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Supporting information for article:

**Structural and functional analysis of Hikeshi, a new nuclear
transport receptor of Hsp70**

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S1. Methods

S1.1. Plasmid construct

Human Hsc70 fragments except for full-length Hsc70 were cloned into bacterial expression vector pET28a (Novagen) modified to a TEV cleavage site with *NdeI/XhoI*. Human full-length Hsc70 was cloned into the pQE80L expression vector (Qiagen) with *BamHI/SalI*. Human Hikeshi and its mutants were cloned into the entry vector, pENTR (Invitrogen). Point and deletion mutants were produced by PCR-based mutagenesis. To construct the Gateway destination vector, the PCR fragment containing PreScission protease site-FLAG-RfB (RfB; Invitrogen) was inserted into the *NdeI-XhoI* site of pCold-ProS2 vector (Takara). The resultant vector is referred to as the pCold-ProS2-PP-FLAG-GW vector. Expression plasmids for the 6xHis-ProS2-FLAG-tag-fused Hikeshi proteins were constructed using Gateway technology. All plasmid inserts were verified by DNA sequencing.

S1.2. Expression and purification of recombinant proteins

The recombinant Hsc70 fragments for the pull-down assay were expressed in BL21(DE3) cells (Stratagene) by 0.3 mM IPTG induction at 18 °C and purified from soluble fraction by Ni-NTA agarose (Qiagen). The purified proteins were quickly exchanged for transport buffer. The expression and purification of recombinant GST- and GFP-fusion Hikeshi proteins and GFP-Hsc70 proteins were performed as described previously (Kose *et al.*, 1997, Kose *et al.*, 2012). To express 6xHis-ProS2-FLAG-tag-fused proteins, the respective expression plasmids were transformed in *E. coli* BL21. The resulting *E. coli* cells were grown at 37 °C in LB medium containing 50 mg/mL ampicillin. After leaving to stand in culture medium for 30 min at 4 °C, protein expression was induced with 0.1 mM IPTG, followed by shaking culture for 18 h at 15 °C. Cultures were harvested by centrifugation and resuspended in lysis buffer (50 mM Tris-HCl, pH 8.3, 500 mM NaCl, 20 mM 2-mercaptoethanol, 0.2 mM PMSF). After cell disruption by freeze-thaw and sonication, the extract was clarified by centrifugation and incubated with Ni-NTA agarose (QIAGEN) at 4 °C. After washing with lysis buffer containing 20 mM imidazole, recombinant proteins were eluted with 50 mM Tris-HCl, pH 7.3, 50 mM NaCl, 20 mM 2-mercaptoethanol, 250 mM imidazole or digested with PreScission protease for cleavage of 6xHis-ProS2-tag at 4 °C. The recombinant proteins were further purified on Hi-TrapQ column (GE Healthcare) using a linear gradient elution of 50–500 mM NaCl in 50 mM Tris-HCl, pH 7.3, 1 mM DTT, and peak fractions were dialyzed against transport buffer (TB; 20 mM HEPES, pH 7.3, 110 mM potassium acetate, 2 mM magnesium acetate, 5 mM sodium acetate, 1 mM EGTA)/1 mM DTT, and concentrated using AmiconUltra-4 (Amicon).

References

Kose S, Furuta M, & Imamoto N (2012). *Cell* 149. 578-589.

Kose S, Imamoto N, Tachibana T, Shimamoto T, & Yoneda Y (1997). *The Journal of cell biology* 139, 841-849.

Table S1 Hydrophobic interactions within 5 angstroms observed in dimer interface via CTD between monomers A and B.

Residue	Position	Chain	Residue	Position	Chain
Phe	138	A	Val	132	B
Phe	138	A	Phe	138	B
Phe	138	A	Phe	141	B
Phe	141	A	Phe	138	B
Phe	141	A	Phe	141	B
Phe	141	A	Phe	184	B
Phe	141	A	Trp	195	B
Phe	149	A	Leu	146	B
Phe	149	A	Phe	149	B
Phe	149	A	Val	176	B
Phe	149	A	Val	177	B
Phe	149	A	Trp	195	B
Phe	152	A	Val	176	B
Phe	152	A	Trp	180	B
Ala	153	A	Ile	172	B
Ala	153	A	Val	176	B
Phe	156	A	Pro	173	B
Phe	156	A	Val	176	B
Ala	157	A	Ile	172	B
Ala	161	A	Pro	167	B
Phe	171	A	Val	158	B
Phe	171	A	Phe	171	B
Phe	171	A	Pro	173	B
Ile	172	A	Phe	149	B
Ile	172	A	Ala	153	B
Ile	172	A	Ala	157	B
Ile	172	A	Ile	172	B

Ile	172	A	Ala	174	B
Pro	173	A	Phe	156	B
Pro	173	A	Phe	171	B
Ala	174	A	Ile	172	B
Val	176	A	Phe	152	B
Val	176	A	Ala	153	B
Val	176	A	Phe	156	B
Val	177	A	Phe	149	B
Val	177	A	Ile	172	B
Trp	180	A	Phe	149	B
Trp	180	A	Phe	152	B
Phe	184	A	Phe	141	B
Leu	188	A	Phe	141	B
Trp	195	A	Phe	141	B

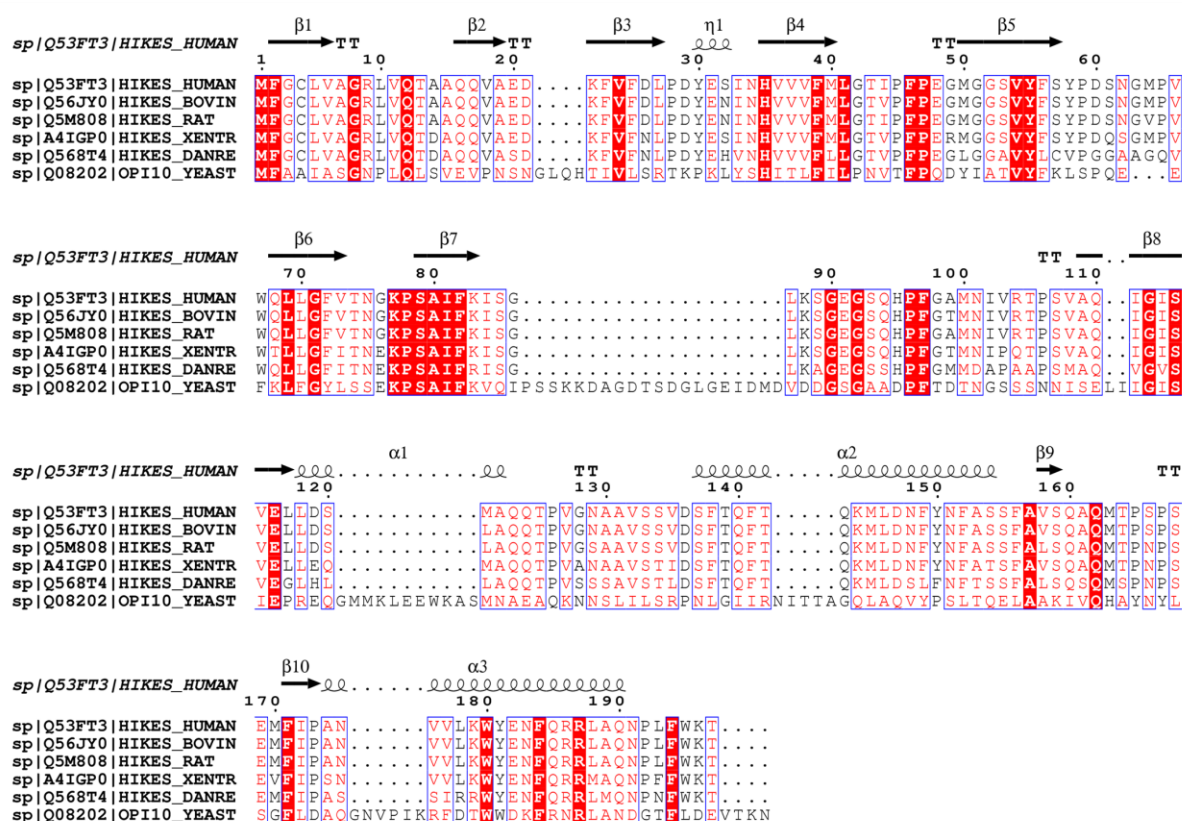


Figure S1 Alignment of Hikeshi protein sequences. Amino acid sequences of selected Hikeshi homologs were aligned using the EBI Clustal-X server. Secondary structure elements for Hikeshi from human are indicated above the sequences by black coloring. The Uniprot codes for the sequences are: *Q53FT3*, *Homo sapiens* (Human); *Q56JY0*, *Bos taurus* (Cattle); *Q5M808*, *Rattus norvegicus* (Rat); *A4IGP0*, *Xenopus tropicalis* (African clawed frog); *Q568T4*, *Danio rerio* (Zebrafish); *Q08202*, *Saccharomyces cerevisiae* (Yeast).

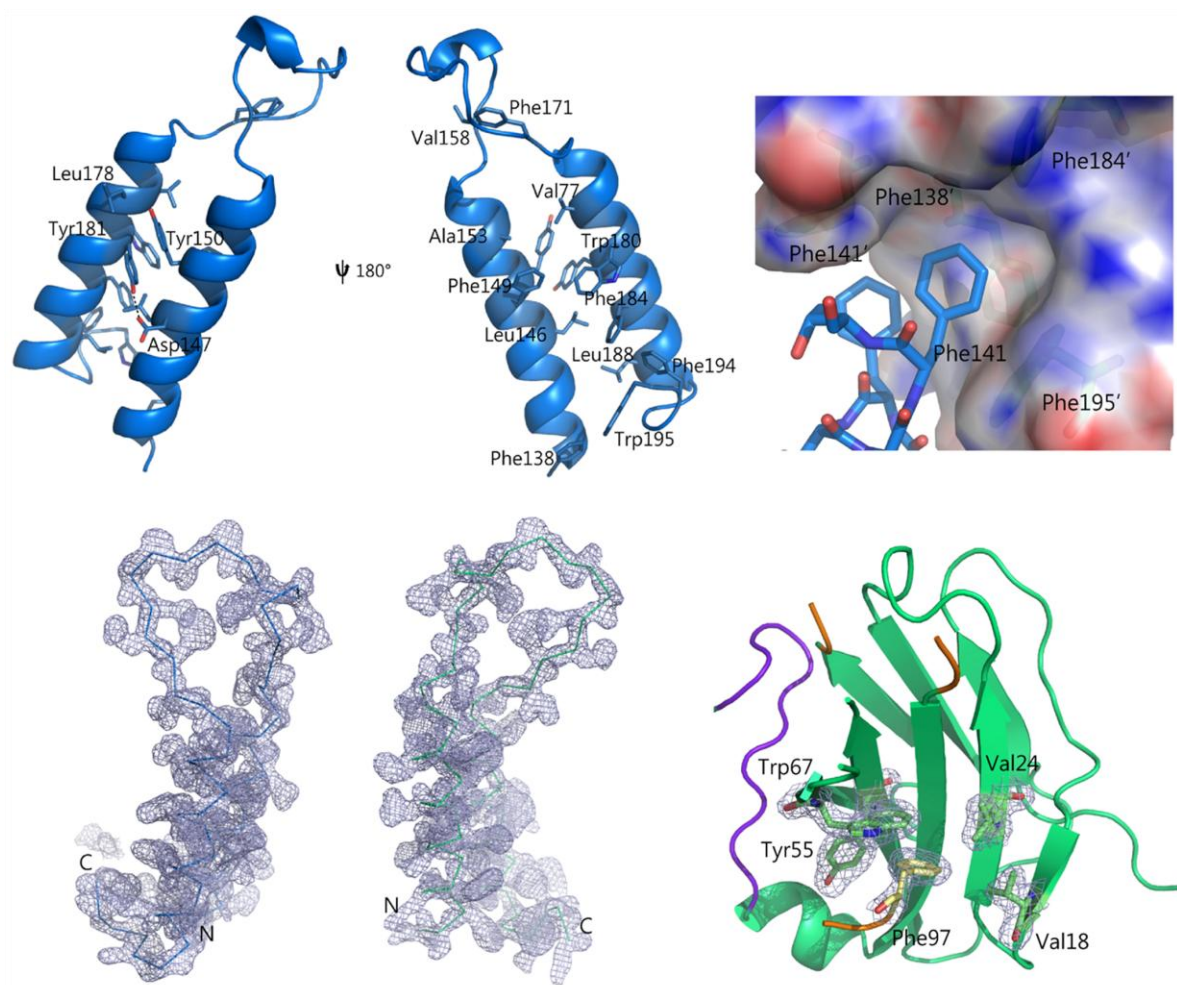


Figure S2 Structural analysis and quality of domain crossover of Hikeshi. (A) Structure of the CTD in monomer A. Front view of the helical hairpin monomer showing residues in the intramolecular interface of two antiparallel helices ($\alpha 2$, $\alpha 4$) and a flexible segment connecting $\alpha 2$ and $\alpha 4$. The amino acids forming the hydrophobic core are indicated as sticks. Hydrogen bond interactions are denoted by black dotted lines (left, colored as in Fig. 1a). A flipped view of the same structure in **a**, roughly related by a 180° rotation (right). (B) Phe141, shown as sticks (O, red; N, blue) lodging in the hydrophobic core formed by residues Phe138, Phe141, Phe184, and W195 on another monomer (surface representation). (C) Stereo view of the domain crossover region (left: monomer A; right: monomer B) of CTDs in the reconstructed density map (violet mesh; 1σ threshold) with an atomic model (ribbon diagram). (D) View of the E-loop and hydrophobic pocket in the NTD of monomer B shown as cartoon representation. It is evident that Phe97A is buried in the hydrophobic pocket although the rest of the E-loop is disordered. The color code is the same as in Fig. 1a.

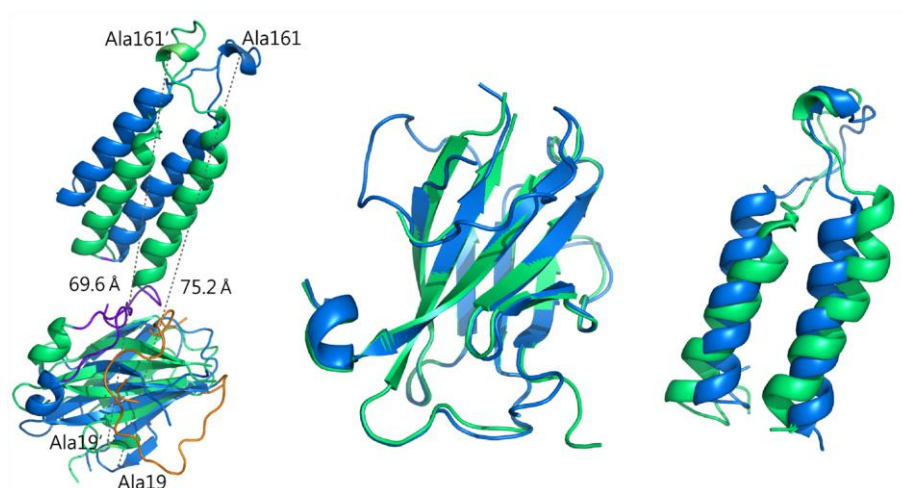


Figure S3 Structural overlay of both monomers from the Hikeshi dimer. (A) Superposition of the full-length structure. The calculated r.m.s.d. of both monomers is 6.7 Å for 193 C α atoms. In addition, the distances between Ala19- and Ala161-C α atoms of each monomer, being on each end of the longest axis of the molecule, are 69.6 Å and 75.2 Å, respectively. (B) Superposition of the NTDs. The NTDs have a high overall topological similarity with an r.m.s.d. of 0.5 Å in 123 C α atoms. (C) Superposition of CTDs showed a remarkable difference with a large r.m.s.d. of 3.1 Å in 60 C α atoms.

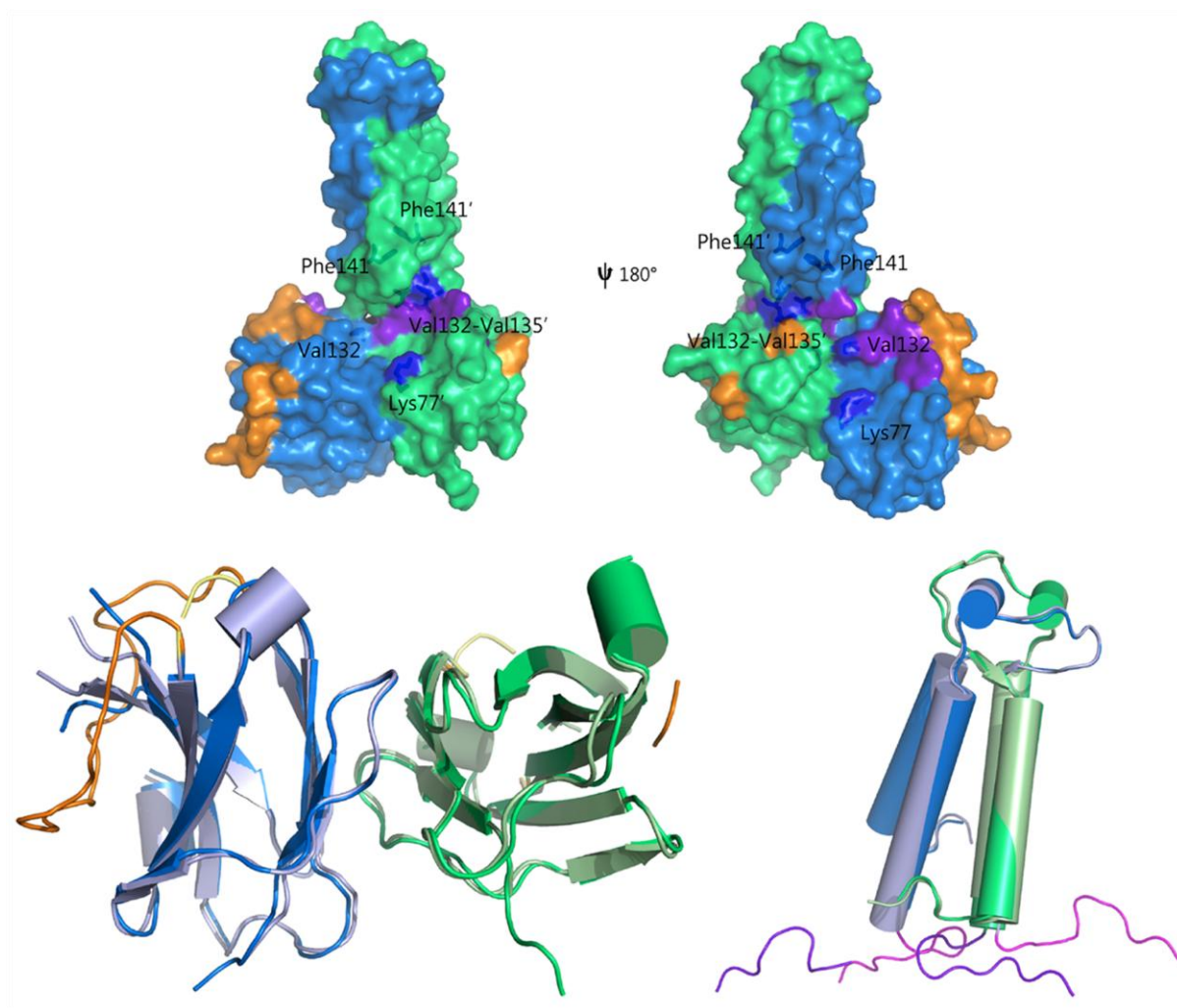


Figure S4 (A) Surface representation showing Hsp70 binding-related residues and region (in blue color). The color code of the secondary structure elements is the same as in Fig. 1A. (B) and (C) Superposition of NTDs of wt and Phe97Ala. The NTDs and CTDs have a high overall topological similarity with an r.m.s.d. of 0.57 Å and 0.45 Å, respectively.

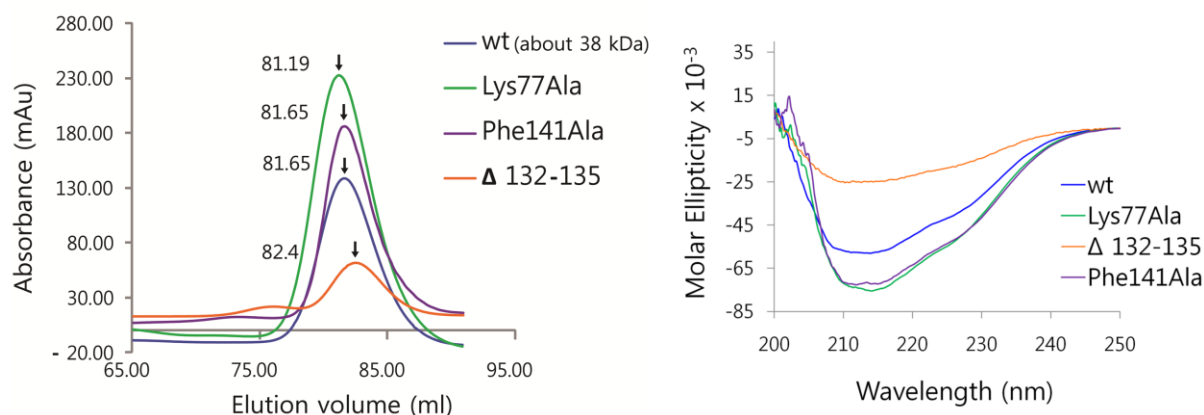


Figure S5 (A) The profiles of gel filtration for the mutant Hikeshi proteins. Gel filtration was performed with a HiLoad 16/60 Superdex 200 (GE Pharmacia) in 20 mM Tris-HCl (pH 8.0), 20 mM NaCl, and 1 mM DTT. Black arrows indicate the top of eluted peak. (B) The secondary structures of the Hikeshi mutants were detected by CD. The characteristic shape and magnitude of the CD spectral trace are all similar to those of wt Hikeshi. Five scans were averaged for each mutant or the wt protein.

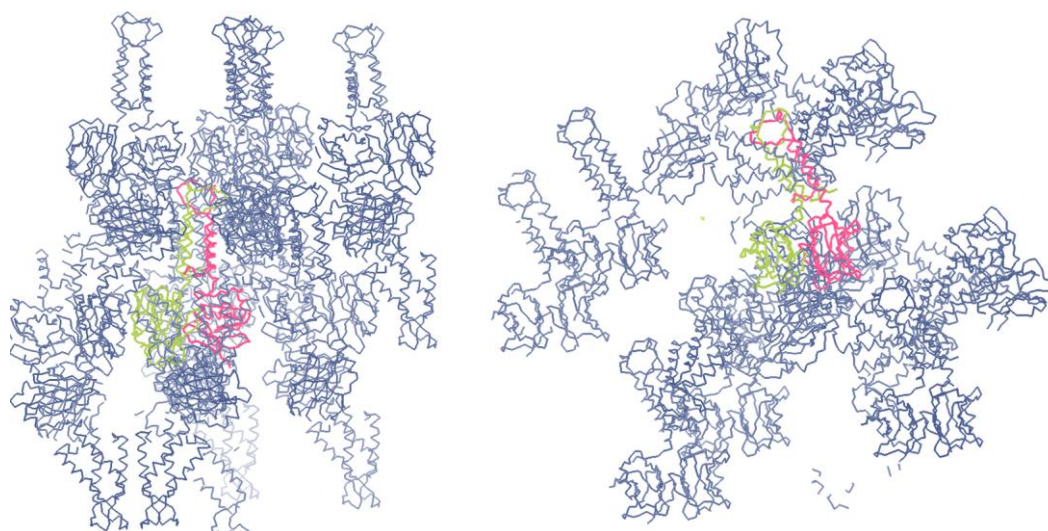


Figure S6 Crystal lattices of wt and Phe97Ala mutant structures in the $C222_1$ (left) and $P3_2$ (right) space groups, respectively.

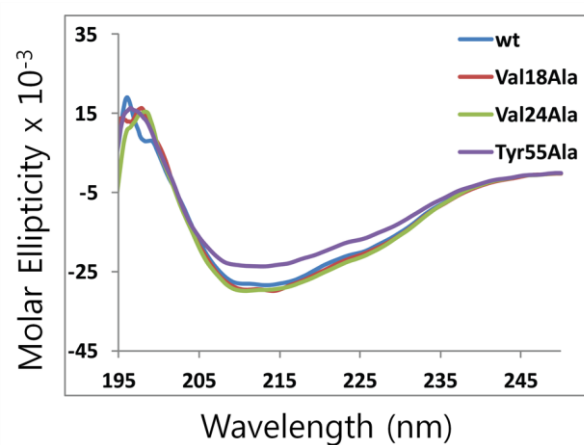


Figure S7 The secondary structures of the Hikeshi mutants were detected by CD. The characteristic shape and magnitude of the CD spectral trace are all similar to those of wt Hikeshi