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Mammalian HECT ubiquitin-protein ligases: Biological and pathophysiological aspects

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A B S T R A C T

Members of the HECT family of E3 ubiquitin protein ligases are characterized by a C terminal HECT domain that catalyzes the covalent attachment of ubiquitin to substrate proteins and by N terminal extensions of variable length and domain architecture that determine the substrate spectrum of a respective HECT E3. Since their discov ery in 1995, it has become clear that deregulation of distinct HECT E3s plays an eminent role in human disease or disease related processes including cancer, cardiovascular and neurological disorders, viral infections, and im mune response. Thus, a detailed understanding of the structure function aspects of HECT E3s as well as the iden tification and characterization of the substrates and regulators of HECT E3s is critical in developing new approaches in the treatment of respective diseases. In this review, we summarize what is currently known about mammalian HECT E3s, with a focus on their biological functions and roles in pathophysiology.This article is part of a Special Issue entitled: Ubiquitin Proteasome System. Guest Editors: Thomas Sommer and Dieter H. Wolf.

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1. Introduction

Covalent attachment of ubiquitin to proteins ("ubiquitination") is involved in the control of many, if not all, eukaryotic processes indi cating that the recognition of proteins by the ubiquitin conjugation system must be a highly specific and adjustable process [1,2]. Sub strate specificity of the ubiquitin conjugation system is mainly medi ated by E3 ubiquitin protein ligases that constitute a large class of proteins, with the human genome encoding more than 600 putative E3s or E3 complexes [1 3]. In a simplified view, E3s are characterized by the presence of at least two functional domains/regions. One domain mediates the interaction with the cognate E2 ubiquitin conjugating enzyme(s), while the other is responsible for the specific recognition of substrate proteins. Based on the identity of the domain involved in E2 interaction, E3 proteins can be grouped into two main families, HECT domain E3s and RING and RING like (e.g., U box) domain E3s [3 6]. While the HECT domain is assumed to have an en zymatic activity and to directly catalyze the covalent attachment of ubiquitin to substrate proteins via a ubiquitin HECT thioester complex intermediate [3 5], most RING/RING like domains do not appear to have an enzymatic activity but rather act as allosteric acti vators of E2s [3,6]. Notably, members of the RING between RING (RBR) subfamily of RING E3s have recently been shown to act as RING/HECT E3 hybrids: one RING functions as docking site for cog nate E2s (UbcH7, UbcH5 family members), while the other RING ac cepts ubiquitin from the E2 in the form of a thioester complex as an obligatory intermediate step in RBR mediated ubiquitination [7 10].

HECT E3s were first reported in 1995 and, thus, were the first fam ily of E3s described [11]. Like ubiquitin, HECT E3s are found in all eukaryotic organisms, with the genome of *Saccharomyces cerevisiae* and the human genome encoding 5 and 28 HECT E3s, respectively [4]. Furthermore, although they do not encode ubiquitin, the ge nomes of some pathogenic bacteria (enterohemorrhagic *Escherichia coli* (EHEC) O157:H7; *Salmonella enterica*) encode HECT domain like E3s that are injected into host cells and presumably exploit the ubiquitin conjugation system for bacterial purposes [12 15]. HECT E3s range in size from approximately 80 kDa to more than 500 kDa and are characterized by the HECT domain, a C terminal region of ap proximately 350 amino acids in length with significant similarity to the C terminus of E6AP (*H*omologous to *E*6AP *C T*erminus) [4,5,11]. While the HECT domain represents the catalytic domain [11,16], the substrate specificity of HECT E3s is mainly determined by their re spective N terminal extensions (Fig. 1). Based on the presence of dis tinct amino acid sequence motifs or domains within these N terminal extensions, human HECT E3s have been grouped into three subfamilies: Nedd4/Nedd4 like E3s, which contain WW domains, HERC (*HE*CT and *R*CC1 like domain) E3s harboring RLDs (*R*CC1 *L*ike *D*omains), and

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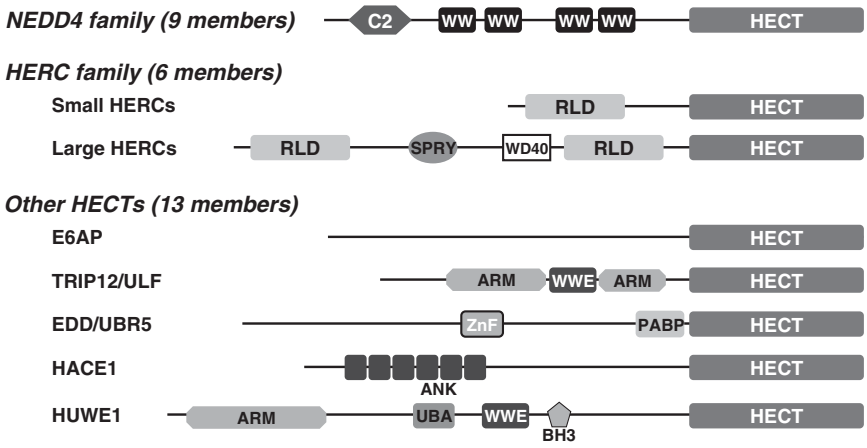

Fig. 1. The human HECT E3 ligases. The human genome encodes 28 members of the HECT E3 family. In all cases, the HECT domain is located at the C terminus of the proteins. The substrate binding is mediated by various domains that are located N-terminal to the HECT domain. The human HECT E3 ligases can be roughly grouped into three families. The NEDD4 family and the HERC family can be readily identified by their distinct domain architectures. NEDD4 members contain an N-terminal C2 domain, 2–4 WW domains and a C-terminal HECT domain. The HERC family members have one or more RCC1-like domains (RLDs). The small HERCs carry a single RLD and large HERCs contain more than one RLD and additional domains. The remaining HECT ligases may contain varied number and types of domains. Schematics are shown for those HECT E3s discussed in this review.

"other" HECT E3s that contain neither RLDs nor WW domains (Fig. 1) [4,5]. Note that this classification is an oversimplification and does not take evolutional considerations into account (for evolutional classification of HECT E3s, see [17]). However, for the sake of convenience, we will use this nomenclature here, when discussing the physiological aspects of selected HECT E3s.

2. Structural and functional aspects of the HECT domain

The HECT domain mediates the interaction with cognate E2s, mainly UbcH7 and members of the UbcH5 subfamily of E2s (for a more comprehensive analysis of the interaction of HECT E3s with E2s, see [18,19]), and forms a thioester complex with ubiquitin via an evolutionally conserved cysteine residue [4,5,11,20]. Since the ability to form ubiquitin thioester complexes in the presence of E2s is necessary for substrate ubiquitination, it is assumed that HECT E3s catalyze the final attachment of ubiquitin to substrate proteins as well as to ubiquitin (in case of ubiquitin chain formation).

2.1. Structure of the HECT domain

Structures of 7 HECT domains (E6AP, WWP1, SMURF2, NEDD4 2/NEDD4L, HUWE1, yeast Rsp5, NEDD4) and the C terminal portion of the HECT domain of UBR5 have been solved [21 28] (note that structures for full length HECT E3s are not yet available). The HECT domain adopts a bilobal structure, with the C terminal lobe containing the catalytic cysteine residue and the N terminal lobe representing the E2 binding domain. The lobes are linked by a flexible hinge region which presumably facilitates proper positioning of the catalytic cysteine towards the ubiquitin E2 thioester bond to allow transthioesterification of ubiquitin to the HECT domain: In the absence of ubiquitin, the distance between the catalytic cysteine residue of an unloaded E2 and the catalytic cysteine of the HECT domain is rather large (e.g., ~41 Å in the case of E6AP and ~16 Å in the case of WWP1) [21 23], in fact too large for transthioesterification. The distance appears to be significantly shortened when a ubiquitin loaded E2 is bound (~8 Å, as shown for the complex between NEDD4 2/NEDD4L and ubiquitin loaded UbcH5B [24]). Thus, the topology of HECT E2 complexes depends on the ubiquitin loading status of the E2 and involves non covalent interactions between the N lobe and the E2 and ubiquitin and the C lobe [24].

2.2. Regulation of HECT domain activity

The activity of HECT E3s can be regulated at two general levels. One level is the association of an HECT E3 with its substrate protein(s), which in most cases is mediated by specific protein protein interaction domains/motifs located N terminal to the HECT domain (Fig. 1). In addition, at least some of the interaction motifs present in HECT E3s bind regulatory proteins that either facilitate ("adaptor and/or auxiliary proteins") or interfere with ("inhibitory proteins") the interaction of substrates with their cognate E3s. For example, Ndfip1 and Ndfip2 as well as several members of the α arrestin protein family (Arrdc) bind to the WW domains of distinct Nedd4 family members (see 3.1) through PY motifs [29 33], thereby assisting the ubiquitination of respective substrate proteins [5,32,33]. In contrast, binding of 14 3 3 proteins to Nedd4 2, which is regulated by hormone induced phosphorylation of Nedd4 2 [5,34 36], precludes the interaction of Nedd4 2 with its substrates (e.g., epithelial sodium channel subunits) [5,34,35]. For more detailed discussions of potential mechanisms involved in regulating HECT E3 substrate interaction, see refs. [4,5,37 39].

The other level concerns the catalytic activity of the HECT domain including the interaction with its cognate E2 (for a review, see [37]). Two illustrative examples for the regulation at the HECT domain level are provided by SMURF2 and Itch [23,40,41], both of which are members of the NEDD4 like family of E3s and contain WW domains and an N terminal C2 domain (Fig. 1). Compared to other HECT domains, the HECT domain of SMURF2 interacts rather ineffectively with its E2 (UbcH7). In fact, for efficient interaction between the HECT domain of SMURF2 and Ubch7 an additional protein SMAD7 which binds to both the HECT domain of SMURF2 and UbcH7 is required [23]. Furthermore, NMR studies showed that in the context of full length SMURF2, the catalytic cysteine of the HECT domain is not accessible for UbcH7, as it is disguised by an intramolecular interaction of the C2 domain with the HECT domain. The inhibitory effect of the C2 domain is also released by SMAD7, the expression of which is regulated by extracellular stimuli (e.g., TGFβ) [40]. With respect to Itch, coprecipitation analyses indicate that the HECT domain interacts with the WW domain rendering Itch inactive. Furthermore, stimulus dependent phosphorylation of distinct serine and threonine residues in the N terminus of Itch induces a conformational change resulting in the disruption of the WW HECT domain interaction and, consequently, the activation of Itch activity [41]. Taken together, the activity of at least some HECT domains can be regulated by intra and/or intermolecular interactions

ensuring that the respective substrate proteins are only targeted for ubiquitination when appropriate.

2.3. Ubiquitin chain formation

In many cases, proteins are modified by ubiquitin chains ("poly ubiquitination") rather than a single ubiquitin moiety ("mono ubiquitination"). Furthermore, ubiquitin contains seven lysine residues and each of these can be used for ubiquitin ubiquitin conjugation. Thus, homo polymeric (i.e. one distinct lysine residue of ubiquitin is used for conjugation throughout a chain, e.g., K48 or K63) and hetero polymeric chains (i.e. different lysine residues of ubiquitin are modified within one chain and/or one ubiquitin moiety can be modified at several lysines) can be assembled. In addition, the alpha amino group of the N terminal residue of ubiquitin can be used for conjugation resulting in so called linear ubiquitin chains. Notably, different types of ubiquitination signal respectively modified proteins for different fates. For example, modification with K48 linked or K11 linked ubiquitin chains targets proteins for proteasome mediated degradation, while mono ubiquitination or modification with K63 linked ubiquitin chains has been associated with non proteolytic roles (for recent reviews on the functions of different types of ubiquitination and how these are po tentially decoded, see [42 46]).

Consistent with their different cellular functions (Section 3), different HECT E3s have been shown to synthesize different ubiquitin chains. For example, E6AP mainly forms K48 linked ubiquitin chains, while mem bers of the Nedd4 family preferentially form K63 linked chains but can also assemble K48 linked chains [47 50]. Thus, important yet not fully re solved issues are: (i) what are the mechanisms that determine which type of ubiquitin chain is built by a given HECT E3 and on which substrate, and (ii) where are ubiquitin chains assembled, on the substrate by a se quential mechanism (i.e. initial mono ubiquitination followed by the stepwise addition of single ubiquitin moieties) or on the catalytic cysteine of the E3 followed by a one step transfer of the fully assembled chain to the substrate. While initial results obtained with KIAA10 (also termed UBE3C or RAUL) and E6AP suggested that both mechanisms are in use [47,51], more recent evidence indicates that ubiquitin chain assembly mainly proceeds via a sequential mechanism [26,27,50,52] and that the ability to build up distinct ubiquitin chains is determined by the C lobe of the HECT domain [50]. Furthermore, studies with yeast Rsp5 and human Smurf2, both of which belong to the Nedd4 family, indicated that besides the covalent interaction between ubiquitin and the catalytic cysteine, at least some HECT domains contain an additional non covalent interaction site for ubiquitin [52,53]. However, while for Rsp5 it was sug gested that the non covalent binding of ubiquitin is required to restrict the length of ubiquitin chains synthesized by Rsp5 [53], the Smurf2 study arrived at a rather different conclusion, namely that the binding site is required for chain formation [52]. This apparent contradiction was recently resolved by crystallographic studies showing that the N lobe of Rsp5 and NEDD4, respectively harbors a non covalent interaction site for ubiquitin supporting a model, in which a growing ubiquitin chain is kept in close vicinity to the catalytic cysteine for ubiquitin conjugation [26,27].

Finally, it was shown that the yeast HECT E3 Ufd4 can team up with Ubr1, a RING domain E3 ligase, in the poly ubiquitination of sub strate proteins [54]. If the ability to interact with other E3s, thereby modulating ubiquitin chain formation and/or widening the substrate spectrum, is a more general feature of HECT E3s remains to be shown.

3. Physiological aspects

In the following, we will limit ourselves on a discussion of those HECT E3s that have been associated with human disease or disease relevant processes.

3.1. Nedd4 like E3s

This group of HECT E3s comprises nine human members: NEDD4, NEDD4 2/NEDD4L, ITCH, SMURF1, SMURF2, WWP1, WWP2, NEDL1 and NEDL2. Nedd4 (*N*eural precursor cell *e*xpressed *d*evelopmentally *d*ownregulated gene *4*) was originally cloned as a developmentally regu lated gene in the mouse central nervous system [55,56]. It contains an N terminal C2 domain (calcium dependent lipid binding domain), 3 (mouse and rat) or 4 (human) WW domains (protein protein interaction domains), and a HECT domain at the C terminus. This modular domain structure, containing sequential C2 WW HECT domains, is characteristic of all Nedd4 family members [57]. The mammalian Nedd4 family mem bers usually contain 2 4 WW domains, although other variants, often due to alternate splicing are also found [5].

As indicated above (2.2), WW domains bind proteins containing PPxY or similar motifs in substrates, adaptors and regulatory proteins [5,58,59]. Given that there are multiple WW domains present, Nedd4 family members can potentially interact with multiple proteins. Below we summarize the functions of individual Nedd4 family members.

3.1.1. NEDD4

Being the first discovered member of the family, NEDD4 (sometimes also called NEDD4 1) has been studied extensively. A large number of the potential substrates of NEDD4 have been reported (for a review, see [60]), including proteins such as Hgs, a sorting adaptor, suggesting that this HECT E3 may have a general role in vesicular sorting and traf ficking [61]. However, in vivo validation is available for only a small number of potential NEDD4 substrates.

The main phenotypes in mice deficient in Nedd4 are growth retar dation, developmental abnormalities in the nervous and cardiovascular systems and defects in neuromuscular junctions and T cell function (see below). The complex phenotypes associated with *Nedd4* knockout sug gest that Nedd4 has multiple targets in vivo. Nedd4 deficient animals die during fetal development between E14 and E18 and are less than half the size of their wild type littermates [62]. Even the mice that are heterozygous for *Nedd4* (*Nedd4*$^{+/-}$) are 15 20% smaller [62].

The growth retardation in Nedd4 deficient animals is at least part ly due to reduced cell surface expression and signaling through insu lin and IGF 1 receptors [62]. The fibroblasts isolated from *Nedd4*$^{-/-}$ embryos show reduced growth and mitogenic activity and increased levels of Grb10, an inhibitor of both receptors [62]. Although not fully elucidated, it appears that Grb10 itself is not a direct target of Nedd4. Nevertheless, Grb10 directly interacts with Nedd4 through the C2 domain of Nedd4 [63,64] and the lethality in *Nedd4*$^{-/-}$ mice can be partially rescued by the maternal inheritance of a disrupted *Grb10* allele [62].

Nedd4 also regulates T cell function. Although *Nedd4*$^{-/-}$ animals display normal T cell development in the thymus, their T cells in the periphery are hyporesponsive, proliferate poorly in response to anti gen and are less likely to produce IL 2 [65]. *Nedd4*$^{-/-}$ B cells undergo class switching with lower frequency [65]. The reduced activation of *Nedd4*$^{-/-}$ T cells is likely to be due to the higher levels of Cbl b, a RING type ubiquitin ligase and a known target of Nedd4. Indeed *Nedd4*$^{-/-}$ T cells contain increased levels of Cbl b protein [65].

Another study using the *Nedd4* knockout mice shows that Nedd4 regulates neurite growth and arborization in neurons [66,67]. Nedd4, the serine/threonine kinase TNIK and Rap2A form a complex that medi ates the Nedd4 mediated ubiquitination of Rap2A [66]. This leads to the reduced Rap2A function and promotion of dendrite growth. Drinjakovic et al [67] found that the downregulation of PTEN by Nedd4 mediated ubiquitination controls branching of retinal ganglion axons. Nedd4 is also required for the proper formation and functioning of the neuromuscular junctions [68]. In *Nedd4*$^{-/-}$ embryos the size of skeletal muscle fibers and the number of motor neurons are greatly reduced, in an apparent non cell autonomous manner. Using a *Nedd4* skeletal

muscle specific knockout mouse, it has been recently demonstrated that the absence of Nedd4 results in an increased mass of the type II fast twitch fibers of denervated gastrocnemius muscle following tibial nerve transection, suggesting that Nedd4 mediates denervation induced skeletal muscle atrophy in vivo [69]. The mechanism by which this occurs remains unknown. Additionally, Nedd4 is critical for vascular development [70]. Increased amounts of thrombospondin 1 (Tsp 1), an inhibitor of angiogenesis, in *Nedd4*$^{-/-}$ mice potentially contribute to the vascular defects in the embryos.

NEDD4 also ubiquitinates and degrades N and c Myc oncoproteins in neuroblastoma and pancreatic cancer cells [71]. The class III histone deacetylase SIRT2 enhances N Myc and c Myc protein stability and promotes cancer cell proliferation by directly binding the *NEDD4* promoter, deacetylating histone H4 lysine 16 and repressing *NEDD4* gene expression. Interestingly, SIRT2 inhibitors reactivate *NEDD4* gene expression, reduce N Myc and c Myc protein expression, and inhibit neuroblastoma and pancreatic cancer cell proliferation [71]. These results suggest that NEDD4 mediated Myc regulation may be targeted therapeutically for the treatment of neuroblastoma and pancreatic cancer.

Lastly, NEDD4 (and also NEDD4 2) has been shown to interact with viral proteins to mediate budding of many viruses including the Ebola virus [72,73] and retroviruses [74 79]. NEDD4 ubiquitinates viral matrix proteins, including the Epstein Barr virus LMP2A and LMP2A associated proteins such as Lyn. This leads to recognition by the cellular ESCRT machinery, trafficking through the host cell vesicular transport machinery and eventually budding from the infected cells [80].

3.1.2. NEDD4 2/NEDD4L

Despite being highly homologous to NEDD4, NEDD4 2 appears to have a more restricted and/or partially different substrate pattern and this is apparent from the phenotype of *Nedd4 2* knockout mice. Mice null for Nedd4 2 develop normally but mostly die at birth due to collapse of the lungs and an inability to breathe [81]. Some *Nedd4 2*$^{-/-}$ animals that survive birth die within 3 weeks due to severe sterile lung inflammation, presumably caused by drying out of alveolar epithelia [81]. One of the main reasons for this phenotype appears to be the increased cell surface expression of the epithelial sodium channel (ENaC), although other factors and targets may contribute [81]. A lung specific conditional knockout of *Nedd4 2* also results in a somewhat similar phenotype and perinatal lethality [82].

Previous electrophysiological and biochemical studies have established NEDD4 2 as a critical regulator of ENaC [83,84]. The WW domains in mouse Nedd4 2 bind the PY motifs present in all three ENaC subunits [85]. This leads to ENaC ubiquitination and targeting for endocytosis and degradation. The disruption of the interaction between Nedd4 2 and ENaC by mutations at the C termini of either the β or the γ ENaC subunit is predicted to be the cause of Liddle's syndrome, an autosomal dominant disorder with severe sodium retention and hypertension [86]. Consistent with this prediction, a *Nedd4 2* mouse knockout line that retains some Nedd4 2 expression ("Nedd4 2 hypomorph") shows hypertension in animals on a normal diet that is further enhanced on high salt diet [87]. These Nedd4 2 hypomorphs were more hypertensive than Liddle syndrome mice, and they do not display the degree of hypokalemia seen in these mice [87], suggesting that Nedd4 2 may also affect blood pressure by other means. Indeed, recent data using an inducible nephron specific deletion of the *Nedd4 2* locus indicates that sodium chloride cotransporter NCC is also upregulated and contributes to salt sensitive hypertension in mice [88].

When coexpressed in *Xenopus* oocytes, Nedd4 2 is also known to negatively regulate a number of voltage gated sodium and potassium channels [89 94]. Voltage gated sodium channels (Na_vs) and potassium channels (KCNQ) play a key role in generating and propagating action potentials in excitable cells, such as the neurons. Most of the Na_vs contain a typical PY motif within the C terminal cytoplasmic region which binds Nedd4 2 WW domains [90]. Several of these PY motif containing Na_vs were shown to be ubiquitinated by Nedd4 2 [90]. KCNQ channels KCNQ1, KCNQ2/3 and KCNQ3/5 interact through their carboxyl terminal region with Nedd4 2 and are inhibited by Nedd4 2 expressed in *Xenopus* oocytes [93,94]. The validation of these findings in the *Nedd4 2* deficient mice has yet to be carried out. Another Nedd4 2 target that is of interest in this context is the dopamine transporter DAT [95]. Nedd4 2 mediated ubiquitination is required for DAT interaction with the adaptor proteins in clathrin coated pits (e.g., epsins Eps15 and Eps15R) and endocytosis [95]. It would be interesting to test whether such regulation of DAT and the dopamine neurotransmission is abrogated in *Nedd4 2*$^{-/-}$ mice. Given the embryonic and neonatal lethality of *Nedd4 2*$^{-/-}$ mice, it would be necessary to generate mice with a conditional dopaminergic neuron specific knockout of the *Nedd4 2* gene.

In addition to ENaC, NCC, Na_vs, and KCNQs there are a number of other proteins, including many channels and transporters, predicted to be regulated by Nedd4 2, although most have not been validated in in vivo models [60]. One protein, the *Drosophila* discs' large member Dlg, also known as the X linked mental retardation gene product requires a mention here. Dlg3 is known to contribute to apical basal polarity and epithelial junction formation in mouse organizer tissues and to planar cell polarity in the inner ear. A recent study demonstrates that Dlg3 is mono ubiquitinated by Nedd4 2 (and Nedd4) and this is required for the apical membrane recruitment and consolidation of the tight junctions [96].

3.1.3. ITCH

Like other NEDD4 family members ITCH interacts with many potential targets and has been implicated in the regulation of various cellular functions. The best known function of ITCH is in the regulation of the immune system. The *Itchy* mutant mice that carry an inversion of the *Itch* locus develop severe inflammatory disease [97]. These mice have T helper 2 (TH2) cell differentiation defects with increased production of IL 4, IL 5 and Ig and exhibit autoimmunity. The deficiency of human ITCH causes syndromic multisystem autoimmune disease and developmental abnormalities, confirming that ITCH plays multiple roles in cellular regulation [98]. ITCH has multiple known targets, including JunB which regulates the transcription of the *IL4* gene. Following T cell activation JNK1 mediated phosphorylation and activation of ITCH result in ITCH dependent ubiquitination and degradation of JunB [99]. This regulatory mechanism is required to negatively regulate TH2 cell activation. Consequently, TH2 cells from Itch deficient mice exhibit resistance to T cell anergy and hence impaired self tolerance [99,100]. Itch, along with the deubiquitinase Cyld also regulates TAK1 to terminate inflammatory signaling via TNF [101].

Another mechanism by which Itch regulates T cell function and allergic response is via Foxp3, a key factor in regulatory T cells (Treg cells) [102]. Foxp3 expression in naive T cells is induced by TGFβ and requires TIEG1 transcription factor. Itch ubiquitinates TIEG1 contributing to a non proteolytic pathway to regulate Foxp3 expression and Treg mediated allergic responses [102].

Recent data suggest that Itch may also regulate inflammation via regulating the ubiquitin editing enzyme A20 which negatively regulates TNF mediated inflammation signaling [103]. In this pathway, TAX1BP1 recruits Itch to A20 and Itch is required for TNFR signaling by controlling the A20 mediated recruitment and inactivation of RIP1 kinase. Interestingly, the Tax oncoprotein of T cell leukemia virus type I caused the inactivation of this complex by disrupting the interaction among TAX1BP1, A20 and Itch [103].

Itch regulates MAVS, a critical adaptor for RIG I like helicases in innate antiviral immunity which is essential for preventing excessive harmful immune response [104]. Viral infection induces the expression of PCBP2 which recruits Itch to ubiquitinate and degrade MAVS [104]. Deficiency of Itch results in prolonged and enhanced antiviral

responses suggesting that Itch acting via PCBP2 and MAVS is critical for immune response in response to viral infection.

Itch is also implicated in Notch and Wnt signaling. The *Itch*$^{-/-}$ mice have more and functionally enhanced hematopoietic stem cells, which show accelerated proliferation rates and sustained pro genitor properties [105]. This was found to be due to the accumula tion of activated Notch1 [105]. Itch regulates Wnt signaling via the regulation of PI 4 kinase type IIα (PI4KIIα) [106]. Itch ubiquitinates PI4KIIα and both proteins colocalize on endosomes to regulate inter nalization and degradative sorting of the Wnt activated frizzled 4 (Fz4) receptor [106].

Finally, Itch ubiquitinates and promotes the degradation of p63 and p73, the members of the p53 family of transcription factors [107,108]. p63 protein levels are significantly increased in Itch deficient keratinocytes [108]. Other targets of Itch include the chemo kine receptor CXCR4, where Itch mediated regulation controls CXCR4 endocytosis and sorting [109], and c FLIP, an inhibitor of caspase 8 in TNF signaling pathway [110].

3.1.4. WWP1

Like human NEDD4 and NEDD4 2, WWP1 also contains 4 WW do mains. In *Caenorhabditis elegans* WWP1 has been shown to be a pos itive regulator of lifespan in response to diet restriction [111]. Although such in vivo studies are not available for mammals, mam malian WWP1 has been implicated in the regulation of a number of proteins including p63, ErbB4, Smad2, Smad4, KLF2, KLF5, RNF11, SPG20, TβR1, EPS15, RUNX2, and JunB, thus potentially regulating many cellular signaling pathways (reviewed in [112]). However, most of these studies have not been validated in mouse knockout studies.

WWP1 gene amplification has been found to occur in a substantial number of breast and prostate cancers, and WWP1 knockdown in cancer cells results in growth arrest [112]. This combined with the possible role of WWP1 in the degradation of p53, KLF2 and KLF5 suggests that WWP1 plays a role in tumor progression. On the other hand, a recent study suggests that WWP1 knockdown in MDA231 breast cancer cells increases tumor area in the bone marrow of the mice. Furthermore, WWP1 knockdown reduces CXCL12 induced CXCR4 lysosomal trafficking and degradation suggesting that WWP1 regulates cell migration and breast cancer metastasis [113].

Another study shows that TNF enhances the expression of Wwp1 in mesenchymal stem cells (MSCs) isolated from mice transgenic for TNF (a model of chronic inflammatory arthritis) [114]. WWP1 medi ates JunB ubiquitination and degradation in MSCs after chronic expo sure to TNF. Importantly, while injection of TNF into wild type mice resulted in decreased osteoblast differentiation of MSCs and in creased JunB ubiquitination, in *Wwp1*$^{-/-}$ mice no such effect was seen. Thus, Wwp1 is important for the control of MSC differentiation into osteoblasts in vivo.

3.1.5. WWP2

WWP2 has been implicated in the regulation of iron transport through the divalent metal transporter DMT1 [115,116], in craniofa cial development through mono ubiquitination of Goosecoid [117], in palatogenesis by interacting with Sox9 and Med25, and in the reg ulation of the tumor suppressor PTEN [118].

DMT1 is the primary non heme iron transporter in mammals that is responsible for iron uptake in the duodenum. It is regulated by the amount of dietary iron and gain and loss of the function of DMT1 re sult in human diseases of iron homeostasis (hemochromatosis and anemia, respectively). WWP2 binds DMT1 through the PY motif containing adaptors Ndfip1 and Ndfip2 to ubiquitinate and potential ly degrade DMT1 [115,119] and this regulation is critical for iron ho meostasis under iron limiting conditions [116].

WWP2 has been shown to ubiquitinate and regulate the transcrip tion factor OCT4 [120,121]. In human embryonic stem cells WWP2 regulates the levels of OCT4 via ubiquitin dependent proteasomal degradation. As OCT4 is essential for maintaining the pluripotency and self renewal of ES cells and also in determining cell fate, WWP2 presumably plays an important role in these processes.

Recent data suggest that the deficiency of Wwp2 in mice leads to defects in the craniofacial region [117]. This phenotype is attributed to the role of Wwp2 in craniofacial patterning through its interactions with the homeobox transcription factor Goosecoid (Gsc) which is known to be involved in craniofacial development. The authors showed that Wwp2 binds and mono ubiquitinates Gsc, which then activates the transcription of cartilage regulatory protein Sox6. Thus Wwp2 function in craniofacial development does not involve ubiquitin dependent degradation of the substrate. Sox9, another transcriptional activator of cartilage specific extracellular matrix genes, regulates Wwp2 expression [122]. Wwp2 also directly inter acts with Sox9 and appears to be important for the nuclear transport and transcriptional activation of Sox9 [122]. This transcriptional acti vation of Sox9 also requires binding of Med25, a component of the Mediator complex. The Wwp2 Med25 Sox9 axis defines the Sox9 transcription and chondrogenesis in the formation of the palate.

Regulation of the tumor suppressor protein PTEN, a phosphatase, by Nedd4 family members has been controversial. While initial re ports suggested that Nedd4 regulates PTEN nuclear transport and reg ulation [123,124], PTEN levels or localization is not affected in Nedd4 deficient cells [125]. More recently, PTEN was shown to be ubiquitinated and translocated into the nucleus in neurons following ischemia [126]. This translocation is dependent on the adaptor pro teins Ndfip1 and Nedd4 or Nedd4 2. In addition to ubiquitination by Nedd4 and Nedd4 2, which primarily appears to regulate PTEN local ization, PTEN is also ubiquitinated by WWP2, and this apparently leads to PTEN degradation [118]. In this case, WWP2 regulates tumor cell survival and tumorigenesis.

3.1.6. SMURF1 and SMURF2

*Sm*ad *u*biquitin *r*egulatory *f*actors SMURF1 and SMURF2 are relat ed Nedd4 family members, both containing 3 WW domains [5]. These ligases are primarily involved in the regulation of signaling by the TGFβ/BMP superfamily members, a function that is conserved in *Drosophila*, which has a single Smurf homologue [5]. Other cellular processes that Smurfs are involved in include cell proliferation, DNA damage response, and tumor suppression (reviewed in [127]).

The TGFβ family members signal through type I and II Ser/Thr ki nase receptors [128,129]. Binding of TGFβ leads to type II receptor mediated phosphorylation of type I receptor. This is followed by the phosphorylation of receptor regulated Smads (R Smads) which then bind the co Smad Smad4. The Smad4 complexes translocate into the nucleus to mediate the transcription of target genes. Inhibito ry Smads (I Smads) terminate TGFβ signaling by blocking R Smads and co Smads [130]. Smurf ligases can regulate TGFβ signaling in multiple ways by directly ubiquitinating Smads, by ubiquitinating TGFβ/BMP receptors themselves or by targeting other downstream components of the pathways [5].

Knockout mouse studies suggest that Smurf1 and Smurf2 play a redundant function in the regulation of TGFβ signaling. *Smurf1*$^{-/-}$ mice develop normally but show an age dependent increase in bone mass [131]. These mice do not show any disruption of the canonical TGFβ or BMP signaling pathways. Rather, they show accumulation of activated protein kinase Mekk2 and downstream activation of Jnk signaling [131]. The authors showed that Mekk2 is a direct target of Smurf1 mediated ubiquitination and degradation, and thus negative ly regulates osteoblast activity.

Smurf2$^{-/-}$ mice also develop normally, are viable and fertile and do not show any overt phenotype [132]. However, *Smurf1*/*Smurf2* double knockout mice die around embryonic day E10.5, suggesting that these two ubiquitin ligases are functionally redundant. The study of the double knockout animals suggested that Smurfs are

involved in planar cell polarity and convergence and extension. As such, the *Smurf1/Smurf2* double knockout embryos show planar cell polarity defects in the cochlea and convergence and extension de fects, such as a failure to close the neural tube [132]. The authors found that Smurfs regulate a non canonical Wnt signaling pathway that leads to the ubiquitination and degradation of the core planar cell polarity protein, Prickle1 [132].

In another study, Tang et al. [133] found that TGFβ mediated tran scriptional responses are elevated in *Smurf2* deficient mice. Surpris ingly, rather than inducing Smad degradation, Smurf2 was found to multiple mono ubiquitinate Smad3. This resulted in the inhibition of Smad3 complex formation. Thus, these results suggest that Smurf2 negatively regulates TGFβ signaling by inhibiting Smad3 ac tivity, but not by causing Smad degradation, as previously thought.

Smurf2 deficient mice are also characterized by early spontaneous tumors, mostly B cell lymphomas, suggesting a tumor suppressor function for Smurf2 in vivo [134]. Interestingly, Smurf2 deficiency leads to an impaired response to senescence in embryonic fibroblasts and premalignant spleen cells. Increased levels of Id1 and decreased p16 expression in Smurf2 deficient cells suggest that Smurf2 regu lates senescence by targeting Id1 for ubiquitination and degradation [134]. Another study suggests a tumor suppressor function of Smurf2 by the mono ubiquitination of histone H2B as well as by the trimethylation of histone H3 by targeting ring finger protein 20 (RNF20) for proteasomal degradation [135]. Thus, the tumor suppres sor function of Smurf2 also appears to be due to its function in the epigenetic control of genomic stability.

SMURF1 has also been implicated in the regulation of tight junc tions and cell polarity by targeting small GTPase RhoA for degradation following TGFβ activation [136]. Numerous other targets of both Smurf1 and Smurf2 have been described and recently reviewed [127]. Many of the Smurf substrates are involved in signaling path ways that participate in tumor progression or tumor suppression. However, the multiple targets and pathways affected by Smurfs sug gest that these ubiquitin ligases have complex cellular functions, and thus may be associated with many pathological scenarios.

3.1.7. NEDL1/HECW1 and NEDL2/HECW2

NEDL1 (NEDD4 like ubiquitin protein ligase 1) is implicated in familial amyotrophic sclerosis (FALS) that arises from germ line muta tions in the *SOD1* gene. NEDL1 binds and ubiquitinates misfolded SOD1 protein [137]. The authors also identified Dishevelled 1 as a target for NEDL1 and suggested that mutant SOD1, NEDL1, Dishevelled 1 and translocon associated protein delta form a complex of ubiquitinated proteins that is part of protein aggregates. These cytotoxic protein ag gregates may function in neuronal cytotoxicity in FALS. In further stud ies the authors generated *NEDL1* transgenic mice expressing human NEDL1 [138]. These mice show motor dysfunctions, degeneration of neurons in the lumbar spinal cord and muscle atrophy, a phenotype showing Amyotrophic lateral sclerosis like symptoms. These studies suggest that NEDL1 is involved in the pathophysiology of some neuro degenerative diseases.

In another study, NEDL1 was shown to bind the C terminus of p53 in neuroblastoma cells [139]. This binding results in an increased transcriptional activity of p53 and the catalytic activity independent enhancement of the proapoptotic activity of p53. The mechanism, by which the enhancement of p53 function by NEDL1 is mediated, re mains unclear. A related study showed that NEDL1 interacts with RNF43, a RING ubiquitin ligase highly expressed in colorectal carcino mas [140]. It was further found that RNF43 interacts with p53 and suppresses the transcriptional activity of p53 in colorectal carcinoma cells, suggesting that NEDL1 association with RNF43 and p53 attenu ates p53 mediated apoptosis.

Finally, ErbB4, a member of the EGF receptor family that is impor tant for mammary epithelial cell proliferation and survival, was found to be a proteolytic target for NEDL1 [141].

Little is known about NEDL2 and its potential functions. In the only published study on this E3 ligase, NEDL2 was found to bind the C terminal PY motifs of p73 and catalyze the ubiquitination of p73 in vitro. Interestingly, the ubiquitination of p73 by NEDL2 led to a sta bilization of p73, resulting in enhanced p73 dependent transcription al activation [142]. Thus, it seems that both NEDL1 and NEDL2 are involved in stabilization and enhancing the transcriptional modulato ry functions of p53 family members.

3.2. HERC E3s

The human HERC E3 subfamily has six members that, based on their molecular mass, can be divided into HERCs with a molecular mass of more than 500 kDa (HERC1, HERC2) and HERCs with a mo lecular mass of approx. 100 120 kDa (this is a purely operational classification that is not based on evolutional considerations) (for re views on HERC E3s, see [143,144]). As mentioned above (Section 1), HERC family members are characterized by the presence of one or more (up to three) RLDs (Fig. 1). A canonical RLD consists of seven re peats of 50 70 amino acids in length and was first described for RCC1 (*R*egulator of *C*hromosome *C*ondensation *1*) [145]. RCC1 adopts a seven bladed β propeller structure with each repeat corresponding to one blade [146]. In RCC1, one side of the propeller binds to the GTP binding protein Ran and acts as a guanine nucleotide exchange factor. The opposite side binds to histones mediating the interaction of RCC1 with chromatin [147,148]. If RLDs of HERC E3s also serve a dual function, remains to be shown.

3.2.1. HERC1

HERC1 is a giant protein of 4861 amino acids and, thus, is rather refractory to biochemical analysis. Nonetheless, it was the first mem ber of the HERC family to be characterized and it was shown to bind to and act as a guanine nucleotide exchange factor for ARF1 [149]. However, as no evidence is available that ARF1 represents a ubiquitination substrate for HERC1, the interaction with ARF1 may not be relevant for the E3 function of HERC1 (i.e. HERC1 may be a multifunctional protein). Furthermore, HERC1 interacts with TSC2, a GTPase activating protein of the Rheb GTPase, and targets it for deg radation [150]. In complex with TSC1, TSC2 negatively affects the mTOR pathway and plays a role in the development of tuberous sclerosis complex (TSC), an inherited disease characterized by hamartoma formation in various organs. However, if the deregulation of HERC1 expression or activity is involved in TSC development, is presently unclear. In addition, the TSC2 TSC1 complex inhibits FRAP1, which is a negative regulator of the protein phosphatase PP2A. One of the substrates of PP2A is the DNA mismatch repair en zyme MSH2, which plays a pivotal role in maintaining genomic integ rity. Recently, it has been reported that a certain percentage of patients with acute lymphoblastic leukemia and sporadic colorectal cancer carries deletions in the *HERC1* gene [151]. Loss of HERC1 re sults in the inappropriate activation of PP2A and, consequently, in the destabilization/inactivation of MSH2 (loss of HERC1 presumably results in the stabilization of TSC2, which in consequence results in more efficient inhibition of FRAP1 resulting in PP2A activation) [151]. Finally, mutations in the mouse *Herc1* gene result in progres sive Purkinje cell degeneration that is associated with a decreased mTOR activity further indicating the physiological relevance of the HERC1 TSC2 interaction [152].

3.2.2. HERC2

HERC2 is also a rather large protein of 4836 amino acids indicating that similar to HERC1, HERC2 is a multifunctional protein. In the late 1990s, it was shown that the so called rjs (runty, jerky, sterile) or jdf2 (juvenile development and fertility 2) mice harbor mutations in the *Herc2* gene [153,154]. Such mice have neuromuscular and spermato genic abnormalities and are characterized by defects in growth,

movement coordination (jerky gait), and fertility. One of the mutant mouse strains expresses a truncated form of Herc2 lacking part of the C terminal HECT domain [154] indicating that loss of the E3 activ ity contributes to the observed phenotype. However, the processes and proteins that are deregulated in the absence of Herc2 activity re main to be determined.

Recently, cell culture studies have implicated HERC2 in the regula tion of DNA metabolism [155 157]. In conjunction with the RING do main E3s RNF8 and RNF168, HERC2 has been reported to coordinate the ubiquitin dependent assembly of DNA repair complexes in re sponse to ionizing irradiation induced DNA damage [155]. In addi tion, the interaction with RNF8 appears to be regulated by the DNA damage induced sumoylation of HERC2 [158]. In contrast to these data, evidence obtained in a chicken cell system indicates that HERC2 is not required for RNF8/RNF168 mediated ubiquitination in response to DNA damage [159]. Furthermore, HERC2 was reported to regulate the stability of the DNA excision repair protein XPA and of the RING domain E3 BRCA1, which also plays a role in DNA repair [156,157]. Finally, in the presence of BRCA1, HERC2 was found to in teract with Claspin, further supporting a role of HERC2 in DNA repair and DNA replication [160]. It should be noted, however, that rjs/jdf2 mice do not display overt DNA repair phenotypes [153,154]. Thus, further studies will be required to fully elucidate the role of HERC2 in DNA metabolism.

The human *HERC2* gene is located on chromosome 15q11 13, which is known as the Prader Willi/Angelman region (see also 3.3.1) [161,162]. This region (~4 megabases in size) contains a bipar tite imprinting center, and consequently maternally and paternally imprinted genes, and is bounded by duplicons of the *HERC2* gene predisposing the region to chromosomal rearrangements. The Prader Willi syndrome (PWS) and the Angelman syndrome (AS) rep resent two clinically distinct neurodevelopmental disorders with PWS resulting from paternal genetic deficiency and AS from maternal genetic deficiency [161,162]. Although the *HERC2* gene is frequently affected in these diseases, it has been assumed that the loss or alter ation of HERC2 function does not contribute to the development of PWS and AS, since the *HERC2* gene itself is not imprinted. However, two recent reports provided unequivocal evidence that a homozy gous missense mutation in the *HERC2* gene is found in patients with a neurodevelopmental disorder that can be described phenotypically as a mild form of AS [163,164]. Furthermore, HERC2 has been shown to have the ability to interact with E6AP and to act as an allosteric ac tivator of the E3 activity of E6AP [165]. Thus, although loss of HERC2, which is frequently observed in AS patients, is not a primary cause for AS development, it seems likely that it contributes to the severity of some features of this disorder (e.g., autistic behavior, seizures, ataxic features) [166,167].

Finally, SNPs (*S*ingle *N*ucleotide *P*olymorphisms) in the *HERC2* gene have been associated with the determination of eye color [168,169]. However, as the SNPs are located in the intronic regions of the *HERC2* gene, the HERC2 protein is most likely not affected and, thus, not involved in this process.

3.2.3. HERC5

With the exception of HERC5, only little is known about the phys iological functions of HERC3 6 [170,171]. In 1999, HERC5 was reported to interact with different Cyclins [172]; the physiological rel evance of these interactions, however, remains unclear. Since then, it was shown that *HERC5* gene expression is upregulated in response to pro inflammatory stimuli and that HERC5 acts as an E3 ligase for ISG15, a ubiquitin like protein that is expressed upon the stimulation of cells with interferon [173 176] (note that in mice, the main ISG15 E3 ligase is Herc6 [177,178]). Indeed, the available evidence indicates that HERC5 plays an important role in the antiviral response [179 181]. Intriguingly, newly synthesized proteins appear to be the main target for the covalent attachment of ISG15 [181]. As HERC5 is associated with polyribosomes [181], this indicates that the "substrate specificity" of HERC5 is determined by its subcellular localization rather than by the recognition of defined amino acid se quences or regions/domains, and with respect to virus infected cells that viral proteins are eliminated before they reach a functional state.

3.3. "Other" HECT E3s

3.3.1. E6AP

E6AP is the founding member of the HECT family of E3s [11,16] and represents an impressive example for the notion that the dereg ulation of components of the ubiquitin conjugation system contrib utes to the development of human disease. In fact, E6AP, which is encoded by the *UBE3A* gene on chromosome 15q11 13, has been as sociated with three distinct disorders: cervical cancer, AS, and autism spectrum disorders (ASD) [182 186].

E6AP was originally isolated as an interacting protein of the E6 pro tein of the so called high risk human papillomaviruses (HPV) that have been etiologically associated with cervical cancer [182,187]. The inter action of E6AP with E6 has been fairly well characterized, and based on various lines of evidence including mouse models, it is commonly assumed that the ability of E6 to interact with E6AP is of critical rele vance for HPV induced carcinogenesis [182,188]. As there are several reviews on the E6 E6AP connection (e.g., [182,189,190]), we will not further discuss the role of E6AP in cervical carcinogenesis, except for noting that in complex with E6, E6AP targets proteins for ubiquitination and degradation (e.g., the tumor suppressor p53 [191]) that are normal ly not recognized by E6AP, thereby contributing to HPV induced cervi cal carcinogenesis.

In contrast to cervical cancer, where the unscheduled activation of E6AP contributes to disease development, development of AS is the re sult of the inactivation of E6AP [166,167,183,184,192,193]. As mentioned above (Section 3.2.2), AS is a genetic neurodevelopmental disorder with an incidence of 1 in 10,000 to 1 in 20,000 and was first described in 1965 [166,167]. AS is characterized by various features including mental retar dation, movement or balance disorders, characteristic abnormal behav iors, severe limitations in speech, and, in some cases, epileptic seizures [166,167]. Intriguingly, studies in mice revealed that the *Ube3a* gene encoding E6AP is biallelically expressed in most somatic cells, while the paternal *Ube3a* allele is silenced in cerebellar Purkinje cells, hippocampal neurons, and mitral cells of the olfactory bulb [194]. Indeed, all of the genetic abnormalities associated with AS development affect the mater nal allele, including deletion of the 15q11 13 region of the maternal chromosome, uniparental paternal disomy, and single point mutations in the maternal *UBE3A* gene, and result in the loss of E6AP expression or expression of mutated forms with a reduced E3 ligase activity [166,167,195]. Since this indicates that constitutive or transient increases in the expression level of substrate proteins of E6AP are critically in volved in AS development, identification and characterization of E6AP substrates do not only provide insight into the processes and pathways underlying AS but may also reveal potential targets for therapeutic strat egies. Several potential substrates of E6AP were reported, including HHR23A and HHR23B, AIB1, PML, α Synuclein, Ring1b, and ARC [196 201]. However, with the exception of ARC [201], the relevance of these interactions for AS development remains unclear.

Experiments with *Ube3A* knockout mice, which display AS like phenotypes, indicate that E6AP is critically involved in the control of synaptic function/plasticity, since for example, such mice have de fects in long term potentiation and impaired experience dependent maturation of the neocortex [202 204]. ARC, on the other hand, is commonly accepted as an important player in synaptic plasticity, in part by facilitating endocytosis of AMPA receptors [205,206]. Thus, the notion that stability, and thus levels, of ARC are regulated by E6AP provides a reasonable explanation for some of the AS features (in the absence of E6AP, ARC levels accumulate resulting in the

increased endocytosis of AMPA receptors and thus impaired synaptic function).

Finally, deregulation of E6AP activity has been associated with ASD [185,186], and experiments with transgenic mice indicate that the amplification of the *Ube3A* gene resulting in increased E6AP levels contributes to ASD [207]. Thus, decreases (AS) and increases (cervical cancer, ASD) in E6AP activity contribute to the manifestation of se vere pathologic conditions, indicating that at least in certain tissues, E6AP activity needs to be tightly regulated. However, with the poten tial exception of HERC2 (see 3.2.2), it is currently unknown how E6AP activity is contained. In this context, it should be noted that the trans genic mice carrying additional *Ube3A* alleles were engineered such that E6AP expressed from these alleles harbors an additional C terminal 3×FLAG tag (E6AP FLAG) [207]. However, fusing a C terminal extension (e.g., FLAG tag) to E6AP or the yeast HECT E3 Rsp5 was previously shown to result in ubiquitination defective proteins [208]. Thus, rather than increasing levels of active E6AP, ex pression of E6AP FLAG may interfere with the E3 function of endoge nous E6AP by acting as a dominant negative variant (in consequence, the ASD phenotype observed in the respective mice would be the result of loss of E6AP function rather than of gain of function). An al ternative but not mutually exclusive possibility is that the ASD phe notype is not related to the E3 function of E6AP. E6AP was reported to affect nuclear hormone receptor mediated transcription, and at least under certain conditions this property of E6AP is independent of its E3 activity [209,210]. Thus, it is conceivable that some of the neuronal functions of E6AP are mediated via nuclear hormone recep tors in a ubiquitin independent manner, while others require the E3 activity of E6AP.

3.3.2. HUWE1/UREB1/HECTH9/ARF BP1/MULE/E3Histone/LASU1

HUWE1 (also termed UREB1, HECTH9, ARF BP1, MULE, E3Histone, LASU1) is a giant protein consisting of 4374 amino acid residues and besides the HECT domain, contains a WWE domain and a BH3 domain (Fig. 1) [20,211 214]. A number of potential substrates for HUWE1 have been identified including p53 [212], histones [214], the anti apoptotic protein Mcl 1 [213], the proto oncoproteins c Myc and N Myc [215,216], the DNA replication regulatory protein Cdc6 [217], the DNA damage and replication checkpoint protein TopBP1 [218], the Myc associated protein Miz1 [219], the circadian heme receptor Rev erb alpha [220], the histone deacetylase HDAC2 [221], the DNA polymerases β and λ [222,223], and Mitofusin 2, an essential compo nent of the mitochondrial outer membrane fusion apparatus [224]. Accordingly, HUWE1 has been implicated in a number of (patho) physiological processes including cell proliferation and apoptosis, DNA repair, tissue homeostasis, and neuronal differentiation.

HUWE1 is overexpressed in various cancers suggesting that it pro motes cancerogenesis and that it may be a promising target for anti cancer therapies [215,225,226]. This notion is supported by the findings that HUWE1 targets the negative growth regulatory proteins p53 and Miz1 for degradation [212,219] and that HUWE1 activates c Myc by modifying c Myc with K63 linked ubiquitin chains (which serve non proteolytic roles) [215]. Furthermore, HUWE1 is negatively regulated by the human tumor suppressor p14ARF [212], which may play an important role in DNA damage repair [227], supporting the no tion that HUWE1 acts as a proto oncoprotein. On the other hand, HUWE1 appears to have pro apoptotic properties: (i) it targets the anti apoptotic protein Mcl 1 for degradation [213], (ii) upon cellular stress, Mitofusin 2 an essential component of the mitochondrial outer membrane fusion apparatus becomes a substrate for HUWE1 resulting in mitochondrial fragmentation and apoptosis [224], (iii) DNA damage induced apoptosis is severely compromised in fibroblasts derived from Huwe1 null mouse embryos [221], and (iv) human high grade gliomas carry hemizygous deletions of the *HUWE1* gene in association with the amplification of the *N MYC* locus [228].

Taken together, the data indicate that HUWE1 has both pro proliferative and anti proliferative properties and the eventual effect of HUWE1 activation or inactivation may depend on the respective tissue and/or additional events that affect HUWE1 function. Along these lines, while the actual importance of HUWE1 for p53 stability regulation in most tissues remains enigmatic (it is commonly as sumed that the RING domain protein Mdm2 represents the main E3 ligase for p53 [229]), data obtained with tissue specific Huwe1 knockout mice showed that Huwe1 is critical for maintaining homeo stasis of B lymphocytes and beta cells of the pancreas and that these roles require its ability to control p53 levels [230,231].

3.3.3. EDD/UBR5

Human EDD (*E*3 identified by *D*ifferential *D*isplay), also termed UBR5, is the ortholog of the *Drosophila h*yperplastic discs tumor sup pressor gene product HYD [232]. It consists of 2799 amino acid resi dues and contains an N terminal UBA domain (UBA domains bind to ubiquitin), a central UBR1 like zinc finger motif, and a PABC do main (a peptide binding domain found in *p*oly (*A*) *b*inding *p*roteins PABP) (Fig. 1) [233,234]. EDD is frequently overexpressed in breast and ovarian cancers [235,236]; however, whether EDD is etiologically associated with human disease, remains to be determined.

A number of potential EDD substrates and interacting proteins have been reported. Substrates include the DNA damage and replication checkpoint protein TopBP1 [237], Paip2 (which interferes with transla tion by displacing PABP from mRNA) [238], the proto oncoprotein beta catenin [239], the microtubule severing enzyme phospho katanin p60 [240], CDK9 (a subunit of the transcription elongation fac tor b) [241], the gluconeogenesis enzyme PEPCK1 [242], and the RING ubiquitin ligase RNF168 [243]; interaction partners include the tumor suppressor protein APC [244], the DNA damage checkpoint kinase CHK2 [245], the progesterone receptor [246], E6AP [247], and the HPV E6 oncoprotein [247]. Accordingly, EDD has been implicated in the reg ulation of various fundamental cellular processes including transcrip tion and translation, DNA damage response, gluconeogenesis, and cellular transformation. Finally, EDD has been reported to regulate miRNA mediated gene silencing but apparently in an E3 independent manner [248], further supporting the notion that at least some HECT E3s are multifunctional proteins.

3.3.4. TRIP12/ULF

ULF or TRIP12, a protein of 1992 amino acids, was originally iden tified as a protein that targets the tumor suppressor p14ARF, a key regulator of p53, for ubiquitination and degradation [249]. According ly, knockdown of ULF expression by RNA interference results in the p14ARF mediated activation of p53 in normal human cells as well as in acute myeloid leukemia cells derived from patients carrying mu tations in Nucleophosmin that ablate its ability to shield p14ARF from degradation [249,250]. The latter observation suggests that ULF may be a potential target in the treatment of AML patients. More recently, experiments with Tradd deficient mice revealed that independent of TNFR1 signaling (Tradd is a central adapter in the TNFR1 signaling complex), such mice are more prone to chemical induced carcino genesis than wild type littermates [251]. Intriguingly, Tradd was shown to modulate the interaction of ULF with p19Arf (the mouse ortholog of human p14ARF) resulting in enhanced p19Arf stability and supporting the notion that ULF is a critical regulator of p14ARF activity. Finally, like EDD, ULF has been reported to regulate the RNF168 mediated ubiquitination of histones upon DNA damage [243].

3.3.5. HACE1

The *HACE1* gene is located on chromosome 6q21 and genetic anal yses indicate that HACE1 has tumor suppressor function insofar as its expression is downregulated in different tumors including Wilms' tu mors and neuroblastoma [252 255]. Besides the findings that HACE1

targets Rac1, which is a critical regulator of cell motility and a target of numerous bacterial virulence factors, for ubiquitination [256,257] and that HACE1 is involved in Golgi biogenesis [258], nothing is known about HACE1 substrates and the cellular processes/pathways that are modulated by HACE1.

4. Conclusion

Since the discovery that HECT domain containing proteins have E3 activity in 1995 [11,16], members of the HECT family have been found to be involved in the regulation of various physiological and pathophys iological processes. However, despite all progress in the identification of the potential substrate proteins of individual HECT E3s, the physiologi cal relevance of many of these potential interactions remains unclear. This shortcoming may be explained by the notions that a given protein is not only recognized as a substrate by a single E3 but by several E3s (i.e. at least some E3s have an overlapping substrate spectrum) and that the E3 mediated ubiquitination of a given protein may be relevant for one tissue, but not for another, or only during certain phases of dif ferentiation and/or development. Thus, to determine whether a given protein represents a substrate for a given HECT E3 or not, biochemical experiments (i.e. binding and ubiquitination experiments) need to be combined with cell culture experiments and with the generation and analysis of conditional, tissue specific *HECT* knockout/knockin mice. Finally, one should keep in mind that as discussed above, at least some HECT E3s may be multifunctional proteins (i.e. having E3 dependent and E3 independent functions) and, thus, an important issue when studying HECT ligases is to determine whether the catalytic function of a HECT protein is indeed involved/required in the process studied.

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