Revisiting the NaCl cotransporter regulation by with-no-lysine kinases

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THE INVITATION OF THE EPITHELIAL TRANSPORT GROUP to present the Steven Hebert Lecture in 2013 came as a very pleasant surprise since it gave me (G. Gamba) the opportunity to make a remembrance of one true scientist, with whom I had the privilege to be trained. I first met Steve at Harvard in the late 1980s when I was looking for a fellow position and he was an assistant professor of medicine. I was 27 and he was 42 years old.

The day I met him in 1989 he explained to me his complicated tubular perfusion techniques, only to conclude by saying: “I am not going to do this anymore because next year I will pursue a transition from perfused tubules to expression cloning and molecular biology.” One year later, with the trust and patience that characterized him as a mentor, there he was, putting such a risky and ambitious enterprise in the hands of two clinicians (Kevin Ho from Ohio and myself from Mexico) with no experience at all in molecular biology, whom he was always on the cutting edge.

Cloning NCC cDNA was the beginning of a new era in the distal nephron physiology that has revealed the key role of DCT in modulating arterial blood pressure, as well as salt and potassium metabolism.

NCC cDNA was identified by expression cloning (Fig. 1) thanks to previous seminal works from Larry Renfro (59), David Ellison (17), and John Stokes (72) that revealed the functional characteristics of the cotransporter and the winter flounder urinary bladder as a unique source of mRNA for expression cloning. Following the predictions of the great Homer Smith, we then went from “fish to philosopher” to identify NCC and NKCC2 from mammalian sources using the flounder probe. Cloning NCC cDNA was the beginning of a new era in the distal nephron physiology that has revealed the key role of DCT in modulating arterial blood pressure, as well as salt and potassium metabolism.

Gene Mutations Affecting NCC Activity

In the year 2000, Melanie Cobb’s laboratory discovered a new family of serine/threonine kinases during their search for new kinases in the central nervous system (89). They named this new kinase with-no-lysine (K) 1 (WNK1) because it lacked a lysine residue within the subdomain II of the kinase domain, which is conserved in all serine/threonine kinases. Soon after the discovery of WNK1, three additional members of the family were identified and named WNK2, WNK3, and WNK4 (79). Without the seminal work of the Lifton group several months later, the discovery of this new family of kinases could have remained unnoticed in the field of ion
transport physiology (85). The Lifton group demonstrated that mutations in the genes encoding WNK1 and WNK4 were associated with the development of a form of salt-sensitive hypertension affecting humans, known as pseudohypoaldosteronism type II (PHAII), familial hyperkalemic hypertension (FHHt), or Gordon’s syndrome (85). PHAII is an autosomal dominant disease featuring arterial hypertension accompanied by hyperkalemia, metabolic acidosis, and hypercalciuria and is characterized by a marked sensitivity to low doses of thiazide-type diuretics (44). The clinical picture of PHAII mirrors that of Gitelman’s syndrome, an autosomal recessive disease featuring salt-remediable hypotension, hypokalemia, metabolic alkalosis, and hypocalciuria. Gitelman’s syndrome is caused by inactivating mutations of NCC (Table 1). Clinical evidence thus suggests that the PHAII phenotype mainly results from the increased activity of NCC by mutated WNKs (Table 1) (18, 66). Over the past 15 years, a tremendous amount of work has been dedicated to the comprehension of the physiological and pathophysiological effects of WNK kinases on this cotransporter. Although our understanding is far from complete, the recent work of many laboratories indicates that we could be reaching the end of the dark ages regarding this matter.

Although WNK1 and WNK4 were associated with PHAII in 2001, it was known over the following decade that mutations in these two genes alone could not explain the occurrence of the disease in over 70% of all the affected families (26). This suspicion indicated that mutations in other genes were likely responsible for the development of a similar phenotype. Potential genomic regions were suggested (26, 42), although without enough power to precisely define which genes were involved. The identification of these genes was delayed for over a decade until the development of exome sequencing techniques. Then, two independent groups successfully applied these techniques and identified that mutations found in a protein known as kelch-like 3 (KLHL3) were causing either a dominant or a recessive form of PHAII (Table 1) (5, 81). One of these groups also identified mutations in another gene, known as culin 3 (CUL3), in a severe form of PHAII that was caused mostly by de novo mutations (Table 1) (5). KLHL3 and CUL3 form a complex, leading to the ubiquitination of their target proteins, thereby marking them for degradation. As discussed below, we now know that the WNKs are targets of the KLHL3-CUL3 complex and that the disruption of this complex causes PHAII.

**Kelch3 and Cul3 Regulate WNK Ubiquitination and Degradation**

The ubiquitination process depends on three components: E1 ubiquitin-activating enzyme, E2 conjugating-enzyme, and E3 ubiquitin ligase (3, 8). Many types of E3 ligases exist, but the Cullin-RING ligases (CRLs) constitute the largest family. CUL3 acts as the scaffolding protein of subfamily CLR3; in other words, it brings together the substrate recognition protein with the rest of the ubiquitination machinery (22, 30). CUL3 binds to an adaptor protein of the BTB (Bric-a-brac, Tramtrack, and Broad complex)-containing family, which in...
Evidence demonstrating that KLHL3 itself is a substrate of CUL3 and that KLHL3 ubiquitination leads to its degradation also exists (45, 52). CUL3 mutations result in the skipping of exon 9 (5), producing a truncated gain-of-function form of the protein that degrades KLHL3 at a much higher rate (45). In the absence of KLHL3, CUL3 cannot bind to and flag the WNKs for degradation, including both WNK4 and WNK1, leading to their overexpression.

The severity of the PHAII phenotype supports the molecular observation that the KLHL3-CUL3 complex lies upstream of the WNKs. CUL3 mutations produce the most severe phenotypes and affect younger patients, followed in order of severity by recessive mutations in KLHL3, dominant mutations in KLHL3, WNK4 mutations and, finally, by WNK1 intronic mutations that produce the less severe clinical form (5).

Overall, PHAII seems to result from increased WNK expression. For WNK1, the increase may be attributable to the elimination of repressive elements within the first intron of WNK1. For WNK4, it could be due to the missense mutations alleviating the kinase sensitivity to the ubiquitin ligase complex formed by KLHL3 and CUL3. Mutations in KLHL3 and CUL3 could lead to PHAII by decreasing the degradation rate of WNK1 and/or WNK4 (or WNK3). Thus, to clarify the pathophysiology of the disease it is critical to understand the effect of WNKs on NCC and other ion transport pathways in the kidney.

### Revisiting the Model of WNK-Mediated NCC Modulation: Evidence That WNK1 Activates NCC

A straightforward explanation of the disease in PHAII patients due to mutations in WNK1 gene is that WNK1 may have a positive effect on NCC activity in a manner such that the elevated expression of WNK1 enhances NCC activation. However, the initial results of Yang et al. (90, 92) and Subramanya et al. (73) describing the role of WNK1 argue against this possibility (Table 2). These studies using *Xenopus laevis* oocytes provided results suggesting that WNK1 had no effect on NCC activity alone.

### Table 2. Studies analyzing the effect of WNK1 on NCC

<table>
<thead>
<tr>
<th>Working Model</th>
<th>Stimulus</th>
<th>NCC Analysis</th>
<th>Effect</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>X. laevis</em> oocytes</td>
<td>Rat L-WNK1</td>
<td>Functional activity</td>
<td>No effect</td>
<td>(90)</td>
</tr>
<tr>
<td><em>X. laevis</em> oocytes</td>
<td>Rat L-WNK1 and mouse WNK4</td>
<td>Functional activity</td>
<td>WNK1 prevented the WNK4 negative effect</td>
<td>(90)</td>
</tr>
<tr>
<td><em>X. laevis</em> oocytes</td>
<td>Rat-KS-WNK1</td>
<td>Functional activity</td>
<td>No effect</td>
<td>(73)</td>
</tr>
<tr>
<td><em>X. laevis</em> oocytes</td>
<td>Rat-L-WNK1</td>
<td>Functional activity</td>
<td>No effect</td>
<td>(73)</td>
</tr>
<tr>
<td><em>X. laevis</em> oocytes</td>
<td>Rat-KS-WNK1/L-WNK1 and WNK4</td>
<td>Functional activity</td>
<td>KS-WNK1 prevents the WNK1 inhibition of WNK4 negative effect</td>
<td>(73)</td>
</tr>
<tr>
<td>Mice</td>
<td>KS-WNK1 overexpression of fragment 1-253</td>
<td>Expression and phosphorylation</td>
<td>↓↓</td>
<td>(39)</td>
</tr>
<tr>
<td>KS-WNK1 knockout mice</td>
<td>KS-WNK1</td>
<td>Expression and phosphorylation</td>
<td>↓↓</td>
<td>(27)</td>
</tr>
<tr>
<td>WNK1/PHAII mice</td>
<td>Overexpression of L-WNK1</td>
<td>Expression and phosphorylation</td>
<td>↑↑↑↑</td>
<td>(81)</td>
</tr>
<tr>
<td><em>X. laevis</em> oocytes</td>
<td>Human L-WNK1 variants</td>
<td>Functional activity, surface expression, and phosphorylation</td>
<td>↑↑</td>
<td>(12)</td>
</tr>
</tbody>
</table>

*X. laevis, Xenopus laevis; KS-WNK1, kidney-specific WNK1; L-WNK1, long WNK1.*
the intermediate kinases, known as the SPAK/OSR1 Ste20-type kinases (49, 61, 62, 82, 83). All WNK kinases activate the SPAK/OSR1 kinases that in turn phosphorylate the cotransporters. However, WNK1 was shown to be at least ten times more potent at activating SPAK/OSR1 than WNK4 (82). Therefore, the results describing the inhibition of NCC by WNK4 (described below) and the lack of effect of WNK1 on NCC activity contradicted the observations that both kinases activate SPAK/OSR1 and that WNK1 does so with a markedly greater potency.

Recent studies have clarified this issue by confirming that WNK1 was a powerful NCC activator (Table 2). Indeed, Vidal-Petiot et al. (81) successfully generated a mouse model harboring an heterozygous deletion in the first intron of endogenous WNK1 that reproduces the human genetic condition observed in PHAII patients affected by an intronic deletion in the WNK1 gene (85). Accordingly, WNK1+/−FHH mice exhibit a twofold increase in WNK1 expression in the DCT, along with an increase in the expression and activation of NCC and a characteristic PHAII phenotype that includes hyperkalemia, hypertension, and metabolic acidosis. This phenotype confirmed that increasing WNK1 expression in mice results in overactivation of NCC and in the development of PHAII. In addition, knowing that the WNK1 gene gives rise to several different isoforms after alternative splicing of exons 9, 11, 12, and 26, the same group described a methodology to quantify the WNK1 protein isoforms lacking these exons (80). This analysis revealed that the variant lacking only exon 11 (WNK1-Δ11) was enriched in the kidneys and was responsible for most of the renal WNK1 expression. However, the previous studies addressing the effects of WNK1 on NCC were performed using the WNK1 variant lacking both exons 11 and 12 (90, 92). More recently, the effect of WNK1 on NCC was analyzed using the human WNK1-Δ11 isoform (12). These results consistently revealed that the WNK1-Δ11 isoform was a powerful NCC activator. In addition, the effect of the human WNK1-Δ11-12 isoform was also assessed and, surprisingly, this isoform was also able to promote a significant activation of the cotransporter, although with a lower potency. In the same experiment, however, the rat WNK1-Δ11-12 isoform that was used in previous studies (90, 92), had no effect on NCC activity. Because of the dramatic difference between the effects of the rat and human WNK1-Δ11-12 isoforms on NCC activation, the rat WNK1 cDNA was fully sequenced and an unexpected mutation was identified. The glycine residue located at position 2120 was substituted by a serine. Site-directed mutagenesis experiments revealed that correcting this mutation in rat WNK1 turned the kinase into a NCC activator, while introducing the G2120S mutation in the human WNK1-Δ11 or WNK1 WNK1-Δ11-12 prevented WNK1 from activating NCC. Therefore, the originally reported lack of effect of WNK1 on NCC was likely due to the presence of an unexpected mutation in the WNK1 cDNA that was used in those studies.

Fig. 2. Simplification of the initial model proposed for NCC regulation by with-nolysine kinases (WNKs). WNK4 has a negative effect on SPAK (Ste20-related proline alanine-rich kinase) and NCC; this can be prevented by the presence of WNK1. In contrast, WNK3 has a direct effect, activating SPAK and thus NCC. In accordance with this model, activation of NCC by increased expression of WNK1 could be the result of overcoming the WNK4 inhibition of NCC. OSR1, oxidative stress response 1.
WNK4 acts upstream of WNK1.

suggest that, as opposed to what was previously suggested (Fig. 2), WNK4 acts upstream of WNK1.

Another possibility is that WNK4 may prevent the activation of endogenous OSR1 by endogenous WNK1, resulting in decreased NCC activity. As shown in Fig. 3A, these observations suggest that, as opposed to what was previously suggested (Fig. 2), WNK4 acts upstream of WNK1.

To prove this hypothesis, WNK1/H11001 mice, which exhibit the PHAII phenotype and an increased phosphorylation level of NCC (81), were crossed with WNK4-null (WNK4/H11001) mice, which exhibit a decreased expression of NCC and a Gitelman’s syndrome-like phenotype (9). It was reasoned that if WNK1 acted upstream of WNK4, as previously suggested (Fig. 2), the removal of WNK4 would rescue the PHAII phenotype in the WNK1/H11001 background. In contrast, if WNK4 acted upstream of WNK1 (Fig. 3A), then the absence of WNK4 would have no effect on the PHAII phenotype in WNK1/H11001 mice. The results showed that the phenotype of the WNK1/H11001, WNK4/H11001 double-mutant mice was similar to that of the WNK1/H11001 mice, including the increased activation of NCC (12). This finding provided in vivo evidence that WNK1 does not need WNK4 to modulate NCC activity.

The negative effect of WNK4 can be prevented by the presence of angiotensin II (Fig. 3B)(10). This effect was demonstrated in Xenopus laevis oocytes, where the peptide hormone angiotensin II did not affect the basal level of NCC activity but exerted an inhibitory effect on WNK4-induced decrease of NCC activity (67). This phenomenon suggested that the previously reported positive effect of angiotensin II on NCC (77) was WNK4-dependent.

In accordance with this hypothesis, angiotensin II induced SPAK and NCC phosphorylation in mDCT cells (67, 74). Conversely, in WNK4-null mice, the increased levels of SPAK/NCC phosphorylation in response to low-salt diet or angiotensin II infusion by subcutaneous minipump were not observed in the absence of WNK4 expression (9). In addition, Na et al. (51) presented evidence that WNK4 activity was sensitive to Ca\(^2+\) ions, with a maximal kinase activity at a Ca\(^2+\) concentration of \(\sim 1\) μM. This finding further supported the possibility of an angiotensin II-mediated modulation of WNK4 activity. In addition, they showed that any of the PHAII-causing mutations found in the acidic motif affected the enzyme’s Ca\(^2+\) sensitivity, which could partly explain the mechanism by which mutated WNK4 induces the disease. Recently, another pathway explaining the angiotensin II-mediated modulation of WNK4 activity was discovered. Shibata et al. (69) observed that phosphorylation of KLHL3 on serine 433 prevented it from interacting with WNK4, thus impeding WNK4 ubiquitylation and degradation. Interestingly, serine 433 is a residue that is frequently mutated in families with PHAII, and the phosphorylation of this site is stimulated by angiotensin II via protein kinase C. Therefore, one mechanism for the angiotensin II-induced phosphorylation of NCC is apparently indirect and mediated by the prevention of WNK4 degradation.

In addition to the full-length WNK1 splice variants mentioned above, a kidney specific-WNK1 (KS-WNK1) variant has been described (16). This variant is exclusively found in the kidney and lacks the first four exons of the WNK1 transcript, yielding a smaller protein with no kinase domain. The
WNK3 is a Powerful Activator of NCC

WNK3 is the family member that has shown the most consistent and robust effect on NCC activity. WNK3 is expressed all along the nephron, and its coexpression with NCC or NKCC2 in Xenopus laevis oocytes resulted in a dramatic increase in the activity of these transporters (63). This positive effect was dependent on the kinase activity of WNK3. Indeed, the catalytically inactive WNK3-D295A mutant not only lost its positive regulatory effect on NCC but also became a powerful inhibitor. This effect suggests that, depending on their catalytic activity, which in turn depends on their auto-phosphorylation capability, the WNK kinases can act in two different modes, either as activators or as inhibitors. As shown in Table 3, all studies on WNK3 have consistently shown the robust activation of NCC. WNK3 is expressed in the brain and the kidneys as differently spliced variants due to the presence or absence of exons 18a or 18b, as well as of exon 22. One study showed that while the renal WNK3 isoforms activate NCC, the brain isoforms actually inhibit the cotransporter (24). However, another study reported that all WNK3 variants displayed the same effect on all members of the SLC12 family, including NCC (13). The WNK3-mediated activation of NCC requires the interaction with SPAK/OSR1 and is associated with an increased phosphorylation of the cotransporter (56). Although WNK3 induces a dramatic activation of NCC, the elimination of WNK3 by knockout in mice generates no apparent consequences or obvious phenotype. Interestingly, however, the WNK3-null mice displayed an upregulation of WNK1 expression in the kidneys (46, 53). Because WNK1 and WNK3 exert the same effect on NCC, the upregulation of WNK1 in WNK3-null mice could be a compensatory response preventing the occurrence of a WNK3 deletion-related phenotype. This type of compensation is often observed in knockout animals. Therefore, it will be important to determine the effect of eliminating WNK3 using a conditional knockout strategy. In conditional knockout mice, the sudden decrease in WNK3 expression might not be compensated by an overexpression of WNK1.

The effect of WNK2 in all members of all SLC12 family, including NCC, is similar to that shown by WNK3 (65). However, by Western blot analysis, WNK2 seems to be expressed only in the central nervous system and the heart, so its interaction with NCC in the kidney is unlikely to occur.

Effect of WNK4 on the NaCl cotransporter activity

Soon after the discovery that mutations in the WNK1 and WNK4 genes were causing PHAII, the role of these kinases in NCC activity was assessed using the Xenopus laevis oocytes heterologous expression system. Two independent groups demonstrated that the coexpression of NCC with WNK4 resulted in a decreased NCC activity that was partly attributable to a reduction in NCC expression levels at the plasma membrane (86, 90). These studies provided evidence supporting that the PHAII-associated mutations in WNK4 were reducing its inhibitory effect on NCC. Accordingly, WNK4 was identified as an endogenous inhibitor of NCC under normal conditions, as depicted in the model proposed in Fig. 2. Furthermore, when mutated in PHAII patients, WNK4 lost its ability to inhibit NCC, thereby causing the disease by favoring an increase in NCC activity. However, as shown in Tables 4 and 5, several studies using similar strategies have reported over the years that WNK4 can act either as an NCC inhibitor or as an NCC activator.

Evidence that WNK4 is an inhibitor of NCC. As discussed above, the initial work on oocytes suggested that WNK4 could inhibit the activity of NCC (Table 4). We observed that coinjection of NCC and WNK4 in Xenopus laevis oocytes resulted in a reduction of NCC activity and that this effect was not present using either the catalytically inactive form of WNK4 (WNK4-D321A) or a WNK4 construct containing one of the PHAII-associated mutations (WNK4-Q562E) (86). This result suggested that the inhibitory effect of WNK4 on NCC was dependent on the catalytic activity of the kinase, which was prevented by a PHAII-associated mutation. Yang et al. (90) simultaneously reported similar observations. In their study, three different PHAII-associated mutations were analyzed: WNK4-E559K, WNK4-D561A, and WNK4-Q562E.

Table 3. Studies that have analyzed the effect of WNK3 on NCC

<table>
<thead>
<tr>
<th>Working Model</th>
<th>Stimulus</th>
<th>NCC Analysis</th>
<th>Effect</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>X. laevis oocytes</td>
<td>WT-human WNK3</td>
<td>Functional activity and surface expression</td>
<td>↑↑↑↑</td>
<td>(63)</td>
</tr>
<tr>
<td>X. laevis oocytes</td>
<td>WT-human WNK3</td>
<td>Functional activity and surface expression</td>
<td>↑↑↑↑</td>
<td>(91)</td>
</tr>
<tr>
<td>X. laevis oocytes</td>
<td>WT-human (fragment 421-1743)</td>
<td>Functional activity</td>
<td>↑↑↑↑</td>
<td>(91)</td>
</tr>
<tr>
<td>X. laevis oocytes</td>
<td>Human WNK3/Mouse WNK4 chimeras</td>
<td>Functional activity</td>
<td>↑↑↑↑</td>
<td>(68)</td>
</tr>
<tr>
<td>X. laevis oocytes</td>
<td>Human “renal” WNK3 variant</td>
<td>Functional activity</td>
<td>↑↑↑↑</td>
<td>(24)</td>
</tr>
<tr>
<td>X. laevis oocytes</td>
<td>Human “brain” WNK3 variant</td>
<td>Functional activity</td>
<td>↑↑↑↑</td>
<td>(24)</td>
</tr>
<tr>
<td>X. laevis oocytes</td>
<td>Human WT-WNK3</td>
<td>Functional activity and phosphorylation</td>
<td>↑↑↑↑</td>
<td>(56)</td>
</tr>
<tr>
<td>X. laevis oocytes</td>
<td>Human “renal” WNK3</td>
<td>Functional activity and phosphorylation</td>
<td>↑↑↑↑</td>
<td>(13)</td>
</tr>
<tr>
<td>X. laevis oocytes</td>
<td>Human “brain” WNK3</td>
<td>Functional activity and phosphorylation</td>
<td>↑↑↑↑</td>
<td>(13)</td>
</tr>
<tr>
<td>X. laevis oocytes</td>
<td>Human WNK3</td>
<td>Functional activity and phosphorylation</td>
<td>↑↑↑↑</td>
<td>(11)</td>
</tr>
<tr>
<td>X. laevis oocytes</td>
<td>Human WNK3</td>
<td>Functional activity</td>
<td>↑↑↑↑</td>
<td>(35)</td>
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<tr>
<td>X. laevis oocytes</td>
<td>Human WNK3</td>
<td>Functional activity and phosphorylation</td>
<td>↑↑↑↑</td>
<td>(12)</td>
</tr>
</tbody>
</table>

WT, wild type.
They observed, however, that only WNK4-Q562E could partially prevent the inhibitory effect of WNK4 on NCC, while the other two mutations had no effect. This finding suggested that not all PHAII-associated mutations necessarily altered WNK4 activity at the level of its inhibitory effect on NCC. In addition, they also observed in this and a follow-up study (90, 92) that while WNK1 alone had no effect on NCC activity, the coexpression of NCC and WNK4 together with WNK1 could prevent the WNK4-induced inhibition of the cotransporter. Therefore, as discussed above, a model was proposed in which WNK4 acts as a NCC inhibitor in a manner that may be prevented by PHAII-associated mutations and WNK1 has no direct effect on NCC but acts as an inhibitor of WNK4 (Fig. 2). This model could explain the activation of NCC in patients with PHAII-associated mutations in the WNK1 gene because, in these families, the deletion of a part of the intron 1 results in the elevation of expression of an otherwise normal WNK1 protein. According to this model, an excess of WNK1 would activate NCC but acts as an inhibitor of WNK4 (Fig. 2). This model was validated using animal models (Table 4). Lalioti et al. (36) constructed two BAC transgenic mice presenting opposite modulations of WNK4 expression: one expressing four copies of the wild-type WNK4 gene (Tg-WNK4wt) and another expressing two copies of the wild-type WNK4 gene and two of the

The inhibitory effect of WNK4 on NCC was corroborated by a third group using *Xenopus* oocytes (25) and by a fourth group using mammalian transfected cells (7) (Table 4). In the latter study using COS-7 cells, Cai et al. (7) observed that NCC expression levels at the plasma membrane were reduced by the presence of wild-type WNK4, but not by the PHAII-associated mutants of WNK4. Considering that the inhibitory effect of WNK4 on NCC observed in expression systems could have been an artifact of the high level of protein expression, it was desirable to corroborate this effect in other systems. In this regard, Ko et al. (33) took the advantage of the mDCT15 cell line that endogenously expresses both NCC and WNK4. They used specific shRNAs to reduce the expression of WNK4 by 68% and reported that following this reduction, NCC activity and cell surface expression were significantly increased by 92% and 117%, respectively. The fact that NCC activity and expression increased as a result of the reduction in endogenous WNK4 expression supported the previous observations showing that overexpression of WNK4 reduced NCC activity.

The inhibitory effect of WNK4 on NCC was also corroborated using animal models (Table 4). Lalioti et al. (36) constructed two BAC transgenic mice presenting opposite modulations of WNK4 expression: one expressing four copies of the wild-type WNK4 gene (Tg-WNK4wt) and another expressing two copies of the wild-type WNK4 gene and two of the

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### Table 4. Studies showing a negative effect of WNK4 on NCC

<table>
<thead>
<tr>
<th>Working Model</th>
<th>Stimulus</th>
<th>NCC Analysis</th>
<th>Effect</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>X. laevis</em> oocytes</td>
<td>Mouse WT-WNK4</td>
<td>Functional activity and surface expression</td>
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<td>(86)</td>
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<tr>
<td><em>X. laevis</em> oocytes</td>
<td>Mouse WNK4-Q562E</td>
<td>Functional activity and surface expression</td>
<td>No effect</td>
<td>(86)</td>
</tr>
<tr>
<td><em>X. laevis</em> oocytes</td>
<td>Mouse WT-WNK4</td>
<td>Functional activity and surface expression</td>
<td>↓ ↓</td>
<td>(90)</td>
</tr>
<tr>
<td><em>X. laevis</em> oocytes</td>
<td>Mouse WNK4-Q562E</td>
<td>Functional activity</td>
<td>↓ ↓</td>
<td>(90)</td>
</tr>
<tr>
<td><em>X. laevis</em> oocytes</td>
<td>Mouse WNK4-E559K</td>
<td>Functional activity</td>
<td>↓ ↓</td>
<td>(90)</td>
</tr>
<tr>
<td><em>X. laevis</em> oocytes</td>
<td>Mouse WNK4-D561A</td>
<td>Functional activity</td>
<td>↓ ↓</td>
<td>(90)</td>
</tr>
<tr>
<td><em>X. laevis</em> oocytes</td>
<td>Rat L-WNK1</td>
<td>Functional activity</td>
<td>No effect</td>
<td>(90)</td>
</tr>
<tr>
<td><em>X. laevis</em> oocytes</td>
<td>WT-WNK4 (diverse fragments)</td>
<td>Functional activity</td>
<td>↓ ↓</td>
<td>(92)</td>
</tr>
<tr>
<td><em>X. laevis</em> oocytes</td>
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<td>Functional activity</td>
<td>↓ ↓</td>
<td>(25)</td>
</tr>
<tr>
<td>M1-Cells</td>
<td>WT-human WNK4</td>
<td>Surface expression</td>
<td>↓ ↓</td>
<td>(7)</td>
</tr>
<tr>
<td>COS-7 Cells</td>
<td>WT-human WNK4</td>
<td>Surface expression</td>
<td>↓ ↓</td>
<td>(7)</td>
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<tr>
<td>COS-7 Cells</td>
<td>Human WNK4-E562K</td>
<td>Surface expression</td>
<td>No effect</td>
<td>(7)</td>
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<tr>
<td>COS-7 Cells</td>
<td>Human WNK4-R1185C</td>
<td>Surface expression</td>
<td>No effect</td>
<td>(7)</td>
</tr>
<tr>
<td>BAC mice</td>
<td>WT-WNK4 overexpression</td>
<td>DCT expression/physiological parameters</td>
<td>↑ ↑ ↑</td>
<td>(36)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>PHAII-like phenotype</td>
</tr>
<tr>
<td><em>X. laevis</em> oocytes</td>
<td>WT-WNK4 (445-1222)</td>
<td>Functional activity</td>
<td>↓ ↓</td>
<td>(91)</td>
</tr>
<tr>
<td><em>X. laevis</em> oocytes</td>
<td>Mouse WT-WNK4</td>
<td>Functional activity</td>
<td>↓ ↓</td>
<td>(68)</td>
</tr>
<tr>
<td><em>X. laevis</em> oocytes</td>
<td>Mouse WT-WNK4</td>
<td>Functional activity</td>
<td>↓ ↓</td>
<td>(67)</td>
</tr>
<tr>
<td><em>X. laevis</em> oocytes</td>
<td>Mouse WT-WNK4</td>
<td>Functional activity</td>
<td>↓ ↓</td>
<td>(23)</td>
</tr>
<tr>
<td><em>X. laevis</em> oocytes</td>
<td>Mouse WT-WNK4</td>
<td>Functional activity</td>
<td>↑ ↑</td>
<td>(24)</td>
</tr>
<tr>
<td>COS-7 cells</td>
<td>Human WNK4 variant</td>
<td>Surface expression and half-like expression</td>
<td>↑ ↑</td>
<td>(95)</td>
</tr>
<tr>
<td>mDCT15 cells</td>
<td>WNK4 shRNA to knock down WNK4</td>
<td>Functional activity and surface expression</td>
<td>↑ ↑</td>
<td>(33)</td>
</tr>
<tr>
<td>Wild-type mice</td>
<td>Isoproterenol-induced decrease of WNK4</td>
<td>Expression and phosphorylation</td>
<td>↑ ↑</td>
<td>(50)</td>
</tr>
<tr>
<td>mDCT cells</td>
<td>Overexpression of WT-WNK4</td>
<td>Protein Expression</td>
<td>↓ ↓</td>
<td>(94)</td>
</tr>
<tr>
<td>mDCT cells</td>
<td>WNK4 knockdown with siRNA</td>
<td>Protein Expression</td>
<td>↓ ↓</td>
<td>(94)</td>
</tr>
<tr>
<td>mDCT cells</td>
<td>WNK4 increase by Per1 blockade</td>
<td>mRNA expression</td>
<td>↑ ↑</td>
<td>(60)</td>
</tr>
<tr>
<td>mDCT15 cells</td>
<td>WNK4 shRNA to knock down WNK4</td>
<td>Functional activity and surface expression</td>
<td>↑ ↑</td>
<td>(12)</td>
</tr>
</tbody>
</table>

DCT, distal convoluted tubule.
PHAIΙ-associated mutated WNK4 gene (Tg-WNK4^PHAII). The Tg-WNK4^wt mice developed low blood pressure and DCT hypoplasia, while the Tg-WNK4^PHAII mice developed arterial hypertension with hyperkalemia and DCT hyperplasia. Thus, in these models, the overexpression of wild-type WNK4 resulted in a phenotype associated with reduced NCC activity, while the overexpression of the PHAIΙ-associated mutant forms of WNK4 resulted in a PHAIΙ-like phenotype with DCT hyperplasia and elevated NCC expression and activity. These observations support the hypothesis that WNK4 works as a molecular switch with distinct functional states. The fact that the Tg-WNK4^PHAII mice harboring two wild-type WNK4 alleles developed PHAIΙ eliminated the possibility that haploinsufficiency could be the mechanism explaining the PHAIΙ disease in the case where mutations confer a loss-of-function effect. Instead, this result suggested that missense mutations in the acidic domain of WNK4 resulted in a gain-of-function effect.

Another study using mouse models further confirmed the inhibitory role of WNK4 on NCC activity. Mu et al. (50) studied a mouse model of norepinephrine-induced salt-sensitive arterial hypertension (Table 4). They observed that the hypertension developed in response to high-salt diet was due to epigenetic changes in the glucocorticoid receptor gene after norepinephrine-induced activation of the renal β2-adrenergic receptor. Interestingly, WNK4 expression was reduced simultaneously with an increase in NCC expression and phosphorylation levels. The results obtained with this animal model were consistent with the idea that NCC activation was associated with a decrease in WNK4 expression, thereby supporting the model involving a negative effect of WNK4 on NCC function. In summary, studies in Xenopus laevis oocytes, two distinct mammalian cell lines (COS-7 and mDCT15), WNK4 BAC transgenic mice and wild-type mice treated with norepinephrine and high-salt diet are all consistent with the hypothesis that WNK4 behaves as an inhibitor of NCC activity.

Evidence that WNK4 is an activator of NCC. Interestingly, some studies have suggested that WNK4 exerts a positive effect on NCC activity rather than a negative one (Table 5). In one study using Xenopus laevis oocytes, Wu et al. (87) observed that the coinjection of NCC and WNK4 cRNAs resulted in increased activity of NCC. One difference from previous studies is that, instead of using the rat or mouse WNK4 cRNA, these authors used human WNK4 cRNA. They also suggested that different conditions during the uptake experiments could explain this difference, although this was not further analyzed. One study in mDCT cells showed that the calcineurin inhibitor cyclosporine induced an increase in WNK4 expression, which was associated with an increase in NCC expression (47) (Table 5). Some results using animal models also suggested that WNK4 acted as a positive activator of NCC (Table 5). Regarding calcineurin inhibitors, Hoorn et al. (29) presented evidence that tacrolimus-induced arterial hypertension was associated with increased WNK4 and WNK3 expression levels, together with an increased NCC phosphorylation level (T53-NCC) and SPAK expression level. In short, opposing studies showed that NCC expression and phosphorylation in wild-type mice were associated with either a decreased expression of WNK4 by norepinephrine (50) or an increased expression of WNK4 by tacrolimus (29).

The results from two genetically engineered mouse models also suggested that WNK4 could activate NCC (Table 5). First, Castañeda-Bueno et al. (9) reported that WNK4-null mice exhibited a dramatic decrease in the expression and phosphorylation of NCC, accompanied by hypokalemia and metabolic alkalosis. In these mice, the blood pressure was normal despite increased plasma renin activity. The fact that the total absence of WNK4 resulted in the decreased expression of NCC and a phenotype resembling Gitelman’s disease suggested that WNK4 was an activator of NCC and that its absence resulted in a decreased expression and activity of the cotransporter. An alternative explanation that was not explored in this work is that the WNK4 knockout strategy could favor the expression of a shorter, truncated isoform of WNK4 that may act as a dominant-negative heterodimer partner with the other WNK isoforms to decrease NCC activity. The second model was developed by Wakabayashi et al. (84) using a similar strategy to that applied by Lalioti et al. (36), consisting of two wild-type WNK4 BAC transgenic mice. They generated two transgenic lines, one with a low copy number and another with a high copy number of WNK4, exhibiting an increased level of WNK4 expression by 1.7- and 9.1-fold relative to control mice, respectively. The increased expression and activity of SPAK/OSR1 and NCC in these transgenic lines was accompanied by the development of a PHAIΙ-like phenotype. In summary, there is evidence showing that wild-type WNK4 BAC transgenic mice can present either an inhibition of NCC and a Gitelman’s syndrome-like phenotype (36) or an activation of NCC with a PHAIΙ-like phenotype (84).

Taken together, these studies used similar strategies and experimental models, such as Xenopus laevis oocytes, cultured mammalian cells, as well as wild-type mice and transgenic

<table>
<thead>
<tr>
<th>Working Model</th>
<th>Stimulus</th>
<th>NCC Analysis</th>
<th>Effect</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>X. laevis oocytes</td>
<td>Mouse WT-WNK4</td>
<td>Functional activity</td>
<td>↑ ✈ ^</td>
<td>(67)</td>
</tr>
<tr>
<td>Wild-type mice mDCT cells</td>
<td>Increased WNK4 expression by tacrolimus</td>
<td>NCC expression and phosphorylation</td>
<td>↑ ✈ ^</td>
<td>(29)</td>
</tr>
<tr>
<td>WNK4 knockout mice</td>
<td>Increased WNK4 expression by cyclosporine</td>
<td>NCC expression and phosphorylation</td>
<td>↑ ✈ ^</td>
<td>(47)</td>
</tr>
<tr>
<td>Wild-type mice BAC mice</td>
<td>Knocking down WNK4</td>
<td>NCC expression and phosphorylation, physiological parameters, response to low-salt diet or ANG II infusion</td>
<td>↑ ✈ ^</td>
<td>(9)</td>
</tr>
<tr>
<td>X. laevis oocytes</td>
<td>WT-WNK4 overexpression</td>
<td>NCC expression and phosphorylation</td>
<td>↑ ✈ ^</td>
<td>(84)</td>
</tr>
<tr>
<td>X. laevis oocytes</td>
<td>Human WT-WNK4 and low chloride hypotonic stress</td>
<td>Functional activity and phosphorylation</td>
<td>↑ ✈ ^</td>
<td>(2)</td>
</tr>
</tbody>
</table>

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mice, and they showed clear evidence for WNK4’s dual behavior towards NCC. Indeed, both inhibition and activation of NCC by WNK4 were reported, suggesting that when both states coexist, one will predominate depending on the specific physiological context. Moreover, it is possible that WNK4 inhibits NCC under a high-salt diet to promote natriuresis but activates NCC under a low-salt diet to maximize salt retention. Recent evidence on WNK4 regulation suggests that this is a reasonable hypothesis.

The Effect of WNK4 on NCC Is Modulated by the Intracellular Chloride Concentration

As discussed above, several lines of evidence suggest that WNK4 may behave as a molecular switch, either activating or inhibiting NCC depending on the physiological state of the organism and/or the cellular environment context at each particular moment. Therefore, the inhibitory or activating effects of WNK4 could coexist and be modulated at the intracellular level.

It is known that the activity of all members of the electroneutral cation chloride cotransporter (SLC12) family are modulated by the intracellular chloride ion concentration ([Cl\textsuperscript{−}]) (14, 15, 41, 55, 58). This family is divided into two branches. The first is composed of three members (the Na\textsuperscript{+}/K\textsuperscript{+}-2Cl\textsuperscript{−} cotransporters NKCC1 and NKCC2 and the Na\textsuperscript{+}/Cl\textsuperscript{−} cotransporter NCC) that couple sodium and chloride transport into the cell and are activated by phosphorylation of key residues in their NH\textsubscript{2}-terminal domain in response to the decrease in [Cl\textsuperscript{−}], (14, 55, 58). The other branch is composed of four members (the K\textsuperscript{+}/Cl\textsuperscript{−} cotransporters, KCC1–KCC4) that couple potassium and chloride transport out of the cell and are inhibited by the phosphorylation of key residues in both the amino and carboxyl terminal domains in response to a decrease in [Cl\textsuperscript{−}] (48, 64). In short, a reduction of cell [Cl\textsuperscript{−}] results in the phosphorylation of SLC12 cotransporters, activating the sodium-coupled and inhibiting the potassium-coupled branch. Because these transporters move chloride into or out of the cell, their activities are key regulators of the [Cl\textsuperscript{−}]. Therefore, a classical feedback mechanism is established between SLC12 cotransporters activity and [Cl\textsuperscript{−}], in which an increased [Cl\textsuperscript{−}], inhibits the influx of chloride in association with a dephosphorylation of the cotransporters, while a decrease in the [Cl\textsuperscript{−}], increases its influx, together with the phosphorylation of SLC12 members. Evidence from our group suggested that the signaling pathway linking the [Cl\textsuperscript{−}] and SLC12 phosphorylation could involve the activation/deactivation of WNK kinases (54, 58).

As shown in Fig. 4, with the recent observation that WNK1 activates NCC, it became evident that exposing oocytes to low-chloride hypotonic stress (LCHS), which is known to be associated with a decreased [Cl\textsuperscript{−}] (2, 4, 31), potentiated the effect of WNK1 on NCC. However, the effect of WNK3, which is already dramatic in control conditions, remained unchanged by LCHS. Therefore, it was reasoned that WNKs could be modulated by [Cl\textsuperscript{−}], and present different affinities for chloride ions. In this scenario, the effect of WNK4 could also be modulated by [Cl\textsuperscript{−}], but with a greater affinity. This hypothesis resulted in the following affinity profile: WNK4 > WNK1 > WNK3. Supporting this idea, in the same series of experiments shown in Fig. 4, the absence or inhibitory effect of WNK4 on NCC activity in the control conditions was noticeably switched to an activating effect in the oocytes exposed to LCHS (2). The hypothesis that WNK4 activity towards NCC is modulated by [Cl\textsuperscript{−}], was further supported by the use of phospho-specific antibodies raised against the conserved WNK autophosphorylation sites (WNK1, S382; WNK3, S308; and WNK4, S335), which demonstrated that LCHS could modulate WNK1 and WNK4 autophosphorylation levels (2). In oocytes under control conditions, WNK4 autophosphorylation was absent, WNK1 was slightly autophosphorylated, and WNK3 was heavily autophosphorylated. LCHS increased WNK4 and WNK1 autophosphorylation, while the level of WNK3 autophosphorylation remained similarly strong in both conditions (2). Piala et al. (57) recently demonstrated that the kinase domain of long WNK1 (L-WNK1) harbors a chloride-binding site and that WNK1 autophosphorylation and activity were inhibited under high [Cl\textsuperscript{−}], when chloride was present on its binding site (with an IC\textsubscript{50} of ~20 mM). Using crystallography techniques, this group identified two key leucine residues located in the DLG motif (L269 and L371) within the kinase domain that form the chloride-binding site. WNK kinases share a common structure with more than 80% identity in their kinase domain. Therefore, these DLG motif leucine residues are conserved among all WNKs, suggesting that the mechanism regulating the L-WNK1 chloride sensor function could be common among the WNKs. In this regard, it was recently demonstrated that mutating/substituting these leucine residues in WNK4 turned the kinase into a constitutively autophosphorylated NCC activator (2). In other words, in the absence of the L322 and L324 residues, WNK4 is no longer inhibited by [Cl\textsuperscript{−}], and acts as a constitutive NCC activator (2). From models in Figs. 3 and 5, it is possible that WNK4 inhibitory and activating effects on NCC could coexist and be modulated by [Cl\textsuperscript{−}]. In a high-chloride environment, WNK4 is not autophosphorylated, a condition in which it may interact with WNK1 and WNK3 and inhibit their activity, while in a low-

![Fig. 4. Effect of WNK1, WNK3, and WNK4 on NCC activity in Xenopus laevis oocytes microinjected with NCC cRNA alone or together with each of the WNKs cRNA. A: uptake in control conditions in which intracellular chloride concentration ([Cl\textsuperscript{−}]) is ~45 mM (2). NCC activity is increased by WNK1 and WNK3 to 150% and 320%, respectively, while it is decreased by the presence of WNK4 (*P < 0.05 vs. black bar). B: oocytes from the same experiments were incubated in a low-chloride hypotonic stress medium for 16 h. This incubation reduces [Cl\textsuperscript{−}] to ~22 mM (2). In these conditions, WNK1 effect on NCC increased when compared with that observed in control conditions. The WNK3 remained unchanged. In contrast to WNK1 and WNK3, WNK4 turned into a positive activator of NCC. Thus, WNK1 and WNK4, but not the WNK3 effect on NCC, was modified by reduction of [Cl\textsuperscript{−}], (*P < 0.05 vs. black bar).]
chloride environment, autophosphorylation turns the kinase to an active mode, making it functional against SPAK and NCC, either as a homodimer or heterodimer with WNK1 and/or WNK3.

The sensitivity of WNK4 to chloride may explain its dual effect in response to [Cl\(^{-}\)], suggesting that the modulator of WNK4 activity has been identified. Interestingly, Terker et al. (75) recently demonstrated that the modulation of NCC phosphoxygenation in response to changes in extracellular potassium concentrations was associated with a potassium-dependent modulation of the membrane voltage in DCT cells, which in turn modulated the intracellular [Cl\(^{-}\)]. They provided evidence showing that when the extracellular potassium concentration was decreased, the resultant hyperpolarization of the DCT cells was associated with a chloride efflux decreasing the [Cl\(^{-}\)], and activating NCC in a WNK1-dependent manner. Similarly, Wang et al. (93) observed that the basolateral potassium conductance in the DCT determines the apical NCC expression and phosphorylation levels via a modulation of the [Cl\(^{-}\)]-dependent SPAK activity. This phenomenon explains the mechanism by which mutations in the basolateral K\(^{+}\) channel KCNJ10 result in NCC inhibition in response to intracellular chloride accumulation.

**Perspectives and Future Directions**

It is now clear that both WNK1 and WNK4 are able to activate NCC but that WNK4 is also able to specifically inhibit the cotransporter. The behavior of the kinases depends on the [Cl\(^{-}\)], modulating their autophosphorylation levels. However, because WNK1 and WNK4 activities are modulated by intracellular chloride concentrations, the increased expression of the kinases in PHAI1 does not appear to be sufficient to explain the development of the disease. Indeed, it is expected that the elevated kinase activity associated with the increased expression of the WNKs would be inhibited by the increase in intracellular chloride caused by NCC hyperactivation. This raises an interesting question: why does the increased WNK1 or WNK4 expression escape the intracellular chloride-mediated regulation? Could WNK4 missense mutations interfere with the capacity of the kinase to bind chloride, thereby modifying its sensitivity to chloride? Further biochemical studies are required to study this possibility and to characterize the chloride sensitivity of different WNK isoforms. Additionally, the relative chloride sensitivity among the WNK isoforms could help us understand the specific roles of each WNK isoform in the different models and assess the correlation between their specific levels and sites of expression.

A second important question concerns the mechanism behind the regulation of the whole WNK-SPAK-NCC complex by the chloride concentration. Does chloride binding to WNK1 or WNK4 affect the conformation of the WNK-SPAK-NCC complex? Alternatively, could it be that the complex is formed properly but that intracellular chloride elevation prevents the SPAK phosphorylation-dependent NCC phosphorylation? The latter potential mechanism could help explain the inhibiting vs. activating effect of WNK4. In a high-chloride environment, the complex may form but remain unphosphorylated, thereby hijacking and reducing SPAK-NCC activity. In contrast, in a low-chloride environment, the phosphorylation of these proteins would result in NCC activation.

The modulation of WNKs by [Cl\(^{-}\)], may represent a central mechanism for the modulation of salt reabsorption in the DCT, as diverse physiological processes appear to converge on the [Cl\(^{-}\)]-dependent regulation of NCC. At least three key pieces of information have recently arisen. First, NCC regulation by extracellular potassium is due to modulations in the [Cl\(^{-}\)], (75). Second, mutations in the potassium channel KCNJ10 result in a Gitelman-like syndrome because of the increased [Cl\(^{-}\)], resulting from KCNJ10 inactivity (93). Third, NCC modulation by angiotensin II could be, at least in part, the consequence of a decrease in [Cl\(^{-}\)], considering that this peptide hormone can promote the opening of the basolateral membrane ion channels. Indeed, Wu et al. (88) provided evidence that angiotensin II promotes NKCC2 activation by stimulating chloride efflux through the opening of the basolateral CLC-KB channels. It will be interesting to determine whether a similar effect also regulates the DCT chloride channels.

In summary, several recent findings have opened the path towards a better understanding of the signaling pathways...
regulating NCC activity, providing many interesting hypotheses to be tested in the upcoming years.

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DISCLOSURES

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

AUTHOR CONTRIBUTIONS

S.B.-V. and G.G. prepared figures; S.B.-V. and G.G. drafted manuscript; S.B.-V. and G.G. edited and revised manuscript; S.B.-V. and G.G. approved final version of manuscript.

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