Vol 456 | 20 November 2008 | doi:10.1038/nature07518 nature

ARTICLES

A role for Rhesus factor Rhcg in renal ammonium excretion and male fertility

Sophie Biver[1]*, Hendrica Belge[2]*, Soline Bourgeois[3,4]*, Pascale Van Vooren[1], Marta Nowik[3], Sophie Scohy[1]†, Pascal Houillier[4], Josiane Szpirer[1], Claude Szpirer[1], Carsten A. Wagner[3], Olivier Devuyst[2] & Anna Maria Marini[1]

The kidney has an important role in the regulation of acid–base homeostasis. Renal ammonium production and excretion are essential for net acid excretion under basal conditions and during metabolic acidosis. Ammonium is secreted into the urine by the collecting duct, a distal nephron segment where ammonium transport is believed to occur by non-ionic NH_3 diffusion coupled to H^+ secretion. Here we show that this process is largely dependent on the Rhesus factor Rhcg. Mice lacking Rhcg have abnormal urinary acidification due to impaired ammonium excretion on acid loading—a feature of distal renal tubular acidosis. *In vitro* microperfused collecting ducts of *Rhcg*$^{-/-}$ acid-loaded mice show reduced apical permeability to NH_3 and impaired transepithelial NH_3 transport. Furthermore, Rhcg is localized in epididymal epithelial cells and is required for normal fertility and epididymal fluid pH. We anticipate a critical role for Rhcg in ammonium handling and pH homeostasis both in the kidney and the male reproductive tract.

Ammonium is a principal nitrogen source for microorganisms and plants, whereas in animals it is best known for its cytotoxic effects that may lead to hepatic encephalopathy for instance[1]. Because more than 98% of ammonium is in the NH_4^+ form at physiological pH, throughout we use 'ammonium' to refer to the sum of ammonium (NH_4^+) and ammonia (NH_3) if not otherwise specified. In mammals, the liver and kidney are involved in ammonium detoxification and excretion, respectively. Ammonium metabolism and transport in the kidney are particularly critical for systemic pH regulation[2–5]. Along the nephron, ammonium, first produced in the proximal tubule, is secreted in the lumen of this segment and then reabsorbed at the level of the thick ascending limb of the loop of Henle. These processes involve nonspecific ammonium transport across several systems in which ammonium competes with natural substrates such as potassium. About 80% of the ammonium produced in the proximal tubule is finally secreted into the urine by the more distal nephron segment, the collecting duct. Since the first description of the concept in the late 1940s, the process of rapid transepithelial transport of ammonium in the collecting duct is thought to occur through non-ionic NH_3 diffusion across the lipid bilayer[6,7]. NH_3 is then trapped as NH_4^+ in the lumen of the collecting duct by buffering protons secreted throughout ATPases present at the apical membrane of acid-secreting (type A) intercalated cells. The inability of the distal nephron to appropriately acidify urine in humans results in complete or incomplete forms of distal renal tubular acidosis (dRTA), with potential associated metabolic acidosis reflecting the altered pH homeostasis[8,9].

In the meantime, genes for specific ammonium transport proteins were identified by functional complementation in yeast, thereby defining the Mep-Amt family of proteins largely represented in microorganisms, plants and invertebrates but absent from vertebrate animals[10,11]. Rhesus (Rh) factors have previously been shown to be related to proteins from the Mep-Amt family[12] and could therefore correspond to their, yet to be described, vertebrate counterpart. Although the main Rhesus antigen (RHD) was identified decades ago[13], the physiological role of Rhesus-type proteins remains largely unknown. Human Rhesus factors comprise the blood-group antigens (RHCE and RHD)[14,15], their associated glycoprotein (RHAG)[16], and two non-erythroid members (RHBG and RHCG)[17–19]. The Rhbg and Rhcg proteins are expressed in distal segments of the mammalian nephron, the connecting tubule and the collecting duct[20–22]. In the acid-secreting intercalated cells of the mouse kidney, basolateral Rhbg coexists with apical Rhcg[20,21]. Human RHAG and RHCG were shown to mediate bidirectional ammonium transport when expressed in yeast[17]. This function of Rhesus proteins in ammonium transport is sustained by several functional expression studies[23–29]. However, genetic ablation of mouse Rhbg yielded no impairment of renal ammonium excretion and no phenotype[30]. Another potential role for Rhesus proteins, including human RHAG, has been suggested in CO_2 transport[31–33] but this role is still questioned[34], and the biological function of Rhesus factors thus remains debated.

Here we targeted the *Rhcg* gene in mouse and show that the Rhcg protein is required for urinary ammonium excretion by being critical for NH_3 transport across the collecting duct epithelium. Hence, the longstanding assumption that this process occurs solely by lipid phase diffusion of NH_3 should be revised.

Decreased ammoniuria in *Rhcg*$^{-/-}$ mice

To address the physiological role of Rhcg in mouse, we disrupted the *Rhcg* gene by homologous recombination deleting exons 3 to 9 coding for transmembrane segments 4 to 12 (Fig. 1a, b). *Rhcg*$^{-/-}$ mice were born with the expected Mendelian ratio. Polymerase chain reaction with reverse transcription (RT–PCR) analysis demonstrated the complete loss of the 2-kb *Rhcg* transcript in *Rhcg*$^{-/-}$ mice (Fig. 1c) and the lack of Rhcg was confirmed by immunoblotting (Fig. 1d) and immunostaining (Fig. 1e). On a standard diet, *Rhcg*$^{-/-}$ mice showed no obvious abnormalities. Broad-spectrum histological analyses revealed no defect, including in kidney structure and the distribution of cell types in the collecting duct (Fig. 1e, Supplementary Figs 1, 2 and

[1]Université Libre de Bruxelles (U.L.B.), Institut de Biologie et de Médecine Moléculaires, Laboratoires de Biologie du Développement et de Physiologie Moléculaire de la Cellule, Rue Professeurs Jeener et Brachet, 12, B-6041 Gosselies, Belgium. [2]Université catholique de Louvain (U.C.L.), Division of Nephrology, Avenue Hippocrate, 54, B-1200 Brussels, Belgium. [3]Institute of Physiology and Zurich Center for Integrative Human Physiology, University of Zurich, Winterthurerstrasse 190, Zurich CH-8057, Switzerland. [4]INSERM UMRS 872; Université Pierre et Marie Curie; Université Paris-Descartes; F-75006 Paris, France. †Present address: Delphi Genetics, B-6041 Gosselies, Belgium.
*These authors contributed equally to this work.

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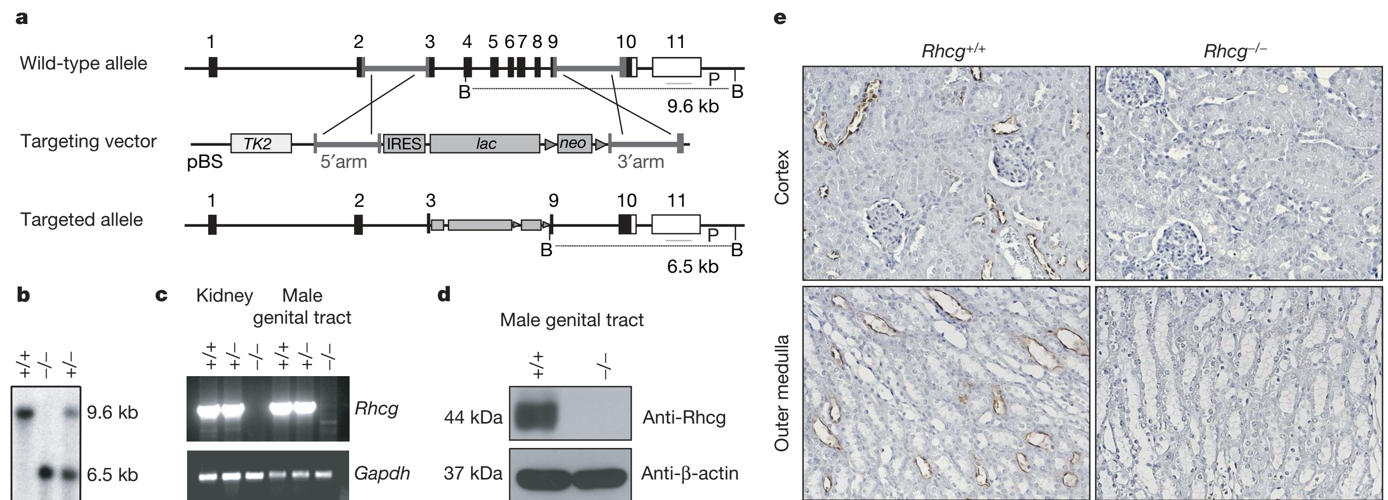

Figure 1 | Gene targeting of *Rhcg*. **a**, The targeting strategy. The IRES-LacZ cassette is the reporter, the pGK-Neo locus is the positive selection marker and thymidine kinase (*TK2*) represents the negative selection marker. The *loxP* sites are shown as triangles, *Rhcg* exons as black boxes. B, BamHI sites used in DNA blot analysis with the exon 11 probe (P). pBS, pBluescript. **b–e**, Demonstration of correct *Rhcg* targeting. **b**, Blot analysis of DNA from littermate progeny of $Rhcg^{+/-}$ crosses. BamHI-digested genomic DNA hybridized with the exon 11 probe produces a shorter fragment in $Rhcg^{-/-}$ DNA (6.5 kb). **c**, RT–PCR analyses on renal and male genital tract RNA with primers amplifying exons 2–7 of *Rhcg* (control: *Gapdh*). **d**, Immunoblotting analysis. Membrane proteins from male genital tract probed with anti-Rhcg antiserum (loading control: β-actin). **e**, Rhcg immunodetection in kidney sections (counterstaining: haematoxylin). Apical Rhcg (brown) detected in intercalated and principal cells of wild-type collecting ducts. Histological analysis of $Rhcg^{-/-}$ kidneys reveals no structural abnormalities. Original magnification, ×320.

Supplementary Table 1). However, $Rhcg^{-/-}$ mice had a significant decrease in urinary ammonium excretion and a more alkaline urinary pH (Table 1, Supplementary Table 2 and Supplementary Table 3).

Metabolic acidosis in $Rhcg^{-/-}$ mice

In humans, defects in distal urinary acidification result in dRTA[8]. Notably, in the incomplete forms of dRTA, the associated metabolic acidosis is not overt but may be revealed by an acid-load challenge. To unmask an incomplete form of dRTA, we investigated the response of $Rhcg^{-/-}$ mice to a short (2 days) and long (6 days) NH_4Cl acid load (Fig. 2a–f, Supplementary Table 2 and Supplementary Table 3). In wild-type mice, the acid load induced a transient decrease of blood pH and bicarbonate levels with partial recovery at day 6 because of adaptation (Fig. 2a, b). In contrast, in $Rhcg^{-/-}$ mice, the decrease in blood pH and bicarbonate levels lasted during the entire treatment, indicating an impaired ability to cope with the acid load. $Rhcg^{-/-}$ mice also showed lower arterial p_{CO_2} levels at day 6, suggesting that respiratory compensation for metabolic acidosis occurs (Fig. 2c).

Both wild-type and $Rhcg^{-/-}$ animals responded to the acid load by increasing the urinary excretion of ammonium and titratable acidity, resulting in a large increase in net acid excretion (NAE) and a concomitant reduction in urinary pH (Fig. 2d–f). However, NAE was considerably lower in $Rhcg^{-/-}$ mice owing to a selective defect in ammoniuria (Fig. 2e, f and Supplementary Table 2). $Rhcg^{-/-}$ mice had a ~40% reduction in urinary ammonium excretion, and were unable to maximally acidify urine (Fig. 2d and Supplementary Table 2). Defective ammonium excretion is not related to impaired ammonium synthesis, because blood glutamine and glutamate levels, and messenger RNA abundance of enzymes involved in renal ammonium production were unaffected by Rhcg deletion (Supplementary Fig. 3). The impaired handling of an acid load in $Rhcg^{-/-}$ mice is thus caused by a decreased ability to excrete acid in the form of NH_4^+.

Table 1 | Baseline parameters in $Rhcg^{-/-}$ and wild-type mice

	$Rhcg^{+/+}$	$Rhcg^{-/-}$
Body weight (g)	43 ± 1 (35)	38 ± 1 (31)*
Arterial blood		
Hematocrit (%)	42 ± 1 (12)	41 ± 2 (11)
pH	7.28 ± 0.01 (12)	7.31 ± 0.01 (11)
p_{CO_2} (mm Hg)	39 ± 2 (12)	37 ± 2 (11)
Plasma		
HCO_3^- (mM)	18 ± 1 (12)	18 ± 1 (11)
Na^+ (mM)	147 ± 1 (12)	146 ± 1 (11)
K^+ (mM)	4.3 ± 0.1 (12)	3.8 ± 0.1 (11)*
Cl^- (mM)	120 ± 1 (12)	117 ± 1 (11)
Urea (mg dl^{-1})	75 ± 6 (9)	69 ± 5 (8)
Creatinine (mg dl^{-1})	0.15 ± 0.01 (9)	0.14 ± 0.01 (8)
Ammonium (μM)	49 ± 5 (10)	47 ± 4 (8)
Urine		
Urinary flow rate (μl min^{-1} g^{-1} BW)	0.035 ± 0.003 (35)	0.034 ± 0.003 (31)
pH	7.42 ± 0.09 (35)	7.68 ± 0.08 (31)*
Na^+ (mM mM^{-1} creatinine)	32 ± 2 (35)	33 ± 1 (31)
Cl^- (mM mM^{-1} creatinine)	36 ± 2 (34)	38 ± 1 (26)
K^+ (mM mM^{-1} creatinine)	59 ± 2 (35)	60 ± 2 (31)
Calcium (mg mg^{-1} creatinine)	0.064 ± 0.01 (32)	0.056 ± 0.01 (27)
Phosphate (mg mg^{-1} creatinine)	1.76 ± 0.13 (31)	1.56 ± 0.17 (21)
Aldosterone (ng mg^{-1} creatinine)	0.074 ± 0.005 (7)	0.083 ± 0.006 (7)
Creatinine (mM)	5.80 ± 0.29 (35)	6.01 ± 0.30 (31)
Ammonium (mM mM^{-1} creatinine)	2.82 ± 0.22 (35)	2.22 ± 0.17 (30)*

Means ± s.e.m. (*n*); BW, body weight.
*, $P < 0.05$ versus wild type.

Acid load and weight loss in $Rhcg^{-/-}$ mice

We also investigated the response of $Rhcg^{-/-}$ mice to HCl, a stronger acid challenge which, in contrast to NH_4Cl, does not modify nitrogen intake. The 7-day HCl load led to a large increase in urinary ammonium excretion in wild-type mice, reaching a plateau after 2 days (Fig. 2g and Supplementary Table 3). $Rhcg^{-/-}$ mice again showed an impaired ability to excrete ammonium, barely reaching 25% of the wild-type response. Chronic metabolic acidosis has been shown to induce weight loss[35]. The HCl load induced a slight and transient reduction of body weight in wild-type mice, whereas $Rhcg^{-/-}$ mice progressively lost about 25% of their initial body weight (Fig. 2h). A significantly reduced body weight of $Rhcg^{-/-}$ mice was already detectable at baseline, and was sustained in $Rhcg^{-/-}$ mice drinking NH_4Cl (Supplementary Table 2). Hence, $Rhcg^{-/-}$ animals have an impaired ability to maximally acidify urine owing to impaired ammonium excretion and are therefore unable to properly handle

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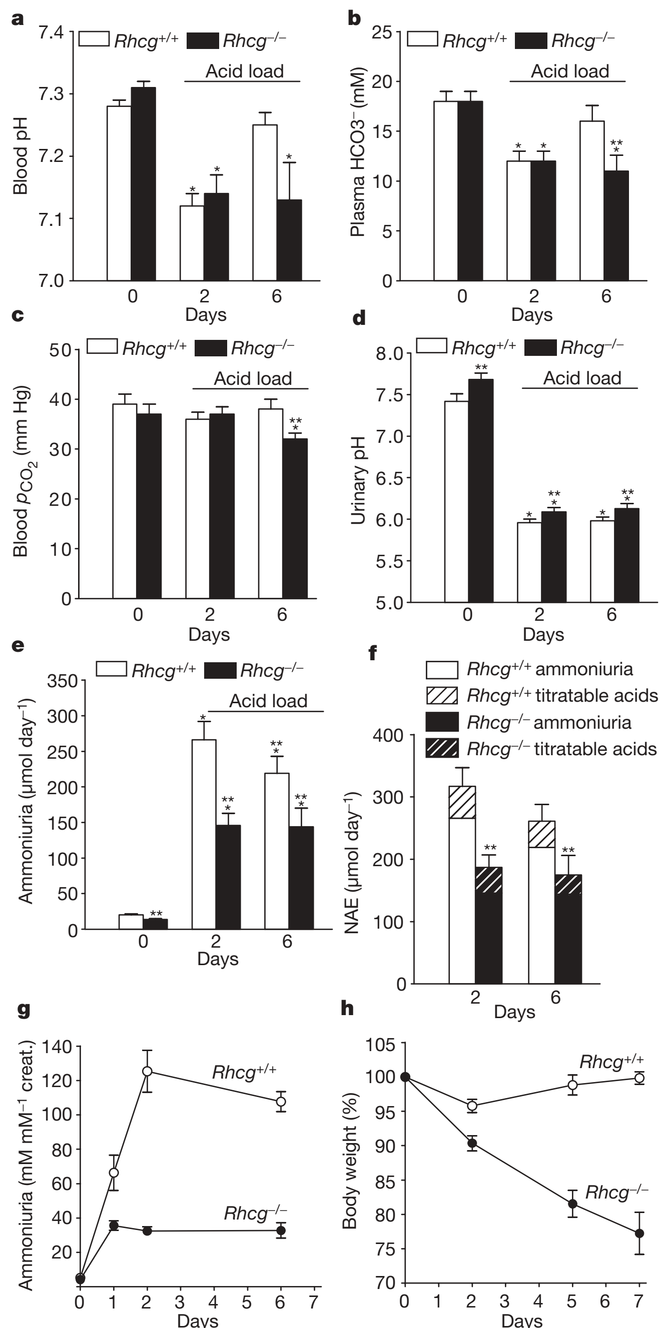

Figure 2 | Impaired acid-stress handling in *Rhcg*$^{-/-}$ mice. a–f, Increased metabolic acidosis and impaired acid excretion in *Rhcg*$^{-/-}$ mice (CD1) exposed to an NH_4Cl load for 0, 2 or 6 days. Data expressed as means ± s.e.m.; $n \geq 11$; asterisk, $P < 0.05$ versus baseline; double asterisk, $P < 0.05$ versus wild type. **a**, Blood pH. **b**, Plasma HCO_3^-. **c**, Blood p_{CO_2}. **d**, Urinary pH. **e**, Ammoniuria. **f**, NAE. The *Rhcg*$^{-/-}$ mice show a strongly reduced NAE owing to a selective defect in ammoniuria, whereas titratable acidity levels are similar to wild type. **g**, **h**, Impaired ammonium excretion and excessive weight loss in *Rhcg*$^{-/-}$ mice (C57BL/6) exposed to an HCl acid-load. **g**, Time course of urinary ammonium excretion; $n \geq 6$. **h**, Adaptation to the acid-loading challenge indicated by body weight loss, expressed as percentages of initial body weights. HCl-induced weight loss is more pronounced in *Rhcg*$^{-/-}$ mice; $n \geq 4$; creat., creatinine.

acid challenges, demonstrating important characteristics of distal renal tubular acidosis.

Rhcg is required for NH_3 transport

To assess the effect of Rhcg on the apical NH_3/NH_4^+ permeabilities of collecting duct cells, we first microperfused pH-sensitive dye-loaded cortical collecting ducts (CCDs) and outer medullary collecting ducts (OMCDs) from *Rhcg*$^{-/-}$ mice after a 2-day acid-load (Fig. 3 and Supplementary Table 4). An inwardly directed ammonium gradient was applied from the lumen (20 mM NH_4Cl; Fig. 3a). The initial rate of intracellular alkalinization of *Rhcg*$^{-/-}$ CCDs and OMCDs was reduced to 29% and 40%, respectively, compared to wild type (Fig. 3b). As the alkalinization phase is associated with rapid NH_3 entry and subsequent protonation to form intracellular NH_4^+ [36], we conclude that the net permeability to NH_3 is impaired in *Rhcg*$^{-/-}$ mice (Fig. 3b). There was no obvious difference in the subsequent acidification rate, reflecting similar rates of NH_4^+ entry and dissociation of NH_3 and H^+ in wild-type and *Rhcg*$^{-/-}$ OMCD and CCD cells (Fig. 3b). We next directly measured diffusive NH_3 permeability across the CCD epithelium of 2-day acid-challenged mice (Fig. 3c and Supplementary Table 5). Imposing a bath-to-lumen NH_3 gradient, in the nominal absence of a NH_4^+ gradient, generated a measurable NH_3 secretion flux across the CCD epithelium that was reduced to 28% in *Rhcg*$^{-/-}$ compared to wild type. This corresponded to a 67% decrease in NH_3 permeability in *Rhcg*$^{-/-}$ CCDs (Fig. 3c).

Both independent *in vitro* methods thus show Rhcg to be critical for NH_3 transport across the apical membrane of CCDs and allow the identification of the mechanism responsible for reduced urinary ammonium excretion in *Rhcg*$^{-/-}$ mice.

Decreased male fertility in *Rhcg*$^{-/-}$ mice

Because *Rhcg* mRNA has been detected in mouse testis[18], we investigated the fertility of *Rhcg*$^{-/-}$ mice. Although the *Rhcg*$^{-/-}$ males fertilized females as quickly as the *Rhcg*$^{+/+}$ littermates, their litters were ~10% smaller than those of the two other male types (*Rhcg*$^{-/-}$: 11.7 ± 0.5 versus *Rhcg*$^{+/-}$: 12.7 ± 0.4 and *Rhcg*$^{+/+}$: 12.9 ± 0.3 pups per litter, $n \geq 40$, $P < 0.05$). Rhcg deletion did not affect the fertility of females. Real-time RT–PCR showed that *Rhcg* is highly expressed in the epididymis and at lower levels in the testis (Fig. 4a). Rhcg staining was detected on the luminal membrane of cells lining a subset of epididymal ducts (Fig. 4b). An important characteristic of the epididymal luminal fluid is that it is maintained acidic to keep spermatozoa immotile during their maturation in the epididymis[37]. Notably, the pH of the epididymal fluid was significantly lower in *Rhcg*$^{-/-}$ mice compared to wild type (Fig. 4c).

Discussion

We have demonstrated that Rhcg is required for maximal urinary acidification and ammonium excretion. This contrasts with the absence of any particular phenotype reported for mice lacking Rhbg[30]. Our data are consistent with a role of Rhcg as an ammonium transport protein mediating the net flux of NH_3 in the collecting ducts. They lead to a new model of renal ammonium excretion occurring at least in part through a protein-mediated pathway, and not only through simple non-ionic diffusion and subsequent luminal trapping as previously suggested[6,7]. Clearly, a channel/carrier-mediated pathway would provide the kidney with the ability to regulate ammonium excretion at this level. Understanding the physiological function of Rhbg and the potential interaction with Rhcg remains a challenge for future investigations.

We have also shown that *Rhcg*$^{-/-}$ mice have features of incomplete human dRTA[8]. Consequently, human *RHCG* may be a candidate gene for inherited forms of dRTA that are not associated with mutations in B1 (*ATP6V1B1*) and a4 (*ATP6V0A4*) subunits of the H^+ V-ATPase or the HCO_3^-/Cl^- exchanger AE1 (*SLC4A1*)[38]. The reduced male fertility and lower pH of epididymal fluid in *Rhcg*$^{-/-}$ mice also indicate that Rhcg has a role in the homeostasis of the male reproductive tract. The impairment of RHCG in humans might likewise be accompanied by male fertility problems. Moreover, low ammonium concentrations were reported to inhibit human spermatozoa motility *in vitro*[39]. Rhcg might thus be involved in ammonium scavenging from the epididymal fluid, consistent with the high

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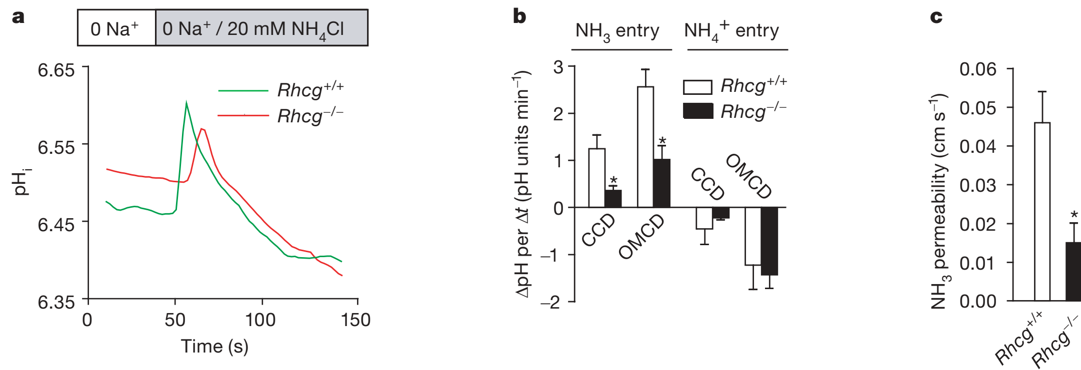

Figure 3 | Reduced NH_3 permeability of microperfused collecting ducts segments from *Rhcg*$^{-/-}$ acid-challenged mice. NH_4Cl was given for 2 days to CD1 mice. **a**, **b**, CCDs and OMCDs were microperfused *in vitro* in the lumen with 20 mM NH_4Cl. Intracellular pH (pH_i) was monitored with the pH-sensitive dye 2′,7′-bis-(2-carboxyethyl)-5-(and -6)- carboxyfluorescein (BCECF) to examine pH_i evolution during luminal NH_4Cl application. **a**, pH_i recording from OMCD cells. **b**, Alkalinization and acidification rates in CCD and OMCD cells. The reduced rate of alkalinization in *Rhcg*$^{-/-}$ cells indicates impaired luminal NH_3 entry in CCD and OMCD cells. Means ± s.e.m.; $n = 4$–7 per genotype or nephron segment; asterisk, $P < 0.05$ versus wild type. **c**, Diffusive NH_3 permeability across *Rhcg*$^{-/-}$ CCD epithelium, measured by imposing a bath-to-lumen NH_3 gradient in microperfused CCDs, was strongly impaired compared to wild type. Means ± s.e.m.; $n = 4$; *, $P < 0.05$.

glutamine synthetase activity of the epithelial cells lining the epididymis head[40] and hence their ability to metabolise ammonium.

By highlighting the role of Rhcg in ammonium disposal and urinary acidification, our study brings new insights in the regulation of acid–base homeostasis, the role of the kidney and the pathophysiology of disease states such as dRTA and male infertility.

METHODS SUMMARY

***Rhcg*$^{-/-}$ mice and *in vivo* studies.** *Rhcg*$^{-/-}$ mice were obtained by homologous recombination, targeting exons 3–9 of *Rhcg*, resulting in a null allele (*Rhcg*^{tm1Ibmm}). Experiments were performed on adult male *Rhcg*$^{+/+}$ and *Rhcg*$^{-/-}$ littermates, matched for age. For acid-loading experiments, a solution of 300 mM NH_4Cl (in 2% sucrose) was given to mice in their drinking water for 2 or 6 days. For HCl acid-loading, a 50:50 mixture (w/v) of normal chow and 0.4 M HCl was given for 7 days with water *ad libitum*. All protocols were conducted in accordance with the National Research Council Guide for the Care and Use of Laboratory Animals and were approved by the local Ethics Committee.

Analytic procedures. Urinary parameters were measured on a Synchron CX5 analyser (Beckman). Blood gases and electrolytes were measured using an ABL77 pH/ blood-gas analyser (Radiometer). Ammonium concentrations were measured by glutamate dehydrogenase determination and by using an ammonium combination electrode (DC218-NH4, Mettler). Titratable acids were measured using a DL 50 titrator (Mettler).

Immunostaining and immunoblotting. Immunostaining was performed on paraffin-embedded sections, and specificity was demonstrated on similar knockout tissues. Membrane proteins were obtained from the male genital tract homogenized in 250 mM sucrose, 10 mM Tris-HCl, pH 7.5, and protease inhibitors. Proteins were separated by SDS–PAGE. The anti-Rhcg antibody was obtained after immunizing rabbits with a carboxy-terminal peptide (EEVNTVYIPEDLAHK) of mouse Rhcg.

***In vitro* microperfusion experiments.** *In vitro* microperfusion of single CCD and OMCD fragments, intracellular pH and transepithelial NH_3 permeability measurements were performed essentially as described previously[30].

Statistics. Data are expressed as means ± s.e.m. Statistical comparisons were tested by Student's *t*-tests using the Graphpad Prism software.

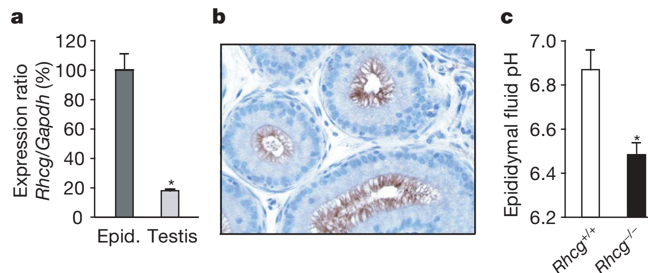

Figure 4 | Rhcg in the male genital tract. **a**, *Rhcg* expression in wild-type testis and epididymis (epid.) by real-time RT–PCR, epididymal expression taken as 100%. *Rhcg* is expressed significantly more in epididymis than in testis; $n = 4$. **b**, Rhcg immunodetection in wild-type epididymis (counterstaining: haematoxylin). Original magnification, ×200. Rhcg (brown) is present on the luminal membrane of epididymal cells. **c**, Significant decrease in *Rhcg*$^{-/-}$ epididymal fluid pH. Means ± s.e.m.; $n = 7$; asterisk, $P < 0.05$.

Received 14 June; accepted 29 September 2008.

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

Acknowledgements We are grateful to P. Drèze, B. André and P. Gabant for their contribution to the initial step of this study. We thank H. Mezdour, L. Pouilly and P. Persoons for microinjection experiments, B. Pajak for help in broad-spectrum histological assessment, V. Beaujean, Y. Cnops, H. Debaix and T. Nishino for excellent technical assistance, and A. Tamah and M.-F. Vincent for amino acid chromatography. These studies were supported by the Belgian agencies FNRS and FRSM (3.4.546.04.F, 3.4.635.05.F, 3.4592.06.F), the 'Fonds Brachet-recherche', the 'Fonds Van Buuren', the 'Fondation Alphonse & Jean Forton', a Concerted Research Action (05/10-328), an Inter-university Attraction Pole (IUAP P6/05), the Swiss National Science Foundation (31-109677), an INSERM grant, and the EuReGene and EUNEFRON (GA#201590) projects of the European Community (FP6 and FP7). S.Bi. was the recipient of a FRIA fellowship; H.B., A.M.M. and C.S. are Research fellow, Research associate and Research director of the FNRS, respectively.

Author Contributions S.Bi., J.S., C.S. and A.M.M. developed the concept of this study; all authors contributed to the intellectual design, execution and interpretation of the experiments; S.Bi. and A.M.M. wrote the paper with specific contribution of all authors and the particular involvement of O.D. and C.A.W. All authors analysed and discussed the data, and commented on the final version of the manuscript. S.Bi., H.B. and S.Bo. share first authorship.

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