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Chapter 11

FROM A CONSERVED STRUCTURE TO REGULATION: CWH UBIQUITIN LIGASES TIGHTLY REGULATE KEY CELLULAR EVENTS

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Abstract

Ubiquitin ligases are key regulators of ubiquitylation reactions, as they establish direct contact with substrate proteins. Ligases of the Nedd-4 family (hereafter referred to as CWHs) form a monophyletic group and are modular proteins composed of an N-terminal C2 domain, two to four WW domains and a catalytic HECT (Homologous to E6-AP Carboxyl Terminus) domain. These ligases are represented in yeasts and metazoa, and are commonly involved in transmembrane protein signaling, trafficking and downregulation. Here, we review the growing body of evidence for the regulation of the CWH ubiquitin ligases.

CWHs ligases are regulated at many levels. Modulating the CWHs transcription and degradation regulate enzyme levels, and consequently their activity. CWHs interact with ubiquitin proteases that reverse their autoubiquitylation and allow fine-tuning of their expression. The catalytic activity of CWHs can be modulated, as the ligases are self-inhibited through intramolecular interaction or dimerization. This inactive state is released by different processes, including calcium binding to the C2 domain, phosphorylation of specific residues or direct binding of regulatory proteins. Inversely, phosphorylation can facilitate the closed conformation or directly inhibit substrate interaction. Some regulatory proteins directly compete with substrate binding. Finally, phosphorylation or binding to regulatory proteins may interfere with E2 recruitment, further limiting the ligase activity. The HECT domain of several CWHs interact with ubiquitin itself, a process suggested to regulate the processivity of the ubiquitylation reaction. Binding of adapter molecules cause the translocation of some CWH ligases to specific subcellular compartments, which may alter their availability to specific substrates. Some of the proteins causing relocalization are also activators, further affecting substrate ubiquitylation.

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Although closely related CWH ligases may show some degree of redundancy in their function, many substrates are recognized by a single ligase. Among CWH ligases, Itch has a uniquely conserved proline rich domain (PRD) allowing it to interact with SH3 domain-containing proteins. Itch thus has access to a completely distinct set of substrates. The PRD contains several SH3 binding motifs and could simultaneously accommodate different proteins. Since the SH3 partners identified to date are all involved in endocytosis and signaling, Itch may act as a scaffold to promote ubiquitylation of substrates and help establish protein networks facilitating surface protein endocytosis. SH3 binding occurs at the baseline level and can be further modulated by extracellular cues. It is interesting to note that the PRD overlaps with sequences involved in Itch regulation, suggesting that SH3 binding could affect the intramolecular inhibition of the ligase or directly interfere with substrate or regulatory protein interactions. Further studies will shed light on the precise functions of PRD-mediated interactions and specificity for substrate recognition among CWHs.

Keywords: HECT-domain, regulation, Nedd4, Rsp5, Itch, Smurf, WWP1, WWP2, HCW, NedL

1. Introduction

Ubiquitin ligases are a very diversified class of enzymes that ensure substrate specificity in the ubiquitylation process. They transfer ubiquitin moieties from an E2 carrier protein to substrates either directly or by bridging the gap between the E2 and the substrate protein. In either case, they need to interact with substrate proteins for the ubiquitylation reaction to proceed. HECT (Homologous to E6-AP Carboxyl Terminus) domain ubiquitin ligases form a thioester intermediate within their catalytic domain prior to ligation of ubiquitin to the substrate [1]. Twenty-eight HECT-domain ubiquitin ligases have been identified in the human genome and are involved in many cellular processes most commonly through the targeting of their substrates to degradation pathways such as the 26S proteasome and lysosome [2, 3, 4].

E6-AP is the founding member of the HECT family. This ligase was found to bind the E6 protein of the cervical cancer-related human papillomavirus (HPV). The E6:E6-AP complex is able to ubiquitylate the p53 tumor suppressor protein targeting it for degradation by the 26S proteasome, thereby inactivating p53 and promoting cellular proliferation and transformation [5]. Only some HPV strains carry an E6 form capable of forming a stable complex with E6-AP, providing a fairly good rational for these strains propensity to induce cervical cancer [6]. EDD (E3 isolated by differential display) is another HECT ligase often overexpressed in breast and ovarian cancers [7], as is HUWE1 and most of the CWH subfamily members [5]. A direct link between HECT ligases and cancer is to be expected as many ligases are directly involved in the regulation of different signaling pathways.

Among the HECT family of ligases, CWH ligases (also referred to as the Nedd4 subfamily) form a monophyletic subgroup of proteins exhibiting a stereotyped succession of domains: a single calcium-phospholipid binding C2 domain, two to four WW protein-protein interaction domains and one HECT domain [8, 9]. Despite a fairly high degree of sequence similarity in the conserved domains, CWH ligases retain some degree of specialization.

2. CWH Family of Ligases

The first mammalian CWH ligase sequence isolated was included in a set of ten novel genes named Nedd1 to Nedd10 (neural precursor cell expressed developmentally down-regulated) discovered by differential screening of a cDNA library from mouse neural precursor cells probed with mRNA prepared from postnatal and adult brain [10]. Nedd4 is particularly expressed during neurogenesis of the mouse central nervous system, and steadily decreases during development [11]. The protein is also detected in many other embryonic tissues where it persists to adulthood [11]. Within a few years, a dozen of other Nedd4-like proteins were discovered in yeasts and mammals [12]. The human genome contains nine sequences classified in this subfamily (Fig. 1).

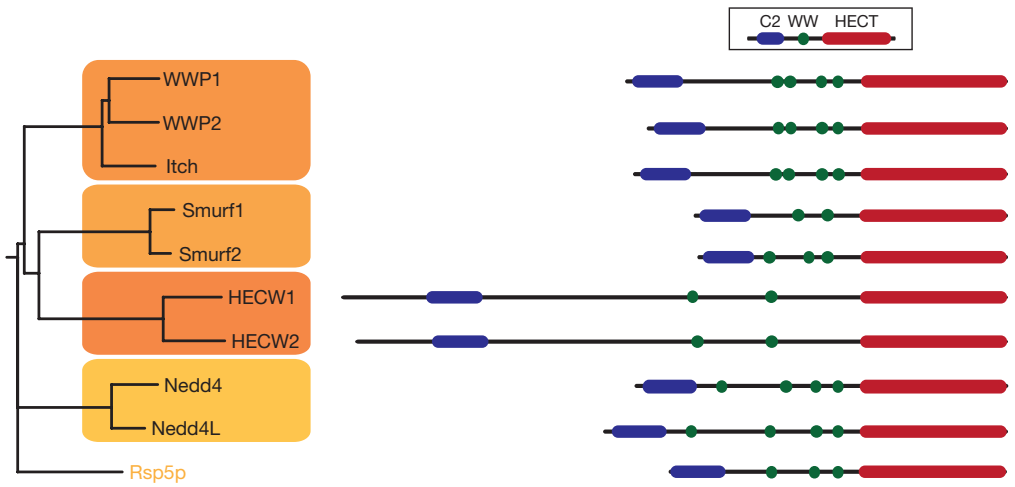

Figure 1. **Cladogram of *H. sapiens* CWH genes and related protein structure.** Rsp5 of *S. cerevisiae* was used as an outgroup to generate the tree. Orange boxes group genes that diverged from one of the four postulated ancestor genes at the emergence of metazoa as proposed by Marin [8]. The tree was based on an alignment of the coding sequences generated with clustalW2 [13] and calculated using the Phylip package [14].

2.1. CWH Ligases Structural Modules

C2 Domain

CWH ligases contain a C2 domain at their N-terminus. This domain has been shown to be required for the ligases subcellular localization [16, 17, 18]. C2 domains are phospholipid or protein interacting domains that were originally identified as the second of four conserved domains found in mammalian Ca^{2+}-dependent protein kinase C (PKC) [19]. This domain is present in many different proteins, mostly involved in signaling and membrane trafficking. Structurally, they share a common overall fold comprising eight antiparallel β-strands assembled in a β-sandwich architecture with flexible loops on the top and bottom

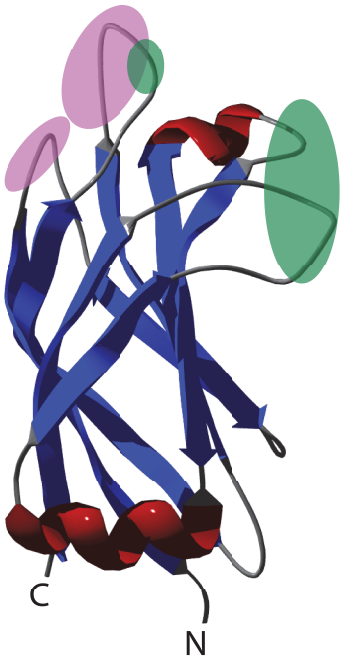

Figure 2. **Smurf2 C2 domain structure.** The β-sandwich fold is represented in blue and the α-helices in red. Because the N and C termini are located at the bottom of the β-sandwich and an α-helix is present between the β6 and β7 strands, this C2 domain is classified as a type IIB domain. The phosphoinositide binding areas are delimited by green circles, while purple circles indicate the HECT binding areas. This model is from the Brookhaven Protein Data Bank (PDB, http://www.rcsb.org) #2JQZ and rendered by the spdbv and POV-Ray software (http://www.expasy.org/spdbv/) [15].

(Fig. 2) [20, 21]. A few Ca^{2+} ions cluster inside the thin loop at one extremity, interacting with negatively charged phospholipid headgroups [21]. Most of the interaction is thus thought to be done through electrostatic forces, although some hydrophobic side chains might also contribute to the binding [21]. Phospholipid binding of many C2 domains is Ca^{2+}-dependent, but some C2 domains with little Ca^{2+} affinity have been identified and are therefore predicted to be Ca^{2+}-independent [22]. The amino acids composition of the Ca^{2+}-binding loops determines the phospholipid preference and consequently impacts on the subcellular localization of the C2 domain-containing proteins [23, 22].

The structure of the Smurf2 C2 domain was determined using nuclear magnetic resonance (NMR) (Fig. 2) [26]. It is tempting to speculate that the C2 domain might be very similar in function for all CWH ligases, but a sequence alignment of C2 domains from *H. sapiens* CWHs shows extensive variability within the family, thereby reflecting their divergent intracellular distributions (Fig. 3). As proposed by Wiesner and colleagues, it is possible that the class IIB fold resolved for Smurf2 is conserved, but this assertion needs more evidence from x-ray crystallography and NMR [26]. In addition to the role of C2

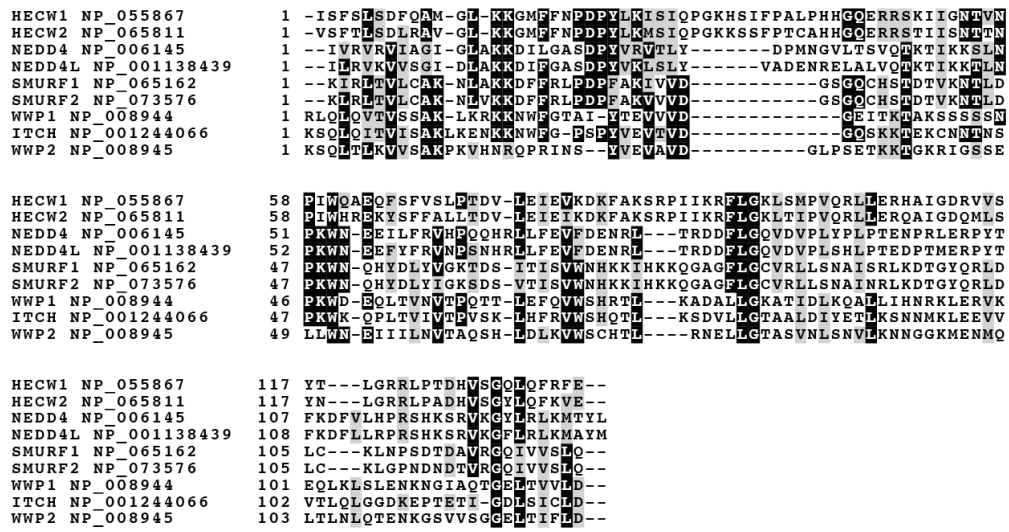

Figure 3. **Alignment of human CWHs C2 domains.** GenBank accession number for each sequence used in the alignment is indicated. Residues highlighted in black are identical, while light gray boxes indicate similar residues. In summary, the C2 domains of CWH ligases show limited similarities. The alignment was produced by the MUSCLE program [24, 25].

as a membrane anchor for CWHs, the ligase activity of some CWHs is inhibited through intramolecular interactions between the C2 and the HECT domain [27, 26]. This is not a general property, as Rsp5p and Itch have been shown not to form such interactions [26, 28].

WW Domain

WW domains are one of the smallest protein module known to fold readily in solution without the need of cofactors or disulfide bounds [29]. The domain binds to short proline-rich sequences, such as PPXY (group I), PPLP (group II), PPR (group III) and (phospho-S/T)P motifs (group IV). Some of these motifs are also recognized by SH3 domains, another small domain with a structure somewhat related to WWs [29, 30, 31, 32]. The WW module is composed of about 40 amino acids containing two highly conserved tryptophan residues (WW) and forms a compact three-stranded anti-parallel β-sheet [33]. The structure provides a shallow hydrophobic binding pocket that accommodates the proline-rich motif-containing ligands [34].

CWH ligases typically recognize their substrates through their WW domains. Although the ligases present several WW domains, usually two to four, they don't participate equally to substrate binding. For example, the second WW domain of Nedd4 is mainly responsible for the recognition of the phosphorylated Cdc25C [35]. The same WW2 domain also binds a PPXY motif found in ENaC (Epithelial Sodium Channel) [36]. Binding affinity of each of the four WW domains of human Nedd4 for ENaC shows a constant of dissociation ranging

from 32 μM to over 100 μM [36]. Point mutation of any residue within the PPXY core motif of ENaC inhibits the interaction. These mutation are responsible for a form of familial hypertension known as the Liddle syndrome, since failure to bind Nedd4 stabilizes the sodium channel at the cell surface, causing excessive sodium uptake [37, 36].

HECT Domain

The C-terminal HECT domain of the CWHs catalyzes the ubiquitylation reaction. HECT ligases differ from the more abundant RING domain ligases by directly binding the ubiquitin moiety through a thioester with a conserved cystein residue within the HECT domain, before attaching ubiquitin to the substrate. To date, 28 HECT E3 ubiquitin ligases are identified in the human genome [8]. The domain typically interacts with either UbcH5 or UbcH7 E2 proteins, but other E2s are also involved [1].

The HECT domain structure was resolved by X-ray crystallography and NMR for a few proteins. As an example, the Nedd4 HECT domain is shown in Figure 4. Typically, the domain is divided into an N- and a C-lobe, the N-lobe being further divided into a small and large subdomain (Fig. 4) [38]. E2 proteins bind to the small N-lobe subdomain [38, 9]. This region is moderately conserved, which explains the variation into E2 preferences among different CWHs [38, 9, 39]. The C-lobe contains the catalytic region and is relatively well conserved. The model indicates that in this conformation, the ubiquitin moiety is positioned too far away from the accepting cystein. This large gap must be compensated to allow ubiquitin transfer from the E2 to the E3 and the substrate. Substrate and ubiquitin binding might provide the structural changes necessary to allow the ubiquitylation reaction [38, 40]. Interestingly, the ligase activity of some CWHs is inhibited through intramolecular binding between the C2 domain and the N-lobe large subdomain [26].

The exact ubiquitylation mechanism catalyzed by the HECT domain is still a subject of hot debate, but two popular models generally prevail. In the first model, the ubiquitin chain is constructed sequentially, with multiple rounds of ubiquitin transfer from E2 to E3, then from E3 to the substrate [40]. In support of this view, recent crystallography data show that the N-lobe large subdomain contains multiple ubiquitin binding sites, that may help fixing the ubiquitin chain on a substrate protein for sequential polyubiquitylation [38]. The second proposed model suggests that the ubiquitin chain is formed on the E2 itself prior to linking it to the substrate. This view was prompted by *in vitro* assays demonstrating the capacity of some E2 proteins to form specific polyubiquitin chains in the absence of any E3 [41, 42]. In both models, binding of ubiquitin to the E3 is an essential step [41].

2.2. Evolution of CWH Ligases

The phylogeny of metazoan HECT E3 ligases reveals that CWH ligases form the only monophyletic group within the HECT family [8]. Whereas most fungi have a single CWH gene in their genome, classification of CWH sequences suggests that four ancestral genes were present when animals emerged. Substantial increase occurred in vertebrates, with most actual species counting nine CWHs. There are notable exceptions to this general pattern that occurred through gene loss (*Drosophila*, *C. elegans*) or gene duplication (teleosts, e.g. *D. rerio*)(Fig. 5). The common modular organization is the hallmark of the CWH subfamily, and was inherited from a common ancestor likely just before fungi and metazoa

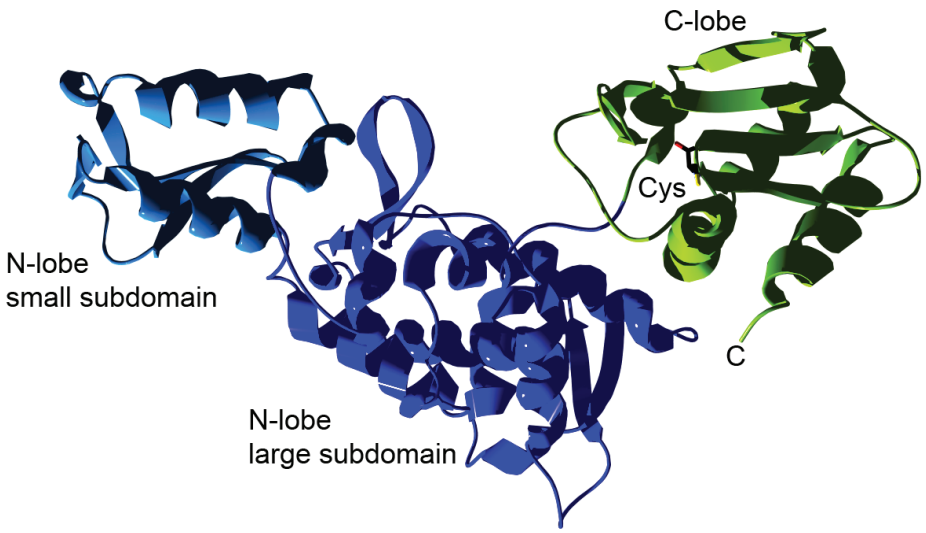

Figure 4. **Structure of Nedd4 HECT domain.** The blue section represents the N-lobe while the C-lobe is drawn in green. The lighter blue subdomain of the N-lobe is responsible for E2 protein binding while the larger dark blue section binds to ubiquitin. The catalytic cystein located in the C-lobe region is indicated. This model was rendered as in Figure 2 from PDB #2XBB data.

diverged [8]. Rsp5p, the *Saccharomyces cerevisiae* sole CWH, is most similar to human Nedd4, but has a Smurf-like C2 domain.

Within each species, CWH paralogs are moderately conserved. Simple sequence alignments of different CWHs from *H. sapiens* performed with the Needle program (EMBOSS package [43]) showed that except for the HECT domain, which is well conserved with 70-75% similarity, the paralogs have a certain degree of variability. The C2 domain is less conserved, in agreement with divergence in properties conferred by this module to different CWHs [27, 26]. To better reflect that variability, the NCBI Conserved Domains Database classifies the C2 domain of CWH ligases into four subfamilies, each corresponding to an ancestral gene as depicted in Figure 1. Hence, the C2 domain of both Smurfs are Smurf-like, whereas the domain of Nedd4 is classified as a Nedd4-Nedd4L C2 domain.

The WW domains are slightly different between paralogs and within the same ligase. In this respect, each WW domain shows specific affinity for a given substrate, and different ligases will prefer different substrates. The number of WW domains vary between paralogs (Fig. 5), but also between orthologs. For example, *M. musculus* and *D. rerio* Nedd4 have three WW domains, compared to four in *H. sapiens* [12, 9]. Interestingly, in respect to human Nedd4, the second WW domain is missing in mouse while the fourth domain is missing in zebrafish (Fig. 5) [12].

Interdomain regions are highly variable and contribute greatly to ortholog variations. However, even in these highly variable stretches, some CWH harbor conserved protein binding motifs and phosphorylation sites. For instance, Itch has a proline-rich region be-

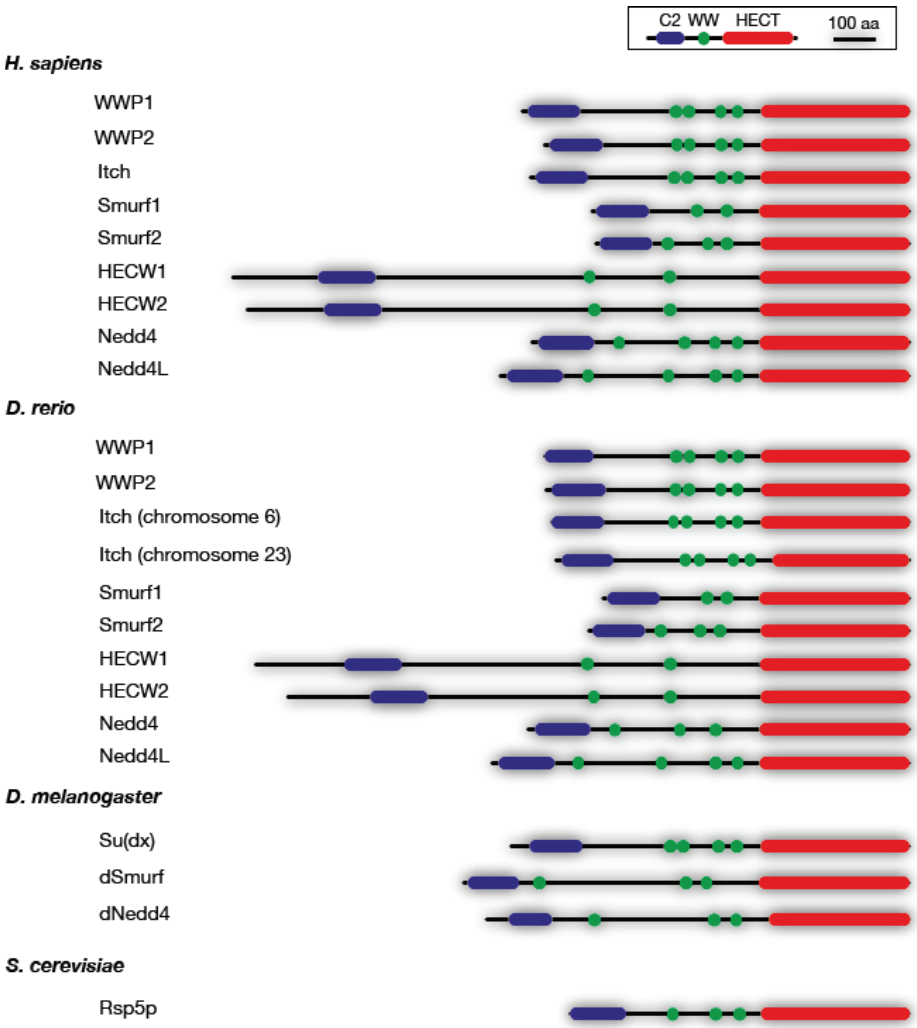

Figure 5. **CWH ligases found in some representative fungi and metazoa organisms.** The same common C2-WW-HECT structure is preserved among all groups, but the variety of ligases, the number of WW domains and the length of the proteins are different. The number of sequences has exploded in vertebrates as compared to invertebrates and fungi. Gene loss is supposed to have occurred in invertebrates, as they normally have four or less CWH ligases. Yeast's Rsp5 is similar to Nedd4, although its C2 domain is more similar to Smurf2 [8, 12].

tween the C2 and the first WW domains which is very similar for all known vertebrate sequences. Nedd4L phosphorylation sites are also conserved between species [44]. Although not categorized as domains, the interdomain regions surely contribute to the specificity of each CWH ligases.

Plants and Other Organisms

The *A. thaliana* genome contains more than 1300 genes associated with the 26S proteasome pathway [45]. Seven genes (UPL1-UPL7) were identified as HECT ligases [46]. UPL3 is involved in trichome development and UPL5 in leaf senescence, but the actual role of the other ligases remain to be determined [47, 46]. Although the 26S proteasome pathway of *A. thaliana* is one of the most elaborate, compared to vertebrates it contains nearly 4 times less HECT ligases [8].

The C2, WW and HECT domains exist in several plant proteins, but no ligase having the typical CWH structure were discovered so far. The C2 domain is found in many proteins involved in signal transduction, such as the phospholipase C in rice [48]. In *Arabidopsis*, the flowering time is controlled partly by the autoregulation of FCA pre-mRNA processing which requires the FCA WW protein interaction domain [49]. Alignment of FCA WW domain sequence with mammalian WW domain-containing proteins like FBP21 and dystrophin suggests that the FCA WW domain might mediate interaction with proline-based motifs similar to those found in their mammalian counterparts [50].

The three domains are likely to be found separately in any of the major eukaryote groups. The Uniprot protein database returns many occurrences of predicted proteins having either domains, but those entries still remain to be confirmed by experimental evidence. Nevertheless, these predictions suggest that the C2, WW and HECT domains appeared early in eukaryotes. However, no evidence suggests the existence of CWH ligases in groups other than fungi and metazoa.

The ubiquitin system only exists in eukaryote, but enzymes from some pathogen strains of bacteria are known to hijack the eukaryote's ubiquitin pathway [51, 52]. SopA of *Salmonella* and NleL of *Escherichia coli* are two HECT-like ligases recently identified to interact with human E2 proteins [53, 54, 55]. Both proteins are not related to CWH ligases however, and nothing is known about their potential influence on ubiquitylation pathways.

2.3. Biological Functions of CWH Ligases

CWH ligases affect key signaling pathways that regulate cellular growth, proliferation, differentiation and apoptosis [56]. These biological processes are at the foundation of tissue development and its uncontrolled counterpart, cancer. Because of their significant implication in development, health and homeostasis, CWH ligases are of great interest.

Both Nedd4 and Itch are implicated in T cell activation and effector differentiation, yet they regulate distinct pathways [57]. Nedd4-/- T cells showed elevated Cbl-b expression, developed normally but proliferated less, resulting in inadequate cooperation with B cells [58]. Itch-/- mice have a strong inflammatory condition phenotype concomitant with a constant itching of the skin [59]. Specifically, Itch negatively regulates the NF- κB pathway and the JunB activity of T cells [57, 60].

The downregulation of the ENaC sodium channel by Nedd4 and Nedd4L is the best described physiological pathway implicating CWHs. Regulating electrolytes is an important aspect of homeostasis. The ENaC channel is located primarily at the apical surface of epithelial cells in the distal nephron and permits sodium uptake. The ENaC of patients with Liddle syndrome don't possess a key region of the channel mediating binding with Nedd4 and Nedd4L, that impairs proper channel downregulation [61]. The ENaC down-

regulation prevents unnecessary sodium absorption and leads to abnormal kidney function and hypertension.

In metazoa, the transforming growth factor beta (TGFβ) superfamily of peptides regulates essential processes in embryogenesis and development. The TGFβ pathway controls cell growth, proliferation, transformation, apoptosis and matrix reorganization [62]. Smads are signal transducers of the TGFβ receptor that are ubiquitylated by the CWH ligases Smurf1 and Smurf2 [12]. Both Smurfs also downregulate the TGFβ receptor through ubiquitylation [12]. Knockdown and overexpression of both Smurfs have profound effects on ectoderm and mesoderm induction and patterning during early frog embryogenesis [63]. Both ligases, in a distinct manner, participate to normal development of *Xenopus* embryos.

Given their profound effects on such fundamental processes, there is no doubt that CWH ligases activity must be finely regulated. This review mainly focuses on CWHs regulatory mechanisms as they are still being rapidly unveiled.

3. Regulation of CWH Ligase Activity

CWHs regulation is highly complex and occurs at transcriptional, posttranscription and posttranslational levels (Fig. 6). Fine-tuning the activity of these ligases is vital for many key processes. Posttranscriptional regulation of CWH ligases involves complex networks of cofactors and activators intimately embedded in the processes the ligases act onto.

3.1. Regulation of CWH Expression

CWH Isoforms

Several CWH ligases are represented by different isoforms produced by alternate promoters or alternative splicing. Although the precise function and expression pattern of these variants remain poorly characterized, a growing body of evidence suggests that isoform diversity has major cellular impacts. Specific isoforms have been shown to have distinct functions and exhibit different regulation mechanisms. Table 1 lists all CWH ligases present in the genome of *H. sapiens* and the number of known and characterized mRNA from isoforms for both *H. sapiens* and *M. musculus* retrieved from GenBank. However, as revealed by some studies described below, it is likely that these numbers are underestimated. We will generally refer to CWHs by the name of the gene indicated in the first column of Table 1.

Among CWHs, Nedd4 products are the most diverse, with two paralogs, Nedd4 and Nedd4L each encoding several transcripts. In humans, Nedd4 is located on chromosome 15q22 [67] while Nedd4L is located on 18q21 and is homologous to mouse Nedd4-2 [68]. Both Nedd4 and Nedd4L bind to ENaC, but with different affinity [69].

An extraordinary number of isoforms are described for Nedd4L in rat, produced by alternate promoter, poly-adenylation sites, internal exon splicing and alternative translation initiation sites [70]. Many Nedd4L transcripts have also been identified in human [71, 72]. Three of these are abundantly expressed: the full-length transcript (FL), a transcript lacking the C2 domain (Nedd4L-ΔC2) and one lacking the C2, WW2 and WW3 domains (Nedd4L-ΔWW2,3). Interestingly, deletion of the N-terminus in Nedd4L-WW2,3 abolishes two out of three Sgk phosphorylation sites important for the regulation of Nedd4L catalytic

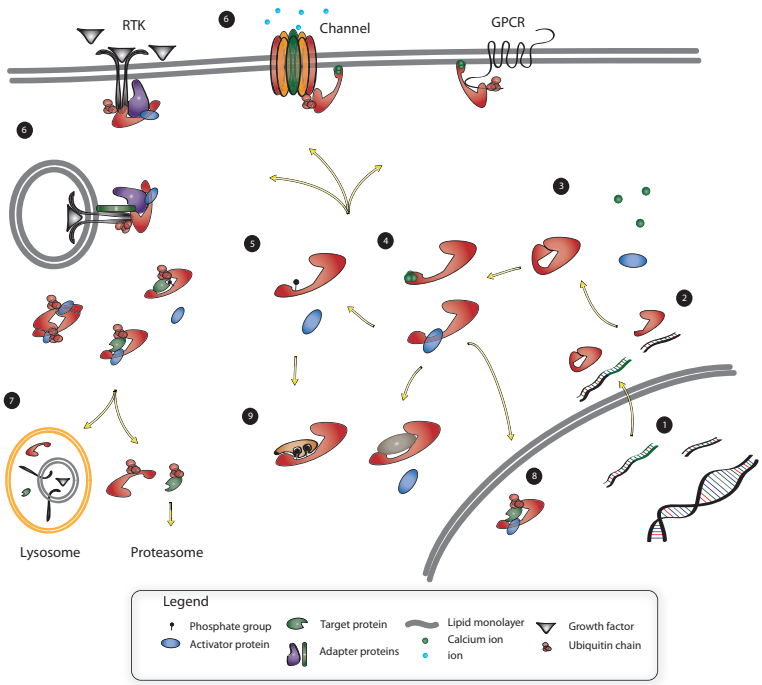

Figure 6. **Summary of regulation mechanisms of CWH ligases.** The regulation network involved is diversified and complex. (1) and (2) Most CWHs are transcribed into multiple isoforms either by alternative promoters or alternative splicing, resulting in different end-products that display specific features. The roles played by most isoforms are still largely unknown. (3) and (4) CWHs fold into an inactive conformation, requiring external activators like calcium or other binding proteins to adopt an active conformation. Calcium clusters inside the C2 domain, activating or enhancing membrane binding properties of this domain. Activator proteins typically interact with the multiple WW domains of CWHs. (5) Phosphorylation of different residues and by different kinases also results in conformation change and activation of the ligases sometimes independently of activators. (6) Once activated, CWHs target many membrane and soluble proteins. The C2 domain, phosphorylation and activator proteins collaboratively participate in the localization of CWHs in specific compartments. The local context greatly influences the binding properties and biological functions of CWHs. CWHs can autoubiquitylate, an efficient negative feedback mechanism to stop their own activity. (7) CWH substrates are usually targeted for degradation either through lysosomal or proteasomal pathways. (8) Some activated CWHs translocate to the nucleus, targeting transcription factors and other nuclear proteins for degradation. (9) Additional phosphorylation and/or binding of inhibitory proteins deactivate CWHs.

activity [73]. Such features are observed in *Drosophila* Nedd4 paralog where alternative splicing eliminates phosphorylation sites, thus preventing Akt-mediated phosphorylation in transfected drosophila cells [74].

Both Nedd4L-FL and C2 are able to regulate ENaC activity in Fisher rat thyroid ep-

Table 1. Known CWH genes for *Homo sapiens*. The data presented in this table are based on information provided by the NCBI gene database. The number of isoforms might be underestimated. For example, experimental evidence shows a higher number of isoforms for WWP1 [64, 65] and WWP2 [66] for both mammals

Gene name	Other common names	Known isoforms for *H. sapiens* (*M. musculus*)
Nedd4	Nedd4-1	2 (1)
NEDD4L	Nedd4-2	7 (2)
WWP1	AIP5	1 (1)
WWP2	AIP2	4 (1)
ITCH	AIP4	3 (2)
SMURF1		3 (2)
SMURF2		1 (1)
HECW1	NEDL1	1 (1)
HECW2	NEDL2	1 (2)

ithelia, whereas Nedd4L-WW2,3 had little effects on sodium currents [71]. These variants also regulate sodium channels in *Xenopus* oocytes and in mouse cortical collecting duct M-1 derived cells. Nedd4L-FL is relocated at the plasma membrane upon calcium entry, but not Nedd4L-ΔC2 since the C2 domain is necessary for calcium response. This suggests that relocalization is not necessary for Nedd4L function towards ENaC [73]. On the other hand, co-immunoprecipitation assays in HEK-293 showed that all three isoforms induced comparable levels of ENaC multiubiquitylation and degradation [75].

Several WWP1 isoforms are present in human and mouse. Northern blot analysis on human tissues show two isoforms expressed mainly in muscle and heart [64, 65] while four isoforms are seen in adult mouse tissues, differently expressed in testis, kidney, liver and heart [76]. Six splice variants differently truncated in the N-terminal region, with some deletions overlapping the C2 domain, were also identified by RT-PCR in human T47D breast cancer cell line [77]. The C2 domain being important to establish intramolecular inhibition in some CWHs, these data raise the possibility that WWP1 splice variants may be differentially regulated. These isoforms also show different tissue distribution [77].

Northern blot analysis on mouse tissues initially identified two WWP2 transcripts [66]. In addition to the full-length (FL) form, two truncated isoforms are found in the Mammalian Gene Collection (MGC) database [78]. WWP2-N is a splice variant truncated after the first WW domain and WWP2-C arises from an alternate promoter within intron 10-11 and comprises the C-terminal portion with WW4 and the HECT domain. These three isoforms have interdependent biological functions as WWP2-N is able to bind and activate WWP2-FL in a TGFβ-dependent manner, increasing its capacity to ubiquitylate Smad2/3 [78].

A splice variant is predicted for Itch although protein expression still needs to be investigated. In addition to the characterized isoform (NP_113671.3), another predicted sequence encodes a protein with an additional 41 amino acids stretch located between the C2 and the first WW domain (NP_001244066.1). Since this region is also known for controlling

the ligase activity, this additional stretch could impact the ligase regulation. This issue has not been investigated. Despite this lack of information on potential Itch isoforms, transcriptional regulation of Itch is known to be achieved through miRNA. Upregulation of miRNA106b has been found to decrease Itch expression in primary leukemia cells treated with deacetylase inhibitors. miRNA-dependent reduction of Itch is further known to impact Itch-induced p73 degradation. These findings indicate that control of Itch expression is an important aspect in the regulation of its activity, and of some substrates [79].

Two isoforms of Smurf1 are described, differing by an insertion of 26 residues between WW2 and WW3 domains. These two WW domains stably couple to jointly bind Smad7 in the shorter isoform. In contrast, this internal coupling is impaired by the sequence insertion in the longer isoform, resulting in reduced affinity for Smad7 and possibly other targets [80].

A splice isoform of Smurf2 deleting 13 residues from the C2 domain has been identified in early passage fibroblasts [81]. This truncation is though to have a major impact on the C2 domain structure, as supported by the accumulation of the truncated isoform in internal organelles, whereas the full-length variant is diffused throughout the cytoplasm in Cos-7 cells [82]. Smurf2 isoforms have antagonistic effects on TGF-β signaling. Full-length Smurf2 is known to downregulate TGF-β. Interestingly, expression of the Smurf2-E2 isoform induced the degradation of the full-length isoform, thereby stabilizing TGF-β signaling, reducing proliferation and stabilizing the production of pro-inflammatory cytokines in T cells [82].

Multiple CWHs isoforms are produced either by alternate promoters or by alternative splicing. Some of these variants display differential localization, affinity for substrates or capacity of regulation. Collectively, these data suggest that production of a variety of isoforms might affect numerous CWH cellular functions.

Developmental Regulation of CWH

CWH ligases are involved in different developmental mechanisms and are therefore also developmentally regulated. Evidence from multiple studies show that misregulation of these ligases leads to several embryonic defects. Thus, shedding light on the cellular processes regulating the CWH activity during development will certainly help to better understand the role played by these ligases in the mechanisms underlying tissue formation itself.

Nedd4 was first identified as a developmentally regulated gene in mouse. Northern analyses show that Nedd4 expression in the central nervous system is highest during neurogenesis and decreases as development progresses. In other tissues, Nedd4 is detected throughout embryogenesis and persists in most adult tissues [11]. Immunohistochemistry further confirms that Nedd4 protein is strongly expressed in many mouse embryonic tissues at various stages [83].

Possibly through regulation of TGFβ signaling, Smurf1 is involved in early embryogenesis as showed by its dorsal localization at gastrula and neural stages in *Xenopus* embryos. It is possibly involved in mesodermal development [84]. Smurf1 and Smurf2 are also differentially expressed in testis during development. Different Smurf1/2 isoforms are detected at various time and cell types during testis development and spermatogenesis [85]. Smurf1 is implicated in lung development and its expression in lung raises during embryogenesis

and then decreases when adulthood is reached. Indeed, overexpression of Smurf1 inhibits branching morphogenesis in cultured mouse lung explants, and this phenotype might be due to a reduction in Smad1 and 5 expression following Smurf1-induced ubiquitylation [86].

Smurf2 expression is initially diffused in blastula and early gastrula *Xenopus* embryo, but is thereafter localized predominantly in dorsal ectoderm and mesoderm. The expression is sustained in neural tissue throughout embryogenesis, suggesting that Smurf2 might be involved in regulation of neural crest and mesendodermal development. Overexpression and downregulation assays further confirm this hypothesis [63]. High levels of Smurf2 mRNA are also observed in chicken embryos during growth of the tibial plate and sterna. High levels of Smurf2 are associated with chondrocytes in proliferative and differentiation stages and decrease as these cells mature [87]. In human fibroblasts, Smurf2 expression is increased during senescence, leading to specific changes in gene expression by recruiting Rb and p53 [81].

WWP1 expression increases during osteoblast differentiation, which correlates with downregulation of Runx2, a known substrate of WWP1 and an important factor in osteoblast differentiation. WWP1 might also regulate Runx2 at other developmental stages [88].

WWP2 might be developmentally regulated in human. Northern blot analysis on various tissues demonstrated high WWP2 levels in undifferentiated ES cells. The expression drastically decreases after ES cells differentiation. These findings suggest developmental regulation of WWP2 abundance [89]. WWP2 transcript level can also be modulated by cell cycle progression. A large scale genome microarray of human fibroblast identified high expression of several ubiquitin-associated transcripts. WWP2 was specifically upregulated during the G1-S transition, indicating a participation of the ligase in cell cycle progression [90].

Most CWHs are thus regulated during development and cell cycle progression. Future work will certainly identify specific regulation mechanisms for CWH in various tissues. This will give a better understanding of the ligases' function in development as well as define a general temporal regulation of CWH ligases.

Transcriptional Regulation by Extracellular Cues

Extracellular cues can trigger numerous cellular responses. Emerging evidence links CWH ligases to signal transduction through degradation of key effectors. Ligase expression should therefore be tightly regulated in order to accomplish such functions. Recent data have indicated transcriptional regulation of several CWH by extracellular cues. This can allow dynamic regulation of CWH through signal integration.

Tumor necrosis factor alpha (TNFα) can regulate the transcription of some CWH ligases. Chronic inflammatory disorders are often associated with elevated TNFα levels and systemic bone loss. Inflammation is thought to cause osteoclast resorption and inhibition of osteoblast function. Expression of Smurf1 mRNA is increased in the metaphyseal region of TNF-transgenic mice overproducing the factor. Smurf1 transcript was also upregulated in calvarial bones injected with TNFα [91]. Further studies demonstrated that chronic exposure of bone marrow cells to TNFα increases Smurf1, but not Smurf2 mRNA. Smurf1 upregulation was also correlated with BMP signaling molecules degradation, notably Smad1/5

and Runx2. These proteins play key roles in bone formation and Smurf1 thus likely regulate this function by inducing their proteasomal degradation [92]. WWP1 can also affect osteoblast differentiation. WWP1 mRNA levels are gradually elevated in bone marrow stromal cells (BMSC) cultured in differentiation medium. WWP1 expression is also induced in TNFα-transgenic mice, unlike other CWH members. WWP1 then ubiquitylates JunB, causing the degradation of this key transcription factor in osteoblast differentiation [93].

TGFβ is another factor with the capacity to increase expression of CWH ligases. RT-PCR on TGFβ responsive HepG2 cells demonstrated an increased Smurf2 expression upon TGFβ treatment. Luciferase reporter assays using the regulatory region of the Smurf2 gene confirmed a dose-dependent transcription in response to TGFβ. In these assays, the PI3K inhibitor LY294002 or kinase negative Akt were able to abolish the TGF β induction of Smurf2 [94]. TGFβ was further demonstrated to influence transcription of WWP1. WWP1 mRNA increases over time and in a dose-dependent manner with TGFβ treatment. These data confirm transcriptional regulation of both Smurf2 and WWP1 by TGF β.

A growing body of evidence suggests that CWH transcription might be linked to other extracellular cues. Zinc has been demonstrated to increase Nedd4 mRNA levels in rat neurons. Upregulation of Nedd4 was associated with PTEN ubiquitylation and subsequent degradation, thereby suggesting that Nedd4 regulates neuronal zinc response via PTEN and regulation of the PI3K pathway [95].

Synthetic androgen can induce transcription of some variants of Nedd4L. Three transcripts have been shown to be upregulated by androgen in LNCaP prostate cancer cells, suggesting that transcriptional regulation of CWH might also be isoform specific [96].

Retinoic acid (RA) regulates the enteric nervous system (ENS) formation. Retinoids have been shown to regulate Smurf1 transcripts. RT-PCR on mouse ENS cells demonstrated a dramatic decrease in Smurf1 levels correlated with RA treatment. Reduction in Smurf1 expression mainly occurred in the cell body and not in neurites shaft or tips. Tight regulation of Smurf1 transcription thus ties the ligase to RA-induced neuronal functions [97].

Sustained calcium signaling can trigger T cell anergy through calcineurin and NFAT transcription factor dependent mechanisms. RT-PCR on T cells demonstrated that Itch mRNA levels are increased when cells undergo ionomycin-induced anergy. Thus, sustained calcium signaling regulates Itch levels and influence T cell anergy [98].

CWH transcription is also regulated in specific cellular contexts. DNA damage regulates WWP1 expression, although there are conflicting reports in regards to the specific mechanism. In different cell types and DNA damage-inducing treatments, WWP1 levels were negatively or positively correlated with p53 expression [99, 88]. Itch is another CWH involved in DNA damage response. Using the luciferase reporter gene under the control of the Itch promoter, Runx was shown to upregulate Itch expression. Runx thus upregulates Itch mRNA and further promotes p73 degradation. Runx effects were abrogated by treatment with cisplatin, that induces Yap1 phosphorylation, disrupting its interaction with the Itch promoter and co-activation of Itch transcription [100]. DNA damage thus seem to differentially regulate CWHs in different cellular contexts.

Together, these data clearly demonstrate the significant role of CWH transcriptional regulation. In addition to posttranslational CWH regulation, their transcriptional regulation offers an alternative mean for fine-tuning CWH functions. Such process can correlate CWHs activity to different cellular contexts by integrating various molecular cues.

CWHs Have a Taste of Their Own Medicine

All CWH ligases exhibit autocatalytic activity. This self targeted ubiquitylation can be achieved by inter or intramolecular interactions and seems to be subjected to the same degree of regulation as substrate ubiquitylation. In turn, this autocatalytic activity can regulate the ligases themselves, much in the same way as they regulate their substrates.

Itch autoubiquitylation is enhanced following JNK-induced phosphorylation on three residues located near the proline-rich domain (PRD) [60, 101]. This reaction has been shown to occur in trans, as ligase inactive mutants are readily ubiquitylated *in vitro* [102, 103]. In HEK-293T cells, Itch self-induced ubiquitylation leads to its proteasomal degradation, a process that is prevented by the deubiquitylating enzyme USP9X [103]. However, similar experiments conducted in Itch-/- mouse embryonic fibroblasts failed to detect any ubiquitylation-induced degradation of Itch, raising the possibility that different ligases, in different cell types, might target the ligase for degradation [102].

Some WW binding proteins can promote CWH ligases autoubiquitylation. NDFIP2 was shown to regulate self-ubiquitylation of Itch, WWP1, WWP2, Nedd4, Nedd4L and Smurf2 [28]. Activating this autoubiquitylating activity could be an efficient way to control the global activity of CWHs.

Some CWHs also regulate one another. Smurf1 and Smurf2 have been shown to interact in co-immunoprecipitation assays. Smurf2 is able to regulate Smurf1 expression, as increasing levels of Smurf2 induce catalytically inactive Smurf1 degradation. Conversely, Smurf1 has no effect on Smurf2 levels [104].

The evidence presented so far demonstrate that the CWH ligases exhibit a simple yet efficient negative feedback mechanism that might be crucial to stop ongoing signaling cascades triggered in some processes. Thus, the autocatalytic activity simultaneously modulates the ligases stability and function.

3.2. Autoinhibition

The general model depicts the CWH ligases as regulated by autoinhibition. They adopt an inactive conformation that can be released by different mechanisms including phosphorylation or binding to coactivators.

The ligase Itch was first found to be activated by a phosphorylation-induced conformational change [60, 101]. This inhibition is driven by the interaction of the ligase HECT domain to a region located within the PRD/WW region. The interaction between the HECT domain and the PRD/WW region is not established through dimerization, as pull-down experiments between Itch lacking the C2 domain and full length Itch showed no intermolecular binding. Furthermore, gel filtration experiments showed no evidence of Itch oligomers [101]. Itch is thus constitutively self-inhibited. The release of its inactive conformation can increase its catalytic activity and enhance its autoubiquitylation as well as ubiquitylation of target proteins [105, 28]. The affinity of the PRD/WW region for the HECT domain is relatively weak, with a Kd of 2 μM measured in ELISA experiments. The autoinhibition could thus be released by the binding of coactivators with relatively stronger affinity, as with spartin that shows a Kd of 0,34 μM [105].

Other CWH ligases are also self-inhibited in such a way, although the precise mechanism slightly differs. Nedd4, WWP2 and Smurf2 show a similar intramolecular interaction,

but the autoinhibition involves binding of the C2 domain to the catalytic HECT domain [26]. Activation of these ligases also causes their auto- and substrate ubiquitylation [26, 28]. It is interesting to note that the autoinhibition of Nedd4L interferes with self-ubiquitylation, but not with ENaC substrate ubiquitylation or degradation [106].

Dimerization has been involved in the inhibition of some CWH ligases. Gel filtration using HCT116 cell extracts revealed Smurf1 oligomers, although the affinity of the HECT domain to either the full length or the C2 domain is quite low in pull-down assays [107]. This could explain the conflicted results showing no evidence of increased self-ubiquitylation when truncating the C2 domain [108]. It is also possible that dimerization affect substrate ubiquitylation but not autocatalytic activity.

Recent evidence also suggests that dimerization plays a role in WWP2 regulation. In this case, alternatively spliced isoforms containing only the N-terminal half of the ligase binds to the full-length form and releases autoinhibition. Interestingly, this activation of WWP2 has more effects on Smad2/3, while ubiquitylation of Smad7 by WWP2 is not activated by WWP2-N binding [78]. Increasing amounts of WWP2 also decrease self-ubiquitylation and ubiquitylation of the transcription factor Oct4 during the differentiation of embryonic carcinoma cells (ECCs), whereas it has no effect in undifferentiated ECCs. It has been proposed that the formation of inhibitory homodimers was responsible for the loss of activity upon increasing the amount of WWP2 ligase [109].

Clearly, interaction of the HECT domain of CWH ligases with sequences located in their N-terminal half seem to generally decrease the ligase activity of most CWH enzymes. Regulation of the inactive conformation is thus a powerful mechanism to ensure regulation of the CWHs activity and substrate specificity.

3.3. Releasing the Inhibition

Calcium

The intramolecular binding of the C2 to the HECT domain of Nedd4 and Nedd4L controls its catalytic activity. Truncated Nedd4 isoforms lacking the C2 domain show increased ubiquitylation in HEK-293 cells, reflecting higher enzymatic activity. Addition of calcium reduces the binding of C2 to the HECT domain, and is sufficient to increase Nedd4 and Nedd4L autoubiquitylation as well as ENaC ubiquitylation. Thus, calcium can regulate Nedd4 and Nedd4L activity by disrupting the C2-mediated autoinhibition. Such calcium-dependent regulatory mechanism has not yet been shown for any other CWH ligase, despite the implication of C2 internal binding in the inhibition of other CWHs [27].

Phosphorylation

JNK phosphorylation occurs on three residues located within Itch PRD. It enhances Itch self-ubiquitylation by releasing its autoinhibitory state, as the C2 domain is not involved in this process [26, 28]. JNK-phosphorylation leads to increased Itch autoubiquitylation as well as ubiquitylation of its substrates c-Jun and JunB. Conversely, inhibition of the JNK pathway decreases c-Jun and JunB turnover [60, 101]. JunB is a transcription factor involved in IL-4 and 6 cytokine production during T cell differentiation [110]. Itch is also known to bind to MEKK1 in the same pathway. Overexpression of inactive MEKK1 or

depletion of Itch in T cells induces enhanced JunB expression and production of IL-4 and IL-6. MEKK1 is thus proposed to act as a scaffold and recruit JNK and Itch in order to promote Itch ligase activity, JunB degradation and impair T cell differentiation [111].

JNK is a stress related kinase activated in many cellular responses. In hepatocytes, JNK activation induces apoptosis by activating Itch. Itch recognizes the antiapoptotic protein c-FLIP, and accelerates its turnover in response to TNFα. C-FLIP degradation is abolished in Itch-deficient cultured fibroblasts as well as in JNK -/- cells that no longer respond to TNF α treatment. JNK phosphorylation of Itch thus regulates its ability to interact with c-FLIP and induce apoptosis [112]. JNK is also an important intermediate in Diallyl trisulfide (DATS) induced cell death. In prostate cancer cells, treatment with DATS leads to reactive oxygen species (ROS) increase and apoptosis. Ferritin degradation is essential for ROS formation, a process known to be dependent on Itch-induced ubiquitylation. DATS treatment activates JNK that phosphorylates Itch and increases ferritin degradation, linking Itch phosphorylation to a second apoptotic pathway [113]. JNK also promotes antiapoptotic pathways, at least one of which involves activation of Itch. Epithelial growth factor (EGF) treatment can activate JNK and Itch, and this effect has been shown to induce the proteasomal degradation of the truncated C-terminal portion of Bid (tBid). Bid is cleaved by TNF α-induced caspase 8 to form tBid. This protein then facilitates Bax and Bak oligomerization, and cytochrome c liberation. Itch binds to and ubiquitylates tBid, but not the full-length Bid. Itch overexpression increases cell survival and decreases tBid level and caspase-3 activation whereas siRNA depletion of Itch leads to the opposite effect [114].

Tyrosine phosphorylation has also been shown to exert a regulatory role in Itch ligase activity. Mass spectrometry coupled with mutagenesis confirmed that Itch is phosphorylated by Fyn on tyrosine 371 located in its third WW domain. Loss of Fyn or Itch-Y371 mutation leads to elevated JunB ubiquitylation, pinpointing the negative regulation exerted by Fyn-mediated phosphorylation of Itch. Interestingly, phosphorylation of Itch Y371 only affects JunB ubiquitylation, as no effect was seen on autoubiquitylation or other substrates [115]. Itch downregulation by Fyn phosphorylation is counteracted by the phosphatase SHP-2 that is known to be induced by CTLA-4 treatment. Interestingly, CTLA-4 deficient mice are associated with a drastic reduction of Itch expression and a phenotype similar to Itchy mice. These results suggest that CTLA-4 could regulate Itch stability and activity although the precise mechanism remains unknown [116].

CISK is a kinase demonstrated to interact with the WW domains of Itch. Phosphorylation induced by CISK occurs mainly on a threonine located in the second WW domain of Itch, although minor phosphorylation on a serine within the third WW domain can also be detected. Since CISK activation inhibits degradation of the CXCR4 receptor, CISK could potentially regulate Itch-induced ubiquitylation and sorting of CXCR4 [117, 118].

Phosphorylation of Nedd4L has been extensively studied. Nedd4L can regulate multiple signals through its impact on channel expression. Regulating the ligase is thus very important to adapt its activity in a variety of cellular contexts.

Nedd4L has two Sgk1 consensus phosphorylation sites conserved in vertebrates sequences. These motifs are flanking the second WW domain. Phosphorylated Nedd4L is unable to bind to ENaC and does not affect its cell surface expression, resulting in increased sodium entry [44]. Aldosterone treatment activates Sgk1 and reduces the interaction between Nedd4L and ENaC. Phosphorylation of Nedd4L promotes binding of 14-3-3

in a region overlapping ENaC binding sites, thus preventing interaction with ENaC [119]. Sgk1 phosphorylation of Nedd4L also negatively regulates the ligase activity towards the NaCl cotransporter NCC and the TGFβ activated Smad3 [120, 121]. Sgk1 phosphorylation affects Nedd4L stability itself, as mutation of the main Sgk1 phosphorylation sites decreases Nedd4L level. This effect is independent of 14-3-3, as inhibition of 14-3-3 binding does not affect Nedd4L turnover. Nedd4L phosphorylation could thus differentially regulate substrate and self-ubiquitylation [122].

Nedd4L has been found to be the target of several other kinases. PKA phosphorylates Nedd4L on the same residues as Sgk1 and was further demonstrated to be involved in ENaC regulation [123]. AMPK phosphorylates Nedd4L and influences ENaC surface expression, although in this case phosphorylation enhanced the ligase and channel interaction [124]. JNK1 phosphorylation sites were identified in Nedd4L by tandem mass spectrometry. Mutation of one JNK phosphorylation site in the HECT domain of Nedd4L drastically decreases autoubiquitylation and ENaC ubiquitylation [125]. GRK2 can phosphorylate the ligase within its WW domains. In vitro phosphorylation of recombinant WW3 domain was demonstrated to reduce its interaction with ENaC [126]. Interestingly, the mapped phosphorylation residue within WW3 is adjacent to a key residue involved in binding to PY-containing proteins [127]. Nedd4L is therefore an important signaling hub integrating multiple phosphorylation events towards channel regulation.

The Smurf1 ligase is phosphorylated by PKA on a threonine residue located within its second WW domain. This modification switches the ligase preference between two proteins with opposite roles in axon development, Par6 and RhoA. The phosphorylated ligase preferably binds to RhoA, reducing its level and promoting differentiation and axon growth. Mutant with inactivated phosphorylation sites are associated with increased Par6 ubiquitylation and stabilization of the growth inhibiting RhoA proteins. Axon development is thus influenced by Smurf1 phosphorylation state [128].

Phosphorylation is thus an important mechanism controlling the activity and substrate binding properties of many CWH ligases.

Activators and Repressors

Binding of repressors or activators to CWH can affect substrate binding or enhance ligase activity through the release of the autoinhibitory state. A variety of these modulators have been identified. Some are specific for a given CWH or bind a wider variety of ligases.

Ndfips are well known for their capacity to regulate ligase activity. Ndfip2 is longer than Ndfip1, with whom it shares 55% identity. Each protein contains 3 PY motifs responsible for their interaction with WW domain-containing proteins like CWHs [129]. Both Ndfip1 and 2 have been shown to increase CWHs autoubiquitylation by releasing their intramolecular interactions [28].

The various roles of Ndfip proteins reflect their regulatory functions towards CWHs. It is interesting to note that the phenotype of Ndfip1 deficient mice is similar to Itchy mice. Ndfip was shown to regulate Itch-induced JunB degradation and T cell activation [130]. Ndfips are also related to DMT1 transporter and iron uptake. Fluorescence quenching assays in CHO cells stably expressing myc-DMT1 demonstrated that WWP2 transfection significantly reduces transporter activity. Similar experiments further demonstrated that this

reduction relies on Ndfip proteins and their PY motifs [131]. Ndfip2 also regulate Nedd4-induced degradation of connexin-43. Intriguingly, Ndfip2 might act differently toward this membrane protein compared to previous findings [28]. It seems that Ndfip2 depletion by siRNA reduced connexin-43 in HeLa cells instead of increasing its abundance by inhibiting Nedd4 activation. Since Ndfip2 localizes to the lysosomes, it might differentially stabilize some membrane proteins by recruiting and sequestering Nedd4 to intracellular compartments [132]. This hypothesis is supported by results indicating that Ndfip2 also stabilizes ENaC [133]. Ndfip proteins are thus important modulators of CWHs activity. These proteins can regulate self-induced ubiquitylation as well as differentially regulate substrates ubiquitylation.

Spartin is a protein involved in hereditary spastic paraplegia (HSP), a neurodegenerative disease causing lower limb weakness. Spartin is absent in several cell lines from patients with Troyer syndrome, a form of HSP. A nucleotide deletion is believed to cause a premature termination of the protein [134, 135]. Spartin can regulate lipid droplet (LD) turnover. These dynamic compartments are involved in lipid storage. Spartin depletion by shRNA increases LD size and number [136]. In HeLa cells, endogenous spartin can be recruited to endosomes following EGF treatment. Stimulation of LD formation through an oleic acid treatment also causes spartin recruitment to these organelles. Immunoprecipitation assays demonstrated that Spartin interacts with Itch and WWP1 via a PY motif located in its C-terminal portion. Spartin is monoubiquitylated on a few sites independently of the interacting ligases [137]. Spartin is not a classic substrate and instead recruits and activates Itch at LD, increasing Itch autocatalytic activity as well as adipophilin ubiquitylation. Itch intramolecular interaction is disrupted by spartin. Translocation and activation of the ligase can regulate LD-associated proteins like adipophilin [105].

Numb is a critical protein for cell fate determination events during neural development. A portion of Numb phosphotyrosine-binding (PTB) domain interacts specifically with Itch WW2 domain. Numb is a potent activator of the ligase through disruption of the intramolecular inhibition. Numb inhibits the interaction between immunoprecipitated Itch incubated with recombinant GST-HECT. Coexpression of increasing amounts of Numb in HEK-293 cells increased Itch self-ubiquitylation. Activation of the ligase also impacts Gli1. This transcription factor is an important regulator of proliferation and development. Itch mediates Gli1 ubiquitylation and degradation. Numb was shown to facilitate Gli1 recruitment together with Itch activation. Numb is therefore an important regulator of Itch functions in hedgehog signaling through regulation of Gli1 [138].

CKIP is a widely expressed protein notably involved in apoptosis [139]. CKIP interacts with Smurf1 and regulate its activity. Coexpression of CKIP with Smurf1 in HEK-293 cells increased Smurf1 autoubiquitylation and proteasomal degradation. CKIP targets specifically the linker region between the WW domains of Smurf1 but not Smurf2. CKIP binding can increase Smurf1 interaction with Smad1/5, probably by driving WW domains in a favourable binding conformation for substrates [140]. Thus, CKIP can modulate Smurf1 activity. Another protein involved in Smurf1 regulation is Cdh1. Expression of these proteins is regulated throughout the cell cycle. Smurf1 inversely correlates with Cdh1, suggesting its role in the ligase regulation. Indeed, Cdh1 stimulates Smurf1 autocatalytic activity *in vitro*. CKIP and Cdh1 bind to the C2 domain of Smurf1, interfering with its dimerization [107].

Like many coactivators, several enveloped viruses use PPXY motifs to recruit ubiquitin ligases involved in viral particle release. It is interesting to note that the PPXY-containing segment of spartin can be substituted with the PPXY segment of the murine leukemia virus (MLV) Gag protein and still effectively release virions [136]. Like Ndfips, UL56 of the herpes simplex virus type 2 (MLV-2) contains three PPXY motifs. UL56 and Ndfip colocalize when overexpressed in HEp-2 cells [141]. UL56 activates Nedd4, increasing its ubiquitylation and degradation in Vero cells [142]. UL56 also interacts with Itch and affects its subcellular localization, an effect unseen with Nedd4. In several cell lines, Itch expression drastically diminishes following infection. UL56 is thus an important regulator for both Nedd4 and Itch [141].

Epstein-Barr virus (EBV) also expresses a protein involved in ligase regulation. Latent membrane protein 2A (LMP2A) possesses two PPXY motifs and is produced upon infection. LMP2A interacts with Nedd4, Itch and WWP2. Expression LMP2A significantly reduces Itch and WWP2, suggesting that it regulates their stability. LMP2A is also associated with decreased Lyn [143]. Viral proteins thus interact with CWH and regulate their activity, thereby exploiting the ubiquitylation machinery to its own benefit.

CWHs activity can be negatively modulated by their interaction with repressors. Among the proteins identified, 14-3-3 is well known for its impact on ENaC. Binding of 14-3-3 to Nedd4L inhibits its binding to ENaC, thereby stabilizing the channel surface expression. This adapter protein thus acts as a repressor for Nedd4L function towards ENaC [144, 145].

Additional proteins can negatively regulate CWHs functions. The protein N4BP1 was found to interact with Nedd4 by screening of a mid-gestation mouse embryo cDNA library [146]. N4BP1 is mono- as well as polyubiquitylated by Nedd4 [147]. N4BP1 was also shown to interact with the WW2 domain of Itch in GST pull-down experiments. Interestingly, Itch does not ubiquitylate N4BP1. Nevertheless, N4BP1 inhibits Itch autoubiquitylation and ubiquitylation of p73α, p63 and c-Jun. N4BP1 is thus a potent repressor of Itch activity [148].

p28GANK was recently identified as a repressor of WWP2. p28GANK attenuates Oct4 ubiquitylation and degradation. The interaction between p28GANK and WWP2 thus restricts the ligase activity towards Oct4 functions [149].

Grb10 is an adapter protein with a complex succession of binding modules. It contains a pleckstrin homology domain for phosphatidylinositol binding and an SH2 domain mediating protein interactions. In the insulin signaling pathway, Grb10 acts as an adapter between Nedd4 and the insulin-like growth factor 1 receptor (IGF1R). The SH2 domain of Grb10 mediates the interaction with Nedd4 C2 domain, leaving IGF1R binding sites accessible [150]. Grb10 is not a substrate of Nedd4 but instead directs multiubiquitylation, internalization and degradation of the IGF1R by Nedd4 [151, 152, 153]. Grb10 is also involved in the regulation of the vascular endothelial growth factor receptor 2 (VEGF-R2). Grb10 thus directs the action of Nedd4 towards the IGF and VEGF receptors [154].

3.4. Regulation by the Ubiquitylation Machinery

E2 Recruitment, Interaction with Ubiquitin and Processisivity

Interfering with specific ubiquitylation processes can also regulate CWHs functions. Smad7 is a negative regulator of TGFβ signaling. Smad7 increases Smurf2 turnover and increases

its affinity for the E2 enzyme UbcH7. Smad7 interaction thus regulates Smurf2-induced ubiquitylation through facilitated recruitment of the ubiquitylation machinery [155].

CWHs can form non-covalent interactions with ubiquitin. Recent evidence shows that the HECT domain large N-lobe subdomain (Fig. 4) interacts with ubiquitin itself. NMR analysis demonstrates such interactions with the N-lobe of Smurf2 HECT domain. Point mutations in the ubiquitin binding surface (UBS) significantly impairs autoubiquitylation *in vitro*. Similarly, Smurf2 mutations impaired polyubiquitylation of RhoA. Fusion of an ubiquitin moiety to RhoA also increases the affinity for Smurf2, indicating a role for ubiquitin binding in ligase-substrate interactions. The UBS region therefore regulates Smurf2 ability to perform different types of ubiquitylation by facilitating the recognition of monoubiquitylated substrates [156]. This represents a conserved regulatory function as it is also found for yeast Rsp5p. The N-lobe of Rsp5p interacts with ubiquitin and mutation of the UBS significantly delays formation of polyubiquitylated Rsp5p.

Non-covalent interactions with ubiquitin further enhance substrate binding. Sna3 is poorly ubiquitylated in vitro by Rsp5p despite the presence of a PY motif and a physiologically relevant ubiquitylation site. Fusion of a single ubiquitin to Sna3 increases its ubiquitylation rate compared to Sna3 alone. This reaction relies on both the PY motif of Sna3 and an intact UBS [157]. Interaction of the HECT domain with ubiquitin has so far been shown for Rsp5p, Nedd4 and Smurf2 [158, 38, 156]. Mutagenesis of the UBS inhibited Nedd4 self-ubiquitylation as well as ENaC substrate ubiquitylation. Non-covalent interaction of CWHs with ubiquitin is thus important to regulate both cis and trans ubiquitylation events. These interactions can also favor interactions with monoubiquitylated substrates [38].

3.5. Ubiquitin Ligases Subcellular Localization

A considerable number of CWH regulators also impact on the ligases intracellular localization. This mechanism represents an effective way to simultaneously recruit and modulate CWH activity towards specifically segregated substrates. Yeast arrestin-related trafficking adapters (ART) is a family composed of nine members with a conserved arrestin domain and two to eight PY motifs. Art1 interacts with the WW domains of Rsp5p. Can1 is a transmembrane protein located at the plasma membrane. Its ubiquitylation is linked to Rsp5p, although it does not directly interact with Rsp5p. Art1 is thought to act as an adapter, bringing Rsp5p to the plasma membrane where it can interact with Can1. Art1 is further recruited to the plasma membrane during cargo downregulation, suggesting its role in endocytosis of surface proteins. It is interesting to note that Rsp5p interacts *in vitro* with all ART proteins except Art9. This family of proteins could therefore regulate signaling events by recruiting Rsp5p to specific surface proteins [159].

In human, ARRDC3 is another protein containing an arrestin domain showed to impact Nedd4 localization. ARRDC3 contains two PY sequences with the C-terminal motif mediating most of the interaction with the ligase. ARRDC3 depletion reduces β2-adrenergic receptor (β2AR) ubiquitylation while increasing receptor expression. This protein interacts with the activated receptor in isoproterenol-treated cells. This complex is then internalized to early endosomes following stimulation. Nedd4 was further demonstrated to colocalize with ARRDC3. Disruption of ARRDC3 PY motifs increased its PM expression and abolished colocalization with the ligase. ARRDC3 thus regulate β2AR through recruitment of

Nedd4 and subsequent ubiquitylation-induced degradation [160].

LITAF has been shown to interfere with Itch localization. LITAF is a small lysosomal proteins related to Charcot-Marie-Tooth neuropathy [161]. This protein was shown to interact with Itch *in vivo* using bioluminescence resonance energy transfer (BRET) assays. Interestingly, LITAF overexpression translocates Itch from the trans-Golgi network to the lysosomes in several cell lines. Mutation of both PY motifs of LITAF was shown to abolish interaction and relocalization. Although Nedd4 can also interact with LITAF, there was no significant change in its intracellular distribution upon coexpression. LITAF thus specifically recruit Itch to lysosomes and may influence substrates interactions and/or regulate Itch catalytic activity [162].

4. Substrates Specificity

Redundancy is a hallmark of biological systems. Signaling pathways involve complex networks of intertwined regulator and activator proteins that assure the robustness and stability of the processes they make up. Because of their identical modular structure and sequence homology, one can assume that CWHs functions are redundant. However, CWH paralogs possess unique characteristics, brought by small conserved sequence divergences and their variable interdomain regions, which provide means of specialization.

4.1. Redundancy and Specificity of WW-domain Binding

CWHs share a common architecture suggesting redundant functions between family members. Indeed, common substrates can be affected by various ligases. For example, ENaC activity is regulated to some extent by Nedd4, Nedd4L and WWP2 [163, 164]. Likewise, p63 is a substrate of both WWP1 and Itch [165, 166]. However, CWHs also display some substrate specificity. Comparison of interactions established by closely related Nedd4 and Nedd4L in a large-scale proteomic array demonstrated that these ligases can interact with distinct ligands. About 8200 human proteins were probed for interactions. 52 hits were common to both ligases while 19 were unique for Nedd4 and 13 for Nedd4L. The WW domains of CWHs thus allow some degree of specificity, although they mostly share binding preferences [167].

4.2. Itch Interacts with SH3 Proteins

The ubiquitin ligase Itch is known to interact with an additional set of proteins, independently of its WW domains, through a conserved proline-rich sequence located just upstream of the first WW domain. SH3 domains are found in a variety of proteins and interact with such proline-rich regions. These domains are composed of roughly 60 amino acids forming six β-strands folded as a β-barrel. SH3 domains generally mediate interactions with minimal PXXP motifs. The few residues flanking this motif are generally implicated in the interaction and the peptide can be presented in two possible orientations. Class I ligands interact with proline-rich peptides containing the (R/K)XXPXXP consensus and class II binds PXXPX(R/K) motifs (Reviewed in [168]). Sequence analysis indicated that although every CWH possesses multiple PXXP motifs, only three class I motifs were found among

them, two of which are within Itch and one in WWP1. Class II motifs are found exclusively in Itch. Two of them partially overlap while a third motif stands only a few amino acids farther [169].

Itch is thus the only CWH with a clearly defined PRD establishing SH3 interactions. This highly conserved 20 amino acids sequence is mainly composed of 10 prolines and 5 arginines, thus creating a variety of possible SH3 binding sites. Itch PRD was first shown to interact with endophilin in pull-down experiments from rat brain extracts. Endophilin is internalised to endosomes following EGFR activation. Itch is located at the trans-Golgi network and endosomal compartments and was found to ubiquitylate endophilin [16]. Itch is phosphorylated following treatment with EGF, leading to its activation. Although endophilin interacts with Itch and is ubiquitylated in unstimulated cells, EGF activation markedly increased this reaction. Itch can thus regulate the stability of key endocytic proteins and provides a possible negative feedback for EGF receptor downregulation [170]. Interestingly, Itch activation by Ndfip2 also increases endophilin ubiquitylation *in vitro* [28]. SH3 partners therefore represent a distinct class of binding molecules that can be influenced by conventional regulatory mechanisms.

Like endophilin, SNX9 is involved in the endocytic process. The SH3 domain of SNX9 interacts with Itch in pull-down experiments. GST fused SNX9 is ubiquitylated by Itch when incubated with extracts from HEK-293 cells overexpressing the ligase. Pulse-chase experiments further demonstrated that SNX9 degradation is accelerated in the presence of Itch. A closely related protein with a common structure, SNX18, also interacts with Itch. These SNX proteins are thus additional SH3 substrates recognized specifically by Itch through its PRD [171].

Itch can interact with another SH3 protein, βPIX. This protein is involved in signal transduction and acts as a guanine nucleotide exchange factor (GEF) for Rac/Cdc42. Isothermal titration calorimetry (ITC) analysis confirmed the SH3 binding sequence located within Itch PRD with an affinity of 7,38 μM. The stoichiometry of the interaction measured by ITC suggests that two SH3 domains interact with one peptide in a heterotrimeric complex. On the other hand, crystallographic data point to a 1:1 relation with a peptide interacting with two faces of the SH3 domain. Although these results together clearly indicate binding of Itch PRD with the SH3 domain of βPIX, the precise molecular mechanism remains elusive. CIN85 is involved in RTK regulation and has three SH3 domains similar to βPIX SH3. CIN85 interacts with Itch and binding is disrupted by increasing amount of βPIX SH3 when immunoprecipitated from HEK-293T [172]. It is interesting to note that in addition to Itch, βPIX also interacts with 14-3-3. Immunoprecipitation assays on mouse cortical cells demonstrated 14-3-3 binding with βPIX at endogenous levels. This interaction was shown to interfere with 14-3-3 binding to Nedd4L and negative regulation of ENaC expression and activity. βPIX could therefore be differentially involved in Nedd4L and Itch functions [173, 174].

Itch interacts with the SH3-domain containing ESCRT-0 protein STAM-1. Both proteins are involved in CXCR4 regulation and trafficking. This interaction might differ from other SH3 binding to Itch as abolition of the SH3 domains only disrupts half of the interaction. Deletion of Itch PRD also allowed residual interactions with STAM-1. Interestingly, STAM-1 has a ubiquitin interaction motif (UIM) located just upstream of its SH3 domain. Non-covalent interactions with ubiquitin could thus participate in Itch recognition.

Further characterization of PRD mediated binding will help understanding how these interactions are established [175]. STAM-1 interacts *in vitro* with two ubiquitin proteases, AMSH and UBPY [176]. These enzymes together with Itch are proposed to control late endosomal/lysosomal degradation processes for GPCRs [177]. Interestingly, STAM can also regulate RTK sorting and degradation through binding of UBPY [178]. STAM-1 could thus regulate trafficking of a variety of receptor through ubiquitin ligase/protease recruitment.

It was previously proposed that Nedd4 also possesses a PRD [171]. This hypothesis is supported by the increased ubiquitylation of immunoprecipitated endophilin when co-expressed with NDFIP activator and Nedd4 [28]. However, there is no direct evidence of Nedd4 binding to an SH3 substrate. *In vitro* pull-down experiments demonstrate that Nedd4 is unable to bind the SH3 domain of Snx9. Our sequence analysis revealed no class I or class II SH3 binding sites in this ligase. In addition, Nedd4 does not have the proline concentration observed with Itch. Analysis with Minimotif Miner indicates several potential SH3 partners for CWH ligases [179]. Yet, only Itch is proven to have direct access to such substrates. These interactions could thus confer specificity for Itch function among CWHs.

Itch PRD is located between its C2 and WW domains. Its interaction with SH3 domains might therefore interfere with ligase binding to PY-motif containing substrates or regulators. The proline-rich region plays additional roles in Itch regulation. Itch intramolecular inhibition involved its HECT domain and the region between the PRD and the first WW domain [28]. Three phosphorylation sites responsible for Itch activation are also located within and around the PRD [101, 60]. SH3 interactions could thus regulate Itch autoinhibition. Alternatively, since many SH3 partners were found to impact endocytosis and trafficking, these binding sites could mediate the formation of molecular scaffolds required during those processes.

5. Conclusion

CWHs form a group of ubiquitin ligases sharing a common structure and mode of operation, yet they are involved in an impressive diversity of physiological processes encompassing embryogenesis, homeostasis, cell signaling and survival. Although their similar structure suggest redundancy in function, CWHs still show remarkable specificity. This is mainly due to the numerous protein interactions established by each member of the subfamily. Perhaps as a direct consequence, the most efficient and powerful way to harness their enzymatic activity is through the regulation of the establishment of these interactions. As CWHs activity is intimately intricate with many aspects of signal transduction, a process largely relying on the formation of protein networks, continued study of CWHs regulation will further our knowledge of cell signaling and protein trafficking.

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