# TGFβ signaling in cancer progression

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# Chapter 2

## Regulation of the TGFβ pathway by deubiquitinases in cancer

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## Abstract

The transforming growth factor β (TGFβ) pathway regulates diverse cellular processes. It signals via serine/threonine kinase receptors and intracellular Smad and non-Smad effector proteins. In cancer cells, aberrant TGFβ signaling can lead to loss of growth inhibition and an increase in invasion, epithelial-to-mesenchymal transition (EMT) and metastasis. Therapeutic targeting of the pro-oncogenic TGFβ responses is currently being explored as a potential therapy against certain invasive and metastatic cancer types. The ubiquitin post-translational regulation system is emerging as a key regulatory mechanism for the control of TGFβ pathway components. In this review, we focus on the role of deubiquitinases (DUBs), which counteract the activity of E3 ubiquitin ligases. We will discuss the mechanisms by which specific DUBs control Smad and non-Smad TGFβ signaling routes, and how perturbation of the expression and function of DUBs contributes to misregulation of TGFβ signaling in cancer.

**Key words**: TGFβ, Smad, Ubiquitin, Deubiquitinase, Cancer.

## 1. Introduction

TGFβ family members, which include TGFβs, activins and bone morphogenetic proteins (BMPs) (1), play prominent roles in regulating cell cycle progression, differentiation, migration/invasion, and survival/apoptosis of a large variety of cell types (2). Their pleiotropic functions are highly dependent on context; in diverse cellular microenvironments they can have different, and even opposing functions (3,4). TGFβ family members play pivotal roles in maintaining tissue homeostasis during development. Aberrant TGFβ family signaling has been associated with multiple human diseases, including fibrosis, immune disorders and cancer (5). TGFβ family members signal via cell surface type I and type II serine/threonine kinase receptors, which mediate intracellular responses via Smad transcriptional regulators (6) and non-Smad pathways (7). Each step of the TGFβ family signaling cascades is intricately controlled by positive and negative regulation. An important mechanism of regulation is via covalent and reversible post-translational modification of TGFβ pathway components, including receptors and Smads, by ubiquitin (8,9).

The ubiquitin system was first described in the late 1970s by Hershko and Ciehanover (10,11). Over the past few decades, this system was identified as one of the most critical and versatile post-translational modifications, which can control a vast range of cellular processes, including cell-cycle control, DNA damage repair and membrane trafficking. While first recognized as a signal for protein degradation (12), ubiquitination has now been found to have much broader roles including regulation of the binding and/or enzymatic activities of proteins involved in cell signaling, trafficking, endocytosis, autophagy, transcription, immunity, and DNA damage response (13,14). Ubiquitination requires ubiquitin-activating enzymes (E1), ubiquitin conjugating enzymes (E2), and ubiquitin ligase enzymes (E3) (15).

Deubiquitinases (DUBs) are isopeptidases that can reverse the ubiquitination process, by removing ubiquitin from their substrate proteins (16). Misregulation of ubiquitin enzymes as well as DUBs has been shown to be closely linked to cancer (e.g. a higher risk of cancer metastasis) as shown by clinical database analysis and animal models (17). DUBs have emerged as critical regulatory mediators of several signaling pathways that are involved in

human cancers, such as tumor protein p53 (p53) signaling (18) and c-Jun NH2-terminal kinase (JNK) signaling (19). In this review we will focus on the role of DUBs in the regulation of TGFβ family signaling and how perturbation of this system may be involved in cancer. We will also discuss the therapeutic value of DUB inhibitors for the treatment of cancer patients.

## 2. The TGFβ pathway

The human TGFβ family of cytokines (TGFβs, activins and BMPs) comprises 33 members (20,21). They are structurally and functionally related, secreted dimeric proteins. They share a characteristic cysteine knot structure and exert pleiotropic effects. There are three human TGFβ isoforms, TGFβ1, TGFβ2, and TGFβ3. TGFβ is a potent growth inhibitor in normal tissues (22,23) and also pre-malignant cells and acts as a tumor suppressor. However, tumor cells can become selectively refractory to the cytostatic effects of TGFβ through the activation of proto-oncogenes or inactivation of tumor suppressor genes. In late phases of tumorigenesis, tumor cells may remain responsive to TGFβ; it can induce the so-called epithelial to mesenchymal transition (EMT) and endow tumor cells with high migratory and invasive potential (24) (25).



Moreover, during tumor progression tumor cells frequently start expressing high levels of TGFβ (26). This may also indirectly contribute to tumor growth by creating a favorable microenvironment through its stimulatory effects on immune suppression and angiogenesis. Consequently, TGFβ can also act as a potent stimulator of metastasis. TGFβ can switch from tumor suppressor in the early phase of tumorigenesis to a tumor promoter at late phases (23).

BMP family members were first discovered as secreted proteins, which induce the formation of bone and cartilage (27-29). Subsequently, BMPs were found to play a role in non-skeleton related processes, including angiogenesis, energy metabolism, neurogenesis and ventral mesoderm specification (30,31).

Activins were initially discovered as regulators of follicle stimulating hormone secretion by pituitary cells. Additionally, activins were shown to exhibit multifunctional activities such as erythroid differentiation in bone, muscle formation, and regulation of endocrine function (32). Like TGFβs, BMPs and activins, as well as other family members such as nodal, anti-mullerian hormone (AMH) and growth and differentiation factors (GDFs), are emerging as important regulators of tumor progression (33-37).

TGFβ family members trigger biological processes by binding to type I and type II single transmembrane spanning serine/threonine kinase receptors (6,38,39). The basic structure of type I receptors is similar to that of type II receptors; both of them have small cysteine-rich extracellular regions and intracellular portions containing kinase domains. One difference is the GS domain, a region rich in glycine and serine residues, which is only found in the juxtamembrane region in the intracellular domain of type I receptors. TGFβ ligands initiate signaling by stimulating the formation of heteromeric complexes of type I and type II receptors. Upon complex formation, the constitutively active type II kinase triggers the phosphorylation of serine and threonine residues in the GS domains of type I receptors (40). This leads to the activation of type I receptor kinases, which phosphorylate specific intracellular Smad effector proteins (6,38,39).

The TGFβ canonical Smad pathways can be divided into two branches (Fig. 1) One is used (predominantly) by TGFβ and activins, which signal through intracellular receptor-regulated (R-) Smad2 and Smad3 effectors (6). The other branch is mainly employed by BMPs, which signal via R-Smad1, Smad5 and Smad8 (41).

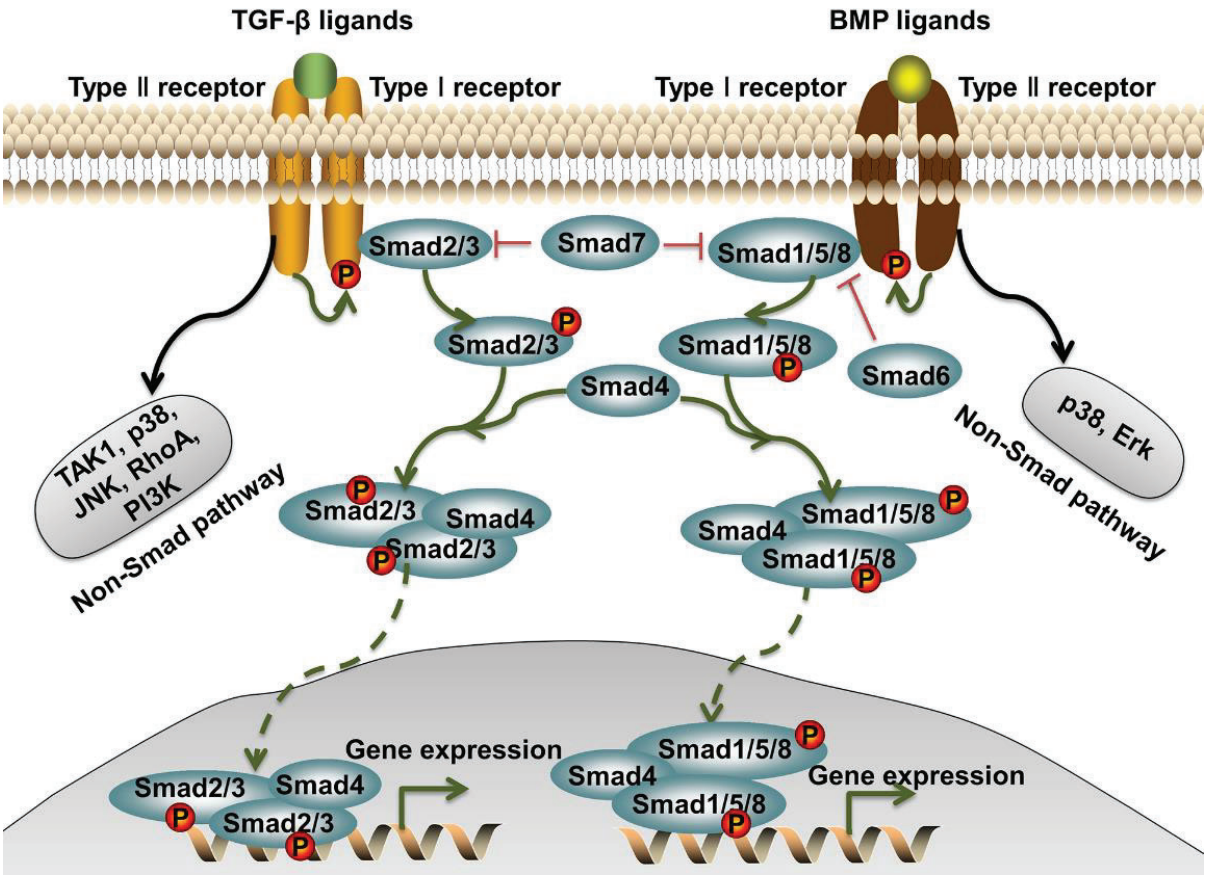


Figure 1. A schematic representation of the Smad and non-Smad TGFβ/BMP pathways. Ligand binding to TGFβ/BMP receptors on the cell surface induces phosphorylation of TGFβ/BMP type I receptors, which induces phosphorylation of Smad2/3 and Smad1/5/8. Phosphorylated Smad2/3 and Smad1/5/8 associate with Smad4, translocate to the nucleus, and bind to DNA to trigger TGFβ/BMP-mediated gene expression. TGFβ receptors also can initiate activation of TGFβ associated kinase 1 (TAK1), p38 and Jun N-terminal kinase (JNK) mitogen-activated protein kinases (MAPKs) pathways, small Rho-like GTPase pathway, and phosphoinositide 3 kinase (PI3K)/Akt-mTOR pathway. BMP receptors also activate the non-Smad p38 and Ras-Erk-MAPK pathways.

R-Smads are phosphorylated by activated type I receptors and form heteromeric complexes with common mediator (Co-) Smad4 (42-44). Subsequently R-Smad-Smad4 complexes translocate to the nucleus, where they regulate gene transcriptional responses, in collaboration with co-activators and co-repressors and DNA-binding transcription factors (45) R-Smads and Smad4 have a conserved N- terminal MH1 and C-terminal MH2 domain. The MH1 domain of Smads can bind to DNA whereas the MH2 domain mediates Smad oligomerization and Smad-receptor interactions. Both MH1 and MH2 domains have been shown to interact with many protein partners.

The two inhibitory (I)-Smads, Smad6 and Smad7, can inhibit canonical Smad signaling by competing with R-Smads for binding to activated receptors (46), thereby suppressing R-Smad

phosphorylation. I-Smads can also interact with Smad4 preventing the interaction between Smad4 and phosphorylated (R)-Smads (47).

Moreover, I-Smads can recruit E3-ubiquitin ligases i.e. Smurf1 and Smurf2, to ubiquitinate type I receptors for subsequent proteasomal degradation (48,49) thereby terminating signaling (50). I-Smads only have an MH2 domain, which mediates the interaction with type I receptors.

In addition to the canonical Smad pathway, TGFβ family members can also activate so-called non-Smad pathways to instigate a multitude of intracellular changes (7). There are various branches including the p38 and Jun N-terminal kinase (JNK) mitogen-activated protein kinases (MAPKs) pathways, ubiquitin ligase tumor necrosis factor (TNF)-receptor associated factor (TRAF6) and TGFβ activated kinase 1 (TAK1). Other branches contain the phosphoinositide 3 kinase (PI3K)/Akt-mTOR pathway, the NF-κB pathway, the Ras-Erk-MAPK pathway, and the small Rho-like GTPase pathway (Fig. 1). There is extensive crosstalk between Smad and non-Smad pathways, e.g. MAP kinases can directly phosphorylate the Smads in their linker regions (51).



## 3. The ubiquitin system

### 3.1. Ubiquitination and deubiquitination

Ubiquitin is an 8.5 kDa, ubiquitously expressed regulatory protein, which contains seven lysine residues (i.e. Lys6, Lys11, Lys27, Lys29, Lys33, Lys48 and Lys63) in its 76 amino acid sequence (52). Ubiquitination (covalent attachment of one or more ubiquitin residues) is an important post-translational modification that modulates protein function, localization, degradation and turnover, thereby serving as a regulator for many aspects of cell physiology in eukaryotes (53).

There are three types of enzymes that play an important role in the conjugation of ubiquitin to its substrates: E1 ubiquitin-activating enzyme, binds to the C-terminal glycine residue of ubiquitin in an ATP-dependent fashion. E2 conjugating enzymes transfer the ubiquitin protein from the E1 to their own cysteine residue, and E3 ligase enzymes catalyse ubiquitin conjugation to the target protein (Fig. 2) (54,55). A group of E3 ligases utilizes their *homologous to the E6AP carboxyl terminus* (HECT) domain to transfer the ubiquitin from E2 to E3, and subsequently to the protein substrate. Another group of E3 ligases can use a *really interesting new gene* (RING) finger domain to directly transfer ubiquitin from E2 to a substrate protein (Fig. 2) (56). Target proteins can be monoubiquitinated or polyubiquitinated (57). Polyubiquitination is the process by which ubiquitin molecules form a polyubiquitin chain through linkage to their internal lysine residues or to the amino terminal methionine residue of the previous ubiquitin (58).

Deubiquitinating enzymes (DUBs) are isopeptidases that can reverse the ubiquitination process by removing ubiquitin from the target protein (16). DUBs have three main functional activities: 1) generation of free ubiquitin from the ubiquitin precursor, 2) reverse the ‘ubiquitin signal’ by removing the ubiquitin from the substrate protein—this ubiquitin is recycled to the free ubiquitin pool to maintain homeostasis, 3) some DUBs edit ubiquitin chains to alter the ubiquitin signal (Fig. 2).

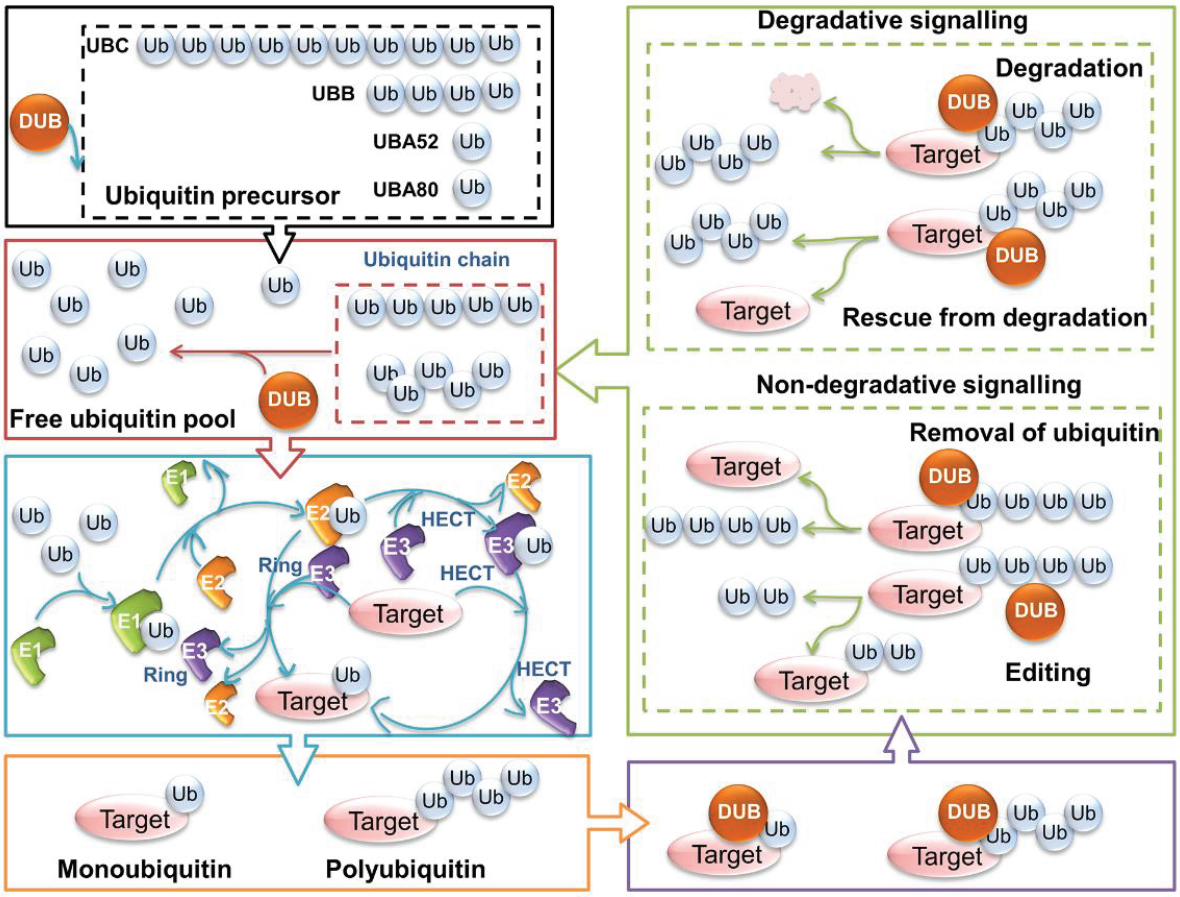


Figure 2. An overview of ubiquitination and deubiquitination processes and the general roles of deubiquitination. Different processes are marked with different colour frames. The black frame represents the generation of ubiquitin (Ub) by its four encoding genes (UBC, UBB, UBA52 and UBA80); deubiquitinases (DUBs) stimulate the generation of free ubiquitin from ubiquitin precursors. The red frame represents the free ubiquitin pool. The blue frame illustrates the conjugation process of ubiquitin to target proteins by the E1 ubiquitin-activating enzyme, E2 ubiquitin-conjugating enzyme, and HECT/Ring E3 ubiquitin ligase enzyme. The orange frame shows the monoubiqutinated and polyubiquitinated target protein. The purple frame shows that DUBs can target proteins with different ubiquitin chains. The green frame explains the function of DUBs in degradative signaling and non-degradative signaling; the removed ubiquitin chains are recycled to the free ubiquitin pool for future use (59,60).

## 3.2. Human deubiquitinating enzymes

There are nearly 100 DUBs encoded by the human genome until 2016. Of these, 79 DUBs them have been shown to have functional activity (16,61,62). They can be divided into five families based on the architecture of their catalytic domains: ubiquitin COOH-terminal hydrolases (UCHs), ubiquitin-specific proteases (USPs), ovarian tumor proteases (OTUs), Machado-Joseph disease proteases (MJDs) and *JAB1/ MPN/MOV34* proteases (JAMMs) (16). The human DUB families are summarized in Figure 3. Members of the UCH, USP, OTU and MJDs are cysteine proteases, which utilize a catalytic triad of conserved amino acids characterized by the classical cysteine protease, papain (63). The JAMM/MPN+ family members are zinc metalloproteases, in which invariant His, Asp, and Ser residues coordinate the catalytic zinc (59).

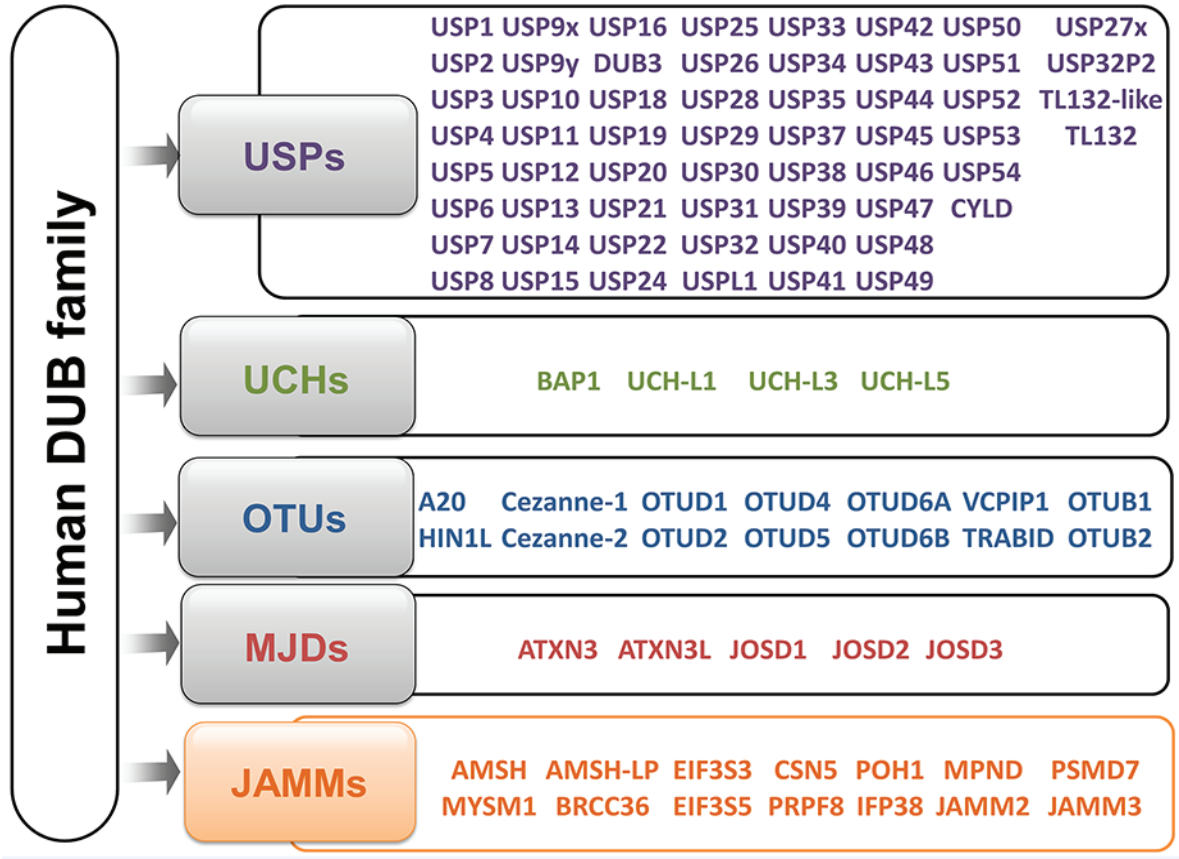


Figure 3. An overview of human DUBs. The 95 putative DUBs can be divided into five families: 58 USPs, 4 UCHs, 14 OTUs, 5 MJDs, and 14 JAMMs. DUBs in the grey frame are cysteine proteases. DUBs in the orange frame are metalloproteases.

The **USP** family is the largest family with around 60 proteases, and the sizes of these proteases range from 50 kDa to 300 kDa. USPs are characterized by a conserved active site composed of a catalytic triad including Cys, His, and Asp (or Asn) residues. Most USPs contain several distinct subdomains within their catalytic domain, such as the zinc finger ubiquitin-specific protease domain (ZnF-UBP), the ubiquitin-associated domain (UBA) and the ubiquitin-interacting motif (UIM) (64). The non-catalytic domains differ at the amino acid sequence level. It has been reported that these non-catalytic domains are important for the localization of individual USPs (65). Most USPs carry a ZnF-UBP binding domain (66), which can specifically recognize the free COOH terminal Gly-Gly motif of free ubiquitin (67,68).

**UCH** family members were the first structurally characterized DUBs. There are four members in humans: UCHL1, UCHL3, UCHL5/UCH37, and BRCA1-associated protein 1 (BAP1) (69). The proteasome associated UCHL5 and the tumor suppressor BAP1 cleave the ubiquitin chains using their more extended cross-over loops (70).

**OTU** family members can be classified in 3 subgroups: OTUBs, OTUDs, and A20-like OTUs (71). A20 (TNFAIP3) has been reported extensively due to its critical function in the NF-κB pathway (72,73).

The **Josephin** family consists of four members: ataxin-3 (ATXN3), ataxin 3-like (ATXN3L), *Josephin domain containing 1* (JOSD1), and *Josephin domain containing 2* (JOSD2).

ATXN3 is mutated in spinocerebellar ataxia type 3 or Machado-Joseph disease (74). It serves as a polyubiquitin chain-editing enzyme and controls the folding and stability of proteins (75). The ubiquitin hydrolase activity of ATXN3 is essential for a normal lifespan. Reportedly, it regulates longevity by controlling insulin like growth factor 1 (IGF-I) signaling (76). ATXN3L, JOSD1 and JOSD2 all have a catalytic triad, consisting of one cysteine and two histidine residues which exhibits deubiquitinating activity.

**JAMM** family members contain a signature 'H-x-H-P-x[6]-S-x[2]-D' motif within the Mpr1-Pad1-N-terminal (MPN) domain. The JAMMs are the only family of DUBs that have zinc-metalloprotease activity (77). *Associated molecule with SH3 domain proteases* (AMSH) can cleave Lys63-linked polyubiquitin chains specifically, and thereby facilitate vesicle trafficking and receptor recycling. *Associated molecule with SH3 domain-like proteases* (AMSH-LP) contains one JAMM core and two conserved insertions. The other members of the JAMM family are *BRCA1/BRCA2-containing complex subunit 36* (BRCC36) (78), *26S proteasome-associated PAD1 homolog1* (POH1/PSMD14), *Myb-like with SWIRM and MPN domains 1* (MYSM1), *MPN domain-containing protein* (MPND), and *COP9 signalosome subunit 5* (CSN5/JAB1) (77,79).

## 3.3. Regulation of the TGFβ pathway by ubiquitin system

Ubiquitination of the receptors and Smads tightly regulate TGFβ signaling. Smad ubiquitin regulatory factors (Smurfs) 1 and 2 are critical E3 ubiquitin-protein ligases negatively regulating the TGFβ pathway by promoting TGFβ type I receptor and R-Smad polyubiquitination and degradation. Smurf1 contains a HECT domain, interacts with the TGFβ type I receptor through Smad7 and triggers receptor degradation (80). Smurf1 can target non-activated Smad1 and Smad5 for proteasomal degradation as well, thereby inhibiting BMP signaling (81). Smurf2 can also be recruited by Smad7 to target the TGFβ type I receptor for degradation (82). Smad1 and Smad2 can be ubiquitinated by Smurf2 under steady-state conditions (83,84).

In addition, the tumor necrosis factor receptor-associated factor (TRAF) family ubiquitin enzymes play a critical role in TGFβ signaling. TRAF4 can associate with the TGFβ receptor complex in a Smad7-independent manner, which can rescue receptors from degradation. TRAF4 can also activate non-Smad signaling by ubiquitinating TAK1. Both of these functions promote metastasis of breast cancer cells in zebrafish and mice (85).

In line with the above mentioned ubiquitin-related functions, DUBs have been reported to play three main roles in TGFβ signaling: 1) protect the receptors, R-Smads and Co-Smads from degradation, leading to enhanced TGFβ signaling; 2) deubiquitinate I-Smads thereby inhibiting TGFβ signaling; 3) regulate non-Smad pathways.

## 4. DUBs in TGFβ pathways and related cancers

Here, we provide a comprehensive review of the DUBs that regulate TGFβ /Smad signaling (schematically depicted in Fig. 4) and discuss DUBs regulation of non-Smad signaling. We will also provide a summary on the action of these DUBs in TGF-β pathways and the gene expression level of them in related cancers in Table 1.

Table 1. Summary of DUBs implicated in TGFβ/BMP signaling and their expression level in cancers.

| DUB | Mode of action in TGFβ pathway | Expression level in cancers (compared with normal tissue) (17,114) | |
|---|---|---|---|
| | | Overexpression | Underexpression |
| **USP4** | Deubiquitinates TGFβ type I receptor (87) and TAK1 (111) | Myeloma, liver, melanoma, brain, bladder, adrenocortical carcinoma (115) (116). | Testicular, lung, head and neck squamous cell carcinoma (HNSCC), renal, brain. |
| **USP11** | Deubiquitinates TGFβ type I receptor (88) | Lung, myeloma, HNSCC, skin, colorectal cancer and melanoma (89). | Brain, renal, testis, pancreas, HNSCC. |
| **USP15** | Deubiquitinates BMP type I receptor, TGFβ type I receptor (90), and (92) R-Smads (98) | Vulva, brain, breast cancer, lymphoma, ovarian cancer and glioblastoma (90). | Brain, bladder, testicular, liver, melanoma. |
| **UCH37** | Deubiquitinates TGFβ type I receptor (94) | Lung, breast, ovarian, vulva, parathyroid. | Brain, pancreas, breast. |
| **OTUB1** | Deubiquitinates pSmad2/3 (99), thereby protecting them from degradation (100) | Bladder, lung, prostate, HNSCC, breast cancer. | Brain, HNSCC, testis, cervical, renal, sarcoma. |
| **USP9x** | Deubiquitinates Smad4 (117) and Smurf (106) | Brain, gastric, cervical, colon, leukaemia, lymphoma, kidney cancer, prostate cancer, sarcoma (118). | Brain, bladder, testicular, leukaemia, lymphoma. |
| **CYLD** | Deubiquitinates SMAD7 (119) deubiquitinates AKT thus reducing stability of Smad3 (112) | Leukaemia, renal, testis, myeloma, breast cancer (120,121), melanoma (122), colon cancer (123), and lung cancer (124). | Brain, ovarian, lung, HNSCC, bladder. |
| **AMSH** | Inhibits Smad6 (107), | Lung, liver, bladder, leukaemia, colon. | Leukaemia. |
| **AMSH2** | Inhibits Smad7 (108) | Kidney, liver, brain, HNSCC. | Brain, testicular, leukaemia. |
| **A20** | Deubiquitinates TAK1, inhibits non-Smad TGFβ pathway MAPK/JNK pathway (113) | HNSCC, leukaemia, lung, brain, cervical. | Bladder, ovary, lung, lymphoma, sarcoma. |

## 4.1. DUBs targeting TGFβ/BMP receptors

**USP4** was found to interact with and deubiquitinate the TGFβ type I receptor, thereby opposing the action of Smad7/Smurf2-mediated ubiquitination. USP4 is a very stable protein, which can deubiquitinate itself to control its own stability (86). USP4 can promote TGFβ-induced invasion and metastasis of breast cancer cells in a zebrafish xenograft model. Moreover, this report showed that USP4 was phosphorylated by AKT, leading to increased USP4 membrane-localization and promoting USP4 self-association, leading to enhanced TGFβ signaling. AKT-induced breast cancer cell migration could be inhibited by depletion of USP4 (87).

**USP11** has been shown to interact with Smad7 and override the negative effects of Smad7 on the TGFβ pathway. It deubiquitinates the TGFβ type I receptor and thereby potentiates TGFβ signaling (Fig. 4). Depletion of USP11 could inhibit TGFβ induced Smad2/3 phosphorylation, TGFββ mediated transcriptional responses and epithelial to mesenchymal transition (EMT) in NMuMG breast cancer cells (88). USP11 downregulation suppressed tumor growth in a HCT116 colon cancer cell xenograft model and in a UACC-62 melanoma cell xenograft model (89). However, the mechanism by which USP11 regulates the TGFβ pathway in colon cancer and melanoma needs further study.

**USP15** has been reported as a key regulator of the TGFβ pathway based on a functional RNAi screen by Seoane's group. USP15 binds to the Smad7-Smurf2 complex and, like USP4 and USP11, deubiquitinates the TGFβ type I receptor, thereby maintaining the stability of this receptor and enhancing TGFβ signaling. A xenograft glioblastoma model showed that the oncogenic capacity of patient-derived glioma-initiating cells could decrease due to the

depletion of USP15. USP15 appears to be a key factor in glioblastoma pathogenesis by regulating the TGFβ pathway (90). Eichhorn et al. found that USP15 not only targets the TGFβ type I receptor complex but also deubiquitinates Smurf2. These authors performed proteomic analysis and found that USP15 deubiquitinates Smurf2 on Lys734, a residue required for Smurf2 catalytic activity, leading to enhanced TGFβ signaling (91). Similar results were reported by Zhang et al, which showed that TRAF4 can promote the recruitment of USP15 to the TGFβ type I receptor, which antagonizes receptor degradation by Smurf2 (85). In addition, USP15 plays a critical role in BMP signaling by interacting with BMP type I receptor and Smad6. Herhaus and co-workers showed that USP15 can interact with and deubiquitinate BMP type I receptors, thereby promoting phosphorylation of Smad1 (92) (Fig. 4). They also showed that depletion of USP15 in Hela cells increased polyubiquitination of BMP type I receptor, and inhibited BMP-mediated Smad1 phosphorylation and BMP target gene transcription. Loss of USP15 in mouse myoblast cells suppressed BMP-induced osteoblast differentiation. Furthermore, they found that USP15 modulates the BMP pathway during *Xenopus* embryogenesis (92).

**USP4, USP11 and USP15** are structurally highly similar and contain significant protein sequence similarity (59). All three DUBs play particularly prominent roles in modulating the ubiquitination of TGFβ type I receptor while USP15 and USP11 can also regulate downstream effectors. USP4 can form stable homodimers and can also interact with USP11 and USP15 (Fig. 4). USP4 has been shown to bind directly to TGFβ type I receptor, and is able to recruit USP15 and USP11 to the TGFβ type I receptor (93).

**UCH37** binds strongly to Smad7 and weakly to Smad2 and Smad3. It subsequently interacts with Smurf ubiquitin ligases to deubiquitinate the TGFβ type I receptor and modify TGFβ-induced transcription (Fig. 4) (94). UCH37 knockdown inhibits transcription of TGFβ target genes and slows lateral cell migration (96). The interplay between Smurf-mediated ubiquitination and UCH37-mediated deubiquitination can influence cancers that are regulated by the TGFβ pathway (94). Interestingly, it has been shown that UCH37 plays a critical role in TGFβ-induced cell migration but not TGFβ-regulated cell proliferation and EMT (95). In human ovarian cancer, higher expression of UCH37 is associated with tumor recurrence after curative resection (96). Also, UCH37 is associated with poor prognosis of esophageal squamous cell carcinoma patients after curative resection (97).

## 4.2. DUBs targeting R-Smads

In addition to its effects on the TGFβ and BMP receptors described above, **USP15** can target the DNA-binding domains of R-Smads and antagonise R-Smad monoubiquitination, leading to enhanced activity of TGFβ and BMP pathways (Fig. 4) (98). As mentioned above, USP15 is required for TGFβ and BMP responses in mammalian cells and *Xenopus* embryos. It has been shown that knockdown of USP15 in immortalized HaCaT keratinocytes can impair TGFβ/SMAD-dependent growth arrest. In MDA-MB-231 metastatic breast cancer cells, USP15 is required for TGFβ-induced cell motility.

**OTUB1** has been shown to interact with E2 enzymes and antagonize efficient ubiquitin transfer from E2 enzymes to E3 enzymes, thereby inhibiting the ubiquitination of Smad2/3 (Fig. 4) (99). It has been shown that OTUB1 interacts with phosphorylated SMAD2/3 at the C-terminus specifically after TGFβ stimulation. Further studies revealed that endogenous

OTUB1 can inhibit the ubiquitination of phosphorylated Smad2/3 and prevent its proteasomal degradation (Fig. 4). OTUB1 is thereby important for TGFβ-induced gene transcription and cell migration. (100).

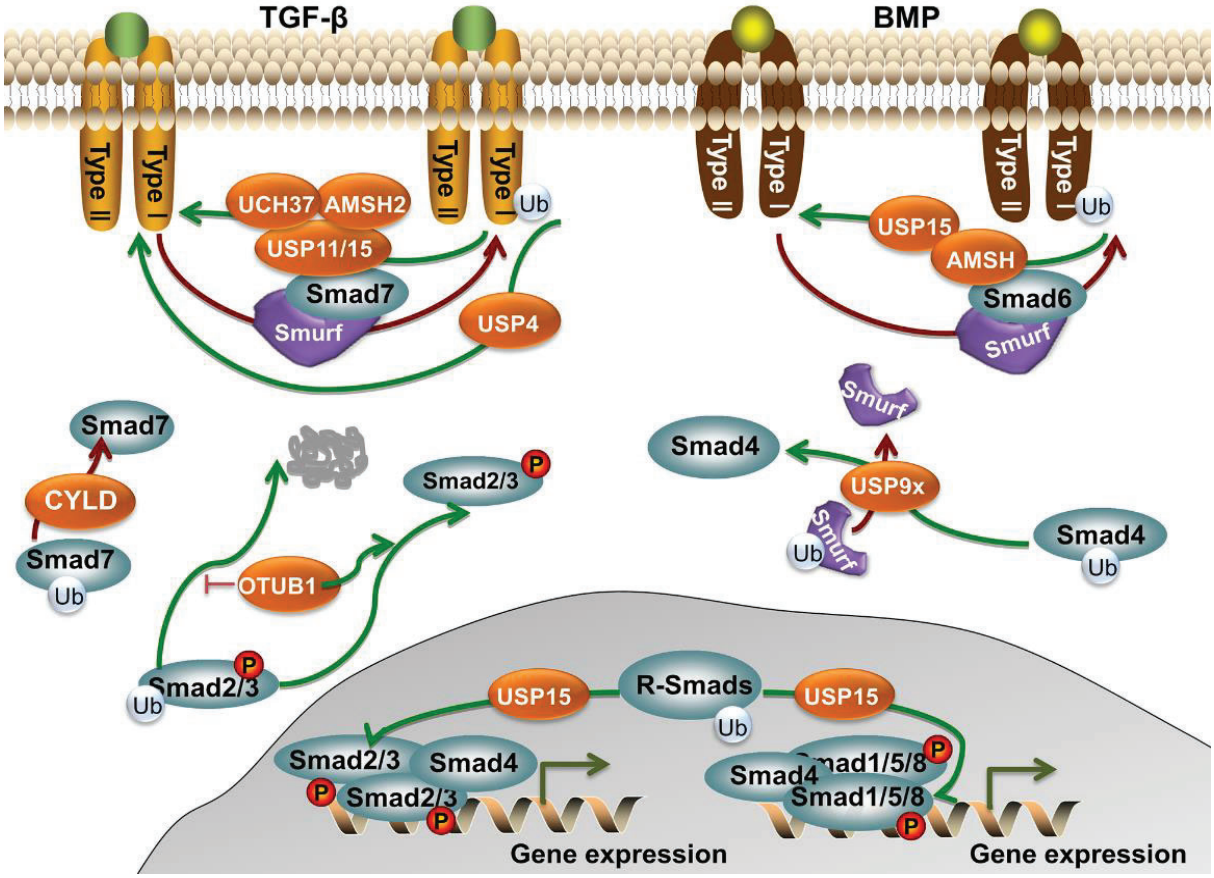


Figure 4. A schematic representation of DUBs regulating Smad signaling. USP4/11/15, UCH37 and AMSH2 deubiquitinate the TGFβ type I receptor which stimulates the activity of the TGFβ/Smad pathway. USP15/AMSH deubiquitinate the BMP type I receptor, which enhances the activity of the BMP pathway. USP15 can target the R-Smad DNA-binding domains and antagonise R-Smad mono-ubiquitination. USP9x deubiquitinates Smad4 and Smurf. CYLD deubiquitinates Smad7. OTUB1 deubiquitinates pSmad2/3 to protect it from proteasomal degradation.

## 4.3. DUBs targeting Co-Smad

**USP9x** is an essential DUB for TGFβ signaling by counteracting Smad4 mono-ubiquitination (101). Its counterpart, Ectodermin (Ecto), was reported as a mono-ubiquitinating factor that blocks Smad4 activity (102) (Fig. 4). It was also shown that Ecto binds to Smad2 and Smad3 and disturbs the association between Smad4 and Smad2/3, leading to inhibition of the TGFβ pathway (103). Lysine K519 was identified as the most principal residue for Smad4 mono-ubiquitination *in vivo*, which can inhibit Smad4 by preventing its association with active Smad2/3. USP9x reverses K519 ubiquitination, augmenting the activity of the TGFβ pathway. USP9x was found to be required for TGFβ-induced growth arrest in colon cancer cells and cell migration in breast cancer cells (101). *Drosophila* and mouse knockout models also revealed important functions of USP9x in TGFβ responses. Loss of the USP9x homologue Fat facets in *Drosophila* inhibits the activity of the Smad4 homologue Medea through ubiquitination of Medea on K738 (equivalent to K519 in human Smad4) (104). In mice,

TGFβ-dependent exogenesis was inhibited when USP9x is knocked out in neural progenitors (105). Interestingly, USP9x also has the potential to negatively regulate the TGFβ pathway by deubiquitinating and stabilizing the Smurf1 E3 ligase, depletion of USP9X destabilizes Smurf1 and blocks Smurf1-dependent cell migration in MDA-MB-231 cells. (Fig. 4) (106).

## 4.4. DUBs targeting I-Smads

**AMSH** has been reported to antagonize Smad6 function, and promote BMP signaling (Fig. 4). AMSH was found to be a direct binding partner of Smad6, and not of R-Smads and Co-Smads. Ectopic expression of AMSH enhanced BMP-mediated Smad1 phosphorylation, and increased BMP-induced reporter activity, growth arrest and apoptosis (107). Besides, **AMSH2** can negatively regulate the function of Smad7. It suppresses Smad7 binding to TGFβ type I receptor, thereby preventing TGFβ type I receptor ubiquitination and degradation by the E3 ubiquitin ligase, Smurf1/2 (Fig. 4) (108).

**CYLD** has been shown to hydrolyse Lys63-linked polyubiquitin chains selectively (109). CYLD also can deubiquitinate Lys63-polyubiquitinated Smad7 (Fig. 4), and thereby inhibit TGFβ signaling and influence the TGFβ-dependent development of regulatory T cells (Tregs). As a result of this, the level of Tregs is increased in CYLD knockout mice (110).

## 4.5. Examples of DUB-mediated non-Smad signaling

As mentioned above, **USP4** binds to and deubiquitinates the TGFβ type I receptor and associates with AKT, leading to enhanced TGFβ signaling and AKT-induced breast cancer cell migration (87). USP4 has multiple functions in non-Smad signaling. It can deubiquitinate transforming growth factorβ-activated kinase 1 (TAK1) *in vitro* and *in vivo*. Tumor necrosis factor-α (TNFα) promotes the interaction between USP4 and TAK1 and the deubiquitination of TAK1, leading to the attenuation of TAK1-mediated NF-κB activation. Furthermore, it was found that overexpression of USP4 can inhibit interleukin-1 β (IL-1β), lipopolysaccharide (LPS), and TGFβ-induced NF-κB-dependent luciferase reporter activity and IκB kinase (IKK) phosphorylation. Knockdown of USP4 promoted IL-1β, LPS, and TGFβ-induced NF-κB activation (111).

Lim and co-workers showed that **CYLD** suppresses TGFβ signaling and prevents lung fibrosis by (indirectly) reducing the stability of Smad3, in an AKT, GSK3β and *E3 ligase carboxy terminus of Hsc70-interacting protein* (CHIP)-dependent manner. They also demonstrated that CYLD can deubiquitinate polyubiquitinated AKT, leading to the inhibition of AKT, resulting in activation of GSK3β, which enhances CHIP-induced Smad3 degradation and suppression of the TGFβ pathway (112) .

**A20** has been reported to be a negative regulator of non-Smad TGFβ signaling. It was shown that Smad6 recruits A20 to deubiquitinate K63-linked polyubiquitination of TRAF6, leading to inhibition of TGFβ1-induced activation of the TRAF6-TAK1-p38/JNK MAPK pathway in AML-12 mouse liver cells and primary hepatocytes. Knockdown of Smad6 or A20 in cell and animal models maintained TAK1 and p38 MAPK/JNK phosphorylation, leading to increased apoptosis (113).

## 5. DUB inhibitors

The first drug to target the ubiquitin system as a cancer therapy was the proteasome inhibitor (PI), *Bortezomib* (125). It was approved as a clinical treatment for multiple myeloma achieving US$1.4 billion in worldwide sales in 2009. However, the toxicity and drug resistance limit its efficacy in the clinic (126). Recently, researchers have begun to develop specific inhibitors of DUBs with therapeutic potential (127). Based on the available preclinical data and reported studies, the DUB inhibitors with potential therapeutic relevance to human cancers are shown in Table 2 (128-130).

Table 2. DUBs inhibitors with possible application in human cancers.

| DUB inhibitor | Target | Mechanism | Effects related to cancer |
|---|---|---|---|
| **b-AP15 (proteasome-inhibitory agent)** | **UCHL5, USP14** | Inhibits the activity of regulatory particle (19S RP) associated UCHL5 and USP14, resulting in accumulation of polyubiquitin (131). | Inhibits tumor progression in human cancer cells and mouse *in vivo* models of solid tumors: breast, lung, colon, and head and neck carcinoma (131). |
| **AC17 (4-arylidene curcumin analogue)** | **UCHL5, USP14** | Irreversibly inhibits the DUB activity of 19S RP-associated UCHL5 and USP14 (132). | Inhibits tumor growth in a lung carcinoma xenograft model with no observable toxicity (132). |
| **Azepan-4-ones (proteasome-inhibitory agent)** | **UCHL5, USP14** | Inhibits the activity of two DUBs, UCHL5 and USP14, that are associated with 19S RP (133). | Effectively treats cancer refractory to conventional chemotherapy and particularly cancers refractory to bortezomib. Examples of cancer types are multiple myeloma and solid tumor malignancies (133). |
| **WP1130 (degrasyn)** | **USP9x, USP5, USP14, UCHL5** | Induces rapid accumulation of polyubiquitinated (K48/K63-linked) proteins into juxtanuclear aggresomes, without affecting 20S proteasome activity (134). | Induces growth arrest and apoptosis in melanoma. Inhibiton of tumor-activated DUBs results in downregulation of antiapoptotic proteins and upregulation of proapoptotic proteins (134). |
| **Tricyclic heterocyclics** | **USP14** | Inhibits the USP14 26S proteasome activity (135) | Inhibits tumorigenesis in cancer by suppressing proteasome activity (136). |
| **P5091** | **USP7** | None reported. | Induces apoptosis in multiple myeloma tumors including bortezomib-relapsed myeloma (137). |
| **USP8i** | **USP8** | Inhibits USP8. | Inhibits USP8 in non-small cell lung carcinoma cells (138). |
| **BA (Betulinic acid)** | **Multiple** | Inhibits multiple DUBs, resulting in accumulation of polyubiquitin, decreased oncoproteins, increased apoptotic cell death (139). | Inhibits tumor growth and promotes apoptosis in a transgenic model of prostate cancer (139). |
| **Isatin O-acyl oximes** | **UCH-L1** | Selectively inhibits UCH-L1 | Selective inhibition of UCH-L1 increases proliferation of the H1299 lung tumor cell line (140). |

## 6. Conclusions

In advanced cancers in which TGFβ acts as a tumor promoter, DUBs that activate the TGFβ pathway are regarded as promising therapeutic targets for the development of specific inhibitors. There is increasing interest in this area of drug discovery to complement ongoing efforts to design drugs specifically targeting the ubiquitin system (141). As we have discussed above, USP4 can target the TGFβ type I receptor and promote invasion and metastasis of breast cancer and high USP15 expression correlated with enhanced pSmad2 expression in tissue samples of glioblastoma patients. Moreover, inhibition of USP15 decreased TβRI and pSmad2 concentrations in these cells, thus corroborating the notion that USP15 stabilizes TβRI and promotes TGFβ/Smad2 signaling (142).

In light of these findings, it could be interesting to investigate and develop drugs that specifically target USP4 and USP15. Inhibition of USP4 would be expected to inhibit the invasion and metastasis of breast cancer and drugs that target USP15 could reduce the oncogenic potential of glioblastomas. One of the main stumbling blocks to developing specific DUB inhibitors is that the active site of many DUBs are quite similar and structurally not optimal for small molecule binding, and it may thus be difficult to generate specific DUB inhibitors that target the protease activity directly.

Another challenge of targeting DUBs for therapeutic purposes is that many of DUBs have multiple substrates. Consequently, inhibiting DUB protease activity may be associated with unwanted side-effects. One approach to overcome these limitations would be to identify inhibitors that target a specific DUB-substrate interaction. Ongoing research in this area is already showing promise by modulating DUB activity through targeting of protein–protein interactions (141).

Other DUBs warrant further research with respect to their potential roles in the TGFβ pathway and cancer. For example, USP22 overexpression can promote EMT and TGFβ expression, whereas depletion of USP22 can reverse EMT and reduce metastasis of lung adenocarcinomas. In 76% of 146 lung adenocarcinoma patient specimens, USP22 expression was positive and correlated with TGFβ expression (143). Moreover, USP22 is an oncogene upregulated in multiple cancers. Knockdown of USP22 was found to suppress cell proliferation *in vitro* and tumor growth *in vivo* by inducing G1 phase cell cycle arrest through synergy with TGFβ1 (144).

Up to now, there have been no reports identifying DUBs that target the TGFβ and BMP type II receptors. This could be an interesting line of investigation. Further systematic functional analysis of DUBs could be performed using CRISPR/CAS9 knock out cell lines or conditional knock out mouse models. The development of selective chemical inhibitors for each DUB will also help to elucidate the functions and mechanisms of action of specific DUBs. Finally, further understanding of the functions and mechanisms of the DUBs targeting TGFβ pathway components in specific cancers may lead to a generation of new cancer therapeutics.

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