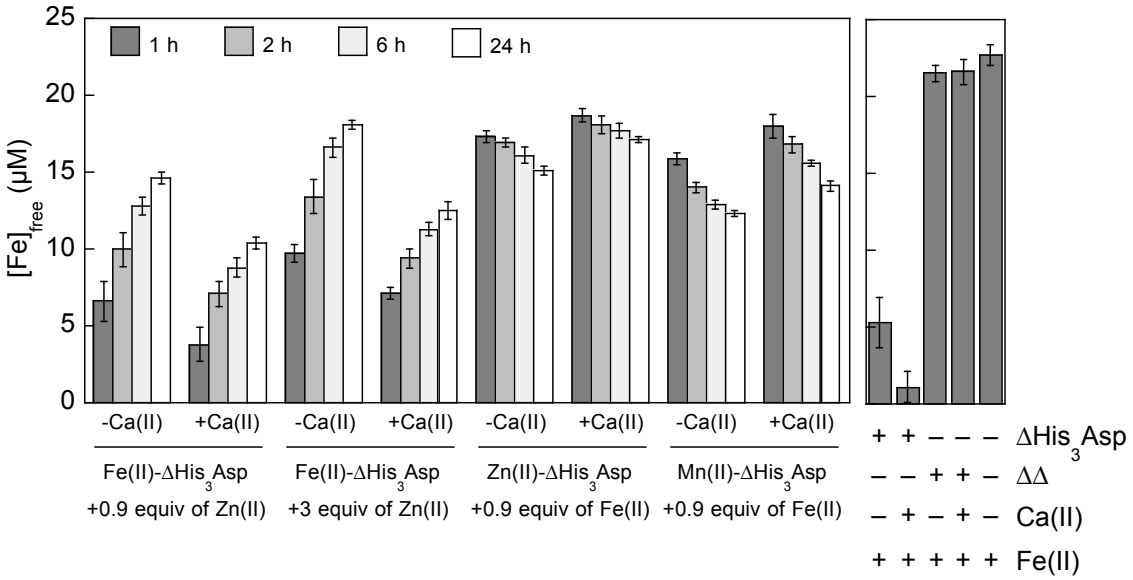
Supplementary Figure 20. ZP1 Fe(II) competition titrations with CP-Ser (75 mM HEPES, 100 mM NaCl, pH 7.0). (**A, B**) The titration with CP-Ser without Ca(II) exhibits similar behavior to the ZP1 only control, signifying that ZP1 outcompetes CP-Ser for Fe(II) under these conditions. CP-Ser and CP-Ser ΔHis_3Asp with 50 equiv of Ca(II) compete with ZP1 for Fe(II). CP-Ser ΔHis_4 and CP-Ser $\Delta\Delta$ with 50 equiv of Ca(II) exhibit outcompetition by ZP1 for Fe(II). Enhanced competition is observed for AAA, AHA, H103A, H104A, and H105A in the presence of 50 equiv of Ca(II). ZP1 emission was recorded after ~30 min of incubation following each addition of Fe(II). (**C**) Relative integrated emission values of ZP1 and protein after addition of 0.5 (white bars) and 1.0 equiv of Fe(II) (gray bars). The integrated emission values were normalized to that of the Fe(II)-free sample (mean ± SDM, *n* = 3).



Supplementary Figure 21. Competition for Fe(II) between ferrozine and CP-Ser or tail variant monitored by absorbance at 562 nm over time in the presence of 50 equiv Ca(II) relative to protein in 75 mM HEPES, 100 mM NaCl, pH 7.0 (mean ± SDM, *n* = 3).



Supplementary Figure 22. ZP1 (1 μM) emission response to Fe(II) in the presence of human S100 proteins (4 μM) in the absence (white bars) and presence (gray bars) of 50 equiv Ca(II) relative to protein after >12 h of incubation (mean ± SDM, *n* = 3).



Supplementary Figure 23. The concentration of unbound Fe(II) in CP-Ser ΔHis_3Asp samples containing Fe(II) and either Zn(II) or Mn(II) in the absence and presence of 50 equiv of Ca(II) in 75 mM HEPES, 100 mM NaCl, pH 7.0 (mean ± SDM, *n* = 3). The metals and the order of addition are described below each plot. The first and third set of samples are described in the main text and are provided here for comparison. Control samples without Zn(II) or Mn(II) are described in the right panel, also provided in the main text.

Supplementary Note

Determination of Fe(II) dissociation constant ($K_{d,Fe(II)}$) of Zinpyr-1 (ZP1). All buffers (75 mM HEPES, 100 mM NaCl, pH 7.0) were deoxygenated using a stream of N_2 or Ar for at least 1 h and stored in an anaerobic glove box. The experiments were conducted under strictly anaerobic conditions. Fe(II) buffering solutions (1 mL) were prepared with 1 mM ethylenediaminetetraacetic acid (EDTA), nitrilotriacetic acid (NTA), tris(2-pyridylmethyl)amine (TPA), or citrate in 75 mM HEPES, 100 mM NaCl, pH 7.0. The ligand was added from 100-mM stock solutions. EDTA, NTA, and citrate were prepared with Milli-Q water and a minimal amount of NaOH for dissolving the compound, and TPA was prepared in DMSO. Fe(II) was added to the buffering solutions from a 100-mM stock solution or a solution serially diluted therefrom. The free Fe(II) concentrations ranged from 10^{-16} to 10^{-7} M. Details of the buffering conditions are described in the Supplementary Information.

To these buffering solutions, 1 μM ZP1 was added from a 2-mM DMSO stock solution, and the samples were allowed to incubate in microcentrifuge tubes for 8 h in the dark. These solutions were transferred to screw-top quartz cuvettes in the anaerobic glove box. The cuvettes were sealed and removed from the glove box, and the emission of each sample was measured. The cuvettes were washed with nitric acid and Milli-Q water prior to each use to prevent metal-ion contamination. For each trial, the emission of a solution containing 1 μM ZP1 without Fe(II) was measured and integrated, and the integrated emission of all other solutions were normalized to this value. Excitation was provided at 490 nm, and the emission was scanned and integrated from 500 to 650 nm (10 nm/sec). The excitation and emission slit widths were set to 1.6 nm.

The relative integrated emission of each solution was plotted against the free Fe(II) concentration, and the data were fit to the following equation using the curve fit program in KaleidaGraph (Synergy Software):

$$F = 1 - R * \frac{R_{\min} * [\mathrm{Fe(II)}]_{\mathrm{free}}}{K_{\mathrm{d1,Fe(II)}} + [\mathrm{Fe(II)}]_{\mathrm{free}}} - (1 - R) * \frac{R_{\min} * [\mathrm{Fe(II)}]_{\mathrm{free}}}{K_{\mathrm{d2,Fe(II)}} + [\mathrm{Fe(II)}]_{\mathrm{free}}}$$

Where *F* is the relative integrated emission,

R is the relative turn-off response of the first binding site,

$R_{\min}$ is the relative turn-off response at minimum fluorescence,

$[\mathrm{Fe(II)}]_{\mathrm{free}}$ is the free Fe(II) concentration,

$K_{\mathrm{d1,Fe(II)}}$ is the Fe(II) dissociation constant at the first binding site, and

$K_{\mathrm{d2,Fe(II)}}$ is the Fe(II) dissociation constant at the second binding site.

A two-site binding model was employed because ZP1 binds 2 equiv Fe(II).

ZP1 Fe(II) Competition Experiments. The following procedures were modified from protocols designed for ZP1 competitions with Mn(II).[5,6] Precautions to prevent oxygen and metal-ion contamination were taken as described above. Samples containing ZP1 were incubated wrapped in aluminum foil and handled in the dark. The excitation wavelength, emission integration, and fluorometer slit widths are given above.

To evaluate the Ca(II) dependence of Fe(II) competition between ZP1 and CP-Ser, Ca(II) was titrated from a 10 mM Ca(II) stock solution into a solution of 1 μM ZP1, 4 μM CP-Ser, and 3.5 μM Fe(II) up to 200 μM Ca(II). After each addition of Ca(II), the mixture was allowed to equilibrate 4–12 h before measuring the emission. At the end of the titration, 4 μM Fe(II) was added to ensure the turn-off response of the sensor. The integrated emission was normalized to that of the ZP1 and CP-Ser solution prior to addition of Fe(II).

For the competition titrations with CP-Ser and mutants, solutions of 1 μM ZP1 and 4 μM CP in the absence or presence of 200 μM Ca(II) were prepared. Fe(II) was titrated into each sample, allowing ~30 min incubation between each point. The metal-ion competition between ZP1 and CP requires >4 h incubation to reach equilibrium. However, the relative binding affinities of CP mutants were apparent after a shorter incubation period, and therefore, this ~30

min incubation time was employed for these competition titrations. The integrated emission of each sample was normalized to that of the Fe(II)-free sample. Titrations of a "ZP1 only" negative control and a "CP-Ser +Ca(II)" positive control were conducted in parallel for every trial. Each titration was conducted three times, and the mean ± SDM are reported (*n* = 3).

For the competition assays with other human S100 proteins, a solution of 1 μM ZP1 and 4 μM protein was incubated with 2 μM Fe(II) in the absence and presence of 200 μM Ca(II). The emission spectra were recorded at 12 h after Fe(II) addition and normalized to that of the Fe(II)-free samples.

For all other ZP1 competition assays, solutions of 1 μM ZP1 and 4 μM protein were incubated with 0, 1, 2, 3, 4, 5, 6, 7, or 8 μM Fe(II) in the presence of 200 μM Ca(II). The emission spectra were recorded >12 h after Fe(II) addition and normalized to that of the Fe(II)-free samples. The mean ± SDM are reported (*n* = 3).

Ferrozine Fe(II) Competition. Solutions (2 mL) of 300 μM ferrozine, 8 μM Fe(II), and 500 mM Ca(II) were prepared in 75 mM HEPES, 100 mM NaCl, pH 7.0, and the samples containing the $[Fe(II)ferrozine_3]^{2+}$ complex were allowed to equilibrate for 30 min. The optical absorption spectrum of each sample was collected (t = 0 h). Buffer-exchanged CP was added to each solution to a final concentration of 10 μM. The optical absorption spectrum of each mixture was recorded at t = 6 h and 24-h intervals over the course of 7 days. To evaluate the competition between ferrozine and CP, the absorbance at λ = 562 nm was plotted against time. Three independent trials are reported (mean ± SDM, *n* = 3). To confirm that ferrozine was still able to bind Fe(II) in the presence of protein at the end of 7 days, 10 μM Fe(II) was added to each sample, and an increase in the absorbance at 562 nm to the original value was observed.

Supplementary References

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