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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REVIEW

Regulation of blood pressure and renal electrolyte balance by Cullin-RING ligases

Shinichi Uchida

Purpose of review
Efforts to explore the pathogenic mechanisms underlying hereditary hypertension caused by a single gene mutation have brought about conceptual advances in our understanding of blood pressure regulation. We here discuss a novel pathogenic mechanism underlying the hereditary hypertensive disease pseudohypoaldosteronism type II (PHAII), caused by mutations in three different genes encoding for Cullin-3, Kelch-like protein 3 (KLHL3), and with-no-lysine kinases (WNKs).

Recent findings
In 2001, mutations in genes encoding for WNKs were identified as being responsible for PHAII. Recent advancements in genetics, in particular whole-exome sequencing, have revealed that mutations in two additional genes encoding for KLHL3 and Cyllin3 also cause PHAII. This discovery contributed to the clarification of the previously unknown regulatory mechanism of WNKs, namely WNK ubiquitination by the KLHL3–Cullin-3 E3 ligase complex.

Summary
Levels of WNKs within cells are regulated via ubiquitination by the KLHL3–Cullin-3 E3 ligase complex and are important determinants of the activity of the WNK-oxidative stress-responsive gene 1 and Ste20-related proline-alanine-rich kinase–SLC12A transporter signaling cascade. The PHAII-causing mutations in WNK4, KLHL3, and Cullin-3 result in the decreased ubiquitination and increased abundance of WNK4 in the kidney, thereby activating the thiazide-sensitive NaCl cotransporter and causing PHAII.

Keywords
blood pressure, Cullin-3, Kelch-like protein 3, kidney, NaCl cotransporter, with-no-lysine kinase

INTRODUCTION

Hypertension is one of the biggest health problems in the industrialized world because it damages critical organs. Studies of monogenic hypertensive diseases, such as Liddle syndrome and pseudohypoaldosteronism type II (PHAII), have provided new insights into the mechanisms of blood pressure regulation in humans. PHAII is an autosomal dominant hereditary hypertensive disease characterized by hyperkalemia, metabolic acidosis, and thiazide sensitivity [1]. Genes encoding for with-no-lysine kinases (WNKs) (WNK1 and WNK4) were identified in 2001 as being responsible for PHAII [2]. Recently, two new genes encoding for Kelch-like protein 3 (KLHL3) and Cullin-3 were also identified as being responsible for PHAII [3▪▪,4▪▪]. Therefore, determining how these causative genes (*WNK*, *KLHL3*, and *Cullin-3*) are orchestrated and how pathogenic mutations in these genes cause a common hypertensive disease would contribute to the understanding of the molecular pathogenesis of hypertension in humans and to the identification of new targets for antihypertensive drugs. We discuss this issue on the basis of the recently published data.

PSEUDOHYPOALDOSTERONISM TYPE II AND WITH-NO-LYSINE KINASES

At the time when *WNK1* and *WNK4* were identified as the causative genes for PHAII, a substrate for WNKs was yet to be identified, but it was expected that NaCl cotransporter (NCC) was regulated by *WNK1* and *WNK4* because PHAII is a thiazide-sensitive disease.

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KEY POINTS

- WNKs are substrates for the KLHL2/3–Cullin-3 E3 ligase complex.
- Impaired degradation of WNK4 and its subsequent increase in the kidney is the common mechanism underlying PHAII, caused by mutations in three different genes encoding for WNK4, KLHL3, and Cullin-3.
- It is important to investigate whether KLHL2/3-mediated regulations of WNKs are involved in the pathophysiology of diseases other than PHAII.

The mutations found in *WNK1* are large deletions in intron 1, which were considered to increase its transcription [2]. The mutations of *WNK4* are four missense mutations, three of which are clustered within a distance of four amino acids in a region termed the 'acidic domain' [2]. This acidic domain is well conserved in all WNK isoforms [5].

WITH-NO-LYSINE KINASE SIGNALING REGULATING SOLUTE CARRIER FAMILY MEMBER 12A TRANSPORTERS

After the identification of *WNK1* and *WNK4* as the causative genes for PHAII, numerous investigations of the effects of the coexpression of WNK1 and WNK4 with transporters, including NCC, were published [6–14]. However, the detailed mechanisms of this regulation, in particular the intracellular signaling cascades involved, were poorly understood. Then, the oxidative stress-responsive gene 1 (OSR1) and Ste20-related proline-alanine-rich kinase (SPAK) were identified as substrates for WNK1 and WNK4 [15,16]. OSR1 and SPAK are related Ser–Thr kinases that belong to the Ste20 kinase subfamily [5], and were already identified as regulators of the SLC12A2 [also known as Na-K-2Cl-cotransporter 1 (NKCC1)] cotransporter [17–20]. Therefore, 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 act as substrates for OSR1 and SPAK. To prove this, and to clarify the molecular pathogenesis of PHAII in the kidney *in vivo*, Yang *et al.* [21] generated a mouse model of PHAII, that is, a knock-in mouse carrying a PHAII-causing missense mutation in *WNK4* (D561A), corresponding to the D564A mutation in patients with PHAII. At the same time, they generated anti-phosphorylated NCC (pNCC) antibodies that recognized potential Ser and Thr phosphorylation sites by OSR1 and SPAK, deduced from sequence alignment with NKCC1. Using the anti-pNCC antibodies, Yang *et al.* [21] demonstrated that NCC phosphorylation 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. SPAK and OSR1 phosphorylation at the specific phosphorylation sites by WNK was also increased in $Wnk^{4D561A/+}$ mice, suggesting that WNK–OSR1/SPAK–NCC signaling was present in the kidney and was activated by the PHAII-causing *WNK4* mutation. The WNK responsible for NCC phosphorylation in the kidney was later identified as WNK4 through the analysis of *WNK1*, *WNK3*, and *WNK4* knockout mice [22–24,25▪].

The mechanism of NCC activation by phosphorylation may be mainly mediated by increased NCC accumulation in the apical plasma membranes of the distal convoluted tubules [21,26,27]. Hossain Khan *et al.* [28] found that phosphorylation of NCC decreased its ubiquitination, and decreased endocytosis and degradation may underlie the increased accumulation of phosphorylated NCC evident in the apical plasma membranes of the distal convoluted tubules.

Apart from NCC regulation, WNK signaling is involved in NKCC1 and NKCC2 regulation. NKCC2 is a target of furosemide and is present on the apical plasma membranes in the thick ascending limb of Henle's loop (TAL). Lin *et al.* [29] generated kidney-specific OSR1 knockout mice, which showed Batter syndrome-like phenotypes with reduced NKCC2 phosphorylation. These data indicate the existence of OSR1–NKCC2 signaling in TAL, although the responsible WNK-regulating OSR1 in TAL remains to be determined (Fig. 1). WNK4 may not be the one as no reduction in NKCC2 phosphorylation was observed in *WNK4* knockout mice (unpublished observation).

In SPAK knockout mice, in addition to the decreased NCC phosphorylation in the kidney, NKCC1 phosphorylation was decreased in the aorta, which showed decreased contractility after phenylephrine administration. Recently, Zeniya *et al.* [30] reported that WNK3 was the WNK responsible for this signaling in the aorta and that this WNK3–SPAK–NKCC1 cascade was regulated by angiotensin II. Thus, WNK may be significantly contributing to blood pressure regulation in extrarenal tissues and the kidney.

NEW GENES CAUSATIVE FOR PSEUDOHYPOALDOSTERONISM TYPE II

Recently, two new genes, *KLHL3* and *Cullin-3*, were identified as being responsible for causing PHAII [3▪▪,4▪▪]. Cullin-3 is one of the six cullins identified in eukaryotes, and cullin-3-based Cullin-RING ubiquitin ligases (CRLs) have been recently identified as being involved in developmental and stress

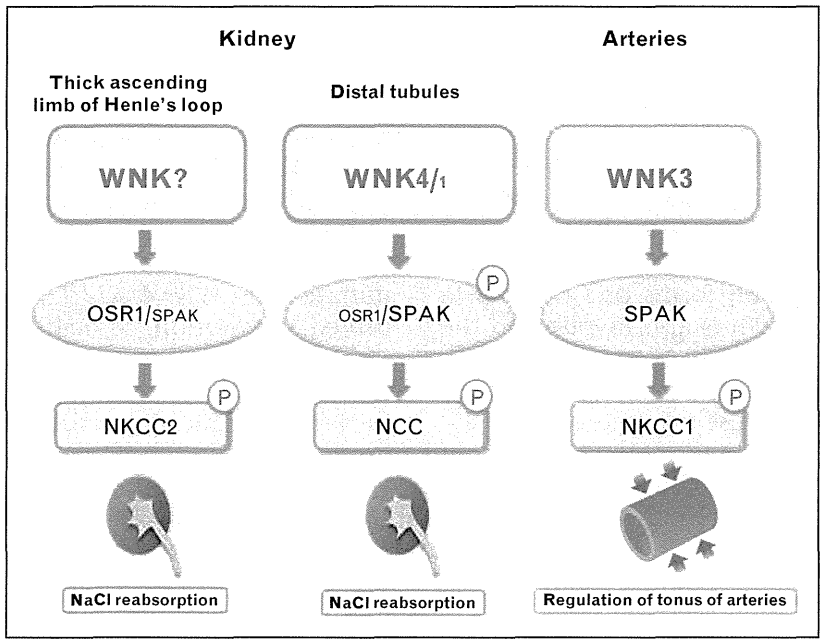

FIGURE 1. WNK signaling in kidneys and arteries. In the signal cascades of WNK–OSR1/SPAK–SLC12A transporters, WNK4 and SPAK may play a dominant role in NCC (SLC12A3) phosphorylation. Likewise, WNK3 (possibly also WNK1) and SPAK (possibly also OSR1) may have similar roles in NKCC1 phosphorylation (SCL12A2) in the smooth muscle cells of arteries. OSR1 was shown to have a major role in NKCC2 (SLC12A1) phosphorylation. The upstream WNK regulating OSR1 in TAL remains to be determined. WNK4 may not be the one as no reduction in NKCC2 phosphorylation was observed in WNK4 knockout mice (unpublished observation).

responses, as well as human hereditary diseases [31▪]. Ubiquitin ligase, also known as E3 ligase, is a key element in the ubiquitin or proteasome system that transfers ubiquitin moieties to substrates. Among several hundreds of E3 ligases identified to date, CRLs constitute the most prevalent class of E3. As shown in Fig. 2, Cullin-3 serves as a scaffold for the catalytic module of a RING finger protein (Rbx1) and a ubiquitin-conjugating enzyme (E2), and a substrate adaptor module. Cullin-3 binds to several substrate adaptor proteins that have BTB domains. The name BTB is derived from a homologous, 115-amino acid domain present in *Drosophila melanogaster* bric a brac 1, tramtrack, and broad complex

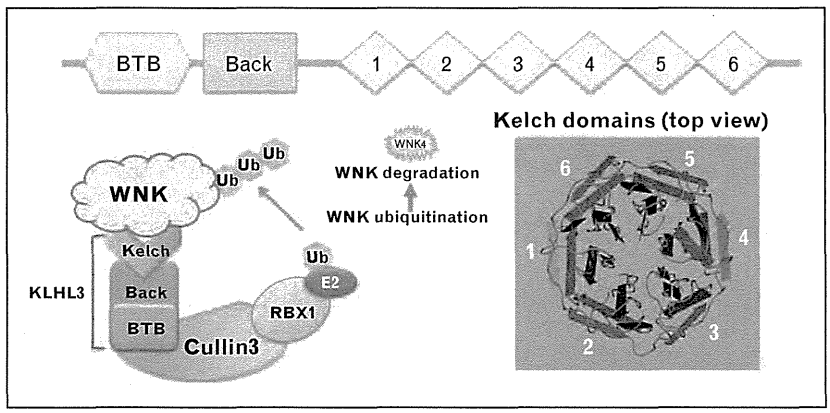

FIGURE 2. Primary and three-dimensional structures of a KLHL protein and its function as a component of CRL3. Upper panel: the primary structure of Kelch-like (KLHL) proteins with N-terminal BTB and BACK domains and five to six C-terminal Kelch domains. The BTB domain is a binding site for Cullin 3, and Kelch repeats constitute a propeller structure, as shown in the right lower panels, to 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. Left lower panel: KLHL3 and Cullin-3 forms an E3 ligase complex with the RING finger protein, RBX1. WNKs are captured by this E3 ligase by binding to KLHL3 and are ubiquitinated and degraded.

proteins that facilitates protein–protein interaction [32]. Several substrate-binding domains, such as Kelch, WD40, and basic leucine zipper, are commonly found in the BTB domain-containing adaptor proteins in CRL3.

The Kelch-like protein family consists of more than 40 members [33▪]. In general, KLHL proteins contain one BTB domain, one BTB and C-terminal Kelch (BACK) domain, and five to six Kelch domains (Fig. 2). The Kelch domain forms one blade of a β-propeller structure, which is also involved in the protein–protein interaction. Kelch domain-containing proteins have been shown to participate in many cellular functions [34] because substrates for KLHL–CUL3 E3 ligases are diverse. A list of the functions of KLHL–CUL3 E3 ligases and their involvement in disease is presented in Table 1 [3▪▪,4▪▪,35,36▪▪,37▪▪, 38–56].

PATHOGENESIS OF PSEUDOHYPOALDOSTERONISM TYPE II THROUGH MUTATIONS IN THREE DIFFERENT GENES

As mutations in *WNK4*, *KLHL3*, and *Cullin-3* cause the same disease, PHAII, it is reasonable to speculate that components of WNK–OSR1/SPAK–NCC signaling cascade, in particular WNK4, could be the substrate for the KLHL3–Cullin-3 E3 ligase complex. In fact, Ohta *et al.* [36▪▪] and Wakabayashi *et al.* [37▪▪] reported that WNK1 and WNK4 were substrates for the KLHL3–Cullin-3 E3 ligase complex, respectively. Then, two further reports [57▪,58▪] demonstrated WNK4 as a target of the KLHL3–Cullin-3 E3 ligase complex. Analyses of PHAII-causing mutations in *WNK4*, *KLHL3*, and *Cullin-3* also confirmed this notion. Wakabayashi *et al.* [37▪▪] and Mori *et al.* [59] showed that binding of KLHL3 to WNK4 was abolished by PHAII-causing mutations in *WNK4*, indicating that the acidic domain is involved in the binding to KLHL3. In contrast to WNK4, mutations in *KLHL3* were not clustered to a single domain, but were present in the BTB, BACK, and Kelch domains. Mutations in the BTB and BACK domains affected the ability of KLHL3 to bind Cullin-3, whereas mutations in the Kelch domains affected the ability of KLHL3 to bind WNK1 and WNK4 [59]. Thus, impaired binding of KLHL3 to Cullin-3 or WNK4 decreased WNK4 ubiquitination, resulting in increased WNK4 proteins within cells.

Almost all PHAII-causing *Cullin-3* mutations are found around the splice donor and acceptor sites of

Table 1. List of KLHL–CUL3 E3s: functions and involvement in disease

Name	Substrate	Function	Disease	Reference
KLHL2	WNK	ND	ND	[35]
KLHL3	WNK1	Regulation of SLC12A transporters	PHAII	[3▪▪,4▪▪,36▪▪,37▪▪]
	WNK4	ND	ND	
KLHL7	ND	ND	adRP	[38]
KLHL8	Rapsyn	AChR clustering	ND	[39]
KLHL9	AuroraB	Mitosis	Distal myopathy	[40,41]
KLHL13	ND	ND	ND	[42]
KLHL21	ND	ND	ND	[43]
KLHL12	Disheveled	Wnt/β-catenin signaling	ND	[44]
	Sec31	Collagen secretion	ND	[45]
KLHL16/Gigaxonin	MAP1B	Regulation of cytoskeletal proteins	Giant axonal neuropathy	[46]
	Intermediate filaments	ND	ND	[47]
				[48]
KLHL19/Keap1	Nrf2	Oxidative stress response	Cancer	[49]
				[50]
				[51]
				[52]
KLHL20	PML	HIF-1 signaling	Progression of prostate cancer	[53]
	DAPK	INF-induced response	ND	[54]
KLHL22	PLK1	Chromosome segregation	ND	[55]
KLHL25	4E-BP	Translational regulation	ND	[56]

AChR, acetylcholine receptor; adRP, autosomal dominant retinitis pigmentosa; DAPK, death-associated protein kinase; ND, not determined; Nrf2, NF-E2-related factor 2; PHAII, pseudohypoaldosteronism type II; PLK1: polo-like kinase 1; PML, promyelocytic leukemia; WNK, with-no-lysine kinase.

exon 9. Osawa *et al.* [60] and Tsuji *et al.* [61] recently verified that exon 9 was skipped in the leukocytes of patients with PHAII-causing *Cullin-3* mutations, as shown by the experiment in cultured cells by Boyden *et al.* [3▪▪]. Overexpression of the mutant *Cullin-3* lacking a portion of exon 9 with KLHL3 showed less ability to reduce the coexpressed WNK4 [37▪▪], suggesting that the mutant Cullin-3 may have less E3 ligase activity. Thus, all PHAII-causing mutations in *WNK4*, *KLHL3*, and *Cullin-3* resulted in a common consequence, that is, decreased WNK4 ubiquitination and increased WNK4 protein within cells (Fig. 3).

This increase in WNK4 protein was confirmed in the kidneys of $Wnk^{4D561A/+}$ mice [37▪▪]. Because WNK4, as well as WNK1, was shown to phosphorylate and activate OSR1 and SPAK *in vitro* [15], the increase in WNK4 must be stimulatory to downstream WNK–OSR1/SPAK–NCC signaling. However, long-standing controversy exists over the influence of WNK4 on NCC function [62]. Initially, WNK4 overexpression experiments in *Xenopus laevis* oocytes showed that WNK4 is a negative regulator of NCC [6,7]. However, Castaneda-Bueno *et al.* [25▪] 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*. Moreover, WNK4 transgenic mice showed robust increases in OSR1, SPAK, and NCC phosphorylation and showed phenotypes similar to PHAII [37▪▪]. Therefore, it is now clear that increased wild-type WNK4 in the kidney activates the WNK–OSR1/SPAK–NCC signaling cascade and causes PHAII. Thus, the long-standing controversy about the influence of WNK4 on NCC was settled by the discovery of the two new causative genes for PHAII. This controversial story gives us an important lesson that it is very risky to make conclusions based on results from a single experimental system, especially from in-vitro overexpression studies. In this regard, the scheme depicted in Fig. 3 should also be validated in mouse models carrying the PHAII-causing mutations in KLHL3 or Cullin-3.

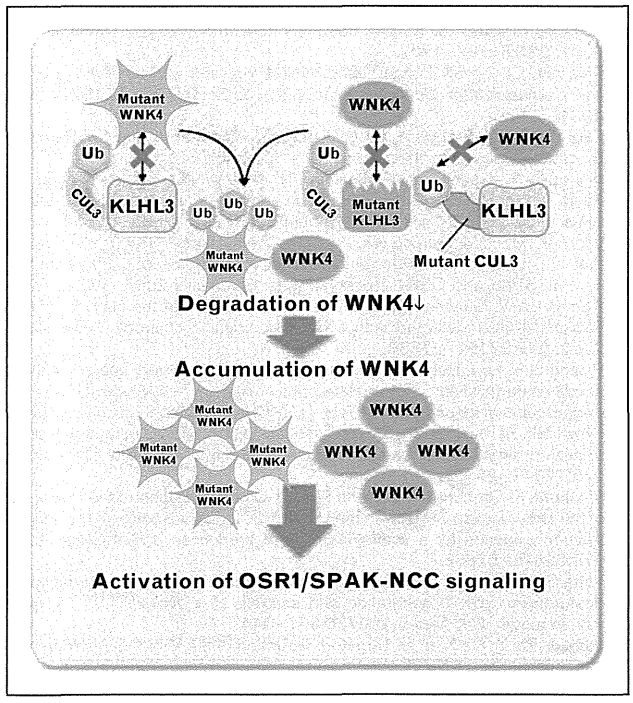

FIGURE 3. Molecular pathogenesis of PHAII. Under normal conditions, WNK 4 proteins within cells are maintained by appropriate degradation after ubiquitination by the KLHL3–Cullin-3 E3 ligase complex. However, PHAII-causing mutations in the acidic domain of WNK4 and in the Kelch domains of KLHL3 affect their binding, thereby reducing the ubiquitination and degradation of WNK4. PHAII-causing mutant *Cullin-3* lacking the portion corresponding to exon 9 is less able to decrease WNK4, probably because of its reduced E3 ligase activity. Thus, PHAII-causing mutations in the three different genes have a common consequence, that is, decreased WNK4 ubiquitination and increased WNK4 protein levels within DCT, leading to the activation of OSR1/SPAK–NCC signaling and to PHAII.

UNANSWERED QUESTIONS AND FUTURE PERSPECTIVES

Thus, impaired ubiquitination and a consequent increase in WNK4 protein were established as the molecular pathogenesis of PHAII, caused by mutations in *WNK4*, *KLHL3*, and *Cullin-3* (Fig. 3). There are several questions to be answered in future. First, is WNK4 the only WNK regulated by the KLHL3–Cullin-3 E3 ligase complex in the kidney *in vivo*? In fact, in-vitro experiments clearly show that both WNK1 and WNK4 proteins were regulated by the KLHL3–Cullin-3 E3 ligase complex [36▪▪,37▪▪]. Therefore, levels of both WNK1 and WNK4 may be increased in the kidneys of patients with PHAII carrying mutations in *KLHL3* and *Cullin-3*, further contributing to the activation of WNK–OSR1/SPAK–NCC signaling and explaining the more severe PHAII phenotypes evident with mutations in *Cullin-3* and *KLHL3* than in *WNK1* and *WNK4* [3▪▪]. Moreover, other WNKs, such as WNK2 and WNK3, could be substrates for the KLHL3–Cullin-3 E3 ligase complex because the KLHL3-binding domain of WNK4 (the acidic domain) is highly conserved in all WNK isoforms. Furthermore, KLHL2, the closest homolog to KLHL3 among KLHL proteins, was shown to behave similarly to KLHL3 in terms of E3 ligase for WNKs [35]. These data suggest that both KLHL2 and KLHL3 may be involved in the regulation of all WNKs in various types of cells. It would also be interesting to confirm

whether there are regulatory mechanisms controlling the interaction between KLHL2/3 and WNKs. Although several regulations of WNKs by diets and hormonal factors have been reported, the detailed mechanisms are largely unknown. Regulated binding of KLHL2/3 and WNKs by phosphorylation or other modifications may be one of the important mechanisms of WNK regulation. Finally, one of the biggest questions may be why mutation in Cullin-3, which is ubiquitously expressed and functions as a scaffold of E3 not only for KLHL3 but also for many other adaptor proteins, induces the kidney-specific disease PHAII. The skipping of exon 9 might occur dominantly in DCT cells in the kidney; however, we can also confirm the skipping in the white blood cells of the patients. Another possibility would be that the mutant Cullin-3 might be functionally defective as E3 ligase only with KLHL3. A knock-in mouse model carrying the same mutations in *Cullin-3* is necessary to answer these questions.

CONCLUSION

Why PHAII-causing missense mutations in *WNK4* are clustered and how these mutations activate downstream signaling to NCC has been a long-standing unanswered question. The recent discovery of two additional genes causing PHAII helped construct a complete picture of the molecular pathogenesis of PHAII and provided definite genetic evidence that WNK4 in the kidney never behaves as a negative regulator of NCC but acts as a positive regulator through WNK–OSR1/SPAK–NCC signaling. Levels of WNKs within cells, regulated via ubiquitination by KLHL2/3–Cullin-3 E3 ligases, would be important determinants of the activity of the WNK signaling cascade.

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Conflicts of interest

There are no conflicts of interest.

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