**REVIEW**

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# Negative regulation of TGF-β signaling in development

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## ABSTRACT

The TGF-β superfamily members have important roles in controlling patterning and tissue formation in both invertebrates and vertebrates. Two types of signal transducers, receptors and Smads, mediate the signaling to regulate expression of their target genes. Despite of the relatively simple signal transduction pathway, many modulators have been found to contribute to a tight regulation of this pathway in a variety of mechanisms. This article reviews the negative regulation of TGF-β signaling with focus on its roles in vertebrate development.

***Keywords:*** TGF-β, BMP, Nodal, Activin, antagonist, development, negative regulation.

## INTRODUCTION

Transforming growth factor beta (TGF-β) and its related factors play vital roles in development and tissue homeostasis by regulating cell proliferation, differentiation, apoptosis and migration. These secreted factors utilize a fairly simple system to relay their signals to these cellular events via modulation of gene expression (Fig. 1). Two kinds of receptors, type I and type II receptors, both of which contain the ligand-binding extracellular domain, a single membrane-spanning domain and the Ser/Thr kinase-containing intracellular domain, are required for their signal transduction. Upon ligand binding, these two types of receptors form a hetero-oligomeric complex. In the complex, the phosphorylation of the type I receptor by the constitutively active type II receptor kinase leads to its activation. The activated type I receptor kinase, in turn, phosphorylates and thus activates downstream signal-mediators Smad proteins. Those receptor-activated Smads then associate with the common Smad, Smad4, and the resulted complexes enter the nucleus to regulate expression of target genes in collaboration with other transcription factors.

Given the important functions of this superfamily members in both embryogenesis and tissue homeostasis in adults, it is not surprising that their relatively simple signal transduction pathways are tightly regulated extracellularly and intracellularly by many factors as well as by inputs from other signaling pathways. There are many review articles on TGF-β signaling and its functions in physiological and pathological conditions [1-8]. This review focuses on current understanding of negative regulation of TGF-β signaling in development.

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## MODULATION OF LIGAND ACTIVITY

The members of the TGF-β superfamily are secreted factors and function in both autocrine and paracrine manners. Their signaling activity is controlled by a variety of ligand-binding proteins or proteins competing for receptor-binding (Fig. 1).

**Chordin and Noggin:** Many growth factors in the TGF-β superfamily, such as Activin, Nodal and bone morphogenetic protein (BMP), function as morphogens that determine different cell fates at different concentrations in formation and patterning of embryos. Morphogenic gradients in embryos may be shaped by ligand-binding proteins [8]. For instance, Decapentaplegic (Dpp), a BMP homolog in *Drosophila*, distinguishes the amnioserosa from the epidermis of the dorsal ectoderm of the *Drosophila* embryo, and the metalloprotease Tolloid (Tld) and the Short gastrulation (Sog) proteins are required to shape a gradient of the Dpp activity [9]. Sog interacts with and prevents Dpp to activate its cognate receptors while Tld processes Sog and thereby liberates Dpp for signaling. Sog also

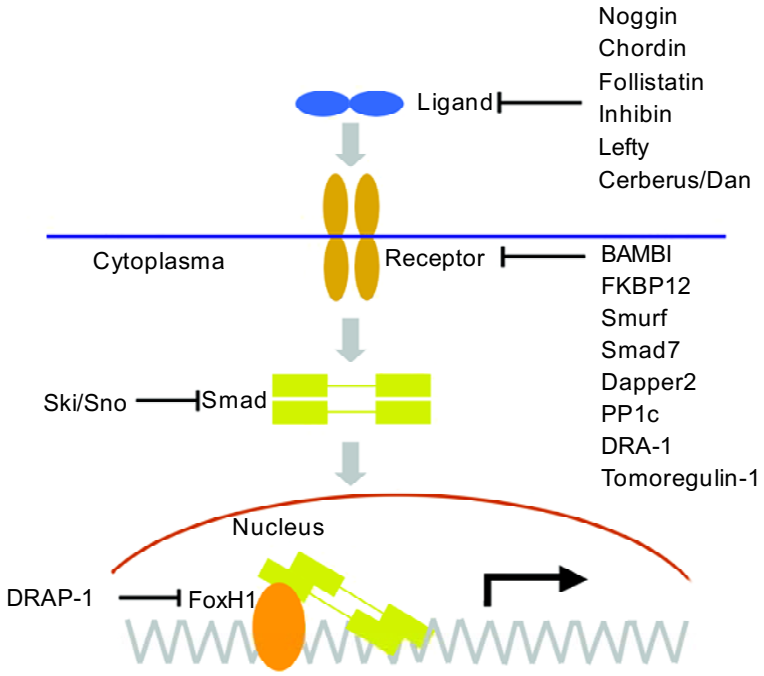


**Fig. 1** The negative regulators of the signaling of TGF-β and its related factors.

contributes to the Dpp gradient formation by promoting Dpp transport, and a coordinative action of Tld, Sog and Dpp are needed to create a sharp boundary of Dpp signal during dorsoventral patterning of the *Drosophila* embryo [10, 11]. Recently, the glypican members of heparin sulfate proteoglycan have been shown to play an important role in formation of a long-range concentration gradient of Dpp to direct the anteroposterior patterning of the insect wing [12].

Chordin and Noggin are BMP-binding proteins secreted in the Spermann's organizer, the dorsal lip of the amphibian gastrula embryo, which is a signaling center for neural induction and mesoderm dorsalization [6]. Both proteins specifically interact with BMP, but not with Activin or TGF-β, and inhibit BMP signaling by interfering BMP binding to its signaling receptors [13, 14]. BMPs are expressed mainly on the ventral side of the embryo and their signaling transforms the ventral ectoderm into the epidermis and induces ventral mesoderm [15, 16]. Inhibition of BMP signals by dorsally-produced Chordin and Noggin enables the dorsal ectoderm to develop into neural tissues and helps to establish the ventralizing BMP signaling gradient for mesodermal patterning [13, 14]. Chordin and Noggin also promote the inductive and trophic activities of rostral organizing centers in forebrain development in the mouse [17]. Besides, Noggin is required for patterning of the neural tube and somites and for proper skeletal development [18, 19]. Identification of heterozygous Noggin missense mutations in patients with two autosomal dominant disorders of joint development, multiple synostosis syndrome and proximal symphalangism, both of which are characterized by bony fusions of joints, has evidently revealed the importance of Noggin in joint formation of limbs [20]. Noggin may be involved in a negative feedback regulation of BMP signaling as its expression is induced by BMP in cultured chondrocytes and osteoblasts [21, 22].

**Follistatin:** Besides an important function in the reproductive system by stimulating follicle-stimulating hormone (FSH) secretion from the pituitary, Activin is able to induce various mesoderm tissues in a concentration-dependent manner in several vertebrate species [23, 24]. Follistatin was originally identified as a secreted glycoprotein to inhibit Activin activity in inducing FSH release [25]. Follistatin can inhibit Activin and myostatin activity by interference of their binding to its type II receptor [26]. Genetic studies in mice have revealed important functions of follistatin in mouse development as follistatin-deficient mice have numerous embryonic defects including shiny and taut skin, growth retardation, and cleft palate leading to death within hours of birth [27]. Overexpression of follistatin in skeleton muscle cells of mouse embryos lead

to dramatic increase in musle mass [28]. Like Chordin and Noggin, follistatin produced in the organizer in *Xenopus* embryos forms complexes with BMPs and inhibit ventralizing and anti-neuralizing activity of the BMP signal [29, 30].

**Inhibin:** Activin activity is also modulated by its structurally related inhibin. Activin functions in the form of homodimer of 14-kD β subunits linked by a disulfide bond whereas inhibin acts in heterodimer of a β subunit and an 18-kD α subunit [23]. Inhibin antagonizes Activin activities in stimulating FSH release, erythroid differentiation and chondrogenesis [31-33]. Inhibin exerts its inhibitory effects on Activin by competing for receptor binding. Although inhibin exhibits a low binding affinity to the Activin type II receptors, betaglycan, a transmembrane cell surface proteolgycan also known as a type III receptor for TGF-β, dramatically increases this binding [34].

**Lefty:** Lefty/Antivin are secreted proteins and can inhibit Activin/Nodal signaling through competition for binding to Activin receptors [7]. One study also found that Lefty proteins could directly bind to Nodal ligand, suggesting a novel mechanism [35]. Nodal proteins have been identified as key endogenous inducers for mesoderm induction and important factors in left-right axis determination in vertebrate development [7]. The products of mouse *Lefty1* and *Lefty2* genes and zebrafish *antivin* (*lefty1*) gene are highly related proteins in structure, and they are divergent members of the TGF-β superfamily and lack a cysteine required for dimer formation [36]. They modulate Nodal signaling during vertebrate gastrulation as feedback inhibitors [37, 38]. Mouse mutants for Lefty2 have an expanded primitive streak and form excess mesoderm, a phenotype opposite to that of mutants for Nodal, whereas overexpression of *antivin* or *lefty2* in zebrafish embryos inhibits head and trunk mesoderm formation, a phenotype identical to that of mutants caused by loss of Nodal signaling. The mesoderm-inhibiting activity of Lefty/Antivin can be suppressed by increasing levels of Activin/Nodal or its receptors [36-38]. A recent study suggested that expression of Lefty/Antivin is dependent on Nodal signaling, indicating a feedback loop wherein Nodal signals induce expression of their antagonists Lefty2 and Antivin to restrict Nodal signaling during gastrulation [39].

**Cerberus and Dan:** The Cerberus/Dan family members were initially identified as BMP antagonists in development processes by binding to BMPs and preventing their interaction with the signaling receptors [3, 40]. The family members include Cerberus, DAN, Gremlin, Caronte, Charon and others, which share a 90-amino-acid cysteine-rich region, a motif known as the cysteine knot that is present in the TGF-β family members [41]. In addition to being involved in the regulation of BMP activity, Cerberus also functions as an antagonist of Nodal and Wnt signals [42]. Cerberus binds these ligands at different sites: BMP4 and *Xenopus* Nodal-related factor Xnr1 bind to the cysteine-rich region of Cerberus, whereas Wnt8 interacts with its amino-terminal half. Misexpression of Charon in zebrafish produced phenotypes similar to those of mutant embryos defective in Nodal signaling or embryos overexpressing Antivin/Lefty1 [43]. Furthermore, Charon also inhibited the dorsalizing activity of all three of the known zebrafish Nodal-related proteins (Cyclops, Squint and Southpaw), and knocking down Charon expression led to a loss of Left/Right polarity, indicating that Charon is a negative regulator of Nodal signaling during left-right patterning.

**Nodal and BMPs:** Nodal can form heterodimers with BMP3 or BMP7 *in vitro*, with a comparable affinity for Nodal itself, resulting in mutual inhibition of Nodal and BMP signals [44]. Since Nodal and BMP signaling pathways play opposite roles in the dorsal-ventral patterning of vertebrate embryos, this finding suggests an interesting mechanism without involvement of downstream mediators Smad2 and Smad3 for Nodal signaling and Smad1 and Smad5 for BMP signaling. However, it needs to test whether this mechanism is used *in vivo*.

## MODULATION OF RECEPTOR ACTIVITY

Many proteins have been found to associate with the signaling TGF-β receptors. It has been known for a while that some accessory proteins such as betaglycan promote TGF-β signaling by facilitating ligand binding to the signaling receptors [24]. The EGF-CFC proteins are essential for signal transduction of the TGF-β superfamily members Nodal, Vg1 and GDF1 [7]. However, many of the receptor-binding proteins have been suggested to attenuate receptor activity (Fig. 1).

**FKBP12:** FKBP12 is an abundant cytosolic protein that is well known for its roles in mediating immunosuppression of small molecules FK506 and rapamycin [45]. Besides binding to the protein phosphatase calcineurin in the presence of FK506 and to the protein kinase FRAP/RAFT in the presence of rapamycin and therefore inhibiting their activities, respectively, FKBP12 also interacts with several other proteins including calcium channels inositol triphosphate receptors and ryanodine receptors as well as TGF-β family type I receptors [3]. Overexpression of FKPB12 attenuates TGF-β signaling [46, 47], and this inhibitory effect needs physical interaction between FKPB12 and the TGF-β type I receptor TβRI/ALK5 [47-49]. FKBP12 associates with ALK5 in the GS domain preceding the Ser/Thr kinase domain in the cytoplasmic domain and physically blocks the type II receptor-mediated phosphorylation in the GS domain, which is required for ALK5

activaiton. These results were supported from the subsequent crystal structure analysis [47, 50]. Although the importance of FKPB12 in modulating the signals of the TGF-β family members in development remains unclear [51], the biochemical studies strongly suggest that FKBP12 controls the basal activity of the receptors, thus functioning as a safe guard to prevent leaky signals resulted from promiscuous formation of receptor heterocomplexes in the absence of ligands [47].

**BAMBI:** BAMBI is a type I transmembrane protein which acts as an pseudoreceptor to inhibit BMP signaling during *Xenopus* embryogenesis [52]. It is co-expressed with the ventralizing morphogen BMP4 during embryonic development of *Xenopus*, zebrafish and mouse, and its expression requires BMP signaling [52-54], indicating that BAMBI plays a role in a negative feedback regulation of BMP signaling. Analysis of BAMBI mRNA expression pattern also suggested a role of BAMBI in modulating TGF-β superfamily signaling in spermatogenesis [55]. In addition, overexpression of BAMBI blocks TGF-β and Activin signals in transcriptional activation [52], which is consistent with its possible role in tumorogenesis. BAMBI, also referred as to nma, negatively regulates TGF-β signaling and induce cell growth and invasion in human gastric carcinoma cell lines [56]. Furthermore, β-catenin interferes with TGF-β-mediated growth arrest by inducing the expression of BAMBI, and this may contribute to colorectal and hepatocellular tumorigenesis [57]. Biochemical analyses suggest that BAMBI does not bind to TGF-β and BMP, instead it interferes with TGF-β signaling by binding to the receptors to prevent the formation of receptor complexes [52].

**Smurf-Smad7:** Two Smurf proteins have been described to date and they are the ubiquitin E3 ligases of the HECT family. Although Smurf-1 was initially found to bind to Smad1 and control the basal level of Smad1 protein via the ubiquitination-proteosome degradation mechanism [58], subsequent studies suggest that both Smurf1 and Smurf2 may control the cell surface receptor level [59, 60]. Indeed, Smurf proteins are able to interact with ALK5 via the inhibitory Smad7. Furthermore, Smurfs negatively regulate TGF-β signaling by targeting activated Smad proteins for degradation [61, 62]. The function of Smurfs has been examined in several organisms. For instance, Smurf1 and Smad6 cooperatively induced secondary axes in *Xenopus* embryos by antagonizing BMP signals as Smurf1 bound to BMP type I receptors via inhibitory Smads and induced ubiquitination and degradation of these receptors [63]. *Drosophila* Smurf regulates fly embryogenesis by controlling the amplitude and the duration of the cellular response to Dpp signals [64], and its overexpression disrupts imaginal disc development [65]. Smurf specifically targets phosphorylated MAD, the Smad1/5 ortholog, to proteasome-dependent degradation [65].

**Dapper2:** Dapper was originally identified as a Dishevelled (Dsh)-binding protein by yeast two-hybrid screening, and it functions as a general antagonist of Wnt signals by inhibiting both the canonical β-catenin pathway and the non-canonical c-Jun N-terminal kinase pathway [66]. Inhibition of maternal Dapper expression results in loss of the notochord and head structures in *Xenopus* embryos, indicating that Dapper is required to modulate Wnt signaling for normal vertebrate development. Interestingly, another Dsh-binding protein, Frodo that was also identified by yeast two-hybrid screening and shares a high homology to Dapper, was shown to be required for Wnt signaling [67]. Frodo synergizes with Dsh in the secondary axis induction in *Xenopus* embryos. Furthermore, interference of Frodo activity blocks axial development in response to XDsh and XWnt8 [67]. A recent study showed that Frodo interacts with the transcription repressor TCF3 and synergizes with Dapper in inducing head formation [68]. Further investigations are needed to clarify the roles of Dapper and Frodo in regulating Wnt signaling during development.

We have found that Dapper2, which is divergently related to Dapper, interferes with Nodal signals in mesoderm induction in zebrafish [69]. Knockdown of Dapper2 expression by morpholino-antisense oligonucleotides enhanced mesoderm markers, whereas its overexpression resulted in eye fusion, a phenotype resembling that of one-eye pinhead mutant with defective Nodal coreceptor. Dapper2 expression depends on Nodal signals. It specifically associates with ALK5 and Activin/Nodal type I receptor ALK4 with a high binding affinity to the activated receptors. Dapper2 protein is mainly localized in late endosomes and targets receptors for lysosomal degradation [69]. Therefore, Dapper2 controls endocytosed activated receptors transport from late endosomes to lysosomes for degradation. By doing so, it functions to finely tune Nodal signaling in mesoderm formation, and possibly in other developmental processes. Interestingly, internalization of the TGF-β type II receptor and the type III receptor (betaglycan) has been shown to be mediated by β-arrestin in cultured cells, leading to down-regulation of TGF-β signaling [70].

**Protein phosphatase 1:** SARA (Smad anchor for receptor activation) was found to bind to TGF-β receptors as well as Smad2/3 and was suggested to work as a scaffold protein to bring Smad substrate to the receptors and thus facilitate Smad activation [71]. SARA also binds the catalytic subunit of type 1 serine/threonine protein phosphatase (PP1c) in *Drosophila* [72]. Disruption of the interaction between SARA and PP1c results in hyperphosphorylation of the type I receptor and enhances Dpp signaling.

These results suggest that SARA targets PP1c to Dpp receptor complexes, where PP1c acts as a negative regulator to control the basal Dpp signaling, presumably by regulating the basal phospholyation level of the type I receptor [72]. Interestingly, PP1c can also be targeted to the TGF-β receptor complexes by GADD34, a regulatory subunit of PP1, and Smad7 [73]. Importantly, GADD34-PP1c recruited by Smad7 inhibits TGF-β-induced cell cycle arrest and confers TGF-β resistance in response to UV light irradiation. Thus, SARA and Smad7-GADD34 may collaborate in recruitment of PP1 to the TGF-β receptor complexes and in modulation of TGF-β receptor activity. The importance of TGF-β receptor dephosphorylation in development waits to be investigated.

**Tomoregulin-1:** Unlike other TGF-β factors, Nodal and Vg1/GDF1 require coreceptor of EGF-CFC proteins, such as Cripto and Cryptic in mouse and Oep in zebrafish, for their signal transduction. Tomoregulin-1, a transmembrane protein with two follistatin modules and an EGF domain, has been found to interact with Cripto and this interaction specifically inhibits Nodal signaling [74]. Tomoregulin-1 also blocks mesodermal induction by BMP2, but its mechanism is not known. While the two follistatin modules and the EGF domain of Tomoregulin-1 is required for its inhibition of Nodal signaling, the cytoplasmic tail is essential for its regulation of BMP activities [75]. In mouse, Tomoregulin-1 mRNA was detected in many mesodermal and ectodermal tissues of 8.5-day-old mouse embryos and in the brain of adult [76]. In *Xenopus*, Tomoregulin-1 is expressed from midgastrula stages onward and is enriched in neural tissue derivatives [75]. Together, these data suggest that Tomoregulin-1 may modulate Nodal and BMP activities during neural patterning.

**Lefty:** In addition to having an ability of binding to Activin/Nodal receptors and Nodal ligands, Lefty proteins have been found to bind to EGF-CFC proteins [35, 77]. This mechanism is adopted for antagonizing EGF-CFC-dependent Nodal and Vg1 signaling, but not for EGF-CFC-independent Activin signaling. It remains unknown where and when one mechanism would be the major one or different mechanisms work coordinately.

**BRA-1/BRAM-1:** TGF-β signaling has a vital function in the regulation of dauer larvae formation in response to starvation and other stress environment in Caenorhabditis elegans. BRA-1 was found to interacts with DAF-1, the type I receptor of the DAF-7 TGF-β pathway, and a loss-of-function mutation of bra-1 suppressed Dauer constitutive phenotype caused by the DAF-7 pathway mutation, indicating that BRA-1 is a negative regulator of the DAF-7 TGF-β pathway [78]. A human homolog of BRA-1, BRAM-1, has shown to associate with a BMP type I receptor ALK3 [79]. However, the physiological function of BRAM-1 awaits further investigation.

Several other proteins have been demonstrated to physically interact with TGF-β receptors and negatively modulate their activities. However, it still remains to be an open question whether these proteins regulate TGF-β signals in development. TβR-I-associated protein-1 (TRAP-1) was first identified to specifically interact with the activated TGF-β type I receptor ALK5 and attenuate its activity [80]. However, the subsequent study indicated that TRAP-1 bind selectively to inactive TGF-β and Activin receptor complexes and may function as a Smad4-chaperone to facilitate the transfer of Smad4 to the receptor-activated Smad proteins [81]. The TRAP-related protein TLP constitutively associates with TGF-β and Activin type II receptors as well as with Smad4 in a similar fashion as TRAP, and it specifies the signaling from the receptors to Smad proteins by promoting Smad2 signaling and suppressing Smad3 signaling [82]. STRAP, a WD domain-containing protein, associates with TGF-β type I, type II receptors as well as Smad7. Overexpression of STRAP inhibited TGF-β induced transcription, and this inhibition was further synergized by Smad7 [83]. STRAP stabilizes the association between Smad7 and the activated ALK5, thus assisting Smad7 in preventing Smad2 and Smad3 from accessing to the receptor [84]. Yes-associated protein (YAP65) associates with Smad7, promotes the interaction of Smad7 with the activated ALK5 and thus potentiates the inhibitory effect of Smad7 on TGF-β singaling (Ferrigno, 4879). In this regard, YAP65 is a functional anolog of STRAP. Another WD domain protein, TRIP-1, which was identified to interacts with the TGF-β type II receptor, selectively represses TGF-β-induced expression of plasminogen activator inhibitor-1, but not cyclin A [85, 86], whereas the recent evidence demonstrated that TRIP mediates the activation of TGF-β/Smad signaling by tartrate-resistant acid phosphatase and participates in bone remodeling [87]. In addition, two PDZ domain-containing proteins, ARIP1 and ARIP2, have been shown to interact with Activin type II receptors (ActRII) and negatively modulate Activin signaling albeit at different ways [88, 89]. ARIP1 is mainly expressed in brain and exists in two forms with a guanylate kinase domain in the NH2-terminal region of the long form, and both forms share two WW domains and five PDZ domains. ARIP1 binds to ActRIIA via its fifth PDZ domain and to Smad3 via its WW domains and suppresses Activin signaling in neuronal cells, likely by sequestering Smad3 in the cytoplasm [88]. In contrast, the single PDZ domain in the NH2-terminal region of ARIP2 associates with ActRII whereas the COOH-terminal region interacts with RalBP1, a binding protein of small GTPase Ral that regulates receptor endocytosis. By enhancing endocytosis of ActRII through the Ral/TalBP1-dependent pathway, ARIP2 sup-

presses Activin signals [89].

## MODULATION OF DOWNSTREAM TRANSCRIPTION MEDIATORS

**Ski/Sno:** Ski and its closely-related Sno are proto-oncogenes whose upregulation promotes tumorigenic transformation [90]. Although they are described to interact with several proteins, the interaction with Smad proteins is better understood. By association with of Smad2, Smad3, Smad4 and Smad complexes (Smad1-Smad4 or Smad5-Smad4), Ski and Sno repress TGF-β and BMP signaling [90]. Studies in model animals have established the importance of Ski in regulating neural and muscle formation [91, 92]. In *Xenopus* embryos, overexpression of Ski induces the secondary neural axis formation and neuronal-specific gene expression in ectoderm explants, and this neural-inducing activity requires the ability of Ski in inhibiting BMP signaling [92]. The ski-null mice show defects in cranial neural tube closure leading to exencephaly and a marked decrease in skeletal muscle mass [93]. The ability of Ski to regulate craniofacial development may be related to its antagonizing effect on BMP signaling as facial clefting and exencephaly have been also observed in transgenic mice overexpressing the BMP target gene Msx2 [94]. Thus, it seems that the function of Ski in development is via antagonizing BMP signaling while its role in promoting oncogenic transformation is mainly through regulating TGF-β signaling.

**FoxG1:** FoxO proteins, members of Forkhead transcription factor family, have diverse functions in metabolism, cell proliferation and differentiation, and neoplasia [95]. FoxOs have been found to form a complex with TGF-β-activated Smad3 and Smad4 in the promoter of p21Cip1, a cell proliferation inhibitory gene, and the complex activates its expression [96]. On the other hand, another Forkhead transcription factor FoxG1, which is essential for the development of the cerebral hemispheres of the brain [97-99], is able to bind to the Smad-FoxO complexes and inhibit the expression of p21Cip1 [96]. It can be speculated that during telecenphalon development FoxG1 functions to antagonize TGF-β signaling and thus to prevent premature growth arrest and differentiation of neuroepithelial progenitor cells.

**DRAP1:** DRAP1 was first identified as a regulatory partner for the transcription repressor Dr1 [100]. Inactivation of Drap1 gene by gene targeting in mouse led to embryonic lethality due to severe defects in primitive streak with excess mesodermal cells, a phenotype resembling one of Lefty 2 knockout [101]. The mesoderm defects in Drap1 mutants were partially suppressed by the reduction of Nodal activity. Further experiments demonstrated that DRAP1 interacts with the winged-helix transcription factor FoxH1 (FAST), which is important for Activin/Nodal signaling [102], and inhibits the DNA binding activity of FoxH1. Thus, DRAP1 may function to limit the morphogenetic signal of Nodal by down-regulating the transcriptional response to the Nodal positive feedback loop [101].

## SUMMARY

The TGF-β signaling pathway is a very conserved pathway utilized by multicellular organisms from worm, fly, fish, amphibian to human. The signaling components and the modulation machineries are also preserved evolutionally: there are two types of receptors, three classes of Smad proteins (R-, Co- and I-Smads). Interestingly, compared to a limited numbers of positive regulators that promote TGF-β/Smad signaling, such as Nodal coreceptors, Arkadia, ARC105, and p53 [7, 103, 104], much more factors have been identified to negatively modulate TGF-β/Smad signaling. This may attribute to the fact that TGF-β and related growth factors can act in both autocrine and paracrine manners. By considering the facts that TGF-β superfamily members play central roles in development and tissue homeostasis and that many of them are almost ubiquitously expressed, the negative regulators are apparently required to prevent unwanted leaky signal propagation. It is vital to control their signal output tightly and precisely by many different modulators. Because TGF-β signaling transduction is a multi-step process, there should exist some more unknown negative regulators that will be identified in the future. For example, little has been known about proteins that control recycling of endocytosed, activated TGF-β receptors. Such proteins can be important players for specific developmental pathways. Furthermore, future efforts should be made to clarify the significance of different mechanisms for regulating TGF-β signaling in a specific developmental process.

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