Phosphorylation Regulates NCC Stability and Transporter Activity In Vivo

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ABSTRACT

A T60M mutation in the thiazide-sensitive sodium chloride cotransporter (NCC) is common in patients with Gitelman’s syndrome (GS). This mutation prevents Ste20-related proline and alanine-rich kinase (SPAK)/ oxidative stress responsive kinase-1 (OSR1)–mediated phosphorylation of NCC and alters NCC transporter activity in vitro. Here, we examined the physiologic effects of NCC phosphorylation in vivo using a novel Ncc T58M (human T60M) knock-in mouse model. NccT58M/T58M mice exhibited typical features of GS with a blunted response to thiazide diuretics. Despite expressing normal levels of Ncc mRNA, these mice had lower levels of total Ncc and p-Ncc protein that did not change with a low-salt diet that increased p-Spak. In contrast to wild-type Ncc, which localizes to the apical membrane of distal convoluted tubule cells, T58M Ncc localized primarily to the cytosolic region and caused an increase in late distal convoluted tubule volume. In MDCK cells, exogenous expression of phosphorylation-defective NCC mutants reduced total protein expression levels and membrane stability. Furthermore, our analysis found diminished total urine NCC excretion in a cohort of GS patients with homozygous NCC T60M mutations. When Wnk4D561A/+ mice, a model of pseudohypoaldosteronism type II expressing an activated Spak/Osr1-Ncc, were crossed with NccT58M/T58M mice, total Ncc and p-Ncc protein levels decreased and the GS phenotype persisted over the hypertensive phenotype. Overall, these data suggest that SPAK-mediated phosphorylation of NCC at T60 regulates NCC stability and function, and defective phosphorylation at this residue corrects the phenotype of pseudohypoaldosteronism type II.


The thiazide-sensitive sodium (Na+)–chloride (Cl−) cotransporter (NCC) is a member of the cation/Cl− cotransporter gene family and has 12 transmembrane domains with both termini and two loops located intracellularly and six loops located extracellularly.1 It is the major NaCl transport pathway in the apical membrane of the distal convoluted tubules (DCTs) and accounts for the reabsorption of 5%–10% of filtered Na+.2 It is also the molecular target of thiazide diuretics and modulates magnesium and calcium reabsorption in the DCT.3–5 Inactivating mutations in SLC12A3 gene encoding NCC lead to Gitelman’s syndrome (GS), an

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autosomal recessive renal tubular salt-losing disorder characterized by hypokalemia, metabolic alkalosis, hypomagnesemia, and hypocalciuria.6–8 Most patients with GS may manifest fatigue, cramps, nocturia, salt craving, muscle weakness, and even paralysis during early childhood or adolescence.9 To date, >180 different mutations have been identified in patients with GS.1,10–13

Of note, a missense threonine (T) to methionine (M) mutation at T60 residue is the most common NCC mutation in Asian patients with GS.9,14–16 T60 in NCC has been well characterized by hypokalemia, metabolic alkalosis, hypomagnesemia, and hypocalciuria.6

Table 1. BP, blood, and urine biochemistries in Ncc T58M mice

<table>
<thead>
<tr>
<th>Genotyping (n)</th>
<th>WT (8)</th>
<th>Homozygous (8)</th>
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<tr>
<td>BP (mmHg)</td>
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<tr>
<td>Systolic</td>
<td>104.5±4.0</td>
<td>90.7±8.2*</td>
</tr>
<tr>
<td>Diastolic</td>
<td>52.5±4.9</td>
<td>53.7±6.4</td>
</tr>
<tr>
<td>Mean</td>
<td>69.7±3.5</td>
<td>62.1±4.5*</td>
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<tr>
<td>Weight (g)</td>
<td>24.6±2.8</td>
<td>24.3±3.2</td>
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<tr>
<td>Plasma</td>
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</tr>
<tr>
<td>Aldosterone (pg/ml)</td>
<td>367±111</td>
<td>837±212*</td>
</tr>
<tr>
<td>pH</td>
<td>7.35±0.03</td>
<td>7.40±0.04*</td>
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<tr>
<td>Na⁺ (mmol/L)</td>
<td>152±1</td>
<td>151±2</td>
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<tr>
<td>K⁺ (mmol/L)</td>
<td>4.2±0.2</td>
<td>3.6±0.3*</td>
</tr>
<tr>
<td>Cl⁻ (mmol/L)</td>
<td>111±1</td>
<td>106±2*</td>
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<tr>
<td>HCO₃⁻ (mmol/L)</td>
<td>22.5±0.6</td>
<td>25.9±0.4*</td>
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<tr>
<td>Total Ca²⁺ (mg/dl)</td>
<td>9.6±0.2</td>
<td>9.5±0.3</td>
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<td>Mg²⁺ (mg/dl)</td>
<td>2.4±0.1</td>
<td>1.9±1.1*</td>
</tr>
<tr>
<td>Cr (mg/dl)</td>
<td>0.18±0.07</td>
<td>0.17±0.06</td>
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<tr>
<td>Urine (ml/d)</td>
<td>1.2±0.5</td>
<td>1.2±0.4</td>
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<tr>
<td>CCr (μl/min per gram)</td>
<td>7.8±3.6</td>
<td>7.9±3.2</td>
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<tr>
<td>FENa (%)</td>
<td>0.31±0.07</td>
<td>0.30±0.07</td>
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<tr>
<td>FEK (%)</td>
<td>15.8±3.2</td>
<td>23.4±4.4*</td>
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<tr>
<td>FEKg (%)</td>
<td>3.2±0.5</td>
<td>5.3±0.4*</td>
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<tr>
<td>Ca²⁺/Cr (mg/mg)</td>
<td>0.22±0.08</td>
<td>0.13±0.03*</td>
</tr>
</tbody>
</table>

*P<0.05 when homozygous versus WT.

RESULTS

Phenotype of the Ncc T58M Knock-In Mice

Ncc T58M knock-in mice were generated by genetic engineering techniques (Supplemental Figure 1) and phenotype was evaluated at the age of 12–14 weeks. On a normal rodent chow diet, BP, serum, and urine electrolytes were not significantly different between Ncc T58M/+ mice and wild-type (WT) mice (data not shown). Compared with WT mice, the Ncc T58M/T58M mice, however, exhibited relative hypotension (P<0.05), high aldosterone levels (P<0.05), hypokalemia (P<0.05) with increased fractional excretion of K⁺ (P<0.05), hypomagnesemia (P<0.01) with increased fractional excretion of Mg²⁺ (P<0.05), hypocalciuria (P<0.01), and metabolic alkalosis (P<0.01) (Table 1), consistent with typical features of GS patients.

Ncc T58M Expression and Localization in the Kidney

Compared with WT mice, the expression of Ncc mRNA measured by quantitative RT-PCR in the kidneys of Ncc T58M/+ and Ncc T58M/T58M mice was not significantly different (Figure 1A). However, the expression of Ncc protein was significantly reduced in Ncc T58M/+ mice (73%±12%, n=4; P<0.01) and almost absent in Ncc T58M/T58M mice (7%±3% n=4; P<0.01) (Figure 1B). In Ncc T58M/T58M mice, immunofluorescence also showed dramatically decreased total and less condensed NCC expression in the apical membrane of DCT cells (Figure 1C). To observe the cellular distribution and localization of the T58M-Ncc protein in detail, immunogold electron microscopic was performed. In WT mice, most of the WT-Ncc was located in the apical membrane of DCT cells (Figure 2A). However, in T58M mice, the sparse T58M-Ncc was primarily located in the cytosol (Figure 2B). Similar to the total Ncc findings, p-Ncc expression (T53, 8%±2%; T58, 2%±0.5%; S71, 9%±4%; P<0.01) was also significantly reduced in Ncc T58M/T58M mice (Supplemental Figure 2).

Morphometric Analyses of DCT Cells

We further examined the change in DCT cellular volume in these Ncc T58M knock-in mice by light microscopy. Strong positive parvalbumin-stained segments were considered early DCT. Because Calbindin-D28k is strongly expressed in the late DCT and cortical connecting tubule and aquaporin 2 (Aqp2) is expressed from the cortical connecting tubule to downstream renal tubules in mice, strong positive Calbindin-D28k but negative Aqp2-stained segments were considered the late DCT (Figure 3A).25,26 In Ncc T58M/T58M mice, early DCT volume in the cortical tubules was not significantly affected (13%±3% versus 14%±5% in WT) but late DCT volume was...
with hydrochlorothiazide (a NCC inhibitor), furosemide (a NKCC2 inhibitor), and amiloride (an ENaC inhibitor). The resulting urine amount and Na⁺, K⁺, and Cl⁻ excretion rates were used as indices of Ncc, Nkcc2, and ENaC function. Urine excretion of Na⁺ and Cl⁻ was markedly increased after a single dose of hydrochlorothiazide, furosemide, or amiloride treatment in WT mice. However, the urine amount and excretion of Na⁺ and Cl⁻ were not significantly affected in Ncc<sup>T58M/T58M</sup> mice after hydrochlorothiazide treatment, indicating that Ncc function was strikingly reduced in Ncc<sup>T58M/T58M</sup> mice (Figure 4A).

An exaggerated response of the urine amount and excretion of Na⁺ and Cl⁻ after furosemide and amiloride challenge suggested that Nkcc2 and ENaC function had increased in the Ncc<sup>T58M/T58M</sup> mice (Figure 4, B and C).

**T60M-NCC Urinary Excretion in GS Patients and Its Expression in MDCK Cells**

Because urinary NCC excretion reportedly correlates with membrane-expressed NCC in the DCT,<sup>27</sup> we also examined urinary NCC excretion in normal healthy participants, GS patients with homozygous NCC T60M mutation, and disease controls (bulimia nervosa) (Supplemental Figure 4). Compared with healthy and bulimic participants, urinary NCC excretion in GS patients with homozygous NCC T60M mutation was markedly decreased (32±8%; P<0.01) (Figure 5).

To evaluate whether the reduced NCC expression in knock-in mice and GS patients was due to a change in translation or inability to phosphorylate the site, exogenous T60M-NCC, T60A-NCC (phosphorylation-defective), and T60D-NCC (phosphorylation-mimicking) were overexpressed in the polarized renal tubule MDCK cell. Decreased total protein abundance of T60M-NCC (25±9%, P<0.01) and T60A-NCC (28±4%, P<0.01) but increased abundance of T60D (185±12%, P<0.01) (Figure 6A) were observed, indicating that reduced NCC expression by the T60M mutation was primarily mediated through post-translational phosphorylation rather than translational change. To further verify whether phosphorylation status of the T60 residue could affect protein stability, we conducted a cycloheximide run-down experiment,<sup>28</sup> which blocks protein synthesis and thus prevents the trafficking of new NCC to the cell membrane. Compared with WT-NCC (t<sub>1/2</sub> 1.7±0.5 hours), both membrane-expressed T60M-NCC (t<sub>1/2</sub> 1.0±0.3 hours; P<0.05) and T60A-NCC (t<sub>1/2</sub> 1.1±0.4

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**Figure 1.** Preserved Ncc mRNA transcripts but diminished Ncc protein expression in the kidneys of Ncc T58M knock-in mice. (A) RT-PCR analysis of Ncc mRNA using primers flanking mutation sites (exon 1) (upper panels). Quantitative gene expression of Ncc in WT, heterozygous (He), and homozygous (Ho) Ncc T58M knock-in mice (n=6/group) are presented as percentage of control (control = 100%) (lower panel). (B) An antibody recognizing the amino terminal portion (amino acids 73–88) of Ncc is applied for detecting Ncc expression (upper panel). Semiquantitative immunoblot of Ncc in crude membrane fractions from WT, He, and Ho mice (middle panels) and its semi-quantification by densitometry analysis (lower panel) (n=4/group). (C) Immunofluorescence staining for Ncc. Representative immunofluorescence micrographs of Ncc in the cortex of WT, He, and Ho mice. *P<0.05 versus WT comparison. Original magnification, ×200 and ×1000 in C.
Figure 2. Total and apically expressed Ncc protein was dramatically attenuated in the distal convoluted tubules (DCTs) of Ncc T58M knock-in mice. Representative immunogold labeling of Ncc in DCT cells of (A) WT and (B) homozygous Ncc T58M knock-in mice (Ho), respectively. Arrows (→) and arrowheads (↑) indicate the apical and cytosol located Ncc (x22.5k), respectively, in DCTs. (C) Semi-quantification of immunogold labeling of Ncc. Total and apical Ncc signals in WT and Ho mice are shown in the figures (n=6/group).

Figure 3. Hypertrophic late DCTs cellular volume in Ncc T58M knock-in mice. (A) Distribution of Ncc, parvalbumin (Pv), Calbindin (Cbp)-D28k, and Aqp2 in the distal renal tubules. (B) Representative early and late DCT segments by double immunofluorescence of Ncc (green) and Pv (red) or Cbp-D28k (red) and Aqp2 (green) (arrows indicate early DCT [upper panels] and late DCT [lower panels] segments) and (C) their semi-quantification of fractional cortical volume in the cortex of WT and Ho mice (n=5/group). *P<0.05 in WT versus Ho comparison. CNT, cortical connecting tubule; CCD, cortical collecting duct. Scale bar, 50 μm.
Ncc T58M Mutation Impaired its Phosphorylation Despite Activated Spak and Reversed the PHAII Phenotype

Because the upstream kinase of Ncc, Spak, is activated on a low-salt diet, phosphorylation of Ncc on a low-salt diet would inform whether the reduction in activated Ncc was due to impaired function or decreased protein abundance alone. Despite increased upstream p-Spak, NccT58M/T58M mice on a low Na⁺ diet (0.05%) could not increase p-Ncc, whereas WT mice on the same low Na⁺ diet did (Figure 7A).

To assess whether defective phosphorylation of Ncc at the T60 residue could correct or reverse the phenotype of PHAII with WNK4 mutation, we crossed the NccT58M/T58M mice with Wnk4D561A/+ mice. Although Wnk4-Spak signaling was constitutively activated in double NccT58M/T58M/Wnk4D561A/+ knock-in mice, total and p-Ncc (T53, T58, and S71) expression were still markedly decreased (Figure 7B). Compared with WT and Wnk4D561A/+ knock-in mice, NccT58M/T58M/Wnk4D561A/+ knock-in mice still exhibited the typical GS phenotype with secondary hyperaldosteronism and hypotension, hypokalemic metabolic alkalosis, hypomagnesemia, and hypocalciuria just like the NccT58M/T58M mice (Figure 8). These findings further suggest that the T58 residue of Ncc is the most important phosphoacceptor site for Spak.
DISCUSSION

In this study, we first created defectively phosphorylated Ncc<sup>T58M/T58M</sup> mice to model human GS caused by homozygous NCC T60M mutation. Ncc<sup>T58M/T58M</sup> mice exhibited typical features of GS and a blunted response to thiazide, confirming that Ncc function was markedly diminished. Despite intact transcription of Ncc mRNA, total and p-Ncc (T53, T58, and S71) abundance in the kidneys of Ncc<sup>T58M/T58M</sup> mice were remarkably reduced and could not be rescued by the activated Spak of a low-salt diet. The impaired phosphorylation of NCC at T60, per se, also caused the decreased NCC membrane stability in MDCK cells. That the PHAII phenotype was overwhelmed by the persistently diminished T58M-Ncc protein expression in Ncc<sup>T58M/T58M/Wnk4<sup>D561A</sup></sup> mice further indicates that T58 of Ncc is the most important Spak/OSR1 phosphoacceptor site involved in Ncc protein expression and function.

Protein phosphorylation is a common post-translational modification that may alter intrinsic protein function, cellular localization, and stability. The T60 residue of NCC is a phosphoacceptor site for SPAK/OSR1 and phosphorylation of T60 has been shown to be crucial in triggering NCC activation because its phosphorylation-defective mutations (T60A or M) alter its intrinsic activity without affecting total and membrane abundance in the heterologous Oocyte expression system. However, impaired T60A NCC membrane expression in nonpolarized HEK cells after hypotonic stress stimulation was found, suggesting that defective phosphorylation of the T60 residue may also affect its membrane expression.

These in vitro findings in nonrenal tubular cells should be further validated in renal tubular cells such as polarized MDCK cells, genetically modulated animal models, or GS patients with homozygous T60M mutation. Reduced T60M-NCC expression in MDCK cells, markedly diminished Ncc abundance in the DCT cells of Ncc<sup>T58M/T58M</sup> mice, and decreased urinary NCC excretion, an index of membrane-expressed NCC, in GS patients all indicated that T60M led to reduced NCC expression primarily through post-translational modification (phosphorylation) rather than translational change. Because increased late DCT volume was found in Ncc<sup>T58M/T58M</sup> mice, similar to our previously reported Ncc<sup>S707X</sup> knock-in mice, the reduced total T58M-Ncc abundance may also be independent of the altered DCT cellular volume. Reminiscent of the markedly reduced total and phosphorylated Ncc expression in Wnk4<sup>−/−</sup> mice and Spak knockout mice as well as kinase-dead Spak knock-in mice, the constitutive inactivation of the Wnk4-Spak cascade may not only decrease Ncc phosphorylation but also reduce its accumulation in apical membranes.

Several hormones such as angiotensin II and aldosterone have been shown to affect Ncc phosphorylation through regulation of the Wnk4-Spak signal cascade. Acute infusion of angiotensin II enhances Ncc phosphorylation (at T53 and T58) and translocates Ncc from subapical areas to the apical membrane without affecting its total abundance. However, chronic infusion of angiotensin II and hyperaldosteronism induced by low Na<sup>+</sup> diet or aldosterone infusion increased both total and p-Ncc abundance through Wnk4-Spak signaling in WT and but not Wnk4 knockout mice. In addition, Ncc<sup>T58M/T58M/Wnk4<sup>D561A</sup></sup> mice still exhibited the GS phenotype with decreased total and p-Ncc expression, implying that even the constitutive activation of the Wnk4-Spak signal cascade could not rescue the expression of Ncc caused by defective phosphorylation at T58. In the cycloheximide run-down experiments, the constitutively phosphorylation-mimicking T60 mutant had much longer <i>t</i><sub>1/2</sub> than the defectively phosphorylated mutants, including T60M. Taken together, these results provide indirect and direct evidence that phosphorylation of NCC on T60 by SPAK affects its membrane stability and expression, akin to the phosphorylation of Aqp2 on S256 by protein kinase A.

The mechanisms underlying reduced NCC expression from defective phosphorylation on T60 residue remain incompletely clear. GS-causing missense mutations might reduce NCC activity through impaired protein processing or sorting, altering protein activity, or accelerating protein removal or degradation. Intact membrane expression of T60M and T60A NCC in the Xenopus Oocyte suggest that reduced antero- or endoplasmic reticulum export
from enhanced endoplasmic reticulum–associated degradation, which has been implicated in several GS-causing NCC missense mutations, is less likely. Adaptor protein (AP) and sortilin-related lysosomal degradation have also been reported to regulate NCC stability. The T60 residue of NCC, of note, is located in one of the putative canonical YXX\(\mu\) (YN\(\text{T58I}\)) binding motifs of the \(\mu\) subunit of AP complexes that are involved in the sophisticated post-Golgi network of protein sorting. It also remains unknown whether NCC phosphorylation participates in protein endocytosis through the NEDD4-2 ubiquitination pathway.

Ncc\(T58M/T58M\) mice also exhibited an exaggerated response to furosemide, which can be caused by the elimination of the compensatory response due to the marked diminution NCC in the DCT and by the enhanced NKCC2 phosphorylation and function in the upstream thick ascending limbs. Similarly, increased compensatory ENaC expression and function in the downstream distal tubules could account for the exaggerated response to amiloride, comparable to the finding in Wnk4 knock-out mice. These findings would also predict that Na\(^+\) reabsorption should be enhanced in the cortical thick ascending limbs and downstream distal tubules in patients with GS.

In summary, Ncc T58M homozygous knock-in mice exhibit the typical phenotype of GS patients and defective Ncc phosphorylation at T58 prevents the development of PHAII in the Wnk4 knock-in mice. Ncc T58M knock-in mice provide a good animal model to decipher the molecular mechanisms of Ncc phosphorylation, protein stability, and membrane expression. Blocking NCC phosphorylation at T60 may be a promising strategy for antihypertensive drug design, especially in conditions with activated WNK4-SPAK/OSR1 signaling.

**CONCISE METHODS**

**Blood and Urine Analyses and BP Measurement**

The mice were raised in a 12-hour day and night cycle, fed with normal rodent chow diet (Na\(^+:\) 0.4% [w/w]; K\(^+:\) 1.0% [w/w]; Ca\(^2+:\) 0.9% [w/w]) and plain drinking water ad libitum for 12–14 weeks. For the low Na\(^+\) diet challenge test, low Na\(^+\) rodent chow diet (Na\(^+:\) 0.05% [w/w]) was fed for 6 days. Mice were placed in metabolic cages for urine collection. Blood was drawn from the submandibular venous plexus under light ether anesthesia. Serum and urine biochemistry analysis was performed as previously described. The BPs of restrained conscious mice were measured with a programmable tail-cuff sphygmomanometer (MK-2000A; Muromachi, Tokyo, Japan). Hydrochlorothiazide, Furosemide, and Amiloride Challenge Studies

After a single dose of intraperitoneal hydrochlorothiazide (12.5 mg/kg), furosemide (15 mg/kg), or amiloride (5 mg/kg) was administered to WT and Ncc\(T58M/T58M\) littermates, urine samples (0–4 hours) were collected for analysis. Fractional excretion rate of a specific ion particle (X) (FEx) is defined as: urine X concentration * plasma Cr concentration/urine Cr concentration * plasma X concentration.
Quantitative Real-Time RT-PCR

Total kidney RNA was isolated by standard RT protocol and the gene expression of Ncc was assessed by real-time RT-PCR as previously described. The primers used for mouse Ncc and \(\beta\)-actin gene RT-PCR were as follows: Ncc: forward 5'-AGG CCA GCC ACT TAA CAC AC-3' (on exon 1), and reverse 5'-GGA TCA CTC CCC AGA TGT TG-3' (on exon 3); and \(\beta\)-actin: forward 5'-ACC ACA CCT TCT ACA ATG AGC-3', and reverse 5'-GGA ACA GTG TGG GTG ACC-3'.

Immunoblotting, Immunofluorescence, and Immunogold Stain

The preparation of kidney tissue for semiquantitative immunoblotting, immunofluorescence, and the analysis of the densitometry data was performed as previously described. The primary antibodies, which have been verified and used by us or other groups, were as follows: rabbit anti-Ncc (×200; Millipore), Nkcc2 (×200; Alpha Diagnostic), ENaC (\(\beta\)) (×200; Alomone), self-generated p-Ncc (T53, T58, and S71) and p-Nkcc2 (T96) (×200), mouse anti-calbindin-D28k (CBP-D28k) (×10,000; Swant), parvalbumin (×10,000; Swant), and goat anti-Aqp2 (×200; Santa Cruz). Immunogold labeling was performed as previously described and electron microscopic images were obtained using H7000 Hitachi electron microscopy. The semi-quantification of immunogold signals was performed as previously described.

HA-NCC Expression and Cell Surface Stability in the MDCK Cells

In brief, cells seeded in a six-well plate with 70%-90% confluence were transfected with pHM6 empty vector or HA-NCC (WT, T60M, T60A, or T60D) by Lipofectamine 2000 Transfection Reagent (Invitrogen) and cells were harvested 24 hours after transfection. To assess the cell surface stability of WT, T60M, T60A, and T60D HA-NCC in MDCK cells, fresh induction medium supplemented with 20 \(\mu\)g/ml cycloheximide was applied for 1, 2, and 4 hours before harvest of the cells. Whole cell lysate without the nuclear portion prepared as reported previously and membrane fraction prepared with the ProteoExtract native membrane protein extraction kit (Merck-Millipore) were applied for semiquantitative immunoblot. Rat anti-HA mAb (9Y10; Roche Diagnostics), mouse anti-\(\beta\)-actin (SC-47778; Sigma), and mouse anti-glyceraldehyde 3-phosphate dehydrogenase (MAB374; Millipore) were used.

Figure 7. Ncc T58M mutation resistant to activated Spak stimulation in vivo. Semi-quantitative immunoblotting of p-Spak, total Ncc, and p-Ncc expression. (A) Low Na\(^+\) (L) diet enhances Spak phosphorylation in both WT and Ncc T58M homozygous knock-in mice. However, enhanced total and p-Ncc abundance are only observed in WT rather than Ncc T58M homozygous knock-in mice. *P<0.05 L diet versus normal Na\(^+\) (N) diet. (B) Constitutive activation of Wnk4-Spak signaling cannot rescue the expression of Ncc in Ncc T58M homozygous with Wnk4 D561A heterozygous double knock-in mice (n=3/group). *P<0.05 versus WT.

NCC Mutation Analyses and Immunoblotting for NCC in Urine

The study protocol was approved by the Ethics Committee on Human Studies at Tri-Service General Hospital, National Defense Medical Center, Taiwan. The participants were given a detailed description of the study before they provided informed consent. Genomic DNA was isolated and purified from peripheral blood of the healthy participants, disease controls, and patients and family members for PCR amplification.
of individual exons of the SLC12A3 and RFLP analysis of the mutated exon of SLC12A3 was performed as previously described.9,15

Fresh first morning voided urine from controls and patients was collected in a bottle that contained a collection solution 25× complete protease inhibitor cocktail (Roche). After measuring the volume, the sample was spun at 1300×g for 15 minutes at 4°C to remove cells, nuclei, and large fragments. The supernatant was then transferred to six 8-ml high-speed tubes for ultracentrifugation (200,000×g for 12 hours at 4°C). Urine creatinine was measured in this supernatant. The resultant pellets were suspended with 120 μl of isolation solution (0.25 mol/L of sucrose, 10 mmol/L of triethanolamine, 1 mM of sodium orthovanadate, 50 mM of sodium fluoride, 1 mM of EGTA, 1 mM of EDTA, pH 7.6 and 1× complete protease inhibitor cocktail [Roche]). Samples were then stabilized by adding 30 μl of 5× Laemmli sample buffer (SDS, 3.75 g; glycerol; 15 ml; 1 mol/L of Tris, pH 6.8, 2.5 ml, bromophenol blue dab, ddH2O to 50 ml), heated to 70°C for 15 minutes, and stored at −80°C until analysis. Just before being loaded for electrophoresis, samples were warmed to 37°C.27

Five milligrams of urine creatinine equivalent of each sample was loaded into each lane of gel and electrophoresed in 7.5% (for NCC) polyacrylamide-SDS minigels. Immunoblotting and instant blue (Genemark) staining were performed as described previously.23

**Statistical Analyses**

All results are expressed as mean ± SD. We used one-way analysis of covariance to compare the difference among three groups (WT, Ncc T58M/+ , and Ncc T58M/T58M knock-in littermates). The Mann–Whitney U test was used when the variables between two groups were not normally distributed. A P value <0.05 was considered statistically significant.

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Supplemental information

Concise Methods

Generation of Ncc T58M knock-in mice

The experimental protocols used in the present study were approved by the Institutional Animal Care and Use Committee of the National Defense Medical Center, Taipei, Taiwan. To generate Ncc T58M knock-in mice, the targeting vector was prepared by the gap-repair technique. Mouse Slc12a3 genomic DNA encoding Ncc was isolated from a 129/Sv genomic DNA BAC library. The nonsense mutation Ncc T58M (TCT to TAG) was introduced by PCR when preparing the targeting vector. The entire sequence was verified by sequencing. The targeting vector was then transfected into R1 ES cells (129X1/SvJ x 129S1) by electroporation. After selection with 240μg/ml G418 and 2μM gancyclovir, correctly targeted ES clones were selected by Southern blotting. The neo cassette was excised by transfecting the Cre-expression plasmid into the targeted Ncc$flox/+ ES cells. The Ncc$^{T58M/+}$ ES cells were selected by PCR (Primers, F: 5’-TGC CCA ACA TGT TCT GTC AT TA-3’; R: 5’-CGG TGC CTG GAC AAT ATG TA-3’), and injected into C57BL/6 blastocysts. Chimeric males were bred with C57BL/6 females to produce heterozygous Ncc$^{T58M/+}$ mice (F1). Homozygous Ncc$^{T58M/T58M}$ mice (F2) were produced by mating Ncc$^{T58M/+}$ mice (F1) with each other. WT, Ncc$^{T58M/+}$ and Ncc$^{T58M/T58M}$ littermates (F2) were bred
and tail genomic DNA applied for genotyping by PCR.

**Results**

**Generation of Ncc T58M knock-in mice**

To generate Ncc T58M knock-in mice, we used homologous recombination in ES cells to create a mutant allele in which exon 1 of the Slc12a3 gene encoding Ncc was replaced by a cassette expressing the selectable marker neomycin transferase (neo) followed by the mutant exon 1 (T58M) (Supplemental Figure S1). Twenty recombinant ES cells were found to be Ncc$^{\text{floxed/+}}$ confirmed by Southern blot (Supplemental Figure S1). The neo cassette was excised by transfecting the Cre-expression plasmid into the selected Ncc$^{\text{floxed/+}}$ ES cells and then confirmed by Southern blot. We obtained 4 chimeric mice from two different selected Ncc$^{\text{T58M/+}}$ clones (D6 and E7) and then crossed them with C57BL/6 mice to produce mutant Ncc$^{\text{T58M/+}}$ progeny. The Ncc$^{\text{T58M/T58M}}$ mice were then generated by crossing Ncc$^{\text{T58M/+}}$ littermates. The genotype of the offspring was verified by PCR amplification (Supplemental Figure 1C) and direct sequencing of exon 1 of the Slc12a3 genomic DNA (Supplemental Figure S1). Because there were no differences in gross appearance and phenotype between D6 and E7 Ncc T58M knock-in mice, the D6 strain was selected for this study. Ncc$^{\text{T58M/+}}$ and Ncc$^{\text{T58M/T58M}}$ mice grew normally and were indistinguishable from wild-type (WT) littermates in appearance, behavior and
fertility.

Reference

**Figure legends**

**Supplemental Figure 1. Generation of Ncc T58M knock-in mice**

(A) Targeting strategy for generating Ncc T58M knock-in mice. The diagram shows the wild-type (WT) Slc12a3 locus encoding Ncc, the targeting construct, and the targeted locus before (flox) and after Cre recombination (T58M). (B) Homologous recombination verified by Southern blotting of TthIII I-digested genomic DNA derived from the selected ES cells (D6 and E7) before deleting neomycin transferase (neo) cassette. The 17.8-kb and 9.6-kb bands are derived from the WT and mutated (flox) alleles, respectively. C: control genomic DNA from WT ES cells. (C) Genomic DNA derived from Ncc T58M knock-in mice were used as template and a primer set flanking the remaining loxP site (arrow) was used for genotyping. The 229bp and 134bp bands represent the mutant (T58M) and WT alleles, respectively. (D) Direct sequencing of the PCR product covering the mutation site in exon 1 from WT, heterozygous (He), and homozygous (Ho) Ncc T58M knock-in mice.

**Supplemental Figure 2. p-Ncc expression in kidneys of Ncc^{T58M/T58M} mice.**

(A) Semiquantitative immunoblot of p-Ncc (T53, T58, and S71) in crude membrane fractions from kidneys of wild-type (WT) and homozygous (Ho) Ncc T58M knock-in mice, and its semi-quantification by densitometry analysis (n = 4/group). * denotes p<0.05 vs. WT comparison. (B) Immunofluorescence (IF) staining for p-Ncc (T53, T58, and S71). Representative IF micrographs of Ncc (low power field 200x and high
power field 1000x) in the cortices of WT and Ho mice.

**Supplemental Figure 3. Total Nkcc2, p-Nkcc2 and ENaC(β) expression in kidneys of Ncc<sup>T58M/T58M</sup> mice.**

(A) Semiquantitative immunoblot of total Nkcc2, p-Nkcc2 (T96) and ENaC(β) in crude membrane fractions from kidneys of wild-type (WT) and homozygous (Ho) Ncc T58M knock-in mice, and its semi-quantification by densitometry analysis (n = 4/group). * denotes p<0.05 vs. WT comparison. (B) Immunofluorescence (IF) staining for total Nkcc2, p-Nkcc2 (T96) and ENaC(β). Representative IF micrographs of total Nkcc2, p-Nkcc2 and ENaC(β) (low power field 200x and high power field 1000x) in the outer medulla of WT and Ho mice.

**Supplemental Figure 4. NCC mutation analysis**

Schematic showing a representative family with NCC T60M mutation diagnosed by exon 1 sequencing directly and restriction fragment length polymorphism (RFLP) by PvuI. The patient number is the same showing in figure 5.
Supplemental Fig. 1

(A) WT Mutant
Human ACG → ATG
Mouse ACC → ATG
Amino acid Thr → Met

WT Slc12a3 locus
Probe
Targeting construct
Targeted locus (floxed)
Targeted locus (T58M)

TthIII I

17.8kb
134bp

Homology recombination

Cre recombination

17.8kb
134bp

229bp

(B) WT

C D 6 E 7

WT (17.8kb)

flox (9.6kb)

(C) WT He Ho

WT (134bp)

T58M (229bp)

(D) WT

Thr58

He

A C C A T G

Met58

A T A

Ho

A A C A T G

Met58
Supplemental Fig. 3

(A) WT and Ho for Nkcc2, p-Nkcc2 (T96), and ENaC(β)

(B) Fluorescent images of WT and Ho for Nkcc2 and p-Nkcc2 (T96)
Supplemental Fig. 4

![Supplemental Fig. 4](image-url)