Trends in Biochemical Sciences

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REVIEWS

Review

Physiological Functions of Nedd4-2: Lessons from Knockout Mouse Models

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Protein modification by ubiquitination plays a key evolutionarily conserved role in regulating membrane proteins. Nedd4-2, a ubiquitin ligase, targets membrane proteins such as ion channels and transporters for ubiquitination. This Nedd4-2-mediated ubiquitination provides a crucial step in controlling the membrane availability of these proteins, thus affecting their signaling and physiological outcomes. In one well-studied example, Nedd4-2 fine-tunes the physiological function of the epithelial sodium channel (ENaC), thus modulating Na^+ reabsorption by epithelia to maintain whole-body Na^+ homeostasis. This review summarizes the key signaling pathways regulated by Nedd4-2 and the possible implications of such regulation in various pathologies.

Highlights

Nedd4-2 (also called NEDD4L) is a HECT-type ubiquitin ligase that is involved in the regulation of multiple membrane proteins.

Nedd4-2 is likely to influence multiple signaling networks via specific targeting of proteins in different tissues, and variants in human *NEDD4L* are associated with developmental disorders, hypertension, and end-stage renal disorders.

Mouse knockout studies indicate that Nedd4-2 is a key regulator of Na^+ homeostasis, and ENaC is one of its most important physiological substrates.

Pathological consequences of dysregulated Nedd4-2 in mice include disorders of the respiratory, renal, cardiac, neural, and immune systems.

Observation that Nedd4-2 deficiency causes kidney disease provides a link between dysregulated Na^+ reabsorption and renal tubular injury.

Nedd4-2 Ubiquitin Ligase: Background

Nedd4-2 belongs to the evolutionarily conserved **Nedd4 family** (see Glossary) of ubiquitin ligases that are characterized by a C2 domain, 2–4 **WW** domains, and a C-terminal **HECT**-type ubiquitin ligase domain [1]. This family comprises of nine members in humans which are involved in regulating numerous signaling pathways [2]. Nedd4-2, closely related to the founding and most ancient member of the family Nedd4, was originally identified in a screen for genes downregulated during development of the central nervous system [3,4].

Many putative substrates of Nedd4-2 have been identified, the best-characterized being the **epithelial sodium channel** (ENaC) [5]. In recent years the physiological importance of Nedd4-2-dependent regulation of substrates has become clear from studies in genetically modified mice. Furthermore, mutations or **single nucleotide polymorphisms** (SNPs) in the human Nedd4-2 gene (*NEDD4L*) are associated with developmental disorders [6], **hypertension** [7], epilepsy [8], and end-stage renal disease [9], highlighting the importance of this gene to human pathophysiology. This review summarizes the biochemical and cellular regulation by Nedd4-2 and the pathological consequences of disruption of such regulation, with a particular focus on studies employing genetically modified mouse models.

Ubiquitination by Nedd4 Ligases and Nedd4-2 (NEDD4L)

Post-translational modification by **ubiquitination** is crucial in many signaling pathways, resulting in the targeted recycling, degradation, or stabilization of proteins [10]. Ubiquitination is a multistep process involving a ubiquitin activating enzyme (E1), ubiquitin-conjugating enzyme (E2), and ubiquitin ligase (**E3**) which transfers ubiquitin onto the substrate protein [11]. There are two main types of E3s; RING E3s that act as a scaffold to facilitate the transfer of ubiquitin from an E2 onto substrates, and HECT E3s that act as an acceptor for ubiquitin, which is then transferred to the substrates. The largest group of HECT E3s is the Nedd4 family, of which Nedd4 is the prototypic member.

Nedd4-2 is the closest homolog of Nedd4 and is present in all vertebrates. Several isoforms of Nedd4-2, derived from alternatively spliced transcripts, have been identified in mouse and

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human tissues [5]. The longest isoform contains a C2 domain, four WW domains, and the HECT domain (Figure 1A). The N-terminal C2 domain is implicated in membrane targeting and protein–protein interactions [12], and the four WW domains (residing between the HECT and C2 domains) bind substrates and regulatory proteins [13,14]. These domains recognize PPxY (PY) or similar motifs in interacting partners. The HECT domain of Nedd4-2 catalyzes polyubiquitin chain assembly in a manner similar to that reported for other HECT domain-containing proteins – a two-step mechanism requiring two linked E2 ubiquitin binding sites [15]. Like other HECT E3s, Nedd4-2 is predicted to assemble substrate-linked ubiquitin chains containing Lys-63, Lys-48, and Lys-11 linkages [16]. Thus, substrate ubiquitination by Nedd4-2 could lead to degradation by either lysosomes or the proteasome, and/or altered cell signaling.

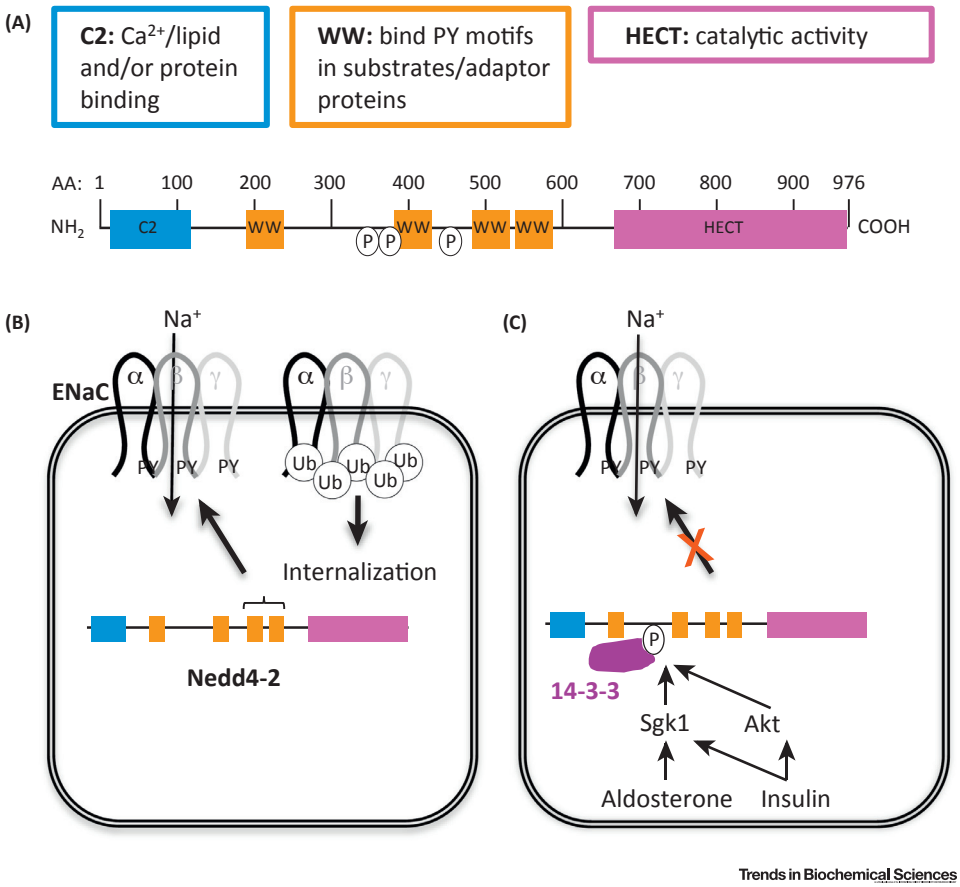


Figure 1. Domain Architecture of Nedd4-2 and Its Action on ENaC. (A) Nedd4-2 comprises an N-terminal C2 domain, four WW domains, and a C-terminal HECT domain. The C2 domain is calcium-dependent, and targets Nedd4-2 to the membrane/lipid (also implicated in protein binding). WW domains facilitate interaction with other proteins. The HECT is the catalytic ubiquitin ligase domain. Phosphorylation sites (P) for kinases Sgk1 and Akt are indicated. (B) ENaC is a membrane-localized channel in epithelial cells that comprises a heterotrimer of α, β, and γ subunits. PY motifs within all subunits of ENaC bind to WW domains (primarily WW3 and WW4) of Nedd4-2. This results in the transfer of ubiquitin onto ENaC and its subsequent internalization from the membrane. (C) Nedd4-2 can be maintained in an inactive form to allow higher ENaC activity. Many factors, including aldosterone, which activates Sgk1, and insulin that activates Akt1 and Sgk1, result in phosphorylation of Nedd4-2 by these kinases. This in turn allows the binding of 14-3-3 and inhibition of the Nedd4-2/ENaC interaction, and thus higher ENaC membrane retention and activity. Abbreviations AA, amino acid; Ub, ubiquitin.

Glossary

Allergic inflammation: a complex biological response to allergen exposure, involving mast cells.
Autophagy: intracellular degradation system that delivers contents of the cytoplasm to the lysosome.
Bradycardia: slower than normal heart rate.
Cystic fibrosis (CF): a genetic disorder primarily affecting the lungs and digestive system, with thick mucus secretions.
Diuretic: a substance that promotes increased production of urine.
E3: ubiquitin protein ligase; transfers ubiquitin from a ubiquitin-conjugating enzyme to a substrate protein.
Epithelial sodium channel (ENaC): the epithelial sodium channel, an inwardly directed Na ion channel on epithelial cell membranes.
Fibrosis: the formation of excess fibrous connective tissue.
HECT (homologous to E6–AP carboxyl terminus): a protein domain found in ubiquitin ligases that accepts ubiquitin from an E2 activating enzyme.
Hypertension: high blood pressure.
Hypertrophy: an increase in organ/tissue volume owing to enlargement of component cells.
Hypomorphic: reduction in gene function, but not a complete loss.
Liddle syndrome: a rare hereditary disorder characterized by early hypertension due to elevated ENaC activity.
Mast cells: immune cells containing secretory granules that mediate inflammatory responses such as hypersensitivity and allergic reactions.
Myocardial infarction: necrosis of cardiac tissue.
Necrosis: death of cells and living tissue caused by acute insult.
Nedd4 family: a group of HECT type ubiquitin ligases that also contain one C2 and 2–4 WW domains.
Neural network synchrony: the simultaneous/synchronous oscillation of membrane potentials in a network of neurons connected with electrical synapses.
Passive cutaneous anaphylaxis: a skin response to an allergen, characterized by increased permeability of vessels.
Pseudohypoaldosteronism type II (PHA-II): a heterogeneous group of

Regulation of Nedd4-2

The substrate specificity and functions of Nedd4 family members, such as Nedd4-2, are determined by phosphorylation, binding of adaptors and accessory proteins to specific regions of the ligase, intramolecular interactions, and deubiquitination.

Phosphorylation can lead to both inhibition and activation of Nedd4-2. The 14-3-3 family of ubiquitous adaptor proteins bind to phosphoserine and phosphothreonine motifs and are well known to regulate Nedd4-2-dependent ENaC ubiquitination [17,18]. In response to insulin and aldosterone, signaling kinases Sgk1 and Akt phosphorylate Nedd4-2 on specific serine residues [19] (Figure 1), which promotes binding of 14-3-3 proteins and prevents Nedd4-2 interacting with ENaC [17,20–22] (Figure 1C). In contrast to Sgk1 and Akt, phosphorylation mediated by AMPK [23] and JNK1 [24] appears to be required for Nedd4-2 activation. Furthermore, phosphorylation of Nedd4-2 by IKKβ is required for IKKβ-mediated regulation of ENaC [25].

Ndfip1 and Ndfip2 are adaptor proteins which contribute to the regulation of Nedd4 family members. These proteins contain three transmembrane domains and localize to membranes in the Golgi, endosomes, and multivesicular bodies [26]. Through their three PY motifs they bind to the WW domains in Nedd4-2 to aid in the recruitment of targets such as the divalent metal ion transporter DMT1 [27], ENaC [28], the water channel aquaporin 2 (AQP2) [29], and the tyrosine kinase Syk in **mast cells** [30]. Ndfip1 also recruits Nedd4 ligases, including Nedd4-2, into exosomes [31], and Ndfip2 binding to Nedd4 E3s has been suggested to activate the ligase function of these enzymes [32]. In addition, arrestin domain-containing proteins (Arrdcs) also act as adaptors for Nedd4-2 and other Nedd4 E3s. These adaptors recruit E3s to plasma-membrane targets, again through their PY motifs, promoting ubiquitination and endocytosis of membrane proteins [33].

WW domains within Nedd4-2 have also been demonstrated to bind to the LPxY motif in its HECT domain [34]. Mutations in the LPxY motif decrease NEDD4-2 binding to ENaC subunits and its ability to inhibit channel activity [34], suggesting that intramolecular interactions inhibit Nedd4-2 autoubiquitination.

Finally, the deubiquitinase Usp2-45 interacts with the N terminus and the HECT domain of Nedd4-2 to position itself for ENaC deubiquitination, thereby increasing ENaC cell-surface retention [35,36]. Thus Usp2-45 modulates Nedd4-2-dependent ENaC regulation via direct interaction with Nedd4-2.

Substrates of Nedd4-2

Most tissues and cell types express Nedd4-2, and high levels are detected in liver, kidney, heart, and lung [1]. The best-studied target of Nedd4-2 is ENaC, which is found primarily in tight epithelia and allows the flow of Na^+ from the lumen across the apical cell membrane and into epithelial cells [37]. Three similar ENaC subunits α, β, and γ are arranged in trimeric stoichiometry in mammals, and are trafficked to the apical membrane to be functional [38]. Nedd4-2 binds to PPxY motifs located towards the C termini of all three ENaC subunits to ubiquitinate and target the channel for removal from the membrane [39,40], thereby affecting normal Na^+ homeostasis (Figure 1B).

In addition to ENaC, many other proteins, both with or without demonstrated PY motifs, have been identified as *in vitro* interacting partners of Nedd4-2 (Table 1). Some have been shown to be downregulated and others directly ubiquitinated by Nedd4-2. Predominantly featured are

disorders of electrolyte metabolism characterized by excess potassium and often high blood pressure.
Single nucleotide polymorphisms (SNPs): relatively rare variations in a single nucleotide that occur at specific genomic locations within a population.
Ubiquitination: the addition of a ubiquitin moiety to a substrate protein to target it for degradation or recognition by specific signaling molecules.
WW: a modular protein domain containing two highly conserved tryptophan resides that bind to proline-rich peptide motifs.

Table 1. Nedd4-2 Interactors and Substrates

Function/ pathway	Protein	Nedd4-2 interaction	PY (or similar) motif	Refs
Regulatory molecules	Nedd4-2	Inhibitory self-ubiquitination	Yes	[34]
	14-3-3	Inhibitory binding partner	No	[17]
	Usp2-45	Binding partner	No	[35]
	ACK-1	Binding partner	Yes	[41]
	Sgk1	Inhibitory phosphorylation	Yes	[17,42]
	Akt	Inhibitory phosphorylation	No	[22]
	PKA	Inhibitory phosphorylation	No	[20]
	AMPK	Activating phosphorylation	No	[23]
	JNK1	Activating phosphorylation	No	[24]
	IKKβ	Activating phosphorylation	No	[25]
	WNK1	Ubiquitination	Yes	[43]
Adaptors	Ndfip1/2	Ubiquitination; adaptors/ regulators	Yes	[2]
	α-Arrestins	Ubiquitination; adaptors/ regulators	Yes	[44]
Sodium channels	ENaC	Ubiquitination	Yes	[45]
	Na_vs 1.2, 1.3, 1.5, 1.6, 1.7, 1.8	Ubiquitination	Yes	[2,46–48]
Chloride channels	CLC-5	Binding partner	Yes	[49]
	CLC-K/barttin	Downregulation	Yes	[50]
	CFTR	Downregulation	No	[51]
	Tweety	Ubiquitination	Yes	[52]
Potassium channels	KCNQ1, 2/3, 3/5	Downregulation	Yes	[53,54]
	hERG	Ubiquitination	Yes	[55]
Other channels and transporters	AQP2	Ubiquitination	No	[29]
	NCC	Ubiquitination	No	[56]
	NKCC1/2	Downregulation	No	[57,58]
	DMT1	Ubiquitination	No	[27]
	ATA2	Downregulation	No	[59]
	DAT	Ubiquitination	No	[60]
	EAAT1/2	Downregulation	No	[61,62]
	SGLT1	Downregulation	No	[63]
	CHT1	Ubiquitination	No	[64]
	OAT1/3	Ubiquitination	No	[65,66]
	NHE3	Ubiquitination	Species-specific	[67]
Neural receptors	TrkA	Ubiquitination	Yes	[68]
	GluA1	Ubiquitination	No	[69]
Wnt signaling	Dvl2	Ubiquitination	Yes	[70]
TGF-β signaling	Smad2, 3, 4, 7, TGF-βR1	Ubiquitination	Yes	[71]
cAMP signaling	CRTC3	Ubiquitination	Yes	[72]

Table 1. (continued)

Function/ pathway	Protein	Nedd4-2 interaction	PY (or similar) motif	Refs
Surfactant protein	SP-C	Ubiquitination	Yes	[73]
Polarity/cell junctions	Dlg3	Ubiquitination	Yes	[74]
	Occludin	Ubiquitination	Yes	[75]
Autophagy	ULK1	Ubiquitination	No	[76]

ion channels (including sodium, chloride, and potassium channels), as well as various other transporters and signaling molecules.

Physiological Functions of Nedd4-2

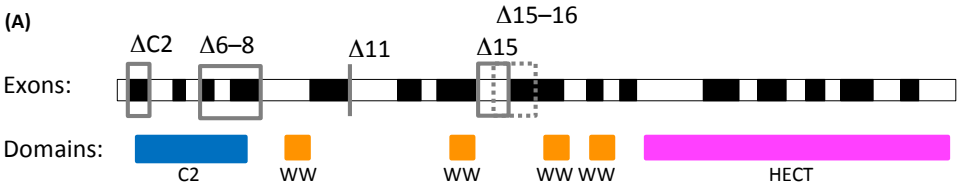
ENaC Is the Key Nedd4-2 Substrate

Evidence generated from several knockout mouse models suggest that ENaC is a key *in vivo* substrate of Nedd4-2 (Figure 2), as well as several voltage-gated Na^+ (Na_v) channels, particularly in cortical brain neurons and dorsal root ganglion (DRG) neurons. The physiological importance of Nedd4-2-dependent ENaC regulation is demonstrated in **Liddle syndrome** (a rare inherited form of hypertension), where mutations of PPxY motifs in human ENaC subunits abrogate Nedd4-2 binding and subsequent removal from the membrane, resulting in increased membrane retention of ENaC [77,78], increased Na^+ reabsorption, and hypertension [79,80].

A **hypomorphic** mouse knockout deleting exons 6–8 of *Nedd4-2* that largely ablates Nedd4-2 expression display an increase in expression of all three (α, β, and γ) ENaC subunits and increased membrane-localized ENaC [81,82], resulting in elevated ENaC activity [81] (Figure 2). Levels of other channels potentially regulated by Nedd4-2 (sodium/hydrogen antiporter 3, NHE3; Na^+/Cl^- cotransporter, NCC; Na/K/Cl cotransporter, NKCC2) are unchanged in these mice [81]. Global knockout of *Nedd4-2*, disrupting exons 15–16, results in increased ENaC in the lung and kidney [83]. A somewhat similar deletion of *Nedd4-2* (exon 15) in lung epithelia ($Nedd4L^{SPC}$) also results in increased membrane retention of αENaC and dramatically increased ENaC activity [84]. ENaC subunits undergo post-translational modification to reach maturity. For α and γENaC this is via proteolytic cleavage, and βENaC requires glycosylation [85,86]. Both full-length and active processed forms of all three ENaC subunits are increased by renal tubule-specific knockout of Nedd4-2 in epithelial cells ($Nedd4\text{-}2^{Ksp1.3}$) [87]. As expected, this correlates with increased membrane retention of ENaC in renal tubules. Blocking ENaC with the **diuretic** drug amiloride rescues the phenotypes in *Nedd4-2* knockout mice [84,87], demonstrating that Nedd4-2 is a crucial physiological regulator of ENaC [88].

In contrast to the above models, an inducible kidney-specific *Nedd4-2* knockout in proximal and distal tubules, and collecting ducts ($Nedd4L^{Pax8/LC1}$) [89] revealed NCC as the primary Nedd4-2 substrate (Figure 2). These mice displayed an increase of β and γENaC after a high-salt diet [89]; however, the phenotype of these mice has been attributed to increased NCC, not ENaC. In a further study, under conditions of long-term K^+ depletion, $Nedd4L^{Pax8/LC1}$ mice showed an increase in full-length α and γENaC, although βENaC levels were unchanged [90]. The ENaC blocker benzamil reversed the phenotype, suggesting the elevated ENaC is responsible for the observed pathology. Thus, in this induced *Nedd4-2* knockout model, ENaC regulation appears to be diet-dependent, and this may explain the variations observed in different models.

(A)



(B)

Targeted region	Global	Tissue specific
ΔC2	*Nedd4-2*$^{-/-}$*ΔC2* (high-Na$^+$ diet only) ↑ENaC expression	
Δ6–8	*Nedd4-2*$^{-/-}$ *Δ6–8* (high-Na$^+$ diet only) ↑ENaC expression and activity	*Nedd4 -2*$^{Pax8/LC1}$ *Δ6–8* (kidney) (high-Na$^+$ diet only) ↑βγENaC,↓αENaC expression ↑NCC *SNS-Nedd4L Δ6-8* (DRG neurons) only analyzed $Na_v1.7$ and $Na_v1.8$ ↑$Na_v1.7$ and $Na_v1.8$
Δ11	*Nedd4-2*$^{-/-}$ *Δ11* only analyzed neural channels $Na_v1.7$, $Na_v1.8$ and TrkA ↑TrkA	
Δ15		*Nedd4-2*$^{Ksp1.3}$ *Δ15*(kidney) ↑ENaC expression *Nedd4-2*^{SPC} *Δ15* (lung) ↑ENaC expression
Δ15–16	*Nedd4 -2*$^{-/-}$*Δ15* ↑ENaC expression and activity	

Trends in Biochemical Sciences

Figure 2. *Nedd4-2* Mouse Knockout Models Used To Study the Regulation of ENaC and Other Ion Channels. (A) Mouse *Nedd4-2* locus (alternative exons are shown in black/white), with corresponding coding regions for various domains. Five regions of Nedd4-2 have been targeted for deletion/disruption (gray boxes/lines). *Nedd4-2* ΔC2 deletes the C2 domain, generating a shorter isoform of *Nedd4-2* [93]. *Nedd4-2* Δ6–8 deletes exons 6–8, generating a hypomorphic *Nedd4-2* allele [81], and has also been used to generate a kidney-specific knockout of *Nedd4-2* using the Pax8/LC1 driver [89], and a dorsal root ganglion neuron-specific knockout using the $Na_v1.8$ promoter (SNS–Cre) [47]. *Nedd4-2* Δ11 inactivates the gene between exons 10 and 11 [99]. Exon 15 was deleted in the *Nedd4-2* Δ15 conditional allele, which also introduces a frameshift [75]. This allele was used for generating an SP-C-driven conditional knockout in the lung [84], as well as a Ksp1.3-driven kidney-specific knockout [87]. *Nedd4-2* Δ15–16 deletes part of exons 15 and 16 and introduces a stop codon in exon 15 (broken box) [83]. (B) Regulation of ENaC in different mouse knockout models. The ΔC2 and Δ6–8 models reveal changes in ENaC expression only after a high-Na$^+$ diet, whereas this is seen on a standard Na$^+$ diet in the Δ15 and Δ15–16 models. Abbreviation: DRG, dorsal root ganglion.

In both humans and mice, there are multiple isoforms of *NEDD4L/Nedd4-2*, and a cryptic splice variant (rs4149601) results in an isoform lacking the C2 domain [91]. Isoforms with the C2 domain have been suggested to be important in ENaC regulation [92], and mice lacking the C2 domain were recently reported to have elevated ENaC on a high-Na^+ diet [93] (Figure 2). In these mice, ubiquitination of βENaC was suppressed after a high-Na^+ diet. Hence, the C2 domain of Nedd4-2 is important for ENaC regulation in mice.

Regulation of Other Ion Channels and Transporters by Nedd4-2

Several transporters are regulated by Nedd4-2, including NCC, as mentioned above. Studies using *Xenopus* oocytes suggest that aldosterone modulates NCC protein expression via a pathway involving Sgk1 and Nedd4-2 [56], and an increase in total and membrane-associated NCC was observed in kidney-specific *Nedd4-2*$^{Ksp1.3}$ mice. A similar increase in NCC, as well as an increase in phosphorylated forms, has been observed in the *Nedd4L*$^{Pax8/LC1}$ mice following a high-Na^+ diet. Loss of Nedd4-2 in this model also resulted in an increase in the abundance and apical localization of the renal outer medullary K^+ channel (ROMK), compensating for the NCC increase. NCC is a downstream target of the with-no-lysine WNK1/WNK4 kinases, and Nedd4-2 regulates WNK1 [43,94]. Indeed, under conditions of long-term K^+ depletion, *Nedd4L*$^{Pax8/LC1}$ mice show increased WNK1 and downstream signaling events such as increased phosphorylation of the STE20/SPS1-related proline/alanine-rich kinase (SPAK) and NCC. However total NCC and its membrane localization remain unchanged [90], suggesting that Nedd4-2 is not directly targeting this transporter under these conditions.

Nedd4-2 knockout specifically in the distal colon of mice results in elevated levels of Na/K/Cl cotransporter (NKCC1) and ENaC, leading to higher steady-state short circuit current [57]. In addition, several PY-motif containing voltage-gated sodium channels (Na_vs) are targeted by Nedd4-2, and Nedd4-2 regulates these Na_vs in DRG neurons and controls intracellular Na^+ mediated inhibition of Na_vs in fetal cortical neurons [47,95] (Figure 2).

Nedd4-2-Dependent Regulation of Neuronal Signaling and Excitability

In addition to regulating Na_vs (discussed above), several studies implicate Nedd4-2 in neuronal survival, signaling, and excitability via trafficking of additional substrates [96]. When neuronal activity is chronically elevated, phosphorylation of the murine double minute-2 (Mdm2) E3 leads to downregulation of its substrate p53 and subsequent upregulation of Nedd4-2. *Nedd4-2*^{andi} mice, that carry a spontaneous mutation resulting in a knockout of the C2 domain containing long-form Nedd4-2 (the predominant form seen in the mouse cortex), show that Nedd4-2 is required for Mdm2–p53-mediated reduction of **neural network synchrony** after activity stimulation [97]. Nedd4-2 also mediates neuronal activity and seizure susceptibility in mice via ubiquitination of the GluA1 subunit of the α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor (AMPAR) [69,98]. Finally, dysregulation of the neurotrophin receptor TrkA has been demonstrated in dorsal root ganglia of *Nedd4-2* knockout embryos, potentially contributing to their increased pain sensitivity [99] (Figure 2).

Nedd4-2 in Mast Cell Activation and Autophagy

Although the majority of Nedd4-2 substrates are membrane-associated proteins, Nedd4-2 can also target some non-membrane proteins, such as the tyrosine kinase Syk. Phosphorylated Syk, that is essential for downstream FcεRI signalosome activity and proinflammatory mediator release in **allergic inflammation**, is a target of Nedd4-2-mediated ubiquitination and degradation [30]. Loss of this negative phospho-Syk regulation by Nedd4-2 and the adaptor protein Ndfip1 leads to mast cell-driven allergic inflammation in mice. In addition, recent work shows that, during **autophagy**, protein levels of the serine/threonine kinase ULK1 are specifically

downregulated by Nedd4-2-mediated ubiquitination, followed by proteasomal degradation [76]. Because ULK1 is the key initiator of autophagy, Nedd4-2 therefore controls activation of autophagy during prolonged stress.

Pathological Consequences of Dysregulated Nedd4-2

Given the importance of Nedd4-2 in regulating multiple ion channels, transporters, and signaling pathways, it is no surprise that loss or dysregulation of Nedd4-2 in mice results in a wide range of pathologies, as discussed below (Figure 3, Key Figure). These are primarily the result of defective Na^+ homeostasis or neuronal excitability.

Respiratory Distress and Cystic Fibrosis-Like Disease

Complete *Nedd4-2* deficiency in mice results in respiratory distress and perinatal death [83]. Premature clearance of embryonic lung fluid resulting in collapsed alveolar spaces is predicted to be due to the elevated ENaC activity in lung epithelia [83]. The failure to inflate lungs results in the death of most pups at birth. Few *Nedd4-2*$^{-/-}$ mice that survive birth die by 22 days with severe sterile lung inflammation, partly due to drying and **necrosis** of alveolar epithelia. The surviving animals resemble mice with conditional lung deletion of Nedd4-2 (*Nedd4L*^{SPC}) [84], although lung-specific deletion also results in mucus plugs and a reduced pericilliary layer reminiscent of **cystic fibrosis** (CF) in human patients. This phenotype is similar to that

Key Figure

Substrates Contributing to Disease Phenotypes in *Nedd4-2*-Deficient Mice

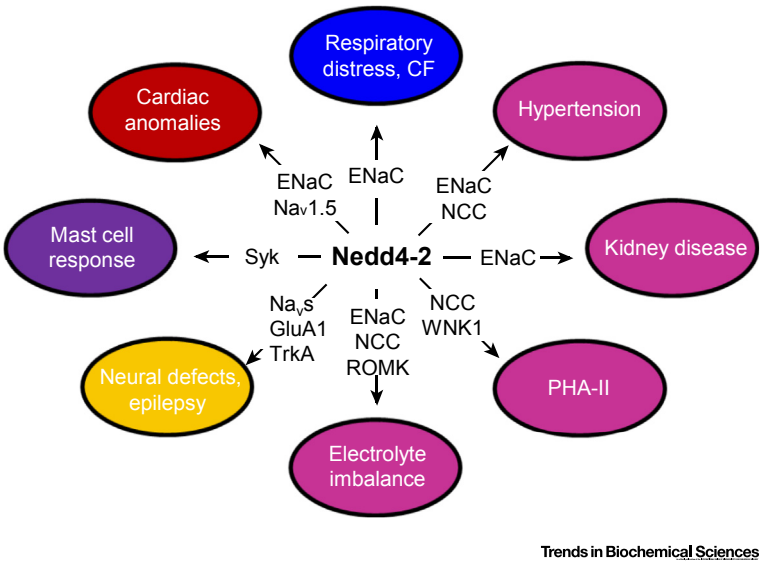


Figure 3. Mouse models of disruption have revealed the importance of Nedd4-2 in safeguarding against many pathological conditions, via regulation of various substrates. Loss of Nedd4-2 results in the following phenotypes: respiratory diseases indicated in blue, renal diseases in magenta, neural defects in yellow, mast cell responses in purple, and cardiac anomalies in red. Abbreviations: CF, cystic fibrosis; PHA-II, pseudohypoaldosteronism type II.

observed in mice overexpressing βENaC in the lung [100]. Importantly, administration of amiloride significantly improves the lung phenotype of *Nedd4L*^{SPC} mice, highlighting the functional importance of elevated ENaC in *Nedd4-2*-deficient animals.

Hypertension

Nedd4-2 plays a key role in Na^+ homeostasis and thus in controlling blood pressure (BP). *Nedd4-2* hypomorphic mice display high-Na^+ diet-dependent hypertension that is due to increased ENaC activity because the ENaC inhibitor amiloride could reduce BP after a high Na^+ diet [81]. Conditional knockout of Nedd4-2 in renal tubular cells in the same background (*Nedd4L*$^{Pax8/LC1}$) also showed high-Na^+ diet-dependent hypertension, although this was attributed to increased NCC [89]. Knockout of the C2 domain of Nedd4-2 also causes increased ENaC expression and hypertension in mice on a high-Na^+ diet [93,101]. Interestingly a kidney-specific deletion of Nedd4-2 using a stronger knockout allele (*Nedd4-2*$^{Ksp1.3}$) showed hypertension on a standard Na^+ diet, largely owing to increased retention of active ENaC at the plasma membrane in renal tubular cells [87]. The nature of the variation in the severity and salt-sensitivity of hypertension in different models of *Nedd4-2* deficiency is unclear. However, it seems that complete and sustained absence of Nedd4-2 in mice results in hypertension irrespective of dietary Na^+. Furthermore, there is a strong correlation between specific human *NEDD4L* variants and hypertension [7], suggesting conservation of this role of Nedd4-2.

Pseudohypoaldosteronism Type II (PHA)-II

The hypertension and increased NCC abundance in *Nedd4L*$^{Pax8/LC1}$ mice has been described as **pseudohypoaldosteronism type II** (PHA-II)-like [89]. PHA-II is a rare disease which is characterized by hyperkalemia, hypercalciuria, metabolic acidosis, low renin and aldosterone levels, and overactive NCC [102]. The elevated NCC is often a result of mutations in the WNK1 and WNK4 kinases which normally negatively regulate NCC levels. Indeed, WNK1 has been identified as a target of Nedd4-2 [43]. Patients with PHA-II often respond well to thiazide diuretics which inhibit NCC. In *Nedd4L*$^{Pax8/LC1}$ mice, thiazides reduced hypertension and the elevated Ca^{2+} excretion in the knockout mice, highlighting the importance of NCC to this pathology. Some elements of PHA-II such as increased NCC and low aldosterone are also apparent in the kidney-specific *Nedd4-2*$^{Ksp1.3}$ mice [87].

Electrolyte Imbalances

Kidney-specific *Nedd4-2*$^{Ksp1.3}$ mice display significant electrolyte imbalances, with increased plasma Na^+, reduced plasma K^+, and increased K^+ excretion even on a standard diet [87]. In addition, aldosterone levels are strongly suppressed, suggesting increased Na^+ retention [87]. Together this manifests as an increase in water consumption and urine output. Some aspects of this have been observed in other *Nedd4-2*-deficient models under high Na^+ intake [89,93].

In addition to Na^+ regulation, the impact of low K^+ in *Nedd4-2* deficiency was recently analyzed. Net urinary K^+ excretion is determined by coordinate responses of different membrane-transport proteins in the distal nephron, including NCC, ENaC, ROMK, and big potassium (BK), many of which are regulated by Nedd4-2. Normally, chronic K^+ dietary restriction causes hypokalemia. Deletion of Nedd4-2 in the kidney (*Nedd4L*$^{Pax8/LC1}$) was found to exacerbate this, primarily by upregulation of ENaC [90].

Kidney Disease

Global *Nedd4-2*$^{-/-}$ mice develop kidney disease within a few days of birth, with cystic tubules, **fibrosis**, necrotic cellular debris, and mesenchymal infiltration [87]. In addition, *Nedd4-2* deletion in a renal tubule-specific model (*Nedd4-2*$^{Ksp1.3}$) produces a similar phenotype. Levels

of both ENaC and NCC are increased in the kidneys of these mice, but only ENaC inhibition ameliorated the kidney disease, suggesting that increased ENaC is the primary cause of kidney disease. Interestingly, this kidney injury has not been reported in other models of *Nedd4-2* deletion, perhaps owing to the hypomorphic nature of alleles producing some functional Nedd4-2 protein and/or to differences in genetic background.

Cardiac Abnormalities

Compromised cardiac function has been associated with loss of Nedd4-2, potentially as a consequence of sustained hypertension. Hypomorphic *Nedd4-2* mice display increased heart weight at 10 months of age on a standard Na^+ diet, which was exacerbated after a long-term high-Na^+ diet [81], suggesting salt-sensitive changes in cardiac morphology with loss of Nedd4-2. Furthermore, high-Na^+ diet reduced heart function in knockout mice. Mice with a knockout of the C2 domain of Nedd4-2 also show **bradycardia** and impaired intracardiac conduction in resting conditions, on a normal Na^+ diet [103]. Impaired renal function in these mice could contribute to cardiac abnormalities; however, the previous study on normal dietary Na^+ implies that these cardiac abnormalities can occur in the absence of hypertension. Artificial generation of **myocardial infarction** also causes changes in cardiac conduction in mice lacking the C2 domain of Nedd4-2, presumably due to impaired regulation of cardiac ion channels. Indeed, regulation of cardiac ion transport is crucial for heart homeostasis, and perturbations in this play an important role in many cardiovascular diseases [104]. For example, increased ENaC activity contributes to cardiac pathology because aldosterone-treated rats develop cardiac pathology, with increased cardiac fibrosis, inflammation, oxidative stress, and **hypertrophy**, as a result of downregulation of Nedd4-2 and a subsequent increase in ENaC [105]. Furthermore, mutations in another well-studied target of Nedd4-2, $Na_v1.5$, can cause conduction disorders such as ventricular arrhythmias and long-QT syndrome, as well as sudden cardiac death [104].

Nervous System Dysregulation and Epilepsy

While global *Nedd4-2*$^{-/-}$ mice are perinatal lethal, heterozygous *Nedd4-2*$^{+/-}$ mice display behavioral and electrophysical defects, including increased locomotor activity and basal synaptic excitability, and an increase in inflammatory pain [99]. This supports studies showing that Nedd4-2 regulates Na_v channels to control neuronal excitability and pain sensitivity [47,95]. In addition, disruption of the long C2 domain-containing Nedd4-2 isoform (*Nedd4-2*^{andi}) suggests a role of Nedd4-2 in regulating neural network synchrony and seizure susceptibility [97]. In support, a variant of *NEDD4L* has been associated with epileptic encephalopathies [106], and three variants are linked to human epilepsy [8,107]. These mutants disrupt GluA1 ubiquitination *in vivo* [69]. Hence, Nedd4-2 dysfunction in mice and humans disrupts proper nervous system regulation.

Immune Response

Nedd4-2 is an important negative regulator of IgE–FcεR1 signaling and proinflammatory mediator release in allergic inflammation via regulation of Syk. Nedd4-2 deficiency in mast cells results in heightened and sustained proinflammatory mediator release *in vitro*, and *Nedd4-2* knockout mice display prolonged IgE-mediated **passive cutaneous anaphylaxis** reactions *in vivo* [30]. Furthermore, recent genetic studies have identified a variant of human *NEDD4L* that is associated with asthma [108], providing additional evidence for a role of Nedd4-2 in mast cell-mediated inflammatory diseases.

Concluding Remarks and Future Perspectives

Knockout mouse models demonstrate that Nedd4-2, through regulation of multiple substrates, plays crucial functions in animal pathophysiology (Figure 3). In particular, the regulation of ion

Outstanding Questions

What are the precise mechanisms governing the specificity of targeting by Nedd4-2?

How is the fate (endocytosis, recycling, lysosomal and/or proteasomal degradation) of Nedd4-2 targets determined?

What is the impact of dietary salt on pathologies, other than hypertension, resulting from Nedd4-2 deficiency?

Do Nedd4-2/NEDD4L variants linked to hypertension in human subjects also result in the complex pathologies seen in mouse models?

channels, primarily ENaC and Na_Vs, by Nedd4-2 is crucial for the maintenance of Na^+ homeostasis and neuronal excitability. Dysregulation of this results in respiratory distress, hypertension, electrolyte imbalances, and kidney disease. In addition, lack of Nedd4-2 in mice causes defects in the cardiac, nervous, and immune systems. Many questions remain as to precisely how Nedd4-2 targeting of specific substrates exerts these effects (see Outstanding Questions). Furthermore, the conservation of these functions of Nedd4-2 in humans is not well studied. Currently, variants of *NEDD4L* have primarily been associated with hypertension and epilepsy; however, given the broad range of mouse pathologies caused by Nedd4-2 deficiency, it is plausible that SNPs in this gene are linked to other human diseases. The challenge now arises as to how to translate these findings from mouse into improving human health outcomes.

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