Supporting Information

Split Ligand for Lanthanide Binding: Facile Evaluation of Dimerizing Proteins

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I. General methods

All Fmoc-protected natural amino acids were purchased from Advanced Chemtech (Louisville, KY). Fmoc-perfluorophenylalanine (Fmoc-Z) was purchased from Chem-Impex Int'l Inc.(Wood Dale, IL). Fmoc-Gly-Wang resin was purchased from Novabiochem (San Diego, CA). All other chemicals were purchased from Sigma-Aldrich (Milwaukee, WI). Peptide synthesis was finished on a Tribute peptide synthesizer (Protein Technologies, Tucson, AZ). ^{1}H-NMR and ^{13}C-NMR data were collected on a Varian Gemini 500 MHz NMR spectrometer. HR-MS data were generated by Boston College Mass-Spec facilities. The concentration of all labeled proteins was determined in Tris buffer (10 mM Tris, 100 mM Sodium Citrate, pH=8.5) by measuring their absorption at 350 nm (ε = 13,405 M^{-1} cm^{-1}) on a Thermo Scientific NanoDrop 2000c (Wilmington, DE). All fluorescence data were collected on a Fluorolog spectrometer (Horiba Jobin Yvon FL3-22).

II. Synthesis of IA-HIP2

2-methoxy-N-methyl-3-(2-thioxothiazolidine-3-carbonyl)benzamide (3, Scheme 1)

2-methoxy-isophthalic acid (**1**, 2.4 g, 10 mmol) was mixed with 2-mercaptothiazoline (**2**, 2.4 g, 20 mmol) in 50 mL dichloromethane (DCM) at room temperature. The mixture was stirred for 5 hrs. Methylamine hydrochloride (670 mg, 10 mmol) was dissolved in 5 mL water, basified with 2 M NaOH to pH=10~11, and then added to the mixture. The resulting mixture was stirred for another 4 hrs. The organic layer was separated, and the aqueous layer was extracted with DCM (3 ×15 mL). The combined organic layer was dried over Na_2SO_4. The solvent was then evaporated under vacuum, and the product was purified by silica gel column with EtOAc as the eluent. The yield for the two steps combined is 30%. ^{1}H-NMR (500 MHz, $CDCl_3$) δ: 3.02 (d, J = 5.0 Hz, 3H), 3.45 (t, J = 7.5 Hz, 2H), 3.87 (s, 3H), 4.68 (t, J=7.5 Hz, 2H), 7.25 (t, J= 7.5 Hz, 1H), 7.40 (br, 1H) 7.42 (dd, J_1 = 7.5 Hz, J_2 = 1.5 Hz,), 8.13

(dd, , J_1 = 7.5 Hz, J_2 =2 Hz,). The NMR spectrum agrees with the previous report: Samuel *et al*.; *Inorganic Chemistry,* **2009**, 48, 687-698.

tert-Butyl (2-(bis(2-(2-methoxy-3-(methylcarbamoyl)benzamido)ethyl)amino)ethyl)carbamate (5)

Tris(2-aminoethyl)amine (75 µL, 0.5 mmol) was dissolve in 5 mL DCM. The solution was stirred in ice bath for 5 min. Compound **3** (311 mg, 1 mmol) was dissolved in 20 mL DCM, and added dropwise to the tris(2-aminoethyl)amine solution over 1 hr. The solution was kept stirring overnight. Boc anhydride (160 mg, 0.75 mnol) was added and the resulting mixture was allowed to react for 3 hrs. The solvent was then evaporated under vacuum, and the product was purified by silica gel column, firstly with EtOAc as eluent and then followed by 5 % methanol in DCM. Yield: 37 %. ^{1}H-NMR (500 MHz, $CDCl_3$) δ: 1.34 (s, 9H), 3.05 (d, J = 5.0 Hz, 6H), 3.55-3.68 (br, 6H), 3.84 (s, 6H), 3.93 (br, 4H), 7.25 (t, J = 8.0 Hz, 2H) 7.44 (br, 2H,), 7.90 (dd, J_1 = 8.0 Hz, J_2 =1.5 Hz, 2H), 8.03 (d, J = 7, 2H), 8.54 (br, 2H). ^{13}C-NMR (125 MHz, CD_3Cl) δ: 27.0, 28.1, 35.6, 54.5, 55.3, 63.6, 125.1, 125.3, 128.2, 134.3, 135.4, 156.4, 160.0, 166.5, 167.3. HRMS (ESI+): *m/z* calculated for $C_{31}H_{45}N_6O_8$ $[M]^+$,629.3299; found 629.3282.

N1,N1'-(((2-aminoethyl)azanediyl)bis(ethane-2,1-diyl))bis(2-hydroxy-N^3-methylisophthalamide) (6)

Compound **5** (30 mg, 0.048 mmol**)** was dissolved in 15 mL DCM under nitrogen, and stirred in ice bath for 5 min. BBr_3 (0.22 mL, 2.3 mmol) was added dropwisely under nitrogen protection. The mixture was stirred overnight and quenched with ice. The aqueous layer was separated and the organic layer was washed with 15 mL water. The combined aqueous layer was loaded onto an ion-exchange column (BIO-RAD AG® 50W-X8 resins). The column was washed with water first to elute the salts, and then followed by 5 - 20% ammonia hydrate to obtain the product. Yield:71 %. ^{1}H-NMR (500 MHz, D_2O) δ: 2.64 (t, J=2.5, 4H), 2.72 (s, 6H), 2.78 (t, J=8.0, 2H), 3.05 (t, J= 6.0, 2H), 3.35 (t, J=5.0, 4H), 6.58 (t, J = 8 Hz, 2H) 7.36 (dd, J_1 = 8.0 Hz, J_2 =1.5 Hz, 2H), 7.52 (dd, J_1 = 8.0 Hz, J_2 =1.0 Hz, 2H). ^{13}C-NMR (125 MHz, D_2O) δ: 25.4, 37.0, 37.2, 51.1, 52.0, 113.3, 119.1, 120.1, 133.2, 133.5, 160.2, 169.7, 170.3. (ESI+): *m/z* calculated for $C_{24}H_{33}N_6O_6$ $[M]^+$, 501.2462; found 501.2465.

N1,N1'-(((2-(2-iodoacetamido)ethyl)azanediyl)bis(ethane-2,1-diyl))bis(2-hydroxy-N^3-methylisophthalamide) (IA-HIP2, 7)

Compound 6 (8.2 mg, 0.016 mmol) and iodoacetic anhydride (6 mg, 0.017 mmol) were mixed in 1 mL methanol. The mixture was stirred for 1 hr and then purified by RP-HPLC with a prep scale C18 column. Yield: 81%. ^{1}H-NMR (500 MHz, CD_3OD) δ: 2.92 (s, 6H), 3.58 (t, J=6.0, 2H), 3.63 (t, J=5.0, 4H), 3.68 (t, J=6.0, 2H), 3.70 (s, 2H), 3.84 (t,

J=5.0, 4H), 6.85 (t, *J* = 8.0 Hz, 2H), 7.86 (t, *J* = 8.5 Hz, 4H). ^{13}C-NMR (125 MHz, CD_3OD) δ: 29.0, 38.7, 39.0, 57.8, 58.7, 120.3, 121.9, 122, 135.8, 137.8, 163.7, 164.0, 172.9, 173.2, 175.3. (ESI+): *m/z* calculated for $C_{26}H_{34}IN_6O_7$ $[M]^+$, 669.1529; found 669.1511.

III. Luminescence characterization of HIP2 and HIP4

HIP4 was synthesized according to the literature reported protocol (Petoud et al. *J. Am. Chem. Soc.* **2003**, *125*, 13324). The negative control HIP2 refers to Compound **6**, whose structure is shown in Scheme 1. To evaluate the lanthanide binding behavior of these compounds, we performed a titration experiment, in which HIP4 and HIP2 were titrated respectively into a terbium solution (Buffer: 10 mM Tris, 100 mM Sodium Citrate, pH = 8.5; $TbCl_3$: 500 μM) and the luminescence data were recorded (Figure S1). The results show a linear luminescence increase with HIP4 concentration in the nanomolar range, as expected given its high affinity to terbium. HIP2 shows greatly reduced terbium binding, affording little luminescence emission with concentrations up to 10 μM.

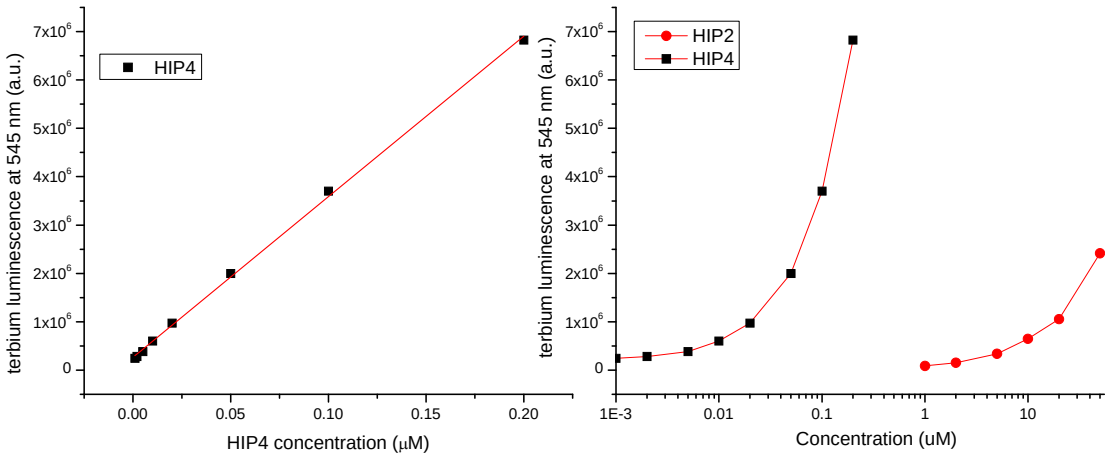

Figure S1. Comparison of HIP4 and HIP2 as sensitizers of terbium emission.

IV. Peptide synthesis, labeling and structural characterization

All peptides (sequences are shown below) were synthesized on Fmoc-Gly-Wang resin (126 mg, 0.10 mmol) using the standard Fmoc/tBu chemistry. Five equivalents of natural amino acids were used for the coupling reaction. Fmoc-pentafluorophenylalanine (Fmoc-Z) was used at three equivalents. The peptides were cleaved off the resin and fully deprotected with reagent K (80% TFA, 5% H_2O, 2.5% EDT, 5% Thioanisole and 7.5% Phenol) at room temperature for two hours. After filtration, the filtrate was treated with cold Et_2O to precipitate out the peptides. The precipitates were collected and dried under vacuum. The crude peptides were purified by using RP-HPLC (Waters Prep LC, Jupiter C4 Column).

α_2D-WT	: GEVEELEKK**F**KELWKGPRRGEIEELHKK**F**CELIKG
α_2D-ZZ	: GEVEELEKK**Z**KELWKGPRRGEIEELHKK**Z**CELIKG
GCN4-WT	: C**L**ENEVARLKKLVG
GCN4-L19P	: C**P**ENEVARLKKLVG

A purified peptide (~1 mg) was dissolved in 1 mL buffer (10 mM sodium phosphate, 100 mM NaCl, pH = 7.5). Concentration of the peptide solution was determined by UV-Vis absorption at 280 nm using the extinction coefficient of 5,680 $M^{-1}\cdot cm^{-1}$ for α_2D and 1,280 $M^{-1}\cdot cm^{-1}$ for GCN4. 10 equivalents of TCEP were added into the peptide solution to reduce the disulfide bond. Then 5 equivalents of IA-HIP2 and 5 μL DIPEA were add to the solution and the resulting mixture was stirred for 2 hrs. The progression of the reaction was monitored by analytical HPLC. After completion, the labeled peptide was purified by using a prep-HPLC with a C4 column. The identity of all labeled peptides was confirmed by ESI-MS analysis.

α_2D-WT	m/z calculated for $[M]^+$	4755,	found 4754
α_2D-ZZ	m/z calculated for $[M]^+$	4935,	found 4937
GCN4-WT	m/z calculated for $[M]^+$	4054,	found 4054
GCN4-L19P	m/z calculated for $[M]^+$	4038,	found 4038

Circular dichroism spectroscopy was used for the structural characterization of the peptide monomers and dimers. The wavelength scans were carried out at room temperature for α_2D and 2 °C for GCN4 (due to the low thermal stability of the GCN4 dimers). Thermal melting profiles in Figure S2 demonstrate that HIP2 labeling causes little perturbation to α_2D folding and dimerization.

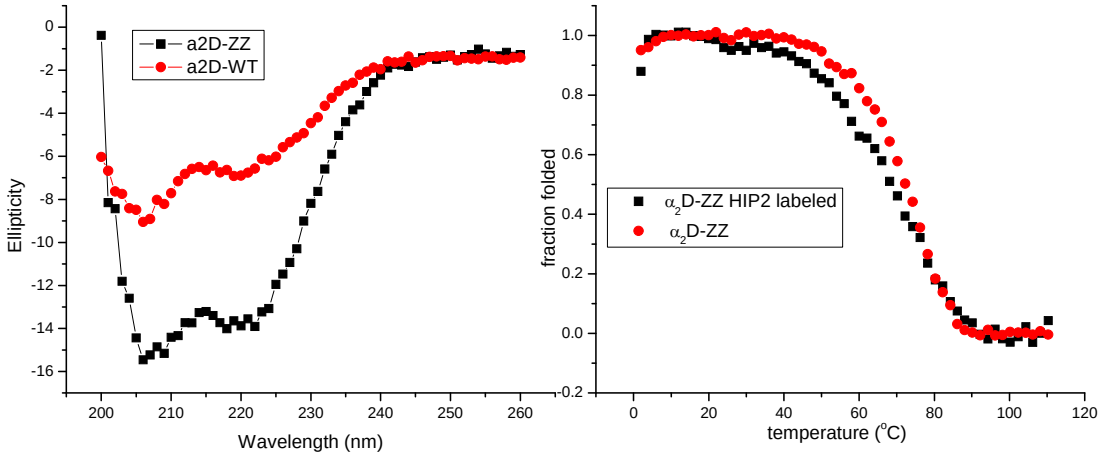

Figure S2. Characterization of the α2D variants with circular dichroism spectroscopy. Peptide concentration = 2 μM, Buffer: 10 mM sodium phosphate, 100 mM NaCl, pH = 7.5

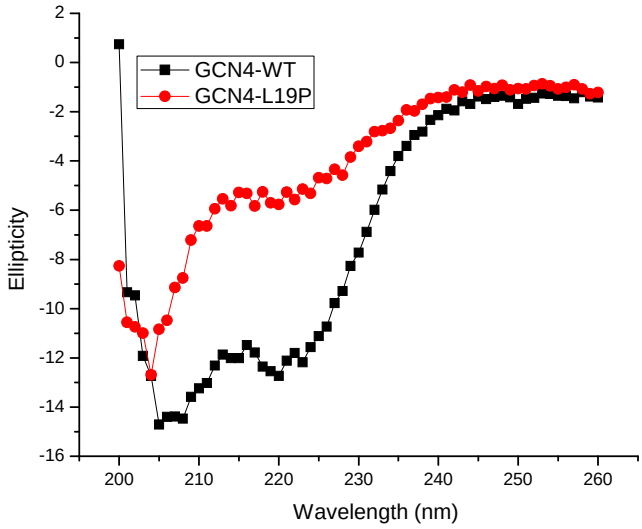

Figure S3. Characterization of the GCN4 variants with circular dichroism spectroscopy. Peptide concentration = 2 μM, Buffer: 10 mM sodium phosphate, 100 mM NaCl, pH = 7.5.

V. Luminescence analysis of dimerizing proteins

The stock solutions of the HIP2-labeled peptides and $TbCl_3$ were made in a Tris buffer (10 mM Tris, 100 mM Sodium Citrate, pH = 8.5) and then diluted and mixed to the concentrations needed. The stock concentration of terbium chloride was 20 mM. The concentration of peptides were determined by UV absorption at 350 nm with extinction coefficient of 13,405 $M^{-1} \cdot cm^{-1}$.

The concentration of terbium needed to saturate the peptide dimers was determined through a set of titration experiments, in which aliquots of the $TbCl_3$ stock were added into a HIP2-labeled peptide and the luminescence emission was recorded. The luminescence intensity at 545 nm was plotted against terbium concentration (Figure S4). The result showed a saturating profile for α_2D-ZZ with an apparent K_d of ~150 μM. The maximum luminescence intensity was achieved at the terbium concentration of ~500 μM. Therefore, this concentration was used for all the rest of experiments in this study. In contrast, α_2D-WT and HIP2 induced minimal luminescence within the concentration range investigated. Similar results were obtained for the GCN4 peptides.

The results of the α_2D system are shown in Figure 2 of the main text. The results of the GCN4 system are shown in Figure 3 and Figure S5 on next page.

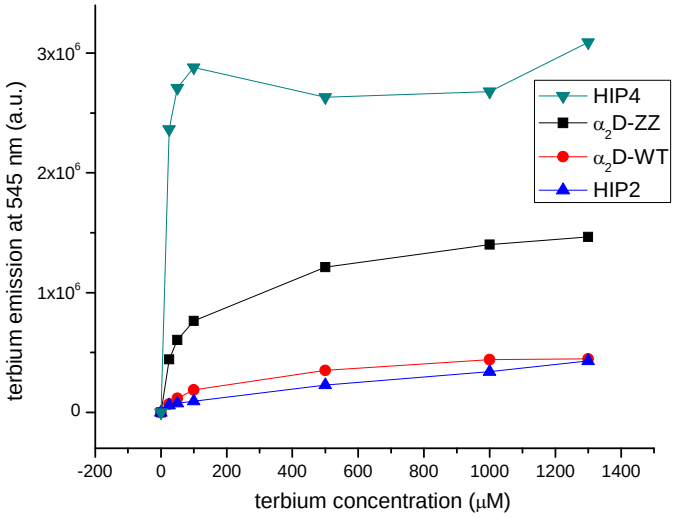

Figure S4. Titration experiments to determine the saturating concentrations of terbium for the peptide dimers.

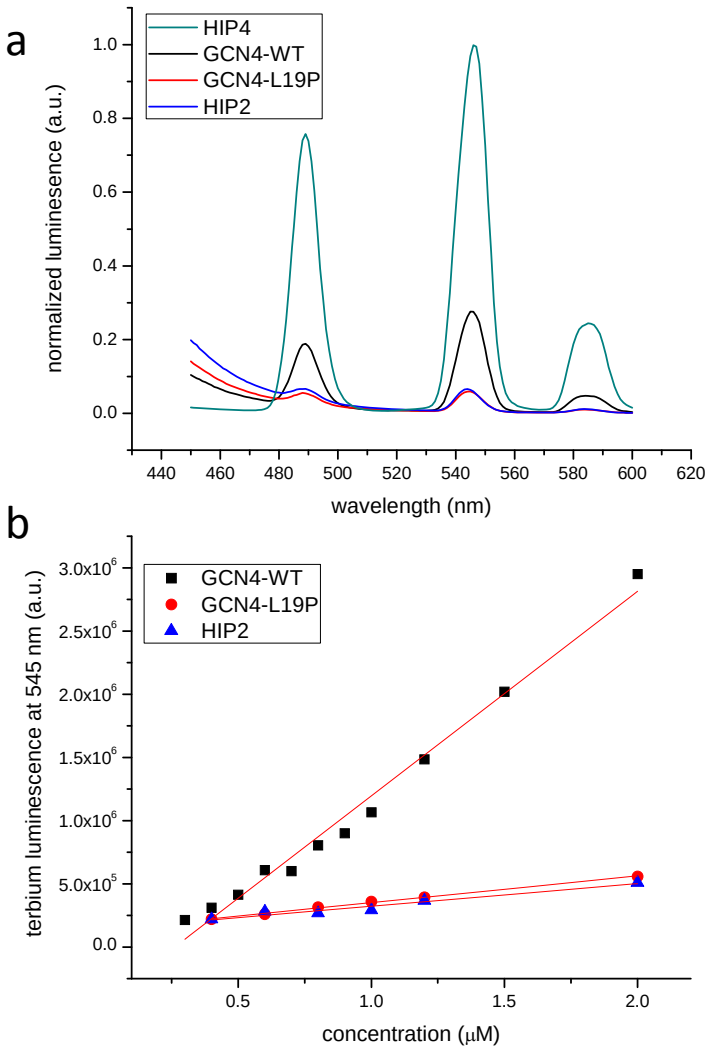

Figure S5. Characterization of GCN4 dimerization with terbium luminescence. a) Emission profiles of the GCN4 peptides and small molecule controls. The luminescence intensities were normalized against that of HIP4 at 545 nm. b) Concentration profiles of terbium luminescence showing a linear increase with the concentration of peptide dimers.

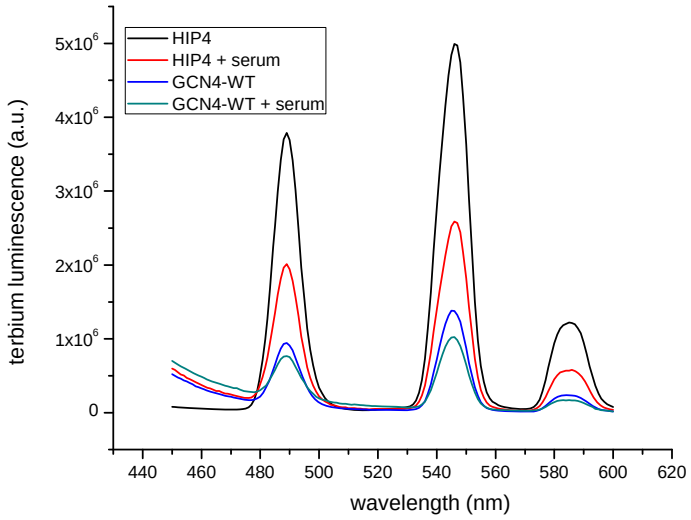

Figure S6. Terbium luminescence in absence and presence of bovine serum. Nonspecific quenching was observed for both HIP4 and GCN4-WT dimer.