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Current perspectives on inhibitory SMAD7 in health and disease

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ABSTRACT

Transforming growth factor β (TGF-β) family members play an extensive role in cellular communication that orchestrates both early development and adult tissue homeostasis. Aberrant TGF-β family signaling is associated with a pathological outcome in numerous diseases, and in-depth understanding of molecular and cellular processes could result in therapeutic benefit for patients. Canonical TGF-β signaling is mediated by receptor-regulated SMADs (R-SMADs), a single co-mediator SMAD (Co-SMAD), and inhibitory SMADs (I-SMADs). SMAD7, one of the I-SMADs, is an essential negative regulator of the pleiotropic TGF-β and bone morphogenetic protein (BMP) signaling pathways. In a negative feedback loop, SMAD7 inhibits TGF-β signaling by providing competition for TGF-β type-1 receptor (TβRI), blocking phosphorylation and activation of SMAD2. Moreover, SMAD7 recruits E3 ubiquitin SMURF ligases to the type I receptor to promote ubiquitin-mediated proteasomal degradation. In addition to its role in TGF-β and BMP signaling, SMAD7 is regulated by and implicated in a variety of other signaling pathways and functions as a mediator of crosstalk. This review is focused on SMAD7, its function in TGF-β and BMP signaling, and its role as a downstream integrator and crosstalk mediator. This crucial signaling molecule is tightly regulated by various mechanisms. We provide an overview of the ways by which SMAD7 is regulated, including noncoding RNAs (ncRNAs) and post-translational modifications (PTMs). Finally, we discuss its role in diseases, such as cancer, fibrosis, and inflammatory bowel disease (IBD).

Abbreviations: AhR: Aryl hydrocarbon receptor; ALK: Activin-receptor-like kinase; AON: Antisense oligonucleotide; BMDC: Bone marrow-derived cell; BMP: Bone morphogenetic proteins; BMSC: Bone marrow mesenchymal stem cell; CD: Crohn's disease; CIA: Collagen-induced arthritis; CircRNA: Circular RNA; CRC: Colorectal cancer; DEN: N-nitrosodiethylamine; DSS: Dextran sulfate sodium; EAE: Experimental autoimmune encephalomyelitis; ECM: Extracellular matrix; EGF: Epidermal growth factor; EMT: Epithelial-to-mesenchymal transition; EV: Extracellular vesicle; GBM: Glioblastoma; GDF: Growth and differentiation factor; HCC: Hepatocellular carcinoma; HDAC: Histone deacetylases; HSC: Hepatic stellate cell; IBD: Inflammatory bowel disease; IFN: Interferon; IL: Interleukin; LncRNA: Long non-coding RNA; LRM: Leucine rich motif; MAPK: Mitogen-activated protein kinase; MD: Molecular dynamics; MH: MAD homology; MIS: Müllerian-inhibiting substance; MiRNA: MicroRNA; NcRNA: Non-coding RNA; OncomiR: Oncogenic miRNA; Pre-miRNA: Precursor miRNA; Pri-miRNA: Primary miRNA; PTM: Post-translational modification; RA: Rheumatoid arthritis; RTK: Receptor tyrosine kinase; SBE: SMAD binding element; SCC: Squamous cell carcinoma; SCLC: Small cell lung cancer; STAT: Signal transducer and activator of transcription; TAK: Transforming growth factor β-activated kinase; TGF-β: Transforming growth factor β; TNF: Tumor necrosis factor; TPA: 12-O-tetradecanoylphorbol-13-acetate; TRAF: TNF receptor-associated factor; UTR: Untranslated region

Introduction

The transforming growth factor beta (TGF-β) family is comprised of over 30 secreted multifunctional polypeptides, including TGF-βs, activins, bone morphogenetic proteins (BMPs), growth and differentiation factors (GDFs) and Müllerian-inhibiting substance (MIS).

Members of the TGF-β family are ubiquitously expressed in metazoans and play an extensive role in cellular communication that orchestrates both early development and adult tissue homeostasis (Massagué 1998). These proteins are involved in a range of cellular processes, including proliferation, cell death, adhesion,

migration and differentiation. Additionally, the biological effects of TGF-β family members are highly cell and context dependent. Aberrant TGF-β family signaling is often associated with a pathological outcome, highlighting the importance of this pathway in maintaining tissue homeostasis. Studies have linked inappropriate functional activation or inhibition of TGF-β family signaling components with a broad spectrum of human diseases, including cancer, fibrosis, developmental-, musculoskeletal-, cardiovascular-, and autoimmune disorders (Akhurst and Hata 2012; Morikawa et al. 2016; Batlle and Massagué 2019; Yu and Feng 2019).

TGF-β family member ligands are initially synthesized as latent precursor proteins with an N-terminal propeptide region. After proteolytic processing, the mature, biologically active ligand is obtained (Derynck and Budi 2019). TGF-β family members signal via heteromeric complexes of single-pass transmembrane type I, also termed activin-receptor-like kinases (ALKs), and type-II serine/threonine kinase receptors (Heldin and Moustakas 2016). Seven type-I receptors have been described in vertebrates: ALK1, ALK2 (also termed ActRI), ALK3 (also termed BMPRIA), ALK4 (also termed ActRIB), ALK5 (also termed TβRI), ALK6 (also termed BMPRIB), and ALK7. Type-I receptors for activin and TGF-β are ALK4 and ALK5, respectively. BMPs signal through ALK1, ALK2, ALK3 and ALK6. Five type-II receptors have been identified: TGF-β type-II receptor (TβRII), activin type II and type IIB (ActRII, and ActRIIB), BMP type-II receptor (BMPRII) and AMH type-II receptor (AMHRII). The type-I and type-II receptors have cysteine-rich extracellular domains through which signaling can be initiated by association with TGF-β family member ligands.

Upon ligand binding, the type-I and type-II receptor intracellular domains associate in a heteromeric complex (Wrana et al. 1994). Type-I receptors contain a glycine-serine repeat motif (GS domain), of which certain serine and threonine residues can become phosphorylated by the constitutively active type-II receptor kinase. Subsequently, an intracellular cascade by means of phosphorylation of downstream SMAD effector proteins is triggered. These SMADs can act as transcription factors and are the central mediators in the canonical TGF-β signaling pathway (Derynck et al. 1998). The eight vertebrate SMADs are divided into three distinct SMAD subtypes: the receptor-regulated SMADs (R-SMADs, SMAD1, 2, 3, 5, and 8), the co-mediator SMAD (Co-SMAD, SMAD4) and inhibitory SMADs (I-SMADs, SMAD6 and 7). Upon receptor activation, specific R-SMADs are phosphorylated at two carboxy (C)-terminal serine residues by the type-I receptor intracellular kinase activity

and undergo formation of heteromeric complexes with SMAD4 (Shi and Massagué 2003). SMAD2/3 mediate TGF-β family signaling, while SMAD1/5/8 respond primarily to the BMP family. The activated SMAD complexes then translocate into the nucleus where they activate or repress the transcription of target genes. With the exception of a common spliced isoform of SMAD2, the above-mentioned R- and Co-SMADs are able to directly interact with DNA (Massagué 2012). The MAD homology (MH)1 domain of these SMADs facilitates their sequence-specific DNA-binding activity. However, SMADs have limited affinity and specificity for DNA and therefore rely on cooperation with other DNA-binding proteins, such as DNA-binding transcription factors and transcriptional coregulators (Hill 2016).

I-SMADs were discovered due to their similarity to previously identified R- and Co-SMADs as they also contain a large conserved MH2 domain (Topper et al. 1997). The I-SMADs, SMAD6 and SMAD7, are potent modulators of the TGF-β family. They inhibit TGF-β and BMP signaling in a negative feedback circuit by blocking type I receptor activity through physical interaction. Moreover, SMAD7 is able to target receptors for proteasomal degradation, preventing R- and Co-SMAD complex formation (Miyazawa and Miyazono 2017). SMAD6 principally regulates BMP signaling, while SMAD7 is able to repress signaling in both the TGF-β and BMP signaling pathway (Itoh and ten Dijke 2007) (Figure 1).

While R- and Co-SMADs are critical in propagating TGF-β family signals from cell surface complexes to the nucleus, inhibition of signaling activity by I-SMADs is essential for maintaining a proper physiological response to TGF-β or BMP signaling. This review focuses on our current understanding of the complex and multifaceted role of SMAD7, as a key player in SMAD signaling inhibition, and as downstream integrator providing crosstalk between the TGF-β family and other signaling pathways. We also highlight recent discoveries focusing on SMAD7 regulation. Particularly, we address the increasing number of publications that demonstrate noncoding RNA-governed regulation of SMAD7. Moreover, we describe its function as revealed by genetic studies in mice and human. And finally, we discuss the role of SMAD7 in disease onset and progression, and possible avenues for therapeutic intervention by targeting SMAD7.

SMAD7 structure and function in TGF-β signaling

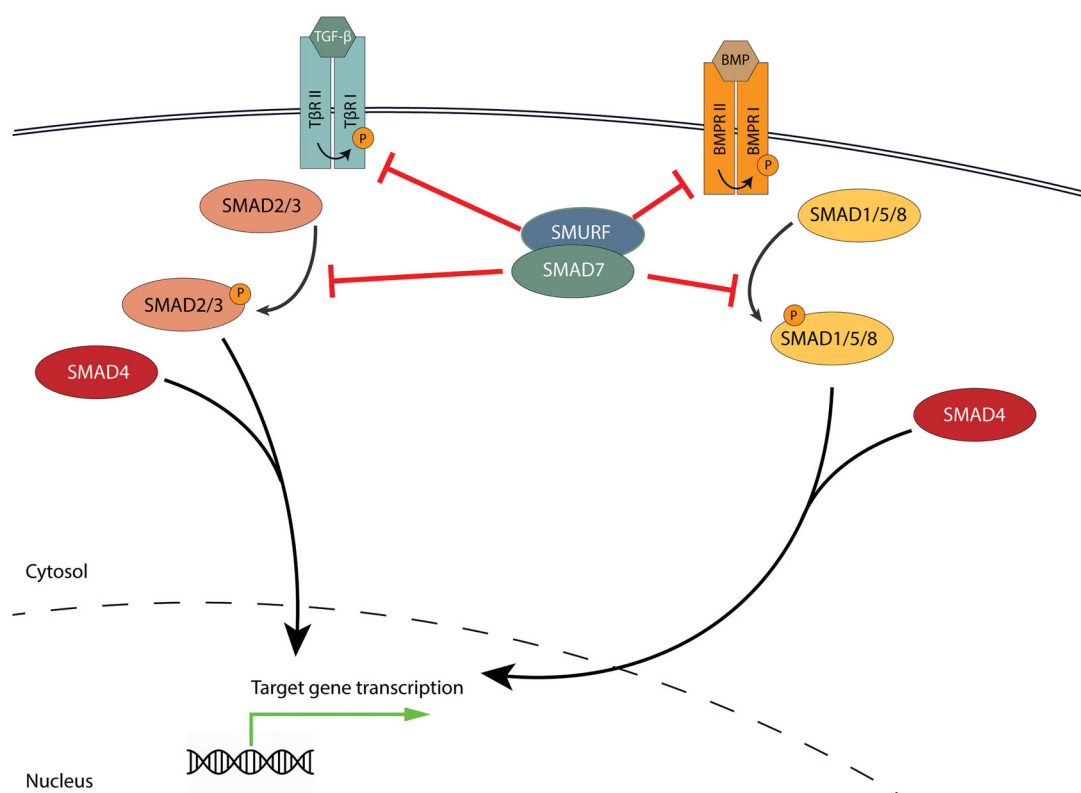Human SMAD7 consists of 426 amino acid residues and is comprised of two globular domains, connected by a

Figure 1. SMAD7 negatively regulates TGF-β and BMP signaling through physical interaction with the type I receptor, preventing R- and Co-SMAD complex formation and promoting proteasomal degradation of receptors.
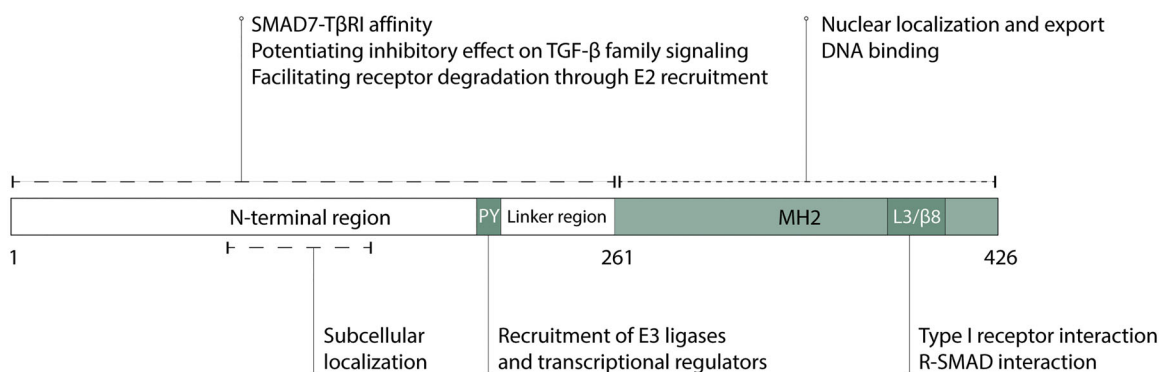

Figure 2. Schematic representation of the structural domains of SMAD7 as they relate to function. PY, proline-tyrosine motif; MH2, Mad homology 2 domain; L3/β8, L3/β8 loop-strand module.

relatively short linker region (Figure 2). SMAD7 interacts with the activated TβRI through its MH2 domain (Hayashi et al. 1997; Nakao et al. 1997). However, SMAD7 lacks a C-terminal SSXS motif, which is required for phosphorylation of SMAD2 and −3 by the TβRI kinase, (Abdollah et al. 1997; Souchelnytskyi et al. 1997) suggesting that it is not a substrate for TβRI phosphorylation. SMAD7 inhibits signaling by providing competition for receptor binding, blocking phosphorylation and activation of downstream SMAD2. Thereby, it prevents SMAD2/4 complex formation and translocation of SMAD2 to the nucleus (Hayashi et al. 1997). Moreover,

SMAD7 recruits E3 ubiquitin ligases such as NEDD4L, SMURF1 and SMURF2 to TGF-β receptors, thereby promoting their ubiquitin-mediated degradation (Kavsak et al. 2000; Ebisawa et al. 2001; Kuratomi et al. 2005). Other mechanisms by which SMAD7 inhibits TGF-β signaling include recruitment of phosphatase GADD34-PP1c to the TβRI and hindrance of SMAD-DNA complex formation (Shi et al. 2004; Zhang et al. 2007).

The MH2 domain of SMAD7 contains a basic region that includes the so-called L3 loop and β8 strand (Yan et al. 2016). Additionally, unlike R- and Co-SMADs, the SMAD7 MH2 domain also includes an α helix containing

K312 and K316, which contributes to the basic character of this domain. SMAD7-TβRI interaction relies on four basic amino acid residues in the basic surface of this C-terminal region: K312, K316, K401, and R409 (Mochizuki et al. 2004). Mutations of these residues abrogate the inhibitory effects of SMAD7 on TGF-β signaling by interrupting SMAD7–TβRI interaction. Furthermore, inhibition of BMP signaling by SMAD7 is likely dependent on two of the above-mentioned SMAD7 residues (K401 and R409), as mutation in either residue abolishes SMAD7-mediated inhibition of the pathway (Mochizuki et al. 2004).

In addition to facilitating SMAD-receptor interaction, the MH2 domain also enables SMAD-protein interaction (Massagué and Wotton 2000). The MH2 domain allows for SMADs to interact with each other and with regulator and effector proteins, including DNA-binding cofactors and chromatin modifiers. The L3/β8 loop-strand module and adjacent lysines comprise key residues that permit SMAD7-SMAD3 association (Yan et al. 2016). This suggests that the MH2 domain is not only a critical structural determinant in establishing SMAD-receptor interaction, but also in establishing SMAD-SMAD interaction. Molecular dynamics (MD) simulations predicted that the L3/β8 loop contributes to the considerable functional flexibility of SMAD7. Hariharan et al. describe that SMAD7 has a flexible overall folding, due to the presence of flexible amino acids in the MH2 domain of the protein, and more specifically in the L3 loop (F411, K401, and C406) (Hariharan and Pillai 2008).

Additionally, the MH2 domain of SMAD7 is able to bind to oligonucleotides containing the minimal SMAD binding element (SBE). Shi *et al.* demonstrated that the isolated MH2 domain of SMAD7 had a higher DNA binding affinity than full-length SMAD7 (Shi et al. 2008). The difference in binding affinity was attributed to an repressive effect of the SMAD7 N-terminal region. The MH2 domain of SMAD7 is able to bind to DNA and thereby suppresses SMAD2/4-DNA complex formation and is able to directly bind to the *plasminogen activator inhibitor 1* (PAI-1) promoter under physiological conditions (Zhang et al. 2007).

As described above, SMAD7 inhibits TGF-β and BMP signaling by promoting receptor turnover through ubiquitination (Kavsak et al. 2000; Ebisawa et al. 2001; Murakami et al. 2003; Wiesner et al. 2007). The PPxY sequence (proline-tyrosine motif, PY motif) in the SMAD7 linker region has a pivotal role in the recruitment of Homologous to E6AP C-terminus (HECT) type E3 ubiquitin ligases, including NEDD4L, and SMURF1/2 (Figure 2) (Aragón et al. 2012). The WW domains of these E3 ligases bind to the PY motif in the linker region of SMAD7. Consistent with this notion, a PY motif deletion or mutation weakens association of SMAD7 with SMURF1/2 (Kavsak et al. 2000; Ebisawa et al. 2001). It has been reported that the SMURF2 WW3 domain binds to an extended binding motif in SMAD7 that includes the PY motif and six C-terminal residues (Chong et al. 2006). Furthermore, as an adaptor protein, SMAD7 antagonizes inhibitory intramolecular interactions between the SMURF2 C2 and HECT domains and relieves C2 domain-dependent autoinhibition, ultimately promoting SMURF2 activity. Besides ubiquitin-mediated regulation of receptors, SMAD7-SMURF2 complexes were recently shown to promote proteasomal degradation of SMAD anchor for receptor activation (SARA), a positive regulator of TGF-β signaling (Wojtowicz et al. 2020). Lastly, the SMAD7 PY motif is involved in binding Yes-Associated Protein (YAP)65 in order to co-repress TGF-β signaling (Ferrigno et al. 2002).

Contrary to the MH2 domain, the N-terminal region of SMAD7 (amino acids 1-260) shows limited similarity with the MH1 domain found in SMAD1 to SMAD5. The SMAD7 N-terminal region has several distinct functions in TGF-β signaling. First, the N-terminal amino acid residues 32-90 were found to be important determinants of inhibitory effect on the receptor signaling. Additionally, even though the SMAD7–TβRI interaction is mainly MH2-mediated, the N-terminal region co-determines the affinity of SMAD7 for TβRI. Furthermore, inhibition of TGF-β signaling is potentiated by physical interaction between the SMAD7 N-terminal region and MH2 domain (Hanyu et al. 2001). In addition, the N-terminal domain was shown to determine subcellular localization of SMAD7 in multiple cell lines (Itoh et al. 1998; Hanyu et al. 2001). Furthermore, the N-terminal region of SMAD7 contains a Leu-rich motif (LRM), which is important in the recruitment of the E2 ubiquitin-conjugating enzyme UbcH7 to the SMURF2 HECT domain (Ogunjimi et al. 2005). Moreover, the SMAD7 N-terminal region has been suggested to contain DNA-binding ability. In R-SMADs, the MH1 domain is responsible for DNA-binding activity. The N-terminal region of I-SMADs, however, lacks the DNA-binding β-hairpin motif. I-SMADs are predicted to contain a zinc atom in their N-terminal region, similar to the zinc atom in R-SMADs that facilitates DNA-binding (Hariharan and Pillai 2008), indicating a possible role for the SMAD7 N-terminal region in DNA-binding. Taken together, these studies demonstrate how divergent structural features of SMAD7, as compared to R- and Co-SMADs, facilitate its function.
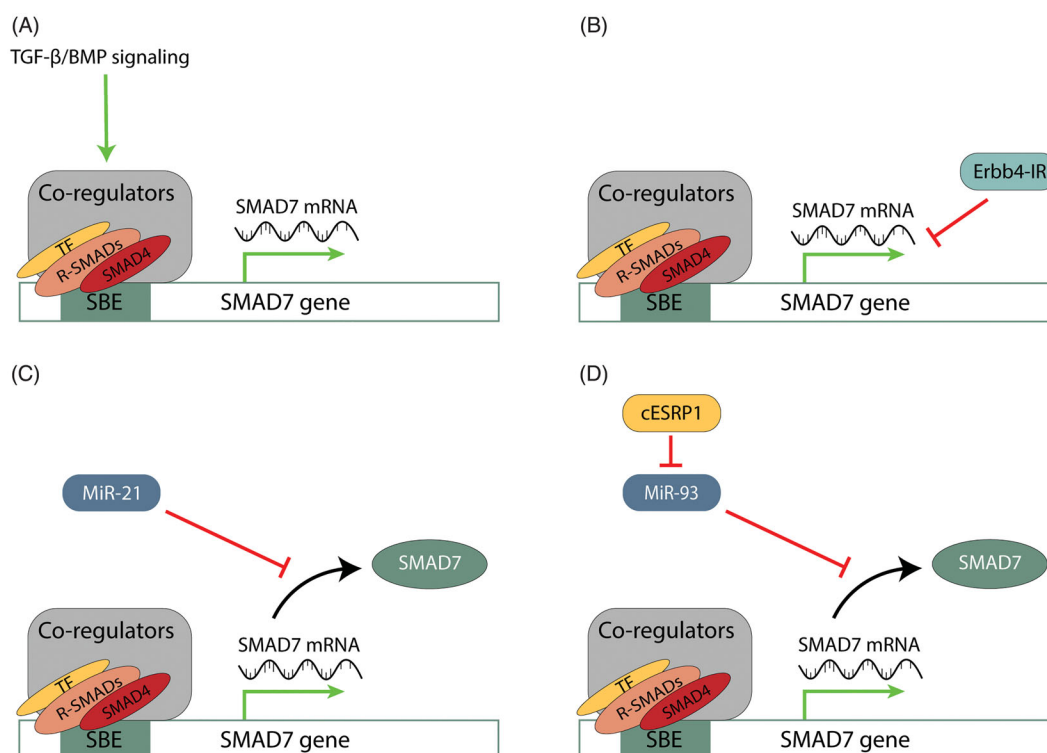
Figure 3. Examples of SMAD7 regulation on a transcriptional and post-transcriptional level. A. TGF-β and BMP signaling activation positively regulate SMAD7 transcription. The *SMAD7* promoter is a target for direct association with R- and Co-SMADs, stimulating promoter activity. B. Long non-coding RNA Erbb4-IR suppresses *SMAD7* transcription by binding to the 3′ UTR of the *SMAD7* gene. C. MiR-21 modulates SMAD7 expression by targeting mRNA for translational repression by pairing to sequence in the mRNA 3′-untranslated region (3′ UTR). D. Circular RNA cESRP1 sponges miR-93, a negative regulator of SMAD7, to inhibit TGF-β signaling. SBE, SMAD binding element; TF, transcription factor.

Regulation of SMAD7 expression and function

SMAD7 is differentially expressed across various tissues and is subject to tight regulation by a range of molecules and a number of regulatory mechanisms. In this section, we will discuss various mechanisms by which SMAD7 expression and function are controlled, including transcriptional, post-transcriptional and post-translational mechanisms (Figure 3).

Transcriptional regulation of SMAD7

SMAD7 mRNA expression is promoted by TGF-β family members (Nakao et al. 1997; Afrakhte et al. 1998). SMAD3 and SMAD4 directly associate with the *SMAD7* promoter, through its consensus SBE, stimulating promoter activity (Figure 3(A)) (Nagarajan et al. 1999; Von Gersdorff et al. 2000). As SMAD7 functions as a TGF-β signaling antagonist, it thereby participates in a negative feedback circuit.

Gene expression can also be modulated by controlling the accessibility of transcription factors to the corresponding gene through covalent histone modification (Strahl and Allis 2000). Histone methylation and acetylation are reversible and their effects are context

dependent. Histone deacetylases (HDACs) regulate the acetylation of histone proteins, for the purpose of gene expression control. TGF-β stimulation has been shown to increase histone H4 acetylation at the *SMAD7* promoter 3.2-fold. This increase corresponded with an induction of SMAD7 mRNA (Alliston et al. 2005). Corepressor Evi-1 and associated corepressor C-terminal-binding protein (CtBP) obstructed TGF-β-induced histone acetylation at the endogenous *SMAD7* promoter, mitigating SMAD7 mRNA expression. In a similar fashion, transcription co-factor Ski, in cooperation with protein arginine methyltransferase 5 (PRMT5) and HDAC3, is present at the *SMAD7* promoter and promotes SMAD7 transcriptional repression (Tabata et al. 2009), suggesting a role for PRMT5 and HDAC3 in maintaining the basal repressed state of SMAD7.

Noncoding RNA-mediated regulation of SMAD7 expression

SMAD7 expression is extensively regulated at the RNA level by noncoding RNAs (ncRNAs), such as microRNAs (miRNAs), circular RNAs (circRNAs) and long noncoding RNAs (lncRNAs). MiRNAs are small, endogenous non-

coding RNA molecules of approximately 22 nucleotides in length, involved in the regulation of gene expression (Bartel 2004; Ambros 2004). In the nucleus, RNA polymerase II or III transcribes primary miRNA (pri-miRNA). Subsequently, 70 nucleotide stem-loop precursor miRNAs (pre-miRNAs) are excised by the RNase III DROSHA microprocessor complex and exported to the cytoplasm (Kim et al. 2009). The cytoplasmic RNase III enzyme Dicer completes the processing of pre-miRNAs to form mature miRNAs. MiRNAs modulate expression by targeting mRNA for translational repression by pairing to sequences in the mRNA 3′-untranslated region (3′ UTR) in target mRNAs or inhibiting translation (Fabian et al. 2010). Hundreds of mammalian miRNAs have been identified as key post-transcriptional regulators in biological processes such as development, cell proliferation and cell death (Landgraf et al. 2007).

MiRNA processing is promoted by TGF-β and BMP signaling (Davis et al. 2010). Following ligand stimulation, R-SMADs associate with the RNA SMAD-binding element (R-SBE) of pri-miRNA transcripts in order to facilitate DROSHA-mediated miRNA maturation. This indicates that R-SMADs modulate gene expression by (A) transcriptional induction and (B) facilitating post-transcriptional processing of miRNAs. Here, we provide an overview of several key miRNAs that regulate SMAD7 expression, and discuss other examples of ncRNA-mediated SMAD7 regulation.

MiR-21

One of the most extensively studied miRNA regulating SMAD7 is human miR-21 (hsa-miR-21). Its role in regulating SMAD7 expression has mainly been examined in the context of disease. MiR-21 was first identified as a regulator of SMAD7 expression in liver fibrosis based on TargetScan predictions (Marquez et al. 2010). Marquez *et al.* established post-transcriptional regulation of SMAD7 by miR-21. They demonstrated direct interaction between miR-21 and the SMAD7 3′UTR using site-directed mutagenesis in the miR-21 seed sequence, which is the primary determinant of miRNA specificity (Marquez et al. 2010). Afterwards, it was confirmed that treatment with miR-21 precursor indeed leads to decreased SMAD7 protein levels in fibroblasts (Liu et al. 2010).

MiR-21 has been extensively studied in a range of diseases, such as cancer, cardiovascular disease, and pulmonary disease. In malignancies, miR-21 is a central player as an oncogenic miRNA (oncomiR) targeting tumor suppressor proteins, including for example PTEN (Feng and Tsao 2016). MiR-21 is upregulated in intraductal epithelial proliferations of breast tissue compared to healthy tissue (Chen et al. 2013). This overexpression correlates with decreased SMAD7 levels. MiR-21 knockdown restored SMAD7 mRNA levels in MCF-7 and Hs578T breast cancer cells. Likewise, SMAD7 mRNA expression was significantly increased in miR-21 knockout HeLa cells (Chen et al. 2015). Wang et al. demonstrated that SMAD7 is also a direct target for transactivated miR-21 in colorectal cancer (Wang et al. 2017). Similar results have been reported for coronary heart disease, autoimmune disease, diabetes and during wound healing (Li et al. 2015, 2018; Murugaiyan et al. 2015; Luo et al. 2017).

Chang et al. have demonstrated a divergent role for hsa-miR-21-5p in the regulation of SMAD7 in hypoxic conditions, compared to normoxic conditions. Here, 5p refers to the 5p strand that is located in the forward (5′-3′) position, whereas the 3p strand is present in the reverse position. In hypoxia, hsa-miR-21-5p expression increased protein expression of SMAD7 in human umbilical vein endothelial cells, whereas at normoxia hsa-miR-21-5p induces downregulation of SMAD7 in these cells, providing a prime example of the effect of context-dependency on functional output (Chang et al. 2017). In conclusion, the fact that SMAD7 is a confirmed target for miR-21 in a plethora of diseases highlights the importance of SMAD7 in maintaining appropriate TGF-β signaling.

SMAD7 regulation by diverse miRNAs

MiRNA-mediated regulation of SMAD7 is not limited to miR-21. The miR-15 family, consisting of six highly conserved miRNAs (miR-15a/b, miR-16, miR-195, miR-497, miR-322), inhibits TGF-β signaling in cardiovascular tissues (Tijsen et al. 2014). Knockdown of the miR-15 family resulted in upregulated SMAD7 mRNA levels and increased SMAD7 3′UTR reporter activity in cardiomyocytes. It remains unclear, however, whether these effects are due to direct interaction between miR-15 family members and SMAD7 3′UTR (Tijsen et al. 2014). Direct regulation of SMAD7 by miR-15b was demonstrated in osteogenic differentiation of bone marrow mesenchymal stem cells (BMSCs) (Fang et al. 2019).

Interestingly, distinct miRNA variants derived from the same precursor can affect *SMAD7* gene expression in different cell types. For example, from the miR-181 precursor four mature miRNAs can be produced: miR-181a, miR-181b, miR-181c or miR-181d, of which MiR-181a was found to induce repression of its functional target SMAD7 in ovarian cancer cells to promote TGF-β-mediated epithelial-to-mesenchymal transition (EMT) (Parikh et al. 2014), whereas MiR-181c downregulated SMAD7 expression in neuroblastoma and osteosarcoma

Table 1. MiRNAs regulating SMAD7 expression in the context of disease[a].

Conditions(s)	MiRNAs	Reference(s)
Cancer	MiR-10b, miR-15a, miR-18a, miR-20a, miR-21, miR-25, miR-32, miR-92a/b, miR-93, miR-106a, miR-106b ~25, miR-130b, miR-132, miR-181, miR-182, miR-185, miR-195, miR-216a, miR-324, miR-367, miR-424, miR-452, miR-497, miR-503, MiR-519d, miR-520g/h, miR-543, miR-663b, miR-1269, miR-2909, miR-4775	(Smith et al. 2012; Chen et al. 2013; Li et al. 2013; Xia et al. 2013; Chang et al. 2013; Li et al. 2014; Parikh et al. 2014; Yu et al. 2014; Chen et al. 2015; Kan et al. 2015; Liu et al. 2015; Tang et al. 2015; Zhu et al. 2015; Bu et al. 2015; Wang et al. 2015; Xu et al. 2015; Hu et al. 2016; Zhuang et al. 2016; Zhou et al. 2016; Liu et al. 2016; Wang et al. 2016; Duan and Chen 2016; Dai et al. 2016; Yu et al. 2016; Ratz et al. 2017; Tong et al. 2017; Yang et al. 2017; Zhao et al. 2017; Wang et al. 2017; Wang 2017; Ayub and Kaul 2017; Hujie et al. 2018; Wang et al. 2018; Wang et al. 2018; Zhang et al. 2018; Chen et al. 2018; Wang et al. 2018; Zhai et al. 2018; Fu et al. 2019; Huang et al. 2019; Shen et al. 2019; Hu et al. 2019; Zhu et al. 2019; Hsu et al. 2020; Li and Zeng 2020; Huang et al. 2020)
Fibrosis	MiR-10a, miR-16, miR-17, miR-21, miR-30, miR-33a, miR-93, miR-96, miR-150, miR-182, miR-195, miR-212, miR-216a, miR-411, miR-877	(Liu et al. 2010; Marquez et al. 2010; Huang et al. 2015; Tu et al. 2015; Yu et al. 2015; Zhu et al. 2015; Wang et al. 2016; Zhou et al. 2016; Song et al. 2017; Zhu et al. 2018; Luo et al. 2018; Che et al. 2020; Tao et al. 2019; Li et al. 2019; Hu et al. 2019; Wang et al. 2019; Gao et al. 2020; Chen et al. 2020)
Cardiovascular disease and repair	MiR-15a, miR-20b, miR-21, miR-92a, miR-125b, miR-195, miR-224, miR-410, miR-424, miR-484, miR-503	(Zhang et al. 2014; Tijsen et al. 2014; Li et al. 2015; Yang et al. 2017; Du et al. 2017; Liang et al. 2018; Wang et al. 2018; Gu et al. 2018; Rong et al. 2018; Zou et al. 2018; Xu et al. 2019; Liu et al. 2020; Pulignani et al. 2020)
Bone development and disease	MiR-15b, miR-16, miR-17, miR-33a, miR-200, miR-590, miR-877	(Jia et al. 2014; Kostopoulou et al. 2015; Vishal et al. 2017; Xu and Xu 2017; Fang et al. 2019; Chen et al. 2019; He et al. 2019; Song et al. 2020)
Muscle development, dysfunction and repair	MiR-125b, miR-542	(Garros et al. 2017; Song et al. 2018)
Eye disease	MiR-187, miR-3175	(Zhang et al. 2015; Zhong et al. 2020)
Chondrogenic differentiation	MiR-526b, miR-590	(Wu et al. 2018)
Pulmonary disease	MiR-15b, miR-195	(Ezzie et al. 2012; Zeng et al. 2018)
Allergy	MiR-17	(Huang et al. 2018)
Angiogenesis	MiR-21	(Luo et al. 2017; Chang et al. 2017)
Inflammatory bowel disease	MiR-195	(Chen et al. 2015)
Keloid formation	MiR-96	(Chao et al. 2019)
Autoimmune disease	MiR-21, miR-181	(Murugaiyan et al. 2015; Ghorbani et al. 2017; Zhang et al. 2018)
Metabolism	MiR-92a	(Zhang et al. 2019)
Kidney disease	MiR-92b, miR-541	(Wei et al. 2019; et al. 2020)
Wound healing	MiR-21	(Li et al. 2018)
Pancreatitis	MiR-216a	(Zhang et al. 2014)
Polycystic ovary syndrome	MiR-423	(Li et al. 2019)
Kawasaki disease	MiR-27b	(Rong et al. 2018)
ECM formation	MiR-590	(Liu et al. 2017)

[a]This table includes SMAD7-regulating miRNAs from *homo sapiens, mus musculus* and *rattus norvegicus*.

(Li et al. 2014; Fu et al. 2019). Additionally, miR-181 variants have been shown to regulate T cell phenotype in autoimmune neuroinflammation by directly targeting SMAD7 mRNA (Ghorbani et al. 2017; Zhang et al. 2018).

Many other miRNAs have been shown to regulate SMAD7 expression (Table 1). Of note, in many cases of miRNA-mediated SMAD7 regulation it remains unclear which variant governs the regulation, and whether the repression of SMAD7 expression occurs due to direct interaction with the SMAD7 3'UTR or through intermediary regulators, such as SMAD7 interacting proteins.

SMAD7 regulation by other types of ncRNAs

In addition to miRNA-mediated regulation, SMAD7 is subject to regulation by circRNAs, and lncRNAs.

CircRNAs, which result from backsplicing of pre-mRNAs, regulate the expression of protein-coding transcripts (Huang et al. 2017). CircRNAs can function as a sponge for miRNAs, obstructing their regulatory function. In TGF-β signaling, circRNA cESRP1 protects SMAD7 from downregulation by sponging miR-93-5p (Huang et al. 2020). Furthermore, Circ-LARP4 has been found to positively regulate SMAD7 expression in non-small-cell lung cancer (Shi et al. 2020).

Finally, lncRNAs have also been attributed with SMAD7 regulatory functions. For example, linc-SMAD7 increases SMAD7 expression by sponging SMAD7-targeting miR-125b (Song et al. 2018). Other instances in which lncRNAs were found to influence SMAD7 expression are summarized in Table 2.

Table 2. LncRNA that positively and negatively regulate SMAD7.

lncRNA	Effect on SMAD7 expression level	Mechanism	Reference(s)
Erbb4-IR	Down	Transcriptional repression of SMAD7	(Feng et al. 2018)
Linc-SMAD7	Up	MiRNA sponge for miR-125b	(Song et al. 2018)
COL1A2-AS1	Up	MiRNA sponge for miR-21	(Nong et al. 2018)
PRINS	Up	Unknown mechanism	(Jiao et al. 2019)
MEG3	Up	Unknown mechanism	(Xu and Xu 2017)
XIST	Up	Reciprocal repression between miR-92b and XIST.	(Zhuang et al. 2016)
HOTAIR	Up	Inhibition of miR-17-5p	(Wei et al. 2017)
Lnc-Ang362	Down	Transcriptional repression	(Chen et al. 2020)
SNHG6	Down	Binding UPF1, a negative regulator of SMAD7	(Chang et al. 2016)

Control of SMAD7 by post-translational modifications

SMAD7 is also known to be extensively regulated by post-translational modifications (PTMs) such as methylation, ubiquitination, acetylation and phosphorylation (Figure 4), which are discussed in more detail below (Xu et al. 2016).

Methylation

Methyltransferases are able to transfer methyl groups to histones, but are also able to target a variety of non-histone proteins, including SMAD7. Histone-lysine N-methyltransferase SET9 promotes TGF-β signaling by methylating SMAD7. This methylation of SMAD7 significantly increased the interaction with E3 ubiquitin ligase Arkadia (also termed RNF111), facilitating SMAD7 ubiquitination and proteasomal degradation (Elkouris et al. 2016). SMAD7 is also a substrate for PRMT1, another methyltransferase (Inamitsu et al. 2006; Katsuno et al. 2018). Arg-57 and Arg-67 methylation of SMAD7 by PRMT1 led to lowered TβRI-binding efficiency and increased degradation of SMAD7. Taken together, these results indicate that methylation of the SMAD7 protein promotes its degradation.

Phosphorylation

In R-SMADs and SMAD4, the linker region is a target for phosphorylation by a large variety of serine/threonine kinases such as mitogen-activated protein kinases (MAPKs), extracellular-signal regulated kinase (ERK), Jun N-terminal kinase (JNK) and p38 kinase (p38) (Kamato et al. 2013). Linker region phosphorylation of R-SMADs regulates SMAD stability, activity and nuclear transport (Matsuzaki 2011). SMAD7 is subject to serine phosphorylation in the linker region, at S249 (Figure 4). Phosphorylation at this site does not inhibit SMAD7 regulatory function in TGF-β or BMP signaling, but does affect SMAD7-dependent transcriptional activity (Pulaski et al. 2001). Conversely, linker region

phosphorylation at S206, directly adjacent to the PY motif, increases binding affinity between SMAD7 and the WW4 domain of E3 ubiquitin ligase WWP2 (Wahl et al. 2019). Besides serine phosphorylation, SMAD7 can also become phosphorylated at T354 (Casado et al. 2013).

Ubiquitination

Ubiquitination (also called ubiquitylation) is a crucial regulator of protein stability. This reversible protein modification occurs through an enzymatic cascade performed in succession by ubiquitin-activating enzymes (E1s), ubiquitin-conjugating enzymes (E2s), and ubiquitin ligases (E3s). The key regulatory roles of E3 ligases in TGF-β signaling have been previously reviewed (Imamura et al. 2013). Here, we highlight two vital E3s that regulate SMAD7 stability. Arkadia is an E3 ligase that promotes TGF-β signaling by inducing polyubiquitination and degradation of SMAD7 (Koinuma et al. 2003). E3 ubiquitin ligase atrophin 1-interacting protein 4 (AIP4) (also termed ITCH) was shown to positively regulate TGF-β signaling via SMAD7 ubiquitination and subsequent degradation (Park et al. 2015). However, AIP4 is also able to affect SMAD7 function independent of its catalytic activity. A catalytic mutant of AIP4 stabilizes the SMAD7-TβRI complex, effectuating TGF-β signaling inhibition (Lallemand et al. 2005). Furthermore, in an extensive mass spectrometry experiment, SMAD7 was found to be ubiquitinated at K101. However, the functional effect of ubiquitination at this site remains unknown (Akimov et al. 2018).

An important SMAD7 function is its role as adaptor for E3 ligase SMURF2. SMAD7 recruits SMURF2 to the cell membrane in order to induce TβRI degradation by means of ubiquitin-mediated proteolysis (Kavsak et al. 2000). Conflicting results have been described on whether SMAD7 itself is a target for ubiquitination by SMURF2. Kavsak et al. showed increased SMAD7 degradation upon overexpression of SMURF2 (Kavsak et al. 2000). In contrast, Zhang et al. report physical interaction between SMURF2 and SMAD7 but no evidence
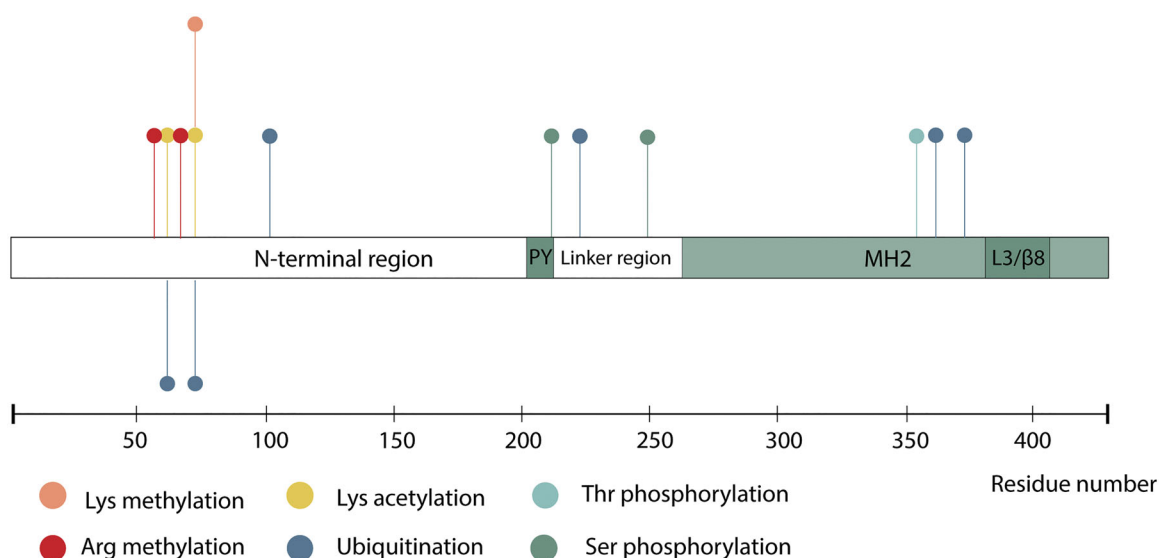
Figure 4. Schematic representation of SMAD7 including identified post-translational modifications. PY, proline-tyrosine motif; MH2, Mad homology 2 domain; L3/β8, L3/β8 loop-strand module.

for SMURF2-mediated ubiquitination and degradation of SMAD7 (Zhang et al. 2001). More recent reports, however, again suggest a role for SMURF2 in SMAD7 proteasomal degradation (Zhao et al. 2016; Liu et al. 2017).

Deubiquitination

Emerging players in the field of SMAD7 regulation are deubiquitinating enzymes (DUBs). In 2004, associated molecule with the SH3 domain of STAM (AMSH2) was discovered to interact with SMAD7 and to positively regulate TGF-β signaling (Ibarrola et al. 2004). Moreover, CYLD deubiquitinates SMAD7 at K360 and K374 and thereby negatively regulates TGF-β-induced TAK1 kinase activity in CD4+ T cells (Zhao et al. 2011). Ubiquitin-Specific Peptidase 26 (USP26) has been found to deubiquitinate and thereby stabilize SMAD7, resulting in reduced TGF-β signaling (Kit Leng Lui et al. 2017). Similarly, OTU Deubiquitinase 1 (OTUD1) cleaves polyubiquitin chains from SMAD7 K220, exposing the PY motif. This in turn enables SMURF2 binding and promotes receptor turnover (Zhang et al. 2017).

Acetylation

SMAD7 stability is regulated by acetylation and the consequential competition between this modification and ubiquitination (Grönroos et al. 2002). SMAD7 interaction with transcription coactivator and acetyltransferase p300 leads to its acetylation at two N-terminal lysines (K64 and K70), preventing ubiquitination at these sites. Additionally, HDAC1-mediated deacetylation of SMAD7

decreases SMAD7 stability by enhancing its ubiquitination and subsequent degradation in the proteasome (Simonsson et al. 2005). In accordance with these findings, Monteleone et al. demonstrated that SMAD7 is not transcriptionally regulated in the human gut. Rather, SMAD7 is post-translationally stabilized by p300-mediated acetylation (Monteleone et al. 2005). Taken together, these results suggest that SMAD7 stability is regulated by acetylation and ubiquitination in a mutually exclusive manner.

Protein interactions govern SMAD7 stability and activity

Another manner of SMAD7 regulation is via protein-protein interactions. E3 ubiquitin ligase SMURF1 directly binds SMAD7 and facilitates cytoplasmic localization of SMAD7, targeting SMAD7 to the plasma membrane through its N-terminal C2 domain. This enables negative regulation of TGF-β signaling independent of its catalytic function (Suzuki et al. 2002). SMAD7 can also be regulated by direct interaction with other proteins, such as Vpr binding protein (VprBP) and REGγ. VprBP was found to interact with SMAD7 to promote SMAD7-SMURF1-TβRI complex formation (Y et al. 2020). Conversely, REGγ, a member of the 11S proteasome activators, binds to SMAD7 in order to promote its turnover (Jiao et al. 2020a). The microtubule actin crosslinking factor 1 (MACF1), a protein highly abundant in bone tissue, has been identified as a SMAD7 interaction partner in mesenchymal stem cells. MACF1 facilitates SMAD7 nuclear translocation to promote osteogenic differentiation (Zhao et al. 2020).
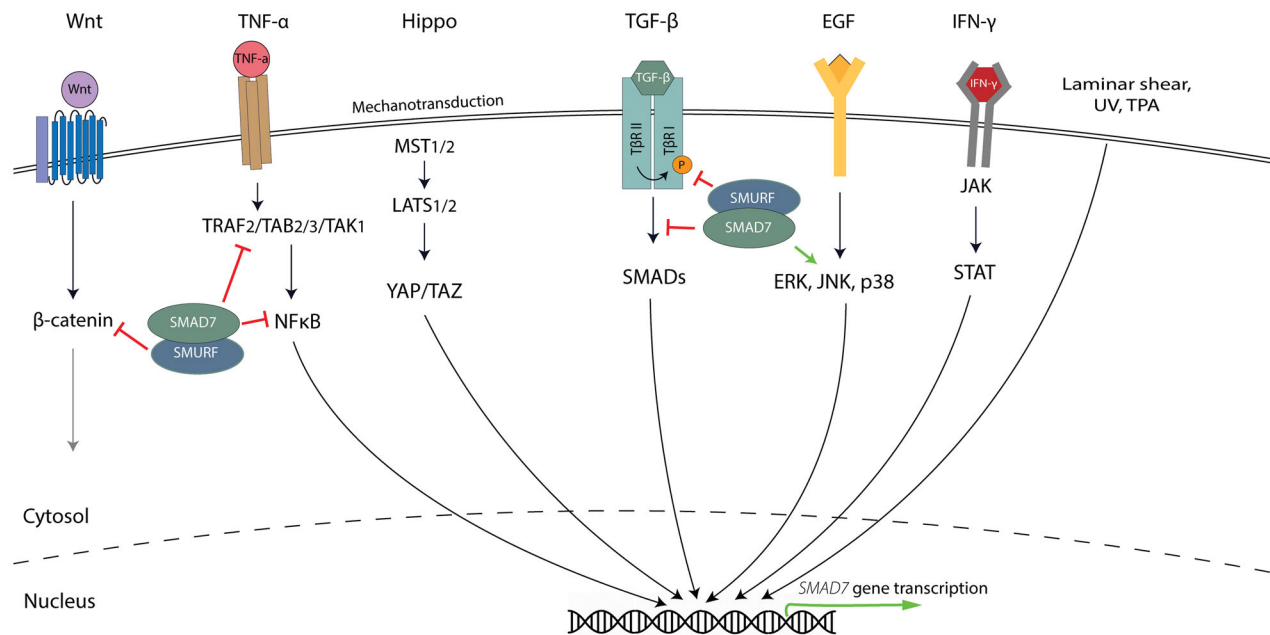
Figure 5. SMAD7 is regulated by and regulates various signaling pathways. In the Wnt signaling pathway, SMAD7 regulates the activity and stability of β-catenin by functioning as a SMURF2 adaptor. Similarly, SMAD7 can affect NFκB signaling by inhibiting phosphorylation and degradation of IκB and disrupting recruitment of the TAK1 complex to TRAF2. SMAD7 expression can be positively regulated by inflammatory cytokines such as TNF-α and IFN-γ, and YAP/TAZ, effectors in the Hippo signaling pathway. Lastly, SMAD7 expression is positively regulated by laminar shear stress, UV light, TPA and EGF. SMAD7 is also important for JNK and p38 activation. ERK, Extracellular-signal-regulated kinase; JAK, Janus kinase; JNK, c-Jun N-terminal kinase; NFκB, Nuclear factor kappa B; STAT, Signal transducer and activator of transcription; TAK, TGF-β-activated kinase; TAZ, Tafazzin; TRAF, TNF receptor-associated factor; YAP, Yes-associated protein.

As discussed, SMAD7 requires interaction with other proteins in order to successfully antagonize TGF-β and BMP signaling. For instance, SMAD7 recruits E3 ubiquitin ligases, such as NEDD4L and SMURF1/2 to induce receptor polyubiquitination and degradative endocytosis (Kavsak et al. 2000; Ebisawa et al. 2001; Kuratomi et al. 2005). SMAD7 activity can, therefore, be regulated by competition for its binding partners. For example, SMURF2 establishes mutually exclusive complexes with SMAD7 or RNF11 (Malonis et al. 2017). Accordingly, RNF11 regulates SMAD7 activity by sequestering SMURF2 at the cell membrane. Moreover, c-SRC-mediated phosphorylation of SMURF2 at W14 and W434 was found to reduce SMAD7 binding, enhance intramolecular interactions within SMURF2, and result in inhibition of SMURF2 activity (Sim et al. 2019).

In addition to being a target for deubiquitination, several DUBs interact with SMAD7 to regulate the TGF-β signaling pathway (Herhaus and Sapkota 2014). Both USP11 and USP15 are able to bind to SMAD7 to deubiquitinate and stabilize the TβRI, enhancing signaling TGF-β signaling (Al-Salihi et al. 2012; Eichhorn et al. 2012). Moreover, SMAD7 recruits ubiquitin C-terminal hydrolase 37 (UCH37) to antagonize the SMURF-mediated ubiquitination and subsequent degradation of the

TβRI (Wicks et al. 2005; Cutts et al. 2011). Taken together, these studies indicate that DUBs can counteract SMAD7 function at the receptor level.

In conclusion, SMAD7 functioning is in part dependent on interaction with interaction partners, such as SMURF1/2 and is affected by competition for and modification of these interaction partners.

SMAD7 as signaling integrator

At any given moment, cells receive a multitude of signals simultaneously that are integrated to determine cell shape and function. A single pathway is often connected at different steps with other pathways, resulting in complicated signaling networks that allow for appropriate spatio-temporal responses. SMAD7 is an important regulatory TGF-β signaling component by which signaling crosstalk is achieved. Initially identified in endothelial cells as a shear stress-inducible gene (Topper et al. 1997), SMAD7 is a potent antagonist of TGF-β and BMP signaling. SMAD7 expression is induced by TGF-β and BMP stimulation, and thereby participates in a negative feedback loop controlling the duration and intensity of the TGF-β family signaling response (Nakao et al. 1997). Efficient induction of SMAD7 by

TGF-β, however, depends on various transcription factors and cofactors, such as activator protein 1 (AP-1), stimulating protein-1 (Sp1), transcription factor mE3 (TFE-3) , activating transcription factor 2 (ATF2), CBP/ p300 and Forkhead box H1 (FOXH1) (Brodin et al. 2000; Hua et al. 2000; Uchida et al. 2001; Jungert et al. 2006; Gohla et al. 2008). Many of these factors are regulated by other signaling cascades besides TGF-β as well. Moreover, SMAD7 expression can be induced by epidermal growth factor (EGF), human epidermal growth factor receptor 2 (HER2/Neu), UV irradiation, and TPA (Afrakhte et al. 1998; Quan et al. 2001; Dowdy et al. 2003; Tsunobuchi et al. 2004). Thus, this suggests a role for SMAD7 as integrator and crosstalk mediator between TGF-β signaling and other signaling pathways. Here, we provide a comprehensive overview of SMAD7 as a signaling integrator.

EGFR signaling and the MAPK/ERK pathways

Crosstalk between TGF-β and other signaling pathways was originally demonstrated by the discovery that EGF increased the expression of SMAD7 mRNA (Afrakhte et al. 1998). The EGF receptor (EGFR) belongs to the receptor tyrosine kinase (RTK) family and is activated by various ligands, including EGF and TGF-α. Luwor et al. reported that EGFR-induced desensitization to TGF-β is dependent on signal transducer and activator of transcription (STAT)3-mediated *SMAD7* promoter activation (Luwor et al. 2013). On the other hand, SMAD7 regulates EGF signaling as well. SMAD7 sequesters E3 ubiquitin ligase c-Cbl, thereby inhibiting ligand-induced EGFR ubiquitination and proteasomal degradation in human HaCaT keratinocytes (Ha Thi et al. 2015). Similarly, SMAD7 overexpression stimulates EGFR-signaling activation and its downstream signaling in skin carcinogenesis (Ha Thi et al. 2019).

Mitogen-activated protein kinases (MAPKs), including ERK1/2, JNK1/2/3, and p38/MAPKs, are serine–threonine kinases and essential mediators of intracellular signaling events involved in regulating cellular processes related to proliferation, differentiation, survival, and death in response to a diverse array of stimuli. Extracellular stimuli, such as EGF, can prompt a phosphorylation cascade from MAP kinase kinase kinase (MAPKKK) to MAP kinase kinase (MAPKK), and ultimately MAPK (Pearson et al. 2001). ERK1/2, JNK1/2/3, and p38 MAPK have all been implicated in the transcriptional regulation of SMAD7 (Figure 5) (Brodin et al. 2000; Uchida et al. 2001; Dowdy et al. 2003). In turn, SMAD7 itself regulates MAPK signaling. Transient and stable SMAD7 expression leads to sustained JNK activation, potentiating apoptosis

(Mazars et al. 2001). Moreover, SMAD7 expression is critical for TGF-β-induced activation of MKK3 and p38 kinases (Edlund et al. 2003). Thus, SMAD7 expression is regulated by EGFR and all MAPK branches and, in turn, SMAD7 influences EGFR and MAPK signaling.

The IL, TNF, and IFN-γ pathways

Interleukines (ILs), tumor necrosis factor-alpha (TNF-α), and interferon-gamma (IFN-γ) are secreted factors that regulate important immune responses, by signaling through the NF-κB and just another kinase (JAK)/STAT pathways in receiving cells. Upon IL- or IFN-γ receptor binding, JAK becomes activated by transphosphorylation and in turn phosphorylates STAT proteins on tyrosine residues. Subsequently, STAT proteins disassociate from the receptor, dimerize and translocate into the nucleus (Rawlings et al. 2004). TNFα can trigger activation of NF-κB proteins, including p50, p52, RelA/p65, and RelB, by inducing degradation of the IκB protein. After entering the nucleus, these activated NF-κB proteins function as transcription factors (Figure 5) (Napetschnig and Wu 2013).

Crosstalk between SMAD7 and the IFN-γ/STAT pathway

The TGF-β and IFN-γ signaling pathways often have distinct opposite effects and reciprocally inhibit each other, for example in the regulation of inflammatory responses. Through JAK1 and STAT1, IFN-γ induces the expression of SMAD7, thereby preventing the interaction of SMAD3 with the TβRI (Ulloa et al. 1999). Further investigation of IFN-γ-mediated reduction of TGF-β signaling showed that Y-box protein-1 (YB-1) activation by JAK1 is necessary for IFN-γ-mediated upregulation of SMAD7 (Dooley et al. 2006). YB-1 binds directly to a recognition site in the *SMAD7* promoter to induce transcription. Moreover, IL-6 treatment results in STAT3-dependent transcriptional induction of *SMAD7* in gp130$^{Y757F/Y757F}$ mice (Jenkins et al. 2005).

Crosstalk between SMAD7 and TNF-α/NF-κB signaling

The proinflammatory cytokines TNF-α and IL-1β induce SMAD7 expression in an NF-κB/RelA-dependent manner in mouse fibroblasts (Bitzer et al. 2000). However, activation of NF-κB by TNF-α stimulation was shown to inhibit the *SMAD7* promoter in HEK293 cells. This inhibition could be reversed by overexpression of the transcription co-activator p300, indicating that NF-κB regulates SMAD7 expression by competition for the common transcription coactivators (Nagarajan et al.

2000). How SMAD7 transcription is modulated by NF-κB might therefore be dependent on the cellular context. Transmembrane protein CD40, member of the TNF-receptor superfamily, promotes SMAD7 expression through the NF-κB pathway (Patil et al. 2000). Conversely, SMAD7 has been reported to have an anti-inflammatory effect by affecting NF-κB signaling. SMAD7 could promote expression of IκB, a specific inhibitor of NF-κB (Wang et al. 2005). Similarly, in colon cancer cells, SMAD7 can inhibit the phosphorylation and degradation of IκB (Grau et al. 2006). Another mechanism through which SMAD7 can regulate NF-κB signaling is by formation of SMAD7-TGF-β-activated kinase (TAK1)-binding protein (TAB)2 and SMAD7-TAB3 complexes, disrupting recruitment of the TAK1 complex to TNF receptor–associated factor 2 (TRAF2), and thereby blocking propagation of proinflammatory signals (Figure 5; Hong et al. 2007).

Wnt signaling

The Wnt signaling pathway initiates when a Wnt-protein ligand binds to a Frizzled family receptor, triggering intracellular signaling cascades via the Disheveled protein. Canonical Wnt signaling leads to transcriptional regulation of target genes via the nuclear translocation of β-catenin, an essential mediator of this pathway (Clevers and Nusse 2012). Edlund et al. demonstrated physical interaction between SMAD7 and β-catenin (Edlund et al. 2005). Additionally, SMAD7 has been shown to bind β-catenin and thereby promote its degradation through SMURF2-mediated ubiquitination (Han et al. 2006). Axin, a key component of the canonical Wnt pathway, suppresses Wnt signal transduction by promoting β-catenin degradation. SMAD7 can bind to Axin in order to stabilize β-catenin (Tang et al. 2008). Conversely, Axin can function as a scaffold protein in TGF-β signaling by formation of a multimeric complex with SMAD7 and Arkadia. This promotes SMAD7 turnover via proteasomal degradation (Liu et al. 2006). Lastly, Yanagida et al. found that supplementing exogenous Dickkopf-related protein (Dkk), a Wnt antagonist, increased SMAD7 expression in a liver fibrosis mice model (Yanagida et al. 2011). These results indicate an intimate relationship between SMAD7 and Wnt/β-catenin signaling.

Hippo signaling

The evolutionarily conserved Hippo pathway is characterized by a kinase cascade, where, in mammals, Macrophage-stimulating protein (MST)1 and 2 and scaffold protein SAV1 form a complex in order to phosphorylate and activate Large tumor suppressor kinase 1 (LATS)1 and 2. In turn, LATS1 and 2 phosphorylate and inhibit two important downstream effectors of the Hippo pathway; Yes-associated protein (YAP) and Tafazzin (TAZ) (Meng et al. 2016). YAP is able to enhance TGF-β signaling inhibition by facilitating recruitment of SMAD7 to the TβRI (Ferrigno et al. 2002). In human skin dermal fibroblasts, YAP/TAZ have been found to inhibit TGF-β signaling by promoting SMAD7 expression via transcription factor AP-1 (Qin et al. 2018). Moreover, loss of tumor suppressor Merlin, an upstream activator of the Hippo signaling pathway, leads to unrestrained YAP/TAZ activity and is concordant with decrease of SMAD7 expression (Mota et al. 2018).

Notch signaling

The Notch signaling pathway is important for regulation of cell differentiation, proliferation, and cell death. The Notch receptor is a cell-surface receptor that transduces signals by interacting with transmembrane ligands, such as Delta-like and Jagged, on juxtaposed cells (Siebel and Lendahl 2017). Tsai et al. determined that SMAD7 is a Notch signaling target in limbal epithelial stem cells, and propose that it may facilitate the function of Notch in mitigating EMT (Tsai et al. 2014). Additionally, Notch signaling activation was found to be essential for maintaining vascular endothelial growth factor (VEGF)165-induced SMAD7 expression in endometrial stromal cells (Lv et al. 2019).

The ATM-dependent DNA damage signaling pathway

As with ultraviolet (UV) light, radiation therapy can induce SMAD7 mRNA (Kruse et al. 2009). Upon TGF-β stimulation, SMAD7 is able to affect the ataxia-telangiectasia mutated (ATM)-dependent DNA damage pathway by acting as a scaffold protein to facilitate interaction between p38 MAPK, ATM and p53, thereby promoting their activation. SMAD7 was found to colocalize with H2A histone family member X (γH2AX) in DNA damage foci. Moreover, SMAD7 is necessary for proper TGF-β induced cell cycle arrest (Zhang et al. 2006). Consistent with these results, Park et al. demonstrated that SMAD7 promotes the interaction between ATM and Nibrin (NBN, also termed NBS1), enhancing DNA repair (Park et al. 2015). Furthermore, SMAD7 localizes to irradiation-induced double strand breaks (Wang et al. 2013). These results indicate a role for SMAD7 in facilitating DNA damage repair.

In summary, SMAD7 is subject to regulation by a myriad of pathways, integrating information from many extracellular stimuli (Figure 5). Accordingly, targeting SMAD7 for therapeutic intervention might produce undesirable outcomes in patients, because of the high degree of connectivity between pathways and the integral role of SMAD7 in a multitude of ways.

Physiological roles of SMAD7 in health and disease

Since the discovery of SMAD7, much research has been conducted to uncover its biological functions in the context of human diseases. In this section, we discuss what genetic studies, in mice and humans (if available), and recent biochemical studies have revealed about the physiological role of SMAD7 in fibrosis, cancer, and inflammatory bowel disease. We also discuss opportunities for therapeutic intervention by targeting SMAD7.

Fibrosis

Genetic studies in mice

TGF-β signaling is an important signaling pathway in tissue fibrosis (Hu et al. 2018). Across numerous fibrosis models, expression or disruption of SMAD7 resulted in reduced or enhanced fibrosis in mice respectively (Nakao et al. 1999; Dooley et al. 2008; Hamzavi et al. 2008; Chung et al. 2009; He et al. 2009). Pancreatic fibrosis is a characteristic hallmark of chronic pancreatitis. Upon induction of chronic pancreatitis, mice with a deficiency in exon I of SMAD7 (SMAD7ΔE1) showed a more severe response characterized by an enhanced accumulation of extracellular matrix (ECM), an increased number of mesenchymal cells, and enhanced infiltration of inflammatory cells (Li et al. 2016). Taken together, these mice studies suggest a protective function of SMAD7 in fibrosis in various organs through inhibition of profibrogenic TGF-β signaling.

Human biological studies

Recent research using human endometrial tissue supports the view of SMAD7 as a protective factor in fibrogenesis. It was reported that SMAD7 and Notch are essential downstream molecules for VEGF165 functioning in endometrial fibrosis. VEGF165 reverses TGFβ1-stimulated fibrotic gene expression. However, this protective effect is SMAD7-dependent (Lv et al. 2019). Collectively, these recent studies provide evidence for the perceived protective role for SMAD7 in fibrotic disease.

Cancer

Genetic studies in mice

The role of SMAD7 in cancer growth and progression has been previously reviewed (Stolfi et al. 2013). TGF-β signaling has a dual role in tumor initiation and progression. In early stages, TGF-β signaling functions as a tumor suppressor. However, in later stages aberrant TGF-β signaling can facilitate EMT, migration, invasion and metastasis (Massagué 2008). In a mouse model for squamous cell carcinoma (SCC), SMAD7 enhanced keratin 8, a marker of malignant conversion (Liu et al. 2003). Contrarily, transgenic expression of SMAD7 was found to reduce 12-O-tetradecanoylphorbol-13-acetate (TPA)-induced hyperplasia in mouse skin (Hong et al. 2007). 1205Lu melanoma cells with stable SMAD7 overexpression showed diminished ability to form tumors by xenograft experiment in nude mice (Javelaud et al. 2005). Moreover, these mice demonstrated prolonged survival and significantly less bone metastasis (Javelaud et al. 2007). Additionally, SMAD7wt transgenic mice showed significantly greater papilloma formation, compared to control and SMAD7mut mice (Ha Thi et al. 2019), while conditional SMAD7 deletion led to highly proliferative and invasive cells and sustained melanoma growth and metastasis (Tuncer et al. 2019). Taken together, these contrary results highlight the complicated, context-dependent role of both SMAD7 and the TGF-β pathway in cancer.

SMAD7 overexpression has been shown to inhibit carcinogenesis and metastasis under certain conditions. In murine osteosarcoma and breast cancer models, SMAD7 overexpression resulted in a decrease in metastasis and longer survival, compared to control mice (Azuma et al. 2005; Lamora et al. 2014). Wang et al. showed that SMAD7 KO mice were more susceptible to N-nitrosodiethylamine (DEN)-induced hepatocellular carcinoma (HCC) than their wild-type counterparts (Wang et al. 2013). Adenoviral overexpression of SMAD7 in SCC cells inhibited growth of xenografts in severe combined immunodeficient (SCID) mice (Leivonen et al. 2006). Similarly, SMAD7 expression in CD4$^+$ T cells was found to prevent colitis-associated colorectal cancer due to increased expression of IFNγ (Rizzo et al. 2011). Even independent of chronic inflammation, T-cell-specific overexpression of SMAD7-protected mice against tumor development (Rizzo et al. 2014).

Conversely, SMAD7 is also able to promote cancer initiation and progression. It has been reported that SMAD7 overexpression in colon adenocarcinoma cells induces tumorigenicity (Halder et al. 2005). Knockdown of SMAD7 using antisense oligonucleotide resulted in

reduced tumor growth in RAG1-/- mice (Stolfi et al. 2014). Moreover, SMAD7 promotes hepatic metastasis in CRC (Halder et al. 2008). Transgenic pancreas-specific expression of SMAD7 induced premalignant ductal lesions in mice (Kuang et al. 2006). Hepatocyte specific deletion of SMAD7 revealed that SMAD7 inhibits IL-6 production in hepatocellular carcinoma, thereby decreasing STAT3 signaling and accelerating tumor growth (Feng et al. 2017). In bronchiolar exocrine cells, present in the mouse airway, constitutively expressed SMAD7 increased incidence of urethane-induced lung cancer (Luo et al. 2010). Altogether, these studies illustrate a complex, multifaceted role of SMAD7 in tumorigenesis.

Genetic studies in humans

In 2007, researchers first demonstrated the importance of SMAD7 in colorectal cancer (CRC) in a genome-wide association study (GWAS) (Broderick et al. 2007). They showed significant association between single nucleotide polymorphism (SNP) rs4939827 of SMAD7 and CRC. Two other SNPs (rs4464148 and rs12953717) were shown to confer modest susceptibility to CRC (Tenesa et al. 2008). In 2014, Fortini et al. discovered four SMAD7 NSPs (rs6507874, rs6507875, rs8085824, and rs58920878) that were in linkage disequilibrium with rs4939827 (Fortini et al. 2014). In normal colon tissues, the SNP rs8085824 C (minor) allele was found to correlate with increased SMAD7 expression. The discovery of these SNPs sparked a new line of investigation into the pathogenesis of CRC, however the functional significance of *SMAD7* variants is not yet clear and provides avenues for future research.

Human biological studies

As in *in vivo* experiments, *in vitro* research demonstrates a dual role for SMAD7 in cancer initiation and progression. SMAD7 is commonly upregulated in CRC tissue compared to healthy adjacent tissue and SMAD7 inhibition causes a block in the S phase of the cell cycle, resulting in reduced CRC cell growth *in vitro* (Stolfi et al. 2014). It was discovered that SMAD7 inhibition promotes phosphorylation and activation of the eukaryotic Initiation Factor 2α (eIF2α) pathway via serine–threonine protein kinase RNA (PKR), leading to enhanced cell death (De Simone et al. 2017).

Conversely, Krüppel-like factor 4 (KLF4) overexpression inhibits TGF-β-induced cell migration and invasion by enhancing SMAD7 transcription in HCC cells (Sun et al. 2017). In glioblastoma (GBM), HECT-domain ubiquitin ligase HERC3 induces ubiquitination-mediated degradation of SMAD7, resulting in TGF-β pathway activation. HERC3-induced downregulation of SMAD7 potentiated TGF-β-induced EMT and was linked to temozolomide chemoradiotherapy resistance in GBM cells (Li et al. 2019). Furthermore, it has been reported that REGγ, the gamma subunit of the 11S regulator, regulates EMT, by mediating ubiquitin-independent degradation of SMAD7 in human lung cancer cells (Tong et al. 2020). Moreover, REGγ has been found to mediate anaplastic thyroid cancer (ATC)-associated dedifferentiation via SMAD7 degradation (Jiao et al. 2020b).

Taken together, these studies suggest that SMAD7 is critical in several cancer hallmarks such as cell death resistance, EMT and altered cellular identity, as well as drug resistance and provide rationale for use of TGF-β inhibitors in (late stage) cancer treatment. However, careful patient selection is warranted in order to ensure the maximum benefit and limit adverse effects.

Inflammatory bowel disease (IBD)

Genetic studies in mice

Inflammatory bowel disease (IBD) is characterized by aberrant TGF-β signaling (Ihara et al. 2017). High expression of SMAD7 in CD4^{+} T Cells was associated with severe colitis. *Recombination activating gene 1* (RAG1)-/- mice reconstituted with SMAD7tg cells displayed more pronounced mucosa hyperplasia and drastic loss of goblet cells (Fantini et al. 2009). Moreover, transfer of SMAD7-overexpressing T cells was inversely correlated with aryl hydrocarbon receptor (AhR) expression and IL-22 production (Monteleone et al. 2016). Recently, a study reported that a short splice form of CYLD (sCYLD) mediates K63-linked ubiquitination and nuclear translocation of SMAD7 in CD4^{+} T cells, inhibiting TGF-β signaling. Furthermore, regulatory T (Treg) cells from sCYLD^{+}/SMAD7^{+} mice failed to prevent the induction of adoptive transfer induced colitis in RAG1^{-/-} mice (Tang et al. 2019). In addition, dendritic cell (DC)-specific deletion of SMAD7 enhances TGF-β responsiveness and expression of programmed death ligand (PDL)1/2, and promotes Treg differentiation, mediating intestinal inflammation in mice (Garo et al. 2019). Sedda et al. demonstrated a novel reciprocal regulatory mechanism between SMAD7 and class III NAD+-dependent deacetylase SIRT1 in the gut using a SMAD7 transgenic mouse model (Sedda et al. 2018), adding further nuance to the regulation of SMAD7 in IBD.

SMAD7 antisense oligonucleotide (AON) treatment in inflammatory bowel disease

For the better part of two decades, the scientific community has followed the development of AON

treatment in various diseases, including IBD (Scarozza et al. 2019). It was reported that SMAD7 AON treatment could potentially restore the endogenous TGF-β signaling pathway and attenuate IBD (Boirivant et al. 2006). In 2014, Celgene Corporation acquired Mongersen (GED-301), a SMAD7 specific AON-containing medication, from Nogra Pharma as a late stage candidate in CD. However, despite encouraging results in pre-clinical and early clinical trials, the phase III clinical trial was prematurely terminated due to lack of efficacy (Ardizzone et al. 2016; Sands et al. 2019). Interestingly, early clinical trials were designed without a control arm (Monteleone et al. 2012; Zorzi et al. 2012; Feagan et al. 2018). However, as the placebo response rate in induction and maintenance trials in CD trials were reported at 28% (95% CI 24–32%) and 26% (95% CI 24–32%) respectively, this demonstrates that it is crucial to establish appropriate controls to exclude placebo effect (Jairath et al. 2017). As CD is a relapsing-remitting disease, it is much more difficult to study than continuous and progressive inflammatory disease. Additionally, between phase II and phase III, there were several notable differences (Bewtra and Lichtenstein 2020). Patients in the phase-II study were enrolled from select Italian centers, and therefore, there is a risk that those results might not translate into favorable outcomes in phase III by enrolling a more heterogeneous population of patients from 34 countries. Moreover, the discrepancy in study results between the two trials, despite their similar primary endpoints, could be due to distinct inclusion criteria. Ultimately, these disappointing results serve to emphasize the importance of careful trial design and nuance the perspective of SMAD7 as a drug target in IBD.

Conclusion and perspectives

TGF-β signaling is involved in cell proliferation, differentiation, survival and death across all metazoans. SMAD7, an I-SMAD, negatively regulates TGF-β and BMP signaling through various mechanisms. Similar to R- and Co-SMADs, SMAD7 contains a conserved MH2 domain, enabling type I receptor and R-SMAD interaction. Conversely, its N-terminal region shares limited similarity with R- and Co-SMADs. Of note, the SMAD7 PY motif is critically important to mediate type-I receptor degradation. Overall, it may be said that the structure of SMAD7 underpins its functional divergence, and allows for efficient inhibition of TGF-β and BMP signaling. SMAD7 is regulated at the transcriptional, post-transcriptional and post-translational level, and is subject to a number of PTMs. As the functional relevance of these

PTMs is often unclear, this provides avenues for future research. Recently, there has been a marked increase in research addressing the regulation of SMAD7 by ncRNAs. However, how these ncRNAs regulate SMAD7 expression, whether through direct interaction or regulation of an intermediary protein often remains unknown. In addition to its role in TGF-β and BMP signaling, SMAD7 is an important integrator and crosstalk mediator between TGF-β signaling and other signaling pathways, such as MAPK signaling. Since SMAD7 can affect many cellular processes, both by antagonizing TGF-β signaling and by facilitating crosstalk with other signaling pathways, it may be interesting, for instance, to investigate the SMAD7 interactome and PTMs in different cell types. This could lead to a more in depth understanding of how such signaling diversity can be accomplished by SMAD7. This inhibitory SMAD plays an integral role in health and disease and is, among others, important in cancer, fibrosis and IBD. Nonetheless, results from recent clinical trials have painted a more nuanced view of the future of SMAD7 targeting AON in CD.

Disclosure statement

The authors report no declarations of interest.

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