

# HECT E3 ubiquitin ligases – emerging insights into their biological roles and disease relevance

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## ABSTRACT

Homologous to E6AP C-terminus (HECT) E3 ubiquitin ligases play a critical role in various cellular pathways, including but not limited to protein trafficking, subcellular localization, innate immune response, viral infections, DNA damage responses and apoptosis. To date, 28 HECT E3 ubiquitin ligases have been identified in humans, and recent studies have begun to reveal how these enzymes control various cellular pathways by catalyzing the post-translational attachment of ubiquitin to their respective substrates. New studies have identified substrates and/or interactors with different members of the HECT E3 ubiquitin ligase family, particularly for E6AP and members of the neuronal precursor cell-expressed developmentally downregulated 4 (NEDD4) family. However, there still remains many unanswered questions about the specific roles that each of the HECT E3 ubiquitin ligases have in maintaining cellular homeostasis. The present Review discusses our current understanding on the biological roles of the HECT E3 ubiquitin ligases in the cell and how they contribute to disease development. Expanded investigations on the molecular basis for how and why the HECT E3 ubiquitin ligases recognize and regulate their intracellular substrates will help to clarify the biochemical mechanisms employed by these important enzymes in ubiquitin biology.

**KEY WORDS:** Ubiquitin, HECT, E3 ubiquitin ligase, Ubiquitylation, Protein-protein interactions, Protein turnover, Cell signaling, Cancer, Neurological disorders, Neurodevelopmental disorders, Neurodegeneration

## Introduction

In 1995, it was discovered that an ~350 amino acid region found near the C-terminus of E6-associated protein (E6AP, also known as UBE3A) showed sequence similarity in a number of related of proteins (Huibregtse et al., 1995). Interestingly, the researchers observed that the homologous to E6AP C-terminus (HECT) domain was able to form a thioester bond with the C-terminus of ubiquitin and catalyze the formation of polyubiquitin chains (Huibregtse et al., 1995). Since that time, these proteins have collectively been referred to as the HECT E3 ubiquitin ligases. There are 28 human members of the HECT E3 ubiquitin ligase family that range in size from ~80 to more than 500 kDa, with each being shown to have prominent roles in disease-relevant processes, including various cancers, neurological disorders, neurodevelopmental and neurodegenerative disease and immunological disease (Scheffner and Kumar, 2014).

Ubiquitylation (also known as ubiquitination) is an essential signaling pathway that involves the covalent attachment of ubiquitin, a highly conserved 8.5 kDa protein, onto a target substrate (Akutsu et al., 2016; Hershko and Ciechanover, 1998; Komander and Rape, 2012; Swatek and Komander, 2016). This highly regulated process is achieved through the sequential action of three enzymes – an ubiquitin-activating enzyme (E1), an ubiquitin-conjugating enzyme (E2), and an ubiquitin ligase (E3). The E3 ubiquitin ligases selectively facilitate the formation of an isopeptide bond between the C-terminus of ubiquitin and the  $\epsilon$ -amino group of a lysine residue on a substrate protein (Deol et al., 2019; Komander and Rape, 2012; Pickart and Eddins, 2004). Owing to the abundance of E3 ubiquitin ligases, it is generally accepted that the different combinations of E2 and E3 enzymes are responsible for substrate specificity and polyubiquitin chain formation (Berndsen and Wolberger, 2014; Zheng and Shabek, 2017).

E3 ubiquitin ligases are classified into three major classes based upon the unique catalytic mechanisms that they employ – really interesting new gene (RING) (Deshaies and Joazeiro, 2009; Metzger et al., 2014), the RING-between-RING or RING-BRcat-Rcat (RBR) (Spratt et al., 2014), and HECT E3 ubiquitin ligases (Berndsen and Wolberger, 2014; Rotin and Kumar, 2009; Scheffner and Kumar, 2014). RING E3 ubiquitin ligases function as scaffolds that bring the E2-ubiquitin complex and substrate into close proximity to facilitate the transfer of ubiquitin onto the substrate protein (Metzger et al., 2014). In contrast, the RBR and HECT E3 ubiquitin ligases play a more catalytic role by forming a thioester bond with a conserved catalytic cysteine residue in the Rcat domain (for RBRs) or the C-terminal lobe of the HECT domain (for HECTs) prior to the transfer of ubiquitin to its destined substrate (Berndsen and Wolberger, 2014; Zheng and Shabek, 2017).

Over the past 15 years, numerous studies have demonstrated the significance of HECT E3 ubiquitin ligases in various biological mechanisms, including protein trafficking (d'Azzo et al., 2005), subcellular localization (Laine and Ronai, 2007), innate immune responses (Liu, 2004), viral infections (Chesarino et al., 2015), the DNA damage response (DDR) (Mohiuddin et al., 2016), transforming growth factor  $\beta$  (TGF- $\beta$ ) signaling (Malonis et al., 2017), Wnt signaling (Flack et al., 2017; Wei et al., 2012) and apoptosis (Kim et al., 2013; Li et al., 2013, 2008b). In addition, recent structural studies on HECT E3 ubiquitin ligases have begun to clarify how these enzymes catalyze the attachment of ubiquitin (as expertly reviewed in Berndsen and Wolberger, 2014; Lorenz, 2018; Scheffner and Kumar, 2014). Dysfunction of HECT E3 ligases have also been linked to the onset of various cancers, neurological disorders and other rare illnesses (Bielskienė et al., 2015; Fajner et al., 2017; Galdeano, 2017); thus, further research on how the HECT E3 ubiquitin ligases work will help lead to breakthroughs in drug discovery. This Review aims to focus, consolidate and summarize the recent findings on the biological

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functions, identified protein–substrate interactions, and disease relevance of each member of the HECT E3 ubiquitin ligase family.

In general, the conserved C-terminal HECT domain catalyzes the transfer of ubiquitin to substrates while the variable N-terminal domains of the HECT E3 ligases are responsible for recognizing and binding to their protein substrates. The 28 human HECT E3 ubiquitin ligases have been classified into three major subfamilies based upon their N-terminal regions (Fig. 1). These comprise the neuronal precursor cell-expressed developmentally downregulated 4 (NEDD4; nine members), HECT and RLD domain-containing (HERC; six members) and ‘other HECTs’ (13 members) subfamilies (Rotin and Kumar, 2009).

The NEDD4 family members all contain an N-terminal single Ca<sup>2+</sup>-binding C2 domain followed by two, three or four WW domains (Ingham et al., 2004). The C2 domain binds to phospholipids in a Ca<sup>2+</sup>-dependent manner (Plant et al., 1997), whereas the WW domain is responsible for substrate recognition (Staub et al., 1996); it is named for the presence of two conserved tryptophan residues separated by 20 to 22 amino acids (Bork and Sudol, 1994).

The HERC family members are characterized by the presence of one or more regulator of chromosome condensation 1 (RCC1) protein-like domains (RLDs) at their N-termini. It has been suggested that the β-propeller blades within the RLD interact with various protein substrates, including the Ras-related GTP-binding protein Ran, which catalyzes guanine nucleotide exchange (Ohtsubo et al., 1989).

Some HECT E3 ubiquitin ligases have variable protein–protein interaction domains at their N-termini and do not contain any RLD or WW domains, so they have been broadly grouped into the ‘other’ HECT E3 ligase family (Rotin and Kumar, 2009) (Fig. 1). However, recent phylogenetic analyses have suggested that it may be more appropriate to further subdivide the remaining HECT E3 ubiquitin ligases into subfamilies based upon their genetic evolutionary histories (Grau-Bové et al., 2013; Marin, 2010). With many sequenced whole genomes of higher organisms becoming available, it will be interesting to see how the classification of these enzymes may change in the coming years.

### Biological functions of HECT E3 ubiquitin ligases

The HECT E3 ubiquitin ligases have been shown to be involved in a myriad of cellular processes and signaling pathways. Many of the links between a specific HECT E3 ubiquitin ligase and an intracellular process were made through the identification of protein–protein interactions. Since the initial discovery of E6AP in 1993 (Huibregtse et al., 1993; Scheffner et al., 1993; Zheng and Shabek, 2017), there have been many studies that have reported protein–protein interactions with HECT E3 ubiquitin ligases, but they have not been consolidated in the literature (see Table S1). To address this, we have summarized all of the experimentally observed protein–protein interactions reported, the method(s) used to observe the interaction, and the specific site(s) of interaction in the HECT E3 ubiquitin ligase over the past 25 years since their original discovery (Tables S1–S4). Using the information detailed in these tables, we discuss here some recent highlights with regard to the different biological functions that HECT E3 ubiquitin ligases regulate and how their interaction with intracellular proteins elicits a cellular response (Fig. 2).

#### Cell growth, proliferation and migration

HECT E3 ubiquitin ligases are involved in regulating cell growth and proliferation. For example, the overexpression of HERC4

facilitates the proliferation of hepatocellular carcinoma (HCC), whereas depletion of HERC4 slows down proliferation and increases the apoptotic rate of HCC (Zheng and Shabek, 2017). Knockdown of UBE3B, of one of the ‘other HECTs’, in glioblastoma cells was shown to cause changes in mitochondrial morphology and physiology and the suppression of cellular proliferation (Braganza et al., 2017). Another study observed NEDD4 upregulation increased cell proliferation, while downregulated NEDD4 in prostate carcinoma cells inhibited colony formation and induced apoptosis (Li et al., 2015). SMURF1, another NEDD4 family member, also regulates cancer cell proliferation by modulating the ubiquitylation and stability of estrogen receptor alpha (Yang et al., 2018) (Table S2).

Some HECT E3 ubiquitin ligases also play a role in controlling cell migration. For instance, HACE1, a member of the ‘other HECTs’ family, reduces cell migration through ubiquitylating Rac1, a GTPase involved in cell migration, angiogenesis and cell growth (Castillo-Lliva et al., 2013) (Table S4). SMURF1 also impacts cell migration through the ubiquitylation of tumor necrosis factor receptor-associated factor 4 (TRAF4), targeting it for 26S proteasomal degradation (Wang et al., 2013) (Table S2). SMURF2 has been shown to be a novel regulator in breast cancer, as overexpressed SMURF2 in nude mouse models leads to cell migration and metastasis of breast and trophoblast cells (Jin et al., 2009; Yang et al., 2009). Consequently, HECT E3 ubiquitin ligases are important regulators in the modulation of cell growth, proliferation and migration and their consequent implications in disease.

#### Regulation of apoptosis

Some HECT E3 ubiquitin ligases are responsible for regulating apoptosis. For instance, it has been shown that HERC1 is necessary to prevent UV-induced apoptosis by promoting Bak1, a pro-apoptotic B-cell lymphoma 2 (Bcl-2) protein (Holloway et al., 2015) (Table S3). The ‘other HECT’ ligase AREL1 is a novel anti-apoptotic protein by targeting the degradation of inhibitor of apoptosis proteins (IAPs), including second mitochondrial-derived activator of caspase (SMAC, also known as DIABLO), high temperature requirement A2 (HtrA2) and endoplasmic reticulum aminopeptidase 1 (ERAP1, also known as ARTS) (Kim et al., 2013) (Table S4). The NEDD4 family members WWP1 and WWP2 are also necessary in regulating apoptosis. Depletion of WWP1 altered Bcl-2-associated X protein (Bax) expression, leading to osteosarcoma cell apoptosis (Wu et al., 2015), while the knockdown of WWP2 promoted G1 cell cycle arrest and apoptosis in liver cancer cells by elevating the expression of apoptosis-associated markers, including caspase-7, caspase-8 and Bax (Xu et al., 2016) (Table S2).

Many HECT E3 ubiquitin ligases also modulate apoptosis by mediating the activity of p53 and p73 (encoded by *TP53* and *TP73*, respectively), key regulators of cell cycle arrest and apoptosis. For example, WWP1 has been shown to increase p53 stability (Laine and Ronai, 2007), while another NEDD4 member, HECW2, promotes the stability of p73 (Miyazaki et al., 2003) (Table S2). WWP2 also interacts with p73 to promote p73 turnover (Chaudhary and Maddika, 2014), and HECW1 increases the transcriptional activity of p53 by binding to RING-finger protein 43 (RNF43) to initiate apoptosis in cancerous cells (Li et al., 2008b; Shinada et al., 2011). SMURF1 and SMURF2 also enhance the degradation of p53 by increasing the activity of the RING E3 ubiquitin ligase mouse double minute 2 (MDM2) to induce ubiquitin-dependent proteasomal degradation of p53 (Nie et al., 2010). Overall, these

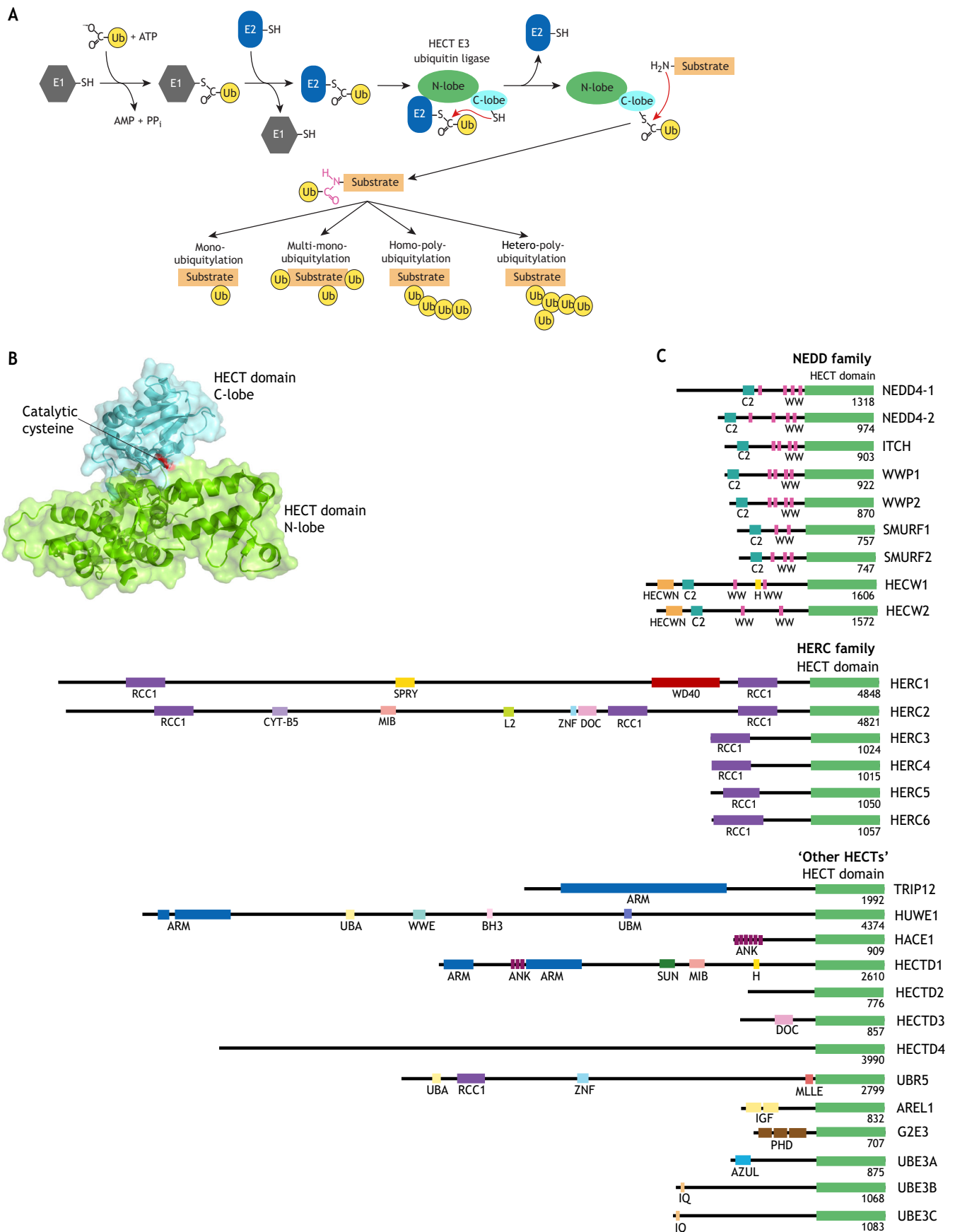


Fig. 1. See next page for legend.

**Fig. 1. HECT E3 ubiquitin ligases.** (A) HECT E3 ligase-dependent ubiquitylation signaling pathway. Target proteins can be mono-, multi- or poly-ubiquitylated. (B) Crystal structure of the HECT domain of HUWE1 (PDB 3H1D; Pandya et al., 2010). The HECT domain, which is found all 28 members of the HECT E3 ubiquitin ligase family, consists of two lobes – the N-terminal lobe (green), which engages with a charged E2–ubiquitin, and the C-terminal lobe (blue), which contains the conserved catalytic cysteine residue (red) required for ubiquitylation activity. (C) HECT E3 ubiquitin ligase domain architecture. HECT E3 ligases can be categorized into three classes. The NEDD4 family members are characterized by their N-terminal Ca<sup>2+</sup>-binding C2 domain and WW domains that recognize PYXP sequences. Members of the HERC family contain one or two RCC1 domains. The ‘Other HECTs’ family members are more diverse and contain a variety of different protein–protein interaction domains, with some similarities and combinations of domains. HECT, homologous to E6AP C-terminus; C2, Ca<sup>2+</sup> domain; WW, WW domain; HECWN, HECW1/2 N-terminal domain; H, helical bundle; SPRY, SPIA and ryanodine receptor domain; WD40, WD dipeptide domain; CYT-B5, cytochrome b<sub>5</sub>-like heme/steroid-binding domain; MIB, MIB/HERC2 domain; L2, ribosomal protein 2; ZNF, zinc finger; DOC, APC10/DOC domain; ARM, armadillo repeat-containing domain; UBA, ubiquitin-associated domain; WWE, WWE domain; BH2, Bcl-2 homology 3 domain; ANK, ankyrin-repeat domain; SUN, SAD1/UNC domain; MLL, Mademoiselle/PABC domain; IGF, immunoglobulin-like fold; PHD, plant homeodomain-type zinc finger; AZUL, Zn-binding N-terminal domain; IQ, IQ motif/EF-hand binding site.

findings establish the important roles of HECT E3 ubiquitin ligase family members play in regulating apoptosis and cell death.

**DNA repair and the DNA damage response**

Genome stability and integrity are crucial for cell survival and normal cell development (McKinnon, 2017). Recent studies have shown that members of the HECT E3 ubiquitin ligase family help in maintaining genome integrity by regulating multiple DNA repair pathways. HERC2 is involved in the nucleotide excision repair (NER) pathway and acts through ubiquitylating the Xeroderma pigmentosum complementation group A (XPA) protein (Kang et al., 2010), which serves as a scaffold for other repair proteins to ensure proper excision at a damaged DNA site (Sugitani et al., 2016) (Table S3). HERC2 also participates in the DDR by orchestrating the assembly of the E2 ubiquitin-conjugating enzyme UBE2N (also

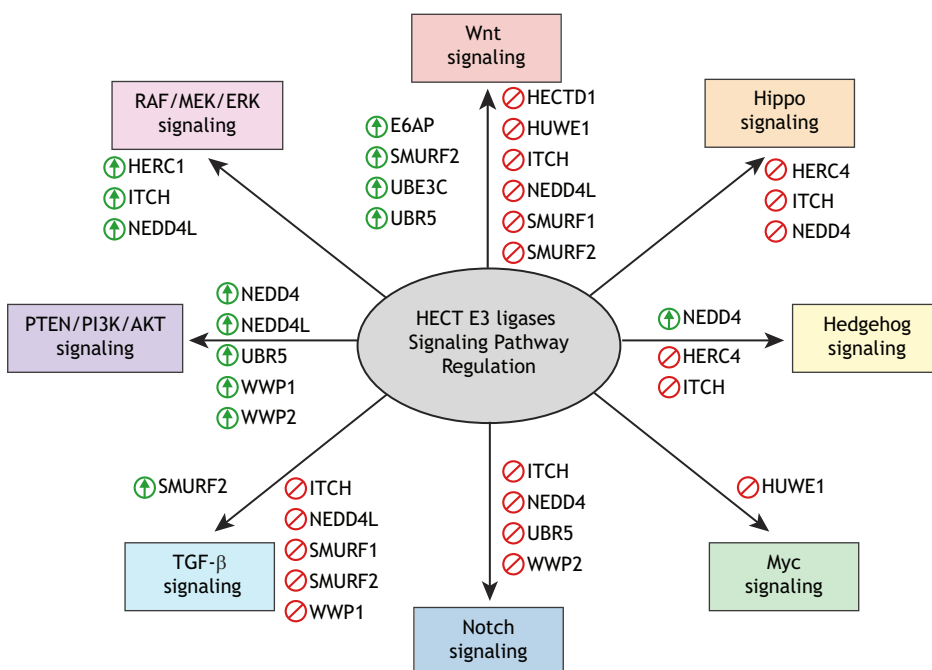
known as UBC13) with RING finger protein 8 (RNF8), a RING E3 ubiquitin ligase that is essential for the retention of repair factors on DNA double-strand breaks (DSBs) (Bekker-Jensen et al., 2010).

TRIP12 regulates ubiquitin-specific protease 7 (USP7)-dependent DDR through ubiquitylation of USP7, a protein known to control stability of p53, tumor suppressor p53-binding protein 1 (TP53BP1), and serine/threonine-protein kinase Chk1 (also known as CHEK1) (Liu et al., 2016) (Table S4). TRIP12 and UBR5, another ‘other HECT’, have been shown to control the accumulation of RING finger protein 168 (RNF168) at DSBs, which ubiquitylates histones to prevent remodeling of damaged chromatin (Gudjonsson et al., 2012). UBR5 also enhances DDR during the G1/S and intra S phase DNA damage checkpoints, while also maintaining G2/M arrest during DSB repair (Munoz et al., 2007).

Furthermore, HUWE1 is involved in DDR by regulating the ubiquitylation of cell division cycle protein 6 (Cdc6), an essential component of pre-replication complexes that assemble at origins of DNA replication during the G1 phase of the cell cycle (Hall et al., 2007) (Table S4). HUWE1 can also control the elimination of myeloid leukemia cell differentiation protein Mcl-1 to trigger DNA damage-induced apoptosis (Zhong et al., 2005). Taken together, these studies demonstrate that several HECT E3 ubiquitin ligases have significant roles in maintaining genome stability.

**Viral infection, viral budding and antiviral activities**

Many viruses exploit the host cellular ubiquitin machinery to promote their replication, with many reports of E6AP and members of the NEDD4 subfamily being specifically targeted by viral proteins. For example, E6AP ubiquitylation activity is hijacked by human papilloma virus (HPV) E6 protein resulting in increased degradation rates of the tumor suppressor protein p53 (Howley, 2006) (Table S1). The L domain of Gag, a key protein involved in viral assembly, from both the human T-cell leukemia virus type 1 (HTLV-1) (Sakurai et al., 2004) and Rous Sarcoma virus (RSV) (Kikonyogo et al., 2001) associates with NEDD4 to promote viral budding (Table S2). The Herpes Simplex virus (HSV) accessory UL56 protein causes NEDD4 autoubiquitylation, targeting it for



**Fig. 2. An overview of intracellular signaling pathway regulation by the HECT E3 ubiquitin ligases.** Signaling pathways that are enhanced by a HECT E3 ubiquitin ligase are denoted with green arrows, while those that are downregulated are highlighted with a red cross. Details on the specific regulatory effect that some of the HECT E3 ubiquitin ligases have in the Wnt, TGF-β and Notch signaling pathways are shown in Fig. 3. There still remain many unanswered questions on the specific role(s) and/or function(s) that the HECT E3 ubiquitin ligases have in each of these pathways.

degradation, which in turn facilitates the cytoplasmic transport of virions from the trans-Golgi network (TGN) to the plasma membrane for virion release (Ushijima et al., 2008). Hepatitis B virus (HBV) recruits cellular  $\gamma$ 2-adaptin and NEDD4 to coordinate its assembly and escape from hepatocytes (Rost et al., 2006). Studies have also shown that binding of NEDD4 to the viral matrix protein (VP40) is essential for budding of Ebola virus (Yasuda et al., 2003), while WWP1-mediated ubiquitylation of VP40 protein enhances the exit of Ebola viral particles (Han et al., 2016, 2017). In contrast, in the presence of viral proteins, SMURF2 inhibits the release of interferon regulatory factors during the antiviral response by ubiquitylating mitochondrial antiviral-signaling protein (MAVS) for degradation (Pan et al., 2014).

HERC5 and HERC6 have also been shown to have antiviral activities by catalyzing the covalent attachment of interferon-stimulated gene 15 (ISG15), a 17 kDa tandem ubiquitin-like protein expressed in the presence of interferons, on to its target proteins (Dastur et al., 2006; Ketscher et al., 2012; Oudshoorn et al., 2012; Takeuchi et al., 2006). For example, in this way, HERC5 inhibits the replication of influenza A virus by conjugating ISG15 to the critical virulence factor non-structural (NS1) protein (Tang et al., 2010) (Table S3). HERC5 also restricts an early stage of HIV assembly by attenuating Gag-particle production to inhibit HIV proliferation (Woods et al., 2011). Likewise, when HERC6 is overexpressed *in vitro*, transcription of the interferon- $\beta$  (IFN $\beta$ ) promoter is enhanced, which initiates the antiviral response against vesicular stomatitis virus and Newcastle disease virus (Oudshoorn et al., 2012). Intriguingly, the ability of NEDD4 to ubiquitylate viral protein VP40 is attenuated by ISG15 and inhibits the budding of Ebola virus (Malakhova and Zhang, 2008; Okumura et al., 2008); this suggests that ISGylation mediated by HERC5 and/or HERC6 may counteract the hijacked HECT ubiquitylation machinery to combat viral infections.

### Immune response

Several members of the NEDD4 subfamily also have important roles in the host immune response. For instance, NEDD4 promotes the ubiquitylation and degradation of Cbl-b, a RING E3 ubiquitin ligase that facilitates destruction of components of T-cell receptor (TCR) signaling to initiate the immune response (Yang et al., 2008) (Table S2). The NEDD4 family member ITCH has been shown to act upstream of B-cell lymphoma 6 (Bcl-6), the master transcription factor involved in coordinating follicular helper T-cell differentiation, germinal center and immunoglobulin G (IgG) in response to an acute viral infection (Xiao et al., 2014). ITCH and WWP2 were recently shown to work in tandem to regulate T-cell differentiation by modulating TCR signal strength (Aki et al., 2018). Likewise, WWP2 negatively regulates Toll-like receptor 3 (TLR3)-mediated innate immune and inflammatory responses by targeting TIR-domain-containing adapter-inducing interferon- $\beta$  (TRIF; also known as TICAM1) for ubiquitylation and degradation (Yang et al., 2013). Furthermore, ITCH controls the stability of critical immune system proteins, such as c-Jun and JunB (Fang et al., 2002; Gao et al., 2004). Accordingly, depletion of ITCH in mice results in the spontaneous development of multisystem autoimmune disease and irregular epidermal development (Lohr et al., 2010; Melino et al., 2008).

The emerging connections between members of the HECT E3 ubiquitin ligases and important cellular processes reinforce the need for continued studies on this family of enzymes to better understand the mechanism that each enzyme uses to elicit a cellular response. By expanding our knowledge on HECT-dependent ubiquitylation and the HECT substrates, the potential for new therapeutic

approaches to treat viral infections, autoimmune disease and other cellular processes can be further explored.

### Intracellular signaling pathways regulated by HECT E3 ubiquitin ligases

HECT E3 ubiquitin ligases are key effectors in several intracellular signaling pathways, including the Notch, TGF- $\beta$  and Wnt pathways (Figs 2 and 3). Connections between these pathways and HECT E3 ligases were determined based on observed interactions and/or changes in activity or protein levels of their respective substrates. While some of these links are well established as discussed below, the specific role of some of the HECT E3 ubiquitin ligase interactors and substrates in these pathways remains unclear. Further research is thus needed to validate the role of HECT E3 ligases and provide further insights into the roles their substrates have in these crucial signaling pathways.

#### Notch signaling

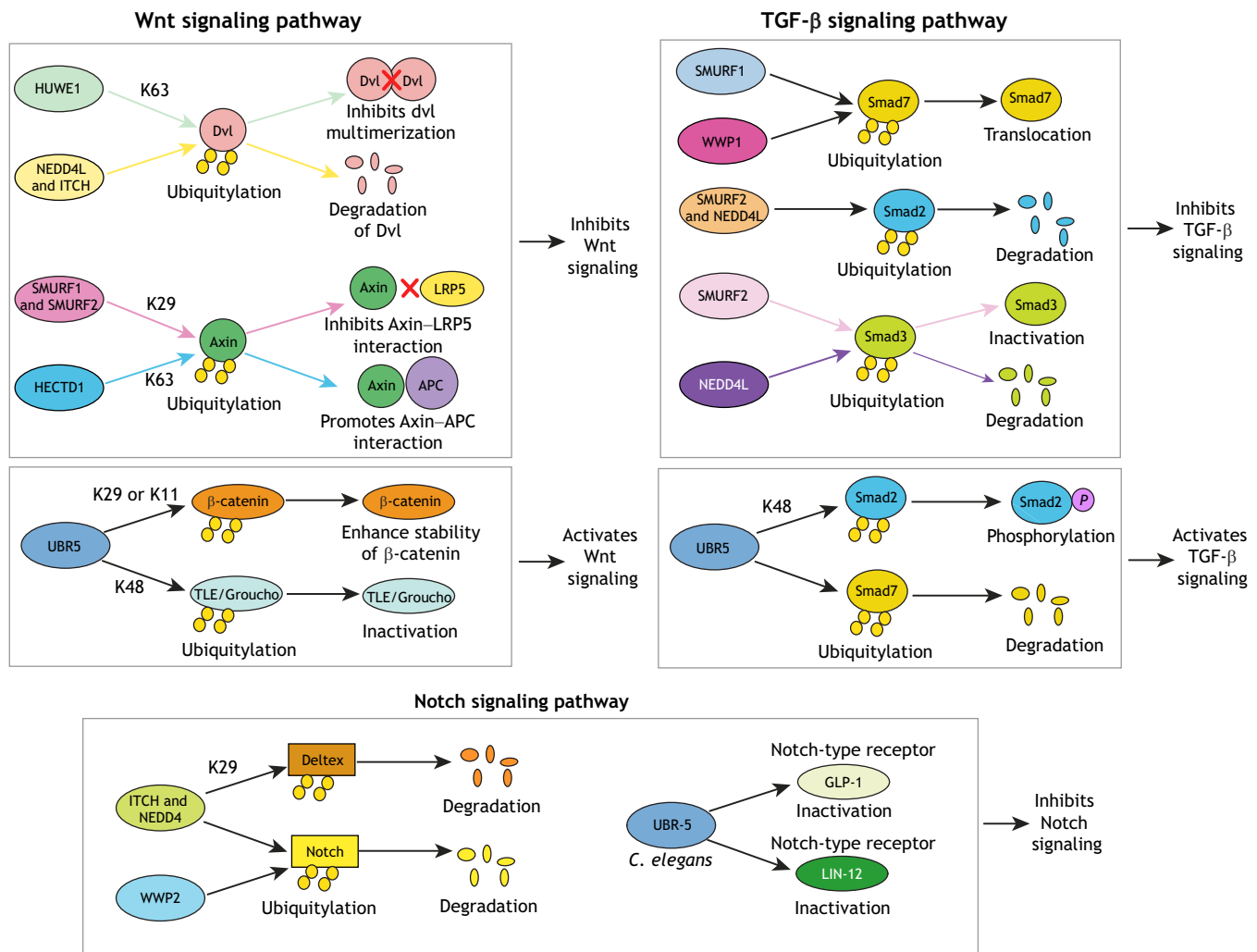
The Notch signaling pathway is involved in regulating many cellular aspects, including cell proliferation, cell fate, cell differentiation and cell death (Kopan, 2012), and dysfunction of this pathway has been shown to be involved in the development of various cancers (Hori et al., 2013). Some HECT E3 ubiquitin ligases have been identified as repressors of the Notch signaling pathway. For example, WWP2 catalyzes the mono-ubiquitylation of the membrane-tethered Notch3 fragment, leading to decreased Notch pathway activity both in cancer cells and during cell cycle arrest (Jung et al., 2014) (Table S2). Moreover, NEDD4 antagonizes Notch signaling by promoting Notch degradation (Sakata et al., 2004), while, in *Caenorhabditis elegans*, UBR-5 limits Notch-type signaling by negatively regulating the activity of GLP-1, a Notch-type receptor (Safdar et al., 2016) (Table S4).

#### TGF- $\beta$ signaling

The transforming growth factor- $\beta$  (TGF- $\beta$ ) signaling pathway regulates cell growth, cell differentiation, apoptosis and cellular homeostasis (Zhang, 2017, 2018). It is well established the members of NEDD4 subfamily play a significant role in TGF- $\beta$  signaling. For example, SMURF1 downregulates TGF- $\beta$  signaling by interacting with inhibitory Smad7 in the nucleus, which causes the translocation of the SMURF1-Smad7 complex to the cytoplasm (Tajima et al., 2003); SMURF1 then associates with the TGF- $\beta$  type I receptor (T $\beta$ R-I, also known as TGFBR1) at the plasma membrane to target it for degradation (Ebisawa et al., 2001; Tajima et al., 2003) (Table S2). Similarly, SMURF2 and WWP1 induce the downregulation of TGF- $\beta$  signaling through the degradation of TGF- $\beta$  receptor via the ubiquitin-proteasome pathway (Kavsak et al., 2000; Komuro et al., 2004; Yang et al., 2009). In contrast, SMURF2 can also target Smad2 and phosphorylated Smad3 for proteasomal degradation (Lin et al., 2000; Wu et al., 2008), as well as ubiquitylating Smad3 to attenuate its activity and enhance TGF- $\beta$  signaling in the cell (Tang et al., 2011). Further studies on the molecular mechanisms employed by the NEDD4 subfamily to enhance and repress TGF- $\beta$  signaling are warranted.

#### Wnt signaling

The Wnt signaling pathway is necessary for the regulation of cell migration, cell polarity, neural patterning, embryonic development and tissue homeostasis; not surprisingly, mutations in members of the Wnt pathway are often linked to birth defects and cancer (Komiya and Habas, 2008; MacDonald et al., 2009). HECT E3 ubiquitin ligases have been shown to positively and negatively



**Fig. 3. Molecular mechanisms used by members of the HECT E3 ubiquitin ligases to regulate Wnt, TGF-β and Notch signaling pathways.** HUWE1, NEDD4L, ITCH, SMURF1, SMURF2 and HECTD1 downregulate Wnt signaling pathway either through mediating the ubiquitylation of Dvl or of Axin proteins. Conversely, UBR5 activates Wnt signaling by regulating the ubiquitylation of β-catenin, which results in enhanced β-catenin stability, as well as that of Groucho/TLE proteins, which inactivates this transcription repressor. SMURF1, SMURF2, WWP1 and NEDD4L E3 ubiquitin ligases suppress TGF-β signaling through promoting the ubiquitylation of Smad family members including Smad2, Smad3 and Smad7. In contrast, UBR5 upregulates TGF-β signaling by ubiquitylating Smad2 and Smad7. ITCH, NEDD4 and WWP2 inhibit Notch signaling by ubiquitylating Deltex (also known as DTX1), an E3 ubiquitin ligase that positively regulates Notch signaling, and targeting Deltex and Notch for proteosomal degradation. NEDD4, ITCH, WWP2 and UBR5 also inhibit the Notch signaling pathway by targeting Notch-like receptors GLP-1 and LIN-12 in *C. elegans* for degradation or inactivation.

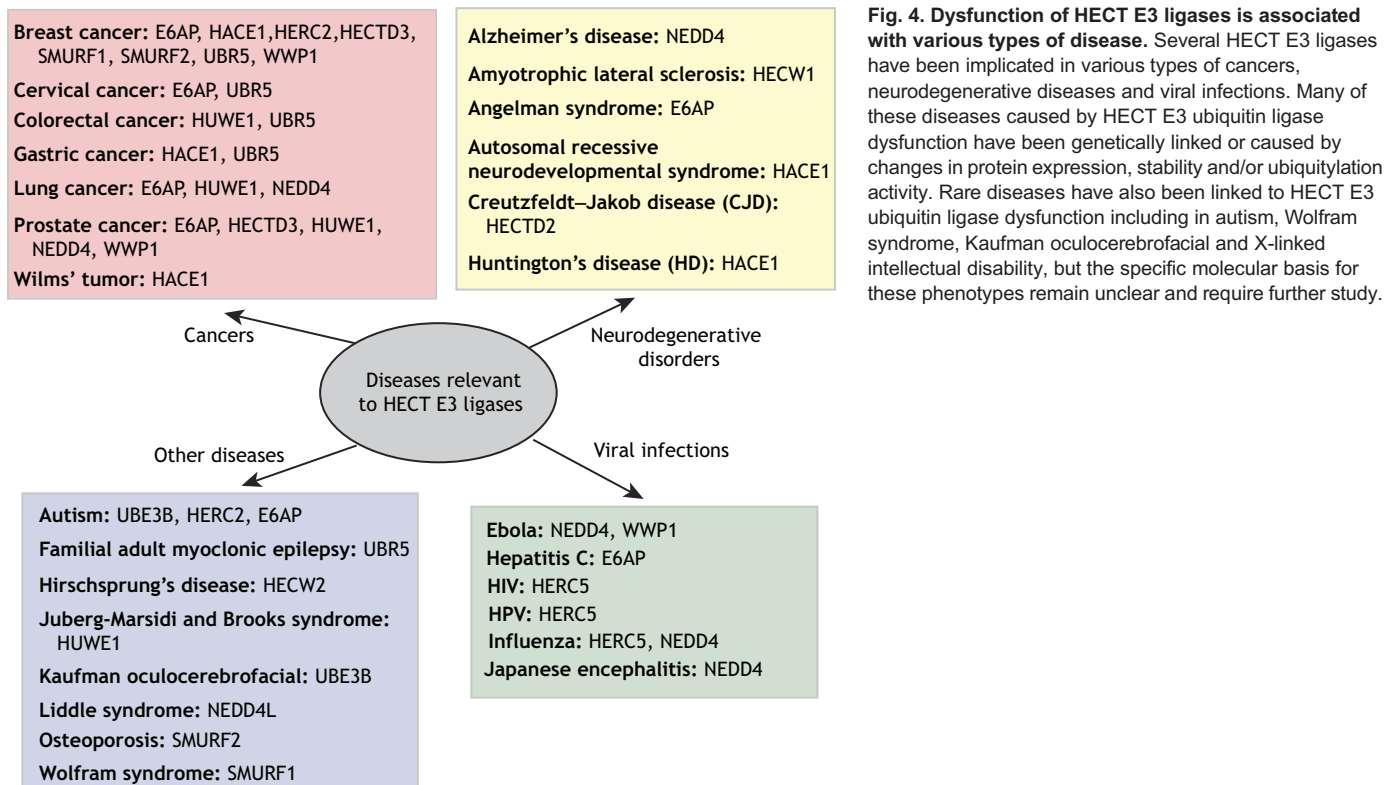
regulate Wnt signaling through binding and inhibiting Axin (herein referring to Axin1), a key regulator of intracellular β-catenin levels (Fei et al., 2013). For example, SMURF1 and SMURF2 both ubiquitylate Axin, which results in disruption of Axin protein-protein interactions and its targeting for degradation (Fei et al., 2013; Kim and Jho, 2010) (Table S2). In addition, both HUWE1 and ITCH can downregulate Wnt signaling by modulating the activity of modulating the activity of dishevelled (Dvl) family proteins, key node proteins in the Wnt signaling pathway. In this context, HUWE1-dependent ubiquitylation of Dvl inhibits its oligomerization and decreases Wnt and β-catenin signaling (de Groot et al., 2014) (Table S4). Likewise, ITCH preferentially ubiquitylates phosphorylated Dvl to induce its subsequent proteasomal degradation (Wei et al., 2012) (Table S2). HECTD1 has also been observed to disrupt Wnt signaling by enhancing the interaction between adenomatous polyposis coli (APC) and Axin (Tran et al., 2013) (Table S4). In contrast, UBR5 has been shown to

increase Wnt signaling by targeting members of the Groucho protein family (also known as TLE proteins), which are Wnt antagonists, for degradation, which in turn enhances β-catenin-dependent transcription (Flack et al., 2017).

Collectively, these findings demonstrate that HECT E3 ubiquitin ligases are critical regulators in the Notch, TGF-β and Wnt signaling. Future studies will help to further clarify how the HECT E3 ubiquitin ligases participate in these important signaling pathways and other various cellular responses critical for cellular homeostasis.

**Disease relevance**

Accumulating evidence suggests that mutations in members of the HECT E3 ubiquitin ligase family are linked to cancers, neurodegenerative disorders and neurodevelopmental syndromes (summarized in Fig. 4), and below we highlight examples for different disease states.



### Cancers

The functional role of some HECT E3 ligases in breast cancer has been extensively investigated over the past decade with numerous studies concluding that there are strong correlations between HECT E3 ligase dysfunction and breast cancer. For instance, the NEDD4 subfamily of HECT E3 ligases have been shown to be primary enzymes involved in the onset of breast cancer. Indeed, expression of the *WWP1* gene is significantly higher in breast tumors than in normal tissues (Chen et al., 2007b, 2009). Elevated *WWP1* expression is also negatively correlated with levels of tumor necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL, also known as TNFSF10), while *WWP1* depletion in breast cancer cells increases TRAIL-induced caspase-8-mediated apoptosis (Zhou et al., 2012). Large tumor suppressor 1 (LATS1) is also targeted for proteasomal-dependent degradation by *WWP1*, which has been linked to breast cancer cell proliferation (Yeung et al., 2013) (Table S2). *SMURF1* has also been shown to support breast cancer cell growth by facilitating estrogen receptor  $\alpha$  signaling, which promotes breast cancer progression (Yang et al., 2018).

Interestingly, there are conflicting reports with regard to the biological role of *SMURF2* in breast cancer. Some studies have shown that *SMURF2* overexpression promotes metastasis and increases migration and invasion of breast cancer cells (David et al., 2014; Jin et al., 2009). *SMURF2* knockdown in human breast cancer cells resulted in increased expression of *SMURF1* to compensate, which, in turn, led to enhanced breast cancer cell migration (Fukunaga et al., 2008). *SMURF2* regulates breast cancer cell proliferation by stabilizing the multi-functional scaffold protein connector enhancer of kinase suppressor of Ras 2 (CNKSR2), which plays an important role in cell proliferation and differentiation, while the knockout of *SMURF2* in breast cancer cells causes enhanced ubiquitylation of CNKSR2, targeting it for proteasomal degradation (David et al., 2018) (Table S2).

Nevertheless, further studies are needed to clarify the exact role of *SMURF2* in breast cancer.

Members of the HERC subfamily have also been linked to breast cancer. A prime example is *HERC2*, which has been shown to mediate the degradation of *BRCA1*, a key breast cancer suppressor protein involved in DNA DSB repair (Wu et al., 2010) (Table S3). *HERC4* expression has also been shown to be elevated in breast cancer cell lines and tissues when compared to a non-tumorigenic cell line and adjacent normal breast tissues (Zhou et al., 2013).

Furthermore, both *UBR5* and *HECTD3* are frequently overexpressed in triple-negative breast cancer and breast carcinomas, respectively (Li et al., 2013; Liao et al., 2017), but the underlying mechanisms are not well defined (Table S4). Further studies are needed to clarify the roles of HECT E3 ubiquitin ligases in breast cancer development.

Several HECT E3 ubiquitin ligases have been linked to prostate cancer. For example, knockdown of *E6AP* attenuates prostate cancer cell growth and promotes senescence (Paul et al., 2016). The overexpression of *HUWE1* was shown to inhibit human prostate cancer proliferation and migration that may be linked to the downregulation of proto-oncogene *c-Myc* (Qu et al., 2018). Deficiency of *WWP1* gene expression in prostate cancer cells has also been observed to significantly suppress cell proliferation and enhance TGF- $\beta$ -mediated growth inhibition (Chen et al., 2007a). *NEDD4* plays a crucial role in the regulation of prostate cancer cell proliferation through its interaction with prostate transmembrane protein androgen induced 1 (PMEPA1) protein; androgen receptor (AR) protein induces *PMEPA1* expression, which in turn causes *NEDD4* to form a complex with *PMEPA1* that ubiquitylates AR for proteasomal degradation (Li et al., 2008a) (Table S2).

HECT E3 ubiquitin ligases have also been shown to be involved in liver cancer development. For example, *HERC4* is overexpressed in hepatoma carcinoma cell lines and leads to increased migration

ability and reduced apoptosis (Zheng et al., 2017). In hepatocellular carcinoma (HCC) cells and tissues, HACE1 expression levels are decreased and depletion of HACE1 accelerates cell proliferation and migration (Gao et al., 2016). Mutations in *UBE3C* promote HCC progression by regulating epithelial–mesenchymal transition in HCC cells (Jiang et al., 2014), and mutant *HUWE1* enhances hepato-carcinogenesis and cell proliferation (Liu et al., 2012).

Lung cancer development is also linked to members of the HECT E3 ubiquitin ligase family. For instance, NEDD4 has shown to have an essential role in the development of non-small-cell lung carcinomas (NSCLCs) (Amodio et al., 2010). Knockdown of NEDD4 markedly reduces NSCLC cell proliferation and tumor growth, whereas NEDD4 overexpression facilitates growth of non-transformed lung epithelial cells and the loss of tumor suppressor phosphatase and tensin homolog (PTEN) (Amodio et al., 2010). Furthermore, levels of HERC4 are elevated in lung tumors when compared to adjacent normal tissues, suggesting that HERC4 could be used as a diagnostic biomarker for lung cancer (Zeng et al., 2015). Lower levels of E6AP have been observed in lung adenocarcinomas that correlate with the reduced expression of the tumor suppressor genes *CDKN2B*, *CDKN2A* and *CDKN2D* (Gamell et al., 2017).

HECT E3 ubiquitin ligases are also implicated in colorectal cancers (CRCs). For example, elevated expression of SMURF1 is associated with CRC progression and poor prognosis (Xie et al., 2014). Additionally, *in vitro* experiments have shown that knockdown of UBR5 prevented proliferation of CRC cells, as well as their colony formation, migration and invasion, suggesting that UBR5 is involved in CRC development (Xie et al., 2017). HECW1 has also been observed to act together with RNF43 to mediate p53-dependent apoptosis in colorectal carcinogenesis (Shinada et al., 2011) (Table S2).

WWP2, HUWE1 and UBR5 also have roles in ovarian cancer development. Indeed, as indicated in the Cancer Genome Atlas (<https://www.cancer.gov/tcga>), the majority of ovarian carcinomas possess a homozygous or heterozygous deletion in the *WWP2* locus, and WWP2 serves as a tumor suppressor by negatively modulating Notch3 signaling in ovarian cancer (Jung et al., 2014). Dysregulation of WWP2 has also been linked to endometrial (Clements et al., 2015) and oral cancers (Fukumoto et al., 2014). In addition, UBR5 expression is enhanced in ovarian cancers, and this has been correlated with its role in regulating modulator of apoptosis protein 1 (MOAP-1) (Matsuura et al., 2017) (Table S4). The *UBR5* gene is also found to be frequently mutated in mantle cell lymphoma (MCL) and may contribute to MCL pathogenesis (Meissner et al., 2013).

Finally, HECT E3 ubiquitin ligases are also involved in more rare cancers, such as gastric cancer, transitional cell carcinoma, esophageal squamous cell carcinoma (ESCC), pancreatic cancer and thyroid cancer. For example, HACE1 expression was shown to be suppressed in gastric cancer tissues, whereas its experimental overexpression can inhibit tumor proliferation, migration and enhance cell apoptosis (Chen et al., 2018). In contrast, NEDD4 expression is upregulated in advanced gastric cancer tissues, with a concurrent increased probability of metastasis (Ye et al., 2014). HECTD3 that has been reported to be overexpressed in ESCC, and facilitates ESCC cell survival and tumor growth (Li et al., 2017). Decreased HUWE1 expression promotes cell proliferation, migration and invasion in thyroid cancer cell lines, whereas overexpression of HUWE1 inhibits thyroid tumor growth (Ma et al., 2016). HUWE1 has also been reported to be downregulated in thyroid cancer tissues and functions as a tumor suppressor by regulating the stability of p53 (Ma et al., 2016) (Table S4).

Taken together, it is evident that members of the HECT E3 ubiquitin ligases play important roles in numerous cancer types. Moving forward, it will be crucial to further compare and contrast how the HECT E3 ubiquitin ligases bind and recognize their interaction partners in normal and cancer cells lines to identify if there are any significant differences that can be exploited for drug intervention. Owing to potential reproducibility issues with some experimental methods, it will be prudent to conduct follow-up studies, such as proteomics and mass spectrometry, to verify substrates of the HECT E3 ubiquitin ligases to further clarify their specific roles in cancer cells.

### Neurodegenerative disorders

The dysfunction of various HECT E3 ligases has also been shown to be a causative agent for neurodegenerative diseases. Currently, links with HECT E3 ubiquitin ligases have been established with Angelman syndrome (AS), Prader–Willi syndrome (PWS), Huntington's disease (HD), Alzheimer's disease (AD), Parkinson's disease (PD), X-linked intellectual disability (XLID), amyotrophic lateral sclerosis (ALS), as well as other more rare diseases.

Both E6AP and HERC2 E3 ubiquitin ligases are involved in AS, a severe neurological disorder characterized by mental retardation, absent speech, ataxia, seizures and hyperactivity (Cooper et al., 2004). Missense and nonsense point mutations as well as chromosomal deletions of the *UBE3A* gene, which codes for E6AP, results in the loss of E6AP ubiquitin ligase activity and is a molecular cause for AS (Cooper et al., 2004; Tomaić and Banks, 2015). Recent proteomic studies indicate that the disruption of HERC2, which has been shown to be a key regulator of E6AP function, results in developmental delay with Angelman-like features (Harlalka et al., 2013).

Large chromosomal rearrangements have also been reported in PWS patients, specifically in the 15q11–q14 region of chromosome 15 that contains the *UBE3A* and *HERC2* genes (Jiang et al., 1998, 2008; Kalsner and Chamberlain, 2015). It is still unknown, however, how the alterations in these genes and their translated proteins lead to the onset of AS and PWS. In addition, *HECTD2* polymorphisms have been shown to be associated with an increased risk of prion infections, including Creutzfeldt–Jakob disease and kuru (Lloyd et al., 2009). Moreover, mutations in *UBE3B* gene have been correlated with the development of Kaufman oculocerebrofacial syndrome (KOS), a rare autosomal recessive neurodevelopmental disorder (Flex et al., 2013; Pedurupillay et al., 2015; Yilmaz et al., 2018).

Since Dvl function is altered in the presence of mutated superoxide dismutase 1 (SOD1), HECW1 plays an important role in ALS pathology through clearing mutated SOD1 by targeting it for proteosomal degradation; this might be clinically relevant as ~20% of patients with familial ALS harbor mutations in the *SOD1* gene (Miyazaki et al., 2004; Zhang et al., 2011). Whole-exome sequencing and homozygosity analysis has also revealed that mutations in the *UBE3B* gene may also be linked to autism spectrum disorders (ASDs) (Chahrouh et al., 2012).

HACE1 has been found to promote the activity of nuclear factor erythroid 2-related factor 2 (NRF2), a known antioxidative stress regulator within the brain that is triggered by oxidative stress (Rotblat et al., 2014). Patients with HD have decreased levels of HACE1 within the striatum, but its role within the antioxidative stress response is still poorly understood. Nevertheless, HACE1 has the potential to serve as a biomarker for HD (Rotblat et al., 2014). NEDD4 can ubiquitylate amino-3-hydroxy-5-methyl-4-isoxazole



propionic-acid receptors (AMPA receptors), which have been observed to be downregulated in mouse models of AD (Rodrigues et al., 2016). NEDD4 can also catalyze the K63-linked polyubiquitylation of  $\alpha$ -synuclein, a major constituent of Lewy bodies, which are neuropathological markers for PD and other  $\alpha$ -synucleinopathies, to prevent its role in synaptic vesicle recycling (Sugeno et al., 2014; Tofaris et al., 2011) (Table S2).

Brain function and neurodevelopment have been shown to be regulated by several members of the HECT E3 ubiquitin ligase family. For example, deleterious missense mutations in *HECW2* have been linked to neurodevelopmental delays and decreased muscle tone (Berko et al., 2017). NEDD4-mediated ubiquitylation of the Ras-related protein Rap2A inhibits its function, leading to a reduction in Rap2A effector kinase activities and enhanced neurite development (Kawabe et al., 2010) (Table S2). Next-generation sequencing (NGS) has also revealed that genetic alterations in *HUWE1* contribute to Juberg–Marsidi and Brooks syndrome, two X-linked intellectual disability syndromes (Bossard et al., 2017; Friez et al., 2016). Mutations in the *HACE1* gene have also been linked to a potentially new autosomal recessive neurodevelopmental syndrome (Hollstein et al., 2015). Deficiency of *HACE1* expression has also been observed to cause decreased synapse counts and promote the neurodevelopmental syndrome spastic paraplegia and psychomotor retardation with or without seizures (SPPRS) (Nagy et al., 2019).

The molecular basis for how each HECT E3 ubiquitin ligase contributes to these different neurological disorders remain largely unclear, with many unanswered questions. To shed further light on this field, multidisciplinary approaches will need to be employed to identify the interactors and/or substrates of the HECT E3 ubiquitin ligases to better understand how the genetic and/or protein function of each enzyme specifically contributes to each neurodevelopmental disorder.

### Other types of diseases

HECT E3 ubiquitin ligases have also been linked to other diseases, including diabetes, Liddle syndrome and laminopathies. For example, UBR5 promotes the ubiquitylation and degradation of phosphoenolpyruvate carboxykinase (PEPCK1), an enzyme that is overexpressed in type II diabetes (Jiang et al., 2011) (Table S4). *HECW2* also has been suggested to play a role in laminopathy pathogenesis by promoting the ubiquitylation and degradation of proliferating cell nuclear antigen (PCNA) and lamin B1, which both bind to lamin A to maintain nuclear envelope integrity and organization (Krishnamoorthy et al., 2018). The partial loss of E6AP can also alleviate Myc-induced B-cell lymphomagenesis (Wolyniec et al., 2012). NEDD4L regulates the turnover of epithelial sodium channel (ENaC), which is essential for preventing Liddle syndrome, a hereditary form of hypertension (Zhou et al., 2007) (Table S2). NEDD4 also regulates denervation-induced skeletal muscle atrophy, as determined by using knockout mice (D’Cruz et al., 2016; Nagpal et al., 2012). These findings showing that HECT E3 ubiquitin ligases are linked to a multitude of diseases underpins the importance of continued research into this class of E3 ubiquitin ligases in order to better understand their etiology.

### Concluding remarks and future perspectives

As outlined here, HECT E3 ubiquitin ligases are involved in numerous cellular pathways, and their dysfunction has been implicated in a myriad of diseases, including cancer, neurodegenerative diseases and neurological syndromes. Over the past decade, our knowledge on

the biological functions and binding substrates for members of the HECT E3 ubiquitin ligase family has expanded considerably.

It is encouraging that high-throughput screening methods are currently being used to identify the full set of substrates for some members of the HECT E3 ubiquitin ligase family, such as mass spectrometry, immunoprecipitation, western blotting, yeast or mammalian two- and three-hybrid screens, and pull-down assays (summarized in Tables S1 to S4). However, outside of E6AP and members of the NEDD4 subfamily, these efforts will need to be expanded to include the so-far understudied members of HECT E3 ligase family. Additional validation experiments are also required to confirm interaction partners or substrates, as well as to determine the mode of interaction, the site(s) of HECT-dependent ubiquitylation, and, importantly, to establish the downstream fate of the substrate after its ubiquitylation.

To that end, the creation of a curated database of validated HECT substrates and binding partners, accessible by the scientific community, would provide an invaluable tool to obtain further insights into the cellular pathways each HECT E3 ubiquitin ligase regulates. It is also worth noting that there is no drug that can specifically target HECT E3 ubiquitin ligases. In this regard, the proteolysis targeting chimera (PROTAC) technology is a promising new development, as this technology can mediate targeted proteolysis of a substrate by using the ubiquitin machinery of the cell and RING E3 ubiquitin ligases (An and Fu, 2018; Paiva and Crews, 2019; Zou et al., 2019), and it might be possible to expand PROTAC to include processive members of the HECT E3 ubiquitin ligase family. Continued biochemical and structural investigations to decipher how exactly HECT E3 ligases interact with their binding partners and substrates will be important in guiding any future drug and therapy development to treat HECT E3 ligase dysfunction in associated diseases.

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The authors declare no competing or financial interests.

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