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(DE) -Ubiquitination in The TGF-β Pathway

Prasanna Vasudevan Iyengar[1] and Pieter Johan Adam Eichhorn[1,2*]

[1]Cancer Science Institute of Singapore, National University of Singapore, Singapore
[2]Department of Pharmacology, Yong Loo Lin School of Medicine, National University of Singapore, Singapore

***Corresponding author:** Pieter Eichhorn, Cancer Science Institute of Singapore, MD6 Centre for Translational Medicine, NUS Yong Loo Lin School of Medicine, 14 Medical Drive #12-01, Singapore 117599; Fax: (65) 6873 9664; Tel: (65) 6516 5475; E-mail: csipjae@nus.edu.sg

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Abstract

Aberrations in the enzymes that modify ubiquitin moieties have been observed to cause a myriad of diseases, including cancer. Therefore a better understanding of these enzymes and their substrates will lead to the identification of prospective druggable targets. Here we discuss the role of ubiquitin modifying enzymes in the canonical TGF-β pathway highlighting the ubiquitin regulating enzymes, which may potentially be targeted by small molecule inhibitors.

Introduction

TGF-β is a multifunctional cytokine that plays a key role in embryogenesis and adult tissue homoeostasis. TGF-β is secreted by a myriad of cell types triggering a varied array of cellular functions including apoptosis, proliferation, migration, endothelial and mesenchymal transition, and extracellular matrix production. Downstream TGFβ responses can also be modulated by other signalling pathways (i.e. PI3K, ERK, WNT, etc.) resulting in a complex web of TGF-β pathway activation or repression depending on the nature of the signal and cellular context. Apart from TGF-β mediated cell-autonomous effects TGF-β can further play an important function in regulating tumour microenvironments effecting the interaction between stromal fibroblasts and tumour cells. Due to the central role of TGF-β in cellular processes it is therefore unsurprising that loss of TGF-β pathway integrity is frequently observed in a variety of human diseases, including cancer. However, the TGF-β pathway plays a complex dual role in cancer. In normal epithelial cells and premalignant cells TGF-β acts a potent tumor suppressor eliciting a cytostatic response inhibiting tumor progression. Supporting this notion, inactivating mutations in members of the TGF-β pathway have been observed in a variety of cancers including pancreatic, colorectal, and head and neck cancer. In contrast, during tumor progression the TGF-β antiproliferative function is lost, and in certain advanced cancers TGF-β becomes an oncogenic factor inducing cellular proliferation, invasion, angiogenesis, and immune suppression. As a consequence, the TGFβ pathway is currently considered a therapeutic target in advanced cancers and several anti- TGF-β agents in clinical trials have shown promising results [1,2]. However, due to the complex dichotomous role of TGF-β in oncogenesis a detailed understanding of TGF-β biology is required in order to design successful therapeutic strategies to identify patient populations that will benefit most from these compounds. The last few years have begun to peel away the many facets of the TGF-β pathway including regulation of TGF-β kinetics by ubiquitination. This review specifically focuses on the role of ubiquitination in the regulation of TGF-β pathway components highlighting the ubiquitin regulating enzymes, which may potentially be targeted in human disease.

TGF-β signal transduction

The TGF-β family members, which include TGF-βs, nodal, and Bone Morphogenetic Proteins (BMPs), are secreted cytokines. TGF-β ligand activation of the pathway is transmitted through a heteromeric receptor complex that includes two type I and two type II receptors both of which are transmembrane Serine/Threonine kinases. Phosphorylation of type I receptors (TβRI) by the activated form of type II receptor (TβRII) permits the type I receptors to transiently interact with and phosphorylate receptor SMADs (R-Smads),

most notably SMAD2 and SMAD3 for TGF-β receptors and SMAD1, SMAD5, and SMAD8 for BMP receptors. SMAD proteins are made up of two globular Mad-homology (MH) domains, MH1 and MH2, separated by a highly regulated linker region [3]. The MH1 domain contains the conserved DNA binding motif capable of recognizing the 5^1-AGAC-3^1 DNA sequence. The variable linker region is enriched in proline residues and contains multiple phosphorylation sites and binding sites for ubiquitin ligases. MH2 is highly conserved and mediates complex binding with other SMADs or SMAD nuclear complexes. Phosphorylation of the MH2 domain in R-Smads on two serine residues in their C-terminal SSXS motif creates an interaction interface that permits them to oligomerize with the co-Smad, SMAD4 [3]. Once formed, the SMAD complexes translocate to the nucleus where they regulate gene expression of hundreds of genes in a cell type and context specific manner [4]. Three major determinants direct the TGF-β transcriptional response in a cell: (1) intensity of the TGF-β signal; (2) transcriptional cofactors; and (3) the epigenetic landscape [5]. The intensity of the TGF-β signal is determined by a number of different pathway regulators such as ligand isoforms, receptor subtypes, inhibitory SMAD proteins, and co-existing parallel pathways like PI3K/mTOR or RAS/RAF/ERK which direct cues from the intercellular and extracellular environment affected by hypoxia, ROS, or glutamine starvation to regulate cellular localization and degradation of canonical TGF-β pathway members [5]. Furthermore, transcriptional cofactors such as forkhead transcription factors and zinc finger proteins may direct activated SMAD proteins to specific genome loci in a lineage and signal specific manner [6-8]. Finally, the epigenetic landscape dictates SMAD transcription factor binding and transcription of downstream target genes. For example, Ectodermin/TIF1γ/TRIM33 (ECTO)-SMAD2/3 complexes recognize specific histone marks promoting an open conformation of the chromatin enabling SMAD dependent transcription [9]. Collectively, these factors tweak the activation signals into proportional levels of transcriptional output. However, to convert quantitative differences of cellular stimuli into desired TGF-β transcriptional responses, mechanisms must exist to continuously regulate TGF-β pathway function and receptor activity. As such, control of desired TGF-β responses is tightly regulated through a number of different mechanisms including phosphorylation. For example, the kinetics of phosphorylated SMAD2 is juxtaposed by the opposing functions of the TGF-β receptor kinase activity and the phosphatase PPM1A [10]. Dephosphorylation of SMAD2 by PPM1A results in nuclear exclusion of SMAD2 and inhibition of the TGF-β stimulus. Like phosphorylation, ubiquitination and deubiquitination of TGF-β pathway components has been identified as a key mechanism of regulating downstream TGF-β activity [5,11-14].

Ubiquitin conjugation and substrate regulation

Ubiquitin is a highly conserved polypeptide and ubiquitylation of proteins by ubiquitin and ubiquitin like molecules has emerged as a critical regulatory process affecting protein stability, activity, and subcellular localisation. Ubiquitin ligase complexes are comprised of E1, E2, and E3 ligases. E1 ligases recruit free ubiquitin from the cell through its active cysteine residue whereby ubiquitin is subsequently transferred to an E2 ligase. The central components of the complex are the E3 ligases, of which several hundred have been identified and accordingly are primarily responsible for substrate recognition through their selective substrate-recognition motifs. Two main families of E3s have been identified: the really interesting new gene (RING) family and the homologues to E6-assosiated protein carboxy terminus (HECT) family. Although both families link E2s with substrates they are functionally different. HECT domain ligases act as an intermediate capable of transferring ubiquitin to its recruited substrate. In contrast, RING domain ligases do not themselves directly transfer ubiquitin but appear to act as scaffolds allowing ubiquitin transfer from the E2 directly to the substrate [15]. However, it has also been proposed that RING ligases may lure ubiquitin from the E2 through allosteric mechanisms independently from its catalytic site [16]. Substrates can be either be monoubiquitinated at a single lysine residue or at multiple lysines simultaneously, multi-monoubiquitination. In addition, the ubiquitin molecule itself contains seven intrinsic lysine residues (K6, K11, K27, K29, K33, K48, K63) permitting some E2/E3 complexes to catalyse further cycles of ubiquitination synthesizing either homotypic linear chains, or heterotypic chains of various topologies [17]. However, to further complicate the issue some ubiquitin chains have been described to contain SUMO moieties while others have demonstrated to exhibit a branched formation. The function of these unique structures remains to be determined but these findings undoubtedly open up a new window into the basic biology of ubiquitin regulation. By all accounts different ubiquitin formations lead to distinct cellular functions. In general K48 chains serves to act as a degradation signal targeting the substrate for proteosomal degradation [18], whereas K63 chains regulate kinase activation and signal transduction among other processes [19]. Like K48 chains, K11 chains appear to choreograph a degradation signal [20]. Although a number of proteins have demonstrated K6, K27, K29, K33 polyubiquitination an accurate role of their true function in protein regulation remains elusive.

Ubiquitination in the canonical TGF-β pathway

A number of ubiquitin modifications have been shown to play key roles in TGF-β signal transduction. The first E3 ligases to be implicated in the TGF-β /BMP pathway were the Smad ubiquitination regulatory factor 1 and 2 (SMURF1/SMURF2). SMURF1 and SMURF2 are closely related C2-WW-HECT-domain ligases capable of binding SMADs through an intermolecular interaction between the SMURFs WW domain and the PPXY sequence (PY) motif in SMADs [21]. SMURF1 was originally demonstrated to target BMP specific SMAD1 and SMAD5 for proteosomal degradation [22]. Similarly, SMURF2 carries out the ubiquitination-mediated degradation of activated SMAD2 and SMAD3 both in the cytoplasm and nucleus [23]. In addition to SMURFs acting downstream in the TGF-β pathway, they also mediate TGF-β kinetics at the receptor level. As part of a negative feedback loop, TGF-β signalling induces the expression of the inhibitor adaptor protein (I-SMAD), SMAD7, which recruits SMURF2 and the E2 li-

gase UBCH7 to the TGF-β receptors facilitating ubiquitin mediated degradation of the SMAD7/SMURF2/ TβRI complex [24]. The binding of SMAD7 to SMURF2 also serves another purpose. SMURF2 autoubiquitinates itself and therefore to maintain its stability and constrain unwanted activity towards its substrates the C2 and HECT domains remain in a tightly closed confirmation. The binding of SMAD7 to the HECT domain of SMURF2 abrogates these inhibitory intramolecular interactions between these domains facilitating SMURF2 ubiquitin ligase activity [25]. Furthermore, SMAD7 can bind to the activated receptor complex preventing access of R-SMADs. This competition between the R-SMADs and I-SMADs maintains a check to ensure desired signalling intensity of the receptor complex. However, recent results by Zhang and colleagues clearly indicate that SMURF2 does not regulate protein stability of these aforementioned proteins in vivo [26]. Engineered Smurf2-/- mouse embryonic fibroblasts displayed similar turnover rates of SMAD 2/3 and TβRI and II. Furthermore, no significant differences were observed in phosphorylated SMAD2 and SMAD3 levels. However, the authors demonstrate that SMURF2 mediated inhibition of TGF-β pathway still appears to be primarily dependent upon ubiquitination of SMAD3 albeit through a proteasomal independent method. SMURF2 induces multi-monoubiquitination of the K333, K378, and K409 in the MH2 domain of SMAD3 blocking formation of both homotrimeric SMAD3 and heterotrimeric SMAD3-SMAD4 complexes invariably limiting these complexes in binding to SMAD motifs on the DNA [26]. The intriguing finding that SMURF2 targets proteins for monoubiquitination is in line with other results showing that neural precursor cell expressed, developmentally down-regulated 4 (NEDD4) family members (of which SMURF1 and SMURF2 are included) are K63 specific enzymes [27]. These findings open up a host of questions regarding the role of SMURF proteins in targeting substrates for degradation. It may exist that SMURF proteins may only prime their targets for entry into early endosomes whereby other ligases targeted these proteins for degradation. Or SMURF proteins might function in a dual role: priming targets through monoubiquitination and later catalysing polyubiquitinated chains depending on other regulatory proteins bound in the complexes.

NEDD4-like or NEDD4-L is another well-studied E3 ligase, which belongs to the same E3 ubiquitin ligase family as the SMURF proteins. It has an N-terminal C2 domain, many WW domains and a C-terminal HECT domain, which confers the E3 activity. As opposed to SMURFs, NEDD4-L is a cytosolic protein and its localization is not affected by TGF-β stimulation. Through its WW-domain, NEDD4-L recognizes activated SMAD2/3 leading to its poly-ubiquitination and subsequently targeting it for degradation [28]. Furthermore, NEDD4-L specifically recognizes and ubiquitinates SMAD2/3 phosphorylated by CDK8/9 and limits the intensity and duration of downstream signalling [29]. Cyclin dependent kinases CDK8 and CDK9 enhance SMAD transcriptional action before being marked for degradation by NEDD4L when activated SMADs cycle through the cytoplasm.

The ubiquitin-proteasome system can also positively regulate the TGF-β cascade. ARKADIA, a RING-finger containing E3, targets multiple negative regulators for proteasomal degradation including, SMAD7, c-Ski, and SnoN [30,31]. C-Ski and SnoN are transcriptional co-repressors that interact with SMAD2 and block binding of SMAD transcriptional activators downregulating TGF-β signaling. Following activation, phosphorylated SMAD2 translocates to the nucleus whereby it forms a complex with ARKADIA targeting SMAD2 bound SnoN for ubiquitin mediated degradation. Incidentally SMURF2 has also been demonstrated to target SnoN via SMAD2 [32]. ARKADIA may also be involved in endocytosis of TβR as it has been shown to bind and ubiquitinate the μ2 subunit of AP-2, which is involved in the formation of clathrin coated pits [33]. Another E3 Ubiquitin ligase, ITCH promotes ubiquitination of SMAD2 but augments SMAD2 phosphorylation enhancing the TGF-β signalling. It is also known that ITCH promotes complex formation between TβRI and SMAD2 [34].

Another well-documented E3 ligase, ECTO is a RING-type ubiquitin ligase targeting SMAD4 for monoubiquitination. Similar to the role of SMURF2 in the nucleus, ECTO ubiquitinates SMAD4 at K519 disrupting the SMAD2/SMAD4 complex inhibiting chromatin binding [35]. The monoubiquitinated form of SMAD4 is exported to the nucleus. Interestingly, it has been proposed that the acetylation of histones in close proximity to the chromatin bound SMAD complexes increases the affinity for ECTO thus disrupting complex formation, DNA binding and resulting in the shutdown of downstream TGF-β target genes.

(De) Ubiquitination in the canonical TGF-β pathway

Like phosphorylation whereby a multitude of phosphatases juxtapose the actions of kinases, ubiquitination is also reversible. Ubiquitin moieties can be removed from polypeptides by deubiquitinating enzymes (DUBs). However, unlike E3 ligases, which catalyse ubiquitin modifications and have been the focus of many studies, the role of DUBs, is less well understood [36,37]. Approximately 80 DUBs have been identified to date with many being implicated in human diseases, including cancer. There are five known DUB families separated into two classes: cysteine proteases and metalloproteases reviewed in Nijman, et al. [36] and Komander, et al. [38]. Cysteine protease DUBs are made up of four families ubiquitin-specific protease (USP), ubiquitin C-terminal hydrolase (UCH), otubain protease, (OTU), and machado-joseph disease protease (MJD). USPs makes up by far the largest family with approximately 55 members. The fifth DUB family all contain a JAMM (JAB1/MPN/Mov34 metalloenzyme) domain. The substrate specificity of the DUBs is determined by sub cellular localisation, complexed binding proteins, and chain topology.

Considering the importance of the TGF-β biology it is unsurprising that this pathway is tightly regulated through multiple mechanisms. One of which, as previously mentioned, is TβRI stability and turnover. TβRI is primarily regulated through a negative feedback loop whereby the inhibitor adaptor protein

SMAD7 acts as scaffold to recruit SMURF2 to the TGFβ receptor complex to facilitate receptor degradation and attenuate TGFβ signalling. Opposing the ubiquitination and degradation of the TβRI no less than five DUBs (USP4, USP11, USP15, USP19, and UCH37) have been identified to date that directly lead to TβRI deubiquitination and stability [39-42]. Utilising functional genetic screens three groups have recently identified USP15 as a critical component of TGF-β pathway regulation. Interestingly, USP15 appears to target a number of different nodes in the TGF-β pathway. Seoane and colleagues recently demonstrated that USP15 forms a complex with SMAD7 and SMURF2 and is recruited to the TβR complex where it deubiquitinates and stabilizes the TβR leading to enhanced TGF-β activity [41]. In this scenario SMAD7 acts as scaffold protein interacting with two enzymes with opposing activities resulting in a constant balancing act between the ligase and DUB in regulating TGF-β output. Intriguingly, through a previously unidentified negative feedback loop TGF-β activity regulates the access of USP15 to the SMAD7-SMURF2 complex. When the TGF-β signal is weak SMAD7 recruits SMURF2 and USP15 to the TβRI and receptor stability is retained protecting low levels of TGF-β output signal. However, at higher concentrations of TGF-β, USP15 gets dissociated from the SMAD7-SMURF2 complex leading to enhanced ubiquitination of TβRI, degradation of the complex and abrogation of TGF-β signal. This generates an elegant rheostat whereby TGF-β regulates its own activity preventing hyperactivation of the signal cascade. However, in scenarios where the levels USP15 are abnormally high, the balance between the SMURF2 activity and USP15 activity shifts, resulting in an enhanced TGF-β signal. This appears to be the case in certain cancers where the USP15 gene was demonstrated to be amplified in glioblastoma, breast and ovarian cancers. Importantly, using a patient derived xenograft glioblastoma model Seoane and colleagues also show that inhibition of USP15 either by shRNA knockdown or chemical inhibition with the DUB inhibitor PR-619 significantly decreased the tumour-initiating capacity of these tumours.

Interestingly, USP15 appears to target other nodes in the canonical TGF-β pathway. Recently, Piccolo and colleagues have identified USP15 as a DUB for monoubiquitinated and polyubiquitinated R-SMADs [43]. Monoubiquitination of R-SMADs targets the DNA binding domains of R-SMADs preventing promoter recognition and aberrant TGF-β signalling. USP15 reverses this modification permitting SMAD transcription factor binding and full TGF-β transcriptional responses. USP15 can also deubiquitinate polyubiquitinated R-SMADs augmenting their stability.

This is where the field becomes slightly ambiguous. SMURF2 regulates both monoubiquitination (see above) and polyubiquitination of R-SMADs, a mechanism which appears to be juxtaposed by USP15. This is in contrast to Polo and colleagues who demonstrate that HECT domain ligases are K63 donors and therefore primarily monoubiquitinate their substrates [27]. Furthermore, Tang et al demonstrated that SMURF2 did not induce turnover of TGF-β pathway components but rather induced multi-monoubiquitnated SMAD3 at K333, K378, and K409 in the MH2 domain. On the other hand, USP15 deubiquitinates mono-ubiquitinated SMAD3 at K81 and to a lesser degree at K33 and K53 suggesting that USP15 deubiquitinates R-SMADs in the MH1 domain at sites independent of SMURF2 function. Incidentally, all of these ubiquitination sites disrupt the R-SMAD/SMAD4 heterodimer. Nevertheless, this leaves a number of questions unanswered, most pertinently of which, what is the E3 ligase responsible for the ubiquitination of R-SMADs within the MH1 domain? And what is the functional relevance of SMURF2 ubiquitination versus this as of yet unidentified ligase in the regulation of R-SMADs? Furthermore, how does overexpression of SMURF2 lead to degradation of TGF-β pathway components? During the course of their experiments Piccolo and colleagues demonstrated that both SMURF2 and the HECT ubiquitin ligase NEDD4 generated mono- di –tri -, and polyubiquitinated forms of SMAD3 in vitro. Interestingly, NEDD4-L was shown to ubiquitinate R-SMADs at both the MH1 and MH2 domains after CDK8/9 phosphorylation in the linker region of SMAD3 [28]. Although primarily cytosolic it is tempting to speculate that NEDD4 may be involved in ubiquitination of SMAD3 in the MH1 domain while SMURF2 may induce ubiquitination in the MH2 domain. The functional relevance of all of this would still be required to be determined. This still leaves the unanswered question on how R-SMADs are polyubiquitinated and targeted for degradation. Undoubtedly, SMURF2 is involved, however, it is as of yet unclear if SMURF2 solely primes R-SMADs for targeting by other ligases or if the enhanced activity of SMURF2 brought on by loss of USP15 or aberrant expression of SMURF2 may lead to the ability to polyubiquitinate its targets.

Recently, ten Dijke and others identified USP4, 11, 15, and 19 as potent regulators of TGF-β signalling cascade [39]. Interestingly, all of these DUBs bound to the TβRI resulting in decreased levels of TβRI ubiquitination, stabilisation of the receptor and enhanced levels of SMAD2 phosphorylation. However, unlike USP15 which utilised the scaffold protein SMAD7 as a recruiter to the TβRI, USP4, 11, and 19 bound to the TβR directly. The authors also demonstrated that USP4 interacts with USP11, 15, and 19 and that USP15 activity towards the TβRI requires USP4 activity, as USP15 was unable to deubiquitinate the TβRI in USP4 deficient cells. This added level of complexity might suggest that TβRI is ubiquitinated at multiple sites resulting in divergent mechanisms of regulation of the receptor. For example, ubiquitination of TβR at the site targeted by USP4 might inhibit ubiquitination of the site targeted by USP15 thereby inhibiting USP15 or SMAD7 binding to the complex and efficient USP15 mediated TβR deubiquitination. But why multiple polyubiquitination sites would be required for efficient targeting of the receptor under different conditions looks unclear. It seems more likely that posttranslational modifications of DUBS by either phosphorylation or ubiquitination events regulate the overall outcome of DUB activity and in this case TβR turnover. Preliminary results by Ten Dijke and colleagues add weight to this theory. They demonstrate that AKT phosphorylation of USP4 enhances the binding of USP4 to USP15 and that overexpression of USP15

increases USP4 stability. One potential scenario is that AKT induced binding may lead to USP15 mediated deubiquitination of USP4 enhancing USP4 activity towards the TβRI.

Like USP15, UCH37, AMSH and AMSH-LP have also been demonstrated to bind to the I-SMADs [42, 44]. However, whereas, USP15 and UCH37 appear to play similar roles in binding to SMAD7 and targeting the TβR resulting in enhanced stability of the receptor, AMSH and AMSH-LP sequester SMAD6 and SMAD7, respectively, suppressing the inhibitory action of these I-SMADs towards their targets. The exact targets of these DUBs are unknown but it is thought that AMSH is required for the regulation of TβR turnover by the endosomal sorting complexes required for transport (ESCRT) formation [45].

DUBs have also been shown to regulate the TGF-β pathway further downstream in the signalling cascade. As previously mentioned the E3 ligase ECTO acts as a disruptase monoubiquitinating SMAD4 at K519 disrupts the binding between phosphorylated SMAD2 and SMAD4 on the chromatin. Monoubiquitinated SMAD4 is exported to the cytoplasm where it is deubiquitinated by the DUB, USP9X permitting SMAD2/SMAD4 complexes to form once again [35]. The activity of ECTO functions in essence as a negative feedback loop, regulating SMAD transcription factor binding and terminating TGF-β transcriptional output. Recent results have demonstrated the importance of this as abrogation of ECTO expression impeded proper embryonic development [37].

Conclusion

Despite the enormous progress that has been made in the last few years in understanding the roles of ubiquitin modifying enzymes in the TGF-β pathway there remain many unanswered questions. However, one thing that has become apparently clear is that ubiquitin modifying enzymes are crucial components in the interplay of TGF-β dynamics and that their deregulation can lead to a number of human diseases (Figure 1). Moreover, mounting evidence has indicated that a number of ubiquitin modifying enzymes are overexpressed in various types of cancer suggesting that effective targeting of these enzymes may be beneficial to patients. Clinical relevance of this is supported by the use of bortezomib, a general proteasome inhibitor, for treatment of multiple myeloma and mantle cell non-Hodgkin's lymphoma. However, due to the broad range of substrates likely targeted by this compound, the effectiveness of bortezomib is limited by a very low therapeutic index and dose limiting toxicities. This has inhibited the usage of bortezomib in combination studies with other active chemotherapies like cisplatin or paclitaxel. More recently, a selective inhibitor of the DUB USP14 has been demonstrated to be effective against neurodegenerative diseases and myeloma [46]. Furthermore, a number of pharmaceutical companies have initiated ambitious programs targeting DUBs as novel therapeutic targets in human disease. However, due to the dichotomous role of TGF-β in cancer and the structural redundancy of E3 ligases and DUBs, effective targeting of these components remains problematic. Before any giant leaps can be made additional structural information and functional relevance of ubiquitin modifying enzymes and their substrates will be required prior to any effective drug design.

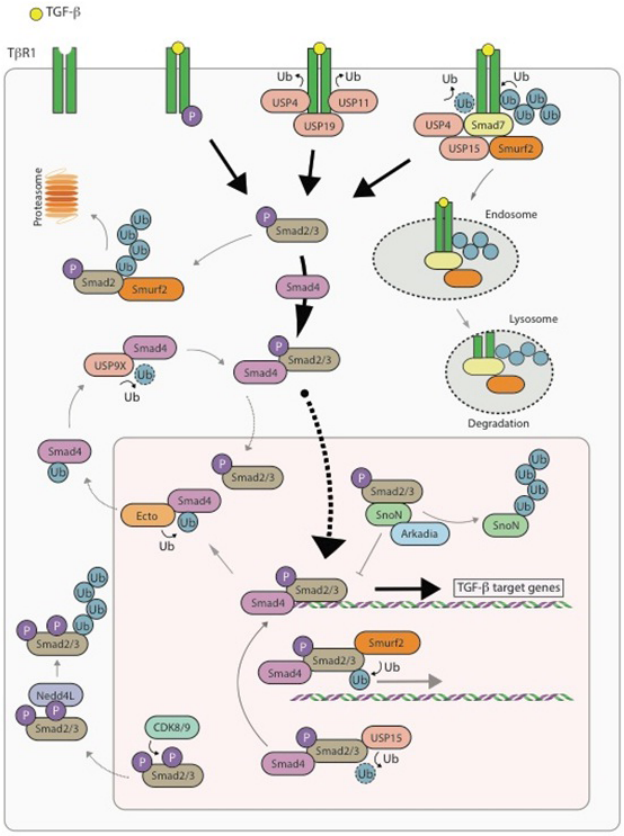

Figure 1: Ubiquitin regulation in the TGF-β pathway. Upon TGF-β stimulation, transforming growth factor β-receptor II (TβRII) binds and phosphorylates type I receptor (TβRI). Phosphorylation of TβRI leads to the recruitment and phosphorylation of regulatory SMADs, SMAD2/3, which then bind to SMAD4. The SMAD2/3-SMAD4 complex is translocated to the nucleus where it binds to SMAD binding elements on the chromatin and initiates transcription of TGF-β target genes. One of the target genes is inhibitory SMAD, SMAD7. Through a negative feedback loop, SMAD7 binds to the E3 ubiquitin ligase, SMURF2, which ubiquitinates the TβR complex leading to ubiquitin- mediated degradation of complex. SMAD7 also recruits the deubiquitinating enzyme USP15, which removes ubiquitin moieties off of TβRI. The balance between USP15 and SMURF2 activities determines the stability of the TGF-β receptor complex. High USP15 concentrations promote TβRI stability, high TGF-β activity and tumour progression in cancer. Like USP15, other deubiquitinating enzymes have been shown to deubiquitinate the TβR complex such as USP4, USP11 and USP19. Activated SMAD2/3 are polyubiquitinated by SMURF2 in the cytoplasm and mono-ubiquitinated in the nucleus, both events lead to attenuation of downstream signalling. Inside the nucleus, SMAD4 can be mono-ubiquitinated by yet another E3 ubiquitin ligase named ECTODERMIN that sends SMAD4 back to the cytoplasm. Herein, the ubiquitin moiety can be removed by USP9X, which recycles Smad4 for further downstream signalling. The transcriptional corepressor SnoN can bind to SMAD2/3 thereby preventing it from transcribing TGF-β target genes. ARKADIA has been shown to positively regulate the TGF-β signalling by binding to and polyubiquitinating SnoN. Activated SMAD2/3 can also be phosphorylated by CDK8/9, an event that temporarily leads to enhanced signalling, however, CDK8/9 phosphorylated SMAD2 is specifically recognized by NEDD4L in the cytoplasm, which mediates its ubiquitin-dependent degradation.

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