The HECT Family of E3 Ubiquitin Ligases: Multiple Players in Cancer Development

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The involvement of the homologous to E6-AP carboxyl terminus (HECT)-type E3s in crucial signaling pathways implicated in tumorigenesis is presently an area of intense research and extensive scientific interest. This review highlights recent discoveries on the ubiquitin-mediated degradation of crucial tumor suppressor molecules catalyzed by the HECT-type E3s. By providing a portrait of their protein targets, we intend to link the substrate specificity of HECT-type E3s with their contribution to tumorigenesis. Moreover, we discuss the relevance of targeting the HECT E3s, through the development of small-molecule inhibitors, as an anticancer therapeutic strategy.

Introduction

Protein ubiquitylation is a highly ordered multistep enzymatic process accomplished by the formation of an isopeptide bond between the C-terminal Gly76 carboxyl group of ubiquitin and the ε-amino group of an internal Lys residue of the substrate (Hershko and Ciechanover, 1998; Ciechanover, 1994, 2005; Hershko, 2005).

After initial ATP-dependent activation by an E1 ubiquitin-activating enzyme (E1), the C-terminal carboxyl group of ubiquitin forms a high-energy thioester bond with an active Cys group of the E1 enzyme. Activated ubiquitin is then transferred to a specific Cys residue of one of a family of E2 ubiquitin-conjugating enzymes (E2s) via a similar thioester linkage. The E3 ubiquitin ligases (E3s) play a critical role in the ubiquitin conjugation cascade by recruiting ubiquitin-loaded E2s, recognizing specific substrates, and facilitating or directly catalyzing ubiquitin transfer to either the Lys residues (in most cases) or the N terminus of their molecular targets. E3s modify protein substrates by either monoubiquitylation or sequential attachment of ubiquitin molecules to form polyubiquitin chains. In contrast to this canonical pathway, monoubiquitylation of ubiquitin-binding domain (UBD)-containing proteins can occur independently of E3s, through direct recruitment of ubiquitin-loaded E2 enzymes by UBDs (Hoeller et al., 2007).

The fate of ubiquitylated proteins is determined by the nature of ubiquitin attachment and the type of isopeptide linkage forming the polyubiquitin chain. When ubiquitin tagging to intracellular substrates occurs through Lys48-linked polyubiquitin chains, proteins are generally labeled for 26S proteasome-mediated recognition and proteolysis. Monoubiquitylation (Mukhopadhyay and Riezman, 2007) and the formation of multiubiquitin chains by isopeptide bonds other than Lys48, such as Lys6 (Nishikawa et al., 2004), Lys29/33 (Chastagner et al., 2006; Al-Hakim et al., 2008), and Lys63 (Deng et al., 2000; Wang et al., 2001; Geetha et al., 2005; Adhikary et al., 2005; Herman-Bachinsky et al., 2007), regulate protein degradation as well as a wide array of cellular activities in a proteolysis-independent manner. In addition, nonproteolytic Lys6 and Lys11 polyubiquitin linkages have been identified in vivo, and their accumulation correlates with the pathogenesis of neurodegenerative disorders (Cripps et al., 2006; Bennett et al., 2007). With few exceptions, single or multiple monoubiquitylation of cell surface receptors triggers receptor internalization and trafficking to the endosomal-lysosomal degradation pathway (Levkowitz et al., 1999; Haglund et al., 2003; Di Fiore et al., 2003).

Based on the sequence homology of their E2-binding domains, E3s can be generally classified into three subfamilies: the homologous to E6-AP carboxyl terminus (HECT) domain-containing E3s, the really interesting new gene (RING) finger domain-containing E3s, and the U box E3s.

The relevance of the E3s in several biological processes is emphasized in vivo by the observation that their genetic alteration, abnormal expression, or dysfunction is often accompanied by the occurrence of pathological disorders, including cancer. Several enzymes belonging to the RING-finger subfamily of E3s have been classified as either tumor suppressors or oncoproteins. It is only recently that various HECT E3s have emerged as crucial regulators of cancer development and therapy. Indeed, in view of their substrate specificity, the E3s represent potentially attractive targets for anticancer treatment. This review will mainly emphasize the oncogenic activity of a few members of the HECT subfamily of E3s.

General Overview of the HECT-Type E3s

The key signature of this E3 subfamily is the HECT domain, a large C-terminal module of approximately 350 amino acids

that was originally characterized in the E6-associated protein (E6-AP). The HECT domain associates with the E2 and provides the catalytic E3 activity (Huibregtse et al., 1995).

The HECT E3s are unique among the E3s in that they possess intrinsic catalytic activity. Their reaction cycle consists of three steps: binding to an E2, loading ubiquitin on themselves through the formation of a ubiquitin-thioester intermediate with the catalytic Cys located at the C terminus of the HECT domain, and transfer of ubiquitin to the target protein. Thus, unlike the RING-finger E3s, which, although able to promote the formation of ubiquitin chains, lack a catalytic site, the HECT E3s directly catalyze substrate ubiquitylation.

The HECT domain consists of a larger N-terminal lobe containing the E2-binding site and a smaller C-terminal lobe including the active-site Cys residue. Structural studies have revealed that these two lobes are connected by a flexible hinge region, which is critical for juxtaposing the catalytic Cys residues of the E2 and E3 (i.e., the HECT domain) during ubiquitin transfer. A conformational change involving an alteration in the relative orientation of the two lobes is thought to facilitate the transthiolation reaction (Verdecia et al., 2003; Huang et al., 1999; G.M. and A. Tramontano, unpublished data).

The substrate specificity of the HECT-type E3s is dictated by protein-protein interaction domains, which account for their classification into three further subfamilies: HERC E3s containing RCC1-like domains (RLDs), C2-WW-HECT E3s possessing tryptophan-tryptophan (WW) domains, and SI(ngle)-HECT E3s lacking either RLDs or WW domains (Scheffner and Staub, 2007).

The C2-WW-HECT E3s likely represent the best characterized subgroup of HECT ligases. They consists of monomeric proteins with a common general modular architecture composed of an N-terminal protein kinase C (PKC)-related C2 domain, two to four WW protein-interacting domains, and a C-terminal HECT domain (Schwarz et al., 1998) (Figure 1).

The C2 domain binds Ca^{2+} and phospholipids and is involved in targeting the HECT E3s to intracellular membranes (Dunn et al., 2004). The C2-WW-HECT E3s are found in several subcellular locations, including the plasma membrane, early and late endosomal compartments, and lysosomes (Marchese et al., 2003; Angers et al., 2004). Some family members can transiently enter the nucleus to target nuclear substrates for protein ubiquitylation (Hamilton et al., 2001; Neumann et al., 2003; Gwizdek et al., 2005; Trotman et al., 2007). The WW domains mediate ligase-substrate associations through interactions with a variety of proline-rich motifs and proline-containing phosphoserine/phosphothreonine sequences of the protein substrate. WW domains display preference for the PPXY (PY) consensus sequence, though atypical interactions with unrelated modular domains in target (Qiu et al., 2000; Marchese et al., 2003; Wegierski et al., 2006) or adaptor and regulatory proteins (Courbard et al., 2002; Oberst et al., 2007) have also been reported.

In addition to the conformational change occurring within the HECT domain, further mechanisms controlling the catalytic properties of the C2-WW-HECT E3s are based on the establishment of intramolecular interactions (Gallagher et al., 2006; Wiesner et al., 2007). As an example, the C2 domain and a region of the HECT module in close proximity to the catalytic Cys of Smurf2 as well as of other C2-WW-HECT E3s are engaged in inhibitory associations. By interfering with ubiquitin thioester formation, these interactions negatively regulate the E3 ubiquitylating activity and ultimately prevent its degradation (Wiesner et al., 2007).

The C2-WW-HECT group is conserved from yeast to mammals, and its evolution is schematically summarized in the phylogenetic tree shown in Figure 1. There is a single Nedd4 homolog in *S. cerevisiae*, while three orthologs exist in *S. pombe* and flies. In mammals, the family has further diverged by generating nine homologs (Figure 1).

The C2-WW-HECT E3s typically regulate endocytosis and trafficking of plasma membrane proteins through monoubiquitylation and the stability of both transmembrane receptors and intracellular substrates via polyubiquitylation. Their subcellular distribution, catalytic activity, and substrate specificity are subjected to many levels of regulation, including posttranslational modifications and interaction with adaptor and accessory proteins (Shearwin-Whyatt et al., 2006). Adaptor proteins generally possess PY motifs, which mediate direct interaction with WW domains of the C2-WW-HECT E3s and facilitate their recruitment to specific substrates (Shearwin-Whyatt et al., 2004; Oliver et al., 2006). Alternatively, adaptor molecules can bridge between C2-WW-HECT E3s and protein targets, which lack canonical binding motifs (Qiu et al., 2000; Kavsak et al., 2000; Marchese et al., 2003; Wegierski et al., 2006).

The oncogenic potential of the HECT-type E3s is highlighted by the identification of a number of tumor suppressor molecules among their protein substrates, as well as by the discovery of genetic aberrations and altered expression patterns of some of the family members in human cancers. Due to the crucial role exerted by the HECT E3 adaptors and protein modifiers, it is possible that dysregulation of their regulators would also influence cellular transformation. The main features of potentially oncogenic HECT E3s and their regulators are summarized in Table 1.

SI(ngle)-HECT E3s

The best studied SI(ngle)-HECT E3s are E6-AP, Huwe1 (HECT, UBA, and WWE domain containing 1), and E3 isolated by differential display (EDD), all of which have been associated with tumor development.

E6-AP

The founding member of the HECT E3 family is E6-AP (also called UBE3A), a 100 kDa polypeptide that interacts with the E6 protein of the cervical cancer-related human papillomavirus (HPV) (reviewed in Narisawa-Saito and Kiyono, 2007). E6-AP forms a stable complex with the adaptor protein E6. The dimeric complex binds to and targets p53 for ubiquitin-mediated proteolysis, thus eventually interfering with the negative growth-regulating activities of this tumor suppressor protein (Scheffner et al., 1990; Huibregtse et al., 1991, 1993a; Scheffner et al., 1994) (Figure 2).

Though E6-independent substrates have been identified (Kumar et al., 1999), E6-AP does not recognize p53 in the absence of the viral oncoprotein E6 (Talis et al., 1998). The recognition of E6 and p53 requires an approximately 200 amino acid region of E6-AP, located at the N-terminal end of the HECT domain (Huibregtse et al., 1993b). The E6:E6-AP complex binds to the DNA-binding domain of p53, which becomes rapidly ubiquitylated and is targeted to proteasomes (Huibregtse et al., 1991).

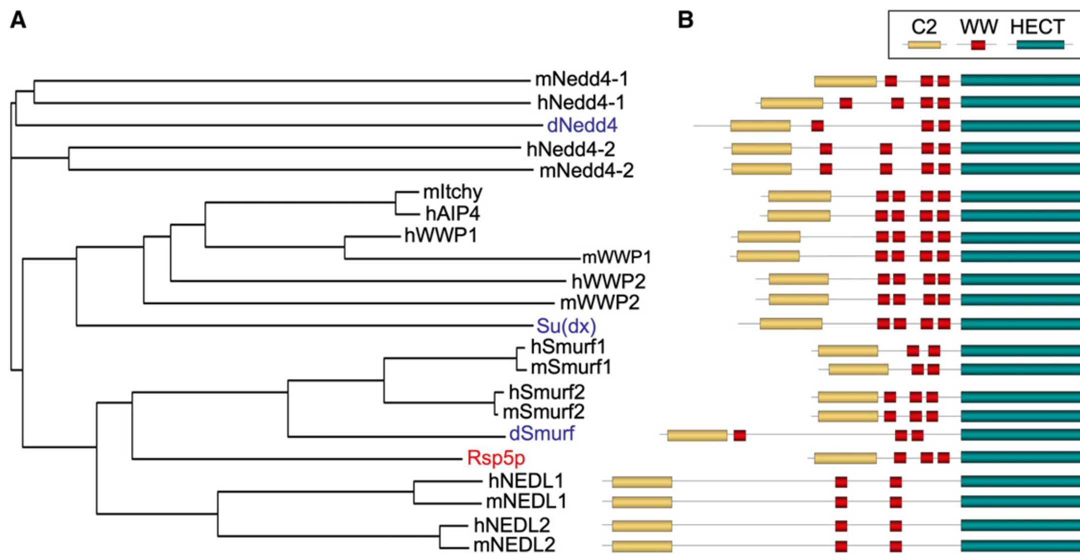

Figure 1. Phylogenetic Relationship Tree of Different Members of the C2-WW-HECT Family of E3s

(A) Orthologs of Nedd4 have been identified in yeast (red), fly (blue), and mouse and human (black). Itch clusters with WWP1 and WWP2, while Nedd4-1 and 2, the Smurfs, and NEDL1 and 2 form separate clusters.

(B) The C2-WW-HECT E3 modular structure consists of an N-terminal Ca^{2+}/lipid-binding (C2) domain (yellow rectangles), a central region containing multiple WW domains (red squares), and a ubiquitin-protein ligase HECT domain (teal rectangles). The HECT domain contains a conserved catalytic Cys residue involved in the formation of a ubiquitin-thioester intermediate.

The ability to promote p53 degradation is an exclusive prerequisite of E6 from the high-risk HPV types (type 16 and 18). On the contrary, the E6 proteins from low-risk HPV types do not stably interact with E6-AP (Huibregtse et al., 1991). This provides a reasonable explanation for the relatively weak interaction of low-risk E6s with p53 and their inability to induce degradation of p53. Consistently, the majority of cervical cancer associated with the high-risk HPV types harbors a wild-type *p53* gene, and the protein levels of p53 are extremely low. E6:E6-AP-induced inactivation of p53 plays a role in the development of more than 90% of human cervical carcinomas.

Although several other targets of E6-AP have been suggested as potential mediators of its tumorigenic activity (Liu et al., 2005a), the major contribution of E6-AP to tumor development is thought to be achieved through the inactivation of p53.

Huwe1

Huwe1 (also named ARF-BP1, E3Histone, HectH9, LASU1, Mule, and Ureb1) is a large protein (~500 kDa) whose function remains controversial. The substrates reported for this E3 include Cdc6 (Hall et al., 2007), histones (Liu et al., 2005b), Mcl-1 (Zhong et al., 2005), c-Myc (Adhikary et al., 2005), and p53 (Chen et al., 2005a). Probably the most puzzling aspect of the controversy surrounding this E3 is apparent from two studies published in the same issue of the journal *Cell* that reported entirely opposite phenotypes (both observed in U2OS cells) following silencing of Huwe1 (Chen et al., 2005a; Zhong et al., 2005). The two divergent effects (i.e., increased survival versus increased apoptosis) were linked to the ubiquitin-mediated degradation of either Mcl-1 (an antiapoptotic protein) or p53 (a proapoptotic protein).

Although Huwe1 targets Mcl-1 for protein ubiquitylation, access of the E3 to Mcl-1 is not evident until cells are exposed to DNA-damaging agents (Zhong et al., 2005). As reported by Chen and colleagues (2005a), under unstressed conditions, Huwe1 directly binds to and ubiquitylates p53 (Figure 2). It was suggested that the tumor suppressor ARF might bind to the HECT domain of Huwe1 and inhibit its ligase activity, thus preventing p53 protein ubiquitylation (Chen et al., 2005a). Experiments conducted in a *HDM2* null genetic background have confirmed that ARF-induced stabilization of p53 also involves Huwe1. However, others have been unable to demonstrate the inhibitory activity of ARF toward Huwe1 (Adhikary et al., 2005).

More recently, Huwe1 was shown to be incapable of controlling p53 abundance in response to DNA-damage stress, while other substrates such as Mcl-1 and Cdc6 are ubiquitylated and degraded (Hall et al., 2007). In addition, the steady-state protein levels of p53 are not increased by depletion of Huwe1 in neuroblastoma cells (Zhao et al., 2008).

Despite this unresolved controversy in the field, it is interesting to note that the *Huwe1* gene is highly expressed in a significant proportion of lung and breast carcinomas (Adhikary et al., 2005; Chen et al., 2005a). Huwe1 overexpression has also been associated with colorectal carcinomas, in which the expression of the E3 directly and inversely correlates with tumor stage and p53 protein levels, respectively (Adhikary et al., 2005; Yoon et al., 2005). The majority of colon cancer samples displaying reduced or absent expression of p53 do not harbor *p53* mutations.

Finally, it was also suggested that Huwe1 assembles Lys63-linked polyubiquitin chains on c-Myc and that this modification is required for gene activation by c-Myc, allowing the interaction of c-Myc with the p300 coactivator (Adhikary et al., 2005). However, other data seem to contradict this hypothesis. First, post-translational modifications of c-Myc do not appear to be required for its interaction with p300, because this complex can be efficiently reconstituted in vitro using bacterially expressed (and therefore unmodified) proteins (Faiola et al., 2005; Vervoorts

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Table 1. The HECT Family of E3s and Their Involvement in Cancer

E3	Substrate(s)	Outcome of Substrate Ubiquitylation	Adaptors/Regulators	Biological Function	Alterations in Cancer
E6-AP	p53	proteasomal degradation	E6	apoptosis	infection by high-risk HPV in cervical carcinomas
Huwe1	p53	proteasomal degradation	ARF	apoptosis, growth arrest	overexpression in breast, lung, and colorectal carcinomas
EDD	TopBP1	proteasomal degradation	unknown	DNA damage	amplification and overexpression in breast and ovarian cancers
Nedd4-1	PTEN, Hgs, Eps15	proteasomal degradation, cytoplasmic/nuclear shuffling	unknown	apoptosis, genome integrity, endocytosis	overexpression in bladder and prostate carcinomas
Nedd4-2	Smad2, Smad4, TβR-I/II	proteasomal degradation	Smad6, Smad7	apoptosis, growth arrest	unknown
Itch	p73, p63, Notch1, c-Jun	proteasomal degradation	Numb	apoptosis, differentiation	unknown
WWP1	p53, Notch1, KLF2, KLF5, Smad2, Smad4, TβR-I/II	proteasomal degradation, nuclear export	Smad2, Smad6, Smad7	apoptosis, growth arrest	amplification and overexpression in prostate and breast cancers
Smurf1	Smad1, Smad4, Smad5, TβR-I/II, BMP-RI/II	proteasomal/lysosomal degradation	Smad6, Smad7	apoptosis, growth arrest	amplification and overexpression in pancreatic cancers
Smurf2	Smad1, Smad2, Smad4, Smad5, TβR-I/II	proteasomal/lysosomal degradation	Smad2, Smad7	apoptosis, growth arrest	overexpression in esophageal squamous cell carcinomas

TopBP1, topoisomerase IIβ binding protein; BMP-R, bone morphogenetic protein receptor.

et al., 2003, 2006). Second, Huwe1 has recently been found to ubiquitylate N-Myc through Lys48-mediated linkages and target it for destruction by the proteasome (Zhao et al., 2008).

As Huwe1 has only recently become a target of research, it is clear that more time is required to resolve the various published discrepancies.

C2-WW-HECT E3s

Nedd4

Nedd4 is the product of the neural precursor cell-expressed developmentally downregulated gene 4-1 (*Nedd4-1*). Nedd4-1 and its closely related homolog Nedd4-2 were originally implicated in regulation of fluid and electrolyte homeostasis by controlling the surface abundance of epithelial cell sodium channel subunits (Staub et al., 1997; Harvey et al., 1999). Recently, however, the identification of the tumor suppressor PTEN as a substrate for Nedd4-1 has extended its role to cancer development. The *PTEN* gene, encoding a plasma membrane lipid phosphatase that antagonizes phosphatidylinositol 3-kinase (PI3K)/AKT survival signaling, is frequently mutated or deleted in human cancers. Nedd4-1-mediated ubiquitylation of PTEN delivers a dual signal for PTEN fate. Polyubiquitylation by Nedd4-1 is thought to target PTEN for proteasomal degradation (Wang et al., 2007), while covalent attachment of a single ubiquitin molecule favors its nuclear translocation (Trotman et al., 2007) (Figure 3). An alternative inactivation pathway of PTEN during tumorigenesis occurs as a result of Nedd4-1 overexpression in human bladder and prostate carcinomas, in which aberrant degradation of PTEN would promote AKT signaling and ultimately provide cell survival advantage (Wang et al., 2007). The oncogenic activity of Nedd4-1 is further corroborated by its ability to cooperate with K-Ras in inducing cellular transformation in a PTEN-dependent fashion.

Similarly to the HDM2:p53 paradigm, Nedd4-1 can also promote PTEN nuclear import and cytoplasmic/nuclear shuffling by targeting Lys289 and Lys13 for monoubiquitylation (Trotman et al., 2007). Nuclear transport of PTEN would potentiate its newly discovered nuclear function in controlling chromosomal integrity and cell death (Trotman et al., 2007; Shen et al., 2007) (Figure 3). The dual behavior of Nedd4-1 may be governed by several factors, including amount, subcellular localization, regulation by posttranslational modifications, and availability of protein adaptors for the E3. The regulatory pathways governing Nedd4 activity were elegantly reviewed by Shearwin-Whyatt et al. (2006). Of note, the enzymatic activity of the HECT E3s can be modulated by their interaction with adaptors. As an example, the ability of the *S. cerevisiae* ortholog of Nedd4, Rsp5 (Figure 1), to switch from mono- to polyubiquitylation of its substrates is strictly dependent on availability of the adaptors Bul1p and Bul2p (Helliwell et al., 2001). No less relevant, modifications of PTEN would also determine its fate by modulating the levels of ubiquitin conjugation.

An additional controversy in the field has been brought to light by a recent report showing that knockout of *Nedd4-1* does not influence degradation or subcellular localization of PTEN (Fouladkou et al., 2008). It remains a challenge for future research to further validate a role for Nedd4-1 as a master regulator of PTEN and to identify the physiological signals that trigger PTEN destruction versus nuclear translocation.

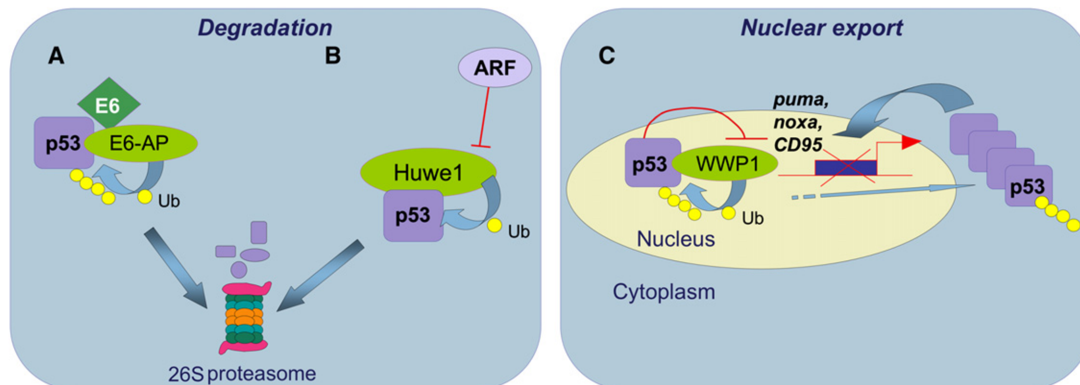

Figure 2. Ubiquitin-Mediated Mechanisms of p53 Inactivation by HECT E3s
p53 inactivation plays a primary role in human tumorigenesis. Besides gene mutation or deletion, accelerated degradation (A and B) and cytoplasmic sequestration (C) of p53 may account for the loss of its tumor-suppressive transcriptional activity.
(A and B) Two SI(ngle)-HECT E3s have been implicated in aberrant ubiquitin-dependent p53 proteolysis in human cancers: E6-AP catalyzes p53 ubiquitylation by acting in concert with the viral oncoprotein E6 as an auxiliary factor (A), and similarly to the ARF-HDM2/p53 axis, Huwe1-induced ubiquitin proteasomal degradation of p53 is repressed by the tumor suppressor ARF (B). Huwe1 possesses a C-terminal HECT domain, which is responsible for its ubiquitin ligase activity.
(C) WWP1-mediated ubiquitylation of p53 is likely to generate multiubiquitin chains by isopeptide bonds other than Lys48. This modification targets p53 for nuclear export and increases its protein stability in the cytoplasmic compartment. Reduced levels of p53 in the nucleus would hence diminish the ability of p53 to transactivate its target promoters.

In addition, it has been proposed that Nedd4 may play a role in regulating the initial sorting events that promote ligand-dependent endocytosis of receptor tyrosine kinases (RTKs). Nedd4-mediated ubiquitylation of endocytic adaptors, involved in coupling ubiquitylated membrane cargo to the endocytic machinery, would promote the disassembly or inactivation of multiprotein endocytic complexes and thus negatively regulate receptor endocytosis (Polo et al., 2002; Katz et al., 2002). These findings provide grounds for speculating that overexpression of Nedd4-1 may contribute to tumorigenesis by inhibiting endocytosis of activated RTKs, thereby enhancing receptor activation.

WWP1

A potential oncogenic role for WW domain-containing protein 1 (WWP1) has been suggested by genetic and functional analyses in human cancers. Gene amplification has been detected in approximately 40% of prostate and breast primary tumors (Chen et al., 2007a, 2007b). The expression of WWP1 has been found to be frequently upregulated at the mRNA and protein level in both tumor types. Knockdown of *WWP1* induces growth arrest and apoptosis in breast epithelial cancer cell lines, substantiating the concept that WWP1 promotes cell proliferation and survival of tumor cells (Chen et al., 2007b).

A recent report has revealed that p53 acts as a substrate for WWP1 (Laine and Ronai, 2007). Binding of WWP1 to the p53 DNA-binding domain results in p53 ubiquitylation. The association is not mediated through canonical WW-PY interactions, though it is reinforced by the presence of the p53 PY motif. In contrast to Huwe1, WWP1-mediated ubiquitylation of p53 promotes its nuclear export and accumulation in the cytoplasm, which results in diminished p53 transcriptional activity (Figure 2). An interesting feedback loop has been proposed according to which p53 represses WWP1 transcription (Laine and Ronai, 2007). Upon stress, inactivation of WWP1 would facilitate complete p53 transcriptional induction. It is thus conceivable that overexpression of WWP1 may contribute to breast and prostate carcinogenesis by attenuating p53 transcriptional activation in response to DNA-damaging insults. Though overexpression of WWP1 has not been associated with known *p53* status in breast cancer cell lines, Chen and coworkers (2007b) have observed that siRNA-induced downregulation of WWP1 does not significantly reduce cell growth in breast cancer cell lines harboring *p53* mutations. These findings suggest that inhibition of WWP1 would sensitize only p53-proficient tumor cells to chemotherapy.

WWP1 has also been reported to target the Krüppel-like factors KLF2 and KLF5 for ubiquitin-mediated proteolysis (Chen et al., 2005b). KLFs are transcription factors that are thought to suppress cell growth in cancer cells, thereby playing a critical role in the progression of several tumors, including breast cancer. Of note, KLF5 is frequently downregulated in breast cancer cell lines (Chen et al., 2002).

Although the closely related WWP1 and WWP2 E3s possess binding specificity for the same PY motif-containing peptides and share at least some common substrates (Martin-Serrano et al., 2005), to date WWP2 has not been similarly implicated in the ubiquitin-dependent regulation of tumor suppressor molecules.

Itch

Itch was initially identified through genetic studies examining the *agouti* locus on mouse chromosome 2 (Perry et al., 1998). The 18H mutation, which is associated with a darker coat color, results from a radiation-induced chromosomal inversion that disrupts the expression of *agouti* and *Itch* genes (Perry et al., 1998).

There are an emerging number of Itch protein targets that have been implicated in tumorigenesis and chemosensitivity (Table 2). Two substrates implicated in human malignancies are the p53 homologs p73 and p63. Beyond their crucial involvement in development and differentiation, both p73 and p63 share functional similarities with p53, being able to mediate cell-cycle arrest and apoptosis in response to DNA damage (Bergamaschi et al., 2003; Irwin et al., 2003; Gressner et al., 2005). Although *p73* and *p63* genes are rarely mutated or inactivated in human cancers (Melino et al., 2002, 2003), they cooperate with p53 in tumor

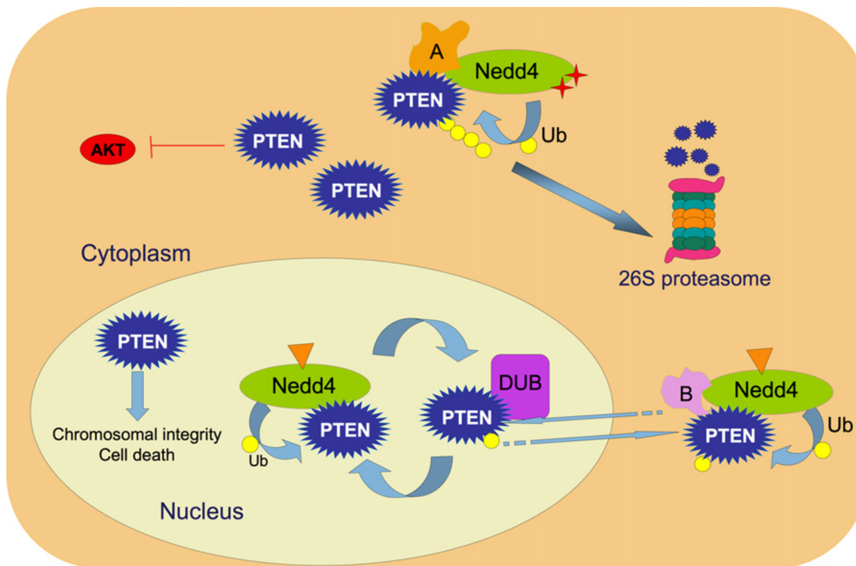

Figure 3. A Model for PTEN Regulation by Nedd4-1
Nedd4-1-mediated polyubiquitylation of PTEN targets the phosphatase for proteasomal degradation. Alternatively, monoubiquitylation of PTEN leads to nuclear import and shuttling back to the cytoplasm. Nuclear availability of ubiquitin-specific proteases would reverse the reaction and allow nuclear accumulation of PTEN. The nuclear PTEN pool can be then monoubiquitylated by Nedd4-1 to be redirected to the cytoplasm. Besides Nedd4-1 localization (cytoplasm versus nucleus) and cellular levels (e.g., oncogenic overexpression), the existence of a distinct set of adaptors (indicated by "A" and "B") and/or posttranslational modifications (indicated by stars and triangles) could select for the outcome of PTEN modification by the E3. DUB, deubiquitylating enzymes.

suppression and chemosensitivity (Flores et al., 2005). Like p53, p73 and p63 expression is maintained at low levels in mammalian cells, and their cellular activation is mainly controlled at the posttranslational level. Both are polyubiquitylated in vivo and degraded via the proteasome (Bernassola et al., 2004; Rossi et al., 2005, 2006). They display a unique C-terminal PY motif, absent in p53, which renders them susceptible to Itch-mediated ubiquitylation and degradation (Rossi et al., 2005, 2006).

Similarly to p53, p73 and p63 accumulate in tumor cell lines in response to γ irradiation or treatment with various chemotherapeutic drugs (Bergamaschi et al., 2003; Irwin et al., 2003; Gressner et al., 2005). Induction of p73 and p63 is at least partially accomplished through Itch downregulation in response to DNA-damaging agents (Rossi et al., 2005) (Figure 4). The molecular mechanisms underlying the decline of Itch protein levels following exposure of tumor cells to chemotherapy await further investigation.

Interestingly, loss or reduced expression of p73 and p63, possibly as result of altered proteasomal proteolysis, has been reported in a number of human tumors, in which it correlated with poor clinical outcome (Puig et al., 2003; Fang et al., 1999; Urist et al., 2002). Thus far, no study has examined the potential association between Itch dysregulation and protein expression levels of p53 family members in human cancers. It is therefore crucial to evaluate whether aberrant upregulation of Itch in tumor cells, or enhanced enzymatic activity, may correlate with low protein expression levels of p73 and p63 and posttranslational suppression of the p53 family members, in a manner similar to the HDM2:p53 paradigm.

On a similar note, the nonagouti 18H or *Itchy* mouse phenotype remains unclear with respect to tumorigenesis. On the C57BL/6J background, *Itch* deficiency causes an autoimmune-like disease that is lethal at 6–8 months of age (Perry et al., 1998). Although no systematic studies have been undertaken to correlate *Itch* ablation with increased resistance to cancer development, possibly due to stabilization of the p53 family members, differences in spontaneous tumor occurrence between normal and mutant mice have not yet been reported. In this respect, it must be remembered that the immune system of the *Itch* mutant mice is seriously compromised and that their life span is shortened by the severe inflammatory defects. Under these circumstances, it might be difficult to reveal potentially relevant differences in tumor latency as well as penetrance between normal and mutant mice. It is likely that chemically induced carcinogenesis or analysis of *Itch* deficiency on a different background would clarify this important issue.

The Nedd4-binding partner-1 (N4BP1) protein, a molecular interactor of Nedd4-1, is a negative regulator of Itch acting as a competitor of its substrate recruitment ability (Oberst et al., 2007). As a result, N4BP1 prevents Itch-mediated transfer of ubiquitin to its protein targets, including p73 and p63, whose transcriptional activity is enhanced by increased protein stabilization (Figure 4). The competition mechanism implies that target selection by Itch could be regulated either by changes in the reciprocal affinity for the interactors (e.g., as a result of posttranslational modifications) or by alterations of their cellular availability. In this scenario, DNA-damage-induced accumulation of p73 or p63 could be achieved either through N4BP1 induction or via chemical/conformational modifications of N4BP1, which in turn would enhance its affinity for the E3 (Figure 4).

An intriguing Itch target crucially involved in controlling cell fate specification and proliferation is the type I transmembrane receptor Notch1. Itch-mediated polyubiquitylation of the intracellular domain (ICD) of membrane-tethered Notch1 results in the degradation of the ICD following receptor activation, in turn antagonizing Notch1 nuclear transcriptional activity (Qiu et al., 2000; McGill and McGlade, 2003). In particular, Notch1 is unique in the manner in which it associates with the HECT-type ligase. The Notch1 ICD does not contain PY motifs but is presumed to interact with Itch through its ankyrin repeats. A key role in the negative regulation of Notch1-dependent signal transduction is played by the adaptor protein Numb, which cooperatively enhances Itch-catalyzed ubiquitylation of the membrane-bound receptor. This effect is achieved through direct and simultaneous binding of Numb to the Notch1 ICD and the WW1 and WW2 domains of Itch (McGill and McGlade, 2003). By this means, Numb

Table 2. Itch Substrates and Regulators

Substrate	Biological Function	Relationship with the *Itchy* Mouse Phenotype
c-Jun, JunB	Th2 cell differentiation/anergy	Th2-mediated allergy/autoimmune disease
JunB	Th2 cell differentiation/anergy	Th2-mediated allergic airway inflammation and atopic dermatitis/autoimmune disease
PLC-γ1/PKCθ	T cell anergy	resistance to anergy induction/autoimmune disease
Notch1	autoimmunity, cancer	autoimmune disease/skin proliferation/ differentiation defects?
Smad2	TβR signaling, cancer	autoimmune disease?
p73	apoptosis, neural development, cancer	unknown
p63	apoptosis, epithelial development, cancer	skin proliferation/differentiation defects?
c-FLIP$_L$	apoptosis	unknown
ErbB4	epithelial kinase receptor, cancer	unknown
CXCR4	agonist-dependent sorting of G protein-coupled receptors	unknown
TRPV4, TRPC4	regulation of channel expression at the cell membrane	unknown
Regulator	**Effect on Itch Catalytic Activity or Substrate Recognition Ability**	**Mechanism of Regulation**
JNK	positive	phosphorylation (Ser/Thr kinase)
Fyn	negative	phosphorylation (Tyr kinase)
USP9X/FAM	positive	deubiquitylation
Itch	positive/negative?	ubiquitylation
N4BP1	negative	binding (adaptor)
Numb	positive	binding (adaptor)
Ndfip1	positive	binding (adaptor)

PLC-γ1, phospholipase C-γ1; PKCθ, protein kinase θ; c-FLIP$_L$, cellular FLICE-inhibitory protein; CXCR4, chemokine receptor 4; Ndfip1, Nedd4 family interacting protein-1.

may act as an adaptor facilitating or stabilizing the WW domain/ ankyrin repeat-mediated interaction between Itch and its substrates.

Notch1 exerts opposite functions in tumor development, being able to act as either an oncogene or a tumor suppressor depending on signal strength, timing, and cell context (Maillard and Pear, 2003). The Notch1 tumor suppressor function seems to be restricted to tissues in which Notch1 signaling orchestrates the spatiotemporal progression of terminal differentiation such as epithelia, including the epidermis. In mouse keratinocytes, Notch1 promotes exit from the cell cycle and entry into the differentiation program by committing basal progenitors to a spinous cell fate. Conditional ablation of *Notch1* in murine epidermis results in skin carcinoma development and increased chemically triggered skin carcinogenesis (Nicolas et al., 2003).

Although a direct correlation with cancer biology has not yet been explored, biochemical and genetic studies have shown that a crucial regulatory mechanism of Itch catalytic activation is its Jun N-terminal kinase 1 (JNK1)-mediated Ser/Thr phosphorylation (Gao et al., 2004). In its unphosphorylated state, Itch enzymatic activity is negatively regulated through intramolecular interactions between the central region, including the WW motifs, and the C-terminal HECT domain ("closed" conformation). Following phosphorylation, Itch undergoes a conformational change ("open" conformation) that destabilizes the self-inhibitory intramolecular interactions, thus allowing substrate recruitment and catalytic activation (Gallagher et al., 2006).

Smurfs

Smad ubiquitylation regulatory factor 1 (Smurf1) and Smurf2 antagonize the transforming growth factor-β (TGF-β) pathway through ubiquitin-mediated degradation of crucial components of its signaling transduction machinery. One of the molecular mechanisms to prevent continuous TGF-β signaling in the absence of ligand stimulation is the Smurf-mediated elimination of phosphorylated TGF-β receptor (TβR)-regulated Smad (R-Smad) proteins by the proteasome (Zhang et al., 2001). The overall basal levels of R-Smads are also regulated by Smurfs (Zhu et al., 1999). This is achieved in order to prevent spurious activation of the TGF-β cascade and to adjust the intensity of responsiveness of a particular cell to TGF-β signaling. With the exception of Smad4 and Smad8, all of the Smads possess a PY motif. Smurf1 specifically targets Smad1 and Smad5, whereas Smurf2 displays a broader specificity toward the family members (see Table 1).

A unique property of the Smads is their ability to function as adaptor molecules for the Smurfs or other members of the HECT family. This mode of action is exemplified by the inhibitory Smad (I-Smad) Smad7, which, acting in concert with Smurf1 and Smurf2, assists the HECT E3s in recruiting the PY-deficient TβR-I (Kavsak et al., 2000). In addition, the auxiliary role of Smad7 is accomplished by presenting the E2 to Smurf2 and enforcing their

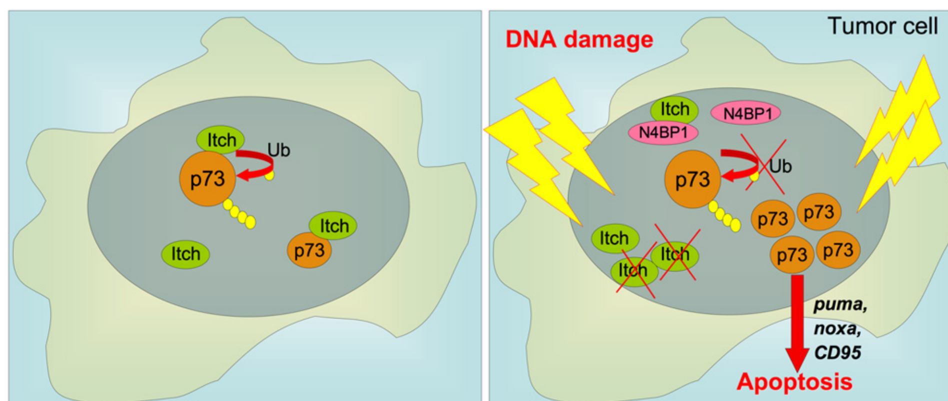

Figure 4. Itch-Mediated Regulation of p73-Induced Apoptosis
In unstressed cancer cells, the steady-state levels of p73 are kept low by the ubiquitylating activity of Itch. p73 upregulation in response to treatment of tumor cells with DNA-damaging agents may arise from (1) Itch downregulation and/or (2) the inhibitory competitive action exerted by N4BP1 on Itch substrate recruitment ability. p73 accumulation leads to increased transactivation of proapoptotic target genes.

interaction (Ogunjimi et al., 2005). This is mediated by the N-terminal domain of Smad7, which binds to both the HECT domain and, via a leucine-rich motif, UbcH7, resulting in enhanced Smurf2 catalytic activity. In addition, Smurf1 recruits activin and BMP receptors through its association with I-Smads. Smad6 and Smad7 also act as a bridge to target non-Smurf HECT E3s, such as Nedd4-2 and WWP1 (see Table 1), to the TβR complexes. Smad7 serves as a protein adaptor for both Smurfs to direct Smad4 for proteasomal destruction (Moren et al., 2005). The Smurf/I-Smad-mediated proteasomal and lysosomal degradation of TGF-β-like receptors and co-Smad contributes to the attenuation or termination of the family signaling events.

A distinct role as an auxiliary molecule is played by Smad2, which upon receptor stimulation and phosphorylation forms a complex with Smurf2 through WW-PY domain-mediated interactions. As a result, Smurf2 is recruited to the transcriptional corepressor SnoN, which in turn is targeted for ubiquitin-mediated proteolysis (Bonni et al., 2001). These findings define temporally distinct roles for Smurf2 in regulating the TGF-β pathway, being the activation or attenuation of TGF-β signaling depending on the association of Smurf with different Smad proteins.

Somatic and germline mutations in cancer cells are present at different levels of the TGF-β signaling pathway, including genes encoding TβR-I, TβR-II, Smad4, and Smad2 (Markowitz et al., 1995; Maurice et al., 2001). Insensitivity to TGF-β results in uncontrolled cell proliferation and contributes to tumorigenesis.

As they are essential modulators of the TGF-β cascade, it is not surprising that dysregulation or dysfunction of Smurfs hampers TGF-β signaling. Aberrant downregulation of TGF-β signaling allows tumor cells to escape TGF-β-induced growth inhibition and thus promotes cancer development. Genetic amplification of *Smurf1* and overexpression of Smurf2 are associated with pancreatic and esophageal squamous cell carcinomas, respectively (Fukuchi et al., 2002). Increased Smurf2 protein expression correlates with higher invasiveness and metastatic potential and poorer prognosis. Interestingly, several *Smad2* and *Smad4* oncogenic inactivating mutations known to interfere with their transcriptional function in cancer cells also affect their protein stability through accelerated degradation (Xu and Attisano, 2000; Maurice et al., 2001). In addition, abnormal expression of the adaptor protein Smad7 has been observed in a subset of human cancers, including follicular thyroid carcinoma cell lines and endometrial cancers (Cerutti et al., 2003; Dowdy et al., 2005). Smad7 overexpression may represent a further mechanism for the abrogation of the TGF-β response in tumor cells.

While the cytostatic actions of TGF-β are a barrier to tumor emergence, during cancer progression tumor cells can acquire the ability to overproduce TGF-β, which in turn starts to behave as an autocrine tumor-promoting factor by enhancing invasion and metastasis. In later stages of the disease, Smurf2 activity is inhibited through interaction with the adaptor protein RNF11, which is highly expressed in invasive breast cancers (Subramaniam et al., 2003). RNF11 is though to promote Smurf2 protein ubiquitylation and proteasomal degradation, thereby enhancing TGF-β signaling and its tumor-promoting activity.

HECT E3s as Potential Cancer Targets

The hierarchical nature of the ubiquitin-proteasome system provides a rich source of molecular targets for anticancer therapies. The clinical relevance of targeting the ubiquitin-proteasome system has been revealed by the use of bortezomib (Millennium Pharmaceuticals, Inc.'s Velcade, also previously known as PS-341), a highly selective and reversible inhibitor of the chymotrypsin-like activity of the proteasome. Bortezomib has been evaluated in a number of preclinical and clinical trials for solid and hematological malignancies (reviewed in Voorhees and Orlowski, 2006). It is presently approved by the US Food and Drug Administration for treatment of relapsed and refractory multiple myeloma, for which manageable side effects have been reported. Unfortunately, the clinical activity of bortezomib in other forms of hematological malignancies as well as solid tumors has so far been less promising, with partial or no response.

Given the lack of specificity of proteasome inhibitors, manipulating less universal features of the ubiquitin-proteasome system, such as the E2s or the neddylation system (which targets certain RING-finger E3s), may in principle be a useful therapeutic

strategy. Moreover, the enzymatic nature, abundance, and specific substrate recognition properties of E3s indicate ubiquitin ligases as even more specific and effective therapeutic targets. Targeting individual E3s would selectively stabilize a particular or limited number of protein substrates, thus possibly limiting untoward side effects. Hence, drugs targeting E3s promise a better therapeutic ratio than proteasome inhibitors. Importantly, E3s show a clear involvement in oncogenesis, as indicated in this review. As one example, downregulation of Itch, with its cancer-related substrates (Table 2), is able to sensitize cells to chemotherapy (Hansen et al., 2007).

However, the advantage of targeting E3s is partially reduced by (1) the promiscuity of enzymes and substrates (each E3 usually degrades more than one substrate, and each substrate can be degraded by several E3s), (2) the unknown nature of the "crucial" oncogenic substrate or substrates for each individual E3, and (3) the intrinsic technical difficulties inherent in high-throughput screenings to identify small-molecule inhibitors of E3s. Blocking E3 functions could be achieved by chemically inhibiting E2 recruitment or, alternatively, by preventing specific substrate recognition by E3s (either directly or allosterically).

In this regard, the HECT family, with its intrinsic catalytic activity, may be easier to inhibit than E3s belonging to the RING-finger family. This is because, in contrast to the RING-finger E3s, HECT E3s have intrinsic enzymatic ubiquitin transfer activity via a mechanism somehow reminiscent of E2s. In fact, HECT E3s, following binding of a ubiquitin-charged E2, form a thioester bond between their own active-site Cys and the ubiquitin C terminus. Subsequently, the HECT domain catalyzes the formation of an isopeptide bond between the ε-N side chain of a Lys residue and the ubiquitin C-terminal carboxylate. Catalysis requires a rotation of the N and C lobes of the HECT domain on a specific polypeptide hinge linking region (Verdecia et al., 2003; Diao et al., 2008).

So far, most high-throughput screenings have been performed with both E3 and substrate, or using the ability of some E3s to self-ubiquitylate. In this latter case, although the E3:substrate recognition inhibitors are missing, there is a stronger chance to inhibit the active catalytic site.

The identification of specific HDM2 inhibitors has been reported with success by several groups (e.g., Yang et al., 2005), showing that inhibitors of E3s are already a reality. Even though this indicates a proof of principle of the feasibility of this difficult road, this is not by any means the end of the story.

Concluding Remarks

Ubiquitin-dependent proteolytic and nonproteolytic pathways play pivotal roles in cancer cell proliferation and apoptosis. The data reviewed here illustrate how certain HECT-type E3s may contribute to tumorigenesis by controlling the ubiquitylation of tumor suppressors and oncoproteins. Inappropriate degradation of these substrates due to aberrant expression, dysfunction, or altered regulation of the HECT E3s would be tightly linked to malignant transformation and chemoresistance. In view of their substrate specificity, the E3s represent attractive targets for cancer therapy. In this scenario, therapeutic strategies that inhibit oncogenic HECT E3s should restore normal expression of tumor suppressors and ultimately induce regression of malignant lesions. A better understanding of the oncogenic potential of the HECT-type E3s will likely lead to the identification and development of biomarkers and drug targets for cancer treatment.

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