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# A Gain of Function NKCC2 Mutation in a Patient with Chronic Cyclic Edema

**Peer review status:**

No

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**Article ID:** WMC005325

**Article Type:** Research articles

**Submitted on:** 21-Sep-2017, 12:27:22 AM GMT **Published on:** 25-Sep-2017, 09:30:38 AM GMT

**Article URL:** [http://www.webmedcentral.com/article\\_view/5325](http://www.webmedcentral.com/article_view/5325)

**Subject Categories:** NEPHROLOGY

**Keywords:** NKCC2, idiopathic edema, transport, gain of function, mutation

**How to cite the article:** Nguyen MK, Rodriguez-Gama A, Moreno E, Borschewski A, Nilson T, Proctor G, et al. A

Gain of Function NKCC2 Mutation in a Patient with Chronic Cyclic Edema. WebmedCentral NEPHROLOGY 2017;8(9):WMC005325

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**Source(s) of Funding:**

IK is supported in part by the Allan Smidt Charitable Fund and the Factor Family Foundation Chair in Nephrology; KM is supported by Â Deutsche Forschungsgemeinschaft Grant MU 2924/2-1; EM and GG are supported by CONACYT-SEP Grant No. 222918 and CONACYT-FOSISS Grant No.261562, respectively.

**Competing Interests:**

None

# A Gain of Function NKCC2 Mutation in a Patient with Chronic Cyclic Edema

**Author(s):** Nguyen MK, Rodriguez-Gama A, Moreno E, Borschewski A, Nilson T, Proctor G, Mutig K, Gamba G, Kurtz I

## Abstract

### Background:

We report a 20 y.o. female with chronic cyclic edema who has a homozygous R116H substitution in the *SLC12A1* gene. The patient was normotensive without any serum electrolyte or metabolic acid-base abnormalities. Urine studies showed significantly reduced 24 hr excretion of Na<sup>+</sup>, K<sup>+</sup>, Cl<sup>-</sup>, Ca<sup>2+</sup>, and Mg<sup>2+</sup> consistent with increased NKCC2 activity. The patient's cyclic edema improved significantly with furosemide therapy.

### Methods:

Phosphorylation and dephosphorylation of NKCC2 was assessed by Western blot in proteins extracted from transiently transfected HEK-293 cells with wild-type and mutant NKCC2-R116H that were exposed to a low-chloride hypotonic buffer (LCHB). In separate experiments examining the mechanisms underlying the impaired dephosphorylation of mutant NKCC2, GST pull-down assays were performed with rat kidney lysates as a source of calcineurin and the N-termini of rat wild-type and mutant NKCC2 fused to GST as baits.

### Results:

Basal phosphorylation was similar between wild-type and NKCC2-R116H. However, following exposure to LCHB for 10, 20 and 30 min (known to activate NKCC2), the mutant transporter was phosphorylated significantly more rapidly than the wild-type transporter. In separate experiments, the rate of dephosphorylation was assessed following the removal of LCHB. The phosphorylation of NKCC2 decreased over 10, 20 and 30 min time points, whereas in mutant NKCC2-R116H at the same time points the decrease in phosphorylation was significantly less. GST pull-down assays revealed that the impaired dephosphorylation of the NKCC2 R116H protein is due to decreased interaction between calcineurin and the NKCC2 R116H mutant.

### Conclusions:

NKCC2 dephosphorylation is defective in the R116H mutation. The R116H mutation decreased the binding

of calcineurin to the mutant NKCC2 R116H cotransporter, thereby impairing dephosphorylation. Since phosphorylation of NKCC2 stimulates its transport activity, the R116H mutation in our patient represents the first reported naturally occurring gain of function NKCC2 mutation reported.

## Introduction

The furosemide-sensitive Na<sup>+</sup>-K<sup>+</sup>-2Cl<sup>-</sup> cotransporter (NKCC2) plays a critical role in sodium, potassium, and chloride reabsorption in the thick ascending limb of the kidney (1). Sodium chloride reabsorption in the loop of Henle is essential for creating the medullary concentration gradient that is required for excretion of maximally concentrated urine in the presence of antidiuretic hormone. Loss of function mutations in the *SLC12A1* gene encoding NKCC2 result in a clinical syndrome known as Bartter syndrome type I which is characterized by renal salt wasting, hypokalemia, a urinary concentrating defect, and metabolic alkalosis (2,3). An activating mutation in the *SLC12A1* gene has not been previously reported. We now report the first gain of function NKCC2 mutation in a patient with a homozygous R116H substitution in the *SLC12A1* gene resulting in chronic cyclic edema.

## Methods

Whole exome sequencing was performed to identify the patient's NKCC2 mutation (HiSeq 2500, Illumina). To determine the clinical effect of the R116H mutation on NKCC2 activity, while ingesting a normal diet, serum chemistries, peripheral renin activity, aldosterone level, and the urinary excretion of Na<sup>+</sup>, K<sup>+</sup>, Cl<sup>-</sup>, Ca<sup>2+</sup>, and Mg<sup>2+</sup> were assessed. The patient was treated with furosemide for her cyclic edema and the response to furosemide was determined with 12 hr urinary collections obtained off and on treatment (20 mg po bid). In order to exclude other causes of peripheral edema, urinalysis, liver function tests and an echocardiogram were performed to rule out underlying nephrotic syndrome, hepatic disease and cardiac dysfunction.

The R116H substitution was introduced into a pCMV5-FLAG1 vector containing human NKCC2 cDNA by site directed mutagenesis using QuickChange II (Agilent Technologies). The mutation was verified by automated direct DNA sequencing.

### Phosphorylation and Dephosphorylation Experiments

It is well known that phosphorylation of key threonine residues in the NKCC2 amino terminal domain correlates with its activity (4-8). Thus, phosphorylation of the transporter has been extensively used as a surrogate of activity and as previously shown (8), is stimulated following exposure to low chloride hypotonic buffer (LCHB). The LCHB used in this study contained 67.5mM sodium gluconate, 2.5mM potassium gluconate, 0.25mM CaCl<sub>2</sub>, 0.25mM MgCl<sub>2</sub>, 0.5mM Na<sub>2</sub>HPO<sub>4</sub>, 0.5mM Na<sub>2</sub>SO<sub>4</sub>, 7.5mM Hepes, pH 7.5. HEK293 cells grown on 6-well plates at a density of 70% were transiently transfected with 1 ug of pCMV5-hNKCC2 WT or the R116H mutant using 20 ul of polyethylenimine (PEI) 1 mg/ml. After 48 hours, the cells were stimulated with LCHB for 10, 20 and 30 minutes and then harvested for Western blotting. To detect changes in NKCC2 phosphorylation, previously characterized antibodies specific for total NKCC2 and phosphorylated NKCC2 at serine 91 (pNKCC2) were used (9) (obtained from Dundee University). To determine if the increased phosphorylation of NKCC2-R116H was due to increased SPAK activity, we evaluated the phosphorylation of SPAK by immunoblotting of cell lysates using a previously characterized antibody against pSPAK S373 (10) (obtained from Dundee University (S670B)). The antibodies were used at 2 ug/ul and an actin-HRP coupled antibody (SC-1616) was used as a loading control at 1:2500 dilution.

To determine the effect of the R116H mutant on NKCC2 dephosphorylation, transfected cells were stimulated with LCHB for 15 minutes, the buffer was then removed and the cells were incubated with isotonic buffer (135mM NaCl, 5mM KCl, 0.25mM CaCl<sub>2</sub>, 0.25mM MgCl<sub>2</sub>, 0.5mM Na<sub>2</sub>HPO<sub>4</sub> and 0.5mM Na<sub>2</sub>SO<sub>4</sub>, 7.5mM Hepes, pH 7.5) for 0, 10, 20, and 30 min to monitor the time course of NKCC2 dephosphorylation. Lysates from these cells were then analyzed by Western blotting at the specified time points.

The phosphorylation and dephosphorylation curves were assessed in two and three different experiments, respectively. Cells were transfected with wild-type or mutant NKCC2-R116H. Phosphorylation observed at time 0 for each experiment was taken as 100% and the remaining time points were normalized accordingly (see figure legends). For statistical analysis ANOVA

followed by multiple comparisons against the control value for each group was performed. The results are reported as mean  $\pm$  SEM.

### GST-Pull Down Assays

It has recently been demonstrated that the dephosphorylation of NKCC2 involves the functional interaction between the ubiquitously expressed calcium-/calmodulin-dependent serine-threonine phosphatase, calcineurin A $\gamma$  (CnA $\gamma$ ), and NKCC2 (11). To test the hypothesis that the substitution of Arg116 for histidine affects the binding of calcineurin A $\gamma$  to NKCC2, the human NKCC2 mutation (R116H) was introduced into the rat NKCC2 N-terminus (aa 1-175) at the corresponding residue (R112H) and also into a phosphorylation-mimicking rat N-terminal NKCC2 mutant (T96D + T101D + R112H) (11). These constructs and the wild-type rat NKCC2 N-terminus (aa 1-175) were cloned into the pGEX-6P1 vector for GST pull-down assays. GST pull-down assays with glutathione beads were performed using rat kidney lysates as a source of CnA $\gamma$  and the GST-fused N-terminal constructs as baits. GST alone (control) and GST-fused rat NKCC2 N-termini were attached to glutathione magnet beads (Invitrogen) and incubated with rat kidney lysates in GST-buffer (200 mM NaCl, 10 mM CaCl<sub>2</sub>, 50 mM Hepes, pH 7.5, with 1 $\times$  complete protease inhibitor cocktail) overnight at 4 $^{\circ}$ C with agitation. The beads were then washed with GST-buffer, boiled in 1 $\times$  Laemmli buffer, and the eluates were analyzed by immunoblotting using an antibody to CnA $\gamma$  (EMD Millipore, Billerica, MA).

UCLA has determined that this work does not meet the definition of Human Subject Research and does not need UCLA IRB review and/or approval. The UCLA IRB's FWA number is 00004642. Our work complies with federal regulations and the Declaration of Helsinki.

## Results

The patient was an adopted 20 y.o. female with chronic cyclic edema (Fig.1). Whole exome sequencing revealed a homozygous c.347G>A variant in the *SLC12A1* gene, resulting in a homozygous R116H substitution (Fig.1). Serum chemistry results were as follows: [Na<sup>+</sup>] 141 mmol/L, [K<sup>+</sup>] 3.7 mmol/L, [Cl<sup>-</sup>] 101 mmol/L, total CO<sub>2</sub> 24 mmol/L, BUN 5 mg/dL, and creatinine 0.6 mg/dL. Plasma renin activity was 1.47 ng/mL/h, and plasma aldosterone level was 6 ng/dL. Liver function tests revealed AST 18 U/L, 12 U/L, alkaline phosphatase 110 U/L, total bilirubin 0.3 mg/dL, albumin 4.8 g/dL, and total protein 7.6 g/dL.

Urinalysis was unremarkable without proteinuria and hematuria. An echocardiogram revealed normal left ventricular size, left ventricular ejection fraction 60-65%, and normal left ventricular diastolic function. Urine studies showed a significantly reduced 24 hr excretion of  $\text{Na}^+$ ,  $\text{K}^+$ ,  $\text{Cl}^-$ ,  $\text{Ca}^{2+}$ , and  $\text{Mg}^{2+}$ :  $\text{Na}^+$  63 meq,  $\text{K}^+$  24 meq,  $\text{Cl}^-$  44 meq,  $\text{Ca}^{2+}$  22 mg,  $\text{Mg}^{2+}$  undetectable, creatinine 710 mg (patient weight 50 kg) consistent with increased NKCC2 activity. Of note, the patient has no history of significant gastrointestinal losses. To evaluate the effectiveness of furosemide in inhibiting NKCC2-R116H, daytime 12 hr urinary collections were obtained off and on furosemide. The 12 hr urine off furosemide contained:  $\text{Na}^+$  24 meq,  $\text{K}^+$  12 meq,  $\text{Cl}^-$  30 meq,  $\text{Ca}^{2+}$  46 mg,  $\text{Mg}^{2+}$  26 mg, creatinine 440 mg. Upon furosemide initiation, the 12 hr urine contained:  $\text{Na}^+$  211 meq,  $\text{K}^+$  27 meq,  $\text{Cl}^-$  245 meq,  $\text{Ca}^{2+}$  148 mg,  $\text{Mg}^{2+}$  70 mg, creatinine 420 mg.

Wild-type NKCC2 (NKCC2-WT) and mutant NKCC2-R116H had baseline phosphorylation levels (Fig.2A). Following exposure to LCHB for 10, 20 and 30 min, the mutant transporter had a significantly faster increase in its phosphorylation level compared to the wild-type transporter (Fig.2A). SPAK phosphorylation (pSPAK S373) levels were similar in both groups, indicating that the WNK/SPAK pathway was equally activated in both groups. Fig.2B shows the values of phosphorylated WT and mutant NKCC2 (pNKCC2-S91) after LCHB stimulation normalized to the respective baseline phosphorylation levels. Fig.2C shows the percentage of phosphorylation levels at different time points during LCHB exposure with each time point normalized to its respective zero time point.

To analyze the dephosphorylation course of NKCC2-WT and NKCC2-R116H after exposure to LCHB, the cells were switched to an isotonic solution and the phosphorylation status was analyzed at 0, 10, 20, and 30 minutes. Fig.3A depicts a representative blot. The normalized values of phosphorylated NKCC2 (pNKCC2-S91) relative to the phosphorylation intensity of wild type or mutant NKCC2 at the end of the LCHB exposure are shown in Fig. 3B. The percentage of phosphorylation levels at different recovery times, with each time point normalized to its respective zero time point is depicted in Fig.3C. NKCC2-WT phosphorylation decreased at 10, 20 and 30 min from  $1.0 \pm 0.01$  (arbitrary units) in the basal state, to  $0.74 \pm 0.06$ ,  $0.59 \pm 0.11$ , and  $0.53 \pm 0.07$  respectively. The differences at 20 and 30 minutes were significant ( $p < 0.05$ ). In contrast, in mutant NKCC2-R116H at the same time points, the decrease

in phosphorylation was significantly less: from  $1.0 \pm 0.01$  at baseline to  $0.95 \pm 0.03$ ,  $0.83 \pm 0.05$ , and  $0.81 \pm 0.09$  respectively (Fig.3C). These change differences versus the baseline were not significant. SPAK dephosphorylation did not differ among the groups. Thus, it is likely that the R116H mutation impairs the dephosphorylation of NKCC2 and thus, after removal of the LCHB phosphorylation stimulus, the mutant cotransporter remains phosphorylated for a longer period of time compared to the wild type.

To elucidate the mechanisms underlying impaired dephosphorylation of the mutant NKCC2-R116H, Western blot analysis using an anti-CnA antibody was performed in GST pull-down assays using GST-fused wild-type rat NKCC2 N-terminus, mutant rat NKCC2 N-terminus (R112H), and the phosphorylation-mimicking (T96D + T101D + R112H) rat NKCC2 N-terminal mutant as baits (Fig.4). The results show that the phosphatase was bound to NKCC2-WT and that this interaction was substantially attenuated by the arginine to histidine substitution. Moreover, the R112H substitution also prevented the binding of CnA to the phosphorylation-mimicking mutant.

## Discussion

We report a patient with a history of chronic cyclic edema of unknown etiology in the absence of nephrotic syndrome, hepatic and cardiac dysfunction. Urine studies showed a significantly reduced excretion of  $\text{Na}^+$ ,  $\text{K}^+$ ,  $\text{Cl}^-$ ,  $\text{Ca}^{2+}$ , and  $\text{Mg}^{2+}$ . Whole exome sequencing uncovered a homozygous c.347G>A variant in the *SLC12A1* gene, resulting in a homozygous R116H substitution. *In vitro* experiments in HEK-293 cells expressing the mutant NKCC2 showed that the R116H substitution decreased the interaction with calcineurin phosphatase, thereby impairing the dephosphorylation of the transporter. All previously documented disease-causing mutations in the NKCC2 gene have been associated with the type I Bartter syndrome characterized by impaired transport function in the thick ascending limb of Henle (1). The R116H mutation in our patient represents the first documented case of a disease-causing activating NKCC2 mutation.

Although there are currently no animal models of gain of function NKCC2 mutations, the clinical findings in our patient are compatible with the *in vivo* consequences of increased NKCC2 activity. The decreased urinary excretion of sodium, chloride, and potassium with low plasma renin activity and aldosterone level in the context of peripheral edema

are consistent with a volume-expanded state due to increased NKCC2 transport in the thick ascending limb. The reduced urinary excretion of calcium and magnesium in our patient are a predicted consequence of increased thick ascending limb divalent cation paracellular flux due to a more favorable lumen positive transtubular electrical gradient. The patient's peripheral edema and impaired urine electrolyte excretion improved significantly following furosemide therapy indicating that the R116H mutation did not affect the sensitivity/binding of furosemide to the transporter.

Prior to the initiation of furosemide therapy, the patient had spontaneous episodes of resolution of her peripheral edema that would be clinically referred to as cyclic edema. This syndrome typically occurs in premenopausal women without concomitant hepatic, renal or cardiac disease (12). It is associated in certain patients with efforts to achieve weight loss (laxatives, self-induced vomiting, diuretic use). The underlying pathogenetic mechanisms are thought to include capillary leak, diuretic-induced edema, and refeeding induced edema (12). The role of other factors such as the renin-aldosterone system, atrial natriuretic factor, dopamine, antidiuretic hormone, and thyroid hormone is unclear. Our findings raise the interesting possibility that other reported patients with idiopathic cyclic edema have gain of function mutations in NKCC2. Although conjectural, the cyclic nature of edema formation in our patient was potentially a result of periods of urinary sodium chloride retention followed by subsequent volume expansion of sufficient magnitude (dependent on sodium chloride intake and hormonal factors) to overcome the gain of function mutation of the transporter and induce a diuresis.

The R116H mutation is located at the N-terminal region of NKCC2 that is not directly involved in ion binding and transport (13). It is well known however that the N-terminal region plays a key role in modulating NKCC2 in which the activity is modulated by the phosphorylation of key threonine and serine residues. The phosphorylation state of NKCC2 by serine-threonine kinases is thought to facilitate both membrane trafficking and intrinsic transport activity (4-6,8). The decrease in intracellular chloride concentration is associated with activation of the NKCC2 via the WNK/SPAK pathway (8,9). Exposing cells to the LCHB promotes chloride efflux and is associated with activation/phosphorylation of the cotransporter (8). After removal of the LCHB, NKCC2 is dephosphorylated as the intracellular chloride concentration returns to normal (14). Thus, changing the LCHB to an isotonic chloride-containing

medium is associated with inactivation/dephosphorylation of the transporter.

The R116H mutant was phosphorylated more rapidly than NKCC2-WT and the rate of its dephosphorylation was reduced. Importantly, the phosphorylation state of NKCC2 at any given time is determined by the rate of phosphorylation versus the rate of dephosphorylation. To determine if the increased rate of phosphorylation of NKCC2-R116H was due to increased SPAK activity, we evaluated the phosphorylation of SPAK after exposing the cells to LCHB. SPAK phosphorylation was similar in both groups, indicating that the increased NKCC2-R116H phosphorylation was not due to a difference in kinase activity. We therefore postulated that the increased phosphorylation of NKCC2-R116H could be due to impaired dephosphorylation of the transporter. To determine the effect of the NKCC2-R116H mutant on NKCC2 dephosphorylation, HEK-293 cells expressing wild-type or mutant NKCC2-R116H were initially stimulated with LCHB and then returned to the regular isotonic chloride-containing medium in order to analyze the rate of dephosphorylation. Whereas NKCC2-WT was significantly dephosphorylated by 20 and 30 minutes, NKCC2-R116H phosphorylation remained unchanged. SPAK dephosphorylation was similar in both groups, demonstrating that the impaired dephosphorylation of the NKCC2-R116H mutant was not due to differences in SPAK dephosphorylation.

Calcineurin inhibitors cause renal sodium chloride retention and hypertension largely due to increased phosphorylation and activity of NCC, the thiazide-sensitive  $\text{Na}^+\text{-Cl}^-$ -cotransporter (NCC) (15), and have also been shown to result in the activation of NKCC2 (16-17). Importantly, calcineurin A (CnA), a serine-threonine phosphatase has been reported to play an important role in NKCC2 dephosphorylation (11). It is unclear at present whether CnA directly binds to and dephosphorylates the N-terminal region of the transporter or whether additional phosphoenzyme complexes are involved as in NKCC1 (18). In pull-down experiments, the mutant NKCC2 N-terminus failed to interact with CnA and the abnormality in CnA binding was still present when a phosphorylated-mimicking NKCC2 mutant that included the arginine to histidine substitution was also used as bait. These findings are compatible with the hypothesis that the increased SPAK-mediated phosphorylation of NKCC2-R116H results from impaired CnA mediated dephosphorylation of the transporter.

## Conclusion(s)

In this study, we reported a homozygous c.347G>A mutation in the *SLC12A1* gene resulting in a NKCC2 R116H substitution that results in chronic cyclic edema. Our patient showed significantly reduced urine excretion of Na<sup>+</sup>, K<sup>+</sup>, Cl<sup>-</sup>, Ca<sup>2+</sup>, and Mg<sup>2+</sup> with secondary suppression of renin and aldosterone secretion, consistent with increased NKCC2 activity. *In vitro* studies of expressed NKCC2 demonstrated increased phosphorylation of the mutant protein due to impaired dephosphorylation by calcineurin, rather than increased phosphorylation by serine-threonine kinases. Impaired NKCC2 R116H dephosphorylation was associated with decreased binding of the CnA's serine-threonine phosphatase to the mutant transporter. Since phosphorylation of NKCC2 is a well-known stimulus of its transport activity (4-6,8), we propose that the R116H mutation in our patient results in its enhanced NKCC2 transport activity representing the first gain of function NKCC2 mutation reported in the literature.

## Acknowledgement(s)

IK is supported in part by the Allan Smidt Charitable Fund and the Factor Family Foundation Chair in Nephrology; KM is supported by Deutsche Forschungsgemeinschaft Grant MU 2924/2-1; EM and GG are supported by CONACYT-SEP Grant No. 222918 and CONACYT-FOSISS Grant No.261562, respectively.

## References

- Gamba G: Molecular physiology and pathophysiology of electroneutral cation-chloride cotransporters. *Physiol Rev.* 85(2):423-493, 2005
- Kurtz I: Molecular pathogenesis of Bartter's and Gitelman's syndromes. *Kidney Int.* 54(4):1396-1410, 1998
- Simon DB, Karet FE, Hamdan JM, DiPietro A, Sanjad SA, Lifton RP: Bartter's syndrome, hypokalaemic alkalosis with hypercalciuria, is caused by mutations in the Na-K-2Cl cotransporter NKCC2. *Nat Genet.* 13(2):183-188, 1996
- Schieffl IM, Castrop H: Regulation of NKCC2 splicing and phosphorylation. *Curr Opin Nephrol Hypertens.* 24(5):457-62, 2015
- Gamba G. Regulation of the renal Na<sup>+</sup>-Cl<sup>-</sup> cotransporter by phosphorylation and ubiquitylation. *Am J Physiol Renal Physiol.* 303(12):F1573-F1583, 2012.
- Gimenez I, Forbush B: Short-term stimulation of the renal Na-K-Cl cotransporter (NKCC2) by vasopressin involves phosphorylation and membrane translocation of the protein. *J Biol Chem.* 278(29):26946-26951, 2003.
- Ares GR, Caceres PS, Ortiz PA: Molecular regulation of NKCC2 in the thick ascending limb. *Am J Physiol Renal Physiol.* 301(6):F1143-F1159, 2018.
- Ponce-Coria J, San-Cristobal P, Kahle KT, Vazquez N, Pacheco-Alvarez D, de Los Heros P, Juárez P, Muñoz E, Michel G, Bobadilla NA, Gimenez I, Lifton RP, Hebert SC, Gamba G: Regulation of NKCC2 by a chloride-sensing mechanism involving the WNK3 and SPAK kinases. *Proc Natl Acad Sci U S A.* 105(24):8458-8463, 2008.
- Richardson C, Sakamoto K, de los Heros P, Deak M, Campbell DG, Prescott AR, Alessi DR: Regulation of the NKCC2 ion cotransporter by SPAK-OSR1-dependent and -independent pathways. *J Cell Sci.* 124(Pt 5):789-800, 2011
- Rafiqi FH, Zuber AM, Glover M, Richardson C, Fleming S, Jovanović S, Jovanović A, O'Shaughnessy KM, Alessi DR: Role of the WNK-activated SPAK kinase in regulating blood pressure. *EMBO Mol Med.* 2(2):63-75, 2010
- Borschewski A, Himmerkus N, Boldt C, Blankenstein KI, McCormick JA, Lazelle R, Willnow TE, Jankowski V, Plain A, Bleich M, Ellison DH, Bachmann S, Mutig K: Calcineurin and sorting-related receptor with A-Type repeats interact to regulate the renal Na<sup>+</sup>-K<sup>+</sup>-2Cl<sup>-</sup> cotransporter. *J Am Soc Nephrol.* 27(1):107-119, 2016
- Kay A, Davis, CL: Idiopathic edema. *Am J Kidney Dis.* 34(3): 405-423, 1999
- Tovar-Palacio C, Bobadilla NA, Cortés P, Plata C, de los Heros P, Vázquez N, Gamba G: Ion and diuretic specificity of a chimeric protein

s between apical  $\text{Na}^+-2\text{Cl}^-$  and  $\text{Na}^+-\text{Cl}^-$  cotransporters. *Am J Physiol Renal Physiol*. :287(3):F570-F577, 2004

14. Gamba G, Miyanoshita A, Lombardi M, Lytton J, Lee WS, Hediger MA, Hebert SC: Molecular cloning, primary structure and characterization of two members of the mammalian electroneutral sodium-(potassium)-chloride cotransporter family expressed in kidney. *J Biol Chem*: 269(26): 17713-17722, 1994

15. Hoorn EJ, Walsh SB, McCormick JA, Fårsténberg A, Yang CL, Roeschel T, Paliege A, Howie AJ, Conley J, Bachmann S, Unwin RJ, Ellison DH: The calcineurin inhibitor tacrolimus activates the renal sodium chloride cotransporter to cause hypertension. *Nat Med*:17(10):1304-1309, 2011

16. Esteva-Font C, Ars E, Guillen-Gomez E, Campistol JM, Sanz L, Jimenez W, Knepper MA, Torres F, Torra R, Ballarín JA, Fernández-Llama P: Cyclosporine-induced hypertension is associated with increased sodium transporter of the loop of Henle (NKCC2). *Nephrol Dial Transplant*: 22(10):2810-2816, 2017

17. Wu MS, Yang CW, Bens M, Peng KC, Yu HM, Vandewalle A: Cyclosporine stimulates  $\text{Na}^+-\text{K}^+-\text{Cl}^-$  cotransport activity in cultured mouse medullary thick ascending limb cells. *Kidney Int*: 58(4):1652-1663, 2000

18. Gagnon KB, Delpire E: Multiple pathways for protein phosphatase 1 (PP1) regulation of Na-K-2Cl cotransporter (NKCC1) function: the N-terminal tail of the Na-K-2Cl cotransporter serves as a regulatory scaffold for Ste20-related proline/alanine-rich kinase (SPAK) and PP1. *J Biol Chem* 285: 14115-14121, 2010

## Figure Legends

Figure 1: A) Cyclic edema: The patient has a history of chronic cyclic edema with intermittent episodes of hand and feet swelling followed by a period of natriuresis and resolution of the edema. Left side (edema phase); Right side (spontaneous resolution phase). B) Amino acid sequence alignment of NKCC2, NKCC1 and NCC shows that the residue R116 is conserved in NKCC2 and NKCC1 across species. Prefixes: h (human); m (mouse); sh (shark).

Figure 2: Cells were subjected to a hypotonic low-chloride stimulus. A) NKCC2-R116H displayed higher levels of phosphorylation compared to the wild

type protein. Note that SPAK phosphorylation occurred similarly in both groups (n=2). B) Normalized values of phosphorylated NKCC2 (pNKCC2) relative to the phosphorylation intensity of wild-type or mutant NKCC2 without stimulus (n=2). C) Percentage of phosphorylation levels at different times of hypotonic low-chloride exposure. Each time point was normalized to its respective 0 time point (n=2). Prefix p: phosphorylated.

Figure 3: A) Western blot of pNKCC2-S91 showed a significant lag in R116H mutant dephosphorylation. Although NKCC2-WT was dephosphorylated by 30 min following isotonic recovery, NKCC2-R116H remained abnormally phosphorylated after 30 min isotonic recovery. Note that SPAK dephosphorylation occurred similarly in both groups. B) Normalized values of pNKCC2 relative to the phosphorylation intensity of wild-type and mutant NKCC2 at the end of the low chloride hypotonic stress phase (n=3). C) Percentage of phosphorylation levels at different recovery times. Each time point was normalized to its respective 0 time point (n=3). Prefix p: phosphorylated.

Figure 4: Representative Western blot of eluates after GST pull-down using GST (control) or GST-fused rat wild-type NKCC2 N-terminus, a mutant rat N-terminus with substituted arginine (R112H) corresponding to the human R116H mutation, and a phosphorylation-mimicking mutant with substituted arginine (T96D + T101D + R112H) as baits. Detection with a CnA antibody showed binding of the phosphatase to NKCC2-WT. However, this interaction was substantially decreased by the arginine to histidine substitution. In addition, the R112H mutation also prevented the binding of CnA to the phosphorylated-mimicking NKCC2 mutant.



## Illustrations

### Illustration 1

Figure 1: A) Cyclic edema: The patient has a history of chronic cyclic edema with intermittent episodes of hand and feet swelling followed by a period of natriuresis and resolution of the edema. Left side (edema phase); Right side (spontaneous resolution phase). B) Amino acid sequence alignment of NKCC2, NKCC1 and NCC shows that the residue R116 is conserved in NKCC2 and NKCC1 across species. Prefixes: h (human); m (mouse); sh (shark).

### Illustration 2

Figure 2: Cells were subjected to a hypotonic low-chloride stimulus. A) NKCC2-R116H displayed higher levels of phosphorylation compared to the wild type protein. Note that SPAK phosphorylation occurred similarly in both groups (n=2). B) Normalized values of phosphorylated NKCC2 (pNKCC2) relative to the phosphorylation intensity of wild-type or mutant NKCC2 without stimulus (n=2). C) Percentage of phosphorylation levels at different times of hypotonic low-chloride exposure. Each time point was normalized to its respective 0 time point (n=2). Prefix p: phosphorylated.

## Illustration 3

Figure 3: A) Western blot of pNKCC2-S91 showed a significant lag in R116H mutant dephosphorylation. Although NKCC2-WT was dephosphorylated by 30 min following isotonic recovery, NKCC2-R116H remained abnormally phosphorylated after 30 min isotonic recovery. Note that SPAK dephosphorylation occurred similarly in both groups. B) Normalized values of pNKCC2 relative to the phosphorylation intensity of wild-type and mutant NKCC2 at the end of the low chloride hypotonic stress phase (n=3). C) Percentage of phosphorylation levels at different recovery times. Each time point was normalized to its respective 0 time point (n=3). Prefix p: phosphorylated.

## Illustration 4

Figure 4: Representative Western blot of eluates after GST pull-down using GST (control) or GST-fused rat wild-type NKCC2 N-terminus, a mutant rat N-terminus with substituted arginine (R112H) corresponding to the human R116H mutation, and a phosphorylation-mimicking mutant with substituted arginine (T96D + T101D + R112H) as baits. Detection with a CnA $\beta$  antibody showed binding of the phosphatase to NKCC2-WT. However, this interaction was substantially decreased by the arginine to histidine substitution. In addition, the R112H mutation also prevented the binding of CnA $\beta$  to the phosphorylated-mimicking NKCC2 mutant.