Regulation of the transforming growth factor β pathway by reversible ubiquitylation

Mazin A. Al-Salihi, Lina Herhaus and Gopal P. Sapkota

Medical Research Council—Protein Phosphorylation Unit, Sir James Black Centre, University of Dundee, Dow Street, Dundee DD1 5EH, UK

1. Summary

The transforming growth factor β (TGFβ) signalling pathway plays a central role during embryonic development and in adult tissue homeostasis. It regulates gene transcription through a signalling cascade from cell surface receptors to intracellular SMAD transcription factors and their nuclear cofactors. The extent, duration and potency of signalling in response to TGFβ cytokines are intricately regulated by complex biochemical processes. The corruption of these regulatory processes results in aberrant TGFβ signalling and leads to numerous human diseases, including cancer. Reversible ubiquitylation of pathway components is a key regulatory process that plays a critical role in ensuring a balanced response to TGFβ signals. Many studies have investigated the mechanisms by which various E3 ubiquitin ligases regulate the turnover and activity of TGFβ pathway components by ubiquitylation. Moreover, recent studies have shed new light into their regulation by deubiquitylating enzymes. In this report, we provide an overview of current understanding of the regulation of TGFβ signalling by E3 ubiquitin ligases and deubiquitylases.

2. The transforming growth factor β signalling pathway

The transforming growth factor β (TGFβ) family of cytokines control a plethora of cellular processes, including proliferation, differentiation, extra-cellular matrix production, motility and survival [1,2]. These translate into critical tissue functions throughout embryogenesis and adult life, achieved by striking a balance between proliferation and differentiation [2–4]. When this balance is perturbed, the TGFβ pathway malfunctions. Aberrant TGFβ signalling is associated with many human diseases including immune disorders, fibrosis, cancer progression and metastasis [5–12]. Therefore, understanding the molecular mechanisms underpinning the regulation of the TGFβ pathway would facilitate novel therapeutic opportunities against these diseases.

TGFβ signalling is initiated when ligands bind to their cognate receptors (figure 1). There are at least 42 different TGFβ ligands, which are divided into two main subgroups: the TGFβ family and the bone morphogenetic protein (BMP) family. Ligand binding induces specific quaternary complex formation of the transmembrane serine threonine kinase receptors. These receptors are divided into type I (ALK1-7) and type II (ACVR-IIA, ACVR-IIB, BMP-RII, AMHG-II and TGFβR-II). SMAD proteins are the intracellular transducers of the pathway; they are divided into specific subgroups: receptor-regulated (R-SMADs; 1–3, 5 and 8), the co-SMAD (4) and the inhibitory (I-) SMADs (6 and 7). Upon ligand binding, the type II receptors phosphorylate and activate the type I receptors. Activated type I receptors phosphorylate the R-SMADs at their C-terminal SXS motif. This induces R-SMAD complex formation with SMAD4 and nuclear translocation, where along with their nuclear cofactors...
they bind DNA and regulate transcription. The vast number of ligands and receptors allows for the formation of unique ligand–receptor complexes in distinct biological settings. In general, the TGFβ receptor subfamily signals through SMADs 2 and 3, while the BMP subfamily signals through SMADs 1, 5 and 8, although some crosstalk between the two pathways has been reported. A negative feedback loop is created by TGFβ- or BMP-induced transcription of the I-SMADs. I-SMADs inhibit the pathway by competing with R-SMADs for association with the type I receptors, or by recruiting E3 ubiquitin ligases and targeting the receptors for degradation.

In the nucleus, a variety of nuclear cofactors are required for the R-SMADs to bind DNA and induce gene transcription (figure 1). Additionally, various histone and DNA modifiers are required for opening or closing sections of DNA to transcriptional regulation by R-SMADs [1,13–18]. While we focus on the role of reversible ubiquitylation in regulating the core components of the TGFβ pathway in this review, they can be further regulated by multiple post-translational modifications, which also impact the outcome of TGFβ signalling. Often it is the integration of all the regulatory inputs that determines the cellular responses to TGFβ signals.

3. Reversible ubiquitylation

Ubiquitylation, also referred to as ubiquitination, is a reversible process by which ubiquitins are attached to proteins, either singly or in chains. This post-translational modification causes target proteins to undergo changes in stability, subcellular localization or activity. Ubiquitin is a member of a conserved family of small eukaryotic proteins (approx. 8.5 kDa) that share the ubiquitin fold structure. Through an iso-peptide bond, ubiquitin is attached to lysine residues on the target, creating mono-ubiquitylated proteins. Attaching more ubiquitin molecules to the lysines of an already protein-bound ubiquitin creates polyubiquitin chains. Depending on which lysine the subsequent ubiquitin molecules are attached to, different fates await the polyubiquitylated proteins. While eight linkage types are possible (through K6, K11, K27, K29, K33, K48, K63 and α-amino group of ubiquitin) as well as mixed chains, not all have been attributed a function. Two linkage types are commonly studied and reported in the TGFβ pathway: K48 chains are known to signal protein degradation, while K63 chains play a role in signalling as well as in protein trafficking and endocytosis.

Ubiquitin attachment is achieved through a three-step process catalysed by an E1-ubiquitin-activating enzyme, specific E2-ubiquitin-conjugating enzymes and a wide array of E3-ubiquitin ligases. E1 enzymes activate and transfer ubiquitin in an ATP-dependant manner to the E2-ubiquitin-conjugating enzyme. This high-energy ubiquitin–E2 conjugate then specifically interacts with the E3-ubiquitin ligase, which could be either a single protein or part of a larger ligase complex. E3s can be divided into three structural groups, U-Box, HECT and Ring E3s, depending on their conserved domains and mode of catalysis. Several ubiquitin-like proteins (UBLs), including SUMO1-3, NEDD8, FUB1, HUB1, ISG15, FAT10, URM1, UFM1, Atg12 and Atg8, share a similar three-step attachment process. However, these UBLs use...
different E1, E2 and E3 enzymes. While SUMO (small ubiquitin-like modifier) has been reported to modulate TGFβ signalling, this review will concentrate on ubiquitin [19–24].

The removal of ubiquitins or polyubiquitin chains from the target protein is catalysed by deubiquitylating enzymes (DUBs). Therefore, DUBs reverse the function of E3 ubiquitin ligases [25]. DUBs remove ubiquitin from cellular adducts, process inactive ubiquitin precursors, proofread ubiquitin–protein conjugates and protect the 26S proteasome from ubiquitin chain accumulation [26]. Furthermore, DUBs generate free ubiquitin by removing and chopping ubiquitin chains from proteins, leading to recycling of ubiquitin, thereby contributing to ubiquitin homeostasis. The fate of ubiquitylated proteins can be further modified by DUBs that edit or trim ubiquitin chains, resulting in a reversal of ubiquitin signalling. This could lead to protein stabilisation by rescue from degradation [25]. Deubiquitylation is implicated in several cellular functions such as gene expression, DNA repair, cell cycle regulation, kinase activation and microbial pathogenesis [27].

DUBs are classified into five distinct functional and structural groups: the zinc metallopeptases JAMM/MPN+, and the cysteine proteases, comprised of ubiquitin C-terminal hydrolases (UCHs), ubiquitin-specific proteases (USPs), ovarian tumour proteases (OTUs) and Josephins [25]. There are also DUBs that resemble the adenovirus protease that cleave interferon-stimulated gene 15 (ISG15) conjugates and ubiquitin-like proteases (ULPs), which belong to the Adenain family of cysteine proteases, that are specific to ubiquitin-like proteins SUMO or NEDD8 [27]. As the human genome encodes less than 100 DUBs, it is evident that DUBs are highly regulated and play a role in diverse signalling pathways in order to oppose the action of over 600 E3 ligases [25,28]. A combination of substrate and target choice determines overall DUB specificity, which is further regulated by conformational/post-translational changes, subcellular localization and interactions with cofactors. DUBs distinguish between ubiquitin-like molecules, isopeptides, linear peptides and different types of ubiquitin linkage and chain structures as well as exo- versus endo- deubiquitylation to ensure specificity. Enzymatic activity of DUBs is often cryptic and regulated by occluding the substrate-binding site of certain DUBs or by inducing conformational changes that activate the catalytic site. Apart from these substrate-induced conformational changes and post-translational covalent modifications, activity can also be regulated by interacting cofactors. Other events, such as transcriptional regulation of DUB expression and subcellular localization, further ensure specific ubiquitin chain cleavage. DUBs are modular and contain multiple domains that mediate protein–protein interactions, apart from their catalytic domains. These domains include ubiquitin-binding domains (UBDs) or ubiquitin-like folds (UBL folds), ubiquitin-interacting motifs (UIMs), zinc finger USP domains (ZnF-UBP domain) and ubiquitin-associated domains (UBA domains). These domains contribute to the binding and recognition of different ubiquitin chain linkages but some DUBs also display direct affinity for their ubiquitylated target protein [25,27,28]. Recent studies have demonstrated that DUBs play critical roles in the TGFβ pathway regulation [7,29,30]. However, the field requires further research in order to identify DUBs that regulate the TGFβ pathway and understand their mode of action. Understanding the precise roles of DUBs in regulating the TGFβ pathway may unravel new opportunities for therapeutic intervention.

4. Regulation of the TGF β pathway components by reversible ubiquitylation

The fundamental steps and the key players in the TGFβ pathway are generally well defined. In this review, we focus on our understanding of how reversible ubiquitylation impacts three groups of key TGFβ pathway mediators: the TGFβ receptors, the SMAD transcription factors and nuclear SMAD cofactors. By integrating multiple signals, reversible ubiquitylation of these components in different biological contexts plays crucial roles in balancing the outcome of TGFβ signalling. Defective ubiquitylation of the TGFβ pathway components has been implicated in many human diseases, especially cancer [7,8,11,12,31–34].

5. Reversible ubiquitylation of TGF β receptors

Receptor complex assembly and activation upon binding TGFβ ligands are central to the activation of intracellular signalling. The activity and integrity of type II and type I TGFβ receptors can be modulated by several strategies: dephosphorylation of the activated receptors, interfering with the receptor/R-SMAD binding, changing receptor localization and/or targeting receptors for proteasomal degradation. I-SMADs play a crucial role in some of these strategies by modulating the activity and stability of active TGFβ receptor complexes. SMAD7 was reported to inhibit the TGFβ pathway by not only interfering with R-SMAD phosphorylation but also recruiting the E3 ubiquitin ligases SMURF1 and SMURF2 to the receptor complex (figure 2) [35,36]. This led to both receptors (ALK5 and TGFβR-II) and SMAD7 being ubiquitylated and targeted for degradation. Similarly, SMAD6/7 has been described to direct SMURF1 to ALK6 and mediates receptor ubiquitylation and degradation [37]. Both I-SMADs and SMURF1/2 are transcriptional targets of TGFβ and BMP signals, thereby creating a negative feedback loop [38,39]. A glycosyl phosphatidylinositol-anchored protein, CD109, further enhances the SMAD7–SMURF2 receptor complex interaction, strengthening the negative feedback [40,41]. Conversely, a recent study demonstrated that a protein named TGF-β-stimulated clone 22 (TSC-22), which is induced by TGFβ, inhibits the SMAD7–SMURF complex from binding, ubiquitylating and degrading the receptor complex. As expected, this leads to enhanced TGFβ signalling that translated physiologically into increased TGFβ-induced cellular differentiation [42]. Tribbles homologue 3 (TRB3) is another TGFβ-induced gene capable of enhancing pathway signalling in a positive feedback loop. TRB3 enhances SMAD3 nuclear localization and induces degradation of SMURF2 promoting cell migration, invasion and epithelial to mesenchymal transition (EMT) [43]. In human renal cell carcinomas, enhanced SMURF2 expression causes the reduction in levels of type II TGFβ receptor by proteasomal degradation [8]. SMURF1 and SMURF2 belong to the NEDD4-like family of HECT E3 ubiquitin ligases and are characterized by the presence of a conserved C2-WW-HECT domain structure [44]. While the C2 domain regulates the subcellular localization, the WW domains are 38–40 residue
SMAD proteins are the intracellular transducers of TGFβ signals. R-SMADs are phosphorylated at their C-terminal SXS motif inducing complex formation with SMAD4 and nuclear translocation. In the nucleus, they induce transcriptional responses of TGFβ target genes. Interfering with R-SMAD phosphorylation, stability, R-SMAD/SMAD4 complex formation or DNA binding would negatively impact TGFβ pathway signalling. Reversible ubiquitylation of SMADs directly impacts one or more of these attributes. Here, we provide an overview of how reversible ubiquitylation of SMAD transcription factors impacts SMAD function and pathway signalling. Figure 3 summarizes the key players regulating reversible ubiquitylation of SMADs.

7. The BMP pathway SMADS

The first E3 ligase reported to ubiquitylate BMP-responsive SMADs was SMURF1 [51]. The WW domain of SMURF1 interacts with the PY motif of SMAD1/5 and targets them for ubiquitylation and proteasomal degradation [51,52]. Studies in Xenopus embryos showed that SMURF1 causes dorsalization of ventral mesoderm and neutralization of ectoderm, phenotypes consistent with inhibition of the BMP pathway [51]. SMURF1-mediated SMAD1/5 ubiquitylation promotes myogenic differentiation of C2C12 cells, blocks BMP-2-mediated osteogenic conversion [52] and modulates the effects of BMP4 on embryonic lung growth [53]. In contrast, SMURF1 has been shown to have little effect on TGFβ-inhibited myogenic differentiation [54]. The PY motif in SMAD1/5 is preceded by a cluster of Ser/Thr residues. Phosphorylation of these residues, catalysed by proline-directed Ser/Thr protein kinases (e.g. MAP kinases and CDK8/9), in response to different stimuli as well as glycogen synthase kinase-3 (GSK-3) is essential for its interaction with SMURF1 [55,56]. BMP-induced sequential linker phosphorylation of SMAD1 by CDK8/9 and GSK-3 primes SMAD1 for transcriptional action and degradation, respectively. While phosphorylation by CDK8/9 induces recruitment of YAP1 mediator through its WW domain, subsequent phosphorylation by GSK-3 displaces YAP1 and recruits SMURF1 [45,55]. YAP1 stability is further regulated by SCF (Skp, Cullin, F-box)–βTRCP-induced ubiquitylation [58]. These studies demonstrate a clear interplay between phosphorylation and ubiquitylation in balancing the outcome of BMP pathway signalling.
8. The TGF β pathway SMADs

Among the SMADs, TGFβ–SMAD ubiquitylation has received the most scrutiny. The evidence for polyubiquitylation and degradation of TGFβ-induced phospho-SMAD2 was first demonstrated in 1999 [62]. Subsequently, several E3 ubiquitin ligases, including SMURF1/2, NEDD4L, and WWP1, have been implicated in mediating the polyubiquitylation and degradation of SMAD2/3 [47, 48, 59, 63]. These NEDD4-like E3 ubiquitin ligase members all use the PY motif present in the SMAD2/3-linker for interaction. However, the recruitment of NEDD4L to SMAD2/3 requires the phosphorylation of the linker region mediated by CDK8/9 as well as the PY motif [64]. A WW-domain-containing protein PIN1 has been implicated in recruiting SMURF2 to linker-phosphorylated SMADs [65]. NEDD4L itself is also subject to further regulation by serum/glucocorticoid-regulated kinase 1 (SGK1), which is itself a transcriptional target of TGFβ signalling [64]. Signal termination is also achieved by other E3 ligases, independent of linker phosphorylation, using SMAD2/3 interactions with transcriptional cofactors. The ROC1–SCF–βTRCP RING E3 ligase complex targets activated SMAD3 for nuclear export and ubiquitin-mediated degradation upon its association with the transcriptional co-activator p300 [66]. The transcriptional regulator TAZ, reported to be required for SMAD2/3/4 complex nuclear accumulation, is also regulated by SCF–βTRCP-induced ubiquitylation and degradation [67, 68]. While the previous examples show SMAD2/3 regulation after TGFβ signal initiation, CHIP has been shown to interact with ubiquitylate and degrade basal SMAD3 levels, resulting in the inhibition of TGFβ signalling [69].

SMURF2 features prominently in reports describing SMAD2/3 degradation. SMURF2-mediated inhibition of TGFβ signalling has been demonstrated across multiple organisms and in obstructive nephropathy [34, 39, 65, 70]. One area of contention is whether SMURF2 polyubiquitylates [39, 65, 70] or monoubiquitylates the TGFβ signal initiation, CHIP has been shown to interact with ubiquitylate and degrade basal SMAD3 levels, resulting in the inhibition of TGFβ signalling [69].

SMURF2 has also been shown to polyubiquitylate SMAD1 and mediate its degradation. Studies in Xenopus embryos confirmed that SMURF2 inhibits SMAD1 responses [59, 60]. SMAD8 lacks the PY motif in its linker region and would be predicted to be resistant to SMURF-mediated ubiquitylation and degradation. A U-box-dependent E3 ubiquitin ligase member carboxyl terminus of Hsc70-interacting protein (CHIP) was reported as an interactor of SMAD1. CHIP was shown to cause ubiquitylation and degradation of SMAD1, resulting in the inhibition of the BMP-induced transcriptional activity [61]. The lysine residues within BMP–SMADs modified by ubiquitylation, the nature of polyubiquitin linkages and the E2-ubiquitin-conjugating enzymes involved remain to be defined. No DUBs for BMP–SMADs have been reported.

Figure 3. Regulation of SMAD transcription factors and nuclear cofactors by reversible ubiquitylation. An overview of how reversible ubiquitylation of SMAD transcription factors and associated nuclear cofactors may impact the SMAD-dependent transcription. Most of the reported E3s and DUBs known to regulate specific proteins are included. The reported mechanisms by which different ubiquitin ligases and DUBs regulate SMAD proteins and associated cofactors are described in the text.
9. SMAD4

Association of SMAD4 with R-SMADs is a critical step in the canonical TGFβ and BMP signalling pathways. Preventing this association or targeting SMAD4 for degradation inhibits TGFβ/BMP signalling. Regulation of SMAD4 by both mono- and polyubiquitylation has been reported [61,79–84]. Despite the lack of an intact PY motif, SMAD4 is polyubiquitylated by SMURF1/2, WWP1 and NEDD4L, which are recruited to SMAD4 by their association with I-SMADs and SMAD2/3 [83]. The E3 ligase CHIP has been implicated in controlling SMAD4 stability; however its role in SMAD4 ubiquitylation is unclear [61]. SCF complexes have been reported to ubiquitylate and degrade SMAD4. β-TRCP1 was initially shown to bind SMAD4 and induce its ubiquitin-mediated degradation through SCF. In the absence of SMAD4, the over-expressed complex was unable to inhibit TGFβ-induced cell cycle arrest [84]. SCF-βTRCP1 complex has been reported to control SMAD4 stability in pancreatic cancer cells [12]. The other SCF complex with SKP2 was also shown to bind and degrade SMAD4 [82]. Interestingly, TGFβ induces destruction of SKP2 in the nucleus, providing a further layer of control in the feedback loop [81].

The RING E3 ubiquitin ligase TRIM33 (also known as Ectodomin/TIF1γ), which also contains a plant homeodomain (PHD)—Bromo domain, has been proposed to interact with and ubiquitylate SMAD4 [80]. Although the critical role of TRIM33 on the TGFβ pathway is not debated, reports on the mechanisms by which it achieves this differ greatly. Two modes of action have been proposed. (i) TRIM33 interacts with phosphorylated SMAD2/3 in competition with SMAD4, thereby interfering with SMAD2/3–SMAD4 binding and creating separate SMAD2/3–SMAD4 and SMAD2/3–TRIM33 complexes, each resulting in distinct functions on cellular processes [85,86]. Furthermore, the PHD-Bromo domain has been demonstrated to be essential for the recruitment of TRIM33 to chromatin [79,86]. (ii) TRIM33 directly interacts with SMAD4 and not SMAD2/3, catalyses its polyubiquitylation [80] or mono-ubiquitylation at Lys519, which inhibits SMAD2/3–SMAD4 complex formation [29]. It has been shown that chromatin binding is required for the E3 ligase activity of TRIM33 in vitro [79]. While targeted disruption of the TRIM33 gene in mice has established the role for TRIM33 in limiting Nodal responsiveness in vivo [87], it has not resolved the debate on its mode of action. A mouse or a cell-line model in which wild-type TRIM33 is replaced by a catalytically inactive mutant with an intact PHD-Bromo domain would resolve definitively the issue of whether the E3 ligase activity of TRIM33 on SMAD4 is necessary for its influence on the TGFβ pathway.

USP9X/FAM is the only deubiquitylase reported to reverse the mono-ubiquitylation of SMAD4 at Lys519 mediated by TRIM33 [29]. Depletion of USP9X resulted in inhibition of TGFβ-induced transcriptional and cellular responses but not phospho-SMAD3. USP9X interacted with and deubiquitylated SMAD4 [29].

10. Inhibitory SMADs 6/7

In the light of multiple reports on the inhibitory effects of I-SMADs, inducing I-SMAD polyubiquitylation and degradation would be predicted to strongly enhance TGFβ/BMP pathway signalling. Although SMAD6/7 interact with the majority of NEDD4-like E3 ubiquitin ligases through their PY motif, these E3s primarily employ SMAD7 as an adaptor to target various substrates, including the TGFβ/BMP receptors. In the process, I-SMADs are often destroyed by proteasomal degradation [35,36]. ARKADIA, an E3 ligase that does not target the receptor complex, has been shown to target SMAD7 for ubiquitylation and degradation, thereby enhancing pathway signalling [88,89]. ARKADIA also targets multiple components of the TGFβ pathway for ubiquitylation and degradation [11,65,71,74–93]. However, selective SMAD7 polyubiquitylation and degradation has been reported in renal fibrosis and hypertension mouse models, causing enhanced pathway signalling [31,94].

Inhibition of I-SMAD ubiquitylation and subsequent degradation would provide a clear way to negatively control the TGFβ pathway. The histone acetyl transferase, p300, has been reported to acetylate SMAD7 at Lys64 and Lys70, the same residues in which ubiquitylation occurs. This prevents SMAD7 from being targeted by E3s for ubiquitylation and degradation [95,96]. It has also been reported that the de-acetylase SIRT1 can reverse this, creating an acetylation/de-acetylation balance controlling SMAD7 fate [97,98].

The only DUB reported to target the I-SMADs is CYLD [99]. The study performed in CYLD-knockout mice reported that CYLD targets SMAD7 protein for deubiquitylation and inhibits TGFβ signalling in the development of regulatory T cells. Moreover, CYLD appears to deubiquitylate SMAD7 at Lys360 and Lys374 but not at Lys64 or Lys70 [99].

11. Regulation of nuclear SMAD cofactors by reversible ubiquitylation

Once the activated R-SMAD–SMAD4 complex is translocated into the nucleus, it must then bind promoter sequences to positively or negatively regulate the expression of TGFβ response genes. However, SMAD proteins on their own
Table 1. A summary of known E3 ubiquitin ligases and deubiquitylating enzymes (DUBs) involved in TGFβ pathway signalling. Asterisks indicate E3s also targeting tail and/or linker-phosphorylated SMAD proteins. Common alternative names for E3 ubiquitin ligases and DUBs in table: ARKADIA = ring finger 111; WWP1 = AIP5, Triu1; NEDD4L = NEDD4-2; TRIM33 = ECTO, TIF1γ; ITCH = AIF4, AIP4; USP9x = FAM; UCHL5 = UCHL5.

| E3 ubiquitin ligases | receptors | BMP SMADs | TGFβ SMADs | SMAD4 | I-SMADs | nuclear cofactors |
|----------------------|-----------|-----------|------------|-------|---------|------------------|
| ARKADIA              | ✓         |           |            |       |         |                  |
| SMURF1               | ✓         | ✓         | ✓          |       |         |                  |
| SMURF2               | ✓         | ✓         | ✓          | ✓     | ✓       |                  |
| WWP1                 | ✓         | ✓         | ✓          | ✓     | ✓       |                  |
| NEDD4L               | ✓         | ✓         | ✓          | ✓     | ✓       |                  |
| CHIP                 | ✓         | ✓         | ✓          | ✓     | ✓       |                  |
| TRIM33               | ✓         | ✓         | ✓          | ✓     | ✓       |                  |
| CBLB                 | ✓         | ✓         |            | ✓     |         |                  |
| SCF-β-TRCP           | ✓         | ✓         |            | ✓     |         |                  |
| SCF-SKP2             | ✓         | ✓         |            | ✓     |         |                  |
| ITCH                 | ✓         | ✓         |            | ✓     |         |                  |
| APC                  | ✓         | ✓         |            | ✓     |         |                  |

| DUBs | receptors | BMP SMADs | TGFβ SMADs | SMAD4 | I-SMADs | nuclear cofactors |
|------|-----------|-----------|------------|-------|---------|------------------|
| UCH37| ✓         |           |            |       |         |                  |
| USP15| ✓         |           |            |       |         |                  |
| USP9x| ✓         |           |            |       |         |                  |

have low DNA-binding affinity and require other cofactors for DNA binding [16]. Additionally, as previously described, some nuclear adaptor proteins actually inhibit SMAD–DNA binding, thereby negatively regulating SMAD transcriptional activity. Therefore, reversible ubiquitylation of nuclear cofactors can modulate TGFβ-induced transcriptional activity. RUNX2 is a transcription factor that promotes R-SMAD/DNA binding in the BMP pathway. SMURF1 has been reported to induce its ubiquitylation and degradation [100]. SMURF1 is recruited to RUNX2 by its association with SMAD6 [101]. Most other reports have concentrated on the regulation of negative nuclear cofactors SKI and SnoN that antagonize SMAD-mediated transcriptional activity. TGFβ-induced SMURF2/SMAD2 binding and targeting of SnoN release the negative regulation of SnoN on nuclear SMAD transcriptional activity in both physiological and pathological pathway signalling [33,102]. ARKADIA is reported to target both SKI and SnoN for ubiquitin-mediated degradation in a similar TGFβ-dependent fashion, leading to activation of transcriptional responses [91,92]. Later reports also identify that SKI ubiquitylation and degradation requires TGFβ signalling and ARKADIA binding to phosphorylated-SMAD2/3 [74,93]. ARKADIA function is itself regulated by binding to proteins such as AXIN and RB1CC1 [89,90]. The anaphase-promoting complex E3 ligase has also been reported to act in a similar manner by targeting SnoN [103,104], while the CDC34 E2 targets SKI and SnoN in a cell-cycle-dependent fashion [105]. Very little is known about the DUBs that reverse the ubiquitylation of the earlier-mentioned nuclear SMAD cofactors.

12. Concluding remarks

The TGFβ family of cytokines influences the behaviour and fate of almost every cell type in vertebrates. The cellular responses to TGFβ signals vary greatly depending on the biological context. Despite this, all cells share the fundamental transduction mechanisms of TGFβ signalling. Various post-translational modifications of key mediators of the TGFβ pathway in response to multiple signals modulate their activity, stability and subcellular localization. The integration of different signals ultimately determines the extent and duration of cellular responses to TGFβ signals. Reversible ubiquitylation of fundamental TGFβ pathway mediators offers a key regulatory balance on the outcome of the pathway. Ubiquitylation confers a versatile modification of target proteins. This versatility is further augmented by the possibility of multiple types of ubiquitin chains that can be formed on target proteins. While K48-linked polyubiquitin chains have been described to cause proteasomal degradation of TGFβ pathway components, the precise nature of polyubiquitin chains remains unexplored. Proteins that contain unique UBDs would be predicted to be essential for interpreting the signals contained within target proteins with unique polyubiquitin chains. In the TGFβ pathway, few such proteins have been identified.

Regulation of the TGFβ pathway by ubiquitylation of key components has been widely reported (table 1). While many candidate E3 ubiquitin ligases have been proposed, little is known about the E2-ubiquitin-conjugating enzymes further upstream. Several members of the NEDD4-like family of E3 ubiquitin ligases have been reported to catalyse the polyubiquitylation and degradation of both TGFβ receptors and SMAD transcription factors. Indeed, SMURF1/2 appears to be transcriptional targets of TGFβ cytokines themselves and inhibit the pathway through a negative feedback loop [18]. The observations that the recognition of SMAD1 and SMAD2/3, by SMURF1 and NEDD4L, respectively, requires phosphorylation of linker regions of SMAD proteins imply an active interplay between phosphorylation and ubiquitylation processes [57,64]. Such crosstalk is likely to happen across
phosphorylation of R-SMADs has resulted in our understanding of the fundamental aspects of TGFβ signalling [1]. The precise ubiquitylation sites within receptors, SMAD proteins or SMAD cofactors as well as the nature of polyubiquitin chains that are attached to the initial ubiquitin are largely undefined. Most of the ubiquitylation sites reported on SMAD proteins thus far have resulted from over-expression and mutagenesis studies, which have the potential of yielding artefacts. Recent technologies capable of identifying ubiquitylated peptides on endogenous proteins hold great promise for investigating reversible ubiquitylation in the TGFβ pathway [108,109]. Indeed one of these studies was able to identify multiple ubiquitylation sites within endogenous type I TGFβ/BMP receptors as well as BMP and TGFβ ligands. That the ligands could themselves be regulated by ubiquitylation is an intriguing observation that has as yet eluded consideration entirely.

Investigation into the regulation of the TGFβ pathway by DUBs is an emerging research field. To date, only three DUBs, namely UCH37, USP9X and USP15, have been attributed a role in deubiquitylating components of the TGFβ pathway (table 1) [7,29,30,50]. The mode of substrate recognition and catalysis of reported TGFβ pathway components are frequently complex. The molecular mechanisms by which reversible ubiquitylation regulates TGFβ signalling may hold some therapeutic promise against these diseases. Amplification of several members of the NEDD4-like E3 ligases, including SMURF1/2, is reported to be associated with tumour progression [44]. Reduced ARKADIA activity is associated with the pathogenesis of colorectal cancers [11]. The efficacy of the proteasome inhibitor Bortezomib against B cell lymphoma demonstrates that ubiquitin ligases and the ubiquitylation system could be exploited as targets for anti-cancer therapies [111]. DUBs, which constitute the largest family of peptidases, are also associated with many human diseases, including cancer and could make attractive therapeutic targets [7,111,112]. Therefore, targeting the TGFβ-pathway-specific E2-ubiquitin-conjugating enzymes, E3-ubiquitin ligases or DUBs for inhibition may provide opportunities for the development of therapies against diseases in which the TGFβ pathway is compromised.

13. Acknowledgements

M.A.A. is a Career Development Fellow supported by the Medical Research Council UK. L.H. is supported by the Medical Research Council UK studenthip. G.S. is supported by the Medical Research Council UK and the pharmaceutical companies (AstraZeneca, Boehringer-Ingelheim, GlaxoSmithKline, Merck-Serono, Pfizer and Johnson & Johnson) supporting the Division of Signal Transduction Therapy Unit at the University of Dundee.

References

1. Massagué J, Gomis RR. 2006 The logic of TGF-beta signaling. Festschrift. 580, 2811 – 2820. (doi:10.1016/S0165-2478(02)00023-8)
2. Shi Y, Massagué J. 2003 Mechanisms of TGF-beta signaling from cell membrane to the nucleus. Cell 113, 685 – 700. (doi:10.1016/S0092-8674(03)00432-X)
3. Moustakas A, Pardali K, Gaal A, Heldin CH. 2002 Mechanisms of TGF-beta signaling in regulation of cell growth and differentiation. Immunol. Lett. 82, 85 – 91. (doi:10.1016/S0165-2478(02)00023-8)
4. Wu MY, Hill CS. 2009 Tgf-beta superfamily signaling in embryonic development and homeostasis. Dev. Cell 16, 329 – 343. (doi:10.1016/j.devcel.2009.02.012)
5. Bierie B, Moses HL. 2010 Transforming growth factor beta (TGF-beta) and inflammation in cancer. Cytokine Growth Factor Rev. 21, 49 – 59. (doi:10.1016/j.cytogfr.2009.11.008)
6. Border WA, Noble NA. 1994 Transforming growth factor beta in tissue fibrosis. New Engl. J. Med. 331, 1286 – 1292. (doi:10.1056/NEJM199411103317907)
17. Santibañez JF, Quintanilla M, Bernabeu C. 2011
16. Ross S, Hill CS. 2008
15. Massague J. 1998
14. Liu IM, Schilling SH, Knouse KA, Choy L, Derynck R, et al.
13. Ikushima H, Miyazono K. 2011
12. Wan M et al. 2005
11. Shima H, Miyazato K. 2011
10. Ross S, Hill CS. 2008
9. Fukasawa H et al.
8. Eichhorn PJ
7. Eichhorn PJ.
6. Pickart CM, Eddins MJ. 2004
5. Komander D, Clague MJ, Urbés S. 2009
4. Liu X, Hu Z. 2011
3. Eriksen PJ
2. Pickart CM, Eddins MJ. 2004
1. Eichhorn PJ.
for degradation. EMBO J. 23, 3780–3792. (doi:10.1093/embj/603958)

49. Ogumijimi AA et al. 2005 Regulation of Smurf2 ubiquitin ligase activity by anchoring the E2 to the HECT domain. Mol. Cell 19, 297–308. (doi:10.1016/j.molcel.2005.06.028)

50. Wicks SJ, Haros K, Maillard M, Song L, Cohen RE, Dijke PT, Chantry A. 2005 The deubiquitinating enzyme UCH37 interacts with Smads and regulates TGF-beta signalling. Oncogene 24, 8080–8084. (doi:10.1038/ccc.2005.394)

51. Zhu H, Kavak P, Abdullah S, Witanja JL, Thomsen GH. 1999 A SMAD ubiquitin ligase targets the BMP pathway and affects embryonic pattern formation. Nature 400, 687–693. (doi:10.1038/32393)

52. Ying S-X, Hussain ZJ, Zhang YE. 2003 Smurf1 facilitates myogenic differentiation and antagonizes the bone morphogenetic protein-2-induced osteoblast conversion by targeting Smad5 for degradation. J. Biol. Chem. 278, 39 029–39 036. (doi:10.1074/jbc.M301193200)

53. Shi W, Chen H, Sun J, Chen C, Zhao J, Wang Y-L, Anderson KD, Warburton D. 2004 Overexpression of Smurf1 negatively regulates mouse embryonic lung branching morphogenesis by specifically reducing Smad1 and Smad5 proteins. Am. J. Physiol. Lung Cell Mol. Physiol. 286, L293–L300. (doi:10.1152/ajnl.00228.2003)

54. Sangadala S, Boden SD, Viggeskwarapu M, Liu Y, Titus L. 2006 LIM mineralization protein-1 potentiates bone morphogenetic protein responsiveness via a novel interaction with Smurf1 resulting in decreased ubiquitination of Smads. J. Biol. Chem. 281, 17 212–17 219. (doi:10.1074/jbc.M511013200)

55. Alarcón C et al. 2009 Nuclear CKD6 drive Smad transcriptional activation and turnover in BMP and TGF-beta pathways. Cell 139, 757–769. (doi:10.1016/j.cell.2009.09.033)

56. Fuentealba LC, Eivers E, Ikeda A, Hurtado C, Kuroda N, Dijke PT, Chantry A. 2005 The deubiquitinating j.molcel.2005.06.028)

57. Sapkota G, Alarcón C, Spagnoli FM, Brivanlou AH, Massague J. 2007 Balancing BMP signaling through integrated inputs into the Smad1 linker. Mol. Cell. 25, 441–454. (doi:10.1016/j.molcel.2007.01.006)

58. Zhao B, Li L, Tumanski K, Wang CY, Duan KL. 2010 A coordinated phosphorylation by Lats and CK1 regulates YAP stability through SCF(beta-TRCP). Genes Dev. 24, 72–85. (doi:10.1101/gad.1843810)

59. Lin X, Liang M, Feng XH. 2000 Smurf2 is a ubiquitin E3 ligase mediating proteasome-degradation of Smad2 in transforming growth factor-beta signaling. J. Biol. Chem. 275, 36 818–36 822. (doi:10.1074/jbc.C0005820)

60. Zhang Y, Chang C, Gehling DJ, Hemmati-Brivanlou A, Derynick R. 2001 Regulation of Smad degradation and activity by Smurf2, an E3 ubiquitin ligase. Proc. Natl Acad. Sci. USA 98, 974–979. (doi:10.1073/pnas.98.3.974)

61. Li L, Xin H, Xu X, Huang M, Zhang X, Chen Y, Zhang S, Fu X-Y, Zhang Z. 2004 CHIP mediates degradation of Smad proteins and potentially regulates Smad-induced transcription. Mol. Cell. Biol. 24, 856–864. (doi:10.1128/MCB.24.2.856-864.2004)

62. Lo RS, Massague J. 1999 Ubiquitin-dependent degradation of TGF-beta-activated smad2. Nat. Cell. Biol. 1, 472–478. (doi:10.1016/j.tcb.2008.07.028)

63. Ito I et al. 2010 Etoxin inhibits transforming growth factor beta signaling by promoting Smad2/3 degradation. J. Biol. Chem. 285, 14 747–14 755. (doi:10.1074/jbc.M109.93039)

64. Gao S et al. 2009 Ubiquitin ligase nedd4L targets activated Smad2/3 to limit TGF-beta signaling. Mol. Cell. 37, 456–467. (doi:10.1016/j.molcel.2009.09.043)

65. Nakano A et al. 2009 Pin1 down-regulates transforming growth factor-beta (TGF-beta) signaling by inducing degradation of Smad proteins. J. Biol. Chem. 284, 6109–6115. (doi:10.1074/jbc.M804659200)

66. Fukuchi M, Imamura T, Kuma K, Miyazono K. 2001 Ligand-dependent degradation of Smad5 by a ubiquitin ligase complex of RDC1 and associated proteins. Mol. Biol. Cell. 12, 1431–1443.

67. Liu CY et al. 2010 The hippo tumor pathway promotes TAZ degradation by phosphorylating a phosphodegron and recruiting the SCF(beta)-TrCP E3 ligase. J. Biol. Chem. 285, 37 159–37 169. (doi:10.1074/jbc.M110.152942)

68. Varelas X, Sakuma R, Samavarchi-Tehrani P, Pecriani R, Rao BM, Dembowey J, Yaffe MB, Zandstra PW, Wanja IL. 2008 TAZ controls Smad nucleocytoplasmic shuttling and regulates human embryonic stem-cell self-renewal. Nat. Cell. Biol. 10, 837–848. (doi:10.1038/ncb1748)

69. Xin H, Xu X, Li N, Ning H, Yang R, Wang Y, Yang W, Fu X-Y, Zhang Z. 2005 CHIP controls the sensitivity of transforming growth factor-beta signaling by modulating the basal level of Smad3 through ubiquitin-mediated degradation. J. Biol. Chem. 280, 20 842–20 850. (doi:10.1074/jbc.m412275200)

70. Runyan CE, Hayashida T, Hubchak S, Curley JF, Schnaper HW. 2009 Role of SARA (SMAD anchor for receptor activation) in maintenance of epithelial cell phenotype. J. Biol. Chem. 284, 25 181–25 189. (doi:10.1074/jbc.M9012847)

71. Mavrakis KJ, Andrew RL, Lee KL, Petropoulou C, Moustakas A. 2005 Degradation of the tumor suppressor Smad4 by WW and HECT domain ubiquitin ligases. J. Biol. Chem. 280, 22 115–22 123. (doi:10.1074/jbc.M410227200)

72. Liang M, Liang Y-Y, Wrighton K, Ungermannova D, Wang X-P, Brunoirdi FC, Liu X, Feng X-H, Lin X. 2004 Ubiquitination and proteolysis of cancer-derived Smad4 mutants by SCF(beta). Mol. Cell. Biol. 24, 7524–7537. (doi:10.1128/MCB.24.17.7524-7537.2004)

73. Morein A, Imamura T, Miyazako K, Heldin CH, Moustakas A. 2005 Degradation of the tumor suppressor Smad4 by WW and HECT domain ubiquitin ligases. J. Biol. Chem. 280, 22 115–22 123. (doi:10.1074/jbc.M410227200)

74. Han M, Tang Y, Tytler EM, Lu C, Jin B, Vickers SM, Yang L, Shi X, Cao X. 2004 Smad4 protein stability is regulated by ubiquitin ligase SCF(beta)-TrCP1. J. Biol. Chem. 279, 14 484–14 487. (doi:10.1074/jbc.C400050200)

75. He W, Dor CM, Cerdan-Bromage H, Tempst P, Moore MAS, Massague J. 2006 Hematopoiesis controlled by distinct TIF-1gamma and Smad4 branches of the TGFbeta pathway. Cell 125, 929–941. (doi:10.1016/j.cell.2006.03.045)

76. Xi Q et al. 2011 A poised chromatin platform for TGF-beta access to master regulators. Cell 147, 1511–1524. (doi:10.1016/j.cell.2011.11.032)
90. Koinuma D, Nagano Y, Liu W, Simonsson M, Heldin C-H, Ericsson J, Groenroos E, Fukasawa H, et al. 2002 Control of Smad7 stability by competition between Smad3 recruitment of the anaphase-promoting complex and ubiquitination. Mol. Cell 10, 483–493. (doi:10.1016/S1097-2765(02)00639-1)
91. Montefuone G et al. 2005 Post-transcriptional regulation of Smad7 in the gut of patients with inflammatory bowel disease. Gastroenterology 129, 1420–1429. (doi:10.1053/j.gastro.2005.09.005)
92. Kume S et al. 2007 SIRT1 inhibits transforming growth factor β-induced apoptosis in glomerular mesangial cells via Smad7 deacetylation. J. Biol. Chem. 282, 151–158. (doi:10.1074/jbc.M605904200)
93. Simonsson M, Heldin C-H, Ericsson J, Gronroos E. 2005 The balance between acetylation and deacetylation controls Smad7 stability. J. Biol. Chem. 280, 21 797–21 803. (doi:10.1074/jbc.M603134200)
94. Zhao Y, Thornton AM, Kinney MC, Ma CA, Spinner JJ, Fuss LJ, Shevach EM, Jain A. 2011 The deubiquitinase CYLD targets Smad7 protein to regulate transforming growth factor β signaling. J. Biol. Chem. 286, 40 520–40 530. (doi:10.1074/jbc.M111.292961)
95. Zhao M, Qiao M, Ohyashi BO, Mundy GR, Chen D. 2003 E3 ubiquitin ligase Smurf1 mediates core-binding factor alpha1/Runx2 degradation and plays a specific role in osteoblast differentiation. J. Biol. Chem. 278, 27 939–27 944. (doi:10.1074/jbc.M304132200)
96. Shen R, Chen H, Wang Y, Kaneki H, Xing L, O’Keefe RJ, Chen D. 2006 Smad6 interacts with Runx2 and mediates Smad ubiquitin regulatory factor 1-induced Runx2 degradation. J. Biol. Chem. 281, 3569–3576. (doi:10.1074/jbc.M605764200)
97. Bonni S, Wang HR, Causing CG, Kovač P, Stroschein SL, Luo K, Wrana JL. 2001 TGF-β induces assembly of a Smad2–SnoN ubiquitin ligase complex that targets SnoN for degradation. Nat. Cell. Biol. 3, 587–595. (doi:10.1038/35078562)
98. Stroschein SL, Bonni S, Wiwa JL, Luo K. 2001 Smad3 recruits the anaphase-promoting complex for ubiquitination and degradation of SnoN. Genes Dev. 15, 2822–2836.
99. Wan Y, Liu X, Kirschner MW. 2001 The anaphase-promoting complex mediates TGF-β signaling by targeting SnoN for destruction. Mol. Cell. 8, 1027–1039. (doi:10.1016/S1097-2765(01)00382-3)
100. Nagano Y, Liu W, Wang W, Roberts E, Cheung TH, Erickson R, Knuessel MT, Liu X. 2004 Control of cell cycle-dependent degradation of c-Ski proto-oncoprotein by Cdh34. Oncogene 23, 5643–5653. (doi:10.1038/sj.onc.1207733)
101. Tamashita M, Ying SX, Zhang GM, Li C, Cheng SY, Deng CX, Zhang Y. 2005 Ubiquitin ligase Smurf1 controls osteoblast activity and bone homeostasis by targeting MEKK2 for degradation. Cell 121, 101–113. (doi:10.1016/j.cell.2005.01.035)
102. Narimatsu M et al. 2009 Regulation of planar cell polarity by Smurf ubiquitin ligases. Cell 137, 295–307. (doi:10.1016/j.cell.2009.02.025)
103. Kim W et al. 2011 Systematic and quantitative assessment of the ubiquitin-modified proteome. Mol. Cell 44, 325–340. (doi:10.1016/j.molcel.2011.08.025)
104. Wagner SA, Beli P, Weinert BT, Nielsen ML, Cox J, Mann M, Choudhary C. 2011 A proteome-wide, quantitative survey of in vivo ubiquitylation sites reveals widespread regulatory roles. Mol. Cell. Proteomics 10, M111.013284. (doi:10.1074/mcp.M111.013284)
105. Sarkari F, Sheng Y, Frappier L. 2010 USP7/HAUSP promotes the sequence-specific DNA binding activity of p53. PLoS ONE 5, e10340. (doi:10.1371/journal.pone.0010340)
106. Cohen P, Tcherpakov M. 2010 Will the ubiquitin system furnish as many drug targets as protein kinases? Cell 143, 686–693. (doi:10.1016/j.cell.2010.11.016)
107. Collard F. 2010 The therapeutic potential of deubiquitinating enzyme inhibitors. Biochem. Soc. Trans. 38, 137–143. (doi:10.1042/BST0380137)