Pathophysiology and treatment of cystinuria

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Pathophysiology and treatment of cystinuria

Josep Chillarón, Mariona Font-Llitjós, Joana Fort, Antonio Zorzano, David S. Goldfarb, Virginia Nunes and Manuel Palacín

Abstract | Cystinuria is a primary inherited aminoaciduria caused by mutations in the genes that encode the two subunits (neutral and basic amino acid transport protein rBAT and $b^{(0,+)}$-type amino acid transporter 1) of the amino acid transport system $b^{0,+}$. This autosomal recessive disorder (in which few cases show dominant inheritance) causes a failure in the reabsorption of filtered cystine and dibasic amino acids in the proximal tubule. The clinical symptoms of this disease are caused by the loss of poorly soluble cystine, which precipitates to form stones. Although rare, the prevalence of cystinuria is sufficiently high that the disease results in a substantial contribution to pediatric renal lithiasis. A thorough understanding of cystine transport processes over the past 15 years and the genetic abnormalities responsible for the disease has led to a new classification of cystinuria and recognition that some cases result from an autosomal dominant etiology with incomplete penetrance. This Review examines the molecular and mechanistic effects of some of the mutations that cause cystinuria based on our current understanding of the structural and cellular biology of system $b^{0,+}$. This Review also describes the current treatments to prevent recurrent cystine lithiasis.

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Introduction

Cystinuria[1] was one of the first inborn errors of metabolism described by Archibald Garrod.[2] The hallmark of cystinuria is hyperexcretion of cystine and dibasic amino acids (that is, lysine, arginine and ornithine) into urine.[3,4] The disease is caused by the defective transport of these amino acids across the apical membrane of epithelial cells of the renal proximal tubule and small intestine.[5–7] In addition to the hyperexcretion of dibasic amino acids and the defective intestinal absorption of these amino acids, cystinuria is characterized by the formation of cystine calculi (lithiasis). The poor solubility of cystine means that this compound can precipitate to form renal calculi that can cause obstruction, infection, and, ultimately, chronic kidney disease. Cystinuria causes 1–2% of all cases of renal lithiasis and 6–8% of renal lithiasis in pediatric patients.[8] Malnutrition does not occur in patients with cystinuria because the intestinal apical transporter solute carrier family 15 member 1 facilitates the apical absorption of dipeptides and tripeptides from dietary protein, which makes up for the loss of cysteine, lysine, and arginine (Figure 1).[9]

Positional genetics[10] and mutational analysis[11] demonstrated that mutations in *SLC3A1*, the gene that encodes neutral and basic amino acid transport protein rBAT, cause cystinuria. Genetic linkage studies, however, indicated that not all cases of cystinuria were caused by mutations in *SLC3A1*[12] and localized an additional gene linked to cystinuria to chromosome 19q13.1.[13,14] Mutational analysis of genes encoding proteins that comprise the light subunits of heteromeric amino acid transporters at 19q13.1 revealed that mutations in *SLC7A9* also cause cystinuria.[15]

Classification of cystinuria

Cystinuria is usually considered to be an autosomal recessive disorder. The average prevalence is 1 in 7,000 births, but it ranges from 1 in 2,500 births in Jewish Israelis of Libyan origin to 1 in 100,000 in Sweden.[3,16] Three phenotypes of cystinuria have traditionally described,[17] on the basis of the urinary excretion of cystine and dibasic (lysine, arginine and ornithine) amino acids of the obligate heterozygous parent—type I, type II, and type III. Obligate heterozygotic relatives of patients with type I cystinuria have normal aminoaciduria, whereas obligate heterozygotic relatives of individuals with type II and III cystinuria had high or moderate hyperexcretion of cystine and dibasic amino acids, respectively. This classification correlates poorly, however, with the extent of hyperaminoaciduria observed in heterozygotic individuals who carry the same mutation, which has resulted in the classification of cystinuria being revised to type I[15] (which includes patients formerly also classified as 'type I'), non-type I[15] (which includes the former type II and type III), and mixed type cystinuria (also known as type I/non-type I).[15]

In type I cystinuria, both parents of affected individuals are type I heterozygotes, which means that they do not have hyperaminoaciduria (that is, the mode of inheritance is recessive). In non-type I cystinuria, patients inherit non-type I alleles from both parents. Non-type I heterozygous individuals have a variable degree of urinary hyperexcretion of cystine and dibasic amino acids; however, production of cystine calculi has

Competing interests
The authors declare no competing interests.

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Key points

- Cystinuria is characterized by the inadequate reabsorption of cystine and dibasic amino acids in the kidney, which leads to the hyperexcretion of these amino acids in urine
- Cystinuria represents 1–2% of all cases of renal lithiasis and 6–8% of cases of pediatric renal lithiasis
- The amino acid transport system $b^{0,+}$ is the main effector of cystine reabsorption in the kidney
- The functional unit of the system $b^{0,+}$ comprises a heterodimer in which the neutral and basic amino acid transport protein rBAT (rBAT) is linked via a disulfide bridge to $b^{(0,+)}$-type amino acid transporter 1 ($b^{0,+}$AT)
- Mutations in either rBAT or $b^{0,+}$AT can cause cystinuria; mutations in the gene that encodes rBAT cause trafficking defects probably due to protein misfolding and mutations in the gene that encodes $b^{0,+}$AT cause trafficking defects or inactivation of system $b^{0,+}$
- Two genetically modified mouse lines exhibit the features of the two main types of cystinuria, type I cystinuria and non-type I cystinuria

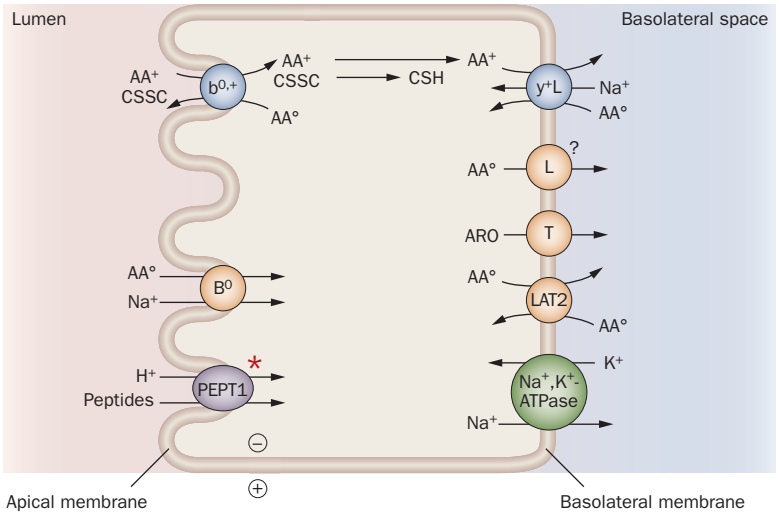

Figure 1 | Trafficking of cystine and dibasic amino acids in epithelial cells of the renal proximal tubule or small intestine. The apical transport system $b^{0,+}$ mediates influx of AA^+ and CSSC in exchange for AA^0. The Na^+-dependent transporter B^0 is believed to be the major apical contributor to the high intracellular content of AA^0 exploited by $b^{0,+}$.[121] In the small intestine (but not in kidney), uptake of dipeptides and tripeptides through the apical H^+-dependent PEPT1 compensates the defective absorption of AA^+ and CSSC. AA^+ exit the basolateral membrane through system y^+L, mutations in which cause lysinuric protein intolerance.[122,123] The basolateral efflux of CSH and AA^0 are not well understood. The general AA^0 exchanger LAT2 and the aromatic transporter T have been suggested to participate in the excretion of CSH and ARO.[124–126] In addition, an unidentified transporter L for unidirectional efflux of AA^0 is believed to have a role in AA^0 reabsorption. *Only in intestinal cells. Abbreviations: AA^+, dibasic amino acids; AA^0, neutral amino acids; ARO, aromatic amino acids; $b^{0,+}$, amino acid transport system $b^{0,+}$; B^0, sodium-dependent neutral amino acid transporter B^0AT1; CSH, cysteine, CSSC, cystine; PEPT1, solute carrier family 15 member 1.

been described very infrequently in these individuals.[18] Non-type I cystinuria should, therefore, be considered an autosomal dominant disease with incomplete penetrance for the cystine lithiasis trait. Patients with a mixed type cystinuria, who inherit type I and non-type I alleles from either parent have also been described.[15,18,19] Of 97 phenotyped families from the International Cystinuria Consortium cohort, which includes mainly patients from Europe and Israel, 37 are type I, 46 are non-type I, and 14 are mixed type.[18]

Genetics of cystinuria

Type I cystinuria is predominantly caused by mutations in *SLC3A1*, but a small proportion of patients with type I cystinuria have two mutated *SLC7A9* alleles.[18] The most frequent *SLC7A9* mutation among silent heterozygotes causes an Ala182Thr mutation in $b^{(0,+)}$-type amino acid transporter 1 ($b^{0,+}$AT). Transfection of HeLa cells and Madin–Darby canine kidney (MDCK) cells with the mutated gene results in a mild phenotype.[20] This apparently straightforward genotype–phenotype correlation is hampered by mutations that are also carried by silent heterozygotes (for example, Gly105Arg in $b^{0,+}$AT) but result in severe phenotypes in transfected cells. In contrast to type I cystinuria, almost all cases of non-type I cystinuria are caused by mutations in *SLC7A9*, with the exception of a few cases that involve a complex, in-frame duplication of *SLC3A1* that results in p.Glu298_Asp539dup (also referred to as dupE5-E9)).[18] This lack of genotype–phenotype correlation has led to the proposal of a novel classification for cystinuria based on genetics.[21] In this classification cystinuria is defined as type A if mutations are found in both *SLC3A1* alleles, type B if mutations are found in both *SLC7A9* alleles, and putative type AB if one mutation is found in each gene.[21] Heterozygous type AB individuals have been identified,[22] but cystinuric patients from families of such individuals have two mutated alleles in the same gene in addition to a mutated allele in the other gene (that is, AAB and BBA 'types').[18] Since type AB double heterozygous individuals do not produce stones, and two mutations in the same gene were found in patients from these families , digenic inheritance of cystinuria was ruled out.[18]

Isolated cystinuria in the absence of urinary hyperexcretion of dibasic amino acids has been described in a German family.[23] The Thr123Met mutant of $b^{0,+}$AT (Figure 2a) in heterozygotes is associated with the isolated cystinuria in this[24] and in other[18] families.

So far, three similar but distinct syndromes associated with type I cystinuria have been described: 2p21 deletion syndrome,[25] hypotonia–cystinuria syndrome (HCS),[26,27] and atypical HCS.[28,29] Genetic analyses have indicated that these are syndromes associated with recessive deletions of *SLC3A1* and genes contiguous to *SLC3A1* (see below).

Mutations that cause cystinuria

133 and 95 mutations in *SLC3A1* and *SLC7A9* have been identified, respectively. Reported mutations include nonsense, missense, splicing, frameshifts, and large sequence rearrangements. In *SLC3A1*, 579 mutated alleles have been reported in patients from 23 countries mainly from Europe (Supplementary Table 1 online). Among patients with cystinuria caused by known *SLC3A1* mutations, the most common mutations are Met467Thr (26% of cases), Thr216Met (12% of cases), p.Glu298_Asp539dup (5% of cases), and Arg270X (4% of cases) (Table 1 and Figure 2b). These mutations have a higher frequency in

some populations than in the overall population. The rBAT Arg270X mutation, for example, has been identified in 73% of Ashkenazi Jewish patients and 11% of North American patients with cystinuria caused by known *SLC3A1* mutations.[30,31]

Eight genomic rearrangements can span the entire *SLC3A1* gene or just its 3' end and may also include some of the neighboring genes, that is, *PREPL*, *PPM1B*, and *C2orf34*. These rearrangements cause HCS and its related syndromes (that is, 2p21 deletion syndrome and atypical HCS).[25–29] Patients with HCS have (partial or total) deletions of *SLC3A1* and *PREPL* in both alleles.[26,27] In atypical HCS, *C2orf34* is partially or totally deleted in addition to *SLC3A1* and *PREPL*,[28] whereas, in patients with the 2p21 deletion syndrome, *PPM1B* is partially or totally deleted alongside *SLC3A1*, *PREPL*, and *C2orf34*.[25] The severity of the phenotype worsens as the number of deleted genes increases.[32] One of the deletions that leads to HCS, DelB (78.5 Kb deletion from exon 2 of *SLC3A1* to exon 13 of the contiguous gene *PREPL*), which is mainly found in Belgian patients, represents 3% of the mutated *SLC3A1* alleles identified worldwide (Supplementary Table 1 online).[26–28]

436 mutated *SLC7A9* alleles have been reported in patients from 18 countries (Supplementary Table 2 online). Among patients with cystinuria caused by known *SLC7A9* mutations, the most frequent mutations are Gly105Arg (21% of cases), Pro482Leu (13% of cases), c.614dupA (7% of cases), Arg333Trp (6% of cases), and Val170Met (4% of cases) (Table 1 and Figure 2b). The mutation Pro482Leu is specific to Japan[33] (except for one allele identified in one Italian patient with cystinuria[34]) and this mutation is present in 88% of Japanese patients with a mutation in *SLC7A9* and in 76% of all Japanese patients with explained cystinuria. In Spanish patients with the *SLC7A9* alleles, c.614dupA is the most frequent mutation (29% of cases), and it is due to a founder effect in Asturias (a region in the north of Spain) (M. Font-Llitjós, M. Palacín, and V. Nunes, unpublished data). The Val170Met mutation is exclusive to Jewish Israeli patients of Libyan origin (94% of patients with the *SLC7A9* alleles.[15,18] Japanese and Israeli Jewish populations have less diversity in cystinuria mutations than other known populations.

Most, but not all, of the alleles that cause cystinuria have been characterized. In the four largest and best-studied cohorts, which account for 618 alleles (half of the total alleles studied worldwide), ~13% of the alleles have not been identified.[18,33–35] These unidentified alleles may involve mutations in nonexplored regions of *SLC3A1* and *SLC7A9*. Although unlikely, mutations in other genes might also account for these unexplained cases of cystinuria. By contrast, mutational analyses in patients with unexplained alleles have not found mutations in the genes that encode cysteine transporters asc-type amino acid transporter 1 (*SLC7A10*)[36] and neutral amino acid transporter B(0) (*SLC1A5*).[37] A third possibility is that hypomorphic (that is, partial loss of function) polymorphisms in *SLC7A9* in combination with mutated *SLC7A9* alleles might explain full-blown cystinuria in some patients.[38,39]

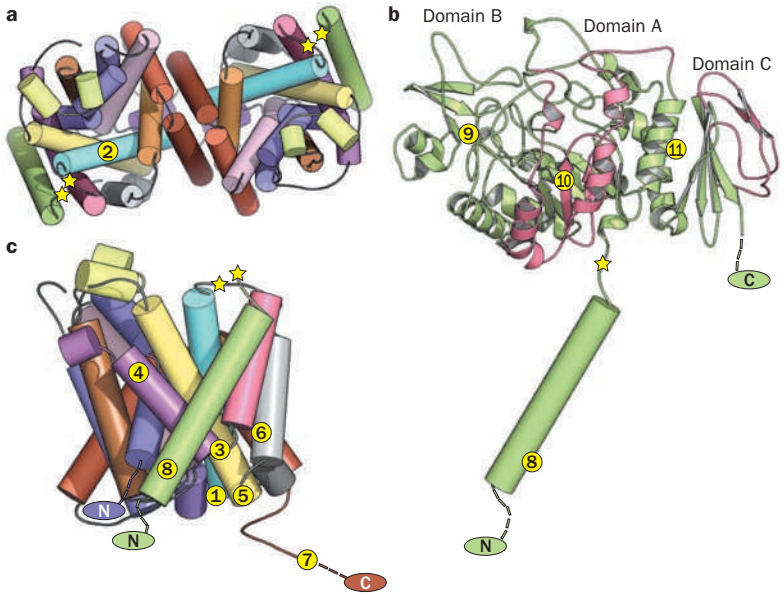

Figure 2 | 3D model of the amino acid transport system $b^{0,+}$. **a** | Structure of $b^{0,+}$AT and of the transmembrane domain of rBAT (shown in green) as inferred from the crystal structure of AdiC.[78] α-Helices are depicted as cylinders. The transmembrane domain of rBAT is tilted to ensure that it is located within the membrane. **b** | Hypothetical structure of human rBAT. The ectodomain structure of rBAT has been predicted based on the ectodomain of 4F2 cell-surface antigen heavy chain[68] and prokaryotic glycoside hydrolases[69–73] using MODELLER[127] (Supplementary Figure 2 online). Domain A comprises a triose phosphate isomerase barrel, domain B, an $\alpha_2\beta_3$ loop, and ectodomain C, eight antiparallel β strands. The area in magenta has a low homology with the available models. **c** | Lateral view of the $b^{0,+}$AT monomer and the transmembrane domain of rBAT. Stars indicate the cysteine residues that bridge $b^{0,+}$AT and rBAT. The location of cystinuria-associated mutations are indicated by circled numbers in panels a, b, and c (1, Gly105Arg; 2, Thr123Met; 3, Val170Met; 4, Ala182Thr; 5, Arg333Trp; 6, Ala354Thr; 7, Pro482Leu for $b^{0,+}$AT and 8, Leu89Pro; 9, Thr216Met; 10, Arg365Trp; 11, Met467Thr/Lys for rBAT). Abbreviations: AdiC, arginine/agmatine exchanger; rBAT, neutral and basic amino acid transport protein rBAT; $b^{0,+}$AT, $b^{0,+}$-type amino acid transport protein.

The transport function of the cystinuria-associated polymorphisms has not been thoroughly characterized, but it is mandatory to establish the role of *SLC3A1* and *SLC7A9* polymorphisms in cystinuria.

Cystinuria mouse models

Cystinuria is known to occur naturally in cats,[40] dogs,[41] and maned wolves.[42] However, these animal species are not suitable for genetic studies. To solve this problem two mouse models of cystinuria have been generated. Mutagenesis with *N*-ethyl-*N*-nitrosourea produced a mouse line that harbors a missense mutation in *Slc3a1* leading to Asp140Gly in rBAT, which parallels human type I cystinuria.[43] *Slc7a9*-knockout mice recapitulate the features of human non-type I cystinuria.[44] Both mouse models produce cystine stones at high rates and have morphological changes in kidney architecture that result from obstruction and inflammation.

Amino acid transport system $b^{0,+}$

Heteromeric amino acid transporters are composed of a heavy subunit and a light subunit linked by a conserved

Table 1 | Most common cystinuria-associated mutations in rBAT*, *SLC3A1*, $b^{0,+}$AT‡, and *SLC7A9*

Mutation	Worldwide frequency	Location in protein	Molecular defect (observed or expected)
rBAT*			
Met467Thr	26% (>40% in Sweden, Czech Republic and Germany)[11,18,22,34,35,38,128–136]	Ectodomain (domain A)	Trafficking[89,137] Folding (expected)[89,137]
Thr216Met	12% (50–70% in former Yugoslavia, Turkey, and Greece)[38,133]	Ectodomain (beginning of domain B)	Trafficking[89] Folding (expected)[89]
p.Glu298_Asp539dup	5% (mainly in Germany and Italy)[18,34,134]	Duplication in frame of part of domain A	Unknown
Arg270X	4% (73% in Ashkenazi Jewish; 11% in North America)[30,31,34,38]	Truncated ectodomain	Folding (expected)[94]
$b^{0,+}$AT (SLC7A9)‡			
Glu105Arg	21% in white patients[15,18,34,38,94,136,138,139]	Just after helix IL1 loop	Protein expression[94] Folding (expected)[94]
Pro482Leu	13% (88% of Japanese patients with cystinuria-associated *SLC7A9* mutations)[33]	C-terminus	Transport[33]
(c.614dupA)	7% (29% in Spain)[18,22,35,38,94,131–136,140]	Stop codon at Phe161	Folding (expected)
Arg333Trp	6% (in white and Japanese patients)[18,33,38,94,136]	TM8	Unknown
Val170Met	4% (94% of Israeli Jews of Libyan origin patients with cystinuria-associated *SLC7A9* mutations)[15,18,94]	TM5	Unknown

*rBAT is encoded by *SLC3A1*. ‡$b^{0,+}$AT is encoded by *SLC7A9*. Abbreviations: $b^{0,+}$AT, $b^{(0,+)}$-type amino acid transporter 1; dup, duplication; rBAT, neutral and basic amino acid transport protein rBAT; TM, transmembrane.

disulfide bridge[45,46] (Figures 2, 3). Two homologous heavy subunits (both members of the SLC3 protein family) have been identified, rBAT[47,48] and 4F2 cell-surface antigen heavy chain (4F2hc), also known as CD98 or fusion regulatory protein 1.[49–51] 10 light subunits (encoded by genes *SLC7A5–SLC7A14*) have been identified, with hLAT1[52,53] and arpAT[54] the first and last reported, respectively. Six of these light subunits heterodimerizes with 4F2hc (LAT1, LAT2, y^+LAT1, y^+LAT2, asc1, and xCT), one heterodimerizes with rBAT ($b^{0,+}$AT), and three (asc2, AGT1 and arpAT) seem to interact with unknown heavy subunits. Heteromeric amino acid transporter-associated amino acid transport activities are discussed elsewhere.[45,54,55]

The heavy subunit of heteromeric amino acid transporters is needed to localize the holotransporter to the plasma membrane.[52,56] In addition, 4F2hc mediates β-integrin signaling,[57] cell fusion,[51] and cell proliferation.[58,59] The role of the 4F2hc-associated amino acid transport activities in these processes is unknown. No function aside from targeting heteromeric amino acid transporters to the plasma membrane has been reported for rBAT. Heteromeric amino acid transporter light subunits comprise the catalytic component of the transporters, as it has been demonstrated by the reconstitution of $b^{0,+}$AT in proteoliposomes in the absence of rBAT.[20]

The rBAT/$b^{0,+}$AT heterodimer mediates the exchange of dibasic amino acids and cystine for other neutral amino acids (except imino acids) in a 1:1 stoichiometry in transfected HeLa cells and in the opossum kidney epithelial cells of renal proximal tubule origin.[20,60–62] rBAT/$b^{0,+}$AT has high apparent affinity for dibasic amino acids and cystine (K_M values of ~100 μmol/l) and a slightly lower affinity for other neutral amino acids. Similar transport activity was detected in the brush border membranes of the small intestine.[63,64] rBAT/$b^{0,+}$AT is expressed in the apical membrane of the epithelial cells of the small intestine and in segments S1–S2 of the proximal tubule,[65] where >90% of cystine reabsorption occurs.[66] Thus, rBAT/$b^{0,+}$AT is the molecular correspondent of the renal and intestinal apical transport system $b^{0,+}$. This transporter is considered to be the agent responsible for the main mechanism of apical reabsorption of cystine in the kidney.[65]

Under physiological conditions, dibasic amino acids and cystine are removed from the intestinal and renal tubular lumen via the transport system $b^{0,+}$ in exchange for neutral amino acids (Figure 1). Active transport through system $b^{0,+}$ is the result of three driving forces: the high intracellular concentration of neutral amino acids caused by active secondary transporters (for example, apical sodium-dependent neutral amino acid transporter B^0AT1), the electric potential across the plasma membrane (that is, the electric charge is negative in the cytoplasm) and the intracellular reduction of cystine to cysteine. Proof for this role of system $b^{0,+}$ in cystine and dibasic amino acid transport comes from the fact that mutations in the system cause cystinuria, which is characterized by urinary hyperexcretion of cystine and dibasic amino acids but not of other neutral amino acids.[3,4]

The structure of system $b^{0,+}$

The heavy subunits (~80–90 kDa) of heteromeric amino acid transporters are type II membrane *N*-glycoproteins with a single transmembrane (TM) domain, an intracellular N-terminus, and a large (~50–60 kDa) extracellular C-terminus (Figure 2b). The amino acid sequence of the ectodomains of rBAT and 4F2hc share identity with bacterial α-amylases,[67] and it is, therefore, unsurprising

that the crystal structure of the extracellular domain of human 4F2hc is similar to that of bacterial α-amylases (domain A, a triose phosphate isomerase [TIM] barrel [$(\alpha/\beta)_8$], and domain C, eight antiparallel β-strands).[68] Despite the sequence and structural similarity between 4F2hc and bacterial α-amylases, the ectodomain of 4F2hc has no glucosidase activity. All attempts to overexpress the ectodomain of rBAT have failed, and its secondary and tertiary structures have, therefore, been predicted based on amino acid sequence similarity with 4F2hc (<30%) and with prokaryotic α-amylases (32%)[68–73] (Figure 2b). The ectodomain of rBAT probably consists of three domains: domains A and C, as in 4F2hc, and domain B (a $\alpha_2\beta_3$ loop), which is characteristic of several α-amylases. Whether the ectodomain of rBAT has any α-amylase activity is not yet clear.

The light subunits of heteromeric amino acid transporters (~50 kDa) are highly hydrophobic and not glycosylated. Biochemical studies suggest that these proteins have a 12-TM-domain topology[74] (Figure 4). Together with their prokaryotic homologs, heteromeric amino acid transporter light subunits form the LAT protein family within the large amino acids, polyamines and organic cations (APC) superfamily of transporters.[75,76] In 2009, the first crystal structures of prokaryotic APC transporters (the arginine/agmatine exchanger [AdiC] and an H^+-dependent amino acid transporter [ApcT]) were reported.[77–79] Despite low amino acid sequence identity with LAT transporters (~18%), AdiC and ApcT structures have similar topological features to LAT transporters,[74] which suggests that they may share similar secondary and tertiary structures.

Surprisingly, APC transporters share the same fold (that is, the secondary and tertiary structure of the protein) with LeuT, a prokaryotic structural paradigm of the Na^+-dependent and Cl^--dependent neurotransmitter transporters,[80] and with transporters from three other families.[81–83] Although these proteins do not have high amino acid sequence identity (~10%), all contain a structural motif dubbed '5+5 inverted symmetry motif', which is exemplified by the arrangement of the TM1–TM10 domains of LeuT. This motif consists of two interior pairs of symmetry-related helices (the TM1/TM6 domains and the TM3/TM8 domains) located in the core of the protein, which are surrounded by an arch of outer helices (also related by the two-fold axis of symmetry; TM2/TM7, TM4/TM9, and TM5/TM10) (Figure 4a). In the outer arch of helices, the TM2/TM7 pair links the TM1 domain and the TM6 domain with the intracellular and extracellular helix-loop structures intracellular loop 1 (IL1) and extracellular loop 4 (EL4).[84]

The two interior pairs of symmetry-related helices (the TM1/TM6 domains and TM3/TM8 domains) largely define the central translocation pathway that contains the binding sites for substrate and ions. TM1/TM6 and TM2/TM7 have been proposed to rotate around an axis almost parallel to the plane of the plasma membrane and located around the center of the protein (Figure 4b).[84] This rotation would explain the conformational changes of these transporters in the transport cycle to translocate the substrate from one side of the membrane to the other (alternative access mechanism). IL1 and EL4 contribute by closing the internal and external gate, respectively, during the transport cycle. The crystal structure of AdiC bound to its substrate L-arginine[85] shows a substrate binding site similar to that of LeuT. Although direct evidence is lacking, the substrate binding site of LAT transporters is assumed to be similar, involving interaction of residues in TM1, TM3, TM6, and TM8.

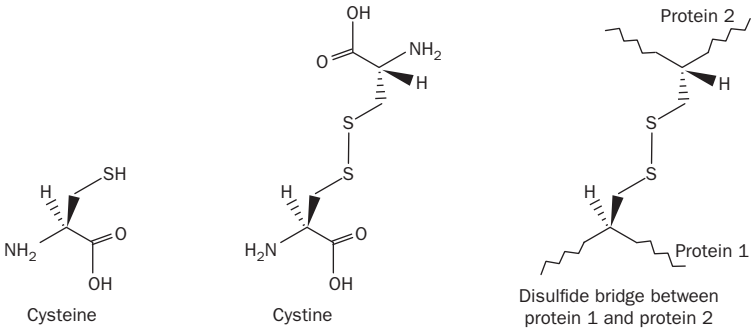

Figure 3 | Chemical structure of cysteine, cystine, and a disulfide bridge linking together two different proteins (the bridge heads are cysteine residues located in either protein).

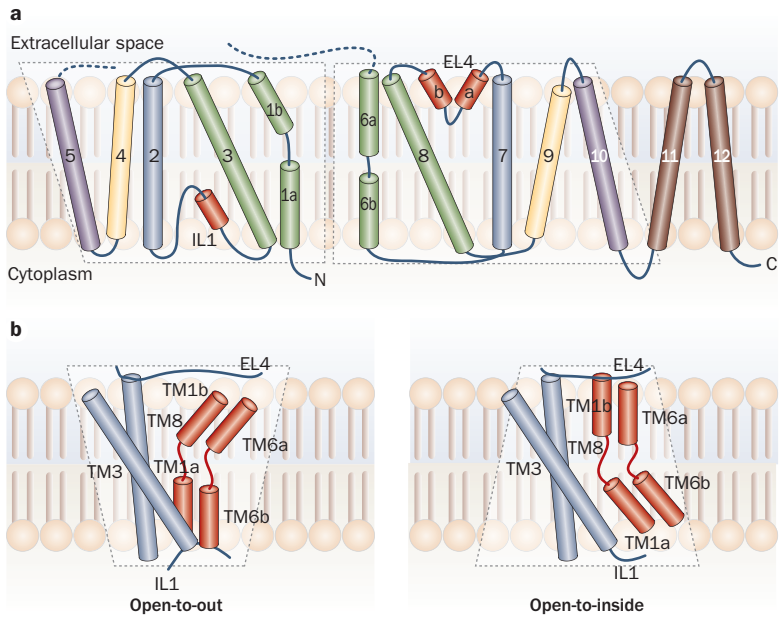

Figure 4 | The '5+5' inverted repeat fold of APC transporters. **a** | Membrane topology scheme of the APC transporters. TM domains 1–5 and 6–10 that are mutually related in the inverted repeat symmetry are depicted in the same color. The APC transporter AdiC interacts with its substrate arginine with residues in the interior TM domains, which are shown in green. **b** | Proposed rotation of TM1 and TM6 domains during the transport cycle in the 'open-to-out' and 'open-to-in' conformations that enable substrate access to the extracellular and the intracellular medium, respectively.[82] Loops IL1 and EL4, which participate in the closing of the internal and external barriers, respectively, are shown in panels a and b. Abbreviations: AdiC, arginine/agmatine exchanger; APC transporters, amino acids, polyamines and organic cations superfamily of transporters; EL, extracellular loop; IL, intracellular loop; TM, transmembrane. Panel a courtesy of Dr Eric Gouaux. Permission to publish panel b obtained from Nature Publishing Group © Krishnamurthy, H. *et al. Nature* **459**, 347–355 (2009).

The low amino acid sequence identity between the LAT transporters on one hand, and AdiC and ApcT on the other, precludes the generation of a robust structural model of $b^{0,+}$AT, but a low resolution model of system $b^{0,+}$ has been developed (Figure 2a,c) with the assumption that the only TM domain of rBAT interacts with $b^{0,+}$AT. This assumption is based on the fact that truncated versions of rBAT devoid of the ectodomain still traffic the light subunit $b^{0,+}$AT to the cell membrane.[86] The cysteine residues involved in the intersubunit disulfide bridge are located in the extracellular loop between TM3 and TM4 of $b^{0,+}$AT and a few residues away from the TM domain of rBAT (Figure 2a,c). The known structure of AdiC means that the rBAT TM domain must be located in the vicinity of TM4 and TM5 domains of $b^{0,+}$AT (pink and violet domains in Figure 2c).

Oligomerization of system $b^{0,+}$

The quaternary structure of system $b^{0,+}$ has been characterized.[87] Two disulfide-linked heterodimers form a noncovalent heterotetramer both in transfected HeLa and MDCK cells and in brush-border membranes of the kidney. Evidence from fluorescence resonance energy transfer studies and crosslinking studies suggest that the two $b^{0,+}$AT molecules homodimerize within the heterotetramer.[87] By contrast, the native structure of the mammalian 4F2hc-associated transporters seems to be a single heterodimer. Of interest, a chimeric functional transporter formed by rBAT and the xCT light subunit does form a heterotetramer. Therefore, rBAT might dictate the oligomerization.

The crystal structure of AdiC[78] might provide a model for $b^{0,+}$AT homodimerization. AdiC forms dimers, and the dimerization interface involves residues in the TM11 and TM12 domains. A similar homodimerization interface for $b^{0,+}$AT would enable interaction of the TM domain of rBAT with the TM5 domain and/or the TM4 domain in $b^{0,+}$AT and enable free movement of the TM1–TM2–TM6–TM7 bundle during the transport cycle.

Experiments with a protein made up of two $b^{0,+}$AT subunits that differed only in their sensitivity to a thiol reagent and were linked by a short peptide sequence support the hypothesis that the single rBAT–$b^{0,+}$AT heterodimer is the functional transport unit of system $b^{0,+}$.[87] Similar results have been obtained with AdiC.[78] Therefore, oligomerization may participate in aspects of system $b^{0,+}$ biology other than the transport mechanism. The oligomerization of membrane proteins has been associated with their traffic through the secretory pathway.[88] In fact, many rBAT mutants associated with type I cystinuria have an endoplasmic reticulum (ER)-retention and degradation phenotype that correlates with the inability to form heterotetramers despite the presence of disulfide-linked heterodimers (see below).[89]

Biogenesis of system $b^{0,+}$

Early work in *Xenopus* oocytes showed delayed traffic of some rBAT mutants associated with type I cystinuria to the plasma membrane.[86] Since then, the biogenesis of system $b^{0,+}$ has been studied by inducing its heterologous expression in MDCK epithelial cells and in HeLa cells.[89–91]

As expected, the co-expression of $b^{0,+}$AT and rBAT is required for the functional expression of system $b^{0,+}$ in the apical plasma membrane of polarized MDCK cells.[90,91] Unbound $b^{0,+}$AT remains in the intracellular space, most likely in the ER, but it can be functionally reconstituted in proteoliposomes.[20] This finding suggests that $b^{0,+}$AT folds in the absence rBAT and can also explain the long half-life of $b^{0,+}$AT in the ER, given that folded proteins are less subject to degradation in the ER than unfolded ones.[89] Like many isolated subunits of oligomeric complexes, free rBAT is retained in the ER and degraded in a proteasome-dependent and a ER-mannosidase-dependent process, both of which are important features of the ER-associated protein degradation pathway.[92] Interaction with the ER chaperone lectins, calnexin and/or calreticulin, may transiently protect rBAT from degradation, because its degradation is accelerated by preventing glucose trimming of rBAT *N*-glycans.[87]

Assembly of $b^{0,+}$AT with the ER-retained core-glycosylated rBAT to form a disulfide-linked heterodimer abolishes degradation of rBAT. The heterodimer rapidly and efficiently travels along the secretory pathway.[89] Indirect evidence suggests that at least part of the folding of the extracellular domain of rBAT, after its assembly with $b^{0,+}$AT is facilitated by calnexin and/or calreticulin.[89] A C-terminal motif in $b^{0,+}$AT (Val480–Pro481–Pro482 [VPP]) is needed for ER-to-Golgi trafficking of the rBAT–$b^{0,+}$AT heterodimer.[91] This motif may interact with rBAT or with ER-cargo receptors. The $b^{0,+}$AT mutant Pro482Leu, which is associated with non-type I cystinuria, is correctly trafficked to the plasma membrane but is not functional, which suggests that the VPP motif has different functions in the ER and plasma membrane.[33] Pro482 may not be important for transport activity *per se*, because substantial transport activity is retained after conversion of this residue to other amino acids or to a STOP codon.[33]

Molecular effects of rBAT mutations

Several rBAT mutants associated with type I cystinuria have been analyzed in HeLa cells in the presence of $b^{0,+}$AT.[89] These mutants have strong trafficking defects, which result from two different mechanisms. The mutant with the mutation Leu89Pro in the TM domain (Figure 2b,c) fails to assemble efficiently with $b^{0,+}$AT, a result that is consistent with studies showing that the rBAT TM domain is needed for $b^{0,+}$AT–rBAT heterodimerization.[86] Of note, a small amount of Leu89Pro-rBAT–$b^{0,+}$AT heterodimers reach the plasma membrane as functional heterotetramers.[86] By contrast, rBAT mutants with mutations in the extracellular domain (ED-rBAT mutants) readily assemble with $b^{0,+}$AT to form disulfide-linked heterodimers but remain core-glycosylated, fail to oligomerize, and are degraded, most likely via the proteasome.[89] We propose that ED-rBAT mutants are misfolded. Notably, the ED-rBAT mutant Arg365Trp recovers transport activity when the

temperature is shifted to 33 °C from 37 °C, which suggests a temperature-sensitive misfolding of the protein.[93]

Consequences of rBAT mutations

How are these mechanisms relevant to protein activity *in vivo*? The mouse models of non-type I and type I cystinuria[43,44] provide important clues. Heterozygous *Slc7a9*-knockout mice express roughly 50% of the $b^{0,+}$AT protein in the kidney that is produced by wild-type mice and, just as human obligate non-type I heterozygous individuals do, hyperexcrete amino acids. This finding indicates that the amount of $b^{0,+}$AT expressed limits by the amount of transport system $b^{0,+}$ produced. By contrast, mice heterozygous for the Asp140Gly mutation in *Slc3a1* are phenotypically normal (importantly human and murine rBAT have 90% amino acid identity),[43] as are human obligate type I heterozygotes, suggesting that the amount of functional rBAT in this case does not limit the synthesis of transport system $b^{0,+}$. The amount of functional rBAT produced by these mice is, therefore, able to cope with the full complement of $b^{0,+}$AT even though Asp140Gly-rBAT might be degraded (human Asp140Gly-rBAT behaves in cell culture as the other ED-rBAT mutants tested; J. Chillarón, unpublished data). This idea critically depends on the assumption that $b^{0,+}$AT proteins initially assembled with ED-rBAT mutants are not degraded together with the mutant rBAT and are able to heterodimerize with new rBAT molecules, either mutant or wild-type. The reason why rBAT does not limit the functional expression of transport system $b^{0,+}$ is unknown.

Molecular effects of $b^{0,+}$AT mutations

All cystinuria-specific mutations in $b^{0,+}$AT that have been studied cause a loss of function, but for only a few of these mutations has the molecular defect been delineated. Expression levels of Gly105Arg mutant are very low after transfection of HeLa cells, which suggests that this mutant is misfolded and degraded (Table 1).[94] Residue Gly105 is located just after IL1 helix-loop (Figure 2c) and is fully conserved in APC transporters and other related transporter families,[20,79] which suggests that this residue has a vital role in the folding of these transporters. The Ala182Thr mutant shows a partial transport defect after transfection of HeLa cells, but has full transport activity upon reconstitution in proteoliposomes, which indicates that this mutant defectively traffics to the plasma membrane.[20] The location of residue Ala182 in the TM5 domain suggests that heterodimerization between Ala182Thr mutant and rBAT might be defective (Figure 2c). The Ala354Thr mutant shows no transport function upon reconstitution in proteoliposomes[20] and the Pro482Leu mutant shows normal expression in the plasma membrane but no transport activity (Table 1).[33]

Despite the low amino acid sequence identity of AdiC and ApcT transporters with $b^{0,+}$AT (~18%), the delineation of the secondary and tertiary structures of these APC transporters,[78–80] might aid analysis of the molecular defects of $b^{0,+}$AT mutants. Nearly 50% of all described cystinuria-specific missense mutations in $b^{0,+}$AT are located in the TM1, TM6, TM3, and TM8 domains (Supplementary Table 1 and Supplementary Figure 1 online). In AdiC[85] and LeuT[80] these domains are involved in substrate binding. This finding suggests that mutations in these four domains cause defects in substrate interaction or conformational changes that occur during the transport cycle. Indeed, the mutation Thr123Met, which causes isolated cystinuria,[25] is located in the TM3 domain, which suggests a role for this residue in cystine recognition.

Lithiasis in cystinuria

Modulators of cystine lithiasis

The urinary hyperexcretion of cystine caused by mutations in *SLC3A1* and *SLC7A9* is a necessary condition for the precipitation of cystine and formation of calculi in the urinary system. However, not all patients with cystinuria develop calculi throughout their lives. In the International Cystinuria Consortium cohort, 6% of individuals with homozygous mutations in *SLC3A1* or *SLC7A9* had not produced calculi.[22] Furthermore, substantial variability existed in the onset of the lithiasis: 35–40% of patients had their first stone between 11 years and 20 years of age, but 14% of female patients and 28% of male patients formed calculi before the age of 3.[22]

Although environmental factors such as dietary intake of fluids, salt, and protein all contribute to stone formation, an individual's genetic background is also likely to contribute. Indeed, the proportion of mice that produce cystine stones increases from ~40% in the second filial generation of *Slc7a9*-knockout mice (on a mixed genetic background)[44] to ~85% in the sixth filial generation obtained by crossing lithiasic mice (M. Font-Llitjós, M. Palacín, and V. Nunes, unpublished data). This phenomenon suggests that the stone-forming tendency of patients with cystinuria has a genetic component beyond mutations in *SLC3A1* or *SLC7A9*. Lithiasis-association studies with *Slc7a9*-knockout mice might help to identify genes that act as modulators of cystine lithiasis.

Treatment of cystinuria-associated lithiasis

Despite increased understanding of the genetic causes and the pathophysiology of cystinuria, methods to reverse the defect in transport that causes the disease have not been developed. Prevention of stone formation instead focuses on reducing the absolute amount and increasing the solubility of the poorly soluble cystine that is excreted in the urine (Box 1). Cystine excretion is reduced by restricting intake of dietary sodium[95–97] and animal proteins. As tubular cystine reabsorption is sodium-independent, the cause of the association of reduced cystine excretion with reduced sodium intake is unknown.[95] A reduction in intake of animal protein reduces the intake of cystine and its precursor methionine[98] and leads to alkalinization of the urine, which also increases cystine solubility. Despite abundant evidence demonstrating that dietary restriction of sodium and animal proteins decreases urinary excretion of cystine, no trials have convincingly demonstrated that these interventions are associated with reductions in stone recurrence.

Box 1 | Methods to prevent recurrent cystine lithiasis*

Decrease cystine excretion

- Limit dietary sodium intake
- Limit animal protein intake (i.e. decrease dietary intake of cystine and methionine)

Decrease cystine concentration

- Increase fluid intake; maintain urine concentration of cystine <1 mmol/l (~250 mg/l)
- Increase cystine solubility
- Increase urinary pH to >7.5

Reduce cystine to the more soluble cysteine

- Thiol drugs: D-penicillamine, α-mercaptoproprionylglycine (tiopronin)
- Captopril (not as effective as thiol drugs)

*The goal of such preventative therapies is to maintain urinary level of free cystine below 100 μmol per mmol of creatinine.

Methods for increasing cystine solubility in urine include increasing urine volume, increasing urine pH, and reducing the relatively insoluble cystine to the more soluble cysteine. Increasing oral fluid intake can be a very effective intervention and should aim to decrease urine cystine concentration to <1 mmol/l (~250 mg/l), with 3–4 l of urine output required daily for excretion of 1 g of cystine per day. Patients should be advised to maintain high urine flow rates throughout the day and to consume fluids before bedtime to decrease the nocturnal aggregation of crystals.

Urine pH is an important determinant of cystine solubility, which increases with increasing pH. The solubility of cystine in urine increases to about 2 mmol/l (~500 mg/l) at ≥pH 7.5.[99] Urinary alkalinization with oral administration of potassium citrate is the most useful means of achieving the desired increase in urine pH and is not associated with the increase in cystine excretion that occurs after administration of sodium citrate.[100] Restricting intake of animal protein also decreases net acid excretion, increasing urine pH, and reducing the dose of potassium citrate (or other bases) required to alkalinize the urine. Although acetazolamide has been recommended as an additional means to increase urine pH, this carbonic anhydrase inhibitor is not well tolerated and has not been shown to be superior to citrate.[101] The long-term effect of acetazolamide to cause metabolic acidosis also makes it a less desirable option than potassium citrate. Although urine alkalinization may be associated with the formation of calcium phosphate stones, citrate inhibits calcium salt precipitation, and in patients without hypercalciuria calcium phosphate stones are an infrequent problem.

Thiol-containing agents can be used in patients who are refractory to the above interventions. The two agents most commonly used are D-penicillamine and α-mercaptopropionylglycine, also known as tiopronin. These drugs work by reducing the disulfide bond that in cystine bridges two molecules of cysteine. The thiol group of the drugs combines with cysteine to form a soluble cysteine–drug product. The solubility of the cysteine–penicillamine complex is up to 50-fold higher than that of cystine.[102] The incidence of adverse effects such as allergy is similar for both agents but is slightly lower with tiopronin.[103] Monitoring of liver enzymes, complete blood cell count, and urinary protein excretion should be performed regularly while patients are on D-penicillamine or tiopronin therapy. The angiotensin-converting-enzyme inhibitor captopril contains a thiol group and can increase solubility of cystine *in vitro*; however, urinary excretion of captopril may not be adequate to cause meaningful changes in cystine solubility.[96] The *Slc7a9*-knockout mouse will be useful in evaluating new therapies for cystinuria. D-Penicillamine reduces cystine excretion and stone mass in this animal model.[104]

Of note, acidic urine causes precipitation of cystine and reduces the accuracy of measurements of the excreted amino acid. Alkalinization of the collected urine and dissolution of crystals after voiding ensures that all excreted cystine is measured.[105] Most assays fail to distinguish between cystine and cysteine–drug complex in the presence of thiol drugs. The use of a solid-phase assay for cystine supersaturation circumvents this problem and may increase the reliability of measurements of treatment results and enable the optimal drug dose to be determined.[96,106] Another approach to distinguish free urinary cystine from drug-bound cystine is the use of high-performance liquid chromatography.[107] Reducing urinary levels of free cystine measured by this assay successfully guided medical therapy in uncontrolled studies of pediatric patients with cystinuria.[108] These assays, which are not widely available, should be validated in prospective, controlled studies to definitively demonstrate that they are more efficacious as clinical tests than more commonly available tests.

Urological interventions are often indicated for the management of cystine stones >5 mm in diameter. The general experience described by both the European Association of Urology and the American Urological Association is that cystine stones are relatively less amenable to successful therapy with extracorporeal shock wave lithotripsy (SWL) than calcium oxalate stones.[109] Some stones have crystalline structures, which may make them resistant to SWL; a rough appearance on X-ray may indicate a crystal structure more easily fragmented by SWL than a stone with a smooth appearance.[110] Other cystine stones have a low degree of radioopacity and may not make easy targets for shock waves. For these reasons, ureteroscopy with a holmium laser may be the preferable modality of stone removal in patients with cystine lithiasis.[111] Unlike SWL, both ureteroscopy and percutaneous nephrostolithotomy permit irrigation of the urinary tract with alkalinizing solutions of tromethamine-E (also known as tris(hydroxymethyl) aminoeathane), although the usefulness of this procedure is only supported by a small number of anecdotal reports.[112]

One promising strategy for the treatment of cystinuria might be chaperone therapy. Cellular molecular

chaperones are ubiquitously expressed proteins that prevent misfolding of other proteins.[113] Pharmacological chaperones are small molecules that, instead of assisting folding, may stabilize an already folded protein by binding it and stabilizing it against proteolytic degradation or thermal denaturation.[114] Active-site-specific chaperones act as folding templates facilitating the folding of mutated proteins in the ER. This process accelerates the escape of mutated proteins from the ER-associated protein degradation pathway, inhibiting protein degradation and increasing the level of residual protein activity.[115] Chaperone therapy is useful for diseases caused by mutations that lead to misfolded proteins.[113] This therapeutic approach is being investigated for the treatment of monogenic diseases such as Gaucher disease, Fabry disease, phenylketonuria, and cystic fibrosis,[115–118] and in the treatment of diseases with a complex genetic component, such as type 2 diabetes.[119] For Gaucher disease, Fabry disease and Pompe disease, phase I and II clinical trials are already being conducted.[120] As several rBAT cystinuria-causing mutations could cause protein misfolding,[89] chaperone therapy could be an attractive treatment strategy for patients with cystinuria in the future.

Conclusions

The management of patients with cystinuria has not improved substantially despite our increasing understanding of the molecular basis of cystinuria over the past 15 years. Thorough characterization of the molecular defects that are associated with cystinuria might help in the development of new therapeutic approaches. Thus, mutations that cause protein misfolding could be targeted by small-chaperone therapy. Moreover, formation of cystine stones may be modulated by genes not directly involved in the onset of the disease. Therefore, studies in cystinuria mouse models might help to identify genes that modulate cystine lithiasis. A large cohort of patients with cystinuria and well-characterized clinical and molecular features will be needed to identify such modulator genes in humans. Prolithiasis and antilithiasis proteins could then become new targets for the management of cystinuria.

Review criteria

Material for this Review was obtained with different strategies, depending on the sections of the Review. For genetic sections, the entries "cystinuria (MIM220100)", "Hypotonia Cystinuria Syndrome (MIM606407)", "solute carrier family 3 (MIM104614)", and "solute carrier family 7 (604,144)" were used in the OMIM database, and PubMed was searched using the terms "cystinuria Type I", "cystinuria non-Type 1", "SLC3A1", and "SLC7A9". For sections related to system $b^{0,+}$, PubMed was searched using the terms "rBAT", "NBAT", "cystinuria", "SLC7A9", "SLC3A1", "cystine transport", "LeuT transporter", "vSGLT", "Mhp1", "BetP", "AdiC transporter", and ApcT. No constrains in language or year of publication were applied.

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Supplementary information

Supplementary information is linked to the online version of the paper at www.nature.com/nrneph