

# Mutations in the beta-subunit of the epithelial Na<sup>+</sup> channel in patients with a cystic fibrosis-like syndrome

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**Cystic fibrosis (CF) is an autosomal recessive disorder of Cl<sup>-</sup> and Na<sup>+</sup> transport. The vast majority of CF patients have deleterious mutations in an epithelial Cl<sup>-</sup> channel called the CF transmembrane conductance regulator (CFTR). In contrast, defects in the epithelial Na<sup>+</sup> channel (*SCNN1*) have been associated with phenotypes dominated by renal disease (systemic pseudohypoaldosteronism type I and Liddle syndrome). We report two non-classic CF patients without *CFTR* mutations who have novel deleterious mutations in the  $\beta$ -subunits of *SCNN1* in the absence of overt renal disease.**

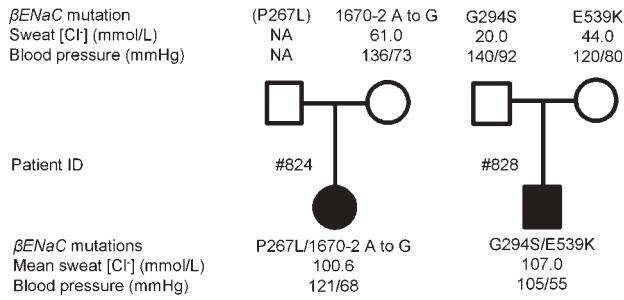
## INTRODUCTION

Cystic fibrosis (CF) is an autosomal recessive disorder that affects Cl<sup>-</sup> and Na<sup>+</sup> transport in the sweat gland, respiratory tract, pancreas and male reproductive system (1). Linkage and positional cloning identified the CF transmembrane conductance regulator (CFTR), a cAMP-activated chloride channel as responsible for CF. The Na<sup>+</sup> transport defect in CF patients is a consequence of the loss of functional interaction between the CFTR and the epithelial sodium channel (ENaC) (1). Several unusual patients with clinical features of CF, including an ion transport defect in the sweat gland, have been reported without mutations in the coding regions of *CFTR* (2–5). In familial cases, linkage analysis has excluded the involvement of *CFTR* (2,4). The presence of a Na<sup>+</sup> transport defect in CF patients and the functional interaction between CFTR and ENaC (6,7) prompted us to explore whether defects in the genes encoding  $\alpha$ ,  $\beta$  or  $\gamma$  ENaC (*SCNN1A*, *B* or *G*) account for features of CF (sweat Cl<sup>-</sup> greater than 60 mmol/l and CF-like pulmonary infections) in 20 non-classic CF patients without mutations in the coding regions of *CFTR* (4,8).

## RESULTS AND DISCUSSION

Sequencing of the exons and the flanking introns of the genes encoding the  $\alpha$ ,  $\beta$  and  $\gamma$ -subunits of ENaC in 20 non-classic CF patients without *CFTR* mutations identified six novel sequence changes. Five are predicted to cause amino acid changes: R181W in *SCNN1A* and S82C, P267L, G294S and E539K in *SCNN1B*, whereas the sixth changes the highly conserved penultimate A in the 3' splice site of *SCNN1B* intron 12 (1670–2 A to G). Four previously reported amino acid polymorphisms in *SCNN1A* at frequencies consistent with those reported for the general population were also found. Four of the five novel *SCNN1B* mutations were found in two patients (824 and 828). Pedigree analysis confirmed that each mutation was inherited on separate genes (Fig. 1). The three amino acids mutated in patients 824 and 828 are completely conserved in ENaC orthologues and paralogues in human, rabbit, mouse and rat (Fig. 2). These substitutions were not found in ethnically matched control alleles (P267L in 256 alleles, G294S in 324 alleles and E539K in 254 alleles) (data not shown). Together, these data indicate that the *SCNN1B* mutations discovered in these patients are likely to be deleterious.

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**Figure 1.** Pedigrees of patients with two novel *SCNNIB* mutations. Parentheses indicate the individual with an inferred genotype. Solid symbols denote individuals who presented with features of CF (elevated sweat  $\text{Cl}^-$  concentration and recurrent lung infections). Blood pressure is the most recent reading obtained in a medical setting. NA, not available.

RT-PCR of nasal epithelial RNA from patient 824 revealed that the 1670-2 A to G mutation results in two stable *SCNNIB* transcripts (Fig. 3). The first retains 83 nucleotides from the 3' end of intron 12 and is predicted to alter the amino acid sequence following codon 514 and terminate after the addition of 188 novel residues. The second transcript lacks the first 33 nucleotides of *SCNNIB* exon 13 leading to a deletion of 11 amino acids that precede the second transmembrane domain. These results confirm that 1670-2 A to G causes aberrant splicing leading to mRNA transcripts with substantial alterations in sequence.

The effect of the three missense mutations on ENaC function was investigated in *Xenopus laevis* oocytes (Fig. 4). Oocytes injected with wild-type  $\alpha$ -,  $\beta$ - and  $\gamma$ -ENaC subunit RNAs displayed inwardly rectifying  $\text{Na}^+$  currents [mean  $-0.89 \pm 0.145 \mu\text{A}$  (SEM) at  $-100 \text{ mV}$ ;  $n = 40$ ; nine batches] that were inhibited by amiloride, as described previously (9). Oocytes injected with E539K  $\beta$ ENaC instead of wild-type  $\beta$ ENaC generated  $\text{Na}^+$  currents ( $-0.26 \pm 0.076 \mu\text{A}$ ;  $n = 12$ ; six batches) that were significantly lower ( $P < 0.001$ ) than those of wild-type. Similarly, co-expression of P267L  $\beta$ ENaC with wild-type  $\alpha$ - and  $\gamma$ -ENaC was associated with  $\text{Na}^+$  currents ( $-0.33 \pm 0.09 \mu\text{A}$ ;  $n = 14$ ; four batches) that were significantly reduced ( $P = 0.002$ ) compared with wild-type. In contrast, the co-expression of G294S  $\beta$ ENaC produced  $\text{Na}^+$  currents ( $-2.08 \pm 0.383 \mu\text{A}$ ;  $n = 27$ ; six batches) that were significantly higher ( $P = 0.004$ ) than those of wild-type. Thus, each of the missense *SCNNIB* mutations found in patients 824 and 828 is associated with abnormal function.

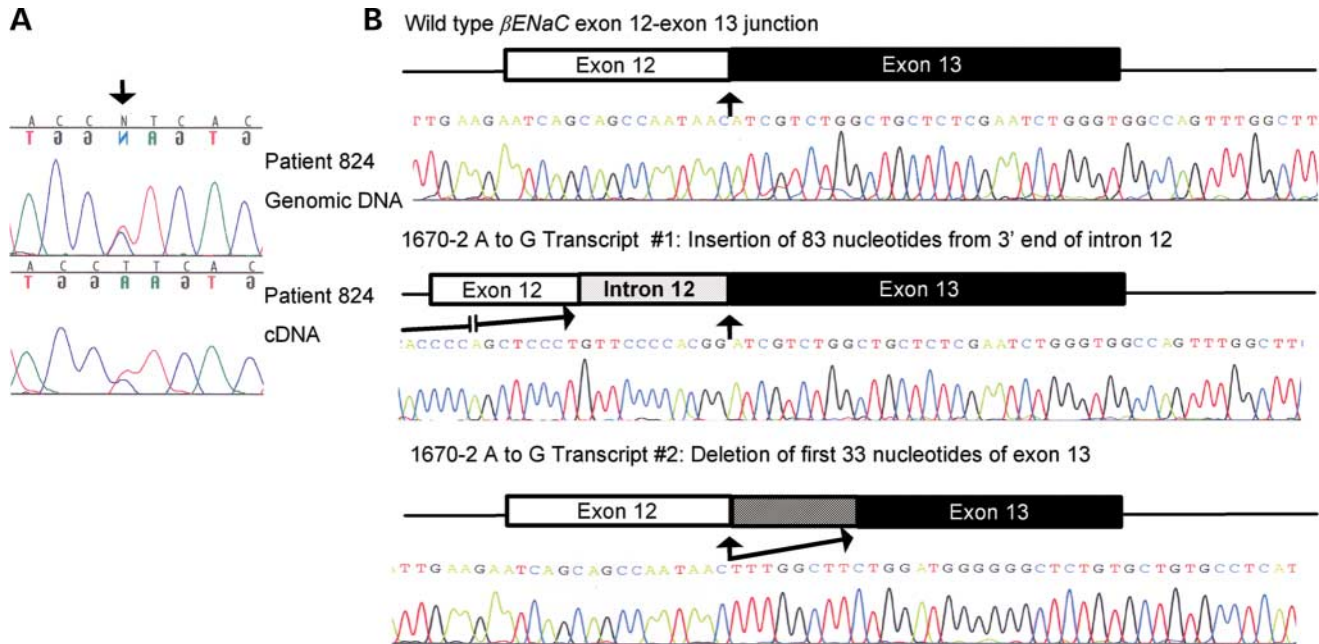
Mutations that cause loss of ENaC function have been associated with systemic pseudohypoaldosteronism type I (PHA I) (10), an autosomal recessive renal salt-wasting disorder, whereas mutations that cause gain of ENaC function occur in patients with Liddle syndrome, an autosomal dominant form of renal hypertension (11,12). Aldosterone and renin, hormones that regulate ENaC activity in the kidney, are elevated in PHA I patients and reduced in Liddle syndrome patients. In addition, urinary, salivary and sweat  $\text{Na}^+$  and  $\text{Cl}^-$  concentrations are elevated in PHA I patients (13). Patient 828 had an episode of dehydration at 6 months of age associated with transient elevation of aldosterone and renin levels; however, neither patient 828 nor patient 824 has abnormal



**Figure 2.** Amino acid sequence alignment of ENaC orthologues and paralogues. Human (NP\_001029, NP\_000327, NP\_001030 and NP\_002969), rabbit (O97741, O97742 and Q28738), mouse (NP\_035454, NP\_035455 and NP\_035456) and rat (NP\_113736, NP\_036780 and NP\_058742)  $\alpha$ ,  $\beta$ ,  $\gamma$  and  $\delta$ ENaC sequences were obtained from GenBank. Residues that are conserved among all orthologues and paralogues are highlighted in black. Amino acids found mutated in this study are indicated in bold. The alignment was generated with Clustal W.

renin or aldosterone levels (Table 1). Both patients have normal concentrations of  $\text{Na}^+$  and  $\text{Cl}^-$  in saliva, urine, and serum, and normal blood pressure measurements (Table 1 and Fig. 1). In contrast, they each have elevated sweat  $\text{Cl}^-$  concentrations and have had multiple pulmonary infections. Elevated sweat  $\text{Cl}^-$  concentrations and pulmonary infections with bacteria that commonly infect the lungs of CF patients (e.g. *Pseudomonas*) have been reported in PHA I patients early in life ( $<5$  years) (14,15). However, neither patient has an excessive volume of airway surface liquid manifest as persistent watery rhinorrhea, which is characteristic of PHA I patients (13). Furthermore, PHA I and Liddle syndrome patients have abnormal amiloride-sensitive  $\text{Na}^+$  absorption across nasal epithelium (16). Measurement of ion transport across the nasal epithelium of patient 824 revealed normal amiloride-sensitive  $\text{Na}^+$  absorption [mean basal potential difference:  $-23.8 \pm 3.05 \text{ mV}$  (SEM);  $n = 2$  and mean percent inhibition with amiloride:  $60\% \pm 7.6$ ;  $n = 4$ ] and robust CFTR-mediated  $\text{Cl}^-$  conductance (mean isoproterenol response:  $-39.8 \pm 7.19 \text{ mV}$ ;  $n = 4$ ). The parents of patient 828 declined nasal potential difference (NPD) measurement testing.

These patients demonstrate that deleterious *SCNNIB* mutations can produce symptoms in the lungs and sweat gland without the renal features of PHA I and Liddle syndrome. Each mutation in patient 824 is predicted to reduce but not to eliminate  $\beta$ ENaC function. Preservation of some ENaC function may allow sufficient  $\text{Na}^+$  reabsorption in the kidney, so that the clinical features of PHA I are avoided when salt is abundant in the diet. Indeed, transgenic mice expressing low levels of  $\beta$ ENaC appear clinically normal, but when placed under salt restriction exhibit acute PHA I symptoms (17). Partial  $\beta$ ENaC function could also explain the normal basal ENaC function in the nasal respiratory epithelia and normal salivary electrolyte concentration. However, the presence of pulmonary infection and elevated sweat  $\text{Cl}^-$  suggest that the level of residual  $\beta$ ENaC function in patient 824 is not sufficient for proper sodium transport in the airway and sweat gland. The concept that decreased ENaC function can lead to lung infections is supported by



**Figure 3.** RT-PCR analysis of *SCNNIB* 1670-2 A to G. (A) To determine whether the transcript resulting from the 1670-2 A to G mutation was stable, RT-PCR was performed using M13 tagged primers designed to amplify the region surrounding the P267L mutation in patient 824. Sequencing of the RT-PCR product revealed biallelic expression at *SCNNIB* nucleotide 927 (indicated by arrow), as seen in genomic DNA from the same individual. This confirms that the transcript resulting from the 1670-2 A to G mutation is stable. (B) To analyse the exon 12/exon 13 junction in transcripts resulting from the 1670-2 A to G mutation, RT-PCR was performed with primers placed in *SCNNIB* exons 12 and 13. RT-PCR products were cloned into pCR 2.1 TOPO (Invitrogen) and sequenced. Two different mutant transcripts were present. The first contains an insertion of 83 nucleotides from the 3' end of *SCNNIB* intron 12. This is predicted to result in frameshift and insertion of 188 novel amino acids after *SCNNIB* exon 12. The second transcript maintains frame, but lacks the first 33 nucleotides of *SCNNIB* exon 13. This is predicted to cause deletion of 11 amino acids preceding the second transmembrane domain.

the pulmonary phenotype of young PHA I patients. In contrast, the two mutations carried by patient 828 (G294S and E539K) each appear to affect ENaC function differently. E539K was associated with reduction of ENaC function in *Xenopus* oocytes, whereas G294S was associated with increased function. As noted earlier, gain of function in ENaC has been associated with hypertension in Liddle syndrome. The father of patient 828, who carries G294S, is hypertensive (Fig. 1). Unfortunately, further characterization of the father's phenotype has not been possible. Intriguingly, the combination of gain and loss of function *SCNNIB* mutations in patient 828 produces a phenotype similar to that of patient 824. Co-expression of the  $\beta$ ENaC mutants carried by patient 828 (G294S and E539K) produced  $\text{Na}^+$  current profiles in oocytes that were very similar to those exhibited oocytes expressing the gain of function mutant (G294S) alone (data not shown). Murine studies reveal that increased  $\beta$ ENaC function can lead to pulmonary disease. Lung-specific over-expression of the  $\beta$  (but not  $\alpha$  or  $\gamma$ ) ENaC subunit increased  $\text{Na}^+$  absorption in the airway and produced CF-like lung disease in transgenic mice in the absence of a  $\text{Cl}^-$  transport abnormality (18). In summary, these patients illustrate that the spectrum of phenotypes associated with mutations in *SCNNIB* is broader than previously recognized. Overlap of this spectrum with features of CF indicates that genetic heterogeneity should be considered in non-classic CF patients without *CFTR* mutations.

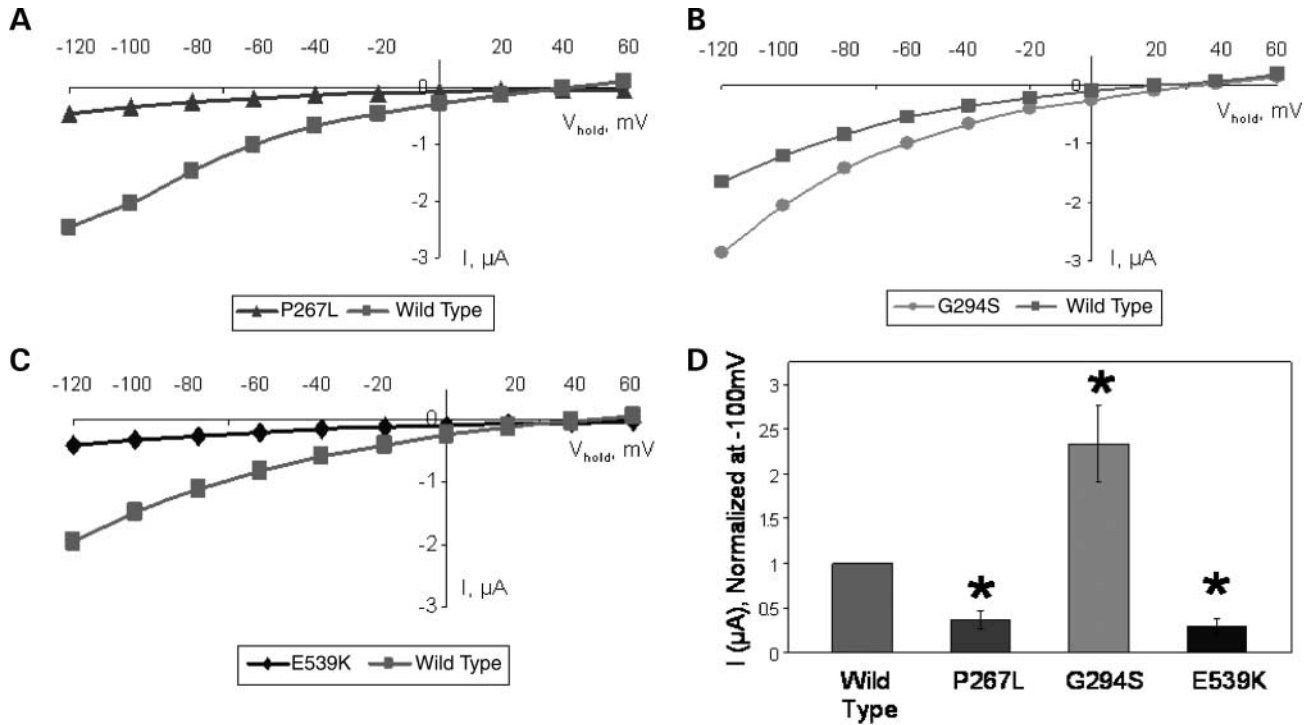
## MATERIALS AND METHODS

### Patient population

One hundred and fifty-eight patients with features of non-classic CF have been studied for mutations in coding regions of the *CFTR* gene (4,8). Of the 73 patients without *CFTR* mutations, 20 had elevated sweat chloride (>60 mmol/l) and pulmonary disease defined by one or more of the following: sputum cultures positive for *Pseudomonas aeruginosa*, chronic productive cough, documented pneumonia, reactive airway disease, abnormal pulmonary function tests, abnormal chest X-ray and/or CT indicating bronchiectasis, infiltrates or blebs, chronic sinusitis and/or nasal polyposis.

### ENaC sequencing and genotyping

Exons and bordering introns of *SCNNIA*, *SCNNIB* and *SCNNIG* were amplified from genomic DNA using polymerase chain reactions with M13 labelled primers (primer sequences may be obtained from the corresponding author). PCR products were purified using the QIAquick PCR purification kit (Qiagen, Valencia, CA, USA) and sequenced using ABI PRISM BigDye Primer Sequencing kit (Applied Biosystems, Foster City, CA, USA). The resulting sequences were analysed bidirectionally by capillary electrophoresis (ABI3100 Genetic Analyzer, Applied Biosystems)



**Figure 4.** Functional studies of *SCNN1B* mutants expressed in *Xenopus* oocytes. The I–V relationship ( $t = 182$  ms) of the amiloride-sensitive currents for oocytes expressing (A) wild-type ENaC (squares) and  $\beta$ ENaC P267L (triangles); (B) wild-type (squares) and  $\beta$ ENaC G294S (circles); (C) wild-type (squares) and  $\beta$ ENaC E539K (diamonds) is shown. Each graph compares the amiloride-sensitive currents from a representative oocyte injected with RNA encoding one of the three  $\beta$ ENaC mutants with wild-type  $\alpha$ - and  $\gamma$ -ENaC against the amiloride-sensitive currents obtained from a paired, wild-type  $\alpha$ -,  $\beta$ - and  $\gamma$ -ENaC-injected oocyte from the same batch. Currents resulting from expression of the three *SCNN1B* mutants with wild-type  $\alpha$ - and  $\gamma$ -ENaC were studied in a masked fashion. These currents reverse at potentials more depolarized than +20 mV, distinguishing them from non-specific background currents. (D) Histogram of normalized mean currents ( $\pm$  SEM) at a membrane potential of  $-100$  mV for oocytes injected with RNA encoding wild-type  $\alpha$ - and  $\gamma$ -ENaC plus wild-type  $\beta$ ENaC,  $n = 40$  from nine batches;  $\beta$ ENaC P267L,  $n = 14$  from four batches;  $\beta$ ENaC G294S,  $n = 27$  from six batches; or  $\beta$ ENaC E539K,  $n = 12$  from six batches. Asterisks indicate  $P$ -values less than 0.005 when compared with wild-type.

**Table 1.** Clinical and laboratory findings in organ systems that are affected in CF, PHA I or Liddle syndrome

Patient	Presenting symptoms	Sweat gland <sup>a</sup>	Pulmonary <sup>b</sup>	GI	GU	Renal <sup>c</sup>	Salivary gland
824 23-year-old- African-American	Sinusitis, mild lung disease	Mean sweat $\text{Cl}^-$ : 100.6 mmol/l (<60) ( $n = 5$ )	FEV <sub>1</sub> : 73% predicted <i>Pseudomonas aeruginosa</i> [23 years old] <i>Staphylococcus aureus</i> [23 years old]	Pancreatic sufficient	Fertile	Normal urine and serum electrolytes; serum aldosterone: 21.8 ng/dl (4–31); renin activity: 2.4 ng/ml/h (0.5–4)	Normal salivary electrolytes
828 7-year-old-Caucasian	Hyponatraemic dehydration with elevated aldosterone and renin at 6 months	Mean sweat $\text{Cl}^-$ : 107.0 mmol/l (<60) ( $n = 4$ )	FEV <sub>1</sub> : 24% Predicted <i>Pseudomonas fluorescens</i> [4 years old] <i>Haemophilus parainfluenza</i> [5 years old]	Pancreatic sufficient	Vas deferens present	Normal urine and serum electrolytes; serum aldosterone: 32.5 ng/dl (2–70); renin activity: 1.45 ng/ml/h (<3.7)	Normal salivary electrolytes

<sup>a</sup>Although  $\text{Na}^+$  and  $\text{Cl}^-$  concentrations are elevated in electrolyte reabsorption disorders such as CF, only  $\text{Cl}^-$  concentration is measured according to clinical guidelines.

<sup>b</sup>Organisms listed were cultured from sputum, and age at most recent positive culture is shown in brackets.

<sup>c</sup>Normal, age-corrected values from the laboratories where the tests were performed are indicated in parentheses.

and the Sequencher analysis program (Gene Codes, Ann Arbor, MI, USA). To determine whether *SCNN1B* missense mutations P267L, G294S and E539K were polymorphisms, ethnically matched control samples were analysed. Briefly,

the regions containing these mutations were amplified from genomic DNA using PCR and screened by allele-specific oligonucleotide hybridization or unidirectional sequencing.

### SCNN1B RNA analysis

Nasal epithelial cells were collected from patient 824. RNA was extracted with the use of RNasee (TelTest, Friendswood, TX, USA) following the manufacturer's protocol. cDNA was synthesized using reverse transcriptase (StrataScript reverse transcriptase, Stratagene, La Jolla, CA, USA) according to manufacturer's protocol. To confirm the presence of transcript from each allele, *SCNN1B*-specific primers were designed to amplify the region containing the mutation P267L in patient 824 by RT-PCR. Resulting products were sequenced as described earlier. The exons 12 and 13 junction was studied in transcripts with the 1670–2 A to G mutation by RT-PCR followed by cloning into pCR2.1 TOPO (Invitrogen, Carlsbad, CA, USA) and dye terminator sequencing (ABI PRISM BigDye Terminator Sequencing Kit, Applied Biosystems).

### Preparation of ENaC mRNA and injection into *Xenopus* oocytes

Plasmids containing wild-type human cDNA encoding the three subunits of the ENaC were obtained from Dr Dale Benos (University of Alabama). Mutations in the  $\beta$ -subunit were generated using the Transformer Site-Directed Mutagenesis Kit (BD Clontech, Mountain View, CA, USA). RNA was synthesized using *in vitro* transcription and capping kits (mMessage mMachine; Ambion, Austin, TX, USA) according to manufacturer's instructions, aliquoted and stored at  $-80^{\circ}\text{C}$ . Mature female *X. laevis* were anaesthetized in 0.2% ethyl *m*-amino benzoate (Tricaine; Sigma Chemical, St Louis, MO, USA) and then placed on ice prior to partial ovariectomy and manual isolation of Stage V and Stage VI oocytes as previously described (19). Oocytes were injected with cRNA encoding wild-type  $\alpha$ - and  $\gamma$ -ENaC, along with wild-type or a given mutant  $\beta$ -ENaC. Oocytes injected with equal volumes of water ( $\sim 50$  nl/oocyte) served as negative controls. Injected oocytes were incubated at  $18^{\circ}\text{C}$  in 50% L-15 media (Invitrogen) media containing  $10\ \mu\text{M}$  amiloride. Wild-type ENaC currents were detectable as early as 1 day after injection.

### Voltage-clamp measurements

Two-electrode voltage-clamp measurements were performed in ND-96 (in mM: 96 NaCl, 2 KCl, 1.8 CaCl<sub>2</sub>, 1 MgCl<sub>2</sub> and 5 NaHEPES; pH 7.5) in the presence and absence of  $10\ \mu\text{M}$  amiloride. Data were acquired using an oocyte voltage-clamp amplifier (Model OC 725A; Warner Instruments, Hamden, CT, USA). The acquisition and analysis of currents were facilitated by pClamp 9.2 software (Axon Instruments, Foster City, CA, USA). Under unclamped conditions, cells expressing wild-type ENaC showed strong membrane depolarization (with  $\Delta V_m$  often as great as  $+60$  mV) on removal of amiloride from the bath solution.

### Nasal potential difference

The function of ENaC in the nasal epithelia of patient 824 was evaluated by performing NPD measurements as previously described (20,21).

### Statistical analysis

Values are expressed as mean  $\pm$  SEM. Two-tailed Student's *t*-test with unequal variance was used to assess statistical significance. *P*-values less than 0.05 were assumed significant.

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*Conflict of Interest statement.* None declared.

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