Title: Ubiquitylation and control of renal Na+ balance and blood pressure.
Authors: Ronzaud C, Staub O
Journal: Physiology (Bethesda, Md.)
Year: 2014 Jan
Issue: 29
Volume: 1
Pages: 16-26
DOI: 10.1152/physiol.00021.2013

Ubiquitylation and Control of Renal Na^+ Balance and Blood Pressure

Caroline Ronzaud and Olivier Staub

[1]Department of Pharmacology and Toxicology, University of Lausanne, Switzerland

Corresponding authors:
Olivier Staub
Department of Pharmacology & Toxicology, University of Lausanne
Rue du Bugnon 27, 1005 Lausanne, Switzerland
Tel. +41-21-692-5407 / Fax. +41-21-692-5355
olivier.staub@unil.ch

Running title: Ubiquitylation and Renal Physiology

Abstract

Ubiquitylation is crucial for regulating numerous cellular functions. In the kidney, ubiquitylation regulates the epithelial Na^+ channel ENaC. The importance of this process is highlighted in Liddle's syndrome, where mutations interfere with its ubiquitylation, resulting in constitutive Na^+ reabsorption and hypertension. There is emerging evidence that NCC, involved in hypertensive diseases, is also regulated by ubiquitylation. Here we discuss the current knowledge and recent findings in this field.

Key words: Ubiquitylation, ENaC, NCC, NEDD4-2, KLHL3/CUL3, WNK, Na^+ balance, blood pressure

Introduction

Hypertension is involved in many diseases like stroke, myocardial infarction, heart and kidney failure. The kidney and most particularly the segments of the post-macula densa that consists in the distal convoluted tubule (DCT), the connecting tube (CNT) and the collecting duct (CD), are crucial for controlling Na^+/K^+ balance and thus extracellular volume and blood pressure (98). In the DCT, Na^+ reabsorption occurs by electroneutral co-transport via the thiazide-sensitive NaCl cotransporter (NCC). In the late part of the DCT (DCT2), Na^+ is reabsorbed via the electrogenic amiloride-sensitive epithelial Na^+ channel (ENaC) in addition to NCC. In the CNT and CD, Na^+ is reabsorbed via ENaC only (75). ENaC provides the driving force for K^+ secretion through the renal outer medullary K^+ channel (ROMK) (27).

The significance of tightly regulating ion transport in the renal nephron segments is underscored by the fact that blockers of the ion channels and transporters are often used to treat hypertension, and by the fact that genetic diseases affecting ENaC and NCC in humans lead to impaired Na^+ balance and blood pressure. Gain-of-function mutations within ENaC cause Liddle's syndrome, characterized by increased ENaC expression and open probability (24) and resulting in salt retention and hypertension (89). Indeed, such mutations in β- and γENaC interfere with the ubiquitylation and degradation of the channel by the ubiquitin-protein ligase NEDD4-2, encoded by the *Nedd4L* gene, and lead to impaired ENaC internalization and degradation (2, 29, 37, 38, 94, 96). Mutations in human proteins leading to overactive NCC result in pseudohypoaldosteronism type II (PHAII, also referred to as Gordon's syndrome or Familial Hyperkalemic Hypertension, FHHt) (9, 48, 105) and are associated with hypertension and a reduced distal delivery of Na^+, with consequent hyperkalemia (36, 49, 82). These mutations were identified in the genes that encode the with-no-lysine kinases WNK1 and WNK4, two main regulators of NCC (57). Recently, the groups

of Lifton and Jeunemaître have independently shown that mutations in humans affecting the genes encoding the kelch-like KLHL3/Cullin 3 (CUL3) ubiquitin-protein ligase complex lead to overactive NCC and thus hypertension (9, 48), pointing at the importance of ubiquitylation in the regulation of renal Na^+ transport. In addition, we have shown both *in vitro* and *in vivo* that NEDD4-2 is involved in NCC regulation (3, 78).

In this review, we summarize what is known about the role of ubiquitylation in the regulation of ENaC and the recent findings about the regulation of NCC by this process. We also discuss the importance of ubiquitylation in the control of Na^+ balance and blood pressure.

Regulation of ENaC-mediated Na^+ transport by the ubiquitin-ligase NEDD4-2

Ubiquitylation is a post-translational modification process that is crucial for regulating many different cellular functions. Indeed, ubiquitylation is used as a signal for membrane protein internalization or degradation by the proteasome or lysosomes (52, 99). Ubiquitin can be conjugated as a monoubiquitin or polyubiquitin chains on the amino group of lysine side chains. Monoubiquitylation on single or several sites on target proteins is involved in numerous biological functions including endocytosis and direction to lysosomal degradation (52). Also polyubiquitin chains may have different roles, but the best characterized canonical function is the targeting to the proteasome via K48-linked ubiquitins (17, 62).

Ubiquitylation is performed by several enzymes: an E1 ubiquitin-activating enzyme, E2 ubiquitin-conjugating enzymes, and E3 ubiquitin-protein ligases. The ubiquitin molecule is first activated by the E1 enzyme using ATP, forming first a thioester with the active site of E1 and then to that of E2. The E3 enzymes, that recognize the target protein, interact with both E2 and the substrate and lead to the formation of an isopeptide bond between a lysine of the

target protein and the C-terminal glycine of ubiquitin. Hundreds of E3 enzymes participate in the target protein recognition and thus provide the specificity to the cascade (65).

There are two main classes of E3 enzymes. The first class involves E3 ubiquitin-protein ligases that contain a RING motif (RING stands for Really Interesting New Gene) that helps transferring the ubiquitin from E2 to the target protein (61). This class includes monomeric proteins, and multimeric complexes comprising Cullin proteins as platforms that assemble E2 enzymes, RING motif containing proteins, and adaptor proteins. KLHL3 and CUL3 belong to this class, whereby CUL3 represents the platform, and KLHL3 an adaptor protein involved in target recognition. The second class is formed by the HECT (homologous to E6-AP C-terminal) domain-containing E3 ubiquitin-protein ligases that form first a thioester with the ubiquitin before transferring it on the substrate (84). The NEDD4/NEDD4-like (Neural precursor cell Expressed Developmentally Down-regulated protein)-like family belongs to the HECT E3 ligase class and regroups nine proteins: AIP4/ITCH, HecW1/NEDL1, and HecW2/NEDL2, NEDD4, NEDD4–2/NEDD4L, SMURF1, SMURF2, WWP1/TIUL1, and WWP2 (79). All these proteins contain an N-terminal (Ca^{2+}-dependent lipid binding) C2 domain for membrane binding (77), a central region containing two to four WW domains for interaction with the target protein (100), and a C-terminal HECT domain for binding to ubiquitin and transfer on the target (79, 84). Ubiquitylation is a reversible process catalyzed by deubiquitylating enzymes (DUBs) (44, 66)

The role of ubiquitylation in the regulation of cell surface expression and endocytosis of ion channels and transporters has emerged with the finding that the WW domains of the NEDD4/NEDD4-like E3 ligases bind to the proline-rich PY motifs of ENaC (core motif: PPXY, where P is a proline, Y a tyrosine, and X any amino-acid), motifs that are deleted in Liddle's syndrome (96) (Figure 1B). In this study, the authors used the PY-containing region

of the βENaC subunit as bait in a two-hybrid screen to identify proteins, which interact with ENaC, and identified NEDD4-2. It was then shown that each of the three ENaC subunits α, β and γ contains a PY motif in the C-terminus (85, 93, 96). Interestingly, NEDD4-2 is expressed in tissues that also express ENaC (37). It was proposed that the enhanced cell surface ENaC expression observed in Liddle's patients might be due to a defect in ubiquitylation and degradation of the channel (Figure 1C). This hypothesis was confirmed in 1999, by the finding that indeed the regulation of ENaC by NEDD4-2 was defective in Liddle's syndrome (2). Another study later showed that the NEDD4-2 WW3-4 domains regulate ENaC (25, 37). In 2006, Knight *et al.* found out that Liddle's syndrome mutations enhance Na^+ transport by acting on ENaC surface expression and open probability (41). One year later, Wiemuth *et al.* demonstrated that ENaC is multi-monoubiquitylated at the cell surface (104), and two other independent groups showed that the PY motifs on ENaC, that are mutated in Liddle's syndrome, regulate the internalization of the channel, its sorting and recycling (50) and that NEDD4-2 catalyzes the plasma membrane ENaC ubiquitylation and degradation (113). However, whether ENaC is mono- or poly-ubiquitylated remains controversial. Some groups have proposed that ENaC is poly-ubiquitylated and targeted to proteasomal degradation (53-55), while others suggested that the channel is either mono- or multimono-ubiquitylated at the surface of the cell (81, 104, 113). One explanation might be that ENaC is polyubiquitylated in the endoplasmic reticulum (ER), in the context of ERAD (ER-associated degradation), where misfolded or unassembled proteins are degraded via ubiquitin-proteasome pathway (10), whereas the assembled channel expressed at the cell surface is likely degraded via endosomal/lysosomal degradation involving multi-monoubiquitylation (97, 112) (Figure 1). Direct evidence of ERAD-based regulation of ENaC has been provided by Brodsky and collaborators (10).

As mentioned above, ubiquitylation is a process that is reversible, catalyzed by DUBs (66). ENaC has been proposed to be regulated by several of these DUBs, including USP2-45, USP8, USP10 and UCH-L3 (8, 11, 21, 114). Although deubiquitylation of ENaC appears to be an appealing concept for ENaC regulation, the physiological relevance of these observations remain to be proven, especially as a recent report of a *Usp2* knockout model provides no evidence for the involvement of USP2 in the control of Na^+ homeostasis or blood pressure control (71).

To elucidate the relationship between the regulation of ENaC and the control of Na^+ balance and hypertension, an *in vivo* model for Liddle's syndrome was generated by mutating the β subunit of the mouse ENaC, resulting in a truncated βENaC as found in Liddle's patients (73). Under high-Na^+ diet, these Liddle mice presented the Liddle phenotype characterized by higher blood pressure, metabolic alkalosis, and hypokalemia, together with heart and kidney hypertrophy. The cell surface density of functional ENaC, but not the open probability, was enhanced in the kidneys of Liddle mice (14). In addition, whereas the γENaC subunit was rapidly internalized in control mice, it was retained at the plasma membrane of CNT in Liddle mice (73). Accordingly, measures in isolated perfused CCD and in confluent primary cultures of microdissected CCD isolated from Liddle mice exhibited higher ENaC activity (73).

NEDD4-2-mediated ubiquitylation of ENaC is under the tight control of the mineralocorticoid hormone aldosterone, which prevents the interaction between the channel and the ubiquitin-protein ligase (Figures 1A and B). This is mediated by the serum- and glucocorticoid-induced kinase 1 (SGK1): under aldosterone stimulus, SGK1 expression is induced and the kinase phosphorylates NEDD4-2, resulting in the recruitment of 14-3-3 proteins that disrupt the NEDD4-2/ENaC interaction and subsequent ubiquitylation and degradation of the channel (6,

13, 15, 33, 46, 91, 92). A number of alternative mechanisms of regulation by SGK1 or NEDD4-2 have also been suggested, including the direct phosphorylation of ENaC by SGK1 (16), or direct phosphorylation of ENaC by ERK kinase which may facilitate the interaction with NEDD4-2 (86). Pearce and collaborators provided evidence for an ENaC regulatory complex that comprises NEDD4-2, SGK1, GILZ, RAF-1 and CNK3. It was proposed that the complex may integrate the activities of various signaling pathways involved in ENaC regulation, including those regarding steroid receptors, PI-3 kinase, mTOR and RAF-MEK-ERK pathways (95).. Very recently, another novel mode of regulation of ENaC, comprising the ubiquitin-like protein NEDD8, was also described (18, 51).

In vivo Nedd4-2 knockout models: new insights in the role of NEDD4-2-mediated regulation of Na^+ transport and blood pressure

As described above, genetic mutations in human and *in vitro* biochemical and overexpression studies suggested that the interaction between NEDD4-2 and ENaC is crucial for regulating Na^+ balance and blood pressure. Moreover, single nucleotide polymorphisms (SNPs) in human *Nedd4L* (that encodes NEDD4-2) have been linked to hypertension (19). To validate the role of NEDD4-2 *in vivo*, several groups have generated in the last few years mouse knockout models of *Nedd4L* (for summary of these mouse models, see Table 1). The first model was developed by the group of Baoli Yang. In these *Nedd4L* knockout mice, the exons 6 to 8 of the *Nedd4L* gene were removed in a constitutive manner(*Nedd4L-Δ6-8* mice) (87). Surprisingly, despite the predicted function of NEDD4-2 as a major regulator of ENaC, the *Nedd4L-Δ6-8* knockout mice were viable and only displayed cardiac hypertrophy and a mild salt-sensitive hypertensive phenotype. The extracellular volume and Na^+/K^+ balance were not impaired (87). The protein expression of the three subunits of ENaC was enhanced in the knockout mice and the increased blood pressure under high-Na^+ diet could be treated by

injecting amiloride to the mice, suggesting overactive ENaC. However, the plasma aldosterone levels were normal under standard and high-Na^+ diet, indicating a rather mild ENaC-overactivation phenotype.

Another *Nedd4L* total knockout model has been generated by Sharan Kumar's group, by deleting exons 15-16 of the *Nedd4L* gene in the mouse (7). In contrast to Yang's mice, the model of Kumar displayed perinatal lethality resulting from premature fetal lung fluid clearance as a consequence of increased ENaC expression and activity in embryonic lungs (7). Expression of the three ENaC subunits α, β and γ was increased in lungs and kidneys of E18.5 embryos and the amiloride-sensitive ENaC-mediated current was strongly enhanced in embryonic knockout lung cells. These data clearly indicate that NEDD4-2 is important for the regulation of ENaC in the kidney and that it is a key regulator of ENaC function in the lung, leading to lethality when deleted (7). These data also confirmed what was observed in the βENaC Liddle mice, in which the mutation in βENaC prevented the down-regulation of the channel by NEDD4-2. Indeed, these mice also showed increased alveolar fluid clearance due to ENaC overactivity (72-74).

The same year, the group of Daniela Rotin published another *Nedd4L* knockout model, but this time by inactivating *Nedd4L* specifically in the lungs (40). This study showed that ablating the exon 15 and the downstream region of the *Nedd4L* gene in mice leads to a phenotype resembling lung cystic fibrosis, with airway mucus obstruction, intense inflammation and lethality at the age of three weeks. These observations were accompanied with increased protein abundance of the three ENaC subunits, enhanced ENaC currents and amiloride-sensitive dehydration, and increased dryness of the lungs (40). These results confirm what was observed in transgenic mice overexpressing ENaC (56) and confirm the crucial role of lung NEDD4-2 in regulating ENaC expression and activity by ubiquitylation. Interestingly, Kimura *et al.* could rescue the lung defects by nasally administrating amiloride

to the newly born lung-specific N*edd4L* knockout mice, suggesting that the observed lung phenotype was resulting from defect in ENaC degradation due to NEDD4-2 ablation (40).

Very recently, Ronzaud *et al.* have published a new *Nedd4L* knockout mouse model, where exons 6 to 8 of the *Nedd4L* gene have been removed (using the same floxed allele as Shi *et al.*) in an inducible and renal tubule-specific manner ($Nedd4L^{Pax8/LC1}$ knockout mice) using a combination of the Cre-loxP and Tet-On systems (78). As expected, these mice displayed salt-sensitive hypertension, decreased plasma aldosterone levels and increased renal β- and γENaC protein expression. However, the urine and plasma Na^+ and K^+ levels were normal in the knockout mice. In addition, the cellular localization of β- and γENaC was mainly cytoplasmic, and γENaC was found in its uncleaved form. Surprisingly, the $Nedd4L^{Pax8/LC1}$ knockout mice showed reduced *αENaC* mRNA levels and proteolytic cleavage, suggesting down-regulation of the channel (Figure 1D). Interestingly, the ENaC down-regulation that accompanied the reduced plasma aldosterone could be rescued by giving aldosterone to the knockouts. Thus, these data confirmed that NEDD4-2 is important for mediating the degradation of β- and γENaC, that aldosterone is crucial for regulating renal αENaC expression (4), and that αENaC is needed for apical trafficking of the two other ENaC subunits (80). Of interest, no mutation in the human αENaC gene was found to cause Liddle's syndrome. In addition, injection of amiloride in $Nedd4L^{Pax8/LC1}$ knockout mice did not reduce the elevated blood pressure, indicating that the hypertensive phenotype is not due to increased ENaC function. Therefore, the $Nedd4L^{Pax8/LC1}$ knockout mice question the previous *in vitro* and *in vivo* studies that showed the implication of NEDD4-2-mediated ubiquitylation in the regulation of αENaC.

It is difficult to reconcile the different phenotypes obtained with all these *Nedd4L* knockout mouse models, as they are differing at several levels: genetic background, part of the *Nedd4L* gene that was deleted, targeted tissue, and knockout strategy (constitutive or inducible).

However, their comparison opens new questions regarding NEDD4-2 function and tissue-specificity. First, it should be noted that Shi *et al.* did not specify whether the observed enhanced α, β and γENaC subunits were expressed apically or within the cell. In addition, the absence of any decrease in plasma aldosterone is in contrast to what is observed in Liddle's patients. It has been proposed that the absence of lung phenotype and lethality in the Shi *et al.*'s model could be explained by the presence of a shorter *Nedd4L* splice isoform (7). Indeed, the region of the *Nedd4L* gene that has been deleted in the Shi *et al.*'s model is known to be subjected to differential splicing (34). However, using different antibodies against NEDD4-2, Ronzaud *et al.* were not able to detect any shorter NEDD4-2 isoform in the $Nedd4L^{Pax8/LC1}$ knockout mice, at least in the kidney (78). It is also plausible that other NEDD4 family members might compensate for the loss of NEDD4-2 in the lung, although Ronzaud *et al.* did not observe any increase in NEDD4-1, the closest family member (78). Another possibility could be that the mixed genetic background used in the Shi *et al.*'s model contributes to the milder phenotype of these mice compared to those developed by Kumar and Rotin (7, 40).

Moreover, it is also difficult to reconcile the phenotype of the $Nedd4L^{Pax8/LC1}$ knockout mice with what has been observed in mouse models of Liddle's syndrome, where the activity of ENaC is increased despite the low plasma aldosterone and where the three ENaC subunits are accumulating in the plasma membrane (72). It is likely that loss of the regulator NEDD4-2 affects other downstream factors like NCC up-regulation and consequent compensatory mechanisms, or other factors involved in the control of ENaC function or trafficking (99), whereas mutations in the ENaC subunits would affect the channel directly. Thus, it would be of interest to generate *Nedd4L* knockout mice specifically in the CNT and CD, without targeting the DCT, to avoid the effects of NEDD4-2 loss on NCC expression and function, and thus see the impact on ENaC only.

Ubiquitylation and regulation of NCC-mediated Na^+ reabsorption and blood pressure: an emerging field

Given the relevance of NCC activity in human hypertension (90), many studies have focused on how NCC is regulated. It was first described in 2006 by Pacheco-Alvarez *et al.* that NCC phosphorylation on T53, T58 and S71 is crucial for its activity (68). Later, it was shown that the WNK pathway is implicated in both NCC phosphorylation and trafficking (59). WNK1, WNK4 and a third family member (WNK3) all activate SPAK/OSR1 that phosphorylate NCC and stimulate its activity (69, 76). In addition, WNK4 down-regulates NCC abundance at the cell surface, by promoting the lysosome-mediated degradation of the cotransporter (26, 42, 103, 108). However, it has long remained unclear how NCC trafficking to the cell surface is regulated. Based on two independent *in vitro* studies, it has been proposed that WNK4 could divert NCC to the lysosomal pathway, possibly by regulating the interaction between the cotransporter and the lysosome-targeting receptor sortilin (111) and the AP3 adaptor complex (101). In PHAII patients, resulting from mutations in both WNK1 and WNK4 kinases, enhanced NCC phosphorylation and cell surface expression are observed, leading to increased Na^+ reabsorption and hypertension (83, 109, 110). In contrast, WNK4-overexpressing transgenic mice develop characteristics of Gitelman's syndrome characterized by decreased NCC expression and function (45). Intriguingly, total WNK4 knockout mice characterized by the group of Gerardo Gamba showed rather an incomplete form of Gitelman's syndrome with decreased NCC expression, phosphorylation and activity, but without any changes in blood pressure and urinary calcium excretion (12).

Aldosterone has been shown to increase NCC protein expression without affecting the *NCC* mRNA levels (1, 39, 63), suggesting a posttranslational mechanism. In the past few years,

many in vitro studies suggested that NCC ubiquitylation plays an important role in the regulation of the cotransporter. Ko *et al.* showed for the first time that NCC is internalized via a dynamin-dependent mechanism (42) and that this process is regulating NCC, resulting in a decreased activity of the cotransporter (43). They showed that the increased NCC ubiquitylation and decreased activity, observed after treating mDCT cells that endogenously express NCC with phorbol-ester, could be prevented using an E1 ubiquitin-activating enzyme inhibitor. Interestingly, a recent study showed that NCC phosphorylation prevents its ubiquitylation (31). In addition, Heise *et al.* showed that WNK1 and WNK4 phosphorylate NEDD4- 2 on the same sites as SGK1 does (30), suggesting that phosphorylation and ubiquitylation could regulate NCC concomitantly. Supporting this hypothesis, Arroyo *et al.* showed *in vitro* that NEDD4–2 co-immunoprecipitates with NCC and induces its ubiquitylation, decreasing the expression of the cotransporter at the plasma membrane and its activity. Moreover, like for ENaC regulation, SGK1 prevented the NEDD4–2 effect on NCC (3). There is also *in vivo* evidence that SGK1 is involved in NCC regulation (22, 23). The implication of NEDD4-2 in NCC regulation has now been confirmed *in vivo* with the generation of the *Nedd4L*$^{Pax8/LC1}$ knockout mouse model. Ronzaud *et al.* showed that NCC protein expression and phosphorylation were elevated in the *Nedd4L*$^{Pax8/LC1}$ knockout mice , as well as NCC function as the high blood pressure and hypercalciuria observed in the knockouts could be corrected by thiazide treatment (78). Taken together with the decreased αENaC and the absence of amiloride effect on the increased blood pressure, it was proposed that the elevated NCC-mediated Na^+ reabsorption resulting from NEDD4-2 ablation is compensated by reduced ENaC function. Similarly, the increased NCC observed in the kidney-specific *ks-wnk1* knockout mice was also compensated by down-regulation of the three ENaC subunits (28). The observation that NEDD4-2 is involved in NCC regulation is

also supported by recent studies of a SNP in the NEDD4-2 gene, which appears to affect thiazide sensitivity (60).

It has been shown that NEDD4 and NEDD4-like proteins can bind to and ubiquitylate target proteins that have no PY motif using PY-motif-containing adaptors (32, 47, 64). Arroyo *et al.* showed that, in contrast to ENaC for which the NEDD4-2-mediated inhibition can be prevented when SGK1 phosphorylates NEDD4-2 on S328, the inhibition of NCC by the SGK1-NEDD4-2 pathway can be prevented by phosphorylation of NEDD4-2 on both S222 and S328 sites (3). The fact that NCC and ENaC are regulated by the SGK1-NEDD4-2 pathway in a different manner and that NCC does not contain any PY motif suggest that NEDD4-2 controls NCC indirectly. Whether NCC and NEDD4-2 interact via an adaptor and its identity represent questions for further investigation.

Interestingly, the *Nedd4L*$^{Pax8/LC1}$ knockout mice did not show all the PHAII characteristics, despite the strong NCC up-regulation. This contrasts with other mouse models for PHAII, like the *PHAII-Wnk4 (Q562E)* transgenic mice developed by Lalioti *et al.* In these mice, the elevated NCC and other PHAII features were abolished after breeding with NCC knockouts (45). Similarly, thiazide treatment of the *Wnk4*-D561A mutant mice developed by Yang *et al.* could prevent the increased NCC expression and phosphorylation and the associated hypertension and hyperkalemia (110). More and more studies suggest that PHAII cannot result from overactive NCC only, but rather from the misregulation of other channels and transporters. For instance, the *ks-wnk1* knockouts described above display some but not all PHAII features (28). Moreover, NCC- overexpressing mice showed no increase in blood pressure or Ca^{2+} excretion (58). Finally, the increased ROMK protein expression in the

Nedd4L$^{Pax8/LC1}$ knockouts (78) may result in elevated K^+ excretion, thus preventing the hyperkalemia normally observed in PHAII patients (28).

Very interestingly, two other groups have recently identified another ubiquitin-protein ligase pathway affecting NCC cell surface abundance and involved in the pathology of PHAII, thus further supporting the importance of ubiquitylation in NCC regulation. Using exome sequencing, Boyden *et al.* identified mutations in two genes encoding the KLHL3 and CUL3 proteins that led to PHAII (9). These two proteins are part of a Cullin-RING E3 ubiquitin-protein ligase complex and, very interestingly, are expressed in DCT like NCC (9, 70). The same findings were reported by an independent group: Louis-Dit-Picard *et al.* also used combined whole-exome sequencing with linkage analysis and identified mutations in *Kelch3* that encodes the KLHL3 protein (48). They confirmed the co-expression of KLHL3 and NCC and showed *in vitro* that KLHL3 down-regulates NCC expression at the cell surface and that the reverse is observed after *Kelch3* silencing. These recent findings strongly support the relevance of ubiquitylation in the control of NCC function.

However, the observations that KLHL3 and CUL3 proteins down-regulate NCC expression at the cell surface do not prove the direct effect of these regulators on the cotransporter. Khan *et al.* have recently shown that phosphorylation of NCC controls its ubiquitylation and might play a role in NCC expression at the cell surface (31). In addition, some light has been very recently shed on the possible involvement of KLHL3/CUL3 in WNK1 or WNK4 ubiquitylation (67, 88, 102, 106). It has been shown *in vitro* that the KLHL3/CUL3 complex interacts with WNK1 and WNK4, but not with NCC, and ubiquitylates and degrades the two WNK isoforms. Very interestingly, the WNK region, which interacts with KLHL3, is also that mutated in PHAII (67, 88).

Taken all these data together, we propose a model for the differential effect of ubiquitylation via NEDD4-2 or via KLHL3/CUL3 on NCC regulation in normal and pathological conditions (Figure 2). In conditions of RAAS activation (increased angiotensin II and aldosterone), NCC could be activated by phosphorylation via the WNKs and NEDD4-2-mediated ubiquitylation of NCC would be inhibited by SGK1 and possibly by the WNK kinases, thus maintaining sufficient NCC expression at the cell surface. In conditions of RAAS inactivation (decreased angiotensin II and aldosterone), NCC could be inactivated via two pathways: the KLHL3/CUL3 complex could interact with and ubiquitylate the WNKs, thus decreasing WNKs expression and resulting in decreased NCC phosphorylation and expression at the cell surface; in parallel, NEDD4-2 would ubiquitylate NCC and thus decrease its cell surface expression. In PHAII patients, mutations in either the WNKs or the KLHL3/CUL3 complex inhibit the interaction between the WNKs and the KLHL3/CUL3. As a result, WNK expression raises, leading to elevated NCC phosphorylation and expression. This results in elevated Na^+ reabsorption, high blood pressure, RAAS inactivation, hypercalciuria and hyperkalemia. How the KLHL3/CUL3 and NEDD4-2 pathways interact with each other remains unknown. One hypothesis could be that the WNKs inhibit NEDD4-2 either directly or via SGK1 activation (30, 107). Finally, in case of NEDD4-2 ablation, NCC cannot be ubiquitylated and degraded, resulting in increased NCC surface expression and function, elevated Na^+ reabsorption, high blood pressure, RAAS inactivation and hypercalciuria. In the *Nedd4L*$^{Pax8/LC1}$ knockout mice, the increased NCC was phosphorylated, indicating that the WNK pathway is activated (78). How the KLHL3/CUL3 and NEDD4-2 pathways interact with each other remains unknown.

Conclusions

The recent studies in the field of ubiquitylation revealed the importance of this process in the regulation of Na^+ reabsorption and blood pressure by the kidney. However, the importance of well-known ubiquitylated targets like ENaC has probably to be redefined, and novel targets like NCC are now emerging.

Indeed, as discussed very recently by Ellison (20), there is more and more evidence suggesting that the absence of NEDD4-2-mediated ubiquitylation is not a prerequisite for up-regulating ENaC (5, 78). The recent data in this field rather suggest that 1) the αENaC abundance (and thus ENaC function) is mainly regulated by aldosterone and that 2) the principal effect of NEDD4-2 on ENaC might be the regulation of the channel degradation inside the cell and not its removal from the cell surface. This last proposal should however be taken with precaution as others have suggested that ubiquitylation plays an important role in driving ENaC endocytosis (35).

In fact, the recent studies have rather demonstrated the critical role of ubiquitylation in renal physiology by modulating the abundance of NCC. However, the underlying mechanisms of how NCC phosphorylation and ubiquitylation interact with each other to regulate NCC remain to be clarified. The observation that NEDD4-2 regulates NCC activity by ubiquitylation and reduction of its surface expression (3, 78), whereas KLHL3/CUL3 seem to target WNK1/4 (67, 88, 102, 106), may lead to the hypothesis that NEDD4-2 regulates NCC activity via direct ubiquitylation, whereas KLHL3/CUL3 may act indirectly by ubiquitylating the NCC regulators, namely the WNKs, thus decreasing NCC phosphorylation. Importantly, inhibiting the NEDD4-2-NCC pathway appears not to be sufficient for resulting in a PHAII phenotype, whereas ablating the KLHL3/CUL3-WNK-NCC pathway does. Of interest, the work of Melanie Cobb has shown *in vitro* that the WNKs can inhibit NEDD4-2 either directly

or via SGK1 activation (30, 107). How exactly and in which physiological conditions the HECT ubiquitin-ligase NEDD4-2 pathway and the RING ubiquitin-ligases KLHL3 and CUL3 pathway are involved in the modulation of NCC represent important questions for further investigation.

Grants

The work performed in OS laboratory has been supported by the Leducq Foundation Transatlantic Network on Hypertension, the Swiss National Science Foundation 31003A-125422/1, the Swiss NCCR Kidney.ch, and the Swiss Kidney Foundation (to CR).

Disclosures

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

Author contributions

CR prepared the figures; CR drafted the manuscript; OS and CR edited and revised the manuscript; OS approved the final version of the manuscript.

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Figure legends

Table 1. *Comparative table of the different Nedd4-2 knockout mouse models.*

Figure 1. M*odel of NEDD4-2-mediated ubiquitylation of ENaC in normal and pathological conditions.* A. In conditions of RAAS activation, aldosterone induces SGK1 expression that leads to phosphorylation and inhibition of NEDD4-2, thus disrupting the NEDD4-2/ENaC interaction and subsequent ubiquitylation and degradation of the channel. In parallel, misfolded or unassembled polyubiquitylated ENaC channels are degraded via the ubiquitin-proteasome pathway in the ER in the context of ERAD (ER-associated degradation). The deubiquitinating enzymes (DUBs) are also involved in deubiquitylation and recycling of ENaC. B. In conditions of RAAS inactivation, aldosterone and SGK1 are low, whereas NEDD4-2 is highly expressed. NEDD4-2 ubiquitylates ENaC and leads either to degradation of the channel or to its recycling. It remains unclear whether NEDD4-2-mediated ENaC ubiquitylation happens on the channel at the cell surface or on the intracellular pool. C. In Liddle patients, mutations in the PY motifs of ENaC result in absence of interaction between ENaC and NEDD4-2 and thus defect in ubiquitylation and degradation of the channel, leading to permanently enhanced cell surface ENaC expression. D. In *NEDD4L*$^{Pax8/LC1}$ KO mice generated by Ronzaud *et al*. (78), it was shown that absence of NEDD4-2 protein expression leads to increased intracellular expression of βENaC and γENaC, but not αENaC. It was proposed that αENaC is regulated at the transcriptional level by aldosterone, thus leading to diminished ENaC activity to compensate increased NCC-mediated Na^+ reabsorption (see Figure 2).

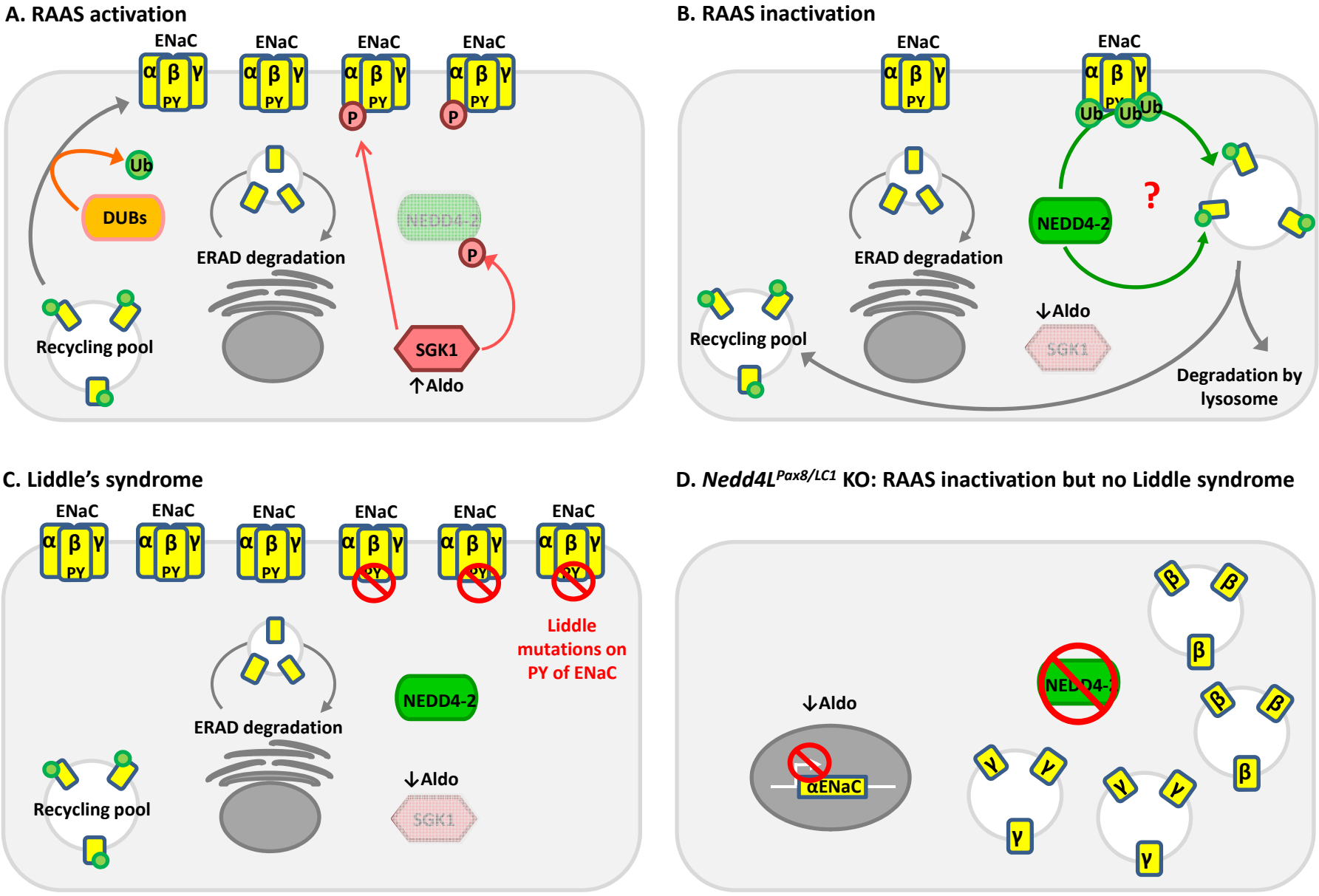
Figure 2. *Proposed model for the differential effect of ubiquitylation via NEDD4-2 or via KLHL3/CUL3 on NCC regulation in normal and pathological conditions.* A. In conditions of RAAS activation, the WNK kinases phosphorylate and activate NCC and at the same time

SGK1, and possibly the WNKs, inhibit the NEDD4-2-mediated ubiquitylation of the co-transporter, thus maintaining sufficient NCC expression at the cell surface. B. In conditions of RAAS inactivation, NCC is inactivated via two pathways: the KLHL3/CUL3 complex ubiquitylates the WNKs, thus decreasing WNKs expression and resulting in decreased NCC phosphorylation and expression at the cell surface; in parallel, NCC is ubiquitylated by NEDD4-2, which decreases its cell surface expression. C. In PHAII patients, mutations in either the WNKs or the KLHL3/CUL3 complex inhibit the interaction between the WNKs and the KLHL3/CUL3. This results in a raise of WNKs expression, leading to elevated NCC phosphorylation and expression. This leads to elevated Na^+ reabsorption andblood pressure, RAAS inactivation, hypercalciuria and hyperkalemia. D. In case of NEDD4-2 ablation, NCC cannot be ubiquitylated and degraded, resulting in increased NCC surface expression and function, elevated Na^+ reabsorption and blood pressure, RAAS inactivation and hypercalciuria.

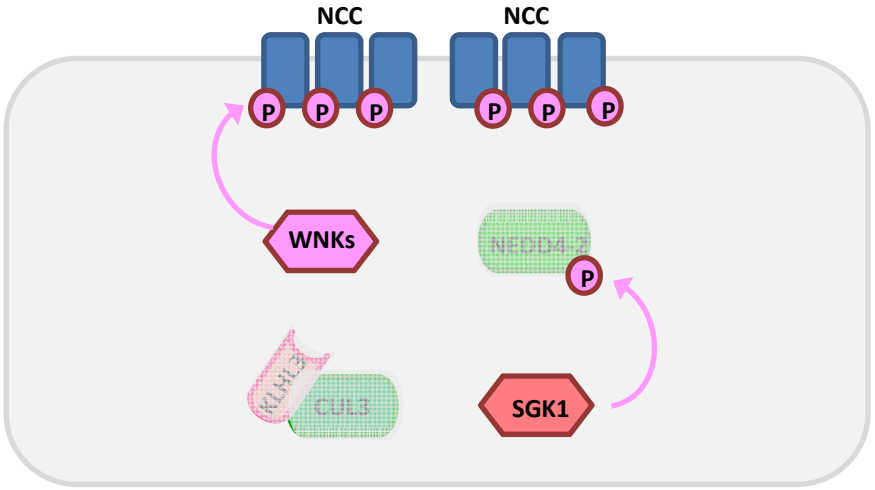
Table 1.

		Shi *et al*, 2009	Boase *et al*, 2011	Kimura *et al*, 2011	Ronzaud *et al*, 2013
Global phenotype		Mild Liddle	Lethal / lung phenotype	Lethal / lung phenotype	Mild PHAII
Knockout	**Type**	Total	Total	Lung-spe cific	Inducible renal tubule-spe cific
	Deleted region	Exons 6-8 (ATG-C2-WW1)	Exon 15 (WW3)	Exon 15 $^{+}$ downstream (WW3-WW4-HECT)	Exons 6-8 (ATG-C2-WW1) (as Shi *et al*, 2009)
Protein expression	**Nedd4-2**	125kD : absent 110kD : present 72kD : n.d. 46kD : present (Boase *et al*, 2011)	125kD : absent 110kD : absent 46kD : absent	n.d.	125kD : absent in microdissected tubules 110kD : absent 40kD : absent
	ENaC	↑ α-, β-, γ-ENaC in kidney	↑ α-, β-, γ-ENaC in lungs and kidney	↑ α-ENaC in lungs	↓ α-ENaC in kidney ↑ β-, γ-ENaC in kidney
	NCC	n.c.	↑ (Ronzaud *et al*, 2013)	n.d.	↑
Function	**ENaC activity**	↑	↑	↑	↓
	NCC activity	n.c. (slight ↑)	n.d.	n.d.	↑
Renal phenotype	**Aldosterone**	Standard / high-Na^{+} : n.c. Low-Na^{+} : ↑	n.d.	n.d.	Standard / high-Na^{+} : ↓
	Blood pressure	ENaC-dependent salt-sensitive HT	Slight HT in $^{+}$/-	n.d.	NCC-dependent salt-sensitive HT
	Calciuria	n.d.	n.d.	n.d.	↑

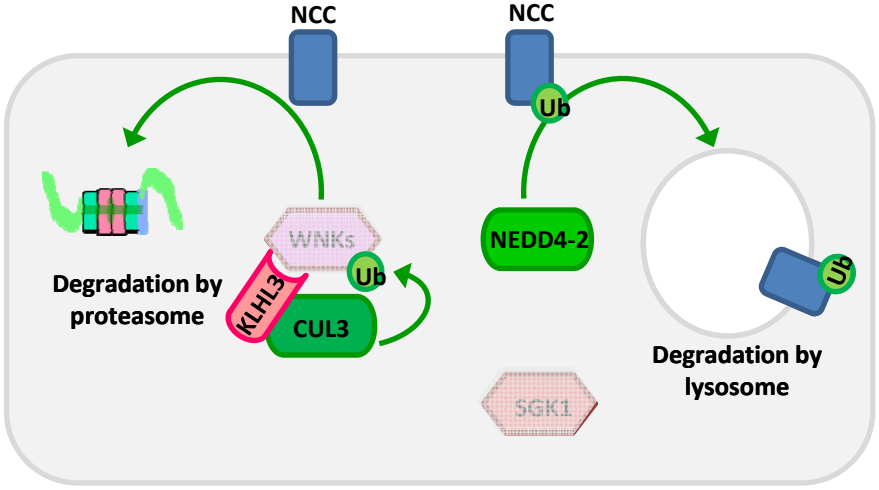
n.d.: not determined; n.c.: not changed; HT: hypertension; $^{+}$/-: heterozygous.


A. RAAS activation
ENaC
α β γ PY
P
Ub
DUBs
ERAD degradation
Recycling pool
NEDD4-2
SGK1
↑Aldo
B. RAAS inactivation
NEDD4-2
?
↓Aldo
SGK1
Degradation by lysosome
C. Liddle's syndrome
Liddle mutations on PY of ENaC
D. Nedd4L Pax8/LC1 KO: RAAS inactivation but no Liddle syndrome
αENaC

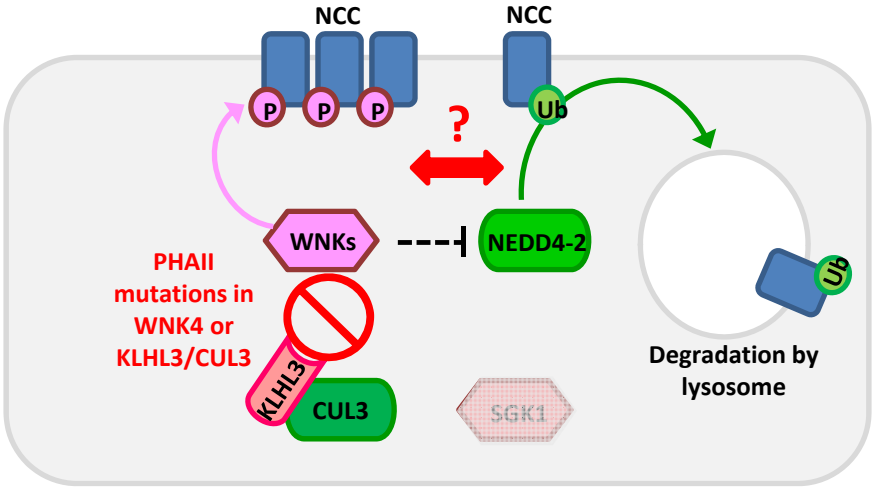
A. RAAS activation



B. RAAS inactivation



C. PHAII mutations : RAAS inactivation



D. Nedd4-2 mutations : RAAS inactivation but no PHAII