

# Distal Renal Tubular Acidosis in Mice Lacking the AE1 (Band3) $\text{Cl}^-/\text{HCO}_3^-$ Exchanger (*slc4a1*)

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Mutations in the human gene that encodes the AE1  $\text{Cl}^-/\text{HCO}_3^-$  exchanger (*SLC4A1*) cause autosomal recessive and dominant forms of distal renal tubular acidosis (dRTA). A mouse model that lacks AE1/*slc4a1* (*slc4a1*<sup>-/-</sup>) exhibited dRTA characterized by spontaneous hyperchloremic metabolic acidosis with low net acid excretion and, inappropriately, alkaline urine without bicarbonaturia. Basolateral  $\text{Cl}^-/\text{HCO}_3^-$  exchange activity in acid-secreting intercalated cells of isolated superfused *slc4a1*<sup>-/-</sup> medullary collecting duct was reduced, but alternate bicarbonate transport pathways were upregulated. Homozygous mice had nephrocalcinosis associated with hypercalciuria, hyperphosphaturia, and hypocitraturia. A severe urinary concentration defect in *slc4a1*<sup>-/-</sup> mice was accompanied by dysregulated expression and localization of the aquaporin-2 water channel. Mice that were heterozygous for the AE1-deficient allele had no apparent defect. Thus, the *slc4a1*<sup>-/-</sup> mouse is the first genetic model of complete dRTA and demonstrates that the AE1/*slc4a1*  $\text{Cl}^-/\text{HCO}_3^-$  exchanger is required for maintenance of normal acid-base homeostasis by distal renal regeneration of bicarbonate in the mouse as well as in humans.

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**N**ormal kidney function is critical for maintenance and control of systemic acid-base homeostasis, as highlighted by several inborn and acquired diseases (1–5). Three main activities contribute to this role: Proximal tubular reabsorption of filtered bicarbonate, proximal tubular synthesis and excretion of ammonia, and collecting duct excretion of protons and regeneration of bicarbonate. Type A acid-secreting intercalated cells (A-IC) in the collecting duct express an apical vacuolar  $\text{H}^+$ -ATPase and a basolateral  $\text{Cl}^-/\text{HCO}_3^-$  exchanger kAE1 (kidney band 3, *SLC4A1*) that respectively mediate proton extrusion into urine and bicarbonate release into blood (2,6). Bicarbonate is formed in the A-IC by the cytosolic carbonic anhydrase. The concerted action of these transport proteins and enzymes in A-IC is important for acid secretion and for parallel *de novo* generation of bicarbonate and/or its reabsorption from the luminal fluid.

AE1 is expressed in red blood cells and in renal A-IC. The kidney-specific kAE1 gene product lacks the N-terminal 79 amino acids (aa) that are present in mouse erythroid AE1 (eAE1), as a result of alternative promoter usage (7,8). Human kAE1 lacks the N-terminal 65 aa that are present in human

eAE1 (6). The N-terminal extension of eAE1 polypeptide is important for interaction with erythroid-specific cytoskeletal and membrane proteins. One group of human AE1 mutations causes dominantly transmitted erythroid-specific abnormalities such as hereditary spherocytosis, Southeast Asian ovalocytosis (SAO), and other “hereditary stomatocytoses with cation leak” (9) accompanied by normal renal function. A distinct set of AE1 mutations can cause distal renal tubular acidosis (dRTA) without apparent erythroid abnormality (1,10–14).

dRTA-associated AE1 mutations are predominantly autosomal dominant among white individuals (13,15,16), with disease characterized by hyperchloremic metabolic acidosis, variable hypokalemia, osteomalacia, childhood growth retardation, nephrocalcinosis, and nephrolithiasis progressing to renal insufficiency (5,15,17). At least two pathophysiologic mechanisms have been proposed to explain autosomal dominant dRTA as a result of AE1 mutations (1,9): A dominant negative intracellular retention phenotype (18) and a dominant or co-dominant mistargeting to the apical membrane (19,20). A distinct set of mutations found among Southeast Asians and Melanesians is transmitted in autosomal or compound recessive patterns (11,14). The recessive disease is often of earlier onset and accompanied by a urinary concentrating defect, with affected infants susceptible to severe dehydration (1,3,5,15,17,21–23). Rare recessive mutations cause combined early-onset hemolytic anemia and dRTA manifest as hydrops fetalis (24). The severity of these symptoms emphasizes the importance of AE1 for normal kidney function. In this study, we examined the renal phenotype of AE1-deficient mice to gain additional insight into the physiologic function of kAE1, the pathogenesis of dRTA

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as a result of AE1 gene mutations, and the mode of inheritance of AE1 mutation-associated dRTA.

## Materials and Methods

### Animals

Generation and breeding of mice that were deficient for anion exchanger 1 (*Slc4a1*  $-/-$ ) on a C57BL/6J background was previously described (25). Mice were maintained on a standard rodent chow (Kliba AG, Kaiseraugst, Switzerland) and had access to drinking water *ad libitum*.

Food and water consumption was monitored, and urine and stool output was quantified in metabolic cages, with urine collection under mineral oil. After an adaptation period to the metabolic cage environment, experiments were performed over periods of 1 to 3 d. During days 1 and 2, mice were kept under control conditions (tap water with 2% sucrose). On day 3, 0.28 M  $\text{NH}_4\text{Cl}$  was added to the drinking water as an oral acid challenge (26,27). All animal studies were according to Swiss Animal Welfare Laws and approved by the Local Veterinary Authority (Kantonales Veterinäramt) Zurich.

### Blood and Urine Analysis

Blood and urine samples that were obtained from metabolic cage experiments were injected into a blood gas analyzer (ABL 505, Radiometer Copenhagen, Copenhagen, Denmark) for determination of pH,  $\text{Pco}_2$ , calculated  $[\text{HCO}_3^-]$ ,  $\text{K}^+$ ,  $\text{Na}^+$ ,  $\text{Cl}^-$  (blood), and  $\text{HCO}_3^-$  (urine). pH of urine that was collected under oil was measured using a pH microelectrode (Metrohm, Herisau, Switzerland). Urine was diluted in distilled water for anion determinations by ion chromatography (Metrohm ion chromatograph) on a SUPP 5-250 column eluted with 3.2 mM  $\text{Na}_2\text{CO}_3/1$  mM  $\text{NaHCO}_3$ . Urine was diluted in 4 mM tartaric acid for determination of  $\text{K}^+$ ,  $\text{Na}^+$ ,  $\text{Ca}^{2+}$ , and  $\text{Mg}^{2+}$  on a METROSEP C-2-150 column eluted with 4 mM tartaric acid/1 mM dipicolinic acid. Serum creatinine concentration was measured using a kit based on the F-Daos method (Wako Chemicals, Neuss, Germany). Urine creatinine was analyzed by the Jaffé method (28,29). Urine ammonium concentration was determined according to the Berthelot Protocol (30). Urinary citrate concentration was measured with the kit from R-Biopharm (Murten, Switzerland). Titratable acids were determined according to Chan (31). Briefly, equal volumes of urine and 0.1 N HCl were mixed and boiled for 2 min. After 10 min of cooling to 37°C, the mix was titrated with 0.1 N NaOH to pH 7.4. A blank sample (distilled water) was identically treated. The difference in volumes of NaOH required to titrate the sample and the blank, multiplied by the normality of NaOH times 1000, revealed the concentration of titratable acids in mmol/L. Osmolarity of blood and urine was determined using a Roebling freezing point osmometer (Auer Bittmann Soulié, Dietikon, Switzerland). GFR, fractional excretion of ions, anion gap in blood and urine, and net acid excretion were calculated from the measured values.

### Statistical Analyses

All data were tested for significance using the ANOVA and *t* test. Only data with  $P < 0.05$  were considered as significantly different.

## Results

The renal phenotype of AE1-deficient mice was not analyzed in early studies because of high perinatal and postnatal lethality (Supplementary Figure 2) (25,32). Crossing of the few surviving *Ae1*  $-/-$  male homozygote mice with *Ae1*  $+/-$  females resulted in AE1-deficient *Ae1*  $-/-$  progeny that exhibited approximately 16% postweaning survival to the age of 10 to 14 wk. AE1-deficient neonates were smaller than their littermates and required 5 to 6 wk of age before they could be successfully weaned. Surviving AE1-deficient mice grew more slowly than heterozygous or wild-type animals, with *Ae1*  $-/-$  male weight at 12 wk of  $22.6 \pm 1.1$  g ( $n = 8$ ) versus *Ae1*  $+/+$  weight of  $26.3 \pm 1.7$  g ( $n = 9$ ).

At age 5 to 7 d, the *Ae1*  $-/-$  mouse hematocrit measured on two separate occasions was  $7.1 \pm 1.0\%$  ( $n = 9$ ) and  $13.3 \pm 1.0\%$  ( $n = 18$ ), with reticulocytosis of  $>70\%$ . In six additional mice of mean age 14 wk, hematocrit was  $10.9 \pm 0.5\%$ . These severely anemic values are nonetheless slightly higher than those originally reported (25).

Twelve-week-old mice were used to assess consequences of the congenital absence of AE1 on renal acid-base transport and systemic acid-base homeostasis. Immunoblotting with an antibody directed against an epitope that was common to the erythrocyte and kidney isoforms confirmed the complete absence of AE1 from kidneys of AE1-deficient mice (Supplementary Figure 1). Kidney function and systemic acid-base balance were evaluated under basal conditions and during a 24-h oral acid load. Under control conditions (Table 1, Figure 1) wild-type and *slc4a1*  $+/-$  mice exhibited no apparent difference in blood or urine parameters. In contrast, *slc4a1*  $-/-$  mice had severe metabolic acidosis with low blood pH and bicarbonate levels and slightly increased blood chloride and phosphate levels (Table 1). Elevated serum urea levels pointed to mild renal insufficiency in AE1-deficient mice, and elevated serum osmolality suggested mild systemic dehydration. The urine of AE1-deficient mice was alkaline, with reduced excretion of net acid and of titratable acids (Table 1, Figure 1). Twenty-four-hour urine volume of *Ae1*  $-/-$  mice was two- to three-fold higher than for wild-type and heterozygous mice, with correspondingly lower osmolality. In *Ae1*  $-/-$  mice, hypercalciuria and hypocitraturia were also evident in basal conditions.

Challenge with an oral acid load ( $\text{NH}_4\text{Cl}$  added to drinking water) induced metabolic acidosis in wild-type and heterozygous mice, as evident from reduced blood pH and bicarbonate levels, hyperchloremia, and decreased urine pH with increased net acid excretion (Table 2, Figure 1). In AE1-deficient mice, this  $\text{NH}_4\text{Cl}$ -loading protocol disproportionately exacerbated the basal metabolic acidosis, decreasing blood pH to  $6.82 \pm 0.02$  (compared with  $7.21 \pm 0.01$  in wild-type and  $7.20 \pm 0.01$  in heterozygous mice;  $n = 7$  to 9). The severely decreased blood bicarbonate concentration in AE1-deficient mice was accompanied by a less than proportionate reduction in  $\text{Pco}_2$ , consistent with a partial respiratory compensation (Tables 1 and 2, Figure 1). Despite the severe metabolic acidosis in *slc4a1*  $-/-$  mice, urine pH remained more alkaline and net acid excretion remained much lower than in wild-type littermates.

$\text{NH}_4\text{Cl}$  loading had to be terminated after 24 h. Longer

Table 1. Control conditions<sup>a</sup>

Genotype	<i>Ae1</i> <sup>+/+</sup>	<i>Ae1</i> <sup>+/-</sup>	<i>Ae1</i> <sup>-/-</sup>
<b>Blood</b>			
pH	7.27 ± 0.02	7.30 ± 0.02	7.12 ± 0.02 <sup>b</sup>
HCO <sub>3</sub> <sup>-</sup>	22.0 ± 0.8	22.2 ± 0.3	17.3 ± 0.5 <sup>b</sup>
Pco <sub>2</sub>	48.8 ± 1.7	44.9 ± 1.0	55.8 ± 2.1
K <sup>+</sup>	3.7 ± 0.1	3.5 ± 0.0	4.4 ± 0.1 <sup>c</sup>
Na <sup>+</sup>	147.3 ± 1.1	145.2 ± 0.8	149.1 ± 1.5
Cl <sup>-</sup>	113.3 ± 1.1	112.2 ± 1.1	116.7 ± 1.2 <sup>c</sup>
P <sub>i</sub>	1.6 ± 0.1	1.6 ± 0.1	2.4 ± 0.3 <sup>c</sup>
anion gap	12.0 ± 0.8	10.8 ± 0.2	15.1 ± 1.1
osmolarity	315.1 ± 2.6	310.2 ± 1.5	336.0 ± 9.1 <sup>c</sup>
creatinine	0.07 ± 0.01	ND	0.11 ± 0.02
urea	6.4 ± 1.6	ND	26.8 ± 3.8 <sup>b</sup>
<b>Urine</b>			
pH	6.48 ± 0.16	6.38 ± 0.13	6.77 ± 0.12
osmolarity	2519.8 ± 176.3	2336 ± 279.1	1085.1 ± 103.1 <sup>b</sup>
creatinine	68.9 ± 3.8	66.2 ± 6.6	27.1 ± 3.4 <sup>b</sup>
HCO <sub>3</sub> <sup>-</sup> /creatinine	0.2 ± 0.1	0.1 ± 0.0	0.5 ± 0.2
K <sup>+</sup> /creatinine	7.4 ± 1.0	2.9 ± 0.4 <sup>c</sup>	6.9 ± 1.1
Na <sup>+</sup> /creatinine	3.5 ± 0.5	1.2 ± 0.2 <sup>c</sup>	3.3 ± 0.5
Cl <sup>-</sup> /creatinine	3.8 ± 0.3	3.7 ± 0.6	4.2 ± 0.3
P <sub>i</sub> /creatinine	0.7 ± 0.1	0.7 ± 0.1	0.5 ± 0.1
NH <sub>4</sub> <sup>+</sup> /creatinine	1.2 ± 0.2	1.0 ± 0.2	1.4 ± 0.2
Ca <sup>2+</sup> /creatinine	0.05 ± 0.01	0.05 ± 0.01	0.11 ± 0.02 <sup>c</sup>
Mg <sup>2+</sup> /creatinine	0.44 ± 0.09	0.19 ± 0.04	0.66 ± 0.19
citrate/creatinine	0.71 ± 0.09	ND	0.25 ± 0.04 <sup>b</sup>
creatinine clearance	1.03 ± 0.17	ND	1.11 ± 0.22
FE Na <sup>+</sup> (%)	0.26 ± 0.05	ND	0.68 ± 0.15 <sup>c</sup>
FE K <sup>+</sup> (%)	21.25 ± 4.11	ND	10.31 ± 2.23
FE Cl <sup>-</sup> (%)	0.31 ± 0.08	ND	0.31 ± 0.05
FE Ca <sup>2+</sup> (%)	0.76 ± 0.09	ND	0.58 ± 0.08
FE Mg <sup>2+</sup> (%)	5.62 ± 2.76	ND	6.88 ± 1.16
FE P <sub>i</sub> (%)	2.09 ± 0.42	ND	1.81 ± 0.44
urinary anion gap	177.8 ± 89.3	21.4 ± 36.3	36.8 ± 24.2
titratable acids	38.7 ± 4.6	28.0 ± 6.1	5.5 ± 6.6 <sup>b</sup>
NEA	120.0 ± 10.2	94.1 ± 14.6	49.1 ± 12.4 <sup>b</sup>
NEA/creatinine	1.9 ± 0.2	1.4 ± 0.1	1.6 ± 0.4

<sup>a</sup>Summary of basal blood and urine data. Urine was collected during 24 h in metabolic cages, and blood was sampled at the end of the collection period. Concentrations of electrolytes, titratable acids, and urea are expressed in units of mmol/L, creatinine in mg/dl, Pco<sub>2</sub> in mmHg, osmolarity in mOsmol/L, creatinine clearance in ml/min; *n* = 8 to 10 animals per genotype. ND, not determined; NEA, net acid excretion.

<sup>b</sup>*P* < 0.001 versus wild-type.

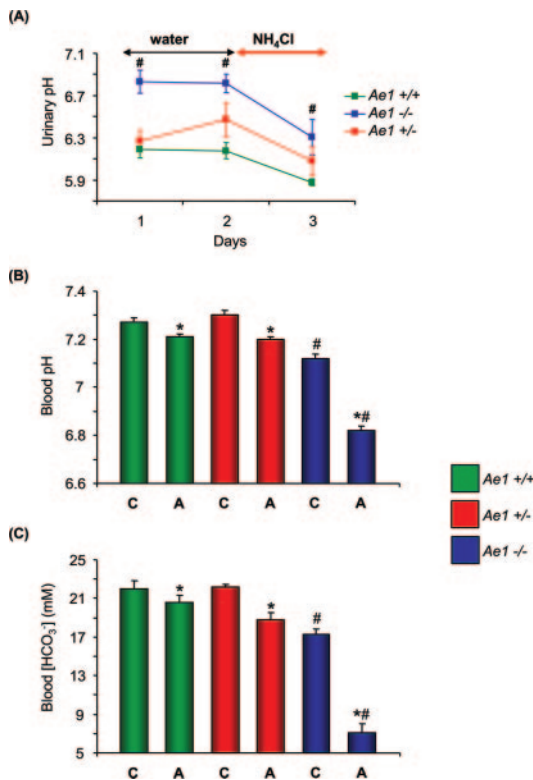
<sup>c</sup>*P* < 0.05 versus wild-type.

periods of acid loading led to death of AE1-deficient mice from apparent dehydration (weight loss) and massive hemolysis (dark urine as a result of hemoglobinuria; Supplementary Figure 3). The severe dehydration in acid-loaded AE1-deficient mice was reflected in elevated serum osmolarity and elevated serum concentrations of sodium and chloride. It is interesting that heterozygous mice failed to display a wild-type urinary concentration response to the oral acid load.

The acid-base parameters of wild-type and heterozygous mice did not differ in their responses to acid loading. Whereas

haploinsufficiency of AE1 did not affect basal systemic acid-base status, complete absence of AE1 led to hyperchloremic metabolic acidosis and to lethal intolerance of an oral acid load easily borne by heterozygotes.

Intercalated cells (IC) were maintained in the collecting ducts of 9-wk-old *Ae1*<sup>-/-</sup> mice. The proportion of cortical collecting duct (CCD) IC that were type A-IC (measured as pendrin-negative, H<sup>+</sup>-ATPase-positive cells) was 48% in 516 A-IC cells from two *Ae1*<sup>-/-</sup> kidneys and 48% in 715 A-IC from one wild-type kidney. The proportion of A-IC was also indistin-



**Figure 1.** Urine and blood gas analysis. Urinary pH and blood gas values of wild-type (*Ae1*<sup>+/+</sup>), heterozygous (*Ae1*<sup>+/-</sup>) and AE1-deficient (*Ae1*<sup>-/-</sup>) mice during metabolic cage experiments under control conditions for 2 d and after oral acid loading with 0.28 M NH<sub>4</sub>Cl for a third day. (A) Urinary pH in AE1-deficient mice was more alkaline than for other genotypes in all conditions but did acidify in response to the oral NH<sub>4</sub>Cl load. Blood pH (B) and [HCO<sub>3</sub><sup>-</sup>] concentration (C) in wild-type and heterozygous mice were indistinguishable in all conditions. However, blood pH and [HCO<sub>3</sub><sup>-</sup>] in *Ae1*<sup>-/-</sup> mice under control conditions revealed metabolic acidosis. This acidosis was severely exacerbated after NH<sub>4</sub>Cl loading (*n* = 8 mice for each genotype and time point). \**P* < 0.001 versus control conditions in the same genotype; #*P* < 0.001 versus wild-type.

guishable when assessed by apical versus diffuse localization of H<sup>+</sup>-ATPase (data not shown) and in kidneys of 5-wk-old mice.

We directly compared Cl<sup>-</sup>/HCO<sub>3</sub><sup>-</sup> exchanger activity in type A-IC of freshly isolated outer medullary collecting ducts from wild-type and AE1-deficient mice. Activity was measured in 2'-7'-bis-(2-carboxyethyl)-5-(and-6)-carboxyfluorescein, acetoxymethyl ester (BCECF)-loaded tubules as the initial rate of intracellular acidification upon chloride readdition to chloride-free bath in the presence of 5% CO<sub>2</sub>/25 mM HCO<sub>3</sub><sup>-</sup> (33). The substantial acidification that was observed in wild-type A-IC was inhibited approximately 60% by 400 nM diBA(5)C4 (34) and by 50 μM 4-4'-diisothiocyanatostilbene-2,2'-disulfonic acid (DIDS), consistent with basolateral membrane AE1-mediated Cl<sup>-</sup>/HCO<sub>3</sub><sup>-</sup> exchange activity (35–37). In contrast, A-IC from AE1-deficient mice exhibited a modestly decreased acidification rate of approximately 80% the wild-type value A-IC (wild-type  $-0.162 \pm 0.011$  units pH/min [*n* = 164 A-IC in six outer medullary collecting ducts

(OMCD) from 4 mice versus AE1 <sup>-/-</sup>  $-0.123 \pm 0.005$  units pH/min [*n* = 246 type A-IC in seven OMCD from four mice; Figure 2). However, acidification by *slc4a1*<sup>-/-</sup> A-IC was insensitive to diBA(5)C4 and showed reduced DIDS sensitivity (inhibition by approximately 20%), suggesting upregulation of a pharmacologically distinct anion exchange activity.

Among candidate anion exchangers that might contribute to this apparent compensation are Ae2/Slc4a2 (38–40), Ae3/slca3, Ae4/Slc4a9 (41), SLC26A7 (37), and (if atypically induced in A-IC) pendrin/Slc26a4 (42–45). Renal levels of AE2 and AE3 transcript variants (Figure 3A) as well as AE4 mRNA abundance (data not shown) were not elevated in *slc4a1*<sup>-/-</sup> mice (Figure 3A). Unchanged renal mRNA levels of AE2 variant transcripts were confirmed by RNase protection assay (data not shown). AE4 polypeptide levels were also unchanged in *slc4a1*<sup>-/-</sup> kidney, consistent with previously reported lack of sensitivity to acid loading in wild-type mice (41) (Figure 3B). However, levels of pendrin polypeptide were markedly decreased, consistent with the previously reported sensitivity of wild-type mouse kidney pendrin to acid loading (42,44,46). In addition, no consistent changes were found in *slc4a1*<sup>-/-</sup> kidney levels of mRNA encoding A-IC gene products Slc26A7 (37), aquaporin-6 (AQP6) (47), the K<sup>+</sup>/Cl<sup>-</sup> co-transporter KCC4 (48), and the renal anion exchanger Slc26A11 (49), whose nephron segment localization is unreported.

Basal 24-h urine volume in AE1-deficient mice was three- to four-fold higher than the wild-type and heterozygote values. Urine osmolality was correspondingly approximately 50% lower than in wild-type and heterozygous mice (Tables 1 and 2, Figure 4). The accompanying elevation in plasma osmolality in *slc4a1*<sup>-/-</sup> mice suggested a urinary concentrating defect. NH<sub>4</sub>Cl loading led to loss of 14% of initial body weight within 24 h and to greatly increased plasma osmolality without the accompanying increase in urine osmolality seen in wild-type mice (Figure 4). Basal creatinine clearance was similar in all genotypes but decreased greatly in AE1-deficient mice during acidosis (Tables 1 and 2), likely reflecting systemic volume depletion and dehydration.

We therefore examined expression and localization of AQP2 under control conditions. In AE1-null mice, AQP2 abundance was increased slightly in cortex and substantially in medulla, consistent with the observed systemic dehydration. Most inner medullary AQP2 was confined to cytoplasmic vesicular structures, in contrast to the predominantly luminal membrane localization in wild-type mice. In contrast, the less dramatically increased AQP2 of OMCD was normally localized to the luminal membrane in both *slc4a1*<sup>-/-</sup> and *slc4a1*<sup>+/+</sup> mice (Figure 5). Gross kidney morphology revealed reduced inner medullary mass, resembling that of chronic hydronephrosis. Thus, the urinary concentrating defect of AE1-null mice is associated with defective inner medullary AQP2 trafficking in the setting of inner medullary atrophy, with a compensatory increase in abundance of outer medullary AQP2.

Distal renal tubular acidosis is often associated with nephrocalcinosis and nephrolithiasis (3,15,23,50). The elevated fractional excretion of calcium in AE1-null mice is (as in humans) a risk factor for nephrocalcinosis (Tables 1 and 2, Figure 6). Urinary citrate is thought to protect against nephrocalcinosis,

Table 2. 24-h NH<sub>4</sub>Cl loading<sup>a</sup>

Genotype	<i>Ae1</i> <sup>+/+</sup>	<i>Ae1</i> <sup>+/-</sup>	<i>Ae1</i> <sup>-/-</sup>
<b>Blood</b>			
pH	7.21 ± 0.01	7.20 ± 0.01	6.82 ± 0.05 <sup>b</sup>
HCO <sub>3</sub> <sup>-</sup>	20.6 ± 0.7	18.8 ± 0.7	7.1 ± 0.9 <sup>b</sup>
Pco <sub>2</sub>	53.3 ± 1.8	49.6 ± 1.5	44.4 ± 3.1 <sup>c</sup>
K <sup>+</sup>	3.7 ± 0.2	4.0 ± 0.1	4.9 ± 0.3 <sup>b</sup>
Na <sup>+</sup>	147.9 ± 1.0	148.1 ± 1.0	156.4 ± 2.8 <sup>c</sup>
Cl <sup>-</sup>	117.0 ± 1.8	119.9 ± 2.0	133.0 ± 4.3 <sup>b</sup>
P <sub>i</sub>	2.0 ± 0.2	2.1 ± 0.2	3.4 ± 0.1 <sup>b</sup>
anion gap	10.1 ± 1.8	9.5 ± 2.3	16.3 ± 2.4 <sup>c</sup>
osmolarity	304.1 ± 1.2	302.4 ± 1.5	346.8 ± 3.4 <sup>b</sup>
creatinine	0.08 ± 0.02	0.10 ± 0.02	0.15 ± 0.02 <sup>c</sup>
urea	10.8 ± 1.6	ND	29.6 ± 5.3 <sup>b</sup>
<b>Urine</b>			
pH	6.07 ± 0.06	6.10 ± 0.10	6.47 ± 0.12 <sup>c</sup>
osmolarity	3302.5 ± 178.4	2388 ± 97.1	1176.5 ± 69.7 <sup>b</sup>
creatinine	83.1 ± 5.8	86.1 ± 13.1	33.2 ± 3.3 <sup>b</sup>
HCO <sub>3</sub> <sup>-</sup> /creatinine	0.0 ± 0.0	0.1 ± 0.0	0.1 ± 0.0
K <sup>+</sup> /creatinine	3.5 ± 0.6	3.1 ± 0.7	5.2 ± 1.0
Na <sup>+</sup> /creatinine	1.8 ± 0.4	1.4 ± 0.3	3.5 ± 0.7
Cl <sup>-</sup> /creatinine	7.6 ± 0.5	7.3 ± 1.0	6.7 ± 1.0
P <sub>i</sub> /creatinine	0.6 ± 0.1	0.5 ± 0.1	0.8 ± 0.2
NH <sub>4</sub> <sup>+</sup> /creatinine	2.1 ± 0.2	2.2 ± 0.3	2.2 ± 0.3
Ca <sup>2+</sup> /creatinine	0.04 ± 0.01	0.05 ± 0.02	0.06 ± 0.01
Mg <sup>2+</sup> /creatinine	0.19 ± 0.04	0.20 ± 0.08	0.26 ± 0.07
creatinine clearance	1.46 ± 0.40	0.80 ± 0.31	0.47 ± 0.06 <sup>b</sup>
FE Na <sup>+</sup> (%)	0.10 ± 0.03	0.09 ± 0.05	0.34 ± 0.08 <sup>c</sup>
FE K <sup>+</sup> (%)	6.78 ± 2.06	3.99 ± 1.05	16.72 ± 4.06
FE Cl <sup>-</sup> (%)	0.44 ± 0.10	0.58 ± 0.22	0.76 ± 0.15
FE Ca <sup>2+</sup> (%)	0.15 ± 0.06	0.24 ± 0.11	0.31 ± 0.07
FE Mg <sup>2+</sup> (%)	4.42 ± 1.69	2.41 ± 1.78	3.80 ± 1.08
FE P <sub>i</sub> (%)	1.98 ± 0.43	3.02 ± 1.01	3.52 ± 0.62
urinary anion gap	-202.3 ± 69.7	-223.2 ± 57.6	80.6 ± 61.8 <sup>c</sup>
titratable acids	37.8 ± 3.2	20.0 ± 4.7	16.1 ± 4.4 <sup>c</sup>
NEA	205.7 ± 9.5	176.6 ± 10.2	85.2 ± 5.2 <sup>b</sup>
NEA/Crea	2.6 ± 0.2	2.9 ± 0.5	2.7 ± 0.3

<sup>a</sup>Summary of blood and urine data after 24 h of oral NH<sub>4</sub>Cl loading. Urine was collected during 24 h in metabolic cages, and blood was sampled at the end of the collection period. Concentrations of electrolytes, titratable acids, and urea are expressed in units of mmol/L, creatinine in mg/dl, Pco<sub>2</sub> in mmHg, osmolarity in mOsmol/L, creatinine clearance in ml/min; *n* = 8 to 10 animals per genotype.

<sup>b</sup>*P* < 0.001 versus wild-type.

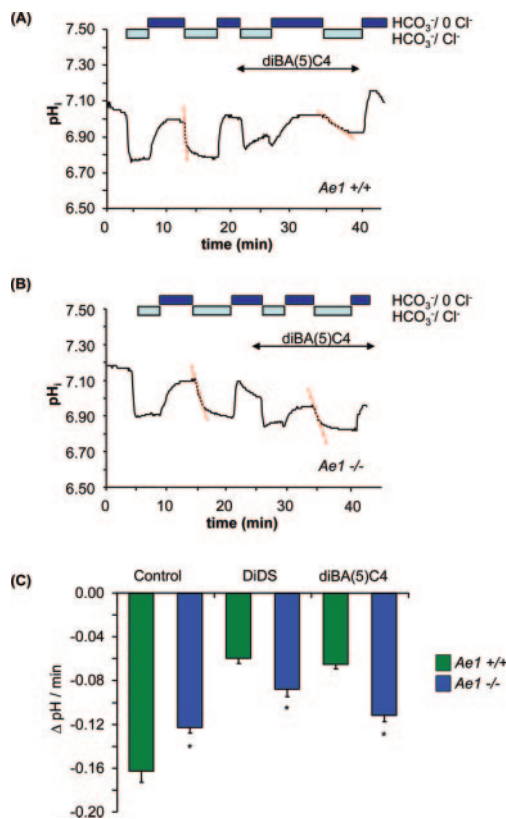
<sup>c</sup>*P* < 0.05 versus wild-type.

and its reduced excretion in acidosis is attributed to upregulation of the NaDC1 Na<sup>+</sup>/citrate co-transporter (51). The hypocitraturia of AE1-deficient mice (Table 1, Figure 6) constitutes an additional risk for nephrocalcinosis. Indeed, Van Kossa staining of *slc4a1*<sup>-/-</sup> kidneys demonstrated predominantly medullary calcium-phosphate deposits (Figure 6), a distribution typical of human dRTA. However, nephrolithiasis was not observed in *slc4a1*<sup>-/-</sup> mice.

## Discussion

Mutations in the human *SLC4A1/AE1* gene cause familial dRTA (1,15,17). The attendant hyperchloremic metabolic acido-

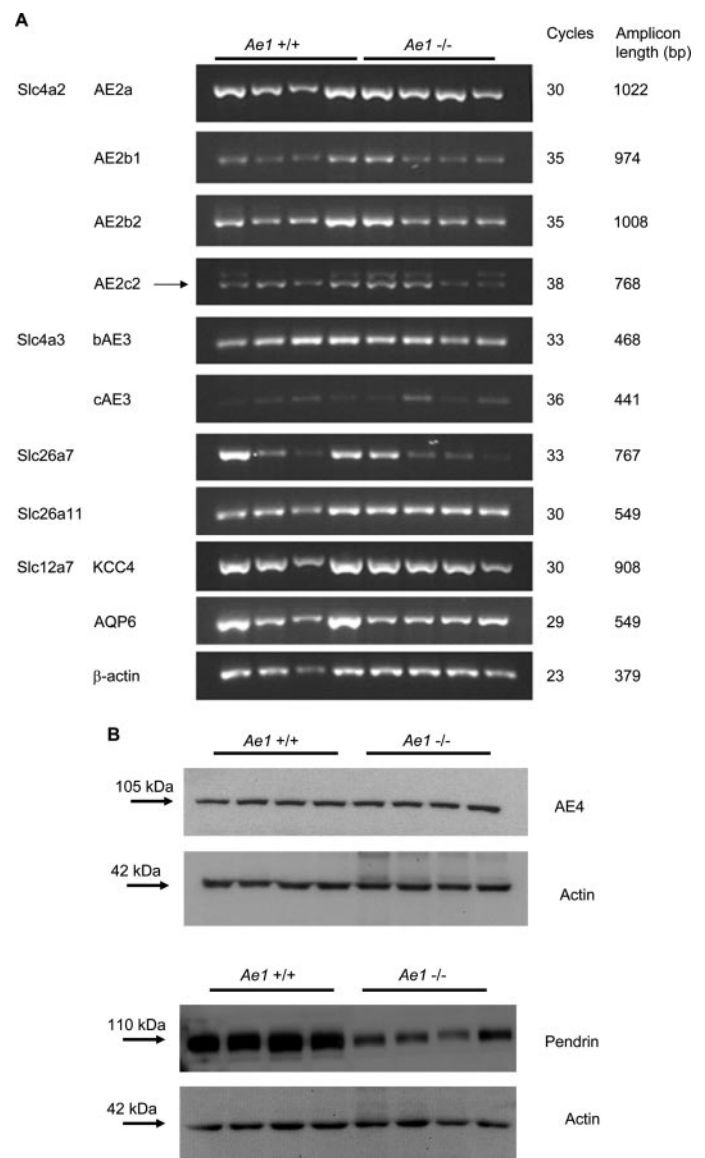
sis often presents as childhood growth retardation with increased risk for dehydration, accompanied by hypokalemia and nephrocalcinosis (1,3,15,17). The AE1-deficient mouse model examined here reproduces many features of the human disease and therefore represents the first mouse model of an inborn form of complete dRTA. A mouse that lacks the IC-specific vacuolar H<sup>+</sup>-ATPase B1 subunit (*Atp6v1b1*<sup>-/-</sup>) shows only incomplete dRTA, with oral acid loading required to unmask a modest urinary acidification defect (52). This mild phenotype contrasts with the severe and often early-onset complete dRTA of patients with mutations in the orthologous human *ATP6V1B1* gene (1,6,53).



**Figure 2.** Measurement of  $Cl^-/HCO_3^-$  exchanger activity in single type A intercalated cells (A-IC) in isolated outer medullary collecting duct (OMCD). (A and B) Representative original tracings of intracellular pH measurements (BCECF ratio fluorimetry) in the presence of  $HCO_3^-/CO_2$  and the alternating presence or absence of extracellular chloride. Addition of chloride caused a rapid acidification as a result of activation of  $Cl^-/HCO_3^-$  exchange activity. The intracellular acidification rate was reduced by 400 nM of the AE1 inhibitor diBA(5)C4. Intracellular acidification was partially reduced in A-IC from *Ae1*<sup>-/-</sup> mice, and the remaining activity was insensitive to diBA(5)C4. (C) Summarized data from all experiments using the inhibitors diBA(5)C4 (400 nM) or DIDS (50  $\mu$ M; *n* = 4 to 5 mice with five to six OMCD for each condition and 100 to 150 A-IC each). \**P* < 0.001 versus wild-type.

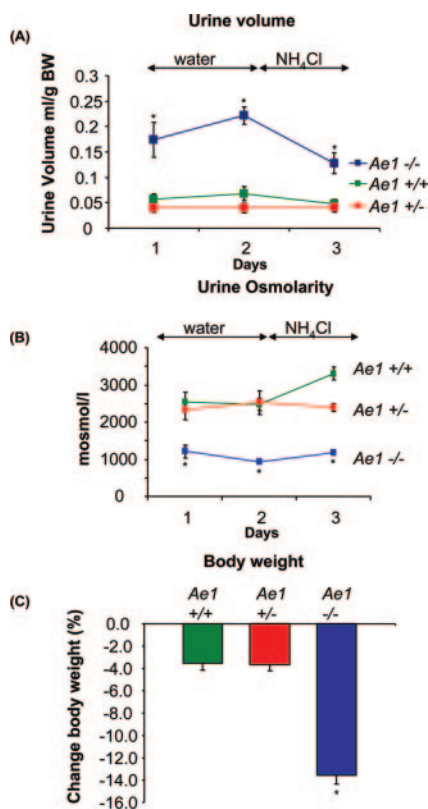
A-IC of the collecting duct are believed to complete the reabsorption of filtered bicarbonate in excess of the reabsorptive capacities of the proximal tubule and the thick ascending limb (2). The absence of bicarbonaturia in the face of the metabolic acidosis of AE1-deficient mice suggests (at least in the presence of reduced blood bicarbonate levels) that AE1 is not critical for reabsorption of filtered bicarbonate. Thus, the main function of A-IC may be rather to replenish body bicarbonate that is newly generated by cytosolic carbonic anhydrase and to release it into blood *via* AE1 and perhaps other basolateral anion exchangers.

Although of considerable magnitude, the residual activity of alternative chloride-bicarbonate exchange activity in A-IC of the *slc4a1*<sup>-/-</sup> mouse apparently does not suffice to support adequate bicarbonate release into the blood. The compensatory

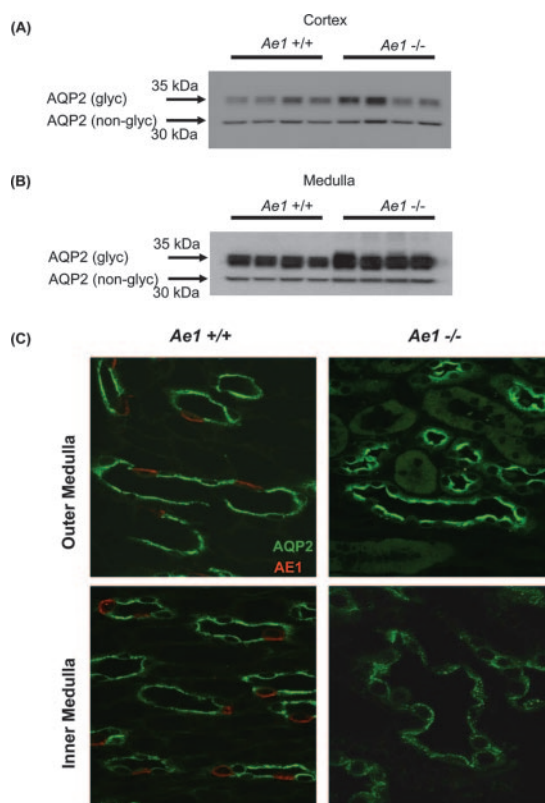


**Figure 3.** Expression of anion exchangers in kidney. (A) reverse transcriptase-PCR with primers detecting specific isoforms of several anion exchanger mRNA as well as KCC4 and aquaporin-6 (AQP6), two transport proteins implicated in A-IC function, did not reveal consistent changes in mRNA abundance in kidneys from *Ae1*<sup>-/-</sup> mice.  $\beta$ -Actin was used as a reference transcript. (B) Immunoblot showed that abundance of AE4/Slc4a9 polypeptide was not changed in kidneys from *Ae1*<sup>-/-</sup> mice. The abundance of pendrin/Slc26a4 anion exchanger polypeptide was strongly reduced in kidneys from *Ae1*<sup>-/-</sup> mice.

anion exchange activity was measured in unperfused OMCD tubules at isotonic bath of pH 7.4 and therefore may not optimally reflect conditions in the acidotic, moderately hypertonic outer medulla of the *Ae1*<sup>-/-</sup> mouse. The molecular identity, regulation, and physiologic function of the alternate anion exchanger(s) remain to be elucidated. Potential candidate anion exchangers reported previously in A-IC basolateral membrane include AE4/Slc4a9 and Slc26a7. Although AE4 has been local-



**Figure 4.** Severe urinary concentrating defect in *Ae1*<sup>-/-</sup> mice. (A) Under control conditions, urine volume normalized to body weight was approximately three-fold elevated in AE1-deficient mice and remained two-fold elevated during the severe dehydration produced by acid loading (see serum osmolarity, Table 2). (B) Urine osmolarity was much lower in AE1-deficient mice and did not increase during acid load-induced dehydration. (C) The acid load-induced loss of body weight in *Ae1*<sup>-/-</sup> mice was greatly increased. \**P* < 0.001 versus wild-type.

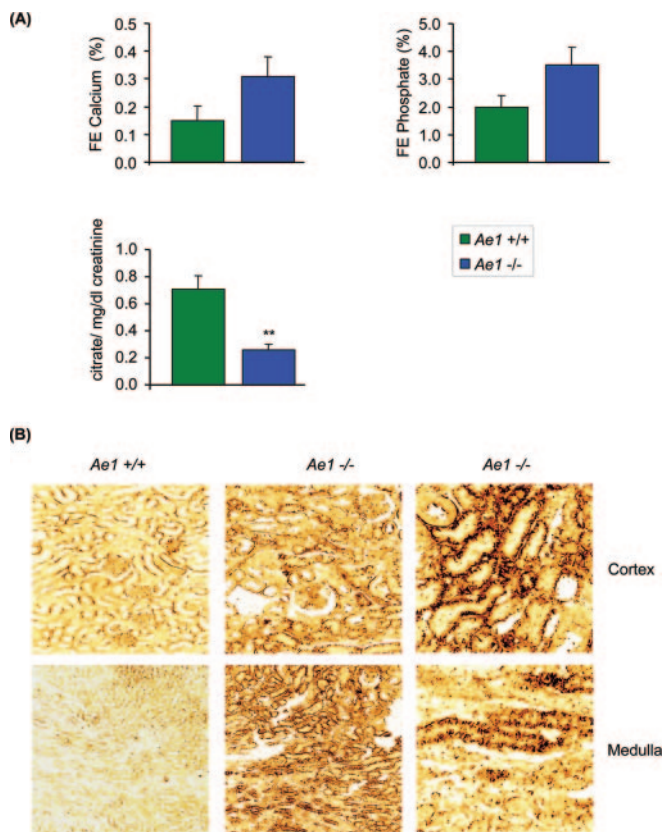


**Figure 5.** Dysregulation of AQP2 water channel in kidney of AE1-deficient mice. Immunoblots reveal increased AQP2 protein abundance in kidney cortex (A) and medulla (B) of *Ae1*<sup>-/-</sup> mice in control conditions. (C) Confocal immunofluorescence microscopy of outer medulla (top) shows increased AQP2 immunostaining (green) with strong apical localization in AE1-deficient mice. In contrast, in the inner medulla of *Ae1*<sup>-/-</sup> kidneys (bottom), AQP2 staining was confined mainly to intracellular vesicular structures, whereas AQP2 in wild-type kidney was predominantly luminal. AE1 staining is in red. Magnification,  $\times 600$ .

ized to the basolateral membrane of rat and mouse A-IC of the CCD (41), it is an apical protein in type B IC of the rabbit CCD (54) and minimally expressed in OMCD. The Slc26a7 anion exchanger has been reported in the basolateral membrane of A-IC of rat OMCD (37) and in an incompletely defined cell subset in human OMCD (55). Slc26a7-mediated Cl<sup>-</sup>/HCO<sub>3</sub><sup>-</sup> exchange is activated by hypertonicity (37) (L. Jiang and S.L.A., unpublished observations, 2006), likely in part by mobilization from intracellular stores to the basolateral membrane (56). Slc26a7 is functionally absent in Brattleboro rats and rescued by their treatment with DDAVP (57). The reduced urinary osmolarity of the *slc4a1*<sup>-/-</sup> mouse suggests that any contribution of Slc26a7 to the residual Cl<sup>-</sup>/HCO<sub>3</sub><sup>-</sup> activity of the superfused *slc4a1*<sup>-/-</sup> OMCD might be similarly reduced. However, the localization and the transport activity of mouse Slc26a7 remain controversial, because the protein has been localized with different antibodies to the proximal tubule rather than to A-IC (58) and has been reported to mediate pH-gated Cl<sup>-</sup> channel activity rather than Cl<sup>-</sup>/HCO<sub>3</sub><sup>-</sup> exchange (59).

dRTA as a result of mutations in *SLC4A1* can be inherited as an autosomal recessive or an autosomal dominant disease.

dRTA in white patients is generally transmitted in an autosomal dominant pattern (13,15,16), whereas patients of Southeast Asian and Melanesian origin more often present recessive or compound heterozygote forms of the disease (11,12,60). Several mechanisms have been proposed to explain the dominant and recessive forms of dRTA. kAE1 forms functional homodimers or hetero-oligomers, and oligomerization of a dominant negative mutant AE1 with a wild-type polypeptide in the heterozygote A-IC likely explains some dominant disease. The mutant/wild-type heterodimer either fails to traffic to the cell surface or, in some cases, traffics normally but may not function normally at the cell surface (18,61,62). Another subset of kAE1 mutant polypeptides seems to cause dominant dRTA by inappropriate targeting to A-IC apical membrane, with resultant apical bicarbonate secretion likely short-circuiting luminal acid secretion (19,20,63). These interpretations of human dRTA are consistent with the normal renal function displayed by mice that are heterozygous for the *Ae1* null allele. The data strengthen the hypothesis that haploinsufficiency of AE1 does not explain the



**Figure 6.** Nephrocalcinosis in kidneys of *Ae1*<sup>-/-</sup> mice. (A) The fractional excretion (FE %) of calcium and phosphate was increased in acid-loaded *Ae1*-deficient mice, whereas citrate excretion was reduced. \*\**P* < 0.001. (B) Van Kossa staining for calcium phosphate deposits (brown-black) detected tubular calcification predominantly in the medulla of *Ae1*<sup>-/-</sup> mice (bottom middle and right). Magnifications: ×100 (left), ×200 (middle), and ×400 (right).

pathophysiology of dominant forms of human dRTA. The reason for the mild concentrating defect that is observed in the heterozygous animals during acid loading remains to be elucidated. This concentrating defect is the only abnormality yet detected in heterozygous mice.

*Ae1*-deficient mice appear as a model for the hydrops fetalis, hemolytic anemia, and dRTA that are exhibited by children who completely lack eAE1 as a result of homozygosity for the autosomal recessive *AE1* mutant V488M, Band 3 Coimbra (24). The severe disease in *Ae1*-deficient mice also resembles the hydrops, anemia, and acidosis in Japanese black cattle that are homozygous for *AE1* mutations that correspond to human *AE1* 646X (64). However, most autosomal recessive dRTA in humans is caused by *AE1* mutations that are compatible with normal eAE1 abundance and near-normal eAE1 anion transport function in erythrocytes. These conditional loss-of-function mutations in human *AE1* are functionally rescued in erythrocytes by the tissue-specific expression of the *AE1*-binding protein glycophorin A, which likely functions as both trafficking chaperon and plasmalemmal subunit of eAE1 (10,14).

Patients who have inborn forms of dRTA are often prone to

dehydration as a consequence of inappropriate renal water loss (3,15,17,21–23). *Ae1*-deficient mice exhibited a urinary concentrating defect, evident as elevated serum osmolality with inappropriately low urine osmolality. These mice were unable to increase urine osmolality and decrease urine output during 24-h  $\text{NH}_4\text{Cl}$  loading, leading to severe dehydration as reflected in further elevation of serum osmolality. This urinary concentrating defect is likely attributable to the intracellular retention of AQP2 water channel polypeptides in the epithelial cells of the inner medullary collecting duct. However, the increased luminal AQP2 expression and total AQP2 abundance in the outer medulla suggest intact regulation by the antidiuretic hormone vasopressin and likely represent compensatory mechanisms. AQP2 in rat inner medullary collecting duct is inhibited by activation of luminal calcium sensing receptor (65). The hypercalciuria of *AE1*-deficient mice may reduce luminal AQP2 expression *via* stimulation of luminal calcium sensing receptor. Thus, the mild diabetes insipidus of the *AE1*-deficient mouse kidney may be associated with defective AQP2 regulation that is spatially restricted to the inner medulla.

Nephrocalcinosis and nephrolithiasis can be associated with all forms of complete and incomplete dRTA and often constitute the initial clinical presentation of disease (3,15,23,50). The associated hypercalciuria may reflect osteomalacia or decreased function and/or expression of renal calcium transporting proteins in the presence of metabolic acidosis (66). *Ae1*-deficient mice similarly exhibited hypercalciuria, hypocitraturia, and hyperphosphaturia, and nephrocalcinosis in a predominantly medullary distribution (3,22,51,63,67,68). We additionally noted higher serum urea levels in mice that lacked *AE1*, suggesting mild renal insufficiency. It remains to be clarified whether the renal failure, which likely has a prerenal component, is secondary to chronic metabolic acidosis, hydro-nephrosis, nephrocalcinosis, or potentially all of these. Conversely, the mild, chronic renal insufficiency may contribute to the metabolic acidosis.

## Conclusion

*Ae1*-deficient mice recapitulate key renal features of inherited forms of dRTA as found in patients with mutations in either *AE1* or the *ATP6V1B1* and *ATP6V0A4* subunits of the vacuolar  $\text{H}^+$ -ATPase. These mice will allow investigation of the underlying mechanisms of secondary manifestations of the disease such as nephrocalcinosis and dehydration as a result of AQP2 dysregulation. The mice will also allow study of the renal tubular adaptations to congenital dRTA, including upregulation of other bicarbonate transport proteins. *Ae1*-deficient *slc4a1*<sup>-/-</sup> mice thus demonstrate that renal kAE1 is critical for systemic acid-base homeostasis. kAE1 mediates the essential step in bicarbonate release from acid-secretory IC into blood.

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## Disclosures

None.

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