Alternatively spliced proline-rich cassettes link WNK1 to aldosterone action

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Introduction

With-No-Lysine (WNK) kinases are large serine-threonine kinases that regulate sodium, potassium, and blood-pressure homeostasis. WNKs participate in these processes by coordinating multiple electrolyte transport pathways in more distal segments of the nephron (reviewed in ref. 1). Mutations in WNK1, WNK4, and the KLHL3/CUL3 complex, which regulates WNK abundance, are 4 known genes that cause familiar hyperkalemic hypertension (FHH1, PHA2, Gordon Syndrome), a Mendelian disorder of thiazide-sensitive NaCl cotransport. Current evidence indicates that hormones that stimulate renal NaCl reabsorption recruit specific WNK kinases to mediate their effects (5). However, due to deficiencies in our understanding of WNK kinase structure and domain organization, the molecular mechanisms that allow these hormones to interact with WNKs and trigger SPAK/OSR1 activation remain obscure.

In this study, we identify a molecular mechanism that explains how aldosterone can interface with the WNK signaling pathway to influence SPAK/OSR1 activity and downstream NCC phosphorylation. We show that WNK1 is an aldosterone-induced protein that contains functional “PY motifs,” which are short-linear proline-containing sequences that bind to an E3 ubiquitin ligase critical for the aldosterone response, NEDD4-2. NEDD4-2 accelerates the rate of WNK1 turnover, and this process is attenuated by the aldosterone-induced kinase SGK1. In gene-edited cells, WNK1 is regulated by aldosterone infusion increased proline-rich WNK1 isoform abundance in WT mice but did not alter WNK1 abundance in hypertensive Nedd4-2 KO mice, which exhibit high baseline WNK1 and SPAK/OSR1 activity toward NCC. Conversely, hypotensive Sgk1 KO mice exhibited low WNK1 expression and activity. Together, our findings indicate that the proline-rich exons are modular cassettes that convert WNK1 into a NEDD4-2 substrate, thereby linking aldosterone and other NEDD4-2-suppressing antinatriuretic hormones to NCC phosphorylation status.

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of these exons into the WNK1 coding sequence converts WNK1 into a NEDD4-2 substrate that allows aldosterone and potentially other hormones to modulate the activity of SPAK, OSR1, and their downstream targets.

Results

WNK1 gene structure and identification of putative NEDD4-2 interaction sites in exons 11 and 12. WNK1 is a large, 32-exon gene spanning approximately 160 kilobases in humans. Three regions downstream of the kinase domain undergo alternative splicing (Figure 1A; ref. 10). Additionally, WNK1 contains multiple promoters that generate 2 functionally distinct classes of isoforms. Two proximal promoters drive the expression of ubiquitously expressed long isoforms (L-WNK1) that contain a full kinase domain and therefore are capable of phosphorylating downstream targets (11). A renal promoter drives the transcription of a kidney-specific transcript (KS-WNK1). Regions of WNK1 that undergo tissue-specific alternative splicing are underlined. Exon 4a, which encodes a short N-terminal sequence unique to KS-WNK1, is shown in red. Exons 11 and 12 are highlighted in blue. (B) Domain structure of L-WNK and KS-WNK1. The location of 3 coiled coil domains (CC) that facilitate WNK complex formation (65), an autoinhibitory domain (AID) that suppresses WNK kinase activity (66), and C-terminal SPAK/OSR1 binding motifs are shown. Downstream of exon 4a, L-WNK1 and KS-WNK1 are identical. (C) Amino acid sequence of rat exons 11 and 12. Two canonical PY motifs are highlighted in yellow. The exons are proline rich and contain numerous PX and PX motives. (D) The PY motifs in exons 11 and 12 are highly conserved in mammals. The exons are not present in nonmammalian organisms, such as Xenopus, zebrafish, Drosophila, and C. elegans.
Notably, this more recent study also found that exon 12 is highly represented in kidney relative to other tissues and is enriched in the aldosterone-sensitive distal nephron (ASDN), suggesting that it plays an important role in renal salt transport physiology.

To determine whether WNK1 proteins containing PY motifs are expressed in the distal nephron, we generated affinity-purified antisera to a peptide epitope located within exon 12 of WNK1 (Figure 2A). The antisera specifically recognized a recombinant Myc L-WNK1 fragment containing exon 12 that was transiently expressed in HEK-293T cells. (C) Immunoblots of dissected rat kidney cortex homogenates (50 μg) probed with the exon 12 antibody revealed a discrete band at approximately 250 kDa, corresponding to the MW of L-WNK1 (L), and shorter species migrating between 150 and 250 kDa, consistent with KS-WNK1 and/or C-terminal proteolytic fragments of L-WNK1 (bracket). Both high-MW bands were not seen when the antibody was preincubated with excess immunizing peptide. Several lower-MW species were also quenched with peptide competition. A nonspecific band was noted between 75 and 100 kDa. (D) Bands of similar MWs were noted in mpkCCDc14 CCD cells. (E) Along with 2 other WNK1-specific antibodies, the antisera recognized a band at approximately 250 kDa in HEK-293T cells that was absent in genetically validated L-WNK1 KO cells (19). (F) Immunostaining of mouse kidney with exon 12 antisera revealed a tubule-specific signal that colocalized with NCC in DCT and aquaporin-2 (AQP2) in CCD. Scale bars: 10 μm. See also Supplemental Figures 1 and 2.
were, however, able to detect both L- and KS-WNK1 at the transcript level in mpkCCD c14 cells. Thus, we decided to perform our initial analysis of the effects of aldosterone on WNK1 function in this model. In mpkCCDc14 cells, dose-response tests indicated that the abundance of WNK1 isoforms increased as the concentration of aldosterone was titrated from 0–500 nM (Figure 3A). This was observed for high-MW species corresponding to long and short forms of WNK1. In addition, treatment with 100 nM aldosterone increased WNK1 abundance over a 16-hour timecourse (Figure 3B). This effect was seen with 3 antibodies targeting different WNK1 epitopes, including an N-terminal L-WNK1–specific antibody to exon 1 and two C-terminal antibodies directed to exons 12 and 28. Aldosterone also increased total SPAK abundance and SPAK/OSR1 phosphorylation, detected with an antibody that recognizes known WNK1 regulatory S-motif phosphosites at serine 383 of SPAK and serine 325 of OSR1, a marker of SPAK/OSR1 activation (3) (Figure 3, C and D).

A previous report found that a noncoding RNA, miR-192, negatively regulated WNK1 mRNA abundance and was suppressed by aldosterone (20). We therefore reasoned that aldosterone-induced downregulation of miR-192 might explain the increase in WNK1 isoforms observed in mpkCCD c14 cells. However, 100 nM aldosterone did not reduce miR-192 expression in these cells over a timecourse of 16 hours, as measured by quantitative PCR (qPCR) (Supplemental Figure 3, A and B). Consistent with this observation, 100 nM aldosterone did not trigger an increase in the abundance of mRNA encoding KS-WNK1, L-WNK1, or exon 12–containing WNK1 isoforms in mpkCCD c14 cells over a timecourse of 16 hours, as measured by quantitative PCR (qPCR) (Supplemental Figure 3, A and B).
Exons 11 and 12 destabilize L-WNK1 in a PY motif-dependent manner. To determine whether the 2 PY motifs in exons 11 and 12 of WNK1 are functional, we assessed the steady-state protein abundance of epitope-tagged L-WNK1 constructs following transient expression in HEK-293T cells, a cell line that endogenously expresses NEDD4-2 and its closely related isoform NEDD4-1 (23). The total protein abundance of Myc-tagged L-WNK1 was compared with L-WNK1 Δ11/12, a previously described PY motif-deficient L-WNK1 splice variant (24), and L-WNK1 Y829A/Y945A, a PY-null mutant lacking the tyrosines that are critical for WW domain interaction (Figure 4A; ref. 25). As shown in Figure 4B, the steady-state abundance of the Δ11/12 variant and the PY-null mutant were both significantly increased relative to full-length L-WNK1. To determine if these differences were due to altered protein stability, we performed a cycloheximide (CHX) chase assay to monitor the timecourse of protein degradation after arresting translation. In these studies, the Δ11/12 and PY-null L-WNK1 constructs were more resistant to protein degradation than the WT full-length protein (Figure 4C), suggesting that the PY motifs in exons 11 and 12 decrease WNK1 stability, possibly via interaction with NEDD4-2.

Nedd4-2 targets PY motif-containing WNK1 isoforms for degradation via the ubiquitin proteasome system. To test whether endogenously expressed NEDD4 E3 ligases regulate WNK1 abundance in HEK-293T cells, we conducted an RNA interference (RNAi) study. Transfection of pan-NEDD4 siRNAs targeting human NEDD4-1 and NEDD4-2 increased the steady-state protein expression of endogenous L-WNK1 isoforms containing exon 12, relative to cells transfected with a scrambled siRNA control (Figure 5A). WNK4 protein expression was unaltered, indicating that the effect was specific for WNK1. In overexpression experiments, WT NEDD4-2 consistently decreased steady-state full-length L-WNK1 abundance at 3 different transfection ratios (Figure 5B); in contrast, a dominant-negative NEDD4-2 mutant lacking catalytic activity had no effect (NEDD4-2 C938S; ref. 26). Consistent with these findings, overexpressed WT NEDD4-2 accelerated L-WNK1 degradation in CHX chase assays, an effect that was attenuated when the WT E3 ligase was substituted with the dominant-negative NEDD4-2 mutant, or when WT NEDD4-2 was coexpressed with PY-null mutant L-WNK1 (Figure 5C). In HEK-293T cells, NEDD4 isoforms coimmunoprecipitated with overexpressed WT L-WNK1 and exhibited decreased interaction with the PY-null mutant and the Δ11/12 L-WNK1 isoform (Figure 5D). Moreover, in mpkCCD c14 epithelia, both long and short forms of WNK1 coimmunoprecipitated in native complexes with NEDD4 isoforms (Figure 5E).
Since intact catalytic activity is required for NEDD4-2 to downregulate WNK1 abundance, we asked whether NEDD4-2 targets WNK1 for degradation via the ubiquitin proteasome pathway. Accordingly, we conducted CHX chases in which L-WNK1 was coexpressed with WT NEDD4-2 in the absence and presence of the proteasomal inhibitor MG-132. As shown in Figure 5F, a 4-hour preincubation of HEK-293T cells with 10 μM MG-132 attenuated the rate of L-WNK1 degradation relative to vehicle-treated controls. In silico analysis using the UbPred algorithm (27) identified numerous potential ubiquitylation acceptor lysines on L-WNK1, both at the N- and C-terminal regions of the protein. Most of these were clustered downstream of exon 12 (Figure 5G). Consistent with this prediction, in vivo ubiquitylation assays detected an appropriately sized high-MW smear in HEK-293T cells expressing a PY motif–containing WNK1 C-terminal fragment, and NEDD4-2 coexpression enhanced this signal (Figure 5H). In contrast, no appreciable change above background was observed in NEDD4-2 transfected cells coexpressing the L-WNK1 N-terminus, suggesting that this region was relatively spared from NEDD4-2 ubiquitylation. Ubiquitylation of the WNK1 C-terminus was dependent on intact NEDD4-2 catalytic activity, as no significant ubiquitylation was apparent when the Halo-tagged C-terminus was coexpressed with dominant-negative NEDD4-2 (Supplemental Figure 4A). In addition, C-terminal fragments lacking exons 11 and 12 or containing mutations within the exon 11/12 PY motifs were ubiquitylated less strongly compared with an intact WT C-terminus (Supplemental Figure 4B). Since this C-terminal WNK1 fragment is present in both L-WNK1 and KS-WNK1, these findings indicate that NEDD4-2 targets both isoforms for degradation (Figure 5I).

The WNK1 cDNAs used for these studies were constructed from the original L-WNK1 clone isolated from brain (AAF74258.1; ref. 24). This clone contains a variant serine located 7 amino acids from the C-terminal end of the protein that replaces a phylogenetically conserved glycine residue (glycine 2368, based on the amino acid numeration for RefSeq XP_008761429.1: the rat L-WNK1 isoform containing exons 9, 11, and 12 studied here). Changing this serine residue back to the conserved glycine enhances its activity toward NCC via SPAK/OSR1 (28). Since serine residues may function as noncanonical sites for ubiquitylation (29), we sought to determine whether this glycine-to-serine variant influences WNK1 stability and NEDD4-2 sensitivity. In CHX chase assays, however, we found that the evolutionarily conserved protein degraded robustly in the presence of NEDD4-2 (Supplemental Figure 5). Degradation of the glycine-amended construct was also dependent on the PY motifs in exons 11 and 12, since inactivating these signatures stabilized the protein significantly (Supplemental Figure 5).

SGK1 inhibits NEDD4-2-mediated downregulation of WNK1. Aldosterone induces the transcription of SGK1, which interferes with NEDD4-2 function by phosphorylating residues that alter PY motif binding, including a canonical “major” site at mouse serine 328 (analogous to Xenopus serine 444) (30). To test whether SGK1 can inhibit NEDD4-2-mediated regulation of WNK1, we first tested the effect of small-molecule SGK1 inhibitors on WNK1 abundance in mpkCCD c14 cells. Pretreatment with the competitive SGK1 inhibitor GSK650394 or the SGK1 activation blocker LY294002 prevented NEDD4-2 major-site phosphorylation and WNK1 induction (Figure 6A). This indicates that aldosterone is incapable of upregulating WNK1 abundance in the absence of SGK1 activity. Consistent with these findings, coimmunoprecipitation studies in mpkCCD c14 cells revealed that the interaction between NEDD4 isoforms and WNK1 was reduced when cells were treated with 100 nM aldosterone (Figure 6B). In CHX chase studies, the rate of L-WNK1 degradation was attenuated when NEDD4-2 was coexpressed with SGK1-S422D, a constitutively kinase-active form of SGK1 (31), when compared with cells transfected with the kinase-dead SGK1 mutant K127M (Figure 6C). Furthermore, coimmunoprecipitation studies with L-WNK1 1-1030, a stable N-terminal L-WNK1 fragment that contains NEDD4-2 binding sites but lacks the C-terminal ubiquitylation hub (Figure 5I), revealed that NEDD4-2 associated more strongly with WNK1 when it was coexpressed with kinase-dead SGK1, rather than the constitutively active S422D form (Figure 6D).

The aforementioned major SGK1 phosphorylation site on NEDD4-2, located at mouse serine 328 (Xenopus serine 444), is also accompanied by other “minor” SGK1 phosphorylation sites at mouse serine 222 and threonine 247 (corresponding to Xenopus serine 338 and threonine 363) (32). These minor sites are believed to be more SGK1 specific (32). All 3 of these phosphorylation sites mediate binding to I4-3-3 proteins, which sequester and inactivate NEDD4-2 (30, 33). Mutation of these residues effectively renders the NEDD4-2 protein resistant to inhibition by SGK1 (14, 34). Coexpression of L-WNK1 and constitutively active SGK1 S422D with a phosphorylation-resistant double–minor site Nedd4-2 mutant (Xenopus Nedd4-2 S338A/T363A; termed NEDD4-2 PR here) decreased steady-state L-WNK1 protein expression, relative to L-WNK1–expressing cells cotransfected with SGK1 and WT NEDD4-2 (Figure 6E). These findings indicate that SGK1 inhibits WNK1 degradation by phosphorylating NEDD4-2 at specific residues previously shown to augment binding to I4-3-3 proteins.
WNK1 is required for the NEDD4-2/SGK1 axis to modulate NCC abundance and phosphorylation status. The observation that NEDD4-2 and SGK1 can control WNK1 abundance suggests that these 2 proteins may exert at least some of their downstream regulatory effects on NCC via WNK1. To test this hypothesis, we initially attempted simultaneous L-WNK1 knockdown and NCC/NEDD4-2/SGK1 overexpression studies in HEK-293T cells. These experiments were technically challenging, how-
ever, due to inefficient WNK1 gene silencing. To overcome this hurdle, we employed CRISPR/Cas-mediated gene-editing technology to generate a WNK1 KO cell line. These previously validated KO cells completely lack endogenous L-WNK1 protein expression and exhibit reduced native SPAK/OSR1 activity (19). We compared the effect of SGK1 on NCC phosphorylation status in WNK1 KO cells and paired unedited controls, in the presence of either WT NEDD4-2 or the SGK1-resistant NEDD4-2 PR mutant. In unedited HEK-293T cells, NCC abundance and phosphorylation status was higher when active SGK1 was coexpressed with WT NEDD4-2, compared with when it was coexpressed with the NEDD4-2 PR mutant (Figure 7, A and B). This is consistent with prior observations that SGK1 can modulate NCC activity through NEDD4-2 phosphorylation (35). The changes in

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**Figure 7. WNK1 is necessary for NEDD4-2 and SGK1 to modulate NCC abundance and phosphorylation in HEK-293 cells.**

(A) WNK1 KO HEK-293T cells and unedited controls were transfected with NCC (1 μg), SGK1-S422D (1 μg), and either WT or phosphorylation-resistant (PR) NEDD4-2 (1 μg). Thirty-six hours after transfection, the cells were lysed and subjected to immunoblotting with the indicated antibodies. (B) Quantification of total NCC, phosphorylated NCC, and phosphorylated SPAK/OSR1 signal in A. n = 4; P values as indicated by 1-Way ANOVA Bonferroni multiple-comparisons post hoc test. (C) Proposed model of the effects of WNK1 deletion on NEDD4-2/SGK1 regulation of NCC in HEK-293T cells. In unedited cells, WT NEDD4-2 interacts with L-WNK1. This interaction can be disrupted by SGK1 phosphorylation at previously defined major and minor sites, increasing L-WNK1 abundance, SPAK/OSR1 phosphorylation, and NCC activation. In WNK1 KO cells, NEDD4-2 and SGK1 cannot alter NCC phosphorylation status. Other regulators, possibly WNK complexes that are less NEDD4-2 sensitive, maintain NCC activity through compensation.
In this regard, WNK3 was recently shown to be capable of activating NCC independently of NEDD4-2 and SPAK/OSR1 (36). Alternatively, the NCC activity may have been maintained by other undefined NCC-specific regulators that compensate for the absence of WNK1 activity. Collectively, these findings indicate that aldosterone-regulated interactions between NEDD4-2 and SGK1 are unable to modulate SPAK/OSR1 and NCC activity when WNK1 is absent (Figure 7C). The data therefore implicate WNK1 as a downstream NEDD4-2 substrate that transduces SGK1-dependent signals that regulate NCC, suggesting a molecular mechanism by which aldosterone-regulated signaling intermediates can interface with the WNK-SPAK/OSR1 signaling pathway.

NCC that were observed in unedited cells were associated with similar changes in SPAK/OSR1 phosphorylation status (Figure 7, A and B), suggesting that signaling through WNK kinases was required for this effect. In WNK1 KO cells, however, we found that the abundance and phosphorylation status of SPAK/OSR1 and NCC was not altered, regardless of whether active SGK1 was coexpressed with WT NEDD4-2 or the phosphorylation-resistant mutant. Interestingly, though SPAK/OSR1 phosphorylation was decreased in WNK1 KO cells (Figure 7, A and B), the abundance and phosphorylation status of NCC was not decreased relative to unedited controls, possibly because of compensatory changes in other endogenous WNK kinases within the passage window studied, such as WNK2 or WNK3 (Figure 7, A and C).
Renal tubular NEDD4-2 and SGK1 deficiency exerts opposite effects onWNK1 abundance and downstream SPAK/OSR1 activation. To determine the in vivo relevance of these findings, we analyzed the WNK-SPAK/OSR1 pathway in an inducible renal tubule–specific Nedd4-2 KO mouse model (Nedd4-2^{fl/fl} Pax8-rtTA TRE-LC1, herein referred to as Nedd4-2^{fl/fl}Pax8/LC1; ref. 37). These mice are homozygous for a floxed Nedd4-2 allele, bred into double-transgeninc mice that express tetracycline-inducible Cre recombinase selectively in renal tubules. Administering doxycycline to these animals enables Cre-mediated excision of the loxP-flanked region, resulting in postdevelopmental Nedd4-2 ablation. On a high-NaCl diet, Nedd4-2^{fl/fl}Pax8/LC1 mice exhibit increased NCC activity relative to controls, despite suppressed aldosterone levels. Consistent with our observations in cell-culture systems, we found that the expression of exon 12–containing WNK1 isoforms was increased in double-transgenic Nedd4-2^{fl/fl}Pax8/LC1 mice, relative to single-transgenic controls (Figure 8A and Supplementary Figure 6A). The 250 kDa L-WNK1 species was strongly increased, and this was associated with enhanced S-motif phosphorylation of SPAK/OSR1, a signature of WNK-mediated activation (38). Total and phosphorylated NCC was also significantly increased, consistent with prior reports (37). Using a C-terminal SPAK antibody (38), we were unable to detect a qualitative directional shift in the expression of previously described long and short SPAK isoforms in Nedd4-2^{fl/fl} KO mice (Supplemental Figure 6A; refs. 39, 40), and in contrast to WNK1, we saw no significant difference in WNK4 abundance between control and KO groups. Collectively, these findings support the in vitro observations that NEDD4-2 negatively regulates WNK1 abundance. Though the ablation of NEDD4-2 in renal tubules could potentially augment NCC activity via multiple mechanisms, these data are consistent with the view that Nedd4-2 deletion activates NCC, at least in part by increasing L-WNK1 protein abundance and downstream SPAK/OSR1 phosphorylation.

We also tested the effect of aldosterone infusion on WNK1 protein abundance in Nedd4-2^{fl/fl} KO mice. In these studies, control and KO animals were subjected to aldosterone infusion or vehicle via s.c. implanted minipump over 3 days. In control single-transgenic animals with intact NEDD4-2 expression, aldosterone infusion increased the protein abundance of WNK1 significantly, relative to vehicle-treated animals (Figure 8B and Supplementary Figure 6B). Consistent with a net increase in L-WNK1 kinase activity, downstream SPAK/OSR1 phosphorylation was increased in control mice. In contrast, Nedd4-2^{fl/fl}Pax8/LC1 animals exhibited high WNK1 exon 12 isoform expression at baseline, and aldosterone did not increase the abundance of these WNK1 isoforms further, suggesting that aldosterone induces WNK1 protein expression in vivo via NEDD4-2 inhibition.

To further evaluate how the NEDD4-2/SGK1 axis influences WNK1 protein expression in vivo, we also analyzed WNK1 protein abundance in Sgk1^{Pax8/LC1} KO mice (Sgk1^{fl/fl} Pax8-rtTA TRE-LC1), the corresponding tet-inducible renal tubule–specific KO model that lacks SGK1 expression. Previous studies have shown that these mice exhibit a phenotype that is a mirror image to that of Nedd4-2^{fl/fl}Pax8/LC1 KOs; i.e., when subjected to a low-NaCl diet, Sgk1^{Pax8/LC1} mice develop relative salt wasting, reduced blood pressure, and elevated aldosterone levels compared with controls (41). These effects are associated with low NCC expression and reduced NEDD4-2 major-site phosphorylation. Because our data suggest that WNK1 acts as a downstream intermediary between NEDD4-2 and NCC, we reasoned that Sgk1^{Pax8/LC1} mice should exhibit low WNK1 protein abundance, compared with controls, when placed under dietary conditions that promote aldosterone secretion. Consistent with this, we found that Sgk1^{Pax8/LC1} mice placed on an NaCl-restricted diet (<0.1% NaCl for 7 days) exhibited significantly lower WNK1 exon 12 isoform abundance compared with single-transgenic controls subjected to the same dietary maneuver (Figure 9, A and B). WK4 abundance was also significantly lower in these mice, possibly due to known direct interactions between SGK1, WNK1, and WNK4 (42–44). Consistent with decreased WNK1 and WNK4 kinase activity, downstream SPAK/OSR1 phosphorylation was also reduced (Figure 9, A and B).
Figure 10. Model of aldosterone-mediated activation of SPAK/OSR1 and NCC via WNK1. In the baseline euclidean state, active NEDD4-2 binds to the PY motifs in exons 11 and 12 of WNK1, ubiquitylating the WNK1 C-terminus and targeting it for proteasomal degradation. This limits total WNK1 protein expression, acting as a closed gate that shuts off downstream SPAK/OSR1 and NCC activity. During states where aldosterone levels are high, such as extracellular fluid volume depletion, SPAK and OSR1 are phosphorylated and active. Aldosterone contributes to this process by driving the transcription of SGK1 in the distal nephron. Active SGK1 phosphorylates and inactivates NEDD4-2, reducing its interaction with exons 11 and 12 of WNK1. This opens the gate, increasing total WNK1 protein expression. Once WNK1 protein is present, SGK1 can directly phosphorylate the N-terminus of L-WNK1, resulting in selective L-WNK1 activation (43). Active L-WNK1 then stimulates SPAK and OSR1 to directly phosphorylate NCC. Previous data suggest that the SGK1 pathway may be particularly active during states that require the conservation of sodium (59, 68). Other stimuli for aldosterone secretion, such as hyperkalemia, may selectively upregulate KS-WNK1 protein expression via additional undefined mechanisms to attenuate L-WNK1 and NCC activity (55, 59, 60). Such effects may act in concert with changes in intracellular [Cl−] to suppress WNK activity (61). Additionally, other SGK1-dependent effects on NCC, including suppression of WNK4-mediated NCC degradation (46) or ubiquitylation of NCC by NEDD4-2 (35), are not shown here to highlight the mechanism of SPAK/OSR1 activation described in this study.
NEDD4-2 and NCC form complexes that could be disrupted by SGK1, the exact nature of the interaction remains undefined, as NCC contains no canonical PY motif. Nevertheless, NCC protein abundance is strongly increased in Nedd4-2 KO mice (37), implicating an important role for NEDD4-2-mediated ubiquitylation of NCC in blood-pressure regulation. Both of these reports potentially explain how aldosterone can posttranslationally alter the abundance of NCC through effects on protein trafficking. However, other studies have suggested that aldosterone also stimulates distal sodium transport via SPAK and OSR1 (6, 47). Thus, aldosterone must somehow signal through WNK kinases to regulate SPAK/OSR1-mediated NCC activation. The data presented here suggest that WNK1 is required for this effect, since WNK1 ablation in cells negates the ability of NEDD4-2 and SGK1 to modulate SPAK/OSR1 and NCC phosphorylation, and in vivo studies indicate that aldosterone can activate SPAK/OSR1 by altering WNK1 protein expression and activity.

Our analyses confirm prior observations that total NCC protein abundance is increased in Nedd4-2−/−/L1 KO mice, relative to Nedd4-2−/−/L1 KO mice. We also observed a comparable increase in the abundance of phosphorylated NCC, consistent with previous reports that the phosphorylated-to-total NCC ratios between controls and Nedd-2 KO animals are similar (37). At first glance, this result may seem at odds with the increase in SPAK/OSR1 phosphorylation seen in Nedd4-2−/−/L1 KO mice. An important issue to consider, however, is the observation that Nedd4-2−/−/L1 KO mice are hypoaaldosteronemic, relative to controls. Since these mice are hypertensive from disinhibition of NCC activity, the low aldosterone levels are consistent with feedback suppression due to extracellular fluid volume expansion. If NEDD4-2 ablation solely mediated this effect by way of a pure effect on cotransporter trafficking, the increased number of NCC molecules at the plasma membrane would be expected to downregulate SPAK and OSR1 activity, and decrease the phosphorylated-to-total NCC ratio due to negative feedback. Clearly, this was not observed in our studies, since we detected an increase in WNK1 abundance and a corresponding increase in SPAK/OSR1 phosphorylation status (Figure 8). It is possible that the lack of change in the phosphorylated-to-total NCC ratio could be due an unidentified compensatory factor that attenuates NCC phosphorylation status in response to volume expansion, such as a phosphatase. The catalytic activity of such a factor might partially mitigate enhanced SPAK/OSR1 activity, resulting in normalization of the phosphorylated-to-total NCC ratio. Indeed, previous work indicates that cation-chloride cotransporters such as NCC can be dephosphorylated (48), and recent studies suggest that such mechanisms may be highly relevant to NCC regulation in vivo (49).

Given the importance of the WNK signaling pathway in the fine-tuning of electrolyte balance (1), the stability of WNK kinases is likely tightly controlled. One key regulator is the KLHL3/CUL3 RING E3 ubiquitin ligase complex, which ubiquitylates and degrades WNK1 and WNK4 (50–52). Mutations of this complex cause FHHt by impairing its ability to dispose of WNK1 and WNK4, either through reduced binding of WNK1 and WNK4 to the KLHL3 adaptor (51) or by CUL3 exon-skipping mutations that augment ubiquitylation and degradation of KLHL3 (53). Given the fact, however, that this E3 ligase complex regulates more than one WNK, other mechanisms must be in place to modulate WNK complex composition and stoichiometry. Here, we identify NEDD4-2 as an E3 ligase that interacts with a specific WNK kinase through a defined, aldosterone-regulated signaling mechanism. Notably, the canonical PY motifs in WNK1 are unique among kidney-expressed WNKs and therefore provide a distinctive binding site for NEDD4-2. Since this E3 ligase is tightly regulated by SGK1, the interaction provides a mechanism by which aldosterone can specifically access and activate the WNK signaling pathway through rapid SGK1 induction. Moreover, the functional PY motifs are the only 2 such motifs contained within the entire WNK1 sequence and are tightly restricted to the same proline-rich region of the protein, within 2 neighboring exons that undergo extensive tissue-specific alternative splicing. Therefore, they reside within an adaptable domain that can change, depending on the physiological requirements of a specific cell type or tissue. WNK1 has been implicated in a variety of processes outside of epithelial ion transport, including cell growth and differentiation, cardiovascular development, and neuronal function (44). Thus, regulated splicing of these proline-rich cassettes may be a mechanism by which cells can limit the diverse actions of WNK1 to a specific subset of operations, independently of KLHL3 and CUL3 regulation.

Previous work has established that L-WNK1 can function as an upstream SGK1 activator (54). In contrast to those results, we report here that NEDD4-2 negatively regulates WNK1 abundance and that this effect can be blocked by SGK1. Therefore, our data identify WNK1 as a downstream SGK1 target. Although these findings may seem contradictory, they can be explained by key structural differences in the WNK1 cDNAs that were used in each of the studies. Notably, all of the experiments that had identified SGK1 as an L-WNK1 target were conducted with either N-terminal L-WNK1 fragments or an L-WNK1 Δ11/12 splice variant lacking PY motifs. Thus, in those studies, the signaling pathway that was characterized was one in which NEDD4-2 interaction was minimized. Once the proline-rich cassettes were incorporated into L-WNK1, the kinase became a robust NEDD4-2 substrate whose abundance could be upregulated by SGK1, thus placing WNK1 downstream of SGK1 action. Collectively, these data suggest that splicing of exons 11 and 12 into the WNK1 sequence reorients the molecular relationship between WNK1, NEDD4-2, and SGK1 in aldosterone-sensitive tissues. As shown in this study and by others (10), WNK1 isoforms containing proline-rich cassettes are highly represented in the ASDN. Therefore, the pattern of WNK1 gene expression in the distal nephron positions WNK1 downstream of SGK1, with NEDD4-2 being an intermediary that suppresses WNK1 protein abundance in the absence of aldosterone. Previous observations that SGK1 directly phosphorylates and activates L-WNK1 through interactions with its N-terminus further support the notion that WNK1 functions downstream of SGK1 in the ASDN (43). Thus, during high aldosterone states, NEDD4-2 inhibition would be expected to increase WNK1 protein abundance, expanding the supply of L-WNK1 kinases that can be activated by SGK1 and trigger downstream signaling through SPAK and OSR1 (Figure 10).

The balance of L-WNK1 and KS-WNK1 isoforms has been proposed to function as a molecular switch that coordinates NaCl reabsorption and K+ secretion in the distal nephron (55). With regards to NCC regulation, L-WNK1 is a stimulator (28, 56), while KS-WNK1...
is an inhibitor that suppresses the catalytic activity of several WNK kinases (57, 58). We found that exons 11 and 12 are represented in both L-WNK1 and KS-WNK1 in human kidney tissue and that NEDD4-2 ubiquitylates the C-terminus common to both isoforms. Thus, aldosterone would be expected to increase the abundance of WNK1 isoforms that exert stimulatory and inhibitory effects toward NCC. Analyses of WNK1 protein expression indeed suggested this, since both L-WNK1 and lower-MW WNK1 species were induced by aldosterone (Figure 3). The net effect of this increase in total WNK1 expression, however, appeared to result in enhanced L-WNK1 activity, since SPAK/OSR1 phosphorylation was increased in aldosterone-treated mpkCCDc14 cells and in Nedd4-2 KO mice (Figure 3 and Figure 8). Since our data suggest that the protein abundance of both L- and KS-WNK1 are increased in the presence of aldosterone, one potential role of the proline-rich cassettes may be to increase total WNK1 availability during high aldosterone states. In other words, NEDD4-2 could function as a regulated gate that interacts with the PRO motifs in WNK1, shutting off WNK1-dependent signaling processes in the absence of aldosterone (Figure 10). By analogy, an increase in circulating aldosterone levels would open the gate via SGK1, inactivating NEDD4-2 and increasing the total number of WNK1 isoforms that are available for regulation. As reported previously, once total WNK1 protein expression is increased, active SGK1 could then selectively phosphorylate the N-terminus of L-WNK1, turning on its kinase activity (Figure 10). By analogy, an increase in circulating aldosterone levels would open the gate via SGK1, inactivating NEDD4-2 and increasing the total number of WNK1 isoforms that are available for regulation. As reported previously, once total WNK1 protein expression is increased, active SGK1 could then selectively phosphorylate the N-terminus of L-WNK1, turning on its kinase activity (Figure 10). By analogy, an increase in circulating aldosterone levels would open the gate via SGK1, inactivating NEDD4-2 and increasing the total number of WNK1 isoforms that are available for regulation. As reported previously, once total WNK1 protein expression is increased, active SGK1 could then selectively phosphorylate the N-terminus of L-WNK1, turning on its kinase activity (Figure 10).

**Methods**

**Molecular methods.** All L-WNK1 clones used in this study were derived from the original rat L-Wnk1 cDNA, isolated from rat forebrain (24) and were generated as described in the Supplemental Methods.

**Exon 12 antibody.** The WNK1 exon 12 antisera were generated by immunizing rabbits to a keyhole limpet hemocyanin-conjugated peptide epitope located within exon 12 of rat WNK1 (SQPAVLSLSQQPPTSSQQC). Peptide synthesis, production of rabbit antisera, and peptide affinity purification were performed by Abgent.

**Cell culture and transfection.** HEK-293T cell culture and transfection protocols were carried out as described previously (62). Cells were analyzed 24–48 hours after transfection. Mouse CCD (mpkCCDc14) cells were cultured using previously established protocols (18). For analyses of aldosterone response, mpkCCDc14 cells were grown to confluence in 6-well dishes and were cultured in basic medium lacking hormones 24 hours prior to incubation.

**Preparation of lysates and immunoblot analysis.** Immunoblot analysis was performed as described previously (62). Freshly dissected kidneys were flash frozen prior to analysis, and homogenates were prepared as described (63). Typically, 50–100 μg protein were analyzed by immunoblotting of kidney lysates.

**RNAi studies.** Transient siRNA-mediated knockdown of NEDD4 isoforms in HEK-293T cells was performed as described in the Supplemental Methods.

**Assays to monitor L-WNK1 degradation.** CHX chase assays in HEK-293T cells were carried out as described previously (62). For chase assays in the presence of the proteasome inhibitor MG-132, transiently transfected cells were pretreated with 10 μM MG-132 or DMSO vehicle control for 4 hours, prior to the initiation of the chase.

**In vivo ubiquitylation assay.** HaloLink resin pulldowns (Promega) were employed to analyze NEDD4-2–mediated ubiquitylation of the WNK1 N- and C-termini in HEK-293T cells, as described in detail in the Supplemental Methods.

**Coimmunoprecipitation studies.** Coimmunoprecipitation studies in HEK-293T and mpkCCDc14 cells were performed with antibody-conjugated resins or protein A/G agarose, as described (62).

**Transcript analysis.** RT-PCR studies in human kidney, and qPCR studies in mpkCCDc14 cells were carried out as described in the Supplemental Methods.

**Immunofluorescence microscopy.** Cryosectioning, blocking, and immunostaining of kidney sections from adult Sprague-Dawley rats was performed as described previously (64). Sections were mounted with Vectashield (Vector Laboratories) and examined using a Leica TCS SP5 confocal microscope.

**Animal studies.** Tetracycline inducible, nephron-specific Nedd4-22lox/fl KO mice (Nedd4-22lox/fl Pax8-rtTA TRE-LC1) and Sgk1fl/fl KO mice (Sgk1fl/fl Pax8-rtTA TRE-LC1) were studied using previously described protocols (37, 41), described in detail in the Supplemental Methods.

**Statistics.** Quantification of Western blots was carried out using NIH ImageJ software. Statistical analysis was performed using GraphPad Prism software. Measurements are presented as mean ± SEM. Comparisons between 2 groups were analyzed by unpaired 2-tailed Student’s or Welch’s t tests. Multiple comparisons were performed by t tests with Bonferroni correction or by 1-way ANOVA followed by the appropriate post hoc tests, as indicated. qPCR data were analyzed by Kruskal-Wallis nonparametric 1-way ANOVA, followed by Dunn’s multiple comparison post hoc test. A base P value of <0.05 was considered statistically significant. Quantification of endogenous WNK1 signal was limited to L-WNK1–specific bands (≥250 kDa) that were detected with the exon 12 antibody. We refrained from quantifying lower-MW species (<250 kDa), as short KS-WNK1 isoforms could not be reliably distinguished from proteolytically processed WNK1 fragments.

**Study approval.** All animal protocols were approved by the IACUC at the University of Pittsburgh School of Medicine, or by the Swiss Federal Veterinary Office, and carried out in accordance to the animal welfare act.
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