PNAS

Supporting Information for

A Na pump with reduced stoichiometry is upregulated by brine shrimp in extreme salinities

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β(S11081)	MADKKP**D**E**Q**FVGSGPK**E**TKWQS**F**K**G**F**V**WNSETS**Q**FMGRT**AGS**WAKITIFY
β1	MADKKP**D**E**Q**FVGSGPK**E**TKWQS**F**K**G**F**V**WNSETS**Q**FMGRT**AGS**WAKITIFY
β2	MADKKP**E**E**F**FVGSGPK**P**TKWQS**V**K**T**F**I**WNSETS**E**FMGRT**GVN**WAKITIFY

β(S11081)	VIFYTLLAGFFAGMLMIFYQTLDFKIPKWQNKDSLIGANPGLGFRPMPPE
β1	VIFYTLLAGFFAGMLMIFYQTLDFKIPKWQNKDSLIGTNPGLGFRPMPPE
β2	VIFYTLLAGFFAGMLMIFYQTLDFKIPKWQNKDSLIGTNPGLGFRPMPPE

β(S11081)	AQVDSTLIQFKHGIKGDWQYWVHSLTEFLEPYETLTSSGQEFTNCDFDKP
β1	AQVDSTLIQFKHGIKGDWQYWVHSLTEFLEPYETLTSSGQEFTNCDFDKP
β2	AQVDSTLIQFKHGIKGDWQYWVHSLTEFLEPYETLTSSGQEFTNCDFDKP

β(S11081)	PQEGKACNFNVELLGDHCTKENNFGYELGKPCVLIKL**T-D**FGWRPEVYNS
β1	PQEGKACNFNVELLGDHCTKENNFGYELGKPCVLIKL**NKI**FGWRPEVYNS
β2	PQEGKACNFNVELLGDHCTKENNFGYELGKPCVLIKL**NKI**FGWRPEVYNS

β(S11081)	SAEVPEDMPADLKSYIKDIETGNKTHMNMVWLSCEGETANDKEKIGTITY
β1	SAEVPEDMPADLKSYIKDIETGNKTHMNMVWLSCEGETANDKEKIGTITY
β2	SAEVPEDMPADLKSYIKDIETGNKTHMNMVWLSCEGETANDKEKIGTITY

β(S11081)	TPFRGFPAYYYPYLNVPGYLTPVVALQFGSLQNGQAVNVECKAWANNISR
β1	TPFRGFPAYYYPYLNVPGYLTPVVALQFGSLQNGQAVNVECKAWANNISR
β2	TPFRGFPAYYYPYLNVPGYLTPVVALQFGSLQNGQAVNVECKAWANNISR

β(S11081)	DRQRRLGSVHFEIRMD
β1	DRQRRLGSVHFEIRMD
β2	DRQRRLGSVHFEIRMD

Fig. S1. Sequence alignment of the two β subunits in the transcriptome (β1 and β2) with the previously reported sequence (S11081). β1 coincides with S11081, except for three residues. β1 and β2 are splice variants that differ in their N-terminus**.**

$\alpha1_{NN}$ (1) **MDSYRVATTSTLADDNRRADGRVK**MAKGKQKKGKDLNELKKELDIDFHKIPIEECYQRLGSNPETGLTNAQARSNIERDGPNCLTPPKTTPEWIKFCKNL
$\alpha2_{KK}$ (1) ----------------------------MGKKQGKQLSDLKKELELDQHKIPLEELCRRLGTNTETGLTSSQAKSHLEKYGPNALTPPRTTPEWIKFCKQL
$\alpha3_{NN}$ (1) ----------------------------MGKKGQNIEELKKELELDGHKISIEELCKRLGTNPKTGLTKAQARENFDRDGPNALSPGKTTPEWLKFLKNL

$\alpha1_{NN}$ (101) FGGFALLLWTGAILCFLAYGIEASSGNEDMLKDNLYLGIVLATVVIVTGIFSYYQENKSSRIMDSFKNLVPQYALALREGQRVTLKAEELTMGDIVEVKF
$\alpha2_{KK}$ (74) FGGFQMLLWIGSILCFIAYTMEKYK-NPDVLGDNLYLGLALLFVVIMTGCFAYYQDHNASKIMDSFKNLMPQFAFVIRDGKKIQLKAEEVTVGDLVEVKF
$\alpha3_{NN}$ (73) FGGFSLLLWIGSILCFIAYYIEVST-AEVPLADHLYLGIVLASVVIVTGCFSYYQENKTSRIMESFRNLVPQYALVVREGHRLTIKAEEVAIGDVVECQS

$\alpha1_{NN}$ (201) GDRVPADLRVLEARSFKVDNSSLTGESEPQARSPEFTNDNPLETKNLAFFSTNAVEGTMRGIVIGIGDNTVMGRIAGLASGLDTGETPIAKEIAHFIHII
$\alpha2_{KK}$ (173) GDRIPADIRITSCQSMKVDNSSLTGESEPQSRSTECTNDNPLETKNLAFFFTNTLEGTGRGIVINVGDDSVMGRIACLASSLDSGKTPIAREIEHFIHII
$\alpha3_{NN}$ (172) GDRIPADVRITEARSFKVDNSSLTGESEPQPRGPEYTNENPLETRNLAFFSTNAVEGAMRGIVINIGDNTVMGRIAVLASGLETGVTPIAKEIDHFIRII

$\alpha1_{NN}$ (301) TGVAVFLGVTFFIIAFVLGYHWLDAVVFLIGIIVANVPEGLLATVTVCLTLTAKRMASKNCLVKNLEAVETLGSTSTICSDKTGTLTQNRMTVAHMWFDG
$\alpha2_{KK}$ (273) TAMAVSLAAVFAVISFLYGYTWLEAAIFMIGIIVAKVPEGLLATVTVCLTLTAKRMAKKNCLVRNLEAVETLGSTSTICSDKTGTLTQNRMTVAHMWFDQ
$\alpha3_{NN}$ (272) TSVAVFLGITLFIIAFILGYHWVDAVVFLIGIIVANVPEGLLATVTVCLTLTAKRMASKNCLVKNLEAVETLGSTSTICCDKTGTLTQNRMTVSHMWFDG

$\alpha1_{NN}$ (401) TITEADTTEDQSGAQFDKSSAGWKALVKIAALCSRAEFKPNQSTTPILKREVTGDASEAAILKCVELTTGETEAIRKRNKKICEIPFNSANKFQVSIHEN
$\alpha2_{KK}$ (373) KIVTADTTENQSGNQLYRGSKGFPELIRVASLCSRAEFKTEHAHLPVLKRDVNGDASEAAILKFAEMSTGSVMNIRSKQKKVSEIPFNSANKYQVSVHER
$\alpha3_{NN}$ (372) SAVKADTTEDQSGVQFDRSSPGWRALVRIAALCSRAEFRPLQQDVPVLKREVIGDASEAAILKCVELCTSQTDAIRWRNRKICEIPFNSTNKFQISIHEN

$\alpha1_{NN}$ (501) EDKSDGRYLLVMKGAPERILERCSTIFMNGKEIDMTEELKEAFNNAYMELGGLGERVLGFCDYLLPLDKYPHGFAFNADDANFPLTGLRFAGLMSMIDPP
$\alpha2_{KK}$ (473) EDKSG--YFLVMKGAPERILERCSTILIDGTEI**L**LDNHMKECFNNAYMELGGMGERVLGFCDFELPSDQYPRGYVFDADEPNFPISGLRFVGLMSMIDPP
$\alpha3_{NN}$ (472) EDRIDGRYHLVMKGAPERILDCCSTIYVNGEERPLDNEAKEAFDDVYMELGGLGERVIGFCDFYLPRDKYPRGYIFNPDDINFQLTGLRFVGLMSMIDPP

$\alpha1_{NN}$ (601) RAAVPDAVAKCRSAGIKVIMVTGDHPITAKAIAKSVGIISEGNETVEDIAARLNIPVSEVNPRDAKAAVVHGGELRDITPDALDEILRHHPEIVFARTSP
$\alpha2_{KK}$ (571) RAAVPDAVSKCRSAGIKVIMVTGDHPITAKAIARQVGIISEGHETVDDIAARLNIPVSEVNPRSAQAAVIHGNDLKDMNSDQLDDILRHYREIVFARTSP
$\alpha3_{NN}$ (572) RAAVPDAVAKCRSAGIKVIMVTGDHPITAKAIAKSVGIISEVSETVEDIAARLNIPVSEVNPEFAKAAVIHGNDLRDYTPERLDYVLRHYSEIVFARTSP

$\alpha1_{NN}$ (701) QQKLIIVEGCQRQGAIVAVTGDGVNDSPALKKADIGVAMGIAGSDVSKQAADMILLDDNFASIVTGVEEGRLIFDNLKKSIVYTLTSNIPEISPFLLFIL
$\alpha2_{KK}$ (671) QQKLIIVEGVQRQGEFVAVTGDGVNDSPALKKADIGVAMGIAGSDVSKQAADMILLDDNFASIVTGVEEGRLIFDNIKKSIAYTLTSKIPELSPFLMYIL
$\alpha3_{NN}$ (672) QQKLIIVEGCQRKGDIVAVTGDGVNDSPALKQADIGVAMGIIGSDVSKEAADMVLLDDNFASIVTGVEEGRLIFDNLKKSIAYTLTSNIPEILPFLMFIL

$\alpha1_{NN}$ (801) FDIPLPLGTVTILCIDLGTDMVPAISLAYEEAESDIMKRRPRNPVTDKLVNERLISLAYGQIGMIQASAGFFVYFVIMAECGFLPWDLFGLRKHWDSRAV
$\alpha2_{KK}$ (771) FDLPLAIGTVTILCIDLGTDVVPAISMAYEGPEAD**LMK**RKPRDPVKEKLVNERLISMAYGQIGVMQAFGGFFTYFVIMGECGFLPNRLFGLRKWWESKAY
$\alpha3_{NN}$ (772) FDIPLPLGTVTILCIDLGTDLVPAISLAYEKPESDIMKRKPRSPITDKLVNERLISMAYGQIGFIQASAGFFTYFTIMAENGFLSGYLFGLRRAWDSRAI

$\alpha1_{NN}$ (901) NDLTDSYGQEWTYDARKQLEYSCHTAYFVSIVIVQWADLIISKTRRNSVFQQGMRNNILNFALVFETCLAAFLSYTPGMDKGLRMYPLKINWWFPALPFS
$\alpha2_{KK}$ (871) NDLTDSYGQEWTWDARKQLEYTCHTAFFISIVIVQWTDLIICKTRRLSLFQQGMKNGTLNFALVFETCVAAFLSYTPGMDKGLRMYPLKIWWWFPPMPFS
$\alpha3_{NN}$ (872) NDLEDSYGQEWTYDARKELEYTCHTAYFISIVVVQWTDLIICKTRRNSLFQQGMGNQPLKFGIFFETFVAAFLSYCPGTDKGLRMYPLKLSWWFPALPFA

$\alpha1_{NN}$ (1001) FLIFVYDEARKFILRRNPGGWVEQETYY
$\alpha2_{KK}$ (971) LLILVYDECRKFLMRRNPGGFLERETYY
$\alpha3_{NN}$ (972) IFILVYDESRKLIMRRNPGGWVERETYY

Fig. S2. Alignment of the three α subunit isoforms found in our transcriptome. $\alpha1_{NN}$ coincides with the previously reported CAA39972 except for its longer N-terminus (shown in bold font). $\alpha2_{KK}$ is almost identical to the previously described (P17326), except where indicated in bold. P17326 has a Pro instead of Leu at 504, and the sequence Leu831/M832/K833 (also in bold font) is substituted by P831. $\alpha3_{NN}$ has not been previously described. Both $\alpha1_{NN}$ and $\alpha3_{NN}$ are canonical NKAs. The asparagine residues of canonical α subunits that are lysine residues in $\alpha2_{KK}$ are highlighted.

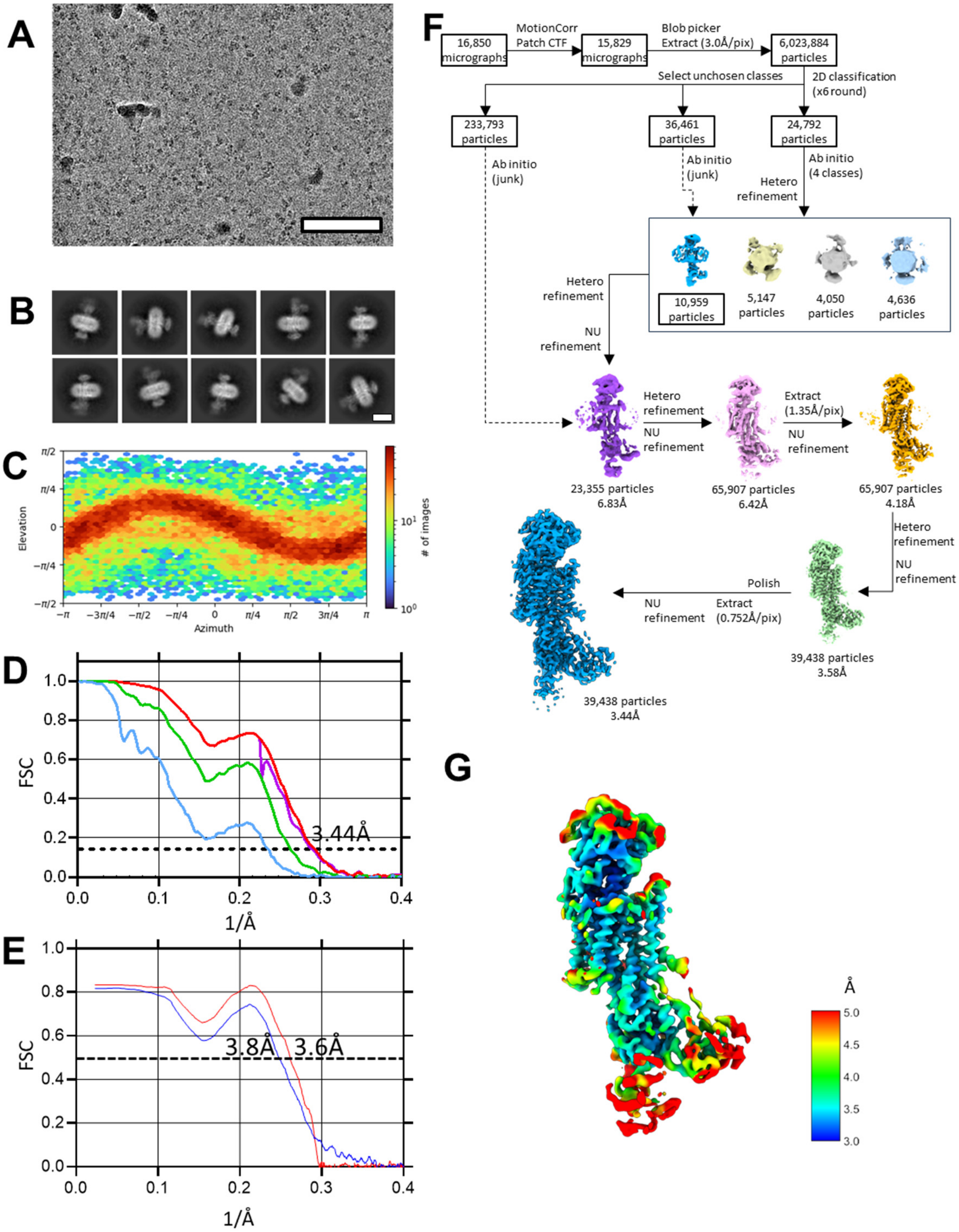

Fig. S3. Cryo-EM analysis. **A)** Representative micrograph. Scale bar 100 nm. **B)** 2D-class averages. Scale bar 70 Å. **C)** Angular distribution plot of particles included in the 3D reconstruction. The number of views at each angular orientation is represented by the color (blue to red). **D)** Fourier Shell Correlation (FSC) plot used for resolution estimation (blue: no mask, green: loose, red: tight, purple: corrected). The dotted line indicates FSC = 0.143. **E)** Correlations between pdb model and EM maps (red: fullmap, blue: half map). The dotted line indicates FSC = 0.5. **F)** Data processing flow chart. See Methods for details. **G)** Unsharpened map colored by local resolution as calculated by cryoSPARC (scale is indicated in the figure).

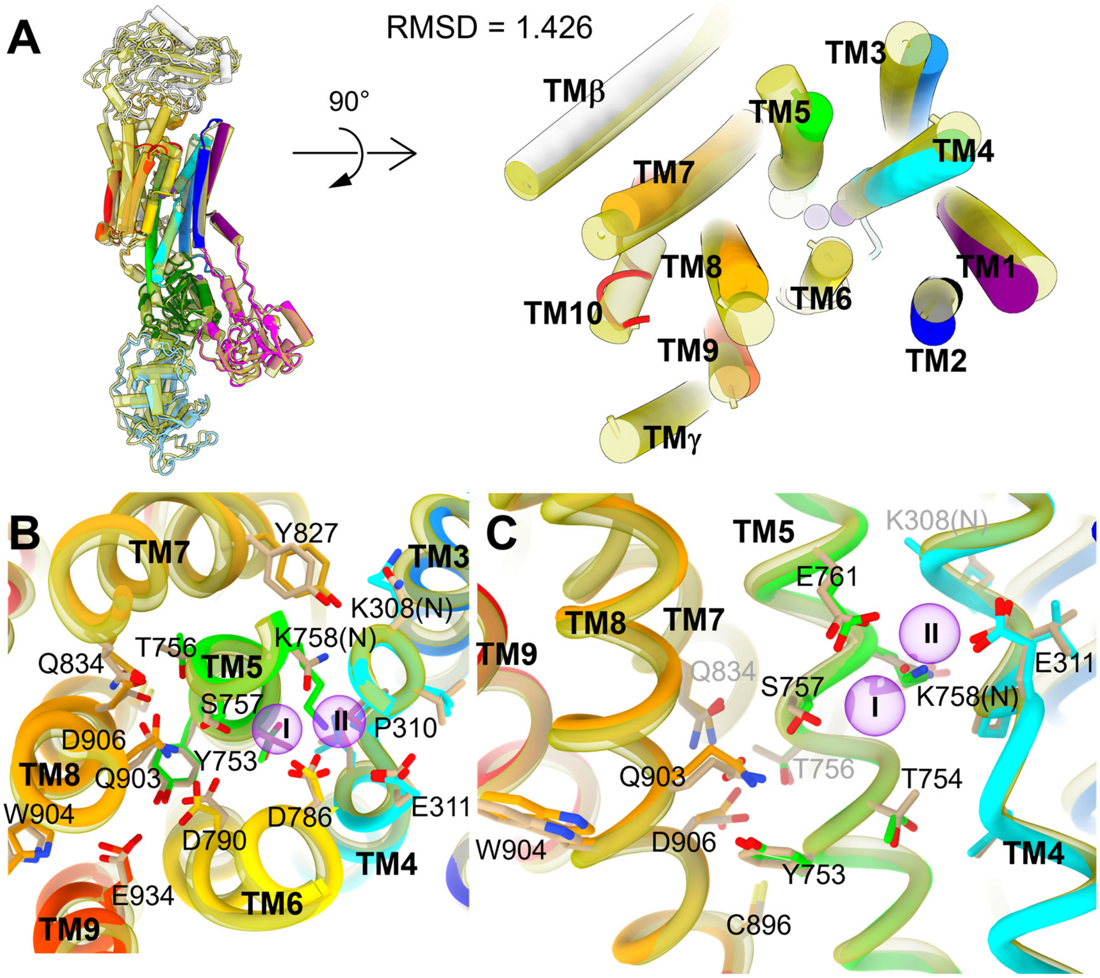

Fig. S4. Structural alignment of *Artemia*'s $\alpha2_{KK}$ in E2-AlF_4 with the canonical pig α1 in E2(2K)-MgF_4. **A)** Overall view of the aligned structures from the membrane plane (*left*) and the transmembrane domain from the extracellular side, perpendicular to the membrane (*right*). **B, C)** Atomic detail of the ion-binding site region viewed perpendicular **(B)** or parallel **(C)** to the membrane. For clarity, TM6 and TM9 were removed in C. Of note, all the residues important for direct or indirect stabilization of Na^+ bound to site **III** in E1 structures of canonical NKAs are conserved in $\alpha2_{KK}$.

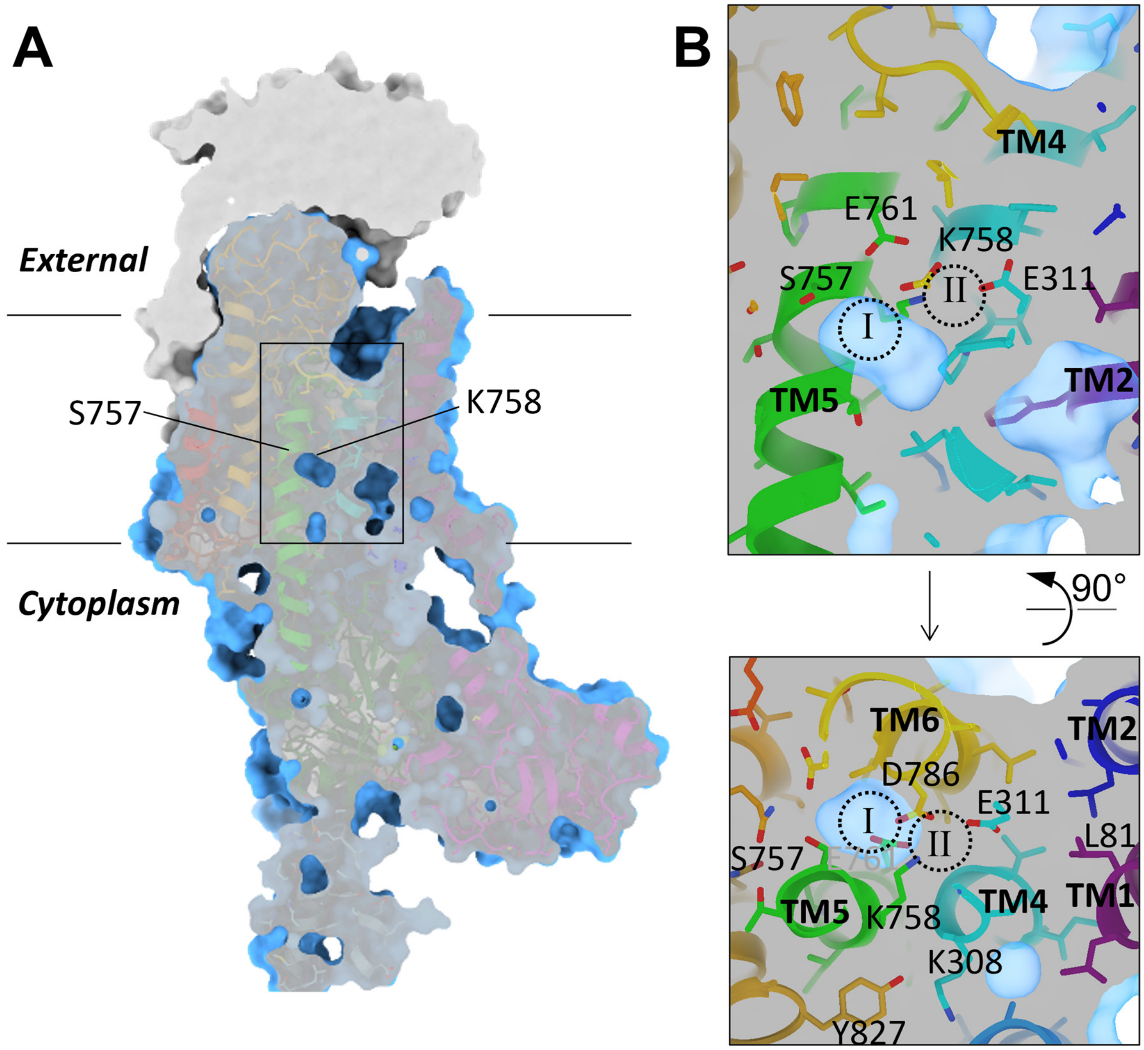

Fig. S5. **A)** Overall clipped membrane slice of *Artemia* $\alpha2_{KK}$ viewed from the membrane plane. **B)** Close-up view at the position indicated by a black box in A, viewed from a plane approximately parallel to the membrane with extracellular side up (*top*), and from cytoplasmic side (*bottom*). Several key amino acids are indicated in the figure. Dotted circles (**I**, **II**) indicate K$^+$-binding site **I** and **II** in the (2K$^+$)E2-P state of the canonical pig α1 (2zxe).

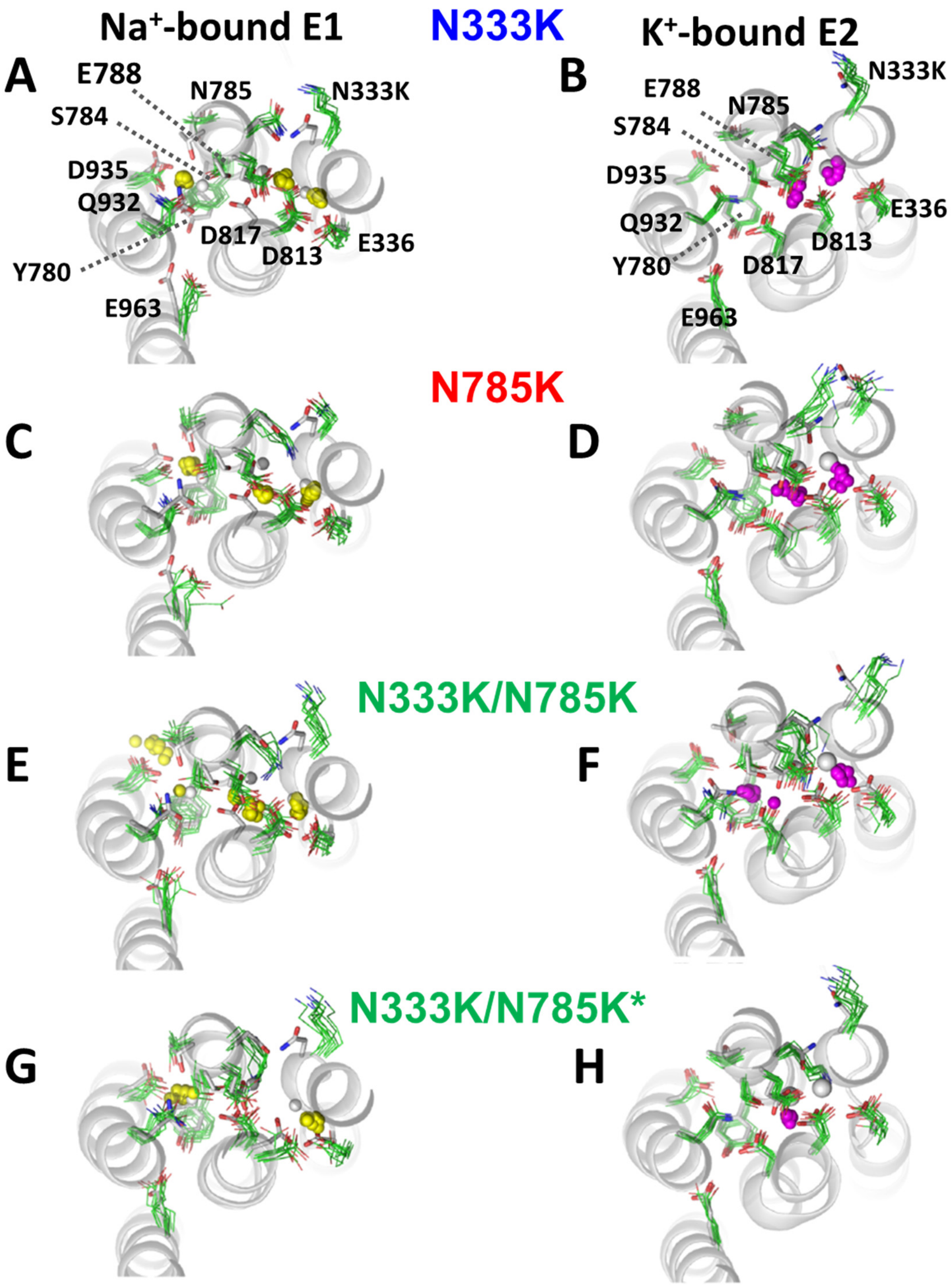

Fig. S6. Representative snapshots from 100 ns-long MD simulations of the mutants introduced in the E1(3Na$^+$), or the E2(2K$^+$) structures, shown on the *left* and the *right*, respectively. **A)** and **B)** N333K, **C)** and **D)** N785K, **E** and **F)** N333K/N785K, **G)** N333K/N785K* with one Na$^+$ removed from site **I**, E1 systems starting with the Na$^+$ removed from site **II** were not stable. **H)** N333K/N785K* with one K$^+$ removed from site **II** in the initial conditions.

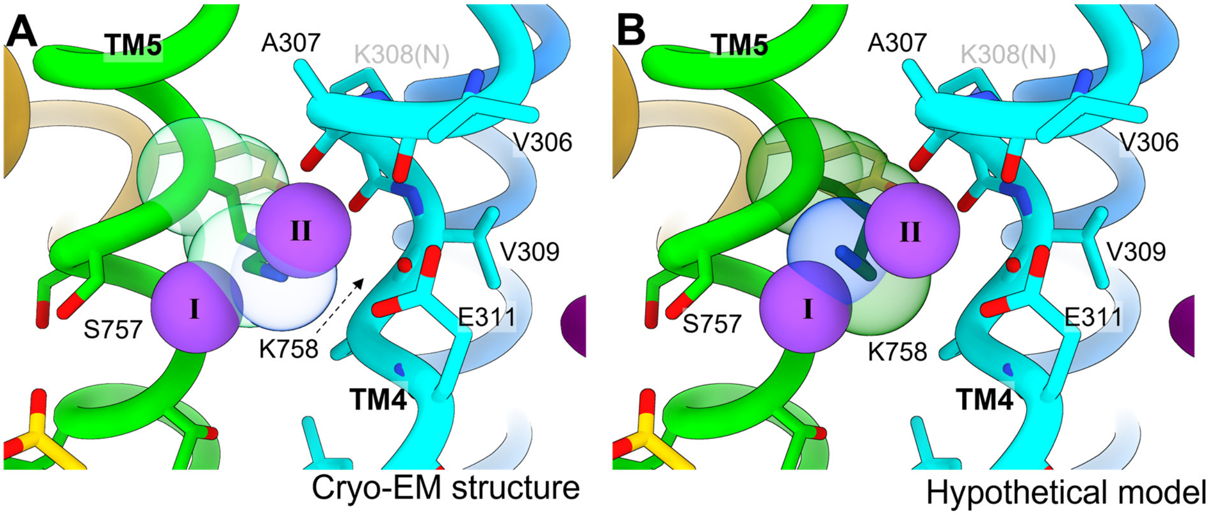

Fig. S7. A hypothetical model of Lys758 rotamer. **A**) Close-up side view (exoplasmic side-up) of the cation-binding site of *Artemia's* α2$_{KK}$ as modeled based on cryo-EM map. **B**) Model using a different rotamer exclusively for Lys758, allowing it to go into site I. Purple spheres represent K^+ ions in the $(2K^+)E2\text{-}P_i$ state of the superimposed canonical NKA (2zxe). Transparent spheres show van der Waals volume of Lys758 side chain. The ε-amine and δ-carbon of Lys758 sterically clash with both K^+ ions in the cryo-EM model in A. If K^+ were to bind to site I (hypothesis 1 in the discussion), Lys746 side chain may be pushed toward the unwound part of TM4 (as noted with a dotted arrow in A) to closely interact with the main chain oxygen atoms of V306, A307 and V309 (sticks). If the K^+ bound structure was closer to B, with a different Lys746 facing site I to bind K^+ to site II (Hypothesis 2 in the discussion) the K^+ in site II still clashes with the Lys758 ε-amine.

Table S1: Transcriptome assembly statistics.

Statistic	**Metric**
Total Trinity ‘transcripts’	764788
Total Trinity ‘genes’	580596
Contig N50 (nt)	719
Median contig length (nt)	327
Mean contig length (nt)	567.51
Total assembled bases	434025290
BUSCO score (metazoa_odb9)	C:97.3% [S:39.8%, D:57.5%], F:1.2%, M:1.5%, n:978

Table S2. Primer identification, sequence, and product size for target *Artemia* genes in quantitative PCR.

Target	Sequence (5’-3’)	Product Size (bp)
$\alpha 1_{NN}$ FWD	CGTATTGCTGGTCTCGCTTC	107
$\alpha 1_{NN}$ RVS	ACACCAAGAAACACACGCAC	
$\alpha 2_{KK}$ FWD	AGGAGGCATGGGTGAAAGAG	180
$\alpha 2_{KK}$ RVS	TTCGAAACGGCATCAGGAAC	
$\alpha 3_{NN}$ FWD	TGTTGAAGGTGCTATGCGTG	163
$\alpha 3_{NN}$ RVS	TTCCCAAGAACACAGCAACG	
NKA β FWD	ACGATTTCAAGTCTGCTGGC	152
NKA β RVS	CGGAGATCATTGCACCAAGG	
NKA ß1 FWD	CTACCAGCTGTCCTTCCCAT	222
NKA ß1 REV	AGCCCAGTTTCACAGTCAGT	
NKA ß2 FWD	TCTTGGCCCAGTTAACACCT	212
NKA ß2 REV	TCACTTGTCTTTCAGCTGTTGA	
EF1α FWD	TCACCAAAGCCGCAGAAAAG	118
EF1α RVS	CGAAAGTGCCGTAGTAACCG	
α-Tubulin FWD	CGAATTTGCCGTCTACCCAG	116
α-Tubulin RVS	TGTCGACCATAAAAGCGCAG	

Table S3 Statistics of the structural analysis.

Conformation	E2-Pi
PDB ID	8K1L
EMDB	EMD-36794
Data collection	
Magnification	60,000
Voltage (kV)	300
Electron exposure (e^-/Å^2)	60
Defocus range (µm)	0.8-1.8
Pixel size (Å/pix)	0.752
Symmetry imposed	*C1*
Movies (no.)	16,850
Initial particles (no.)	6,023,884
Final particles (no.)	39,483
Box size (extract/final, pix)	320/450
Map resolution (Å)	3.44
Map sharpening B-factor (Å^2)	-92.7
FSC threshold	0.143
Refinement	
Initial model used (PDB)	2zxe
Model resolution (Å)	3.6
FSC threshold	0.5
Model composition	
Non-hydrogen	9,895
Protein residues	1,255
Waters	0
Ligands	AlF
B-factor (mean value, Å^2)	
Protein	110.72
Ligand	94.35
Water	-
R.m.s. deviations	
Bond length (Å)	0.004
Bond angles (°)	0.734
Validation	
MolProbity score	2.18
Clashscore	13.29
Poor rotamers (%)	0.37