Mutation affecting the conserved acidic WNK1 motif causes inherited hyperkalemic hyperchloremic acidosis

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Gain-of-function mutations in with no lysine (K) (WNK) and WNK4 genes are responsible for familial hyperkalemic hypertension (FHHt), a rare, inherited disorder characterized by arterial hypertension and hyperkalemia with metabolic acidosis. More recently, FHHt-causing mutations in the Kelch-like 3–Cullin 3 (KLHL3-CUL3) E3 ubiquitin ligase complex have shed light on the importance of WNK’s cellular degradation on renal ion transport. Using full exome sequencing for a 4-generation family and then targeted sequencing in other suspected cases, we have identified new missense variants in the WNK1 gene clustering in the short conserved acidic motif known to interact with the KLHL3-CUL3 ubiquitin complex. Affected subjects had an early onset of a hyperkalemic hyperchloremic phenotype, but normal blood pressure values. Functional experiments in Xenopus laevis oocytes and HEK293T cells demonstrated that these mutations strongly decrease the ubiquitination of the kidney-specific isoform KS-WNK1 by the KLHL3-CUL3 complex rather than the long ubiquitous catalytically active L-WNK1 isoform. A corresponding CRISPR/Cas9 engineered mouse model recapitulated both the clinical and biological phenotypes. Renal investigations showed increased activation of the Ste20 proline alanine–rich kinase–Na+–Cl– cotransporter (SPAK-NCC) phosphorylation cascade, associated with impaired ROMK apical expression in the distal part of the renal tubule. Together, these new WNK1 genetic variants highlight the importance of the KS-WNK1 isoform abundance on potassium homeostasis.

Introduction

Familial hyperkalemic hypertension (FHHt), also known as Gordon syndrome and pseudohypoaldosteronism type 2, is a rare disease associated with net positive Na+ balance and renal K+ retention resulting in hypertension, hyperkalemia, and hyperchloremic metabolic acidosis (1). Mutations causing FHHt were first identified in the WNK1 and WNK4 genes that encode 2 members of the with no lysine (K) (WNK) serine-threonine kinase family (2). More recently, we and others have identified disease-causing mutations in the Kelch-like 3 (KLHL3) and Cullin 3 (CUL3) proteins belonging to a ubiquitin-protein ligase complex (3, 4). This complex has been shown in vitro to interact with WNK1 and WNK4, induce their ubiquitination, and regulate their protein levels through proteasomal degradation (5–8). Ohta and collaborators mapped the interaction site in WNK1 to a region containing a short acidic motif, which is highly conserved among the members of the WNK family (5). Interestingly, most of the FHHt-causing WNK4 variants cluster in this motif and are charge changing. WNK4 proteins carrying the same mutations fail to interact with the KLHL3 adaptor protein in vitro (5–7). This causes increased WNK4 abundance with secondary activation of the Na+–Cl– cotransporter (NCC) and development of FHHt (6, 7).
We took advantage of one 4-generation family to combine linkage analysis and whole exome sequencing (WES). This family included 7 affected individuals (all with metabolic acidosis and hyperkalemia, but with normal BP) and 6 unaffected individuals (Figure 1A and Supplemental Table 1A; supplemental material available online with this article; https://doi.org/10.1172/JCI94171DS1). Using a SNP-based linkage approach, 7 suggestive linkage regions (maximum logarithm of odds [LOD] score = 1.8 in all linked regions) were identified in this panel (Supplemental Table 1B). In total, linked regions spanned 69 Mb and included 829 protein-coding genes. After filtering, WES performed in 1 unaffected and 2 affected individuals identified 71 possible disease-causing coding variants (Supplemental Table 1C). Four missense variants mapped to the linkage regions and were predicted in silico to be damaging. These variants were located in solute carrier family 30 member 7, a zinc transporter (SLC30A7), kinesin family member 11 (KIF11), tectonic family member 3 (TCTN3), and WNK1. The WNK1 variant (c.1905T>A; p.Asp635Glu) (Figure 1B) was located in exon 7 (ex7), which encodes the conserved acidic motif, previously shown to mediate the interaction with the substrate adaptor KLHL3 (14).

**Figure 1. Missense variant in the WNK1 acidic motif in the FHHt pedigree 29.** (A) Kindred 29: family affected by FHHt composed of 7 affected (black) and 6 unaffected (white) members. Arrow indicates the index case. Asterisks indicate exome-sequenced individuals. (B) Electrophoregram obtained by Sanger sequencing showing the double-peak A/T corresponding to the WNK1 heterozygous mutation (c.1905T>A; p.Asp635Glu).

The genetic heterogeneity of FHHt is reflected in its phenotypic heterogeneity, ranging from severe cases presenting in childhood to mild and sometimes asymptomatic cases presenting in late adulthood (9, 10). A similar spectrum exists for the more mild phenotypes. FHHt-causing WNK4 mutations are located in ex7 and ex17, encoding highly conserved acid and base motifs, respectively. Thus, we screened the homologous motifs of WNK1 encoded by ex7 and ex25, respectively, in 26 unrelated affected cases previously found as negative for the classical mutations in WNK4, KLHL3, CUL3, or the intron 1 deletion in WNK1. Direct sequencing identified 5 additional nonsynonymous heterozygous variants in ex7 in 8 unrelated subjects (Figure 2, A–C). The in silico pathogenicity of these variants is described in Supplemental Table 2. All were located within the acidic motif, but only positions 631 and 636 of the L-WNK1 protein, and were predicted to be pathogenic. Four of the 6 missense variants were charge changing (E631K, D635N, Q636R, Q636E); 2 affected residues (D635, Q636) were also found mutated in the homologous acidic motif of WNK4 (D564 and Q565, Figure 2, C and D).

**Clinical and biochemical characteristics: hydrochlorothiazide-sensitive hyperkalemic acidosis without hypertension.** Detailed clinical and biological characteristics of index cases are given in Table 1. The circumstances of discovery and the clinical symptoms of these index patients are detailed in Supplemental Table 3. In most of the cases, patients were asymptomatic and showed no signs of hyperkalemia on an electrocardiogram. All displayed the electrolyte abnormalities typical of FHHt, including marked hyperkalemia (median, 5.9 mmol/L; IQR, 5.3–6.3), hyperchloremia (median, 108 mmol/L; IQR, 106–110), and metabolic acidosis (total CO₂, 20 mmol/L; IQR, 19–21) despite a normal glomerular filtration rate (GFR) (median creatinine, 58 μmol/L; IQR, 47–74). For the 7 index cases with prospective reliable clinical data, hyperkalemia and hyperchloremia were rapidly corrected with low doses of hydrochlorothiazide (HCTZ) (6.25 to 25 mg/d; Supplemental Figure 1). In comparison, an average drop in potassium of only 0.7 mmol/L was observed in normal, healthy subjects administered a much higher dose of HCTZ (50 mg for 3 weeks) (15). Compared with reference values (16), we also observed a tendency for significant hypercalcemia in infancy and adulthood (Supplemental Figure 2).

Surprisingly, most of the index cases had casual BP values in the normal range, except a 25-year-old woman with associated obesity (K3-1, BMI, 32 kg/m²) and a 22-year-old woman (K88-I) without other cardiovascular risk factors, but who had short stature and mild mental retardation (Table 1). This tendency for normal
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Figure 2. Acidic motif WNK1 mutations. (A) Schematic representation of the WNK1 gene. The coordinates of the different exons are indicated above or below the structure with ex7 and ex25 (gray), which code for the conserved acidic and basic motifs. The proximal promoter (pP) drives the expression of the long ubiquitous kinase active isoform (L-WNK1), whereas the renal promoter (rP) drives the expression of the kinase defective kidney-specific isoform (KS-WNK1). (B) Schematic linear structure of LWNK1 and KS-WNK1. The kinase domain is represented in full black, the autoinhibitory domain (AID) is striped, coiled-coil domain 1 (CC1) and CC2 are represented in gray, and the conserved acidic motif (AM) and basic motif (BM) are represented in red. (C) Location and sequences of the mutated residues clustering in the acidic motif. The brackets indicate the number of unrelated affected subjects for each mutation. On the lower right are shown the Sanger sequencing electrophoregrams showing the various missense WNK1 mutations. (D) Conservation of the acidic motif showing residues mutated in FHHt among human WNK family members. The previously described WNK4 mutations are indicated in red; those at WNK1 and identified in this study in blue. All are located at completely conserved residues. The bottom part shows the conservation of the WNK1 acidic motif across species. The mutated residues are indicated in bold.

BP values was confirmed in the first-degree relatives harboring the familial mutations, since only 2/10 were mildly hypertensive (Supplemental Table 4). Overall, the 20 mutated individuals with measured BP (23 ± 18 years) belonging to 9 affected families had normal systolic BP (SBP) (116 ± 20 mmHg) and diastolic BP (DBP) (73 ± 21 mmHg). Low or suppressed plasma renin levels were observed with normal to slightly elevated plasma aldosterone concentrations (Table 1 and Supplemental Table 4), as has been observed with other FHHt genotypes.

We then compared the clinical phenotypes of FHHt patients with WNK1 ex7 missense mutations with those who had WNK4 missense variants affecting the same acidic motif collected in our center and in the literature (Table 2). Interestingly, WNK4-related patients consistently had the strongest BP and electrolyte phenotype, whatever the subclassification (index cases, adults or males only); BP was much higher in patients with WNK4 mutations (SBP and DBP averaged -20 mmHg and -10 mmHg higher) compared with that in patients with WNK1 ex7 mutations.

The WNK1 ex7 mutations selectively abolish the ubiquitination and degradation of the KS-WNK1 isoform in vitro. The acidic motif common to WNK1 and WNK4 has been reported as a binding site for the CUL3 and KLHL3 E3-ubiquitin ligase complex (14). Therefore, we tested the effect of KLHL3 on the abundance of WT and mutant L- and KS-WNK1 isoforms (Figure 3). The expression of KLHL3 in X. laevis oocytes resulted in a small (about 30%), but significant, decrease in the amount of L-WNK1 and almost completely abolished KS-WNK1. We then analyzed the recurrent WNK1 A634T variant, found in the K58 and K75 kindreds. No significant change in L-WNK1 abundance was observed between the WT and mutant isoforms, whereas the KS-WNK1-A227T mutant
Table 1. Basic clinical and biochemical characteristics of index cases with ex7 missense variants

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<th>Age (yr)</th>
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<th>SBP (mmHg)</th>
<th>DBP (mmHg)</th>
<th>Na (mmol/L)</th>
<th>K (mmol/L)</th>
<th>Cl (mmol/L)</th>
<th>CO₂t (mmol/L)</th>
<th>Creatinine (μmol/L)</th>
<th>Renin (mU/L)</th>
<th>Aldo (pmol/L)</th>
<th>Urinary CA/creatine (mmol/mol)</th>
<th>Urinary anion gap (UAG) (μmol/L)</th>
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<th>Urinary K/Cl</th>
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<td>226</td>
<td>15 (108)</td>
<td>15 (108)</td>
<td>0.5</td>
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CO₂t, total bicarbonates; F, female; M, male. ACreatinine reference values in children: newborn: 21–75 μmol/L; 2 months–3 years: 15–37 μmol/L. BUrinary anion gap (UAG) mol/L; 3–7 years; 27–52 years, 37 μmol/L. CUrinary Ca/creatine reference values in children: <6 months: 0.10–2.6 mmol/mol; 6–12 months: 0.09–2.2 mmol/mol; 1–2 years: 0.07–1.5 mmol/mol; 2–3 years: 0.06–1.4 mmol/mol. DUnit in ng/mL/h. EMean and SD values calculated on plasma renin values expressed in mU/L.

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was highly expressed in comparison with the WT KS-WNK1 isoform, suggesting that the mutation abrogates the degradation of KS-WNK1 (Figure 3A). We also tested the other observed WNK1 missense variants of the acidic motif using the same experimental system (Supplemental Figure 3A). All were protective with regard to KLHL3/CUL3-mediated degradation.

Since both WNK1 isoforms are physiologically coexpressed in the distal nephron (12), we sought to characterize how KLHL3/CUL3-mediated degradation affects KS- and L-WNK1 abundance in coexpression experiments (Figure 3B). On KLHL3 expression, a 70%–80% decrease in WT KS-WNK1 abundance was observed, while there was no change in L-WNK1. This major difference was not observed after transfection with the mutated isoforms. The coexpression of WT and mutant isoforms led to an intermediate decrease in KS-WNK1 abundance, suggesting that only the WT KS-WNK1 was degraded. Overall, these results suggest that human mutations of the WNK1 acidic motif selectively increase KS-WNK1 abundance in the distal nephron where KLHL3 is exclusively expressed.

We also tested the effect of KLHL3 on the WT and mutant WNK1 isoforms in human tissue culture HEK293T cells. Since we found that this cell line expresses high levels of CUL3, but undetectable levels of KLHL3, we first established a stable tetracycline-inducible cell line expressing KLHL3 (see Supplemental Methods). The induction of KLHL3 resulted in a dramatic decrease in the level of transfected KS-WNK1, but not L-WNK1 (Figure 4A). The introduction of the D635N mutation (D228N for the KS-WNK1 isoform) prevented this decrease. Interestingly, all other mutations were also protective with regard to KLHL3/CUL3-mediated degradation, with the exception of KS-WNK1 D228E (Supplemental Figure 3B). The substitution of an aspartic acid (D) by a glutamic acid (E) was not charge changing, which might explain the milder phenotype. The coexpression of L- and KS-WNK1 in HEK293T cells confirm the observations made in Xenopus oocytes (Figure 4B). Next, we immunoprecipitated L- and KS-WNK1 and assayed their ubiquitination status in denaturing conditions to exclude any ubiquitination signal linked to WNK1 interactors. KLHL3 induction resulted in heavy KS-WNK1 ubiquitination, whereas it had little or no effect on L-WNK1 or D635N (D228N) mutants (Figure 4C). The induction of KLHL3 resulted in a dramatic decrease in the level of transfected KS-WNK1, but not L-WNK1. This major difference was not observed after transfection with the mutated isoforms. The coexpression of WT and mutant isoforms led to an intermediate decrease in KS-WNK1 ubiquitination, whereas it had little or no effect on L-WNK1 or D635N (D228N) mutants (Figure 4C). KS-WNK1 ubiquitination by the KLHL3/CUL3 complex suggests that the E3 ligase adaptor (KLHL3) and the substrate (WNK1) interact. Indeed, both L-WNK1 and KS-WNK1 coimmunoprecipitated KLHL3 in native conditions. However, KS-WNK1 did so with a much higher efficiency (Figure 4D). As expected, the D635N mutation in the acidic domain (D228N in KS-WNK1) reduced the interaction with KLHL3. Taken together, our in vitro studies strongly suggest that KS-WNK1 is the preferential WNK1 isoform target of the KLHL3/CUL3 ubiquitin complex and that the mutations identified in WNK1 ex7 prevent this ubiquitination.

A mouse model bearing a mutation at the WNK1 acidic motif confirms the absence of arterial hypertension despite a typical electrolyte phenotype. To understand why WNK1 ex7 missense mutations lead to a hyperkalemic metabolic acidosis with normal BP in most of the affected patients, we sought to generate a mouse model bearing the D635E mutation at the WNK1 acidic motif using CRISPR/Cas9 genome-editing technology (Supplemental Figure 4A). The knockin of the point mutation was not found in 300 microinject-
Table 2. Comparison of the main clinical and biological features of patients with missense variants at ex7 of either the WNK1 or the WNK4 gene

<table>
<thead>
<tr>
<th>Case 1</th>
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<td><strong>cases</strong></td>
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</tr>
<tr>
<td><strong>Number (n)</strong></td>
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<td>21 (±10)</td>
<td>17 (±10)</td>
</tr>
<tr>
<td><strong>Age (yr)</strong></td>
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<td>45 (±20)</td>
<td>45 (±16)</td>
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<td>25 (±18)</td>
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<td><strong>CRE (μmol/L)</strong></td>
<td>60 (±17)</td>
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Data are shown as mean ± 1 SD. Index cases with WNK4 mutations. Israeli family described by Mayan et al. (58) as bearing the Q560E WNK4 mutation. All affected members had hyperkalaemia (5.2 to 6.0 mmol/L). Six out of the 18 affected subjects were normotensive, all female, but with similar biological abnormalities. Hyperkalaemia of mild to severe degree was found in most affected subjects, associated with an abnormal occurrence of stroke in our cases as well as in the family described by Mayan et al. (58). Such a family history of stroke was not present in any of the 9 WNK1-E7 families described here.

Increased activation of the SPAK-NCC phosphorylation cascade. The extreme sensitivity of FHH patients to thiazide diuretics initially suggested that the syndrome resulted from an increased activity of the Na⁺-Cl⁻ cotransporter NCC. This hypothesis was confirmed by the demonstration that NCC abundance and phosphorylation are increased in all FHH mouse hypertensive models (13, 18–20). Despite the absence of arterial hypertension, we observed the same phenomenon in the renal cortex of Wnk1(delE631) mice compared with Wnk1(delE631) mice (Figure 5G, Table 3 and Supplemental Table 6). The results confirm that there were no differences in basal BP between the 2 groups (SBP, 124.5 ± 10.4 in Wnk1(delE631) mice vs. 125.2 ± 8.4 mmHg in Wnk1(delE631) as well as no significant BP increase on a high-sodium diet (SBP: 120.6 ± 10.1 in Wnk1(delE631) vs. 128.1 ± 10.1 mmHg in Wnk1(delE631); Supplemental Table 6).

Using telemetry, we found that basal SBP and DBP were similar in heterozygous Wnk1(delE631) mice and WT littermates (Figure 5A) as well as on HCTZ (Figure 5B). Tail-cuff measured SBP on a larger set of adult males also revealed the absence of hypertension (113 ± 5 mmHg, n = 20 Wnk1(delE631) vs. 108 ± 5 mmHg, n = 20 Wnk1(+/−), NS). The homozygous Wnk1(delE631delE631 mice were perfectly viable, with no difference from the expected Mendelian proportions when heterozygous mice were crossed. The biological phenotype of these homozygous mice did not differ from that of the heterozygous mice, with the exception of SBP, which was significantly higher than in heterozygous or WT littermates when measured by tail cuff (Supplemental Table 5). We focused our studies on the heterozygous model, since it corresponds to the human autosomal dominant phenotype.

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dative stress–responsive kinase 1 (OSR1). WNK4 abundance was not significantly changed in the kidney of Wnk1+/delE631 mice (Figure 6). However, due to the lack of a suitable antibody, we could not measure the phosphorylation level of the T-loop serine, which reflects the activation state of the kinase (23).

To further characterize the activation of the WNK1-SPAK/OSR1 cascade in the kidney of Wnk1+/delE631 mice, we performed immunofluorescence experiments with an antibody recognizing both L- and KS-WNK1 isoforms (a KS-WNK1–specific antibody was not available). While the DCT of Wnk1+/+ mice contained small WNK1-positive puncta, large WNK1-positive structures, resembling the previously described WNK bodies, were observed in Wnk1+/delE631 NCC-positive DCTs (Figure 7). These bodies were also found in AQP2-positive, but not in NKCC2-positive, tubules. Previous studies demonstrated that KS-WNK1 is required for the formation of these WNK1 bodies, which are dynamic membraneless structures (24). Containing the components of the WNK/SPAK pathway, they are usually not present at baseline conditions, but form under conditions of low potassium intake when WNKs become activated (25). The fact that we observed them at baseline in Wnk1+/delE631 kidneys provides further support that the WNK1/SPAK cascade is activated in Wnk1+/delE631 mice.

Potassium secretory capacity is diminished without change of ENaC expression in Wnk1+/delE631 mice. Basal urinary K⁺ excretion and urinary/plasma ratio of K⁺ concentration were lower in Wnk1+/delE631 mice, indicating a defect in urinary potassium excretion (Figure 8, A and B). We calculated the transtubular potassium gradient (TTKG) to estimate the potassium secretory capacity of the aldosterone sensitive distal nephron (ASDN), connective tubule and collecting duct (26). Wnk1+/delE631 mice exhibited significantly lower TTKG (8.6 ± 0.3, n = 24) than Wnk1+/+ mice (10.1 ± 0.3, n = 24, P = 0.0003), despite higher aldosterone levels, consistent with a potassium secretory defect (Figure 8C). As observed in patients, HCTZ rapidly corrected the hyperkalemia in the Wnk1+/delE631 mice (see above) and partially abolished the TTKG difference between the Wnk1+/+ and Wnk1+/delE631 mice (Figure 8C), suggesting the defect develops as a consequence of increased Na⁺ reabsorption by NCC and reduced sodium delivery to ASDN. Metolazone, a thiazide diuretic suggested as having less carbonic anhydrase inhibitory effect, did not correct the phenotype when administered intraperitoneally at a dose of 50 μg/kg/d over 4 days (Supplemental Figure S5).

Decreased activity of the epithelial sodium (Na⁺) Channel ENaC and the renal outer medullary potassium (K⁺) channel ROMK are thought to contribute to the hyperkalemia in other forms of
Abundance of ENaC α and γ subunits in the membrane-enriched fraction of the renal cortex was assessed as a surrogate for channel function (28, 29). No significant increases in the abundance in full-length or cleaved forms of the ENaC subunits were observed in Wnk1+/delE631 mice compared with Wnk1+/+, except for the short fragment of α subunit (Figure 8D). We then acutely treated Wnk1+/delE631 and Wnk1+/+ mice with amiloride, the specific pharmacological inhibitor of ENaC. Six hours after the amiloride injection, the natriuresis increased very significantly and similarly in both groups that had similar Na and K intake (Figure 8E). Urinary K+ excretion also decreased similarly in Wnk1+/+ and in Wnk1+/delE631 (Figure 8F), the difference in postamiloride absolute values reflecting the difference in basal values. Together, these results make it seem unlikely that alterations in ENaC contribute to the potassium secretory defect.

Altered regulation of ROMK in Wnk1+/delE631 mice. We then analyzed ROMK expression in the kidney cortex and at the apical pole of the...
protein abundance of the Na-K-2Cl cotransporter (NKCC2) (Fig -
ure 9, C and D), which has been shown to be regulated in vivo by
KS-WNK1 (31), was unchanged. In addition, a significant increase
in NKCC2 phosphorylation was observed (Figure 9D) in accor-
dance with the observed increase in SPAK and OSR1, the main
kinases involved in the phosphorylation of NKCC2 (32), and oppo-
site of the expected inhibitory effect KS-WNK1. We did not detect
an increase in KS-WNK1 in NKCC2-positive tubules (Figure 7),
suggesting that other regulatory mechanisms are at play (33).

Discussion
This is the first report, to our knowledge, of missense mutations
in the WNK1 gene leading to an autosomal dominant tubulopa-
thy. The syndrome is striking in that it resembles more an isolat-
distal part of the DCT (DCT2) and CNT cells, where the channel is
most significantly upregulated in response to dietary potassium
loading (30). At the basal level, Western blots showed that ROMK
protein expression was not increased in Wnk1+/delE631 mice compared
with Wnk1+/+ littermates despite significant hyperkalemia (5.1 ± 0.5 vs. 4.3 ± 0.2 mmol/L, P < 0.0001; n = 20) (D); hyperchloremia (114 ± 2 vs. 110 ± 3 mmol/L, P < 0.0001; n = 20) (E); and metabolic acidosis (HCO3−, 22.8 ± 2.1 vs. 24.6 ± 2.5 mmol/L, P < 0.05; n = 20) (F) were observed together with normal creatinine
values (not shown). Data are represented as mean ± SEM. Statistical comparisons were made using unpaired Student’s t test. (G and H) Renin expression.
Levels of renin mRNA were measured by RT-qPCR in the kidney cortex of Wnk1+/+ (n = 7) and Wnk1+/delE631 (n = 7) mice in baseline conditions (G) or of Wnk1+/+
(n = 6) and Wnk1+/delE631 (n = 6) mice fed a low (0%) K+ diet (H). Results (mean ± SEM) are expressed in arbitrary units relative to the expression of ubc. The expression level in Wnk1+/+ mice under basal conditions was arbitrarily set to 1. *P < 0.05; **P < 0.01; ****P < 0.0001, unpaired Student’s t test.

We also studied the large-conductance Ca++-activated K+ (BK)
channel, the expression of which was unchanged. Likewise, the
protein abundance of the Na-K-2Cl cotransporter (NKCC2) (Fig-
ure 9, C and D), which has been shown to be regulated in vivo by
KS-WNK1 (31), was unchanged. In addition, a significant increase
in NKCC2 phosphorylation was observed (Figure 9D) in accor-
ance with the observed increase in SPAK and OSR1, the main
kinases involved in the phosphorylation of NKCC2 (32), and oppo-
site of the expected inhibitory effect KS-WNK1. We did not detect
an increase in KS-WNK1 in NKCC2-positive tubules (Figure 7),
suggesting that other regulatory mechanisms are at play (33).
sic FHHt. Our cases are very similar to the 3 published cases of Spitzer-Weinstein syndrome, characterized by normotension and early onset hyperkalemic tubular acidosis and sensitive to HCTZ (34–36). The mutations are all located in the acidic motif, which is highly conserved across members of the WNK family and pivotal for their recruitment by KLHL proteins for ubiquitination by the CUL3-based E3 ligase ubiquitin complex (5, 37). Our in vitro experiments suggest that WNK1 mutations result in a differential regulation of the 2 major WNK1 isoforms, with an increase in abundance of KS-WNK1 being preferentially affected compared with the kinase-active L-WNK1.

Since the discovery that mutations in KLHL3 and CUL3 cause FHHt (3, 38), several studies have demonstrated that the WNK kinases are substrates for the KLHL3-CUL3 E3 ligase complex (37). Ohta et al. mapped the WNK interaction site to a region containing a motif of 10 amino acids, EPEEPEADQH, called the acidic motif because of the predominance of negatively charged residues (5). Most of the FHHt-causing mutations in WNK4 cluster in this highly conserved motif (2). The analysis of the Kelch domain of KLHL3 crystal structure in complex with the WNK4 acidic motif revealed close polar contacts between several residues at this motif and other conserved residues at the surface of the Kelch domain β-propeller (14). In particular, the D635 residue of L-WNK1 (equivalent to D654 in WNK4) establishes ionic interactions with R528 of KLHL3, both residues being mutated in FHHt. Mutations in this degron motif abolish the interaction of WNK4 with the ubiquitin-ligase complex, thereby preventing their ubiquitination and proteasomal degradation. Accordingly, WNK4 protein abundance increases in the kidney of WNK4<sup>−/−D635A</sup> mice, which carried 1 of the FHHt mutations (7, 39).

We found that KS-WNK1 abundance is much more affected by the acidic motif mutations than L-WNK1. While the expression of KLHL3 only slightly reduced L-WNK1 abundance, the expression of KS-WNK1 was drastically decreased in both X. laevis oocytes and mammalian cells (HEK293T). We also found that KS-WNK1 is more ubiquitinated than L-WNK1 when KLHL3 is overexpressed. Each of the mutations in the acidic motif abolished the degradation of KS-WNK1, with the exception of KS-WNK1 D228E. Ohta et al. previously demonstrated that L-WNK1 interacts with KLHL3 and that this interaction is prevented by mutations in either KLHL3 or the acidic motif of L-WNK1 (5). However, the effect of KLHL3 overexpression on KS-WNK1 abundance and ubiquitination was not tested. Therefore, the potential difference in sensitivity of WNK1 isoforms to KLHL3-induced degradation was not analyzed. The acidic motif is present in both L- and KS-WNK1 isoforms. Thus, one might have expected that the missense mutations would have the same consequence on both isoforms. However, the 2 proteins have a different 3D structure, the conformation of which is probably regulated by different factors. Piala et al. showed that L-WNK1 is maintained in an inactive conformation by the binding of chloride to the catalytic site (39). KS-WNK1 is insensitive to this regulation because it lacks the major part of the kinase domain. Under physiological conditions, KS-WNK1 mRNA is markedly more abundant because it lacks the major part of the kinase domain. Under physiological conditions, KS-WNK1 mRNA is markedly more abundant because it lacks the major part of the kinase domain. Under physiological conditions, KS-WNK1 mRNA is markedly more abundant because it lacks the major part of the kinase domain. Under physiological conditions, KS-WNK1 mRNA is markedly more abundant because it lacks the major part of the kinase domain. Under physiological conditions, KS-WNK1 mRNA is markedly more abundant because it lacks the major part of the kinase domain. Under physiological conditions, KS-WNK1 mRNA is markedly more abundant because it lacks the major part of the kinase domain. Under physiological conditions, KS-WNK1 mRNA is markedly more abundant because it lacks the major part of the kinase domain. Under physiological conditions, KS-WNK1 mRNA is markedly more abundant because it lacks the major part of the kinase domain. Under physiological conditions, KS-WNK1 mRNA is markedly more abundant because it lacks the major part of the kinase domain. Under physiological conditions, KS-WNK1 mRNA is markedly more abundant because it lacks the major part of the kinase domain. Under physiological conditions, KS-WNK1 mRNA is markedly more abundant because it lacks the major part of the kinase domain. Under physiological conditions, KS-WNK1 mRNA is markedly more abundant because it lacks the major part of the kinase domain. Under physiological conditions, KS-WNK1 mRNA is markedly more abundant because it lacks the major part of the kinase domain. Under physiological conditions, KS-WNK1 mRNA is markedly more abundant because it lacks the major part of the kinase domain. Under physiological conditions, KS-WNK1 mRNA is markedly more abundant because it lacks the major part of the kinase domain. Under physiological conditions, KS-WNK1 mRNA is markedly more abundant because it lacks the major part of the kinase domain.
in the last 10 years, we now believe that increased NCC activity in KLHL3–/– mice, ref. 18; mia (Wnk4-PHAII transgenic mice ref. 19; in which NCC is stimulated display hypertension and hyperkalemia (22).) This new interpretation is supported by the fact that all the other mouse models of renin expression (our unpublished observations). This new interpretation of the phenotype resulting from KS-WNK1 inactivation (42) was guided by the early oocyte studies, which suggested that this WNK1 isoform exerted a dominant negative effect on L-WNK1 (46). However, we have since shown that an unfortunate mutation in the L- and KS-WNK1 cDNA used in these oocyte studies caused spurious loss of function (47). A newer study in oocytes, using the corrected cDNA, now indicates that KS-WNK1 is necessary for the physiological stimulation of ROMK currents in response to a dietary potassium load (49), a condition known to stimulate KS-WNK1 transcriptions (40). We speculate that KS-WNK1 may affect ROMK differently depending on the circumstance and where it is expressed. In Wnk1+/delE631 mice and patients, overexpressed KS-WNK1 in the DCT may indirectly inhibit ROMK in the CNT through distal nephron remodeling processes, as have been observed in a DCT-specific mouse model of FHHt (45). But when KS-WNK1 is physiologically activated in the CNT, its direct positive effects on ROMK would be completely opposite. Obviously, a test of these ideas will require further studies, perhaps with nephron-specific KS-WNK1 knockout and overexpression models.

The absence of hypertension in the patients and Wnk1+/delE631 mice is puzzling, given the increase in NCC phosphorylation. In that regard, it was interesting to compare the phenotypes of our FHHt patients depending on the WNK1 or WNK4 gene affected with similar genetic variants at the acidic motif of the kinase. Despite a relatively small number of affected subjects and difference in median age of the 2 groups (39.5 vs. 23.0 years), it was clear that WNK4 patients had a much higher BP than those with similar mutations at the WNK1 gene (median 163/102 mmHg vs. 111/75 mmHg, respectively). In contrast, these 2 groups had a similar biological profile, characterized by marked hyperkalemia, hyperchloremia, and metabolic acidosis. In addition, the Wnk1+/delE631 mice are normotensive, even on a high–sodium chloride diet. Only homozygous mice displayed a higher basal SBP (Supplemental Table 5). These in vivo observations highly suggest a differential effect on NaCl reabsorption between the 2 genes, independent of NCC stimulation. Interestingly, renin secretion was low in the patients with WNK1 mutations at the acidic motif and renin transcription was reduced in Wnk1+/delE631 mice, as expected from the negative feedback loop of increased intravascular volume. However, ENaC expression was unchanged and natriuresis sensitivity to acute amiloride was not affected in mutant mice, suggesting that the hypertensive phenotype in FHHt may require increased ENaC activity. Finally,
ian robust linear model with Mahalanobis distance classifier (BRLMM) and only analyzed SNPs with BRLMM = 0.15. We applied the 1 Mb to 1 centimorgan conversion before analysis as recommended (50). Checks for Mendelian errors and parametric linkage analyses were computed by MERLIN (51) under a rare dominant model with full penetrance (100%) and a disease allele frequency of 0.0005.

WES. IntegraGen performed library preparation, capture, sequencing, and variant detection and annotation. Exons of genomic DNA samples were captured using Agilent in-solution enrichment methodology with their biotinylated oligonucleotides probes library, followed by paired-end 75 bp massively parallel sequencing on Illumina HiSeq 2000 (see ref. 52 for detailed explanation of the process). The bioinformatics analysis of deep-sequencing data was based on the Illumina pipeline (CASAVA 1.8).

Direct Sanger sequencing
PCR amplification and Sanger sequencing from genomic DNA was performed using standard methods. The sequencing of the WNK1 ex7 and ex25 was performed using the primers listed in Supplemental Table 7A.

In vitro functional characterization
Mutant proteins in X. laevis oocytes. Vectors used for expression in Xenopus oocytes and HEK293 cells are described in the Supplemental Methods. cRNA for Xenopus oocyte injections were prepared by in vitro transcription of the corresponding human clones after plasmid linearization using the T7 RNA polymerase m MESSAGE kit (Ambion). We used X. laevis oocytes as an expression system. Oocyte preparation protocol has been described before (53). In brief, oocytes were obtained from female frogs under anesthesia and the follicular layer was removed. After 24 hours, oocytes were injected with 50 nL of H2O alone or containing 0.2 μg/μl of each clone cRNA. After 48 hours of incubation, protein extracts were obtained using lysis buffer containing 50 mM Tris-HCl (pH 7.5), 1 mM EGTA, 1 mM EDTA, 50 mM sodium fluoride, 5 mM sodium pyrophosphate, 1 mM sodium orthovanadate, 1% (wt/vol) Nonidet P-40, 0.27 M sucrose, 0.1% (vol/vol) 2-mercaptoethanol, and protease inhibitors (Complete Tablets; Roche). Protein extracts equivalent to 1 oocyte (60 μg) were resolved using SDS-PAGE and transferred to PVDF membranes for Western blot analysis. The antibodies used were commercial c-myc (catalog 11814150001, Roche/MilliporeSigma) and Flag antibody (M2, catalog A6592; MilliporeSigma) at 1:1000 and 1:5000 as well as commercial anti–β-actin (catalog sc47778; Santa-Cruz Biotechnology Inc.) at 1:2500. Immobilized antigens were detected by chemiluminescence using the Luminata Forte Western HRP substrate (Merck Millipore).

Methods

Human genetic studies

Linkage analysis. Linkage in Ped29 was analyzed using markers generated by the 250K Affymetrix array, as previously performed (38). We excluded nonpolymorphic and low-frequency (minor allele frequency [MAF] < 0.1) SNPs. To decrease the SNPs list to a manageable set of 49,187 SNPs for linkage analysis, we applied a filter using the Bayes-
Figure 8. Abnormal K+ handling in Wnk1+/delE631 mice. (A–C) Decreased UK+ excretion, Uk/Pk ratio, and TTKG. (A) Urinary K+ excretion was lower in Wnk1+/delE631 mice (n = 7, 52 ± 5.3 mmol/mmol creatinine) than in Wnk1+/+ mice (n = 7, 69.3 ± 7.9 mmol/mmol creatinine). *P < 0.05, unpaired Student’s t test. (B) Basal urinary/plasma ratio of K+ concentration was lower in Wnk1+/delE631 mice (n = 24, 37.2 ± 2.1) than in Wnk1+/+ mice (n = 24, 56.3 ± 3.8). ***P < 0.0001, unpaired Student’s t test. (C) TTKG was significantly lower in Wnk1+/delE631 mice (n = 24, 8 ± 0.3) than in Wnk1+/+ mice (n = 24, 10.1 ± 0.3). ***P = 0.0003. Following 4-day HCTZ oral (240 mg/kg/d) administration, the difference in TTKG between Wnk1+/+ and Wnk1+/delE631 remained the same (9.5 ± 0.9 versus 11.8 ± 0.8, respectively) although no more significant (P = 0.068), likely because of the smaller number of animals studied (n = 9 and n = 11, respectively). Statistical comparisons were made using unpaired t tests. (D) ENaC expression. Representative immunoblots with the indicated antibodies performed on the membrane-enriched fractions of the renal cortex of mice of each genotype. Densitometric analysis. The abundance of the cleaved form of the α-subunit of ENaC was significantly increased in Wnk1+/delE631 mice compared with Wnk1+/+. The expression level in Wnk1+/+ mice was arbitrarily set to 100. Data are represented as mean ± SEM.*P < 0.05, unpaired Student’s t test. (E and F) Natriuretic and kaliuretic response to amiloride. Wnk1+/+ and Wnk1+/delE631 males (n = 7 in each group) were housed in metabolic cages and received 1 injection of vehicle or amiloride on 2 consecutive days. Urine was collected 6 hours after injection. Data are represented as mean ± SEM. *P < 0.05; **P < 0.01; ****P < 0.0001 versus vehicle, unpaired Student’s t test.
Mutant proteins in HEK293T cells

Cell culture and transfection. Flp-InTM T-RExTM 293 cells (Invitrogen) were stably transfected with pCDNA5/FRT/(His)6-protein C-Flag-hKLHL3 WT or R528H vector following the manufacturer’s instructions. Conditions of culture and induction are described in Supplemental Methods. For expression of WNK1-myc constructs (pcDNA vectors described in Supplemental Methods) and ubiquitin-HA, cells were transiently transfected using Effectene (QIAGEN) following the manufacturer’s instructions.

Immunoprecipitation. Cells were harvested 48 hours after transfection, washed in cold PBS, and frozen in liquid nitrogen. Lysis/immunoprecipitation of cell pellets in denaturing or native conditions were performed according to classical procedures described in Supplemental Methods.

Figure 9. Expression of ROMK, BK channel, and NKCC2. (A) ROMK protein abundance. No change in cortical ROMK expression was observed in Wnk1+/delE631 (n = 4) vs. littermate Wnk1+/+ (n = 4) mice, despite the latter group having significantly higher plasma K+ levels (4.4 ± 0.08 mM, n = 20 vs. 5.1 ± 0.12 mM, n = 20). Conversely, a more than 2-fold increase was observed in Wnk1+/+ mice treated by amiloride (25 mg/kg/d for 4 days) achieving a similar rise in plasma potassium (Wnk1+/+ vehicle: 3.8 ± 0.06 mM, n = 4 vs. Wnk1+/+ amiloride: 5.0 ± 0.11 mM). Quantification of cortical ROMK expression (n = 4 per group). *P < 0.05, t test. (B) ROMK immunofluorescence in the distal tubule. Left panel: immunolocalization of ROMK in the DCT2 of Wnk1+/+ and Wnk1+/delE631 mice. Right panel: analysis of membrane labeling intensity showed no change in ROMK apical expression in the DCT2 and CNT of Wnk1+/delE631 mice (n = 4 per genotype). Scale bar: 10 microns. (C) Basal BK α channel protein abundance in Wnk1+/delE631 mice and Wnk1+/+ littermates. BK α immunoblots (left panel) and quantification (right panel) demonstrate that cortical BK α expression is unchanged between Wnk1+/+ (n = 4) and Wnk1+/delE631 mice (n = 5). (D) Basal NKCC2 and P-NKCC2 protein abundance in Wnk1+/delE631 mice and Wnk1+/+ littermates. (n = 7 per group). NKCC2 and pNKCC2 immunoblots (left panel) and quantification (right panel) demonstrate that NKCC expression is unchanged between Wnk1+/+ (n = 7) and Wnk1+/delE631 mice (n = 7), but pNKCC2 is significantly increased in Wnk1+/delE631 mice compared with Wnk1+/+. ****P < 0.0001, unpaired Student’s t test.
Immunoblotting. Lysates and immunoprecipitates were analyzed by SDS-PAGE (6% or 8% gels), transferred to nitrocellulose membrane, and immunoblotted with primary antibodies including anti-HA (Cell Signaling Technology), anti-myc (clone 9B11, Cell Signaling Technology), anti-protein C (clone hpc4, Roche), rabbit anti-GAPDH (catalog ab37168; Abcam), and goat anti-actin (catalog sc47778; Santa Cruz Biotechnology Inc.). Thereafter, the membranes were incubated with a horseradish peroxidase-conjugated secondary antibody (1:5000 dilution). Images were obtained with chemiluminescence (Pierce ECL Western Blotting Substrate) using a luminescent image analyzer (LAS-4000 mini, Fujifilm) and quantified with MultiGauge software.

Mouse experimental studies

Crispr/Cas9 engineered mice. In order to produce the c.1905T>A, P.D635E mutation at ex7 of the mouse Wnk1 gene using the CRISPR/Cas9 system, we chose three 20 mer sgRNAs (Supplemental Table 7C), using the CRISPR Design algorithm (http://zlab.bio/guide-design-resources). sgRNAs were produced with Cas9 SmartNuclease RNA System (SBI Ozyme), and quality was controlled by the Experion Automated Electrophoresis System (Bio-Rad).

One-cell zygotes (3 series of 100 oocytes) were coinjected with CAS9WT protein (New England Biolabs), 1 of the 3 sgRNAs, and a single-strand 160 mer nucleotide (ssODN) carrying the acidic motif of WNK1 and ROMK1 immunolocalization in the cortical nephron. WNK1 and ROMK1 immunofluorescence experiments were performed on 14 C57BL6J male mice divided in 2 subgroups (7 control animals and 7 Wnk1+/+ mice). Mice were implanted with BP and heart-rate measuring telemetric probes (DSI). The precise monitoring of the animals is described in Supplemental Methods.

Radiotelemetry monitoring of BP and heart rate. The study was performed on 14 C57BL6J male mice divided in 2 subgroups (7 control Wnk1+/+, 7 Wnk1−/− mice). Mice were implanted with BP and heart-rate measuring telemetric probes (DSI). The precise monitoring of the animals is described in Supplemental Methods.

Basal conditions. Animals were housed in metabolic cages and fed a standard diet (0.3% NaCl) with free access to tap water. After a 3-day adaptation period, urine was collected daily for electrolyte measurements for 2 days.

Diet. The standard diet contained 0.3% NaCl and 1% KCl; low diet contained 0.3% NaCl and 0% KCl; high-salt diet contained 3% NaCl and 1% KCl.

Amiloride. After a 3-day adaptation period, physiological saline was injected intraperitoneally at 10:00 am and urine was collected 6 hours after the injection. The same procedure was repeated the day after with an injection of amiloride (1.45 mg/kg body weight) dissolved in physiological saline. For the analysis of the variations in physiological ROMK expression response to blood potassium increase as well as for the measurement of TTKG, amiloride was administered intraperitoneally at the same dose over 4 days.

HCTZ. Animals were fed with a specific diet supplemented with HCTZ (240 mg/kg/d, oral) over 4 to 7 days such as done in previous studies (13, 19) for analysis of BP response and analysis of TTKG variations.

Metolazone. Metolazone (50 µg/kg/d) was administered intraperitoneally over 4 days.

Blood and urine measurements. Plasma electrolytes were measured using an i-STAT system (Abbott) and EC-8 cartridge test. Blood samples were collected under isoflurane anesthesia by retroorbital puncture. Creatinine was determined using an Olympus AU400 analyzer. Urinary aldosterone was measured by competitive chemiluminescent immunoassay (LIAISON Aldosterone Kit, Diasorin). TTKG was calculated as (K+urine/[K+]blood) × (osm/blood/urine), as has been used widely before in animal model studies to estimate the potassium secretory capacity of the distal nephron. Although urea recycling has been argued as making TTKG greater than the actual value in vivo (55), the error is small (~10%) because the urea reabsorption is offset by passive potassium reabsorption in the inner medullary collecting duct (56). This is in agreement with experimental measurements that have validated the TTKG calculation as a reasonable estimate of potassium secretory capacity in rodents (57). Although we cannot rule out that urea recycling or passive potassium reabsorption is affected by the WNK1 ex7 mutation, it seems unlikely. Nevertheless, we acknowledge this as a limitation.

RNA extraction and RT-qPCR for renin expression. The extraction of total RNA from mouse kidneys, reverse transcription and quantitative PCR (RT-qPCR) were performed as described in ref. 12. Primer sequences are indicated in Supplemental Table 7D. Ubiquitin c (ubc) was used a reference gene, and comparative quantification of the gene of interest between the 2 genotypes was obtained using the 2−ΔΔCT method.

Mouse kidney immunoblotting. At the end of the experimental period, animals were sacrificed with ketamine and xylazine (0.1 and 0.01 mg/g of body weight, respectively). Renal cortex or total renal samples were homogenized, extracted, and submitted to SDS-PAGE electrophoresis, and immunoblotting was performed as detailed in Supplemental Methods.

WNK1 and ROMK1 immunolocalization in the cortical nephron. WNK1 and ROMK1 immunofluorescence experiments were performed on kidneys embedded in paraffin. All technical details are given in Supplemental Methods.

Statistics

Human studies. Data were analyzed using Fisher’s exact and t tests. All tests were 2 sided. P values of less than 0.05 were considered significant.

Mouse studies. When analyzing 2 groups of mice, we used an unpaired Student’s t test. One-way ANOVA followed by Holm-Šidák’s multiple comparisons test was used to analyze more than 2 groups of 5 or more while a Kruskal-Wallis test followed by a Mann-Whitney test was used to analyze more than 2 groups of 5 or less. Data are given as mean ± SEM. Differences between groups were considered significant at P < 0.05.

Study approval

Affected individuals were recruited at the Department of Genetics of the Hôpital Européen Georges Pompidou as well as at other Departments of Nephrology located in France, Italy, and the United Kingdom. Genetic testing and research were performed according to the French ethical laws published in 2001 (articles L.1100-4 al 1 CSP and R 1131-14 CSP). Informed, written consent was obtained from all study participants. All mouse studies were conducted on 3- to 5-month-old male mice and performed in accordance with the European Communities Council Directive. The project was approved by the French Ministry of Research (no. 02650.02). Work with X. laevis was approved and performed following the guidelines set by the Institutional Animal Care and Use Committee of the University of Nice-Sophia Antipolis.
Care Committee of our institution. The Xenopus oocytes studies were approved by the IACUC from the Instituto Nacional de Ciencias Médicas y Nutrición Salvador Zubirán in Mexico City.

Author contributions
HLDP and NT conducted the human genetic studies, IK, WAK, CR, TM, and MCC performed the in vitro cellular experiments from mouse models. ERA, SV, OS, and GG performed the Xenopus oocytes studies. RC, RG, and PAW performed the ROMK1 experiments. ILF, MDC, SB, TM, CR, and JH conducted in vivo experiments from mouse studies. MH, SD, XG, KO, PM, GR, IT, RU, and RVP acquired and analyzed clinical human data. PAW, GG, EC, JH, and XJ designed the research studies, assembled the figures and tables, and wrote the manuscript. XJ coordinated the entire study.

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