REVIEW

Regulation of the renal NaCl cotransporter by the WNK/SPAK pathway: lessons learned from genetically altered animals

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1Molecular Physiology Unit, Instituto de Investigaciones Biomédicas, Universidad Nacional Autónoma de México, Mexico City, Mexico; 2PECEM, Facultad de Medicina, Universidad Nacional Autónoma de México, Mexico City, Mexico; 3Department of Nephrology and Mineral Metabolism, Instituto Nacional de Ciencias Médicas y Nutrición Salvador Zubirán, Mexico City, Mexico; and 4Tecnológico de Monterrey, Escuela de Medicina y Ciencias de la Salud, Monterrey, Mexico

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Ostrosky-Frid M, Castañeda-Bueno M, Gamba G. Regulation of the renal NaCl cotransporter by the WNK/SPAK pathway: lessons learned from genetically altered animals. Am J Physiol Renal Physiol 316: F146–F158, 2019. First published August 8, 2018; doi:10.1152/ajprenal.00288.2018.—The renal thiazide-sensitive NaCl cotransporter (NCC) is the major salt transport pathway in the distal convoluted tubule of the mammalian nephron. NCC activity is critical for modulation of arterial blood pressure and serum potassium levels. Reduced activity of NCC in genetic diseases results in arterial hypotension and hypokalemia, while increased activity results in genetic diseases featuring hypertension and hyperkalemia. Several hormones and physiological conditions modulate NCC activity through a final intracellular complex pathway involving kinases and ubiquitin ligases. A substantial amount of work has been conducted to understand this pathway in the last 15 yr, but advances over the last 3 yr have helped to begin to understand how these regulatory proteins interact with each other and modulate the activity of this important cotransporter. In this review, we present the current model of NCC regulation by the Cullin 3 protein/Kelch-like 3 protein/with no lysine kinase/STE20-serine-proline alanine-rich kinase (CUL3/KELCH3-WNK-SPAK) pathway. We present a review of all genetically altered mice that have been used to translate most of the proposals made from in vitro experiments into in vivo observations that have helped to elucidate the model at the physiological level. Many questions have been resolved, but some others will require further models to be constructed. In addition, unexpected observations in mice have raised new questions and identified regulatory pathways that were previously unknown.

INTRODUCTION

The distal convoluted tubule (DCT) is the region of the nephron that is located between the end of the cortical thick ascending limb, few cells after macula densa (50), and the connecting tubule (CNT) and is divided into two portions known as early and late, or DCT1 and DCT2, respectively. Salt reabsorption in DCT1 is due exclusively to the thiazide-sensitive NaCl cotransporter (NCC). In DCT2, in addition to NCC, the epithelial Na⁺ channel (ENaC) also reabsorbs sodium at the apical membrane, which is where the expression of ROMK begins in the distal nephron.

The importance of NCC for the regulation of arterial blood pressure, potassium secretion, and acid-base metabolism has been clearly demonstrated by the presence of clinical syndromes in which the activity of NCC is affected. In addition, thiazide-type diuretics, specific inhibitors of NCC, have been widely used as effective antihypertensive agents for more than 50 yr.

CLINICAL SYNDROMES DUE TO ALTERED ACTIVITY OF NCC

Gitelman’s syndrome (GS; OMIM 263800) is an autosomal recessive illness that features hypokalemic metabolic alkalosis and hypocaliuria, accompanied by a salt-remediable decrease in blood pressure levels (18, 23). More than 100 different point mutations in the SLC12A3 gene that encodes for NCC have been described in families with GS (31). Interestingly, family members with one affected SLC12A3 allele exhibit lower blood pressure than those with no mutations (18), and healthy subjects from the Framingham Study with heterozygote mutations within the coding region of the SLC12A3 gene that reduce the activity of NCC are asymptomatic but protected against hypertension and cardiovascular mortality (1, 30).
The SeSAME or EAST syndrome (OMIM 614491), also known as pseudohypaldosteronism type II (PHAII) or Gordon syndrome, is a disease that is the mirror image of GS, featuring arterial hypertension, with hyperkalemia metabolic acidosis and hypercalciuria (36, 37). The disease can be dominant, recessive, or sporadic, depending on the mutated gene. All clinical features respond to low doses of a thiazide-type diuretic and current evidence strongly suggests that an increase in NCC function is the first order event that drives FHHT (16, 26, 32, 64, 77).

Mutations in four genes have been shown to be associated with FHHT in different kindreds: two genes encoding the with no lysine kinases, WNK1 and WNK4 (74), and two genes encoding for proteins that form a RING-type ubiquitin ligase complex known as Kelch-like 3 protein (KLHL3) and Cullin 3 protein (CUL3) (10, 35). As shown below, the WNK1 and WNK4 kinases modulate the activity of NCC indirectly by activating intermediate kinases known as STE20-serine-proline alanine-rich kinase (SPAK) and oxidative stress response kinase 1 kinase (OSR1), which in turn phosphorylate NCC (42, 72). WNKs are substrates of the KLHL3-CUL3 complex for ubiquitylation and thus degradation (45, 62, 73). Consistent with KLHL3-CUL3 being upstream of WNK1 and WNK4, mutations in KLHL3 or CUL3 produce a more severe phenotype than mutations in WNK1 or WNK4 (10).

After the discovery of WNKs as a cause of hypertension in humans (74), a tremendous amount of work has been conducted in many laboratories to understand the mechanisms by which this occurs. Here we present the authors’ view about how this occurs. What we know, what we do not know, and what is still confusing in the field.

**NCC REGULATION BY THE WNK/SPAK PATHWAY**

It has been demonstrated that both WNK1 and WNK4 act as positive regulators of NCC activity, and they do so by phosphorylating and promoting activation of SPAK/OSR1 that phosphorylate NCC in residues that are key to its activation and located in the cytoplasmic NH2-terminal domain (6, 11, 15, 46, 52). A chloride-binding site exists within the conserved kinase domain of WNK kinases, which lies close to the kinases’ active site. Chloride binding to this site stabilizes an inactive conformation and inhibits kinase activity (49). Thus the activity of WNK kinases relies on each WNK’s affinity for chloride and the intracellular chloride concentration ([Cl\(^-\)]i) of each particular cell model. In this regard, it has been suggested that the chloride affinity of WNK4 is higher than that of WNK1 and WNK3 (66), but the affinity was assessed only in in vitro kinase assays performed with the kinase domains of each kinase, and thus, no definitive in vivo data exist. However, when expressed in a cellular model with high [Cl\(^-\)]i, like Xenopus laevis oocytes, WNK4 displays the highest Cl\(^-\) sensitivity since in this system WNK4 mainly remains inactive and exerts an inhibitory effect on NCC, while WNK1 and WNK3 are able to activate NCC (WNK1 < WNK3) (6, 15). In contrast, when [Cl\(^-\)]i is reduced, all three WNKs promote similar levels of NCC activation (6, 7), thus suggesting that affinity for chloride could be WNK4 > WNK1 > WNK3.

It is well known that NCC activity is regulated in response to changes in dietary K\(^+\) intake, which leads to mild changes (within the physiological range) in the extracellular K\(^+\) concentration ([K\(^+\)]e) (12, 66, 68). NCC activation in the face of low dietary K\(^+\) intake plays an important role in the achievement of low urinary K\(^+\) excretion levels. DCT cells appear to be extremely sensitive to changes in [K\(^+\)]e due to the presence of K\(^+\) channels conformed of Kir4.1 and Kir5.1 subunits in the basolateral membrane (19). Studies using transfected cells in culture have shown that when [K\(^+\)]e decreases, intracellular K\(^+\) exits through Kir4.1/Kir5.1 channels promoting membrane hyperpolarization, which in turn stimulates Cl\(^-\) exit through Cl\(^-\) channels (67). The resulting decrease in [Cl\(^-\)]i leads to WNK4–SPAK–NCC activation. The opposite phenomenon is observed when [K\(^+\)]e increases (Fig. 1). It is worth to mention that no Cl\(^-\) measurements have been done in the DCT cells, and one study suggests that high potassium-mediated dephosphorylation of NCC uses probably both Cl\(^-\)–dependent and -independent pathways (48). In addition, it is still not clear if the effect of NCC activity modulates K\(^+\) secretion beyond the DCT because of a change in Na\(^+\) concentration or in the

![Fig. 1. Current model for NaCl cotransporter (NCC) regulation via the Kelch-like 3 protein/with no lysine kinase 3/STE20-serine-proline alanine-rich kinase (KLHL3-3WNK-SPAK) pathway. CaSR, calcium sensing receptor; AT1, angiotensin receptor type 1; Kir, inward rectifier potassium channel; CLCKB, chloride channel Kb; ANG II, angiotensin II; PKC, protein kinase C; Ub, ubiquitin; RRXS, PKA/PKC phosphorylation motif; P, phosphorylation; KS, kidney specific. *Data suggest that WNK1 may not be present in distal convoluted tubule (DCT) in physiological conditions, but in the familial hyperkalemic hypertension (FHHT) due to intronic deletions of WNK1 gene, it is expressed in DCT and activates SPAK-NCC, independently of WNK4. **Recent data suggest that KS-WNK1 can interact with WNK4, reducing its sensitivity to Cl\(^-\), and that KS-WNK1 is part of WNK bodies that are associated with activation of SPAK and that WNK bodies perhaps contain also WNK4 or L-WNK1.](https://www.ajprenal.org/content/187/2/R300/fig1)
volume of the fluid delivered to the CNT or collecting duct. Early studies using in vivo microperfusion suggested that luminal Na⁺ concentration play no role in modulating K⁺ secretion (24, 25). However, these studies were performed using acute tubule perfusion. It has been proposed that in the long term Na⁺ delivery to the aldosterone sensitive distal nephron could be a factor promoting remodeling of the K⁺ secretory machinery (26).

In the DCT, WNK4 activity also responds to changes in circulating angiotensin II (ANG II) in the face of extracellular fluid volume alterations (13, 14). This effect of ANG II is aldosterone independent (69). The ANG II-induced AT1 receptor activation in DCT cells leads to increased PKC-mediated phosphorylation of WNK4 at several sites, at least two of which are important for kinase activation (S64 and S1196) (11). Through a similar mechanism, activation of the calcium-sensing receptor also increases the activity of NCC via WNK4-SPAK pathway (8) (Fig. 1).

Another layer of regulation of NCC activity involves modulation of NCC or WNK expression levels by ubiquitin ligases. In vitro experiments suggest that NCC is a substrate of the HECT-type ubiquitin ligase Nedd4-2, which also regulates ENaC (5), by ubiquitinating specific sites on the cotransporter (56). A conditional knockout model to eliminate the Nedd4-2 expression in the nephron was associated with increased expression and phosphorylation of NCC (55). However, the inhibition of Nedd4-2 that is observed in clinical conditions such as primary hyperaldosteronism features hypertension accompanied by hypokalemia, not by hyperkalemia, indicating that the effect of Nedd4-2 on ENaC predominates. The elucidation of the role of Nedd4-2 in the regulation of NCC will require the generation of additional in vivo models.

The WNK kinases are substrates of the CUL3-KLHL3-RING E3 ubiquitin ligase complex (10, 35). This complex binds to WNK kinases through the β-propeller structure of KLHL3, which serves as the substrate adaptor of the complex and establishes an interaction with the acidic motif of WNK kinases (60). Indeed, all but one FHHt causative mutation in WNK4 occurs within the acidic motif and decreases the interaction with the ubiquitin ligase complex leading to WNK4 overexpression (45, 62, 73, 74). Since a mutation in only one WNK4 allele is necessary to observe overexpression, these mutations present an autosomal dominant pattern of inheritance (74).

FHHt mutations in the KLHL3 and CUL3 genes also affect the activity of the ubiquitin ligase complex and provoke a decrease in WNK degradation. Binding experiments have shown that dominant KLHL3 mutations affect either binding to CUL3 (41, 45) (mutations located in the BTB or BACK domains of KLHL3) or binding to WNK kinases (mutations located in the Kelch repeats that form the β-propeller structure) (41, 45). Only one recessive mutation has been functionally characterized (S410L), and it was shown to result in decreased intracellular stability compared with that in WT KLHL3 (41). Thus a low KLHL3 abundance may be responsible for FHHt caused by recessive mutations in KLHL3.

To date, all FHHt mutations occurring in CUL3 result in skipping of exon 9, and thus, they generate a mutant protein lacking residues 403–459 (CUL3-D9). Structural studies suggest that this mutant displays altered flexibility that may confer distinct functional properties (60). Indeed, in vitro studies have shown that while retaining critical interactions with KLHL3 and the RBX1 E3 ligase and forming an active Cullin-RING ligase complex, CUL3-D9 displays increased auto-ubiquitination that may lead to degradation but also increased neddylation (which is associated with complex activation) that may be related to its increased ability to promote ubiquitination and degradation of KLHL3, thus compromising the complex’s ability to ubiquitylate WNKs (40, 59). However, as discussed below, in vivo mouse studies have not been able to confirm the precise molecular mechanism by which the CUL3-D9 mutations lead to FHHt.

At the physiological level, a mechanism has been described that regulates CUL3-KLHL3-RBX1-mediated degradation of WNK kinases. A phosphorylation site has been described within the substrate binding domain of KLHL3 (S433) that is a target for phosphorylation by PKC and PKA and whose phosphorylation level increases in the context of DCT stimulation by ANG II or in the face of low dietary K⁺ intake. This phosphorylation prevents WNK interaction with the ubiquitin ligase complex and leads to WNK upregulation (29, 61).

**ANIMAL MODELS EXHIBITING A GITELMAN-LIKE SYNDROME**

Even though GS in humans is due to inactivating mutations of NCC, elimination of WNK or SPAK expression or function in mice produces a similar phenotype, indicating the importance of the WNK/SPAK pathway for the baseline activity of NCC.

The first GS model produced in mice was a total NCC knockout generated by Schultheis et al. (58). Although the mice exhibited a complete absence of NCC transcripts in the kidney, the phenotype was very mild (Table 1). Interestingly, two knockin models in which SLC12A3 mutations causing GS were introduced completely recapitulated the fully GS syndrome (Table 1). One model harbored a serine 707X substitution that resulted in premature truncation of NCC (76), and the other had a T58M substitution (75) that eliminated the master phosphorylation site of NCC (46).

Complete elimination of WNK4 expression in mice resulted in a GS-like phenotype (13) but with normal blood pressure at the expense of activation of the renin angiotensin system (Table 1). The expression and phosphorylation of NCC were remarkably reduced in this model, indicating the major role of WNK4 as a positive modulator of NCC expression and indicating that the total absence of WNK4 cannot be compensated by other WNKs (15, 53, 54). One important exception is when L-WNK1 has an intronic deletion producing FHHt, in which the absence of WNK4 has no effect on reverting the FHHt phenotype (15). One possible interpretation of these data is that perhaps under physiological conditions the presence of L-WNK1 in DCT is probably very low to none and thus L-WNK1 has an intronic deletion producing FHHt, in which the absence of WNK4 in the WNK4-KO mice, while in the presence of intronic deletions, the expression of L-WNK1 in the DCT is upregulated, producing FHHt, regardless of the presence of WNK4.

Hypomorphic WNK4 mice lacking the fragment encoded by exons 7 and 8 expressed a shorter WNK4 variant, with reduced phosphorylation activity, but the resultant GS-like phenotype was absent, with normal expression of NCC (Table 1) (44). Thus the total absence of WNK4 is associated with profound
Table 1. Genetic mouse models that present a Gitelman-like phenotype

| Affected Gene (Protein) | Model | Genetic Modification | Hypotension | Hypokalemia | Hypochloremia | Metabolic Alkalosis | Hypocalciuria | Molecular and Morphological Observations | Phenotype Corrected by | Ref. No. |
|-------------------------|-------|----------------------|-------------|-------------|--------------|---------------------|--------------|-----------------------------------------|-----------------------|---------|
| Slc12a3 (NCC)           | NCC−/−| NCC knockout mice    | Only with low-salt diet | X           | X           | X                   | ✓            | ↓ DCT cells; ↑ Developed mitochondria | Ref. No. 58           |         |
|                         |       |                      |             |             |             |                     |              |                                         |                       |         |
|                         |       |                      |             |             |             |                     |              |                                         |                       |         |
|                         |       |                      |             |             |             |                     |              |                                         |                       |         |
|                         |       |                      |             |             |             |                     |              |                                         |                       |         |
|                         |       |                      |             |             |             |                     |              |                                         |                       |         |

NCC, NaCl cotransporter; NKCC2, Na-K-Cl cotransporter; DCT, distal convoluted tubule; ENaC, epithelial Na⁺ channel; FHHt, familial hyperkalemic hypertension; WNK, with no lysine kinase; SPAK, STE20-serine-proline alanine-rich kinase; OSR1, oxidative stress response 1 kinase; ND, not determined.
downregulation of NCC and a GS-like phenotype, while the presence of a truncated version of WNK4 did not result in NCC downregulation or a GS-like phenotype.

The requirement of SPAK for normal NCC and DCT function has been clearly established by two models eliminating SPAK activity: the total SPAK knockout model (38) and the SPAK knockin model in which the phosphorylation site threonine 243, targeted by WNKs (78), was substituted by alanine, thus preventing SPAK activation by WNKs (51). In both models, NCC expression and phosphorylation were decreased. A different situation seems to occur with the Na-K-2Cl cotransporter (NKCC2). Interestingly, the activity of NKCC2 was increased in the SPAK knockout model, decreased in the SPAK knockin model (Table 1), and decreased in the kidney-specific OSR1 knockout model (33). Thus under physiological conditions, NKCC2 is under the control of OSR1 (33), while NCC is under the control of SPAK (27, 51). However, inactive variants of SPAK play a dominant negative role inhibiting OSR1 (47). Thus, a complete absence of SPAK in the knockout mice allows OSR1 to upregulate NKCC2, while in the SPAK knockin, inactive SPAK exerts a dominant negative effect on OSR1-NKCC2, reducing its activity (22).

As expected, elimination of Kir4.1 in mice results in a GS-like phenotype with downregulation of SPAK and NCC activity (79) because potassium cannot leave the intracellular fluid, decreasing the chloride driving force, thus retaining chloride inside the cell, which shuts down the system by keeping WNK4 inactive (19).

ANIMAL MODELS OF FHH

**WNK4 Models**

Three different models have been generated that exhibit FHHI due to a genetic modification of WNK4 (Table 2). 1) Transgenic mice harboring two extra copies of the WNK4 gene with the FHHI mutation Q562E (TgWnk4PHAII) (32) displayed the full FHHI phenotype, with notably high serum K+ levels when mice were placed on a high K+ diet (8.35 ± 0.36 mM). Hyperkalemia and all phenotypic alterations were completely corrected by thiazides or genetic deletion of NCC. Hypertrophy of the DCT was reported to occur as a consequence of NCC overactivation. 2) WNK4 knockin mice carrying the D561A mutation in a heterozygous fashion (WNK4<sup>D561A/+</sup>) (77) presented the full FHHI phenotype and high phosphorylation levels of SPAK, OSR1, and NCC. The phenotypes of these mice were completely corrected by thiazides (77) or in a Spak<sup>T243A/T243A</sup>Osrt1<sup>T185A/+</sup> background (16). It was later shown that in these two models WNK4 is overexpressed due to decreased degradation by the CUL3-KLHL3-RING E3 ubiquitin ligase complex (62, 73). 3) Transgenic mice that overexpressed WNK4 due to the introduction of extra copies of the WNK4 gene also exhibited the FHHI phenotype (73).

**WNK1 Models**

Mice harboring a heterozygous deletion in the first intron of WNK1, an identical modification to the one that causes FHHI in humans, develop a full FHHI phenotype with increased NCC expression and phosphorylation (Table 2) (71). All phenotypic alterations are corrected by thiazide treatment. Most importantly, the generation of this model was useful to characterize the consequences of the intrinsic deletion on WNK1 expression. A higher L-WNK1 expression level was observed in the DCT, and a slightly higher L-WNK1 expression level was also observed in the CNT, with no changes in KS-WNK1 levels throughout the different nephron segments. Thus it was concluded that the increase in L-WNK1 expression in the DCT and CNT was responsible for the development of the disease. Interestingly, when these mice were crossed with WNK4-KO mice (described above), the FHHt phenotype was preserved, indicating that in the context of L-WNK1 DCT/CNT overexpression, elimination of WNK4 expression has no effect on the high activity level of the WNK1-SPAK/OSR1-NCC pathway caused by L-WNK1 overexpression (15).

**KLHL3 Models**

Two models of FHHI carrying mutations in KLHL3 have been generated, one that harbors an autosomal dominant mutation (KLHL3<sup>R528H/+</sup>) (63) and a KLHL3 knockout mouse strain (KLHL3<sup>−/−</sup>) in which the disease presents only when both KLHL3 alleles are knocked out (57). Both strains (KLHL3<sup>R528H/+</sup> and KLHL3<sup>−/−</sup>) exhibit an FHHI phenotype, but increased blood pressure was only observed when mice were exposed to a high salt diet. High protein expression levels of WNK1 and WNK4 were observed, with no changes in WNK1 or WNK4 mRNA levels. High NCC and SPAK protein expression and phosphorylation were reported. The KLHL3<sup>−/−</sup> model resembles the scenario observed with certain KLHL3 mutations that present a recessive pattern of inheritance, confirming that KLHL3 haploinsufficiency is not enough to produce the disease and that dominant mutations must have a dominant negative effect. Interestingly, elimination of WNK4 in the KLHL3<sup>R528H/+</sup> mice (WNK4<sup>−/−</sup>/KLHL3<sup>R528H/+</sup> or WNK4<sup>−/−</sup>/KLHL3<sup>R528H/R528H</sup> mice) resulted in downregulation of NCC and SPAK, to levels similar to those observed in the WNK4<sup>−/−</sup> mice (13), despite the fact that these mice showed increased renal WNK1 protein levels (64). This result clearly contrasts with what is observed in WNK4<sup>−/−</sup>Wnk1<sup>+/FHHt</sup> mice in which the FHHt phenotype prevails (with high NCC and pNCC) (15), suggesting the KLHL3-R528H mutation has a different effect on the expression of WNK1 than the deletion of a segment of intron 1 of the WNK1 gene.

**CUL3 Models**

Some attempts have been made to generate FHHI models harboring CUL3 mutations. First, McCormick et al. (40), after observing that in vitro the CUL3-Δ9 mutant had an impaired ability to promote WNK degradation, hypothesized that CUL3 loss in the mouse kidney should phenocopy FHHI. Thus nephron-specific, doxycycline-inducible CUL3 knockout mice were studied. Higher WNK1, WNK3, WNK4, and pNCC levels were observed in agreement with the role of CUL3 in the regulation of WNK degradation. However, other unexpected effects were observed, namely, lower NKCC2 phosphorylation (only medullar in the chronic setting) and lower aquaporin 2 (AQP2) expression. As a consequence, the electrolytic abnormalities characteristic of FHHI were not observed. Signs of kidney damage developed after chronic deletion, which included smaller kidneys, increased plasma creatinine, inflammation, and fibrosis.
| Affected Gene (Protein) | Model | Genetic Modification | Hypertension | Hyperkalemia | Hyperchloremia | Metabolic Acidosis | Hypercalcemia | Molecular and Morphological Observations | Phenotype Corrected by | Ref. No. |
|-------------------------|-------|----------------------|--------------|--------------|---------------|-----------------|---------------|----------------------------------------|------------------|---------|
| Wnk4 (WNK4)             | TgWnk4P1A11 | Mice carrying 2 copies of endogenous Wnk4 and 2 transgene copies of WNK4-Q562E | ✓            | ✓            | ✓            | ✓              | ✓             | DCT hypertrophy and hyperplasia, NCC, parvalbumin, and calbindin-1 mRNA WNK4 protein | Thiazides NCC-KO | (32, 62) |
| WNK4D561A/+             | Knockin of WNK4-D561A mutation. Characterization of heterozygotes. | ✓            | ✓            | ✓            | ✓             | ✓              | ND            | NCC protein expression and membrane abundance. pNCC, pSPAK/pOSR1 WNK4 protein | Thiazides Spak1243A/1243A Ost1185V/+ NOT corrected in WNK1+/− context. | (16, 17, 77) |
| TgWNK4WT                | Mice carrying 2 transgene copies (additional to the endogenous copies) or 30 transgenic copies of wild type Wnk4 | ✓            | ✓            | ✓            | ✓             | ✓              | ND            | NCC protein expression. pNCC, pSPAK/pOSR1 | | (73) |
| Wnk1 (WNK1)             | WNK1+/−FHHt | Heterozygous deletion in the endogenous first intron of WNK1 | ✓            | ✓            | ✓            | ✓             | ✓             | NCC protein expression. pNCC | Thiazides NOT corrected by WNK4 deletion (FHH phenotype prevails) | (15, 71) |
| Klhl3 (KLHL3)           | KLHL3R528H/+ | Knockin of KLHL3-R528H mutation. Characterization of heterozygotes. | ✓ (under high salt diet) | ✓ | ✓ | ✓ | ✓ | WNK1, WNK4, NCC, and SPAK protein pNCC, pSPAK/pOSR1 When crossed with WNK4+/−, levels of NCC, pNCC, pSPAK/pOSR1 are comparable to those of WNK-/- | | (63, 64) |
| KLHL3−/−                | KLHL3 knockout mice | ✓ (under high salt diet) | ✓ | ✓ | ✓ | ✓ | | WNK1, WNK4 protein (only in kidney) NCC and SPAK protein pNCC, pSPAK/pOSR1 No phenotypic or molecular changes observed in KLHL3+/− | | (57) |

Continued
Table 2.—Continued

| Affected Gene (Protein) | Model                                                                 | Genetic Modification                                                                 | Hypertension | Hyperkalemia | Hyperchloremia | Metabolic Acidosis | Hypercalciuria | Molecular and Morphological Observations                                                                 | Phenotype Corrected by | Ref. No. |
|------------------------|----------------------------------------------------------|--------------------------------------------------------------------------------------|--------------|--------------|-----------------|-------------------|---------------|----------------------------------------------------------------------------------------------------------|------------------------|----------|
| Cul3 (Cullin3)         | KS-CUL3<sup>−/−</sup> (nephron specific, inducible)      | Nephron-specific, doxycycline-inducible, CUL3 knockout mice (Cul3<sup>fl/fl</sup> | X            | X            | X               | X                 |               | ▶ WNK1, WNK3, and WNK4 protein +KLHL3 protein +pNCC Signs of kidney damage after chronic deletion (>6 wk): smaller kidneys ▶ Plasma creatinine, inflammation, and fibrosis. ▶ pNKCC2 and AQP2 (causes hypochloremic alkalosis, diabetes insipidus, and salt sensitive hypotension) |                        | (40)     |
|                        |                                                                 | Pax8-rTA/TRE-LC1)                                                                   |              |              |                 |                   |               |                                                                                                          |                        |          |
| CUL3<sup>−/−</sup> and |                                       | Mice harboring mutations in the 3′-end of CUL3 intron 8 (that in humans result in  | X            | X            | X               | X                 | X             | No skipping of exon 9 resulted from these mutations in mice Slight ↓ in CUL3 expression with no phenotypic or molecular consequences |                        | (3)      |
| Cul3<sup>(−6)G/−6G</sup> |                                       | skipping of exon 9 and thus a mutant CUL3<sup>−/−</sup> protein)                   |              |              |                 |                   |               |                                                                                                          |                        |          |
| CUL3<sup>−/−</sup>     |                                       | Mice in which exon 9 was deleted by Flp-mediated recombination. Heterozygotes        | ✓            | ✓            | ✓               | ✓                 | ND            | ▶ WNK4 protein +KLHL3 protein +pNCC, pSPAK/pOSR1 ▶ NCC protein Puncta become evident in the DCTs in SPAK, pSPAK, and WNK4 immunofluorescence |                        | (60)     |
|                        |                                       |                                                                                        |              |              |                 |                   |               |                                                                                                          |                        |          |

Continued
Table 2.—Continued

| Affected Gene (Protein) | Model | Genetic Modification | Hypertension | Hyperkalemia | Hyperchloremia | Molecular and Morphological Observations | Phenotype Corrected by | Ref. No. |
|-------------------------|-------|----------------------|--------------|--------------|---------------|----------------------------------------|------------------------|----------|
| CUL3-HET/D9             |       |                      | ✓            | ✓            | ND            | ND                                     | Thiazides              | (21)     |
|                         |       | Mice with nephron- specific, doxycycline inducible-deletion of 1 CUL3 allele, which also carries a transgene that directs inducible expression of CUL3-D9 in the nephron. |
|                         |       |                      |              |              |               | ND                                     | Thiazides              | (21)     |
| S6.39 (SPAK)            | SPAK-CA (DCT-specific SPAK T243E, S383D/T243E, S383D) | The ORF coding for a constitutively active version of SPAK was introduced within exon 1 of the SPAK gene, after a Lox-P flanked selection cassette. When crossed with mice expressing Cre in the DCT (under parvalbumin promoter control), the construction results in mice lacking SPAK expression in all renal cell types but expressing constitutively active SPAK in DCT. | ✓            | ✓            | ✓            | ND                                     | Thiazides (even under high-K⁺ diet). A delay in plasma K⁺ correction is observed compared with blood pressure. | (26)     |

NCC, NaCl cotransporter; FHHt, familial hyperkalemic hypertension; CUL3, CulIn 3 protein; KELCH3, Kelch-like 3 protein; WNK, with no lysine kinase; SPAK, STE20-serine-proline alanine-rich kinase; OSR1, oxidative stress response 1 kinase; ORF, open reading frame; DCT, distal convoluted tubule; CNT, connecting tubule; KO, knockout; KS, kidney specific; AQP, aquaporin; ND, not determined.
Schumacher et al. (59) generated a mutant mouse strain harboring deletion of exon 9. These mice (CUL3<sup>∆9/+</sup> mice) displayed a full FHHt phenotype with the classic electrolytic alterations and high blood pressure levels, with increased protein levels of WNK4 and NCC, and markedly higher NCC and SPAK/OSR1 phosphorylation levels. Immunofluorescent staining of kidney sections revealed the formation of WNK4, SPAK, and pSPAK-positive puncta specifically in the DCT. Notably, no DCT hypertrophy was reported, contrasting with the observations made by Laloti et al. (32) in the TgWnk4<sup>PHAI</sup>H FHHt model. Abundance of WT-CUL3 decreased by 50%, as expected, while CUL3<sup>∆9</sup> was almost undetectable. No changes in KLHL3 expression were observed. It was proposed that the mechanism leading to FHHt may not involve a decrease in KLHL3 abundance, as suggested by in vitro observations (40, 60), but instead may be due to reduced levels of CUL3 expression, i.e., haploinsufficiency.

More recently, however, another work has questioned the haploinsufficiency hypothesis (21). It was shown that mice harboring a deletion of one CUL3 allele in renal epithelial cells (CUL3-Het) did not develop FHHt. In contrast, when the CUL3-Het mice also expressed CUL3<sup>∆9</sup> from a Cre-induced transgene, they developed FHHt. These observations favor the idea that CUL3<sup>∆9</sup> exerts a dominant negative effect on the activity of the CUL3-KLHL3-RING complex toward WNKs.

Finally, it has recently been suggested that the increased severity of the hypertensive phenotype in FHHt patients carrying CUL3 mutations, in comparison with those carrying other FHHt mutations, may be due to altered CUL3 activity in vascular smooth muscle cells, which leads to arterial stiffness contributing to hypertension (2). This would add up to the hypertensive effect of salt retention caused by NCC activation. Agbor et al. (2) showed that selective CUL3<sup>∆9</sup> expression in smooth muscle cells impairs the ability of CUL3 to promote RhoA degradation, which in turn leads to vascular dysfunction. This scenario, however, does not explain the increased severity of other phenotypic features, such as hyperkalemia, observed in CUL3-FHHt patients (10).

### Other Models Exhibiting FHHt

An interesting FHHt model was produced by expressing a constitutively active version of SPAK, specifically in DCT1 (SPAK-CA mice) (26). These mice lack SPAK expression in all other cell types, and thus the full FHHt phenotype is strictly the consequence of SPAK actions in the early portion of the DCT. Mice display higher NCC protein expression and phosphorylation, DCT1 hypertrophy, and CNT hypotrophy, and these are all reversed by thiazide treatment. However, in contrast to what occurs with hypertension, the correction of hyperkalemia and normalization of urinary K<sup>+</sup> excretion is gradual, suggesting that distal nephron remodeling plays an important part in the development of this trait.

### ANIMAL MODELS WITH UNEXPECTED OR UNCLEAR PHENOTYPES

Genetically modified mice often develop unexpected compensations or phenotypes that are difficult to reconcile with the current knowledge. Here, we discuss models in which the phenotype is still unclear or has been misinterpreted or models that need further information to understand them (Table 3).

| Table 3. Genetic mouse models with mutations in the WNK-SPAK/OSR1-NCC pathway that present unexpected phenotypes |
|---------------------------------------------------------------|
| **Affected Gene** | **Protein Model** | **Molecular and Morphological Observations** | **Corrected by** |
| Slc12a3 (NCC) | TgNCC WT | Mice carrying 5 extra copies of the NCC gene | Serum Chloride Low | Ref. No. (32) |
| Wnk4 | TgWnk4 WT | Mice carrying 2 transgene copies (additional to the endogenous copies) of wild-type Wnk4 | Urinary Calcium Low | |
| Wnk4 | KS-WNK1 | Insertion of loxP sites flanking exon 4a (expression of L-WNK1 remains intact) | Elevated DBP and mean serum K | | (34) |
| Wnk4 | KS-WNK1<sup>1–253</sup> | Replacement of exon 4a with a DND cassette containing the selectable marker gene neomycin transferase | Low | | (34) |

NCC, NaCl cotransporter; FHHt, familial hyperkalemic hypertension; DBP, diastolic blood pressure; KS, kidney specific; ND, not determined; Ni, Normal.
NCC Models

McCormick et al. (39) produced transgenic mice that overexpressed NCC but had no signs of FHHt. Some authors have used this model as an argument against the role of NCC in blood pressure regulation. However, in this model, although NCC expression was increased, the phosphorylated NCC and the amount of NCC at the apical membrane were similar to wild-type mice. Because the NCC activity depends on its membrane expression and phosphorylation (46, 52), it is possible that although NCC expression was increased, its regulation by intracellular chloride via the WNK4-SPAK pathway is intact. Thus only the NCC that is required for physiological activity is phosphorylated, and therefore, the increased expression does not result in increased activity.

WNK4 Models

Two mice colonies with overexpression of wild-type WNK4 exhibited completely opposite phenotypes. Lalioti et al. (32) prepared BAC transgenic mice that in addition to their normal wild-type WNK4 alleles harbored two extra WNK4 copies, either wild type (TgWNK4WT) or FHHt (TgWNK4PHAII). The latter exhibited an FHHt phenotype and was discussed above. The mice harboring four genes of wild-type WNK4 exhibited a GS-like phenotype with hypotrophy of DCT. Because at that time it was thought that WNK4 had a negative effect on NCC, this phenotype apparently made sense. The protein levels of total or phosphorylated WNK4, SPAK or NCC were not reported. In contrast, Wakabayashi et al. (73) also developed BAC transgenic mice harboring 2 or 30 extra copies of the wild-type WNK4 gene. These animals developed a full FHHt phenotype, with increased expression and phosphorylation of NCC and SPAK. No histological analysis of DCT was presented. Interestingly, the FHHt phenotype was similar in both colonies, although the animals with 30 copies exhibited a greatly increased expression level and phosphorylation of SPAK and NCC than those with only two extra copies. Thus these two models that are apparently similar in genotype are completely opposite in phenotype. It would be interesting to complete a molecular and histological analysis of these colonies to understand the source of the difference. For instance, it could be that in Lalioti’s wild-type WNK4 BAC transgenic mice, the overexpression of WNK4 occurred outside of DCT, actually producing a mouse with a WNK4 deficiency in DCT, thus resulting in a GS-like phenotype, as has been described for the WNK4-KO mice (13).

KS-WNK1 Models

This WK1 short, kidney-specific isoform (KS-WNK1), which lacks the kinase region and thus, catalytic activity, was identified more than a decade ago, and it has been shown to be uniquely expressed in the kidney, where it is mostly present in the DCT (43). In addition, the KS-WNK1 transcript is ~80 times more abundant than L-WNK1 in the DCT (20, 70). The KS-WNK1 isoform lacks a large segment of the kinase domain because its transcription starts from an alternative promoter at exon 4a, replacing exon 4 (20).

Two recent reports have provided evidence suggesting that KS-WNK1 could be an activator of NCC. Ariga et al. (4) observed that coexpression of KS-WNK1 with NCC in X. laevis oocytes promotes activation of NCC that is accompanied by increased SPAK/OSR1 phosphorylation as well as NCC surface expression and phosphorylation. The fact that the specific WNK inhibitor WNK463 prevented the effect of KS-WNK1 on NCC suggested that this effect was due to interactions with and activation of an endogenous WNK kinase. When coexpressed with WNK4, KS-WNK1 increased WNK4 phosphorylation at S335, a modification that is translated into WNK activation. This effect was dependent on the WNK4-KS-WNK1 interaction supporting the hypothesis that interaction with KS-WNK1 promotes WNK4 phosphorylation and activation, despite no changes in the [Cl\textsuperscript{-}] (4). Another study characterized the punctae formation induced by WNKs in DCT cells in vitro and in vivo (9). It was observed that punctae formation requires the presence of WNKs, and thus, punctae were renamed as WNK bodies. The formation of WNK bodies was observed to occur in opposite situations that promote activation (low K\textsuperscript{+} diet) or inactivation (high K\textsuperscript{+} diet) of the WNK-SPAK pathway. In both cases, the WNK bodies formation absolutely required the presence of KS-WNK1, since they were not present in the KS-WNK1 knockout mice. Thus it was proposed by the authors that KS-WNK1 could be part of the machinery required to form an active WNK body.

To elucidate the role of KS-WNK1, three different models have been developed: two KS-WNK1 knockout mice strains (28, 34) and one transgenic mouse colony that over-expressed a fragment containing amino acid residues 1–253 of KS-WNK1 (KS-WNK1-TG-1–253) (34). These models apparently support the hypothesis of KS-WNK1 as an inhibitor of NCC because the KS-WNK1 knockout mice did not develop the FHHt phenotype. Their blood pressure and serum electrolytes were all normal (28, 34). The models with WNK4 FHHt-type mutations (32, 77) and the CA-SPAK model expressing a constitutively active SPAK only in DCT1 (26) feature increased NCC phosphorylation, together with the FHHt phenotype, supporting the hypothesis that primary activation of NCC produces FHHt. The fact that KS-WNK1 knockout mice resulted in increased expression and phosphorylation of NCC, without the FHHt phenotype, raises the possibility that the increased NCC was secondary, that is, a compensatory increase in pNCC and NCC, perhaps to compensate for a urinary potassium loss. On the other hand, now that is known that both WNK1 and WNK4 are potent activators of NCC, it is possible that the small 1–253 fragment of KS-WNK1 in the KS-WNK1-TG-1–253 exerted a dominant negative effect on WNK1 and/or WNK4 (4), thus reducing NCC with the consequent GS-like phenotype. It would be interesting to reanalyze these mice to define the status of WNK1, WNK4, and SPAK expression and phosphorylation.

PERSPECTIVES

The use of genetically altered mouse models guided by data acquired in in vitro experiments has been a helpful tool to understand the regulatory pathway of NCC by the WNK/SPAK pathway. It is presently clear that WNK4 is the principal kinase in the DCT, which is inhibited by [Cl\textsuperscript{-}], and activated by the PKA and PKC kinases that are regulated by the angiotensin receptor type 1 and calcium-sensing receptors. It is also known...
that on the one hand, WNK4 phosphorylates SPAK, which in turn phosphorylates and activates NCC and, on the other hand, WNK4 half-life is modulated by ubiquitination by the CUL3-KLHL3-RING E3 ubiquitin ligase complex. Uncompleted, unexpected, or counterintuitive observations in some transgenic models need to be resolved. Some examples are 1) the mechanism by which deletion of exon 9 in CUL3 results in FHHt is still unclear; 2) the mechanism by which mutant WNK4 remains active in FHHt despite the hyperkalemia and FHHt is still unclear; and 3) the physiological role of KS-WNK1. Thus although clear advances have occurred in the field in the last few years, there is still a lot of work to do to fully understand the molecular nature of NCC regulation by the KLHL3-WNK-SPAK pathway.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS

M.O.-F., M.C.-B., and G.G. prepared figure; M.O.-F., M.C.-B., and G.G. edited and revised manuscript; M.O.-F., M.C.-B., and G.G. approved final version of manuscript. M.O.-F., M.C.-B., and G.G. drafted manuscript; M.O.-F., M.C.-B., and G.G. edited and revised manuscript; M.O.-F., M.C.-B., and G.G. approved final version of manuscript.

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