

Supplementary Information for

Structural basis for amino acid exchange by a human heteromeric amino acid transporter

Di Wu¹, Tamara N. Grund¹, Sonja Welsch², Deryck J. Mills³, Max Michel¹, Schara Safarian^{1*}, Hartmut Michel^{1*}

*Corresponding authors: Hartmut Michel and Schara Safarian

Email: Hartmut.Michel@biophys.mpg.de; Schara.Safarian@biophys.mpg.de

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Movies S1 to S2

Fig.	S1.
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Fig. S1. Sample preparation and validation. **(A)** Fluorescence micrographs of uninduced and induced Flp-InTM T-RExTM293-b(0,+)AT1-eGFP-rBAT cells. The results confirmed the successful production and trafficking to the plasma membrane. **(B)** SDS PAGE analyses of fractions from the tandem affinity purification procedure. **(C)** SEC profile of purified b(0,+)AT1-rBAT complex. **(D)** Native PAGE and Western immunoblotting results from the peak fraction of the SEC run. Samples subjected to electrophoresis were additionally treated with10 mM DTT to confirm the inter-subunit disulfide bond. **(E)** DSF profile and T_m value of purified b(0,+)AT1-rBAT complex indicate for a

stable complex preparation. The measured T_m is 65.5 ± 0.1 °C. Data is mean \pm SD, n = 5 replicates. (F) Cell based uptake assay of 50 µM L-[³H]Arginine (54.5 Ci/mmol, 0.5 Ci/well) using different cell lines (G) Cell based competition assay of 50 µM L-[³H]Arginine (54.5 Ci/mmol, 0.5 Ci/well) with varying concentrations of cystine using different cell lines. Data in (F) and (G) are mean \pm SEM. n = 3 replicates. The results confirm the activity of the heterologously produced b^(0,+)AT1-rBAT complex.



Fig. S2. Cryo-EM data processing workflow. (A) Exemplary micrograph and 2D class averages from an initial negative staining EM experiment. **(B)** Exemplary micrograph and CTF estimation (Gctf) of the cryo-EM dataset. **(C)** 2D class averages from the cryo-EM dataset. **(D)** Schematic workflow of pre-processing, classification and refinement of cryo-EM data (see Supplementary Methods for details). Different processing approaches are indicated by color. Reported resolution values correspond to FSC = 0.143.



Fig. S3. Map quality analysis of the b^(0,+)**AT1-rBAT structure.** (**A**) Local resolution map of the heterotetrameric b^(0,+)**AT1-rBAT complex with corresponding (B)** 3DFSC plot, and (**C**) Euler angle distributions. (**D**) Local resolution map of the heterodimeric b^(0,+)**AT1-rBAT complex with corresponding (E)** 3DFSC plot, and (**F**) Euler angle distributions. (**G**) Map-to-model correlation curve of maps from three different focused refinement jobs.





Fig. S4. Comparison of overall conformation and substrate-binding sites between b^(0,+)**AT1, LAT1, GkApcT and AdiC.** The overall structure of b^(0,+)AT1 was captured in an inward-facing conformation. Its overall fold resembles that of LAT1, GkApcT and AdiC. The putative binding pocket of b^(0,+)AT1 is characterized by a local environment which is common among SLC7 family amino acid transporters. LAT1 (PDB 6IRT), GkApcT (PDB 5OQT) and AdiC (PDB 3L1L) are colored green, red and yellow, respectively.



Fig. S5. The inward-facing conformation of $b^{(0,+)}$ **AT1.** Schematic illustration of extracellular and cytoplasmic barriers of $b^{(0,+)}$ **AT1** in the inward-facing conformation.

Fig. S6.

LAT1_H.sapiens_SLC7A5 y+LAT2_H.sapiens_SLC7A6 y+LAT1_H.sapiens_SLC7A7 LAT2_H.sapiens_SLC7A8 b(0,+)AT1_H.sapiens_SLC7A9 ASC1_H.sapiens_SLC7A10 ASC1_H.sapiens_SLC7A11 AGT1_H.sapiens_SLC7A13

LAT1_H.sapiens_SLC7A5 y+LAT2_H.sapiens_SLC7A6 y+LAT1_H.sapiens_SLC7A7 LAT2_H.sapiens_SLC7A8 b(0,+)AT1_H.sapiens_SLC7A8 ASC1_H.sapiens_SLC7A10 ACT_H.sapiens_SLC7A11 AGT1_H.sapiens_SLC7A13

LAT1_H.sapiens_SLC7A5 y+LAT2_H.sapiens_SLC7A6 y+LAT1_H.sapiens_SLC7A7 LAT2_H.sapiens_SLC7A8 b(0,+)AT1_H.sapiens_SLC7A8 ASC1_H.sapiens_SLC7A10 ASC1_H.sapiens_SLC7A11 AGT1_H.sapiens_SLC7A13

LAT1_H.sapiens_SLC7A5 y+LAT2_H.sapiens_SLC7A6 y+LAT1_H.sapiens_SLC7A7 LAT2_H.sapiens_SLC7A8 b(0,+)AT1_H.sapiens_SLC7A9 ASC1_H.sapiens_SLC7A10 xCT_H.sapiens_SLC7A11 AGT1_H.sapiens_SLC7A13

LAT1_H.sapiens_SLC7A5 y+LAT2_H.sapiens_SLC7A6 y+LAT1_H.sapiens_SLC7A7 LAT2_H.sapiens_SLC7A8 b(0+)AT1_H.sapiens_SLC7A8 ASC1_H.sapiens_SLC7A10 ASC1_H.sapiens_SLC7A11 AGT1_H.sapiens_SLC7A13

LAT1_H.sapiens_SLC7A5 y+LAT2_H.sapiens_SLC7A6 y+LAT1_H.sapiens_SLC7A7 LAT2_H.sapiens_SLC7A8 b(0+)AT1_H.sapiens_SLC7A9 ASC1_H.sapiens_SLC7A10 xCT_H.sapiens_SLC7A11 AGT1_H.sapiens_SLC7A13

LAT1_H.sapiens_SLC7A5 y+LAT2_H.sapiens_SLC7A6 y+LAT1_H.sapiens_SLC7A7 LAT2_H.sapiens_SLC7A8 b(0+1)AT1_H.sapiens_SLC7A8 b(0+1)AT1_H.sapiens_SLC7A10 xCT_H.sapiens_SLC7A11 AGT1_H.sapiens_SLC7A13

LAT1_H.sapiens_SLC7A5 y+LAT2_H.sapiens_SLC7A6 y+LAT1_H.sapiens_SLC7A6 LAT2_H.sapiens_SLC7A8 b(0,+)AT1_H.sapiens_SLC7A9 ASC1_H.sapiens_SLC7A10 ASC1_H.sapiens_SLC7A11 AGT1_H.sapiens_SLC7A13

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282 275 267 273 260 273 274 246	L P MP I P I P I P MA L P	V T V T V T V T V T V T V T	LVY IIY FVY ACY FVY IGY VVY	VL 1L VF 1L VF 1L VL	TNL TNV ANV MNV TNI TNV	AYI AYY AYY SYI AYI AYI	= T T Y T V Y T V Z T A = T V = T A = T T L T V	L ST L D I MS F MT A MS F I N A L T F			5 S D 5 S D 4 S D 4 S D 5 S N 5 S N 5 S D	AV AV AV AV AV AV	A V A V A V A V A V A V A V		G N N A D C A D C G E F G E F A D F			MS FN MA FS FS LA	W W W W V W V W V L A V W N	PV PL 1PL 1PV 1PV 1PF	FV SV SV FV FV FV AIS	C L S A L S A L S A L S S T S	CF CF CF TF CF CF CF CF CF	GSV GGL GGV GAA GGI GSM GSNL	NG NA NG NG NG	SLF SIV SLF TCF YLF SVF SIF	TS AS TS TA TY AV KS	SRL SRL SRL SRL SRL SRL SRL SRP	FFV FFV FFA IYV CF <mark>S</mark> FYV IYL	G 3 G 3 G 3 G 3 G 3 G 3 G 3 A 3 A 3 A 3	53 46 38 44 31 44 45 17
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495 490 482 486 472 486 487 455	LCC GTC VSC HLC WGC TLC YLC		MQV CFC CM <mark>S</mark> CVV MEV CFV LEV	VP VL VA VP VP VP VP CL			A E - E D G B S G E	TEE	ANI	E DN	MEE 	QQ EE	Q <mark>P</mark> N <mark>G</mark>	MY(PC	- GE QPT 	- E MP T	K K K Q K D P A	DE RD KD	RKT PKS VA <mark>G</mark> K <mark>P</mark> S 	D - N - Q P K P 	Q P Q -									5 5 5 4 5 4 5 4	07 15 35 87 23 01 70

Fig. S6. Sequence alignment of human SLC7 family members. Red stars indicate the

conserved G(S/A)G motif and residues involved in formation of substrate binding sites.

Fig. S7.

rBAT(H.sapiens) Trehalose_Synthase(D.radiodurans) Oligo-1,6-Glucosidase(B.cereus) Alpha-Glucosidase(Geobacillus_sp.HTA-462) Neopullulanase(G.stearothermophilus) 4F2hc(H.sapiens)

rBAT(H.sapiens)

IGA (IT.Sapiens) Trehalose_Synthase(D.radiodurans) Oligo-1,6-Glucosidase(B.cereus) Alpha-Glucosidase(Geobacillus_sp.HTA-462) Neopullulanase(G.stearothermophilus) 4F2hc(H.sapiens)

rBAT(H.sapiens)

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rBAT(H.sapiens)

Trehalose_Synthase(D.radiodurans) Oligo-1,6-Glucosidase(B.cereus) Alpha-Glucosidase(Geobacillus_sp.HTA-462) Neopullulanase(G.stearothermophilus) 4F2hc(H.sapiens)

rBAT(H.sapiens) Trehalose_Synthase(D.radiodurans) Oligo-1,6-Glucosidase(B.cereus) Alpha-Glucosidase(Geobacillus_sp.HTA-462) Neopullulanase(G.stearothermophilus) 4F2hc(H.sapiens)

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rBAT(H.sapiens) Trehalose_Synthase(D.radiodurans) Alpha-Glucosidase(B.cereus) Alpha-Glucosidase(Geobacillus_sp.HTA-462) Neopullulanase(G.stearothermophilus) 4F2hc(H.sapiens)

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Trehalose_Synthase(D.radiodurans) Oligo-1,6-Glucosidase(B.cereus) Alpha-Glucosidase(Geobacillus_sp.HTA-462) Neopullulanase(G.stearothermophilus) 4F2hc(H.sapiens)

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rBAT(H.sapiens) IDA I (n.sapiens) Trehalose_Synthase(D.radiodurans) Oligo-1,6-Glucosidase(B.cereus) Alpha-Glucosidase(Geobacillus_sp.HTA-462) Neopullulanase(G.stearothermophilus) 4F2hc(H.sapiens)

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1	MRKEAIYHR <mark>P</mark> ADNFAYAYDSETLHLRLRTK <u>KD</u> DIDRVELLH <mark>G</mark> D <mark>P</mark> YDWQNGAWQFQMM <mark>P</mark> MRK	61
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151 42 39 39 184 107	Y I TALNIK TVWI TSFYKSSLKDFRYGVEDFREVDPIFGTMEDFENLVAAIHDKGLKLIIDF YLKNLGVDCLWLLPWFPSPLRDDGVDVADYRGIHPDLGTLDDFKVFLREAHARGLRVIGDL YLKELGIDVIWLSPVYESPNDDNGYDISDYCKIMNEFGTMEDWDELLHEMHERNMKLMMDL YLVELGVDIVWICPIYRSPNADNGYDISDYXAIMDEFGTMDDFDELLAQAHRRGLKVILDL YLVELGVDIVWICPIYRSPNADNGYDISDYYAIMDEFGTMDDFDELLAQAHRRGLKVILDL YLVELGVDIVWICPIYRSPNADNGYDISDYYAIMDEFGTMDDFDELLAQAHRRGLKVILDL YLVELGVDIVWICPIYRSPNADNGYDISDYYAIMDEFGTMDDFDELLAQAHRRGLKVILDL YLVELGVDIVWICPIYRSPNADNGYDISDYYAIMDEFGTMDFDELLAQAHRRGLKVILDL YLVELGVDIVWICPIYRSPNADNGYDISDYYAIMDEFGTMDFDELLAQAHRRGLKVILDL YLVELGVDIVWICPIYRSPNADNGYDISDYYAIMDEFGTMDFDELLAQAARRGLKVILDL	211 102 99 99 243 167
212 103 100 100 244 168	I PNHTS DKHIWFQLSETRTGKYT DYY IWH DCTHENGKT I PPNNWLS VYGNSSW VTNHTS DHPWFQAARRGPT L PDG. SPNEYHDYY VWSDECKEYA DTRIIFTDIEVSNW VVNHTS DEHNWFIESEKS	264 159 149 149 281 228
265 160 150 150 282 229	H H D E VRNQCYF H Q FMKE Q P D L NF R NP D V QE E I K E I L R FWL - T K G V D G F SL D A V K - F L L Ê A K T L D E Q A G K Y WH R F A S Q P D L NY D N R K V E E L H G A A R FWL - D L G L D G F R V D A V P - Y L I E R E Q Y D E M T D E Y Y L H L F S K K Q P D L NWD N E K V R Q D Y Y E M K FWL - E K G I D G F R MD V I N - F I S K E Q Y D E M T G Q Y Y L H L F S K K Q P D L NWE N S E V R Q A L Y E M V NIWL - D K G I D G F R I D A I S - H I K K K P C Y D E T G Q Y Y L H I F D V K Q P D L NWE N S E V R Q A L Y E M V NIWL - D K G I D G F R I D A I S - H I K K K P L P R P N Y D T F R F V P Q M P K L NT A N P E V K RY L L D V A T Y WI R E F D I D G WR L D V A N	323 218 208 208 331 288
324 219 209 209 332 289	HLRDEIQVNKTQIPDTVTQYSELYHDFTTTQVGMHDIVRSFRQT GTSCENLPETHEILKGFRAMVDRPYGRLLAEANOWPEEVVEYFG GLPTVETEE-EGYVSGHKHFMNGPNIHKYLHEMNEEVLSHYDIMTVGEMPGVTEEAKLYT GLPDLPNPKGLKYVPSFAGHMNQPGIMEYLRELKEQTFARYDIMTVGEANGVTVDEAEQWV EIDHEFWREFRQEVKAKPDVYILGEIWHDAMP DFDSLLQSAKKKSIRVILDLTPNYRGENSWFSTQVDTVATKVKDALE	367 264 268 269 364 335
368 265 269 270 365 336	MDQYSTEPGRYRFMGTEAYAESIDRTVMYYGLPFIQEADFPFNNYLSMLDTV TEAEPEHMCFNFPVMPRLYMSLKREDTSSIREIMGRLPKIPSFGQWCTFLRH GEERKELQMVFQFEHMDLDSG.EGGKWDVKPCSLLLKENLTKWQKALEHTGWNSLYWNNH GEERGVFNMIFQFEHLGLWERRADGSIDVRRLKRTLTKWQKGLENRGWNALFLENH WLRGDQFDAYMNYPFTDGVLRFFAKEEISARQFANQMMHVLHSY FWLQAGVDG.FQVRDIENLKDASSFLAEWQNITKGFSEDRLLIAGTNSSDLQQILSLLESN	419 318 328 325 408 395
420 319 329 326 409 396	S GNSVYEVITSWMENMPEGKWPNWMIGGPDS RLTSRL GNQYVNVMNMLLFTLPGTP DELTLEMVTDDERAFMYAAYA PDARMKINVGIRRLAFLLDNDRRRIELINTVLLALPGSP DQPRVVSRFGNDGMYRIESAKMLATVLHMKGTPYIYQGEIGMTNVRFESIDEYRDIET DLPRSVSTWGNDRDYWAESAKALGALYFFMQGTPFIYQGQIGMTNVRFDDIRDYRDVSAL PN	476 379 389 386 453 456
477 380 390 387 454 457	ITYYGEEIGMGNIVAANLNESYDINTLRSKSPMOWDNSSNAGFSEASNTWLPTNS ILYYGDEIGMGDLGLPDRNGVRTPMOWNAGTSGGFSTAQPSDCFFPPIODPVY NMYKEKVMERGEDIEKVMQSIYIKGRDNARTPMOWDQNHAGFTTGEPWITVNP RLY-ELERAKGRTHEEAMTIIWKTGRDNSRTPMOWSGASNAGFTGTPWIKVNE CIYYGDEIGMTGGNDPECEKCMVWDPTPWIKVNE LFTLPGTPVFSYGDEIGLDAAALPGQPMEAPVMLWDESSFPDIPGAVS.	531 433 443 439 479 504
532 434 444 440 480 505	: DYHTVNVDVQKTQPRSALKLY-QDLSLLHANELLLNRGWFCHLRNDSHYVVYTRELDGIDR GFGRVNVOSQLQDPSSLLKWTARQLELRRAHPAFAHGDLTFIETGNPAILAFTRQYDGE NYKEINVKQAIQNKDSIFYYYKILELRKNNEIVVYGSYDLILENNPSIFAYVTTYGVG NYRTINVEAERROPNSVWSFYRQMIQLRKANELFVYGTYDLLLENHPSIYAYTTLGRD 	591 492 502 498 529 561
592 493 503 499 530 562	: IFIVVLNFGESTLLNLHNMISGLPAKMRIRLSTNSADKGSKVDTSGIFLDKGEGL ITLLIVSNFAGNAQAGLDLAPFVGRAPVTLSGASPLPVVTGNGVPVVMGKYDYY KLLVIANFTAECIFELPEDISYSEVELLIHN-YDVE-NGPIENITLRPYEAM RALVVVNLSDRFSLYRY-DGFRLOSSDLALSN-YPVRHKNATRFKLKPYEAR ITVLVIINRSDQKADIPIPLDARGTWLVNLLTGERFAAEAETLCTSLPPYGFV RFLVVLNFGDVGLSAGLQASDLPASASLPAKADLLLSTQPGREEGSPLELERLKLEPHEGL	646 547 553 549 581 622
647 548 554 550 582 623	IFEHNTKNLLHRQTAFRDRCFVSNRACYSSVLNILYTSC WLRLNLEHHHHHH VFKLK VYKLK LYAIEHW- LYAIEHW- LRFPYAA	685 560 558 555 588 630

Fig. S7. Full length sequence alignment of human rBAT, 4F2hc and GH13 α -amylase family

members. Red stars indicate conserved residues participating in Ca²⁺ binding and coordination.

588 630

Fig. S8.

GCQTNNGFVHNEDILEQTPDPGSSTDNLKHSTRGILGSQEPDFKGVQPYAGMP GCRTNNGFVQNEDIQEQDPD---SRDTPQSNAVSIPAPEEPQLKVVRPYAGMP GCQTNNGFVQNEDIPELDLD-----PGSSEHIGPEEPALKAVIQPYAGMP GCQTNNGFVQNEDIPEQDPDG-SRDTPQPNAVSIPAPEEPHLKAVPYAGMP EGQTNNGFVQNEDIRETDLDPSSPVVGPQHNTVDILGPGEPDVKDVRPYAGMP MAED<mark>KS</mark>K MNEDKDK MAEEGSK MDEDKGK MAED<mark>KS</mark>K RDS I EMSMK RDS I QMSMK RDS I KMNMK 70 67 62 rBAT(H.sapiens) rBAT(R.norvegicus) rBAT(O.cuniculus) 69 70 70 rBAT(M.musculus) R D P I Q M S L P rBAT(B.taurus) DS rBAT(O.aries) GOT DLDPSSPAAGPQHNTVD I LGPGE rBAT(H.sapiens) 140 71 REILFWLTVVSVFLLIGATIAIIISP REILFWLVVSVFLLIGATIAIIISP REILFWLVVSVFLLIGATIAIIAISP REILFWLTVVSVFLLIGATIAIIAISP EVLFQFSGQARY EVLFQFSGQARY EVLFQFSGQARY EVLFQFSGQACY RVP RVP RVP KCLDWWQAGPMYQI KCLDWWQAGPMYQI KCLDWWQAGPIYQI KCLDWWQAGPNYQI RSF RSF RSF RSF CDGNG CDGNG CDGNG CDGDG DSD DSD DSD DSN rBAT(R.norvegicus) rBAT(O.cuniculus) 68 137 132 63 rBAT(M.musculus) rBAT(B.taurus) 139 140 70 71 rBAT(O.aries) 71 EVLEW SVLLLIAA<mark>T</mark>IAIIAISP CLDWWQAGPMY 140 rBAT(H.sapiens) 141 210 E I DP IF GTMKDFENLVAAVHDKGL EI IDP IF GTMEDFENLLAAIHDKGL EI IDP IF GTMKDFENLVAAIHDKGL EI IDP IF GTMKDFENLVAAIHDKGL KLDYI1 KLDYI1 KLDYI1 KLDYI1 T I WI T T I WI T T L WI T RYAVEDF RYGVEDF RYAVEDF RHGVEDF 207 202 rBAT(R.norvegicus) 138 GIQE SFY SFY SFY SFY rBAT(O.cuniculus) 133 SSL SSL SSL rBAT(M.musculus) 140 141 209 210 rBAT(B.taurus) GIQD vw rBAT(O.aries) 141 DL GIQD 210 TMKDFENLVAAIHD * rBAT(H.sapiens) 211 YTDYYIWHDCTHENGKII YTDYYIWHCTHANGVT YTDYYIWHCAHENGIT YTDYYIWHCHENGTT YTDYYIWHCRHCNRENGTTI 280 CHPWFQSSRTRSG HAWFQLSRTRTG CHPWFQSSRTRSG KHAWFQWSRNQTG PPNNWLSVYGNSSWUFDEF PPNNWLSVYGNSSWUFDEV PPNNWLSVYGNSSWHFDEVF RKQCYFHQFLK RNQCYFHQFLK RKQCYFHQFLK RKQCYFHQFLK rBAT(R.norvegicus) 208 277 NHTSD 203 F I PNHTSD 210 F I PNHTSD 211 F I PNHTSD rBAT(O.cuniculus) 272 rBAT(M.musculus) 279 rBAT(B.taurus) 211 NWL 280 rBAT(O.aries) 211 HAWEOW IWHDONYENG 280 FIPNHTSD NPDVQEEI NPAVQEEI NPDVQEEI NPDVQEEI GVDGFSLDAVKFLLEAKHLRDEIQVI GVDGFSFDAVKFLLEAKDLRNEIQVI GVDGFSFDAVKFLLEAKDLRNEIQVI GVDGFSFDAVKFLLEAKHLRNEIQVI GVDGFSFDAVKFLLEAKHLRDEAQVI rBAT(H.sapiens) rBAT(R.norvegicus) 281 278 EQPDLNFF EQPDLNFF EQPDLNFF EQPDLNFF EQPDLNFF 350 CETTKFWLS CETTKFWLS CETTFWLS CETTFWLS QIPDTV QIPDTV QIPDTV 347 SEL 273 280 SELY 342 349 rBAT(O.cuniculus) rBAT(M.musculus) rBAT(B.taurus) 281 350 rBAT(O.aries) 281 350 FNAL YRFMGTEAYAESIDRTVMYYGLPFIQEADFPFNNYLSMLDTVS 420 YRFMGTEVSAESTERTMVYYGLSFIQEADFPFNKYLATLDTLS 417 YYRFNGAEASAESIDRTMRYYGLSFIQEADFPFNKYFTTLGTLS 412 YRFMGAEASAESIERTMMYYGLPFIQEADFPFNKYFTTLGTLS 419 YRFMGTEAHGESITKTMVYYGLPFIQEADFPFNSYLSKLDKPS 420 YRFMGTEAHGESITETMVYYGLPFIQEADFPFNSYLSKLDKPS 420 rRAT(H saniens) 351 VGMHDI MDC TE TTQVGMHDIVRSF TTQEGMHDLVRSF TTQEGMHDLVRSF TTQVGMHDIVRSF TTQVGMHDIVRSF MNQFSREP MDKYSREP MNQYSREP rBAT(R.norvegicus) 348 rBAT(O.cuniculus) 343 350 rBAT(M.musculus) MNQ rBAT(B.taurus) 351 351 rBAT(O.aries) WPNWMIGGPDSSRLTSRLGNQYVNVMNMLLFTLPGTPITYYGEEIGMGNIV 490 WPNWMIGGPETSRLTSRVGSEYVNAMNMLLFTLPGTPITYYGEEIGMGDIS 487 WPNWMTGGPDITRLTSRLGNQYVNIMNMLLFTLPGTPITYYGEEIGMGNIL 482 WPNWMTGGPETPRLTSRVGSEYVNAMHMLLFTLPGTPITYYGEEIGMGNIL 480 WPNWMTGGPDNVRLTSRLGEKYVNIMNMLVFTLPGTPITYYGEEIGMRNIL 490 rBAT(H.sapiens) 421 GN WMENM 418 GHT VYEA I TSWMENMPEG 413 GHT VYEA I TSWMENMPEG 420 GHT VYEV I TSWMENMPEG 421 GNSVSEI I TSWMENMPEG rBAT(R.norvegicus) rBAT(O cuniculus) rBAT(M.musculus) rBAT(B.taurus) rBAT(O.aries) 421 491 AANLNESYD INTLRSKSPMQWDNSSNAGF SEASNTWLPTNSDYHTVNVDVQKTQPRSALK 488 ITNLNERYDTNALLSKSPMQWDNSSNAGF TEANHTWLPTNSDYHTVNVDVQKTQPSALK 483 ATNLNESYDVNTLLSKSPMQWDNSSNAGF SEGNHTWLPTSSDYHTVNVDVQKTQPTSALK 490 VTNFRESYDSTLVSKSPMQWDNSSNAGF TEANHTWLPTNSDYHTVNVDVQKTQPSALK 491 AANLNETYDAGTLFSKSPMQWDNSSNAGF SEGNHTWLPTSSDYHTVNVDVQKTQPRSALK 491 AANLNENYDTGTLFSKSPMQWDNSSNAGF SEGNHTWLPTSSDYHTVNVDVQKTQPRSALK rBAT(H.sapiens) 560 KLYQDISLIHA RLYQDISLIHA KLYQAISLIHA RLYQDISLIHA KLYQEISLIHA KLYQEISLIHA 557 rBAT(R.norvegicus) rBAT(O.cuniculus) 552 rBAT(M.musculus) 559 rBAT(B.taurus) 560 rBAT(O.aries) 560 561 NELLLN RGWFCHLRNDSHYVVYTRELDGIDRIFIVVLNFGES-TLLNLHNMISGLPAKMRIRLSTNSADK 629 558 RELLLSRGWFCLLRDDHHSVVYTRELDGIDKVFLVVLNFGESSTVLNLQETISDVPTKLRIRLSTNPASK 627 553 NELLLSRGWFCLLRDSRVLVYTRELDGIDRVFIVVLNFGES-TLLNLQEMISGLPVRLSIKLSTNSAST 621 560 TELVLSRGWFCLLRDSHSVVYTRELDGIDNVFLVVLNFGESSTVLNLQGIISDLPPELRIRLSTNSASK 629 561 NELLLGRGWFCFLGNYNHSINYTRELDGINNFFLVVLNFGESSTVLNLQGIISDLPPELRIRLSTNSAYG 629 rBAT(H.sapiens) rBAT(R.norvegicus) rBAT(O.cuniculus) rBAT(M.musculus) rBAT(B.taurus) rBAT(O.aries) 561 ELLLSR s 629 rBAT(H.sapiens)

 630
 GSKVDTSGIFLDKGEGLIFEHNTKNLLHRQTAFRDRCFVSNRACYSSVLNILYTSC

 628
 GSCVDTHAVSLEKGEGLILEHSMKTLLHQKAFRDKCFISNRACYSSVLDLLYSSC

 622
 GSQVDTRGIFLERGEGVLEHSMKTLLHRQTAFRDRCFISSRACYSSALDILYSSC

 630
 GSAVDTRAISLEKGEGLVLEHSKKAPLUGAAFRDRCFVSSRACYSSALDILYSSC

 630
 GREVDTHAVTLASGEGLVLEHSTKAPLHQAAFRDRCFVSSRACYSSALDILYSSC

 630
 GREVDTHAVTLASGEGLILEYNTRNLLHRQTAFKERCFVSNRACYSRALVILYSLC

 630
 GREVDTHAVTLASGEGLILEYNTRNLLHRQTAFKERCFVSNRACYSRVLNILYSLC

630 GSKVDTSGIFLD 685 683 677 rBAT(R.norvegicus) rBAT(O.cuniculus) rBAT(M.musculus) 685 rBAT(B.taurus) 685 rBAT(O.aries) 685

Fig. S8. Full length sequence alignment of mammalian rBAT homologs. Red stars indicate conserved residues participating in Ca²⁺ binding and coordination.



b^(0,+)AT1 Domain A of rBAT 📕 Domain B of rBAT 📕 Domain C of rBAT Fig. S9. Cystinuria causative mutations of the b^(0,+)AT1-rBAT complex. (A) Overall structure of the heterodimeric b^(0,+)AT1-rBAT complex. Locations of cystinuria-related mutations are indicated in red. (B) Closeup view of domain C mutations. The M467 mutation is found at the interface between domains B and C. Residues C666 and C673 are involved in formation of disulfide bonds within the C-loop of rBAT. (C) Closeup view of domain A and B mutations. T216 is in immediate vicinity of the Ca2+-bind site. (D) Closeup view of b^(0,+)AT1 mutations. W230 completes the substrate binding site of b^(0,+)AT1and acts as a gating residue. G105 locates in the IL1 at a close position to

TM3. A detailed summary of the respective mutations and their physiological effects is given in the Table. S2.



Fig. S10. Interaction interfaces of rBAT and $b^{(0,+)}AT1$. (A) Atomic model and density map of the C-terminal loop of rBAT and EL2 of $b^{(0,+)}AT1$. (B) Atomic model and density map of the β_4/α_5 loop of rBAT and EL2 and EL4b of $b^{(0,+)}AT1$. EL = extracellular loop.



Fig. S11. Conformational heterogeneity of the b^(0,+)**AT1-rBAT complex revealed by principle component analysis.** (**A**) The most dominant component of the conformational heterogeneity is shown. We observe a relative rotational movement between the two HAT units along the membrane axis. The rotation center is found to be at the interaction interface between of the two extracellular rBAT domains. For a more comprehensive illustration of the movement see movies S1 and S2. The initial conformational is colored in blue. The highest rotational sampling angle is colored in red.



Fig. S12. Structural comparison of the β_4/α_4 extension between human rBAT and the bacterial trehalose synthase. Ribbon representation of the extracellular domain of human rBAT and the trehalose synthase from *D. radiodurans* (PDB 4TVU). The β_4/α_4 extensions of the two structures highlighted in red indicate that the rBAT domain possesses a larger loop segment than the rehalose synthase. Despite the structural resemblance between rBAT and the trehalose synthase, the bacterial member of the glycoside hydrolase (GH13) family is lacking critical residue required for dimer formation.

	rBAT	b ^(0,+) AT1	b ^(0,+) AT1-rBAT
	(homodimer)		(heterotetramer)
Data collection	· · ·		
Accession number	EMD-10936	EMD-10940	EMD-10933
Magnification	105k	105k	105k
Voltage / kV	300	300	300
Dose / e ⁻ Å ⁻²	40	40	40
Pixel size / Å	0.837	0.837	0.837
Defocus range / um	-1.1 to -2.2	-1.1 to -2.2	-1.1 to -2.2
Recorded movies	1780	1780	1780
Initial particle images	223706	223706	223706
Final particle images	92789	185560	92789
Camera	Gatan K3	Gatan K3	Gatan K3
Microscope	Titan Krios G3i	Titan Krios G3i	Titan Krios G3i
Image processing			
Symmetry imposed	C2	C1	C2
Resolution (FSC _{0.143}) / Å	2.82	3.43	2.88
Applied B-factor / Å	-30	-10	-30
Map resolution range	2.7-3.7	3.2-4.4	2.7-4.7
Model refinement			
PDB accession	6YUZ	6YV1	6YUP
Validation			
FSC ^{map-to-model} (0.5) / Å	2.9	3.7	3.4
MolProbity score	1.95	1.63	2
Composition	-	-	
Atoms	9782 ⁺	2496+	14774
Protein residues	1188	328	1844
Ligands	8 NAG, 2 CA		8 NAG, 2 CA
Bonds (R.M.S.D.)			
Length (Å)	0.005	0.002	0.003
Angles (°)	0.54	0.58	0.909
B-factors (min/max/mean)			
Protein	39.2/138.4/60.7	15.63/79.6/40.11	1563/138.2/53.96
Ligand	36.71/82.83/77.06		36.71/82.83/77.06
Clashscore	7.9	9.39	
Ramachandran plot (%)	-	-	
	05.00	00.04	00.04
	90.08	99.04	90.84
Allowed	3.9	0.96	2.88
Outliers	0.4	U 1 10	0.28
Rotamer outliers (%)	1.95	1.48	1.8

Table S1. Cryo-EM data collection, refinement and validation statistics

Mutation	Location	Structural comments	Mutation defect summary				
b ^(0,+) AT1							
Gly105Arg Gly105Glu	IL1	In the IL1 region and is close to TM3	Mutated charge and/or larger side chains may interfere with the conformation switches				
Thr123Met	ТМЗ	In the middle of TM3 and near the binding pocket zone	Mutated hydrophobic side chain may form van der Waals contacts with residues from neighbour helix and affect its behaviour even the overall conformation				
Trp230Arg	TM6 (binding pocket)	Act as occlusion gate of the binding pocket on the extracellular side	Loss of hydrophobic interaction and steric hindrance would prevent the successful transport of substrates				
Tyr232Cys	TM6	Locates right in the binding pocket zone	Mutated smaller side chain may result in loose packing and disrupt the substrate interaction				
Asp233Glu	TM6 (binding pocket)	Play a crucial dual role as part of the binding pocket	A slightly larger residue may affect the tight packing pattern of the binding pocket				
Ser379Arg	TM10	In the middle of TM10 and near the binding pocket	Mutated charge and/or larger side chain may change the size of the binding pocket and disrupt substrate interaction				
Ala382Thr	TM10	In the middle of TM10 and near the binding pocket	Mutated polar and/or larger side chain may change the size of the binding pocket and disrupt the substrate interaction				
rBAT							
Thr216Met	Domain B	Adjacency of Asn214 and forms hydrogen bond with Tyr237	Loss of hydrogen bond and mutated longer residue may disrupt the stabilization of the ion binding site				
Arg270X Arg270Leu	Domain B	Forms hydrogen bond network along with Thr234, Gln272 and Asp241	Arg270X: Truncated ectodomain Arg270Leu: Loss of hydrogen bond network				

Table S2. Representatives of cystinuria-associated mutations in b^(0,+)AT1 and rBAT

Arg362Cys Arg362His	Domain B	Forms charge interaction with Asp359 from another protomer	Arg362Cys: Loss of charge interaction and may destabilize the dimerization Arg362His: Shorter residue leads to weaker charge interaction and may destabilize the dimerization				
Arg365Trp Arg365Gln Arg365Pro Arg365Leu	TM6	TIM $\alpha 4$ in domain A					
Met467Thr Met467Lys	At the interface between domain A and C	Forms hydrophobic interaction network as described in main text	Met467Thr/Lys: Mutated polar and charge side chain may disrupt hydrophobic interactions network				
Cys666Trp	C-terminus loop	Forms disulfide bond with Cys571	Loss of disulfide bond and may destabilize the c-terminus which also participates in the interaction with $b^{(0,+)}AT1$				
Cys673Trp Cys673Arg	C-terminus loop	Forms disulfide bond with Cys685	Loss of disulfide bond and may destabilize the c-terminus which also participates in the interaction with $b^{(0,+)}AT1$				

Movie S1 (separate file). Principle components of multibody analyses (part I)

Repositioning of the reconstructed body densities along three major motion for the heterotetrameric complex reveals the flexibility of the TMH domain over the extracellular domain. Soft masks for extracellular domain and TMH domain were applied for conducting this multibody refinement.

Movie S2 (separate file). Principle components of multibody analyses (part II)

Repositioning of the reconstructed body densities along three major motion for the heterotetrameric complex reveals the flexibility between two heterodimeric subunits. Soft masks for heterodimeric bodies were applied for conducting this multibody refinement.