*Supporting information*

# Controlling leucine-zipper partner recognition in cells through modifications of a-g interactions

Yusuke Azuma,[a] Tim Kükenshöner,[b] Guangyong Ma,[c] Jun-ichiro Yasunaga,[c] Miki Imanishi,[a] Gen Tanaka,[a] Ikuhiko Nakase,[a] Takahiro Maruno,[d] Yuji Kobayashi,[d] Katja M. Arndt,[b]* Masao Matsuoka,[c]* Shiroh Futaki [a] *

a. Institute for Chemical Research, Kyoto University
b. Institute for Biochemistry and Biology, University of Potsdam, Germany
c. Institute for Virus Research, Kyoto University
d. Graduate School of Engineering, Osaka University

## Materials and Methods

Materials

Fmoc (=9-fluorenylmethoxycarbonyl)-amino acids, 2-(1H-Benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU) and 1-hydroxybenzotriazole (HOBt) for peptide synthesis were purchased from Peptide Institute (Osaka, Japan). Other chemicals were purchased from Wako Pure Chemicals (Osaka, Japan), Sigma-Aldrich (MO, USA), Merck KGaA (Darmstadt, Germany), and VWR (PA, USA) unless otherwise stated. Restriction enzymes and buffers were from New England Biolabs (MA, USA). Primers to construct libraries were obtained from Microsynth (Balgach, Switzerland). Other primers were purchased from Invitrogen (CA, USA) and Sigma-Aldorich.

Cloning

*Plasmids for HiT selection*

The construction of the vectors used for the present work (pAK630_TorA-Helix and pAK632-Helix-bLac) were described elsewhere[1,2]. The gene of leucine zipper segment in *HBZ* was amplified from pME-ΔHBZ(WT)[3] by PCR using FW_*Nhe*I_HBZ (CACCATCACCATGCTAGCACTAGACAGTTGGAAGGCG; restriction site for *NheI* is underlined) and RV_*Asc*I_HBZ (GGACTTGGCGCGCCCGCCTCCAGCCTCCC; restriction site for *AscI* is underlined) as primers and then cloned into the pAK632-Helix-bLac via *Nhe*I and *Asc*I, resulting in pAK632-HBZ-bLac. The cJun library gene was constructed by partially randomized oligonucleotides; FW_*Nhe*I_cJunL: (GCATTAGCTAGCATTGCGCGCCTGGAAGAAAAA**R**TAAAAACCCTGAAAGCG**M**AAA **W**TTATGAACTGGCGAGC; restriction site for *NheI* is underlined) and RV_*Asc*I_cJunL: (CGATATCGGCGCGCCCATCACTTTCTGTTTC**WS**CTGCGCTA**Y**TT**K**TTCGCGCAGCAT GTTT**RY**T**KB**GCTCGCCAGTTCATAA; restriction site for *AscI* is underlined). Here, **R**, **M**, **W**, **S**, **Y**, **K**, and **B** represent mixtures of A/G, A/C, A/T, C/G, C/T, G/T, and C/G/T, respectively.

These fragments were annealed and filled-in by Klenow-fragment, and then cloned into pAK630-TorA-Helix via *Nhe*I and *Asc*I, resulting in pAK630-TorA-cJunL. The plasmid was transformed into *E.coli*. XL1 blue (Agilent Technologies, Inc., CA, USA) to assess the library quality and size. Sequencing revealed an approximately equal distribution of varied amino acids and a 10-fold overrepresentation of the theoretical library size.

*Plasmid for mammalian cells*

The JWH DNA sequence, with an additional SV40 nucleus localization signal (NLS) in the N-terminus, was synthesized and cloned into the pMD19 vector by TaKaRa (Shiga, Japan). JWH was further subcloned into the pCAG-HA expression vector[4] to obtain an HA-Tag at the N-terminus. Expressed HA-JWH protein is about 10 kD in size while the empty vector encoded a protein of around 8 kD. pCMV-HA-cJun was constructed by cloning full-length of the human cJun coding sequence into the pCMA-HA expression vector (TaKaRa). PathDetect AP1-luc was purchased from Agilent. pcDNA-MycHis-HBZ and pPolIII-Renilla have been described previously[5].

HiT selection[1]

The vector pAK632-HBZ-bLac and pAK630-TorA-cJunL were co-transformed into *E. coli*. BL21(DE3) (Agilent Technologies, Inc.), and 10-fold overrepresentation of the theoretical library size was confirmed. The resulting colonies were pooled and used for the selection. For the 1st selection, 10 μL of overnight culture grown from the pooled library in Lysogeny broth (LB) medium containing 25 mg/L Chloramphenicol (Cm), 50 mg/L Kanamycin (Kan) and 1% Glucose was used to inoculated 1 mL of LB containing 25 mg/L Cm, 50 mg/L Kan and precultured at 37ºC for 3 h. The cells were collected by centrifugation and washed with 1 mL of LB medium containing 25 mg/L Cm, 50 mg/L Kan and 1mM Isopropyl b-D-1-thiogalactopyranoside (IPTG) 3 times to get rid of β-lactamase leaked from dead cells. The cells were re-suspended in LB medium containing 25 mg/L Cm, 50 mg/L Kan and 1mM IPTG and diluted to an $OD_{600}$ of 0.0002. 100 μL of the diluted culture (approx. $1.6 \times 10^5$ cells) was plated on LB-agar plates containing 25 mg/L Cm, 50 mg/L Kan, 1mM IPTG and several concentrations (0, 10, 25, 50, 100, 200, 400 mg/L) of Ampicillin (Amp). Sequences of winner variants were checked from the clones survived on the plate containing 400 mg/L Amp. The colonies surviving on plates containing 100, 200, 400 mg/L Amp were pooled and used for the next selection round. Further selection rounds were performed with the same protocol except for using 0.5 mM (2nd selection) and 0.3 mM IPTG (3rd selection).

Peptide synthesis

Peptides used in the study were synthesized basically as reported before.[6] The peptide chain was constructed using the 9-fluorenylmethoxycarbonyl (Fmoc) strategy, with a TGS-RAM resin (Shimadzu, Kyoto, Japan) in the PSSM-8 Shimadzu SPPS synthesizer. The peptides were deprotected and cleaved from the resin by treatment with a trifluoroacetic acid (TFA)-

ethanedithiol mixture (95:5), followed by reverse-phase high performance liquid chromatography (RP-HPLC) purification, yielding the desired peptides with > 95% purity (RP-HPLC). The fidelity of the purified peptides were confirmed by matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) mass spectrometry using Microflex (Bruker Daltonics, MA, USA): cJun-ZIP 4778.7 [calcd for $(M + H)^+$ 4778.5]; cFos-ZIP 4791.6 [calcd for $(M + H)^+$ 4792.4]; HBZ-ZIP 4941.1 [calcd for $(M + H)^+$ 4941.5]; JWH-ZIP 4822.1 [calcd for $(M + H)^+$ 4820.6]. Peptide concentration was determined as reported by Pace *et al*[7].

Circular Dichroism (CD) spectrometry[8]

CD spectra and thermal melts were performed at 50 μM total peptide concentration in 10 mM potassium phosphate buffer containing 100 mM KF (pH 7.0), using a J-810 CD instrument (Jasco, Tokyo, Japan). The spectra were measured at 20°C. For thermal denaturing, the temperature was ramped from -8 to 95 °C at a rate of 0.5 °C/min. Baselines for fully folded dimers of cFos and HBZ were estimated by a melting curve of these peptide at pH 2.0 which is a condition where these peptides form stable coiled-coil homodimer.[9] Melting profiles were fitted to a two-state model to yield the melting temperature (Tm) using Kaleida graph program (Abelbeck software):

$$\Delta G = \Delta H - (T_A/T_m) \times (\Delta H + R \times T_m \times \ln(P_t)) + \Delta C_p \times (T_A - T_m - T_A \times \ln(T_A/T_m)) \quad (1)$$

where $\Delta H$ is the change in enthalpy, $T_A$ is the reference temperature; $R$ is the ideal gas constant, $P_t$ is the total peptide concentration, and $\Delta C_p$ is the change in heat capacity.

Analytical ultracentrifugation (AUC)

Sedimentation equilibrium studies at a final peptide concentration of 50 μM were performed as reported previously,[10] using a Beckman Coulter Optima XL-A analytical ultracentrifuge with a double-sector centerpiece and sapphire windows, at rotor speeds of 30 000 and 42 000 rpm at 10°C. Absorbance scans were carried out at 240 nm in the radial step mode at 0.001-cm intervals and the data were collected as the average of 16 measurements at each radial distance. Equilibrium was considered to have been reached when replicate scans separated by 6 h were indistinguishable. The density of solvent (10 mM HEPES containing 500 mM NaCl (pH 7.4)) was determined using an Anton Paar DMA-5000 density meter to be 1.02526 g/cm$^3$. The average of the partial specific volumes of the respective peptides were employed when weight-averaged molecular weight of a 1:1 peptide mixture were calculated.

Size-exclusion chromatography with on-line light-scattering (SEC-LS)

SEC-LS measurement was performed using a Viscotek TDA 305 (Triple Detector Array) with a GPCmax sample delivery tool as reported reported.[2] 150 μL of peptides (1 mg/mL) in 25 mM Hepes 150 mM NaCl pH 7.4 was loaded on a Superdex75 10/300 GL (GE Healthcare) column and eluted with same buffer at 23 °C. UV280, refractive index and Right Angle Light Scattering

(RALS) were monitored. An apparent molecular mass of each sample was calculated from refractive index and RALS using the Viscotek software OmniSEC.

Isothermal titration calorimetry (ITC)

ITC experiments were carried out with a MicroCal $ITC_{200}$ (GE Healthcare). A peptide solution (100 μM) was titrated into another peptide solution (10 μM) at 37 °C. All samples were in 10 mM sodium phosphate buffer (pH 7.0) containing 100 mM NaCl and the buffer was degassed under vacuum before use. The cell volume was 200 μL and the syringe volume was 40 μL. Each titration consisted of 19 injections (2.0 μL each) at 120 s intervals. The stirring rate was 1000 rpm throughout the experiments. Data were analyzed using the Origin software supplied by MicroCal.

Fluorescence microscopy

Immunofluorescence experiments were performed as described previously[5]. Briefly, HeLa cells were transfected with pCAG-HA-JWH and pcDNA-MycHis-HBZ. 48 hours later, cells were stained at 4°C overnight with anti-HA-Alexa488 (Cell Signaling Technology, MA, USA) and anti-Myc-Cy3 (Sigma-Aldrich) and observed under a Leica confocal microscope (Wetzlar, Germany).

Immunoprecipitation (IP)

pCMV-HA-cJun, pcDNA-MycHis-HBZ and pCAG-HA-JWH were transfected into 293FT cells and 48 hours later, immunoprecipitation was performed as reported previously[5]. pCAG-HA empty vector was also transfected to balance the total plasmid amount. Antibodies used were anti-Myc (9E10, Sigma-Aldrich), anti-HA-HRP (12CA5, Roche, IN, USA) and anti-α-tubulin (DM1A, Sigma-Aldrich). HRP-conjugated anti-mouse IgG and normal mouse IgG were purchased from Sigma-Aldrich and Santa Cruz (CA, USA) respectively.

Luciferase reporter assay

Generally, $2\times10^5$ cells/well of Jurkat were seeded in a 12-well plate and transfected with indicated combinations of plasmids using Lipofectamine LTX reagent (Life technologies, NY, USA). 24 hours later, cells were harvested and luciferase activities were measured using the Dual-Luciferase Reporter Assay System (Promega, WI, USA) according to the manufacturer's instructions. All luciferase values were normalized with the activity of Renilla luciferase and represented as the mean of a triplicate set of experiments.

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