Supplemental data

A METHOD FOR OVEREXPRESSION OF MEMBRANE PROTEINS IN *E.COLI*

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Supplemental Methods

Cloning of α and β into pET28a(+). Cloning is described at supplemental table 1.

Cloning of membrane proteins into the 8 overexpression combination cassettes. DNA encoding the specific membrane protein was amplified by PCR using available DNA or cDNA (Supplemental table 2). The amplified DNA was ligated at room temperature for 1 h into the pGEM T-easy (Promega) plasmid and amplified in DH10B *E.coli* cells. Purified plasmids were digested with the relevant restriction enzymes (usually EcoRI and XhoI) and the purified fragments were cloned into pET-28a(+) (Novagene) containing the 9 construct combinations (Fig. 1 in manuscript). The resulting plasmids were amplified and subsequently transformed into *E.coli* C43 competent cells (commercially available at Over*Express* TM).

Cloning of MISTIC into pET-28(a)+ expression vector. The MISTIC gene was kindly provided by Dr. Senyon Choe at the Salk Institute. Inducible bacterial expression vector was generated to encode MISTIC at the N-terminus of the pET-28a(+) polylinker region and to encode His-tags at both termini. DNA encoding the MISTIC was amplified by PCR, digested with NdeI and BamHI and ligated into linearized pET-28a(+). The resulting plasmid, MISTIC-pET-28a(+), was digested with EcoRI and XhoI and chosen membrane proteins genes were ligeted into it. The resulting plasmids were amplified and subsequently transformed into *E.coli* C43 competent cells.

Cloning of the TEV protease sites into the 8 pET-28a(+) expression cassettes. Two TEV protease sites were generated: (i) at the N-terminus, inserted at the BamHI site, downstream to the N-terminus fusion domain, and (ii) at the C-terminus, inserted at the XhoI site, upstream to the C-terminus fusion domain. For each site, two complementary oligonucleotides were generated (Sigma-Genosys) to create the TEV protease site, together with two partially complementary enzyme-digestion sites (supportal Fig 5f). Each oligo was resuspended in DDW (1.35 μg/μl), and 1 μl was added to 48 μl of annealing solution (100 mM K-acetate, 30 mM HEPES pH 7.4, 2 mM Mg-acetate (Sigma)), heated to 95°C for 4 min, slowly equilibrated to room temperature (1ºC/min), and diluted for ligation. The inserts and vectors (digested with BamHI or XhoI accordingly) were ligated and used to transform DH10B *E.coli* cells.

Reconstitution. The purified β-rVGLUT2-α, β-hSialin-β and β-mSialin-β (for aspartate transport assay) were co-reconstitution into liposomes with bacterial F-ATPase. Proteoliposomes for phosphate transport assay of β-NPT1-β, sialic acid transport assay of β-hSialin-β and β-mSialin-β and nucleotide transport assay of β-hVNUT were prepared without F-ATPase. Reconstitution was carried out by the freeze/thaw method as described (8-10). In brief, 10 μg (for VGLUT, VNUT, NPT1) or 40 μg (for Sialin) of proteins were mixed with prepared liposomes (0.5 mg lipid) and 90 μg F-ATPase (when indicated above), frozen at -80 °C and left at this temperature for at least 5 min. The mixture was thawed quickly by holding the tube in hands and diluted 60-fold (for VGLUT, VNUT, NPT1) or 30-fold (for Sialin) with reconstitution buffer containing 20 mM MOPS-Tris pH 7.0, 0.5 mM DTT, 5 mM Mg-acetate, 0.1 M K-acetate (for VNUT 0.15M Na-acetate instead of K-acetate). Reconstituted proteoliposomes were pelleted by

centrifugation at 200,000 X g for 1 h at 4 °C and suspended in 0.4 ml of 20 mM MOPS-Tris pH 7.0, 5 mM Mg-acetate, 0.1 M K-acetate (0.15 M Na-acetate for VNUT).

Asolectin liposomes were prepared as follows. Soybean lecithin (20 mg; Sigma type IIS) was suspended in 2 ml of 20 mM Mops_NaOH (pH 7.0) containing 0.5mM DTT. The mixture was sonicated in a bath-type sonicator until clear, divided into small aliquots, and stored at -80°C until use.

*ATP Transport assay***.** Reconstituted proteoliposomes (0.5 µg protein per assay) were suspended in 20 mM Mops Tris pH 7.0, 5 mM Mg-acetate, 4mM KCl, 0.15M K-acetate and incubated for 3 min at 27°C. Valinomycin was added to give a final concentration of 2 µM, and when tested, inhibitors were added to give a final concentration of 2 µM for DIDS and 1 µM for Evans Blue. The mixture was incubated for additional 3 min. The assay was initiated by addition of 0.1mM[α-32P]ATP (3.7 GBq/mmol), and 130 µl aliquots were taken after 2 min or at the times indicated and centrifuged through a Sephadex G-50 (fine) spin column at 760 X *g* for 2 min. Radioactivity of the eluate were measured. For dose-dependence measurements, upon the addition of [α-32P]ATP, samples were taken after 1 min.

*Na+ driven Pi transport assay***:** Reconstituted proteoliposomes (0.3 µg protein per assay) were suspended in 20 mM MOPS-Tris, pH 7.5, 5 mM Mg-acetate, 4 mM KCl and 0.1 M K-acetate and incubated for 3 min at 27 °C. Reaction was started by the addition of proteoliposomes to the reaction mixture containing 20 mM MOPS-Tris, pH 7, 5 mM Mg-acetate, 0.1 M Na-acetate, 4 mM sodium chloride and 100 µM [^{32}P] Na_2HPO_4 (3.7 MBq/µmol). 130 µl aliquots were taken after 2 min or at the times indicated and centrifuged through a Sephadex G-50 (fine) spin column at 760 X g for one min. Radioactivity of the eluate was measured. For dose-dependence measurements, upon the addition of [^{32}P] Na_2HPO_4, samples were taken after 0.5 min

Glutamate Transport assay. Reconstituted proteoliposomes (0.55 µg protein per assay) were suspended in 20 mM MOPS-Tris, pH 7.5, 5 mM Mg-acetate, 4 mM KCl, 0.1 M K-acetate and incubated for 3 min at 27 °C. ATP was added to give a final concentration of 2 mM and when tested, inhibitors were added to give a final concentration of 2 µM for DIDS and 1 µM for Evans Blue. The mixture was incubated for a further 3 min. The assay was initiated by addition of 100 µM [2,3-^{3}H] L-glutamate (0.5 MBq/µmol) and 130 µl aliquots were taken after 2 min or at the times indicated and centrifuged through a Sephadex G-50 (fine) spin column at 760 X g for one min. Radioactivity of the eluate was measured. For dose-dependence measurements, upon the addition of the listed concentrations of [2,3-^{3}H] L-glutamate, samples were taken after 1 min.

*H^+/sialic acid co-transport assay***.** Proteoliposomes (0.32 µg protein per assay) were suspended in natural buffer (20 mM Mops-Tris pH 7.0, 5 mM Mg-acetate, 4 mM KCl, 0.1 M K-acetate) or acidic buffer (40 mM Mes pH 5.6, 5 mM magnesium acetate, 0.1 M potassium acetate, 4 mM potassium chloride) and incubated for 3 min at 27°C. When tested, inhibitors were added to give a final concentration of 1 µM for DIDS, 5 mM L-aspartate and 5 mM L-lactate. The mixture was incubated for additional 3 min. The assay was initiated by addition of 100 µM [6-^{3}H]sialic acid (0.5 MBq/µmol) and 130 µl aliquots were taken after 2 min or at the times indicated and centrifuged through a Sephadex G-50 (fine) spin column at 760 X g for one min. Radioactivity of the eluate was measured. For dose-dependence measurements, upon the addition of the listed concentrations of [6-^{3}H]sialic, samples were taken after 1 min

Aspartate transport Assay. Proteoliposomes (0.6 µg protein per assay) were suspended in 20 mM Mops-Tris pH 7.0, 5 mM Mg-acetate, 4 mM KCl, 0.1 M K-acetate and incubated for 3 min at 27°C. ATP was added to give a final concentration of 2 mM, and when tested, inhibitors were added to give a final concentration of 2 µM for DIDS and 1 µM for Evans Blue. The mixture was incubated for a further 2 min. The assay was initiated by the addition of 100 µM L-[2,3-^{3}H]aspartate (0.5 MBq/µmol) and 130 µl aliquots were taken after 2 min or at the times indicated and centrifuged through a Sephadex G-50 (fine) spin column at 760 X *g* for 2 min.

Radioactivity in the eluate was measured. For dose-dependence measurements, upon the addition of the listed concentrations of [L-[2,3-^{3}H]aspartate, samples were taken after 1 min

Yield Calculation. Yield (in mg of purified protein per liter of E.coli culture) was calculated by dividing the final amount of purified protein (mg) by the volume (liters) of the E.coli culture used.

Western Blot Analysis. Samples were denatured by SDS sample buffer (with no boiling) and electrophoresed on 12.5% polyacrylamide mini-gels (BioRad). Following electrotransfer at 0.5 A for 30 min, the nitrocellulose filters were blocked for 30 min in a solution containing 100 mM NaCl, 100 mM sodium phosphate pH 7.5, 0.1% Tween-20, and 3% bovine serum albumin (BSA; Sigma). Antibody (mouse anti-His-tag-peroxidase; Roche) was incubated for 1.5 h at room temperature at a dilution of 1:1000 in a similar solution containing 3% BSA. After three washes in the same solution but with 2% dry milk, and one wash with a solution lacking the milk, the nitrocellulose filters were subjected to antibody detection by enhanced chemiluminescence (ECL; Amersham). Filters were exposed to Kodak X-Omat AR film for 10 s - 2 min.

Supplemental Table 1: Cloning of the two fusion domains (α and β) into the pET-28a(+) expression vector, creating the 8 overexpression combination.

Cloning vector	Recombinant GOI (gene of interest) configuration	Cloning scheme
α-pET-28a(+)	α-GOI	YaiN (α) was amplified by PCR, using a primer containing the restriction site NdeI and a primer containing the restriction site BamHI, and cloned into pGEM T-easy plasmid. The segment was cloned into pET-28a(+) plasmid, which contained a 6-His tag at both the N-terminus and the C-terminus. The resulting cloning vector contained YaiN (α) with the two restriction sites conserved.
pET-28a(+)-α	GOI- α	YaiN (α) was amplified by PCR, using a primer containing the restriction sites NotI and XhoI and a primer containing the restriction site SalI, and cloned into pGEM T-easy plasmid. The segment was cloned into pET-28a(+) plasmid, which contained a 6-His tag at both the N-terminus and the C-terminus. The resulting cloning vector contained YaiN (α) with the two restriction sites (NotI and XhoI) conserved. (SalI is compatible with the XhoI site of pET-28a(+), and is canceled.)
β-pET-28a(+)	β-GOI	YbeL (β) was amplified by PCR, using a primer containing the restriction site NdeI and a primer containing the restriction site BamHI, and cloned into pGEM T-easy plasmid. The segment was cloned into pET-28a(+) plasmid, which contained a 6-His tag at both the N-terminus and the C-terminus. The resulting cloning vector contained YbeL (β) with the two restriction sites conserved.
pET-28a(+)-β	GOI-β	YbeL (β) was amplified by PCR, using a primer containing the restriction sites NotI and XhoI and a primer containing the restriction site SalI, and cloned into pGEM T-easy plasmid. The segment was cloned into pET-28a(+) plasmid, which contained a 6-His tag at both the N-terminus and the C-

		terminus. The resulting cloning vector contained YbeL (β) with the two restriction sites (NotI and XhoI) conserved. (SalI is compatible with the XhoI site of pET-28a(+), and is canceled.)
α-pET-28a(+)-β	α-GOI-β	PET-28a(+) plasmid containing YaiN (α), prepared as described in the cloning scheme for cloning vector α-pET-28a(+), was digested with NdeI and BamHI. The resulting insert was ligated into the pET-28a(+) plasmid containing YbeL (β) (as described in the cloning scheme for cloning vector pET-28a(+)-β), which was digested with the same restriction enzymes (NdeI and BamHI). The resulting cloning vector contained YbeL (β) and YaiN (α) with the restriction sites conserved as described in the cloning scheme for α-pET-28a(+) and pET-28a(+)-β.
α-pET-28a(+)-α	α-GOI-α	pET-28a(+) plasmid containing YaiN (α), prepared as described in the cloning scheme for cloning vector α-pET-28a(+), was digested with NdeI and BamHI. The resulting insert was ligated into the pET-28a(+) plasmid containing YaiN (α) (as described in the cloning scheme for construct pET-28a(+)-α), which was digested with the same restriction enzymes (NdeI and BamHI). The resulting cloning vector contained YaiN (α) with the restriction sites conserved as described in the cloning scheme for cloning vectors α-pET-28a(+) and pET-28a(+)-α.
β-pET-28a(+)-α	β-GOI-α	YaiN (α) was amplified by PCR as described in the cloning scheme for pET-28a(+)-α, and cloned into pGEM T-easy plasmid. The segment was cloned into the pET-28a(+) plasmid containing YbeL (β) (as described in the cloning scheme for construct β-pET-28a(+)), which was digested with NotI and XhoI. The resulting cloning vector contained YbeL (β) and YaiN (α) with the restriction sites conserved as described in the cloning scheme for cloning vectors pET-28a(+)-α and β-pET-28a(+).
β-pET-28a(+)-β	β-GOI-β	pET-28a(+) plasmid containing YbeL (β), prepared as described in the cloning scheme for cloning vector β-pET-28a(+), was digested with NdeI and BamHI. The resulting insert was ligated into the pET-28a(+) plasmid containing YbeL (β) (as described in the cloning scheme for pET-28a(+)-β), which was digested with the same restriction enzymes (NdeI and BamHI). The resulting cloning vector contained YbeL (β) with the restriction sites conserved as described in the cloning scheme for cloning vectors β-pET-28a(+) and pET-28a(+)-β.

Supplemental Table 2: Selected membrane proteins cloned into the 8-expression vectors cassettes.

Organism	Gene	Function	No of TM	Topology	Provided by	Ref
E.coli	MntH	Divalent metal-ion transporter	11	N' cytoplasmic C' periplasmic		3
Listeria monocytogenes	KvLm	Potassium channel	6	N' cytoplasmic C' cytoplasmic	Dr. Maurice Montal, University of California, San Diego, CA, USA	4
Mus musculus	GAT1,2, 4	GABA neurotransmitter transporters	12	N' cytoplasmic C' cytoplasmic		5
Homo sapiens	SERT	Serotonin transporter	12	N' cytoplasmic C' cytoplasmic	Dr. Gary Rudnick, Yale University School of Medicine, New Haven, CT, USA	6
Rattus norvegicus	Vmat2	Vesicular monoamine transporter	12	N' cytoplasmic C' cytoplasmic	Dr. Shimon Schuldiner, The Hebrew University of Jerusalem, Israel	7
Homo sapiens	VNUT	Vesicular nucleotide transporter	12	N' cytoplasmic C' cytoplasmic	Dr. Yoshinori Moriyama, Okayama University, Japan	8
Rattus norvegicus	VGLUT2	Vesicular glutamate transporter	12	N' cytoplasmic C' cytoplasmic	Dr. Yoshinori Moriyama, Okayama University, Japan	9
Mus musculus	NPT1	Na+/Pi co-transporter	12	N' cytoplasmic C' cytoplasmic	Dr. Yoshinori Moriyama, Okayama University, Japan	9
Homo sapiens	Sialin	Sialic-acid and Aspartate transporter	12	N' cytoplasmic C' cytoplasmic	Dr. Yoshinori Moriyama, Okayama University, Japan	10
Mus musculus	Sialin	Sialic-acid and Aspartate transporter	12	N' cytoplasmic C' cytoplasmic	Dr. Yoshinori Moriyama, Okayama University, Japan	10

Mesocricetus auratus	HMGR	HMG-CoA reductase	8	N' cytoplasmic C' cytoplasmic	Dr. Joseph Roitelman, Sheba Medical Center, Tel Hashomer, Israel	11
Homo sapiens	Glut4	Glucose transporter	12	N' cytoplasmic C' cytoplasmic	Dr. Amira Klip, The Hospital for Sick Children, University of Toronto, Canada	12
Rattus norvegicus	rKv4.2	Potassium Channel	6	N' cytoplasmic C' cytoplasmic	Dr. Lily Yeh Jan Howard Hughes Medical Institute, San Francisco, USA.	13

FIGURE LEGEND

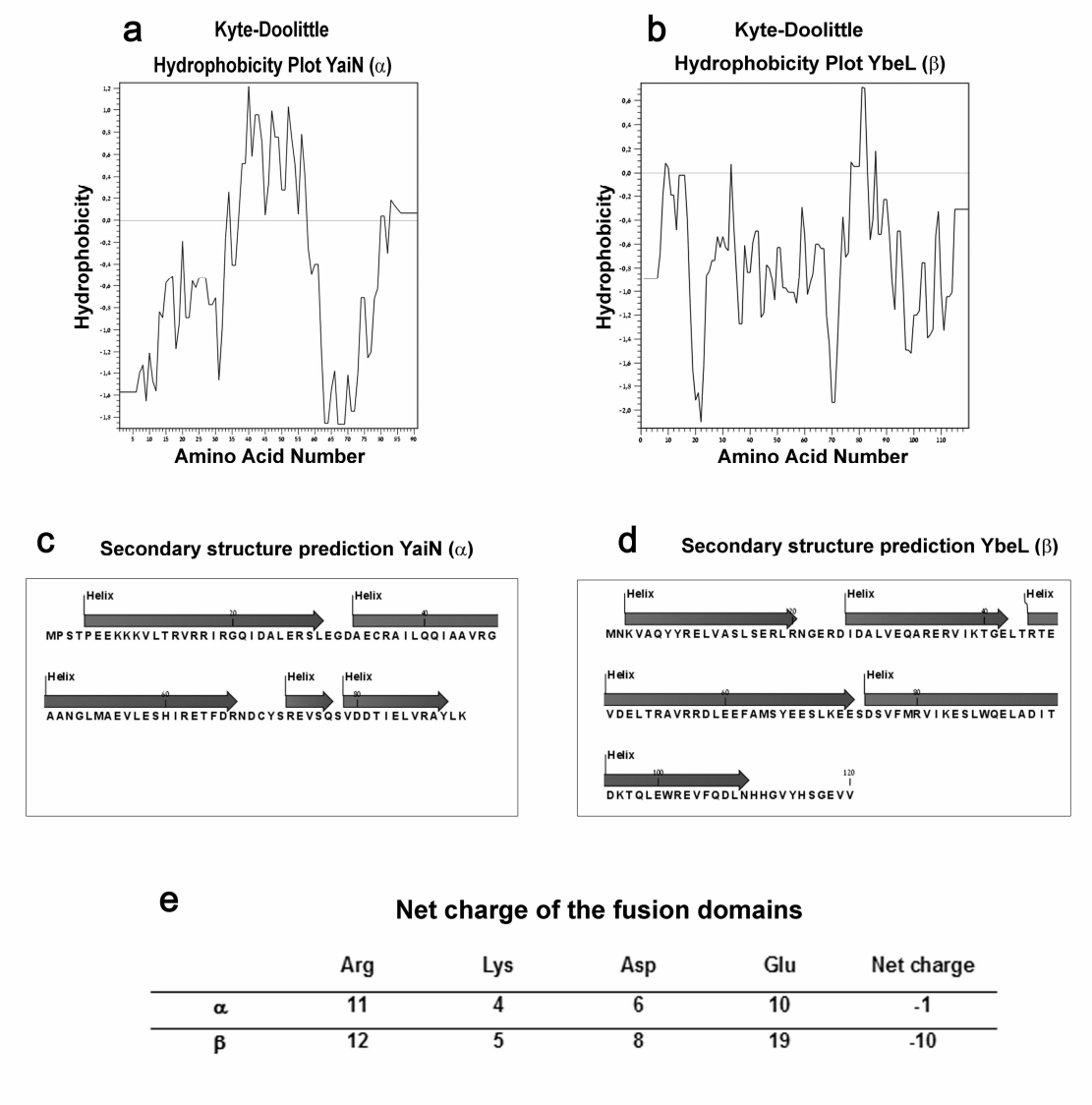
Supplemental Fig 1: Bioinformatic characterization of the fusion domains. Calculations were computed at the PredictProtein[1] website (http://www.predictprotein.org/) using the CLC Combined Workbench software (http://www.clcbio.com). (a) Kyte-Doolittle[2] hydrophobicity plot of YaiN (α). (b) Kyte-Doolittle hydrophobicity plot of YbeL (β). (c) Secondary structure prediction of YaiN (α). (d) Secondary structure prediction of YbeL (β). (e) Net charge of the fusion domains: Arg and Lys contributes +1 and Asp and Glu contributes –1.

Supplemental Fig. 2. Gel-filtration UV traces of the purified overexpressed membrane proteins. 120 ml Size-exclusion gel filtration column (HiLoad 16/60 Superdex 200 prep grade) was equilibrated with buffer containing 20mM Tris pH 7.5, 70mM NaCl, 10mM KCl and 0.02-0.05 % detergent. Flow rate was 1ml/min. (a) β-SERT-β̲ solubilized in Fos-choline-14, followed by detergent exchange to DTM with PE + Cholesterol. (b) β-hVNUT solubilized in Fos-choline-14, followed by detergent exchange to DTM with PE. (c) β-NPT1-β̲ solubilized in Fos-choline-14, followed by detergent exchange to DTM with PE + Cholesterol. (d) β-hSialin-β̲ solubilized in Fos-choline-14, followed by detergent exchange to DDM with PE + Cholesterol. (e) β-mSialin-β̲ solubilized in Fos-choline-14, followed by detergent exchange to DDM with PE+ Cholesterol. (f) β-KvLm-β solubilized in Fos-Choline-16, followed by detergent exchange to DDM. (g) β-MntH-β solubilized in DDM.

Supplemental Fig. 3. Proteolysis of β-MntH-β, β-MntH , and α-GAT4. (a) β-MntH-β in 0.02% Fos-choline-16 was incubated with TEV protease (1:25) at 4°C for 15 h, followed by size-exclusion chromatography. (b) β-MntH in 0.02% DTM was incubated with TEV protease (1:25) at 4°C for 15 h, followed by size-exclusion chromatography. (c) α-GAT4 in 0.1% DTM was incubated with TEV protease (1:3) at 4°C for 15 h, followed by size exclusion chromatography. (d) Trypsin proteolysis of β-KvLm-β in 0.02% DDM. β-KvLm-β was incubated with TEV protease (1:1000) at 4°C for 10 min, 30 min, and for 1,2,3,15 h. (e) Oligomerization state analysis of the

purified Trypsin-digested KvLm. Potein was loaded on 7%-60% linear sucrose gradient. Gradient fractions were collected and analyzed by CBB stainin. Molecular weight marker size from the top, in kDa: 118, 85, 48, 36, 26, 20. (f) TEV proteolysis site generation via complementary oligonucleotides annealing.

Supplementary Figure 1: Bioinformatic characterization of the fusion domains.



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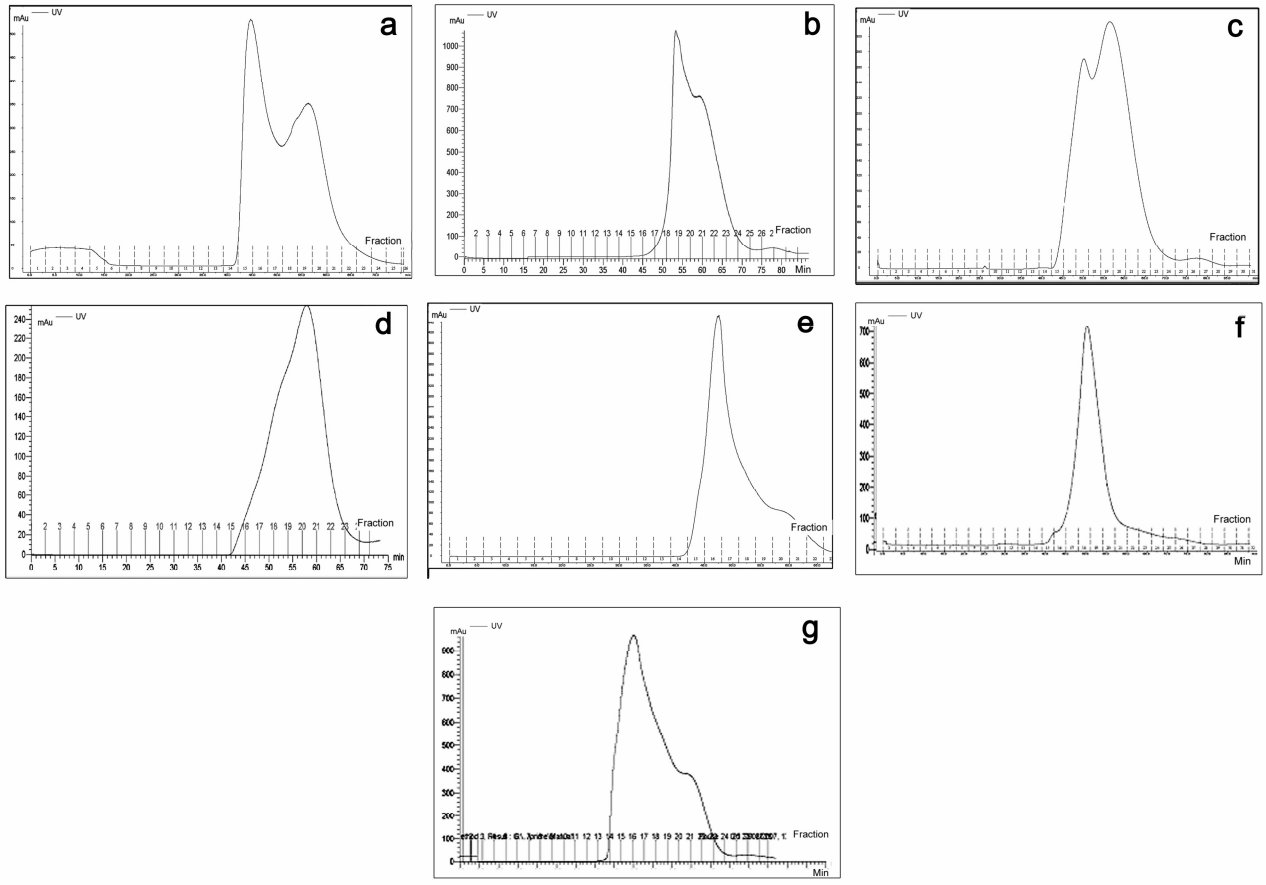
Net charge of the fusion domains

	Arg	Lys	Asp	Glu	Net charge
α	11	4	6	10	-1
β	12	5	8	19	-10

Supplementary Figure 2: Gel-filtration UV traces of the purified overexpressed membrane proteins.



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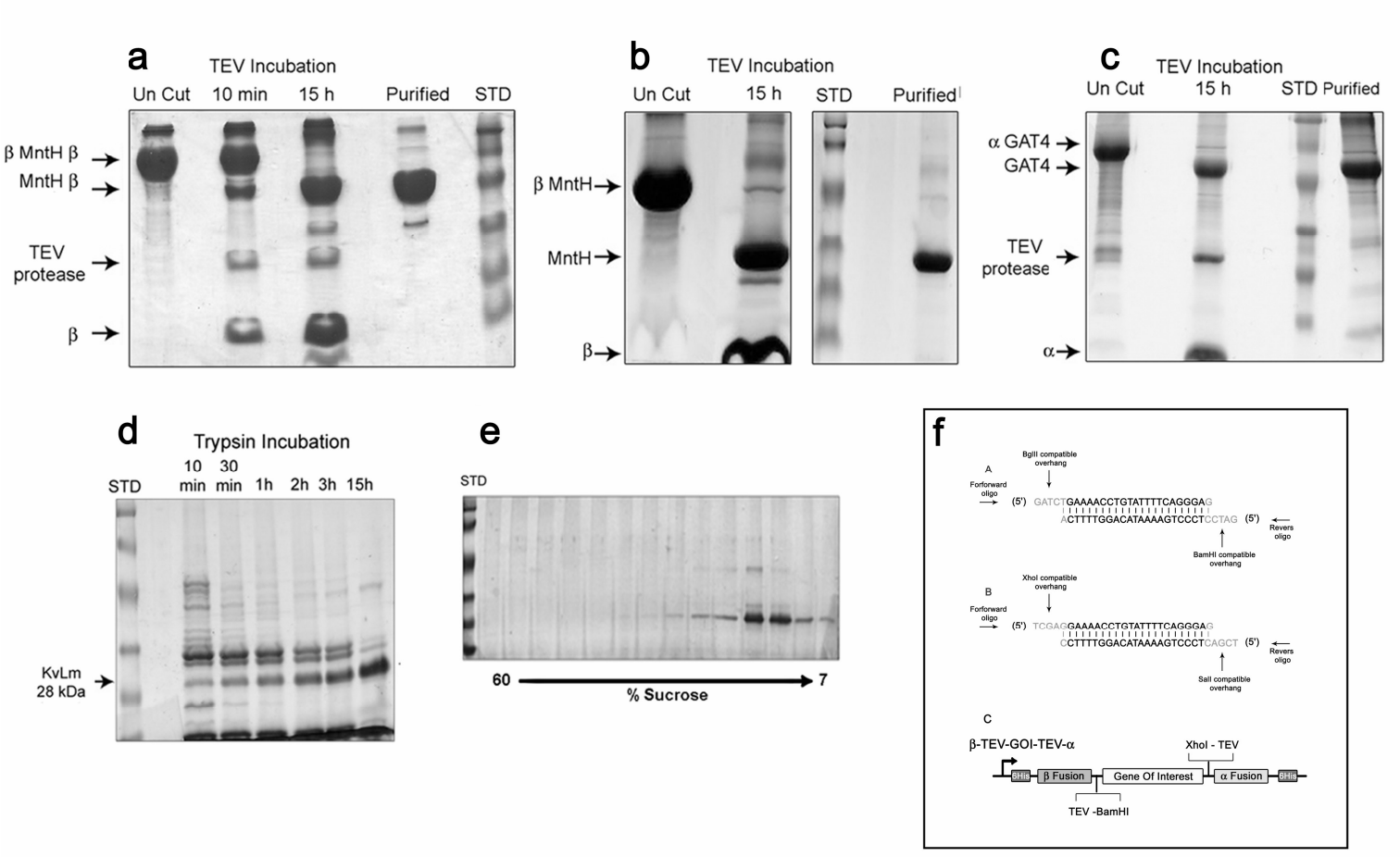
Supplementary Figure 3: TEV and Trypsin proteolysis.



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