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NEDD4-2 (NEDD4L): The ubiquitin ligase for multiple membrane proteins

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Abstract

NEDD4–2 (also known as NEDD4L, neural precursor cell expressed developmentally down-regulated 4-like) is a ubiquitin protein ligase of the Nedd4 family which is known to bind and regulate a number of membrane proteins to aid in their internalization and turnover. Several of the NEDD4–2 substrates include ion channels, such as the epithelial and voltage-gated sodium channels. Given the critical function of NEDD4–2 in regulating membrane proteins, this ligase is essential for maintenance of cellular homeostasis. In this article we review the biology and function of this important ubiquitin-protein ligase and discuss its pathophysiological significance.

Keywords

Ubiquitin; Ubiquitin protein ligases; NEDD4 family; Epithelial sodium channel (ENaC); Voltage-gated sodium channels (Navs); Hypertension

1. Introduction

Ubiquitination is a well-characterized protein modification system that leads to substrate degradation, stabilization or relocalization (Bonifacino and Weissman 1998; Rieser et al., 2013). A wide range of proteins are degraded by the proteasome or the lysosome following ubiquitination. These proteins include many membrane proteins, cell cycle regulators, transcription factors, tumor suppressors and oncogenes (Hershko and Ciechanover 1998; Hicke 2001; Glickman and Ciechanover 2002). Defects in the ubiquitination machinery have been implicated in cancer and other human pathologies (Ciechanover et al., 1998).

Ubiquitination is a stepwise process involving three classes of enzymes. Ubiquitin activating enzyme (E1) activates the ubiquitin molecule in an ATP dependent manner resulting in a thioester bond formation between the carboxyl terminus of ubiquitin and the internal cysteine (Haas et al., 1983). Ubiquitin is then transferred to the ubiquitin conjugating enzyme (E2) with an active cysteine (Jentsch 1992; Hershko and Ciechanover 1998).

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Following this, ubiquitin is transferred to the substrate via the ubiquitin protein ligases (E3). In humans, there is only one E1 protein, 36 E2 proteins and several hundred E3s (Hershko and Ciechanover 1998). E3s provide substrate specificity to the ubiquitin system and recognize multiple substrates through different protein-protein interactions. There are two types of E3s, the HECT (homologous to the E6-AP C terminus) and RING (really interesting new gene). While most E3s belong to the RING family, there are 29 human members of the HECT family. The largest group of HECT is the NEDD4 family, with 9 members. The HECT members E6-AP and NEDD4 were the first E3's discovered (Huibregtse et al., 1995; Scheffner and Kumar 2014). This review focuses on NEDD4–2, also known as KIAA0439 and NEDD4L (NEDD4L mostly used for human gene/protein) (Yang and Kumar 2010).

2. NEDD4–2 structure and expression

NEDD4–2 belongs to the Nedd4 family of ubiquitin ligases and is the closest homologue of NEDD4, the prototypic member of the family (Harvey and Kumar 1999; Yang and Kumar 2009). NEDD4 and NEDD4–2 share similar E2 specificities (Fotia et al., 2006). NEDD4 is an evolutionary conserved E3, with homologues in yeast (Kumar et al., 1992; Kumar et al., 1997; Kumar and Harvey 1999). It was originally identified in the early embryonic central nervous system (CNS) as a developmentally down-regulated gene (Kumar et al., 1992). The NEDD4 family members are characterized by a unique modular domain architecture comprising the amino terminal Ca^{2+} phospholipid binding (C2) domain, 2–4 WW domains (protein-protein interaction domains) and the HECT domain at the carboxyl terminus (Harvey and Kumar 1999). WW domains consist of 35–40 amino acids and have two conserved tryptophan residues (Andre and Springael 1994; Hofmann and Bucher 1995). These domains generally bind PY (PPxY) motifs and to a lesser degree LPSY motifs in substrates and regulatory proteins. The multiple WW domains in NEDD4 members suggest that they can potentially interact with several proteins at once. The C2 domain binds lipid membranes, but has also been shown to bind proteins (Scheffner and Kumar 2014). The HECT domain is the catalytic domain in NEDD4 ligases. Structural studies suggest that HECT is a bilobular domain with the C lobe containing the acceptor cysteine and N lobe interacting with the ubiquitin-charged E2 (Maspero et al., 2013; Rotin and Kumar 2009).

In addition to NEDD4 and NEDD4–2, other family members include ITCH, SMURF1, SMURF2, WWP1, WWP2, NEDL1 and NEDL2 (Rotin and Kumar 2009). The difference in the number of WW domains characterizes the variability among the family members along with substrate specificity and involvement in distinct biological processes. This could also be dependent on the C2 domain, which may be absent in some alternately spliced isoforms of NEDD4 family members (Plant et al., 2000, Scheffner and Kumar 2014).

While NEDD4 homologues are found in all eukaryotes, NEDD4–2 is likely to have evolved by gene duplication much later in evolution as NEDD4–2 orthologues are only found in vertebrates (Yang and Kumar 2010). All NEDD4–2 proteins share a similar modular structure containing 4 WW domains, in addition to the C2 and HECT domains (Figure 1A). The phylogenetic analysis shows that NEDD4–2 is highly conserved in the vertebrates (Figure 1B). NEDD4–2 transcripts are present in many tissues, with particularly high

expression in the liver, kidney, heart and lung (Harvey et al., 2001, Araki et al., 2008). Human NEDD4–2 gene is located on chromosome 18q21.31. It contains 38 exons, which give rise to multiple spliced mRNA (Chen et al., 2001; Dunn et al., 2002; Araki et al., 2008). In the NCBI database there are at least 17 predicted isoforms of human NEDD4–2. However, in most tissues NEDD4–2 (both mouse and human) appears as two protein bands, one of which may vary slightly in a tissue specific manner. The most commonly expressed protein contains a C2 domain and all 4 WW domains, in addition to the HECT domain (Harvey et al., 2001; Fotia et al., 2003).

3. NEDD4–2 targets and function

The best known and most studied target of NEDD4–2 is the epithelial sodium channel (ENaC) (Harvey et al., 2001, Kamynina et al., 2001). In addition to ENaC, multiple proteins have been shown to bind NEDD4–2 and some shown to be ubiquitinated (Table 1) (Persaud et al., 2009). Fewer have been validated to be genuine NEDD4–2 targets in vivo. Here we summarize main regulatory functions and relevant targets of NEDD4–2.

3.1. ENaC regulation by NEDD4–2

ENaC is a heterotrimeric channel composed of three subunits (α, β and γ) with each subunit having a conserved protein structure with two transmembrane regions, an extracellular loop and cytoplasmic N and C termini (Canessa et al. 1994; Mano and Driscoll 1999; Staruschenko et al. 2005). It is a member of the DEG/ENaC family of ion channels shown to be widely expressed in epithelial tissues such as the kidney, distal colon, lung, skin and trachea (Kashlan and Kleyman 2012). Normal ENaC function in renal sodium transport is critical as it is necessary for body sodium balance and maintenance of blood pressure (Garty and Benos 1988). In the lung ENaC is responsible for normal fluid clearance from alveolar spaces and subsequently normal exchange of gases (Collawn et al., 2012).

ENaC is down-regulated by both NEDD4 and NEDD4–2 *in vitro,* and NEDD4–2 *in vivo* mediated by direct binding of the WW domains to PY motifs of ENaC (Staub et al., 1996; Dinudom et al., 1998; Harvey et al., 1999; Harvey et al., 2001; Kamynina et al., 2001; Fotia et al., 2003; Snyder et al., 2004). This leads to ENaC ubiquitination and removal from the membrane (Figure 2). Abnormal NEDD4–2 mediated ENaC regulation is seen in Liddle's syndrome where ENaC hyperactivity along with increased blood pressure with aberrant Na^+ reabsorption in the kidney are observed (Bhalla and Hallows 2008). This is due to abrogation in the interaction of NEDD4–2 with ENaC due to mutations or deletions that affect the PY motifs of the βENaC or γENaC subunits. Potential inhibitors of sodium transport in the cellular pathways that affect ENaC via NEDD4–2 include phosphorylation, feedback inhibition by intracellular sodium and dietary sodium intake through aldosterone (Bhalla and Hallows 2008) (Figure 2).

3.2. NEDD4–2 mediated regulation of renal Na^+-Cl^- cotransporter (NCC)

The Na^+-Cl^- cotransporter (NCC), critical for sodium absorption in the distal convoluted tubule is also regulated by NEDD4–2. NEDD4–2 interacts with and ubiquitinates NCC at the surface of transfected cells and in Xenopus oocytes. Coexpression of NEDD4–2 with

NCC reduces NCC activity and surface expression (Arroyo et al., 2011). As NCC does not have a classical PY motif and a PY-like motif in NCC is unable to bind NEDD4–2, it remains unclear how the two proteins interact. Surprisingly, the mutations of NEDD4–2 at either serine 328 or 222 (sites that are known to be phosphorylated by Sgk1) failed to affect Sgk1 action (Arroyo et al., 2011). Importantly, a deficiency of NEDD4–2 in mouse renal tubules and in cultured mDCT cells increases NCC activity, suggesting that NEDD4–2 is an *in vivo* regulator of NCC. Furthermore, as discussed below, the regulation of NCC by NEDD4–2 in kidney is critical for Na^+ absorption and NEDD4–2 deficiency specifically in kidney leads to increased blood pressure (Ronzaud et al., 2013; Lagnaz et al., 2014). These results indicate that NEDD4–2 is an important regulator of NCC.

3.3. NEDD4–2 as a regulator of voltage-gated sodium channels (Na_Vs)

Voltage-gated sodium channels (Na_Vs) are essential for the generation of action potentials in electrically excitable cells via mediating the influx of Na^+ in response to local depolarizing stimuli. These channels are expressed in many cell types especially in the nervous system as they play a critical role in development, plasticity and tissue integrity. There are 9 members of the Na_V family and the carboxyl termini of seven of these contain PY motifs, which can interact with the WW3 and WW4 domains of both NEDD4 and NEDD4–2 (Fotia et al., 2004). Several of these NEDD4–2 interacting Na_V channels are also ubiquitinated by NEDD4–2 and in Xenopus oocytes, Na_V currents are strongly inhibited by the coexpression of NEDD4–2, and to a lesser extent by Nedd4 (Fotia et al., 2004; van Bemmelen et al., 2004). The cardiac Na_V ($Na_V1.5$) expressed in HEK cells is also strongly inhibited by NEDD4–2 coexpression, accompanied by reduced cell surface expression of $Na_V1.5$ (van Bemmelen et al., 2004). Furthermore Na_Vs were reported to be downregulated by NEDD4–2 at cell surface in adult dorsal root ganglion neurons (Cachemaille et al., 2012). In animal models of neuropathic pain where Na_Vs play an important role in neuronal excitability, NEDD4–2 was shown to be downregulated, and NEDD4–2-deficiency resulted in hyperexcitability in DRG neurons, which contributed to pathological pain (Cachemaille et al., 2012; Laedermann et al., 2013). These studies collectively suggest a critical role for NEDD4–2 in post-translational regulation of Na_Vs.

3.4. Chloride channels as NEDD4–2 targets

CFTR (Cystic fibrosis transmembrane conductance regulator) is a cAMP stimulated Cl- channel that transports chloride ions across epithelial cell membranes (Pilewski and Frizzell 1999). Ubiquitination is important for CFTR regulation and it has been reported that various ubiquitin ligases such as c-cbl, RMA1/RNF5 and GP78 are responsible for WT and mutant CFTR degradation (Morito et al., 2008; Ye et al., 2010). NEDD4–2 was also reported to be a ubiquitin ligase for CFTR *in vitro* (Caohuy et al., 2009). NEDD4–2 co-immunoprecipitates with both WT and mutant ΔF508 (the most common mutation of three nucleotides leading to loss of the amino acid phenylalanine at the 508 position), which leads to predisposition to loss of protein at the membrane of airway cells. It was shown that NEDD4–2 interaction with both WT and ΔF508 CFTR leads to increased CFTR activity (Caohuy et al., 2009). However, a recent study in Xenopus oocytes showed that NEDD4–2 had no effect on CFTR chloride channel current (Koeppen et al., 2012). These studies need to be further validated *in vivo* to confirm that NEDD4–2 interaction is indeed possible with CFTR in a physiological

setting. Since CFTR does not have a PY motif, the mechanism by which it interacts with NEDD4–2 also requires further investigation.

Another chloride channel CLC-5 is expressed in renal and epithelial tissues. Defects in this Cl^-/H^+ exchanger leads to impairment of endocytosis in renal proximal tubules causing kidney stones and Dent's disease (Rickheit et al., 2010). Although *in vitro* studies suggest a role for NEDD4–2 in regulating CLC-5, *in vivo* data in mice are inconsistent (Hryciw et al. 2004; Rickheit et al., 2010). Mice and humans lacking ClC-5 are reported to have hypercalciuria and hyperphosphaturia. Unlike CLC-5 knockout mice, CLC-5 PY knock-in mice display no abnormal proteinuria or hyperphosphorylation with endocytosis being normal (Rickheit et al., 2010). Thus, the physiological function of NEDD4–2 in regulating CLC-5 remains to be validated.

CLC-Ka/Barttin, a chloride channel involved in renal tubular transport is also a possible NEDD4–2 target (Embark et al., 2004). CLCka and CLCkb chloride channels require coexpression of an essential beta subunit of these channels called Barttin (Embark, Bohmer et al. 2004). Mutations in these genes have been shown to cause Barters syndrome variant BSND with congenital deafness and renal salt wasting (Simon et al., 1997). NEDD4–2 binds to the PPxY motif of Barrtin leading to its degradation (Embark et al., 2004). The Tweety-family of chloride channels consist of 3 members identified in humans (TTYH1–3). NEDD4–2 binds to the TTYH2 AND 3 family members containing the PPxY motif but not TTYH1 since it lacks this motif. Also NEDD4–2 is reported to ubiquitinate TTYH2 and 3 (He et al., 2008). Neither Barttin nor Tweety channels have been validated as *in vivo* targets of NEDD4–2 as physiological data on the levels and regulation of these channels in NEDD4–2 knockout mice are currently not available.

3.5. Potassium channels as putative NEDD4–2 targets

KCNQs are a family of potassium channels. In the heart, KCNQ1 alpha subunit binds to the KCNE beta subunit to form a channel complex. KCNQ1/KCNE levels have been shown to be reduced upon expression with NEDD4–2 (Jespersen et al., 2007). Further *in vivo* evidence indicates that expression of the catalytically inactive form of NEDD4–2 in guinea pig cardiomyocytes leads to increased KCNQ1 current. NEDD4–2 coexpression decreases total levels of KCNQ1 in HEK293 cells due to ubiquitination (Jespersen et al., 2007). This was corroborated by a study in Xenopus oocytes and in collecting duct epithelial cells (Alzamora et al., 2010). The activities of voltage gated KCNQ2/3 and KCNQ3/5 channels, which play pivotal roles in regulating neuronal excitability, are also shown to be affected by NEDD4–2 coexpression in Xenopus oocytes (Schuetz et al., 2008).

NEDD4–2 has also been implicated in ubiquitinating and regulating the potassium channel encoded by the human ether a go go related gene (hERG), which is important for cardiac repolarisation (Albesa et al., 2011; Guo et al., 2012; Cui and Zhang 2013). NEDD4–2 mediated degradation of hERG channels was found upon disrupting the NEDD4–2 binding domain in hERG channels which completely ablated the effect of muscarinic receptor on hERG channel expression (Wang et al., 2014). These studies suggest that NEDD4–2 can potentially regulate a number of potassium channels. However, the conclusions derived

largely from *in vitro* studies, have not yet been validated in genetically modified animal models.

3.6. Surfactant protein C (SP-C) as a NEDD4–2 substrate

NEDD4–2 has been shown to bind to the surfactant protein C (SP-C), an interaction involving NEDD4–2 WW domains and a PPxY motif in SP-C (Kotorashvili et al., 2009; Conkright et al., 2010). SP-C is released as a phospholipid rich film synthesized by alveolar type 2 epithelial cells and is involved in reducing surface tension. As such, a lack of this surfactant causes respiratory distress in premature infants and mutations in the SP-C gene have been shown to cause interstitial lung disease (Nogee et al., 2002). Mutation of the SP-C PY motif abrogated binding to NEDD4–2 and reduced secretion of SP-C. However, a physiological role of NEDD4–2 in SP-C regulation is not fully established as NEDD4–2-deficient mice fail to show any defects in the levels of surfactant protein C in the lungs (Boase et al., 2011).

3.7. Other channels and transporters

Other potential membrane protein substrates/targets of NEDD4–2 include EAAT1/2, the glial excitatory amino acid transporters that are responsible for glutamate and aspartate uptake (Boehmer et al., 2003; 2006). In separate studies these transporters were shown to be inhibited by coexpressed NEDD4–2 in Xenopus oocytes (Boehmer et al., 2003, Boehmer et al., 2006). This inhibitory effect of NEDD4–2 could be reversed upon additional expression with SGK1, SGK3 and PKB but not by an inactive mutant of SGK1, suggesting that phosphorylation inhibits NEDD4–2 function (Figure 2) (Lang et al., 2006). The amino acid transporter ATA-2, Na^+ glucose transporter 1 (SGLT1), divalent metal ion transporter DMT1, calcium channels Orai1/STIM1, neurotrophin receptor TrkA and hyperpolarization activated cyclic nucleotide gated HCN1 channel have also been shown to be inhibited by NEDD4–2 (Dieter et al., 2004; Hatanaka et al., 2006; Howitt et al., 2009; Eylenstein et al., 2011; Georgieva et al., 2011; Lang et al., 2012; Wilkars et al., 2014).

Another potential substrate of NEDD4–2 is the dopamine transporter (DAT), which is necessary to terminate dopamine neurotransmission (Sorkina et al., 2006). A model for NEDD4–2 mediated DAT endocytosis has been proposed where the PKC-induced ubiquitination of DAT is mediated by NEDD4–2. This leads to interaction of DAT with adaptor proteins in the coated pits, such as epsin and Eps15, promoting DAT endocytosis (Georgieva et al., 2011; Vina-Vilaseca et al., 2011). *In vivo* physiological significance to these studies awaits experiments in animal models.

3.8. Regulation of TGFβ signalling by NEDD4–2

Several studies propose a role for NEDD4–2 mediated ubiquitination in the control of TGFβ signaling, however *in vivo* validation of these studies requires further work. NEDD4–2 has been shown to downregulate TGFβR1 through Smad7, which acts as PY motif containing adaptor and induces the ubiquitin-mediated degradation of TGFβR1 (Kuratomi et al., 2005). Following receptor activation, NEDD4–2 was also shown to bind to regulatory Smad2 but not Smad3, leading to its degradation (Gao et al., 2009). Overexpression of NEDD4–2 in the HepG2 cell line decreases TGFβ signaling whereas silencing NEDD4–2 increases it

(Kuratomi et al., 2005). Thus NEDD4–2 can potentially regulate TGFβ signaling by targeting more than one component of the signaling cascade.

3.9. NEDD4–2 in virus budding

Nedd4–2 has been implicated in budding of enveloped RNA viruses, such as the HIV-1 (Martin-Serrano et al., 2005). These viruses interact with NEDD4–2 through its WW domains leading to ubiquitination of the viral matrix proteins and in turn recognition by the ESCRT (endosomal sorting complex required for transport) machinery that the viruses recruit for budding. The late domains within the HIV-1 $p6^{Gag}$ recruits two ESCRT proteins, TSG101 and ALIX, to facilitate virus budding (Segura-Morales et al., 2005; Putz et al., 2008; Dussupt et al., 2009; Usami et al., 2009; Sette et al., 2010). Overexpression of NEDD4–2 promotes release of the variant HIV-1 that is deficient in TSG101 and ALIX-binding late domains, whereas the knockdown of endogenous NEDD4–2 inhibited the release (Chung et al., 2008). These results suggest a direct and critical role NEDD4–2 plays in budding through interaction with the viral proteins and components of the ESCRT machinery.

3.10. Other substrates of NEDD4–2

Dishevelled-2 (Dvl2) is a critical player in the Wnt signaling and is important for embryogenesis and tissue homeostasis (Ding et al., 2013; Zhang et al., 2014). NEDD4–2 has been proposed to play a role in Wnt signalling by Dvl2 degradation. In Xenopus embryos NEDD4–2 inhibits Dvl2 induced dorsal axis duplication upon activation of Wnt signalling (Ding et al., 2013; Zhang et al., 2014). Another novel NEDD4–2 interactor is Drosophila disc large scaffolding protein (Dlg3), which is a tumor suppressor (Lickert and Van Campenhout 2012). Dlg3 binds NEDD4–2 through its PPxY motif, resulting in Dlg3 monoubiquitination, regulating apical membrane recruitment and tight junction consolidation (Van Campenhout et al., 2011).

Occludin is an integral membrane protein in tight junctions responsible for tight junction integrity and paracellular barrier (Feldman et al., 2005). NEDD4–2 has been shown to interact with occludin through a PY motif, reduce occludin levels and inhibit tight junction formation (Raikwar et al., 2010). In mpkCCD cells NEDD4–2 overexpression leads to reduced levels of occludin in tight junctions and increased levels of transient paracellular conductance with delay in tight junction formation. On the other hand, NEDD4–2 depletion results in increased levels of occludin and paracellular conductance (Raikwar et al., 2010). Thus NEDD4–2 may play a role in the controlling tight junctions in epithelia.

4. Studies with NEDD4–2 knockout (KO) mice

At least three independent NEDD4–2 KO mice lines have been generated to study the *in vivo* function of NEDD4–2. The KO mice reported by Shi *et al.* are developmentally normal, viable and live a normal life span (Shi et al., 2008). However they show a slight increase in ENaC expression and mild salt sensitive hypertension, suggesting that ENaC regulation by NEDD4–2 is critical, confirming *in vitro* data (Shi et al., 2008).

In contrast to the mice generated by Shi *et al.*, a NEDD4–2 KO mouse model in a C57B16 pure genetic background showed fetal or perinatal lethality (Boase et al., 2011). These mice appear to be null as both NEDD4–2 mRNA and protein were found to be absent (Boase et al., 2011). Most of the KO mice died just prior to birth due to collapsed alveolar spaces resulting in respiratory failure, and show elevated ENaC expression in the lung and kidney. Consistent with the higher expression, ENaC currents in alveolar type II lung cells from E18.5 KO mice were found to be greatly elevated and this presumably is the cause of premature lung fluid clearance and failure to inflate lungs (Boase et al., 2011). Few NEDD4–2 KO animals that survive birth live for up to 22 days. They also show increased α and β ENaC expression in the kidneys and the lung and die due to severe lung inflammation. The phenotypic differences between the Shi mice and the KOs reported by Boase *et al.* may be due to the presence of a small amount of functional NEDD4–2 protein present in the Shi KO allele.

Many of the observations from the Boase *et al.* studies were recapitulated in an independently generated NEDD4–2 KO line by Kimura *et al.*, which reported that NEDD4–2 deletion specifically in lung epithelia results in a cystic fibrosis-like disease, characterized by airway mucus obstruction in the airways and severe inflammation (Kimura et al., 2011). Like the Boase mice, Kimura mice showed elevated ENaC levels and activity in primary alveolar type II cells and animals died within three weeks of birth due to severe lung inflammation (Kimura et al., 2011). Importantly, the lung phenotype could be rescued by the administration of amiloride (an inhibitor of ENaC) into the lungs of neonates, suggesting that the defect is directly due to increased ENaC function in the NEDD4–2 KO mice (Kimura et al., 2011). The two studies combined confirm that NEDD4–2 is a critical regulator of ENaC in the lung and its absence results in lethality in mice.

In another study Ronzaud et al. generated an inducible renal tubule-specific NEDD4–2 deletion (Ronzaud et al., 2013). When fed a high Na^+ diet these mice show hypercalciuria and hypertension. Interestingly these defects could be reversed by thiazide (an inhibitor of NCC) treatment. Consistent with this, NCC protein levels were increased in KO kidneys, along with increased βENaC and γENaC, as well as the renal outer medullary K^+ channel (ROMK). These observations confirm previous *in vitro* studies that NEDD4–2 is a regulator of NCC and indicate that NEDD4–2-deficiency in adult renal tubules is sufficient to cause mild, salt-sensitive hypertension without hyperkalemia (Arroyo et al., 2011). They also suggest that NEDD4–2 is a key regulator of renal Na^+ homeostasis through a number of ion channels.

Using neuron-specific gene KO in mice and gene ablation experiments in Xenopus, NEDD4, the prototypic member of the family, has been shown to be essential for dendrite growth and arborization (Kawabe et al., 2010; Drinjakovic et al. 2010). A recent study using post mitotic neuron-specific NEDD4–2 conditional KO mice now shows that NEDD4–2 is also required for neurite growth, as NEDD4–2 deletion resulted in a reduction of neurite complexity (Hsia et al., 2014). While NEDD4 targets Rap2 for ubiquitination to modulate dentrite growth, as yet the substrate(s) of NEDD4–2 that may explain the neurite complexity phenotype in the KO mice is not known (Kawabe et al., 2010; Hsia et al., 2014).

Finally, a recent study tested if the neurons derived from NEDD4–2 KO mice show any aberrations in the control of Na_v (Ekberg et al., 2014). Using patch-clamping of neurons from NEDD4–2 KO mice, surprisingly it was noted that at steady-state, the expression of Na_v channels on the plasma membrane of DRG and cortical neurons is not regulated by NEDD4–2. However, it was demonstrated that NEDD4–2 is involved in activation-induced down-regulation of Na_vs in cortical neurons isolated from the NEDD4–2 KO embryos but not in embryonic DRG neurons. The activation-induced down-regulation involving NEDD4–2 occurs in response to increased intracellular Na^+, which suggests that like ENaC, Na_vs are regulated by a feedback inhibitory mechanism dependent on NEDD4–2 mediated ubiquitination, endocytosis and subsequent degradation (Ekberg et al., 2014). The pathophysiological consequences of such a mechanism of Na_v regulation require further study, perhaps using a conditional tissue specific NEDD4–2 KO model.

5. NEDD4–2/NEDD4L in human disease

5.1. Hypertension

Consistent with *in vitro* and mouse studies, NEDD4–2 is important in maintaining blood pressure and single nucleotide polymorphisms (SNPs) in NEDD4L gene on chromosome 18q21 are linked to familial hypertension (Dunn et al., 2002; Russo et al., 2005; Svensson-Färbom et al., 2011). There are three main isoforms of Nedd4L: that containing a novel C2 domain (isoform I), an intact conserved C2 domain (isoform II), or with an alternate start codon and a C2 domain lacking Nedd4L (isoform III) (Itani et al., 2005). Isoform 1 is abundant in kidney and adrenal gland and isoform 2 abundant in the lungs. Differences in isoform expression in multiple tissues could potentially alter NEDD4–2 function in regulating membrane proteins (Araki et al., 2008; Raikwar and Thomas 2008).

There are numerous SNPs of which rs4149601 is the most common, carrying a G/A variation in exon 1 of isoform I (Dunn et al., 2002; Ishigami et al., 2010). Depending on ethnicity this variation is found in 16–41% individuals and leads to expression of C2 domain (Luo et al., 2009). The G allele of this isoform is involved in hypertension and enhanced salt sensitivity, whereas the A allele is linked to hypertension due to increased Na^+ absorption (Fava et al., 2006). Carriers of rs4149601GG genotype along with rs2288774 CC genotype have increased salt sensitivity and increased blood pressure. In Xenopus oocytes, isoform I (G allele) is shown to downregulate other NEDD4L isoforms and prevent ENaC downregulation (Araki et al., 2008, Svensson-Farbom et al., 2011).

Recently Dahlberg *et al.* (2014) reported genetic variations in NEDD4L associated G allele with increased blood pressure leading to cardiovascular death. In another study on Chinese Han population, genetic variation of NEDD4L was found to be associated with essential hypertension and related phenotypes with three representative polymorphisms in Nedd4L (rs228874, rs3865418 and rs149601), which was attributed to sex dependent effect in hypertension development of Han Chinese (Wen et al., 2008). The NEDD4–2 allelic variants rs12606138 and rs8094327 have also been implicated as risk factors in dyslexia, a disease characterized by impaired reading and writing (Mueller et al., 2014). However the mechanisms of how these variants affect functions that lead to the risk remain unclear.

5.2. Cancer

Through its potential functions in regulating key signalling pathways such as EGFR, TGFβ and Wnt, NEDD4L has been suggested to act as tumour suppressor, however, direct experimental evidence for such a function is still lacking. Both increased and reduced expression of NEDD4L transcripts in human prostate cancer has been reported (Qi et al., 2003; Hu et al., 2009; Hellwinkel et al., 2010). Lower levels of NEDD4L have been seen in non-small cell lung cancer, gastric cancer, gliomas with poor prognosis, colorectal cancer where NEDD4L has been reported to inhibit Wnt signalling, and gall bladder cancer where NEDD4L has been reported to modulate the transcription of MMP-1 and MMP-13 involved in gall bladder cancer invasion (Takeuchi et al., 2011; Gao et al., 2012; He et al., 2012; Sakashita et al., 2013). Patients with Sezary syndrome, a cutaneous T cell lymphoma, show an increase in the transcripts of NEDD4L (Booken et al., 2008).

Recent reports indicated the possible involvement of NEDD4L in melanoma. The knockdown of NEDD4L in cultured melanoma cells reduces growth, whereas NEDD4L overexpression in xenograft models promotes melanoma tumour growth (Kito et al., 2014). In pancreatic ductal adenocarcinoma (PDAC) NEDD4L was amongst the genes targeted by miR21, miR23a and miR27a, which aid in reduction of cell proliferation in PDAC cells (Frampton et al., 2014). The N-myc downstream regulated gene-1 (NDRG1), a negative regulator of tumor progression in multiple neoplasms and pancreatic cancer, upregulates NEDD4L (Kovacevic et al., 2013). Hepatocellular carcinoma caused by altered Wnt/β-catenin signaling in human hepatoma cells show an apparent increase in levels of NEDD4L (Kovacevic et al., 2013). Thus altered levels of NEDD4L are often associated with multiple tumour types. That said, there is no data directly showing that NEDD4–2 (NEDD4L) loss *in vivo* is responsible for tumour growth or suppression and much mechanistic and *in vivo* animal work is required before the function of NEDD4–2 in cancer can be fully established.

6. Regulation of NEDD4–2 function

6.1. Intramolecular interactions

NEDD4–2 weakly binds to the LPxY motif in its HECT domain via its WW domain suggesting this intramolecular interaction could inhibit its auto ubiquitination (Bruce et al., 2008). Mutations in the LPxY motif decreases NEDD4–2 binding to ENaC subunits and its ability to inhibit ENaC activity (Bruce et al., 2008). This is further supported by the fact that WW domain mediated HECT intramolecular interaction stabilizes NEDD4–2 thus preventing its auto ubiquitination which upon binding to substrate proteins could lead to NEDD4–2 ubiquitination (Bruce et al., 2008).

6.2. Deubiquitinase (DUB) Usp2–45

The DUB Usp2–45 is known to decrease ubiquitination of ENaC and to increase its cell surface expression in *Xenopus* oocytes (Krzystanek et al., 2012; Oberfeld et al., 2011; Pouly et al., 2013). Both the catalytic domain and the N-terminal tail of Usp2–45 physically interact with the HECT domain of NEDD4–2 (Krzystanek et al., 2012; Oberfeld et al., 2011). This interaction with NEDD4–2 is thought to favourably position Usp2–45 for ENaC deubiquitination (Krzystanek et al., 2012; Oberfeld et al., 2011). Thus it seems that this

DUB regulates NEDD4–2 mediated ENaC control through binding the E3 and deubiquitinating the substrate in the complex.

6.3. Ndfip adaptors

While most proteins interact with the NEDD4 family of ubiquitin ligases directly through the PY motif-WW domain mediated binding, other targets use adaptors that bind the WW domains of the NEDD4 E3s. Two types of such adaptors have been described, the NDFIPs (NDFIP1 and NDFIP2) and Arrestin domain containing proteins (Arrdcs) (Lin et al., 2008; Nikko et al., 2008; Mund and Pelham 2009). NDFIP1 (N4WBP5) was discovered in a screen to identify proteins that bind NEDD4 WW domains (Harvey et al., 2002). NDFIP2 (N4WBP5A) was identified as a protein closely related to NDFIP1 (Konstas et al., 2002; Shearwin-Whyatt et al., 2004, 2006). These proteins contain 3 transmembrane domains and localize to intracellular membranes in the Golgi, endosomes and the multivesicular bodies. Both proteins also contain 3 PY motifs which interact with NEDD4 family members via their WW domains. Through NDFIP1, NEDD4–2 and WWP2 ligases regulate divalent metal ion transporter DMT1 (the primary non-heme iron transporter) (Foot et al., 2008). In addition, overexpression of NDFIP1 in the neurons is shown to recruit NEDD4, NEDD4–2 and ITCH proteins into the exosomes (Howitt et al., 2009). More recently both NDFIP1 and NDFIP2 were shown to be adaptors for the water channel aquaporin 2 (AQP2) (Velic et al., 2005; de Groot et al., 2014). In transfected cells NDFIP1 and NDFIP2 bound AQP2 and were essential for NEDD4/NEDD4–2-mediated ubiquitination and degradation of AQP2. In mpkCCD cells, downregulation of NDFIP1 not NDFIP2, increased AQP2 levels and in mouse kidney, NDFIP1, but not NDFIP2, colocalized and coimmunoprecipated with AQP2. These results indicate that NDFIP1 is more likely an adaptor for the AQP2-NEDD4/NEDD4–2 interaction (de Groot et al., 2014).

While a role for NDFIP2 as an adaptor or regulator of NEDD4–2 is less well established, it is suggested to be both a recruiter and an activator of several NEDD4 family members (Mund and Pelham 2010). In *Xenopus* oocytes NDFIP2 increases surface expression and activity of ENaC possibly by interfering with the xNedd4–2-mediated regulation of ENaC. NDFIP2 is highly expressed in renal collecting duct along with ENaC, and thus may modulate NEDD4–2-mediated ENaC regulation in vivo (Konstas et al., 2002).

6.4. The 14-3-3 proteins

14-3-3 are a family of ubiquitiously expressed adaptor proteins that bind to phosphoserine and phosphothreonine motifs (Bhalla et al., 2005; Bridges and Moorhead 2005; Ichimura et al., 2005). They regulate signaling by preventing interaction of target proteins with their downstream interacting proteins. 14-3-3 proteins have been shown to bind to one of the phosphoserine motifs in NEDD4–2. Several kinases like Sgk1 and Akt phosphorylate NEDD4–2 on specific serine residues, which promote recruitment and binding of 14-3-3 proteins (Snyder et al., 2002; 2004; Bhalla et al., 2005; Nagaki et al., 2006; Lee et al., 2007, Lee et al., 2009). The binding of 14-3-3 inhibits NEDD4–2 function by preventing it from interacting with its substrates, such as the ENaC (Nagaki et al., 2006). The kinase Sgk1 is itself shown to be a target of NEDD4–2, in addition to mediating NEDD4–2 phosphorylation (Bhalla et al., 2005).

7. Conclusions

The work summarized in this review indicate that the ubiquitin ligase NEDD4–2/NEDD4L, binds and regulates, though not exclusively, membrane associated proteins, particularly ion channels and transporters. Although the function of the C2 domain in NEDD4–2 has not been delineated in great detail, it is likely that the presence of this domain is the main determinant of NEDD4–2 preference in targeting membrane proteins. The best studied substrate of NEDD4–2 is ENaC and the critical role of NEDD4–2 in regulating this important channel is amply clear from gene KO studies in mice and correlative linkage data in human hypertension. The work on NEDD4–2 is a nice example where the prediction from basic biochemical studies carried out during the past 15 years could be validated in animal models and human pathophysiology.

The review also summarises studies that link NEDD4–2 to the regulation of NCC and Na_Vs *in vivo*. NEDD4–2 interacts with numerous other proteins and may regulate several other substrates, based on *in vitro* observations. The perinatal lethal phenotype associated with NEDD4–2 KO mice has limited the detailed study of the validation of other pathways that may be defective in the absence of NEDD4–2. However, as more conditional and tissue specific KO work is carried out, we are likely to learn about additional physiological functions of this essential E3 in coming years.

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Abbreviations

NEDD4L	NEDD4-like
KO	knockout
ENaC	Epithelial Sodium Channel
NCC	Na^+-Cl^- cotransporter
Na_V	Voltage-gated sodium channels
HECT	homologous to the E6-AP C terminus
CFTR	Cystic fibrosis transmembrane conductance regulator
ESCRT	endosomal sorting complex required for transport

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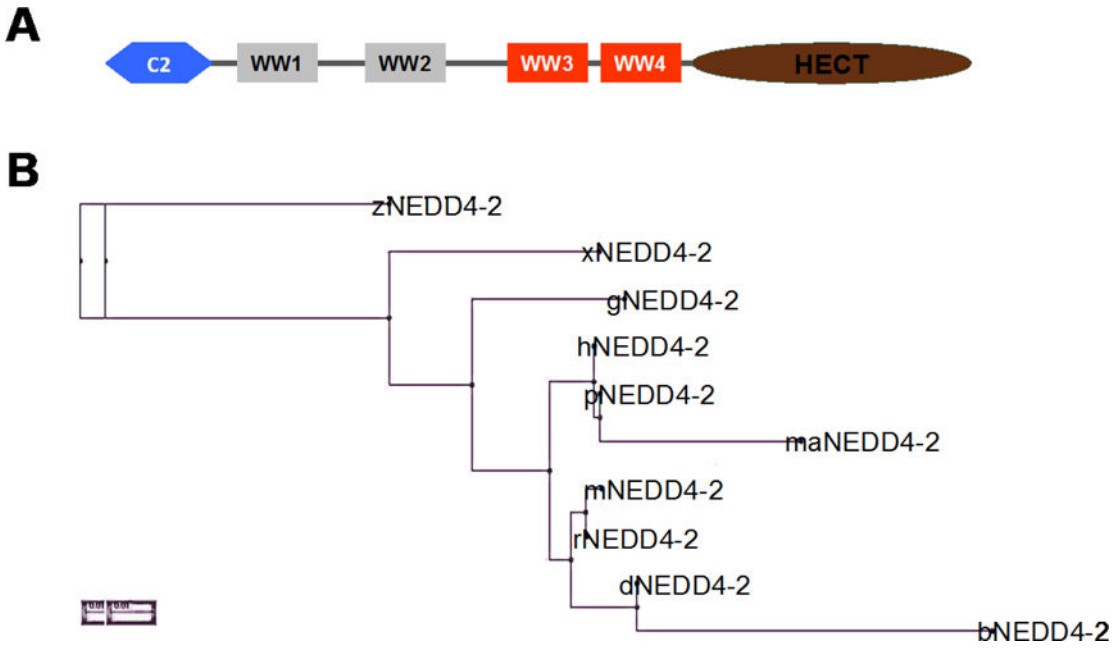

Figure 1. (A) The primary structure of NEDD4–2 protein.

NEDD4–2 protein comprises an amino-terminal C2 domain, 4 WW domains and a carboxy terminal HECT domain. C2 mediates lipid membrane binding, as well as acting as a protein-protein interaction motif. The WW domains are the main mediators of NEDD4–2 interaction with its substrates and adaptors. The HECT is the catalytic domain responsible for binding E2 and for transferring the ubiquitin to the substrate.

(B) The phylogenetic relationship between NEDD4–2 proteins from various species. The neighbour joining phylogenetic tree construction was carried out using NCBI phylogenetic tree view (http://www.ncbi.nlm.nih.gov/blast/treeview/treeView.cgi). b: Cattle (*BosTaurus*); d: Dog (*Canis lupus familaris*); ma: Monkey (*Macaca mulatta*); p: Chimpanzee (*Pan troglodytes*); g: Chicken (*Gallus gallus*); z: Zebrafish (*Danio rerio*); h: Human (*Homo sapiens*); m: Mouse (*Mus musculus*); r: Rat (*Rattus novergicus*); and x: Frog (*Xenopus tropicalis*).

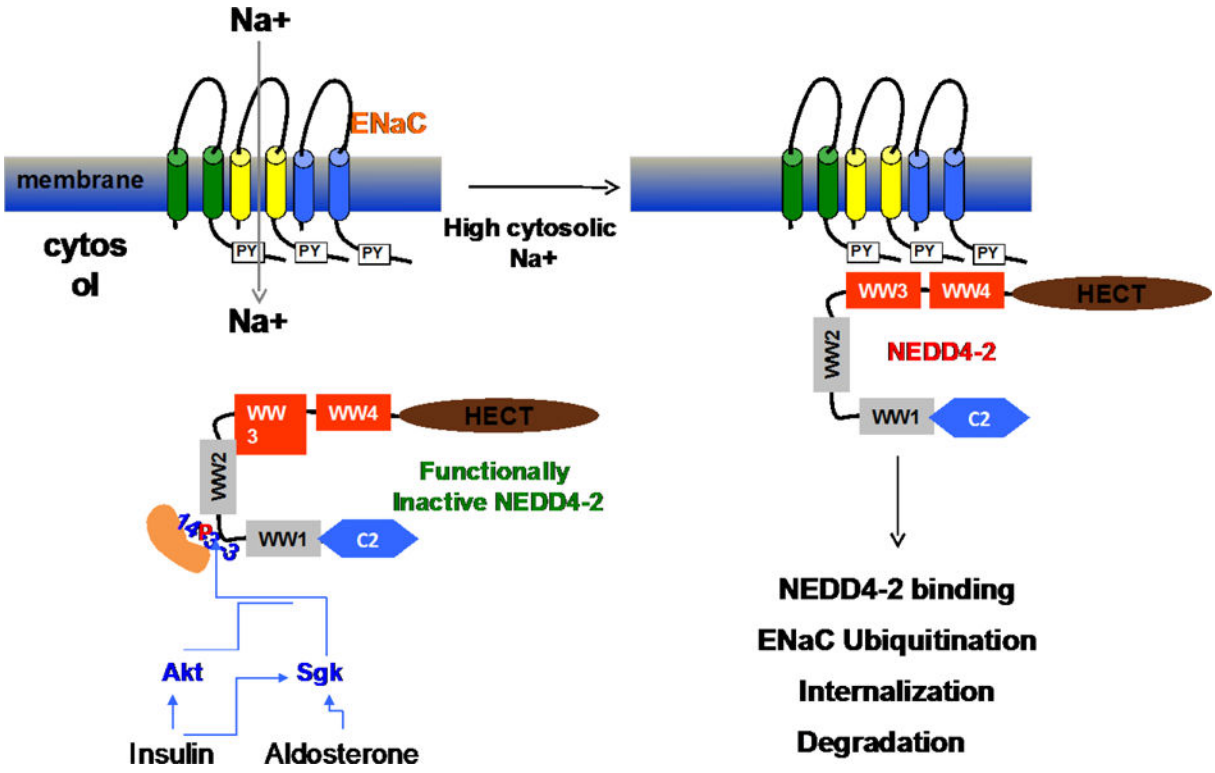

Figure 2. NEDD4–2 is a critical regulator of ENaC.

Through the WW domains 3 and 4 NEDD4–2 directly interacts with the PY motifs found in the carboxyl termini of the three ENaC subunits. Under conditions where ENaC mediated Na^+ uptake is not required (such as when intracellular Na^+ concentration is high) the ubiquitination of ENaC by NEDD4–2 leads to channel endocytosis and degradation. A disruption of this control mechanism results in increased cell surface ENaC and high ENaC activity, as seen in Liddle's syndrome and the NEDD4–2 KO mice. Under conditions where Na^+ intake through ENaC is active NEDD4–2 acts (at least in part) as a converging point for hormones, such as aldosterone and insulin, which stimulate ENaC activity (directly or indirectly through inactivating NEDD4–2 function). Under this scenario, signaling kinases Sgk1 (serum glucocorticoid-inducible kinase) and Akt (PKB), phosphorylate NEDD4–2. This promotes binding of NEDD4–2 to 14-3-3, thus inhibiting the ability of NEDD4–2 to bind ENaC subunits.

Table 1.

Potential substrates and/or binding partners of NEDD4–2

Interacting Protein	Functional Groups	References
14-3-3	Inhibitor of NEDD4–2	(Bhalla et al., 2005)
ACK-1	Activated Cdc42 associated kinase	(Chan et al., 2009)
ATA2	Amino acid transporter	(Hatanaka et al., 2006)
AQP2	Aquaporin channel	(de Groot et al., 2014)
CLC-5	Chloride channel	(Simon et al., 1997; Rickheit et al., 2010)
CLC-Ka/Barttin	Chloride channel	(Embark et al., 2004)
CFTR	Chloride channel	(Caohuy et al., 2009, Koeppen et al., 2012)
DAT	Dopamine transporter	(Sorkina et al., 2006, Vina-Vilaseca and Sorkin 2010)
EAAT1/2	Glutamate transporter	(Boehmer et al., 2003, Boehmer et al., 2006)
ENaC	Epithelial sodium channel	(Harvey et al., 2001, Kamynina et al., 2001)
Dvl2	Dishevelled homolog	(Ding et al., 2013; Zhang et al., 2014)
Dlg3	Disks large homolog 3	(Van Campenhout et al., 2011)
KCNQ1	Voltage gated potassium channels	(Ekberg et al., 2007; Jespersen et al., 2007, Schuetz et al., 2008)
KCNQ2/3 and		
KCNQ3/5		
Na_vs (many)	Voltage gated sodium channels	(Fotia et al., 2004; van Bemmelen et al., 2004; Rougier et al., 2005)
Sgk1	Serum glucocorticoid kinase	(Bhalla et al., 2005)
SGLT1	Glutamate transporter	(Dieter et al., 2004)
SP-C	Surfactant protein C	(Conkright et al., 2010)
Smad 2,3,4,7	TGFβ signaling	(Kuratomi et al., 2005; Moren et al., 2005, Gao et al., 2009)
TGFβR1	TGFβ signaling	(Kuratomi et al., 2005)
Trk	Neutrophin receptor	(Georgieva et al., 2011; Yu et al., 2014)
Tweety	Chloride channels	(He et al., 2008)
NCC	Sodium chloride co- transporter	(Arroyo et al., 2011)
Ndfip1/2	Adaptors	(Harvey et al., 2002; Shearwin-Whyatt et al., 2004; de Groot et al., 2014)
Occludin	Tight junction protein	(Raikwar et al., 2010)
NEDD4–2	HECT ubiquitin ligase	(Bruce et al., 2008)
USP2–45	Deubiquitiylating enzyme	(Oberfeld et al., 2011; Krzystanek et al., 2012)