Title: TGF-β Signalling Regulation: Ubiquitination Isoforms-new players with new potential.

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Subtitle: TGF-β regulation by NEDD4 E3s are discussed, while emphasising the potential role of isoform-derivatives of E1-E3 proteins in ubiquitination.

Key Words: TGF beta, Smad, Ubiquitination, Uniquitinome, EMT, cancer

Abstract
Ubiquitination of protein species in regulating signal transduction pathways is universally accepted as fundamental during normal development and has been implicated in the progression of many human diseases, such as cancer. One particular pathway that has received much attention in this context is TGF-β signalling, particularly during the regulation of EMT and tumour progression. While E3-ubiquitin ligases offer themselves as potential therapeutic targets (based on their ability to confer substrate specificity), much remains to be unveiled regarding mechanisms that culminate in their regulation. With this in mind, the focus of this review highlights the significance of a recently described group of E3-ubiquitin ligase isoforms in the context of the TGF-β pathway regulation. Moreover, we now broaden these observations to incorporate a growing number of protein-isoforms within the ubiquitin ligase superfamily as a whole, and discuss what relevance they may have in defining a new 'iso-ubiquitinome'.

Introduction

Physiological Transforming Growth Factor-beta (TGF-β) cytokine signalling is quite pleiotropic in it's activity, whereby it plays a key role in modulation of the immune response in addition to regulating tissue homeostasis by determining the balance between cell loss and renewal [1]. Of significance are its effects on the cell cycle and apoptosis (as in the instance of genotoxic stress and cytotoxic chemotherapy), thereby contributing to the neoplastic transformation of cells in addition to modulating their motility and invasiveness thereafter by driving Epithelial-Mesenchymal Transition (EMT) [2]. TGF-β initiates signalling by occupying TGFβ-receptor II (TGFβ-RI1), to which TGFβ-receptor I (TGFβ-RI) is recruited. The Intracellular Domain (ICD) of TGFβ-RI is phosphorylated by TGFβ-RII kinase activity, which then phosphorylates and activates regulatory-Smad2 and Smad3 (rSmads) resulting in their nuclear translocation, in a Smad4-dependant manner [3,4]. Subsequently, the modulation of gene expression by the Smads then ensues with the aid of specific co-activators such as p300 and CBP [5] and co-repressors such as Ski [6], SnoN [7] and TGIF [5]. Down-regulation of TGF signalling is under strict control by inhibitory Smad7, which is induced after continual TGF-β stimulation [8] and can act by physically associating with TGFβ-R1, thereby blocking receptor-mediated activity [9].

Physiologically, while TGFβ-RII activation represents one key signalling step in TGF-β signalling, Smad4-mediated translocation of rSmads and the subsequent dampening of TGF-β signalling by Smad7 (Figure 1) are also fundamental regulatory steps shown to be instrumental in the development of TGF-β mediated disease [10,11]. Recently, these intermediates have therefore received much attention in light of how they are regulated by the ubiquitination-proteasomal pathway. Moreover, supported by the observation that certain E3-Ubiquitin ligases such as Smurf2, WWP1 and WWP2 [12-14] have been linked to TGF-β signalling pathway activity and tumour progression, highlighting the importance of the ubiquitination pathway in disease and potentially providing an 'open niche' for the development of intervening therapeutics. For example, while the drug Velcade/Bortezomib [15,16] broadly targets the 26s subunit of the proteasome, as upstream regulators of the proteasome, E3-ubiquitin ligase-directed therapeutics may hold greater potential in being more specific to certain signalling pathways and therefore may demonstrate greater efficacy with possibly less side effects. Additionally, further understanding the mechanistic and biological input of the E1/E2/E3 protein isoforms in modulating the ubiquitination pathway and the 'ubiquitinome', may lead to identification of alternative targets and a new generation of therapeutic agents that may offer even greater specificity in the treatment of disease.

TGF-β and Epithelial-Mesenchymal Transition of cells

EMT is a key reprogramming process that regulates morphological plasticity upon modulating the delamination of epithelial cells resulting in them undertaking a mesenchymal phenotype, thus enhancing their migratory potential. Moreover, mesenchymal cells have the ability to repolarise by MET (Mesenchymal-Epithelial Transition), which (along with EMT) has been recognised as being fundamental during embryogenesis, development and evolution [17]. However, during disease progression, EMT and MET can be devastating (as in the case of cancer), where transformed epithelial cells can spread as tumour metastases and become established at distal sites throughout the host.

Underpinning this is TGF-β signalling, which has received particular attention over the recent years, which can suppress uncontrolled proliferation in normal cells while conversely promoting tumour metastasis by stimulating cells to undergo EMT. Typically, EMT induction arises by normal epithelial cells (and fibroblast cells) responding to TGF-β by synthesising and secreting numerous cytokines, growth factors and Extra-cellular Matrix (ECM) components into the tumour cells microenvironment aiding tumour progression [18,19]. At the molecular level, this occurs by a number of changes in gene expression levels of a number of key products [20-23], namely matrix metalloproteases [24,25], N-CAM [26], UPA [27,28], PAI-1 [29,30], Collagen IV [31], Fibronectin [18], E-Cadherin [32], Vimentin [33] and α-Smooth Muscle Actin [34].

While TGF-β potently activates signalling through the Smad proteins, other signalling networks that directly result in the modulation of the above genes central to EMT, are also activated which result in the modulation of key signalling intermediates and pathways such as Rho GTPases [35,36], PI3K/AKT [37], NF-κB [38], MAP Kinases [39,40].

The Ubiquitination Pathway

The ubiquitination of proteins is evolving to be intrinsic in modulating not just the stability of targeted proteins, but also their sub-cellular localisation and consequently their ability to modulate signal transduction pathways in mammalian systems. Broadly, a three-tiered system exists, which results in the sequential transfer of activated ubiquitin to the target protein-which is colloquially referred to as the 'Ubiquitinome' (Figure 2).

In addition to ubiquitin, small molecules termed 'Ubiquitin-Like Modifiers' (ULMs) have also been described, which contain ubiquitin-like domains and also target substrate proteins *via* the conserved E1-E3 enzyme system of the 'ubiquitinome' with differing outcomes. Examples of these include SUMO, NEDD, ISG15, ATG8, ATG12, FAT10, URM1, UFM1, MNSF-β, which have been shown to modulate intracellular protein localisation, turnover, signal transduction, and transcriptional activation [41].

While ULMs have also been recognised for their ability to regulate the steady-state levels of targeted proteins, for simplicity and clarity, the focus here shall be centred around ubiquitin (for other ULMs, see [41]).

E1 Activating Enzymes

The first step in ubiquitination of target proteins involves the Mg^{++}/ and ATP-dependant activation of the 76 amino-acid ubiquitin protein [42] by the process of Adenylation, which is driven by an E1-Ubiquitin activating enzyme [43]. Eight known mammalian members of the E1 activating enzyme family exist, all of which share significant sequence conservation (see [44]). However, UBE1 [45] [46,47] and the recently

discovered UBA6 (also named UBE1L2 or E1L2 [48-50] are the only 2 'true' E1 ubiquitin activating enzymes, as the rest can activate other ULMs [44].

Structurally, E1s are composed of an Adenylation domain containing 2 ThiF homology motifs (which bind ATP and Ubiquitin), the Catalytic Cysteine Domain (CCD) and the Ubiquitin Fold Domain (UFD, also known as the β-grasp fold) [51-53] The UFD is particularly important as it is responsible for recognition and binding the relevant cognate E2-Conjugating Enzyme [52 49]. To date the regulation of these key components of the ubiquitination pathway, particularly in the context of how such E1 proteins have the ability to be charged by their cognate ULM, have been intensely studied and structurally very revealing [44].

E2 Conjugating Enzymes

Next, the activated ubiquitin is transferred from the catalytic-cysteine of the E1 to the active-site cysteine of the E2 ubiquitin-conjugating enzyme. E2s are at the centre of the ubiquitination cascade of which there are over 30 in number and characterised by the presence of a highly conserved catalytic UBC (Ubiquitin-Congugating enzyme) domain of 150-200 amino-acids [54]. In a recent study addressing the activation of ubiquitin by specific E1- and E2-enzymes, 29 E2s accepted ubiquitin from UBE1 and UBA6 of which 14 were charged by UBE1 (but not by UBA6), 9 were charged by both UBE1 and UBA6 and 1 by UBA6 (but not UBE1), thus highlighting that each E1 has a distinct preference for a specific E2 [49]. Within the E2, it is the UBC domain that is of particular importance as it is responsible for specific binding to the relevant E3-ligase [55]

Structurally, UBC domains consists of α-helices, β-sheets and variable loop regions that surround an active-site catalytic domain [55]. Amino (N-Ex, N-) and Carboxyl (C-Ex, C-) terminal extensions have also been discovered to flank the catalytic domain (Figure 2) and shown to be important in steps such as substrate selection, subcellular localisation and dimerisation. For example, with UBE2C, the N-terminal extension regulates the number of substrate lysines that become modifed by the E3 complex, whereas the N-terminal extension of E2-UbcH10 may also be important for ULM substrate selection and modulating UbcH10 catalytic activity [56,57]. Alternatively, the C-terminal extensions may be generally involved in mediating protein-protein interactions with other members of the ubiquitination pathway. For example, this region in E2-Ubc2 is important in mediating E3-selectivity for the Ubr1 E3-ligase [58]. Also, in the case of (cdc34/UBE2R2) the acidic C-terminal extension, can be targeted for phosphorylation by CK2, which modulates SCF mediated Sic1 ubiquitination and possibly localisation of this E2 [59,60]. Concurrently, E2s can be divided into 4 classes according to the presence of these extensions [61,62].

E3 Ubiquitin Ligases

The final step during the ubiquitination of proteins is mediated by a number of E3-ligases, which result in the transfer of mono- or poly-ubiquitin to target proteins giving rise to the addition of K48-linked ubiquitin chains (where at least 4 are responsible for the 26S proteasomal degradation of proteins) [63], K63-linked chains-which have been implicated in the regulation of a number of effects including protein trafficking, endocytosis and signal transduction [64,65].

E3-ligases can be categorised into 3 distinct groups, the RING- (Really Interesting New Gene), the U box- and the HECT- (Homologous to E6-AP COOH Terminus) family of proteins, based on the presence of these conserved domains-which not only contain the active-sites for these proteins but also serve as domains

that can interact with ubiquitin-charged E2 enzymes [66].

Within the SCF system, the RING (and U-box) E3s exists as an active polypeptide in a multi-component complex, for example the Skp1-Cullin-F-box protein complex-SCF [67]. Based upon their substrate-specificity and the discovery of genetic alterations, E3 ligases have been described to possess tumour suppressive (BRCA1 and Fbw7) and oncogenic properties (Mdm2, Skp2) and indeed do therefore play a key role in tumorigenesis [68]. Mechanistically, the RING finger E3s (to some extent) behave as substrate specific protein adaptors, due to them lacking a catalytic activity and consequently aid the transfer of active-ubiquitin from the E2 to the acceptor protein by positioning the substrate lysine residue in proximity to the E2-ubiquitin conjugate. Ubiquitin transfer occurs in a cyclical manner permitting liberating the E2 to be recharged with ubiquitin in a mechanism distinct to the HECT E3s [69].

To date, 30 HECT E3-ligases have been identified and can be further sub-classified into the NEDD4 E3-ligase family, based on the presence of WW-domains [70]. Structurally, the HECT-E3s are composed of an amino-terminal C2-domain (responsible for calcium, lipid and protein interaction domain- [71], the WW-(which confers substrate specificity and mediates E3-substrate interactions) and the carboxyl HECT-domain (which interacts with the charged E2 [72], thereby physically accepting charged ubiquitin prior to transferring it to the substrate (Figure 3). More recently, it has been demonstrated that sequences other than the PxxY motif may also be responsible for binding the E3 HECT domain, as in the case of the Smad7 and Smurf2 [72].

Structural information regarding E3 HECT ligase domains is limited and, to date, is available for E6AP[73], WWP1 [74], Smurf2[72] and Nedd4-L1 (PDB; Protein Data Bank). In all cases, the HECT domain was seen to be bi-lobal and segregated into two distinct sub-domains comprising an amino- terminal lobe (N-lobe) of about 250 amino acids that contains the E2 binding site and an approximately 100 amino acid C-lobe containing the critical active site cysteine residue. A short linker between the N- and C-lobes provides rotational flexibility, an essential architectural facet that is required for stepwise transfer of ubiquitin from the charged E2 to the E3 and then onto the target substrate. More recently, the structure of the Nedd4 HECT:Ubiquitin complex suggests that non-covalent association with the growing ubiquitin chain at key residues within the HECT domain is flexible enough to promote both ubiquitin transfer and polyubiquitin processivity[75,76]. Undoubtedly, it is likely that further such detailed mechanistic insight into the key catalytic and tripartite ubiquitin transfer step within E3 ligases will prove to be the 'holy-grail' in addressing the future challenge to rationally design and develop any new E3/substrate specific inhibitors.

Regulation of TGF-β signals by ubiquitination

Recently, significant research has focused on defining key regulatory intermediates in TGF signalling and the identification of their specific E3-ubiquitin ligases. More specifically, the Smad7/TGFβ-R1 axis of regulation has been studied the most intensely due to this being a key negative regulatory step responsible for shutting down TGF signals. Consequently, findings here have been extrapolated to define the modulation of the rSmads, which has culminated in revealing novel mechanisms that are spatially and temporally dependant.

Regulatory SMADs2 and 3

Initial studies clearly defined substrate localisation as being a key factor in mediating TGF signalling. For example, rSmad activation is shown to be regulated by adaptor and anchoring proteins such as SMAD Anchor for Receptor Activation (SARA) [77] and Disabled-2 (Dab2) [78] and tubulin [79]. SARA was cloned as a binding partner to Smad2 and unveiled to modulate sub-cellular rSmad and its interaction and activation by TGFβRI. Alternatively, Dab2 can interact with Smad2, TGFβ-RI and -RII and was observed to restore the correct activity of Smad signalling in a TGF-signalling-defective cell line. The tubulin:Smad interaction connects TGFβ signalling to the microtubule (MT) network, and it was shown that disruption of the MT network significantly enhances Smad2 phosphorylation. As to whether these regulatory intermediates can modulate the ubiquitination of rSmads or the TGF-receptors remains to be demonstrated.

Initial regulation of TGF signalling by E3s was derived from a study published in 1999, whereby the E3-ligase Smurf1 was cloned and identified to destabilise Smads-1 and -5 in a proteasomal-dependant manner [80]. Thereafter, by using a combination of database [81] and yeast 2 hybrid-screening [82], Smurf2 was cloned and shown to interact with Smads-1, -2, -3 and -4 and demonstrated to ubiquitinate Smad2 causing it's proteasomal breakdown.

Subsequently, Smad2 has been shown to be ubiquitinated by the E3 NEDD4-2 and which (more importantly) was demonstrated to ubiquitinate and degrade Smad7 and TGFβ-R1 [83]. Moreover, Smad2 can be degraded in a ubiquitination-dependant manner by Tui1/WWP1, an effect that can be potentiated by the expression of TGIF [84]. Conversely, ITCH (or AIP4) has been demonstrated to be required for maximum TGF-β signalling by mediating the phosphorylation of Smad2 (in addition to regulating Smad2/TGFβ-R1 binding [85]), highlighting that E3s do not always negatively modulate TGF-β signalling and that auxiliary proteins can be involved. Additionally, a new tier of Smad2/3 regulation by E3s has recently been elucidated involving their association with Nedd4L following TGFβ-induced Smad linker phosphorylation at a conserved WW domain binding phospho-Thr-Pro-Tyr motif [86]. Moreover, this study demonstrated an extra level of hierarchical control involving by the kinase SGK1, which was shown to phosphorylate Nedd4L to block it's binding to Smads2/3. This highlights a new aspect of E3-ligase regulation of Smads that fine-tunes the potency and timescale of TGF-β activity, and also implicates a broader role for other kinases linked to unrelated signalling pathways in the direct control of E3 ligases associated with TGF-β biological function.

As well as receptor-regulated rSmads, Smad4 as a central component of the Smad signalling pathway, has been seen to be targeted by ubiquitination by the SCF-βTRCP E3-complex [87,88] and by the E3, Jab1 [89]. Here Smad4 was shown to undergo degradation, resulting in diminished cell cycle arrest in response to TGF-β [87]. Thus highlighting that the modulation of TGF signalling may be regulated simultaneously in an orchestrated manner by the NEDD and RING E3s.

More recently, a new E3 regulator of TGF-β signalling known as WWP2 was demonstrated to modulate TGF-β signalling upon interacting with rSmads, resulting in their ubiquitination and degradation [14]. Here, an N- terminal isoform of WWP2 (WWP2-N) was also reported and found to interact with the rSmads, and interestingly this complex was rapidly disrupted in a the presence of TGF-β. Following over-expression of WWP2-N prior to TGFβ treatment, this culminated in reduced TGF-β/Smad-dependent transcriptional activity. Mechanistically, this was proposed to be by 2 proposed mechanisms; 1) by binding and activating full-length

WWP2 in the absence of TGF-β to restrict rSmad levels; and/or 2) by directly binding rSmads and directing activated full-length WWP2 to them for ubiquitination in the presence of TGF-β (Figure 4a). Since Smad2/3 steady-state levels were rapidly depleted upon overexpression of WWP2-FL together with WWP2-N, it was postulated that the former might be the overriding mechanistic explanation for the action of WWP2-N on rSmad stability/activity. Although, an alternative possibility worth exploring in the future is that WWP2-N binding to rSmad in the presence of TGF-β could hinder association with other key signalling intermediates such as Smad4 or prevent rSmad binding to DNA. Interestingly, WWP2-N overexpression was not able to prevent EMT following a prolonged 4-day exposure to TGF-β. Thus the prime functional role for WWP2-N in this context might be to act to prevent or perhaps moderate the onset of TGF-β induced EMT. In this regard, expression profiling of WWP2-N by RT-PCR during TGF-β dependent EMT revealed that it disappeared at day-4 as this particular differentiation programme comes to an end. Thus highlighting that WWP2-isoforms, and WWP2-N in particular, may serve to co-ordinate and orchestrate key biological outputs such as EMT, and that this activity might ultimately be globally determined by external input from other related and/or un-related signalling pathways that determine temporal patterns of E3-ligase isoform gene expression.

Collectively, the above observations highlight the importance of Smad-specific E3s, in addition to unveiling the mechanisms underlying how these E3s may be regulated by accessory proteins such as E3-ligase isoforms, and what biological relevance these intermediates may possess. Whether such protein isoforms (or transcription spliced variants) exist for other E3s (or even E1 and E2s) is certainly an interesting area of research that may hold many answers to how the 'ubiquitinome' may be mechanistically regulated at the molecular level.

Inhibitor SMAD 7 and TGFβ-R1

Within the TGF-β signalling system, all of the Smad proteins (with the exception of Smad4) contain the PxxY motif, which can be recognised by the WW-domains of the relevant substrate-specific Nedd E3-ligase [90]. Consequently, numerous E3s from the Nedd family have been unveiled to be important modulators of these key TGF signalling intermediates, the most characterised being the Smurf and WWP E3s.

While originally cloned as a protein that can block the signalling of TGF-β receptors, Smad7 [8,9] was later revealed to regulate ubiquitin-mediated TGFβ-R1 degradation [91,92]. Subsequently, a number of studies have added to these findings by identification of Smurf1 as a key E3 involved in this-a protein from the Nedd4 family of E3s. For example, Ebisawa et al. identified Smurf1 as a protein that interacts with TGFβ-R1 via Smad7, destabilising both of these proteins, thus highlighting a novel mechanistic role for Smad7 as an adaptor protein in down-regulation of Smurf1-mediated TGF-β signalling [92]. Similarly, Smad7 can also act as a scaffolding protein for WWP1- and Nedd4-mediated ubiquitination of Smad4 [93].

Following the identification of another member of this E3 family, Smurf2 was observed to be less active in destabilising Smad7 but still potently destabilised TGFβ-R1 adding further support to the role played by Smad7 as a scaffolding protein [91]. Mechanistically, recent findings whereby a C-terminal Nuclear Export Signal (NES) present in Smad7 has been reported, highlights how Smad7 may utilise Crm1 to achieve efficient export of Smurf1 to TGFβ-R1 [94]. Indeed, in the case of Smad7 and

Smurf1, while proteins may act cooperatively by the modulation of the nuclear export of this complex being mediated by Smad7, the C2-domain of Smurf is believed to direct this complex to the plasma membraine, where the degradation of TGFβ-R1 can be mediated [95]. Similarly, WWP1 [96] has been shown to stimulate the nuclear export of Smad7 followed by enhanced TGFβ-R1 binding and degradation, resulting in reduced TGF-mediated Smad2 phosphorylation and activation [97]. Consequently, this highlights an important role for the C2-domain of these Nedd4 E3s. More recently, the Smurf1 C2 domain was shown to play a role in substrate selection as well as sub-cellular localization [98], and the calcium binding properties of this domain could provide an important regulatory role within the Nedd4 family [99], representing an interesting avenue worth pursuing in the future.

More recently, we have reported that the C2 domain may also stimulate the intermolecular activity of the full length E3, as in the case of WWP2-N and full length WWP2-FL [14]. Moreover, in this study the WWP2-C isoform, which lacks the C2-domain was observed to be potently active in ubiquitination of Smad7, thereby highlighting an additional intramolecular inhibitory role for the C2-domain in modulation of WWP2-FL [14] and this adds a new layer of complexity of WWP2 isoform and Smad regulation relevant to EMT and cancer (Figure 4b). These observations are also in strong agreement with the regulatory mechanism proposed for Smurf2, where an intramolecular interaction between the C2- and the HECT- domain results in the inhibition of Smurf2 activity [100], thereby tightly regulating the activity of this E3-ligase and thus preventing the auto-activation and the uncontrollable loss of substrate.

Collectively, these observations indeed highlight an important and complex regulatory role for the C2- and HECT-domains of the above E3s, in addition to emphasising the importance of E3-derived isoform proteins and their expression. Moreover, in the case of WWP2-N, expression studies were shown to modulate WWP2-mediated ubiquitination activity and Smad/TGF-β dependent gene expression, thus highlighting a novel paradigm in regulation of WWP2-FL and isoform-specific biological output [14].

Another member of the Nedd family of HECT E3s recently demonstrated to enhance Smad7-TGFβ-R1 binding in the absence of ubiquitination activity, which results in down-regulation of TGF signals, is AIP4 (ITCH), highlighting that E3s may also take on the role as scaffolding proteins [101]. Moreover, a mechanism involving the autoubiquitination and degradation has also been suggested for ITCH [102], which may also be triggered upon JNK-mediated phosphorylation of this E3-ligase [103]. Similar autoubiquitination of E3s, as a ubiquitination-derived mechanism for self-regulation has also been proposed for WWP1 [104] and WWP2 [14].

With the above in mind, other E3s from the RING-family of proteins have been demonstrated to modulate TGF-β signals. In this context, Arkadia was found to negatively regulate Smad7 [105,106]; by the ubiquitination pathway (in the absence of TGFβ-R1 binding) causing the amplification of TGF-β signals, highlighting a mechanism distinct to the Smurf proteins [105] during EMT and renal fibrosis [107]. Moreover, substrate specificity for Arkadia may also be modulated by Axin behaving as an accessory protein that aids the nuclear export of Smad7 [108]. However, it is worth noting that Arkadia also acts independently of Smad7 in TGF-β pathway regulation to further regulate signal intensity by inducing ubiquitinantion and turnover of other substrates, including transcriptional co-repressors SnoN and c-Ski [109,110].

Collectively, these findings highlight insight into how the Nedd and RING E3-ligases may be regulated mechanistically by auxiliary and E3-derived isoform

proteins. In the case of WWP2-FL, the WWP2-N and WWP2-C proteins and their function in E3-mediated ubiquitination and destabilisation of SMAD-2, -3 and -7 highlights that these isoforms do indeed play a very apparent role in TGF signalling which themselves may also be viewed as auxiliary proteins that work in cooperation with their full-length counterpart, WWP2-FL. Moreover, identification and characterisation of such isoforms for other E3s (or even E1 and E2s) and their role in substrate-directed ubiquitination in addition to how these proteins may be regulated by the charged dynamics of E1 and E2 probably warrants further investigation.

Regulation of the E1-E3 Ubiquitination pathway intermediates

Whereas hundreds of E3-ligases have been identified and described, outside of the structural requirements for substrate recognition, events that physically modulate the biochemical activity of E3s are poorly understood and could therefore open up a novel regulatory niche that predisposes this pathway to therapeutic intervention in disease. While we have touched upon, protein trafficking as being one possible mechanism of E3-protein regulation and the role of Smads behaving as scaffolding proteins, other modes of regulation of the ubiquitin pathway do indeed exist, such as by caspase cleavage [111].

In this section we would like to focus on introducing the potential involvement of key ubiquitination protein isoforms and the regulatory roles they have been (or may have been) recognised to exhibit during ubiquitination. An interesting and important feature of HECT E3s is their ability to catalyze both substrate poly-/mono-ubiquitination as well as auto-ubiquitination. In some cases, and dependent on the type of ubiquitin-chain linkage, this can culminate in protein turnover as well other non-proteasomal biological functions. This has particular relevance to not just highlighting targeted substrate recognition, but auto-ubiquitination of E3s as a key regulatory step in addition to the interplay and dynamics between E1-E3 proteins, which in this context is relatively poorly understood.

To establish whether protein isoforms may potentially exist for the E1-E3 enzymes as potential modulators of the ubiquitinome, we scrutinised the ENSEMBL database for the presence of alternative transcriptional (validated or non-validated) and protein-isoform variants for specific E1-E3 enzymes (Tables 1-3).

In conjunction with recent findings reported by Jin et al [49] we firstly searched for variants for UBE1, UBA6 (E1 activating enzymes), secondly for their cognate E2-conjugating enzymes which accept activated ubiquitin and thirdly, we specifically searched for spliced-variants for members of the Nedd4 E3 family. Surprisingly, our findings highlighted that indeed protein isoforms do exist for this pathway and in most cases for which, very little has been reported.

E1 Activating Isoforms

In the case of the two E1s, UBE1 and UBA6, scrutinising the ENSEMBL database revealed transcription spliced variants that may encode protein isoforms which may have carboxyl-terminal deletions-with respect to the full-length UBE1 protein (Table 1). As these derivatives may posses defective UFDs, it is likely that the interaction of these predicted E1s with their cognate E2s may be impaired. The existence of these isoforms is supported by findings which reported that total E1-ubiquitin activating enzyme UBE1 can exist as two isoforms, E1a (117kd) and E1b (110 kDa)-arising from possible alternative start sites within the mRNA [47,112]. Both of these isoforms were observed to be exclusively compartmentalised in the nucleus (Ela) or the cytoplasm (E1b) [113], primarily reflecting the essential requirement for E1a

activity during distinct compartmentalised phases of the cell cycle. Also, E1a was modified post-translationally by phosphorylation [114], in a cell cycle-dependant manner [115]). How these isoforms modulate the mechanistic activation of ubiquitin and the subsequent transfer of charged ubiquitin to the E2 conjugating enzyme remains to be demonstrated.

In the instance of UBA6, no validated protein isoforms derived from this gene locus have been described in the literature. However, 2 alternative transcripts were detected within the ENSEMBL database (Table 1), predicted to encode the first 389 aa, which putatively encode the inter-domain dimerisation motif (as part of the conserved Ube1 repeat)-but lack the CCD and UFD domains (UBA6-002). Whereas, UBA6-005 may encode a protein theoretically lacking a significant amount of the N- and C-region of UBA6, while still retaining the ATP binding motifs and the active-site cysteine residue. As differences in the N-terminal ThiF region may alter adenylation for the ULM by the E1 and taking into consideration that some E1s require the N-terminal region for dimerisation, the prediction would be that UBA6-005 may certainly exhibit some unique biochemical properties with respect to whether it is functionally active or may behave in a dominant-inhibitory manner.

E2 Conjugating Enzyme Isoforms

For the E2 Ubiquitin conjugating enzymes, the ENSEMBL database revealed in the instance of UBE2-A, -C, -D2, -D3, -E1, -G2, -H, -J2, -Q2 and -W2 that validated protein-isoforms of these intermediates do indeed exist (Table 2), which predictably may have altered specificity for the E1 activating enzyme UBE1. Additionally, predicted and non-validated transcription spliced-variants of UBE2 -E2, -E2B, -E3, -D4 and -L3 were also identified for which protein isoforms are yet to be confirmed.

Similarly, for E2s that are specific for the E1 UBA6, validated protein-isoforms were detected for UBE2-E2A, -G2, -2C, -D2, -D3, -E1 and non-validated transcription-isoforms for UBE-2B, -D4, -E2, -E3, -L3, -2Z (Use1), (see Table 2).

Here, of interest is the E2 isoform (which lacks the N-or C-terminal regions) that is shared by UBE1 and UBA6, such as UBE2D (-see Jin et al [49]), for which 4 validated protein-isoform variant exists. One of these, called UBE2D2-002 contains subtle amino-acid substitutions and lacks the N-terminal 30aa. Bearing in mind the possible function of the N-terminal extensions present in the E2s, could the UBE1 and UBA6 proteins have different specificities for this E2 protein-isoform, in addition to this isoform possessing impaired biochemical properties?

Moreover, UBE2B-005 and -008 transcripts encode potential proteins of respective sizes 138 and 141 aa, that lack the N- and C- terminal residues. May these potential isoform proteins consequently have impaired affinity for UBE1 and UBA6 E1-activating enzymes (or even E3-ligases)?

E3 Ligase Isoforms

For the E3s, there is accumulating literature recognising the presence of protein isoforms within the Nedd family, including Nedd4L [116], Nedd4-2 [117], WWP1 [118] and WWP2 [14]. While expressed as paralogues [116], and unlike Nedd4, Nedd4L contains an N-terminal deletion, which results in it having a partial C2-domain and is catalytically more active than Nedd4. Not only could this be due to the N-terminal having defective intramolecular inhibitory properties it could also be due to the incomplete C2-domain being unable to target its substrate protein to the plasma membrane (as in the case of Smad7/Smurf1). However, other regulatory mechanisms

such as whether it can form homodimers and whether it's ability to be modulated correctly by the E2 conjugating enzyme remains to be seen. No such validated protein-isoforms or splice-variants have been detected for Smurf2, whereas several additional non-validated transcription splice-variants have been identified for WWP1, ITCH, NEDL1, NEDL2 and await to be confirmed for their protein products (Table 3).

As demonstrated by Soond et al, WWP2 proteins isoforms WWP2-N and –C are expressed in addition to full-length WWP2 [14]. While their mechanistic interplay in ubiquitination of Smads -2, -3, -7 and WWP2 autoubiquitination have been highlighted, very little is known regarding their biochemical regulation by the E2s. For example do they modulate any regulatory mechanism for the E2s? Is the specificity of WWP2-C altered for any particular E2 and ULM-charged E2 in relation to WWP2-FL? Do these WWP2-isoforms have differing subcellular localisation that may imply protein trafficking as being fundamental in their regulation? Do these isoforms modulate the stability of TGFβ-R1 in a Smad7-dependent manner, as in the case of Smad7-Smurf? Is phosphorylation important for WWP2 isoform regulation, auto-ubiquitination and/or substrate recognition of WWP2? Are other accessory proteins such as Nedd4 family-interacting proteins (NDFIPs[119]) differentially controlled by distinct E3 isoforms? These are all very interesting and valid questions for which the answers are eagerly awaited. At the same time, it is also worth considering the diverse substrate specificities of E3-ligases, perhaps linked to isoform-specific inter-regulation, and particularly in the context of disease-specific function. For example, the WWP2 E3-ligase has a growing list of known physiological substrates including Oct4 [120,121], DMT1 [122], RNA Polymerase II [123], Goosecoid [124], and most recently PTEN [125]. Thus, it is also timely to begin to address the question of E3-isoform substrate preference *in vivo*, and in appropriate physiological settings.

In contrast to the HECT E3s, the RING E3s and their isoforms are extensive in number and in some cases have been well documented. For example, βTRCP is encoded by 2 genes (βTRCP1 and 2), which give rise to 2 protein variants, -beta and –gamma, which are present in the cytoplasm and nucleus, respectively [126], highlighting subcellular specific activities. Fbw7 [127], SIAH [128], RUNX1 [129], BRCA [130], RBCK [131] and Pir2B [132] are also other RING E3-ligases that protein-isoform have been reported for. As to how these variants may be modulated by the E2s remain largely uncharacterised.

In TGF-β signalling, while some of the regulatory E3-isoforms identified to date may not have been validated (Table 3), some protein-isoforms certainly have and warrant further investigation into the precise mechanistic role played by them within the ubiquitination pathway using model systems. Taken with what little is known about how the alternative variants of these proteins are generated, the transcriptional regulation of such variants certainly does also warrant further study and may consequently hold great mechanistic potential. This is based upon the unique properties the protein variants may exhibit in the different stages of tumour progression, such as EMT and cancer cell invasiveness.

Conclusions and Future Directions

In the rapidly growing field of ubiquitination research, where great strides are being made in extrapolating the findings of basic research into translational biology, it certainly seems that the future holds great hope. With this in mind, and while initial observations point to a simple sequence of events that lead to protein ubiquitination, the reporting and characterisation of E1-E3 isoform variants is set to change the way we view the underlying regulation of the ubiquitination pathway and their respective substrates. Based on our initial findings, reported herein and in the case of E3-isoforms (like WWP2-N or WWP2-C), protein-isoforms of this pathway may have a unique biological and regulatory role to play during onset of disease and development. Consequently, is it too premature to ask 'whether the E3 (or even the E1 and E2) isoforms may modulate the 'ubiquitinome?'. While research into this phenomenon is in its infancy, the answer to this is question at this juncture in time is 'quite possibly'. Indeed the literature does suggest that structurally, the UBE1 isoforms [112] may have differing biochemical properties based upon their sub-cellular location and phosphorylation status. In the case of E2-isoforms, these may modulate E3 enzymatic activity directly or as competitive inhibitors towards activated E1 and E3 isoforms.

With this in mind and taken with how fast this field of research is developing, it is therefore justified that the nomenclature in this rapidly exciting field should also be permitted to evolve from contextualising all that embodies ubiquitination *via* the colloquial 'ubiquitinome' to possibly the 'isoubiquitinome'-all that embodies ubiquitination in light of the existence of protein-isoforms. Consequently, this should paint a more accurate (and tangible) picture of how the ubiquitination pathway is regulated and thus highlight more specific and potential targets to aid therapeutic development within this exciting field of research.

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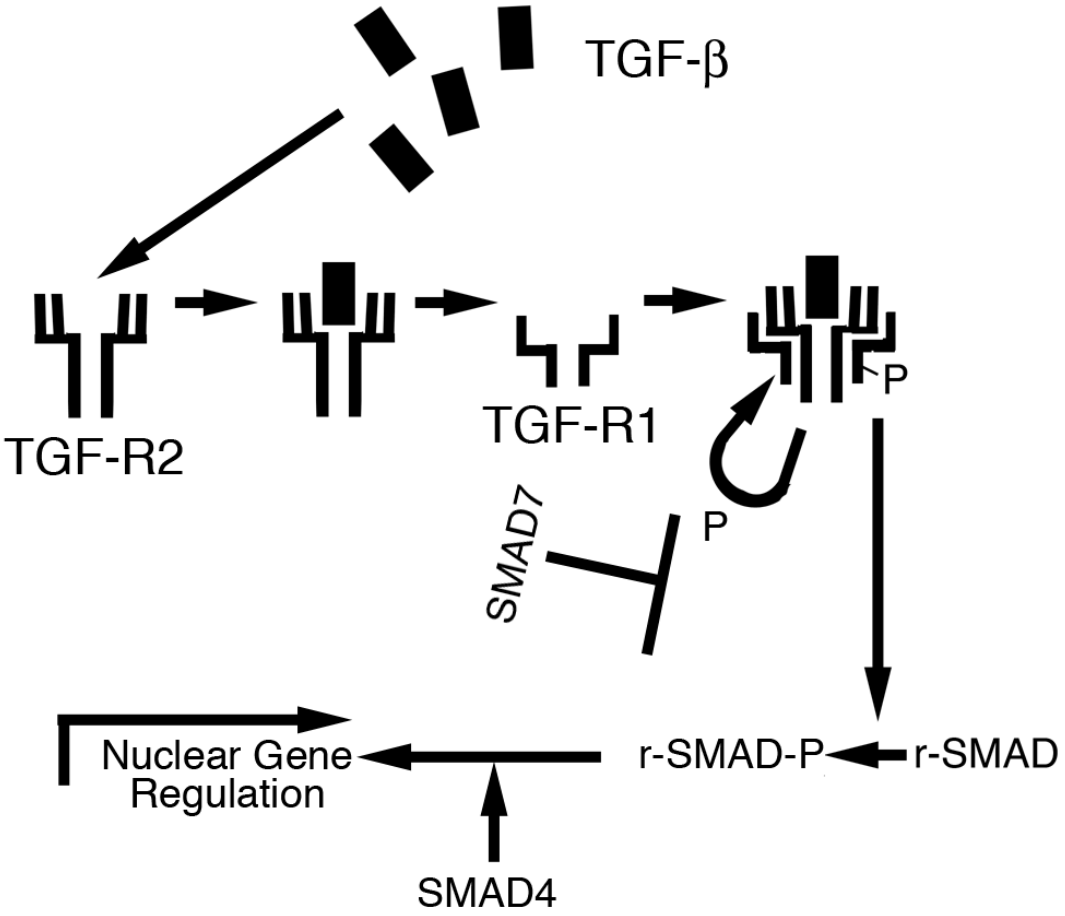

Figure 1: The TGF signalling pathway highlighting TGFβ-RII receptor occupation by TGF-β, the trans-phosphorylation of TGFβ-R1 and Smad4-mediated gene regulatory events dictated by Smads2 and 3. Smad7 is highlighted as a central negative-regulator of signalling.

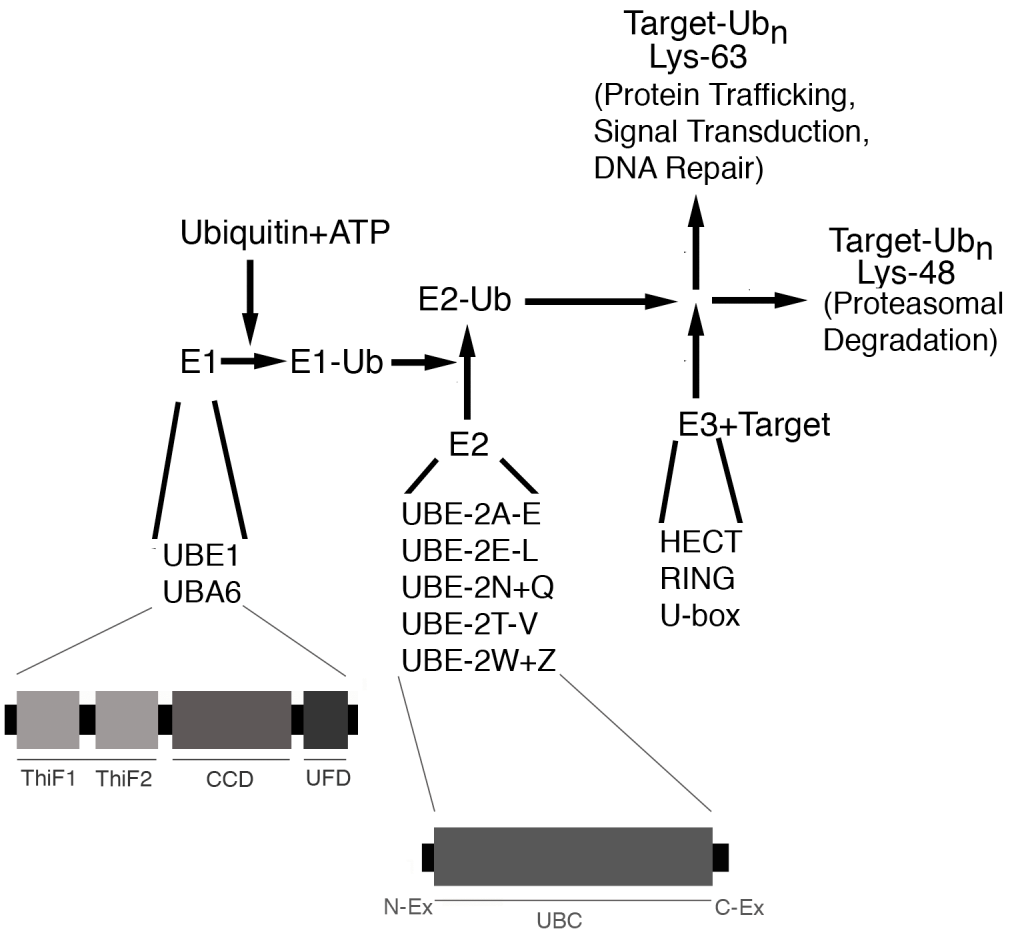

Figure 2: The ubiquitination pathway highlighting the identity of the E1-E3 enzymes involved, their structural motifs and the functional role of the resulting ubiquitinated-substrate species.

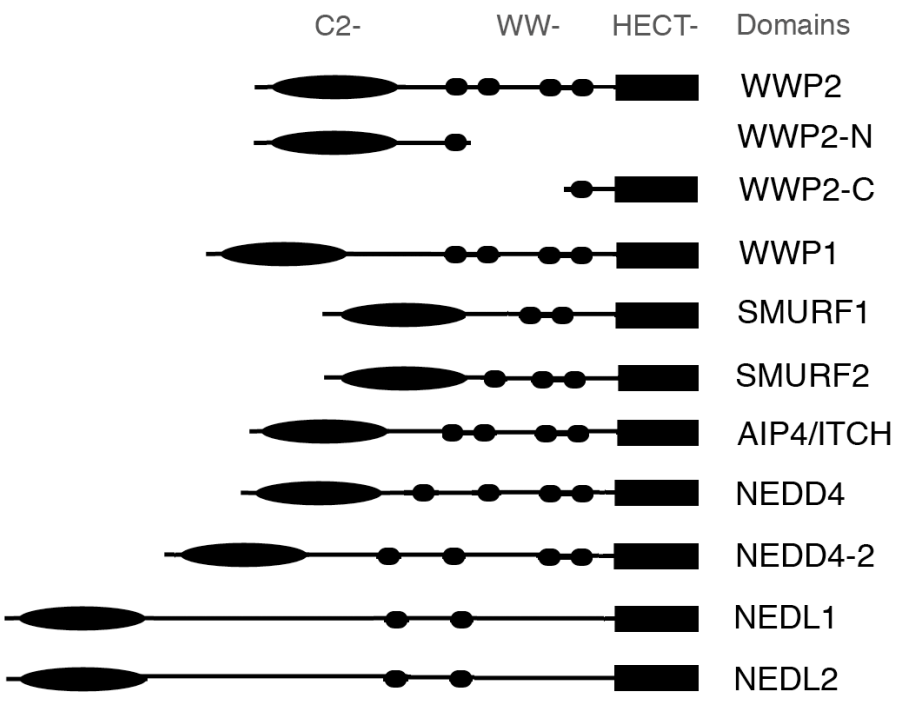

Figure 3: The structural architecture of the Nedd4 family of E3-ligases.

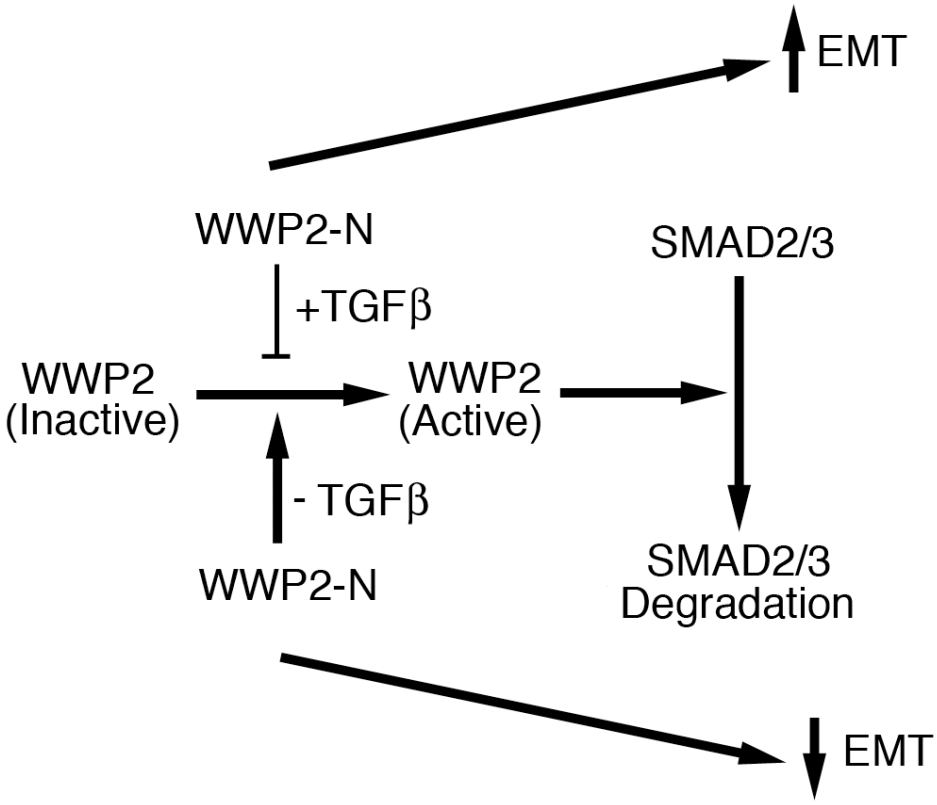

Figure 4a: Proposed modulation of Smad-ubiquitination by the WWP2 E3-ligase isoforms in regulating TGF-β-dependent EMT. WWP2-N has been proposed to be an intermolecular activator of WWP2-FL (in the absence of TGF-β stimulation), which reduces EMT by destabilising the rSMADs. However, in the presence of TGF-β stimulation, this regulatory step can be downregulated, giving rise to less active WWP2 and enhanced rSMAD-mediated EMT.

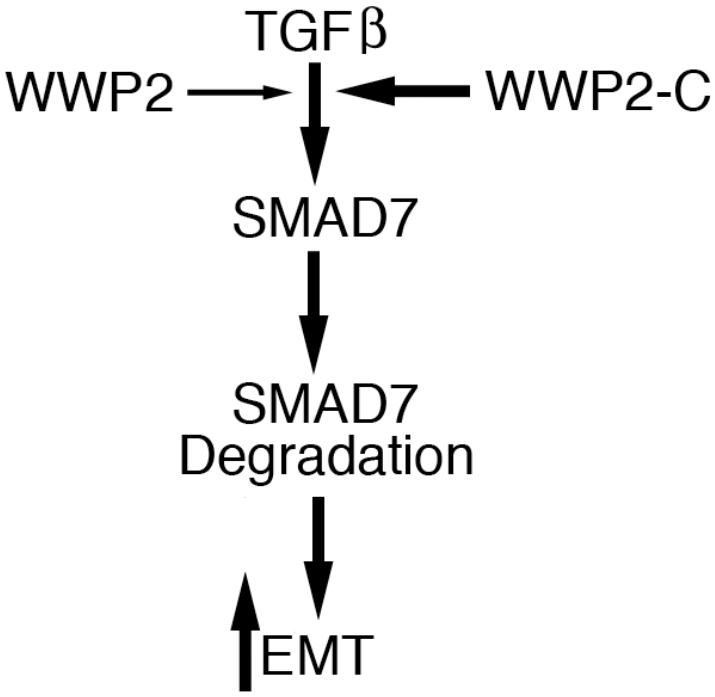

Figure 4b: WWP2-C was observed to have enhanced Smad7 destabilisation properties in comparison to WWP2-FL, highlighting an intramolecular inhibitory role for the C2 domain. Consequently, expression of WWP2-C results in enhanced EMT of cells in comparison to the WWP2-FL protein.

E1-Name	Transcript ID	Length-bp	Protein ID	Length-aa	CCDS
UBA1-002	ENST00000377351	3466	ENSP00000366568	1058	CCDS14275
UBA1-003	ENST00000377269	2497	ENSP00000366481	506	-
UBA1-004	ENST00000412206	1148	ENSP00000415033	271	-
UBA1-005	ENST00000427561	671	ENSP00000397816	173	-
UBA1-006	ENST00000457753	809	ENSP00000404796	195	-
UBA1-007	ENST00000442035	1054	ENSP00000389583	284	-
UBA1-008	ENST00000451702	1706	ENSP00000401101	234	-
UBA6-001	ENST00000322244	6455	ENSP00000313454	1052	CCDS3516
UBA6-002	ENST00000420827	1664	ENSP00000399234	389	-
UBA6-005	ENST00000505673	816	ENSP00000421984	272	-

Table 1: Identification of transcriptional splice variants of E1-ubiquitin activating enzymes. ENSEMBL and Consensus CDS (CCDS) accession numbers are highlighted in blue.

E2-Name	Transcript ID	Length-bp	Protein ID	Length-aa	CCDS
UBE2A-001	ENST00000371558	1796	ENSP00000360613	152	CCDS14580
UBE2A-002	ENST00000346330	612	ENSP00000335027	122	CCDS14581
UBE2A-003	ENST00000371569	2643	ENSP00000360624	77	CCDS14582
UBE2B-001	ENST00000265339	2515	ENSP00000265339	152	CCDS4174
UBE2B-005	ENST00000506787	500	ENSP00000426364	138	-
UBE2B-008	ENST00000507277	644	ENSP00000425137	141	-
UBE2C-001	ENST00000356455	850	ENSP00000348838	179	CCDS13370
UBE2C-003	ENST00000335046	737	ENSP00000335674	161	CCDS13371
UBE2C-004	ENST00000372568	914	ENSP00000361649	140	CCDS13374
UBE2C-005	ENST00000243893	476	ENSP00000243893	50	CCDS13373
UBE2C-006	ENST00000352551	665	ENSP00000333975	150	CCDS13372
UBE2D2-001	ENST00000398733	2702	ENSP00000381717	147	CCDS43369
UBE2D2-002	ENST00000505548	1113	ENSP00000424941	118	CCDS47275
UBE2D3-001	ENST00000453744	3990	ENSP00000396901	147	CCDS3660
UBE2D3-002	ENST00000343106	2389	ENSP00000345285	148	CCDS3661
UBE2E1-001	ENST00000306627	1511	ENSP00000303709	193	CCDS2638
UBE2E1-002	ENST00000346855	1398	ENSP00000329113	176	CCDS2639
UBE2G2-001	ENST00000345496	3106	ENSP00000338348	165	CCDS13714
UBE2G2-005	ENST00000330942	721	ENSP00000331384	137	CCDS33586
UBE2H-001	ENST00000355621	5151	ENSP00000347836	183	CCDS5814
UBE2H-006	ENST00000473814	535	ENSP00000419097	152	CCDS47710
UBE2J2-001	ENST00000349431	1733	ENSP00000305826	259	CCDS14
UBE2J2-015	ENST00000400930	1021	ENSP00000383719	275	CCDS15
UBE2J2-016	ENST00000400929	1057	ENSP00000383718	207	CCDS16
UBE2Q2-001	ENST00000267938	3130	ENSP00000267938	375	CCDS10286
UBE2Q2-202	ENST00000426727	2971	ENSP00000400960	359	CCDS45309
UBE2W-001	ENST00000517608	8426	ENSP00000428813	151	CCDS47874
UBE2W-002	ENST00000419880	2281	ENSP00000397453	162	CCDS47875

Table 2: Identification of the E2-conjugating enzyme transcription splice-variants and protein isoforms. ENSEMBL and Consensus CDS (CCDS) accession numbers are highlighted in blue.

E3-Name	Transcript ID	Length-bp	Protein ID	Length-aa	CCDS
SMURF1-001	ENST00000361125	5737	ENSP00000354621	757	CCDS34690
SMURF1-002	ENST00000361368	5581	ENSP00000355326	731	CCDS34689
SMURF2-201	ENST00000262435	3866	ENSP00000262435	748	CCDS32707
NEDD4-001	ENST00000338963	7019	ENSP00000345530	1247	CCDS10156
NEDD4-006	ENST00000435532	5760	ENSP00000410613	900	CCDS45265
NEDD4L-204	ENST00000357895	8378	ENSP00000350569	967	CCDS45874
NEDD4L-205	ENST00000382850	8413	ENSP00000372301	955	CCDS45873
NEDD4L-206	ENST00000400345	8473	ENSP00000383199	975	CCDS45872
NEDD4L-207	ENST00000431212	8300	ENSP00000389406	854	CCDS45875
NEDD4L-208	ENST00000435432	8406	ENSP00000393395	834	CCDS45876
WWP1-201	ENST00000265428	3362	ENSP00000265428	922	CCDS6242
WWP1-202	ENST00000341922	2972	ENSP00000340564	792	-
WWP1-203	ENST00000349423	2708	ENSP00000342665	704	-
WWP1-204	ENST00000436619	2163	ENSP00000415672	180	-
ITCH-001	ENST00000374864	6401	ENSP00000363998	862	CCDS13234
ITCH-002	ENST00000262650	3730	ENSP00000262650	903	-
HECW1-001	ENST00000395891	6839	ENSP00000379228	1606	CCDS5469
HECW1-002	ENST00000453890	5169	ENSP00000407774	1572	-
HECW1-007	ENST00000429529	415	ENSP00000413336	139	-
HECW1-201	ENST00000265522	6781	ENSP00000265522	346	-
HECW2-001	ENST00000260983	6926	ENSP00000260983	1572	CCDS33354
HECW2-002	ENST00000409111	6690	ENSP00000386775	1216	-
HECW2-003	ENST00000452031	620	ENSP00000409918	133	-
HECW2-004	ENST00000427457	568	ENSP00000395770	118	-

Table 3: Identification of the NEDD E3-ligase transcription splice-variants and protein isoforms. ENSEMBL and Consensus CDS (CCDS) accession numbers are highlighted in blue.