

Role of the ubiquitin system in regulating ion transport

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Abstract Ion channels and transporters play a critical role in ion and fluid homeostasis and thus in normal animal physiology and pathology. Tight regulation of these transmembrane proteins is therefore essential. In recent years, many studies have focused their attention on the role of the ubiquitin system in regulating ion channels and transporters, initiated by the discoveries of the role of this system in processing of Cystic Fibrosis Transmembrane Regulator (CFTR), and in regulating endocytosis of the epithelial Na⁺ channel (ENaC) by the Nedd4 family of ubiquitin ligases (mainly Nedd4-2). In this review, we discuss the role of the ubiquitin system in ER Associated Degradation (ERAD) of ion channels, and in the regulation of endocytosis and lysosomal sorting of ion channels and transporters, focusing primarily in mammalian cells. We also briefly discuss the role of ubiquitin like molecules (such as SUMO) in such regulation, for which much less is known so far.

Keywords Aldosterone · Anion channel · Cation channel · Cystic fibrosis transmembrane conductance regulator · Electrophysiology · Epithelial Na channels · Ion channel · Potassium channel · Signal transduction · Sodium channel

Introduction

Ion channels play a major role in regulation of the electric properties of virtually every cell. Because of their capability to allow the passage of more than 10⁶ ions per second across a membrane, their activity and expression at the plasma membrane is tightly regulated by a plethora of different cellular mechanisms. In recent years it has become evident that the ubiquitin system plays a major role in this control [3]. The modification of target proteins with ubiquitin often (although not always) targets them for degradation by the proteasome or lysosomes [67, 177], and is now recognized as a critical mechanism for regulation of many cellular functions. Ubiquitin is a 76 amino acid long polypeptide, which becomes conjugated via an isopeptide bond on the ε amino groups of lysine. This is achieved by an enzymatic cascade, involving an E1 (ubiquitin-activating) enzyme, an E2 (ubiquitin-conjugating) enzyme, and an E3 ubiquitin-protein ligase. E3 enzymes provide the specificity to the cascade, as they recognize the relevant target proteins and promote the conjugation of ubiquitin. The process is reversible and involves the activity of deubiquitylating (DUB) enzymes. The two major E3 classes known are RING E3s that facilitate transfer of ubiquitin from an E2 to substrates, and Hect E3s, which directly bind ubiquitin and transfer it onto the substrate. In mammals, there are over 1000 E3 enzymes and over 100 DUBs. Different types of ubiquitin modifications can be generated: Ubiquitin can be conjugated as a mono-ubiquitin on a lysine residue. However, frequently poly-ubiquitin chains are generated that link ubiquitins via internal lysines present in ubiquitin. Indeed, it is now known that polyubiquitin chains are formed that can involve any of the lysines on ubiquitin (K6, K11, K27, K29, K33, K48, K63) [218, 223] and hence largely increase

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the amount and versatility of ubiquitylation. Moreover, a number of ubiquitin-like (UBL) proteins have been discovered that modify proteins. Similar, but not identical enzymatic cascades are involved in UBL's conjugation, with the final outcome often not resulting in protein degradation. In this review we summarize the role of the ubiquitin system and UBLs in the regulation of ion channels and transporters both during their processing and their endocytosis and sorting (Table 1).

Role of the ubiquitin system in regulating ion channels in ER/Golgi

The ERAD system

Proteins destined for the secretory pathway and plasma membrane proteins are inserted co-translationally into the

endoplasmic reticulum (ER) membrane, representing approximately one third of all newly synthesized proteins [141]. In the ER, these proteins are subject to regulation by the quality control system, ensuring that proteins fold properly or multimeric complexes assemble correctly. Proteins that do not, are recognized, retrotranslocated into the cytosol, ubiquitylated and degraded by the proteasome, a process known as ER associated degradation (ERAD). As is evident, the nature of the many client proteins is very diverse (soluble proteins, mono- or polytopic membrane proteins, protein complexes), and consequently the machinery is complex. Misfolded or unassembled proteins are recognized by chaperones [143], chaperone-like lectins [29], or protein disulfide isomerase homologues [201]. They assist with folding the client proteins and release them either for ER export or for ERAD (if folding/assembly fails). Proteins are then transferred to secondary quality control machinery, which includes the ubiquitin-protein

Table 1 Ion channels and transporters regulated by the ubiquitin system

Transport protein	Cellular function of ubiquitylation	Involved enzymes and proteins of the ubiquitin pathway	References
ENaC	ERAD	Doa10, Hrd1p	[26]
CFTR/ Δ F508-CFTR	ERAD/Endocytosis	RMA1, CHIP, gp78, SCF ^{Fbs}	[137, 139, 146, 229]
ENaC	Endocytosis, recycling	Nedd4-2, UBE2E3, Usp2, Usp10, UCH-L3	[2, 23, 27, 42, 54, 99, 191]
NavS, Nav1.2, Nav1.3, Nav1.5, Nav1.7, Nav1.8	Endocytosis	Nedd4-2, Nedd4/Nedd4-like proteins	[61, 170, 204]
KCNQ1/KCNE1	Endocytosis	Nedd4-2	[95]
KCNQ2/3, KCNQ3/5	Endocytosis	Nedd4-2	[50, 175]
KCNA3/KCNA5	Endocytosis	Nedd4-2	[17, 79]
CACNB1	Endocytosis	Nedd4-2	[155]
TRPV4, TRPC4	Endocytosis	AIP4/Itch	[213]
TRPV6	Endocytosis	Nedd4-2	[232]
CIC5	Endocytosis	WWP2, Nedd4-2	[84, 176]
CIC2	Endocytosis	Nedd4-2	[150]
CIC-Ka/Barttin	Endocytosis	Nedd4-2	[51]
TTHY2	Endocytosis	Nedd4-2	[77]
EAAT2	Endocytosis	Nedd4-2	[17]
DMT1	Endocytosis	Nedd4-2/Ndfip1	[59, 83]
ASIC	Endocytosis	Parkin/Pick1	[96]
AQP2	Endocytosis	?	[98]
PC1, PC2	Sorting to lysosome	?	[85]
PC2	Degradation by proteasome	SCF ^{β-Trep}	[198]
ROMK1	Endocytosis	POSH	[123]
Gap1	Endocytosis	Rsp5/Bul1 or Bul2	[189]
Smf1, Smf2	Endocytosis	Bsd2	[124]
Connexin 43	Endocytosis	Nedd4-2	[115]
TRPM4	Endocytosis	SUMO	[110]
K2P1	Intrinsic activity	SUMO	[161]
K _v 2.1	Intrinsic activity	SUMO	[40]
K _v 1.5	Intrinsic activity	SUMO	[11]

ligases Hrd1 [107] and its homologue gp78 [35]. Hrd3 and SEL1L associate with Hrd1 and contribute to ERAD substrate processing [121, 140]. Derlin-1, -2 and -3, possibly with Sec61, have been suggested to form the retrotranslocation channel in the ER membrane, and the p97-Ufd1-Npl4 complex assists with “retrotranslocating” and delivering the proteins to the proteasome [121, 145, 160, 227, 228]. Ubiquitylation may take place during or after retrotranslocation. A number of additional ubiquitin-protein ligases can participate in ERAD, including Doa10 [196], RMA1 [139], CHIP (in combination with Hsp70) [137] or SCF^{Fbx} ligases [229].

ERAD of transmembrane proteins

The quality control of transmembrane proteins in the ER is a challenging task, as these proteins have three topologically distinct regions that have to be monitored to ensure that the folding of the protein is correct: extracellular, transmembrane and intracellular domains. Consequently, it has been proposed that there are three different types of ERAD mechanisms with respect to transmembrane proteins [33, 196, 206]. ERAD-C (cytoplasmic pathways) monitors and recognizes the cytoplasmic regions of the protein (as is the case for the Cystic Fibrosis Transmembrane Regulator (CFTR) channel, where the cytoplasmic Hsp70/CHIP act; see below), whereas the ERAD-L system detects luminal folding defects (with the help of Bip, calnexin/calculin, OS-9, XTP3-B and others). Alternatively membrane proteins and especially multimeric transmembrane protein complexes may have folding defects in the membrane-spanning region, qualifying these as ERAD-M class of molecules. These pathways have been primarily characterized in yeast [33], where different ERAD complexes with different E3 ubiquitin-protein ligases play a role. It is currently believed that at least in yeast, ERAD-C substrates require Doa10, whereas ERAD-L and ERAD-M substrates require the Hrd1p ubiquitin-protein ligase. In mammalian cells it is likely that other ligases are involved as well. It is possible that depending on the nature of the protein several of these mechanisms are implicated in parallel, as seems to be the case for CFTR (see below). There is evidence for a large number of multimeric transmembrane complexes and channels whose assembly is controlled by ER and which involve ERAD. Examples include the T-cell receptor [19, 226], the nicotinic acetylcholine receptor [38, 207], GABA_A receptors [71], voltage-gated Na⁺ channels [174], connexin 32 [205], NMDA receptors [104, 190], K⁺-channels [65, 132, 133, 179, 199, 224, 231], Ca²⁺-channels [209], Inositol 1,4,5-triphosphate receptors [211, 219] and ENaC [103, 202]. The mechanisms by which these proteins are recognized by ERAD vary widely, including intra-membrane charges [19], C-terminal PDZ binding motifs

[190, 199], or positive charges in the cytoplasmic domains [231]. It is becoming clear that these events can be regulated. This is the case for KCNK3 channels, in which 14-3-3 binding to phosphorylation sites can overcome the ER retention by dibasic signals [144], the IP3 receptors which become degraded upon stimulation, an event that involves the ERAD protein SPFH2 [154], or Kv1.2 K-channel in which phosphorylation plays a role in ER exit [224]. Here, we discuss two selected examples, namely the quality control and ERAD of CFTR, which is one of the best characterized transmembrane ERAD substrate, and ENaC, which is an example of a multimeric transmembrane protein assembled in the ER (Fig. 1).

CFTR as a model for integral membrane proteins subjected to ERAD

The cystic fibrosis transmembrane regulator (CFTR) is the gene product responsible for cystic fibrosis (CF) when mutated [167]. The large interest into ERAD of this multi-membrane spanning protein is due to a mutant CFTR (CFTR Δ F508), which is present approximately in 90% of

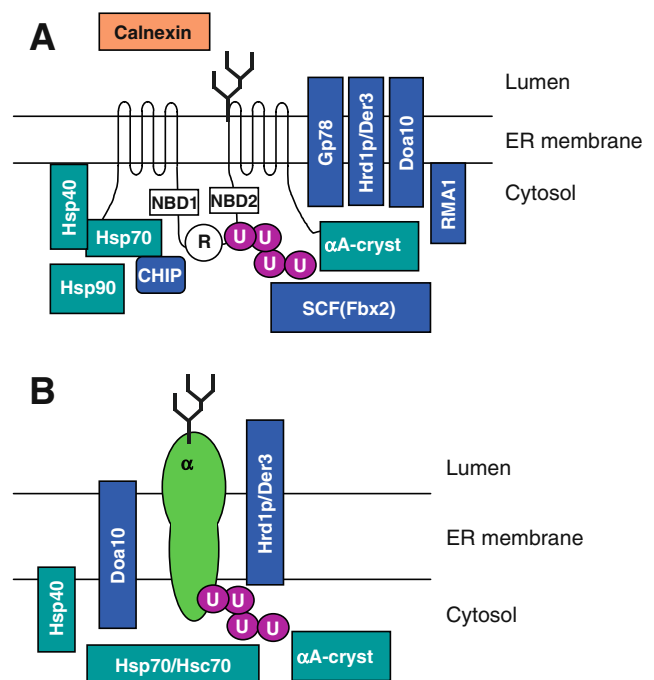


Fig. 1 Chaperones and ubiquitin-protein ligases involved in ERAD of CFTR and ENaC. **a** ER associated degradation of CFTR. On the cytosolic side, Hsp40, Hsp70 and Hsp90, as well as α A-crystallin (cryst), are involved in recognition of the unfolded cytosolic regions, whereas calnexin is involved in luminal control. Hsp70 recruits the ubiquitin-protein ligase CHIP. Other ubiquitin-protein ligases are also involved, such as Gp78, Hrd1p/Der3, Doa10, as well as the cytosolic RMA1 and SCF(Fbx). **b** The ERAD machinery for the 2-TM domain ENaC subunits is less well characterized, and possibly less complex. The heatshock proteins Hsp70/Hsc70, Hsp40 and α A-crystallin have been shown to play a role, as well as Doa10 and Hrd1p/Der3

all Caucasian CF patients, and which represents a folding mutant that is retained in the ER and degraded by ERAD [109]. Indeed, it was one of the first ERAD substrates to be shown to be degraded in a ubiquitin-dependant fashion by the proteasome [93, 210]. CFTR is a member of the ATP binding cassette (ABC) transporter superfamily and contains 1,480 amino acids. It is composed of two membrane spanning domains (with six transmembrane (TM) each), two cytosolic nucleotide binding domains (NBD1 and NBD2), and a regulatory R region. The assembly of this protein into a functional ion channel is complex, as the different domains have to be folded in a coordinated fashion, whereby they are thought to interact in a co- and post-translational fashion. The complexity of this process is illustrated by the fact that only 25% of the wild-type channel are properly folded and exported to the cell surface, whereas the rest is degraded via ERAD; in the case of CFTR Δ F508 the entire pool is degraded by ER [37]. As with other proteins chaperones (Hsp40, Hsp70, Hsp90, α A-crystallin, calnexin) [5, 233] contribute to the recognition of CFTR by ERAD, as do lectins [147]. ERAD involves the participation of various complexes, such as Derlin-1, either to retain CFTR in the ER membrane or to serve as retrotranslocon, and promoting the interaction with the ubiquitin-protein ligase RMA1 that acts in concert with the E2 enzyme Ubc6e [139]. It is believed that RMA1 is capable of recognizing folding defects in CFTR in a co-translational fashion, whereas CHIP, another E3 ligase, appears to interact together with Hsp70 in a post-translational manner with misfolded NBD2 [137]. Other ubiquitin-protein ligases are also important, such as gp78 [139], or SCF^{Fbs} [229]. As well, the extracting activity of p97/Ufd1/Npl4 is necessary for CFTR degradation [68].

ENaC, an example of a multimeric protein controlled by ERAD

The amiloride-sensitive epithelial Na⁺ channel (ENaC) is composed of three subunits, $\alpha\beta\gamma$, and is expressed primarily in the apical membrane of salt-absorbing epithelia, such as those in the distal nephron, distal colon and lung [47], where it participates in salt and fluid absorption (see below). It is well established that efficient functional expression of this channel at the cell surface requires all three subunits [28, 57, 87, 135, 136]. $\alpha\beta$ and $\alpha\gamma$ dimers are also able to generate small amiloride-sensitive currents, whereas $\beta\gamma$ channels are rather weakly expressed at the cell surface [20]. Study of such “dimeric” channels is important for both physiological and pathophysiological reasons. ENaC is highly regulated by the steroid hormone aldosterone, which positively regulates this channel. At least in the kidney, this is achieved by

regulating the expression level of α ENaC; without aldosterone stimulation α ENaC is expressed at only very low levels; under such conditions the β and γ subunits are retained in an as yet undefined compartment [126, 134]. Aldosterone stimulation leads to α ENaC expression, assembly with the $\beta\gamma$ subunits and translocation of the channel to the plasma membrane [126]. It is not understood how and where the $\beta\gamma$ dimers are retained, and how they are protected from degradation. Moreover, there is a pathological condition, pseudohypoaldosteronism type I, in which mutations in the genes encoding either α -, β - or γ ENaC lead to inactivation of these subunits, leaving the cell with only two subunits [34]. As for many other multimeric membrane complexes it is believed that the ENaC channel subunits assemble in the ER and are exported to the plasma membrane, and that at least part of the unassembled subunits are retained in the ER and eventually degraded by ERAD. It is not clear how subunits assemble but they do so early in biosynthesis, possibly prior to glycosylation. It was shown earlier that the N-terminus of γ ENaC plays a crucial role in channel assembly [4], that each subunit can interact with each other to form dimers, and that multiple ENaC domains participate in its assembly [25]. Processing/assembly of ENaC is inefficient, with less than 1% of synthesized subunits reaching the plasma membrane [202, 214], suggesting that the bulk of newly synthesized proteins are eliminated by ERAD. Little is known how unassembled ENaC chains are retained in the ER, and how they are targeted for ERAD. No ER retention signal has been identified, but a C-terminal exit signal was characterized recently [103]. Beside the fact that ENaC needs to assemble all three subunits in order to be efficiently targeted to the cell surface, the evidence (sometimes indirect) for ERAD of ENaC subunits is as follows: (1) Intracellular ENaC subunits are polyubiquitylated [13, 23, 54, 90, 130, 171, 192, 234], whereas ENaC at the cell surface appears to be either mono- or multi-mono-ubiquitylated [171, 216]. (2) Proteasome, but not lysosomal inhibition, interferes with turnover of individually expressed ENaC subunits in MDCK cells, whereas the turnover of ENaC subunits in cells expressing all three subunits is inhibited by both inhibitors. This implies that there are 2 populations of ENaC subunits in such cells, assembled ones (multi-mono-ubiquitylated) that traffic to the plasma membrane and later to the lysosomes, and the unassembled ones (polyubiquitylated) that are degraded by ERAD and the proteasome [192]. (3) It has been shown that proteasome inhibition can increase ENaC activity in A6 cells, most likely by inhibiting ERAD of ENaC subunits, and increasing the level of unassembled subunits, consequently increasing the chance of channel assembly in the ER [129]. Recently, some light has been

shed on the machinery that appears to be involved in ERAD. First, it has been shown that Hsp70, a stress-induced protein, and Hsc70 (a constitutively expressed Hsp70 paralogue), both cytosolic chaperone involved in ER quality control, play an important role in ENaC regulation [69]. Interestingly, when co-expressed in *Xenopus laevis* oocytes, Hsp70 stimulates cell surface expression of ENaC, whereas Hsc70 decreases it. This difference is not very well understood, but it suggests that Hsc70 targets ENaC for ERAD, whereas Hsp70 may promote its folding. The same group also identified α -crystallin as a protein involved in ERAD [103]. Similar to what was done for CFTR, α ENaC was expressed in yeast cells, and its turnover tested in various mutants, including *Hsp26* and *Hsp42* mutants, in which the protein was stabilized. It was then shown that α -crystallin (the *Hsp26* orthologue) reduces ENaC activity and cell surface expression when co-expressed in *Xenopus* oocytes. Evidence was provided that it was the delivery pathway to the plasma membrane (i.e. secretory pathway) that was involved and not the endocytic pathway. Recently, the expression of ENaC subunits in yeast cells has led to additional characterization of the ERAD mechanisms involved in ENaC degradation [26]. It was shown that singly expressed ENaC subunits display several defects in different compartments (cytosolic, luminal and possibly membrane), as evidenced by the observation that both Doa10 (a ubiquitin-protein ligase important for cytosolic substrates), as well as Hrd1p (luminal and membrane regions) were required for turnover of the ENaC subunits. Moreover, it was shown that luminal Hsp40 proteins (Jem1 and Scj1), but not Bip (a luminal Hsp70 chaperone), were involved in turnover and ubiquitylation of ENaC. This was further supported in *Xenopus* oocytes, in which the Hsp40 orthologue ERdj3 and ERdj4 inhibited amiloride-sensitive currents [26]. However, it remains to be seen if these mechanisms are also valid in more physiological cells, such as renal epithelia.

Role of the ubiquitin system in endocytosis, sorting and recycling of ion channels and transporters

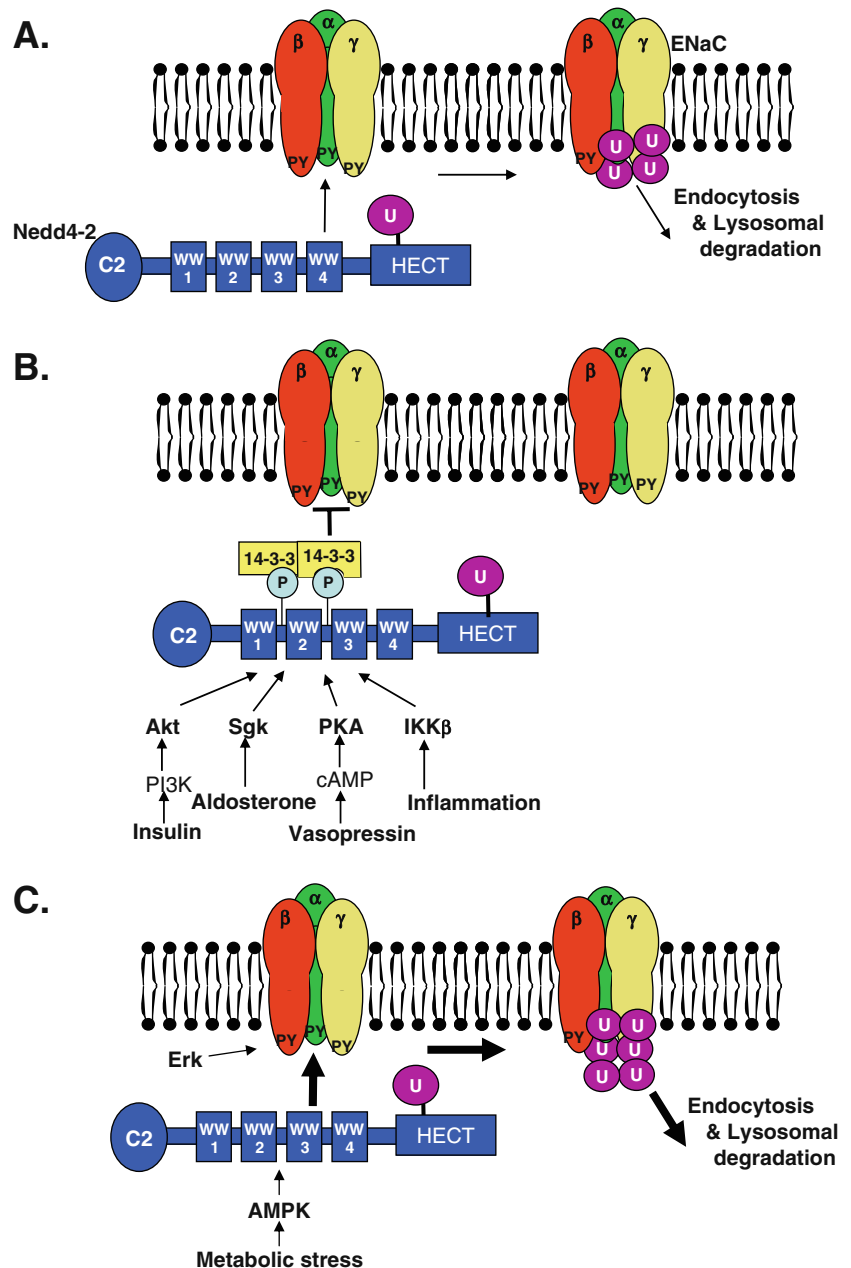
The role of ubiquitin in endocytosis and sorting of transmembrane (TM) proteins has received much attention over the past 15 years, initially from work in yeast [64, 78, 82] and in mammalian cells [114, 191]. Relevant to this review, our initial observation that ENaC binds the Nedd4/Nedd4-2 family of E3 ligases [191] opened the field to many subsequent studies that have focused on the role of ubiquitin system, and in particular the Nedd4 family proteins, in the regulation of cell surface stability and endocytosis of ion channels and transporters in mammals.

Regulation of ENaC by Nedd4-2

Perhaps the best-studied example of the regulation of ion transport by ubiquitin is that of ENaC regulation by Nedd4-2. As indicated above, ENaC regulates salt and fluid absorption in epithelia of several organs, including kidney, lung and colon. The critical role of ENaC in salt and fluid absorption in these tissues/organs is underscored by the findings that loss or gain of function mutations in this channel in humans causes pseudohypoaldosteronism Type I (PHA1) or pseudoaldosteronism (Liddle syndrome- see below), respectively, diseases that are also recapitulated in mouse models bearing the same mutations [89, 120]. In the kidney, ENaC function is up-regulated by the hormone aldosterone, which binds the mineralocorticoid receptor (MR), while in the lung it is up-regulated by glucocorticoids and their cognate receptor (GR) [125]. As indicated above, ENaC is comprised of 3 subunits, $\alpha\beta\gamma$ [28], with a short proline-rich sequence, called the PY motif (PPxYxx \emptyset , minimal consensus = L/PPxY), present at the cytosolic C-terminus of each subunit [173, 187, 191]. The PY motif of β or γ ENaC is deleted or mutated in Liddle syndrome [74, 75, 120, 183], a hereditary hypertension caused by increased activity and number of ENaC channels at the plasma membrane [57]. These PY motifs serve as binding sites for Nedd4 proteins [191], in particular Nedd4-2 [2, 76, 99, 188]. *Nedd4-2* (Nedd4L) possesses a C2 domain that can be spliced out [92], 4 WW domains that bind PY motifs [102, 191], and an E3 ligase Hect domain. The third WW domain, present in all Nedd4-2 but absent in most Nedd4-1 proteins (except human, zebrafish and *Drosophila* Nedd4-1), appears to provide higher affinity interactions to PY motifs [80, 101], but both WW3 and WW4 of Nedd4-2 are essential for interaction with and regulation of ENaC [60, 92, 100]. It is curious that human Nedd4-1, which also possesses 4 WW domains including WW3, can only inhibit ENaC function upon removal of its inhibitory C2 domain [100, 185], in line with a recent demonstration of inhibition of Hect catalytic activity by the C2 domain of the Nedd4 family relative Smurf2 [217] and Nedd4-1 and -2 [208], which in the latter can be relieved by elevated Ca^{2+} levels. However, it should be emphasized that the data with Nedd4-1 have been acquired in heterologous expression systems; to date there is no *in vivo* evidence that endogenous Nedd4-1 is involved in ENaC regulation.

Given the ability of Nedd4-2 to interact with the PY motifs of ENaC, which are deleted/mutated in Liddle syndrome, it was proposed [191] and later demonstrated [2, 99] that the increased retention of channels at the plasma membrane in this disease may be caused by impaired ubiquitylation and endocytosis of the mutant channels (Fig. 2a). Indeed, ENaC's cell surface stability was shown earlier to be negatively regulated by ubiquitylation on its α

Fig. 2 Regulation of ENaC by Nedd4-2. **a** Nedd4-2 WW domains binds to the PY motifs of ENaC, leading to channel ubiquitylation and endocytosis. **b** Hormones and factors that inhibit the ability of Nedd4-2 to suppress ENaC, including aldosterone, vasopressin, insulin and inflammatory mediators. These factors promote Nedd4-2-mediated phosphorylation via kinases such as Sgk1, Akt, PKA or IKK β , leading to binding of 14-3-3 proteins to the phosphorylated Nedd4-2 and preventing this E3 ligase from interacting with ENaC. **c** Nedd4-2 mediated suppression of ENaC can be stimulated by metabolic stress that leads to activation of AMPK and enhanced Nedd4-2 interaction with ENaC. *U* ubiquitin



and γ subunits [192]. In support, recent studies have demonstrated that Nedd4-2 can ubiquitylate ENaC at the plasma (apical) membrane [127, 216, 234]. Moreover, the increased retention of the PY motif mutant ENaCs at the plasma membrane is primarily caused by impaired internalization of the mutant channels, with some defects also observed in channel sorting and cAMP-dependent mobilization of channels from a sub-apical pool [127]. The nature of ENaC ubiquitylation is not entirely clear, but several reports recently suggested that the channel is mono or multi-mono ubiquitylated at the cell at the cell surface [171, 216, 234], a finding that is in accord with the known role of mono or oligo-ubiquitylation in endocytosis and sorting of

transmembrane proteins [81]. Another group proposed that native ENaC in A6 cells is regulated by polyubiquitylation and is degraded by the proteasome [129, 130] although it needs to be established whether the polyubiquitylated pool is the one at the plasma membrane. The type of ubiquitin linkage employed is not known, but one would expect to identify at least some K63-linked ubiquitylation of ENaC, a linkage often detected in proteins destined for endosomal sorting and lysosomal degradation, and was described in transmembrane proteins that are target for Rsp5 (the yeast Nedd4 orthologue)—mediated ubiquitylation and endocytosis/sorting [63, 111, 149]. In addition to regulating cell surface stability, the PY motifs (Nedd4-2 binding sites) of

ENaC also regulate its response to intracellular Na^+ concentrations, since feedback inhibition by Na^+ exhibited by WT ENaC is inhibited in a PY motif mutant of the channel [45, 106, 151].

The clear prediction from these findings is that the increased retention of active ENaC at the plasma membrane would increase Na^+ reabsorption in the kidney, leading to hypertension, as seen in Liddle syndrome patients. Indeed, knock-in mice bearing the Liddle syndrome mutation in β ENaC exhibit salt-induced hypertension, metabolic alkalosis and hypokalemia, leading to renal and cardiac hypertrophy which resembles the human Liddle disease [159]. In accord, these Liddle mice exhibit enhanced alveolar fluid clearance following pulmonary edema [162]. Interestingly, recently generated knockout (KO) mice of *Nedd4-2* exhibit a similar phenotype, with salt-induced hypertension (which was inhibited by amiloride) and cardiac hypertrophy, suggesting upregulation of ENaC. Indeed, ENaC protein expression in the kidney of these mice was elevated, and rectal PD measurements indicated excessive ENaC activity also in their colon [181]. While the potential effect of loss of *Nedd4-2* on lung function of these KO mice was not described, a separate study using RNAi to knockdown *Nedd4-2* in rat lungs immediately after birth, which led to ~60% reduction in level of *Nedd4-2*, has demonstrated increased ENaC expression along with reduced extravascular lung water following knockdown [116]. This suggests that *Nedd4-2* suppresses ENaC function in the lung, similar to its function in the kidney and colon.

It has long been recognized that CFTR regulates ENaC function, in most cases leading to inhibition of ENaC activity by reducing its open probability [194]. In cystic fibrosis (CF), where CFTR is defective, Na^+ and fluid absorption is elevated, leading to dehydration of airway surface fluid, mucus accumulation and repeated bacterial infections [22, 46]. Recent work by Mall, Boucher and co-workers provided further support for this model, by demonstrating that overexpression of ENaC (β ENaC) in mouse airways leads to a CF-like phenotype, a process that can be ameliorated by treatment of the mice with amiloride [131, 235]. An important issue that needs to be addressed is, therefore, why do Liddle syndrome patients, exhibiting excessive ENaC activity, not develop CF-like disease?

The elevated blood pressure (BP) in the *Nedd4-2* KO mice suggests that mutations or variation in the *NEDD4-2* gene in humans may be involved in the regulation of blood pressure [168]. In support, a recent study suggested that a cryptic splice variant (generated by the SNP rs4149601) that generates Isoform I of *Nedd4-2* (*NEDD4-2* A allele, aka *Nedd4-2C8* [48]) is associated with hypertension in a Chinese group of patients [128]. This splice isoform, which generates an inactive protein [48], was proposed to interact

with other *Nedd4-2* isoforms and block their function in a dominant negative fashion, thus leading to enhanced ENaC activity [8]. Another variant, encoding *NEDD4-2-P355L*, was shown to be less effective at suppressing ENaC than the native protein, likely due to its enhance phosphorylation [62] (see below).

Role of Nedd4-2 in hormone-mediated regulation of ENaC

As indicated above, aldosterone and vasopressin (ADH), as well as insulin, enhance ENaC function. In search for transcripts and proteins that exhibit early response to aldosterone and glucocorticoids, several groups identified serum and glucocorticoid-regulated kinase 1 (*Sgk1*), a relative of Akt (PKB) [212]; *Sgk1* was shown to enhance ENaC function by stimulating an increase in ENaC abundance at the plasma membrane in response to aldosterone, including in renal collecting ducts [7, 36, 126, 142, 182]. Subsequent work, initially from Staub and co-workers, demonstrated that *Sgk1* phosphorylates *Nedd4-2* on Ser residues (S444, S338), a process that involves a PY motif present in *Sgk1* and suggests a *Nedd4-2*:*Sgk1* interaction (most likely of low affinity [80, 163]); This phosphorylation leads to interference with the ability of *Nedd4-2* to interact with ENaC, resulting in increased abundance of channels at the plasma membrane, as demonstrated in a *Xenopus* oocyte model system, tissue culture cells and cortical collecting ducts [41, 58, 186] (Fig. 2b). Unlike *Nedd4-2*, *Nedd4-1* does not possess *Sgk* phosphorylation sites, and is not known to be regulated by aldosterone. In addition to *Sgk1*, it appears that cAMP-activated PKA can also phosphorylate *Nedd4-2*, on the same sites as those used by *Sgk1*, leading to increased ENaC abundance due to its inability to interact with phosphorylated *Nedd4-2* [184]. Since cAMP is generated and PKA activated in response to vasopressin release and its binding to its V2 receptor, these collective results suggest that *Sgk1* and PKA serve as convergent points to phosphorylate *Nedd4-2* and block its ability to suppress ENaC in response to both aldosterone and vasopressin (Fig. 2b), the two major hormones regulating fluid absorption, blood volume and BP.

In addition to the regulation of *Sgk*/PKA/*Nedd4-2* by aldosterone and vasopressin, Insulin was recently shown to increase ENaC function by activating its downstream effectors Akt1 and *Sgk1*, thus feeding into the same *Nedd4-2* pathway and connecting cellular metabolism and growth to ENaC [112]. Likewise, $\text{II-1}\beta$ -induced cortisol production was shown to stimulate fluid absorption (ENaC activation) via *Sgk*-mediated *Nedd4-2* inhibition in fetal guinea pigs lungs [117]. Additionally, the WNK kinase was also shown to activate *Sgk1*, resulting in suppression of *Nedd4-2* and elevated ENaC function [222]; mutations in

WNK cause PHAIL, a form of hypertension. Interestingly, another player, IkappaB kinase β (IKKB), was recently shown to phosphorylate Nedd4-2 on S444 (the same site as that phosphorylated by Sgk1), leading to enhanced accumulation and function of ENaC at the plasma membrane [49]. These latter results provide another arm to the regulation of ENaC by Nedd4-2, connecting it to inflammation and the immune system (Fig. 2b).

How does phosphorylation of Nedd4-2 by Sgk/PKA prevent it from interacting with ENaC? Recent studies identified 14-3-3 proteins as binding partners to phosphorylated Nedd4-2; 14-3-3 maintains phosphorylated Nedd4-2 in a “blocked” form that cannot bind ENaC [13, 90]. Recent work also suggested that a heterodimer of the 14-3-3 β :14-3-3 ϵ is required for this interaction [118] and that these 14-3-3 isoforms are themselves induced by aldosterone [119]. The exact mechanism of this “blockage” is still unknown, since the phosphorylation sites on Nedd4-2 are located outside the WW domains that bind the PY motifs of ENaC; it is possible that a 14-3-3 dimer binds to Nedd4-2 and alters its conformation, thus masking the binding sites on its WW domains. This, however, remains speculative until the structure of Nedd4-2 in complex with the PY motifs of ENaC, in the presence or absence of 14-3-3, is solved.

While the Sgk1/Nedd4-2 pathway can lead to enhanced ENaC function, it should be kept in mind that aldosterone, vasopressin and Sgk1 can also activate ENaC independently of Nedd4-2. Evidence for this is provided by the observations that (1) aldosterone and Sgk1 can increase cell surface levels of ENaC channels bearing the Liddle syndrome mutations (which cannot properly bind Nedd4-2) [7, 9, 43, 182], (2) Cortical collecting tubules harvested from mice bearing the Liddle syndrome mutation reveal normal response to aldosterone [39], (3) ENaC activity in the colon of these Liddle mice is enhanced by aldosterone [12], and (4) ENaC was shown to be inhibited by WNK4 kinase, a process inhibited by Sgk1, which phosphorylates WNK4 [165, 166].

Interestingly, recent reports provide a connection between ENaC and the metabolic state of the cell, by demonstrating that the metabolic sensor AMP kinase (AMPK) inhibits ENaC function in a PY motif-dependent manner, by enhancing its ability to interact with Nedd4-2, thus reducing cell surface stability of the channel [6, 14, 30] (Fig. 2c); the latter effect did not involve Sgk or PKA, and overall suggests that under metabolic stress, reducing ENaC function by activated AMPK, leading to enhanced channel endocytosis, would prevent cellular Na⁺ overload. Likewise, Erk (part of the MAPK pathway) downregulates ENaC by phosphorylating it on Thr residues in the C termini of β and γ ENaC, thus facilitating ENaC interaction with Nedd4/Nedd4-2 [138, 180, 225], although it is not

clear yet how such phosphorylation would increase ENaC: Nedd4/Nedd4-2 binding given the hydrophobic nature of WW domain:PY motif interaction [101, 102].

Two knockout models of Sgk1 have been generated and the role of Sgk1 in ENaC regulation has been evaluated in these mice. Wulff et al. found that Sgk1 (−/−) mouse displayed a salt-losing phenotype only when mice were kept under low Na⁺ diet. Under such conditions, ENaC was also weakly expressed, as evidenced by potential difference (PD) measurement in isolated cortical collecting ducts and by immunohistochemistry [221]. Somewhat different data were obtained by Fejes-Toth et al. Although they also found a salt-losing phenotype under low Na⁺ diet, they observed by in situ patch clamping CCD cells that amiloride-sensitive Na⁺ currents were upregulated under these conditions, suggesting that other Na⁺ transporters must be upregulated, including the thiazide-sensitive Na⁺/Cl[−] cotransporter [55]. Hence the precise role of Sgk1 *in vivo* in the kidney remains to be further characterized, and the mechanism of aldosterone regulation continues to remain obscure.

De-ubiquitylating enzymes and ENaC

The critical role of the E3 ubiquitin ligase Nedd4-2 and ubiquitylation in the regulation of ENaC endocytosis and cell surface stability prompted a search for de-ubiquitylation enzymes (DUBs) that may participate in this regulation as well. Recently, a DUB induced by aldosterone, Usp-45, was identified by Verrey and colleagues [54]. Usp-45 deubiquitylates ENaC and stimulates ENaC cell surface stability and function; this effect is dependent on the presence of the PY motifs of ENaC and on its prior ubiquitylation by Nedd4-2, but is independent of Sgk1 [54]. Another DUB that was described to regulate ENaC is the endosomal DUB, UCH-L3 [27]. UCH-L3 increases cell surface expression of ENaC by de-ubiquitylating it, presumably in an endosomal compartment, and enhancing its recycling to the plasma membrane. Given the known role of cAMP in inducing ENaC recycling [127], it would be interesting to test whether UCH-L3 participates in this process. In addition, the DUB Usp10 was recently identified as a transcript/protein whose expression is elevated following vasopressin stimulation, an increase that leads to enhanced ENaC abundance and function at the plasma membrane [23]. Usp-10 utilizes an adapter protein, SNX3, to carry out its effect on ENaC, as detailed below.

Thus, specific DUBs appear to oppose the action of Nedd4-2 (directly, or indirectly via an adapter protein), leading to increased ENaC stabilization at the plasma membrane (Fig. 3), especially in response to hormones that are released into the blood stream to promote ENaC function and Na⁺/fluid absorption.

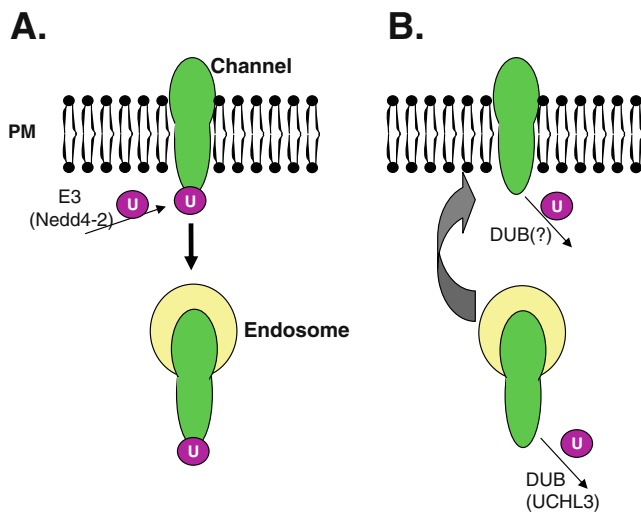


Fig. 3 Opposing actions of E3 ligases and DUBs on channel abundance at the cell surface. **a** Ubiquitin ligases such as Nedd4-2 ubiquitylate ion channels (e.g., ENaC) at the plasma membrane, leading to its endocytosis. This action can be opposed (**b**) by de-ubiquitylating enzymes (DUBs), such as USP2-45 or UCHL-3, which remove ubiquitins from the channel, thus increasing its cell surface abundance by promoting recycling (e.g., bin the case of UCHL-3) or possibly by decreasing endocytosis. *U* ubiquitin

Relationship between regulation of ENaC ubiquitylation and its proteolytic cleavage

Proteolytic cleavage of the ectodomain of ENaC by serine proteases leads to activation of the channel by increasing its open probability (P_o) [88, 169, 203]. In particular, the α and γ subunits are the targets for such cleavage: α ENaC is cleaved by Furin at two sites, releasing an inhibitory peptide/tract, whereas γ ENaC is cleaved by Furin at one site and by another protease, such as prostasin/CAP1, elastase or plasmin, at a second site, to promote release of the inhibitory tract [24, 31, 32, 86, 153]. It appears that the inhibitory tract provides higher affinity for Na^+ [178] and may mediate the well-known self-inhibition of ENaC by extracellular (luminal) Na^+ [151]; thus, removal of the inhibitory tract by proteases promotes channel activation (increased P_o) by decreasing sensitivity to luminal Na^+ [178]. Curiously, while proteases increase ENaC P_o , their effect is connected to ubiquitylation, Nedd4-2 and Usp-45. Work from Snyder and colleagues has demonstrated that Nedd4-2 facilitates rapid endocytosis of proteolytically cleaved ENaC, leading to increased degradation of the cleaved, active channel [97]. This endocytosis was dependent on the presence of the PY motifs of ENaC. In accord, mutations of these PY motifs (mimicking those in Liddle syndrome) or mutation of the ubiquitin acceptor sites in ENaC increased the fraction of cleaved, active channels at the plasma membrane [108]. In agreement with these observations, Staub and colleagues have recently demon-

strated that Usp-45—mediated de-ubiquitylation of ENaC enhances its proteolytic cleavage (on α and γ ENaC), thus stimulating its activation [171]. These data thus suggest that ubiquitylation may regulate the conformation of the extracellular domain, and likely the accessibility of protease sites for cleavage.

The strong reliance of ENaC on Nedd4-2-mediated downregulation (channel ubiquitylation and endocytosis) has been a long-standing puzzle, since intuitively it would be much faster to close the channel rather than to internalize it when its function is no longer needed; the recent findings that protease cleavage releases a segment of the channel (the inhibitory tract), leading to irreversible opening of the channel, helps explain this puzzle, since such constitutively active channels have to be removed from the plasma membrane to stop the influx of Na^+ .

Regulation of endocytosis of other ion channels by Nedd4 family ubiquitin ligases

Following the discovery of ENaC: Nedd4/Nedd4-2 interactions, several groups have identified PY motifs in numerous other channels, prompting investigations into their potential regulation by Nedd4 proteins, as described below.

Voltage-gated Na^+ channels

Voltage-gated Na^+ channels, Na_v s, are responsible for generating action potential in excitable cells, primarily in cardiac and neuronal tissues. They are comprised of pore-forming α subunits assembled together with regulatory β subunits [230]. These channels play a critical role in cardiac and neuronal functions, and mutations in some of them cause human diseases [230]. For example, mutations in $\text{Na}_v1.5$ (*SCN5A*) that impair its inactivation in the heart lead to long QT (type 3) and Brugada syndromes [105] and inactivation of the *Scn5a* gene in mice leads to cardiac function defects [152]. Likewise, gain of function mutations in $\text{Na}_v1.1$ (*SCN1A*) causes generalized epilepsy with febrile seizures (GEFS+) by enhancing inward Na^+ current in brain neurons [52]. Of the ten known Na_v s, seven ($\text{Na}_v1.1$ – 1.3 and $\text{Na}_v1.5$ – 1.8) possess a conserved PY motif (PPSYxxØ) at the cytosolic C termini of their α subunits [61, 170]. Much like ENaC, cell surface stability of the cardiac $\text{Na}_v1.5$ was demonstrated to be negatively regulated by Nedd4-2, a process that requires the PY motif of this channel, as well as active Nedd4-2, suggesting channel ubiquitylation regulates its endocytosis [1, 204]. Similar results were obtained for other PY-motif containing Na_v s, $\text{Na}_v1.2$, 1.3, 1.7 and 1.8 [61, 170]; while Nedd4-2 was the predominant E3 ligase involved in suppressing these Na_v s, WWP2 and Nedd4-1 may contribute to the regulation as well. A recent

search for *NEDD4-2* variants in ~250 families with photosensitive generalized epilepsy (IGE) identified several such variants, although their expression in *Xenopus* oocytes did not affect their ability to interact with $\text{Na}_v1.2$, nor alter channel function [44], suggesting that the physiological significance of these variants need to be further explored.

Recently it was shown that Nav1.6 is regulated by Nedd4-2 as well. Interestingly Nedd4-2 appears to interact with 2 motifs, one of which is a PGSP motif that needs to be phosphorylated by the p38 kinase in order to interact with Nedd4-2 [66].

Voltage-gated K^+ channels and Ca^{2+} channels

Voltage-gated K^+ channels comprise several subfamilies, including the KCNQ (K_v7), KCNA (K_v1), KCNB (K_v2) and others. These channels play a key role in neuronal excitability, and mutations in several of them cause neurological and cardiac impairments. For example, loss of function mutations in KCNQ2 ($\text{K}_v7.2$) or KCNQ3 ($\text{K}_v7.3$) cause epilepsy [164], while such mutations in KCNQ1 ($\text{K}_v7.1$) or its associated subunit KCNE1 cause Long QT (Type I) syndrome (cardiac ventricular arrhythmia) [105].

KCNQ1 possesses a PY motif (LPxYxxØ) at its intracellular C terminus, which is conserved across species and serves as binding site for human Nedd4-2, Nedd4-1, or WWP2. Accordingly, the current produced by the KCNQ1/KCNE1 channel (I_{ks}) is reduced by overexpression of these Nedd4 family members and is suppressed by their catalytically inactive mutants, or upon mutating the PY motif of KCNQ1 [95]. These Nedd4 family members thus affect channel ubiquitylation and internalization. Likewise, the muscarinic-sensitive K^+ current (M-current), mediated by heterotetrameric channels consisting of KCNQ3 associated with either KCNQ2 or KCNQ5 (i.e., KCNQ2/3 or KCNQ3/5), are regulated by Nedd4-2, ubiquitylation and Sgk1 [50, 175]. The C termini of KCNQ2 and KCNQ3 contain an atypical “PY motif” (PPxPPY). However, while Nedd4-2 can bind the C termini of these channel subunits, binding is not mediated via this motif [50]; thus, Nedd4-2 either interacts with another C-terminal sequence, or via an adapter protein. Active Nedd4-2 is required to suppress function of these channels, suggesting that ubiquitylation regulates their stability, likely at the cell surface [50]. Similar to ENaC, the regulation of KCNQ2/3 and KCNQ3/5 via Nedd4-2 can be modulated by Sgk1 mediated phosphorylation of this E3 ligase [175].

The K^+ channels $\text{K}_v1.3$ (KCNA3) and $\text{K}_v1.5$ (KCNA5), which are stimulated by insulin or IGF-1, have been demonstrated to be inhibited by Nedd4-2 and up-regulated by Sgk1, which suppresses Nedd4-2, using *Xenopus* oocyte expression system [17, 79]. Nedd4-2 was shown to

ubiquitylate $\text{K}_v1.5$, thus inducing its endocytosis [17]. Interestingly, while $\text{K}_v4.3$ (KCND3) assembled with its accessory protein KChIP, was stimulated by Sgk1, this stimulation was not mediated via Nedd4-2 and was not associated with increased cell surface stability of this channel [10].

In addition to the above-described examples, a recent proteome array screen aimed at identifying substrates for mammalian Nedd4 protein, identified other K^+ channels as *in vitro* substrates for Nedd4-2: KCNAB1, KCNAB3 and KCNB1 ($\text{K}_v2.1$), with the latter two possessing PY motifs [155]. The role of these *in vitro* substrates needs to be examined *in vivo* now to test if these channels are regulated by Nedd4-2 in a physiological setting.

The same proteome array screen also identified the Ca^{2+} channel, CACNB1, as a substrate for Nedd4-2, an observation that was also validated in mammalian cells [155]. Interestingly, a subset of the TRP Ca^{2+} channel, such as TRPV4 and TRPC4, have been shown to be ubiquitylated and AIP4/Itch, a Nedd4-like protein; this ubiquitylation leads to endocytosis of these channels [213], thus suppressing their function. This AIP4-mediated ubiquitylation requires the N terminal region of the TRP channels, although a variant PY motif (GPxYxxL) present within the N-terminus was dispensable [213]. Recently, evidence was also provided that the calcium channel TRPV6 may be regulated by Nedd4-2 [232].

Collectively, these results point to a major role of Nedd4 proteins, primarily Nedd4-2, in promoting ubiquitylation and endocytosis of numerous cell surface cation channels, thus suppressing their function.

Cl^- channels/transporters

Much like cation channels, several anion channels have been shown to be regulated by Nedd4 family members, with Cl^- channels (CIC) family most extensively studied. The CIC family comprises both Cl^- channels and Cl^-/H^+ antiporters [94, 215]. CIC5 is a Cl^-/H^+ antiporter that is expressed primarily in endosomal membranes in the proximal tubules and is responsible for endosomal acidification, a role it carries out in collaboration with V-ATPase. This acidification is required for re-absorption of small molecule proteins, minerals and vitamins by the kidney [72]. Loss of function mutations in *CLC5* leads to Dent disease, a disorder characterized by excessive loss of proteins (proteinuria), calcium and phosphate in the urine, resulting in kidney stones [220]. In accord, CIC5 knockout mice exhibit proteinuria and defects in both receptor mediated and fluid phase endocytosis, resembling Dent disease in humans [156]. A carboxy terminal PY motif (PPLPPY) was identified earlier in CIC5, and shown to be required for endocytosis of the channel [176]. It was

postulated that WWP2 could be mediating the endocytosis of CIC5 by interacting with its PY motif [176], although a later study proposed that Nedd4-2 is the E3 ligase involved [84]. In the latter study, Nedd4-2 was shown to regulate CIC5 ubiquitylation and albumin uptake into endosomes. Similar to CIC5, CIC2 was also shown to be regulated by Nedd4-2 [150]. CIC2, a *bone fide* Cl⁻ channel, is ubiquitously expressed and is localized to the plasma membrane. Disruption of *CIC2* in mice causes testicular and retina degeneration, as well as wide-spread vacuolation in the white matter of the CNS, suggesting that regulation of ionic environment via CIC2 in these cells/tissues is necessary for their normal maintenance [16, 21]. One study also suggested that the *CIC2* gene is included in a chromosomal region associated with susceptibility to epilepsy [172], however, the putative role of CIC2 in this increased susceptibility needs to be further investigated. CIC2 was shown to be positively regulated by Sgk1-3, and negatively regulated by Nedd4-2, which reduces its cell surface expression [150], although a direct interaction between Nedd4-2 and CIC2 was not demonstrated in that study, nor does CIC2 possess a PY motif. CIC-Ka is another *bone fide* Cl⁻ channel that along with CIC-Kb is a basolateral channel expressed in the ascending loop of Henley and regulates Na⁺, Cl⁻, and fluid absorption. In accord, knockout of *Clck1* (CIC-Ka) in mice leads to severe urinary loss [200]. The CIC-Ka and CIC-Kb channels require co-expression of an accessory protein, barttin, to become functional [53]. Mutations in *BSND* (the barttin gene) causes a severe form of Bartter's syndrome, characterized by severe renal salt wasting and deafness [15]. Interestingly, Barttin contains a PY motif and its mutation enhances activity of the CIC-Kb/Barttin channel [53]. It was subsequently shown that Nedd4-2 can suppress CIC-Ka/Barttin function when co-expressed in *Xenopus* oocytes, an effect prevented upon mutation of the PY motif in barttin [51]. Here, too, Sgk1 and Sgk3 were able to phosphorylate Nedd4-2 and block its suppression of the channel.

In addition to the CIC family, other Cl⁻ channels appear to be regulated by Nedd4 proteins. The Tweety (TTYH) family are maxi Cl⁻ channels that includes three members in humans, TTYH1-3, with TTYH2 and TTYH3 (but not TTYH1) activated by Ca²⁺ [195]. TTYH2 and TTYH3 possess a PY motif, which a recent study has suggested is involved in binding to Nedd4-2; accordingly, Nedd4-2 was shown to ubiquitylate TTYH2 and regulate its cell surface stability [77]. An additional Cl⁻ channel, CLIC2 (intracellular Cl⁻ channel 2), could potentially be another target for regulation by Nedd4-2, as it was identified in the above-described proteome array screen as a target for ubiquitylation by Nedd4-2 (and Nedd4-1) and possesses a PY motif [155].

The suppression of channel function by Nedd4 family members was also reported in several transporters in addition to CIC5. For example, the excitatory amino acid transporter EAAT2 (which does not contain a PY motif) was shown to be suppressed by Nedd4-2 and stimulated by Sgk1-3 or Akt [18]. Likewise, the divalent metal transporter, DMT1, was shown to be regulated by WWP2 and Nedd4-2; this was mediated by recruitment of the adapter protein Ndfip1 [59, 83], as described below. Moreover the connexin 43, which contains a PY-motif, was also demonstrated to be regulated by Nedd4-2 [115] by one group, whereas the Staub's group found that the extended PY-motif was rather part of a tyrosine-based sorting signal not regulated by Nedd4 ubiquitin-protein ligases [197].

In addition to the regulation of CFTR and its Δ F508-CFTR trafficking mutant by ERAD in the biosynthetic pathway (described above), it was recently found that "rescued" Δ F508-CFTR that is able to reach the plasma membrane exhibits a short half life there and is targeted for degradation by the E3 ligase CHIP, with the aid of several chaperones and co-chaperones [146].

Overall, it appears that cell surface stability of numerous ion channels and transporters is regulated by Nedd4 proteins, mainly Nedd4-2 (and sometimes WWP2, Nedd4-1, or AIP4). This regulation often involves the Sgk/Akt family of kinases, which phosphorylate Nedd4-2 thus preventing it from interacting with its targets. The regulation by these kinases (and others) help link Nedd4-2 and the channels its ubiquitylates to hormone and growth factors signaling (e.g. aldosterone, vasopressin, insulin), which play critical roles in maintaining homeostasis of ion and fluid composition and overall normal physiological functions of the organism.

Regulation of endocytosis of channels/transporters by other (or unknown) E3 ligases

While most studies to date have focused on the regulation of ion channels/transporters by Nedd4 family proteins, several others have described regulation of such channels by different (or still unknown) E3 ligases. For example, function of the ENaC-related channel, ASIC, is suppressed by the E3 ligase Parkin, in a process that includes the adapter protein, PICK1 [96] (see below). Another example is the water channel AQP2, which is oligo-ubiquitylated with ub(K63) on one Lys residue upon withdrawal of vasopressin/forskolin or PKC activation, which targets it for endocytosis, MVB sorting and lysosomal degradation [98]. The E3 ligase that mediates this reaction is not yet known.

In cilia of the mammalian kidney and sensory cilia in *Caenorhabditis elegans*, polycystin-1 (PC1, encoded by PKD1) and PC2 (TRPP2, encoded by PKD2) function

together as a mechanosensory Ca^{2+} channel. Using *C. elegans*, it was recently shown that this channel complex is sorted for lysosomal degradation by the STAM-Hrs system in a process that involves mono-ubiquitylation of PC2 by as yet an unknown E3 ligase [85]. The physiological importance of these channels and their regulation is underscored by the observation that mutations in PKD1 or PKD2 cause autosomal dominant polycystic kidney disease (ADPKD), an end stage renal disease [91]. In a separate study, it was discovered that knockout of the transcription factor TAZ in mice led to increased levels of PC2 [198]; upon phosphorylation, TAZ interacts with $\text{SCF}^{\beta\text{-Trcp}}$ to form an E3 ligase complex, which then targets PC2 for degradation by the proteasome [198]. It is unlikely that the TAZ: $\text{SCF}^{\beta\text{-Trcp}}$ complex (and polyubiquitylation) is also involved in targeting of the PC1:PC2 channel for sorting by STAM-Hrs and lysosomal degradation, and thus suggests that regulation of this Ca^{2+} channel is mediated by more than one E3 ligase. In addition to regulation by E3 ligases, a role for DUBs was also described for the regulation of TRP channels. The TRPA1 channel, which responds to cold temp and pungent compounds, was shown to associate with the DUB CYLD, which is a tumor suppressor; this leads to de-ubiquitylation of TRPA1 and its stabilization, thus connecting TRPA1 to oncogenesis [193].

Analogous to regulation of several K^+ channels by Nedd4 family members (described above), a recent paper describes the suppression of ROMK1 (Kir1.1) by the RING E3 ligase POSH [123]. POSH binds the N terminus of ROMK1, leading to channel ubiquitylation via the POSH-RING domain and endocytosis via a clathrin-independent pathway [123].

Ubiquitin-mediated regulation of ion channels and transporters via adapter proteins

In most of the examples described above, Nedd4-2 (or its family relatives) was able to directly interact with its target channel/transporter, especially in those cases where these transmembrane proteins possess a PY motif that can bind the WW domains of Nedd4 proteins (eg in ENaC, several Na_v s, K^+ channels and CIC5). In other cases, such interactions may be indirect, via adapter proteins, which themselves possess PY motifs (Fig. 4). This has been observed earlier in yeast TM proteins that are regulated by Rsp5, the *S. Cerevisiae* orthologue of Nedd4/Nedd4-2. For example, Bul1 and Bul2 serve as adapters to connect between Rsp5 and the general amino acid permease, Gap1, thus facilitating Rsp5-mediated ubiquitylation of Gap1 and its subsequent endocytosis from the PM or sorting from the Golgi to vacuoles [189].

Likewise, several arrestin related proteins (ARTs) were recently identified as PY motif-containing adapters that connect between Rsp5 and numerous PM proteins (mostly transporters), thus promoting Rsp5-mediated ubiquitylation and endocytosis of these proteins [122]; for reasons not yet clear, the adapter proteins themselves are target for Rsp5-mediated ubiquitylation [73, 122], which is required for their function. An interesting example, also conserved in mammals, is the adapter Bsd2. One function of this ER protein is to regulate ubiquitin-mediated trafficking to the PM of the metal ion transporters, Smf1 and Smf2, in yeast [124]. The mammalian homologues of Bsd2 are Ndfip1 (N4WBP5) and Ndfip2 (MDWBP5a), and are PY motif-containing adapters for Nedd4 proteins that reside in intracellular vesicles. Similar to Bsd2 in yeast, they serve as adapters to connect between WWP2 and the divalent metal transporter DMT1 in mammals [59]. DMT1 is responsible for iron uptake in enterocytes and its release from vesicles in hepatocytes. Ndfip1&2 facilitate ubiquitylation of DMT1 by WWP2, resulting in degradation of this metal transporter (Fig. 4A). Accordingly, knockout of Ndfip1 in mice leads to increased DMT1 activity and hepatic iron accumulation [59], suggesting a key role for this adapter in regulating iron homeostasis. More recently, Ndfip1 was shown to protect human neurons from iron and cobalt toxicity, and knockdown or knockout of this adapter resulted in accumulation of these metals in neurons [83]; In this case, Ndfip1 serves as an adapter to connect neuronal DMT1 to Nedd4-2.

As indicated above, the vasopressin-inducible DUB, Usp-10, enhances ENaC abundance and function at the plasma membrane [23]. Here, however, Usp10 does not directly de-ubiquitylate ENaC, but rather a third partner, sorting nexin 3 (SNX3), a protein that when co-expressed with ENaC increases cell-surface stability of the channel. Thus, Usp-10 mediated de-ubiquitylation of SNX3 leads to increased stability of SNX3, which in turn promotes ENaC stabilization [23]. (Fig. 4B).

ASIC, the acid-sensing ion channel that is expressed in neurons, belongs to the ENaC/degenerin family, but lacks the C-terminal region present in ENaC that contains a PY motif (and thus does not interact with Nedd4-2). Instead, ASIC binds PICK1, a PDZ-containing protein that normally potentiates ASIC function following PKC stimulation. PICK1 is a substrate for Parkin, a RING finger E3 ligase, which mono-ubiquitylates PICK1; this mono-ubiquitylation (and thus protection from proteasomal degradation) appears to block the ability of PICK1 to potentiate ASIC [96] (Fig. 4C). In accord, loss of parkin in hippocampal neurons by its knockout in mice leads to enhanced ASIC activation, thus potentially promoting neuro-degeneration, as seen in Parkinson's disease [96].

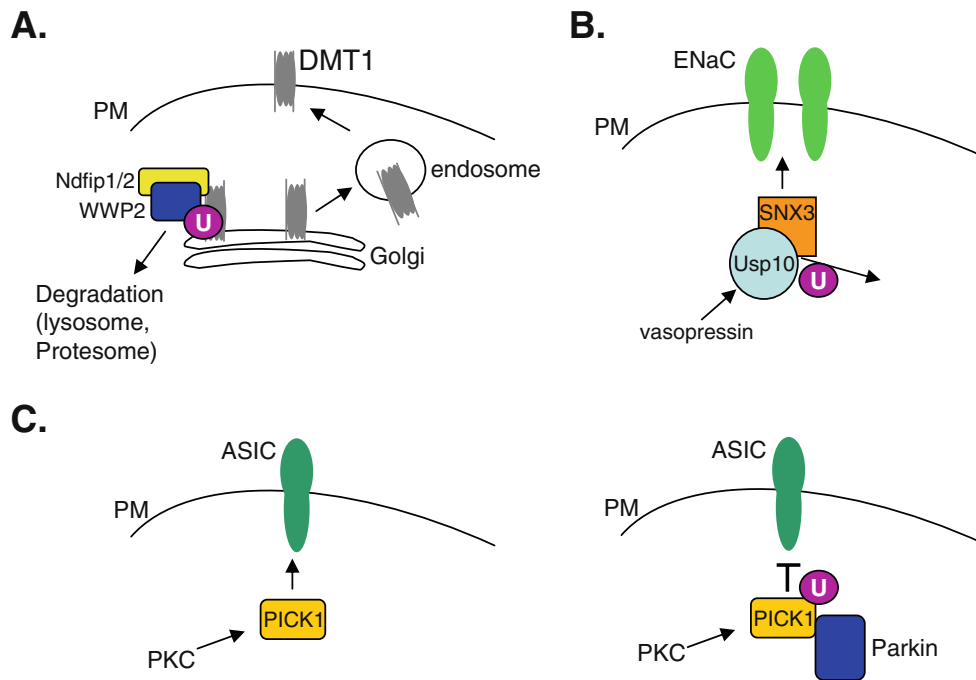


Fig. 4 Role of adapter proteins in ubiquitin-mediated regulation of ion transporters or channels. **A** The metal ion transporter DMT1 is targeted to the plasma membrane PM via recycling endosomes. This delivery can be inhibited by the WWP2, which is recruited to DMT1 at the Golgi via the adapter proteins Ndfip1/2 (that possess PY motifs), leading to ubiquitylation of the transporter and its degradation

by the lysosome and/or proteasome. **B** The vasopressin-sensitive DUB, Usp10, de-ubiquitylates sorting nexin 3 (SNX3), a process that stabilizes SNX3. SNX3, in turn, leads to increased stabilization of ENaC at the plasma membrane. **C** PKC-stimulated potentiation of ASIC function mediated by PICK1 (*left panel*) is inhibited by mono-ubiquitylation of PICK1 by Parkin (*right panel*). U ubiquitin

Regulation of ion channels by UBLs (SUMO)

As indicated in the “Introduction” section, posttranslational modification by UBLs has emerged as an important factor in the regulation of function of numerous cellular proteins. One of the better-studied UBLs is SUMO. Several recent studies have focused on the role of SUMOylation in regulating ion channels.

The Ca^{2+} channel TRPM4, expressed in cardiac Purkinje fibers, was recently demonstrated to harbor a mutation, E17K, which is associated with progressive familial heart block type I (PFHB1), an autosomal dominant disease; the mutant channel exhibits an increased channel SUMOylation and accumulation at the plasma membrane, leading to enhanced function of this channel [110]. It was proposed that such enhanced SUMOylation prevents channel ubiquitylation and endocytosis, although this hypothesis was not experimentally tested (i.e., ubiquitylation of the mutant vs. WT channel was not investigated).

Several K^{+} channels were also reported to be regulated by SUMOylation. The K2P1 (TWIK1) leak channel was previously shown to be a weak (or inactive) channel [70, 113, 148]. A report by Goldstein and colleagues then demonstrated that this channel is maintained in an inactive state by SUMOylation on one specific Lys (K274); upon de-SUMOylation by SENP-1, the channel becomes active,

exhibiting K^{+} selectivity and sensitivity to extracellular pH [161]. A subsequent paper challenged these findings, suggesting that the K274 residue may not be involved in SUMOylation of the channel [56]. Recently it has been shown that one SUMO is sufficient to silence the K2P1 channel [157]. More recently, other studies provided evidence for SUMO modification of other K^{+} channels. $\text{K}_{\text{v}}1.5$, which mediates the I(kur) depolarizing current in human atrial myocytes, was shown to be directly SUMOylated by Ubc9 (the E2 for SUMOylation reaction) at two sites [11]; removal of this SUMO conjugation by SENP2 or by mutating the SUMOylation sites led to hyperpolarizing shift in voltage dependence of steady-state inactivation of the channel. The voltage-dependent channel $\text{K}_{\text{v}}2.1$ regulates pancreatic beta cell excitability and insulin secretion. Recent work demonstrated that SUMOylation of $\text{K}_{\text{v}}1.5$ inhibits its native current by accelerating channel inactivation and inhibiting recovery from inactivation, resulting in decreased firing frequency [40]. These effects were augmented by overexpression of Ubc9 and alleviated by SENP1. It remains to be investigated how exactly (by what mechanisms) does SUMOylation of K^{+} channels, such as $\text{K}_{\text{v}}1.5$ or $\text{K}_{\text{v}}2.1$, regulate their biophysical properties and activity.

It is curious that KChAP, which was originally described as a chaperone that increases protein expression of several

K⁺ channels, was later identified as PIAS3 β , a SUMO E3 ligase [158]. So far it is not known if/how SUMOylation may affect the chaperone activity of KChAP/PIAS3 β towards these channels.

Thus, relative to ubiquitylation of ion channels, much less is known about the mechanisms by which SUMOylation regulates function of such channels, nor is it known (yet) whether modifications by other UBLs can affect ion channel function.

Summary/perspectives

Over the past 15 years much has been learned about the role of the ubiquitin system in regulating ion channels and transporters, particularly during their processing in the ER and during endocytosis or sorting to MVBs and the lysosome. Much of the work has focused on the Nedd4 family of E3 ligases, and more recently on other E3 ligases, DUBs and several adapter proteins. In spite of these great advances, there are critical outstanding issues that need to be addressed:

- (a) To date, many studies demonstrating regulation of endocytosis of ion channels and transporters by E3s, especially Nedd4 family members, have been carried out by overexpression in *Xenopus* oocytes or tissue culture cells. It is essential that these initial studies are validated *in vivo* in the relevant physiological tissues. In that regard, recent advances in studying the role of Nedd4-2 in regulating ENaC in mouse kidney (and lung) represent important advances, and hopefully will be followed by similar studies on other ion channels/transporters.
- (b) So far, in most studies the nature of the ubiquitin attachments to the ion channels/transporter, especially at the plasma membrane, is not clear. For example, are the channels/transporters mono-, oligo- or poly ubiquitylated? Are the ubiquitins attached utilize K63, K48 (or others) linkages? Accordingly, is the cargo channel/transporter targeted to the lysosome, proteasome or both? Likewise, very little is known about the functions of UBLs in regulating ion transport, nor the role of other modifications, such as acetylation.
- (c) As detailed in the review, there are numerous diseases that are associated with malfunction of some of the ion channels described, including defects that involve the ubiquitin system. To develop drugs that targets these defects, it would be useful to solve the structure of the E3 ligase involved in complex with their cognate channel/transporter, or at least the binding interface of these binding partners. Given the recent advances in solving structures of transmembrane proteins, this difficult task

may now become a reality. Such an approach could lead to the development of compounds that block or enhance these interactions in a specific manner, which could have an important therapeutic value.

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