Review Article

Regulation of TGF-β family signaling by E3 ubiquitin ligases

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Members of the transforming growth factor-β (TGF-β) family, including TGF-β, activin and bone morphogenetic proteins (BMPs), are multifunctional proteins that regulate a wide variety of cellular responses, such as proliferation, differentiation, migration and apoptosis. Alterations in their downstream signaling pathways are associated with a range of human diseases like cancer. TGF-β family members transduce signals through membrane serine/threonine kinase receptors and intracellular Smad proteins. The ubiquitin–proteasome pathway, an evolutionarily conserved cascade, tightly regulates TGF-β family signaling. In this pathway, E3 ubiquitin ligases play a crucial role in the recognition and degradation of target proteins by the 26S proteasomes. Smad degradation regulates TGF-β family signaling; HECT (homologous to the E6-accessory protein C-terminus)-type E3 ubiquitin ligases, Smad ubiquitin regulatory factor 1 (Smurf1), Smurf2, and a RING-type E3 ubiquitin ligase, ROC1-SCF^{Fbw1a} have been implicated in Smad degradation. Smurf1 and Smurf2 bind to TGF-β family receptors via the inhibitory Smads, Smad6 and Smad7, to induce their ubiquitin-dependent degradation. Arkadia, a RING-type E3 ubiquitin ligase, induces the ubiquitination and degradation of Smad7 and corepressors, c-Ski and SnoN, to enhance TGF-β family signaling. Abnormalities in E3 ubiquitin ligases that control components of TGF-β family signaling may lead to the development and progression of various cancers. (*Cancer Sci* 2008; 99: 2107–2112)

Transforming growth factor-β (TGF-β) family signaling

Members of the transforming growth factor-β (TGF-β) family including TGF-β, activin and bone morphogenetic proteins (BMPs) are multifunctional proteins that regulate a diverse set of cellular responses, including proliferation, differentiation, migration and apoptosis.[1] TGF-β family members transduce signals as parts of heteromeric complexes containing type I and type II serine/threonine kinase receptors and intracellular Smad proteins (Fig. 1).[2–5] Following ligand binding, the type II receptor phosphorylates the type I receptor, which in turn phosphorylates the receptor-regulated Smads (R-Smads), typically at a C-terminal SXS motif (Fig. 2a). Smad1, Smad5 and Smad8 serve as substrates for the BMP receptors, whereas Smad2 and Smad3 are substrates for TGF-β and activin. Activated R-Smads associate with Smad4, a common-partner Smad (Co-Smad), and translocate into the nucleus. Once there, Smad complexes bind to transcriptional factor(s), such as FoxH1, Mixer, Runx-related proteins and E2F, as well as transcriptional coactivators (e.g. p300 and CBP) and corepressors (e.g. TGIF, c-Ski, and SnoN) to regulate transcriptional target genes.[6] The third class of Smads, inhibitory Smads (I-Smads) like Smad6 and Smad7, are induced by TGF-β family ligands. I-Smads compete with R-Smads for binding to type I receptors, resulting in the inhibition of TGF-β family signaling.[7,8]

Ubiquitin-proteasome system

The ubiquitin-proteasome pathway is a major pathway for the targeted degradation of proteins. This process plays critical roles in a wide range of biological processes, including cell-cycle progression, signal transduction, transcriptional regulation, receptor down-regulation and endocytosis.[9,10] In general, protein ubiquitination is catalyzed by a cascade of enzymes, including an ubiquitin-activating enzyme E1, an ubiquitin-conjugating enzyme E2 and an ubiquitin ligase E3. E3 ubiquitin ligases are crucial in the selective recognition of target proteins and also function in subsequent protein degradation by the 26S proteasomes.[11] E3 ubiquitin ligases exist and act a single peptide (such as Mdm2 and XIAP) or as a multiple-component complex (such as Skp1-Cullin-F-box protein [SCF]). Frequently, genetic alterations and aberrations in the expression of E3 ubiquitin ligases result in cancer development.[12,13]

Smad ubiquitin regulatory factors (Smurfs)

Smad ubiquitin regulatory factor 1 (Smurf1) and Smurf2 are HECT (homologous to the E6-accessory protein C-terminus)-type E3 ubiquitin ligases that regulate TGF-β and BMP signaling.[14–19] Smurfs contain an N-terminal C2 domain for membrane binding, a central region containing two or three WW domains for protein-protein interaction and a C-terminal HECT domain for ubiquitin protein ligation (Fig. 2b). Smurf1 was originally identified as an E3 ubiquitin ligase that interacted with Smad1 and Smad5 through a specific interaction between the Smurf1 WW domain and the PY motif in linker region of Smad1, and induces degradation of Smads (Fig. 3).[19] In addition to regulating the degradation of R-Smads, Smurf1 and Smurf2 facilitate the inhibitory activities of I-Smads (see below for details). Phosphorylation of the Smad1 linker primes Smad1 for proteasome-dependent degradation by facilitating the Smurf1-dependent polyubiquitination of Smad1.[20] In addition to inducing Smad1 ubiquitination, Smurf1 binding inhibits Smad1 from interacting with the nuclear translocation factor Nup214.[20] Thus, linker phosphorylation-dependent Smurf1 binding results in Smad1 degradation or cytoplasmic retention. Smurf2 interacts with both Smad1 and Smad2 to induce their ubiquitin-mediated degradation.[16,18]

Smurf1 and Smurf2 interact with Smad7 with higher affinities than those for the R-Smads, inducing the ubiquitin-dependent degradation of Smad7 and the associated receptors for members

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Abbreviations: TGF-β, transforming growth factor-β; BMPs, bone morphogenetic proteins; HECT, homologous to the E6-accessory protein C-terminus; Smurfs, Smad ubiquitin regulatory factors; Nedd4-2, neural precursor cells-expressed developmentally down-regulated 4-2.

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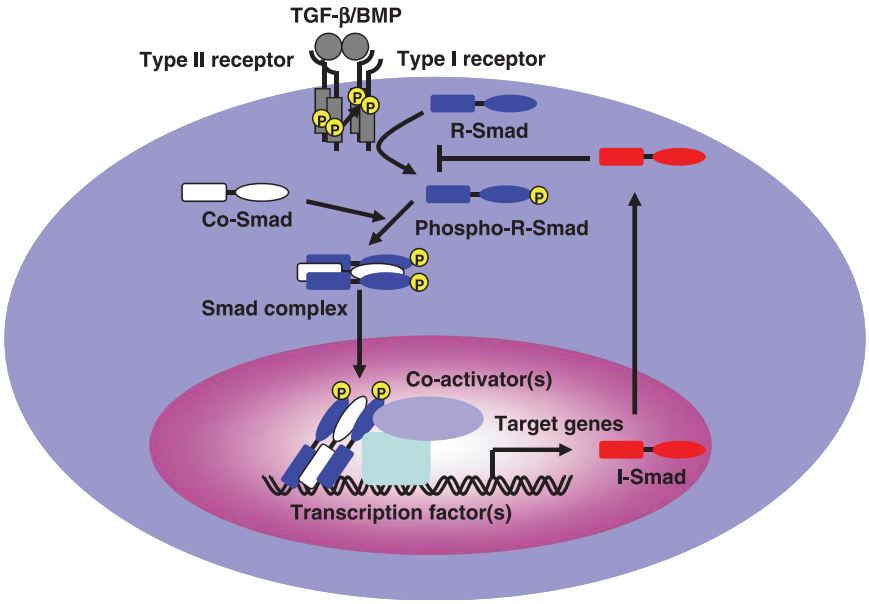

Fig. 1. The transforming growth factor-β (TGF-β) family signaling pathway. Binding of TGF-β family ligands induces the association of the type II and type I receptors into a heterodimeric complex. The type II receptor kinase phosphorylates the type I receptor, inducing its serine/threonine kinase activity. Receptor-regulated Smads (R-Smads) are then activated by phosphorylation by the type I receptor kinase. Activated R-Smads form complexes with common-partner Smads (Co-Smad), and translocate into the nucleus. Once there, these proteins bind other transcriptional factors, including both transcriptional coactivators and corepressors.

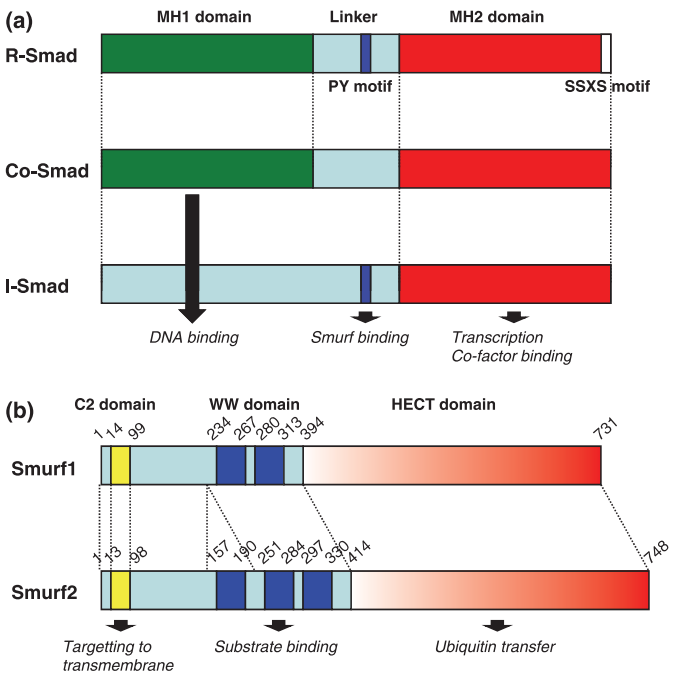

Fig. 2. Schematic diagram displaying the structural organization of Smad proteins and Smad ubiquitin regulatory factor (Smurf) proteins. (a) Diagrammatic representation of the three subfamilies of Smads. Smad proteins consist of two conserved domains, the MH1 and MH2 domains, and the linker region. The PY motif of the linker that is recognized by the homologous to the E6-accessory protein C-terminus (HECT)-type E3 ubiquitin ligases, and receptor-mediated phosphorylation occurs at the carboxy-terminal SSXS motif of R-Smads. (b) Diagrammatic representation of Smurf1 and Smurf2. The C2 domain is responsible for localization of Smurfs to the plasma membrene in a Ca^{2+}-dependent manner. The WW domain binds substrate proteins containing PY motifs. The HECT domain catalyzes the transfer of ubiquitin to target substrates.

of the TGF-β family.[14,15,17] Although Smurf1 and Smurf2 are localized to the nucleus, their binding to Smad7 induces their export and recruitment to activated receptors, resulting in the degradation of the receptors and Smad7. Smurf1 also binds to CRM1 (chromosomal region maintenance 1) through a C-terminal nuclear export signal (NES).[21] Smurf1 induces the export of Smad7 from the nucleus to the cytoplasm, allowing Smad7 to associate with activated type I receptors at the plasma membrane. This localization of Smad7 to the plasma membrane requires the C2 domain of Smurf1.[22]

Although the C-terminal MH2 domains of Smad6 and Smad7 are essential for the inhibition of TGF-β and BMP signaling, the N domain of Smad7 (Smad7N) is required for efficient inhibition of TGF-β signaling by Smad7 (Fig. 2a).[23] Smad7N physically interacts with the MH2 domain of Smad7, which may induce a conformational change in the MH2 domain that enhances its affinity for the TGF-β receptor complex. Smad7N also positively regulates the catalytic activity of the Smurf2 HECT domain by facilitating E2 recognition.[24] The interaction of the Smurf2 HECT domain with the UbcH7 E2 is weak; Smad7N strongly enhanced the interaction of Smurf2 and UbcH7, stimulating Smurf2 ligase activity. Thus, Smad7 both regulates Smurf2 activity by promoting E2 binding to its HECT domain and aids in recruiting Smurf2 to membrane receptors.

E3 ubiquitin ligases catalyze the ubiquitination of both their substrates and themselves. In many cases, E3 autoubiquitination induces their proteasome-dependent degradation, suggesting that autoubiquitination controls E3 ubiquitin ligase abundance. Wiesner *et al*. demonstrated that intramolecular interactions between the C2 and HECT domains of Smurf2 inhibited its catalytic activity and stabilized Smurf2 levels.[25] This interaction inhibited Smurf2 ligase activity by interfering with ubiquitin thioester formation, which served to stabilize steady-state levels of both Smurf2 and the substrate. The Smad7N domain disrupted the interaction between the C2 and HECT domains. These data suggest that autoinhibition of the Smurf2 HECT domain by the C2 domain maintains the steady-state levels of this E3 ubiquitin ligase and can be relieved by adaptor-mediated substrate targeting.[25]

In addition to Smad7, Smad2 plays an important role in regulating the activity of Smurf2.[26] In response to TGF-β ligation, Smad2 forms a complex with Smurf2, which mediates the recruitment of Smurf2 to the transcriptional corepressor SnoN. Smurf2 subsequently promotes the ubiquitination and proteasomal degradation of SnoN.[26] The anaphase-promoting complex (APC) similarly uses Smad3 as an adaptor for SnoN recruitment, resulting in the ubiquitin-dependent degradation of SnoN.[27,28] SnoN degradation is an essential initial step in TGF-β signaling.[29] Therefore, the TGF-β-dependent degradation of SnoN, either through the Smurf2 pathway or the APC pathway, is thought to be

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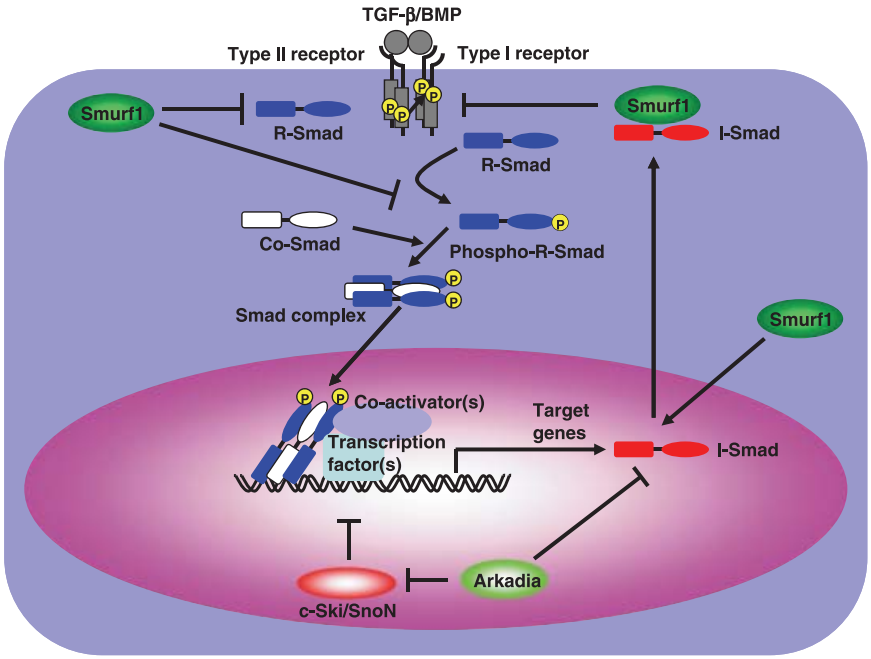

Fig. 3. Smad ubiquitin regulatory factors (Smurfs) and Arkadia regulate transforming growth factor-β (TGF-β) family signaling. Smurfs induce the ubiquitination and degradation of R-Smads. Smurfs also mediate the degradation of type I receptors by associating with inhibitory-Smads (I-Smads) in response to ligands, leading to the repression of TGF-β family signaling. In contrast, Arkadia enhances TGF-β signaling by down-regulating the negative regulators Smad7 and SnoN/c-Ski.

required for activation of TGF-β signaling in a context-dependent manner.

WWP1, Nedd4-2 and Itch

In addition to Smurf1 and Smurf2, WWP1/TGIF-interacting ubiquitin ligase 1 (Tiul1), neural precursor cells-expressed developmentally down-regulated 4 (Nedd4)-2, and Itch/atrophin-1 interacting protein 4 (AIP4) also regulate TGF-β family signaling. These molecules share a characteristic domain organization with Nedd4 and the Smurf proteins. We identified WWP1 and Nedd4-2 as Smad7-binding proteins by yeast two-hybrid screening.[30,31] Both proteins interact with the TGF-β type I receptor (TβR-I) via Smad7 to induce ubiquitin-mediated degradation of TβR-I. Although Nedd4-2 is capable of enhancing the ubiquitination and degradation of Smad2 in the presence of activated TβR-I, a similar function for WWP1 remains controversial. Smad2 ubiquitination by WWP1 may require TGIF and other factors involved in TGF-β signaling.[32] Smad7 also functions as an adaptor for WWP1 and Nedd4-2 in the ubiquitination of Smad4.[33]

Itch/AIP4, which is involved in immune responses,[34,35] also regulates TGF-β signaling. Loss of Itch from MEF results in reduced susceptibility to TGF-β-induced cell growth arrest and decreased Smad2 phosphorylation, without any alterations in the protein levels of either Smad2 or TβR-I.[36] Itch mediates the TGF-β-induced ubiquitination of Smad2, which enhances the interaction of Smad2 with activated TβR-I in a manner dependent on E3 ubiquitin ligase activity. In contrast, Lallemand *et al*. demonstrated that Itch interacts with Smad7 to inhibit TGF-β signaling.[37] Itch enhances the association of Smad7 with the activated TβR-I, independent of ubiquitin ligase activity. This difference may be due to tissue- or cell-type-specific effects; further studies will need to delineate the molecular mechanism by which Itch participates in the regulation of TGF-β signaling.

Arkadia

Arkadia was originally identified as a protein that enhances nodal signaling, inducing mammalian nodes during embryonic development.[38,39] Arkadia possesses multiple nuclear localization signals in its N-terminus and a RING-finger domain at its C-terminus. Arkadia, which is widely expressed throughout mammalian tissues, enhances the signaling activities of both BMP and TGF-β. Arkadia interacts with Smad7 and induces its ubiquitination and degradation (Fig. 3).[40] In contrast to the Smurf proteins, Arkadia does not interact with TβR-I and fails to induce receptor degradation. Liu *et al*. demonstrated that Axin activates TGF-β signaling upon forming a multimeric complex containing Smad7 and Arkadia.[41] Axin, a scaffold protein in the Wnt pathway, is required for constitutive degradation of β-catenin.[42] Axin also enhances TGF-β signaling in an Arkadia-dependent manner. By promoting Smad7 polyubiquitination, Axin cooperates with Arkadia to reduce Smad7 stability. Wnt-1 attenuates Axin-induced Smad7 ubiquitination, which is consistent with the observation that Wnt-1 down-regulates Axin protein levels.[41] Thus, Axin acts as an intrinsic regulator in both Wnt and TGF-β signaling, which may play an important role in regulating the cross-talk between these two signaling pathways.

Recently, several groups have reported that Arkadia targets SnoN and c-Ski as well as Smad7 for degradation.[43,44] SnoN and c-Ski are potent negative regulators that inhibit activated Smad complex.[45] Arkadia therefore enhances TGF-β signaling by inducing the ubiqitination and degradation of Smad7, SnoN, and c-Ski, all of which are independently acting negative regulators of TGF-β signaling.

Others

The SCF complexes are multisubunit RING-type E3 ligases that participate in the degradation of a wide variety of proteins. The SCF complex contains three invariable components, ROC1/Rbx1 (RING-finger protein), Cul1 (scaffold protein), and Skp1 (adaptor protein), as well as the variable component that confers specific substrate recognition, known as an F-box protein, that binds to Skp1 via its F-box motif. ROC1-SCF^{Fbw1a} interacts with Smad3 to trigger the degradation of Smad3 in a ligand-dependent manner.[46] Transcriptional coactivator p300 potentiates the transcriptional activity of Smad3, but also induces the interaction of Smad3 with the ROC1-SCF^{Fbw1a} complex. ROC1-SCF^{Fbw1a}-induced proteasomal degradation may be necessary to terminate Smad3-mediated transcriptional activity. Wan *et al*. reported that SCF^{Fbw1a} also regulates Smad4 protein stability.[47]

Smad4 mutants isolated from cancer cells exhibit accelerated induction of ubiquitin-dependent proteasomal degradation in

Table 1. E3 ubiquitin ligases implicated in family signaling pathway

E3 ubiquitin ligase	Target proteins	Adaptor	Regulatory mechanisms	Reference
Smurf1	Smad1/5		Smurf1 ubiquitinates Smad1/5 in a ligand-independent manner	(19)
	TβR-I	Smad6/7	Smurf1 induces the degradation of Smad7 and the associated receptors	(14)
	Smad7			(14)
Smurf2	TβR-I	Smad7	Smurf2 induces the degradation of Smad7 and the associated receptors	(15)
	Smad1		Smad1 ubiquitination by Smurf2 is a ligand-independent manner	(18)
	Smad2		The interaction between Smad2 and Smurf2 is enhanced after TGF-β ligation	(16)
	SnoN	Smad2	Smurf2 interacts with SnoN via Smad2 to induce ubiquitin-dependentdegradation of SnoN in response to TGF-β	(26)
Nedd4-2	TβR-I	Smad7	Nedd4-2 interacts with TβR-I via Smad7 to induce ubiquitin-dependent degradation of TβR-I	(31)
	Smad2		Smad2 ubiquitination by Nedd4-2 is enhanced after TGF-β ligation	(31)
	Smad4	Smad7	Smad7 functions as an adaptor for Nedd4-2 in the ubiquitination of Smad4	(33)
WWP1/Tiul1	TβR-I	Smad7	WWP1 interacts with TβR-I via Smad7 to induce ubiquitin-dependent degradation of TβR-I	(30,32)
	Smad2		Smad2 ubiquitination by WWP1 may require TGIF	(32)
	Smad4	Smad7	Smad7 functions as an adaptor for WWP1 in the ubiquitination of Smad4	(33)
Itch/AIP4	Smad2		The interaction between Smad2 and Itch is enhanced after TGF-β ligation	(36)
Arkadia	Smad7	Axin	Axin cooperates with Arkadia to reduce Smad7 stability	(40,41)
	SnoN/c-Ski		Arkadis enhances TGF-β signaling by inducing ubiquitin-dependent degradation of SnoN and c-Ski	(43,44)
ROC1-SCF^{Fbw1a}	Smad3		ROC1-SCF^{Fbw1a} interacts with Smad3 to induce the degradation of Smad3 in a ligand-dependent manner	(46)
	Smad4		SCF^{Fbw1a} decreases Smad4 protein stability	(47)
SCF^{Skp2}	Smad4		Several cancer-associated Smad4 mutants exhibit a significantly increased affinity for Skp2	(49)
CHIP	Smad1/4		CHIP decreases the protein levels of Smad1 and Smad4 in a ligand-independent manner	(50)
	Smad3		CHIP decreases total Smad3 levels independent of TGF-β activation	(51)
Ectodermin/TIF1γ	Smad4		Ectodermin binds to Smad4 and may induce Smad4 ubiquitination and degradation	(52)
APC	SnoN	Smad3	APC uses Smad3 as an adaptor for SnoN recruitment to induce the ubiquitin-dependent degradation of SnoN	(27,28)

comparison to wild-type Smad4.[48] Liang *et al*. demonstrated that the SCF^{Skp2} complex physically interacts with Smad4; several cancer-associated Smad4 mutants exhibit a significantly increased affinity for Skp2. Skp2 promotes the ubiquitination-dependent degradation of these Smad4 cancer mutants, but not the wild-type protein.[49] Skp2, which targets tumor suppressor proteins such as p27 for degradation, is up-regulated in a multitude of human cancers.[12] Thus, the ubiquitin-dependent degradation of cancer-associated Smad4 mutants by the SCF^{Skp2} complex may be the molecular mechanism mediating both the oncogenic role of Skp2 and the tumor suppressor function of Smad4.

CHIP (carboxyl terminus of Hsc70-interacting protein), a U-Box-dependent E3 ligase, interacts with Smad1/4 to regulate the BMP signaling pathway.[50] CHIP also associates with Smad3 to function as a negative regulator of TGF-β signal transduction.[51] Unlike ROC1–SCF^{Fbw1a} however, CHIP decreases total Smad3 levels independent of TGF-β activation. Overexpression of CHIP attenuates the cytostatic effects of TGF-β. Reductions in endogenous CHIP protein levels by knock-down enhance cellular sensitivity to TGF-β signaling. CHIP may desensitize the cell to TGF-β by decreasing basal Smad3 levels.

Ectodermin/TIF1γ, a RING-type E3 ligase, is essential for the specification of the ectoderm. This protein acts by restricting the mesoderm-inducing activity of TGF-β signals to the mesoderm, which favors neural induction.[52] Ectodermin/TIF1γ binds to Smad4 and induces Smad4 ubiquitination and degradation. Depletion of Ectodermin/TIF1γ from human cancer cell lines enhances the cytostatic effects of TGF-β. Ectodermin/TIF1γ mediates these biological responses by down-regulating Smad4 expression, which results in the repression of both TGF-β and BMP signaling. He *et al*. identified Ectodermin/TIF1γ as a protein that selectively bound only the receptor-activated subset of Smad2 and Smad3 proteins.[53] In these experiments, overexpression of Ectodermin/TIF1γ inhibited the binding of Smad2/3 to Smad4, whereas its depletion augmented the binding of Smad2/3 to Smad4. The converse was also true when Smad4 levels were manipulated. Although Ectodermin/TIF1γ inhibited Smad4-dependent gene responses, He *et al*. were not able to demonstrate that Ectodermin/TIF1γ targets Smad4 for ubiquitination and degradation. The two models are not mutually exclusive, each scenario may dominate in different cellular contexts.

Genetic aberration and alterations in expression of E3 ubiquitin ligases in human cancer

As TGF-β signaling is tightly regulated by numerous E3 ubiquitin ligases, dysregulated expression or functionality of such E3 ubiquitin ligases may affect the proper transmission of TGF-β signaling, contributing to cancer development. In support of this theory, misregulated expression or aberrant function of E3

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ligases, such as Smurfs, Arkadia, WWP1, Ectodermin/TIF1γ, Skp2, and Fbw1a, is observed in several human cancers.

High expression levels of Smurf2 correlates with increased depth of invasion and lymph node metastases and poor survival.[54] An inverse correlation between Smurf2 expression and phospho-Smad2 levels is also observed in cancers. In patients with esophageal squamous cell carcinoma, elevated expression levels of Smurf2 correlated with tumor development and a poor prognosis, suggesting that the repression of TGF-β signaling by Smurf2 occurs during tumor development in humans. The *Smurf1* gene, mapping to 7q21.1-31.1, was amplified and overexpressed in pancreatic cancer.[55]

SnoN is overexpressed in a variety of tumors. Several esophageal cancer cell lines have lost the ability to degrade SnoN following TGF-β ligation. Although all the components of the TGF-β pathway are present and functional in SEG-1 cells, a Barrett's-associated esophageal adenocarcinoma cell line, this cell line is resistant to TGF-β-mediated growth inhibition.[56] Levy *et al*. demonstrated that SEG-1 cells have lost Arkadia expression and exhibit deficient SnoN degradation in response to TGF-β.[43] Reintroduction of Arkadia restored TGF-β-induced Smad3/4-dependent transcription and SnoN degradation. These results suggest that the loss of Arkadia may contribute to tumorigenesis by increasing SnoN expression.

The amplification of 8q21 occurs in a large percentage of prostate and breast cancers; WWP1 is located at this region. WWP1 is frequently overexpressed in prostate and breast cancers; the degree of overexpression correlates significantly with copy number.[57,58] Forced overexpression of WWP1 enhanced cell proliferation, whereas gene silencing of WWP1 mRNA suppressed it. Therefore, WWP1 likely functions as an oncogene in prostate and breast cancers.

Conclusion and perspectives

Recent progress studying TGF-β signaling mechanisms has revealed the important role for ubiquitin-dependent proteasomal degradation in regulating TGF-β signaling (Table 1). Disruption of ubiquitin-dependent degradation of the components of TGF-β signaling can lead to cancer development. These E3 ubiquitin ligases regulating the TGF-β signaling pathway may be candidates for pharmacological cancer therapies. Small molecule inhibitors of E3 ubiquitin ligases (e.g. Mdm2 and TRAF6) have recently been developed.[59] Further research examining the role of the ubiquitin–proteasome system in regulating TGF-β signaling will provide additional insight into the development of small-molecule or peptide-based inhibitors for future therapeutic treatments.

Acknowledgments

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