

Transforming growth factor- β signaling and ubiquitinators in cancer

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Abstract

Transforming growth factor- β (TGF- β) represents a large family of growth and differentiation factors that mobilize complex signaling networks to regulate cellular differentiation, proliferation, motility, adhesion, and apoptosis. TGF- β signaling is tightly regulated by multiple complex mechanisms, and its deregulation plays a key role in the progression of many forms of cancer. Upon ligand binding, TGF- β signals are transduced by Smad proteins, which in turn are tightly dependent on modulation by adaptor proteins such as embryonic liver fodrin, Smad anchor for receptor activation, filamin, and crkl. A further layer of regulation is imposed by ubiquitin-mediated targeting and proteasomal degradation of specific components of the TGF- β signaling pathway. This review focuses on the ubiquitinators that regulate TGF- β signaling and the association of these ubiquitin ligases with various forms of cancer. Delineating the role of ubiquitinators in the TGF- β signaling pathway could yield powerful novel therapeutic targets for designing new cancer treatments.

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Introduction

Transforming growth factor- β (TGF- β) signaling

TGF- β represents a large family of growth and differentiation factors that include activin/inhibins and bone morphogenetic proteins (BMPs; Moses & Serra 1996, Massague 1998, Sporn 1999, Hu *et al.* 2001, Derynck & Zhang 2003, Lange *et al.* 2004). These proteins mobilize complex signaling networks to regulate cellular differentiation, proliferation, motility, adhesion, and apoptosis. TGF- β signals are conveyed through types I and II serine/threonine kinase receptors to specific intracellular mediators known as Smad proteins (Kimchi *et al.* 1988, Chen *et al.* 1997*b*, Feng & Derynck 1997, Sporn 1999). Activation of the Smad signaling pathway results in nuclear translocation and activation of gene expression (Massague *et al.* 2000). Vertebrates possess at least nine Smad proteins (Moses & Serra 1996, Heldin *et al.* 1997, Sporn 1999, Massague *et al.* 2000, Derynck & Zhang 2003, Tang *et al.* 2003*a*) falling into three functional classes: i) receptor-activated Smads (R-Smads): Smad1, Smad2, Smad3, Smad5, and Smad8; ii) co-mediator Smads: Smad4 and Smad 10; and

iii) inhibitory Smads (I-Smads): Smad6 and Smad7. Activation of the TGF- β receptor complex by ligand binding results in the phosphorylation of type I receptors by the serine/threonine kinase activity of the type II receptor. R-Smads, such as Smad2 and Smad3, act as direct substrates of specific activated type I receptors, and are phosphorylated on the last two serines at the carboxyl terminus within a highly conserved SXS motif (Moses & Serra 1996, Heldin *et al.* 1997, Sporn 1999, Massague *et al.* 2000, Hu *et al.* 2001, Weinstein *et al.* 2001, Derynck & Zhang 2003, Tang *et al.* 2003*b*, Lange *et al.* 2004). Regulation of R-Smads by the receptor kinase confers specificity in this system. Thus, Smad2 and Smad3 are substrates for activin and TGF- β receptors and mediate signaling by these ligands (Kimchi *et al.* 1988, Chen *et al.* 1993, Wrana *et al.* 1994, Feng & Derynck 1997), whereas Smad1, 5, and 8 are targets of BMP receptors and propagate BMP signals (Moses & Serra 1996, Heldin *et al.* 1997, Kretschmar *et al.* 1997). Once phosphorylated, R-Smads associate with the co-mediator Smad, Smad4, and the heteromeric complex then translocates into the nucleus (Abdollah *et al.* 1997, Heldin *et al.* 1997). In the nucleus, Smad complexes activate specific genes such as integrins and E-cadherin through

cooperative interactions with other DNA-binding and coactivator (or co-repressor) proteins.

Smad proteins consist of two globular Mad homology (MH) domains separated by a flexible linker region. In the basal state, MH1 and MH2 domains are responsible for intrinsic, reciprocal inhibition. MH1 domain inhibition of Smad function is relieved by agonist-induced phosphorylation of the SSXS motif (Massague 1998). The MH2 domains are responsible for homomeric and heteromeric interactions between Smads (Janknecht *et al.* 1998). Additionally, the MH2 domain is involved in protein–protein interactions such as those between Smad2 and the winged-helix transcription factor fork-head activin signal transducer-1 (FAST-1) (Labbe *et al.* 1998, Germain *et al.* 2000). Similar interactions occur between Smad3 and the transcriptional co-activator CREB binding protein (CBP)/p300. TGF- β -mediated transactivation of the cyclin-dependent kinase (cdk) inhibitors p21 (Moustakas & Kardassis 1998) and p15INK4b (Li *et al.* 1995) involves the Sp1 transcriptional regulator (SP1), whereas TGF- β induction of the plasminogen activator inhibitor (PAI-1) and human collagenase promoters involves transcriptional regulators such as activator protein 1 (AP1) (Zhang *et al.* 1998). In *Xenopus laevis*, FAST-1 binds a hexanucleotide repeat motif within the activin response element (ARE) of the activin-inducible *Mix.2* promoter (Chen *et al.* 1997a). In response to activin stimulation, FAST-1 becomes part of a larger ARE-bound complex known as the activin response factor (ARF) that also contains Smad2 and Smad4. Smad2 interacts directly with FAST-1 recruiting Smad4 to the complex. Similarly, in both *X. laevis* and zebrafish, the activin-inducible promoter of the LIM-homeodomain transcriptional regulatory gene, *lim-1*, is activated by an ARF that contains FAST-1, Smad2, and Smad4 (Watanabe *et al.* 2002). This interaction is significant because FAST-1 is involved in primary DNA-binding activity and Smad4 stabilizes DNA binding through its MH1 domain and activates transcription via its MH2 domain (Zhou *et al.* 1998). A functional interaction between SP1 and a Smad3–Smad4 complex has also been illustrated in TGF- β -mediated activation of the p21 promoter (Koutsodontis *et al.* 2002).

Direct DNA binding by the MH1 domain has also been demonstrated in *Drosophila melanogaster*, where the MH1 domain of Mothers of Decapentaplegic (Dpp; Mad), the Smad ortholog, is necessary and sufficient for binding to the vestigial (*vg*) ‘quadrant’ enhancer (Kim *et al.* 1997). Similarly, Mad binds to the Dpp response element in the Ultrabithorax (*ubx*) promoter via its MH1 domain. Additionally, in mammals, direct interaction of Smad3 and Smad4 with three CAGA-box repeats in the TGF- β responsive PAI-1 promoter requires the MH1

domains, as well as agonist stimulation or MH2 domain deletion. Termination of Smad-mediated transactivation by oncoproteins, such as Ski (Smad3) and SnoN (Smad4), as well as Ras, results in negative feedback regulation of TGF- β signaling (Kretzschmar *et al.* 1999, Stroschein *et al.* 1999, Sun *et al.* 1999).

Extrinsic regulatory pathways such as the Erk, c-Jun N-terminal kinase (JNK), and p38 mitogen-activated protein kinase (MAPK) pathways contribute to Smad regulation (Denhardt 1996, Kretzschmar *et al.* 1997, Massague *et al.* 2000, Saha *et al.* 2001). In addition, there is a Ca²⁺-dependent interaction between calmodulin (CaM) and several Smad family members. CaM binds to the N-terminal half of Smad2 with subsequent phosphorylation inhibiting TGF- β -induced nuclear import and transcriptional activity of Smad2 (Zimmerman *et al.* 1998). Therefore, CaM influences Smad protein function in response to agents that regulate intracellular Ca²⁺ flux. Other TGF- β -induced signaling pathways involve activation of Rho-like GTPases, including RhoA, Rac, and cdc42 through RhoA-specific guanine exchange factor or Ras activation (Shen *et al.* 2001, Derynck & Zhang 2003).

TGF- β signaling pathway and malignancy

Growth inhibition by TGF- β , associated with inhibition of c-myc, cdk, reduction in cyclin D1 levels, and inhibition of cdk-4-associated Rb kinase activity as well as induction of cdk inhibitors p15 and p27, has been noted in intestinal epithelial cells (Kurokawa *et al.* 1987, Ko *et al.* 1995, 1998). Loss of responsiveness to growth inhibition from TGF- β occurs in many cell types including breast (Arteaga *et al.* 1988), colorectal carcinoma cells, and pancreatic carcinoma (Beauchamp *et al.* 1990). Mutational inactivation of T β RII represents one mechanism for the loss of TGF- β -mediated growth inhibition, which in many cases leads to the development of gastrointestinal cancer (Arteaga *et al.* 1988, 1993, Kimchi *et al.* 1988, Sun *et al.* 1994). Thirteen percent of colorectal carcinomas are associated with a replication error (RER) or microsatellite instability phenotype with inactivation of T β RII (Markowitz *et al.* 1995) and restoration of T β RII by stable transfection results in decreased tumorigenicity (Wang *et al.* 1995). Accordingly, mutations with loss of function of Smad2 and Smad4 have been noted in 40–50% of pancreatic cancers and 30% of human colorectal cancers (Eppert *et al.* 1996, Takagi *et al.* 1996, Thiagalingam *et al.* 1996). Heterozygous germline mutations in Smad4 are also responsible for a subset of familial juvenile polyposis, a disorder characterized by predisposition to hamartomatous polyps, and gastrointestinal cancer (Howe *et al.* 1998).

Transgenic mice that are null for *Smad3* develop invasive and metastatic colorectal cancers at an early age (Zhu *et al.* 1998). Additionally, *Smad3* is needed to establish the gastrointestinal mucosal immune response to TGF- β signals (Tang *et al.* 2003b). Hence, *Smad3*-deficient mice frequently develop gut abscesses and die between 1 and 10 months because of impaired mucosal immunity (Yang *et al.* 1999). *Smad4* is required for gut endoderm lineage, and *Smad4*^{+/-} mice develop gastric tumors after 12 months (Xu *et al.* 2000). Furthermore, anaphase-promoting complex (APC Δ^{716}) mice, a model for human familial adenomatous polyposis, intercrossed with *Smad4*^{+/-} mice develop larger and more invasive colorectal tumors (Takaku *et al.* 1998).

Adaptor and receptor interacting proteins

Adaptor proteins are important regulators of the TGF- β signaling pathway. For example, Smad2/3 and Smad4 are thought to be distributed along the microtubule (MT) network, and MT stability may be involved in Smad inactivation (Dong *et al.* 2000, Wrana 2000). Thus, MTs represent a large group of TGF- β signaling adaptor proteins. Additional adaptor proteins include Smad anchor for receptor activation (SARA; Tsukazaki *et al.* 1998), embryonic liver fodrin (ELF; Tang *et al.* 2003b), filamin (Sasaki *et al.* 2001), CrkL (Wurdak *et al.* 2005), FK506-binding protein 12 (FK506BP12; Wang *et al.* 1996, Chen *et al.* 1997b), Jab1/CSN5 (Wan *et al.* 2002, Kim *et al.* 2004), disabled-2 (Dab2; Hocevar *et al.* 2001, 2005, Itoh *et al.* 2003), *Drosophila* inhibitor of apoptosis-1 and -2, T β RI-associated protein-1 and -2, T β RII binding WD-domain protein TGF β receptor-interacting protein 1 (TRIP-1; Chen *et al.* 1995), X-linked inhibitor of apoptosis (Yamaguchi *et al.* 1999, Birkey Reffey *et al.* 2001, Harlin *et al.* 2001), Death domain-associated protein (Daxx; Yang *et al.* 1997, Perlman *et al.* 2001), associated molecule with the SH3 domain of STAM-2 (AMSH-2; Ibarrola *et al.* 2004), and CD2-associated protein (CD2AP; Schiffer *et al.* 2004).

SARA is a scaffolding protein that regulates the subcellular localization of unactivated R-Smads, potentially scaffolding the TGF- β receptor kinase to the Smad2 substrate (Tsukazaki *et al.* 1998, Wu *et al.* 2000). Filamins are a family of actin polymerization proteins that also form scaffolds for a range of signaling proteins including SAP kinases such as MKK-4, small GTPases Rho and Ras, as well as Smad2 and Smad5 (Sasaki *et al.* 2001).

ELF, a β -Spectrin, is a component of TGF- β signaling that functions to recruit Smads to the receptor by controlling the subcellular localization of Smad3 and Smad4 (Mishra *et al.* 1998, 1999). ELF plays a

critical role in Smad4 localization upon TGF- β stimulation, suggesting that these molecules interact and translocate to the cell nucleus. ELF does not appear to interact with SARA or filamin, and in *elf* mutants, SARA and filamin distribution is the same as in wild-type mice (Tang *et al.* 2003b). Thus, TGF- β signaling through R-Smad/ELF interactions may work by way of a different mechanism than that of SARA and filamin.

Adaptors and malignancy

The role of adaptor proteins may prove to be critical for tumor development. However, ELF is one of the few adaptor proteins known to serve as a tumor suppressor. In *elf*^{-/-} homozygotes, liver development is defective with loss of gastrointestinal epithelial cell shape and polarity. This phenotype is similar to that of *Smad2*^{+/-}/*Smad3*^{+/-} double heterozygous mice (Weinstein *et al.* 2001). Interestingly, there is a dramatically high prevalence of hepatocellular carcinoma in *elf*^{+/-} heterozygotes. *Smad4*^{+/-} mice were found to develop hyperplasia of the fundus and antrum, and intercrosses with *elf* mutants exacerbated the phenotype. Interestingly, *elf*^{+/-}/*Smad4*^{+/-} mutants also develop colonic adenomas (Tang *et al.* 2005). None of the other mutants in the TGF- β family, including Smad3 or Smad4 mutants, develop HCCs in the absence of a carcinogen, establishing that ELF has sufficient anti-oncogenic activity on its own.

Loss of ELF in human gastric cancer is a significant aspect of gastric tumor suppression by TGF- β . Little or no ELF is expressed in three out of six well-defined human gastric cancer cell lines. Importantly, ELF is inactivated in one (NCI-N87) cancer cell line in which the rest of the TGF- β pathway is intact. In contrast, Smad4 expression is decreased in only one cell line.

Control of TGF- β signaling by ubiquitinators

Interactions involving ubiquitination are an integral part of the TGF- β /Smad signaling pathway (Fig. 1). The ubiquitin–proteasome system is composed of two discrete steps. First, multiple ubiquitin molecules are attached to the target protein. Second, the polyubiquitinated protein is degraded by the 26S proteasome complex. Ubiquitination is mediated by at least three enzymes: 1) ubiquitin-activating enzyme (E1); 2) ubiquitin-conjugating enzyme (E2); and 3) ubiquitin ligase (E3). E3 ubiquitin ligases are primarily responsible for the recognition of specific target proteins and deregulation of E3 ubiquitin ligases is linked to the development of cancer (Nakayama & Nakayama 2006). E3 ubiquitin ligases are categorized into four major

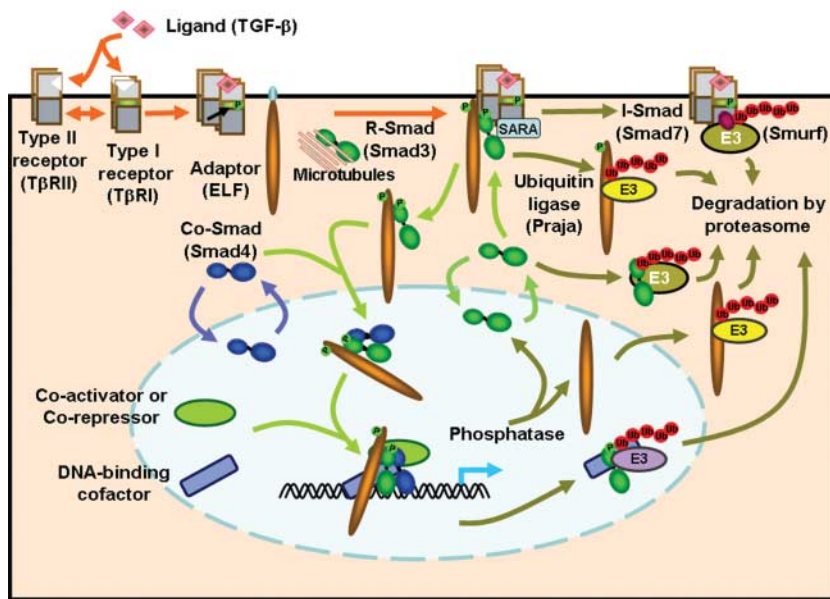


Figure 1 The TGF- β signaling pathway is regulated at multiple points by ubiquitin-mediated targeting and proteasomal degradation. Receptors, adaptor proteins, Smads, co-activator/co-repressor and DNA binding co-factors are targeted by E3 ubiquitin ligases for ubiquitination and degradation by the proteasome. These protein degradation pathways impart precise and specific control over the cellular response to TGF- β signaling.

groups based on specific structural motifs. These are the homologous to the E6-AP carboxyl terminus (HECT)-type, really interesting new gene (RING)-finger type, U-box type, and plant homeo domain (PHD)-finger type proteins. HECT-type ligases that are involved in TGF- β signaling include Smurf1 and Smurf2, Neural precursor cell expressed, developmentally down-regulated 4-2 (Nedd4-2), WW domain containing protein 1 (WWP1)/Tiul1, Itch, Cbl-b, and Arkadia. A single U-box type ligase, carboxyl terminus of hsc70-interacting protein (CHIP), is additionally implicated in this pathway. RING-finger type, the largest group of E3 ligases, includes Skp1/Culin/F-box protein (SCF)-Skp2, SCF- β -TrCP1, Roc1-SCF- β -TrCP1, anaphase promoting complex (APC)-CDH1, Ectodermin, and PRAJA. The role of these ubiquitinators and their association with development of cancer is presented below and in Tables 1 and 2.

Smurf1

Smurf1 modulates BMP signaling through multiple mechanisms thereby influencing a variety of BMP-induced cellular behaviors. Smurf1 ubiquitinates BMP-regulated R-Smads. In *Xenopus*, Smurf1 inhibits BMP signaling through Smad1, Smad5, and Smad8, while potentiating activin signaling through Smad2 and Smad3 (Zhu *et al.* 1999). In mouse lung, Smurf1 over-expression reduces levels of Smad1 and Smad5, but not

Smad8. In addition, Smurf1 inhibits lung epithelial branching, which is rescued by over-expression of Smad1 or BMP4 (Shi *et al.* 2004). In bone, LIM mineralization protein-1 (LMP-1) directly interacts with the Smurf1 WW2 domain and can effectively compete with Smad1 and Smad5 for binding, thus potentiating BMP signaling (Sangadala *et al.* 2006). In C2C12 cells, Smurf1 promotes myogenic differentiation, while blocking BMP-induced osteogenic conversion, yet Smurf1 has no effect on TGF- β -induced differentiation arrest. In these cells, elevated Smurf1 reduces the level of endogenous Smad5, but not Smad2, Smad3, or Smad7. Adding back Smad5 to the Smurf1 over-expressing C2C12 cells restores BMP-mediated osteoblast conversion. Conversely, depletion of the endogenous Smurf1 through RNA interference blocks myogenic differentiation and promotes BMP-induced osteogenic conversion (Ying *et al.* 2003).

Smurf1 negatively regulates BMP signaling together with the I-Smads, Smad6/7. Smurf1 interacts with nuclear Smad7 and induces Smad7 ubiquitination and translocation into the cytoplasm. Additionally, Smurf1 associates with BMP type I receptors (T β RI) via Smad7, with subsequent enhancement of degradation of both the receptor and Smad7. Thus, Smad7 functions to induce the degradation of T β RI by recruiting the E3 ubiquitin ligase, Smurf1, to the receptor (Ebisawa *et al.* 2001). Further, Smurf1 induces ubiquitination and degradation of Smad1/5. Moreover, Smurf1 can associate with

Table 1 E3 ubiquitin ligases implicated in transforming growth factor- β (TGF- β) signaling

Target proteins	E3 ubiquitin ligase	Adaptor	Reference
T β RI	Smurf1	Smad7	Ebisawa <i>et al.</i> (2001)
	Smurf1	Smad6/7	Murakami <i>et al.</i> (2003)
	Smurf1	Smad7 & FKBP12	Yamaguchi <i>et al.</i> (2006)
	Smurf2	Smad7	Kavsak <i>et al.</i> (2000)
	NEDD4-2	Smad7	Kuratomi <i>et al.</i> (2005)
	WWP1/Tiul1	Smad7	Komuro <i>et al.</i> (2004) and Seo <i>et al.</i> (2004)
Smad1/5/8	Smurf1		Zhu <i>et al.</i> (1999)
Smad1/5	Smurf1		Shi <i>et al.</i> (2004)
	Smurf1	LMP-1	Sangadala <i>et al.</i> (2006)
	Smurf1	Smad6/7	Murakami <i>et al.</i> (2003)
Smad1	Smurf2		Zhang <i>et al.</i> (2001)
	CHIP		Li <i>et al.</i> (2004, 2005)
Smad5	Smurf1		Ying <i>et al.</i> (2003)
Smad2	Smurf2		Lo & Massague (1999), Lin <i>et al.</i> (2000) and Zhang <i>et al.</i> (2001)
	Nedd4-2		Kuratomi <i>et al.</i> (2005)
	WWP1/Tiul1	TGIF	Seo <i>et al.</i> (2004)
	Itch/AIP4		Bai <i>et al.</i> (2004)
	Cbl-b		Wohlfert <i>et al.</i> (2006)
	CHIP		Xin <i>et al.</i> (2005)
Smad3	ROC1–SCF– β -TrCP1		Fukuchi <i>et al.</i> (2001)
	PRAJA		Saha <i>et al.</i> (2006)
Smad4	Smurf1	Smad2/6/7	Moren <i>et al.</i> (2005)
	Smurf2	Smad2/6/7	Moren <i>et al.</i> (2005)
	NEDD4-2	Smad7	Moren <i>et al.</i> (2005)
	WWP1	Smad7	Moren <i>et al.</i> (2005)
	CHIP		Li <i>et al.</i> (2004)
	SCF–Skp2		Liang <i>et al.</i> (2004)
	SCF– β -TrCP1		Wan <i>et al.</i> (2004, 2005)
Ecto		Dupont <i>et al.</i> (2005)	
Smad6/7	Smurf1		Murakami <i>et al.</i> (2003)
Smad7	Smurf1		Ebisawa <i>et al.</i> (2001)
	Smurf2		Kavsak <i>et al.</i> (2000)
	Arkadia	Axin	Liu <i>et al.</i> (2006)
	Arkadia		Koinuma <i>et al.</i> (2003)
ELF	PRAJA		Saha <i>et al.</i> (2006)
SnoN	Smurf2	Smad2	Bonni <i>et al.</i> (2001)
	APC	Smad3 > Smad2	Stroschein <i>et al.</i> (2001)
	APC–CDH1	Smad3	Wan <i>et al.</i> (2001)
HEF1	Itch/AIP4	Smad3	Feng <i>et al.</i> (2004)
	APC–CDH1	Smad3	Liu <i>et al.</i> (2000) and Nourry <i>et al.</i> (2004)
Runx2	Smurf1	Smad6	Shen <i>et al.</i> (2006)

Smad1/5 indirectly through I-Smads and induce their ubiquitination and degradation. Thus, Smurf1 controls BMP signaling with and without I-Smads through multiple mechanisms (Murakami *et al.* 2003).

In activin signaling, the cytoplasmic immunophilin FKBP12, a 12 kDa FK506-binding protein, interacts with Smad7 in an activin-dependent manner and forms a complex with Smad7 on the type I receptor. The interaction of FKBP12 and Smad7 enhances the ubiquitination of the type I receptor by Smurf1. Thus, FKBP12 acts as an adaptor molecule for the Smad7–Smurf1 complex to regulate the duration of the activin signal (Yamaguchi *et al.* 2006).

Smurf1, which otherwise cannot directly bind to Smad4, mediates ubiquitination of Smad4 in the presence of Smad6 or Smad7. Smad2 can also act as an adaptor for Smurf1 binding and ubiquitination of Smad4. Ternary complexes of Smad4, Smad7, and Smurf1 primarily co-localize in the cytoplasm and in peripheral cell protrusions. Smad2 or Smad7 mutants, defective in Smad4 interaction, fail to induce Smurf1-mediated down-regulation of Smad4. Likewise, Smad4 mutants defective in Smad2 or Smad7 interaction are not effectively down-regulated by Smurf1 (Moren *et al.* 2005).

Smurf1 also modulates transcription by inducing the ubiquitination and degradation of Runx2.

Table 2 E3 ligases associated with transforming growth factor- β (TGF- β) signaling and cancer

E3 ligase	Cancer cell lines	Altered expression in cancer
Smurf1	RhoA – tumor cell migration, Boyer <i>et al.</i> (2006) and Sahai <i>et al.</i> (2007)	Amplified – pancreatic adenocarcinomas, Loukopoulos <i>et al.</i> (2007); amplified – pancreatic cancer, Bashyam <i>et al.</i> (2005)
Smurf2		High expression correlates with poor prognosis of esophageal squamous cell carcinoma, Joazeiro <i>et al.</i> (1999) and Fukuchi <i>et al.</i> (2002)
Smurf2		p14ARF mutant melanoma, Packer <i>et al.</i> (2007)
Nedd4-2	Lung, colon, gastric, breast, ovary, and melanoma, Kuratomi <i>et al.</i> (2005)	
WWP1	Breast, Chen <i>et al.</i> (2007)	Breast cancer, Chen <i>et al.</i> (2007)
PRAJA	Gastrointestinal, Saha <i>et al.</i> (2006)	
SCF- β -TrCP1	Pancreatic, Wan <i>et al.</i> (2005)	Pancreatic ductal adenocarcinomas, Wan <i>et al.</i> (2005)
Ecto	Colorectal and breast, Dupont <i>et al.</i> (2005)	

Runx2 is a bone-specific transcription factor that plays a critical role in bone development, postnatal bone formation, and chondrocyte maturation. Smad6 enhances Smurf1-induced Runx2 degradation in an ubiquitin–proteasome-dependent manner. Thus, Smurf1 induces Runx2 degradation in a Smad6-dependent manner and serves as a negative regulatory mechanism for the BMP–Smad–Runx2 signaling pathway (Shen *et al.* 2006).

Smurf2

Smurf2 interacts with R-Smads, including Smad1, Smad2, and Smad3 but not Smad4. Smurf2 over-expression is sufficient to reduce the steady-state levels of Smad1 and Smad2 but not Smad3 or Smad4. Significantly, Smurf2 exhibits higher-binding affinity to activated Smad2 and displays a preference for Smad2 as its target for degradation (Lin *et al.* 2000). Upon TGF- β activation, phosphorylated Smad2 translocates to the nucleus, where its accumulation results in Smad2 ubiquitination and degradation, thereby terminating TGF- β signaling (Lo & Massague 1999). On the other hand, Zhang *et al.* report that Smurf2 preferentially targets Smad1 for ubiquitination- and proteasome-mediated degradation. At higher Smurf2 expression levels, Smad2, but not Smad3, also decreases. In *Xenopus* embryos, ectopic Smurf2 specifically inhibits Smad1 responses and thereby affects embryonic patterning by BMP signals (Zhang *et al.* 2001). As seen with Smurf1, Smurf2 can also mediate ubiquitination and degradation of Smad4 in the presence of Smad7 or Smad2 (Moren *et al.* 2005).

Smad7 associates constitutively with Smurf2. Smurf2 is nuclear, but binding to Smad7 induces export and recruitment to activated T β RI, where it causes degradation of receptors and Smad7 via proteasomal and lysosomal

pathways. IFN γ , which stimulates expression of Smad7, induces Smad7–Smurf2 complex formation and increases TGF- β receptor turnover. TGF- β receptor turnover is stabilized by blocking Smad7 or Smurf2 expression. Furthermore, Smad7 mutants that interfere with recruitment of Smurf2 to the receptors are compromised in their inhibitory activity. Thus, Smad7 acts as an adaptor in an E3 ubiquitin ligase complex that targets activated T β RI for degradation (Kavsak *et al.* 2000).

In the presence of TGF- β signaling, Smad2 interacts through its proline-rich PPXY motif with the tryptophan-rich WW domains of Smurf2. Activated Smad2 mediates association of Smurf2 with the transcriptional co-repressor SnoN (Ski-related novel protein N). This allows the HECT domain of Smurf2 to target SnoN for ubiquitin-mediated degradation by the proteasome, thus relieving SnoN-mediated repression of transcription (Bonni *et al.* 2001).

NEDD4-2

NEDD4-2 is a direct-binding partner of Smad7. NEDD4-2 associates with TGF- β type I receptors via Smad7 and induces its ubiquitin-dependent degradation. Additionally, NEDD4-2 binds to activated Smad2 and Smad3, and induces degradation of Smad2, but not Smad3 (Kuratomi *et al.* 2005). Furthermore, NEDD4-2, in the presence of Smad7, can mediate ubiquitination of Smad4 (Moren *et al.* 2005). In contrast to Smurf2, NEDD4-2 fails to induce ubiquitination of SnoN, although NEDD4-2 binds to SnoN via Smad2 more strongly than Smurf2. Over-expression of NEDD4-2 prevents transcriptional activity induced by TGF- β and BMP, whereas silencing of NEDD4-2 by siRNA enhances the responsiveness to TGF- β superfamily cytokines (Kuratomi *et al.* 2005). Thus, NEDD4-2 is a negative regulator of TGF- β signaling.

WWP1/Tiul1

WW domain-containing protein 1, WWP1, also known as TGIF-interacting ubiquitin ligase 1, Tiul1, is structurally related to Smurf E3 ubiquitin ligases. WWP1/Tiul1 associates with Smad7, induces Smad7 nuclear export, and enhances the binding of Smad7 to activated T β RI resulting in their ubiquitination and degradation, without affecting the expression levels of Smad7. WWP1/Tiul1 can also mediate ubiquitination of Smad4 in the presence of Smad7 (Moren *et al.* 2005). Over-expression of WWP1/Tiul1 suppresses TGF- β -induced growth arrest and transcriptional responses, and inhibits TGF- β stimulated phosphorylation of Smad2 (Seo *et al.* 2004). Thus, WWP1/Tiul1 over-expression, in cooperation with Smad7, negatively regulates TGF- β signaling. However, unlike Smurfs, WWP1/Tiul1 fails to ubiquitinate R-Smads and SnoN. Importantly, WWP1/Tiul1 and Smurfs are expressed in distinct patterns in human tissues and carcinoma cell lines, suggesting unique pathophysiological roles of WWP1/Tiul1 and Smurfs (Komuro *et al.* 2004).

Upon activation of TGF- β signaling, WWP1/Tiul1 interacts with Smad2 and the nuclear co-repressor TGIF. Steady-state levels of TGIF are not affected by WWP1/Tiul1, but the interaction of WWP1/Tiul1 and Smad2 with TGIF, targets Smad2 for degradation. Silencing of WWP1/Tiul1 or TGIF by siRNA suppresses TGF- β -dependent degradation of Smad2 and enhances TGF- β -mediated gene expression. Thus, WWP1/Tiul1 and TGIF act as negative regulators of TGF- β signaling (Seo *et al.* 2004).

Itch

Itch, also known as atrophin-1-interacting protein 4 (AIP4), is an E3 ligase for Notch and JunB (Qiu *et al.* 2000, Gao *et al.* 2004). Itch also regulates TGF- β signaling, where it facilitates complex formation between T β RI and Smad2 and enhances TGF- β -induced transcription. In addition, Itch promotes ubiquitination of Smad2. Moreover, Itch augments Smad2 phosphorylation, which requires intact ligase activity. In mouse embryonic fibroblasts (MEFs), loss of Itch reduced susceptibility to TGF- β -induced cell growth arrest and decreased phosphorylation of Smad2, without altering protein levels for Smad2, Smad4, and Smad7. Thus, Itch positively regulates TGF- β signaling via proteolysis-independent ubiquitination (Bai *et al.* 2004).

HEF1, human enhancer of filamentation 1, functions as a multidomain docking protein implicated in signaling pathways such as those mediated by integrin, T-cell receptor, and B-cell receptor. HEF1 is also involved in TGF- β signaling pathways by interacting

with Smad3. The interaction of Smad3 with HEF1 induces HEF1 proteasomal degradation, which is further enhanced upon TGF- β stimulation. Itch functions as an ubiquitin E3 ligase for HEF1, forming a complex with both Smad3 and HEF1 through its WW domains in a TGF- β -independent manner. This complex regulates HEF1 ubiquitination and degradation, and can be enhanced by TGF- β stimulation. Smad3-regulated proteasomal degradation of HEF1 by Itch therefore serves to broaden the network of cross-talk between TGF- β signaling and those pathways involving HEF1 and Itch (Feng *et al.* 2004).

Arkadia

Arkadia was originally identified as a protein that enhances signaling activity of Nodal and induces mammalian nodes during early embryogenesis. Arkadia is widely expressed in mammalian tissues, and it enhances both TGF- β and BMP signaling. Arkadia physically interacts with Smad7 and induces its ubiquitination and degradation. In contrast to Smurfs, however, Arkadia does not associate with T β RI and does not induce degradation of TGF- β receptors. TGF- β down-regulates Arkadia, while it up-regulates Smad7. Silencing of Arkadia by siRNA results in repression of the transcriptional activities induced by TGF- β and BMP, and in the accumulation of Smad7 protein. Arkadia therefore, may play a role as an amplifier of TGF- β signaling by reducing levels of inhibitory Smad7 (Koinuma *et al.* 2003).

Axin, a pivotal player in Wnt signaling that is required for the constitutive degradation of β -catenin, also coordinates TGF- β signaling by forming a multimeric complex consisting of Smad7 and Arkadia. Specific knockdown of Axin or Arkadia demonstrates that Axin and Arkadia cooperate with each other in promoting Smad7 ubiquitination. Coexpression of Wnt-1, which downregulates Axin levels, reduces Smad7 ubiquitination by Arkadia. Thus, Axin acts as an intrinsic regulator in TGF- β as well as in Wnt signaling, and therefore may participate in cross-talk between these signaling pathways (Liu *et al.* 2006).

Cbl-b

In T cells, loss of Cbl-b results in defective TGF- β -mediated Smad2 phosphorylation. Cbl-b loss also prevents TGF- β -mediated induction of Foxp3+ functional regulatory T cells. Cbl-b^{-/-} mice show significantly enhanced responses to a tumor that is strictly TGF- β regulated. Thus, the E3 ubiquitin ligase Cbl-b plays an integral role in T-cell TGF- β signaling, and its

absence results in multifunctional TGF- β -related defects that have important implications in cancer.

CHIP

CHIP is a U-box-dependent E3 ubiquitin ligase that directly binds and interacts with Smad1 (Li *et al.* 2005). Over-expression of CHIP results in ubiquitin-mediated degradation of Smad1 and Smad4. Conversely, reduction of CHIP levels with RNAi results in enhanced BMP signaling (Li *et al.* 2004).

CHIP directly mediates ubiquitination and degradation of Smad3 independently of TGF- β signaling, thereby regulating the basal level of Smad3. In cell culture luciferase assays, over-expression of CHIP inhibits TGF- β signaling, whereas silencing CHIP by siRNA increases sensitivity to TGF- β signaling. In cell lines with stably over-expressed CHIP, Smad3 is greatly decreased and TGF- β signaling is abolished, based on cell proliferation assays and JunB expression. Thus, CHIP can modulate the sensitivity of the TGF- β signaling by controlling the basal level of Smad3 through ubiquitin-mediated degradation (Xin *et al.* 2005).

SCF-Skp2

The SCF is a multi-protein E3 ubiquitin ligase complex, containing Skp, Cullin, and an F-box protein. SCF-Skp2 mediates the metabolic instability of cancer-derived mutant Smad4. Skp2, the F-box component of SCF-Skp2, physically interacts with Smad4. Several cancer-derived unstable Smad4 mutants exhibit significantly increased binding to Skp2, which leads to increased ubiquitination and proteolysis (Liang *et al.* 2004).

SCF- β TrCP1

SCF- β TrCP1 is a critical determinant for Smad4 degradation. F-box protein β TrCP1 in this E3 ligase interacts with Smad4, but has no interaction with Smad2 and has weak interaction with Smad3. The β TrCP1/Smad3 interaction is abolished by Smad4 gene silencing, indicating that the interaction is indirect and is through Smad4. Ectopic expression of SCF- β TrCP1 induces the ubiquitination and degradation of Smad4, while suppression of β TrCP1 by siRNA increases expression of Smad4. Consistent with these results, cells that over-express SCF- β TrCP1 have reduced TGF- β -dependent transcriptional activity and an impaired cell cycle arrest function. Thus, SCF- β TrCP1 abrogates TGF- β function *in vivo* by decreasing Smad4 stability (Wan *et al.* 2004).

SCF- β TrCP1 inhibits TGF- β biological activity in pancreatic cancer cells by decreasing Smad4 stability. In human pancreatic ductal adenocarcinoma cells, Smad4 levels are very low as determined by immunohistochemistry. In pancreatic tumor-derived Smad4 mutants, most point-mutated Smad4 proteins, except those within or very close to a mutation cluster region, exhibit higher interaction affinity with β -TrCP1 and significantly elevated ubiquitination. Two cancer cell lines harboring Smad4 point mutations, AsPC-1 and Caco-2, exhibit rapid SCF- β TrCP1-mediated Smad4 degradation. Conversely, reduction of SCF- β TrCP1 expression by siRNA in pancreatic cancer cells results in elevated levels of Smad4 and TGF- β signaling. Therefore, inhibition of Smad4-specific E3 ligase might be a target for therapeutic intervention in pancreatic cancer (Wan *et al.* 2005).

ROC1-SCF- β -TrCP1

Activated Smad3 is degraded by the ubiquitin-proteasome pathway through an interaction with the C-terminal MH2 domain of ROC1, a RING finger protein. An E3 ubiquitin ligase complex regulator of cullins 1 (ROC1)-SCF- β -TrCP1 consisting of ROC1, Skp1, Cullin1, and β -TrCP1 (also known as Fbw1a) induces ubiquitination of Smad3. Recruitment of p300, a transcriptional coactivator, to nuclear Smad3 facilitates the interaction with ROC1-SCF- β -TrCP1 and triggers export from the nucleus to the cytoplasm for proteasomal degradation (Fukuchi *et al.* 2001).

APC

The APC is a multi-subunit ubiquitin E3 ligase that mediates Smad2- or Smad3-induced degradation of SnoN. SnoN is an important negative regulator of TGF- β signaling, which functions to maintain the repressed state of TGF- β target genes in the absence of ligand. Upon TGF- β stimulation, Smad3 and Smad2 translocate into the nucleus and induce a rapid degradation of SnoN, allowing activation of TGF- β target genes. Smad3 and to a lesser extent, Smad2, interact with both the APC and SnoN, resulting in the recruitment of the APC to SnoN and subsequent ubiquitination of SnoN in a destruction box (D box)-dependent manner. In addition to the D box, efficient ubiquitination and degradation of SnoN requires the Smad3-binding site in SnoN as well as key lysine residues necessary for ubiquitin attachment. Mutation of either the Smad3-binding site or the lysine residues results in stabilization of SnoN and in enhanced antagonism of TGF- β signaling (Stroschein *et al.* 2001, Wan *et al.* 2001).

The APC activator CDH1 forms a quaternary complex with SnoN, Smad3, and APC, in which CDH1 and Smad3 synergistically regulate SnoN degradation (Wan *et al.* 2001). CDH1 also regulates HEF1 levels. HEF1, CDH1, Smad3, and APC10, a component APC, physically interact. Distinct subdomains within the MH2 domain of Smad3 bind to APC10 and HEF1, suggesting the formation of a complex of HEF1, Smad3, APC10, and CDH1. In addition, over-expression of APC10 and CDH1 leads to a reduction in HEF1 protein levels. Thus, Smad3 may recruit the APC complex via a direct interaction with APC10 to regulate the ubiquitination and degradation of HEF1 by the CDH1 subunit of the APC complex (Nourry *et al.* 2004). Over-expression of Smad3 triggers the proteasomal degradation of HEF1. In addition, TGF- β stimulation induces rapid degradation of endogenous HEF1 in different TGF- β -responsive cell lines. Interestingly, in TGF- β -treated epithelial cells, the degradation of HEF1 is followed closely by an increase in HEF1 mRNA, resulting in a time-dependent increase in HEF1 protein. Elevated HEF1 protein levels inhibit TGF- β -induced gene responses. Thus, the TGF- β signaling pathway is regulated by HEF1 via a negative feedback mechanism (Liu *et al.* 2000).

Ecto

Ecto, Ectoderm, is a RING-type E3 ubiquitin ligase for Smad4. In *Xenopus* embryos, Ecto is essential for the specification of ectoderm and acts by restricting mesoderm-inducing activity of TGF- β signals to the mesoderm thus favoring neural induction. Depletion of Ecto in human cells enforces TGF- β -induced cytosclerosis. Ecto additionally plays a causal role in limiting the antimitogenic effects of Smad4 in tumor cells. Thus, Ecto may act as a switch in the control of TGF- β gene responses during early embryonic development and cell proliferation (Dupont *et al.* 2005).

PRAJA

PRAJA, a RING-H2 protein, interacts with ELF in a TGF- β -dependent manner. PRAJA manifests substantial E3-dependent ubiquitination of ELF and Smad3, but not Smad4. Treatment with potent proteasomal inhibitors MG132 results in the accumulation of ELF, indicating that PRAJA ubiquitinates ELF and that its degradation is mediated via the proteasomal pathway. Deletion of the RING finger domain at the C terminus of PRAJA, Δ -PRAJA, abolishes ubiquitination of ELF. In a cell line that stably over-expresses PRAJA, ELF expression

is low compared with normal controls. In contrast, in a Δ -PRAJA stable cell line, ELF expression is high compared with normal controls. Moreover, in PRAJA-transfected hepatocytes, the degradation rate of ELF is substantially higher in TGF- β stimulated cells compared with unstimulated cells. After 30 min of TGF- β induction, ELF associates with PRAJA, which is approximately when the ELF–Smad heteromeric complex translocates into the nucleus. This raises possibilities that PRAJA interaction and degradation of ELF could result in the disruption of Smad4 signaling, with a potential role in development of cancer.

There is a fivefold increase of PRAJA expression and a subsequent decrease in ELF and Smad4 expression in gastrointestinal cancer cell lines. Therefore, alteration of ELF and/or Smad4 expression in the TGF- β signaling pathway may be induced by enhancement of ELF degradation, which is mediated by a high-level expression of PRAJA in gastrointestinal cancers (Saha *et al.* 2006).

Overall, these studies suggest a unique mechanism by showing that the pathway and growth of cells that are dependent on the TGF- β adaptor protein, ELF, are inactivated by the RING E3 ubiquitin ligase, PRAJA. Also, multiple other cancers derived from meso-endodermally derived epithelium are associated with the TGF- β /BMP pathway inactivation, where it may regulate progenitor cell fate (Souchelnytskyi *et al.* 2002, Siegel & Massague 2003, Tang *et al.* 2003b). Indeed, the functions of TGF- β are more complex than simply inhibiting cell growth, as TGF- β can induce the growth of mesenchymal cells and alter synthesis of extracellular matrix components as well as metalloproteases involved in cell invasion (Sporn & Roberts 1990, Heldin *et al.* 1997, Derynck *et al.* 1998, Souchelnytskyi *et al.* 2002, Itoh *et al.* 2003, Tang *et al.* 2003a). TGF- β signals also modulate the immune response to tumors and are thought to play a role in tumor angiogenesis (Wieser 2001). The development of gut tumors in *elf*^{+/-} and *elf*^{+/-} *Smad4*^{+/-} mutants points to the critical role played by ELF as an essential adaptor protein for the proper transmission of signals generated by the TGF- β pathway. Furthermore, modification of the TGF- β signaling pathway by loss of expression of ELF through PRAJA could play a significant role in the development of gastrointestinal tumors. Further research into the role of the ubiquitin–proteasome system in modulating TGF- β signaling could be the key to unlocking the mechanisms behind many cancers, and lead to unique therapeutic approaches to treating these diseases.

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