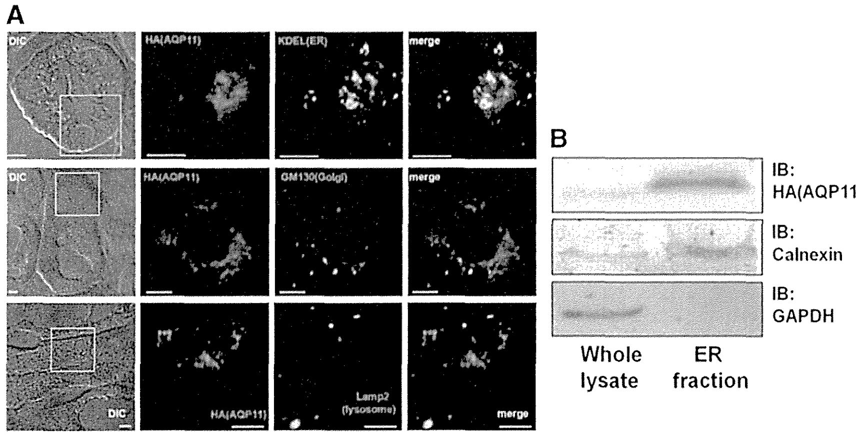

Figure 4. Subcellular localization of AQP11 in the Tg^{AQP11} mouse. (A) Immunofluorescence of Tg^{AQP11} mouse kidney with anti-HA antibody and organelle markers. The AQP11 immunostaining overlapped with KDEL (an ER marker) and not GM130, a Golgi marker, and Lamp2, a lysosome marker. Scale bar, 5 μm. (B) Immunoblotting analysis of the ER fraction from the Tg^{AQP11} mouse kidney. Calnexin was used as the ER marker. Robust expression of AQP11 was found in the ER.

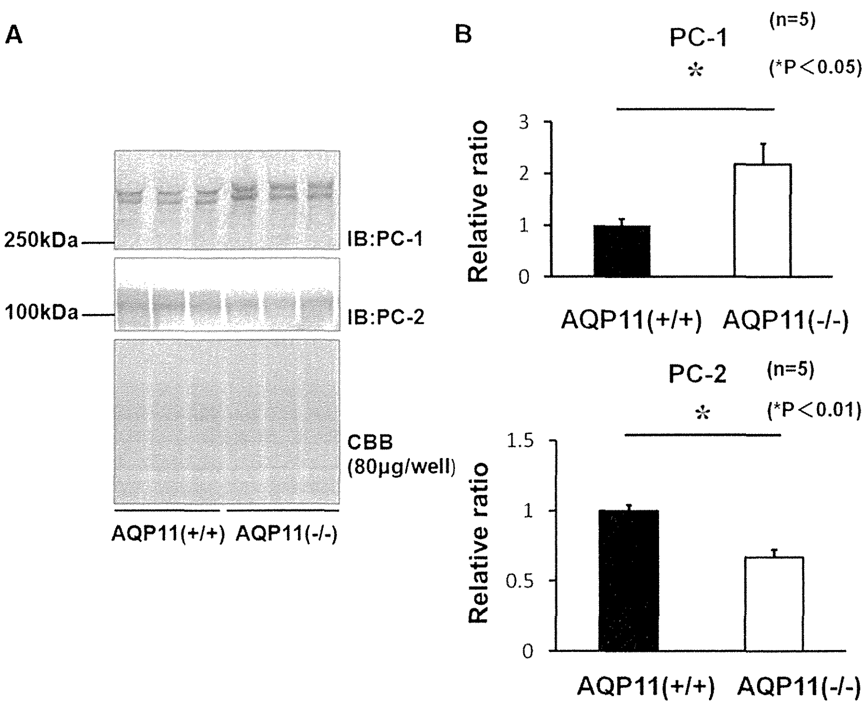

Figure 5. PC-1 and PC-2 in the AQP11(−/−) kidney. (A) Western blot of PC-1 and PC-2 in 2-week-old WT and AQP11(−/−) mouse kidneys. Western blot of PC-1 detected an altered electrophoretic mobility of PC-1 in AQP11(−/−) mouse kidneys compared with WT littermates, suggesting aberrant protein modification. (B) Densitometric analyses of PC-1 and PC-2 in AQP11(−/−) mouse kidneys. The relative levels of PC-1 and PC-2 expressions were determined by normalization to overall CBB staining in each lane. The PC-1 protein level in the kidneys of AQP11(−/−) mice was increased compared with that of WT littermates, and the PC-2 protein level in the kidneys of AQP11(−/−) mice was decreased compared with that of WT littermates. (Upper panel) $*P<0.05$. (Lower panel) $*P<0.01$.

glycosylation processing of PC-1 at the ER.[9,22] However, both kidney homogenates from GIIβ knockout mice and immortalized epithelial cells from kidney tubules of SEC63p knockout mice showed decreased protein expression of PC-1, which was opposite to the increased expression of PC-1 in AQP11(−/−) mice.[22] Although GIIβ protein was checked in AQP11(−/−) mouse kidneys, no difference was found in the GIIβ protein expression level in AQP11(−/−) mouse kidneys (data not shown). Taken together, AQP11 may play a role in glycosylation processing of PC-1 at the ER in a different manner from GIIβ and SEC63p. However, the reduction of EndoH-resistant PC-1 and impaired trafficking of PC-1 were observed in both AQP11(−/−) and GIIβ knockout mice.[22] It is possible that aberrantly glycosylated PC-1 in the AQP11(−/−) mouse is retained in the ER, similar to the GIIβ knockout mouse. Although PC-1 protein levels in the kidneys of AQP11(−/−) mice were increased, loss of function of PC-1, due to, impaired membrane localization, is considered to be the main cause of renal cystogenesis in AQP11(−/−) mice, which could represent a common mechanism of cystogenesis for ER proteins. In addition, PC-1 mRNA levels were not increased.[23] These data indicated that aberrantly glycosylated PC-1 might fail to enter the degradation pathway in AQP11(−/−) kidney because of the inability of PC-1 to exit the ER in the AQP11(−/−) kidney. Additional investigation will be required to clarify this issue.

In this study, it was shown that PCs are involved in the mechanism of cystogenesis in AQP11(−/−) mice; however, the true function of AQP11 at the ER remains unclear. It has been reported that proximal tubule cells exhibited ER vacuolization in AQP11(−/−) mouse kidneys.[4] In addition, increased ER stress response and oxidative stress in AQP11(−/−) mice were recently reported.[23–25] Therefore, AQP11 may play an important role in the homeostasis of the ER, and the absence of AQP11 could lead to ER dysfunction. However, to the best of our knowledge, there are no reports showing that renal cystogenesis is caused by nonspecific disruption of ER function. In this study, we clarified a

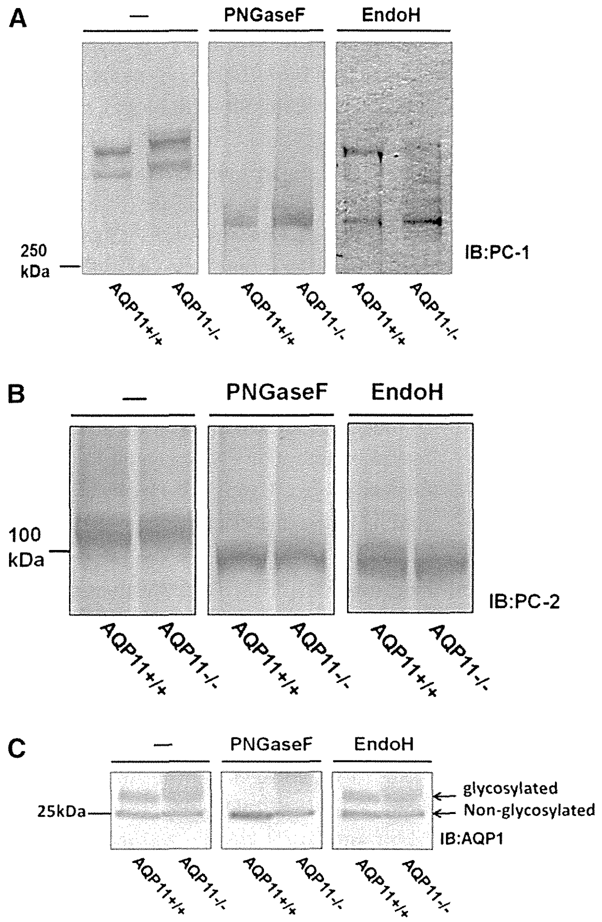

Figure 6. Aberrant glycosylation of PC-1 in AQP11(−/−) mouse kidney. (A) Treatment of PC-1 with PNGaseF reduced the size of the two products of abnormally modified PC-1 in AQP11(−/−) mice to the same molecular mass as the deglycosylated PC-1 product of WT mice. In addition, the upper PC-1 band in WT mice was EndoH-resistant, whereas the lower band was EndoH-sensitive. In contrast, the upper and lower bands in AQP11(−/−) kidney were EndoH-sensitive, with both bands being reduced in size to that of the EndoH-sensitive lower band from WT mice. (B) Treatment of PC-2 with PNGaseF and EndoH made no differences in molecular mass between WT and AQP11(−/−) mice both before and after treatment. To more readily detect the effect of deglycosylation, a greater amount of AQP11(−/−) kidney homogenate was loaded. (C) Treatment of AQP1 with PNGaseF and EndoH made no differences in molecular mass between WT and AQP11(−/−) mice both before and after treatment.

defective glycosylation in PC-1 in the proximal tubules of AQP11(−/−) mice but not other proteins, such as AQP1 and PC-2. Therefore, the absence of AQP11 in the ER might specifically lead to aberrant glycosylation of PC-1.

In this study, in contrast to PC-1, we observed decreased protein expression levels of PC-2 in the AQP11(−/−) kidney. It was previously reported that PC-2 mRNA levels are not altered significantly in AQP11(−/−) kidneys compared with wild type.[23] PC-2 could be misfolded, less stable, and susceptible to degradation because of the loss of AQP11 protein in the ER, and the mechanism of degradation for PC-1 and PC-2 could differ. In addition, although it is well known that inactivation of PKD2 leads to renal cystogenesis,[26–29] Wu *et al.*[26] reported that Pkd2(+/−) mice showed only limited renal cytogenesis in the age range of 9–17 weeks. Considering that the membrane trafficking of PC-2 is still observed in AQP11(−/−) mouse kidneys, the decreased PC-2 protein level in the kidneys of AQP11(−/−) mice at 2 weeks old might not be the main cause of renal cystogenesis in the AQP11(−/−) kidney. Additional investigation will be required.

Interestingly, although AQP11 does not localize to primary cilia, elongated primary cilia of proximal tubules in AQP11(−/−) mouse kidneys were observed (Figure 9). To the best of our knowledge, this case is the first in which ER protein was found to regulate the ciliary length of kidney tubules. Hopp *et al.*[11] recently reported that the ciliary length increase could be a compensatory response to reduced functional PC-1, suggesting that elongation of cilia is caused by loss of PC-1 function in AQP11(−/−) mice. However, the Pkd1(+/−) background did not change the ciliary length of proximal tubules in AQP11(−/−) mice (Figure 9). These data indicate that elongation of cilia in AQP11(−/−) mice might not be solely dependent on PC-1. In addition, these data also indicate that elongated primary cilia of proximal tubules in the kidneys of AQP11(−/−) mice might not play a major role in the mechanism of renal cystogenesis, although it is very interesting that an ER protein was found to regulate the ciliary length of kidney tubules. Additional investigation will be required.

In summary, impaired glycosylation processing and aberrant membrane trafficking of PC-1 could be key mechanisms of cystogenesis in AQP11(−/−) mice. The pathogenesis underlying PKD phenotypes remains unclear. It has been reported that aberrant PC-1 post-translational modifications, such as glycosylation, phosphorylation, and cleavage, are related to cyst formation.[10,22,30–33] Additional investigation of post-translational modifications of PC-1 will be required to clarify the pathogenesis of cystogenesis in PKD.

CONCISE METHODS

Mouse Lines

AQP11 BAC transgenic mice were generated by PhoenixBio (Utsunomiya, Tochigi, Japan). BMQ33M09, a 160-kb BAC clone containing the whole exons of mouse AQP11 with its promoter region was prepared. The galactokinase selection system was used for BAC recombineering,[34] and a 3×HA tag flanked by the N terminus of AQP11 of BAC was inserted. To select transgenic mice by Southern blotting and select AQP11(−/−)Tg^{AQP11} mice by PCR and *Eco*RI digestion, the modified BAC also contained a new *Eco*RI site in intron 1. The transgene was injected into fertilized eggs of C57BL/6J mice, and transgenic mice were obtained. Founder transgenic mice were identified by PCR and Southern blot analysis, and offspring were

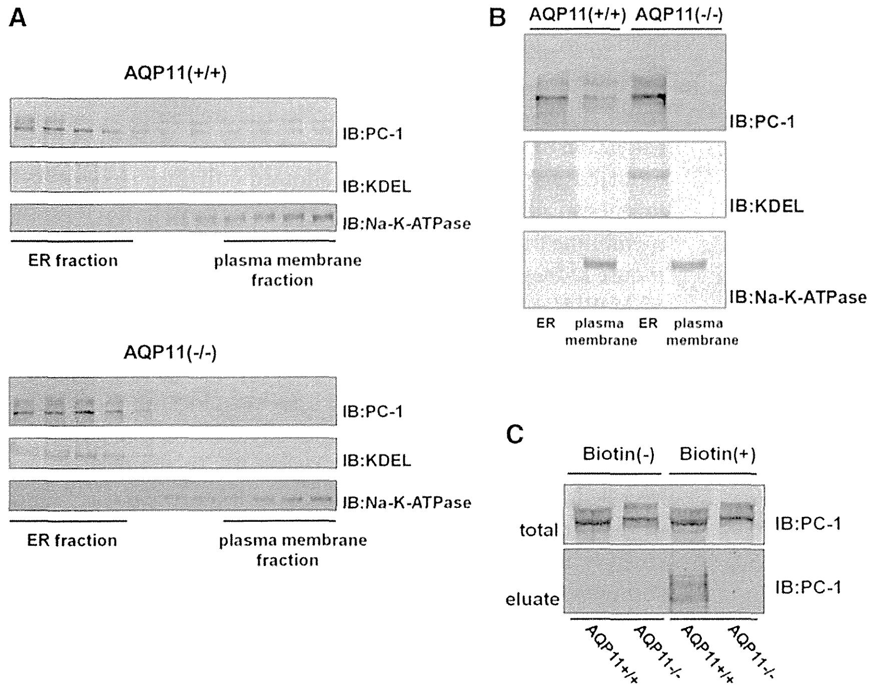

Figure 7. Impaired membrane trafficking of PC-1 in AQP11(−/−) mouse kidneys. After subcellular fractionation of kidney homogenates, samples of (A) individual fractions or (B) ER and plasma membrane fractions were analyzed by immunoblotting with antibodies against PC- 1, KDEL, and Na-K-ATPase. PC-1 protein expression in the plasma membrane fraction was clearly decreased in AQP11(−/−) kidneys compared with that of WT kidneys. (C) *In vivo* protein biotinylation showed that cell surface PC-1 protein expression *in vivo* was clearly decreased in AQP11(−/−) kidneys compared with WT.

genotyped by PCR. PCR primers were designed on both sides of the 3×HA tag. The forward primer was 5-AGGTCACATCTGCACAGCGC-3, the reverse primer was 5-ACGGGCCTGTGTAGCTGTTG-3, and the resulting amplification product was 380 bp. AQP11(+/−) mice[4] and Pkd1(+/−) mice[35,36] were used as previously reported. AQP11(−/−)Tg^{AQP11} mice were genotyped by PCR and *Eco*RI digestion. PCR primers were designed on both sides of the new *Eco*RI site. The forward primer was 5-TACTGCTGTGGCATGAGCAG-3 and the reverse primer was 5-GTTCCAAGGTATCCAGGGC-3. All animal studies were performed using the procedures approved by the Institutional Animal Care and Use Committee at the Tokyo Medical and Dental University.

Immunoblot Analyses

Kidneys were dissected as previously reported.[37] Kidney homogenates of 2-week-old mice without the nuclear fraction (600 g) were prepared to measure the levels of PC-1, PC-2, and AQP1. The crude membrane fractions (17,000-g pellet) of 3-week-old mouse kidneys were prepared to measure the levels of AQP11. Semiquantitative immunoblotting was performed as described previously.[38] The relative intensities of immunoblot bands were determined by densitometry with YabGelImage (free software). The primary antibodies used were rat anti-HA (Roche Applied Science, Penzberg, Germany), goat anticalnexin (Santa Cruz Biotechnology, Santa Cruz, CA), mouse anti-glyceraldehyde 3-phosphate dehydrogenase (Santa Cruz Biotechnology), mouse anti–PC-1 (7e12; Santa Cruz Biotechnology), rabbit anti–PC-2 (H-280; Santa Cruz Biotechnology), mouse anti-KDEL (Enzo Life Sciences, Farmingdale, NY), rabbit anti–Na-K-ATPase (Santa Cruz Biotechnology), rabbit anti-AQP1 (Sigma-Aldrich, St. Louis, MO), mouse anti-GM130 (BD Transduction Laboratories, San Jose, CA), guinea pig anti-NCC,[37] and rabbit anti–UT-A1 antibodies.[39] Alkaline phosphatase-conjugated anti-IgG antibodies (Promega, Madison, WI) and WesternBlue (Promega) were used to detect the signals. Deglycosylation assays by PNGaseF and EndoH (New England Biolab, Beverly, MA) were performed according to the manufacturer's protocol.

Immunohistochemical Analyses

Immunohistochemical analyses were performed on cryostat sections of Tg^{AQP11} and control littermate kidneys as well as formalin-fixed sections of AQP11(−/−) mice and their counterparts as previously reported.[40] The primary antibodies included rat anti-HA (Roche Applied Science), rabbit anti-HA (EMD Millipore, Billerica, MA), rabbit anti-AQP1 (Sigma-Aldrich), guinea pig anti-NCC,[37] mouse anti-KDEL (Enzo Life Sciences), mouse anti-GM130 (BD Transduction Laboratories), rat anti-Lamp2 (Developmental Studies Hybridoma Bank, Iowa City, IA), and mouse anti–α-acetylated tubulin (Sigma-Aldrich). Alexa fluor (Molecular Probes; Invitrogen, Carlsbad, CA) was used for secondary antibodies. Fluorescent *dolichos biflorus* agglutinin and *lotus tetragonolobus* lectin were purchased from Vector Laboratories (Burlingame, CA). Immunofluorescent images were obtained using an LSM510 laser-scanning confocal microscope system (Carl Zeiss, Oberkochen, Germany).

Isolation of ER from the Kidney

For detailed analysis of the intracellular localization of AQP11, ER was isolated from Tg^{AQP11} mouse kidneys, and immunoblotting was performed. To obtain the ER from the mouse kidneys, an ER extraction kit (Imgenex, San Diego, CA) was used following the manufacturer's instructions as previously reported.[41]

Subcellular Fractionation

Kidney lysates of 2-week-old mice were fractionated by density gradient centrifugation as previously reported.[13] After kidney homogenization in ice-cold homogenization buffer (250 mM sucrose, 1 mM EDTA, and 10 mM Tris-HCl, pH 7.5) containing protease inhibitors (protease inhibitor cocktail; Roche), a postnuclear supernatant was prepared by centrifugation at 1000 rpm for 10 minutes. Postnuclear supernatant was then layered onto a continuous 0%–15% Optiprep (Axis Shield PoC AS, Oslo,

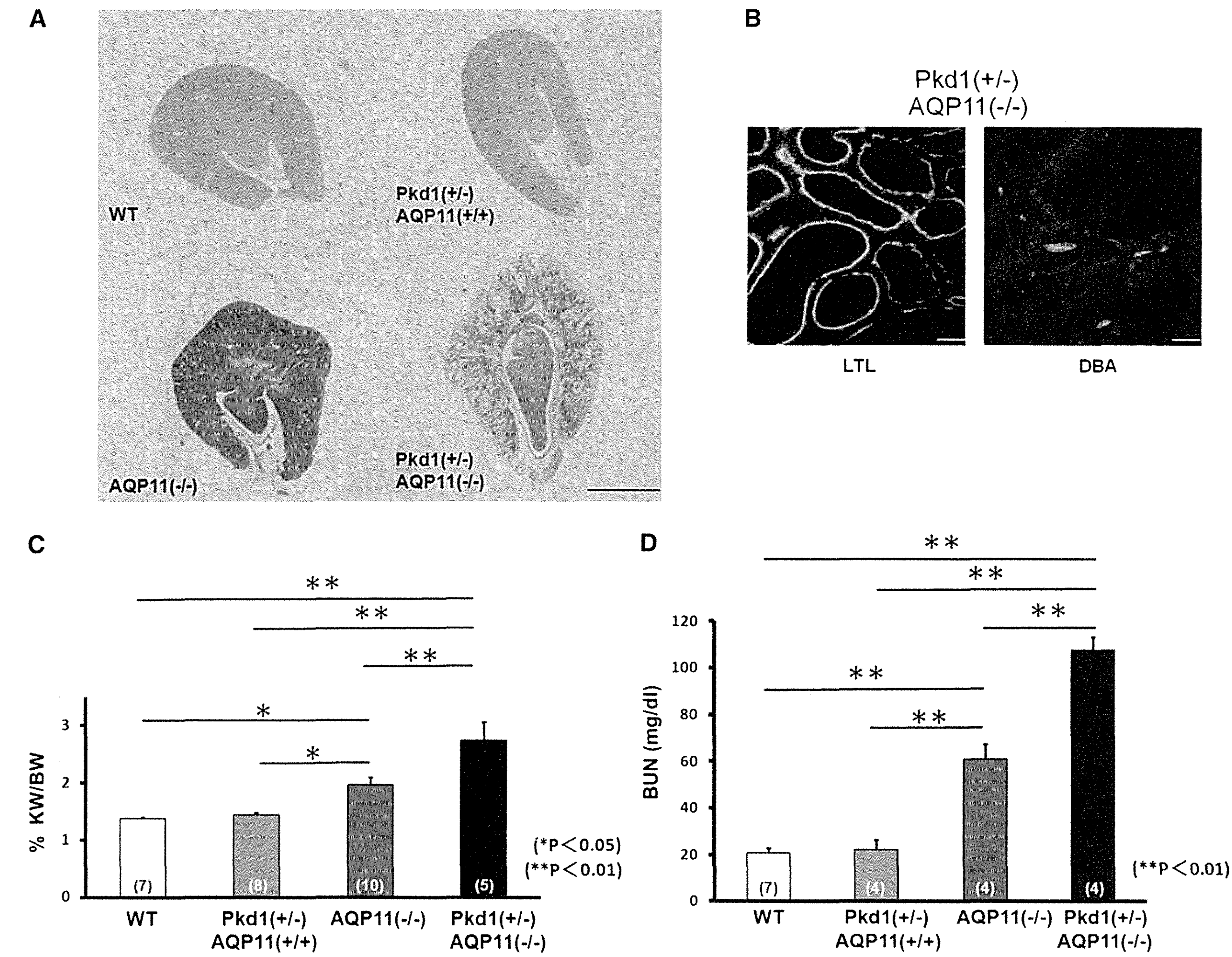

Figure 8. Pkd1(+/−) background resulted in increased severity of PKD in AQP11(−/−) mice. (A) Histologic kidney sections from littermates of the indicated genotypes at postnatal day 12. WT and Pkd1(+/−) mice showed no cysts. The Pkd1(+/−) background resulted in increased severity of PKD in AQP11(−/−) mice. Scale bar, 1 mm. (B) Fluorescent staining with LTL and DBA in 3-week-old Pkd1(+/−)AQP11(−/−) kidneys. The segmental origins of the cysts were mainly localized in the proximal tubules, which is consistent with AQP11(−/−) kidney. Scale bar, 50 μm. (C) The kidney-to-body weight ratio (KW/BW) and (D) BUN showed significant increases in Pkd1(+/−)AQP11(−/−) mice compared with their counterparts. *$P<0.05$; **$P<0.01$.

Norway) gradient and centrifuged at 200,000×*g* for 3 hours at 4°C using a swinging bucket rotor. The individual fractions were recovered from the top with a piston gradient fractionator. Equal amounts of samples were loaded and analyzed for PC-1, PC-2, KDEL, and Na-K-ATPase expressions. Finally, the ER and plasma membrane fractions were analyzed for PC-1, PC-2, KDEL, and Na-K-ATPase expressions.

In Vivo Protein Biotinylation

Proteins from 2-week-old mice were biotinylated *in vivo* as previously reported.[42] In brief, mice were anesthetized, the chest was opened, the left ventricle of the heart was punctured with a perfusion needle, and a small cut was made in the right atrium to allow outflow of the perfusion solutions. After blood components were washed away with prewarmed PBS supplemented with 10% (wt/vol) dextran 40 (GE Healthcare, Little Chalfont, United Kingdom), the mouse was perfused with biotinylation solution. The perfusion solution contained 1 mg/ml sulfo-NHS-LC-biotin (Pierce, Rockford, IL) in PBS (pH 7.4) and 10% (wt/vol) dextran 40. To neutralize unreacted biotinylation reagent, the mouse was then perfused with 50 mM Tris in PBS with 10% (wt/vol) dextran 40. After perfusion, kidneys were excised and freshly snap-frozen for preparation of organ homogenates. After homogenization with ice-cold homogenization buffer (1% Triton X-100, 150 mM NaCl, and 10 mM Tris-HCl, pH 7.5) containing protease inhibitor (protease inhibitor cocktail), homogenates were centrifuged at 16,100×*g* for 5 minutes at 4°C, and 3 mg total protein extract was added to a Streptavidin-Sepharose slurry (100 μl/sample; GE Healthcare). The biotinylated proteins were

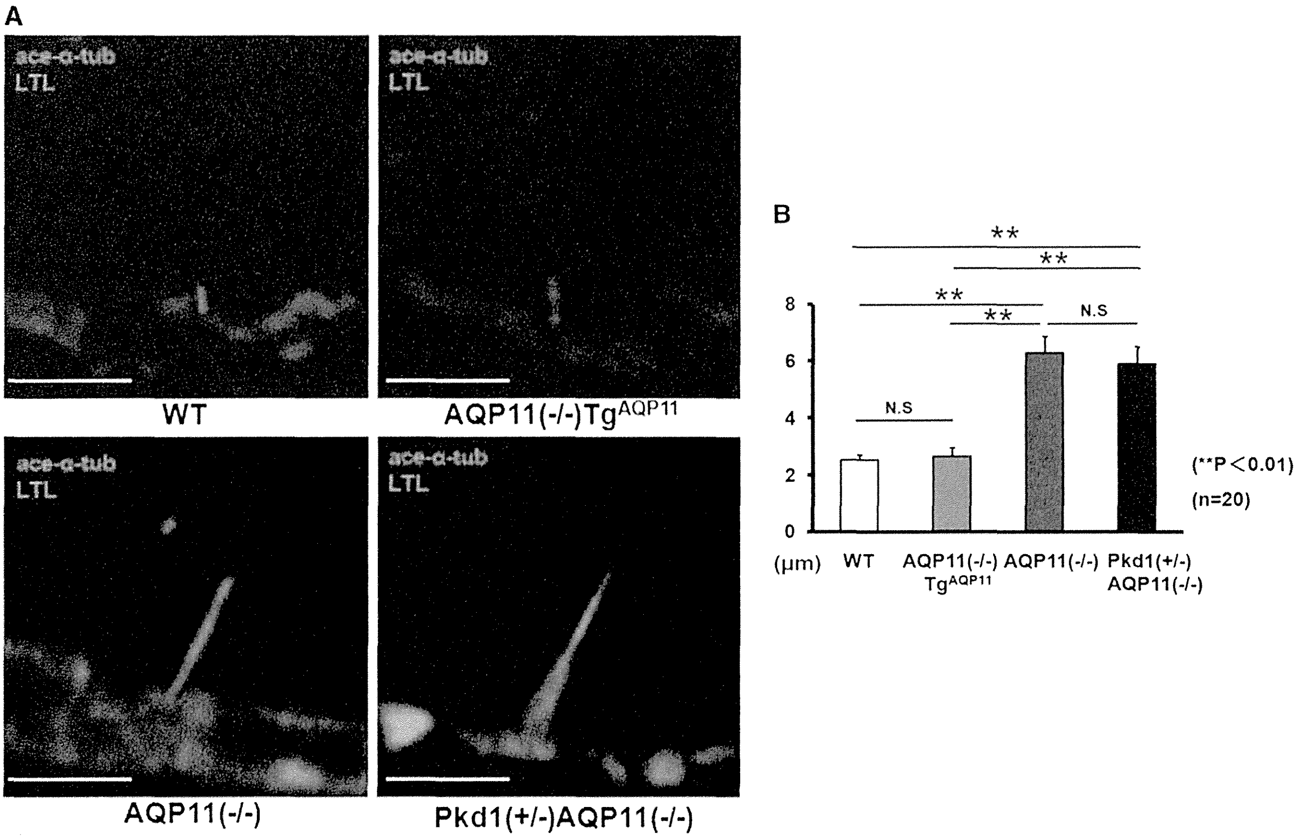

Figure 9. Primary cilia in proximal tubules were elongated in AQP11(−/−) mice. (A) Immunofluorescent labeling of kidney sections from 3-week-old WT, AQP11(−/−)Tg[AQP11], AQP11(−/−), and Pkd1(+/−)AQP11(−/−) mice with antiacetylated α-tubulin (red) and LTL (green). Scale bar, 5 μm. (B) Quantification of cilia length in kidney sections. The cilia lengths of proximal tubules in AQP11(−/−) and Pkd1(+/−)AQP11(−/−) mice were significantly longer than in WT mice. In addition, Tg[AQP11] rescued abnormal ciliary length in AQP11 (−/−) mice. **P<0.01.

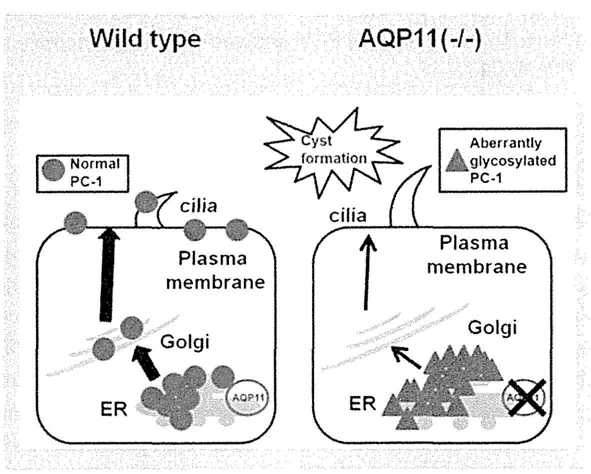

Figure 10. Schematic illustration of the mechanism of renal cystogenesis in the AQP11(−/−) kidney. Aberrant glycosylation processing and defective membrane trafficking of PC-1 in AQP11 (−/−) mice resulted in kidney cyst formation.

captured overnight at 4°C in a rotating mixer. The resin was washed three times and resuspended in 100 μl 2× SDS sample buffer at 60°C for 30 minutes.

Statistical Analyses

Comparisons between the two groups were performed using unpaired t tests. An ANOVA with Tukey *post hoc* test was used to evaluate significance in comparisons among multiple groups. P values <0.05 were considered significant. Data are presented as means±SEMs.

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DISCLOSURES

None.

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Impaired degradation of WNK1 and WNK4 kinases causes PHAII in mutant KLHL3 knock-in mice

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Pseudohypoaldosteronism type II (PHAII) is a hereditary disease characterized by salt-sensitive hypertension, hyperkalemia and metabolic acidosis, and genes encoding with-no-lysine kinase 1 (WNK1) and WNK4 kinases are known to be responsible. Recently, Kelch-like 3 (KLHL3) and Cullin3, components of KLHL3-Cullin3 E3 ligase, were newly identified as responsible for PHAII. We have reported that WNK4 is the substrate of KLHL3-Cullin3 E3 ligase-mediated ubiquitination. However, WNK1 and Na–Cl cotransporter (NCC) were also reported to be a substrate of KLHL3-Cullin3 E3 ligase by other groups. Therefore, it remains unclear which molecule is the target(s) of KLHL3. To investigate the pathogenesis of PHAII caused by KLHL3 mutation, we generated and analyzed KLHL3$^{R528H/+}$ knock-in mice. KLHL3$^{R528H/+}$ knock-in mice exhibited salt-sensitive hypertension, hyperkalemia and metabolic acidosis. Moreover, the phosphorylation of NCC was increased in the KLHL3$^{R528H/+}$ mouse kidney, indicating that the KLHL3$^{R528H/+}$ knock-in mouse is an ideal mouse model of PHAII. Interestingly, the protein expression of both WNK1 and WNK4 was significantly increased in the KLHL3$^{R528H/+}$ mouse kidney, confirming that increases in these WNK kinases activated the WNK-OSR1/SPAK-NCC phosphorylation cascade in KLHL3$^{R528H/+}$ knock-in mice. To examine whether mutant KLHL3 R528H can interact with WNK kinases, we measured the binding of TAMRA-labeled WNK1 and WNK4 peptides to full-length KLHL3 using fluorescence correlation spectroscopy, and found that neither WNK1 nor WNK4 bound to mutant KLHL3 R528H. Thus, we found that increased protein expression levels of WNK1 and WNK4 kinases cause PHAII by KLHL3 R528H mutation due to impaired KLHL3-Cullin3-mediated ubiquitination.

INTRODUCTION

Pseudohypoaldosteronism type II (PHAII) is a hereditary disease characterized by salt-sensitive hypertension, hyperkalemia, and metabolic acidosis (1,2). Mutations in with-no-lysine kinase 1 (WNK1) and WNK4 genes were reported to be responsible for PHAII (3). It was previously demonstrated that the WNK kinase family phosphorylates and activates oxidative stress-responsive kinase 1 (OSR1) and STE20/ SPS1-related proline/alanine-rich kinase (SPAK) (4,5), and that activated OSR1/SPAK kinases could phosphorylate and activate Na–Cl cotransporter (NCC), constituting the WNK-OSR1/SPAK-NCC phosphorylation signaling cascade. This regulation of NCC by WNK-OSR1/SPAK signaling was confirmed *in vivo* using various genetically engineered mouse models (6–14). Through analysis of the WNK4 knock-in PHAII mouse model, constitutive activation of this WNK-OSR1/SPAK-NCC phosphorylation cascade in kidney was found to be the major pathogenic mechanism of PHAII.

Recently, two additional novel genes, Kelch-like 3 (KLHL3) and Cullin3, were identified as responsible for PHAII (15,16). KLHL3 is a member of the BTB–BACK–Kelch family, which are known as substrate adapters of Cullin3-based E3 ubiquitin ligase complexes (17–20). We and others have reported

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that KLHL3 interacts with Cullin3 and WNK4, induces WNK4 ubiquitination and reduces WNK4 protein levels in cultured cells and *Xenopus laevis* oocytes (21–24). Interestingly, it was also reported that WNK1 could be a substrate of KLHL3-Cullin3 E3 ubiquitin ligase (21,24). Moreover, another group reported that KLHL3 was able to bind to NCC and regulate its intracellular localization in cultured cells (16). Therefore, it remains unclear which molecule involved in the pathogenesis of PHAII is the *in vivo* target of KLHL3. In addition, the above experiments were performed in cultured cells. Thus, it is necessary to clarify the role of KLHL3 mutation in PHAII pathogenesis *in vivo*.

In this study, to answer these questions, we generated KLHL3$^{R528H/+}$ knock-in mice that carry the same mutation as autosomal dominant type PHAII patients. This KLHL3$^{R528H/+}$ knock-in PHAII model mouse revealed that increased protein expression levels of WNK1 and WNK4 kinases, due to impaired binding of KLHL3 with WNK kinases, cause PHAII *in vivo*. These results also indicated that both WNK1 and WNK4 are physiologically regulated by KLHL3-Cullin3-mediated ubiquitination *in vivo*.

RESULTS

Generation of KLHL3$^{R528H/+}$ knock-in mice

KLHL3$^{R528H/+}$ knock-in mice were generated using homologous recombination in Balthal embryonic stem (ES) cells to create a mutant allele (25). Exon 15 of the Klhl3 gene was replaced by a cassette expressing a neomycin selective marker flanked by loxP sites, which was followed by the mutant exon 15 (R528H) (Fig. 1A). Recombinant ES cell clones were injected into morula to generate chimeric mice. The neo cassette was deleted by crossing the mutant KLHL3$^{flox/+}$ mice with CAG promoter-Cre transgenic mice. Successful generation of KLHL3$^{R528H/+}$ knock-in mice was confirmed by genomic sequencing (Fig. 1B–E). In addition to the generation of KLHL3$^{R528H/+}$ heterozygous mice, we generated KLHL3$^{R528H/R528H}$ homozygous mice to more readily detect the pathological effects of mutant KLHL3 R528H.

PHAII phenotypes of KLHL3$^{R528H/+}$ knock-in mice

There were no significant differences in body weight and physical appearance between KLHL3$^{R528H/+}$ and wild-type mice. To confirm the KLHL3$^{R528H/+}$ mouse as an accurate model of PHAII, we measured systolic blood pressure of mice fed a normal-salt diet. The systolic blood pressure in the KLHL3$^{R528H/+}$ mice fed a normal-salt diet did not differ from that of wild-type mice (Table 1). Since PHAII shows salt-sensitive hypertension, we then measured blood pressure in mice fed a high-salt diet, which revealed that a high-salt diet produced significantly higher systolic blood pressure in KLHL3$^{R528H/+}$ mice compared with wild-type mice (133.5 ± 1.6 mmHg versus 120.1 ± 5.1 mmHg, respectively; $n = 5$ and 4, $P < 0.05$). Moreover, as shown in Table 2, KLHL3$^{R528H/+}$ mice exhibited hyperkalemia and metabolic acidosis similar to PHAII patients. These data clearly indicate that the KLHL3$^{R528H/+}$ mouse is an ideal model of PHAII caused by KLHL3 mutation. The severity of hyperkalemia and metabolic acidosis was not changed under a high-salt diet (Supplementary Material, Fig. S1). We also performed blood pressure measurement and analysis of blood biochemical characteristics of KLHL3$^{R528H/R528H}$ homozygous knock-in mice (Tables 1 and 2). Although KLHL3$^{R528H/R528H}$ mice also exhibited salt-sensitive hypertension, hyperkalemia and metabolic acidosis, the blood pressure and blood biochemistries did not significantly differ from those of KLHL3$^{R528H/+}$ mice.

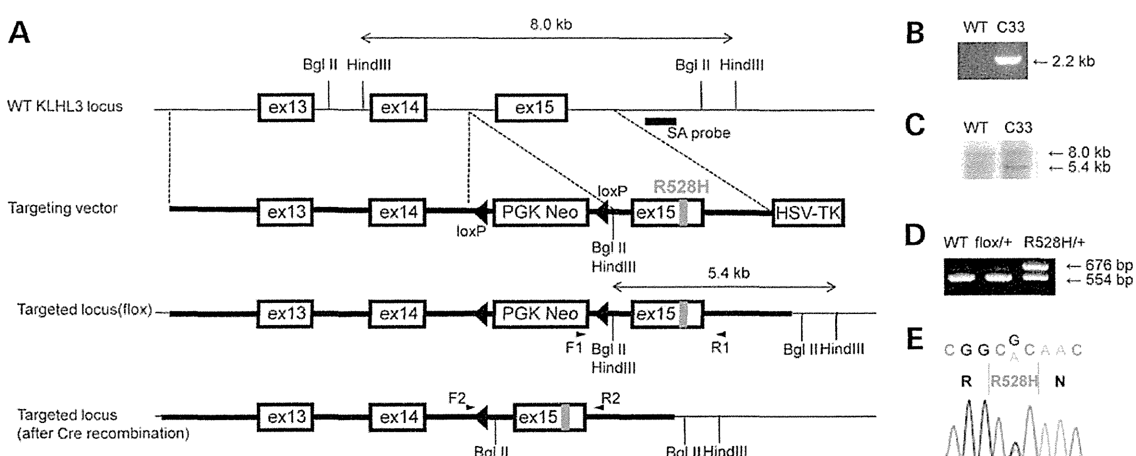

Figure 1. Generation of KLHL3$^{R528H/+}$ knock-in mice. (**A**) Targeting strategy for generating KLHL3$^{R528H/+}$ knock-in mice. The diagram shows the wild-type KLHL3 locus, the targeting construct and the targeted locus before and after Cre recombination. (**B**) Verification of homologous recombination by PCR of genomic DNA of the selected ES cell clones; primers F1 and R1 were as shown in (A). The 2.2 kb band is from the mutated allele. The primer set was designed to prevent amplification of the wild-type KLHL3 gene. C33 is the name of the selected ES cell clone. WT, host ES cells. (**C**) Verification of homologous recombination by Southern blotting of Hind III-digested genomic DNA derived from mouse tails. The 8.0 kb band is from the wild-type allele and the 5.4 kb band is from the mutated allele. (**D**) Genotyping PCR after Cre recombination using a primer set (F2 and R2) flanking the remaining loxP site. The 676 bp band represents the mutant allele containing the remaining loxP site, while the 554 bp band represents the wild-type allele. WT, wild-type mice; flox/+, KLHL3$^{flox/+}$ (R528H) mice; R528H/+, KLHL3$^{R528/+}$ mice. (**E**) Direct sequencing of the PCR product covering the mutation site.

Table 1. Blood pressure of wild-type, KLHL3$^{R528H/+}$, and KLHL3$^{R528H/R528H}$ mice

	WT	KLHL3$^{R528H/+}$	KLHL3$^{R528H/R528H}$
Systolic blood pressure (mmHg)			
Under normal diet	122.5 ± 2.0 ($n = 7$)	125.3 ± 3.0 ($n = 7$)	124.2 ± 1.6 ($n = 4$)
Under high-salt diet	120.1 ± 5.1 ($n = 4$)	133.5 ± 1.6* ($n = 5$)	136.3 ± 2.0* ($n = 4$)

*$P < 0.05$ compared with wild-type mice. No significant difference between KLHL$^{R528H/+}$ and KLHL3$^{R528H/R528H}$ mice was observed.

Table 2. Blood biochemical characteristics of wild-type, KLHL3$^{R528H/+}$, and KLHL3$^{R528H/R528H}$ mice

	WT	KLHL3$^{R528H/+}$	KLHL3$^{R528H/R528H}$
Blood biochemistries			
Na^+ (mmol/l)	149.0 ± 0.4	149.7 ± 0.4	149.9 ± 1.0
K^+ (mmol/l)	4.1 ± 0.1	4.8 ± 0.1*	4.8 ± 0.3*
Cl^- (mmol/l)	113.1 ± 0.4	116.0 ± 0.6*	115.8 ± 1.0*
BUN (mg/dl)	24.1 ± 1.4	22.9 ± 0.9	27.5 ± 2.4
Glu (mg/dl)	221.8 ± 8.3	219.3 ± 11.4	192.3 ± 17.9
pH	7.321 ± 0.008	7.287 ± 0.011*	7.261 ± 0.025*
pCO_2 (mmHg)	44.5 ± 1.4	46.1 ± 1.6	47.1 ± 3.1
$HCO3^-$ (mmol/l)	23.5 ± 0.4	21.7 ± 0.4*	20.7 ± 0.4*
Hb (g/dl)	14.7 ± 0.3 ($n = 12$)	14.6 ± 0.2 ($n = 12$)	14.5 ± 0.3 ($n = 8$)

BUN, blood urea nitrogen; Glu, glucose; Hb, hemoglobin. No significant difference between KLHL$^{R528H/+}$ and KLHL3$^{R528H/R528H}$ mice was observed in any values.
*$P < 0.05$ compared with wild-type mice.

Increased protein expression levels of WNK1 and WNK4 in KLHL3$^{R528H/+}$ mouse kidney

To investigate the pathogenesis of PHAII caused by the R528H mutation of KLHL3 *in vivo*, we examined the protein expression and phosphorylation of molecules constituting the WNK signaling pathway. As shown in Figure 2A and B, protein expression levels of WNK1 and WNK4 were significantly increased 1.8- and 1.4-fold, respectively, in the kidney of KLHL3$^{R528H/+}$ mice compared with those of wild-type mice. Accordingly, phosphorylation of OSR1, SPAK and NCC was also increased in KLHL3$^{R528H/+}$ mice. The KLHL3$^{R528H/R528H}$ homozygous mouse showed obvious increases of WNK1 and WNK4 protein levels (6.9- and 2.4-fold, respectively, compared with wild-type mice) and increased phosphorylation of OSR1 and SPAK. However, we also found that the protein level and the phosphorylation status of NCC in KLHL3$^{R528H/R528H}$ homozygous knock-in mice were not significantly increased compared with those in KLHL3$^{R528H/+}$ heterozygous knock-in mice, suggesting that the levels of increased WNK1 and WNK4 in the KLHL3$^{R528H/+}$ heterozygous knock-in mice might be high enough to fully phosphorylate and activate NCC. Considering that constitutive activation of NCC is the cause of PHAII, this saturated phosphorylation status of NCC could explain why the blood pressure and blood chemistries in KLHL3$^{R528H/R528H}$ homozygous knock-in mice did not differ from those of KLHL3$^{R528H/+}$ mice (Tables 1 and 2).

In addition, we confirmed that mRNA levels of WNK1 and WNK4 were not increased in the KLHL3$^{R528H/+}$ heterozygous mouse kidney (Fig. 2C), indicating that the increased protein levels of WNK1 and WNK4 were due to impaired degradation rather than transcriptional activation. To confirm that protein expression levels of WNK1 and WNK4 were increased in distal convoluted tubules (DCTs) where NCC is present, we performed double immunofluorescence of mouse kidney. As shown in Figure 3A and B, most of the KLHL3, WNK1 and WNK4 signals were colocalized with NCC, and signal intensities of WNK1 and WNK4 at DCT were apparently higher in the KLHL3$^{R528H/+}$ mouse kidney. Considering that both the WNK1 and WNK4 transgenic mice were reported to cause activation of the WNK-OSR1/SPAK-NCC phosphorylation cascade (9,21), these data clearly indicated that the essential pathogenesis of PHAII caused by KLHL3 mutation is due to increased WNK1 and WNK4 in DCT, leading to activation of the WNK-OSR1/SPAK-NCC phosphorylation signaling cascade.

Defective binding between the acidic motif of WNK1/WNK4 and mutant KLHL3 R528H

We had previously reported that KLHL3 mutation in the Kelch domain decreased its binding to the acidic domain of WNK4, leading to impaired ubiquitination and reduced WNK4 protein levels in HEK 293 cells (21). To confirm that the increased protein levels of WNK1 and WNK4 in KLHL3$^{R528H/+}$ mouse kidney were caused by impaired binding between WNK kinases and the mutant KLHL3 R528H, we measured the diffusion time of the TAMRA-labeled acidic motif of WNK1 or WNK4 peptide using fluorescence correlation spectroscopy (FCS) in the presence of different concentrations of GST-fusion proteins of wild-type and mutant KLHL3 R528H; the binding of KLHL3 to these peptides is observed as increased diffusion time of the fluorescent peptide (21,26,27). Wild-type KLHL3 increased the diffusion time of the TAMRA-labeled WNK1 and WNK4 peptides, confirming that wild-type KLHL3 can interact with the acidic motif of WNK1 as well as that of WNK4 (Fig. 4). On the

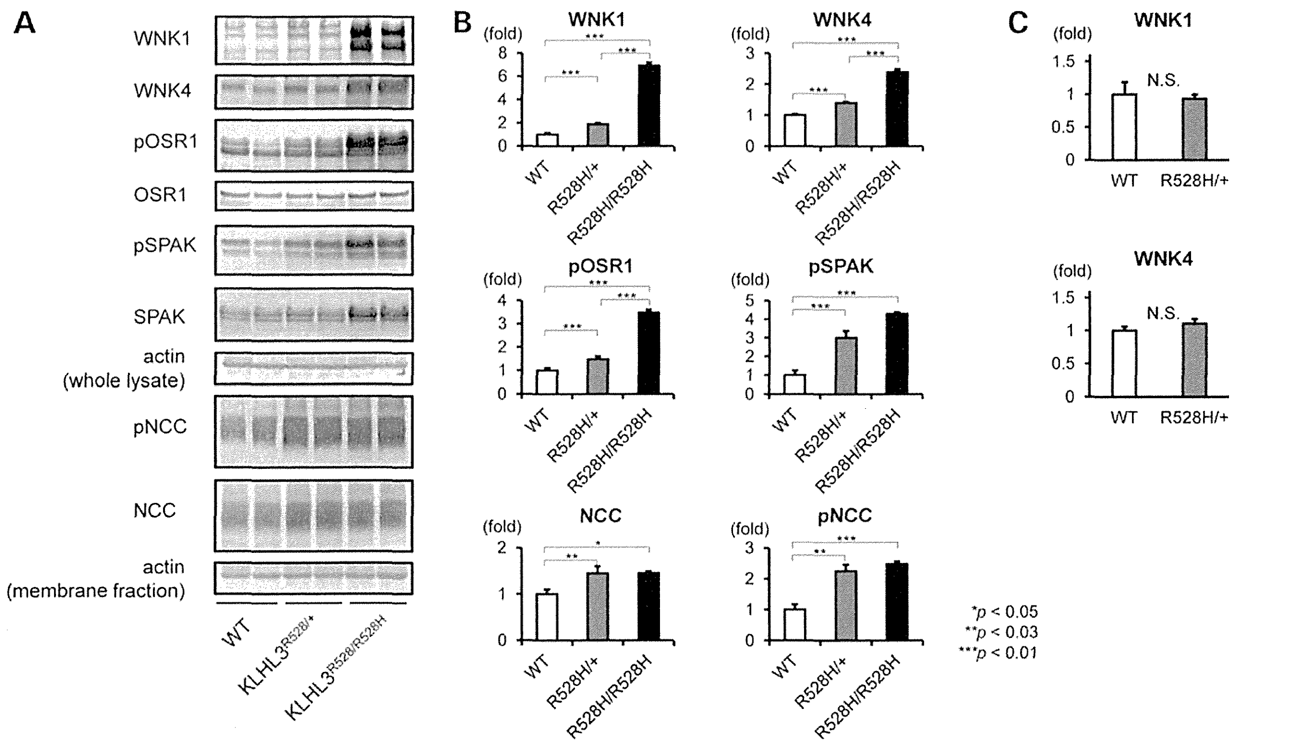

Figure 2. Increased WNK1 and WNK4 protein levels and activation of the WNK-OSR1/SPAK-NCC phosphorylation signaling cascade in KLHL3 mutant mouse kidney. (**A**) Representative immunoblots of the WNK-OSR1/SPAK-NCC signaling cascade in the kidneys of wild-type mice (WT), KLHL3$^{R528H/+}$ heterozygous knock-in mice and KLHL3$^{R528H/R528H}$ homozygous knock-in mice. (**B**) Densitometry analysis; values are expressed as a ratio of the average signal in wild-type mice. KLHL3$^{R528H/+}$ heterozygous knock-in mice and KLHL3$^{R528H/R528H}$ homozygous knock-in mice showed significantly increased protein levels of WNK1 and WNK4. The mutant KLHL3 mice also showed increased phosphorylation of OSR1, SPAK and NCC. WT, wild-type mice; R528H/+, KLHL3$^{R528/+}$ mice; R528H/R528H, KLHL3$^{R528/R528H}$ mice. $n = 6$ to 9. $^{*}P < 0.05$. $^{**}P < 0.03$. $^{***}P < 0.01$. (**C**) Quantitative PCR analysis of WNK1 and WNK4 mRNA levels. SYBR Green quantitative PCR was used to quantify mRNA levels in the kidneys of wild-type mice and KLHL3$^{R528H/+}$ mice. KLHL3$^{R528H/+}$ mice did not show significant differences of WNK1 and WNK4 mRNA levels in the kidney. WT, wild-type mice; R528H/+, KLHL3$^{R528/+}$ mice. $n = 5$.

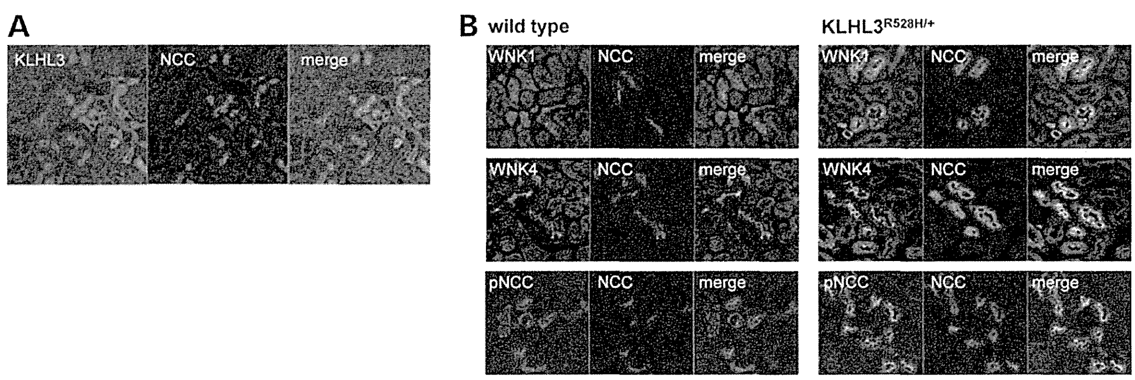

Figure 3. Immunofluorescence of WNK1, WNK4 and NCC in the kidney cortex. (**A**) Double immunofluorescence of KLHL3 and NCC in the kidney cortex of wild-type mice. The KLHL3 signal is colocalized with NCC. (**B**) Double immunofluorescence of WNK1 and NCC (upper), WNK4 and NCC (middle), and phosphorylated NCC and NCC (lower) in the kidney cortex of wild-type mice (left) and KLHL3$^{R528H/+}$ mice (right). Signals of WNK1, WNK4, NCC and pNCC are increased in KLHL3$^{R528H/+}$ mice.

other hand, the diffusion time of TAMRA-labeled WNK1 and WNK4 peptides was not affected by the addition of mutant KLHL3 R528H protein, indicating that neither WNK1 nor WNK4 bind to mutant KLHL3 R528H. The defective binding between WNK kinases and mutant KLHL3 R528H could result in impaired KLHL3-Cullin3 mediated ubiquitination of WNKs, as we previously reported, leading to increased protein expression of WNK kinases and activation of WNK signaling in the KLHL3$^{R528H/+}$ mouse kidney.

Regulation of epithelial Na^+ channels and ROMK in KLHL3$^{R528H/+}$ mice

We investigated epithelial Na^+ channels (ENaC) and renal outer medullary K^+ channels (ROMK), two important channels for Na^+ reabsorption and K^+ secretion in cortical collecting ducts (CCD), in KLHL3$^{R528H/+}$ mice. As shown in Figure 5, although KLHL3$^{R528H/+}$ mice did not show a significant change in the protein levels of total ENaC α subunit (85 kDa) compared

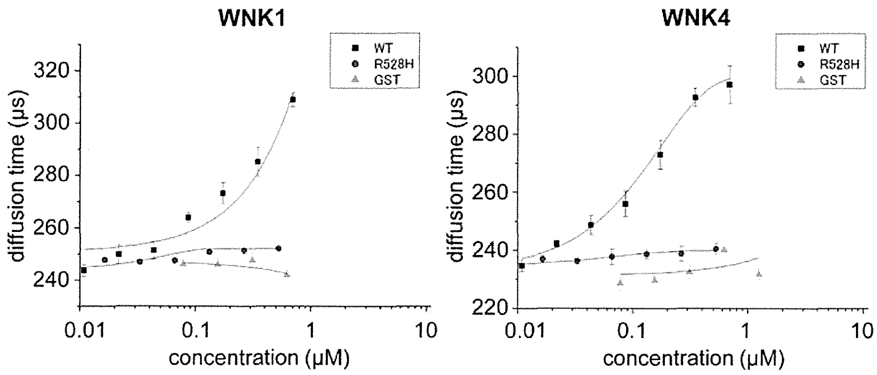

Figure 4. FCS assay of the binding between wild-type/mutant KLHL3 and WNK1/WNK4. The diffusion time of TAMRA-labeled WNK1 and WNK4 peptides containing the acidic motif was measured using FCS in the presence of different concentrations of GST fusion proteins of the wild-type KLHL3 and KLHL3 R528H mutant. The acidic motif of both WNK1 and WNK4 bound to the wild-type GST-KLHL3. On the other hand, GST alone and the KLHL3 R528H mutant did not affect the diffusion time, indicating that the KLHL3 R528H mutant could not bind to the acidic motif of WNK1 and WNK4.

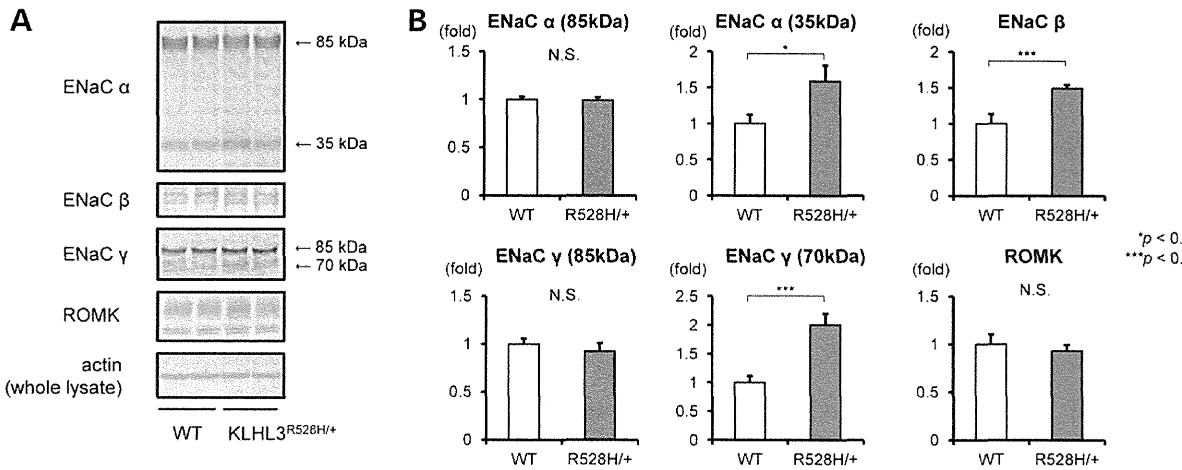

Figure 5. ENaC and ROMK in KLHL3$^{R528H/+}$ mice. (**A**) Representative immunoblots of ENaC α, ENaC β, ENaC γ subunits and ROMK in the kidneys of wild-type mice and KLHL3$^{R528H/+}$ mice. (**B**) Densitometry analysis of ENaC α, ENaC β, ENaC γ subunits and ROMK in the kidneys; values are expressed as a ratio of the average signal in wild-type mice. WT, wild-type mice; R528H/+, KLHL3$^{R528/+}$ mice. $n = 6$ to 9. $^{*}P < 0.05$. $^{***}P < 0.01$.

with wild-type mice, the levels of cleaved ENaC α subunit (35 kDa) were increased in KLHL3$^{R528H/+}$ mice. The level of ENaC β subunit was also increased in KLHL3$^{R528H/+}$ mice. Similar to ENaC α subunit, KLHL3$^{R528H/+}$ mice showed no significant change in the total protein level of ENaC γ subunit (85 kDa). However, a significant increase of cleaved ENaC γ subunit (70 kDa) was found in the KLHL3$^{R528H/+}$ mouse kidney. Similar to WNK4$^{D561A/+}$ knock-in mice (7), these results indicate that ENaC is activated in the KLHL3$^{R528H/+}$ mouse kidney. On the other hand, KLHL3$^{R528H/+}$ mice did not show a significant difference in protein levels of ROMK in immunoblot analysis of whole kidney (Fig. 5).

DISCUSSION

Investigation of the pathophysiology of PHAII is extremely important, not only to increase knowledge of this rare inherited disease, but also for the discovery of novel mechanisms of salt handling in the kidney. Although KLHL3 was identified as responsible for PHAII, several molecules have recently been reported as substrates that interact with KLHL3 in cultured cells. It was demonstrated that the loss of interaction between KLHL3 and WNK4 induced impaired ubiquitination of WNK4 and increased protein levels of WNK4 (21–24). In addition, WNK1 was also reported to be bound to KLHL3 (21,24). In contrast, Louis-Dit-Picard *et al.* (16) reported that KLHL3 is responsible for direct regulation of NCC membrane expression. Therefore, the pathophysiological role of KLHL3 in PHAII required investigation of *in vivo* kidney. For this purpose, we generated KLHL3$^{R528H/+}$ knock-in mice that carry a mutation found in human PHAII patients (15,16). This KLHL3$^{R528H/+}$ knock-in mouse exhibited salt-sensitive hypertension, hyperkalemia and metabolic acidosis, which are characteristic symptoms of PHAII patients. Moreover, increased NCC phosphorylation was also observed. These results clearly confirmed that our KLHL3$^{R528H/+}$ mouse is an ideal model of mutant KLHL3-induced PHAII.

To investigate the mechanisms of PHAII in KLHL3$^{R528H/+}$ mice, we assessed the protein levels and phosphorylation status of the WNK-OSR1/SPAK-NCC phosphorylation signaling cascade. Interestingly, KLHL3$^{R528H/+}$ heterozygous mice showed increased protein levels of both WNK1 and WNK4 in the kidney, and KLHL3$^{R528H/R528H}$ homozygous mice more

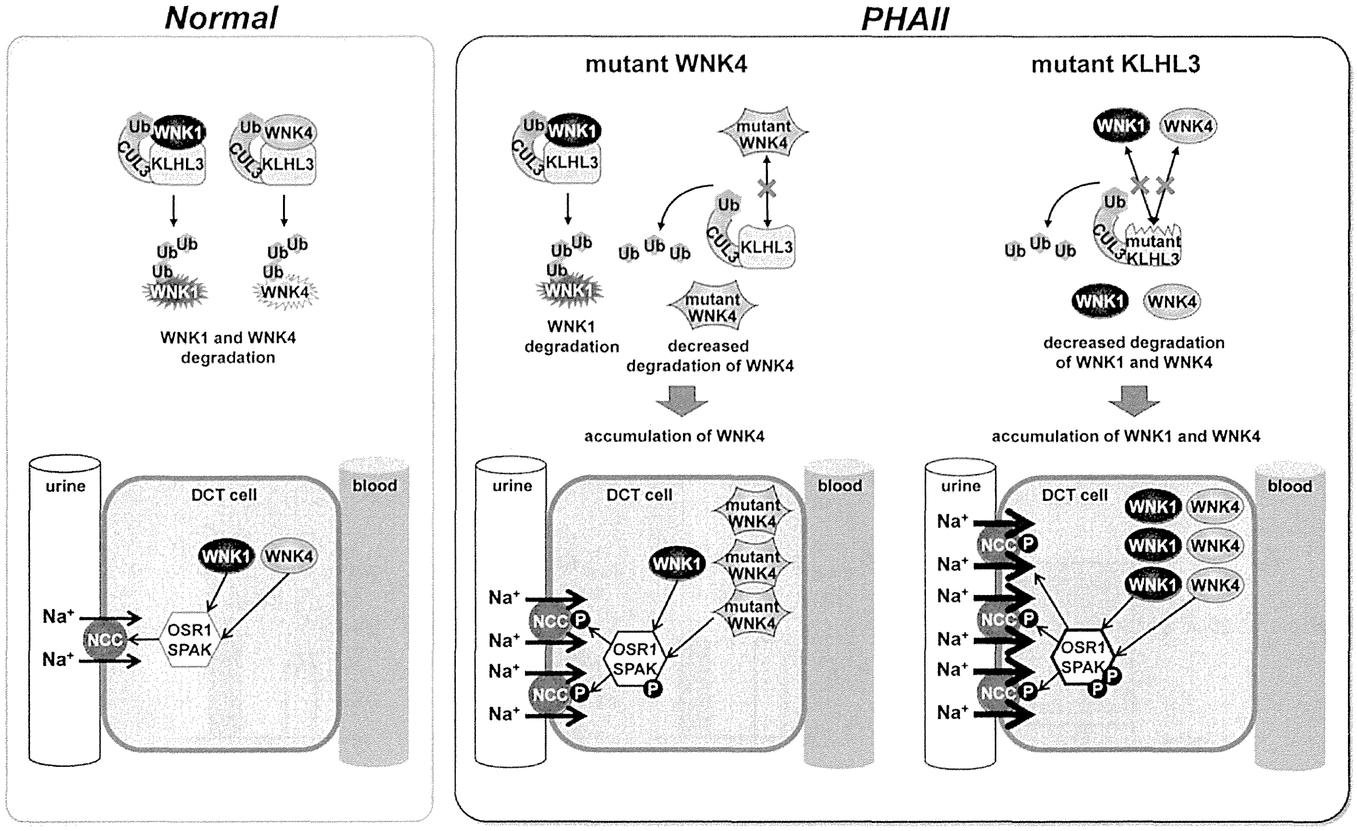

Figure 6. Schematic representation of the mechanism of PHAII caused by KLHL3 mutation.

clearly demonstrated increased WNK1 and WNK4 protein levels. We further demonstrated using an immunofluorescence assay that protein levels of both WNK1 and WNK4 are increased in DCT in KLHL3$^{R528H/+}$ mice. We also confirmed by FCS assay that mutant KLHL3 R528H did not bind to the acidic motif of either WNK1 or WNK4. Considering these observations, we have demonstrated for the first time that the essential mechanism of mutant KLHL3-induced PHAII involves impaired ubiquitination and increased protein levels of both of WNK1 and WNK4 in DCT *in vivo* (Fig. 6).

Importantly, these facts suggest that Cullin3-KLHL3 E3 ligase complexes physiologically regulate WNK-OSR1/SPAK-NCC phosphorylation cascades *in vivo*, indicating that KLHL3 plays an important role in the physiological mechanisms of sodium handling in the kidney. To date, physiological regulators of the WNK-OSR1/SPAK-NCC phosphorylation signal cascade, such as insulin (28–31), angiotensin II (10,32–34) and aldosterone (32,35–37), have been reported. However, the regulatory mechanism of these factors in WNK signaling remains unknown. The novel KLHL3-mediated regulation of WNK signals might be involved in these mechanisms. Further investigation is required to clarify this issue.

It was reported that KLHL3 was able to bind to NCC and regulate its intracellular localization in cultured cells (16). However, WNK and OSR1/SPAK were activated in KLHL3$^{R528H/+}$ mice, but were not down-regulated by increased NCC phosphorylation. Moreover, it was reported that simple over-expression of NCC did not produce the PHAII phenotype in NCC transgenic mice (38), indicating that increased phosphorylation, but not increased protein expression, of NCC is required for the PHAII phenotype *in vivo*. Considering these *in vivo* observations, the essential pathogenesis of PHAII caused by KLHL3 mutation is not due to impaired ubiquitination or regulation of NCC, but to the impaired ubiquitination of WNK kinases. This discrepancy could be due to the experimental system, the genetically engineered mouse model or cultured cells.

Boyden *et al.* (15) reported that symptoms of human PHAII patients caused by KLHL3 mutation are more severe than those caused by mutation in WNK1 or WNK4. WNK4$^{D561A/+}$ knock-in (7) and WNK1$^{+/FHHt}$ (9) PHAII mouse models exhibited increases of only a single kind of WNK kinase that carries a mutation. The increased severity of KLHL3 mutation-induced PHAII in human patients may be explained by the physiological difference between the increase of 'both WNK1 and WNK4 kinases' and 'a single WNK kinase'. Accumulation of both WNK1 and WNK4 kinases by KLHL3 mutation could result in further increases in phosphorylation of downstream components compared with the accumulation of a single WNK kinase (Fig. 6). However, the KLHL3$^{R528H/+}$ mouse shows a less severe phenotype than other mouse models of PHAII previously reported (7,9), which could be explained by the difference of origin of ES cells used for the generation of the knock-in mice, i.e. the difference of genetic background of these mice. In this study we utilized Balthal ES cells derived from C57BL/6 mouse, which is a one-renin-gene mouse strain. However, the other mouse models of PHA II were established with ES cells derived from two-renin-gene mouse strains, 129Sv (7,9). It was reported that 129Sv mice showed increased blood pressure response to salt intake, compared with C57BL/6 mice (39). Further investigation to compare the phenotypes of three PHAII model mice under the same genetic background will be required.

Finally, we discuss the involvement of ENaC and ROMK in PHAII caused by KLHL3 mutation. ENaC was activated in KLHL3$^{R528H/+}$ heterozygous knock-in mice, similar to WNK4$^{D561A/+}$ mice (7). However, inconsistent with the KLHL3$^{R528H/+}$ and WNK4$^{D561A/+}$ mice, WNK1$^{+/FHHt}$ mice are reported to show no activation of ENaC (9). This difference might be explained by whether WNK4 is increased or not. On the other hand, in the case of ROMK, we did not find differences in ROMK expression in the kidney between wild-type and KLHL3$^{R528H/+}$ mice. However, we were unable to perform microdissection of kidney, and the available anti-ROMK antibody detects all ROMK variants. Therefore, to clarify the *in vivo* effect of KLHL3 on ROMK in CCD, further investigation is required.

In summary, we established and analyzed KLHL3$^{R528H/+}$ knock-in mice, and clarified the essential pathogenesis of mutant KLHL3-induced PHAII. Mutant KLHL3 causes accumulation of both WNK1 and WNK4 at DCT due to loss of binding ability of KLHL3 to WNK kinases. This regulation of the WNK-OSR1/SPAK-NCC phosphorylation cascade by Cullin3-KLHL3 E3 ligase complexes plays important physiological and pathophysiological roles for sodium handling in the kidney *in vivo*.

MATERIALS AND METHODS

Generation of KLHL3$^{R528H/+}$ knock-in mice

To generate KLHL3$^{R528/+}$ knock-in mice, the targeting vector was prepared using the BAC recombineering system (40). The point mutation (R528H) was introduced into exon 15 of the targeting vector by *galK* selection system (41). The targeting vector was then transfected into Balthal ES cells (25), which are derived from C57BL/6 mice, by electroporation as previously reported (42). After selection with 150 μg/ml G418 and 2 μM ganciclovir, targeted ES clones were selected by PCR with a sense primer F1 (5′-ATA GCA GAG CCG TCT CTG TG-3′) located within the neo cassette and an antisense primer R1 (5′-ACT TGT GTA GCG CCA AGT GC-3′) located following exon 15, Southern blotting and sequencing of the mutation site. Selected ES clones were injected into C57BL/6 morula. Chimeric males were bred with C57BL/6 females to produce mutant KLHL3$^{flox/+}$ (R528H) mice, and the neo cassette was then deleted by crossing these mutant KLHL3$^{flox/+}$ mice with transgenic mice expressing Cre recombinase under the control of the CAG promoter (43). Offsprings were genotyped by PCR with sense primer F2 (5′-CAC AGG GTA ACT GGG GCT GGT-3′) and antisense primer R2 (5′-GGA AGA ACT GTG ACC CCC GC-3′) flanking the remaining loxP site and exon 15.

Animals

Studies were performed using 10-week-old mice that had free access to food and water. Mice of each genotype were placed on a normal-salt diet (NaCl 0.4% w/w) or a high-salt diet (8.0% w/w) for 1 week. All experiments were performed 1 week after dietary change. The Animal Care and Use Committee of Tokyo Medical and Dental University approved the experimental protocol.

Measurement of blood pressure

We measured blood pressure by using a radiotelemetric method in which a blood pressure transducer (Data Sciences International, St. Paul, MN, USA) was inserted into the left carotid artery. Seven days after transplantation, each mouse was housed individually in a standard cage on a receiver under a 12 h light–dark cycle. Systolic and diastolic blood pressure was recorded every minute via radiotelemetry. For each mouse, we measured blood pressure values for more than 3 consecutive days and calculated the mean ± SE of all values. These experiments were performed under a normal-salt (NaCl 0.4% w/w) or high-salt (8.0% w/w) diet.

Blood analysis

Blood was drawn from the retro-orbital sinus under light ether anesthesia. Serum data were determined using the i-STAT system (FUSO Pharmaceutical Industries, Osaka, Japan).

Immunoblot analysis

Immunoblot analyses were performed on kidney homogenates. Kidneys were dissected from mice. Homogenates of whole kidney without the nuclear fraction (600 g) were prepared, and the crude membrane fraction (17 000 g) was used to measure the levels of NCC as described previously (7). Blots were probed with the following primary antibodies: anti-WNK1 (A301–516A; Bethyl, Montgomery, TX, USA), anti-WNK4 (36), anti-total OSR1 (M10; Abnova, Taipei, Taiwan), anti-phosphorylated OSR1 (44), anti-total SPAK (Cell Signaling Technology, Danvers, MA, USA), anti-phosphorylated SPAK (33), anti-total NCC (29), anti-phosphorylated NCC (pSer71) (28), anti-ENaC α subunit (kindly provided by M. Knepper), anti-ENaC β subunit (Alomone, Jerusalem, Israel), anti-ENaC γ subunit (kindly provided by M. Knepper), and anti-ROMK antibodies (kindly provided by J. B. Wade and P. A. Welling) (45), anti-actin (Cytoskeleton, Denver, CO, USA). Alkaline-phosphatase-conjugated anti-IgG antibodies (Promega, Madison, WI, USA) and Western Blue (Promega) were used to detect the signals. The relative intensities of immunoblot bands were determined by densitometry with YabGelImage free software.

Quantitative PCR analysis

Quantitative PCR analysis was performed on kidney as previously described (46). Total RNA from mouse kidneys was extracted using TRIzol reagent (Invitrogen, Carlsbad, CA, USA), according to the manufacturer's instructions. Total RNA was reverse transcribed using Omniscript reverse transcriptase (Qiagen, Hilden, Germany). Quantitative real-time PCR by Thermal Cycler Dice (Takara Bio, Otsu, Japan) was performed using the primer sets shown in a previous report (47).

Immunofluorescence

Kidneys were fixed by perfusion with periodate lysine (0.2 M) and paraformaldehyde (2%) in PBS. Immunofluorescence was performed as previously described (42). The primary antibodies used were anti-KLHL3 (Proteintech, Chicago, IL, USA), anti-WNK1 (A301–516A; BETHYL), anti-WNK4 (36), anti-NCC (29) and anti-pNCC (pSer71) (28). Alexa 488 or 546 dye-labeled (Molecular Probes; Invitrogen) secondary antibodies were used for immunofluorescence. Immunofluorescence images were obtained using an LSM510 Meta Confocal Microscope (Carl Zeiss, Oberkochen, Germany).

Fluorescence correlation spectroscopy

Fluorescent TAMRA-labeled WNK1 and WNK4 peptides covering the acidic motif were prepared (Hokkaido System Science Co., Ltd., Hokkaido, Japan). Human full-length KLHL3 (wild-type and R528H mutant) was cloned into pGEX6P-1 vectors. The recombinant GST-fusion KLHL3 protein expressed in BL21 *Escherichia coli* cells was purified using glutathione Sepharose beads. The TAMRA-labeled WNK peptides were incubated at room temperature for 30 min with different concentrations of GST-KLHL3 (0–2 μM) in 1× PBS containing 0.05% Tween 20 (reaction buffer). FCS measurements using the FluoroPoint-light analytical system (Olympus, Tokyo, Japan) were performed as previously described (26,27). The measurements were repeated five times per sample.

Statistics

Data are presented as means ± SE. A student's *t*-test was used for comparisons between groups. ANOVA and Tukey's test were used for multiple comparisons.

SUPPLEMENTARY MATERIAL

Supplementary Material is available at *HMG* online.

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Conflict of Interest statement. The authors declare that they have no conflict of interest.

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WNK4 is the major WNK positively regulating NCC in the mouse kidney

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Synopsis

By analysing the pathogenesis of a hereditary hypertensive disease, PHAII (pseudohypoaldosteronism type II), we previously discovered that WNK (with-no-lysine kinase)–OSR1/SPAK (oxidative stress-responsive 1/Ste20-like proline/alanine-rich kinase) cascade regulates NCC (Na–Cl co-transporter) in the DCT (distal convoluted tubules) of the kidney. However, the role of WNK4 in the regulation of NCC remains controversial. To address this, we generated and analysed WNK4$^{-/-}$ mice. Although a moderate decrease in SPAK phosphorylation and a marked increase in WNK1 expression were evident in the kidneys of WNK4$^{-/-}$ mice, the amount of phosphorylated and total NCC decreased to almost undetectable levels, indicating that WNK4 is the major WNK positively regulating NCC, and that WNK1 cannot compensate for WNK4 deficiency in the DCT. Insulin- and low-potassium diet-induced NCC phosphorylation were abolished in WNK4$^{-/-}$ mice, establishing that both signals to NCC were mediated by WNK4. As shown previously, a high-salt diet decreases phosphorylated and total NCC in WNK4$^{+/+}$ mice via AngII (angiotensin II) and aldosterone suppression. This was not ameliorated by WNK4 knock out, excluding the negative regulation of WNK4 on NCC postulated to be active in the absence of AngII stimulation. Thus, WNK4 is the major positive regulator of NCC in the kidneys.

Key words: angiotensin II, distal convoluted tubule, hypertension, kidney, Na–Cl co-transporter, with-no-lysine kinase (WNK).

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INTRODUCTION

PHAII (pseudohypoaldosteronism type II) is a hereditary hypertensive disease characterized by hypokalaemia, metabolic acidosis and thiazide sensitivity [1]. In 2001, *WNK (with-no-lysine kinase) 1* and *WNK4* were identified as the genes responsible for PHAII [2]. The high thiazide sensitivity of PHAII suggested that activation of the NCC (Na–Cl co-transporter) in the DCT (distal convoluted tubules) of the kidneys was responsible for its pathogenesis. Therefore, several investigations on the regulation of NCC by WNKs were performed [3,4]. Initially, some of these studies reported that wild-type WNK4 negatively regulated NCC in *Xenopus* oocytes [5,6], which was reportedly not kinase activity-dependent function of WNK4 [7,8]. However, we generated and analysed PHAII model mice (*WNK4*$^{D561A/+}$) carrying a PHAII-causing mutation in WNK4, and discovered a novel signalling cascade between WNK and NCC via OSR1 (oxidative stress-responsive 1) and SPAK (Ste20-like proline/alanine-rich kinase) in the kidney [9,10]. We reported that constitutive activation of this signalling cascade caused the molecular pathogenesis of PHAII induced by WNK4 mutation [10,11]. Because both WNK1 and WNK4 were demonstrated to positively influence OSR1 and SPAK [12], this kinase activity-dependent effect of WNK4 must be acting positively on NCC in the kidneys. Thus, two opposing methods of NCC regulation by WNK4 were postulated, and the identity of the major mechanism of NCC regulation in the kidneys *in vivo* remained unclear. Previously,

Abbreviations: Akt, also called protein kinase B (PKB); AngII, angiotensin II; BAC, bacterial artificial chromosome; BP, blood pressure; DCT, distal convoluted tubule; ENaC, epithelial Na^+ channel; ES, embryonic stem; KLHL3, Kelch-like family member 3; NCC, Na–Cl co-transporter; NKCC2, Na-K-Cl co-transporter isoform 2; OSR1, oxidative stress-responsive 1; PHAII, pseudohypoaldosteronism type II; RAA, renin–angiotensin–aldosterone; ROMK, renal outer medullary K^+ channel; SPAK, Ste20-like proline/alanine-rich kinase; TG, transgenic; WNK, with-no-lysine kinase.

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Castañeda-Bueno et al. [13] reported the analysis of WNK4 knockout mice. However, this group focused on the role of WNK4 in AngII (angiotensin II)- or low-salt diet-induced activation of NCC, and did not completely investigate the controversial role of WNK4 in NCC regulation.

Mutations in two additional genes, KLHL3 (*Kelch-like family member 3*) and *Cullin-3*, were recently reported to cause PHAII [14,15]. We clarified that WNK4 is a substrate of the KLHL3–Cullin-3 E3 ligase complex, and that the impaired ubiquitination of WNK4 protein and, subsequent, increase of WNK4 protein abundance in DCT would represent a common molecular pathogenesis of PHAII caused by mutations in *WNK4*, *KLHL3* or *Cullin-3* [16]. In fact, we demonstrated that WNK4 protein is increased in $WNK4^{D561A/+}$ PHAII model mice [16]. These recent data strongly support a positive regulatory role for WNK4 in NCC regulation.

To obtain a definite conclusion regarding the physiological role of WNK4 in the kidneys, a more detailed examination of WNK4 knockout mice would be necessary. Previously, we generated hypomorphic WNK4 knockout mice, with the reduced function of WNK4, and obtained results suggesting that WNK4 acts positively on NCC in the kidneys [17]. However, a definite conclusion must be presented by analysing complete WNK4 knock out; to address this, we generated $WNK4^{-/-}$ mice and examined them under various conditions. Because we had clarified that NCC is regulated by diets and some hormonal factors other than AngII, we first attempted to clarify whether NCC regulations other than that by AngII are WNK4-dependent. In addition, we investigated whether there was any evidence in the kidneys that supports a negative role of WNK4 in NCC regulation.

MATERIALS AND METHODS

Generation of WNK4 knockout mice

To generate $WNK4^{-/-}$ mice, we prepared a BAC (bacterial artificial chromosome) clone bMQ42809 containing the mouse genomic *WNK4* locus. The targeting vector was then transfected into J1-6 ES (embryonic stem) cells by electroporation, as previously described [18]. After selection with 150 μg/ml G418 and 2 μM ganciclovir, the targeted ES cell clones were selected by PCR. Chimeric male mice were bred with C57BL/6J females to produce heterozygous floxed ($WNK4^{flox/+}$) mice, and the neo cassette was then deleted by crossing the $WNK4^{flox/+}$ mice with Cre recombinase-expressing TG (transgenic) mice [19]. Genotyping of the mice was performed by PCR using the sense primer F (5′-ACAAAGGCGCTATTGAGTGC-3′) and the antisense primer R1 (5′-CGTCTGGGTCGGAAAGAAACT-3′) to detect the flanked exon 2. Further PCR analysis was performed using the sense primer F and the antisense primer R2 (5′-CAAGAAGAGCATGGGACATC-3′) to detect the upper loxP site.

Studies were performed on 12–16-week-old $WNK4^{+/+}$, $WNK4^{+/-}$ and $WNK4^{-/-}$ littermates. The mice were raised under a 12-h day and night cycle, and were fed a normal rodent diet and plain drinking water. This experiment was approved by the Animal Care and Use Committee of the Tokyo Medical and Dental University, Tokyo, Japan.

Blood measurements

Blood for electrolyte analysis was obtained as previously described [9]. Electrolyte levels were determined using an i-STAT® Portable Clinical Analyzer (Fuso Pharmaceutical Industries Ltd). Samples for plasma aldosterone measurement were obtained from the inferior vena cava under anaesthesia with pentobarbital. Plasma aldosterone levels were measured by SRL Clinical Laboratory Services.

Immunoblotting and immunofluorescence

Extraction of kidney protein samples, semi-quantitative immunoblotting and immunofluorescence were performed as previously described [9]. For semi-quantitative immunoblotting, we used entire kidney samples without the nuclear fraction (600 ***g***) or the crude membrane fraction (17000 ***g***). The relative intensities of immunoblot bands were analysed and quantified using ImageJ (National Institutes of Health) software. The primary antibodies used in this study were as follows: rabbit anti-WNK1 (A301-516A; Bethyl Laboratories); anti-WNK4 [17]; rabbit anti-phosphorylated SPAK [20]; rabbit anti-SPAK (Cell Signaling Technology, Inc.); anti-phosphorylated OSR1/SPAK [17]; anti-OSR1 (M10; Abnova Corporation); rabbit anti-phosphorylated NCC (S71) [21]; guinea pig anti-NCC [22,23]; rabbit anti-ENaC (epithelial Na^+ channel) α subunit (Chemicon International); rabbit anti-ENaC γ subunit (provided by M. Knepper, NIH) [24]; rabbit anti-ROMK (renal outer medullary K^+ channel; provided by P. A. Welling and J. B. Wade, Maryland University) [25]; rabbit anti-actin (Cytoskeleton, Inc.); rabbit anti-phosphorylated NKCC2 (Na-K-Cl co-transporter isoform 2) [26]; guinea pig anti-NKCC2 (provided by K. Mutig, Department of Anatomy, Charité-Universitätsmedizin, Berlin) [27]; rabbit anti-phosphorylated serine/threonine protein kinase Akt [PKB (protein kinase B: also called Akt); Cell Signaling Technology, Inc.]; and rabbit anti-Akt (Santa Cruz Biotechnology, Inc.). ALP (alkaline phosphatase)-conjugated anti-IgG antibodies (Promega Corporation) were used as secondary antibodies and Western Blue® (Promega Corporation) was used to detect the signal.

Blood pressure (BP) measurement

We measured the systolic BP using implantable radiotelemetric devices. The equipment for conscious, freely moving laboratory animals was purchased from Data Sciences International, which included an implantable transmitter (model TA11PA-C10), a receiver (model RPC-1), a data-processing device (Data Exchange Matrix) and an APR-1 (ambient pressure reference monitor). All data were computed using an analysis program (Dataquest ART4.31).

Table 1 Blood biochemistry of WNK4$^{+/+}$ and WNK4$^{-/-}$ mice
Values are the means ± S.E.M.

	WNK4 WT ($n=16$)	WNK4 KO ($n=17$)	P
Na (mM)	146.3 ± 0.4	144.4 ± 0.3	<0.05
K (mM)	4.58 ± 0.15	4.59 ± 0.15	0.958
Cl (mM)	113.1 ± 0.6	109.2 ± 0.8	<0.01
Glu (mg/dl)	206.9 ± 8.1	203.4 ± 7.3	0.737
pH	7.237 ± 0.01	7.298 ± 0.01	<0.001
PCO_2 (mmHg)	57.0 ± 1.8	52.8 ± 1.6	0.084
HCO_3^- (mM)	24.2 ± 0.5	25.9 ± 0.8	0.073
BE	− 3.3 ± 0.5	− 0.6 ± 0.9	<0.05
Hb (g/dl)	15.1 ± 0.2	15.6 ± 0.2	0.092

Statistical analysis

Comparisons between the two groups were performed using unpaired *t*-tests. One-way ANOVA with Tukey's *post hoc* test was used to evaluate statistical significance in the comparisons between multiple groups. *P*-values <0.05 were considered statistically significant. Data are presented as the means ± S.E.M.

RESULTS

Generation of WNK4$^{-/-}$ mice

To generate WNK4$^{-/-}$ mice, we designed a targeting vector to delete a single exon (exon 2) of the *WNK4* (Figure 1A). Homologous recombination was confirmed by PCR in ES cell lines, which were used to generate WNK4$^{flox/+}$ mice. Next we crossed WNK4$^{flox/+}$ mice with Cre recombinase-expressing TG mice. The Cre-mediated excision of exon 2 in heterozygous (WNK4$^{+/-}$) mice was verified by PCR. Homozygous knockout (WNK4$^{-/-}$) mice were successfully generated by crossing WNK4$^{+/-}$ mice (Figure 1B). Heterozygous and homozygous WNK4 knockout mice exhibited no gross anatomic or behavioural abnormalities, and presented a normal birth rate. Absence of WNK4 protein in WNK4$^{-/-}$ mice was confirmed by immunoblotting of the kidney proteins (Figure 1C).

General characteristics of WNK4$^{-/-}$ mice

First, we compared venous blood chemistry between WNK4$^{-/-}$ mice and their wild-type littermates fed with a normal diet (Table 1). WNK4$^{-/-}$ mice exhibited higher plasma pH and lower plasma Na^+ and Cl^- than the wild-type littermates. There was no significant difference in plasma K^+ between the two groups; this was in contrast to the findings of Castañeda-Bueno et al. [13].

Next, we analysed the effect of dietary salt intake on the BP in WNK4$^{-/-}$ mice. We used radiotelemetric devices to measure the BP of WNK4$^{-/-}$ mice and their wild-type littermates at different dietary sodium levels. Male mice were fed normal [0.9 % NaCl (w/w)], high- [4 % NaCl (w/w)] or low- [0.01 % (w/w)] sodium diets for 7 days. As shown in Figure 2, no significant difference was evident while they received the normal or high-sodium diets. However, on the low-salt diet, the systolic BP of WNK4$^{-/-}$ mice was significantly lower than that of WNK4$^{+/+}$ mice during the resting period (day time; 105.9 ± 5.84 versus 84.1 ± 8.07 mm Hg; $n=3$; $P<0.05$; Figure 2A).

Expression of proteins associated with the WNK–OSR1/SPAK–NCC phosphorylation cascade

We investigated the expression of proteins associated with the WNK–OSR1/SPAK–NCC phosphorylation cascade in the kidneys of the mice receiving a normal diet. In the WNK4$^{+/-}$ mice, although the level of WNK4 protein expression in the kidneys was about half that of WNK4$^{+/+}$ mice, levels of SPAK, OSR1 and NCC expression were not significantly altered by this degree of reduction in WNK4 expression. However, total NCC and NCC phosphorylated at S71 were almost completely absent in WNK4$^{-/-}$ mice (Figure 2C). In contrast with a previous report [13], a significant decrease in total and phosphorylated SPAK protein was evident in WNK4$^{-/-}$ mice, and total OSR1 was increased. Because WNK1 and WNK3 are known to phosphorylate OSR1/SPAK, we also examined their expression. WNK1 expression in the kidneys was significantly increased in WNK4$^{-/-}$ mice. Despite this, NCC phosphorylation remained almost absent. To clarify the segment of the kidney in WNK4$^{-/-}$ mice with increased WNK1 expression, we performed double immunofluorescence staining of WNK1 and total NCC. As shown in Supplementary Figure S1 (available at http://www.bioscirep.org/bsr/034/bsr034e107add.htm), the DCT were dilated, cell height was decreased and staining for NCC was reduced in the WNK4$^{-/-}$ mice. The WNK1 antibody used does not usually detect significant WNK1 expression in the kidneys of WNK4$^{+/+}$ mice. However, increased WNK1 expression, co-localized with NCC expression, was evident in WNK4$^{-/-}$ mice, suggesting that a compensatory increase in WNK1 occurred in the DCT, but that this was insufficient to maintain NCC activity. This compensation might be caused by the increased transcription of the *WNK1* gene and/or by the reduced degradation of WNK1 protein. WNK3 protein expression was not detected by immunoblotting in the kidneys of either WNK4$^{+/+}$ or WNK4$^{-/-}$ mice (results not shown), confirming that WNK3 is not a major WNK in the kidneys *in vivo* [28,29], even in the absence of WNK4.

We also investigated other transporters and channels thought to be regulated by WNK4. The levels of expression of both the full-length and cleaved forms of the α and γ subunits of ENaC were similar between WNK4$^{+/+}$, WNK4$^{+/-}$ and WNK4$^{-/-}$ mice. Castañeda-Bueno et al. [13] did not present immunoblots for ENaC. However, they did perform functional assays to estimate ENaC activity, and showed that ENaC was activated to compensate for NCC inactivation resulting from WNK4 knockout. We thought that ENaC should be activated to compensate for NCC inactivation in our WNK4$^{-/-}$ mice, but the magnitude of difference may be insufficient for detection by immunoblotting, possibly because the phenotype of our WNK4$^{-/-}$

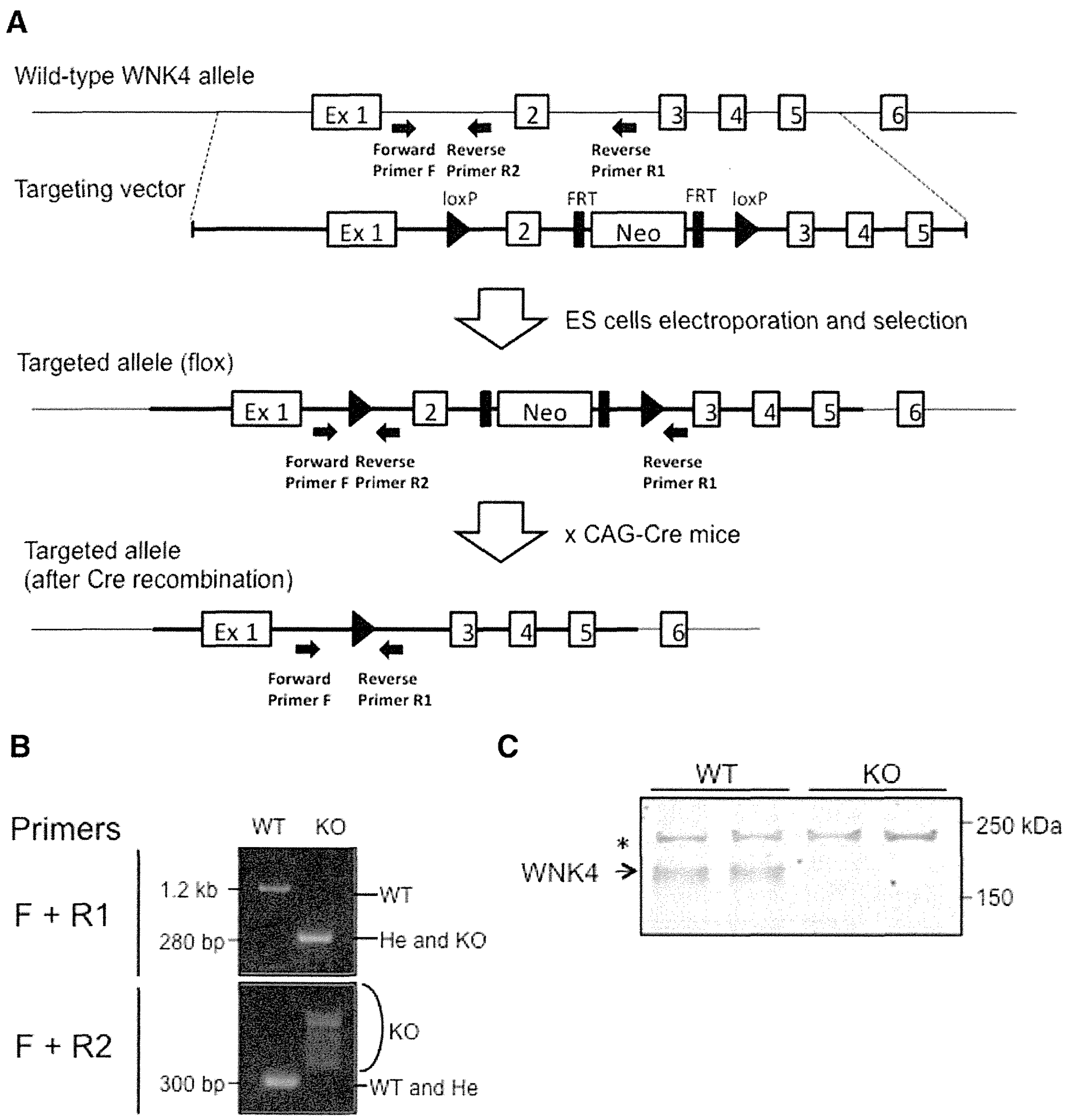

Figure 1 **Generation of WNK4$^{-/-}$ mice**
(**A**) Targeting strategy to generate WNK4 knockout mice. This diagram shows the targeting construct, the wild-type WNK4 locus, and the targeted locus before and after Cre recombination. Exon 2 was flanked by two *loxP* sites. (**B**) Genotyping PCR after Cre recombination using primer sets flanking exon 2 (Primer F and R1) and flanking the upper *loxP* site (Primer F and R2). In the upper panel, the 280-bp band represents the mutant allele containing the remaining loxP site, whereas the 1.2-kb band represents the wild-type allele. In the lower panel, the 300-bp band represents the wild-type allele, whereas the smeared band represents the homozygous knockout alleles. (**C**) Immunoblotting of WNK4 from the kidneys of WNK$^{+/+}$ and WNK4$^{-/-}$ mice. We used 40 μg of total protein per lane. The asterisk indicates non-specific bands [22].

is milder than those analysed by Castañeda-Bueno et al. [13] given their serum potassium levels. However, ROMK was significantly increased in our WNK4$^{-/-}$ mice. NKCC2 expression and phosphorylation were not significantly affected by WNK4 knockout, consistent with our previous finding that the major WNK4 signal in the mouse kidney does not co-localize with NKCC2 [22].

Involvement of WNK4 in low-potassium diet- and insulin-induced NCC activation

We previously identified regulators of the WNK–OSR1/SPAK–NCC signal cascade, including salt [11] and potassium intake [30], aldosterone [11], AngII [31,32] and insulin [20,33]. However, the identity of the WNK responsible for their regulation of NCC remains unclear. Low-salt diet- and