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The Nedd4-like family of E3 ubiquitin ligases and cancer

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Abstract Accumulating evidence suggests that E3 ubiquitin ligases play important roles in cancer development. In this article, we provide a comprehensive summary of the roles of the Nedd4-like family of E3 ubiquitin ligases in human cancer. There are nine members of the Nedd4-like E3 family, all of which share a similar structure, including a C2 domain at the N-terminus, two to four WW domains in the middle of the protein, and a homologous to E6-AP COOH terminus domain at the C-terminus. The assertion that Nedd4-like E3s play a role in cancer is supported by the overexpression of Smurf2 in esophageal squamous cell carcinoma, WWP1 in prostate and breast cancer, Nedd4 in prostate and bladder cancer, and Smurf1 in pancreatic cancer. Because Nedd4-like E3s regulate ubiquitin-mediated trafficking, lysosomal or proteasomal degradation, and nuclear translocation of multiple proteins, they modulate important signaling pathways involved in tumorigenesis like TGFβ, EGF, IGF, VEGF, SDF-1, and TNFα. Additionally, several Nedd4-like E3s directly regulate various cancer-related transcription factors from the Smad, p53, KLF, RUNX, and Jun families. Interestingly, multiple Nedd4-like E3s show ligase independent function. Furthermore, Nedd4-like E3s themselves are frequently regulated by phosphorylation, ubiquitination, translocation, and transcription in cancer cells. Because the regulation and biological output of these E3s is such a complex process, study of the role of these E3s in cancer development poses some challenges. However, understanding the oncogenic potential of these E3s may facilitate the identification and development of biomarkers and drug targets in human cancer.

Keywords Nedd4 · WWP1 · Smurf · AIP4/Itch · E3 · Ubiquitination · Cancer

Abbreviations

AIP	atrophin-1 interacting protein
AR	androgen receptor
BMP	bone morphogenetic protein
CHX	cycloheximide
CIN85	Cbl-interacting protein of 85 KDa
CISK1	cytokine independent survival kinase 1
CXCR4	chemokine (C-X-C motif) receptor 4
DAT	dopamine transporter
E6-AP	E6 associated protein
HECT	homologous to E6-AP COOH terminus
EGFR	epidermal growth factor receptor
EMT	epithelial-mesenchymal transition
ENaC	epithelial sodium channel
EPS15	epithelial growth factor receptor substrate 15
FLIP	FLICE (pro-caspase-8)-inhibitory protein
GR	glucocorticoid receptor
Hrs	hepatocyte growth factor-regulated tyrosine kinase substrate
IGF-1R	insulin-like growth factor 1 receptor
KLF	krüppel-like factor
MEF	mouse embryonic fibroblasts
mUb	mono-ubiquitination
MVB	multi-vesicular bodies
Nedd4	the neural precursor cells-expressed developmentally down-regulated 4
NGF	nerve growth factor

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PR	progesterone receptor
RING	the really interesting new gene
SCF	Skp1-Cul1-F-box; SDF-1: stromal cell-derived factor-1
SGK	serum and glucocorticoid-inducible kinase
Smurf	Smad ubiquitin regulatory factor
SOD1	superoxide dismutases-1
TβR1	TGF-β receptor 1
Tiul1	TGIF interacting ubiquitin ligase 1
TNFα	tumor necrosis factor-alpha
TRAP-δ	translocon-associated protein δ
TSG	tumor suppressor genes
UIM	ubiquitin interaction motif
UPS	ubiquitin proteasome system

1 Introduction

1.1 Ubiquitination, E3 ligases, and cancer

Protein ubiquitination is a post-translational modification that can direct proteins for degradation by the 26S proteasome or plasma membrane proteins for endocytosis, sorting, and destruction in the lysosome. Ubiquitination can also mediatate proteolysis-independent effects like modulation of signal transduction, transcription, or DNA repair. The differing fates of ubiquitinated proteins depend on the length and architecture of ubiquitin chain [1]. Ubiquitin is an evolutionarily conserved 76 amino acid protein that is covalently conjugated to target proteins. K48-linked polyubiquitin chains are well known to function in protein degradation by the 26S proteasome. K63-linked polyubiquitin chains have been proposed to function in DNA repair, IκB kinase activation, translational regulation, and endocytosis [2]. Mono-ubiquitination (mUb) has been implicated in the endocytosis and trafficking of plasma membrane proteins [3]. Protein ubiquitination is generally catalyzed by the sequential activity of three enzymes: a ubiquitin activation enzyme (E1), ubiquitin conjugation enzymes (E2s), and ubiquitin ligases (E3s). It has been well established that E3s control the substrate specificity in this process. E3 ubiquitin ligases are important in cellular regulation because E3s specifically recognize a substrate for modification temporally and spatially. In mammalian cells, there are more than 500 E3s. Most single peptide E3s contain either a RING (the really interesting new gene) finger domain or a HECT (homologous to E6-AP COOH terminus) domain.

E3 ligases could be attractive targets for cancer therapy because of their substrate specificity. FDA approval of the general proteasome inhibitor, Velcade, for the treatment of multiple myeloma speaks to the promise of targeting the ubiquitin-proteasome system in anti-cancer therapy. However, proteasome inhibitors are not specific to cancer cells, and thus have obvious side effects. The E3 ligases could be better targets for cancer therapy due to their specificity [4]. Indeed, frequent genetic alterations and aberrations in the expression of E3s have been documented in human breast cancer [5]. Many E3s have been identified as either tumor suppressors (i.e., BRCA1 and Fbw7) or oncoproteins (i.e., Mdm2 and Skp2).

1.2 Structures and members of the Nedd4-like family

The *neural precursor cells-expressed developmentally down-regulated 4* (*Nedd4*) gene encodes a HECT type E3 ligase with three functional domains: an N-terminal C2 domain for membrane binding, a central region containing WW domains for protein–protein interaction, and a C-terminal HECT domain for ubiquitin protein ligation [6] (Fig. 1). The C2 domain is a calcium-binding domain that is approximately 120 amino acids in length. Upon Ca^{2+} binding, the C2 domain can bind to phospholipids, inositol polyphosphates, and some proteins [7, 8]. WW domains are 35–40 amino acid long domains containing two conserved tryptophan (W) residues spaced 21 amino acids apart. WW domains interact with PY(PPXY) motifs or phospho-serine/threonine residues in substrate proteins [9]. The HECT domain is comprised of ~350 residues and is responsible for ubiquitin transfer from a conserved cysteine residue to a lysine residue in a substrate protein. Protein structures for C2, WW, and HECT domains have also been solved separately (reviewed in [10]).

Since the original discovery of the *Nedd4* gene, eight related proteins, including Nedd4–2/Nedd4L, WWP1/Tiul1, WWP2, AIP4/Itch, Smurf1, Smurf2, HecW1/NEDL1, and HecW2/NEDL2 (Table 1) have been identified in human and the mouse. Although the similar structure and expression patterns of the Nedd4 family members suggest functionally redundant roles for these proteins, recent studies have begun to define specific functions for individual members. The evolution of Nedd4-like E3s has been described in three excellent review papers [11–13]. Here we mainly focus on their potential roles in cancer.

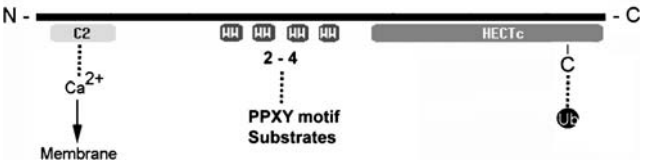

Fig. 1 Schematic diagram showing the structural organization of the nine mammalian Nedd4-like E3 ubiquitin ligases. The C2 domain translocates the protein to the membrane upon calcium binding. The two to four WW domains are known to bind substrate proteins containing PY motifs. The catalytic cysteine within the HECT domain is responsible for ubiquitin transfer

2 Genetic aberrations and alterations in expression of the Nedd4-like E3s in human cancer

To date, the genetic data suggest that several Nedd4-like E3s could function as oncogenic proteins. Smurf2, WWP1, Nedd4, and Smurf1 have all been found to be overexpressed in cancer cells (Table 1).

2.1 Smurf2

Smurf2 has been implicated in suppressing TGFβ signaling by targeting Smad2 and TGFβ receptor 1 (TβR1) for ubiquitin-mediated degradation [14, 15] (see below in detail). From immunohistochemical staining, Fukuchi et al. found that Smurf2 protein expression in esophageal squamous cell carcinoma correlated with lack of Smad2 phosphorylation, depth of invasion, lymph node metastasis, and survival [16]. Thus, increased Smurf2 expression results in more aggressive esophageal squamous cell carcinoma and a poorer prognosis. While TGFβ is known to inhibit epithelial cell proliferation, it also stimulates proliferation of fibroblasts. Consistent with this, when Smurf2 is overexpressed in fibroblasts, TGFβ signaling is inhibited and a senescent phenotype results [17].

2.2 WWP1

The *WWP1* gene maps to 8q21, a region that frequently displays a gain of copy number in human cancers, including prostate and breast cancer. The copy number of *WWP1* was shown to increase in 44–51% of prostate and breast cancer cell lines [18, 19] Consistent with this observation, the amplification of the *WWP1* gene was confirmed in 31–41% of primary tumors. Although *WWP1* was also mutated in two prostate cancer samples, the frequency is relatively low. In agreement with the reported gene copy number gain, the expression of WWP1 is also upregulated at the mRNA and protein levels in 58–60% of cancer samples from the prostate and breast. The overexpression of WWP1 significantly correlated with the gene copy number gain in both cancer types [18, 19] These findings strongly suggest that WWP1 gene could be an oncogene in prostate and breast cancer.

Table 1 The Nedd-like E3 ubiquitin ligases and cancer

E3	Gene locus	Alterations in cancer	Substrates	Function	Expression
Nedd4	15q21	Overexpression in prostate and bladder cancer	ENaC, pTEN, IGF-1R, p63, VEGF-R2, Cbl-b, EPS15, Hrs	Hypertension, genome integrity	Kidney, liver, muscle, brain, and heart
Nedd4–2/ Nedd4L	18q21	Alternative splicing in prostate and breast cancer cell lines	ENaC, TrKA, TβR1, Smad2, Smad4, EPS15	Hypertension, TGFβ signaling, apoptosis	Kidney, testis, liver, lung, brain, and heart
WWP1/Tiul1	8q21	Amplification and overexpression in prostate and breast cancer, alternative splicing	TβR1, Smad2, 4, KLF2, KLF5, RUNX2 p53, EPS15	TGFβ signaling, apoptosis, bone development	Heart, muscle, placenta, kidney, liver, pancreas and testis
WWP2	16q22.1		OCT4, ENaC		Heart, brain, placenta, muscle, and pancreas
AIP4/Itch	20q11.22-q11.23		NF-E2, JunB, c-Jun, p63, p73, RNF11, Cbl, EPS15, Hrs, endophilinA1, CXCR4, Notch, C-FLIP$_L$, HEF1, Gli	TGFβ signaling, apoptosis, immuno cell differentiation	Heart, kidney, brain, lung, spleen, testis, liver, placenta, muscle, and pancreas
Smurf1	7q21.1-q31.1	Amplified and overexpressed in pancreatic cancer	MEKK2, RUNX2, RUNX3, Smad1-5, RhoA	Cell motility, bone development TGFβ/ BMP signaling	Placenta, pancreas, and testis
Smurf2	17q22-23	Overexpressed in esophageal squamous cell carcinoma	TβR1, SnoN, Smad1, 2, 4, 5, RUNX2, RUNX3, RNF11	TGFβ signaling, Senescence, bone development	
HecW1/NEDL1	7p14.1-p13	Differential expression in neuroblastoma	SOD1, Dvl1	Protein quality control, Wnt signaling	
HecW2/NEDL2	2q32.3-q33.1		p73		

Functional analysis of WWP1 further supports the notion that WWP1 promotes prostate and breast epithelial cell proliferation and survival [18, 19]. RNAi mediated *WWP1* knockdown significantly suppressed PC-3, BT474, MCF7, and HCC1500 cancer cell proliferation. In the latter two breast cancer cell lines, WWP1 inhibition caused significant amounts of cell apoptosis [19]. Our results are consistent with two previous reports that shRNA directed against WWP1/Tiul1 (TGIF interacting ubiquitin ligase 1) sensitizes kidney-derived MDCK cells to TGFβ-induced growth arrest [8] and WWP1 siRNA decreased cell viability of kidney-derived HEK293T cells [20]. In contrast, forced overexpression of WWP1 in two immortalized breast epithelial cell lines, MCF10A and 184B5, enhances cell proliferation [19]. Interestingly, WWP1 appears to promote cell proliferation in a manner independently of ligase activity [19].

CeWWP1 is essential for *Caenorhabditis elegans* development [21]. However, *Wwp1* knockout mice are viable and fertile with no gross or histological abnormalities, suggesting that there is functional redundancy among members of the Nedd4 family. In support of this hypothesis, the combination of loss-of-function *Wwp1* and *Itch* results in postnatal lethality within 72 h of birth due to lung hemorrhage (Matesic et al., manuscript in preparation). Since *WWP1* is amplified and overexpressed in prostate and breast cancer, tissue specific transgenic mouse models may better mimic the genetic alteration of *WWP1* in human cancer.

Similar to the genetic alteration of *WWP1* in breast and prostate carcinomas, the *Smurf1* gene, mapping to 7q21.1-31.1, was reported to be amplified and overexpressed in pancreatic cancer [22, 23]. Most recently, Nedd4 was specifically shown to be upregulated in invasive bladder cancer and to negatively regulate pTEN activity [24] (See below in detail).

3 The Nedd4-like E3s regulate membrane growth factor receptors

The first substrate identified for Nedd4 was the epithelial sodium channel (ENaC), which plays an important role in hypertension [25]. The regulation of ENaC by Nedd4-like E3s has been studied extensively. *Nedd4–2* null mice develop hypertension due to the lack of ENaC downregulation [26]. A growing body of evidence suggests that Nedd4-like E3s also regulate endocytosis and degradation of multiple growth factor receptors, including insulin-like growth factor 1 receptor (IGF-1R), vascular endothelial growth factor receptor 2 (VEGF-R2), chemokine (C-X-C motif) receptor 4 (CXCR4), Notch, TβR1, epidermal growth factor receptor (EGFR), and the Trk receptor. These growth factor receptors play important roles in tumorigenesis. The deregulation of these receptors results in cell transformation and invasive growth.

It is well established that many growth factor receptors undergo ligand-dependent endocytosis and degradation in lysosomes. This is one of the most important cellular mechanisms to prevent continuous receptor activation in response to growth factor ligands. Receptors are usually recruited to clathrin-coated pits and internalized into the early endosome. Internalized receptors are either recycled back to the plasma membrane or sorted to multi-vesicular bodies (MVB) and subsequently to the lysosome for degradation. It is the ubiquitin moiety that serves as a sorting signal for receptor endocytosis and sorting (reviewed in Hicke and Dunn [3]).

3.1 The Nedd4-like E3s regulate the degradation of multiple membrane receptors

3.1.1 TGFβ receptor 1

Smurf1, Smurf2, WWP1, Nedd4–2, and AIP4 have been found to negatively regulate the TGFβ signaling pathway (reviewed in Izzi and Attisano [27]). It is well documented that the TGFβ signaling pathway regulates epithelial cell proliferation, differentiation, migration, and apoptosis [28]. Transformed epithelial cells usually lose sensitivity to TGFβ-induced inhibition. In contrast, TGFβ can promote cancer cell invasion and metastasis at late stages. Several Nedd4-like E3s induce ubiquitination and degradation of TβR1, a receptor serine/threonine kinase. Since TβR1 does not have a PY motif, Smad7 was reported to function as an adaptor for Smurf1 [29, 30], Smurf2 [15], Nedd4–2 [31], and WWP1 [32] to recruit TβR1 for ubiquitination. Recently, AIP4 and WWP1 were demonstrated to promote formation of Smad7/TβR1 complex through a ubiquitin-independent mechanism [33]. In parallel, Smurf1 also targets bone morphogenetic protein (BMP) type 1 receptor for degradation [34]. BMP receptors are known to utilize some of the same Smads for signal transduction. These Smads are also targeted by Nedd4-like E3s for ubiquitin-mediated proteasomal degradation (See below in detail).

3.1.2 Notch receptors

AIP4/Itch was documented to negatively regulate the Notch1 receptor. Notch is an important transmembrane receptor that regulates cell fate and oncogenesis. Misregulation of Notch toward either direction is associated with tumor formation [35, 36]. Upon ligand binding, the Notch receptor is cleaved and the soluble cytoplamic domain translocates into the nucleus to activate gene transcription. Recent observations have demonstrated the importance of Notch endocytosis and degradation by multiple ubiquitin ligases. Itch was first shown to mono- and polyubiquitinate Notch1 [37]. Itch can directly bind to the N-terminal region

of the Notch1 receptor, which has no PY motif [37]. Interestingly, Itch-mediated Notch ubiquitination and degradation can be enhanced by Numb [38]. Further, it was recently discovered that the increased amounts of full length Notch1 found in *itchy* mice can generate an AKT-cell survival signal that contributes to the genesis of autoimmune disease [39]. This increase in full length Notch1 was specific to the activity of Itch, as similar changes in Notch1 levels were not observed in loss-of-function Wwp1 animals (LE Matesic, NG Copeland, and NA Jenkins, unpublished observations). Accumulating evidence from *C. elegans* and *Drosophila* also support the hypothesis that Nedd4-like E3s inhibit Notch signaling. CeWWP1 has been shown to promote Notch degradation but not internalization [40]. In Drosophila, dNedd4 and Su (dx) were shown to negatively regulate Notch by directly ubiquitinating the receptor and altering the amount of full length Notch available at the cell surface [41]. In addition to the Notch receptor, Itch was reported to promote ubiquitination and lysosomal degradation of DTX, a RING finger E3 that has been shown to promote Notch signaling in certain cellular contexts [42].

3.1.3 Insulin-like growth factor 1 receptor

Nedd4 was reported to downregulate IGF-1R [43]. IGF-1R has been implicated in the initiation and development of many different human cancers [44]. It is also a promising drug target because many tumor cells undergo apoptosis when the IGF-1R is downregulated [45]. Vecchione and colleagues found that Nedd4 forms a complex with Grb10 and IGF-1R in mouse embryo fibroblasts (MEF). This interaction is mediated by the SH2 domain of Grb10 and the C2 domain of Nedd4 [46]. Interestingly, IGF-1R, but not Grb10, was shown to be ubiquitinated, suggesting that Grb10 serves as an adaptor to mediate the interaction between Nedd4 and IGF-1R. In support of this notion, overexpression of a catalytically inactive form of Nedd4 decreased IGF-1R ubiquitiantion and degradation upon IGF-1 stimulation [43].

3.1.4 Vascular endothelial growth factor receptor 2

Nedd4 was also demonstrated to downregulate VEGF-R2 [47]. VEGF is an important angiogenic factor which stimulates cell proliferation, migration, and angiogenesis [48]. Binding of VEGF to the tyrosine kinase receptor VEGF-R2 induces receptor dimerization, autophosphorylation, and signaling. VEGF-R2 is also ubiquitinated in response to VEGF. Overexpression of Nedd4 was shown to promote VEGF-R2 degradation [47]. Murdaca et al., proposed that Nedd4 indirectly regulates the ubiquitination of VEGF-R2 in response to VEGF because ectopic overexpression of a catalytically inactive form of Nedd4 does not inhibit VEGF-R2 ubiquitination [47]. Interestingly, Grb10 was confirmed to interact with Nedd4 in this study. In contrast to the role of Grb10 in the ubiquitination of IGF-1R by Nedd4, overexpression of Grb10 suppressed the degradation of VEGF-R2 by Nedd4. However, whether Nedd4 regulates IGF-1R and VEGF-R2 degradation under physiological conditions remains to be determined.

3.1.5 Chemokine (C-X-C motif) receptor 4

AIP4 was shown to downregulate CXCR4 [49]. Binding of stromal cell-derived factor-1 (SDF-1) to the G protein coupled receptor CXCR4 activates a variety of intracellular signal transduction pathways that promote cell survival, proliferation, chemotaxis, migration and adhesion [50]. Overexpression of CXCR4 in tumor cells is strongly associated with increased metastatic potential [51]. AIP4, but not Nedd4 and Nedd4–2, specifically promotes SDF-1 induced CXCR4 mUb and lysosomal degradation [49]. It was proposed that AIP4 indirectly regulates CXCR4 through hepatocyte growth factor-regulated tyrosine kinase substrate (Hrs) and Vps4 [49].

3.1.6 Epithelial growth factor receptors

There is some data suggesting that Nedd4-like E3s can regulate EGFRs, although these data are far from conclusive. It is well established that the abnormal activation of the EGF pathway is a common theme in epithelial cancer [52]. Although genetic amplification and mutation are involved in over-activation of the EGFR family, misregulation of the degradation of this family also contributes to cancer development. It has been shown that Nedd4 inhibits EGF-independent EGFR endocytosis and degradation through its antagonization of Cbl-b, a RING E3 that normally functions to monoubiquitinate the EGFR and target it for turn over by the lysosome [53]. It has also been proposed that Nedd4 regulates ligand-independent EGFR degradation by ubiquitinating Hrs [54]. Thus, by these mechanisms, Nedd4 effectively increases the amount of EGFR available at the cell surface, and lowers the required signaling threshold. AIP4/Itch has also been shown to interact with Cbl-c [53, 55]. Angers et al., found that Itch regulates EGFR by ubiquitinating endophilin A1 [56]. In contrast, Marchese and colleagues observed that AIP4 siRNA has no effect on EGF induced EGFR endocytosis and degradation in HeLa cells [49]. Most recently, Itch was shown to target Erbb4 for degradation [57].

In addition to the receptors noted above, nerve growth factor (NGF)-dependent TrkA receptor [58] was reported to be a substrate of Nedd4–2. Nedd4–2 was demonstrated to constitutively bind to an unphosphorylated PY motif on

TrkA through its WW domains. Interestingly, Nedd4–2 overexpression caused apoptosis of NGF-dependent neurons.

The data reviewed here indicate that a number of receptors or membrane proteins are regulated by Nedd4-like E3s. Some of these results require further validation since the overexpression of Nedd4-like E3s can induce profound consequences on the intracellular trafficking machinery (see below). It is therefore essential that the results obtained from such experiments be validated with RNAi and *in vivo* approaches. Further, there is the consideration of how the Nedd4-like E3 function in a given cellular context. Signaling pathways targeted by the Nedd4-like E3s, including the TGFβ and Notch pathways, play context-dependent roles in tumorigenesis. Therefore, the action of an individual Nedd4-like E3 in cancer could also be context-dependent.

3.2 The Nedd4-like E3s regulate the endocytosis machinery

The molecular determinants mediating the recruitment of various Nedd4-like E3 to different membrane receptors are not clear. Some Nedd4-like E3s have been demonstrated to directly ubiquitinate growth factor receptors. However, under normal conditions, Nedd4-like E3s do not directly interact with these receptors. Nedd4-like E3s have been shown to ubiquitinate multiple members of the endocytosis machinery such as Cbls, Endophilin A1, epithelial growth factor receptor substrate 15 (EPS15), Hrs, and N4WBPs.

3.2.1 Cbls

The RING finger E3 ligases Cbl-b and Cbl-c are regulated by Nedd4 and AIP4 [53, 55]. The endocytosis and degradation of EGFR have been extensively studied. Binding of EGF to EGFR results in dimerization and autophosphorylation of several tyrosine residues in the cytoplasmic tail of the EGFR. The phosphorylation of EGFR at Tyr1045 provides a binding site for the SH2 domain of the Cbl E3 ligases [59]. Subsequently, Cbls are phosphorylated and activated to monoubiquitinate the EGFR. This step was proved to be necessary and sufficient for EGFR endocytosis and degradation. Additionally, Cbls are negative regulators of several receptor tyrosine kinases such as PDGFR [60] and c-Met [61]. Nedd4 was reported to directly ubiquitinate Cbl-b and target it for proteasomal degradation. This has the effect of concomitantly blocking Cbl-b mediated EGFR endocytosis and degradation [53].

3.2.2 Endophilin

The endophilin-Cbl-interacting protein of 85 KDa (CIN85)-Cbl complex is required for ligand-stimulated receptor internalization [61, 62]. Angers et al. found that AIP4 interacts with and ubiquitinates Endophilin A1 at the endosome upon EGF treatment [56]. This finding is also supported by a study in yeast. Rvs167p and Sla1p, orthologues of the mammalian endophilin and CIN85 proteins, were shown to be ubiquitinated by Rsp5p (the only ortholog of the mammalian Nedd4-like E3s in yeast *Saccharomyces cerevisiae*) [63]. Ubiquitination of Endophilin and CIN85 may affect rapid EGFR internalization because these proteins play an important role in regulating clathrin-coated vesicle budding.

3.2.3 Epithelial growth factor receptor substrate 15

EPS15 is regulated by multiple Nedd4-like E3s. It has been reported that ubiquitinated EGFR can be recognized by the ubiquitin-binding protein EPS15 or by Epsin, which promotes EGFR internalization to the early endosome [64]. Interestingly, EPS15 and Epsin undergo mUb mediated by their own ubiquitin interaction motif (UIM) [65, 66]. Recently, mUb was proposed to inactivate the function of EPS15 since the intermolecular interaction between ubiquitin and the UIM supplants the association between the ubiquitinated receptor and the UIM [67]. Nedd4, Nedd4–2, AIP4, and WWP1, but not WWP2, were reported to ubiquitinate EPS15 [66]. We found that WT WWP1, but not the C890A mutant, specifically increased EPS15 ubiquitination (Fig. 2). EPS15 does not contain a PY motif. Instead, it was proposed that the ubiquitination of an E3 ligase itself provides a platform for EPS15 binding to the E3 through their UIM domain [66]. Most recently, the E3 ligase Parkin was reported to mono-ubiquitinate EPS15 and delay EGFR degradation [68]. The Nedd4-family of E3s may also decrease EGFR endocytosis through a similar mechanism, although this hypothesis awaits more direct experimental proof.

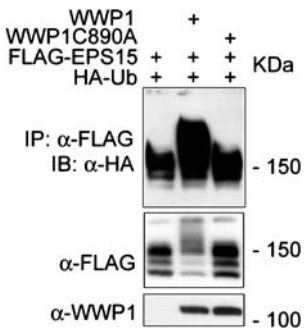

Fig. 2 WWP1 ubiquitinates EPS15. WT or catalytic inactive WWP1C890A were co-transfected with FLAG tagged EPS15 (kindly provided by Dr. Ivan Dikic) and HA tagged Ub into HEK293T-derived LinX cells in 60-mm plates. Cell lysates were immunoprecipitated with FLAG-M2 beads (Sigma) under denaturing conditions and detected using the anti-HA antibody. Five percent of the cell lysate used in the immunoprecipitation was loaded on a gel for Western blot analysis with anti-FLAG and anti-WWP1 antibodies

3.2.4 Hepatocyte growth factor-regulated tyrosine kinase substrate

In addition to the Cbls, Endophilin, and EPS15, Nedd4 and AIP4 have also been shown to ubiquitinate Hrs, another ubiquitin-binding protein [49, 54]. Upon delivery to early endosome, the EGFR is either recycled back to the plasma membrane or sorted to MVB and then the lysosome. Hrs was found to promote the sorting of EGFR and CXCR4 to the lysosome for degradation [49, 54].Similar to EPS15, mUb of Hrs by Nedd4 or AIP4 may cause the loss of Hrs endocytic activity by the intramolecular interaction between the ubiquitin and UIM. Interestingly, yeast two hybrid analysis demonstrated that Hrs does not interact with WWP1 and WWP2 [20], indicating a specificity in this effect.

3.2.5 N4WBPs

Furthermore, Nedd4-like E3s may regulate several other proteins in the MVB or Golgi network such as N4WBP4/PMEPA1 [69], N4WBP5 (Ndfip1) [70], and N4WBP5A (Ndfip2) [71]. All of these Nedd4-interacting proteins contain PY motifs. Nedd4, Nedd4–2, WWP2, AIP4, but not Smurf1, were shown to interact with N4WBP5A [71]. N4WBP5A protein was shown to regulate EGFR endocytosis in HeLa cells [71]. Additionally, Nedd4 and Nedd4–2 ubiquitinate N4WBP5A but do not target N4WBP5A for degradation. Interestingly, the expression of N4WBP4/PMEPA1 is induced by androgen and downregulated in prostate cancer, and its tumor suppressor function requires the PY1 motif [69]. It was proposed that PMEPA1 serves as an adaptor that allows Nedd4 to recruit androgen receptor (AR) for ubiquitination and degradation [69]. This negative regulatory loop may be disrupted in prostate cancer due to the loss of expression of PMEPA1 [72]. Whether Nedd4-like E3s regulate receptors by ubiquitinating N4WBP proteins under physiological conditions needs to be investigated further.

AIP4 and Smurf2 were found to interact with the RING finger E3 ligase RNF11 [73, 74]. RNF11 has also been found to localize to MVB and to promote EGFR endocytosis and degradation [75]. Thus, we speculate that Nedd4-like E3s regulate receptor trafficking via multiple mechanisms by targeting proteins involved in endocytosis such as Cbls, EPS15, Hrs, N4WBP5, or RNF11. Importantly, these lines of evidence imply that Nedd4-like E3s upregulate EGFR. However, direct experimental evidence in support of this hypothesis is still lacking. Regardless of the mechanism, it is well documented that impaired downregulation of growth factor receptors is strongly associated with cancer [76]. It will be interesting to see whether Nedd4-like E3s promote cancer development by regulating the degradation of multiple growth factor receptors.

4 Regulation of cancer-related transcription factors

Multiple cancer-related transcription factors are regulated by the Nedd4-like E3s through ubiquitin-mediated degradation and relocalization. Since transcription factors regulate the transcription of numerous genes, the abnormal regulation of those same factors plays an important role in tumorigenesis. The first hint that Nedd4-like E3s regulate transcription factors came from the observation that the localization of these E3s is shuttling from the cytoplasm to the nucleus. In recent years, Nedd4-like E3s have been demonstrated to regulate multiple transcription factor families, such as Smad, p53, krüppel-like factor (KLF), RUNX, and Jun.

4.1 Smad family

It is well established that Smad proteins play crucial roles in the TGFβ and BMP signaling pathways. The activated TGFβ receptors phosphorylate Smad2 and Smad3. Then, phosphorylated Smad2 and Smad3 form complexes with Smad4 and translocate into the nucleus to regulate gene transcription. The TGFβ pathway plays an important role in tumor development. Consistent with this hypothesis, Smad4 is frequently inactivated in a variety of cancer types [77].

Nedd4-like E3s target several Smad proteins for ubiquitin-mediated degradation. All Smads except for Smad4 and Smad8 contain a PY motif. Not surprisingly, Smurf1, Smurf2, and WWP1 directly interact with all Smads (except Smad4 and Smad8) with slightly different affinities [8, 32]. Smurf1 and Smurf2 have been reported to target Smad1, Smad2, and Smad5 for degradation [14, 78–81]. WWP1 has also been demonstrated to promote Smad2 ubiquitin-mediated degradation [8, 82]. Komuro *et al.* found that WWP1 can ubiquitinate Smad6 and Smad7 but not Smad2 [32]. In fact, Smad2 ubiquitination by WWP1/Tiul1 requires TGIF and TGFβ signaling [8]. Thus, Nedd4-like E3s strongly inhibit the TGFβ and BMP signaling pathways by targeting Smads for degradation.

Several Smad proteins have been reported to serve as adaptors for Nedd4-like E3s in the targeting of substrates for ubiquitination. For example, Komuro and colleagues demonstrated that Smad7 helps WWP1 and Smurf1 recruit TβR1 [8, 32]. However, whether the Smad7 adaptor protein is ubiquitinated by WWP1 or not is controversial [8, 32]. Smad7 was also demonstrated to function as an adaptor for Smurf2 in targeting β-catenin for degradation [83]. Similar to what has been observed with Smad7, Moren et al. found that Smad2 functions as an adaptor for WWP1 in the ubiquitination of Smad4 [8, 84]. The use of Smads as adaptors has also been noted with other members of the Nedd4 family of E3 ligases. For instance, Smurf2 uses Smad2 as an adaptor to target SnoN [85]. Similarly, Smurf1

and WWP1 use Smad6 as an adaptor to target RUNX2 for degradation [86]. Itch uses Smad3 to target Cas^{130} family protein HEF1 for degradation [87]. The usage of adaptor proteins makes the regulation of the TGFβ pathway more fine-tuned and also expands the repertoire of substrates for Nedd4-like E3s.

4.2 p53 family

The p53 transcription factor is the most extensively studied tumor suppressor in human cancer. p53 activation up-regulates growth arrest- and apoptosis-related genes in response to stress signals, thereby leading to either cell cycle arrest, senescence, or apoptosis [88]. Two p53 homologues, p63 and p73, have overlapping and distinct functions in the regulation of gene expression [89]. Indeed, both p73 and p53 can be induced by DNA damage and activate some common genes to suppress growth or induce apoptosis. Accumulating evidence supports the idea that the full-length transactivation (TA) isoforms (i.e., those containing the TA domain) of p63 and p73 have pro-apoptotic properties, whereas the ΔN isoforms of p63 and p73 generally have anti-apoptotic properties [90]. The ΔN isoforms of p63 and p73 have also been shown to suppress cell proliferation [91, 92]. The p53 family of transcription factors are tightly regulated by multiple mechanisms including ubiquitin-mediated degradation and subcellular relocalization [90, 93, 94].

WWP1 was shown to ubiquitinate p53, the major form being mUb [95]. It is well established that Mdm2 can mono-ubiquitinate p53 which results in the exportation of nuclear p53 to the cytoplasm [96]. Nuclear export of p53 can also be mediated by WWP1 [95]. Consequently, p53 transcriptional activity is inhibited by WWP1, despite elevations in the p53 protein level [95].

The Nedd4-like E3 NEDL2/HecW2 was identified as the first E3 that binds and ubiquitinates p73 via a WW/PY interaction [97]. However, NEDL2 does not bind to p53, which lacks the PY motif. NEDL2 has been shown to stabilize p73 and increase its transcriptional activity although the precise mechanism by which this occurs remains unclear [97]. It was also reported that Itch targets p73 for ubiquitin-mediated proteasomal degradation [98, 99]. In contrast to NEDL2, Itch negatively regulates the transcriptional activity of p73 [98]. It was proposed that Itch is rapidly downregulated upon DNA damage, allowing the TA-p73 protein to accumulate and induce cell growth arrest and apoptosis [98]. Recently, YAP1 was demonstrated to stabilize p73 by displacing Itch binding to p73 [100].

In addition to p53 and p73, p63 activity has also been shown to be modulated by Nedd4 [101] and Itch [102–104]. Itch interacts with TA-p63 and ΔNp63 via a WW/PY interaction and targets them for ubiquitin-mediated proteasomal degradation [103]. However, whether Nedd4-like E3s modulate tumor development through the regulation of the abundance of various p63 and p73 isoforms has not yet been determined.

4.3 KLF family

KLF is a transcription factor family which consists of over 20 members in humans, and is structurally characterized by three tandem zinc-finger domains at the C-terminus [105, 106]. The members of this family form a network that regulates a diverse range of biological processes, including cellular proliferation, cell cycle, apoptosis, differentiation, and angiogenesis [105, 106]. Several members of the KLF family, such as KLF2 [107] and KLF5 [108], have been implicated in the development of a variety of human cancers.

In 2001, work from Jerry Lingrel's laboratory first suggested that WWP1 inhibits the transcriptional activity of KLF2 [109]. KLF2 inhibits cell growth [107], angiogenesis [110], and sensitizes cells to DNA damage-induced apoptosis [111]. Further investigation revealed that WWP1 targets KLF2 for ubiquitin-mediated proteasomal degradation. The inhibitory domain of KLF2 is essential for interacting with WWP1 because KLF2 has no PY motif. Surprisingly, WWP1 promotes KLF2 ubiquitination independent of E3 ligase activity and may serve as an adaptor to recruit another E3 ligase.

Recently, KLF5 was demonstrated to be a substrate of WWP1. KLF5 is a key regulator of embryonic development, tissue remodeling, angiogenesis, adipoctye differentiation, epidermal development and tumorigenesis. The KLF5 protein turns over rapidly via the ubiquitin-proteasome pathway [112]. This is mediated by WWP1, which specifically interacts with KLF5 via the PY motif of KLF5 and targets KLF5 for proteasomal degradation [113]. Interestingly, the expression of WWP1 is negatively correlated with KLF5 protein expression in human prostate and breast cancer. Although the exact role of KLF5 in cancer remains to be elucidated, these findings suggest that WWP1 may contribute to tumorigenesis through promoting KLF5 ubiquitination and degradation.

4.4 RUNX family

The three mammalian Runt homology domain transcription factors (RUNX1, RUNX2, RUNX3) control genes involved in the differentiation of distinct tissues. The RUNX family can also function as cell context-dependent tumor suppressors or oncogenes. Both RUNX2 and RUNX3 contain conserved PY motifs.

RUNX2 is a bone-specific transcription factor that functions in regulating bone development and metastasis

[114]. RUNX2 is frequently overexpressed in invasive breast and prostate cancer [114]. Smurf1, Smurf2, and WWP1 were reported to target RUNX2 for ubiquitin-mediated degradation via either WW/PY motifs or a Smad6 adaptor [86, 115, 116]. WWP1 was demonstrated to regulate RUNX2 *in vivo* [117]. Another adaptor protein Schnurri-3 was shown to enhance Runx2 ubiquitination by Wwp1 [117]. Transgenic mice that express Smurf1 specifically in osteoblasts show reduce bone formation during postnatal life due to a decrease in Runx2 [118]. However, Runx2 is not upregulated in Smurf1 null osteoblasts *in vivo* [119].

In contrast to RUNX2, *RUNX3* functions as a gastric tumor suppressor. The expression of *RUNX3* is lost in about 60% of primary gastric cancer specimens [120]. Both Smurf1 and Smurf2 have been demonstrated to promote RUNX3 ubiquitination and degradation [121]. Interestingly, p300 mediated RUNX3 acetylation protects RUNX3 from ubiquitin-mediated degradation [121].

4.5 Jun family

JunB and c-Jun were identified as direct targets of Itch [122, 123]. JunB has been shown to play an important role in T cell differentiation [124] while c-Jun may function in T cell activation [125]. The regulation of JunB and c-Jun by Itch is further refined by phosphorylation (detailed later) and by interaction with other proteins. Itch has recently been shown to immunoprecipitate with *N4WPBP5/Ndfip1* and to co-localize in T cells [126]. *Ndfip1*$^{-/-}$ mice have a similar phenotype to *itchy* mice, including severe inflammation and a Th2 bias in T cell differentiation. Further, levels of JunB are increased in *Ndfip1*$^{-/-}$ T cells, suggesting that Ndfip1 is required for efficient ubiquitination of JunB by Itch. Since Ndfip1 is a membrane-bound protein, it may act to recruit Itch to the appropriate subcellular compartment for Itch to exert its effects.

As targets of the TGFβ pathway [127], JunB and PAI1 are inhibited by WWP1/Tiul1 in MDCK cells through an indirect mechanism [8]. Work from the Zhang lab has demonstrated that JunB is upregulated in Smurf1 null osteoblasts [119]. However, an upstream kinase of JunB, MEKK2, was identified as the authentic, direct physiological target of Smurf1 but not Smurf2. MEKK2 interacts with Smurf1 via the PY motif of MEKK2 and first two WW domains of Smurf1. Interestingly, the auto-phosphorylation of MEKK2 is a prerequisite for Smurf1 mediated MEKK2 ubiquitination. Consistent with these observations, Smurf1-deficient mice exhibit an age-dependent increase of bone mass [119].

NF-E2 is a hematopoietic transcription factor containing two subunits. The large subunit p45 is homologous to c-Jun and bears PY motifs. The p45 protein was found to bind to WWP1 and Nedd4 [128]. Yeast two hybrid experiments showed that p45 could interact with Itch. Further analysis demonstrated that Itch might function as a transcriptional co-repressor of NF-E2 [129].

Additionally, Nedd4-like E3s may target other transcription factors for degradation. For example, Itch has been shown to ubiquitinate and degrade the transcription factor Gli1, which plays an important role in Hedgehog signaling in cell development and tumorigenesis [130]. As with the regulation of Notch, Numb can promote Itch-dependent Gli1 ubiquitination and degradation. Oct-4 is an important transcription factor that affects the fate of mammalian embryonic stem cells. WWP2 has been shown to promote Oct-4 ubiquitin-mediated proteasomal degradation [131]. Finally, Nedd4/hRPF1 has been shown to potentiate the transcriptional activity of nuclear receptors including progesterone receptor and glucocorticoid receptor [132].

5 Other substrates of the Nedd4-like E3s in cancer

5.1 Nedd4 and pTEN

pTEN is an important tumor suppressor protein in various types of cancers. The pTEN phosphatase negatively regulates PI3K/Akt signaling [133], which is critical for cancer cell survival. Additionally, nuclear localization of pTEN has been proposed to maintain genome stability and tumor suppression [134]. pTEN is frequently inactivated by gene deletion or mutation [135]. Intriguingly, pTEN is also ubiquitinated and degraded through the proteasome although it is a relatively stable protein [136]. Recently Nedd4 was identified as a specific E3 ligase for pTEN. Nedd4 not only targets pTEN for proteasomal degradation through polyubiquitination but also transports pTEN into the nucleus through mUb [137].

By decreasing the level of pTEN protein, Nedd4 was shown to promote Akt signaling [24]. Overexpression of Nedd4 collaborates with K-Ras to transform p53 deficient MEF. Additionally, *Nedd4* RNAi inhibited DU145 (pTEN positive) but not PC-3 (pTEN negative) xenograft growth. Consistently, the mRNA level of Nedd4 was shown to be upregulated in invasive bladder cancer samples [24]. Taken together, Nedd4 was proposed to be an oncoprotein and a potential target for pharmacological intervention in pTEN positive tumors.

5.2 HecW1/NEDL-1 and dishevelled-1

Miyazaki et al. identified a novel, differentially expressed Nedd4-like E3, NEDL1, in brain tumors [138]. *NEDL1* mRNA is preferentially expressed in neuronal tissue and is

highly expressed in neuroblastomas with favorable prognosis. Through yeast-two hybrid screening, NEDL1 was demonstrated to be associated with two proteins, dishevelled-1 (Dvl1) and translocon-associated protein δ (TRAP-δ), which bind to mutant but not WT superoxide dismutases-1 (SOD1). As a result, NEDL-1 targets mutant Dvl1 and SOD-1, but not TRAP-δ, for ubiquitiantion and degradation. Because TRAP-δ localizes to the ER, NEDL-1 was proposed to regulate protein quality control through an ER-associated degradation pathway [138]. Interestingly, Dvl1 is one of the key transducers in the Wnt/β-catenin signaling pathway. Thus, NEDL-1 may play a role in motor neuron differentiation and apoptosis through its regulation of Dvl1 degradation [138]. The role of NEDL1 in brain or other types of tumors remains to be elucidated.

5.3 Itch and apoptosis

Itch was demonstrated to target long FLICE (pro-caspase-8)-inhibitory protein (c-FLIP$_L$) for ubiquitin-mediated proteasomal degradation [139]. FLIP can be upregulated by NF-κB and specifically inhibits caspase-8 activation and tumor necrosis factor-alpha (TNFα) induced apoptosis [140]. Chang et al. found that c-FLIP$_L$ ubiquitination by Itch is regulated by JNK mediated Itch phosphorylation [139]. However, the work fails to identify the domain of Itch that interacts with the CASP domain of c-FLIP$_L$. This is important because c-FLIP$_L$ does not have a PY motif, yet no other Nedd4-like E3s have been reported to have similar function on c-FLIP$_L$ turnover.

Loss of function *Itch* (*itchy*) mice display a variety of immunological and inflammatory disorders as they age, including inflammation of the lung and stomach and hyperplasia of lymphoid and hematopoietic cells [141]. It was noticed that the *itchy* mice have a persistent itching behavior. Part of this can be attributed to the Th2 bias in differentiation of T cells from animals, which results in an allergic response with increased IgG1 and IgE levels in the serum, as well as eosinophil activation [122]. Additionally, it was reported that *itchy* mice have increased levels of full length Notch1 which may complex with PI3K and p56^{lck} in T cells. This results in increased amounts of phospho-AKT and decreased apoptosis in developing thymocytes [39]. These data speak to the fact that Itch likely plays a physiological role in mediating apoptosis through a variety of different pathways under the influence of a number of molecular signals.

5.4 Smurf1, RhoA, and invasion

Accumulating evidence points to the fact that Smurf1, but not Smurf2, promotes RhoA ubiquitination and degradation and regulates cell motility [142–145]. RhoA, Rac, and Cdc42 proteins belong to Ras superfamily of GTPases. RhoA binds and hydrolyzes the conversion of GTP to GDP. The GTP-bound form of RhoA is active. The active form regulates cell morphology, differentiation, migration, and division. Smurf1-mediated RhoA degradation is crucial for TGFβ-induced dissolution of tight conjunctions during epithelial-mesenchymal transition [143]. Boyer et al. demonstrated that the induced degradation of RhoA is blocked in Smurf1 null MEF cells and Smurf1 selectively ubiquitinates GTP-bound active RhoA [144]. However, total RhoA protein levels are not increased in Smurf1 knockout mice and Smurf siRNA transfected cells [119, 145]. Sahai et al. demonstrated that Smurf1-mediated RhoA degradation only occurs at the cell periphery [145]. Furthermore, Smurf1 inhibition in cancer cells increases cell motility and favors intravasation, but is not sufficient to promote metastasis *in vivo* [145].

5.5 Nedd4, Cdc25, and the cell cycle

The Pub1 E3 ligase from *Schizosaccharomyces pombe* is one of the three orthologues of mammalian Nedd4-like E3s. Pub1p was demonstrated to target Cdc25 for ubiquitin-mediated degradation [146]. Cdc25 (Cdc25A, Cdc25B and Cdc25C in mammals) is a key phosphatase regulating the cell cycle through its activation of the Cdks (reviewed in [147]). Cdc25A is ubiquitinated and degraded by the E3 ligase APC/C^{Cdh1} and possible other Skp1-Cul1-F-box E3 complexes [148]. Interestingly, Cdc25A contains a PY motif; and Cdc25C has been demonstrated to bind to the second WW domain of Nedd4 in HeLa cells [9]. It will be interesting to further investigate whether Nedd4 or other family members target Cdc25 for degradation since Cdc25 is frequently overexpressed in human cancer.

6 Ligase independent function of the Nedd4-like E3s

Although the ligase activity of Nedd4-like E3s is very important for their cellular functions, several lines of evidence suggest that Nedd4-like E3s may have ligase-independent functions as well. Overexpression of various Nedd4-like E3-derived WW domains strongly inhibits viral budding, a process requiring protein trafficking [20] Itch and WWP1 can inhibit TGFβ signaling independently of their ligase activities [33]. Smurf2 overexpression has been shown to arrest fibroblasts in a ligase- and C2 domain-independent manner [17]. Recently, we found that catalytically inactive WWP1 promotes breast epithelial cell proliferation as efficiently as the WT WWP1 [19]. These findings suggest that Nedd4-like E3s could regulate trafficking and other cellular processes independently of their ligase activities.

Because E3 ligase activity appears not to be necessary for the function of Nedd4-like E3s under some conditions, it is tempting to speculate that these E3s could affect the action of their partners through just binding. Several WW domain-containing proteins, such as WWOX and YAP, function by binding to PY motif-containing substrates [149]. Zhang et al. mapped the Smurf2 domains required for causing fibroblast senescence and found that although both the WW domains and the HECT domain contribute to Smurf2's senescence-inducing function, the E3 ligase activity is not required.

7 Regulation of the Nedd4-like E3s

7.1 Phosphorylation

AIP4/Itch has been shown to be phophorylated upon EGFR [55] and IGFR activation [46]. Additionally, JNK was shown to phosphorylate the proline-rich motif of AIP4/Itch which is active toward JunB, c-Jun, and c-FLIP$_L$ [123, 139]. In contrast, the phosphorylation at Y371 of AIP4 by Fyn significantly reduced the ability of AIP4 to ubiquitinate JunB [150]. An auto-inhibition model between the WW domain and HECT domain was proposed to explain this interesting phenomenon [151]. Recently, Nedd4 was reported to be phophorylated by Src which is essential for EGF-induced mUb of EPS15 [66].

Nedd4–2 was shown to be serine/threonine phosphorylated in response to several different stimuli, such as insulin, insulin growth factor, aldosterone, vasopressin and NGF [58, 152, 153]. Nedd4–2 is phophorylated by serum and glucocorticoid-inducible kinase (SGK1), a target of the PI3K/PDK1 pathway. This modification decreases the ligase activity toward ENaC since 14-3-3 will bind to phosphorylated Nedd4–2 and block substrate recognition. Nedd4–2 phosphorylation can also be mediated by vasopressin-activated PKA [152]. Similarly, cytokine independent survival kinase 1 (CISK1), another target of the PI3K/PDK1 pathway, phosphorylates AIP4 and blocks AIP4-mediated CXCR4 degradation induced by SDF-1 [154]. Both SGK1 and CISK1 contain a PY motif, which is essential for their interaction with Nedd4–2 or AIP4, respectively [154]. Interestingly, the SGK1/CISK1 phosphorylation sites on Nedd4–2 or AIP4 have been identified and are located in the WW domains [154]. Similar mechanisms may exist for other Nedd4-like E3s.

7.2 Ubiquitination and degradation

Itch was found to be rapidly degraded in primary T cells in response to CD3 and CD28 stimulation [122]. Itch degradation may be mediated by self-ubiquitination and enhanced by JNK-mediated phosphorylation [123]. Similarly, we found that the proteasome inhibitor MG132 significantly increased WWP1 protein levels in the MCF10A breast epithelial cell line (Fig. 3(a)). Further, the degradation of WWP1 was almost completely blocked by MG132 in 22Rv1 prostate cancer cells (Fig. 3(b)). As assayed by cycloheximide chase, the half-life of WWP1 is about 3 h in 22Rv1. Other Nedd4-like E3s may be subject to similar autoregulation.

Besides ubiquitin-mediated proteasomal degradation, Nedd4-like E3s may be degraded by cleavage. For example, Nedd4 was shown to be cleaved by multiple caspases during apoptosis [155]. However, the significance of this observation is not known.

7.3 Subcellular localization

Nedd4-like E3s have been found to localize to the plasma membrane, the cytoplasm, and the nucleus. Nedd4-like E3 proteins have been observed on multiple components of the endocytic pathway including the plasma membrane, the early and late endosome, and the Golgi. For example, AIP4 has been reported to localize to various endosomal subdomains [49, 56]. The C2 domain is necessary but not sufficient for endosomal localization. Wwp1 has been detected on early endosomes in the murine skeletal muscle cell line C2C12 [156]. Interestingly, Nedd4 is translocated to mitochondria upon IGF-1R activation, raising the possibility that Nedd4 may mediate the anti-apoptotic signaling of the IGF-1R [157]. Finally, the Nedd4-like proteins have also been localized to the nucleus [20, 113]. A Rev-like nuclear export signal has been identified in Nedd4 [158] and Smurf1 [159].

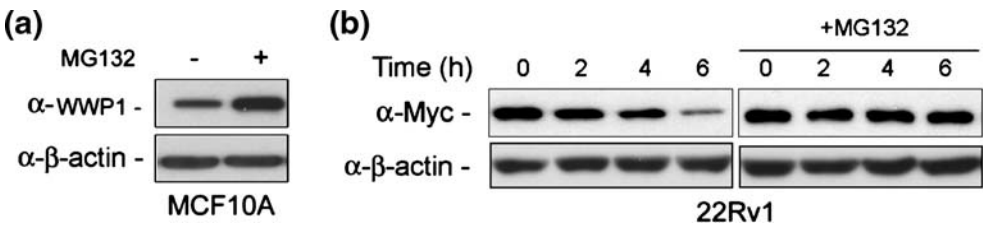

Fig. 3 WWP1 degradation by the proteasome. (**a**) The MCF10A cell line stably expressing WWP1 was treated with 10 μM of the proteasome inhibitor MG132 for 12 h. WWP1 protein was detected by Western blot. β-actin was used as a loading control. (**b**) Myc tagged mouse Wwp1 was transfected into the 22Rv1 prostate cancer cell line. The half-life of Wwp1 was determined in the absence or presence of 10 μM MG132 by cycloheximide (CHX) chase assay. One hundred micrograms per milliliter CHX was used in this experiment

Different subcellular locations of Nedd4-like E3s may be dependent upon which proteins the ligases interact with. For example, upon TGFβ stimulation, Smad7 accumulates in the nucleus where it interacts with Smurf2 or WWP1. These complexes then translocate out of the nuclei and interact with TβR1 [29, 32]. Similarly, Notch was reported to exclude WWP1 out of the nucleus and instead localize to the early endosome [156]. The HECT domain is necessary and sufficient for WWP1 nuclear localization and regulation by Notch. After inducing C2C12 cell differentiation by serum depletion, nuclear WWP1 relocalized to the cytoplasm [156]. These findings highlight the fact that changing subcellular localization is an important way to control the accessability and activity of Nedd4-like E3s toward different substrates.

7.4 Transcription

Nedd4-like E3s are usually ubiquitously expressed in multiple tissues but some show distinct tissue distributions (Table 1). *Nedd4* is widely expressed in mouse kidney, liver, muscle, brain, and heart [6]. *Nedd4–2* was shown to be expressed in rat testis, kidney, liver, lung, brain, and heart [160]. *Smurf1* is mainly expressed in human placenta, pancreas, and testis [32]. *WWP2* and *AIP4* mRNA are widely expressed in human heart, brain, placenta, muscle, and pancreas [161]. *WWP1* mRNA is strongly or modestly expressed in human heart, muscle, placenta, kidney, liver, pancreas and testis [32, 128]. The expression pattern of *Wwp1* and *Itch* mRNA seems to be conserved in mouse [21, 141]. Interestingly, multiple *Wwp1* isoforms were observed in several mouse tissues.

RNA splicing may be another way to modulate the activity of Nedd4-like E3s. In a breast cancer cell line, T47D, six isoforms of *WWP1* have been identified [162]. Expression of *WWP1* splice variants was detected in multiple tissues and the ratios among the *WWP1* isoforms showed tissue-specific distribution. This observation is reinforced by our recent discovery that *WWP1* mRNA with different molecular sizes was detected in several prostate and breast cancer cell lines by Northern blot and Western blot analysis [18, 19]. Different splice variants of *AIP4* and *Nedd4–2* have also been reported [162, 163], suggesting that RNA splicing could be a common regulatory mechanism for Nedd4-like E3s.

The transcripts of Nedd4-like E3s are responsive to multiple growth factor signals. The mRNA expression of *Smurf1* and *Smurf2* was demonstrated to be induced by TGFβ and BMP [164]. Similarly, we found that *WWP1* is upregulated by TGFβ in HaCaT and PC-3 cells in a dosage and time dependent manner (Fig. 4). As mentioned earlier, Smurfs and WWP1 are potent inhibitors of TGFβ signaling; therefore, this feedback control loop aids in precisely regulating TGFβ signal transduction. Further, *Smurf1/2* and *Nedd4* can be induced by the pro-inflammatory factor TNFα [116, 165]. In fibroblasts, *Smurf2* mRNA is upregulated when cells undergo senescense [17].

Several mRNA isoforms of *Nedd4–2* were upregulated by androgen in the AR positive LNCaP prostate cancer cell line [163]. Interestingly, *WWP1* was also reported to be upregulated by androgen and is highly expressed in LNCaP cells [18, 166]. In parallel, a high level of *WWP1* expression is more frequently detected in ER positive breast cancer cell lines [19] and tumors (Oncomine.org). Additionally, progesterone receptor and Glucocorticoid receptor activity are potentiated by Nedd4/hRPF1 [132]. Thus, it is tempting to speculate that Nedd4-like E3s and hormone signaling are mutually regulated.

Upon DNA damage, levels of the endogenous Itch protein were shown to significantly decrease, allowing the TA-p73 protein to accumulate and induce cell cycle arrest and apoptosis [98]. Similarly, WWP1 is also decreased in response to γ-irradiation in p53 WT, but not p53 null, MEF cells [95]. These findings suggest that the transcription of Nedd4-like E3s can be regulated by various DNA damage-response signaling cascades.

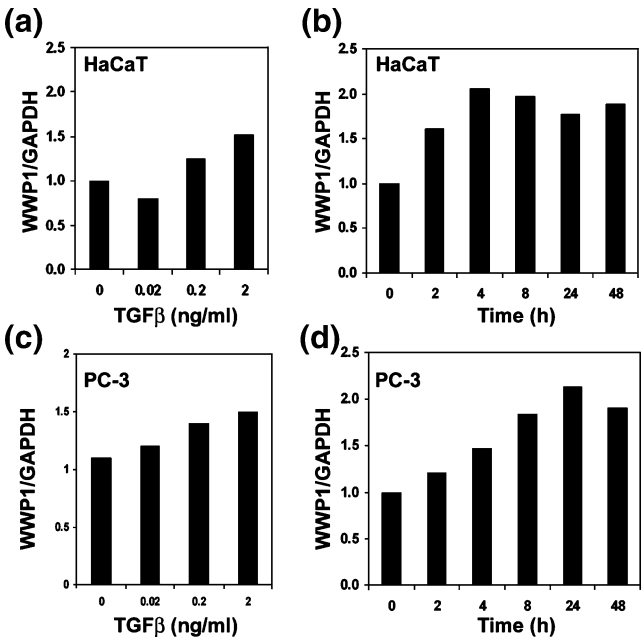

Fig. 4 TGFβ induces the expression of *WWP1* mRNA in HaCaT and PC-3 cells. Expression of *WWP1* mRNA was detected by SYBR real time RT-PCR. GAPDH was used as a control to normalize the cDNA input. The primers have been described in [18, 19]. Cells were serum starved overnight before adding TGFβ. (**a**, **c**) Different amounts of TGFβ were used to treat HaCaT or PC-3 cells for 24 h. (**b**, **d**) Two nanograms per milliliter TGFβ were used to treat HaCaT or PC-3 cells for different durations

8 Concluding remarks

After more than a decade of investigation, we are certain that Nedd4-like E3 are critical regulators of multiple cancer-related growth factor receptors and transcription factors. Several Nedd-4 like E3s, such as Smurf2, WWP1, Nedd4, and Smurf1 are emerging as oncogenic factors due to their frequent deregulation in human cancer. Whether Nedd4-like E3s can be used as diagnostic markers or drug targets remains to be determined because the exact role and mechanisms of action of these E3s in different cancer types remains unclear.

Smurf2 levels have been shown to correlate with poor prognosis in patients with esophageal squamous cell carcinomas [16]. WWP1 also has the potential to be a biomarker in cancer. Gene amplification and overexpression have been detected in around half of those prostate and breast cancer samples studied [18, 19]. In breast cancer, the overexpression of *WWP1* mRNA appears preferentially in ER positive and noninvasive tumors. However, immunohistochemical staining of WWP1 in a large number of tumor samples is definitely required to confirm these promising results.

siRNA inhibition of WWP1 was shown to induce growth arrest and apoptosis [19], suggesting that WWP1 could be a potential molecular target for an anti-cancer drug. However, WWP1 showed ligase-independent proliferative activity *in vitro*, implying that an inhibitor which targets ligase activity may not be effective. In contrast, targeting protein–protein interaction between WW domains and PY motifs using non-peptide small molecular inhibitors might be a better strategy, although assuring the specificity of such constructs will be a challenge. Additionally, characterization of the mechanism by which WWP1 promotes cell proliferation and survival will aid in more ration drug design for cancer therapy. Because of their documented roles in human cancers, Nedd4 and Smurf1 also warrant further investigation as potential drug targets using similar strategies.

Nedd4-like E3s may play context-dependent roles in cancer development for a number of reasons. First, Nedd4-like E3s are ubiquitously expressed in multiple tissues. The regulation of the expression of Nedd4-like E3s under physiological and pathological conditions in different tissues remains poorly understood. Second, each Nedd4-like E3 may have multiple substrates, which, in turn, mediate different functions. Previous work indicates that different WW domains in Nedd4-like E3s display different substrate preferences *in vitro*. It is plausible that each of the Nedd4-like E3 regulate a number of different target proteins. Even though there is functional redundancy among some of the members of the Nedd4-like family of E3's, there are almost as many examples of how each of these proteins specifically acts upon certain substrates. It will take some time to clearly delineate the physiological substrates of each specific Nedd4-like E3. Finally, multiple receptors may use the same endocytic and protein trafficking machinery that are regulated by multiple Nedd4-like E3s. How these E3s specifically affect different receptors is not clear. It is widely accepted that TGFβ plays a context-dependent role during tumor development, and several Nedd4-like E3s are known to antagonize TGFβ signaling. Thus, it is not surprising that different, if not opposite, roles of Nedd4-like E3s in various cancer stages and types have been observed.

As further understanding leads to the development of potential anti-cancer therapeutics that target the activities of Nedd4-like E3s, it will be necessary to validate these drugs. To that end, transgenic and gene-targeted mouse models are especially powerful tools in dissecting the physiological role of Nedd4-like E3s *in vivo*. In this regard, a high throughput gene trap library (tigm.org) will accelerate *in vivo* research efforts. All nine Nedd4-like E3s knock out ES cells are available from this library. We expect more transgenic, knockout, and knockin mouse models will be generated for characterizing the function of this interesting family in the development of cancer. In conclusion, although still in its infancy, the study of Nedd4-like E3s has the potential to provide much insight into the development of cancer diagnostics and therapeutics, which could have enormous impact on human health.

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