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KLHL2 interacts with and ubiquitinates WNK kinases

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ABSTRACT

Mutations in the WNK1 and WNK4 genes result in an inherited hypertensive disease, pseudohypoaldosteronism type II (PHAII). Recently, the KLHL3 and Cullin3 genes were also identified as responsible genes for PHAII. Although we have reported that WNK4 is a substrate for the KLHL3–Cullin3 E3 ligase complex, it is not clear whether all of the WNK isoforms are regulated only by KLHL3. To explore the interaction of WNKs and other Kelch-like proteins, we focused on KLHL2 (Mayven), a human homolog of *Drosophila* Kelch that shares the highest similarity with KLHL3. We found that KLHL2, as well as KLHL3, was co-immunoprecipitated with all four WNK isoforms. The direct interaction of KLHL2 with WNKs was confirmed on fluorescence correlation spectroscopy. Co-expression of KLHL2 and Cullin3 decreased the abundance of WNK1, WNK3 and WNK4 within HEK293T cells, and a significant increase of WNK4 ubiquitination by KLHL2 and Cullin3 was observed both in HEK293T cells and in an in vitro ubiquitination assay. These results suggest that KLHL2–Cullin3 also functions as an E3-ligase for WNK isoforms within the body.

1. Introduction

Mutations in the with-no-lysine kinase 1 (WNK1) and WNK4 genes are responsible for pseudohypoaldosteronism type II (PHAII) [1], which is characterized by hypertension, hyperkalemia, and metabolic acidosis [2]. Numerous studies have been performed to clarify the molecular pathogenesis of PHAII [3]. We have found that increased phosphorylation of oxidative stress-responsive kinase 1 (OSR1) and STE20/SPS1-related proline/alanine-rich kinase (SPAK), which are substrates of WNK kinases, results in the activation of the Na-Cl cotransporter (NCC) in vivo [4–6]. The analysis of WNK4$^{-/-}$ mice was concordant with our hypothesis that WNK4 kinase is a major regulator of NCC phosphorylation in kidney [7].

In 2011, additional genes responsible for PHAII (KLHL3 and Cullin3) were identified [8,9]. KLHL3 is a member of the BTB-Kelch protein family, which includes components of the Cullin-RING E3 ubiquitin ligases and a substrate adaptor for ubiquitination [10–12]. Recently, we reported that KLHL3 interacted with Cullin3 and WNK4, induced WNK4 ubiquitination, and reduced WNK4 protein abundance. We also revealed the pathophysiological role of PHAII-causing mutations of the WNK4, KLHL3 and Cullin3 genes [13]. Namely, the mutations caused impaired ubiquitination and a subsequent increase of WNK4 in the kidney, which activate the WNK-OSR1/SPAK-NCC signal cascade and cause PHAII [13]. Two independent reports have supported our findings [14,15]. In addition to WNK4, Alessi's group reported that WNK1 interacts with the KLHL3–Cullin3 complex in vitro [16]. This data is quite reasonable since we identified that the domain within WNK4 responsible for the binding to KLHL3 is an acidic domain that is highly conserved among WNK kinases [13]. Therefore, it would be possible that WNK2 and WNK3 could also be targets of KLHL3–Cullin3 E3 ligase. However, WNK isoforms are widely expressed in various cell types within the body, whereas KLHL3 expression might be relatively limited in specific cell types. In this respect, we hypothesized that there may be other ubiquitin ligases for WNK kinases. Here, we focused on KLHL2 (Mayven), another human homolog of *Drosophila* Kelch, since the Kelch domain (WNK-binding domain) of KLHL2 is highly similar (86% identity) to that of KLHL3. Initially, KLHL2 was identified as an actin-binding protein predominantly expressed in brain [17]. Later, it was found that KLHL2 formed a complex with Cullin3 and bound to and increased the ubiquitination of neuronal pentraxin with chromo domain (NPCD), suggesting its role as E3 ubiquitin ligase [18]. In the present study, we report that KLHL2 binds to WNKs at their acidic domain and functions as an E3 ubiquitin ligase for WNK kinases.

2. Materials and methods

2.1. Plasmids

Expression plasmids for 3xFLAG-tagged human WNK1, WNK4, Cullin3, Halo-tagged human WNK4 and KLHL3 have been described previously [13,19,20]. The cDNA encoding Halo-tagged

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human WNK1, WNK2, WNK3 and GAPDH in pFN21A vector were purchased from Promega. Human KLHL2 cDNA was isolated by reverse transcription-polymerase chain reaction using human brain mRNA from human total RNA master panel II (Clontech) as a template. Sequence of the amplification primers employed is as follows: KLHL2 sense, 5′-ATC GAT CGA TAT GGA GAC GCC GCC GCT GCC-3′; KLHL2 antisense, 5′-CCG GCG CGC CGT TTA AAC TCA TAA TGG TTT ATC AAT AAC-3′. The cDNA was cloned into pFN21A vector (Promega). HA_4-tagged ubiquitin expression vector was kindly provided by T. Ohta (St. Marianna University, School of Medicine).

2.2. Reverse transcription polymerase chain reaction

RNAs from human total RNA master panel II (Clontech) were reverse-transcribed by using Omniscript RT kit (QIAGEN). The target cDNAs were amplified by PCR, which was performed by using primers described below. The primers for GAPDH were purchased from Roche Diagnostics. Sequence of the amplification primers employed is as follows: KLHL2 sense, 5′-TAA TAC CGA AAA ACA CTG CC-3′; KLHL2 antisense, 5′-TTC TAA CTC TCT TTG CTC GG-3′; KLHL3 sense, 5′-TGA CAA GAA CCA GAG GAC GA-3′; KLHL3 antisense, 5′-AAC TGA GAC TGC AGG AAG-3′.

2.3. Cell culture and transfections

HEK293T cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% (v/v) fetal bovine serum (FBS), 2 mM L-glutamine, 100 U/ml penicillin, and 0.1 mg/ml streptomycin at 37 °C in a humidified 5% CO_2 incubator. HEK293T cells (3×10^5 cells/6-cm dish) were transfected by the indicated amount of plasmid DNA with Lipofectamine 2000 reagent (Invitrogen).

2.4. Immunoprecipitation

HEK293T cells transfected with the indicated amount of DNA were lysed in a buffer (50 mM Tris–HCl (pH 7.5), 150 mM NaCl, 1% Nonidet P-40, 1 mM sodium orthovanadate, 50 mM sodium fluoride, and protease inhibitor cocktail) for 30 min at 4 °C. When the cells were transfected with the HA-ubiquitin expression plasmid, the cells were treated with 1 μM epoxomicin (specific and irreversible proteasome inhibitor; Peptide Institute, Osaka, Japan) for 3 h before harvesting. After centrifugation at 12,000×*g* for 15 min, the protein concentration of the supernatants was measured and equal amounts of the supernatants were used for immunoprecipitation with anti-FLAG M2 beads (Sigma–Aldrich) for 1 h at 4 °C. Thereafter, the precipitants were washed with the lysis buffer and the immunoprecipitates were eluted in SDS sample buffer after boiling for 5 min. To detect ubiquitination of WNK4 in denatured samples, the cells transfected with various plasmids were lysed in 2% SDS buffer (2% SDS, 150 mM NaCl, 10 mM Tris–HCl, pH 8.0, 2 mM sodium orthovanadate, 50 mM sodium fluoride, 1× protease inhibitors) and boiled for 10 min followed by sonication. Before immunoprecipitation, the lysates were diluted 1:10 in a dilution buffer (10 mM Tris–HCl, pH 8.0, 150 mM NaCl, 2 mM EDTA, 1% Triton X-100), incubated at 4 °C for 1 h with rotation, and centrifuged at 12,000×*g* for 15 min.

2.5. Immunoblotting

Cells transfected with the indicated amount of plasmid DNA were lysed in lysis buffer [50 mM Tris–HCl, pH 7.5, 150 mM NaCl, 1% Nonidet P-40, 1 mM sodium orthovanadate, 50 mM sodium fluoride, and protease inhibitor cocktail (Roche Diagnostics)] for 30 min at 4 °C. After centrifugation at 12,000×*g* for 15 min, the supernatants were boiled with SDS sample-buffer (Cosmo Bio, Inc.) and subjected to SDS–PAGE. Blots were probed with the following primary antibodies: anti-FLAG (Sigma–Aldrich), anti-Halo (Promega), anti-HA (Merck Millipore), anti-T7 (Merck Millipore) and anti-actin (Cytoskelton) antibodies. Alkaline-phosphatase-conjugated anti-IgG antibodies (Promega, Madison, WI) and WesternBlue (Promega) were used to detect the signals.

2.6. In vitro ubiquitination assay

GST-WNK4(490–626) in pGEX6p-1 vector was described previously [13]. Recombinant GST-fusion WNK4 protein was expressed in BL21 *Eschericia coli* cells and purified by using glutathione sepharose beads. KLHL2 or KLHL3 complexes were immunoprecipitated from the lysates of HEK293T cells transiently expressing FLAG-KLHL2 or FLAG-KLHL3. Then, the complexes were incubated in a 20 μl volume of reaction buffer (50 mM Tris–HCl, pH 7.4, 2.5 mM $MgCl_2$, 0.5 mM DTT, 2 mM ATP) for 2 h at 30 °C with purified GST-WNK4-His (1 μg), 100 ng recombinant human E1 (Boston Biochem), 500 ng recombinant human UbcH5a/UBE2D1 (Boston Biochem), and 2.5 μg recombinant human ubiquitin (Boston Biochem). The reaction was terminated by addition of SDS–PAGE sample buffer, followed by boiling for 5 min. The reaction mixtures were then subjected to immunoblot analyses with anti Ub (Cell Signaling Technology) or His (Abcam) antibodies.

2.7. Fluorescence correlation spectroscopy

Fluorescent TAMRA-labeled WNK1, 2, 3 and WNK4 peptides covering the PHAII mutation sites were prepared (Hokkaido System Science Co., Ltd., Hokkaido, Japan). Kelch-repeats of human KLHL2 or KLHL3 were cloned into pGEX6P-1 vector. Recombinant GST-fusion KLHL proteins were expressed in BL21 *E. coli* cells and purified by using glutathione sepharose beads. The TAMRA-labeled WNK peptides were incubated at room temperature for 30 min with different concentrations of GST-KLHL proteins (0–2 μM) in 1× PBS with 0.05% Tween 20 reaction buffer and the fluorescence correlation spectroscopy (FCS) measurements of single-molecule fluorescence were performed using the FluoroPoint-light analytical system (Olympus, Tokyo, Japan) [21]. The assay was performed in a 384-well plate. All experiments were performed in 10 s of data acquisition time and the measurements were repeated five times per sample. The amino acid sequence of TAMRA-WNKs is as follows: WNK1, TAMRA-SVSTQVEPEEPEADQHQQLQYQQPSISVLS (30 aa); WNK2, TAMRA-GQPGPPEPEEPEADQHLLPPTLPTSATSLA (30 aa); WNK3, TAMRA-AQQTGAECEETEVDQHVRQQLLQRKPQQHC (30 aa); WNK4, TAMRA-PSVFPPEPEEPEADQHQPFLFRHASYSSTT (30 aa). Acidic domains of each WNK isoform are underlined.

2.8. Statistics

Statistical significance was evaluated by ANOVA test with multiple comparisons using Tukey's correction. The results with p values <0.05 were considered statistically significant. Data are presented as mean ± SEM.

3. Results

3.1. Expression pattern of KLHL2 and KLHL3 in human organs

To examine the distribution of KLHL2 and KLHL3 expression, we performed RT-PCR of KLHL2 and KLHL3 cDNA in human organs (Fig. 1). cDNA from the indicated human tissues was used as a template for PCR by using primers specific for KLHL2, KLHL3 and GAPDH mRNA. KLHL2 as well as KLHL3 was expressed differently in various human organs.

3.2. Overexpressed KLHL2 interacts with WNK kinases

We first confirmed the interaction of KLHL2 and Cullin3 by co-immunoprecipitation (Fig. 2A) as previously reported [18]. To investigate whether KLHL2 interacts with WNK kinases in mammalian cells, we then performed co-immunoprecipitation assays of full-length KLHL2 and KLHL3 with WNK kinases. Because the expression level of Halo-tagged WNK1 was low, we used FLAG-tagged WNK1 in co-immunoprecipitation assays (Fig. 2B). Other WNKs were Halo-tagged (Fig. 2C). We found that KLHL3 could interact with WNK2 and WNK3 as well as WNK1 and WNK4 and that KLHL2 could also interact with all WNK kinases. As in the case with KLHL3, KLHL2 was not co-immunoprecipitated with other components of the WNK-OSR1/SPAK-NCC signal cascade, i.e., OSR1, SPAK, and NCC (Fig. S1).

To investigate whether the conserved acidic domains of WNKs could be the common binding sites for KLHL2 and KLHL3, we prepared TAMRA-labeled WNK peptides covering each acidic motif (Fig. 2D), and the binding to the Kelch-repeat of KLHL2 or KLHL3 proteins was assayed in vitro as we described previously [13]. As shown in Fig. 2D, the diffusion time of TAMRA-labeled peptide became slower as the concentration of GST-KLHLs increased, indicating that all WNK peptides could bind to both GST-KLHL2 and GST-KLHL3. GST alone did not affect the diffusion time. It is notable that the WNK3 peptide showed the slowest diffusion time both with KLHL2 and KLHL3. The amino acid sequence of the WNK3 acidic motif (ECEETEVDQH) was different from those of other WNK kinases (EPEEPEADQH) (Fig. 2D), which might be responsible for the difference in diffusion times.

3.3. KLHL2 regulated the abundance of WNK kinases

To determine the effect of KLHL2 on WNK kinases, we overexpressed KLHL2 or KLHL3 plus Cullin3 along with WNK kinases in HEK293T cells. As shown in Fig. 3, co-expression of KLHL2 or KLHL3 with Cullin3 decreased the levels of WNK1, WNK3, and WNK4 proteins as compared to the expression of Cullin3 alone. In contrast to WNK1, 3, and 4, the effect of KLHL2 on WNK2 was not clear, even after repeating the experiments more than three times.

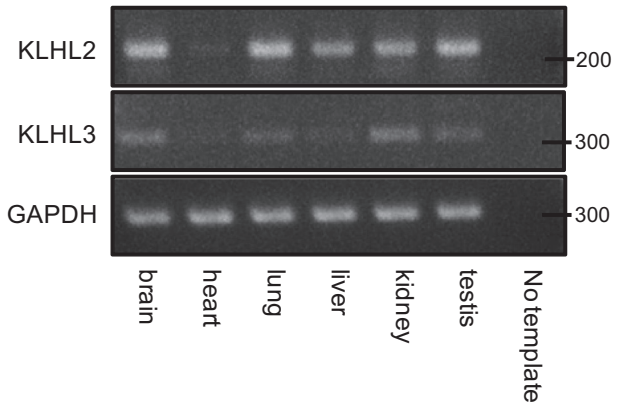

Fig. 1. Expression of KLHL2 and KLHL3 mRNAs in human organs. Complementary DNAs from the indicated human organs were used as a template for PCR using primers specific for KLHL2, KLHL3 and GAPDH mRNA. The expression patterns of KLHL2 and KLHL3 are different.

3.4. KLHL2 increases the ubiquitination of WNK4

To investigate whether KLHL2 ubiquitinates WNK kinases, we first performed an in vitro ubiquitination assay. FLAG-tagged KLHL2 or KLHL3 expressed in HEK293T cells was immunoprecipitated with M2-agarose and mixed with recombinant E1, E2, ubiquitin, and WNK4 protein (residues 490–626). We did not overexpress Cullin3, since we knew from the preliminary experiments that there was a sufficient amount of endogenous Cullin3 in HEK293T cells to perform this assay (data not shown). We confirmed that KLHL2 directly ubiquitinated WNK4 protein (residues 490–626) (Fig. 4A) as KLHL3 did in our previous study. We also confirmed WNK4 ubiquitination in vivo by immunoprecipitation under a denaturing condition. As shown in the ubiquitin (HA) immunoblot (Fig. 4B), the ubiquitination signals appeared as a smeared band over the apparent molecular size of WNK4 (200 kDa). This ubiquitination was increased by co-expression of KLHL2 or KLHL3, although the immunoprecipitated WNK4 was decreased by co-expression. These results clearly indicate that KLHL2 functions as an E3 ubiquitin ligase of WNK4.

4. Discussion

In the present study, we reported the interaction between KLHL2 and WNK kinases that induced the ubiquitination of WNK protein, leading to a reduced level of WNK protein in cells. KLHL2 was initially identified as an actin-binding protein highly expressed in the brain [17], and it was implicated in oligodendrocyte process outgrowth as well as transcriptional regulation of growth-promoting factors in breast cancer cells [22–24]. However, several members of the Kelch-like protein family have been recently described as components of multi-protein complexes known as Cullin-RING E3 ubiquitin ligases (CRLs) [10,11,25]. CRLs are involved in the identification and targeting of proteins for ubiquitination. Kelch-like proteins function as substrate adapters, recruiting proteins destined for ubiquitination into the CRL complex. In line with this notion, neuronal pentraxin with chromo domain (NPCD) was reported to bind to KLHL2, and its ubiquitination was increased by KLHL2 co-expression [18]. However, no direct evidence showing that KLHL2 is an E3 ligase has been demonstrated.

We observed a highly similar function of KLHL2 to that of KLHL3 as an E3 ubiquitin ligase to WNK kinases. KLHL2 is the closest homolog of KLHL3 among the KLHL proteins, and it is also the closest homolog of drosophila Kelch (63% identity). The Kelch repeats among these three proteins are highly conserved. The four β-strands found in each Kelch repeat are named ‘a’ to ‘d’. It is surprising that not only all of the mutated residues reported in PHAII patients [8,9] are conserved between these three Kelch-like proteins, but also that KLHL2 shares almost perfect identity (98% identity) with KLHL3 when focused on its ‘b–c’ loops and ‘d–a’ loops, in which most of the PHAII-causing KLHL3 mutations cluster [8,9]. This high identity between KLHL2 and KLHL3 is not seen between KLHL3 and other Kelch-like proteins [26]. The function of each loop of the KLHL3 Kelch repeat has not been evaluated yet, but considering that the ‘d–a’ loops and ‘b–c’ loops of Keap1 form the top face of the β-propeller and that this face is the substrate (Nrf2)-binding pocket [27], the extensive shared identity in these domains between KLHL2 and KLHL3 may support the identity of substrate specificity between KLHL2 and KLHL3.

This study clearly shows that not only WNK1 and WNK4 but all WNK kinases could be regulated by KLHL3. In addition, KLHL2 could regulate all WNKs as well. In the case of WNK4, the dysregulation of WNK4 degradation significantly affected the downstream signaling and caused PHAII. In this respect, the regulation

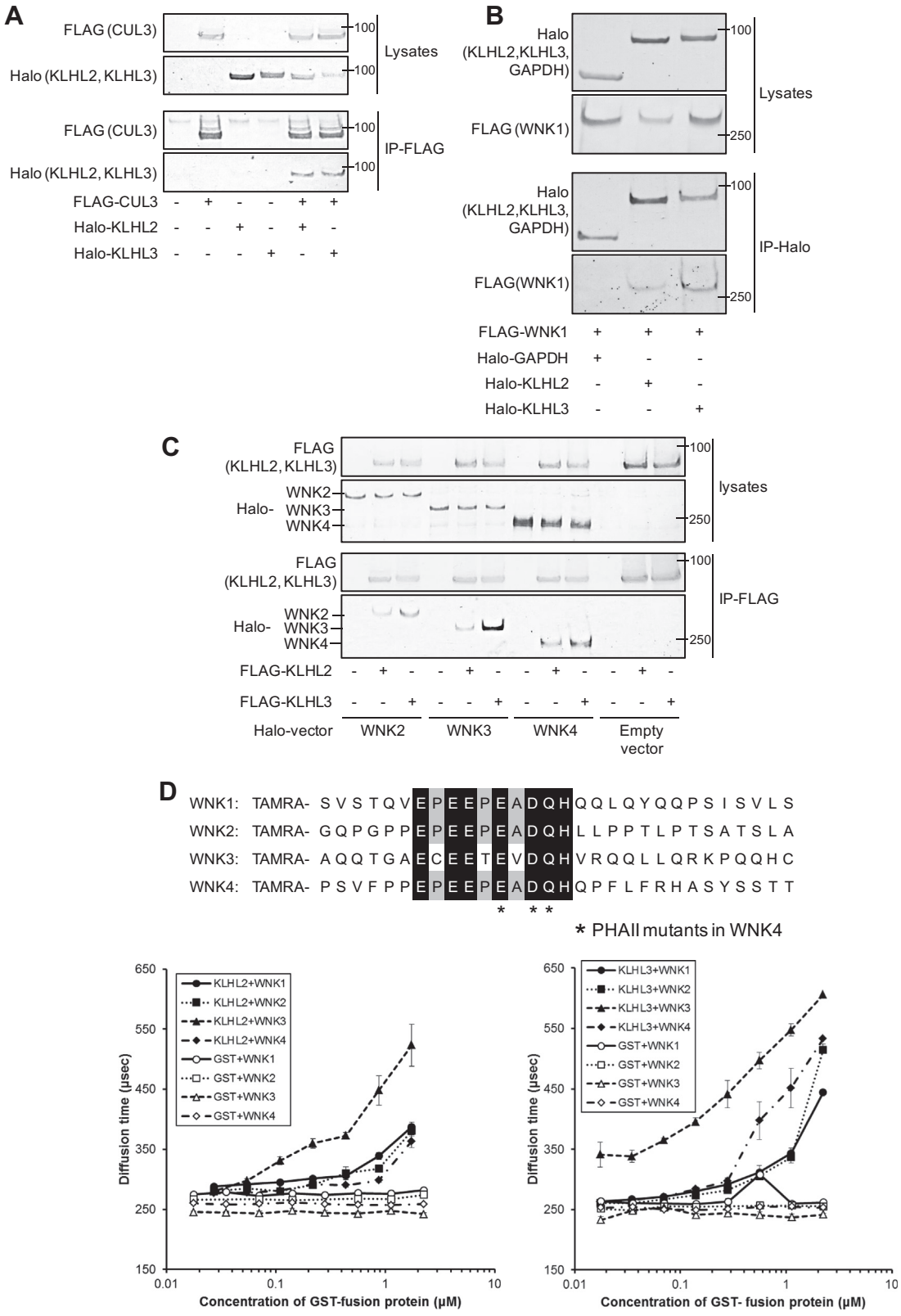

Fig. 2. KLHL2 interacted with Cullin3 and WNK kinases. (A) FLAG-tagged Cullin3 was co-immunoprecipitated with Halo-tagged KLHL2 as well as KLHL3 in HEK293T cells. (B) Halo-tagged KLHL2 and KLHL3 were co-immunoprecipitated with FLAG-tagged WNK1. (C) FLAG-tagged KLHL2 and KLHL3 were co-immunoprecipitated with Halo-tagged WNK2, WNK3 and WNK4. (D) Diffusion time of a single-molecule TAMRA-labeled peptide corresponding to the acidic domain of wild-type WNK kinases was measured by fluorescence correlation spectroscopy. The diffusion time of each peptide was significantly ($p < 0.05$) increased with KLHL2-GST at 0.21 μM or higher and KLHL3-GST at 0.14 μM or higher, but not with GST alone. These data indicate the direct binding of the Kelch-repeat of KLHL2 and KLHL3 to the acidic domain of WNK kinases.

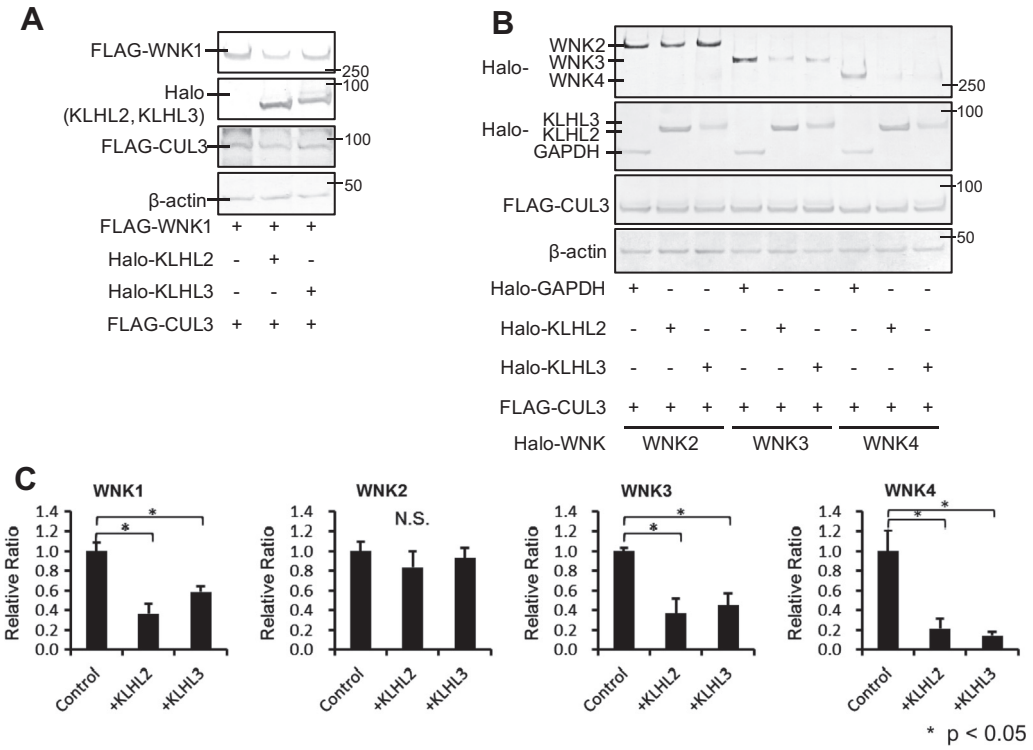

Fig. 3. KLHL2 regulated WNK kinase abundance. (A) FLAG-tagged WNK1 and FLAG-tagged Cullin3 were co-expressed with Halo-tagged KLHL2 or KLHL3. Co-expression of KLHL2 as well as KLHL3 decreased the abundance of WNK1. (B) Halo-tagged WNK kinases and FLAG-tagged Cullin3 were co-expressed with Halo-tagged GAPDH, KLHL2 or KLHL3. The cellular WNK3 and WNK4 abundance were decreased by co-expression of KLHL2 or KLHL3. The WNK2 was not affected by the co-expression. (C) Densitometric analysis of the results of (A) and (B). $^{*}p < 0.05$ compared with control.

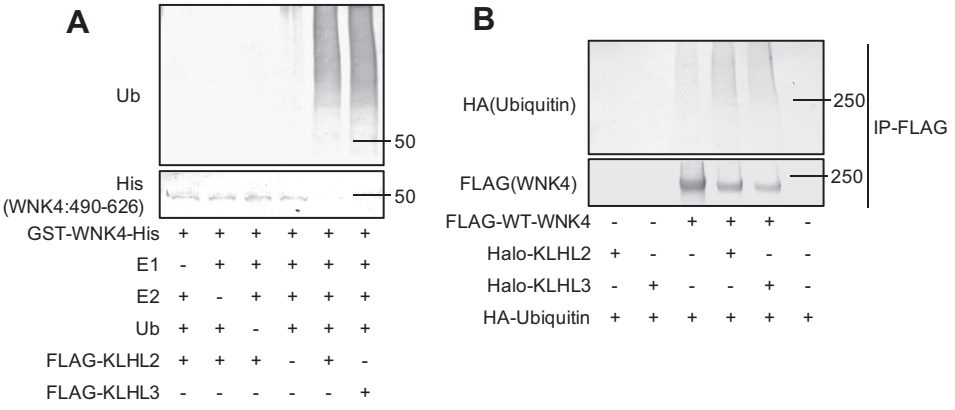

Fig. 4. KLHL2 increased WNK4 ubiquitination. (A) In vitro ubiquitination assay of WNK4. WNK4 (490–626) (50 kDa) was incubated with ubiquitin, E1 and E2 (UbcH5a/UBE2D1) with or without KLHL2–Cullin3 or KLHL3–Cullin3 complex. KLHL2 as well as KLHL3 significantly ubiquitinated WNK4 (490–626) in vitro. (B) WNK4 was expressed with HA-ubiquitin and KLHL2 or KLHL3, and immunoprecipitated under a denaturing condition. As shown in the ubiquitin (HA) immunoblot, the ubiquitination signals were observed as a smeared band over the apparent molecular size of WNK4 (200 kDa). The ubiquitination of WNK4 was increased by co-expression of KLHL2 or KLHL3.

of other WNKs by KLHL2 and/or KLHL3 may be involved in certain pathophysiological conditions in extrarenal tissues.

In summary, we have identified the function of KLHL2 as an E3 ubiquitin ligase for WNK kinases. Different combinations of KLHL2 and KLHL3 with WNKs could regulate WNK kinase signaling in different kinds of cells.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2013.06.104.

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