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Interdependence of free zinc changes and protein complex assembly – insights into cellular zinc signal regulation

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Contribution from

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Experimental details

Materials

Dansyl chloride, 5(6)-carboxyfluorescein (FAM), 5(6)-carboxytetramethylrhodamine (TAMRA), Fmoc-Asp(OtBu)-(Dmb)-Gly-OH dipeptide and 1-cyano-2-ethoxy-2-oxoethylidenaminooxy)dimethylamino-morpholino-carbenium hexafluorophosphate (COMU) were purchased from NovaBiochem. Thioanisole, 1,2-ethanedithiol (EDT), anisole, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), *N*-(2-hydroxyethyl)ethylene-diamine-*N*,*N*′,*N*′-triacetic acid (HEDTA), ethylene-bis(oxyethylenenitrilo)tetraacetic acid (EGTA), $ZnSO_4 \cdot 7H_2O$, 4-(2-pyridylazo)resorcinol (PAR), 5,5'-dithiobis(2-nitrobenzoic acid (DTNB) and isopropyl β-D-1-thiogalactopyranoside (IPTG) were purchased from Sigma-Aldrich. Tryptone, yeast extract, glycerol, anhydrous K_2HPO_4, anhydrous KH_2PO_4, guanidine hydrochloride (GdnHCl) and DTT were from BioShop. The metal-chelating resin Chelex 100 was from Bio-Rad. Tris base and DTT were obtained from Carl Roth GmbH + Co. KG. Chitin resin was purchased from New England Biolabs. NaOH, acetonitrile (ACN), NaCl, NaOH, HCl, acetic acid, ethyl ether (Et_2O) and ethylenediaminetetraacetic acid (EDTA) were from Avantor Performance Materials Poland (Gliwice, Poland). Tris(2-carboxyethyl)phosphine hydrochloride (TCEP), *N,N*-dimethylformamide (DMF), dichloromethane (DCM), 1-methyl-2-pyrrolidinone (NMP), *N,N,N′,N*′-tetramethyl-*O*-(1*H*-benzotriazol-1-yl)uronium hexafluorophosphate (HBTU), trifluoroacetic acid (TFA), *N,N*-diisopropylethylamine (DIEA), piperidine, TentaGel R Ram and all Fmoc-protected amino acids were obtained from Iris Biotech GmbH (Marktredwitz, Germany).

Peptide synthesis

The following peptides were synthesized on TentaGel R RAM resin (substitution 0.19 mmol/g, Rapp Polymere GmbH) using a Liberty 1 microwave-assisted synthesizer (CEM) as described before[1] with some modifications: LIM (LIM domain fragment of human PDLIM1 protein) amide with N-terminal acetyl group,[2] ZF133 (11th zinc finger of ZF133 protein) amide with N-terminal dansyl moiety and Lys to Trp residue substitution (Table S1),[3,4] HK (14-amino acid zinc hook fragment from pfRad50 protein) amide with N-terminal dansyl moiety and C-terminal Trp residue,[5] CD4 (38-amino acid fragment of cytoplasmic tail from CD4 protein) amide with N-terminal FAM, and Lck (29-amino acid fragment of Src-family tyrosine kinase Lck) amide with N-terminal TAMRA.[6] Fmoc-Asp(OtBu)-(Dmb)-Gly-OH dipeptide was used for the synthesis of Lck peptide in order to prevent aspartimide formation.[7] HK and ZF133 peptides were labeled on-resin using 4 eq. dansyl chloride, 6 eq. HBTU and 6 eq. DIEA in DMF. CD4 and Lck peptides were N-terminally on-resin labeled with 4 eq. of 5(6)-carboxyfluorescein (FAM) or 5(6)-carboxytetramethylrhodamine (TAMRA), respectively using 4 eq. COMU and 6 eq. DIEA in DMF. Peptide cleavage was carried out using a mixture of 90% trifluoroacetic acid, 5% thioanisole, 3% EDT and 2% anisole over a period of 2 h, followed by precipitation of cold (-80°C) Et_2O. Peptide crudes were purified on HPLC (Dionex Ultimate 3000) on a Phenomenex C18 column using a gradient of 0.1% TFA in water and ACN. The identity of peptides was finally confirmed by mass spectrometry using an API 2000 Applied Biosystems instrument.

Table S1. Peptides used in this work, shortcut names, amino acid sequences, found and calculated molecular weights.

Peptide /protein	Amino acid sequence[a]	MW Found	MW Calculated
CD4	FAM-RCRHRRRQAERMSQIKRL LSEKKTCQCPHRFQKTCSPI-NH_2	5054.2	5054.9
Lck	TAMRA-SHPEDDWMENIDVCEN CHYPIVPLDGKGT-NH_2	3725.6	3726.1
HK	Dns-AKGKCPVCGRELTDW-NH_2	1894.8	1896.1
ZF	Dns-PMVCGECGRGFSQWSNLVA HQRTHSGER-NH_2	3362.0	3362.7
LIM	Ac-LPMCDKCGTGIVGVFVKLRD RHRHPECYVCTDCGTNLKQK GHFFVEDQIYCEKHARERV-NH_2	6908.5	6909.0
Apo-MT2a	MDPNCSCAAGDSCTCAGSCKCK ECKCTSCKKSCCSCCPVGCAKC AQGCICKGASDKCSCCA	6042.0	6042.3

[a] FAM-, TAMRA-, Dns- and -NH_2 denote 5(6)-carboxyfluorescein, 5(6)-carboxytetramethylrhodamine, dansyl and amid moiety, respectively. The underlined Trp residues refers to the additional C-terminal one and mutated from Lys amino acid residue in the sequence of HK and ZF, respectively.

Table S2. Raw fluorescence intensity (arbitrary units) data from FRET measurements of ZF formation in different concentrations in EDTA and HEDTA metal buffers.

Total Zn(II)	10 μM		3 μM		1 μM		0.5 μM	
(mM)	354 nm	540 nm	354 nm	540 nm	354 nm	540 nm	354 nm	540 nm
0.025	867420	619586	300314	157232	823384	437970	839860	443198
0.05	840858	830858	299514	158473	822093	434970	827909	438047
0.1	832280	535066	291066	152906	842082	442970	804474	422901
0.2	798056	529284	277078	155154	774176	445424	784214	445084
0.3	766438	546278	266926	158752	726418	453410	751423	457693
0.4	727890	533622	265194	164734	699778	456986	741983	472432
0.5	692582	566688	251366	170000	675800	471454	692607	475681
0.6	658336	591318	228324	170306	664618	478824	635079	465482
0.7	597534	570782	212802	174926	636416	485052	610564	482930
0.8	525652	814814	190416	179884	559768	498326	525301	481520
0.9	462666	616372	171294	183866	501860	517956	462203	486389
0.1	567652	609300	203980	174390	537290	501046	567555	506290
0.2	468414	635516	175324	186392	546564	513374	506607	505276
0.3	430558	618324	158416	191340	460140	524472	459710	539164
0.4	404976	661842	153410	194126	444014	527272	445826	546236
0.5	389920	634868	150650	196002	503964	530712	415000	483060
0.6	368484	661610	145592	190848	440826	537856	400968	506763
0.7	366294	676806	140140	196210	424310	528606	408090	538047
0.8	346180	653292	136386	196844	422566	542020	380068	516229

Table S3. Raw CD data (mdeg) measurements of LIM domain formation in different concentrations in EDTA and HEDTA metal buffers.

Total Zn(II) (mM)	5 µM	10 µM	15 µM	20 µM
	220 nm	220 nm	220 nm	220 nm
0.025	-0.411	-1.336	-2.209	-4.489
0.05	-0.467	-1.537	-2.581	-5.093
0.1	-0.650	-2.025	-3.342	-6.378
0.2	-0.919	-2.858	-4.628	-8.202
0.3	-1.241	-3.124	-5.543	-9.459
0.4	-1.484	-3.585	-5.993	-11.466
0.5	-1.653	-3.830	-6.626	-11.811
0.6	-1.673	-3.906	-6.950	-12.351
0.7	-1.727	-4.020	-6.969	-12.698
0.8	-1.751	-4.091	-7.208	-12.844
0.9	-1.736	-4.070	-7.174	-12.937
0.1	-1.691	-3.981	-7.095	-12.375
0.2	-1.698	-3.984	-7.088	-12.696
0.3	-1.703	-4.030	-7.052	-12.965
0.4	-1.695	-4.049	-7.031	-12.787
0.5	-1.698	-4.059	-7.088	-13.039
0.6	-1.672	-4.089	-7.319	-12.961
0.7	-1.699	-4.073	-7.185	-12.892
0.8	-1.717	-4.059	-7.176	-12.920

Table S4. Raw fluorescence intensity (arbitrary units) data from FRET measurements of zinc hook metalation process in different concentrations in EDTA and HEDTA metal buffers.

Total Zn(II)	10 μM		5 μM		1 μM		0.5 μM	
(mM)	354 nm	540 nm	354 nm	540 nm	354 nm	540 nm	354 nm	540 nm
0.025	1359740	510270	889014	320480	1007880	345458	924702	331979
0.05	1322310	534288	856648	333342	988118	380045	884484	347093
0.1	1219140	616194	823750	339918	968188	356066	859818	362232
0.2	1125590	694926	724748	373548	944380	391942	841212	422763
0.3	1003560	708390	670170	396810	903014	394028	826034	459135
0.4	929558	724544	617104	412300	866638	415160	815160	502444
0.5	900274	749954	587076	431916	819456	420126	780234	518982
0.6	845360	754166	555334	439228	802196	445810	727926	521851
0.7	845762	790428	523170	445284	751992	457054	692050	533684
0.8	784914	781798	486826	457114	706856	469858	670312	562753
0.9	762532	809746	450824	469940	647766	478944	646506	596132
0.1	816458	758850	510260	455576	698424	457190	688064	554355
0.2	753030	804482	458646	471138	657542	483146	621234	569854
0.3	730004	833632	440000	486150	591540	496086	595712	601087
0.4	693432	846070	425175	480424	600208	503696	559770	579832
0.5	693432	844974	415750	484212	605084	510342	568558	595367
0.6	697752	849294	408138	484149	568240	511896	554816	599880
0.7	676692	846070	405854	490933	575958	523124	542970	597217
0.8	678212	849092	399412	495768	561346	530914	540104	608748

Table S5. Raw fluorescence intensity (arbitrary units) data measurements of zinc clasp metalation process in different concentrations in EDTA, HEDTA and EGTA metal buffers.

Total Zn(II)	1 μM		0.5 μM		0.25 μM		0.1 μM	
(mM)	525 nm	573 nm	525 nm	573 nm	525 nm	573 nm	525 nm	573 nm
0.025	465974	127518	778348	217870	885050	351468	823932	253651
0.1	469760	129228	787502	221248	1083580	409178	834638	254393
0.3	446502	124188	782068	220472	1125100	422506	827122	253058
0.4	435740	121952	765030	217430	1159470	432160	827861	254087
0.1	346878	102784	654494	192664	1089150	411704	823805	262432
0.2	311576	94084	587144	177272	1038970	400944	803617	261620
0.3	289846	88054	540286	167912	987376	394306	784299	261043
0.4	271394	84874	499484	158104	983104	375360	776436	259506
0.5	256968	81740	462400	148998	917780	378090	722672	249929
0.6	244420	78338	432792	143356	978680	389302	712578	247051
0.7	233364	75882	400678	134616	781524	336014	687393	246152
0.8	220014	72790	362054	128306	694228	321438	604697	226644
0.9	220280	73414	328870	118390	429666	240854	539009	213827
0.1	209116	68540	302498	109964	329868	213716	383858	156775
0.2	222574	72104	326892	117898	444056	244488	374644	146426
0.3	221918	72910	324506	117810	430198	234318	368960	144990
0.4	215846	71346	325326	117050	398826	232840	365581	145890
0.5	219488	71332	321472	115334	370954	218016	355275	140874

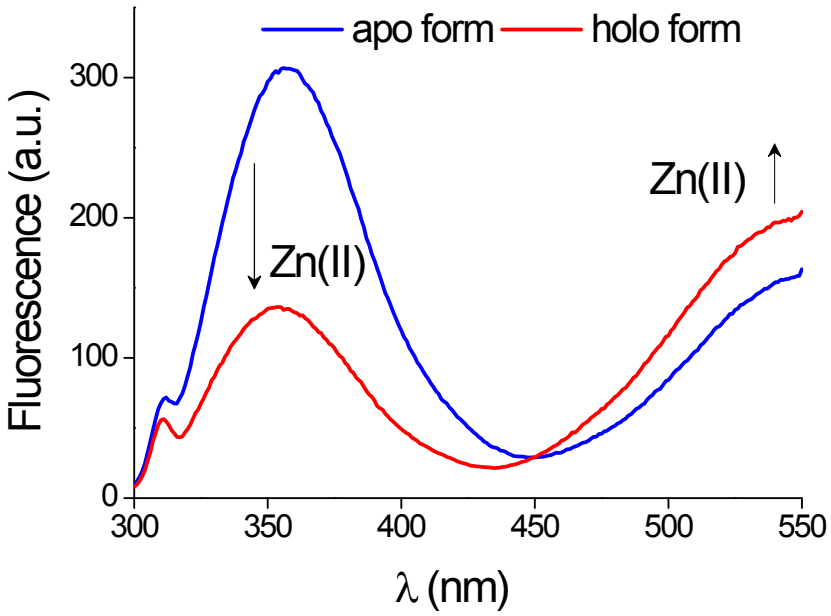

Figure S1. Fluorescence spectra of zinc finger domain measurements in the apo (black) and holo (red) form.

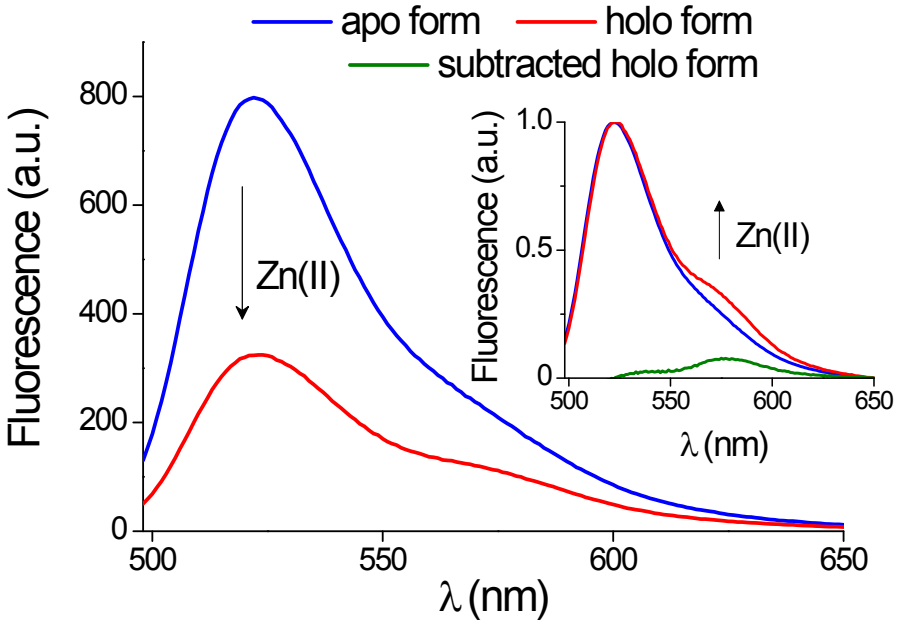

Figure. S2. Fluorescence spectra of zinc clasp domain measurements. Spectra of the apo and holo form are indicated in black and red, respectively. Blue color represents the subtracted spectra. The inset presents spectra with normalized fluorescence, where the growth of acceptor emission in the holo form is more noticeable.

All of the pZn (-log[Zn(II)]$_{free}$) calculations were obtained based on the published protonation and Zn(II) stability constants of EDTA

$$\beta_{HL} = 10.17, \beta_{H_2L} = 16.28,\ \beta_{H_3L} = 18.96, \beta_{H_4L} = 20.96, \beta_{H_5L} = 22.47, \beta_{ZnHL}^{EDTA} = 19.4$$
$$= 16.44), HEDTA\left(\beta_{HL} = 9.81, \beta_{H_2L} = 15.18,\ \beta_{H_3L} = 17.78, \beta_{ZnL}^{HEDTA} = 14.6\right)$$
$$and\ EGTA\ (\beta_{HL} = 9.40, \beta_{H_2L} = 18.18, \beta_{ZnL}^{EGTA} = 12.60$$

) and were performed using MINEQL 4.6 software.[8, 9]

Table S6. Composition of metal ion buffers used for the equilibration of fluorogenic zinc finger peptide (ZF133-11) at various concentrations (0.5–10 μM). Buffers contained 1.0 mM EDTA or 1.0 mM HEDTA and $ZnSO_4$ in the range of 0.025–0.9 mM in 50 mM HEPES, 100 mM NaCl, 200 μM TCEP, pH 7.4.

Total Zn(II) (mM)	Initial pZn values of metal buffers	Corrected pZn values for Zn(II) transferred to ZF			
		10 μM	3 μM	1 μM	0.5 μM
0.025	15.20	15.21	15.20	15.20	15.20
0.05	14.89	14.90	14.89	14.89	14.89
0.1	14.56	14.57	14.56	14.56	14.56
0.2	14.21	14.21	14.21	14.21	14.21
0.3	13.98	13.98	13.98	13.98	13.98
0.4	13.78	13.78	13.78	13.78	13.78
0.5	13.61	13.61	13.61	13.61	13.61
0.6	13.43	13.43	13.43	13.43	13.43
0.7	13.24	13.25	13.24	13.24	13.24
0.8	13.00	13.02	13.01	13.00	13.00
0.9	12.65	12.68	12.66	12.65	12.65

0.1	13.14	13.17	13.15	13.14	13.14
0.2	12.79	12.81	12.79	12.79	12.79
0.3	12.55	12.57	12.55	12.55	12.55
0.4	12.36	12.38	12.36	12.36	12.36
0.5	12.18	12.20	12.18	12.18	12.19
0.6	12.01	12.02	12.01	12.01	12.01
0.7	11.82	11.83	11.82	11.82	11.82
0.8	11.58	11.61	11.59	11.58	11.58

Table S7. Composition of metal ion buffers used for the equilibration of LIM domain (LIM) at various concentrations (5–20 μM). Buffers contained 1.0 mM EDTA or 1.0 mM HEDTA and $ZnSO_4$ in the range of 0.025–0.9 mM in 50 mM HEPES, 100 mM NaCl, 200 μM TCEP, pH 7.4.

Total Zn(II) (mM)	Initial pZn values of metal buffers	Corrected pZn values for Zn(II) transferred to LIM			
		20 μM	15 μM	10 μM	5 μM
0.025	15.20	15.22	15.21	15.19	15.22
0.05	14.89	14.91	14.89	14.90	14.91
0.1	14.56	14.58	14.58	14.59	14.60
0.2	14.21	14.23	14.24	14.25	14.25
0.3	13.98	14.00	14.00	14.00	14.00
0.4	13.78	13.81	13.81	13.81	13.82
0.5	13.61	13.63	13.63	13.63	13.64
0.6	13.43	13.46	13.45	13.46	13.47
0.7	13.24	13.27	13.27	13.28	13.27
0.8	13.00	13.05	13.05	13.07	13.07
0.9	12.65	12.70	12.70	12.69	12.70

0.1	13.14	13.20	13.16	13.19	13.17
0.2	12.79	12.79	12.81	12.83	12.81
0.3	12.55	12.56	12.54	12.55	12.54
0.4	12.36	12.35	12.34	12.35	12.34
0.5	12.18	12.17	12.15	12.16	12.17
0.6	12.01	12.04	12.01	12.03	12.03
0.7	11.82	11.84	11.83	11.81	11.81
0.8	11.58	11.64	11.60	11.62	11.60

Table S8. Composition of metal ion buffers used for the equilibration of fluorogenic zinc hook peptide (HK) at various concentrations (0.5–10 μM). Buffers contained 1.0 mM EDTA or 1.0 mM HEDTA and $ZnSO_4$ in the range of 0.025–0.9 mM in 50 mM HEPES, 100 mM NaCl, 200 μM TCEP, pH 7.4.

Total Zn(II) (mM)	Initial pZn values of metal buffers	Corrected pZn values for Zn(II) transferred to ZH			
		10 μM	5 μM	1 μM	0.5 μM
0.025	15.20	15.21	15.21	15.20	15.20
0.05	14.89	14.90	14.90	14.89	14.89
0.1	14.56	14.57	14.56	14.56	14.56
0.2	14.21	14.22	14.21	14.21	14.21
0.3	13.98	13.98	13.98	13.98	13.98
0.4	13.78	13.79	13.78	13.78	13.78
0.5	13.61	13.61	13.61	13.61	13.61
0.6	13.43	13.44	13.43	13.43	13.43
0.7	13.24	13.25	13.25	13.24	13.24
0.8	13.00	13.02	13.01	13.00	13.00
0.9	12.65	12.67	12.66	12.65	12.65

0.1	13.14	13.16	13.15	13.14	13.14
0.2	12.79	12.80	12.80	12.79	12.79
0.3	12.55	12.56	12.55	12.55	12.55
0.4	12.36	12.37	12.36	12.36	12.36
0.5	12.18	12.19	12.18	12.18	12.18
0.6	12.01	12.02	12.01	12.01	12.01
0.7	11.82	11.83	11.82	11.82	11.82
0.8	11.58	11.59	11.58	11.58	11.58

Table S9. Composition of metal ion buffers used for the equilibration of equimolar Lck and CD4 peptides (ZC domain) at various concentrations (0.25–1 μM). Buffers contained 1.0 mM EDTA, 1 mM HEDTA or 1.0 mM EGTA and $ZnSO_4$ in the range of 0.025–0.9 mM in 50 mM HEPES, 100 mM NaCl, 50 μM TCEP, pH 7.4.

Total Zn(II) (mM)	pZn values of metal buffers	Corrected pZn values for Zn(II) transferred to ZC			
		1 μM	0.5 μM	0.25 μM	0.1 μM
0.025	15.20	15.20	15.20	15.20	15.20
0.1	14.56	14.56	14.56	14.56	14.56
0.3	13.98	13.98	13.98	13.98	13.98
0.4	13.78	13.78	13.78	13.78	13.78
0.1	13.14	13.14	13.14	13.14	13.14
0.2	12.79	12.79	12.79	12.79	12.79
0.3	12.55	12.55	12.55	12.55	12.55
0.4	12.36	12.36	12.36	12.36	12.36
0.5	12.18	12.18	12.18	12.18	12.18
0.6	12.01	12.01	12.01	12.01	12.01
0.7	11.82	11.82	11.82	11.82	11.82
0.8	11.58	11.58	11.58	11.58	11.58

0.9	11.23	11.23	11.23	11.23	11.23
0.1	10.16	10.16	10.16	10.16	10.16
0.2	9.80	9.81	9.81	9.81	9.81
0.3	9.57	9.57	9.57	9.57	9.57
0.4	9.38	9.38	9.38	9.38	9.38
0.5	9.20	9.20	9.20	9.20	9.20

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