

Regulation of the renal Na⁺-Cl⁻ cotransporter by phosphorylation and ubiquitylation

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Gamba G. Regulation of the renal Na⁺-Cl⁻ cotransporter by phosphorylation and ubiquitylation. *Am J Physiol Renal Physiol* 303: F1573–F1583, 2012. First published October 3, 2012; doi:10.1152/ajprenal.00508.2012.—The activity of the renal thiazide-sensitive NaCl cotransporter (NCC) in the distal convoluted tubule plays a key role in defining arterial blood pressure levels. Increased or decreased activity of the NCC is associated with arterial hypertension or hypotension, respectively. Thus it is of major interest to understand the activity of NCC using in vivo models. Phosphorylation of certain residues of the amino-terminal domain of NCC has been shown to be associated with its activation. The development of phospho-specific antibodies against these sites provides a powerful tool that is helping to increase our understanding of the molecular physiology of NCC. Additionally, NCC expression in the plasma membrane is modulated by ubiquitylation, which represents another major mechanism for regulating protein activity. This work presents a review of our current knowledge of the regulation of NCC activity by phosphorylation and ubiquitylation.

WNK4; distal tubule; ion transport; hypertension; diuretics

THE RENAL THIAZIDE-SENSITIVE NaCl cotransporter (NCC) is the major salt transport and limiting step for salt reabsorption in the distal convoluted tubule (DCT) of mammalian kidneys. The role of NCC in the regulation of arterial blood pressure and in the renal ability for potassium, calcium, and proton excretion has been firmly established by the clinical effects of reduced or augmented activity of the cotransporter. Inactivating mutations in the *SLC12A3* gene encoding NCC results in Gitelman's disease, in which patients exhibit hypokalemic metabolic alkalosis, arterial hypotension, and hypocalciuria. In contrast, an increased activity of NCC, as a result of mutations in the with no lysine kinases *WNK1* or *WNK4* genes, produces the mirror image condition: hyperkalemic metabolic acidosis, accompanied by arterial hypertension, and hypercalciuria. This condition is known as Gordon syndrome, pseudohypoaldosteronism type II (PHAII), or familial hyperkalemic hypertension (FHHT) (27). The thiazide-type diuretics that specifically inhibit NCC have been used for years and are recommended as the first-line pharmacological therapy for arterial hypertension (16). Finally, in an open population, rare inactivating mutations in one allele of NCC, which reduce the activity of the cotransporter (3), are associated with reduced blood pressure, lower risk for arterial hypertension, and no cardiovascular mortality (3, 40). Thus the activity of NCC plays a fundamental role in cardiovascular physiology and pathophysiology.

The study of NCC was practically impossible for years because of the lack of a native cell model purified from the DCT that exhibited thiazide-sensitive Na⁺ transport activity.

The first tool for studying NCC using in vivo models was developed by Fanestill and coworkers (7) at the end of the 1980s: binding of the tracer [³H]metolazone to crude plasma membranes extracted from rat or mouse renal cortex was assessed. The authors demonstrated that [³H]metolazone binds to both a low- and a high-affinity site in the renal cortex. The high-affinity site showed several binding characteristics, demonstrating that the tracer was actually binding to NCC, then called the thiazide receptor. These characteristics included the absence of high-affinity sites in any other tissue, including the renal medulla; displacement of the tracer by different thiazide-type diuretics, but not by any other tested drug, with a similar potency to thiazide in clinical studies; and the localization of these sites only to the DCT as confirmed using autoradiography (22). A few years later, however, better tools were developed to study NCC in both in vitro and in vivo models. First, expression cloning was used to identify the cDNA encoding the NCC from winter flounder (*Pseudopleuronectes americanus*) urinary bladder (30). Next, the mammalian NCC cDNA was isolated using a homology approach, first from rat kidney (29) and later from human (53, 80), mouse (48), and rabbit (91) kidney. With the molecular identification of NCC, it became possible to generate antibodies against the cotransporter that allowed the immunolocalization of NCC specifically to the DCT, with more prominent expression in the early portion of the DCT (DCT1) than in the later portion (DCT2) (69). Together with probes and specific primers for assessing mRNA expression, the effects of several stimuli on NCC mRNA and protein expression levels (44) were studied using animal models to understand the role of NCC in physiological and pathophysiological conditions. NCC, and therefore DCT, was identified as a key site for modulation of NaCl transport and hence,

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for blood pressure regulation (21). However, it quickly became obvious that many stimuli do not affect NCC expression levels. Thus assessing the activity of the cotransporter, in addition to the expression level, would be required for a better understanding of the role of the cotransporter in many situations.

NCC Activity Correlates with Phosphorylation

The regulation of NCC activity by the phosphorylation of conserved threonine and serine residues in the amino-terminal domain was first demonstrated by Pacheco-Alvarez et al. (65). They first demonstrated in *Xenopus laevis* oocytes expressing

NCC that the activity of NCC dramatically increased when the intracellular chloride concentration was lowered using two different techniques (Fig. 1A). One method involved low chloride hypotonic stress, in which incubation of oocytes in a light hypotonic medium (170 instead 210 mosmol/kgH₂O) without chloride (substituted by isotheaionate) promotes the opening of endogenous chloride channels (2) that efficiently decrease the intracellular chloride concentration (42). The other was the coinjection of oocytes with NCC cRNA, together with the cRNA of K⁺-Cl⁻ cotransporter KCC2. This isoform of KCC remains active in isotonic conditions

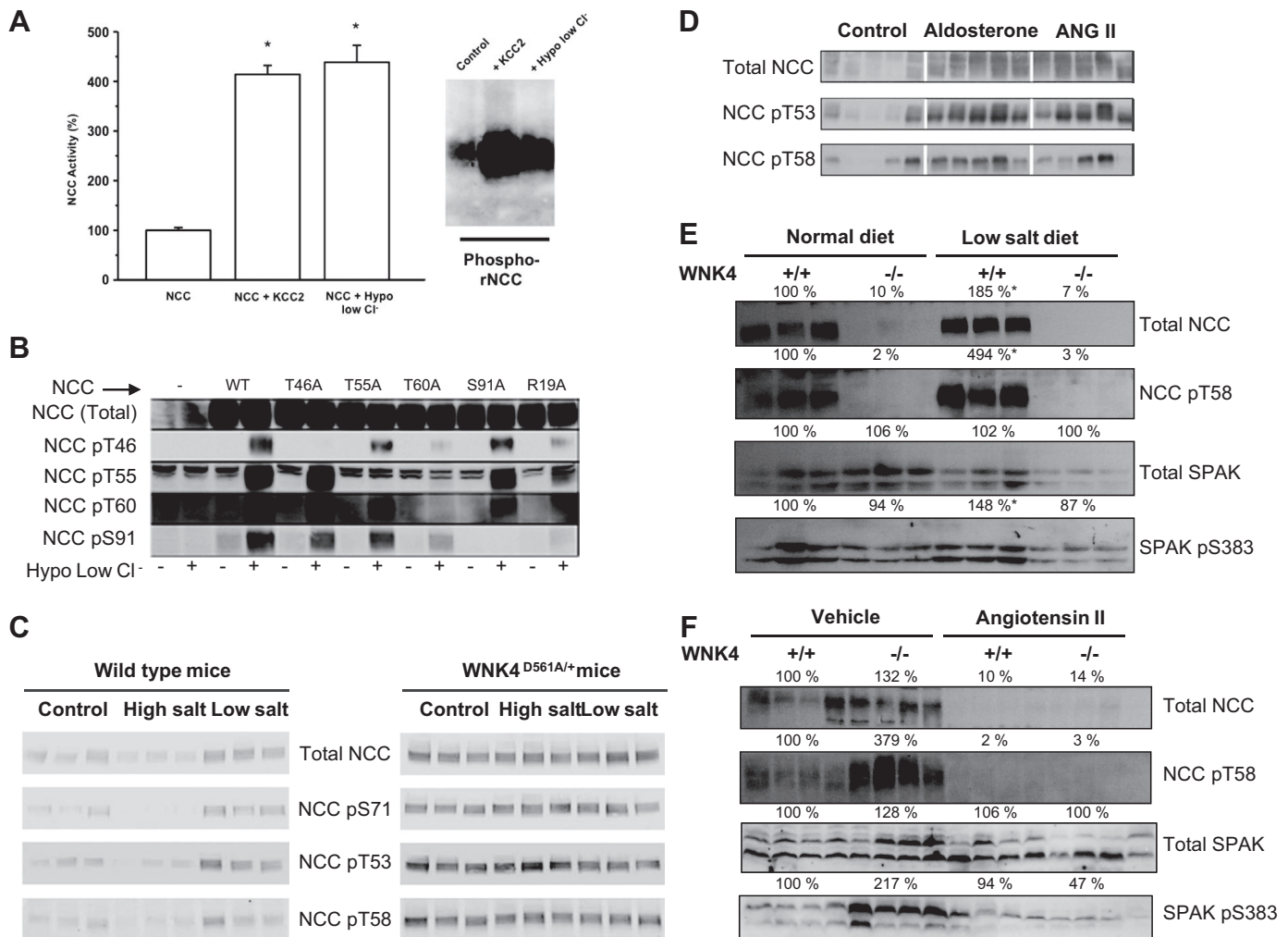


Fig. 1. Activity of the renal thiazide-sensitive NaCl cotransporter (NCC) is modulated by phosphorylation. *A*: percent NCC activity in the heterologous expression system of *Xenopus laevis* oocytes is increased by 2 different maneuvers inducing intracellular chloride depletion (**P* < 0.001 vs. NCC alone). This activation is associated with increased phosphorylation of threonine residues 53 and 58, as detected with the phospho-specific antibody R5 (modified from Ref. 65). *B*: detection of total NCC or phosphorylated NCC in threonine residues 46, 55, 60 or serine residue 91 using specific phospho-antibodies rose against each site, in extracted proteins from HEK-293 cells transiently transfected with wild-type or mutant NCC, as stated. The blots are shown in control conditions or after exposing of the cells to low chloride hypotonic stress, as stated (modified from Ref. 72 with permission). *C*: Western blot analysis of renal proteins from wild-type or with no lysine kinase WNK4^{D561A/+} mice exposed to control, a high-salt, or low-salt diet. Western blots were performed using the total anti-NCC antibody, or specific antibodies against phosphorylated threonine 53, threonine 58, or serine 71, as stated (modified from Ref. 15 with permission). *D*: NCC expression and phosphorylation at residues T53 or T58 in adrenalectomized rats treated with vehicle, aldosterone, or angiotensin II infusion (modified from Ref. 89 with permission). *E*: NCC expression and phosphorylation at T60 and Ste20-related proline/alanine-rich kinase (SPAK) expression and phosphorylation at S383, as stated, in wild-type or WNK4 total knockout mice exposed to a normal- or low-salt diet, as stated (modified from Ref. 12). *F*: NCC expression and phosphorylation at T60 and SPAK expression and phosphorylation at S383, as stated, in wild-type or WNK4 total knockout mice infused with vehicle or angiotensin II (modified from Ref. 12).

(83) and was therefore expected to promote a continuous extrusion of chloride ions from the oocytes during the incubation period. The efficiency of KCC2 in decreasing the intracellular chloride concentration was later demonstrated by Bertram et al. (8). At the time of these findings, there were no phospho-specific NCC antibodies. Therefore, the NKCC1 phospho-specific antibody (R5) developed by Forbush and coworkers (26) was used. However, for the R5 antibody to recognize phospho-NCC, a single amino acid change was made to rat NCC. A tyrosine residue in position 56 was changed to a histidine, which is the residue in the same position of $\text{Na}^+\text{-K}^+\text{-2Cl}^-$ cotransporters NKCC1 and NKCC2 that is recognized by R5 antibodies. It was observed that this single point mutation did not change the functional properties of NCC or its activation by intracellular chloride depletion.

As shown in Fig. 1A, the increased activity of NCC induced by intracellular chloride depletion was associated with increased phosphorylation of threonine residues 53 and 58, as detected with the R5 antibody (65). An increased signal in activated NCC was not observed in the presence of alkaline phosphatase, indicating that it was a result of phosphorylation. Eliminating threonine residues 53 and 58, as well as serine residue 71, was associated with decreased basal activity of NCC and the complete prevention of the activation by intracellular chloride depletion, particularly when T58 was eliminated. Interestingly, the decreased activity observed in the triple mutant of NCC (T53A, T58A, and S71A) was not associated with a change in the expression level at the cell surface. Thus this study demonstrated that increased phosphorylation of amino-terminal threonine/serine residues in NCC are correlated with increased activity of the cotransporter. Therefore, "NCC activity" could be indirectly assessed by analyzing NCC phosphorylation with *in vivo* models. Moreover, a subsequent study showed that, in *X. laevis* oocytes, NCC activity is significantly reduced by coexpression with protein phosphatase 4 and that T58 is the target threonine for dephosphorylation by this phosphatase (32).

After this observation, specific phospho-antibodies for detecting equivalent threonine/serine residues in human or mouse NCC were raised (72, 99). Richardson et al. (72) used mass spectrophotometric analysis of NCC protein extracted from NCC-transfected HEK-293 cells and observed that low chloride hypotonic stress resulted in the phosphorylation of threonine residues 46, 55, and 60 and serine residue 91 of human NCC. This finding corroborated the importance of T53 and T58 of rat NCC (equivalent to 55 and 60 in human NCC) and added two new sites. Specific phospho-antibodies were raised for each site and used in transfected HEK-293 cells and in mpkDCT cells endogenously expressing NCC. Thus it was confirmed that the low chloride hypotonic stress increased the phosphorylation of these sites (Fig. 1B). There were two additional important observations from this study. One was that the R19A mutation, which eliminated a Ste20-related proline/alanine-rich kinase (SPAK) binding site in NCC, prevented the phosphorylation of these sites, implicating SPAK as the responsible kinase (Fig. 1B). The other was that eliminating threonine 60 (NCC-T60A) prevented or reduced the phosphorylation of the other sites, supporting a previous suggestion (65) that this threonine residue was key in the regulation of NCC activity.

Yang et al. (99) raised specific phospho-antibodies against serine 71. These antibodies were used to show in a knockin model of PHAII that, indeed, the mutation D561A in one allele of WNK4 resulted in increased phosphorylation of NCC in this residue (Fig. 1C). $\text{WNK4}^{\text{(D561A/+)}}$ mice exhibited increased expression of NCC in the apical membrane of DCT cells and increased phosphorylation of SPAK in renal tissue. These findings suggested that WNK4 mutations causing PHAII produce an increase in SPAK phosphorylation, which in turn induces an increase in NCC phosphorylation at serine 71 and in the surface expression of the cotransporter.

Two additional phosphorylation sites have been observed in NCC by using large-scale proteomics from human urine or rat renal cortex. In humans exosomes from urine, phosphorylation of NCC at serine 811 was observed (34). This serine is part of an exon that is present in humans, but not in the rat or mouse. Its significance is not known. In rat renal cortex, phosphorylation of serine 124 of NCC was also detected by this methodology (24). A recent study shows that this serine phosphorylation is increased by vasopressin and a low-salt diet (74).

Activation of NCC by a Low-Salt Diet

The studies discussed above revealed the close correlation between NCC activity and phosphorylation (65) and generated the tools for detecting NCC phosphorylation (72, 99). Thus it became possible to measure NCC activity using *in vivo* models. Table 1 shows a compilation of the studies in which the phosphorylation of NCC has been assessed. One of the first issues for researchers in the field was to analyze the role of NCC in salt handling when the salt content of a diet changed. Thus NCC phosphorylation was assessed in animals subjected to low- or high-salt diets. The first such study was performed by Chiga et al. (15), who observed that high- and low-salt diets were associated with decreased and increased phosphorylation of NCC, respectively, in threonine residues 53 and 58, as well as in serine residue 71 (Fig. 1C). The phosphorylation of serine 71 during a low-salt diet was partially prevented by spironolactone, suggesting that aldosterone modulates the phosphorylation of NCC. However, the ratio between phosphorylated NCC and total NCC was not presented. Thus the effect observed with spironolactone could be solely a consequence of NCC expression levels, which are affected by aldosterone (44). In another study, Vallon et al. (88) observed that the increased phosphorylation of NCC at the same residues during a low-salt diet was attenuated in serum glucocorticoid 1 (SGK1)-knock-out mice. Because SGK1 is involved in the effects of aldosterone in the distal nephron, this observation supported the idea that increased phosphorylation of NCC during a low-salt diet is partially a result of aldosterone's effects. It was subsequently observed that NCC phosphorylation could be achieved independently by both angiotensin II and aldosterone. Angiotensin II modulates NCC trafficking in DCT cells from rats (78, 79) and increases the activity and phosphorylation of NCC in oocytes (77). Therefore, to distinguish between the effects of angiotensin II and aldosterone, Van der Lubbe et al. (89) performed a study in rats in which the adrenal glands were removed and subsequently treated with vehicle, aldosterone, low nonpressor doses of angiotensin II, or pressor doses of this hormone. Without adrenal glands, NCC expression and phosphorylation and SPAK phosphorylation in the rats were in-

Table 1. Phosphorylation of NCC in different sites and conditions

Experimental System/Model	Stimulus	Effect	Phospho-Site*	Kinase Involved	Reference No.
<i>Xenopus</i> oocytes	Intracellular Cl ⁻ depletion	↑↑	T ⁵⁵ , T ⁶⁰ , S ⁷³	?	65
Mouse	WNK4 ^(D561A/+) knockin	↑↑↑	S ⁷³	WNK4	99
HEK-293 cells	Intracellular Cl ⁻ depletion	↑↑↑	T ⁴⁶ , T ⁵⁵ , T ⁶⁰ , S ⁹¹	SPAK	72
Rat	Low-salt diet	↑↑	T ⁵⁵ , T ⁶⁰ , S ⁷³	?	15
Human urine	None	↑↑	S ⁸¹¹	?	34
<i>Xenopus</i> oocytes/mpkDCT cells	Angiotensin II	↑↑	T ⁵⁵ , T ⁶⁰	WNK4-SPAK	77
Mouse	Low-K ⁺ diet	↑↑	T ⁵⁵ , T ⁶⁰ , S ⁷³	SGK1	88
Mouse	WNK4 hypomorphic	↓	T ⁵⁵ , T ⁶⁰ , S ⁷³	WNK4	64
Brattleboro rats	dDAVP	↑↑↑	T ⁵⁵ , S ⁷³	?	60
Brattleboro rats/Wistar rats	dDAVP	↑↑↑	T ⁵⁵ , T ⁶⁰	SPAK	67
Mouse	SPAK knockin	↓↓↓	T ⁵⁵ , T ⁶⁰ , S ⁹¹	SPAK	71
mpkDCT cells/mouse	Angiotensin II/aldosterone	↑↑↑	T ⁵⁵ , T ⁶⁰ , S ⁷³	SPAK	86
Adrenalectomized rats	Angiotensin II/aldosterone	↑↑↑	T ⁵⁵ , T ⁶⁰	WNK4-SPAK	89
Mouse	KS-WNK1 knockin	↑↑↑	T ⁴⁵ , T ⁵⁵ , T ⁶⁰ , S ⁷³	WNK1/WNK4	36
Mouse	KS-WNK1 knockout	↑↑	T ⁶⁰	WNK1/WNK4	51
In tube	Incubation with MO25	↑↑	T ⁴⁵ , T ⁵⁵ , T ⁶⁰ , S ⁹¹	SPAK/OSR1	25
Mouse	SPAK knockout	↓↓↓	T ⁵⁵ , T ⁶⁰ , S ⁷³	SPAK	98
Mouse	SPAK knockout	↓↓↓	T ⁵⁵ , S ⁷³	SPAK	54
mDCT cells/rats	Cyclosporine	↑↑	T ⁵⁵ , T ⁶⁰ , S ⁷³	?	56
Mouse	Tacrolimus	↑↑	T ⁵⁵	WNK3/WNK4	38
Mouse	NCC transgenic	↔	T ⁵⁵	?	55
Mouse	WNK4 Knockout	↓↓	T ⁶⁰	WNK4/SPAK	12
mpkDCT cells/mouse	Insulin	↑↑	T ⁵⁵ , T ⁶⁰ , S ⁷³	WNK4/SPAK	81
<i>Xenopus</i> oocytes Ex vivo kidney	Insulin	↑↑	T ⁶⁰	WNK3	14
ZO obese Zucker rats	Hyperinsulinism	↑↑	T ⁵⁵	WNK4	47
Mouse	KS-OSR1 knockout	↑↑	T ⁵⁵	OSR1/SPAK	50
Mouse	Isoproterenol-salt-sensitive hypertension	↑↑	T ⁵⁵ , S ⁷³	WNK4	58
In tube	WNK4/Ca ²⁺	↑↑↑		WNK4	61
<i>Xenopus</i> oocytes	WNK3	↑↑↑	T ⁶⁰	WNK3/SPAK	66
Rats	Large-scale proteomics	↑↑	S ¹²⁴	?	24
Brattleboro rats	dDAVP	↑↑	S ¹²⁴	?	74

*Numbers correspond to human Na-Cl cotransport (NCC) sequence. DCT, distal convoluted tubule; WNK, with no lysine kinase; SPAK, Ste20-related proline/alanine-rich kinase.

creased by either angiotensin II or aldosterone, indicating that angiotensin II affects NCC independently of aldosterone. Thus the increased phosphorylation of NCC during a low-salt diet is most likely a result of the combined effects of angiotensin II and aldosterone on DCT cells (Fig. 2). The aldosterone-sensitive distal nephron begins at DCT2 because 11- β -hydroxysteroid dehydrogenase type II enzyme is absent in DCT1, where NCC is more heavily expressed. This enzyme eliminates cortisol, preventing the promiscuous effect of the glucocorticoid on the aldosterone receptor (6). Thus the angiotensin II effects on NCC phosphorylation might occur in both DCT1 and DCT2, while the effect of aldosterone occurs only in the NCC of DCT2. No study thus far has specifically address this issue.

Regulation of NCC by the WNKs-SPAK Complex

A decade ago, it was revealed that PHAII is a result of mutations in two genes encoding WNK1 and WNK4 (94). The PHAII clinical picture is the mirror image of Gitelman's disease and is reversed by low doses of thiazide-type diuretics. Therefore, it was suggested that WNK1 and WNK4 regulate NCC and that the pathophysiology of PHAII may be caused by impaired regulation of NCC by mutant WNK kinases. PHAII resulting from mutations in the *PRKWNK1* gene is caused by intronic deletions that apparently increase the expression of wild-type WNK1. In contrast, PHAII associated with mutations in the *PRKWNK4* gene is caused by missense mutations in a highly conserved acidic region of WNKs. Initial observations suggested that wild-type WNK4 reduces NCC activity

and that this effect did not occur in WNK4 harboring PHAII-type missense mutations (11, 95, 96). Subsequently, it was demonstrated using in vivo models that extra-wild-type WNK4 activity (transgenic mice with 4 wild-type WNK4 alleles) is associated with reduced activity of NCC (Gitelman-like phenotype) (49). In contrast, mice with WNK4-PHAII-type mutant alleles (99), even in the presence of two normal WNK4 alleles (49), developed the PHAII phenotype associated with hypertrophy of the DCT and increased phosphorylation of NCC at serine 71. WNKs were found to lie upstream of other serine/threonine kinases of the STE-20 type known as SPAK and OSR1 (92), which appear to be the kinases that actually phosphorylate NCC (Fig. 2) (72). Supporting this hypothesis, WNKs induce phosphorylation of SPAK at threonine residue 243 (93). Additionally, the knockin mice in which this site in SPAK was substituted with alanine, SPAK^{T243A/T243A} mice, developed a Gitelman-like syndrome, with arterial hypotension and a decreased phosphorylation of NCC at residues T53, T58, and S71 (71). Similar observations were obtained with the complete SPAK knockout colony (54, 98).

Experimental evidence in several models including *X. laevis* oocytes, mammalian epithelial cells, and genetically altered mice consistently shows that under certain circumstances, WNK4 behaves as an inhibitor of NCC. However, in the presence of PHAII-type mutations, WNK4 acts as an activator (13). This can be explained, at least in part, by the observation that the WNK4 effect on SPAK/NCC is modulated by angiotensin II (Fig. 2). This peptide hormone produces a positive

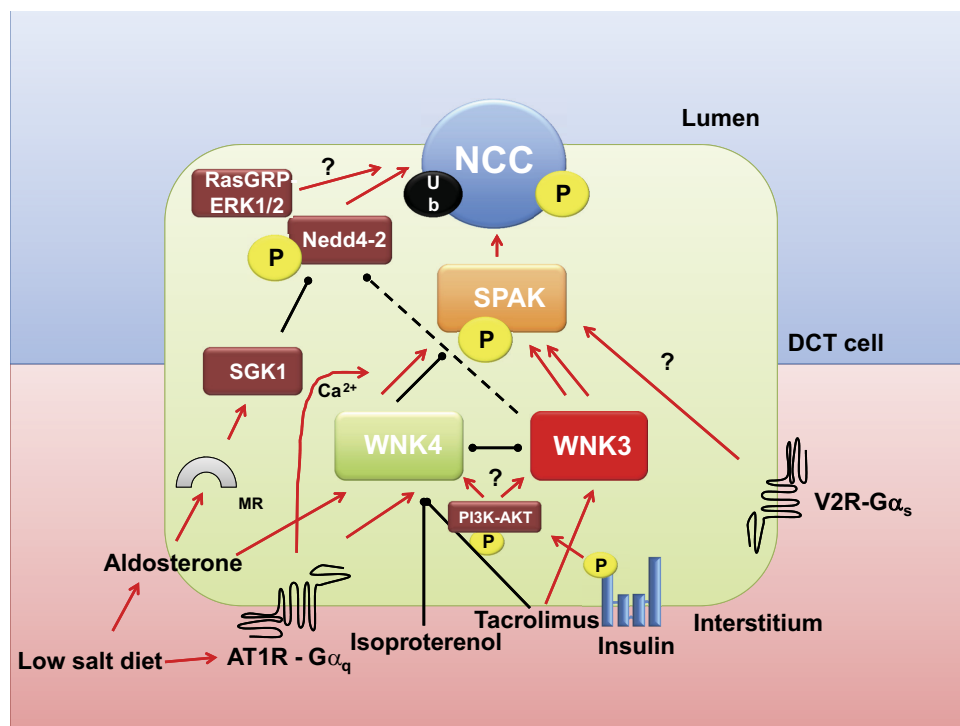


Fig. 2. Regulation of NCC by ubiquitylation and phosphorylation. See text for explanations. MR, mineralocorticoid receptor; red arrows, activation; black lines, inhibition. To simplify the figure, WNK1 effects were not included.

effect on NCC trafficking toward the apical membrane of DCT cells (79) and induces increased thiazide-sensitive salt reabsorption in the distal nephron (101). In the *X. laevis* oocyte expression system, coexpression of WNK4 and NCC results in reduced activity of NCC (95, 96). This effect is reversed in the presence of angiotensin II and can be prevented by losartan, a specific AT₁ receptor blocker (77). Interestingly, angiotensin II increased the activity of NCC when oocytes were coinjected with WNK4, but not in the absence of WNK4. This finding suggested that the effect of angiotensin II on NCC is WNK4 dependent. The activation of NCC by angiotensin II in the presence of WNK4 was also SPAK dependent and associated with increased phosphorylation of both SPAK and NCC, not only in oocytes but also in the mpkDCT cell line (77, 86). The requirement of WNK4 for the angiotensin II-induced activation of NCC has been confirmed in vivo. The NCC-induced phosphorylation in threonine 60 and SPAK at serine 383 by a low-salt diet or by chronic angiotensin II administration in wild-type animals was not observed in the WNK4^{-/-} knockout mice (12) (Fig. 1, E and F). A potential biochemical explanation has been proposed. The WNK4-induced SPAK/OSR1 and NCC phosphorylation appears to be modulated by the calcium ion concentration. The NCC phosphorylation is higher when incubated in high (1 μM) than in low (10 nM) calcium concentrations. Interestingly, this difference does not occur when WNK4 constructs with PHAII-type mutations are used (61). Because the AT₁ receptor is coupled to a Gα_q type of G protein, angiotensin II binding induces an increase in calcium concentration in the cytoplasm by promoting the production of IP₃ that stimulates the release of calcium by the smooth endoplasmic reticulum.

Considering all of these studies together, we have suggested that PHAII-type mutations in WNK4 are gain-of-function mutations, mimicking the effect of angiotensin II on the WNK4-

SPAK-NCC (6, 12, 13, 77) and providing a pathophysiological explanation for PHAII. Because PHAII is a dominant disease, having one constitutively active allele of WNK4, the DCT behaves as if angiotensin II is present and explains the salt retention and hence arterial hypertension. This explanation also applies to hyperkalemia because increased salt reabsorption in the DCT reduces the salt delivery to the collecting duct, decreasing the possibility for Na⁺/K⁺ exchange via the epithelial sodium channel (ENaC)/renal outer medullary potassium channel (ROMK). Additionally, angiotensin II reduces the activity of ROMK by a WNK4-dependent mechanism (100).

Phosphorylation of NCC by WNK4/SPAK may be involved in at least two known models of arterial hypertension. Mu et al. (58) presented evidence supporting the hypothesis that salt-sensitive hypertension induced by isoproterenol administration is caused by an epigenetic-induced decrease in WNK4 expression, leading to an increased phosphorylation of NCC, thus increasing salt reabsorption in the DCT (Fig. 2). Similarly, two independent groups have shown that the arterial hypertension associated with calcineurin inhibitors, such as cyclosporine or tacrolimus, that are extensively used to prevent allograft rejection in organ transplantation, is associated with changes in WNK3 and WNK4 expression, and consequently in NCC phosphorylation (Fig. 2). This finding suggests that the arterial hypertension induced by these compounds is a result of increased salt reabsorption in the DCT (38, 56).

The effect of WNK1 on NCC activity remains elusive. No evidence of WNK1 effect on NCC has been reported. Instead, it was observed in *X. laevis* oocytes that WNK1 prevented the WNK4-induced inhibition of NCC activity (96), thus suggesting that increased WNK1 expression could exaggeratedly prevent the WNK4 inhibition of NCC, thus increasing the activity of the cotransporter. Later on it was observed that WNK1

produces a specific variant that is exclusively expressed in the kidney, particularly in DCT. This variant, named KS-WNK1 for kidney-specific WNK1, lacks the entire kinase domain and prevents the WNK1-induced inhibition of WNK4's negative effect on NCC (18, 64, 85). Thus it was proposed that under normal circumstances, KS-WNK1 modulates the effect of the longer WNK1 on WNK4-NCC. In this case, PHAII could be explained by deletion of intron 1 of WNK1 that results in increased expression of the longer form of WNK1, surpassing the inhibitory effect of KS-WNK1 and thus preventing the inhibitory effect of WNK4 on NCC. This proposal, however, awaits confirmation in animal models. On the one hand, deletion of intron 1 in mouse results in overexpression of both the long and the short WNK1 variants (17). On the other hand, however, KS-WNK1 knockout mice exhibit increased expression and phosphorylation of NCC (Table 1) (36, 51).

WNK3 kinase is a powerful activator of NCC and consequently also phosphorylates threonine residue 58 in NCC (Fig. 2) (66). The positive effect is also associated with increased expression of NCC in the plasma membrane (33, 73). One group suggested that this effect is SPAK independent because coexpression of WNK3 and NCC with a dominant negative version of SPAK did not prevent the activation of NCC by WNK3 (33). However, the elimination of the SPAK binding site located at phenylalanine 242 of WNK3 completely prevents the activation of NCC, suggesting that a WNK3-SPAK interaction is required (66). Moreover, the requirement of SPAK for WNK3 modulation of other members of the SLC12 family, including NKCC1, NKCC2, and KCC4, has also been observed (66, 70).

Vasopressin Modulates NCC Activity

Vasopressin is a neuropeptide hormone produced in the hypothalamus and released in the posterior pituitary gland in response to increased plasma osmolarity or decreased blood pressure. Consequently, vasopressin promotes the formation of concentrated urine and increases blood pressure. Similarly to many other hormones (catecholamines, atrial natriuretic peptide, angiotensin II, aldosterone, etc.), vasopressin modulates blood pressure by modifying both vascular smooth muscle contraction and urinary salt and water reabsorption. For vasopressin, these effects are transduced in the smooth muscle and renal tubular cells through the G α -coupled receptors V1 (G α_q) and V2 (G α_s), respectively. The role of antidiuretic hormone in modulating the activity of NKCC2, urea transporters, and aquaporin 2 in the kidney is very well known. However, these actions modulate extracellular osmolarity rather than arterial pressure. Early micropuncture studies from Elalouf et al. (20) in homozygous Brattleboro rats suggested that vasopressin increases salt reabsorption in the DCT. In addition, the expression of the V2 receptor in the DCT has been documented using in situ hybridization and immunohistochemistry of human, mouse, and rat kidney (59). Furthermore, by analyzing the phosphorylation of NCC, two groups have independently confirmed the effect of vasopressin on NCC (60, 67). Administering dDAVP to Brattleboro rats resulted in increased phosphorylation of NCC at residues T53, T58, and S71. Both groups showed that the effect occurs in the DCT, independently of other potential hormones such as angiotensin II or aldosterone. Pedersen et al. (67) suggested that vasopressin's effect could

be through SPAK activation (Fig. 2). An interesting discrepancy regarding the effect of phosphorylation on NCC membrane trafficking is discussed below.

Insulin Modulates NCC Activity

There is a clear association between blood pressure levels and body weight. The higher the body mass index, the higher the prevalence of arterial hypertension. Metabolic syndrome, obesity, and diabetes mellitus type II are risk factors for the development of hypertension. One possibility is that hyperinsulinism that is often seen in these syndromes plays a critical role in the development of hypertension. It is known that obesity and hyperinsulinemic states are associated with increased expression of the distal nephron salt transporters (9, 43, 82, 87). In this regard, it has recently been shown by three independent groups that insulin or hyperinsulinemic states are associated with increased activity and phosphorylation of NCC (Table 1). Shoara et al. (81) observed in mpkDCT cells that insulin induces phosphorylation of SPAK and NCC by a phosphoinositol 3-kinase (PI3K)-dependent pathway and that this is reduced by knocking down WNK4, suggesting that it is WNK4 dependent. They also observed that intraperitoneal injection of insulin is associated with increased phosphorylation of SPAK and NCC in the kidney of wild-type mice but not in a WNK4 hypomorphic mouse. Komers et al. (47) demonstrated in obese Zucker rats that response to hydrochlorothiazide is increased, together with the phosphorylation of NCC at threonine 53. They also observed in vitro that insulin's effect on phosphorylating NCC can be prevented by PI3K blockers and suggested that WNK4 could be implicated in the insulin effect on NCC. Finally, the observation of Chavez-Canales et al. (14) in *X. laevis* oocytes showed that insulin increases the activity of NCC, assessed by thiazide-sensitive $^{22}\text{Na}^+$ uptake and that this is associated with increased phosphorylation of threonine 60. Inhibitors of the PI3K, akt1, and mammalian target of rapamycin (mTOR)2 pathway prevented the insulin effect on NCC. It was also observed by using a kidney ex vivo perfusion technique that insulin perfusion into the kidney induces phosphorylation of the cotransporter. Thus insulin is an activator of NCC by inducing its phosphorylation in the amino-terminal domain acceptor sites (Fig. 2).

Do NCC Phosphorylation and Activity Correlate with Surface Expression?

A subject of debate is the location at which the phosphorylation that activates NCC occurs. Does it occur in the vesicles containing NCC copies, promoting their trafficking toward the plasma membrane, thus increasing surface expression of the cotransporter? Alternatively, does it occur at the NCC molecules that are already present in the plasma membrane, increasing their transport capacity as a consequence of the phosphorylation? As shown in Table 2, a few studies have addressed this issue. The data obtained from NCC cRNA-transfected *X. laevis* oocytes, a robust NCC expression system used to clone and study NCC properties (28), suggest that amino-terminal threonine phosphorylation occurs in the proteins that are already in the membrane. Intracellular chloride depletion increases the activity and phosphorylation of NCC, without changing the level of expression at the cell surface. Eliminating threonine 53 and 58, as well as the serine residue 71, dramatically reduced the basal activity of the cotransporter without affecting its

Table 2. Phosphorylation and cell surface expression of NCC

Experimental Model	Stimulus	Phosphorylation/Activity	Surface Expression	Reference No(s).
<i>Xenopus</i> oocytes	Intracellular chloride depletion	↑ ↓	↔	65
<i>Xenopus</i> oocytes	Protein phosphatase 4	↓ ↓	↔	32
<i>Xenopus</i> oocytes	WNK3	↑ ↑ ↑	↑ ↑ ↑	66, 73, 97
<i>Xenopus</i> oocytes	Mutant NCC-T58D	↑ ↑ ↑	↔	32
<i>Xenopus</i> oocytes	Mutant NCC-T58A	↓ ↓ ↓	↔	32, 65
Mouse	WNK4 ^(D561A/+) knockin	↑ ↑ ↑	↑ ↑	99
Brattleboro rats	dDAVP	↑ ↑ ↑	↑ ↑ (no shift)	67
Brattleboro rats/Wistar rats	dDAVP	↑ ↑ ↑	↑ ↑ (shift)	60
Mouse	SPAK knockout	↓ ↓ ↓	?	98
Mouse	SPAK knockout	↓ ↓ ↓	?	54

cell surface expression (65). Supporting these observations, another study demonstrated that protein phosphatase 4 reduces NCC activity, presumably by dephosphorylation, without affecting NCC surface expression. In the same study, phosphorylation that mimicked NCC mutation T58D increased the basal activity of the cotransporter without affecting its expression levels at the cell surface (32).

The data from *in vivo* models are less clear regarding the relationship between NCC plasma membrane expression and phosphorylation. Using immunogold electron microscopy, Pedersen et al. (67) showed in rats that the vasopressin-induced increase in NCC phosphorylation apparently occurs in the NCC that is already present in the plasma membrane. In contrast, Mutig et al. (60) proposed that vasopressin-induced NCC phosphorylation occurs in the intracellular vesicles that then move toward the plasma membrane. This last possibility is supported by a previous study showing that vasopressin activation and phosphorylation of NKCC2 in the thick ascending limb of Henle's loop in mice is associated with increased expression of the cotransporter in the apical membrane (31). Thus in the heterologous expression system it appears as if phosphorylation occurs in NCCs that are already at the cell surface. However, the *in vivo* data do not yet allow a definitive conclusion.

With regard to the regulation of NCC by the WNKs-SPAK pathway, it appears as if both phosphorylation and trafficking occur, most likely because WNKs modulate either independently of each other. In oocytes, the activation of NCC by WNK3 is associated with both increased phosphorylation and cell surface expression (66, 73). Preliminary evidence suggests that WNK3, in addition to phosphorylating NCC (most likely via SPAK), also reduces the Nedd4-2-induced ubiquitylation of the cotransporter. Therefore, the increase in NCC expression in the plasma membrane could be explained by decreased NCC ubiquitylation (see below) rather than by increased NCC phosphorylation (5). In the WNK4^(D561A/+) knockin mouse resembling PHAII, there is a clear increase in phosphorylation of NCC and in the expression at the apical surface of DCT cells (99). The elimination of the kinase SPAK, either by a knockout (54, 98) or by a knockin model in which SPAK is present but is unable to be activated by WNKs (71), is associated with a reduction in total NCC expression. Thus interpreting the decrease in both cell surface expression and phosphorylation of NCC in these models is difficult.

Modulation of NCC by Trafficking

In the cell, NCC resides within intracellular vesicles that undergo exocytosis and endocytosis. In this regard, one proposed mechanism is that WNK4, which is known to reduce

NCC activity in basal conditions (95), reduces the delivery of NCC to the plasma membrane by redirecting NCC molecules from the Golgi complex to lysosomes, in which NCC is then degraded. Supporting this view, WNK4 stimulates the interaction between NCC and the AP3 protein complex that promotes the switching of proteins from endosomes to lysosomes (84). Another study showed that WNK4 also promotes the interaction between NCC and sortilin, a DCT protein that redirects peptides from the Golgi and other compartments to lysosomes (102). This degradation-promoting effect of WNK4 could explain, at least in part, the Gitelman-like effect in which wild-type WNK4 is overexpressed in a BAC transgenic model (49) that exhibits remarkable DCT hypotrophy. In addition, accelerated degradation in the endoplasmic reticulum appears to be the major mechanism for reducing the expression of NCC in the plasma membrane in Gitelman's disease (48, 62, 76).

Protein Ubiquitylation: A Novel Regulatory Mechanism for NCC

Adding the 76-amino acid protein ubiquitin to lysine residues in target proteins is a powerful way to modulate the activity of proteins because ubiquitylation marks the protein for destruction, modulating its half-life (90). There are two major types of ubiquitin ligases: HECT (for homologous to the E6-AP carboxyl terminus) and RING (for really interesting new genes). These ligases contain several members that, in conjunction with a variety of interacting proteins, constitute hundreds of possibilities for highly specific ubiquitylation of proteins at lysine residues, just as kinases induce phosphorylation of proteins at specific threonine, serine, or tyrosine residues. Several membrane proteins, including transporters and channels in the kidney, are regulated by ubiquitylation (75).

A well-known example of ubiquitylation-mediated regulation occurs with ENaC. This channel in the collecting duct is ubiquitylated by a HECT-type ubiquitin ligase known as Nedd4-2. This protein directly interacts with ENaC via a specific PY motif in the channel, inducing ubiquitylation of the channel. This ubiquitylation marks the channel to be removed from the apical plasma membrane and thus reduces sodium reabsorption. The mineralocorticoid hormone aldosterone increases ENaC activity, at least in part, by stimulating the expression of SGK1. SGK1 phosphorylates Nedd4-2 at serine residues 222 and 328, preventing Nedd4-2-ENaC interaction and thus ENaC ubiquitylation. As a consequence, the expression of the channel in the apical membrane is increased, as is the sodium reabsorption. The loss of the PY motif of the β - or γ -subunit of ENaC by specific mutations that occur in Liddle's

syndrome provides a pathophysiological explanation for the disease. The absence of the PY motif in just one of the ENaC subunits is sufficient to preclude the Nedd4–2-induced ubiquitylation of the channel and thus chronically increase the expression/activity of the channel (41).

Modulation of NCC activity by ubiquitylation is an emerging field that is attracting the interest of researchers. The first observation suggesting that NCC is modulated by ubiquitylation was performed by Ko et al. (46) by searching for the mechanism by which phorbol esters reduce NCC activity via RasGRP1 and ERK1/2 activation (Fig. 2) (45). In that study, mDCT cells endogenously expressing NCC and Madin-Darby canine kidney cells transiently transfected with NCC cDNA were used. Exposing the cells to TPA resulted in increased ubiquitylation of NCC that could be prevented with UBEI-41, a compound that inhibits the activity of the E1 ligase, thus preventing TPA-induced ubiquitylation and reduction of NCC activity. The ubiquitin ligase responsible for NCC ubiquitylation was not elucidated. Subsequently, the SGK1-Nedd4–2 pathway that modulates ENaC activity was found to also regulate NCC (Fig. 2) (4). Aldosterone promotes increased NCC protein expression (44) by a mechanism that does not affect the NCC mRNA levels (1, 57), strongly suggesting that aldosterone increases NCC by a posttranslational effect. Supporting this hypothesis, Arroyo et al. (4) recently showed that Nedd4–2 induces an increase in NCC ubiquitylation, reducing the cotransporter in the plasma membrane, and thus its activity. Consistent with this observation, Nedd4–2 conditional knockout mice developed a remarkable increase in NCC expression a few days after the administration of doxycycline to induce the loss of Nedd4–2 in the kidney. Similarly to ENaC regulation, SGK1 prevented the Nedd4–2 effect in NCC. Supporting this, Faresse et al. (23) recently showed that inducible renal tubule-specific SGK1 knockout mice exhibit impaired Na⁺ reabsorption on a low-NaCl diet, and this was associated with decreased NCC abundance. Thus it is probable that the aldosterone activation of NCC is caused, at least in part, by decreasing the Nedd4–2-induced ubiquitylation of NCC in an SGK1-dependent mechanism. A recent study suggests that phosphorylation and ubiquitylation of NCC could interact with each other in such a way that phosphorylating NCC prevents ubiquitylation (39), and it has been shown in vitro that WNK1 and WNK4 are able to phosphorylate Nedd4–2 at sites overlapping with SGK1 (37). The significance of this finding is not known but could represent a possibility for regulating phosphorylation and ubiquitylation of NCC simultaneously.

Evidence suggests that NCC could be a substrate for other types of ubiquitin ligases. Degradation of NCC in the endoplasmic reticulum has been shown to be associated with the E3-ubiquitin ligase Hrd1 (62) and, as discussed below, NCC could be a target for the RING-type ubiquitin ligases.

Ubiquitylation is Also Involved in the Pathophysiology of PHAII

After the discovery of WNK1 and WNK4 as the causative genes of PHAII, it became clear that in several families this disease could occur in the absence of WNK1 or WNK4 mutations. Therefore, one or more unknown genes were capable of producing the same clinical outcome (19, 35). The causative genes have now been uncovered. Boyden et al. (10) observed that two genes encoding proteins that form a RING-type complex of ubiquitin ligase produce PHAII. These genes are known as Kelch-like 3 and Cullin 3. The human genome contains at least seven different cullin proteins; each constitutes the backbone of several multisubunit ubiquitin ligase complexes. In forming the complex, Cullin 3 specifically binds proteins, for example Kelch 3, that contain BTB domains. These two proteins, Kelch-like 3 and Cullin 3, form a complex that associates with a RING-type ubiquitin ligase (68). Kelch 3 or any of the BTB-containing domain proteins serves as the specific substrate recognition site of the complex, and the RING-type protein promotes the transfer of ubiquitin residues from E2 ligases onto the substrate recognized by the BTB protein. Using exome sequencing, mutations in Kelch 3 or Cullin 3 in 41 unrelated families with PHAII were identified (10). Although Kelch 3 mutations were either recessive or dominant, the Cullin 3 mutations were predominantly de novo. Interestingly, the PHAII patients with Cullin 3 mutations exhibited a more severe disease. As shown in Table 3, the severity of PHAII syndrome in terms of the mutated gene is Cullin 3 > Recessive Kelch 3 > Dominant Kelch 3 > WNK4 > WNK1. This ranking accounts for the age of diagnosis, the percentage of affected members under 18 yr of age exhibiting hypertension, the level of hyperkalemia, and the level of metabolic acidosis at the time of diagnosis.

For the reasons discussed above, it is generally accepted that the level of activity of NCC plays a key role in the development of PHAII. Thus these observations suggest that Cullin 3 and Kelch 3 must be regulators of NCC, by ubiquitylation of the cotransporter or of proteins that in turn modulate NCC activity (such as WNKs, SPAK, and phosphatases). Louis-Dit-Picard et al. (52) also reported on PHAII families resulting from mutations in Kelch 3 and presented preliminary evidence suggesting that Kelch 3 may modulate NCC. They showed that Kelch 3 in the nephron is mostly expressed in the DCT. Additionally, in HEK-293 cells transiently transfected with NCC cDNA, treatment with Kelch 3 siRNA increased the presence of NCC protein in the plasma membrane. These observations do not necessarily probe the direct effect of Kelch 3 on NCC. However, these findings do support the hypothesis that by a direct or indirect pathway, NCC expression is most likely affected by the Cullin 3/Kelch 3 complex. Therefore, the disruption of NCC regulation by Cullin 3/Kelch 3 would most

Table 3. Severity of PHAII stratified by genotype¹

Mutated Gene	No. of Families	No. of People Affected	HTA ⁴ <age 18 yr, %	Age at Diagnosis, yr	K ⁺ , ⁵ mM	HCO ₃ ⁻ , ⁶ mM
Cullin 3	17	21	94	9 ± 6	7.5 ± 0.9	15.5 ± 2.0
Kelch Rec ²	8	14	14	26 ± 14	6.8 ± 0.5	17.6 ± 1.5
Kelch Dom ³	16	40	17	24 ± 18	6.2 ± 0.6	17.2 ± 2.5
WNK4	5	15	10	28 ± 18	6.4 ± 0.7	20.8 ± 2.3
WNK1	2	23	13	36 ± 20	5.8 ± 0.8	22.4 ± 4.6

PHAII, pseudohypoaldosteronism type II; 1, modified from Boyden et al. (10); 2, recessive; 3, dominant; 4, hypertension; 5, serum potassium; 6, serum bicarbonate.

likely provide a pathophysiological explanation for PHAII. Because Cullin 3/Kelch 3 patients exhibit a more severe phenotype than WNK4 or WNK1 patients, it is possible that the effect of these ubiquitin ligases on NCC is more pronounced than that of WNKs or, alternatively, that in addition to NCC, ubiquitin ligases may affect other unknown players in the distal nephron for the development of PHAII, which has not become evident from studying WNKs. A decade has elapsed since the discovery of WNKs as causative genes for PHAII, but there are still several important unanswered questions about the mechanisms by which WNKs produces PHAII. Thus exciting times are ahead of us in defining the molecular mechanisms by which mutations in Cullin 3/Kelch 3 produce the disease.

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DISCLOSURES

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

AUTHOR CONTRIBUTIONS

Author contributions: G.G. provided conception and design of research; G.G. performed experiments; G.G. analyzed data; G.G. interpreted results of experiments; G.G. prepared figures; G.G. drafted manuscript; G.G. edited and revised manuscript; G.G. approved final version of manuscript.

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