HHS Public Access

Author manuscript

Hypertension. Author manuscript; available in PMC 2018 March 01.

Published in final edited form as:

Hypertension. 2014 November ; 64(5): 1047–1053. doi:10.1161/HYPERTENSIONAHA.114.04036.

The WNK-SPAK-NCC cascade revisited: WNK1 stimulates the activity of the NaCl cotransporter via SPAK, an effect antagonized by WNK4

María Chávez-Canales[1,2,9], **Chong Zhang**[3,9], **Christelle Soukaseum**[4,5], **Erika Moreno**[2], **Diana Pacheco-Alvarez**[6], **Emmanuelle Vidal-Petiot**[4,5,10], **María Castañeda-Bueno**[1,2,11], **Norma Vázquez**[1,2], **Lorena Rojas-Vega**[1,2], **Nicholas P. Meermeier**[3], **Shaunessy Rogers**[3], **Xavier Jeunemaitre**[3,4,7], **Chao-Ling Yang**[3], **David H Ellison**[3,8], **Gerardo Gamba**[1,2,12], and **Juliette Hadchouel**[4,5,12]

[1]Molecular Physiology Unit. Instituto de Investigaciones Biomédicas, Universidad Nacional Autónoma de México, México City, Mexico

[2]Department of Nephrology and Mineral Metabolism, Instituto Nacional de Ciencias Médicas y Nutrición Salvador Zubirán. Mexico City, Mexico

[3]Division of Nephrology & Hypertension, Oregon Health & Science University, Portland, Oregon, USA

[4]INSERM UMR970 - Paris Cardiovascular Research Center - Paris, France

[5]University Paris-Descartes, Sorbonne Paris Cité, Faculty of Medicine, Paris, France

[6]Escuela de Medicina, Universidad Panamericana, Mexico City, Mexico

[7]AP-HP, Department of Genetics, Hôpital Européen Georges Pompidou, Paris, France

[8]Veterans Affairs Medical Center, Portland, Oregon, USA

Abstract

The With No lysine kinases WNK1 and WNK4 are key regulators of blood pressure. Their mutations lead to Familial Hyperkalemic Hypertension (FHHt), associated with an activation of the Na-Cl cotransporter, NCC. Although it is clear that WNK4 mutants activate NCC via SPAK (Ste20 Proline-Alanine rich Kinase), the mechanisms responsible for WNK1-related FHHt and alterations in NCC activity are not as clear. We tested whether WNK1 modulates NCC through WNK4, as predicted by some models, by crossing our recently developed WNK1-FHHt mice ($WNK1^{+/FHHt}$) with $WNK4^{-/-}$ mice. Surprisingly, the activated NCC, hypertension, and

Corresponding authors: Juliette Hadchouel, Paris Cardiovascular Research Center, INSERM U970, 56, rue Leblanc, 75015 Paris, Tel : +33.1.53.98.80.73, Fax : +33.1.53.98.79.52, juliette.hadchouel@inserm.fr; Gerardo Gamba, Molecular Physiology Unit, Biomedical Research Institute, Vasco de Quiroga No. 15, Tlalpan 14000, Mexico City. Mexico, Tel: 5255-5513-3868, gamba@biomedicas.unam.mx.

[9]These authors contributed equally to this work.

[10]Present address: AP-HP, Physiologie - Explorations Fonctionnelles Multidisciplinaires, Hôpital Bichat Claude Bernard, Paris, France

[11]Present address: Department of Genetics and Howard Hughes Medical Institute, Yale University School of Medicine, New Haven, USA

[12]These authors co-directed the work

Conflict(s) of Interest/Disclosure(s). None

hyperkalemia of *WNK1*$^{+/FHHt}$ mice remain in the absence of WNK4. We demonstrate that WNK1 powerfully stimulates NCC in a WNK4-independent and SPAK-dependent manner. Moreover, WNK4 decreases the WNK1 and WNK3-mediated activation of NCC. Finally, the formation of oligomers of WNK kinases through their C-terminal coiled-coil domain is essential for their activity towards NCC. In conclusion, WNK kinases form a network in which WNK4 associates with WNK1 and WNK3 to regulate NCC.

INTRODUCTION

Arterial hypertension is now the largest contributor to premature morbidity and mortality worldwide[1]. Although increased renal salt reabsorption is recognized as a crucial pathogenic factor, the underlying pathophysiological mechanisms remain unknown in most patients. In order to identify pathways, the disturbance of which could cause hypertension, one of the strategies used is the genetic analysis of rare monogenic hypertensive or salt-losing disorders, such as Familial Hyperkalemic Hypertension (FHHt; OMIM 145260; Gordon's syndrome or pseudohypoaldosteronism type II). FHHt is a rare disease of hypertension, hyperkalemia and metabolic hyperchloremic acidosis[2]. Genetic analysis of FHHt patients showed that the disease results from mutations in genes encoding the serine/threonine kinases WNK1 or WNK4 (With No lysine (K) 1 and 4)[3] and components of an ubiquitin-ligase complex, KLHL3 (Kelch-like 3) and Cullin3[4]. These proteins seem to belong to the same blood pressure regulating pathway, as WNK1 and WNK4 are recruited by KLHL3 as substrates for ubiquitination by the Cul3-based E3 RING-type ubiquitin-ligase complex[5].

The high sensitivity of patients to thiazides suggested that FHHt was caused by an activation of NCC, the Na^+-Cl^- transporter of the Distal Convoluted Tubule (DCT) inhibited by this class of diuretics. Subsequent to the discovery of WNK kinases, the efforts of several groups focused on characterizing the mechanisms underlying the regulation of NCC by WNK1 and WNK4. The results were however often contradictory and did not allow the establishment of a clear and unifying model to explain how WNKs regulate the expression and/or activity of the co-transporter.

We first reported that WNK4 inhibits NCC activity in *Xenopus laevis* oocytes[6, 7], an effect reversed by angiotensin II[8]. This inhibitory effect was also observed in mouse DCT cells[9], endogenously expressing WNK4 and NCC, suggesting that it is not an overexpression artifact. The mechanisms by which WNK1 regulates NCC are less well defined. Biochemical studies showed that the catalytically active WNK1 isoform (L-WNK1) activates the Ste20 Proline-Alanine rich Kinase SPAK[10, 11], responsible for phosphorylating and activating NCC directly[12]. However, these data were not supported by studies performed in *Xenopus* oocytes. In this system, L-WNK1 failed to activate NCC but relieved NCC from WNK4-mediated inhibition[7], suggesting that L-WNK1 lies upstream of WNK4 to activate NCC. The apparent discrepancies between results obtained using different model systems have generated substantial confusion concerning WNK effects.

In order to identify the pathway(s) involved *in vivo* in WNK1-dependent activation of NCC, we generated a mouse model of FHHt caused by *WNK1* mutations (*WNK1*$^{+/FHHt}$ mice)[13]. This model exhibited a full FHHt phenotype, resulting from an increased expression of L-

WNK1 in the DCT and, to a lesser extent, in the CNT. As expected, the abundance of total and phosphorylated NCC at the apical membrane of DCT cells was enhanced. We also showed that phosphorylated SPAK abundance is increased near the apical membrane of DCT cells of mutant mice. These data support the existence of a WNK1/SPAK/NCC cascade, but do not indicate whether SPAK/NCC is stimulated directly by WNK1, or whether the effects result from relief of WNK4 inhibition.

The present study therefore tested the role of WNK1-WNK4 interaction in vitro and *in vivo*. Our results provide evidence that WNK1 is a powerful activator of NCC, independent of WNK4. In addition, we show that WNK4 antagonizes the activating effect of WNK1 on the cotransporter. Together, these results help resolve existing controversies and elucidate the mechanisms by which L-WNK1 and WNK4 signal to NCC.

METHODS

A detailed Methods section is available in the online Data Supplement at http://hyper.ahajournals.org.

RESULTS

WNK1-FHHt mice retain the full FHHt phenotype in the absence of WNK4

Mutations identified at the *WNK1* locus in FHHt patients are large deletions of the first intron[3]. We recently developed a mouse model for *WNK1*-FHHt by deleting the first intron of mouse *WNK1* (*WNK1*$^{+/FHHt}$ mice)[13]. This model recapitulates the FHHt phenotype, with elevated arterial pressure, hyperkalemia and hyperchloremic metabolic acidosis. We used these mice to test the interaction between WNK1 and WNK4 *in vivo*, by crossing the *WNK1*$^{+/FHHt}$ animals with *WNK4*$^{-/-}$ mice. The latter displayed decreased abundance of total and phosphorylated NCC, associated with hypochloremic metabolic alkalosis and increased renin and hematocrit[14, 15]. If WNK1 can activate SPAK and NCC, independent of WNK4, as suggested by biochemical studies, the phenotype of *WNK1*$^{+/FHHt}$ might persist when WNK4 is inactivated. As shown in Table 1 and Figure 1, *WNK1*$^{+/FHHt}$*:WNK4*$^{-/-}$ animals retained the FHHt phenotype, including elevated systolic blood pressure (*WNK1*$^{+/i1lox}$: 98 ± 2.6, *WNK1*$^{+/FHHt}$: 109 ± 3 and *WNK1*$^{+/FHHt}$*:WNK4*$^{-/-}$: 112 ± 8, $P<0.05$). The increased extracellular volume was also evidenced by a decreased renin expression in both *WNK1*$^{+/FHHt}$ and *WNK1*$^{+/FHHt}$*:WNK4*$^{-/-}$. Moreover, the increased NCC expression and phosphorylation seen in *WNK1*$^{+/FHHt}$ mice were still present in the double mutants (Figure 1B). These data show that *WNK1* mutations cause FHHt by increasing the level of expression of L-WNK1 in the distal nephron, which then activates NCC independently of WNK4. As prior studies by others and ourselves[7, 16] found that L-WNK1 did not regulate NCC directly in *Xenopus* oocytes, we decided to revisit the effects of L-WNK1 in this system and in cells.

The kidney-enriched L-WNK1 isoform activates NCC in a SPAK-dependent manner

Several isoforms of WNK1 are generated by alternative splicing of eight exons in humans and mice, and we showed that the isoform lacking only exon 11 (L-WNK1-Δ11) is the major variant in the nephron (about 70% of all WNK1 isoforms)[17]. This isoform was, however, not

the one used in the studies aimed at characterizing the regulation of NCC by L-WNK1[7], which used the isoform lacking both exons 11 and 12[18]. Therefore, the lack of L-WNK1 effect on NCC in expression systems could be due to the use of this isoform. We tested the effect of the L-WNK1 variants upon NCC activity in *Xenopus* oocytes and HEK-293 cells. L-WNK1-Δ11 induced a 3.25 fold increase in NCC activity (Figure 2A), associated with increased membrane abundance of total and phosphorylated NCC (pNCC; Figure S1). With the exception of L-WNK1-Δ9, variants containing all exons or a combination of the alternatively spliced exons 9, 11 and 12 also activated NCC, but significantly less than L-WNK1-Δ11 (Figure 2A). This effect was kinase dependent as it was absent when WNK1 D368A, known to lack kinase activity[18], was employed (Figure 2B). Previous studies did not observe the direct activation of NCC by the rat L-WNK1-Δ11-12 variant first because it is less potent than L-WNK1-Δ11 (Figure 2A) and, second, because it carries a previously unsuspected mutation in the C-terminal domain, identified by sequencing (G2120S; Figure S2A). This mutation reduced the effect of L-WNK1 on the cotransporter. Replacing the serine residue by a glycine in the rat L-WNK1-Δ11-12 cDNA restored NCC activation (Figure S2B), while mutating the glycine into a serine in the human L-WNK1-Δ11 cDNA prevented NCC activation (Figure S2C).

In order to test the requirement for SPAK in L-WNK1-mediated activation of NCC, we disrupted the L-WNK1/SPAK interaction by eliminating a SPAK-binding motif in L-WNK1-Δ11. Disruption of a similar motif in WNK3 blocks its ability to activate NCC[19]. This mutant (L-WNK1-Δ11-F316A) not only failed to stimulate NCC, but also inhibited the co-transporter (Figure 2B). Because oocytes express L-WNK1[20] and L-WNK1 oligomerizes[21], we hypothesized that L-WNK1-Δ11-F316A exerted this effect through interactions with endogenous L-WNK1. Thastrup and colleagues showed that a small HQ motif (HIQEVVSLQT), located in the C-terminal coiled-coil domain, is essential for the formation of homo and heteromers of WNK kinases[22]. The replacement of the HIQEVVSLQT sequence by AIQEVVSLAT impairs the interaction of WNK kinases[22]. Introduction of these mutations into L-WNK1-Δ11 (Δ11HQ/AA) prevented the activation of NCC. Moreover, mutation of the HQ motif of L-WNK1-Δ11-F316A abrogated its inhibition (Δ11-F136A-HQ/AA; Figure 2B). This suggests that basal NCC activity *in vitro* is dependent on endogenous L-WNK1 and that L-WNK1-Δ11-F316A acts as dominant negative form. To confirm that basal NCC activity *in vitro* requires endogenous L-WNK1 activity, we used NCC transfected mammalian kidney cells, which express, glycosylate, and phosphorylate NCC in a manner that resembles mammalian kidney (Figure S3). Overexpression of human L-WNK1-Δ11 increased the abundance of total and phosphorylated NCC (Figure 2C). Conversely, L-WNK1 siRNA-mediated knockdown (Figure 2D) reduced pNCC abundance. These results demonstrate that kidney-enriched isoform of L-WNK1 is a powerful activator of NCC through a WNK4-independent mechanism, since WNK4 is not expressed in HEK293 cells (Figure S3).

L-WNK1 effect on NCC is antagonized by WNK4

WNK4 inhibits NCC in *Xenopus* oocytes[6, 7], mammalian cells[23] and mice[24]. Additionally, WNK4 prevents the positive effect exerted by WNK3 on NCC[25] (Figure 3A). We observed the same effect when WNK4 and L-WNK1-Δ11 were co-expressed, in *Xenopus* oocytes and

HEK293 cells (Figure 3A-B). The decreased NCC activity observed when WNK4 and L-WNK1Δ11 are co-expressed could result from the inhibition of L-WNK1-Δ11 by WNK4 or from the parallel action of an activator (L-WNK1-Δ11) and an inhibitor (WNK4). The former could be caused by a reduced L-WNK1-Δ11 abundance mediated by WNK4. Figures 3C and 3D show that WNK4 significantly reduced the abundance of L-WNK1-Δ11 in both oocytes and HEK293 cells. The effects to reduce L-WNK1 were not simply the result of an overexpression artifact. Indeed, overexpression of WNK3 did not modify L-WNK1-Δ11 (Figure S4A) and overexpression of WNK4 did not affect SPAK or pSPAK abundance in HEK293 cells (Figure S4B).

We next assessed the requirement for the formation of WNK4 oligomers by mutating the C-terminal HQ motif[22]. The resulting WNK4-HQ/AA mutant lost the capacity to inhibit NCC (Figure 3E) and precluded the ability of WNK4 to inhibit L-WNK1- or WNK3-induced activation of NCC in oocytes (Figure 3E). These functional observations indicate that interactions of WNKs kinases via the HQ motif are necessary for their effects on NCC.

DISCUSSION

Since the discovery of WNK kinases and their relation to FHHt, an inherited disease featuring arterial hypertension, a great deal of effort has been directed at understanding how WNK kinase mutations produce the disease. As all FHHt symptoms are corrected by a thiazide treatment and as the expression of the Na-Cl cotransporter NCC is increased in FHHt mouse models, several groups aimed at understanding how the WNK kinases modulate NCC activity. However, the results thus obtained failed to generate an unifying model. In the present study, we show data supporting a revised model of WNK kinase action in signalling to NCC, in which WNK1 and WNK3 mediate activation of NCC through SPAK, and WNK4 antagonizes WNK1 and WNK3 (Figure 4).

This model can explain several contradictory observations. First, the demonstration that L-WNK1 is a strong stimulator of NCC activity clarifies the pathophysiology of FHHt (Figure S5), since increased L-WNK1 expression in the DCT is sufficient to promote the development of the FHHt phenotype[13]. Second, it fits with the *in vitro* observations that L-WNK1 is a more powerful activator of SPAK than WNK4[11]. Third, the new model provides a plausible explanation for the discrepancy between the mild phenotype consequent to WNK3 deletion *in vivo*, and the powerful ability of WNK3 to activate NCC *in vitro*[25, 26]. Since both WNK1 and WNK3 can activate NCC, the increased WNK1 expression observed in *WNK3*$^{-/-}$ mice likely compensates for the loss of WNK3[27, 28].

Previous studies failed to demonstrate the WNK1 effect on NCC for two reasons. First, those studies did not use the L-WNK1 isoform lacking exon 11, the most abundant isoform along the nephron[17] and the most powerful NCC activator. Instead, the studies used an isoform lacking both exons 11 and 12, which represents only 20% of WNK1 isoforms in the kidney[17]. Second, the previously used rat L-WNK1-Δ11-12 cDNA carries a mutation in the C-terminal region of the protein that reduces its effect on NCC. This cDNA corresponds to the first isolated L-WNK1 cDNA, which was subcloned from a rat brain library[18], where the variant missing both exons 11 and 12 is the predominant variant[17].

Co-expression of L-WNK1-Δ11 and WNK4 results in a decreased NCC activity compared to L-WNK1-Δ11 alone. This could result from the inhibition of L-WNK1-Δ11 by WNK4 or from the parallel action of an activator (L-WNK1-Δ11) and an inhibitor (WNK4). WNK kinases can form homo or heteromers via a conserved carboxyl-terminal domain[21, 29]. Co-immunoprecipitation experiments indicated that L-WNK1 interacts with both WNK4 and WNK3 in HEK293 cells through a C-terminal conserved HQ motif[22]. We show here that the interaction between L-WNK1-Δ11, WNK3 and WNK4 is essential for the regulation of NCC, as the mutation of their HQ motif abrogates the effect of all three kinases. Our study does not however allow us to determine which homo or heteromers are required for NCC regulation. The disruption of the interaction by mutating the HQ motif results in a decreased capacity of WNK3 to autophosphorylate and phosphorylate WNK1 on the T-loop residue (Ser382)[22], required for WNK1 activation. Inhibiting the formation of activating WNK3 homomers and/or L-WNK1/WNK3 heteromers could therefore prevent the activation of NCC by L-WNK1 or WNK3 in oocytes. Similarly, preventing the formation of WNK4 homomers reduced its autophosphorylation. Interestingly, Thastrup and colleagues showed that, while WNK4 can interact with WNK1, it is unable to phosphorylate its T-loop residue[22]. The expression of WNK4 in oocytes could thus result in the formation of inhibitory WNK4 homomers and/or L-WNK1/WNK4 or WNK3/WNK4 heteromers, in which L-WNK1 or WNK3 is not activated (Figure 4). The expression of WNK4-HQ mutant in *Xenopus* oocytes would then restore NCC activity by preventing the formation of these inhibitory homomers and/or heteromers.

The present study reconciles *in vivo* and *in vitro* observations about the mode of L-WNK1 action towards NCC, but controversy still remains concerning intrinsic WNK4 activity. Most *in vitro* studies, including the present one, concluded that WNK4 is an inhibitor of NCC, either when overexpressed in *Xenopus* oocytes and mammalian cells[6, 7, 23] or when endogenous WNK4 is knocked down in mDCT15 cells[9]. These results are supported by some but not all *in vivo* studies. Lalioti *et al.* first reported that WNK4 overexpression in transgenic mice results in Gitelman-like syndrome[24], caused by inactivating mutations of NCC, again suggesting that WNK4 inhibits the cotransporter. This conclusion is however not supported by two subsequent studies. First, WNK4 inactivation in mice also leads to the development of a mild Gitelman syndrome[14, 15]. Second, Wakabayashi and colleagues observed that WNK4 overexpression caused FHHt in mice[30], even though they used the same transgene than the one used in Lalioti's study. These two studies suggest that WNK4 is an activator of NCC, *in vivo*.

How could we reconcile the WNK4 inhibitory effect observed *in vitro and in vivo* with the activating effect observed *in vivo*? NCC inhibition by WNK4 in *Xenopus* can be abrogated by angiotensin II[8]. Similarly, the phosphorylation of SPAK by WNK4 is stimulated by an increased Ca^{2+} concentration[31]. One could therefore speculate that WNK4 inhibitory and stimulatory actions coexist and can be modulated by intracellular signals and/or that the environment of *in situ* DCT cells at baseline favours the activating mode of WNK4 while the culture conditions of oocytes or mammalian cells mostly favours its inhibitory mode.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

The authors would like to thank Dominique Eladari and Régine Chambrey for stimulating discussions as well as all the technicians of the E.R.I. (Elevage Rongeurs Inserm).

Sources of Funding

This work was supported by the Institut National de la Santé et de la Recherche Médicale (Inserm), the Fondation pour la Recherche pour l'Hypertension Artérielle (CS, XJ, JH), a collaborative funding from CONACYT - Agence Nationale pour la Recherche (Conacyt 188712-ANR-12-ISVS1-0001-01), the Transatlantic Network on Hypertension funded by the Fondation Leducq (both XJ, GG, JH), grants No. 165815 from the Mexican Council of Science and Technology, IN200513 from PAPIIT-DGAPA, and No. 091415 from Wellcome Trust to GG; grant No. NIH DK51496 to DHE and funds from a VA Merit Review. M.C-C was supported by a scholarship from CONACYT-Mexico.

References

1. Narayan KM, Ali MK, Koplan JP. Global noncommunicable diseases–where worlds meet. N Engl J Med. 2010; 363:1196–1198. [PubMed: 20860499]
2. Gordon, RD., Klemm, SA., Tunny, TJ., et al. Gordon's syndrome: A sodium-volume-dependent form of hypertension with a genetic basis. In: Brenner, JHLaBM., editor. Hypertension: Patholgy, diagnosis and management. New York: Raven Press Ltd; 1995. p. 2111-2113.
3. Wilson FH, Disse-Nicodeme S, Choate KA, Ishikawa K, Nelson-Williams C, Desitter I, Gunel M, Milford DV, Lipkin GW, Achard JM, Feely MP, Dussol B, Berland Y, Unwin RJ, Mayan H, Simon DB, Farfel Z, Jeunemaitre X, Lifton RP. Human hypertension caused by mutations in wnk kinases. Science. 2001; 293:1107–1112. [PubMed: 11498583]
4. Uchida S. Regulation of blood pressure and renal electrolyte balance by cullin-ring ligases. Curr Opin Nephrol Hypertens. 2014
5. Uchida S, Sohara E, Rai T, Sasaki S. Regulation of with-no-lysine kinase signaling by kelch-like proteins. Biol Cell. 2014; 106:45–56. [PubMed: 24313290]
6. Wilson FH, Kahle KT, Sabath E, Lalioti MD, Rapson AK, Hoover RS, Hebert SC, Gamba G, Lifton RP. Molecular pathogenesis of inherited hypertension with hyperkalemia: The na-cl cotransporter is inhibited by wild-type but not mutant wnk4. Proc Natl Acad Sci USA. 2003; 100:680–684. [PubMed: 12515852]
7. Yang CL, Angell J, Mitchell R, Ellison DH. Wnk kinases regulate thiazide-sensitive na-cl cotransport. J Clin Invest. 2003; 111:1039–1045. [PubMed: 12671053]
8. San Cristobal P, Pacheco-Alvarez D, Richardson C, Ring AM, Vazquez N, Rafiqi FH, Chari D, Kahle KT, Leng Q, Bobadilla NA, Hebert SC, Alessi DR, Lifton RP, Gamba G. Angiotensin ii signaling increases activity of the renal na-cl cotransporter through a wnk4-spak-dependent pathway. Proc Natl Acad Sci US A. 2009; 106:4384–4389.
9. Ko B, Mistry AC, Hanson L, Mallick R, Cooke LL, Hack BK, Cunningham P, Hoover RS. A new model of the distal convoluted tubule. Am J Physiol Renal Physiol. 2012; 303:F700–710. [PubMed: 22718890]
10. Moriguchi T, Urushiyama S, Hisamoto N, Iemura S, Uchida S, Natsume T, Matsumoto K, Shibuya H. Wnk1 regulates phosphorylation of cation-chloride-coupled cotransporters via the ste20-related kinases, spak and osr1. Journal of Biological Chemistry. 2005; 280:42685–42693. [PubMed: 16263722]
11. Vitari AC, Deak M, Morrice NA, Alessi DR. The wnk1 and wnk4 protein kinases that are mutated in gordon's hypertension syndrome, phosphorylate and active spak and osr1 protein kinases. Biochemistry Journal. 2005; 391:17–24.

12. Richardson C, Rafiqi FH, Karlsson HK, Moleleki N, Vandewalle A, Campbell DG, Morrice NA, Alessi DR. Activation of the thiazide-sensitive na+-cl- cotransporter by the wnk-regulated kinases spak and osr1. Journal of Cell Sciences. 2008; 121:675–684.
13. Vidal-Petiot E, Elvira-Matelot E, Mutig K, Soukaseum C, Baudrie V, Wu S, Cheval L, Huc E, Cambillau M, Bachmann S, Doucet A, Jeunemaitre X, Hadchouel J. Wnk1-related familial hyperkalemic hypertension results from an increased expression of l-wnk1 specifically in the distal nephron. Proc Natl Acad Sci US A. 2013; 110:14366–14371.
14. Castaneda-Bueno M, Cervantes-Perez LG, Vazquez N, Uribe N, Kantesaria S, Morla L, Bobadilla NA, Doucet A, Alessi DR, Gamba G. Activation of the renal na+:Cl- cotransporter by angiotensinII is a wnk4-dependent process. Proc Natl Acad Sci US A. 2012; 109:7929–7934.
15. Takahashi D, Mori T, Nomura N, Khan MZ, Araki Y, Zeniya M, Sohara E, Rai T, Sasaki S, Uchida S. Wnk4 is the major wnk kinase positively regulating ncc in the mouse kidney. Biosci Rep. 2014
16. Golbang AP, Cope G, Hamad A, Murthy M, Liu CH, Cuthbert AW, O'Shaughnessy KM. Regulation of the expression of the na/cl cotransporter (ncct) by wnk4 and wnk1: Evidence that accelerated dynamin-dependent endocytosis is not involved. Am J Physiol Renal Physiol. 2006; 291:F1369–F1376. [PubMed: 16788137]
17. Vidal-Petiot E, Cheval L, Faugeroux J, Malard T, Doucet A, Jeunemaitre X, Hadchouel J. A new methodology for quantification of alternatively spliced exons reveals a highly tissue-specific expression pattern of wnk1 isoforms. PloS one. 2012; 7:e37751. [PubMed: 22701532]
18. Xu B, English JM, Wilsbacher JL, Stippec S, Goldsmith EJ, Cobb MH. Wnk1, a novel mammalian serine/threonine protein kinase lacking the catalytic lysine in subdomain ii. Journal of Biological Chemistry. 2000; 275:16795–16801. [PubMed: 10828064]
19. Pacheco-Alvarez D, Vazquez N, Castaneda-Bueno M, de los Heros P, Cortes-Gonzalez C, Moreno E, Meade P, Bobadilla NA, Gamba G. Wnk3-spak interaction is required for the modulation of ncc and other members of the slc12 family. Cell Physiol Biochem. 2012; 29:291–302. [PubMed: 22415098]
20. San Cristobal P, Ponce-Coria J, Vazquez N, Bobadilla NA, Gamba G. Wnk3 and wnk4 amino terminal domain defines their effect on the renal na+:Cl- cotransporter. Am J Physiol Renal Physiol. 2008; 295:F1199–F1206. [PubMed: 18701621]
21. Lenertz LY, Lee BH, Min X, Xu BE, Wedin K, Earnest S, Goldsmith EJ, Cobb MH. Properties of wnk1 and implications for other family members. Journal of Biological Chemistry. 2005; 280:26653–26658. [PubMed: 15883153]
22. Thastrup JO, Rafiqi FH, Vitari AC, Pozo-Guisado E, Deak M, Mehellou Y, Alessi DR. Spak/osr1 regulate nkcc1 and wnk activity: Analysis of wnk isoform interactions and activation by t-loop trans-autophosphorylation. Biochemistry Journal. 2012; 441:325–337.
23. Cai H, Cebotaru V, Wang YH, Zhang XM, Cebotaru L, Guggino SE, Guggino WB. Wnk4 kinase regulates surface expression of the human sodium chloride cotransporter in mammalian cells. Kidney International. 2006; 69:2162–2170. [PubMed: 16688122]
24. Lalioti MD, Zhang J, Volkman HM, Kahle KT, Hoffmann KE, Toka HR, Nelson-Williams C, Ellison DH, Flavell R, Booth CJ, Lu Y, Geller DS, Lifton RP. Wnk4 controls blood pressure and potassium homeostasis via regulation of mass and activity of the distal convoluted tubule. Nat Genet. 2006; 38:1124–1132. [PubMed: 16964266]
25. Yang CL, Zhu X, Ellison DH. The thiazide-sensitive na-cl cotransporter is regulated by a wnk kinase signaling complex. J Clin Invest. 2007; 117:3403–3411. [PubMed: 17975670]
26. Rinehart J, Kahle KT, De Los Heros P, Vazquez N, Meade P, Wilson FH, Hebert SC, Gimenez I, Gamba G, Lifton RP. Wnk3 kinase is a positive regulator of nkcc2 and ncc, renal cation-cl- cotransporters required for normal blood pressure homeostasis. Proc Natl Acad Sci US A. 2005; 102:16777–16782.
27. Mederle K, Mutig K, Paliege A, Carota I, Bachmann S, Castrop H, Oppermann M. Loss of wnk3 is compensated for by ks-wnk1 in the kidney of the mouse. AmJ Physiol Renal Physiol. 2013; 304:F1198–F1209. [PubMed: 23427142]
28. Oi K, Sohara E, Rai T, Misawa M, Chiga M, Alessi D, Sasaki S, Uchida S. A minor role of wnk3 in regulating phosphorylation of renal nkcc2 and ncc co-transporters in vivo. Biology Open. 2012; 1:120–127. [PubMed: 23213404]

29. Yang CL, Zhu X, Wang Z, Subramanya AR, Ellison DH. Mechanisms of wnk1 and wnk4 interaction in the regulation of thiazide-sensitive nacl cotransport. J Clin Invest. 2005; 115:1379–1387. [PubMed: 15841204]

30. Wakabayashi M, Mori T, Isobe K, Sohara E, Susa K, Araki Y, Chiga M, Kikuchi E, Nomura N, Mori Y, Matsuo H, Murata T, Nomura S, Asano T, Kawaguchi H, Nonoyama S, Rai T, Sasaki S, Uchida S. Impaired klhl3-mediated ubiquitination of wnk4 causes human hypertension. Cell reports. 2013; 3:858–868. [PubMed: 23453970]

31. Na T, Wu G, Peng JB. Disease-causing mutations in the acidic motif of wnk4 impair the sensitivity of wnk4 kinase to calcium ions. Biochemical Biophysical Research Communications. 2012; 419:293–298. [PubMed: 22342722]

Perspectives

We present evidence that the kidney-enriched isoform of L-WNK1 stimulates NCC expression and activity in a SPAK-dependent manner and not through modulation of an inhibitory effect of WNK4, as previously reported. In addition, we show that WNK4 antagonizes WNK1- or WNK3-mediated activation of the co-transporter. A lot of questions, however, remain open before we can fully understand how the WNK kinases interact with each other to regulate the activity of the Na-Cl cotransporter. We believe that ones of the most important issues are to determine the WNKs relative expression level in the DCT and the characterization of the oligomers truly formed by WNK1, WNK3 and WNK4 *in vivo*.

Novelty and Significance

Since the discovery of WNK kinases and their relation to FHHt, an inherited disease featuring arterial hypertension, numerous studies have aimed at understanding how WNK kinase mutations produce the disease. Most of the FHHt phenotype is due to activation of NCC. Yet none of the models for WNK actions on NCC fully account for experimental results. In the present study, we show data supporting a revised model of WNK kinase action in signalling to NCC, in which WNK1 and WNK3 activate NCC through SPAK, and WNK4 antagonizes WNK1 and WNK3.

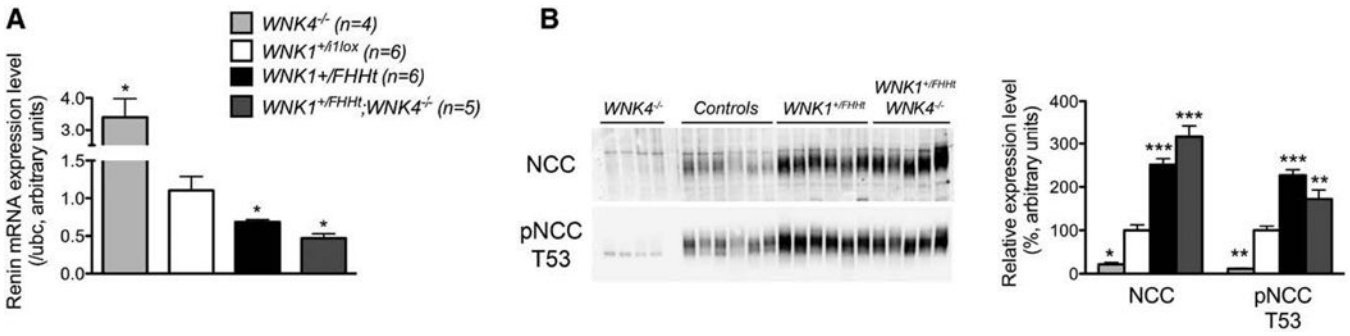

Figure 1. L-WNK1 induces hyperkalemic hypertension and NCC phosphorylation independently of WNK4 in mice

(A) Renin transcripts level was measured by quantitative real-time PCR in the kidney cortex of *WNK4*$^{-/-}$ (n=4), *WNK1*$^{+/i1lox}$ (n=5), *WNK1*$^{+/FHHt}$ (n=6) and *WNK1*$^{+/FHHt}$:*WNK4*$^{-/-}$ (n=3) males. Results are expressed in arbitrary units relative to the expression of ubc. The control group (*WNK1*$^{+/i1lox}$ mice) has been arbitrarily set to 1. Data are mean ± s.e.m. *: $P<0.05$ compared to the control group. **(B)** Left: Immunoblots performed on membrane-enriched fraction of renal cortex dissected from *WNK4*$^{-/-}$ (n=4), *WNK1*$^{+/i1lox}$ (n=6), *WNK1*$^{+/FHHt}$ (n=6) and *WNK1*$^{+/FHHt}$:*WNK4*$^{-/-}$ (n=5) males, incubated with antibodies against anti-NCC or its T53 phosphorylated residue (pNCC-T53). Right: Densitometric analysis of the immunoblots. Data, expressed as a percentage of the average signal intensity measured in the control group, set arbitrarily to 100%, are mean ± s.e.m. *: $P<0.05$, **: $P<0.01$ and ***: $P<0.001$ compared to the control group (Student's unpaired t-test).

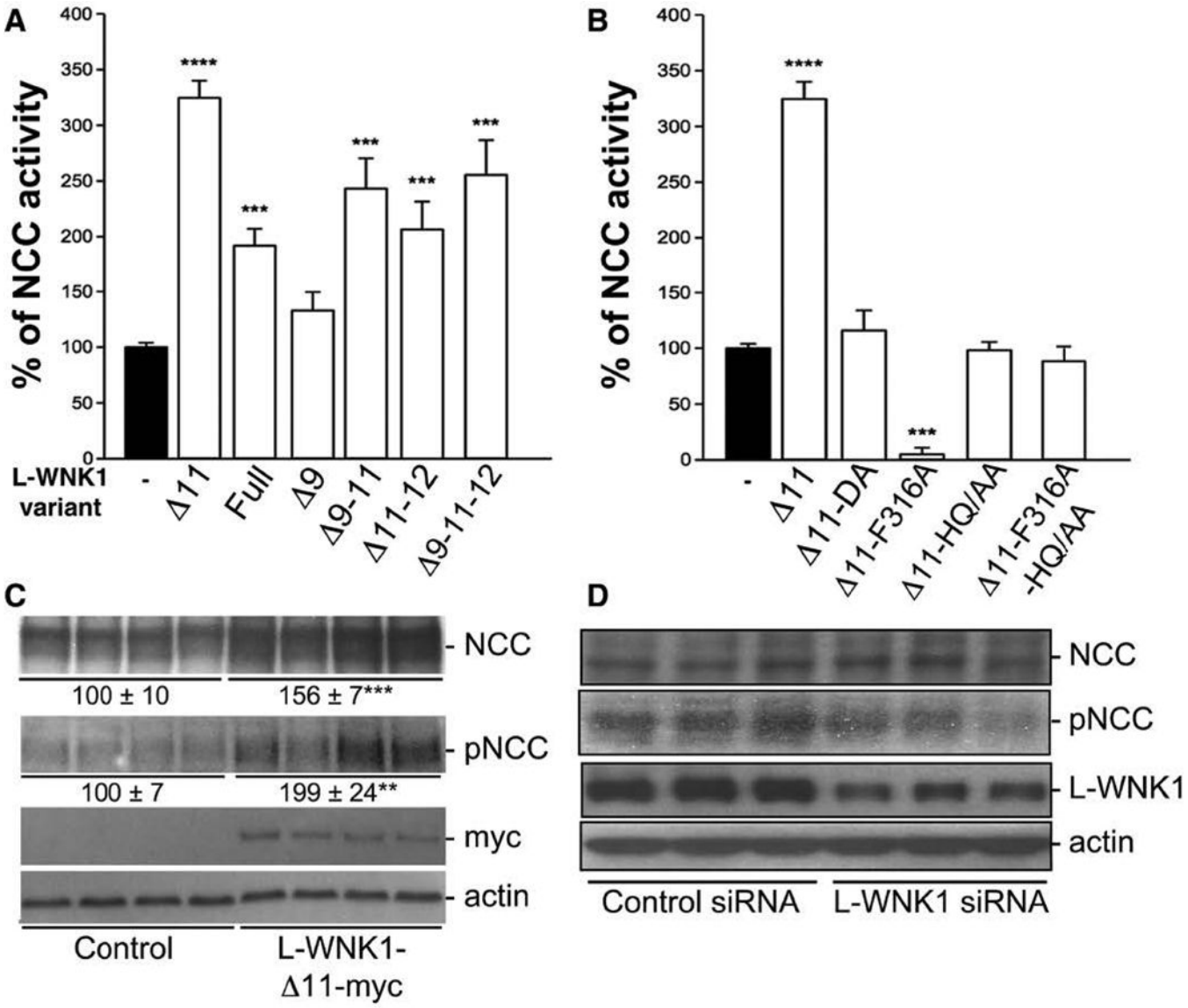

Figure 2. L-WNK1 is a powerful activator of NCC activity in *Xenopus laevis* oocytes

(A) NCC activity was measured in *Xenopus* oocytes overexpressing NCC alone or in combination with different L-WNK1 splicing variants. All L-WNK1 variants, except the one deleted from exon 9 (L-WNK1-Δ9), stimulated NCC activity. **(B)** Effect of L-WNK1-Δ11 point mutations affecting its kinase activity (Δ11-DA), SPAK binding (Δ11-F316A), dimer formation (Δ11-HQ) or both (Δ11-F316A-HQ) on NCC activity. For **(A)** and **(B)**, NCC activity in oocytes injected with NCC cDNA alone (black bar) was arbitrarily set to 100%. Data are mean ± s.e.m. ***: $P<0.001$ and ****: $P<0.0001$ vs. NCC alone (Student's unpaired t-test). **(C)** Immunoblots from NCC-HEK293 cells transiently transfected with NCC and human L-WNK1-Δ11-myc constructs, and probed with antibodies indicated. Data, indicated below the blots, are mean ± s.e.m. **: $P<0.01$ and ***: $P<0.001$ vs. the control (Student's unpaired t-test). **(D)** The inhibition of L-WNK1 expression by RNA-interference

decreased NCC phosphorylation. Immunoblots from NCC-HEK-293 cells transfected with control and WNK1-specific siRNA, probed with the antibodies indicated.

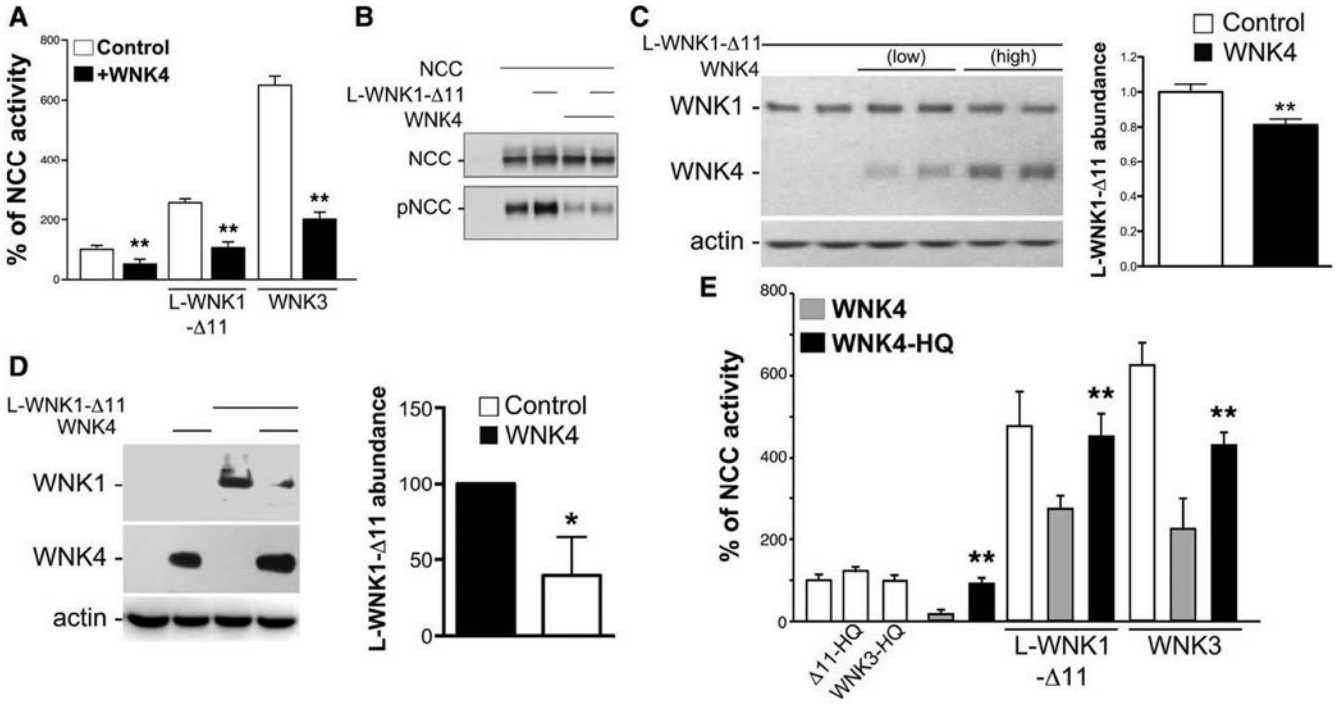

Figure 3. WNK4 inhibits the activation of NCC by L-WNK1 and WNK3

(A) NCC activity was measured in *Xenopus* oocytes injected alone or in combination with L-WNK1-Δ11 (WNK1), WNK3 and/or WNK4 as stated. WNK4 not only inhibited NCC activity but also prevented the L-WNK1-Δ11 or WNK3-dependent stimulation of NCC (N=15 independent experiments per combination). **(B)** Immunoblots from NCC-HEK293 cells transiently overexpressing L-WNK1-Δ11 and WNK4. WNK4 overexpression prevented the activation of NCC by L-WNK1-Δ11. **(C-D)** Immunoblots from NCC HEK293 cells transiently transfected with myc-tagged L-WNK1-Δ11 and WNK4 constructs **(C)** or from *Xenopus* oocytes injected with myc-tagged L-WNK1-Δ11 alone or in combination with WNK4 **(D)**. WNK4 overexpression significantly decreased abundance of L-WNK1-Δ11 in both systems. *: $P<0.05$ and **: $P<0.01$ for the comparison between L-WNK1-Δ11 alone and L-WNK1-Δ11 + WNK4 (Student's unpaired t-test). **(E)** NCC activity alone or in combination of wild type or mutant-HQ WNKs as stated. The inhibition of the formation of WNKs dimers by mutating the C-terminal HQ motif relieved L-WNK1-Δ11 or WNK3 from WNK4-mediated inhibition. For oocytes experiments, the activity measured in oocytes injected with NCC alone was arbitrarily set to 100% and data are mean ± s.e.m. **: $P<0.01$ for the comparison between wild type WNK4 and WNK4-HQ (Student's unpaired t-test).

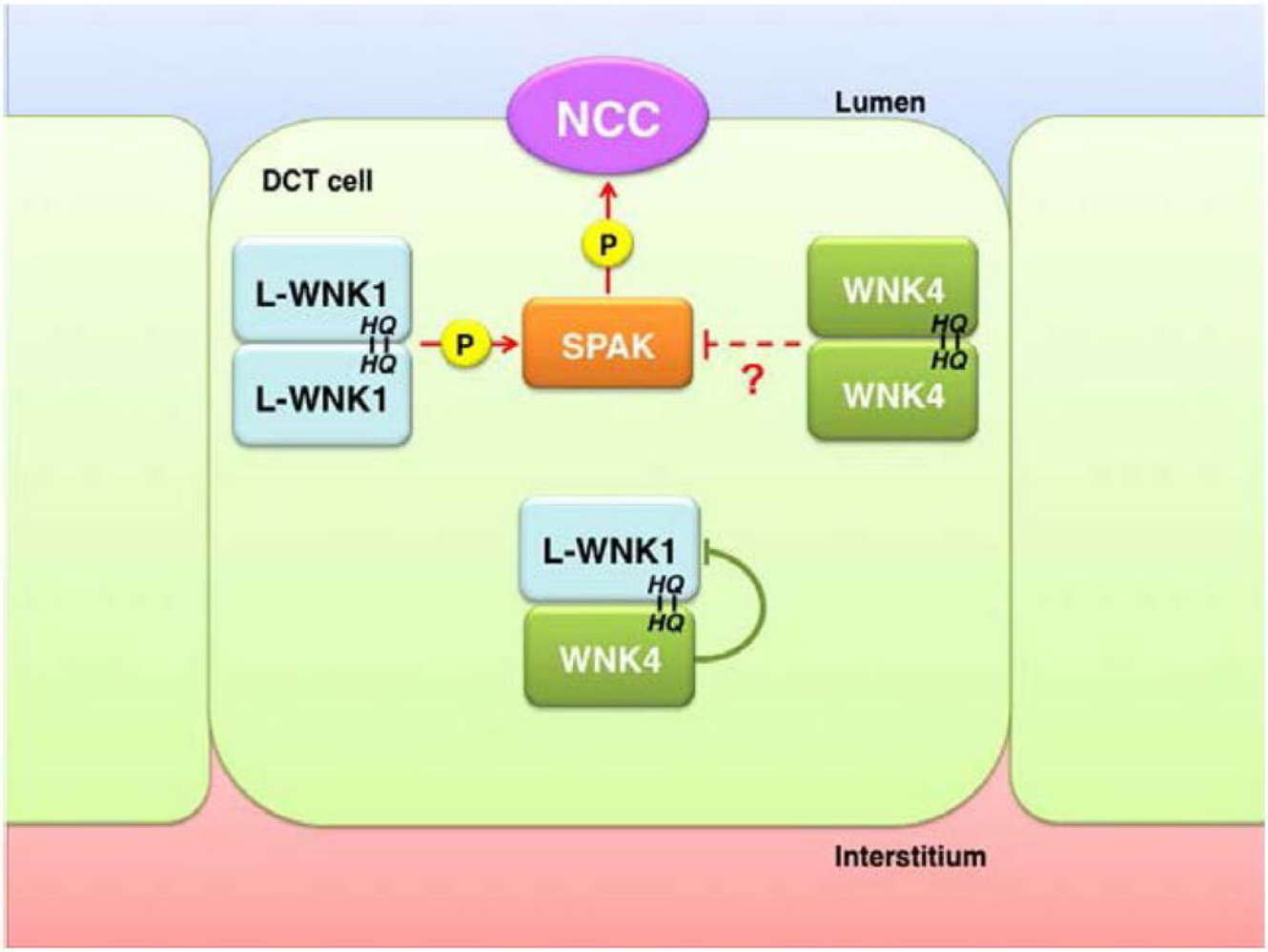

Figure 4. Proposed new model for the regulation of NCC by WNKs

Note that WNK3 is not shown and that L-WNK1 is indicated instead of L-WNK1-Δ11 to simplify the Figure. At baseline, L-WNK1-Δ11 forms homomers that activate NCC in a SPAK-dependent manner. WNK4 could antagonize the effect of L-WNK11-Δ11 on NCC by either forming inactive WNK4-WNK1 heteromers or inhibitory WNK4 homomers.

Table 1

WNK1$^{+/FHHt}$:*WNK4*$^{-/-}$ mice still exhibit FHHt metabolic abnormalities

Blood was sampled from the retro-orbital sinus and blood gas and electrolytes were measured using a Radiometer® ABL80 Flex analyzer.

Plasma	*WNK4*$^{-/-}$ (n=4-5)	Controls (n=14-22)	*WNK1*$^{+/FHHt}$ (n=15-29)	*WNK1*$^{+/FHHt}$:*WNK4*$^{-/-}$ (n=13)
Na^+ (mmol/L)	144.5 ± 0.5	147.4 ± 0.4	147.7 ± 0.3	149.0 ± 0.4 *
K^+ (mmol/L)	3.9 ± 0.1	4.1 ± 0.1	4.9 ± 0.1 ***	5.0 ± 0.1 ***
Cl^- (mmol/L)	107.0 ± 0.7 ***	111.1 ± 0.4	114.6 ± 0.3 ***	115.3 ± 0.5 ***
pH	7.39 ± 0.01 **	7.32 ± 0.01	7.28 ± 0.01 **	7.28 ± 0.02 *
HCO_3^- (mmol/L)	27.2 ± 1.5 **	24.6 ± 0.4	22.7 ± 0.4 *	23.6 ± 0.6
Hematocrit	47.2 ± 0.5 **	42.8 ± 0.6	39.7 ± 0.5 ***	39.3 ± 0.6 **

* *P*<0.05,

** *P*<0.01 and

*** *P*<0.001 compared to controls (One-way ANOVA followed by Dunnet's multiple comparisons test).