KLHL3 Knockout Mice Reveal the Physiological Role of KLHL3 and the Pathophysiology of Pseudohypoaldosteronism Type II Caused by Mutant KLHL3

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ABSTRACT Mutations in the with-no-lysine kinase 1 (WNK1), WNK4, kelch-like 3 (KLHL3), and cullin3 (CUL3) genes are known to cause the hereditary disease pseudohypoaldosteronism type II (PHAII). It was recently demonstrated that this results from the defective degradation of WNK1 and WNK4 by the KLHL3/CUL3 ubiquitin ligase complex. However, the other physiological in vivo roles of KLHL3 remain unclear. Therefore, here we generated KLHL3<sup>−/−</sup> mice that expressed β-galactosidase (β-Gal) under the control of the endogenous KLHL3 promoter. Immunoblots of β-Gal and LacZ staining revealed that KLHL3 was expressed in some organs, such as brain. However, the expression levels of WNK kinases were not increased in any of these organs other than the kidney, where WNK1 and WNK4 increased in KLHL3<sup>−/−</sup> mice but not in KLHL3<sup>+/−</sup> mice. KLHL3<sup>−/−</sup> mice also showed PHAII-like phenotypes, whereas KLHL3<sup>+/−</sup> mice did not. This clearly demonstrates that the heterozygous deletion of KLHL3 was not sufficient to cause PHAII, indicating that autosomal dominant type PHAII is caused by the dominant negative effect of mutant KLHL3. We further demonstrated that the dimerization of KLHL3 can explain this dominant negative effect. These findings could help us to further understand the physiological roles of KLHL3 and the pathophysiology of PHAII caused by mutant KLHL3.

KEYWORDS kelch-like 3 (KLHL3), distal convoluted tubule, hypertension, kidney, NaCl cotransporter, with-no-lysine kinase (WNK)

Pseudohypoaldosteronism type II (PHAII) is a hereditary disease that is characterized by salt-sensitive hypertension, hyperkalemia, metabolic acidosis, and thiazide sensitivity (1). Mutations in the with-no-lysine kinase 1 (WNK1) and WNK4 genes are known to cause PHAII (2). Furthermore, it has generally been considered that overactivation of the thiazide-sensitive Na-Cl cotransporter (NCC) is the main cause of PHAII (3).

Many studies have demonstrated that WNK kinases are at the top of the signaling cascade, along with oxidative stress-responsive gene 1 (OSR1), Ste20-related proline-alanine-rich kinase (SPAK), and the solute carrier family 12a (SLC12a) transporter family, which includes the NCC and Na-K-Cl cotransporter (NKCC). WNK phosphorylates and activates OSR1/SPAK, which in turn phosphorylate and activate the SLC12a transporters (4–6). The regulation of NCC by WNK-OSR1/SPAK signaling was confirmed in vivo using various genetically engineered mouse models (7–14) and overactivation of this WNK-OSR1/SPAK-NCC phosphorylation signal in the kidney causes PHAII (5, 6, 15, 16).
In 2012, mutations in KLHL3 and cullin3 (CUL3) genes were newly identified as causing PHAII (17, 18). KLHL3 is a member of the BTB-BACK-Kelch family, and combines with Cul3 to form the E3 ubiquitin ligase complex. KLHL3 binds with the acidic motif of WNK kinases and functions as a substrate adapter protein for Cul3-based E3 ligase-mediated ubiquitination (19–22). Recently, we generated KLHL3R528H/+ mice carrying autosomal-dominant type PHAII, which contained mutant KLHL3 R528H that had a reduced binding ability to WNK4. This PHAII mouse model revealed that the defective binding of WNK kinases to mutant KLHL3 results in impaired degradation of WNK kinases, leading to increased protein expression of WNK1 and WNK4 (23). These findings clearly indicated that KLHL3 is a key physiological regulator of WNK signaling via the protein degradation mechanism. However, although phosphorylation of KLHL3 is reported to regulate its binding ability to WNK4 (24, 25), the pathophysiological roles of KLHL3 aside from its involvement in PHAII remain unclear. Moreover, other substrates of the Cul3-KLHL3 E3 ligase complex are not yet known.

Previous research has largely focused on the role of WNK signaling in renal epithelial ion homeostasis. However, more recent studies have demonstrated that WNK signaling also occurs in extrarenal tissues. For example, in vascular smooth muscle cells, WNK1 and WNK3 regulate the vascular tonus through the phosphorylation of SPAK and NKKC1 (9, 26, 27), and in neuronal and brain cells, WNK kinases regulate the phosphorylation of NKCC1 and K-Cl cotransporter (KCC2) via SPAK activation, controlling intracellular Cl− levels via γ-aminobutyric acid (GABA) (28–30). In addition, several types of cross talk between WNK kinases and other signaling pathways have been reported (31–34). Thus, the WNK signaling pathway is also expected to be essential in organs other than the kidney, which raises the question whether KLHL3 also regulates the expression level of WNK kinases in these organs. However, to date, clinical features in organs other than the kidney have not been reported in patients with PHAII caused by mutant KLHL3, and the tissue distribution and intratissue localization of KLHL3 have not been well examined.

In this study, we aimed to answer these questions by generating and analyzing KLHL3−/− mice that expressed β-Gal under the control of the endogenous KLHL3 promoter. We took advantage of the expression of β-galactosidase (β-Gal) by the Klhl3 knockout allele in this mouse model to investigate the tissue distribution and intratissue localization of KLHL3, and then examined how WNK kinases are regulated in KLHL3-expressing organs. After this, we further investigated the dominant negative effect of mutant KLHL3 R528H, which causes autosomal dominant type PHAII.

RESULTS

Generation of KLHL3 knockout/lacZ knock-in mice. In this study, we generated KLHL3−/− mice whose knockout allele expressed β-Gal via the lacZ gene under the control of the endogenous KLHL3 promoter (i.e., KLHL3 knockout/lacZ knock-in mice). To generate these mice, we used a targeting vector that had been designed to delete exon 3 of KLHL3 but which also led to the expression of the lacZ gene under the control of the endogenous KLHL3 promoter. As shown in Fig. 1, these mice were crossed with CAG promoter-Cre transgenic mice to successfully generate KLHL3−/− mice. KLHL3 heterozygous (+/−) and homozygous (−/−) mice exhibited no gross anatomical abnormalities and no significant differences in body weight or height and also presented a normal birth rate. The absence of the KLHL3 protein in KLHL3−/− mice was confirmed by the immunoblotting of protein samples from the brain and kidney. In addition, we confirmed β-Gal protein expression by the Klhl3 knockout allele in KLHL3 knockout/lacZ knock-in mice (Fig. 2).

Tissue and cellular distribution of KLHL3. Next, we used the KLHL3 knockout/lacZ knock-in mice to investigate the tissue and cellular distribution of KLHL3. Since the KLHL3 antibody we used could only detect the KLHL3 protein in the kidney and brain, we performed immunoblots of β-Gal to investigate the distribution of KLHL3 in other tissues. This demonstrated that KLHL3 was strongly expressed in the brain and kidney, weakly expressed in the eye, testis, lung, heart, liver, stomach, and colon, and not
expressed in the muscle (Fig. 3A). We then took advantage of the β-Gal expression in the knockout allele of the mouse to perform LacZ staining of the KLHL3-expressing tissue from KLHL3 knockout/lacZ knock-in mice (Fig. 3B). Although the cellular distribution of KLHL3 could not be determined in the tissues with lower levels of expression due to the poor LacZ signal, strong LacZ staining signals were observed in the hippocampus and cortex of the brain, and in the distal convoluted tubules (DCT) of the kidney, which was also confirmed by immunofluorescence with NCC. KLHL3−/− mice showed hyperplasia of the DCT, as previously reported in WNK4 transgenic PHAI1 model mice (35) (Fig. 3C).

Protein levels of WNK kinases in the tissues of KLHL3−/− mice. Since KLHL3 is known to play a role in the ubiquitination and degradation of WNK kinases, we expected that an absence of KLHL3 might result in increased expression levels of WNK kinases in various organs. Therefore, we investigated the protein levels of WNK1, WNK3, and WNK4 in the KLHL3-expressing organs from KLHL3−/− mice (Fig. 4A). We found that the expression levels of WNK1, WNK3, and WNK4 were not increased in the brain and other tissues that exhibited lower expression levels of KLHL3 even in the KLHL3−/− mice, but the expression of WNK1 and WNK4 was dramatically increased in the kidneys

**FIG 1** Generation of KLHL3 knockout/lacZ knock-in mice. (A) Targeting strategy for generating KLHL3 knockout/lacZ knock-in mice. The diagram shows the wild-type KLHL3 locus, the targeting vector, and the targeted locus before and after Cre recombination. The targeting vector was designed to delete exon 3 of KLHL3. In addition, this vector led to the expression of the lacZ gene under the control of the endogenous KLHL3 promoter. EN2-SA, Engrailed-2 splice acceptor. (B) Verification of homologous recombination and genotyping PCR of the genomic DNA of the mice.

**FIG 2** Confirmation of the absence of KLHL3 expression and the presence of β-Gal expression by the knockout allele. Representative immunobLOTS of KLHL3 and β-Gal in the kidneys and brains of KLHL3+/+, KLHL3+/−, and KLHL3−/− mice are shown, confirming the absence of the KLHL3 protein and expression of the β-Gal protein by the Klhl3 knockout allele.
Furthermore, we separated and prepared samples of the hippocampus and cortex of the brain, where KLHL3 was expected to be present, but no significant differences in WNK1 and WNK4 protein levels were detected between wild-type and KLHL3–/– mice, as shown in Fig. 4B.

![FIG 3 Tissue and intratissue distribution of KLHL3 was confirmed by an immunoblot of β-Gal and LacZ staining. (A) Immunoblotting to detect the β-Gal protein level in each organ from KLHL3 knockout/lacZ knock-in mice. Representative immunoblots of β-Gal in each of the organs of wild-type and KLHL3 homozygous knockout/lacZ knock-in mice are shown. β-Gal was strongly detected in the kidney and brain and weakly detected in the eye, testis, lung, heart, liver, stomach, and colon. (B) LacZ staining of the kidney and brain in KLHL3 knockout/lacZ knock-in mice. Strong LacZ staining was observed in the hippocampus and cortex of the brain and in the distal convoluted tubules of the kidney. (C) Double immunofluorescence of β-Gal and the Na–Cl cotransporter (NCC) in the kidneys of wild-type and KLHL3 knockout/lacZ knock-in mice. The β-Gal signal colocalized with NCC, indicating that KLHL3 was present in the distal convoluted tubules. Scale bars, 10 μm.](image)

![FIG 4 WNK kinases only increased in the kidneys of KLHL3–/– mice. (A) Representative immunoblots of β-Gal and WNK1, WNK3, and WNK4 in KLHL3-expressing tissues in KLHL3+/+ and KLHL3–/– mice. In KLHL3–/– mice, the expression levels of WNK1, WNK3, and WNK4 did not increase in any organ other than the kidney, where WNK1 and WNK4 increased. N.D., not detected. (B) Immunoblotting of WNK1 and WNK3 protein levels in hippocampus and cortex from KLHL3–/– mice. No significant differences in WNK protein levels were detected between wild-type and KLHL3–/– mice. We repeated the same experiments three times with consistent results.](image)
**TABLE 1** Blood chemistry bioparameters

| Blood biochemistry parameter | Mean ± SEM<sup>b</sup> |
|-----------------------------|------------------------|
| Na<sup>+</sup> (mmol/liter)  | KLHL3<sup>++/+</sup> mice | KLHL3<sup>++/-</sup> mice | KLHL3<sup>+-/-</sup> mice |
| 149.5 ± 0.6                 | 148.8 ± 0.4            | 150.4 ± 1.9                |
| K<sup>+</sup> (mmol/liter)  |                        |                        |
| 4.5 ± 0.1                  | 4.6 ± 0.1              | 5.2 ± 0.2*                |
| Cl<sup>-</sup> (mmol/liter) |                        |                        |
| 112.0 ± 0.8                | 111.4 ± 0.6            | 116.1 ± 1.8*              |
| Glu (mg/dl)                |                        |                        |
| 228.0 ± 18.8               | 239.9 ± 9.5            | 222.5 ± 11.6              |
| pH                         |                        |                        |
| 7.297 ± 0.011              | 7.286 ± 0.009          | 7.235 ± 0.015*            |
| pCO<sub>2</sub> (mmHg)     |                        |                        |
| 43.7 ± 1.1                 | 43.7 ± 1.4             | 45.1 ± 2.4                |
| HCO<sub>3</sub>- (mmol/liter)|                      |
| 21.6 ± 0.6                 | 20.6 ± 0.4             | 19.0 ± 0.8*               |
| Hb (g/dl)                  |                        |                        |
| 14.9 ± 0.3                 | 15.0 ± 0.1             | 14.5 ± 0.2                |

<sup>a</sup>Glu, glucose; Hb, hemoglobin.
<sup>b</sup>, *P < 0.05 compared to KLHL3<sup>++/+</sup> mice.

**KLHL3<sup>+-/-</sup> mice exhibited a PHAII-like phenotype.** To confirm that KLHL3<sup>+-/-</sup> mice are a good model of PHAII, we first checked their blood chemistry. As shown in Table 1, KLHL3<sup>+-/-</sup> mice exhibited lower plasma pH and higher plasma K<sup>+</sup> and Cl<sup>-</sup> levels than wild-type mice. However, there was no significant difference in the blood chemistry data between KLHL3<sup>++/-</sup> heterozygous mice and KLHL3<sup>++/+</sup> mice, different from KLHL3<sup>+-/-</sup> mice. We also measured the BP of KLHL3<sup>+-/-</sup> mice and KLHL3<sup>++/+</sup> mice that had been fed a normal diet and then repeated these measurements in the same mice after feeding them a high-salt diet for more than 3 days. As shown in Fig. 5, no significant difference in BP was detected under the normal diet, but the delta systolic blood pressure was significantly higher in KLHL3<sup>+-/-</sup> mice than in KLHL3<sup>++/+</sup> mice after consuming the high-salt diet, demonstrating an increased salt sensitivity of KLHL3<sup>+-/-</sup> mice. Thus, KLHL3<sup>+-/-</sup> mice clearly exhibited a PHAII-like phenotype.

The WNK-OSR1/SPAK-NCC phosphorylation cascade in the kidneys of KLHL3 knockout mice. Since KLHL3<sup>+-/-</sup> mice exhibited increased WNK1 and WNK4 expression levels in their kidneys, we next examined the protein expression and phosphorylation of molecules constituting the WNK signaling pathway. As shown in Fig. 6, the protein expression levels of WNK1 and WNK4 were significantly higher in the kidneys of KLHL3<sup>+-/-</sup> mice compared to KLHL3<sup>++/+</sup> and KLHL3<sup>++/-</sup> mice. Accordingly, the phosphorylation of OSR1, SPAK, and NCC also increased in KLHL3<sup>+-/-</sup> mice. However, although the protein expression of KLHL3 appeared to be lower in the kidneys of KLHL3<sup>++/-</sup> heterozygous mice than in KLHL3<sup>++/+</sup> mice (Fig. 2), there was no significant difference in the protein levels of WNK1 and WNK4 in the kidneys between KLHL3<sup>++/+</sup> and KLHL3<sup>++/-</sup> mice.

Dimer formation of KLHL3 could explain the dominant negative effect of mutant KLHL3 R528H. Interestingly, we found that the WNK-OSR1/SPAK-NCC phos-

**FIG 5** Increased salt sensitivity in KLHL3<sup>+-/-</sup> mice. (A) The blood pressure of KLHL3<sup>++/+</sup> and KLHL3<sup>+-/-</sup> mice after being fed a normal or a high-salt diet. (B) The observed blood pressure increased as a result of the high-salt diet. No significant difference in blood pressure was detected under a normal diet, whereas the increase of systolic blood pressure was significantly higher in KLHL3<sup>+-/-</sup> mice than in KLHL3<sup>++/+</sup> mice under a high-salt diet.
Phosphorylation cascade was not activated in the kidneys of KLHL3/H11001/H11002 mice. This finding clearly demonstrates that the heterozygous deletion of KLHL3 was not sufficient to cause PHAII in the kidney, indicating that the PHAII phenotypes previously observed in KLHL3R528H/H11001/H11002 heterozygous mice were caused by the dominant negative effect of the R528H KLHL3 mutant. To further investigate the mechanisms of this dominant negative effect of mutant KLHL3, we compared the protein expression levels of WNK1 and WNK4 in the kidneys of KLHL3/H11001/H11002 mice.

![Graph](image1.png)

**FIG 6** Increased WNK1 and WNK4 protein levels, and the activation of the WNK-OSR1/SPAK-NCC phosphorylation signaling cascade in the kidneys of KLHL3 +/- mice. (A) Representative immunoblots of WNK1 and WNK4 in the kidneys of KLHL3 +/-, KLHL3+/+, and KLHL3 -/- mice. The expression levels of WNK1 and WNK4 and the phosphorylation of SPAK, OSR1, and NCC were significantly higher in the kidneys of KLHL3 -/- mice but not in KLHL3 +/- mice. (B) Densitometric analysis. Values are expressed as a ratio of the average signal in wild-type mice. n = 3 to 6; *, P < 0.05.

![Graph](image2.png)

**FIG 7** The protein levels of WNK1 and WNK4 were higher in KLHL3 -/- mice than in KLHL3R528H/+ mice. (A) Protein expression levels of WNK1 and WNK4 in kidneys from wild-type, KLHL3 -/-, and KLHL3R528H/+ mice. The ability to degrade WNK kinases. This was conserved in KLHL3R528H/+ mice but lost in KLHL3 -/- mice. (B) Densitometric analysis. Values are expressed as a ratio of the average signal in KLHL3R528H/+ mice. n = 3 to 9; *, P < 0.05.
in KLHL3<sup>R528H</sup> heterozygous mice, indicating that KLHL3<sup>R528H/+</sup> mice still retained some capacity for the degradation of WNK kinases.

It has previously been reported that the polymerization of a disease-causing protein could explain the dominant negative effect in some cases (36). In this case, small amount of normal polymers that consist only of wild-type proteins will occur, preserving their normal function. Actually, some of the KLHL family proteins, such as Keap1, have been shown to form dimers (37). Therefore, we hypothesized that one of the mechanisms behind the dominant negative effect of mutant KLHL3 could be the formation of KLHL3 dimers. To test this hypothesis, we performed an immunoprecipitation experiment to see whether wild-type KLHL3 and mutant KLHL3<sup>R528H</sup> could form dimers. As shown in Fig. 8A, wild-type KLHL3 could form a homodimer and heterodimer with wild-type and mutant KLHL3<sup>R528H</sup>, respectively. IP, immunoprecipitation. (B, upper panel) Coexpression experiments of KLHL3<sup>R528H</sup> and wild-type KLHL3. Compared to the cells transfected with wild-type KLHL3 alone, the degradation of WNK4 protein was significantly decreased by the addition of KLHL3<sup>R528H</sup>. In addition, this effect was cancelled when the BTB-BACK domain, containing the binding site for dimer formation, was deleted from the mutant KLHL3<sup>R528H</sup>, suggesting that the dominant negative effect of mutant KLHL3<sup>R528H</sup> required dimer formation of KLHL3. These results clearly indicate that mutant KLHL3<sup>R528H</sup> has a dominant negative effect due to dimer formation.

**DISCUSSION**

In this study, we generated KLHL3<sup>−/−</sup> mice and confirmed that the absence of KLHL3 resulted in increased WNK1 and WNK4 levels in the kidney due to their defective degradation, which led to the activation of the downstream OSR1/SPAK-NCC phosphorylation cascade and a PHAII-like phenotype.
Recent studies have focused on WNK signaling in extrarenal tissue and have found that WNK signaling also plays a role in other tissues, including vascular smooth muscle cells (9, 26, 38), the brain (39–41), neurons (42), the intestine (43), and the heart (44). However, the tissue distribution and intratissue localization of KLHL3 remained unclear. Therefore, to investigate the physiological roles of KLHL3 in organs other than the kidney, we first investigated the tissue distribution of KLHL3. Unfortunately, the KLHL3 antibody we used could only detect KLHL3 by immunoblotting in samples from the kidney and brain. Therefore, instead, we used our KLHL3 knockout mouse model to answer these questions, in which the knockout allele expressed β-Gal as a result of the lacZ gene under the control of the endogenous KLHL3 promoter. Taking advantage of this system, we confirmed that there is strong β-Gal expression by the KLHL3 promoter in the brain and kidney and a lower expression in the eye, testis, lung, heart, liver, stomach, and colon. As expected, the expression of WNK1 and WNK4 was significantly higher in the kidneys of KLHL3−/− mice than in KLHL3+/+ mice. However, in the brain and other tissues that had lower expression levels of KLHL3, there was no difference in the expression of WNK1, WNK3, and WNK4 between KLHL3−/− and KLHL3+/+ mice.

These findings suggest that WNK protein levels in KLHL3-expressing tissues may not be solely governed by KLHL3. It is possible that the expression levels of WNK kinases are regulated only in specific KLHL3-expressing cells rather than across the entire organ, although we detected no significant differences in the WNKs of the hippocampus and cortex in the brain between wild-type and KLHL3−/− mice. Therefore, to exclude this possibility, it may be necessary to assess WNK protein levels in specific KLHL3-expressing cells in the future. Since the KLHL3 antibody we used was unable to detect a good KLHL3 signal by immunohistochemistry in extrarenal organs, the use of LacZ staining of tissue samples from β-Gal-expressing KLHL3−/− mice may be an appropriate method for detecting the expression of KLHL3 in specific types of cells in each organ. However, unfortunately, the LacZ staining of organs other than the kidney and brain has not been successful to date, likely due to the lower expression level of β-Gal. Therefore, further investigation will be required to determine the role of KLHL3 on WNK degradation in specific types of cells in each tissue.

Alternatively, it is possible that other KLHL proteins, such as KLHL2, are involved in controlling WNK protein levels. Similar to KLHL3, KLHL2 directly interacts with WNK kinases, leading to their ubiquitination and degradation (45, 46). Zeniya et al. recently reported that ubiquitination by KLHL2 is the major regulator of WNK3 protein levels in response to angiotensin II stimulation, although other in vivo roles of KLHL2 were not determined (38).

In the present study, dominant LacZ staining was observed in the hippocampus and cortex of the brain and in the distal tubules of the kidney. It was reasonable that KLHL3 was expressed in the distal convoluted tubules where NCC was localized, and this finding was consistent with previous reports (17, 23). In the brain, the WNK signal is believed to regulate the Cl− current via GABA (29, 30). The fact that GABA in the hippocampus regulates memory function (47) suggests that KLHL3 may also be important for memory through the regulation of WNK signaling. We also found that KLHL3 localized in the cortex. A recent study showed that WNK3 knockout mice were protected from focal ischemia in a middle cerebral artery occlusion-reperfusion model (48). Furthermore, a few case reports have shown that intellectual impairment occurred in sporadic cases of PHAII (1), probably due to CUL3 mutation, indicating that WNK signaling may play a role in the brain, although we cannot exclude the possibility that severe acidosis simply causes some development disorders of the brain. However, there are no reports of intellectual impairment in patients with PHAII caused by mutant KLHL3, and we did not detect an increase in the expression levels of WNK kinases in the whole lysate of the brain. Therefore, the role of KLHL3 in the brain of PHAII patients remains unclear and requires further careful investigation in the future.

We recently found that p62 interacts with KLHL3 and that WNK4 is degraded not only by proteasomes but also by p62-KLHL3-mediated selective autophagy (49). However, other proteins that interact with the CUL3-KLHL3 E3 ligase complex remain
unknown. We expected that the phenotypes of KLHL3−/− mice (aside from PHAII) would give us an indication of novel substrates of KLHL3. However, unfortunately, we have not yet found any other obvious phenotypes of KLHL3−/− mice. Therefore, to investigate the novel roles of KLHL3 and new substrates of the Cul3-KLHL3 E3 ligase complex, further analysis of the KLHL3−/− mice is required, with a particular focus on the KLHL3-expressing organs that were found in the present study.

Interestingly, KLHL3+/− heterozygous mice did not show increased protein levels of WNK1 and WNK4 and did not exhibit PHAII-like phenotypes (i.e., hyperkalemia and metabolic acidosis). The lack of PHAII phenotypes in these mice clearly shows that the heterozygous deletion of KLHL3 was not sufficient to cause PHAII in the kidney, indicating that the PHAII-like phenotypes that we previously observed in KLHL3R528H+/+ model mice were caused by the dominant negative effect of the mutant R528H KLHL3. It is known that KLHL family proteins, such as Keap1 and KLHL7, form a dimer by themselves (37, 50). It was previously reported that Keap1 mutations found in lung tumors suppress wild-type Keap1 activity, leading to Nrf2 activation, due to heterodimerization of mutant Keap1 with wild-type Keap1, demonstrating the mechanism of dominant negative effect of mutant Keap1 (51). However, to date, there has been no molecular evidence of the direct interaction of KLHL3, although structural analysis of its BTB-BACK domain suggests that it could form a dimer (52). In this study, we confirmed that wild-type KLHL3 forms a dimer and that wild-type and mutant R528H KLHL3 bind with each other, which constitutes dimer formation. We also demonstrated that the dominant negative effect of KLHL3 R528H required dimer formation. As shown in Fig. 9, assuming that dimers that contain at least one mutant KLHL3 exhibit a defective binding ability to WNK1 and WNK4, one-quarter of all dimers will consist only of wild-type KLHL3 and will have a normal ability to catch WNK kinases. Although mutant KLHL3 dimerization cannot be quantitatively measured in KLHL3R528H+/+ heterozygous mice, the speculation presented above fits with the finding that the protein levels of WNK1 and WNK4 were higher in KLHL3−/− mice than in KLHL3R528H+/+ mice (Fig. 7), since a small amount of normal dimers that consist of two wild-type KLHL3 will still occur in KLHL3R528H+/+ mice. Therefore, we believe that the dimer formation of wild-type and R528H KLHL3 could explain the dominant negative effect of this mutant.

The majority of KLHL3 mutations in the Kelch domain show an autosomal dominant
cascade. In contrast, increased WNK protein levels in the kidney, leading to activation of the WNK signaling pathway, might fail to form the fully functional Cul3-KLHL3 E3 ligase complex.

KLHL3 mutations in BTB domain also shows autosomal dominant inheritance pattern; however, the precise mechanism of dominant negative effect of BTB domain mutation is not clear. One explanation might be defect of interaction of KLHL3 with Cul3. We previously showed that BTB mutation (E85A) cannot interact with Cul3 (53). Even if KLHL3 with BTB mutation could form a dimer with wild-type KLHL3, this dimer might fail to form the fully functional Cul3-KLHL3 E3 ligase complex.

KLHL3 mutations in BACK domain mainly exhibit recessive inheritance. We speculate that this could be simply explained by defective dimer formation of KLHL3 mutant at the BACK domain. Heterozygous mutation of KLHL3 BACK domain does not show any obvious phenotype in the majority of cases, similar to that of KLHL3+/− heterozygous knockout. However, a rare case of KLHL3 mutation in BACK domain exhibiting dominant inheritance has been reported, and the reason for this is still unclear.

Thus, multiple speculations can be made considering the molecular pathogenesis of KLHL3 mutations with many questions left to be clarified. Our report is the first to successfully elucidate the mechanism of dominant negative effect of KLHL3 R528H by in vivo data. There could be still unknown mechanisms in the other type of KLHL3 mutation. Further investigations are required.

In conclusion, the KLHL3 knockout mice exhibited PHAII-like phenotypes and increased WNK protein levels in the kidney, leading to activation of the WNK signaling cascade. In contrast, KLHL3+/− heterozygous mice did not show an increase in WNK kinases and activation of WNK signaling, even in the kidney. This lack of a PHAII phenotype in these mice clearly indicates that the dominant type of mutant KLHL3 has a dominant negative effect, likely due to dimer formation. These findings could be valuable for further understanding the physiological roles of KLHL3 and the pathophysiological mechanisms of PHAII caused by mutant KLHL3.

MATERIALS AND METHODS
Generation of KLHL3 knockout mice. In this study, we generated KLHL3−/− mice that expressed β-Gal under the control of the endogenous KLHL3 promoter. To generate KLHL3−/− mice, we purchased a targeting vector containing the mouse genomic KLHL3 locus from the Knockout Mouse Project (KOMP) Repository (Davis, CA). The targeting vector was then transfected into Balb1 embryonic stem (ES) cells (23) derived from C57BL/6 mice by electroporation, as previously reported (54). After selection with G418 at 150 μg/ml and 2 μM ganciclovir, the targeted ES cell clones were identified by PCR. Verification of homologous recombination by PCR of genomic DNA of the selected ES cell clones was performed; primers F1 (5′-GAGATGGGGCAACGGCAATTAATG-3′) and R1 (5′-GGTTTTAAGCAAAACAGGAGGTC-3′) show an ~3-kb band, primers F2 (5′-GGGATCTCATGGGAGTTCTTCG-3′) and R1 show an ~5-kb band, and primers F3 (5′-CTGATTGAAAGGAGCGCTTCAG-3′) and R2 (5′-GGTTGTTGAGGAAAGG GTTCAGAAG-3′) show an ~7-kb band.

To generate KLHL3+/− mice that expressed β-Gal under the control of the endogenous KLHL3 promoter, chimeric male mice were bred with C57BL/6J females to produce heterozygous floxed (KLHL3floxed−) mice. The neo cassette was then deleted by crossing the KLHL3floxed− mice with Cre recombinase-expressing transgenic (TG) mice (55). Genotyping of the mice was performed by PCR using the sense primer F (5′-AATCTAGGGGACCGCTGGAGAAG-3′) and the antisense primer R3 (5′-CTGACACAGG TTTACAGGTGACG-3′) to detect the wild-type allele. Further PCR analysis was then performed using the sense primer F4 (5′-GGTCTAATTACCAGTGTCGGTGTA-3′) and the antisense primer R3 to detect the upper LoxP site.

Animals. The experiments were performed on 8- to 16-week-old mice that had been raised under a 12-h day/night cycle and had been 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.

Plasmids. The expression plasmids for 3×FLAG-tagged human KLHL3, Halo-tagged human KLHL3, and Halo-tagged R528H human KLHL3 have been described previously (19). The expression plasmid encoding Halo-tagged human KLHL3 R528H with deletion of BTB-BACK domain was obtained by PCR site-directed mutagenesis with the primers 5′-TGGGGAAAACGCTGAAAGAAGAGGTTG-3′ (forward) and 5′-CTTCTCACAACCTTGGAAAGTTCC-3′ (reverse).
**Cell culture and transfections.** HEK293T cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% (vol/vol) fetal bovine serum, 2 mM L-glutamine, penicillin at 100 U/ml, and streptomycin (2%) in a humidified 5% CO₂ incubator. In the coimmunoprecipitation experiments, the cells were placed in six-well plate (2.0 ml of medium/well, 60% confluence) and transiently transfected with the indicated amounts of plasmid DNA using Lipofectamine 2000 (Invitrogen). For each transfection, wild-type KLHL3 and/or mutant KLHL3 were used. In the coexpression experiments to investigate the dominant negative effect of KLHL3 R528H, the cells were placed in a 6-cm dish (4 ml of medium/dish, 60% confluence) and transfected with combinations of FLAG-tagged wild-type KLHL3 with Halo-tagged KLHL3 R528H or Halo-tagged KLHL3 ΔBTB-BACK plasmid. FLAG-tagged CMV and Halo-tagged GAPDH (glyceraldehyde-3-phosphate dehydrogenase) were used as control. Bio-ease-FLAG-tagged human WNK4 and FLAG-tagged human Cul3 were also transfected to all the experiments of the dominant negative effect of KLHL3 R528H. The cells were then incubated for 24 h after the transfection.

**Coimmunoprecipitation experiments.** The HEK293T cells transfected in combination with FLAG-tagged wild-type KLHL3 and Halo-tagged wild-type KLHL3 or Halo-tagged mutant KLHL3 were harvested in lysis buffer composed of 50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1% NP-40, 1 mM sodium orthovanadate, 50 mM sodium fluoride, and protease inhibitor cocktail for 30 min at 4°C. After centrifugation at 15,000 x g for 10 min, the supernatant was immunoprecipitated with anti-FLAG M2 beads (Sigma-Aldrich) for 2 h at 4°C. The beads were then washed with lysis buffer and the immunoprecipitates were eluted by boiling for 10 min in sodium dodecyl sulfate (SDS) sample buffer.

**Blood measurements.** Blood for electrolyte analysis was obtained as previously described (8) and electrolyte levels were determined using an i-STAT portable clinical analyzer (Fuso Pharmaceutical Industries, Ltd.). Samples were obtained from the venous plexus near the mandible under anesthesia with light ether.

**Immunoblotting.** Protein samples were extracted from the kidney and other organs, and semiquantitative immunoblotting was performed as previously described (56). For semiquantitative immunoblotting, we used entire kidney samples without the nuclear fraction (600 μm sections of the kidney) were prepared using a cryostat microtome. The protein samples were extracted in lysis buffer composed of 50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1% NP-40, 1 mM sodium orthovanadate, 50 mM sodium fluoride, and protease inhibitor cocktail for 30 min at 4°C. After centrifugation at 15,000 x g for 10 min, the supernatant was immunoprecipitated with anti-FLAG M2 beads (Sigma-Aldrich) for 2 h at 4°C. The beads were then washed with lysis buffer and the immunoprecipitates were eluted by boiling for 10 min in sodium dodecyl sulfate (SDS) sample buffer.

**BP measurement.** We measured the systolic blood pressure (BP) using implantable radiotelemetric devices, as previously reported (61). 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 ambient pressure reference monitor (APR-1). All data were analyzed using Dataquest ART4.31.

**LacZ staining.** LacZ staining was performed using the LacZ Tissue staining kit (InvivoGen, San Diego, CA). We dissected the kidneys and brains from 12-week-old KLHL3 knockout/lacZ knock-in and wild-type (control) mice into phosphate-buffered saline (PBS)–MgCl₂ solution on ice. The samples were fixed in 4% paraformaldehyde (PFA) (24 h) and 10% formalin (72 h) in 0.1 M sodium phosphate buffer, pH 7.4. The tissues were then cut in half and inserted into X-Gal (5-bromo-4-chloro-3-indolyl-galactopyranoside) solution (InvivoGen) for 24 h at 37°C in the dark. Next, serial frozen sections (10-μm sections of the brain and 5-μm sections of the kidney) were prepared using a cryostat microtome. The frozen sections were incubated in X-Gal solution for 12 h and observed under a microscope.

**Immunofluorescence.** The kidneys were fixed by perfusion with periodate lysine (0.2 M) and paraformaldehyde (2%) in PBS. Immunofluorescence was then performed as previously described (54) using the primary mouse anti-β-Gal (Promega Corporation) and guinea pig-anti-NCC (62) antibodies and Alexa Fluor 488 or 546 dye-labeled (Molecular Probes; Invitrogen) secondary antibodies. Immunofluorescence images were obtained using an LSM510 Meta Confocal Microscope (Carl Zeiss, Oberkochen, Germany).

**Statistical analysis.** The two groups of mice were compared by using unpaired t tests. In addition, one-way analysis of variance, followed by Tukey's post hoc test, were used to evaluate the statistical significance of comparisons between multiple groups. P values of <0.05 were considered statistically significant. Data are presented as means ± the standard errors of the means.

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