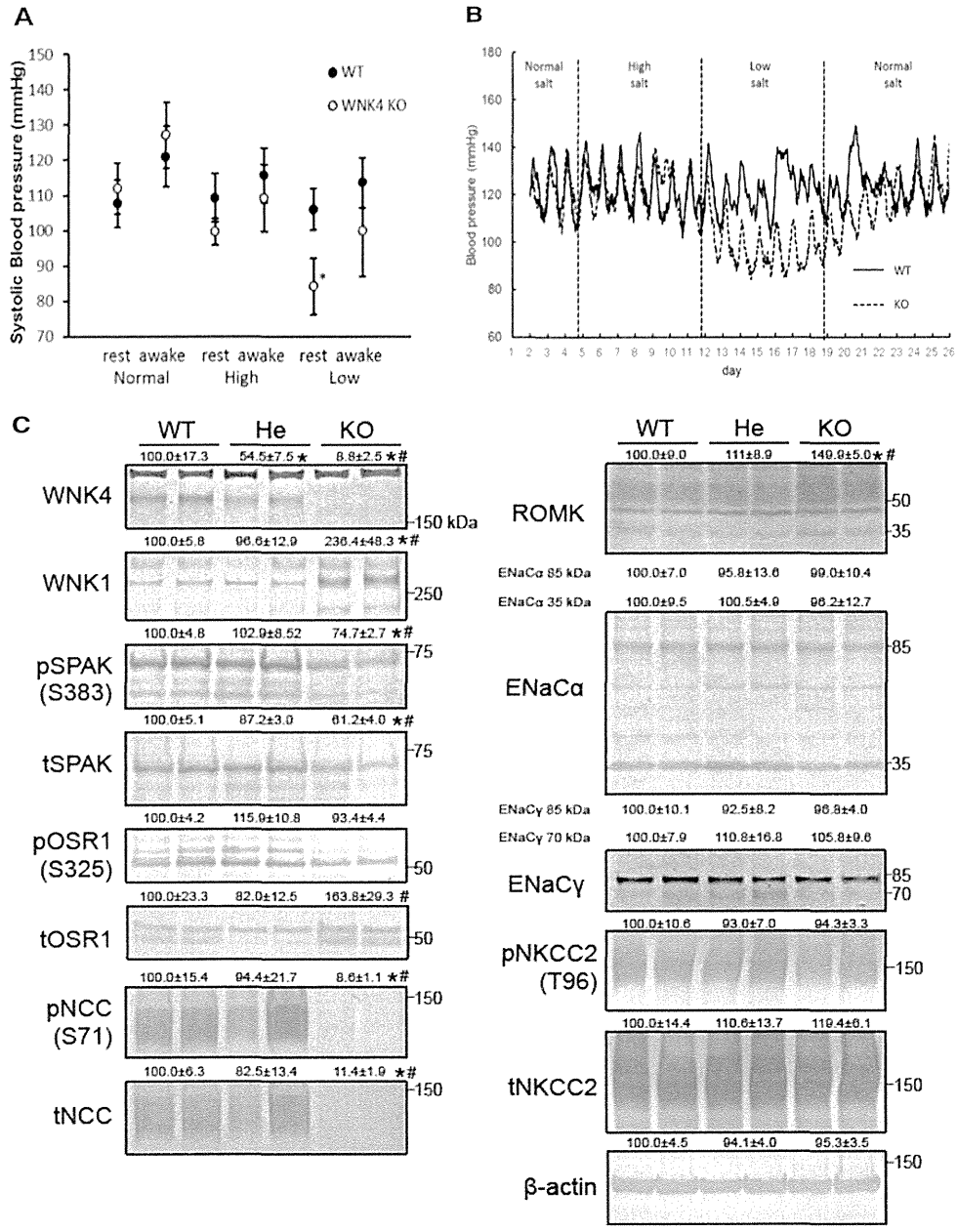

Figure 2 **General characteristics of WNK4$^{-/-}$ mice**
(**A**) Radiotelemetric analyses of BP in WNK4$^{+/+}$ and WNK4$^{-/-}$ male mice receiving diets with differing salt contents. Systolic BP in WNK4$^{+/+}$ mice remained stable in response to diets with differing salt contents. In contrast, WNK4$^{-/-}$ mice exhibited a significantly lower BP when fed a low-salt diet ($*P < 0.05$). (**B**) Representative radiotelemetric measurements of BP. Although WNK4$^{+/+}$ mice were unaffected by diets with differing salt contents, WNK4$^{-/-}$ mice exhibited lower BP when fed a low-salt diet. BP returned to normal when dietary salt reverted to normal levels. (**C**) Representative immunoblotting analyses of kidney proteins in WNK4$^{+/+}$ (WT), WNK4$^{+/-}$ (He), and WNK4$^{-/-}$ (KO) mice fed a normal diet. The numbers reflect the results of semi-quantification by densitometry ($*P < 0.05$ versus WNK4$^{+/+}$ mice, $\#P < 0.05$ versus WNK4$^{+/-}$ mice; $n = 5$, expressed as percentages, means ± S.E.M.). We used 40 μg of total protein per lane. WNK4 in WNK4$^{+/-}$ mice was about half that in wild-type mice, and was absent in WNK4$^{-/-}$ mice. WNK1 increased in WNK4$^{-/-}$ mice. Total and phosphorylated SPAK expression decreased in WNK4$^{-/-}$ mice. Total OSR1 expression increased in WNK4$^{-/-}$ mice. NCC expression did not decrease in WNK4$^{+/-}$ mice, but NCC was almost completely absent in WNK4$^{-/-}$ mice. ROMK expression increased in WNK4$^{-/-}$ mice.

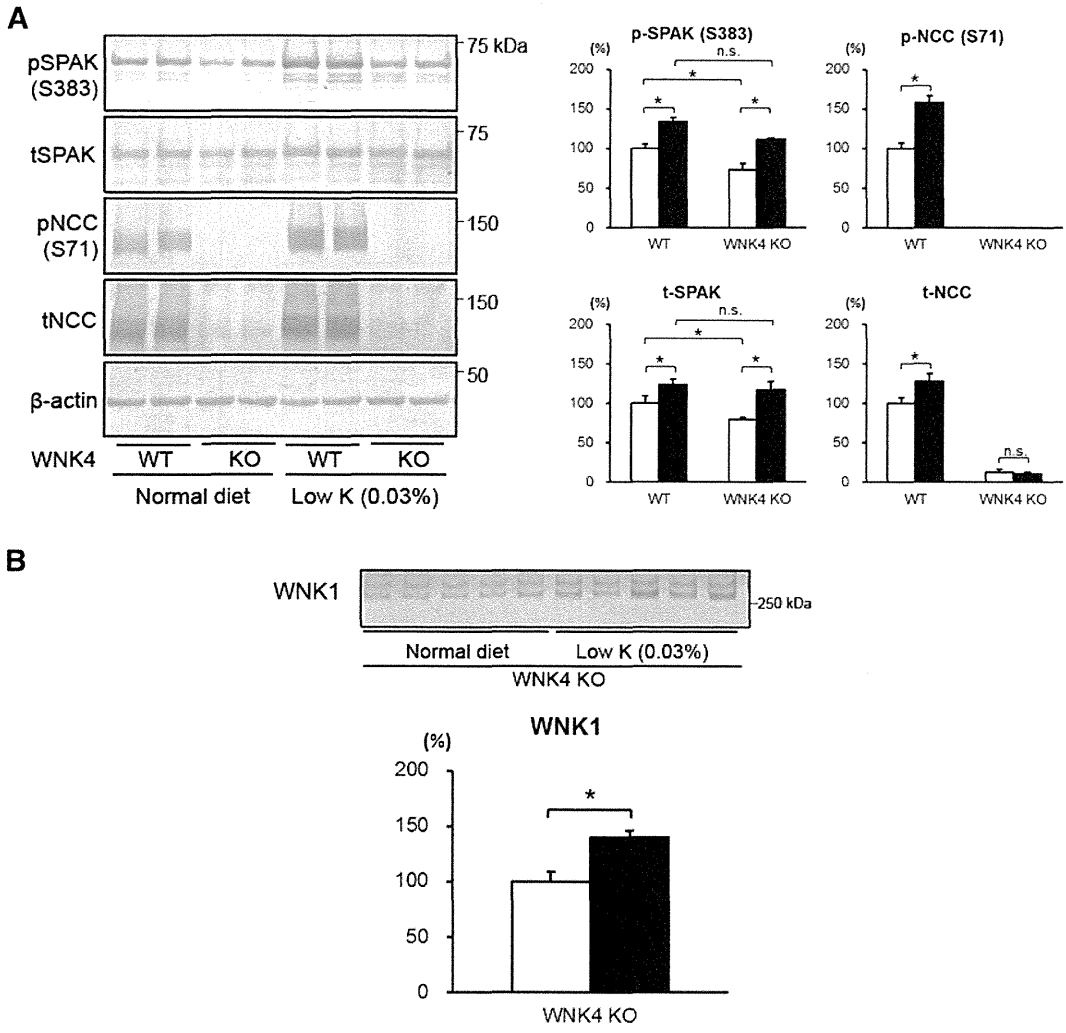

Figure 3 **Responses to a low-potassium diet in WNK4$^{-/-}$ mice**
(**A**) Immunoblots of the kidney proteins of WNK4$^{+/+}$ and WNK4$^{-/-}$ mice fed normal (open bars) or low-potassium (black bars) diets for 7 days before experiments (*$P < 0.05$; $n = 5$–6). Both WNK4$^{+/+}$ and WNK4$^{-/-}$ mice showed increased phosphorylation of SPAK when fed the low-potassium diet. However, WNK4$^{-/-}$ mice did not exhibit increased phosphorylation of NCC. (**B**) In WNK4$^{-/-}$ mice, WNK1 increased with a low-potassium diet (black bar) compared with a normal diet (open bar; *$P < 0.05$; $n = 5$).

AngII-induced NCC activation are reduced in WNK4 knock-out mice [13]. Therefore, we investigated the involvement of WNK4 in the regulation of NCC by a low-potassium diet and insulin.

First, we fed the mice a low-potassium diet [0.03% (w/w) KCl] for 7 days. A low-potassium diet-induced increase in total and phosphorylated NCC observed in WNK4$^{+/+}$ mice was not evident in WNK4$^{-/-}$ mice. In contrast, both WNK4$^{+/+}$ and WNK4$^{-/-}$ mice exhibited increased SPAK phosphorylation, when fed a low-potassium diet (Figure 3A). We hypothesized that other WNKs may be involved in SPAK activation in nephron segments other than the DCT in WNK4$^{-/-}$ mice, and examined WNK1 expression in these mice. As expected, a further increase in WNK1 expression was evident in WNK4$^{-/-}$ mice fed a low-potassium diet (Figure 3B).

We then performed an acute insulin infusion experiment in WNK4$^{-/-}$ mice by administering insulin intraperitoneally at a dose of 5 U/kg, and killed the mice 60 min after injection. As shown in Figure 4, insulin-induced SPAK and NCC phosphorylation in the kidneys did not occur in WNK4$^{-/-}$ mice, indicating that the effect of insulin signalling on SPAK and NCC is exclusively mediated by WNK4.

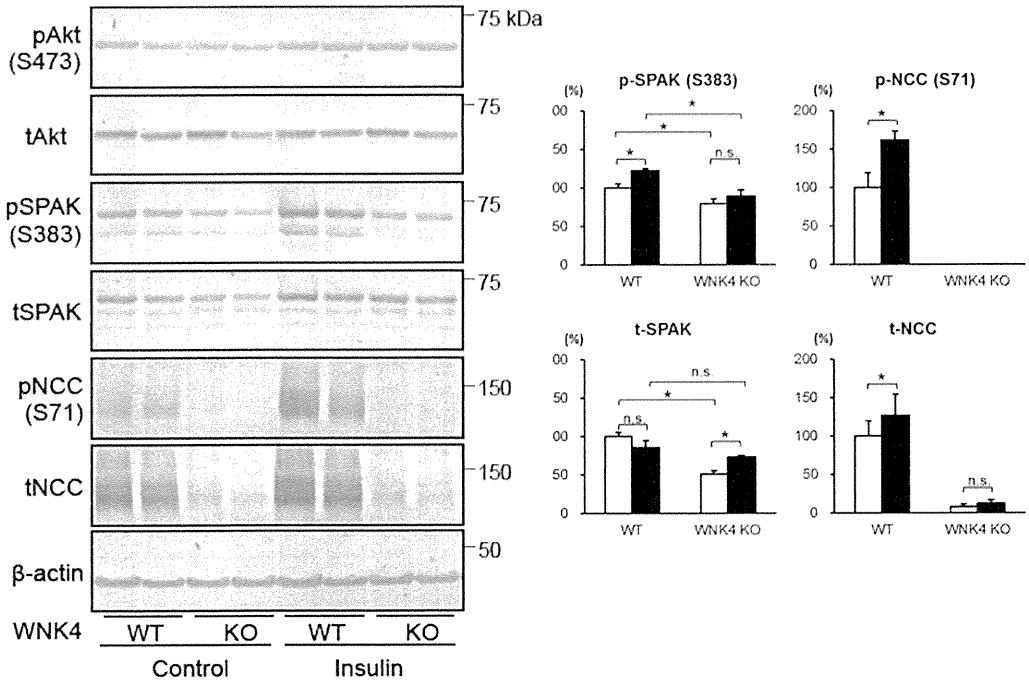

Figure 4 **Response to acute insulin administration in WNK4$^{-/-}$ mice**
Immunoblots of kidney proteins from WNK4$^{+/+}$ and WNK4$^{-/-}$ mice infused with vehicle (open bars) or insulin (black bars). Insulin-induced SPAK and NCC phosphorylation were not observed in WNK4$^{-/-}$ mice. Insulin was administrated intraperitoneally at a dose of 5 units/kg. Control mice received vehicle alone. Mice were killed 60 min after insulin injection (*$P < 0.05$; $n = 5$).

Role of WNK4 in the regulation of NCC in mice fed a high-salt diet

We showed that WNK4 behaves as a positive regulator of NCC through the activation of SPAK and OSR1 in the kidneys *in vivo* [10,17]. However, some still believe that WNK4 is a negative regulator of NCC, switching to positive regulation only in the presence of AngII signalling [32]. Therefore, we investigated the role of WNK4 in NCC regulation under suppression of the RAA (renin-angiotensin-aldosterone) system affected by a high-salt diet. We fed the mice a normal [0.9% (w/w) NaCl] or high-sodium [4% (w/w) NaCl] diet for 7 days before the extraction of the kidney proteins and collection of blood samples. Aldosterone levels were significantly decreased by the high-salt diet in WNK4$^{+/+}$ and WNK4$^{-/-}$ mice (Figure 5A), indicating that the RAA system was suppressed. As shown previously [11] and in Figure 5(B), a high-salt diet suppressed phosphorylation of SPAK and NCC and reduced total NCC in WNK4$^{+/+}$ mice. If WNK4 is a negative regulator of NCC in the absence of AngII signalling in WNK4$^{+/+}$ mice, NCC should be activated by a mechanism independent of WNK–OSR1/SPAK signalling in WNK4 knockout mice [8]. However, phosphorylated and total NCC did not increase in WNK4$^{-/-}$ mice compared with WNK4$^{+/+}$ mice, remaining almost undetectable. Furthermore, BP in WNK4$^{-/-}$ mice did not increase compared with WNK4$^{+/+}$ mice, even with a high-salt diet (Figure 2A), suggesting that NCC is never activated in the absence of WNK4. Thus, there is no evidence for a negative regulatory role of WNK4 in NCC regulation in the kidneys, even when RAA signalling is suppressed.

DISCUSSION

In hypertension research, the mechanism of renal sodium transport regulation is a controversial issue. In this study, we clarified the essential role of WNK4 as a positive regulator of NCC in the kidneys using the WNK4 knockout mice. We demonstrated that NCC expression and phosphorylation are highly dependent on the presence of WNK4, and that the absence of WNK4 does not promote NCC expression under any of the conditions tested in this study.

There is now general consensus that the phosphorylation status of NCC reflects its *in vivo* activity [34]. NCC phosphorylation at specific N-terminal sites was shown to be important for its transport activity [34], and also for its localization at the apical plasma membrane [9,35]. We have often observed that a decrease in NCC phosphorylation is accompanied by a decrease in total NCC in the kidneys [10,11]. We believe that the primary change was in phosphorylation because we often observed the magnitude of change in phosphorylation was greater than that in total NCC. We recently showed that phosphorylation inhibits NCC

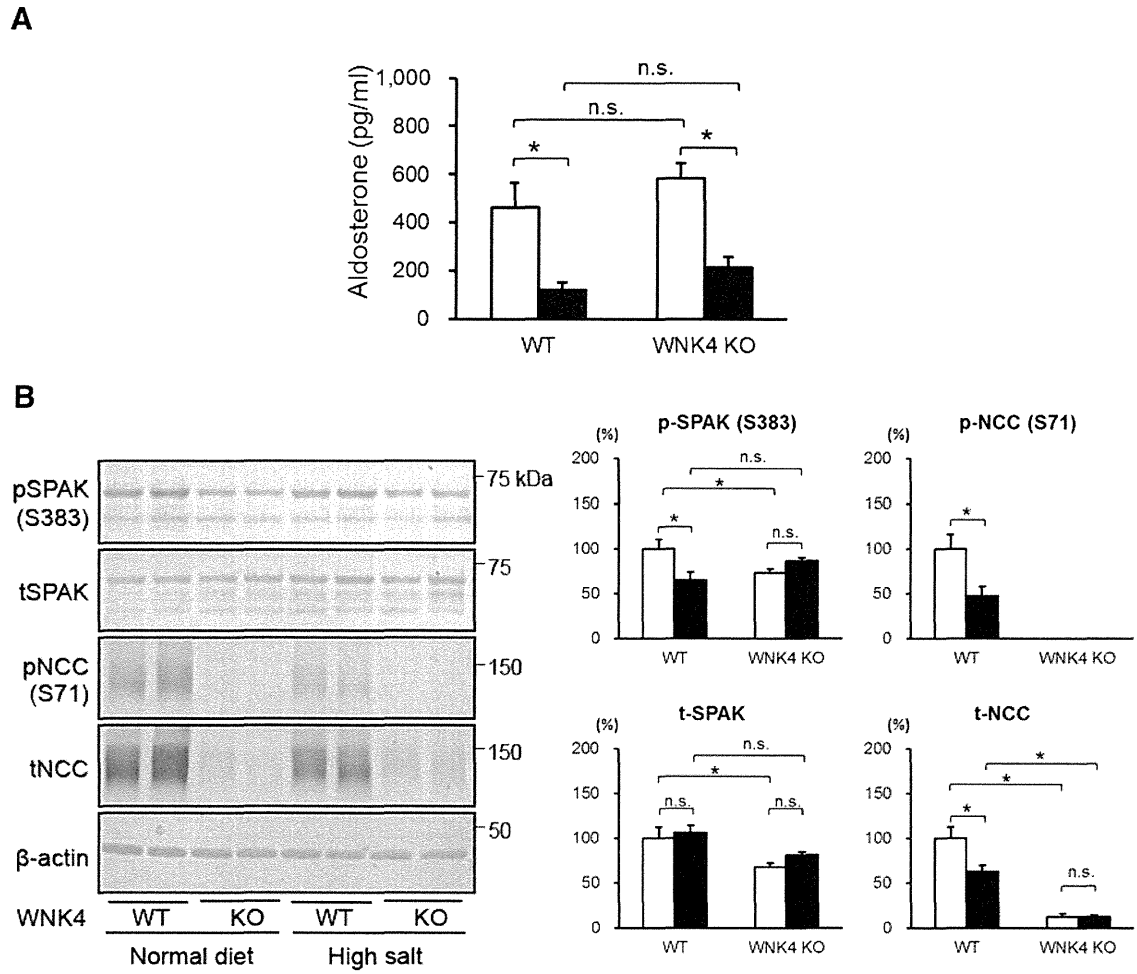

Figure 5 **Response to a high-salt diet in WNK4$^{-/-}$ mice**
(**A**) Plasma aldosterone level in WNK4$^{+/+}$ and WNK4$^{-/-}$ mice on a normal (open bars) or high-salt (black bars) diet. There was no difference between WNK4$^{+/+}$ and WNK4$^{-/-}$ mice. A high-salt diet significantly decreased aldosterone levels in both groups ($*P < 0.05$; $n = 5$–6). (**B**) Immunoblots of the kidney proteins of WNK4$^{+/+}$ and WNK4$^{-/-}$ mice fed a normal (open bars) or high-salt (black bars) diet for 7 days ($*P < 0.05$; $n = 5$). WNK4 knock out did not ameliorate the decreased phosphorylation and total amount of Na–Cl cotransporter caused by a high-salt diet.

ubiquitination, supporting this theory [36]. In this respect, NCC regulation by phosphorylation as a result of WNK–OSR1/SPAK signalling is the major mechanism of NCC regulation, and WNK4 must be considered an important positive regulator of NCC, as both WNK4 and WNK1 were shown to phosphorylate OSR1 and SPAK *in vitro* [12]. Furthermore, the discovery of new genes (*KLHL3* and *Cullin-3*) responsible for PHAII [14,15] supports the notion that WNK4 positively regulates NCC in the human kidney. Recently, we also generated several lines of wild-type WNK4–BAC TG mice, and showed that overexpression of WNK4 in the kidneys robustly increased SPAK and NCC phosphorylation and induced PHAII phenotypes [16]. Before this study, Lalioti et al. [37] reported that a single line of WNK4 TG mice appeared to show inhibition, rather than activation, of the NCC function. This data was the sole *in vivo* evidence validating the negative effect of wild-type WNK4 on NCC suggested by *in vitro* studies [5,6]. However, Lalioti et al. data [37] should be interpreted with caution, considering the pitfalls inherent in studies using TG mice [16].

In this study, WNK4$^{-/-}$ mice exhibited an almost complete absence of total and phosphorylated NCC on immunoblots, consistent with the data of Castañeda-Bueno et al. [13]. Although this group focused on the role of WNK4 in NCC regulation in the presence of AngII stimulation, their analyses of WNK4 knockout mice gave us further opportunity to investigate the role of WNK4 in NCC regulation under other conditions, thereby establishing a complete picture of the role of WNK4 in NCC regulation *in vivo*. As shown by our results, NCC was never up-regulated or activated by the deletion of WNK4 even in the absence of AngII stimulation. We are not denying the inhibitory effect of wild-type WNK4 on NCC observed in *Xenopus* oocytes [5,6] and cultured cells [38]. However, these inhibitory effects may

be minimal in the kidneys *in vivo*, overwhelmed by the strong positive regulation of NCC by WNK4 via its phosphorylation. The importance of phosphorylation to the regulation of NCC is confirmed by the absence of PHAII phenotypes in NCC TG mice [39]. An increase in the abundance of NCC alone, without upstream WNK–OSR1/SPAK signalling, does not result in NCC activation.

We observed some minor phenotypic differences between our WNK4$^{-/-}$ mice and those used by Castañeda-Bueno et al. [13]. WNK4 knockout mice used by Castañeda-Bueno et al. [13] exhibited hypokalaemia, but our WNK4$^{-/-}$ mice did not exhibit hypokalaemia. We observed a slight but significant decrease in total and phosphorylated SPAK in our WNK4$^{-/-}$ mice, and an increase in total OSR1, but those used by Castañeda-Bueno et al. [13] did not exhibit any significant changes in SPAK and OSR1. The origin of these disparities is unclear, but differences in genetic background may provide one explanation, as both WNK4 knockout mice have a mixed background (C57BL6/J and 129Sv), and the contribution of these strains to each WNK4 knockout mouse may differ. Regarding serum potassium, even NCC knockout mice did not exhibit hypokalaemia [40], suggesting that normokalaemia in our WNK4$^{-/-}$ mice is not necessarily unexpected. It is also true that data on serum potassium in mice vary significantly depending on how blood samples are collected (after killing versus alive but under anaesthesia). As for the differences in SPAK and OSR1, these may originate from the antibodies used in the two studies.

We also obtained data on WNK-related molecules not described in the study by Castañeda-Bueno et al. [13]. We demonstrated that WNK1, but not WNK3, underwent a compensatory increase in WNK4$^{-/-}$ mice, further establishing the minor involvement of WNK3 in NCC regulation in the kidneys. However, this increase in WNK1 in the DCT may be insufficient to compensate for WNK4 deletion, as NCC phosphorylation was significantly decreased in WNK4$^{-/-}$ mice. Although insufficient to compensate for the absence of WNK4, this finding suggests that WNK1 has the same role as WNK4 in the DCT. As no controversy surrounds the positive influence of WNK1 on NCC, this data also supports the positive role of WNK4 in NCC regulation. An increase in WNK1 expression was also evident in WNK4$^{-/-}$ mice fed a low-potassium diet (Figure 3B). Increased WNK1 expression may explain the increase in SPAK phosphorylation, but did not contribute to NCC phosphorylation, suggesting that the increased phosphorylation of SPAK in WNK4$^{-/-}$ mice fed a low-potassium diet occurs in nephron segments other than the DCT.

For the first time, we have presented the status of ROMK in WNK4$^{-/-}$ mice. Previously, ROMK was shown to be inhibited by wild-type WNK4, and strongly inhibited by PHAII-causing mutant WNK4 [41]. Through the analysis of *WNK4*$^{D561A/+}$ PHAII model mice crossed with *Osr1* and *Spak* knock-in mice, we showed that the pathogenesis of hypokalaemia is also the result of NCC activation by WNK–OSR1/SPAK signalling, but is not caused by a direct effect of mutant WNK4 on ROMK. However, the effect of wild-type WNK4 on ROMK has not been clarified *in vivo*. We observed a clear increase in ROMK in the immunoblots of the total kidney samples from WNK4$^{-/-}$ mice exhibiting normal serum potassium levels. Although further detailed analyses focusing on intracellular localization are necessary, our data suggest that wild-type WNK4 behaves as a negative regulator of ROMK in the kidneys, consistent with *in vitro* studies [41].

In conclusion, data obtained from our WNK4$^{-/-}$ mice under various conditions clearly show that WNK4 is a strong positive regulator, and never a negative regulator, of NCC in the mouse kidney *in vivo*.

AUTHOR CONTRIBUTIONS

Daiei Takahashi performed the mice studies, and wrote the manuscript. Takayasu Mori, Naohiro Nomura, Muhammad Zankir Hossain Khan, Eisei Sohara and Tatemitsu Rai generated WNK4$^{-/-}$ mice. Yuya Araki, Moko Zeniya and Sei Sasaki helped in general experimental procedures and contributed to data discussion. Shinichi Uchida designed and directed the project and wrote the manuscript.

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SUPPLEMENTARY DATA

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

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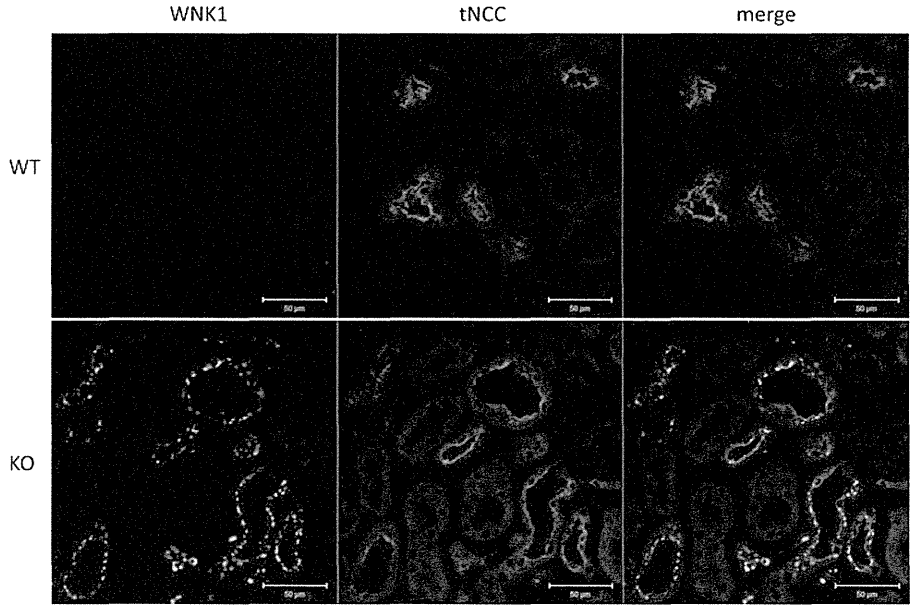

Figure S1 **Immunofluorescence of WNK1 and NCC in the kidney**
The immunofluorescence of WNK1 and NCC in the kidneys of WNK4$^{+/+}$ and WNK4$^{-/-}$ mice. An apparent increase in WNK1 in the kidneys of WNK4$^{-/-}$ mice was only observed in the distal convoluted tubules (DCT). However, the DCT appeared to be dilated with a reduced cell height and NCC immunofluorescence.

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Review

Regulation of with-no-lysine kinase signaling by Kelch-like proteins

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In 2001, with-no-lysine (WNK) kinases were identified as the genes responsible for the human hereditary hypertensive disease pseudohypoaldosteronism type II (PHAII). It took a further 6 years to clarify that WNK kinases participate in a signaling cascade with oxidative stress-responsive gene 1 (OSR1), Ste20-related proline-alanine-rich kinase (SPAK), and thiazide-sensitive NaCl cotransporter (NCC) in the kidney and the constitutive activation of this signaling cascade is the molecular basis of PHAII. Since this discovery, the WNK–OSR1/SPAK–NCC signaling cascade has been shown to be involved not only in PHAII but also in the regulation of blood pressure under normal and pathogenic conditions, such as hyperinsulinemia. However, the molecular mechanisms of WNK kinase regulation by dietary and hormonal factors and by PHAII-causing mutations remain poorly understood. In 2012, two additional genes responsible for PHAII, *Kelch-like 3* (*KLHL3*) and *Cullin3*, were identified. At the time of their discovery, the molecular mechanisms underlying the interaction between these genes and their involvement in PHAII were unknown. Here we review the pathophysiological roles of the WNK signaling cascade clarified to date and introduce a new mechanism of WNK kinase regulation by *KLHL3* and *Cullin3*, which provides insight on previously unknown mechanisms of WNK kinase regulation.

With-no-lysine kinases and pseudohypoaldosteronism type II

Polymerase chain reaction (PCR)-based homology cloning of mitogen-activated protein kinases (MAP) and MEK kinase initially identified WNK1 kinase (Xu et al., 2000). Subsequently, a database search revealed the existence of homologous kinase genes in mammals and in other species: four homologues (*WNK1–4*) were discovered in mammals, one in *Drosophila melanogaster*, one in *Caenorhabditis elegans*, and eight in *Arabidopsis thaliana*, but none was discovered in yeast (Verissimo and Jordan, 2001). The kinases were named "with-no-lysine" (WNK) kinases because the lysine (K) residue present in subdomain II of most kinases was not conserved in WNK kinases but instead replaced with a cysteine residue. As shown in Fig. 1, a kinase domain exists at the N-terminus of WNK kinases, followed by an autoinhibitory domain (Xu et al., 2002) and a coiled-coil domain. Another coiled-coil domain is present at the C-terminus.

In 2001, *WNK1* and *WNK4* were identified as the genes responsible for the autosomal dominant hereditary hypertensive disease pseudohypoaldosteronism type II (PHAII; Wilson et al., 2001). In addition to hypertension, PHAII is characterised by hyperkalemia, metabolic acidosis and thiazide sensitivity (Gordon, 1986). Thiazide is widely used as an antihypertensive drug: It induces salt excretion into the urine as it is a specific inhibitor for NaCl cotransporter (NCC) in the distal tubules of the kidney. NCC is responsible for the reabsorption of approximately 5%–10% of filtered NaCl in the glomeruli. At the time of this discovery, a substrate for WNK kinases was yet to be identified, but it was expected that NCC was regulated by *WNK1* and *WNK4* because the activation of NCC was considered the major pathogenesis of PHAII.

The mutations found in the *WNK1* gene comprised large deletions in intron 1, which were considered

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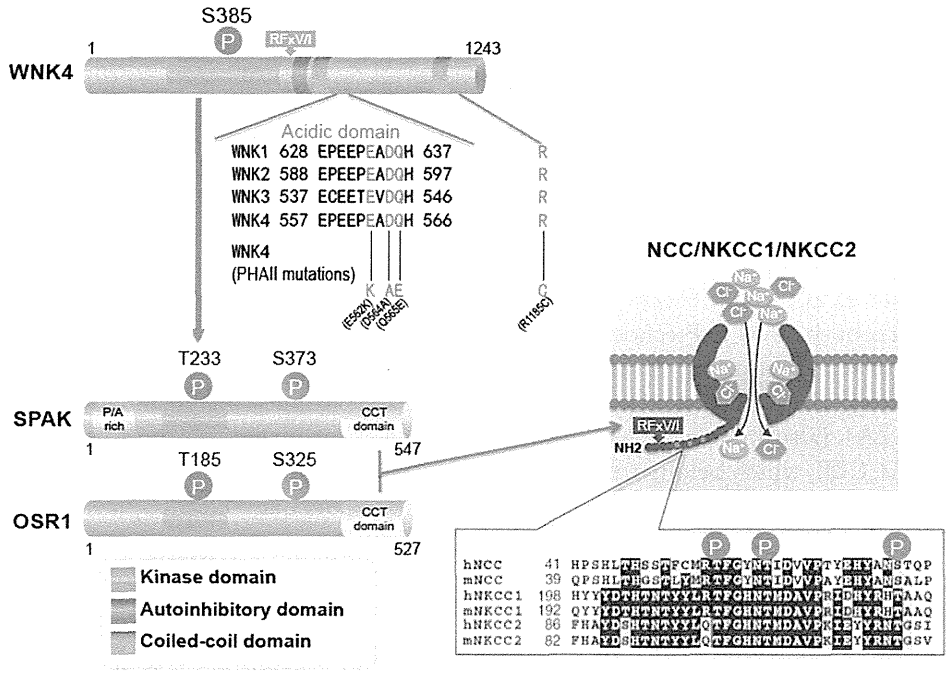
Abbreviations: KLHL3, Kelch-like 3; NCC, NaCl cotransporter; OSR1, oxidative stress-responsive gene 1; PHAII, pseudohypoaldosteronism type II; SPAK, Ste20-related proline-alanine rich kinase; WNK, with-no-lysine.

Figure 1 | Structures of WNK, OSR1, and SPAK kinases

Acidic domains are located downstream of the first coiled-coil domain and conserved in all WNK kinases. Three of four pseudohypoaldosteronism type II-causing mutations in *WNK4* are located in the acidic domain. WNK kinases activate OSR1 and SPAK by phosphorylating threonine residues in their kinase domains (T185 and T233). Serine residues (S325 in OSR1 and S373 in SPAK) in the S motif are also phosphorylated by WNK kinases, but their phosphorylation is not involved in the activation of the kinases. Conserved C-terminal domains in OSR1 and SPAK (shown in yellow) bind to the RFx[V/I] motif in WNK and solute carrier family 12 transporters. The N-terminal regions of NCC, NKCC1, and NKCC2 around the sites phosphorylated by OSR1 and SPAK are highly conserved.



to increase its transcription based on reverse transcription PCR analysis of *WNK1* mRNA levels in the leukocytes of patients with PHAII (Wilson et al., 2001). However, after the initial report, the existence of two isoforms in WNK1, full-length WNK1 and a kidney-specific WNK1 lacking the kinase domain, was clarified (Delaloy et al., 2003; O'Reilly et al., 2003). Exactly which isoform is increased in patients with PHAII, and whether WNK1 expression is indeed increased in the human kidney, remains undetermined (Delaloy et al., 2008). In the case of *WNK4*, four missense mutations were identified in patients with PHAII, three of which are clustered within a distance of four amino acids in a region termed the "acidic domain" (Wilson et al., 2001). As shown in Fig. 1, this domain is well conserved in all WNK kinase isoforms.

Discovery of the WNK–oxidative stress-responsive gene 1/Ste20-related proline–alanine-rich kinase–solute carrier family 12a transporter signaling cascade

After the identification of *WNK1* and *WNK4* as the genes underlying PHAII, numerous investigations of the effects of coexpressing WNK1 and WNK4 with transporters, including NCC, were published (Kahle et al., 2003; Wilson et al., 2003; Yang et al., 2003; Kahle et al., 2004; Yamauchi et al., 2004, 2005; Cai et al., 2006; Gamba, 2006; Garzon-Muvdi et al., 2007; Ring et al., 2007; Yang et al., 2007a). In most studies, WNK4 was demonstrated to exert an inhibitory effect on the transporters. However, the detailed mechanisms of this regulation, in particular the intracellular signaling cascades involved, were poorly understood. Then, in 2005, two groups

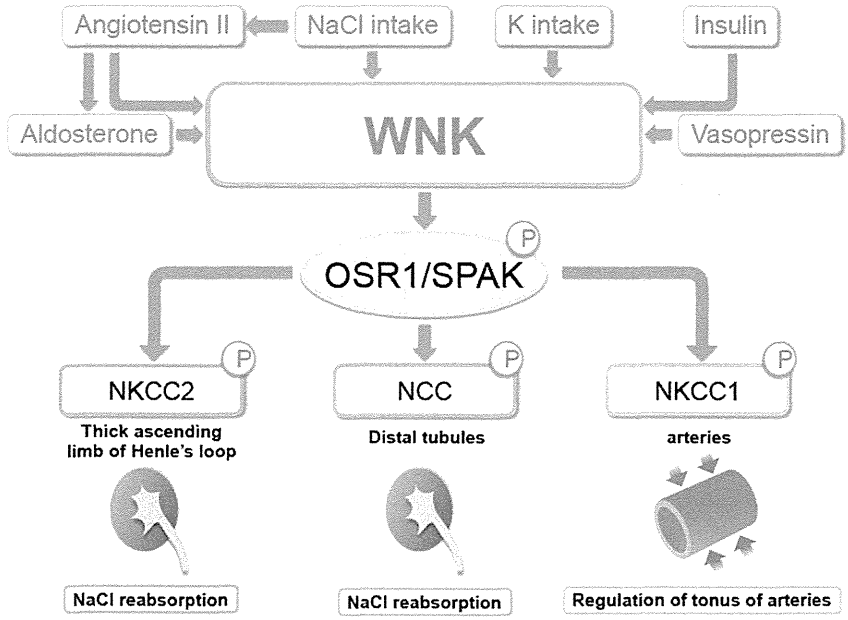
identified that oxidative stress-responsive gene 1 (OSR1) and Ste20-related proline-alanine-rich kinase (SPAK) were substrates of WNK1 and WNK4 (Moriguchi et al., 2005; Vitari et al., 2005). OSR1 and SPAK are related serine–threonine kinases that possess an N-terminal catalytic domain similar to those of other members of the Ste20 kinase subfamily, and two conserved regions known as the serine motif (S motif) and conserved C-terminal (CCT) domain. SPAK also possesses a unique 48-amino-acid N-terminal extension that primarily consists of alanine and proline. The CCT domains of OSR1 and SPAK were shown to interact with the RFv[V/I] motif in WNK kinases and solute carrier family 12 (SLC12) transporters (Fig. 1). OSR1 and SPAK were already identified as regulators of the SLC12A2 [also known as Na-K-2Cl-cotransporter 1 (NKCC1)] cotransporter (Flemmer et al., 2002; Piechotta et al., 2002; Dowd and Forbush, 2003; Piechotta et al., 2003): through *in vitro* experiments, Moriguchi et al. (2005) demonstrated that SLC12A3 (also known as NCC) and SLC12A1 [also known as Na-K-2Cl-cotransporter 2 (NKCC2)], which belong to the same transporter family as NKCC1, could also be substrates of OSR1 and SPAK. To prove this notion in the kidney *in vivo*, Yang et al. (2007c) generated anti-phosphorylated NCC (pNCC) antibodies that recognised potential serine and threonine phosphorylation sites deduced from sequence alignment with NKCC1. They also generated a mouse model of PHAII: a knock-in mouse carrying a PHAII-causing missense mutation of *WNK4* (D561A), corresponding to the D574A mutation in patients with PHAII (Yang et al., 2007c). $Wnk4^{D561A/+}$ mice exhibited a PHAII phenotype, including increased thiazide sensitivity, indicating that NCC is activated in the kidneys of the mutant mice. Using anti-pNCC antibodies, Yang et al. (2007c) demonstrated that NCC phosphorylation at three sites (Thr53, Thr58 and Ser71 in mouse NCC) was significantly increased in the kidneys of PHAII model mice, and that pNCC was concentrated on the apical plasma membranes of the distal convoluted tubules. Phosphorylation of SPAK and OSR1 was also increased in $Wnk4^{D561A/+}$ mice, suggesting that WNK–OSR1/SPAK–NCC signaling was present in the kidney and activated by the PHAII-causing *WNK4* mutation. Subsequently, by crossing $Wnk4^{D561A/+}$ mice with *SPAK* and *OSR1* knock-in mice, in which the T-loop Thr residues in SPAK (Thr243) and OSR1 (Thr185) were mutated to Ala to prevent activation by WNK kinases, Chiga et al. (2011) demonstrated that NCC phosphorylation and PHAII phenotypes in $Wnk4^{D561A/+}$ mice were dependent on WNK–OSR1/SPAK signaling. Thus, the WNK–OSR1/SPAK–NCC signaling cascade in the kidney was established, and its activation was shown to be the pathogenic mechanism underlying PHAII. The WNK kinase responsible for NCC phosphorylation in the kidney was later identified as WNK4 through the analysis of *WNK1*, *WNK3* and *WNK4* knockout mice (Ohta et al., 2009; Oi et al., 2012; Castaneda-Bueno et al., 2012; Susa et al., 2012).

The mechanism of NCC activation by phosphorylation was initially investigated by Pacheco-Alvarez et al. (2006) using the *Xenopus laevis* oocyte expression system. Phosphorylation-incompetent mutant NCC molecules were present on the plasma membrane, but their transport activity was significantly decreased, suggesting that phosphorylation of NCC is important for its transport activity. As previously mentioned, analysis of NCC phosphorylation in the kidney *in vivo* clarified that phosphorylated NCC was exclusively present on the apical plasma membranes of the distal convoluted tubules (Yang et al., 2007c; Pedersen et al., 2010; Lee et al., 2013), suggesting that phosphorylation regulates the plasma membrane expression of NCC. Hossain Kahn et al. (2012) found that phosphorylation of NCC decreased its ubiquitination: decreased endocytosis and/or degradation may underlie the increased phosphorylated NCC accumulation evident in the apical plasma membranes of the distal convoluted tubules.

Regulators of WNK signaling

After the discovery of the WNK–OSR1/SPAK–NCC signaling cascade in the kidney and its involvement in PHAII, its pathophysiological roles outside of PHAII were investigated (Fig. 2). Salt intake regulates this cascade, partly through aldosterone (Chiga et al., 2008; Vallon et al., 2009). High and low salt intake decreased and increased the phosphorylation of OSR1/SPAK and NCC in the kidney, respectively, adjusting the excretion of NaCl according to its intake. This regulation was abolished in $Wnk4^{D561A/+}$ mice (Chiga et al., 2008): A high-salt diet did not down-regulate WNK–OSR1/SPAK–NCC signaling

Figure 2 | Regulators and effectors of WNK–OSR1/SPAK kinase signaling
NaCl and K intakes regulate WNK kinase–OSR1/SPAK-NCC signaling in the kidney. Angiotensin II, aldosterone, vasopressin, and insulin also regulate WNK–OSR1/SPAK–NCC signaling in the kidney. A WNK1/WNK3–SPAK–Na-K-2Cl-cotransporter 1 cascade regulates arterial tonus.



in PHAII model mice. Elucidation of the mechanism of this dysregulation was one of the important unanswered questions in the molecular pathogenesis of PHAII. Potassium intake also regulates this cascade; high and low potassium intake decreased and increased WNK–OSR1/SPAK–NCC signaling, respectively (Vallon et al., 2009; Sorensen et al., 2013; van der Lubbe et al., 2013). As the initial phenotype of PHAII is hyperkalemia rather than hypertension, WNK–OSR1/SPAK–NCC signaling must also regulate potassium homeostasis in the body. In this regard, it is reasonable to predict that this signaling cascade is regulated by potassium intake. Although Naito et al. (2011) reported that extracellular potassium levels directly regulated WNK1 activity in cultured cells; the mechanisms of WNK kinase regulation by dietary potassium remain unclear. Hormonal factors also regulate WNK signaling. In addition to aldosterone, angiotensin II (San-Cristobal et al., 2009; Talati et al., 2010; van der Lubbe et al., 2011; Castaneda-Bueno et al., 2012; Castaneda-Bueno and Gamba, 2012) and vasopressin (Mutig et al., 2010; Pedersen et al., 2010; Rieg et al., 2013; Saritas et al., 2013) reportedly activated this signaling cascade. However, the details of intracellular signaling from these hormones to WNK kinases are poorly understood. Recently, insulin was identified as a powerful activator of this signaling cascade, and the phosphatidylinositol 3-kinase/Akt pathway was shown to mediate the signal from insulin to WNK4 (Sohara et al., 2011; Nishida et al., 2012; Chavez-Canales et al., 2013). Constitutive activation of this cascade caused by hyperinsulinemia may underlie the pathogenesis of salt-sensitive hypertension in metabolic syndrome (Nishida et al., 2012; Komers et al., 2012).

Extrarenal roles of WNK–OSR1/SPAK kinase signaling

In addition to NaCl and K homeostasis in the kidney, WNK–OSR1/SPAK signaling has been shown to be involved in the regulation of arterial tonus. In this context, the transporter involved is not NCC but NKCC1. *SPAK* knockout mice showed a decreased response to phenylephrine and decreased phosphorylation of NKCC1 (Yang et al., 2010). Similarly,

heterozygous *WNK1* knockout mice exhibited reduced phosphorylation of NKCC1 and reduced arterial tonus (Bergaya et al., 2011; Susa et al., 2012). Zeniya et al. (2013) reported the existence of WNK3–SPAK–NKCC1 signaling in vascular smooth muscle cells, which was regulated by salt intake through angiotensin II. Thus, WNK–OSR1/SPAK signaling is involved in the regulation of blood pressure by modulating both NaCl excretion in the kidney and vascular tonus in the arteries (Fig. 2).

In addition, mutation of the *WNK1* gene was shown to be responsible for human neuropathy (Shekarabi et al., 2008). WNK kinases were also shown to regulate KCl cotransporters (KCC family; Kahle et al., 2005; de de Los Heros et al., 2006; Garzon-Muvdi et al., 2007; Rinehart et al., 2009). The reciprocal regulation of NKCC1 and SLC12A5 (also known as KCC2) by WNK kinases is postulated to regulate intracellular chloride concentration, thereby regulating the excitability of neuronal cells (Kahle et al., 2006). Although data supporting this idea are accumulating, further validation by *in vivo* experiments is necessary.

Discovery of *Kelch-like 3* and *Cullin3* as pseudohypoaldosteronism type II causing genes

Although several upstream regulators of this cascade have been identified (Fig. 2), exactly how these regulators regulate WNK kinase activity remains largely unknown. Similarly, how PHAII-causing mutations of *WNK4* activate the cascade remained unelucidated. Recently, two new genes [*Kelch-like protein 3* (*KLHL3*) and *Cullin3*] were identified as genes responsible for causing PHAII (Boyden et al., 2012; Louis-Dit-Picard et al., 2012). However, how these genes were involved in causing PHAII was unknown. Determining how these genes (*WNKs*, *KLHL3* and *Cullin3*) interact and how their mutation causes a common hypertensive disease would contribute to the understanding of the molecular pathogenesis of human hypertension, and also to the identification of new targets for anti-hypertensive drugs.

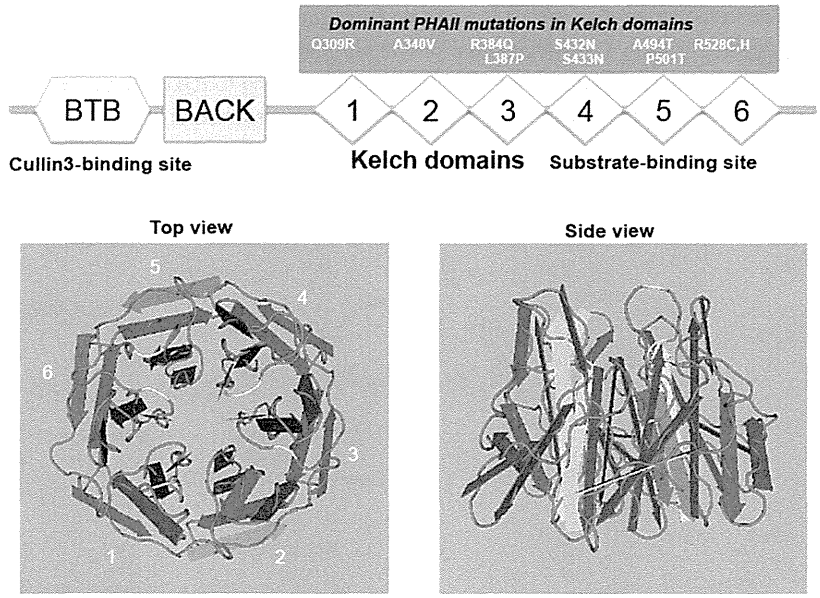
KLHL3 is a member of the Kelch-like protein family, which consists of 42 members (Dhanoa et al., 2013). Kelch-like ECH-associated protein 1 (Keap1), known as the E3 ligase to NRF2, also belongs to the KLHL family and is designated KLHL19 (Dhanoa et al., 2013). In general, KLHL proteins contain one BTB domain, one BTB and C-terminal Kelch (BACK) domain, and five to six Kelch domains (Fig. 3). The BTB domain was named based on a homologous, 115-amino-acid domain present in *D. melanogaster* bric a brac 1, tramtrack, and broad complex proteins and facilitates the protein–protein interaction (Zollman et al., 1994). The Kelch domain forms one blade of a β-propeller structure, as shown in Fig. 3. This domain is also involved in the protein–protein interaction. Kelch domain-containing proteins have been shown to participate in many cellular functions, such as the regulation of cell morphology and gene expression (Adams et al., 2000). Mutations in *KLHL* genes reportedly cause multiple human diseases. *KLHL7* mutations cause autosomal dominant retinitis pigmentosa (Friedman et al., 2009; Kigoshi et al., 2011), and a missense mutation in *KLHL9* causes distal myopathy (Cirak et al., 2010). Mutations in *KLHL16* are linked to human giant axonal neuropathy (Bomont et al., 2000). In investigations of the molecular pathogenesis of these diseases, Kigoshi et al. (2011) clarified that KLHL7 assembles with Cullin3 and exerts E3 ligase activity. Likewise, KLHL20 was also reported to function as an E3 ligase in combination with Cullin3 on death-associated protein kinase (Lee et al., 2010), PDZ-Rho guanine nucleotide exchange factor (Lin et al., 2011) and promyelocytic leukemia protein (Yuan et al., 2011). KLHL7 and KLHL20 proteins bind to Cullin3 via their BTB domains and capture their substrates with their Kelch repeats. Therefore, it has been speculated that the KLHL3-Cullin3 complex also acts an E3 ligase on an unknown target protein.

WNK kinases are substrates of Kelch-like protein 3-Cullin3 E3 ligase

As mutations in *WNK4*, *KLHL3* and *Cullin3* cause the same disease, PHAII, and the activation of WNK–OSR1/SPAK–NCC signaling underlies its pathogenesis, it is reasonable to speculate that components of this signaling cascade, in particular WNK4, could be the substrate of KLHL3-Cullin3 E3 ligase. A French group reported that KLHL3 was able to bind to NCC and regulate its intracellular localisation (Louis-Dit-Picard et al., 2012). They did not investigate whether NCC was ubiquitinated by

Figure 3 | Structure of Kelch-like proteins

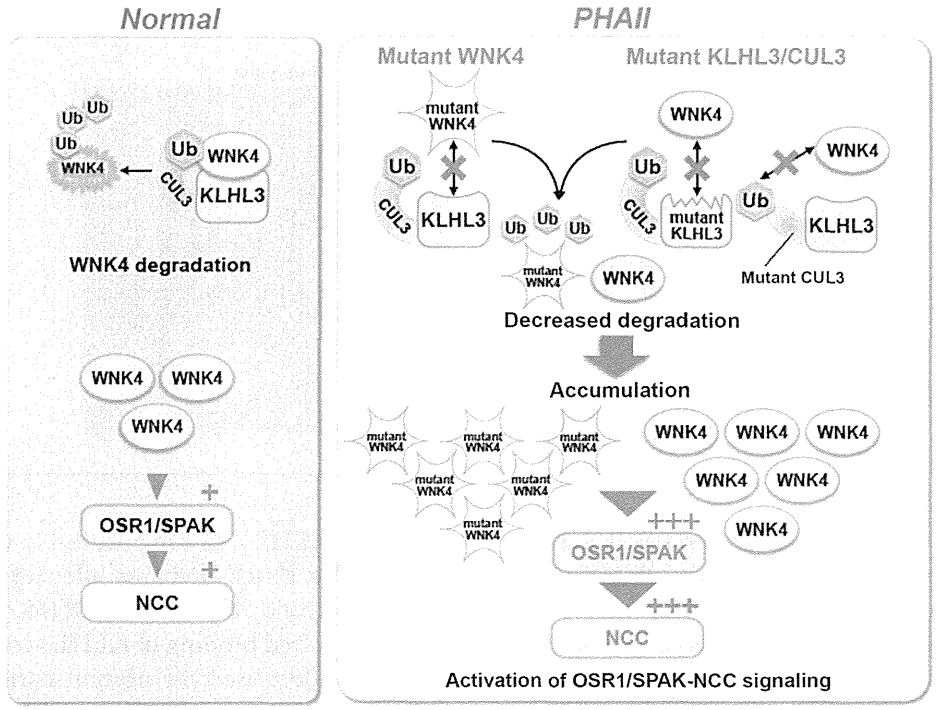
The upper panel shows the structure of Kelch-like (KLHL) proteins with N-terminal BTB and BACK domains and five to six C-terminal Kelch domains, and most autosomal dominant mutations causing pseudohypoaldosteronism type II (PHAII). The BTB domain is a binding site for Cullin 3 and Kelch repeats constitute a propeller structure, as shown in the lower panels, and capture a substrate. Each Kelch domain forms a blade, and most PHAII-causing mutations (shown in yellow lines) are located in the loop regions linking each blade, which may be involved in substrate binding.



KLHL3. Then, Ohta et al. (2013) and Wakabayashi et al. (2013) reported that WNK1 and WNK4 were substrates of KLHL3-Cullin3 E3 ligase, respectively. In both studies, the binding of KLHL3 to NCC was not reproduced. Subsequently, two further reports (Shibata et al., 2013; Wu and Peng, 2013) supported WNK4 as a target of KLHL3-Cullin3 E3 ligase.

Analyses of PHAII-causing mutations in *WNK4*, *KLHL3* and *Cullin 3* also clearly disclosed how these three proteins interact. As previously mentioned, PHAII-causing mutations in *WNK4* were clustered in the acidic domain, which is highly conserved in all WNK kinases (Fig. 1). Wakabayashi et al. (2013) and Mori et al. (2013) showed via fluorescent correlation spectroscopy that binding of KLHL3 to WNK4 was abolished by PHAII-causing mutations in *WNK4*, indicating that the acidic domain is involved in binding KLHL3. In contrast to WNK4, mutations in *KLHL3* were not confined to a single domain, but present in the BTB, BACK and Kelch domains. Mutations in the BTB and BACK domains affected the ability of KLHL3 to bind Cullin3, whereas mutations in the Kelch domains affected the ability of KLHL3 to bind WNK1 and WNK4 (Mori et al., 2013). Impaired binding of KLHL3 to either Cullin3 or WNK4 decreased the ubiquitination of WNK4, resulting in increased WNK4 within cells. PHAII-causing *Cullin3* mutations are clustered around the splice donor and acceptor sites of exon 9. Boyden et al. (2012) showed via experiments in cultured cells that these mutations resulted in the skipping of exon 9. Osawa et al. (2013) and Tsuji et al. (2013) verified that exon 9 was skipped in the leukocytes of patients with PHAII. Mutant *Cullin3* lacking a portion of exon 9 did not show reduced binding to KLHL3, but E3 ligase activity towards WNK4 was significantly decreased (Wakabayashi et al., 2013). Thus, all PHAII-causing mutations in *WNK4*, *KLHL3* and *Cullin3* resulted in a common consequence: reduced ubiquitination of WNK4 and increased WNK4 protein within cells (Fig. 4). This increase in WNK4 protein was confirmed in the kidneys of $Wnk4^{D561A/+}$ mice (Wakabayashi et al., 2013).

Figure 4 | Molecular pathogenesis of pseudohypoaldosteronism type II
Under normal conditions, WNK 4 protein within cells are maintained by appropriate degradation after ubiquitination by KLHL3-Cullin3 E3 ligase. However, PHAII causing-mutations in the acidic domain of WNK4 and in the Kelch domains of KLHL3 affect their binding, thereby reducing the ubiquitination and degradation of WNK4. PHAII-causing mutant *Cullin3* lacking the portion corresponding to exon 9 exhibits lower E3 ligase activity in combination with KLHL3 toward WNK4. Thus, PHAII-causing mutations in three different genes have a common consequence: decreased ubiquitination and increased WNK4 protein levels within cells. The increase in WNK4 protein was confirmed in the kidneys of *Wnk4*$^{D561A/+}$ PHAII model mice. Furthermore, increased WNK4 protein levels in the kidneys of *WNK4* transgenic mice activated OSR1/SPAK–NCC signaling. Although WNK4 is the major WNK kinase regulating NCC in the kidney, other WNKs normally expressed at low levels could also be increased in kidneys with PHAII caused by *KLHL3* and *Cullin3* mutations, thereby contributing to the more severe phenotypes resulting from these mutations compared with those resulting from *WNK1* or *WNK4* mutations alone.



Increased WNK4 in kidney activates OSR1/SPAK–NCC signaling and causes PHAII

Long-standing controversy exists about the influence of WNK4 on NCC function (McCormick and Ellison, 2011). Initially, WNK4 overexpression experiments in *X. laevis* oocytes showed that WNK4 is a negative regulator of NCC (Wilson et al., 2003; Yang et al. 2003). Further analyses by Yang et al. (2005, 2007b) showed that the inhibitory effect of WNK4 on NCC was not kinase activity dependent. Therefore, this inhibitory effect cannot be mediated by OSR1/SPAK–NCC signaling. Casteneda-Bueno et al. (2012) reported that *WNK4* knockout mice exhibit a phenotype reminiscent of Gitelman syndrome (Gitelman syndrome is caused by the loss of function of NCC), indicating that WNK4 is a positive regulator of NCC *in vivo*. In fact, NCC phosphorylation, and even NCC protein abundance, was markedly decreased in the kidneys of *WNK4* knockout mice. Thus, it is barely possible that a decrease in WNK4 levels activate NCC, and there is little evidence that WNK4 is a negative regulator of NCC *in vivo*, except that *WNK4* BAC transgenic mice harboring a single copy of the wild-type *WNK4* transgene exhibited a Gitelman syndrome-like phenotype (Lalioti et al., 2006). The results of this transgenic mouse study were obtained through analysis of a single line

of wild-type *WNK4* transgenic mice, and whether WNK4 protein abundance was indeed increased in the kidney was not shown. Data from transgenic mouse studies should be interpreted with caution, as there is no guarantee that transgenes are expressed in the same manner as endogenous genes. Sometimes, transgenes disrupt endogenous genes by homologous recombination. To circumvent the problems inherent in transgenic mouse studies, analysis of multiple lines of transgenic mice with different copy numbers is necessary. Proof that an observed phenotype is dependent on the level of the protein overexpressed is very important to draw a definite conclusion. Wakabayashi et al. (2013) reproduced the method of transgenic mouse generation used by Lalioti et al. (2006) to generate several lines of *WNK4* BAC transgenic mice. They showed that, as WNK4 protein levels in the kidney increased, phosphorylation of OSR1, SPAK and NCC robustly increased. Furthermore, their *WNK4* transgenic mice mimicked the phenotype of PHAII model mice. These results indicate that increased wild-type WNK4 in the kidney activates the OSR1/SPAK–NCC signaling cascade and causes PHAII.

Thus, impaired ubiquitination and a consequent increase in WNK4 protein was established as the molecular pathogenesis of PHAII caused by mutations in *WNK4*, *KLHL3* and *Cullin3* (Fig. 4). However, WNK kinases other than WNK4 may also be regulated by the KLHL3-Cullin3 complex. The amino acid sequence of the KLHL3 binding site in WNK4 is highly conserved in other WNK kinases (Fig. 1), and both the WNK1 and WNK4 proteins were shown to be regulated by KLHL3-Cullin3 (Ohta et al., 2013; Wakabayashi et al., 2013). Therefore, levels of both WNK1 and WNK4 may be increased in the kidneys of patients with PHAII carrying the *KLHL3* and *Cullin3* mutations, further contributing to the activation of OSR1/SPAK–NCC signaling and explaining the more severe PHAII phenotypes evident with *Cullin3* and *KLHL3* mutations than with *WNK1* and *WNK4* mutations (Boyden et al., 2012). PHAII-causing mutations in *WNK1* consist of large deletions in intron 1 (Wilson et al., 2001): This deletion was recently discovered to increase full-length *WNK1* transcription in the kidneys of a mouse model of the *WNK1* mutation (Vidal-Petiot et al., 2013). The mechanism elucidated in this study may not be directly related to the pathogenesis of PHAII caused by *WNK1* mutations. However, PHAII should be considered a disease caused by increased WNK kinase caused by the dysregulation of either transcription or the ubiquitination of WNK kinases.

Future perspectives

Analyses of PHAII pathogenesis suggest that the regulation of levels of WNK kinase protein is an important regulatory mechanism of WNK–OSR1/SPAK–SLC12 signaling. In addition to WNK1 and WNK4, it is hypothesised that other WNKs, such as WNK2 and WNK3, could be substrates of KLHL3-Cullin3 E3 ligase because the KLHL3-binding domain of WNK4 (the acidic domain) is highly conserved in all WNK isoforms. Furthermore, KLHL2 is the closest homolog to KLHL3 among KLHL proteins, and it is also the closest homolog to *D. melanogaster* Kelch (63% homology; (Soltysik-Espanola et al., 1999). Kelch repeats in these three proteins are highly conserved. KLHL2 shares almost perfect homology (98%) with KLHL3 in the loop regions of the Kelch repeats connecting each blade, in which most of the PHAII-causing *KLHL3* mutations cluster (Boyden et al., 2012; Louis-Dit-Picard et al., 2012). The high degree of homology between KLHL2 and KLHL3 is not evident between KLHL3 and other Kelch-like proteins (Prag and Adams, 2003). The function of the loops connecting the blades of the Kelch repeats has not yet been evaluated in KLHL3, but given that these loops form the top face of the β-propeller (Fig. 3) and that this face is considered the substrate-binding pocket, extensive homology in these loop domains between KLHL2 and KLHL3 supports the theory of shared substrate specificity between KLHL2 and KLHL3. Takahashi et al. (2013) verified that KLHL2 in combination with Cullin3 could function as an E3 ligase for all WNK isoforms. These data suggest that all WNK kinases could be regulated by KLHL2 and KLHL3 in multiple cell types. Regulation of WNK kinases by KLHL2 and KLHL3 could be involved in PHAII and in other contexts where WNK kinases are regulated. The hormones and diets known to regulate WNK–OSR1/SPAK signaling (Fig. 2) may not directly regulate WNK but rather regulate KLHLs, thereby regulating WNK kinase. In addition, the binding of WNKs to KLHL2 and KLHL3 could be regulated by external stimuli, such as the phosphorylation of serine and threonine residues in Kelch domains. Further analyses focusing

on these points are necessary, in addition to the confirmation of PHAII pathogenesis *in vivo* in PHAII model mice carrying *KLHL3* and *Cullin3* mutations.

Conclusions

Why PHAII-causing missense mutations in *WNK4* are clustered and how these mutations activate downstream signaling to NCC remained undetermined. Recent advancements in genetics, in particular whole-exome sequencing, revealed two additional genes responsible for causing PHAII, and their discovery helped to construct a complete picture of the molecular pathogenesis of PHAII. Levels of WNK kinases within cells, regulated via ubiquitination by KLHL proteins, are important determinants of the activity of the WNK–OSR1/SPAK–SLC12A signaling cascade. Consequently, KLHL2 and KLHL3 could represent new targets for drug discovery to regulate WNK kinase activity.

Conflict of interest

The authors have declared no conflict of interest.

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