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LETTERS

Essential role for collectrin in renal amino acid transport

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Angiotensin -converting enzyme 2 (ACE2) is a regulator of the renin angiotensin system involved in acute lung failure, cardiovascular functions and severe acute respiratory syndrome (SARS) infections in mammals[1–3]. A gene encoding a homologue to ACE2, termed collectrin (Tmem27), has been identified in immediate proximity to the *ace2* locus[4]. The *in vivo* function of collectrin was unclear. Here we report that targeted disruption of *collectrin* in mice results in a severe defect in renal amino acid uptake owing to downregulation of apical amino acid transporters in the kidney. Collectrin associates with multiple apical transporters and defines a novel group of renal amino acid transporters. Expression of collectrin in *Xenopus* oocytes and Madin–Darby canine kidney (MDCK) cells enhances amino acid transport by the transporter B^0AT1. These data identify collectrin as a key regulator of renal amino acid uptake.

ACE2 is a chimaeric protein that has emerged from the duplication of two genes: homology with ACE at the catalytic domain and homology with collectrin in the membrane proximal domain (Fig. 1a). In the mouse, *collectrin* is expressed in the kidneys[4] and pancreas[5,6] and to a lesser extent in the intestine (duodenum, jejunum and ileum), liver, heart and stomach (Supplementary Fig. 1). To assess the *in vivo* function of collectrin, we generated *collectrin* knockout mice (Fig. 1b). Mice were crossed with the transgenic Cre *deleter* strain to ubiquitously delete *collectrin*[7] (Supplementary Fig. 2a). The null mutation of *collectrin* was verified by the absence of *collectrin* messenger RNA transcripts and protein (Supplementary Fig. 2b, c). ACE2 expression in the kidneys was not altered in *collectrin* mutant mice (Supplementary Fig. 2c). *Collectrin* null mice were born at the expected mendelian frequency, indistinguishable from their heterozygous and wild-type littermates. *Collectrin* mutant males ($collectrin^{-/y}$) and females ($collectrin^{-/-}$) were fertile and we failed to observe any overt morphological alterations in all organs analysed, including kidney, for up to six months of age (Fig. 1c).

Collectrin was originally reported to be expressed in collecting ducts[4] and β-islet cells of the pancreas[5,6]. We therefore assessed whether collectrin is involved in renal salt and/or glucose balance. Na^+ excretion in urine was comparable in $collectrin^{+/y}$ and $collectrin^{-/y}$ mice. Loss of collectrin also did not affect the levels of calcium, potassium, phosphate, chloride, urea, uric acid or creatinine in urine and blood (Supplementary Table 1). *collectrin* mutant mice had the same glucose concentration as their wild-type littermates in serum and urine. Surprisingly, storage of the urine samples collected from mutant mice at 4 °C resulted in the formation of large white precipitates (Fig. 1d). Morphological analysis revealed large needle-like crystals in the urine of the *collectrin* null mice whereas such crystals were never observed in wild-type mice (Fig. 1e). Biochemically, these crystals were not composed of common urinary crystal components such as ammonium urate, sodium urate, calcium oxalate dihydrate or calcium phosphate. High performance liquid chromatography (HPLC) analyses showed that these crystals contained ~10% phenylalanine (Phe) and 90% tyrosine (Tyr) (see Fig. 1f). When animals were fed a tyrosine-free and phenylalanine-free diet or when the animals were fasting, no crystals were formed in the urine of *collectrin* null mice (Fig. 1d), indicating that the observed changes are not caused by tyrosinaemia[8,9]. Thus, loss of collectrin expression results in the formation of tyrosine/phenylalanine crystals in the urine.

To determine whether collectrin deficiency caused a more general defect in amino acid handling in the kidneys, we assessed urinary amino acid contents. Whereas in wild-type mice almost all amino acids are reabsorbed from the urine, deletion of *collectrin* resulted in severe leakage of all amino acids (Fig. 1g). Concentrations of the amino acids were also lower in the serum of mutant mice, although amino acids were still present (Supplementary Fig. 3). Urinary output was higher in $collectrin^{-/y}$ mice (3.31 ± 0.25 ml urine per 24 h) as compared to $collectrin^{+/y}$ littermates (1.65 ± 0.28 ml urine per 24 h) which may be owing to the higher concentration of osmotically active solutes in urine from *collectrin*-deficient mice. Water restriction for 24 h demonstrated that *collectrin*-deficient mice were not able to concentrate urine to the same degree, confirming that the higher urinary output is due to a primarily renal defect (Supplementary Fig. 4). Thus, collectrin is critical for normal amino acid reabsorption in the kidney.

Amino acid reabsorption occurs in the proximal tubules of the kidney[10]. Immunohistochemical staining showed that collectrin is indeed expressed at the luminal side of brush border membranes in proximal tubules (Fig. 2a), which is a specialized microenvironment involved in amino acid reabsorption[11]. Biochemically, we confirmed specific expression of collectrin in brush border membrane vesicles (BBMV) isolated from the kidney of $collectrin^{+/y}$, but not $collectrin^{-/y}$ mice (Fig. 2b). BBMV isolates were free from contamination (assessed by the absence of the collecting duct marker aquaporin 2; Fig. 2b). In addition, collectrin mRNA was found to be highly expressed in proximal tubules (Fig. 2c). Thus, collectrin is localized in brush border membranes of proximal tubules.

Because collectrin is predicted to be a type I transmembrane protein having only one transmembrane helix[4], it was unlikely that collectrin itself forms an amino acid transport pore. Because collectrin expression parallels that of the B^0 cluster of the solute carrier SLC6 proteins B^0AT1, XT3s1/SIT1, XT2 and XT3, a recently identified subfamily of Na^+-dependent transporters of neutral amino acids[12–17], we assessed whether loss of collectrin may affect these

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amino acid transporters. Immunoblots of BBMVs revealed that the neutral amino acid transporter B^0AT1 was markedly downregulated in *collectrin*$^{-/y}$ mice (Fig. 2d and Supplementary Fig. 5a, b). Protein expression of the three other members of the SLC6 family XT3s1/SIT1, XT2 and XT3, and expression of EAAC1, the apical transporter responsible for glutamate/aspartate reabsorption[18], was also markedly downregulated (Fig. 2e). By contrast, expression of $b^{0,+}AT$ (also known as slc7a9) (Fig. 2e), the exchanger mediating apical uptake of cystine, arginine, lysine and ornithine[19], and expression of the basolateral amino acid transporter subunits 4F2hc and LAT2 (ref. 10) remained unchanged (Supplementary Fig. 5b). Expression of the sodium/phosphate co-transporter Na^+/Pi IIa was also not affected in *collectrin* null mice (Fig. 2d). mRNA expression of all these transporters was comparable in *collectrin*$^{+/y}$ and *collectrin*$^{-/y}$ mice (Supplementary Fig. 6). Immunoblotting of total kidney membranes demonstrated that the abundance of these amino acid transporters was also decreased to a similar extent as observed in BBMVs (Supplementary Fig. 5b).

To test whether reduced expression resulted in decreased amino acid transport activity, we isolated BBMVs and measured Na^+-dependent amino acid uptake rates. As expected from the urinary loss of B^0AT1 substrates, the uptake rate of glutamine and phenylalanine in BBMV from kidneys of *collectrin* null mice was decreased compared to control littermates, whereas uptake of phosphate was not affected (Fig. 2f). These data show that loss of collectrin is associated with reduced expression and impaired activity of apical, Na^+-dependent amino acid transporters in the kidney.

To identify the molecular pathway by which collectrin regulates amino acid transporters, we first examined whether collectrin can associate with B^0AT1 and/or other transporters. Immunoprecipitations from BBMV lysates showed direct binding between collectrin and B^0AT1, XT2 and XT3 in *collectrin*$^{+/y}$, but not *collectrin*$^{-/y}$ mice (Fig. 3a). Moreover, collectrin co-localized with B^0AT1 in the early proximal S1 tubule (Fig. 3b). As a control, collectrin did not associate with $b^{0,+}AT$ (Fig. 3a). The specificity of the collectrin band was confirmed by pre-incubation with the antigenic peptide (Supplementary Fig. 7a, b). Renal amino acid transporters such as LAT2 or y^+LAT1 form functional heterodimers with a second subunit, 4F2hc (ref. 10). In all known instances, this heterodimerization is stabilized by disulphide bonds[20]. Under reducing conditions (+DTT), collectrin appears as a single band with gel mobility of ~44 kDa, whereas B^0AT1 can be detected as a single band that corresponds to a monomeric form of

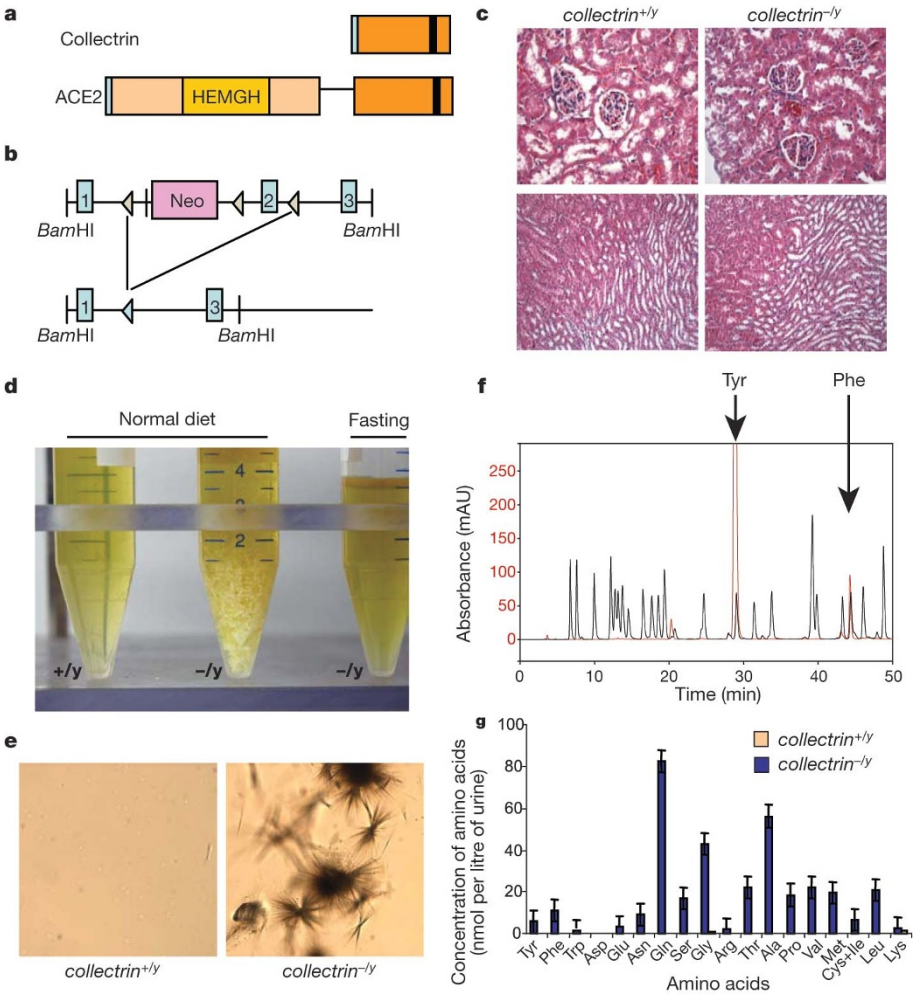

Figure 1 | Tyrosine crystals and renal amino acid loss in *collectrin*$^{-/y}$ mice. **a**, Schematic outline of homologous regions between collectrin and ACE2. Collectrin lacks the HEMGH carboxypeptidase domain. The black boxes indicate the transmembrane domains. Leader peptides are shown in light blue. **b**, Gene targeting (flox-and-delete) strategy for the generation of *collectrin* mutant alleles. Upon breeding with Cre *deleter* mice, animals with excision of exon 2 were selected. **c**, H&E-stained sections of kidney isolated from six-month-old *collectrin*$^{+/y}$ and *collectrin*$^{-/y}$ mice. Top panels, overview over cortex; bottom panels, normal medullary structures. **d**, At 4 °C, large white precipitates form in the urine of *collectrin* null (−/y) but not control wild-type (+/y) mice. These precipitates disappear upon fasting for 24 h. **e**, Formation of needle-like crystals in the urine of *collectrin* null mice. **f**, HPLC analysis of crystals isolated from the urine of *collectrin*$^{-/y}$ mice. The crystals co-elute with tyrosine and phenylalanine. mAU, milli-absorbance units. **g**, Excretion of all amino acids tested in *collectrin*$^{-/y}$ mice. Urine was collected from four-month-old *collectrin*$^{+/y}$ and *collectrin*$^{-/y}$ littermates and analysed by HPLC. Values are mean ± s.e.m. of ten mice per group. $P < 0.05$ (Student's *t*-test and analysis of variance, ANOVA).

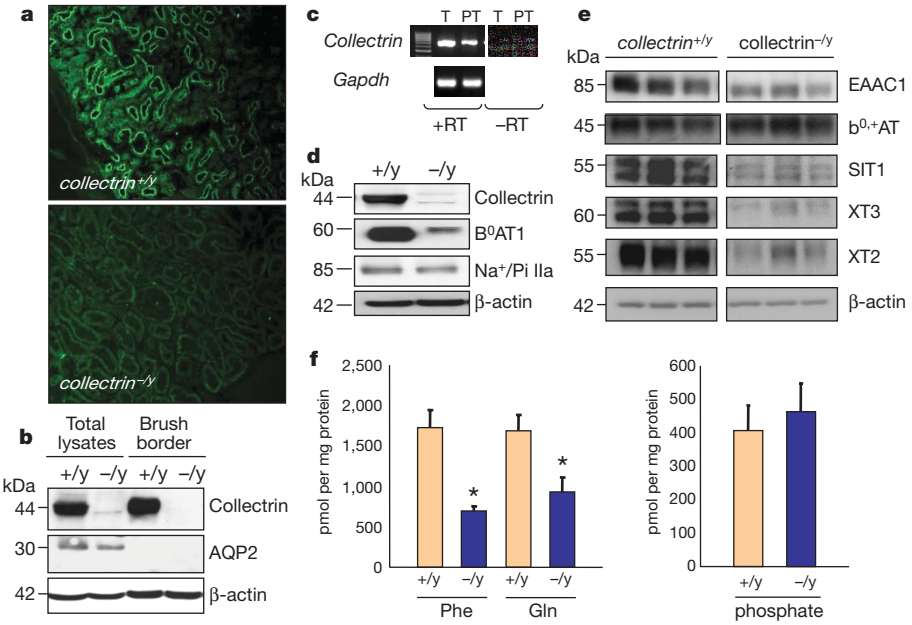

Figure 2 | Defective expression of amino acid transporters in *collectrin* mutant mice. **a**, Immunodetection of collectrin (green) in kidneys of *collectrin*$^{+/y}$ and *collectrin*$^{-/y}$ mice. Note that collectrin localizes to proximal tubules. **b**, Western blot analysis of brush border membranes from *collectrin*$^{+/y}$ and *collectrin*$^{-/y}$ mice. Protein extracts from total lysates (50 μg per lane) and renal brush border membranes (10 μg per lane) were analysed using antibodies to collectrin, aquaporin (AQP2), and β-actin. **c**, Collectrin mRNA expression in proximal tubules (PT) and total kidneys (T). RNA was isolated from wild-type mice and reverse transcribed (+RT). Equal loading was confirmed by Gapdh. **d**, **e**, Western blot analysis of proteins (50 μg) from brush border membranes isolated from *collectrin*$^{+/y}$ and *collectrin*$^{-/y}$ mice. In **d** and **e**, data from three different mice are shown for each genotype. Molecular sizes are indicated in kDa. **f**, Na^+-dependent uptake of the amino acids phenylalanine (Phe) and glutamine (Gln) and uptake of phosphate into BBMVs from *collectrin*$^{+/y}$ and *collectrin*$^{-/y}$ mice. All experiments were done in triplicates from each kidney. Values are mean ± s.e.m. of four mice per group. $^*P < 0.001$ (Student's *t*-test).

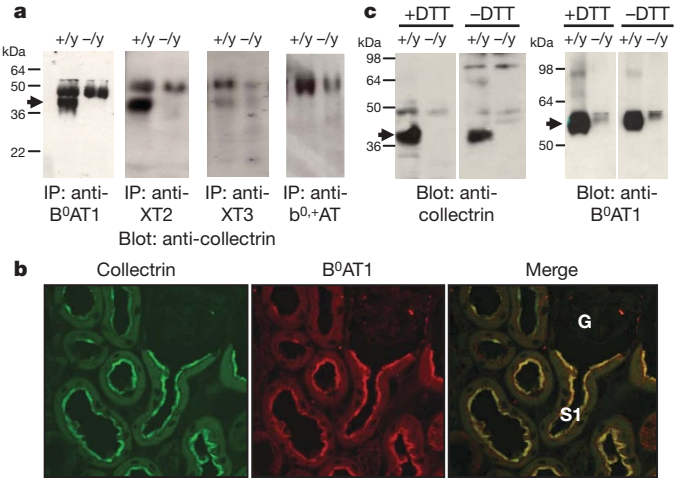

Figure 3 | Collectrin associates with apical amino acid transporters through non-covalent interactions. **a**, Renal brush border membrane proteins (100 μg) from *collectrin*$^{+/y}$ and *collectrin*$^{-/y}$ mice were incubated overnight with anti-B^0AT1, anti-XT2, and anti-XT3 and anti-$b^{0,+}AT$ antibodies. Immunoprecipitated (IP) complexes were analysed by western blot using an anti-collectrin antibody (arrow). **b**, Collectrin co-localizes with B^0AT1 in kidney proximal tubules. Kidney sections from wild-type mice were stained with antibodies to collectrin (green) and B^0AT1 (red). Yellow indicates co-localization. S1, initial part of the proximal tubule. G, glomerulus. Magnifications, ×400. **c**, Western blot analysis of isolated renal brush border membranes under reducing (+DTT; 100 mM) and non-reducing (−DTT) conditions from *collectrin*$^{+/y}$ and *collectrin*$^{-/y}$ mice using antibodies directed against collectrin and B^0AT1. Under reducing as well as under non-reducing conditions collectrin and B^0AT1 appear as bands of ~44 and ~60 kDa, respectively (arrows). Molecular sizes are indicated in kDa.

B^0AT1 (Fig. 3c). Unlike 4F2hc and its catalytic subunits, B^0AT1 and collectrin migrate under non-reducing (no dithiothreitol, −DTT) conditions with the same mobility as under reducing conditions (Fig. 3c), indicating that the association between collectrin and B^0AT1 as well as other transporters occurs through non-covalent interactions. Thus, collectrin co-localizes and specifically associates with a subgroup of renal amino acid transporters.

To define the molecular mechanism of collectrin-regulated amino acid transport, we examined the effect of collectrin on B^0AT1 function using *Xenopus laevis* oocytes and polarized MDCK cells. Two days of expression of B^0AT1 alone in *Xenopus* oocytes conferred only a low transport activity for the B^0AT1 substrate L-isoleucine[16] (Fig. 4a). Collectrin alone did not exhibit any transport activity. However, co-expression of B^0AT1 with collectrin resulted in a striking increase in L-isoleucine uptake (Fig. 4a). As a control, co-expression of collectrin with the Na^+/phosphate IIa co-transporter in oocytes did not affect the rates and kinetics of phosphate uptake (Fig. 4b). To assess whether collectrin changes binding of L-isoleucine to its transporter B^0AT1, we measured the apparent affinity (K_m) for L-isoleucine uptake (Fig. 4c). Co-transport of L-isoleucine with Na^+ generated a saturable, reversible inward current with an apparent K_m for L-isoleucine of 0.99 ± 0.17 mM when B^0AT1 was expressed alone, and of 0.78 ± 0.10 mM when B^0AT1 was co-expressed with collectrin. Thus, the collectrin-induced increase in L-isoleucine transport is not due to a change in apparent affinity, but due to an increase in the maximal transport rate of B^0AT1, probably as a result of increased surface expression. Indeed, expression of collectrin allowed functional surface expression of B^0AT1 and apical uptake of L-isoleucine in polarized kidney MDCK cells (Fig. 4d). Thus, collectrin enhances functional surface expression of the SLC6 family transporter B^0AT1.

We conclude that genetic inactivation of *collectrin* in mice results in a major defect in renal amino acid reabsorption. Collectrin controls protein expression and function of apical amino acid transporters in the brush border membranes of proximal tubules.

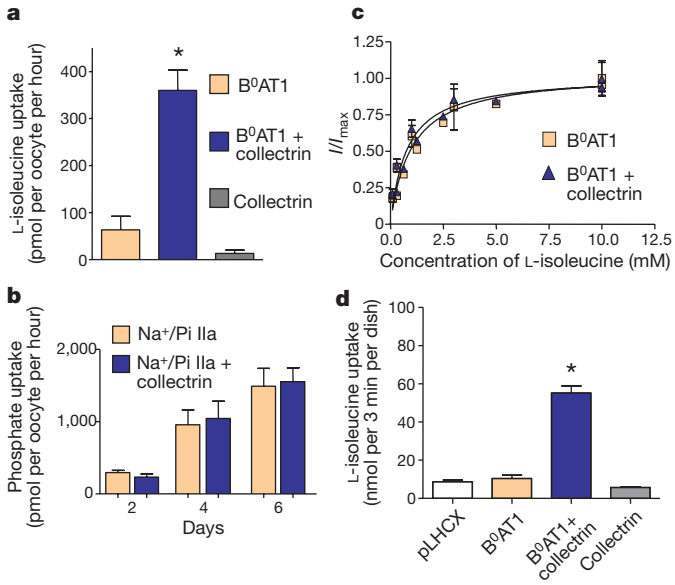

Figure 4 | Collectrin enhances B^{0}AT1 transport activity. **a**, *Xenopus laevis* oocytes were injected with murine B^{0}AT1, collectrin, or B^{0}AT1 plus collectrin RNA. L-isoleucine transport was determined two days after injection. Each bar represents mean transport activity ± s.d. ($n = 15$ oocytes analysed). **b**, *Xenopus laevis* oocytes were injected with murine Na^+/Pi IIa alone or Na^+/Pi IIa plus collectrin; phosphate transport (mean ± s.d.) was then determined at the indicated time points. $n = 7$ oocytes per sample. **c**, The half-maximal uptake rate K_m of L-isoleucine by B^{0}AT1 at six days of expression in the absence or presence of collectrin. K_m was determined using two electrode voltage clamps at −50 mV using 0–10 mM of L-isoleucine in the presence of 100 mM Na^+. Data was normalized to 10 mM L-isoleucine for $n = 11$ oocytes per group. **d**, MDCK cells were transduced with murine B^{0}AT1, collectrin, B^{0}AT1 plus collectrin, or empty vector (pLHCX). Bars represent the mean uptake of 300 μM L-isoleucine in 3 min ± s.d. *$P < 0.001$ (Student's *t*-test).

Mechanistically, collectrin associates with several amino acid transporters, enhances their surface expression, and thereby increases amino acid transport function. These data identify the ACE2 homologue collectrin as a regulator of renal amino acid uptake and may provide a molecular explanation for aminoaciduria in Hartnup disease, Fanconi syndrome, or diabetes.

METHODS

***collectrin* knockout mice.** A gene-targeting vector containing three *loxP* sites flanking exon 2 and a Neo selection cassette was constructed. Chimaeric progeny were crossed to the C57BL/6J Cre *deleter* line[7] to generate complete null animals.

mRNA and protein analyses. Real-time polymerase chain reaction (PCR) was performed as described[12]. For protein analyses, total cell lysates and BBMVs of adult mouse kidney were prepared[21] and probed with antibodies to mouse b$^{0,+}$AT, NaPi IIa, aquaporin 2, collectrin, XT2, XT3, SIT1, B^{0}AT1, ACE2, LAT2 and 4F2. For immunoprecipitations, brush border membrane proteins were probed with antibodies to B^{0}AT1, XT2, XT3 and b$^{0,+}$AT.

Urine and plasma analyses. Urine was collected over 24 h in a metabolic cage. Urinary crystals were observed under a magnification of ×1,000. For HPLC analysis[19], crystals were dissolved in 0.1 M HCl. Na^+, K^+ and Cl^- were assayed by indirect potentiometry. Phosphate was determined using the phosphomolybdate method. Calcium levels were obtained by cresolphthalein. Glucose, urea and uric acid were measured using Reflovet (Roche).

Histology and immunohistochemistry. Paraffin sections were stained with haematoxylin and eosin (H&E). Immunohistochemistry was performed with an affinity purified anti-collectrin antibody. B^{0}AT1 was detected using a rabbit anti-mouse B^{0}AT1 antibody.

Amino acid transporter activities. Uptake of ^{3}H-Phe, ^{14}C-Gln and $^{32}PO_4$ was determined in BBMVs at 60 s. The final amino acid concentration was 1 mM. Measurements were done in parallel in the presence of either Na^+ or K^+. Expression studies and uptake measurements in *Xenopus laevis* oocytes and kidney MDCK cells were performed as described[22,23].

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