## **Supporting Information**

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**Fig. S1.** Interactions between Eh-D and Eh-F. (A) Binding measurement was performed using the Biacore system. Sensorgrams for the binding of various concentrations of the analyte (Eh-F) to the ligand (Eh- $A_3B_3D$ ) are shown. (B) Surface representation of the interface between Eh-D and Eh-F: Polar residues (N, Q, S, T) are colored in green, positively charged residues (H, K, R) are blue, negatively charged residues (D, E) are red, and hydrophobic residues (A, C, F, G, L, M, P, V, W, Y) are dark gray. The residues with buried surface area of >10 Å<sup>2</sup> are mapped on the surface.

LKDK LKKKI LKEK LKDK LKRK KKQ TRI НК Eh-D . MF LNVN ....MRLNVNP ....MAQQDVKP ...MAQQDVKP ...MPEILKIKP ....MRLNVNP MSG.NREQVFP Tt-D Mm-D Ph-D Cf-D Sc-D NLLQRRGQ ELINLKKK ELLKLKRR ELSRLKKR НK LGLMKTK Hs-D MSGKDRIEI H2 20000000 8 9 → H3 110 β1 β2 222 200000000 90 100 120 
 7 0
 8 0
 90
 10 0
 11 0
 12 0
 13 0

 LAKSTVEEAFIDELLALPAEN.VSISVVEKNIMSUKUVPLUSUPUDETLNETPLEYGYLHSNAELDRSIDG
 LASAVNGNVAVRSTAFTAFS.PEIQLSGHNIMSUKUVPLUSSTGVRK.SLYERGYGIIGTNSYIDETADA

 LASAVNGMVAVRSTAFTAKES.PEIQLSGHNIMGVVVPLISSTGVRK.SLYERGYGIIGTNSYIDETADA

 RAQVDVGALRLKELAIGVKN.KEIEIKTRNIMGVVVPLISSTGVRK.SLYERGYGIIGTNSYIDETADA

 MAKAVMGALFLQEAISMPAET.IKLEVTRKNIMGVSVPUPUTEVPELKR.KASERGYAFVSTTSTVDMAAEK

 EVSYATGENIGYQVQESVSTAFFKVRARQENVSGVYLSQFESYIDPE.INDFRLTGLGRGGQQVQRAKEI

 EAKFTAGD.FSTTVJQNVNKAQVKIRAKKDNVAGVTLPVFEHYHEG.TDSYELTGLARGGEQLAKLKRN
Eh-D Tt-D Mm-D Ph-D Cf-D Sc-D Hs-D 200 TYOLUP KILKLAEVEKTCOLMAE FRRYAEALIR VANTETRIKKIGEEI YEDLVEKIITAAELETTMKRILDEI FESVIELAIRLAEVESLKRIGKEI LYDIMPOLLELAEVEKACOLMADEI YSRAVETLVELASLOTSFVTLDEAI Eh-D Tt-D Mm-D Ph-D Cf-D Sc-D Hs-D 210 MGTEE. KIEAREAEEEGGRPNPQVEIGAGL. RMRD. ILEARQSM. MMOK. Eh-D Tt-D Mm-D

Sc-D Hs-D	KKONETAKLDAEMKLKRDRAEQDASEVAADEEPQGETLVADQEDDVIF. KKKILKEKSEKDLEQRRAAGEVLEPANLLAEEKDEDLLFE
. Sequence alignment of t	he D subunits from different species. The primary sequences of the D subunits of V-ATPase were aligne
Tt-D; Thermus thermophilu	s HB8; Mm-D; Methanosarcina mazei Gö1; Ph-D, Pyrococcus horikoshii; Cf-D, Clostridium fervidus; Sc-I

Fig. S2 d: Eh-D, Enterococcus hirae; D, Saccharomyces cerevisiae; and Hs-D, Homo sapiens. The secondary structures of Eh-D are shown above the sequences. The deleted region (90–108) of the mutation is indicated in the red box. The Dm mutant was constructed by the deletion of the  $\beta$ -hairpin region (90–108) and the insertion of two linker residues (GS).

Ph-D



Fig. S3. Structural similarity of Eh-D with the  $\gamma$ -subunit and FliJ. (A) Eh-D (green) is superimposed with the  $\gamma$ -subunit (orange) of bovine F<sub>1</sub>-ATPase [Protein Data Bank (PDB) ID 1E79]. (B) Eh-D (green) is superimposed with FliJ (light blue) of Salmonella flagellar type III protein export apparatus (PDB ID 3AJW).



**Fig. S4.** Effect of the  $\beta$ -hairpin region of the D subunit. Binding measurements were performed using the Biacore system. Sensorgrams for the binding of various concentrations of analyte to ligand are shown. (*A*) Analyte, Eh-DF; ligand, Eh-A<sub>3</sub>B<sub>3</sub>. (*B*) Analyte, Eh-DmF; ligand, Eh-A<sub>3</sub>B<sub>3</sub>. (*C*) ATP concentration dependence of the ATPase activity of reconstituted Eh-A<sub>3</sub>B<sub>3</sub>DF (circle, black) and Eh-A<sub>3</sub>B<sub>3</sub>DmF (triangle, red). (*D*) The double reciprocal plot for the ATPase activity assay.



Fig. S5. Interactions between Eh-D and Eh-F $\Delta$ C $\alpha$ . Binding measurements were performed using the Biacore system. Sensorgrams for the binding of various concentrations of the analyte (Eh-F $\Delta$ C $\alpha$ ) to the ligand (Eh-A<sub>3</sub>B<sub>3</sub>D) are shown. The inset shows the kinetic plot and binding isotherm for binding.



**Fig. S6.** Interaction of Eh-d. (*A*) Binding measurements were performed using the Biacore system. Sensorgram for the binding order of two analytes to the ligand is shown. After the immobilization of the ligand (Eh-A<sub>3</sub>B<sub>3</sub>D) onto the sensor chip, Eh-d and Eh-F were manually injected. Black bars indicate the injection period of each analyte. In the first injection of Eh-d, no specific binding response between Eh-A<sub>3</sub>B<sub>3</sub>D and Eh-d was observed. A specific binding response between Eh-A<sub>3</sub>B<sub>3</sub>D and Eh-F was observed in the injection of Eh-F. An additional binding response in the second injection of Eh-d corresponded to the interaction between Eh-A<sub>3</sub>B<sub>3</sub>DF and Eh-d. (*B*) Binding measurements were performed using the Biacore system. Sensorgrams for the binding of various concentrations of the analyte (Eh-d) to the ligand (Eh-A<sub>3</sub>B<sub>3</sub>DF) are shown.



**Fig. 57.** Amino acid sequence similarity between Eh-d and Tt-d and homology modeling of Eh-d structure. (*A*) Sequence alignment of Eh-d and Tt-d. The charged residues are shown with red squares (positively charged) and blue triangles (negatively charged) below the sequence. The disulfide bonded cysteine residues are indicated with an orange box and line. (*B*) The homology model of Eh-d (orange) is superimposed with Tt-d (light blue). Electrostatic potential surface of Tt-d (*C*) and homology model of Eh-d (*D*) were produced in the same manner as in Fig. 5.

## Table S1. Data collection and refinement statistics

	SeMet crystal*				
Data collection Space group Cell dimensions a, b, c, Å $\alpha, \beta, \gamma, °$ Wavelength, Å Resolution, <sup>†</sup> Å $R_{merge}^{+}$ $I/\sigma I^{+}$ Completeness, <sup>†</sup> % Redundancy <sup>†</sup>	C2				
	105.79, 68.43, 51.15 90, 114.99, 90 Peak (reprocessed) Peak Inflection Remote				
	0.9791 2.00 (2.11-2.00) 0.090 (0.561) 10.7 (2.8) 97.2 (96.3) 6.1 (6.1)	0.9791 2.10 (2.21-2.10) 0.084 (0.394) 14.9 (3.8) 97.4 (96.4) 6.1 (6.1)	0.9794 2.10 (2.21-2.10) 0.048 (0.245) 11.0 (3.0) 97.0 (96.0) 2.0 (2.1)	1.000 2.10 (2.21-2.10) 0.045 (0.300) 11.6 (2.9) 96.9 (95.9) 2.0 (2.0)	
Refinement Resolution, <sup>†</sup> Å No. reflections <sup>†</sup> $R_{work}/R_{free}^{\dagger}$ No. atoms Protein Ligand/ion	2.00 (2.05-2.00) 21,696 (1,503) 19.4(26.3)/25.2(29.0) 2,341 4				
Water B factors Protein Ligand/ion Water rmsd Bond lengths, Å Bond angles, °	152 41.8 56.7 47.7 0.011 1.276				

\*All datasets were obtained from one selenomethionine (SeMet)-labeled crystal.

<sup>†</sup>Values in parentheses are for the highest-resolution shell.

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