Acta Biochim Biophys Sin, 2018, 50(1), 12–36
doi: 10.1093/abbs/gmx126
Advance Access Publication Date: 27 December 2017
Review

Review

TGF-β family co-receptor function and signaling

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Received 1 November 2017; Editorial Decision 6 November 2017

Abstract

Transforming growth factor-β (TGF-β) family members, which include TGF-βs, activins and bone morphogenetic proteins, are pleiotropic cytokines that elicit cell type-specific effects in a highly context-dependent manner in many different tissues. These secreted protein ligands signal via single-transmembrane Type I and Type II serine/threonine kinase receptors and intracellular SMAD transcription factors. Deregulation in signaling has been implicated in a broad array of diseases, and implicate the need for intricate fine tuning in cellular signaling responses. One important emerging mechanism by which TGF-β family receptor signaling intensity, duration, specificity and diversity are regulated and/or mediated is through cell surface co-receptors. Here, we provide an overview of the co-receptors that have been identified for TGF-β family members. While some appear to be specific to TGF-β family members, others are shared with other pathways and provide possible ways for signal integration. This review focuses on novel functions of TGF-β family co-receptors, which continue to be discovered.

Key words: activin, BMP, co-receptor, SMAD, TGF-β

Introduction

Transforming growth factor-β (TGF-β) family members are secreted cytokines that regulate a plethora of diverse biological responses, including cell proliferation, migration, survival, and differentiation. Thirty-three different human genes have been identified that encode structurally related homomeric (and also heteromeric) TGF-β family members, which are highly conserved over evolution (for a review: [1]). Members of this family play pivotal roles in embryogenesis and in maintaining tissue homeostasis in the adult lives of all multicellular animals. Genetic changes that induce misregulation in TGF-β family signaling have been causally linked to various human diseases, including cancer, fibrosis, bone-, muscle-, cardiovascular-, and auto-immune diseases (for a review: [2]).

TGF-β family members exert their cellular function via two structurally related transmembrane proteins, i.e. Type I [also termed activin receptor-like kinases (ALKs)] and Type II receptors, which are endowed with intrinsic serine/threonine kinase domains (for a review: [3]). In humans, five Type II receptors and seven Type I receptors have been identified. Each family member selectively binds and signals via its own repertoire of Type I/Type II combinations. In a simplistic mechanistic model, the ligand mediates and stabilizes the formation of heteromeric Type I/Type II complexes at the plasma membrane, with the receptor assembly comprising two Type I and two Type II receptors possibly each of the same kind. As a result, the intracellular domains interact with each other enabling the Type II kinase to phosphorylate the Type I receptor on specific serine and threonine residues

in a glycine-serine rich domain termed the GS-box [4]. This transphosphorylation then activates the Type I receptor kinase, which subsequently phosphorylates cytoplasmic effectors to initiate intracellular signaling cascades such as the SMAD-signaling pathway (for a review: [5]). This seemingly well-defined sequence of events leads to the transduction of an extracellular signal across the plasma membrane into the cytosol and finally to the nucleus to regulate TGF-β factor-mediated gene transcription. However, this accepted textbook model is rather oversimplified. Data from Rik Derynck's group show for instance that bone morphogenetic protein (BMP)-mediated SMAD signaling requires an arginine methylation step for the removal of an otherwise signaling-inhibiting SMAD6 protein ahead of transphosphorylation of the TGF-β/BMP receptor kinases [6]. Also on the extracellular side, the above-presented model is certainly not fully accurate. For example, structural biology and biochemistry have always drawn pictures of a symmetrical hetero-tetrameric receptor complex, which results from the symmetrical homodimeric nature of the growth factor that mediates the assembly of the receptors (for recent reviews: [1,7]). However, the long-claimed existence of heterodimeric TGF-β factors [8] with their skewed architecture and hence dissymmetrical receptor-binding preferences predicts the formation of TGF-β ligand-receptor complexes composed of up to four different Type I and Type II receptors. The latter of which are often inferred to explain the vastly different activities (and sometimes, the unique biological functions) of such heterodimeric TGF-β ligands (see also: [1,9]). However, even for homodimeric TGF-β ligands, a symmetrical assembly is not an indispensable condition given the high promiscuity with which TGF-β ligands can usually bind various Type I and Type II receptors (see also: [10]; for reviews: [11–13]). Given the small differences in binding affinities between the various receptors bound by promiscuous TGF-β ligands the composition/stoichiometry of a TGF-β receptor assembly might therefore only be a consequence of the different binding affinities of a given TGF-β ligand and the expression levels of the receptors on the cell surface. In line with this hypothesis, data on BMP6 receptor activation suggested that even for homodimeric TGF-β ligands asymmetric receptor assembly may be required for some signaling events [14]. Whereas non-glycosylated BMP6 incapable to bind ALK2, but capable to interact with ALK3 and ALK6, was found inactive to induce alkaline phosphatase (ALP) expression in C2C12 cells, glycosylated BMP6 capable to bind ALK2 and ALK3 as well, was however able to do so [14,15]. As the ALP expression mediated by glycosylated BMP6 could be blunted by addition of an ALK3-neutralizing antibody, both ALK3 and ALK2 seem to be part of the receptor assembly mediated by BMP6, however, a BMP6 receptor assembly comprising only of ALK3 as the Type I receptor is incapable to deliver the signal [15]. Furthermore, for some cellular activities, it was demonstrated that the presence of only one Type I receptor is sufficient to fully activate the required subcellular signaling cascades, thus leaving room for speculations as to whether the unoccupied Type I receptor-binding epitope, the so-called wrist epitope, participates in the interaction with other membrane bound and/or membrane-associated structures [10,16].

The SMAD cascade is the most prominent signaling pathway regulated by TGF-β members and separates all TGF-β members into two distinct classes (for a review: [5]). While activated TGF-β, activin and nodal Type I receptors, i.e. ALK5, ALK4, and ALK7, respectively, induce phosphorylation of receptor-regulated (R-) SMAD2 and -3, activated BMP Type I receptors, i.e. ALK1, ALK2, ALK3, and ALK6 mediate R-SMAD1, -5, and -8. Activated R-SMADs then form heteromeric complexes with common mediator (Co)-SMAD4, and these complexes can travel to the nucleus, where together with other DNA-binding transcription factor and co-repressors/co-activators and chromatin modulators, they regulate specific gene transcriptional responses (for a review: [17]). In addition to the canonical SMAD pathway that is unique for TGF-β family receptor-initiated signaling, non-SMAD-signaling pathways can also be activated, including ERK, JNK and p38 MAP kinases, PI3K/AKT and GTPases (for a review: [18]). Each step of the TGF-β family pathway at the extra- and intracellular levels is carefully controlled and provides means for other pathways to crosstalk (for a review: [19]). There is thus a striking conversion in signaling: over 30 TGF-β family ligands signal via five Type II and seven Type I receptors and two main SMAD pathways, and this observation appears to be at odds with the multifunctional properties of TGF-β family members. Hence, the key determinants that control intensity, duration, specificity and diversity are cell surface co-receptors (or accessory receptors); they interact with TGF-β family ligands and/or signaling Type I and Type II receptors.

Co-receptors are distinct from signaling Type I and Type II receptors in that they do not possess a functional enzymatic motif (for a review: [20]). They can be transmembrane proteins that only contain a very short intracellular region, or can be proteins that are associated with the membrane by a glycosylphosphatidylinositol (GPI) anchor and are completely devoid of intracellular domains. Recently, however, co-receptors, such as the receptor tyrosine kinase MuSK (muscle-specific kinase), have been identified whose intrinsic enzymatic activity is not regulated by TGF-β family members [21]. Co-receptors may share other ligands besides TGF-β family members and thereby provide means for signal integration [22]. Co-receptors may also perform a more structural function, e.g. by mediating cell adhesion of adjacent cells through extracellular protein–protein interactions [23], or by direct interaction of intracellular domain with cytoskeletal proteins [24].

Frequently, co-receptors demonstrate lower affinities for TGF-β family members, but are more abundant than the signaling receptors. When a ligand engages the cell, it may initially interact with co-receptors, which thereafter present it to (or sequester it from) the signaling receptors [25], and determine signaling intensity and duration. Co-receptors have been shown to determine whether a certain cell responds to a ligand [26] or they can act as a decoy receptor and thereby antagonize signaling [27]. The intracellular domains of co-receptors can be involved in the recruitment of intracellular components and thereby specify, modulate and even initiate signaling responses. Co-receptors may determine the subcellular localization, internalization and trafficking of signaling receptors and thereby modulate their activity and stability. Moreover, for many co-receptors soluble extracellular forms exist [28], which are produced by enzymatic-mediated shedding or by alternative mRNA splicing. Soluble forms may antagonize or regulate TGF-β family signaling differently from the membrane-associated forms or can even function as ligands and initiate signaling responses on their own. This review provides an overview of the TGF-β family co-receptors that have been identified, and focuses on their novel emerging functions.

Do Co-receptors Act Differently on TGF-β Ligands Activating a Different SMAD Pathway?

On the basis of the phylogenetic relationships of the ligands the members of the TGF-β family can be separated into four main ligand families, the TGF-βs, the activins and inhibins, the BMPs and GDFs and a forth subgroup comprising all residual members that do

not fit into one of the other three subfamilies. However, a simpler classification is also possible from the major signaling pathway induced by TGF-β family members. Here, TGF-βs, activins and inhibins, nodal and a few BMPs and GDFs (e.g. GDF1, -3, -10, BMP3, and GDF8/myostatin and GDF11) activate the SMAD2/3 branch of the SMAD-signaling pathway, whereas a large group of BMPs and GDFs signal through the SMAD1/5/8 branch. Except for TGFβ-s, the selection of which SMAD branch will be activated seems to be an exclusive either/or choice and depends on the Type I receptor present in the heteromeric ligand-receptor assembly. While this binary allocation might appear oversimplified various signaling properties support by this classification and suggest that this paradigm and its consequences are evolutionarily based. For example, signaling by the SMAD2/3-activating activins, GDF8 and 11, and in part also of TGF-βs displays some signaling features reminiscent of circulating protein hormones, whereas most SMAD1/5/8-activating BMPs and GDFs (except for BMP9 and 10) act in the predominant cases like paracrine factors (often in a morphogenic fashion). While TGF-βs and a few SMAD2/3-activating GDFs are latent, i.e. the processed proprotein complex is sufficiently stable to demand active release of the activity-bearing C-terminal mature growth factor domain, SMAD1/5/8-activating BMPs and GDFs seem to be readily active despite these factors are also secreted as proprotein complexes. Third, for TGF-βs, activins and GDF8 only one secreted antagonist family (i.e. follistatin and follistatin-like) is known, suggesting that regulation of their activity in the extracellular space is less stringently or otherwise controlled. In contrast, for the group of SMAD1/5/8-activating BMPs and GDFs, nature has developed a plethora of secreted antagonists that tightly regulate the activities of these factors and actively take part in the formation of morphogenic gradients; however, despite similarities in their mechanistic function, these antagonists seem to lack any structural similarities. It seems therefore reasonable to assume that co-receptors modulate the functionalities of TGF-β factors possibly differently between these two major subgroups. Conformingly and noteworthy, a number of co-receptors have been described for TGF-βs, activins (and the activin antagonists inhibins) and nodal, whereas co-receptors for the SMAD1/5/8-activating BMPs and GDFs have remained unknown and mysterious for a long time. Hence, in this review, the co-receptors and their significance to TGF-β members are separated into two major chapters, which focus first on co-receptors for SMAD2/3-activating TGF-β members and then address the co-receptors modulating the activities of SMAD1/5/8-activating BMPs and GDFs.

The Co-receptors of SMAD2/3-activating TGF-βs, Activins and GDFs

Endoglin: a SMAD-signaling pathway switch for TGF-βs

Endoglin and structurally related betaglycan represent transmembrane proteins with large extracellular domains (561 and 766 amino acids, respectively), hydrophobic transmembrane domains, and short cytoplasmic domains (only 47 and 42 residues in endoglin and betaglycan, respectively) [29,30] (**Fig. 1**). Both glycoproteins are mainly expressed as homodimers, and heteromeric endoglin-betaglycan complexes have been identified as well [35]. In the case of endoglin, both monomers are covalently linked by disulfide bonds, whereas the betaglycan dimer is stabilized by non-covalent interactions. Endoglin and betaglycan share stretches of high sequence similarity and consequently they presumably also share similar biological functions. This similarity, especially within the cytoplasmic domain, constitutes the most highly conserved region among homologous members from different mammalian species [36] and is also demonstrated by the alternative splice forms found in mouse and human tissue [37–39]. The cytoplasmic domains of both co-receptors can be phosphorylated by serine/threonine kinases such as TGF-β Types I and II receptors [40–44].

Phosphorylation of endoglin does not seem to regulate downstream SMAD signaling. Instead it seems to be involved in SMAD-independent signaling processes, which regulate the influence of endoglin in cellular processes, such as cell growth and cell adhesion. Patterning of endoglin phosphorylation by TGF-β receptors appears to be rather complex, as addressed by studies utilizing constitutively active (ca) forms of TGF-β Type l receptors either alone or in combination with the wild-type form of the Type II receptor TGF-βRII. A site-directed mutagenesis approach identified serines 634 and 635 in endoglin's cytoplasmic domain as targets for ALK5 and TGF-βRII phosphorylation, whereas ALK1 preferentially phosphorylates endoglin only at threonine residues. Further mutagenesis studies revealed that these threonine residues can only be phosphorylated after prior phosphorylation of the serine residues mentioned before [42]. Endoglin, like betaglycan, contains the so-called PDZ-binding motifs at its C-terminal end. In the case of endoglin, removal of this PDZ-binding motif results in hyper-phosphorylation of distal threonine residues [42], indicating a serial mechanism of starting serine followed by secondary threonine phosphorylation. Interactions of some cytosolic proteins, such as zyxin and the zyxin-related protein 1 (ZRP-1) [24,45], with the cytosolic domain of endoglin are likely regulated by these phosphorylation events [42]. In contrast to zyxin and ZRP-1, which solely bind to endoglin, β-arrestin2 also interacts with betaglycan. The interaction of β-arrestin2 with both co-receptors is known to regulate the internalization of both co-receptors [43,46] and thereby weakens TGF-β signaling. Endoglin also interacts with Tctex2b, a cytosolic dynein light chain protein that links TGF-β signaling to the microtubule transport [47]. This interaction is of special interest since the related Tctex1 protein can be phosphorylated by BMP Type II receptor (BMPRII) [48], highlighting a possible connection between TGF-β and BMP receptor signaling by the cytosolic part of these co-receptors.

The extracellular domains of endoglin and betaglycan share little homology, a common feature between both co-receptors is their so-called zona pellucida (ZP) motif in juxtaposition to the cell membrane. The ZP domain consists of approximately 260 amino acids with eight conserved cysteine residues [49,50] and is probably essential for receptor oligomerization [50,51]. A unique feature of endoglin is the presence of an RGD-motif within the ZP domain, indicating a potential interaction with integrins or other substrates specifically interacting with RGD sequences [30]. Noteworthy, this RGD-motif seems to be species-specific since in contrast to human endoglin, it is absent in murine [51,52] and porcine endoglin [53]. Endoglin's extracellular domain (ECD) contains four N-linked glycosylation motifs (NXS/T) and additional O-linked glycosylation motifs proximal to the transmembrane region [30]. Glycosylation of endoglin occurs in multiple stages as observed upon overexpression in COS cells, indicating that partially and fully glycosylated protein species exist at the cell surface [54]. A first glimpse into the three-dimensional structure of the ECD of endoglin was obtained by single-particle electron microscopy at a resolution of 25 Å [51]. However, from these structural data, no further information in terms of protein–protein interactions with other membrane-bound structures has been elucidated so far.

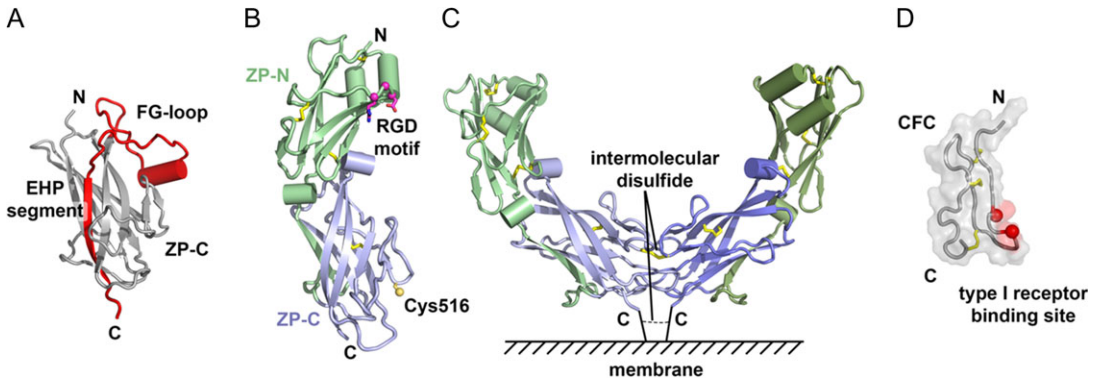

Figure 1. Structural domains in betaglycan, endoglin and crypto-1 (A) Structure/function analysis of the C-terminal region of the zona pellucida (ZP-C) domain of betaglycan revealed that binding of the ZP domain to TGF-β and BMPs is mediated by the EHP (external hydrophobic patch, marked in red) region at the C-terminus of the ZP-C domain [31]. The EHP element together with the FG-loop were also indicated in ZP-mediated protein polymerization [32]. (B) Structure of the ZP domain of endoglin comprising the N-terminal (ZP-N, marked in light green) and C-terminal (ZP-C, marked in light blue) zona pellucida subdomains highlight the high similarity between the ZP-C domains of endoglin and betaglycan (A) [33]. (C) In contrast to betaglycan, endoglin can form covalent dimers through formation of intermolecular disulfide bonds via cysteine 516 and 582. A theoretical model was built on the basis of the crystal structure of the endoglin ZP domain (see also [33]). The intermolecular disulfide between Cys516 is shown, and the disulfide bond involving Cys582 is indicated by a stippled line. (D) A ribbon representation (the solvent-accessible surface is indicated semi-transparent) of the CFC domain of cripto-1 shows a compact, secondary-structure less architecture stabilized by three disulfide bonds (indicated by yellow sticks). Residues identified in the interaction of cripto-1's interaction with the Type I receptor ALK4 are marked in red (only Cα atoms are shown) [34].

Endoglin is predominantly expressed in vascular endothelial cells, and due to the apparent sequence similarities of the cytosolic part of endoglin and betaglycan [36], it can be suggested that endoglin takes part in the signaling response of at least a subset of the same ligands. In fact, endoglin binds to TGF-β1 and TGF-β3, but astonishingly not to TGF-β2, whereas betaglycan binds all three isoforms [55]. Due to this difference, it was not a surprise that both functional differences and similarities exist between the two co-receptors. However, aside from TGF-β1 and -3 endoglin also binds to activin A, BMP7, and BMP2; however, importantly, with the exception of TGF-β1 and TGF-β3 all other ligands bind to endoglin only in the presence of the corresponding Type II receptor [56,57]. This suggests that endoglin only interacts with these ligands in the context of the particular signaling receptor complexes and probably explains, why for example, only a subfraction of endoglin that is present at the cell surface binds TGF-β1 [55]. In contrast, high-affinity interactions of membrane-anchored endoglin with BMP9 can occur in the absence of Type I and Type II receptors [58]. Ligand-bound endoglin can be isolated if it is in a complex with certain Type I and Type II receptors [55], including ALK1, the BMP receptors ALK2, ALK3 and ALK6, ALK5 and the activin Type I receptor ALK4 and the Type II receptors TGF-βRII, ActRII or BMPRII [59–61]. The interactions of endoglin with several different TGF-β, activin and BMP ligands via the corresponding signaling receptors might indicate that the two canonical SMAD1/5/8 and SMAD2/3 signaling pathways can be specifically modulated. Indeed, it was demonstrated that endoglin overexpression enhanced BMP7-mediated signaling via SMAD1/5/8, whereas TGF-β1-induced SMAD2/3 signaling was inhibited [62,63]. For TGF-β signaling, more molecular details were provided in that binding of endoglin with ALK5 and TGF-βRII involves both, the extracellular and cytoplasmic domains of endoglin, but in the case of ALK5 interaction with endoglin, binding only occurs if the kinase domain of the Type I receptor is inactive. Upon ALK5 activation the cytoplasmic domain of endoglin gets phosphorylated, which results in the dissociation of ALK5 from the complex [41], thus explaining the inhibitory effect of endoglin on TGF-β1-mediated signaling as mentioned before. At a cellular level, this mechanism might explain how endoglin expression counteracts the known inhibitory effect of TGF-β on the proliferation of, for example, endothelial cells [64,65]. Interestingly, the impact of endoglin on TGF-β-mediated signaling seems to differ in some cases if SMAD2- and SMAD3-activation is compared. Although endoglin inhibits ALK5-SMAD3-mediated cellular responses [56,62,66–68], it can likely enhance ALK5-SMAD2 signaling [41,69,70] by stabilizing SMAD2 via reducing the expression levels of the SMAD ubiquitination response factor 2 (Smurf2) [70]. Additionally, the involvement of ALK1 in TGF-β-mediated signaling, which most likely requires the presence of endoglin, strongly indicates that a balance of ALK5 and ALK1 expression might play an important role in the regulation of cell growth and differentiation in cells that express endoglin.

Taken together, endoglin seems to interact with the ligand:receptor complexes of various TGF-β members, potentially reflecting endoglin's role in the modulation of these interactions and consequently its role in activated downstream signaling pathways.

Betaglycan: a TGF-β2 facilitator and activator of inhibin antagonism

While the previous section already mentioned some common features of endoglin and betaglycan, this section will focus on the properties that are unique to betaglycan. Betaglycan represents a transmembrane proteoglycan with an average molecular weight of 280–330 kDa (**Fig. 1**). About two-thirds of the mass corresponds to glycosaminoglycans (GAGs), including heparan and chondroitin sulfates in varying proportions [71], and another 10 kDa corresponds to N-linked glycosylation of the ECD [72]. Betaglycan can form dimers; however, in contrast to endoglin, these dimers are not covalently linked. Furthermore, no alternatively spliced isoforms have been identified so far. In contrast to endoglin, whose presence seems more restricted to endothelial cells, betaglycan is more ubiquitously distributed. It is thus the major TGF-β-binding molecule at the cell surface and besides binding all three TGF-β isoforms, it also interacts with inhibins, BMP2, -4, -7, and GDF5 [73,74]. Betaglycan's

large extracellular domain comprises two similarly sized subdomains, an N-terminal, membrane-distal (sometimes referred to as E-domain) and a C-terminal, membrane-proximal domain known as ZP or zona pellucida domain [36]. Both subdomains are involved in the binding to TGF-βs [75–78]. Betaglycan's cytoplasmic domain lacks kinase activity, but harbors interaction motifs for the adapter protein GAIP-interacting protein, C-terminus (GIPC) and β-arrestin2 [43,79]. Structure analyses of the ZP domain have revealed that this domain itself is modular and consist of a (smaller) N-terminal (ZP-N) and a (larger) C-terminal (ZP-C) substructure (**Fig. 1**). Mapping studies showed that within the ZP domain only the C-terminal ZP-C is involved in TGF-β/BMP-binding [76–78]. In two structure/function studies a construct harboring the so-called ZP-C domain and the EHP region, which presents a hydrophobic stretch between the ZP-N and the ZP-C domain, was shown to confer binding of TGF-βs and BMPs [31,32]. Using short peptides segments of the C-terminus of the ZP-C domain, the major binding site for TGF-β/BMP ligands could be located in a surface exposed loop of the ZP-C domain [31]. Besides binding of TGF-β ligands, betaglycan was also shown to directly interact with the Type II receptor TGF-βRII, but in contrast to endoglin, binding of the ligands occurs independently of the presence of the corresponding Type II receptors. Since it enhances binding of all three TGF-β isoforms to their corresponding signaling receptors its main role in the TGF-β signaling complex most-likely relies on the presentation of the ligand to the Type II receptor ([25,75,80], for a review: [81]). As TGF-β2 has a low affinity for its 'capturing' Type II receptor TGF-βRII, recruitment and presentation via betaglycan might be essential for efficient TGF-β2-signaling suggesting a TGF-β2-specific co-receptor function of betaglycan [25,75,82]. The betaglycan-mediated presentation of TGF-β ligands to cell surface receptors can then result in altered signaling outcomes by hitherto unknown mechanisms [25,36,80,81,83]. While the TGF-β ligand-presentation function strictly requires the membrane-located form of betaglycan, also soluble betaglycan forms exist due to proteolytic cleavage of the ectodomain, similar as described for endoglin [75]. The resulting soluble form can compete for ligand binding to the cell surface receptors and thus antagonizes TGF-β activities [28,75,84]. Consequently, betaglycan acts as a modulator with dual functionality: located at the cell membrane, it enhances TGF-β-mediated signaling, whereas it acts as an inhibitor in the soluble form [75,84].

As mentioned above, betaglycan directly interacts with another SMAD2/3-modulating TGF-β members despite TGF-βs itself: i.e. inhibins [76]. In contrast to TGF-βs, binding of inhibins to betaglycan only involves the ZP domain [78]. Both, inhibins and activins, share essential functions in maintaining the functionality of various tissues. While activins, which consist of two inhibin β-chains, activate SMAD2/3 signaling, inhibins are composed of one inhibin α- and one inhibin β-chain and have no intrinsic SMAD-signaling activity [85]. As suggested from their naming, inhibins can however antagonize activin-mediated signaling by displacing activins from their corresponding Type II receptor. As inhibins bind to activin Type II receptors with significantly lower affinities than activins, antagonistic efficacy of inhibins is limited if not facilitated by a third component [86]. Consequently, binding of inhibins to betaglycan via their unique α-subunit enhances binding to activin Type II receptors and thereby strongly increases their antagonistic efficacy [73]. This mechanism seems important, for example, for the biology of tumor progression, as the down-regulation of betaglycan often result in resistance to inhibin-mediated tumor suppression [87–89]. However, the function of betaglycan in this scenario is reminiscent of that of nodal, which requires the presence of, e.g., co-receptor cripto-1 to exert its biological functions via a highly similar mechanism (see below).

Connections to other signaling cascades are provided by the cytoplasmic domain of betaglycan, which interacts with not only the autophosphorylated form of TGF-βRII, but also with the Gα-interacting protein, thereby stabilizing betaglycan on the cell surface [79]. Another interaction with β-arrestin2 regulates the internalization and degradation of betaglycan, as also described for endoglin [43]. Interestingly, betaglycan undergoes both clathrin-mediated and clathrin-independent endocytosis. However, only inhibition of the clathrin-independent lipid raft pathway decreased TGF-β1-induced SMAD2 and p38 MAP-kinase phosphorylation. This observation suggests a specific role for the clathrin-independent endocytosis of betaglycan in regulating both SMAD-dependent and SMAD-independent TGF-β signaling [90]. Indeed, betaglycan was found to modulate NFκB activity in correlation with the invasiveness of tumor cells [91,92], and to be involved in the activation of p38MAPK signaling [93].

Neuropilins: an important link between TGF-β and VEGF signaling in angiogenesis

Neuropilins are single-pass transmembrane proteins composed of a large extracellular domain and a short cytoplasmic domain that lacks enzymatic activity [94,95]. Nrp1 and closely related Nrp2 share approximately 40% sequence homology and have a similar domain structure [96]. The extracellular domain of Nrp1 comprises a set of subdomains (a1, a2, b1, b2, and c) that are crucial for the binding of several ligands, such as class 3 semaphorins (Sema3), vascular endothelial growth factors (VEGFs), and placental-like growth factor (PLGF), but they are used for binding to structures of the extracellular matrix (ECM), such as heparin [97,98]. Nrp1 can form oligomers via interactions involving the c domain and the transmembrane region. The C-terminus harbors a PDZ-binding motif, which binds to the PDZ-domain of the neuropilin-interacting protein (NIP) also known as synectin or RGS–GAIP-interacting protein (GIPC)-1 [99]. NIP can also bind to myosin VI thereby driving endosomal trafficking [100,101]. Binding of VEGF165, an isoform of VEGFA, to Nrp1 induces complex formation with the VEGF receptor 2 (VEGFR2). These complexes are subsequently internalized and transported to specific endosomal compartments, which is an essential step for proper VEGFR2 signaling [102–104]. Interestingly, if Nrp1 is presented in trans, i.e. Nrp1 is expressed on one cell, while VEGFR2 and NIP are localized on another cell, VEGFR2 signaling is impeded. Despite being devoid of inherent catalytic activities, Nrp1 can interact with various receptors at the cell surface, thus enabling the modulation of different intracellular signaling pathways by interacting with, for example, phosphoinositide 3-kinase (PI3K)/AKT, ERK (extracellular signal-regulated kinase), p130cas (Crk-associated substrate), Src (SRC proto-oncogene, non-receptor tyrosine kinase), and p38 mitogen activated protein kinase (p38MAPK) [105]. Importantly, Nrp1 is also capable to modulate TGF-β signaling, as non-canonical TGF-β signaling (among other signaling pathways) also addresses PI3K, ERK, p38MAPK-mediated signaling (for a review: [106]). Nrp1 binds to both the active mature TGF-β growth factor domain and to its latent form (i.e. the mature part associated with the prodomain, which is also known as latency-associated protein (LAP)) [107]. Both latent and active TGF-β

compete with VEGF165 for Nrp1 binding, which indicates that the binding epitopes for both ligands possibly overlap. Binding of Nrp1 to LAP-TGF-β is mediated via the b2 subdomain, involving a core RKFK amino acid sequence. In addition to ligand binding, Nrp1 also forms complexes with TGF-β Type I receptor ALK5 and with TGF-β Type II and Type III receptors (betaglycan). As demonstrated for endoglin, Nrp1 also promotes the oligomerization of TGF-β Type I and Type II receptors [108,109]. Since both Nrp1 and endoglin contain a PDZ-binding motif, both proteins might share similar functions in TGF-β signaling. Interestingly, in addition to TGF-β-promoting activities, Nrp1 can also provide inhibitory activities, as demonstrated for angiogenic endothelial cells. In these cells, ablation of Nrp1 results in increased pSMAD3 levels, indicating enhanced TGF-β signaling [110]. Since deletion of the PDZ-binding motif does not affect SMAD2 activation, this effect seems to be mediated via the extracellular domain of Nrp1 [111]. The effect of Nrp1 on SMAD1/5/8 signaling is not well investigated, but the context-dependent dual functionality of this co-receptor might rely on the presence of either ALK1 or ALK5 on the target cell. However, it remains unclear whether both activities are primarily translated by canonical SMAD signaling and/or via other non-SMAD pathways.

SCUBE proteins: a new class of TGF-β receptor interactors?

More recently, another class of proteins were identified that could also be linked to TGF-β signaling, the so-called SCUBE (signal peptide-complement protein C1r/C1s-Uegf-BMP1-EGF domain containing) proteins [112]. The three members (SCUBEs1–3) identified to date [112–119] represent secreted oligomer-forming glycoproteins composed of approximately 1000 amino acids that can be stably anchored on the cell surface. SCUBE proteins could be functionally linked to a number of cellular processes related to cancer progression [120], e.g. cell migration and epithelial mesenchymal transition (EMT) [121], but they also seem to play an important role during embryogenesis as, for example, SCUBE3 expression is found prominently in developing tissues [122].

While little is known about the molecular mechanisms by which SCUBE proteins trigger these processes, a link to TGF-β-mediated signaling was reported by Wu and co-workers [123]. These studies revealed that SCUBE3 can be cleaved by the matrix metalloprotease-2 (MMP-2) and MMP-9 producing an N-terminal, EGF-like domain-containing fragment and a C-terminal fragment comprising the CUB domain. The latter fragment and full-length SCUBE3 were shown to directly bind to the TGF-β Type II receptor thereby activating TGF-β signaling by SMAD2/3 phosphorylation. Importantly, this activation seems to function in a ligand-independent manner. Thus, SCUBE3 might act as an autocrine and paracrine regulator of TGF-β receptor-mediated signaling. Whether this mechanism represents a common feature of SCUBE protein family members remains unclear.

Integrins and ECM: co-receptors in TGF-β activation

As multicellular organisms appeared in the course of evolution individual cells needed to communicate with each other in order to get information from their individual microenvironment. Thus, it is a prerequisite that signaling molecules being secreted from a donor cell are transported within an interstitial fluid to an acceptor cell, subsequently resulting in the generation of specific cellular responses. However, since in higher organisms various cell types are specifically arranged in order to produce functional tissues, the communication between the individual cells requires more than the information, which is transported via soluble signaling molecules. Particularly, mechanical stimuli need to be perceived, which requires an interface for the conversion of mechanical forces being applied to extracellular structures at the cell surface, e.g. the extracellular matrix (ECM), into (bio-)chemical signals inside the cells. In the acceptor cells these signals are then translated into specific cellular activities, such as cell adhesion, migration/invasion, proliferation, survival, and apoptosis. As these aspects of signal integration seem to be important for the activation of TGF-β ligands they should also be mentioned here briefly.

The best characterized proteins with such converter functions are represented by integrins. Integrins are assembled, heterodimeric transmembrane glycoproteins, consisting of an α- and a β-subunit. As these intracellular activities can also translate to mechanical forces at the cell exterior, they seem to be capable of a bi-directional signaling, referred to as 'outside-in' and 'inside-out' signaling [124–127]. Since integrins are linked to the cytoskeleton, they mainly function as mechano-transducers exhibiting force-sensing capabilities [128,129]. As a minimal integrin-binding motif a short, highly conserved peptide sequence Arg-Gly-Asp (RGD) was identified first in the matrix protein fibronectin [130]. Then this motif was also found in many other ECM proteins such as vitronectin [131], von Willebrand factor [132], osteopontin [133], and laminin [134]. Although the short RGD-motif seems sufficient for integrin-binding, the generation of binding specificity in this system appears to be rather complex. It could be demonstrated that binding specificity relies on the distinct conformational and spatial presentation of the RGD-sequence as well as on regions flanking this motif [135–137]. Furthermore, integrins specifically interact with other receptor and anchor proteins that are arranged as a multiprotein network within focal adhesions [138,139]. In summary, the ECM and its connected structures not only provides physical support for residing cells, it further serves as structure providing information, which is interpreted by the cells via multiple lines of interacting sensors and mediated via growth factors and their receptors, respectively, and the integrins [140], and thus meet all criteria to function as co-receptor.

The best investigated example of a direct integrin:growth factor interaction can be seen in the binding of latent TGF-β (**Fig. 2**). The binding of TGF-β (i.e. TGF-β1 and -β3; the β2 isoform lacks the RGD consensus sequence) essentially requires a RGD-motif being located within the growth factor's prodomain [142,143]. All three TGF-β isoforms are expressed in an inactive form in which the prodomain, termed latency-associated peptide (LAP), shields the so-called mature part of the protein thereby preventing the interaction with cell surface receptors. The mature part (the active growth factor) and LAP form a complex, which upon cleavage by furin proteases remains non-covalently assembled. Integrin-binding to LAP seems crucial for TGF-β activation, since transgenic animals in which in both alleles the RGD-motif of LTGFβ is converted to RGE display similar phenotypes as TGF-β1 knockout mice [144]. *In vitro* studies revealed that six different integrins (αVβ1, αVβ3, αVβ5, αVβ6, αVβ8, and α8β1) can interact with LAP, but αVβ6 and αVβ8 appear to be the most important activators of LTGFβs as shown by studies using knockout mice [145]. These studies also reveal that these integrins can at least partially compensate for any deficiency of the other. For αVβ5 integrin, a role in the transition of fibroblasts to myofibroblasts was reported by Scotton and co-workers [146]. But, the *in vivo* function of the other integrins remains unclear [147,148]. Activation of LTGFβ1 by αVβ6 is independent from

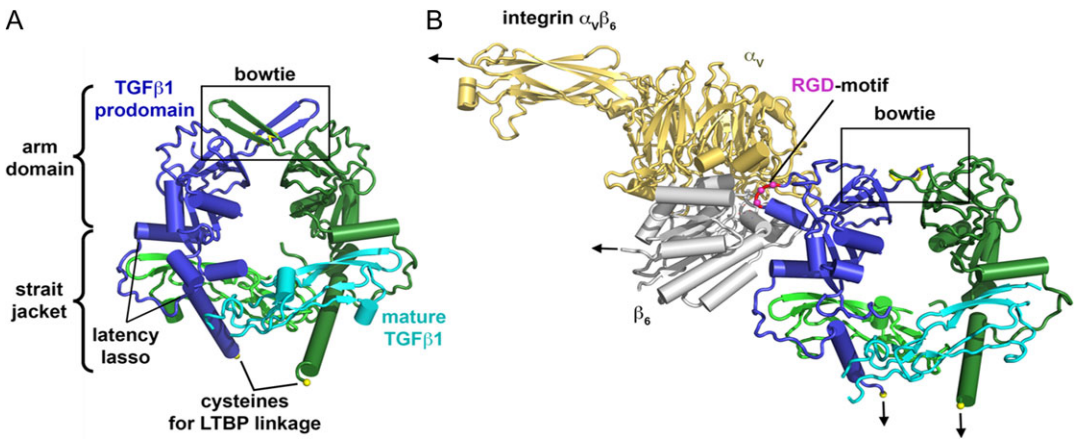

Figure 2. Structural determinants in the activation of latent TGF-β (A) The proprotein complex of TGF-β1 reveals the molecular mechanism of latency. The prodomain (indicated in dark blue and dark green) wraps around the growth factor domain (marked in green and cyan) like a clamp. Elements of the prodomain, i.e. the N-terminal helix, the latency lasso and the second helix, form the so-called strait jacket, which shields the growth factor domain from receptor binding. The stability of the prodomain clamp is likely enhanced by the intermolecular disulfide bonds (indicated as yellow sticks) in the bowtie region. (B) Via their head piece $\alpha_V\beta_6$ integrins bind to the RGD-motif present in the arm domain of the TGF-β proprotein complex. Binding (without further traction force by activating integrins) already results in a major shift of elements in the TGF-β prodomain, namely the RGD-motif and the β-strands forming the bowtie region, which shift by almost 20 Å and lead to local unfolding of the prodomain [129]. Due to the dimeric nature of TGF-β, possibly two integrin moieties can simultaneously bind. Since also the N-terminus of the TGF-β prodomain is additionally anchored via disulfide bonds to LTBPs, which themselves tightly interact with the ECM, exertion of traction force by the integrin β-subunit via the actin cytoskeleton leads to removal of the prodomain and hence release of the non-covalently associated TGF-β growth factor domain (for a review: [141]).

proteolytic activities and does not cause release of TGF-β to the culture medium [143]. This observation strongly indicates that integrin-binding might induce conformational change(s) within LTGFβ, thereby probably unmasking the binding site(s) being required for the interaction with the TGF-β receptors, which are located in close proximity to the anchored LTGFβ. Interestingly, latent TGF-β-binding protein LTBP [149] as well as cell contraction [150] also seem to be crucial for LTGFβ1 activation by αVβ6 integrin. Taken together, the function of LTBP most-likely relies on anchoring TGF-β to the ECM, while mechanical forces produced by the contracting cell alters the integrin-bound LAP structure, thereby releasing the active TGF-β ligand (**Fig. 2**). The mechanism by which LTGFβ gets activated by αVβ8 integrin completely differs from what is described for αVβ6 integrin. Here, the LTGFβ:αVβ8 integrin interaction seems not to cause a conformational change within LTGFβ, but possibly exposes it to the membrane-type matrix metalloproteinase (MT1-MMP) hereby promoting the release of TGF-β to surrounding tissues [151]. Thus αVβ6-mediated activation of LTGFβ seems to limit TGF-β signaling to the close surrounding, i.e. neighboring cells, whereas activation of TGF-β by αVβ8 produces a long-range activity, in that the released TGF-β may diffuse further away from the site of activation.

ECM: involved in presentation, storage and activation

TGF-β superfamily members can be recruited to the cell surface by multiple mechanisms not involving their cognate receptors. Particularly for the TGF-βs, a transfer and coupling of the inactive factor to the ECM seems to be crucial for proper function. In addition to the discussed surface immobilization via integrins, TGF-βs and other TGF-β members, such as GDF8 (also known as myostatin), can be brought to the cell surface though interaction with LTBPs. These proteins represent a group of four (LTBP1 to -4) extracellular proteins that share various modular domains, such as calcium-binding domains and the so-called 8-Cys type domains. The latter can be further classified into TGF-β-binding and hybrid domains representing an assembly of both, TGF-β- and calcium-binding domains [152]. All four LTBPs can interact with fibrillins, but in contrast to LTBP1, -2, and -4 the binding of LTBP3 seems to occur indirectly [153–155]. LTBPs also interact also with other matrix components, such as fibronectin, ADAMTSL proteins, and the fibulins, and hence seem to function as adapter in order to connect fibrillin-fibers with other matrix proteins. LTBPs were identified as part of a large latent complex with TGF-β and LAP [156] and were thus called latent TGF-β-binding proteins. Disulfide-linked complex formation of LTBPs and LAP occurs in the endoplasmic reticulum (ER) prior to secretion. After furin-protease cleavage the mature part remains associated with LAP due to strong non-covalent interactions. The association with LTBPs possibly also assists the proper folding of the TGF-β precursor protein within the Golgi apparatus [157,158] thereby supporting secretion and directing association of TGF-β with the ECM. This interaction seems crucial for TGF-β function since a functional disruption produces a phenotype being similar to that of TGF-β1 knockout mice [159]. One mechanism, by which LTBPs might trigger TGF-β activity, relies on an assisting role in integrin-mediated LTGFβ activation. LTBP1 facilitates TGF-β association with the ECM and generates traction when LAP is bound by integrins. Application of mechanical forces might induce conformational changes in LAP resulting finally in the release of the active growth factor [149,150,160]. However, regulating the biological activity of TGF-βs is not the sole function of LTBP family members. For instance, LTBPs1, -2, and -3 also interact with other TGF-β family members including pro-GDF8 and -11, suggesting that LTBPs also trigger the bioactivity of these factors [161,162]. The interaction of LTBPs, i.e. LTBP2 and LTBP3 with GDF8 indicates their importance for GDF8-mediated muscle mass regulation. LTBP3 interacts non-covalently with pro-GDF8 and inhibits GDF8 maturation consequently limiting GDF8 levels. Accordingly, ectopic expression of LTBP3 in adult mouse muscles results in an increase in skeletal muscle fibers [161]. Additionally,

binding of LTBP3 to the proprotein convertase 5/6A (proPC5/6A)in the endoplasmic reticulum prevents the conversion of proPC5/6A into the active convertase PC5/6A, thus preventing the release of active GDF8 in muscular tissue [162]. The few examples discussed above already provide insights into the complex regulation of TGF-β family members by cell surface structures. The involvement of integrins and LTBPs seem not to be restricted to signaling of the three TGF-β isoforms, but appears preferentially required for ligands signaling via the SMAD2/3 pathway.

Cripto: a co-receptor to switch between activin and Nodal signaling

Cripto, cryptic, fibroblast growth factor receptor-related ligand (FRL)-1, and zebrafish one-eyed pinhead (oep) belong to the epidermal growth factor (EGF)-cripto-1/fibroblast growth factor-related ligand (FRL1)/Cryptic (CFC) gene family. Cripto-1, the member of this protein family best investigated, has been identified only in vertebrates [163,164]. It is expressed in non-differentiated pluripotent human and mouse embryonic stem (ES) cells and in early vertebrate embryos [165] and plays a pivotal role during embryonic development especially in the left-right specification of body axes [166,167]. The strong, widespread expression of cripto-1 observed during embryonic development is strongly reduced in adult tissue to subpopulations of adult stem cells [168,169]. Upregulation of cripto-1 is observed in several neoplasia, which also highlights its importance as a regulatory trigger for the activation and integration of different subcellular signaling cascades [164]. Structurally, cripto-1 represents a cell membrane-associated protein, comprising a N-terminal EGF-like domain, a cysteine-rich the so-called cripto-FRL1-Cryptic (CFC) motif, and a C-terminal consensus sequence motif for attachment of a glycosylphosphatidylinositol (GPI) anchor moiety for membrane localization [170,171] (**Fig. 1**).

In the past, a variety of signaling pathways have been reported to directly interact or crosstalk with cripto-1. For example, interaction of cripto-1 with glypican-1 results in a nodal/ALK4-independent activation of mitogen activated protein kinase (MAPK) and phosphatidylinositol 3-kinase (PI3K)/Protein Kinase B (AKT) signaling pathways [172]. Signaling via cripto-1 seems to be connected also to the canonical Wnt/β-catenin/T-cell factor (Tcf) signaling pathway, which is activated by the binding of secreted Wnt ligands to cell surface receptors, such as frizzled (Fzd), and co-receptors, such as LRP5/6. Cripto-1 has been identified as a primary target gene in Wnt/βcatenin signaling [173], but several studies have also demonstrated a direct interaction between cripto-1 and the Wnt co-receptor LRP5/6 [174].

However, cripto-1's primary task seems to be its function as co-receptor for TGF-β family ligands. In the case of nodal, cripto-1 acts as an obligate cell surface co-receptor. It directly interacts with a ligand:receptor complex composed of the ligand, the activin Type II receptors ActRIIA or -IIB and the Type I receptors ALK4 or 7 (for a review: [175]). Cripto-1 simultaneously binds to the pre-helix loop of nodal via its EGF-like domain [176] and to parts within ALK4 via its CFC domain [177,178]. Although cripto-1 seems essential for nodal signaling, cripto-1 independent nodal functions have also been reported [179,180]. Consequently, these activities must be also independent of ALK4 or -7 activation, indicating that another receptor-independent mechanism that enables downstream SMAD2/3 signaling is stringently required. This mechanism might hence act intracellularly, as cripto-1 was shown to be involved in pro-nodal processing in the early endosome. In this compartment, cripto-1 functions as a chaperone for furin and for the furin-like protease PACE-4 by facilitating the translocation of processed nodal to endosomes, thereby enabling SMAD2/3 signaling [181–183].

Interaction of cripto-1 with either ALK4 or -7 occurs in two different ways. While binding of cripto-1's CFC domain to ALK4 is essential for nodal-mediated signaling [178], activation of ALK7 by nodal can occur also in the absence of cripto-1 [184]. In this scenario, cripto-1 significantly enhances the sensitivity of ALK7 and ActRIIB for nodal by probably cooperatively increasing the apparent affinities for the interaction of nodal with ALK7 and/or ActRIIB. However, both modes indicate that ALK7 and ALK4 cooperate with cripto-1 in modulating nodal signaling to different extents [184]. Aside from nodal, EGF-CFC proteins have also been reported to mediate the signaling of other SMAD2/3-activating TGF-β family members, such as GDF1 and -3. As in the case of nodal, both GDF1 and -3 also signal through complex formation with the receptors ALK4 and ActRIIB only in the presence of EGF-CFC family proteins [185,186]. Taken together, the mechanism by which nodal, GDF1 and -3 are integrated into the network of SMAD-dependent signaling appears *prima facie* relatively simple. If cripto-1, or possibly another EGF-CFC protein family member, is present, signaling of these factors is initiated or at least enhanced, whereas the absence of these co-receptors renders the addressed cell less sensitive or even insensitive to the particular ligand. However, it has to be taken into account that other TGF-β ligands, such as activin A and B, also use combinations of ALK4 with ActRII or -IIB for signal transduction. Both activin ligands can interact with cripto-1 either via cripto-1's EGF-like domain (activin A) or via its CFC domain (activin B), respectively [187,188]. Interestingly, in contrast to nodal, binding of cripto-1 to activin A or -B results in signaling inhibition, although cripto-activin-receptor complexes are formed that appear structurally and functionally similar to those of nodal [189]. Furthermore, cripto-1 also binds to TGF-β1 via its EGF-like domain, thereby reducing the binding of this ligand to ALK5 [190]. In summary, the signaling capabilities of the above-mentioned TGF-β family members strongly depend on the presence of cripto-1 or cripto-1-related proteins, involving different regulatory mechanisms. In the case of TGF-β1 signaling, ligand binding to the receptors is impeded by cripto-1, resulting in non-occupied receptors that are accessible to other ligands. For the other TGF-β members, ligand binding is either initiated by or facilitated in presence of the co-receptor. However, despite a similar mechanism in assisting receptor assembly, the effect of cripto on signaling can just be contrariwise: for activin A and -B, cripto-assistance leads to inhibition, in the case of nodal or GDF1/3, involvement of cripto promotes signaling activity.

Co-receptors of SMAD1/5/8-activating BMPs and GDFs

Betaglycan: also a directly acting co-receptor for SMAD1/5/8-activating BMPs?

For a long time co-receptors for SMAD1/5/8-activating BMPs and GDFs were unknown or considered non-existent: in contrast, TGF-βs and activin members were shown to strongly interact with co-receptors, such as cripto, betaglycan, and endoglin (see above). Betaglycan, whose actions on TGF-βs and inhibins have been described above, was shown to also interact with various SMAD1/5/8-activating BMP and GDF ligands, for example, BMP2, -4, -7, and

GDF5 utilizing the same epitopes as the TGF-βs [31,32,74,76–78]. The binding affinities of betaglycan for BMPs are significantly lower than those for TGF-βs and inhibins, however, raising questions as to whether its interaction with BMPs might be physiologically significant and whether its interaction is indeed capable to modulate BMP signaling. Lee *et al.* [191] have shown that betaglycan can differentially direct trafficking of the BMP Type I receptors ALK3 and ALK6 with betaglycan mediating ALK6 internalization in a β-arrestin2-dependent manner, whereas ALK3 was retained at the cell surface by a betaglycan-dependent mechanism. Thereby betaglycan could indeed potentially influence BMP signaling by directly altering Type I receptor availability.

Furthermore, BMP signaling might be additionally modulated, albeit indirectly, through the interaction of betaglycan with inhibins (see also above). Inhibins are signaling-inactive heterodimers that do not activate any of the canonical TGF-β Type I receptors [85]. Inhibin A and inhibin B, however, form highly stable ternary complexes with betaglycan and activin Type II receptors from which they cannot be competitively released by other TGF-β ligands [73]. Therefore by sequestering ActRIIA and -IIB into these complexes, activin Type II receptors are 'depleted' at the cell surface and blocked from binding to activins (or other TGF-β members) explaining how inhibins efficiently antagonize activins [73]. As BMPs also utilize activin Type II receptors ActRIIA and -IIB, inhibins can also antagonize BMPs by the very same mechanism [192]. Since inhibin can also sequester BMP Type II receptor BMPRII in the presence of betaglycan and BMPRII serves as another BMP Type II receptor in addition to the activin Type II receptors, inhibins act as a full antagonist to BMPs and activins. Thus, while the significance of betaglycan as a BMP co-receptor is unclear with its direct interaction with BMPs (in contrast to its defined presentation function for TGF-βs), it can nevertheless modulate BMP signaling either by altering BMP Type I receptor endocytosis or in the presence of inhibin, it can function as a negatively regulating non-interacting co-receptor for BMPs. Additional complexity in the regulation of BMP activity by betaglycan might be due to the fact that betaglycan also exists in soluble form upon proteolytic cleavage near the transmembrane region [28,193]. While soluble betaglycan exhibited an antagonizing effect of TGF-β signaling as expected [75], it is still unclear how the soluble isoform affects BMP and inhibin functions.

Endoglin: a high-affinity co-receptor for BMP9 and BMP10

The transmembrane glycoprotein endoglin was initially discovered as a co-receptor for TGF-βs and reported to bind TGF-β1 and -β3, but not TGF-β2 [55]. However, Barbara *et al.* [57] showed that endoglin can also bind the SMAD1/5/8-activating BMPs 2 and 7. For BMP2, the presence of either of the Type I receptors ALK3 or ALK6 seemed to be necessary, whereas in the case of BMP7 co-expression of an activin Type II receptor, either ActRIIA or -IIB, was required for interaction with endoglin. While the interaction of endoglin with TGF-βs and TGF-β receptors results in altered phosphorylation of Type I and Type II receptors and hence modulates the downstream signaling of TGF-βs, the consequences of its interaction with SMAD1/5/8-activating BMPs, in particular for BMP2 and BMP7, are unknown. In 2007, a search was conducted to unravel the molecular roles of Type I receptor ALK1 and endoglin, which are both mutated in the autosomal-dominant vascular disorder hereditary hemorrhagic telangiectasia (HHT, also known as Osler-Weber-Rendu disease). Here, endoglin was shown to serve as a co-receptor for the SMAD1/5/8-activating BMP9 (as well as BMP10, see [194]) and facilitates complex formation of BMP9 (and BMP10) with ALK1. Mapping studies revealed that binding of BMP9 (and BMP10) is confined to the membrane-distal orphan domain, whereas in this context, the ZP domain only serves to stabilize the endoglin-BMP9 complex, but does not contribute to BMP binding [195]. A very recent structure analysis of a complex of BMP9 bound to the orphan (OR) domain of endoglin provides insight into the endoglin:BMP9 interaction, showing that the orphan domain binds to the Type II receptor-binding site of BMP9, thereby allowing Type I receptor (ALK1) binding to BMP9 (**Fig. 3**), but blocking the interaction of BMP9 with Type II receptors, such as ActRIIB [33]. While the overlapping binding sites of endoglin and Type II receptors at BMP9 explain, how soluble endoglin ectodomain antagonizes BMP9 signaling, it remains to be elucidated why membrane-localized endoglin functions as an activator of BMP9 signaling and does not interfere with receptor activation, although endoglin must be released from the complex to allow Type II receptor recruitment for canonical BMP9 signaling [33] (**Fig. 3**).

Glycosaminoglycan-containing proteoglycans as BMP co-receptors: more than just distributing BMPs to form a morphogenic gradient?

In addition to betaglycan and endoglin, of which the latter is considered rather a glycoprotein and not a proteoglycan (no O-linked glycosaminoglycans (GAG) are described for endoglin), other proteoglycans, including (but not limited to) aggrecan, agrin, biglycan, decorin, fibromodulin, perlecan, versican, and members of the syndican or the glypican family are known, and many were shown to modulate BMP activities [196,197]. The likely most important function of proteoglycans is in establishing and maintaining morphogen gradients, which includes the formation of BMP gradients, to facilitate patterning during embryogenesis [196]. The impact of GAG-containing proteoglycans on TGF-β/BMP signaling is therefore frequently seen from function-impairing mutations appearing in patients or in animal models leading to skeletal defects/malformation or defects in organogenesis [198–201].

All SMAD1/5/8-activating BMPs and GDFs (except for BMPs9 and 10) have accumulated basic amino acid residues in the N-terminus of their mature region just ahead of the cystine-knot growth factor domain. Hence, these mature BMP and GDF growth factors can interact through charge-charge interactions with the acidic groups, e.g. sulfate or carboxylic acid groups, present in the poly-anionic glycosaminoglycan (GAG) structures, and these interactions likely contribute to the morphogenic properties of these BMPs [202,203]. It is important to note that TGF-βs, activins/inhibins, nodal and other SMAD2/3-activating GDFs, lack these highly positively charged sequences at the N-terminus of their mature growth factor domains and hence (as mature factors) do not bind significantly to ECM components. This remarkable architectural difference possibly explains why SMAD2/3-activating TGF-β members can exert also long-range (besides autocrine and paracrine) activities like protein hormones, such as interleukins, whereas the activities of the above-listed SMAD1/5/8-activating BMPs and GDFs are usually spatially highly restrained.

Since these ionic interactions between GAG-containing proteoglycans and BMPs/GDFs are however unlikely to yield a structurally defined complex (in contrast to the interactions of betaglycan and endoglin), the mechanisms, how these proteoglycans affect BMP activity on a molecular level, other than just by targeting and shaping a BMP concentration gradient, must differ from those based on direct

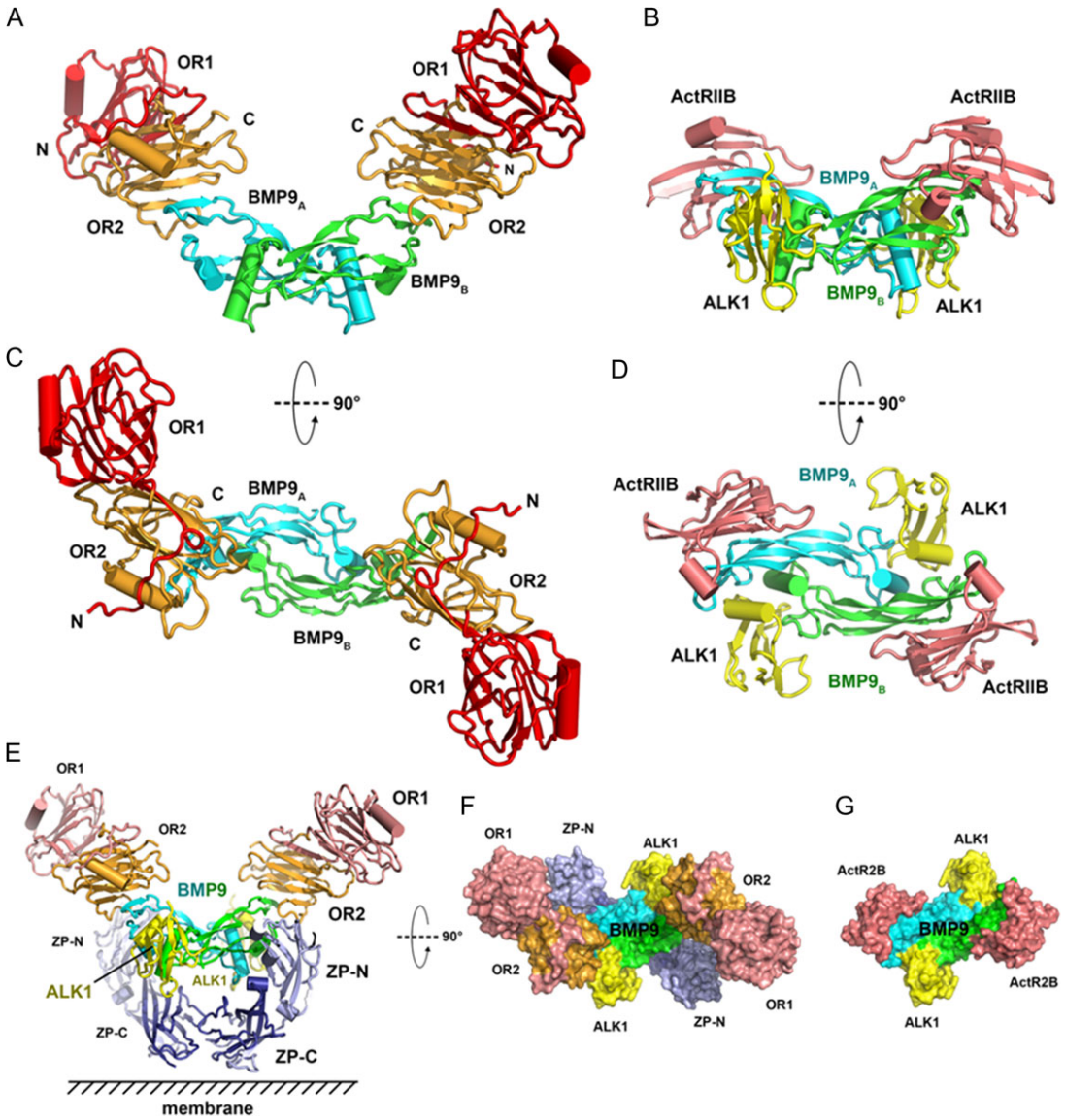

Figure 3. Structural determinants in the interaction of BMP9 with cell surface co-receptors (A) Interaction of endoglin and BMP9 (as well as BMP10) is mediated by the orphan domain of endoglin, which comprises of two structurally highly similar subdomains (marked as OR1 and OR2). Interaction of the subdomain OR2 with the ligand blocks a large part of the Type II receptor-binding epitope of BMP9 as highlighted by comparing the co-receptor:BMP9 structure with that of the BMP9 ternary ligand:receptor complex comprising BMP9, ALK1 and ActRIIB (B). (C,D) as in (A) and (B), respectively, but rotated by 90° counterclockwise around the *x*-axis. (E) A theoretical model of the ectodomain of endoglin comprising the OR subdomains OR1 and OR2 and the zona pellucida domain comprising ZP-N and ZP-C bound to a complex of BMP9 and Type I receptor ALK1 (see also [33]). (F) The model of this ternary ligand:co-receptor:Type I receptor complex (a surface representation is shown with BMP9 marked in green/cyan, the co-receptor domains color-coded as in (F) and the Type I receptor ALK1 shown in yellow) suggests that interaction of endoglin is compatible with simultaneous binding to ALK1, while the overlap of the OR2 subdomain with the binding site for the Type II receptor as shown in (G) clearly indicates that simultaneous binding of endoglin and a Type II receptor to BMP9 seems impossible.

and highly defined protein–protein interactions. The GAG-binding motifs (often termed heparan/heparan-sulfate binding sites) in these BMPs indeed directly modulate *in vitro* and *in vivo* activities, and this modulation was, for example, shown in a mutational study on BMP2 [203–205]. The N-terminus of mature BMP2 contains three characteristic triplets of basic amino acid residues (K^{285}HK, R^{289}KR and K^{297}RH), of which the last triplet motif, which is located just past the first cysteine of the cystine-knot, is highly conserved among BMPs2, -4, -5, -6, -7, -8, GDF5, -6, and GDF7. Upon exchanging five of the six basic residues in the first two triplet motifs, mature BMP2 lost its capability to bind to heparin *in vitro* [203]. In a cellular assay measuring sulfate incorporation via BMP-induced proteoglycan synthesis, the heparin-binding deficient BMP2 variant exhibited a 15–20-fold enhanced specific activity, suggesting that the heparin binding to wild-type BMP2 attenuates BMP activity. Conformingly, an increase in BMP activity could be also observed when cells were stimulated with wild-type mature BMP2 in either the presence of the N-terminal peptide harboring these basic triplets or in the presence of soluble heparin. Noteworthy, the (enhanced) activity of the heparin-binding deficient BMP2 variant could not be further increased upon addition of the N-terminal peptide or heparin, showing that this effect is saturable and hence due to a direct interaction between ECM sites at the cell

surface and the heparin-binding sites located in the N-terminus of BMP2 [203]. In a follow-up study, additional basic motifs were introduced and two variants with three or four triplet motifs were analyzed *in vitro* and *in vivo* [204]. Interaction analyses confirmed enhanced heparin binding of these two BMP2 variants, and cell-based assays indicated that these variants seemingly have lower specific BMP activity. However, when applied either into muscular tissue of rat hindlimbs or used in a calvarial critical size defect model, these variants performed superior and 'produced' a more compact bone with significantly higher bone density than obtained with wild-type BMP2. Conformingly, the BMP2 variant with attenuated heparin binding failed completely at lower doses and required high protein doses to induce bone formation [204]. The observation of heparin-mediated increased BMP activity might be explained with a recruitment of BMP proteins into ECM-rich cell clusters/tissues, thereby potentially enhancing local BMP concentrations from BMP levels being too low to directly initiate BMP signaling. This localization could furthermore warrant elevated BMP concentrations over an extended period of time, thereby enabling longer BMP signaling; without ECM interaction, the BMP factors might diffuse away from their site of action or might get cleared by endocytosis. Cell-based assays showing attenuated activity upon heparin interaction are however in conflict with this mechanism explaining enhanced activity *in vivo* and suggest a rather inhibitory function for the BMP-ECM interaction instead. Thus, a cell context-dependent mechanism possibly might switch between both activities observed: a release of BMP proteins from an ECM storage could result in enhanced (paracrine) activity, while by depleting BMP proteins due to ECM-mediated internalization into cells (thereby decreasing the local concentration of BMP ligands) could lead to attenuated activity (e.g. [206,207], for a review [196]). While the above-described example was obtained from specifically altered BMP2 variants, similar (partially conflicting) results were obtained by other groups by studying signaling of other BMPs in the presence of soluble heparan sulfate, removing GAGs from the cell surface prior to BMP stimulation, or recombinantly overexpressing ECM components [199,202,206–211].

This remarkable complexity in ECM:BMP interactions and its impact on signaling of SMAD1/5/8-activating BMPs might be further increased by the fact that also BMP ligands are synthesized as proproteins. After proteolytic processing, the active C-terminal (mature) growth factor domain must be released from the proprotein complex. For various SMAD2/3-activating TGF-β members, e.g. TGF-βs, GDF8, and GDF11, this is an active process and allows to use the proprotein complex for storage of an transiently inactivated growth factor, which is also termed latency. In these cases, the release of the mature growth factor domain involves ECM components and (for TGF-βs) integrins (for a review: [212]). In contrast, the proprotein complexes of most SMAD1/5/8-activating BMPs/GDFs seem not to be latent (to the same degree as they are for TGF-βs), indicating that the prodomain might be released more easily. However, these BMPs are nevertheless secreted as non-covalent proprotein complexes, and the prodomains of some SMAD1/5/8-activating BMPs were shown to also directly interact with ECM components, such as fibrillin [213]. While the ECM-binding capacity of these BMP proprotein complexes might purely serve to target BMP factors to ECM-rich tissues and cell clusters, it was shown that the ECM-BMP proprotein interaction also modulates release and therefore regulates BMP activity [214–217].

In addition to the pro- and growth factor domain of BMPs, also BMP antagonists, such as noggin, chordin, follistatin, or members of the DAN family, bind to heparan/heparan sulfate and thus are likely to strongly interact with ECM compounds (e.g. [218–221], for reviews: [222,223]). Furthermore, many other ligands from functionally related growth factor families, such as the FGF, Wnt, IGF, or Notch families, which are known to also tightly bind to glycosaminoglycans and ECM components (for a review: [224]), might be co-expressed with BMPs/GDFs at the same site at the same time and might therefore compete with the latter for binding to ECM components with unpredictable consequences for the signaling of this mixture of growth factors. In summary, the overlapping and likely competing ECM-binding capacities of all these proteins will certainly modulate the interactions, targeting, and functionality of the above-mentioned BMPs and GDFs, and predicting how particular ECM components act as co-receptors for certain BMPs is impossible. This highly interwoven network with a multitude of mutual impacting interaction partners also highlights why our molecular understanding of how morphogenic gradients form is still very limited.

The pseudo-receptor BAMBI: co-receptor or universal TGF-β/activin/BMP decoy?

An expression screen for components involved in BMP4 signaling in *Xenopus* led to the discovery of the transmembrane protein BAMBI [27]. It proved to be an ortholog of human NMA, which was first found in a screen of differentially expressed genes in melanoma cell lines [225]. Here, the inverse correlation of its expression with the metastasis level of melanoma cells leading to its name NMA for non-metastatic gene A. In another screening, NMA was also identified in zebrafish as a gene, whose restricted expression pattern during embryogenesis follows that of BMP [226]. A similar strict co-expression for BAMBI and BMP4 was also confirmed during limb development in mice [227]. BAMBI encodes a single-span transmembrane protein with an extracellular domain of approximately 130 amino acid residues in length and a short, less than 90-residue long intracellular domain. Its extracellular domain was reported to share similarity with the extracellular ligand-binding domains of TGF-β and BMP Type I receptors [27], although strictly speaking, only ten cysteine residues involved in the formation of the three-finger toxin fold in TGF-β/BMP Type I receptors were found to be invariant. In contrast, the short intracellular domain neither harbors nor shares similarity with the serine/threonine kinase domain of TGF-β/BMP receptors [27,226]. To unravel the function, Onichtchouk *et al.* analyzed the effects of BAMBI on *Xenopus* development by mRNA injection into oocytes, showing that it interferes with BMP and activin activity, which also led to its name as BMP and activin membrane-bound inhibitor. The inhibitory activity was also confirmed *in vitro* by employing cell-based assays using SMAD reporter gene assays, which indicated that BAMBI negatively regulates TGF-β, activin and BMP signaling and thus presents a rather indiscriminate pseudo- or decoy receptor for SMAD2/3- and SMAD1/5/8-activating TGF-β family ligands. To reveal the molecular mechanism of its inhibitory action, the authors showed that a truncation variant lacking the extracellular domain was no longer capable of attenuating BMP signaling in cell-based assays, pointing towards a ligand-dependent inhibition mechanism. However, as this truncation variant also reversed the inhibitory effect of wild-type BAMBI in a dominant-negative manner, a ligand-independent mode of action seemed also possible. But as the presence of BAMBI abrogated TGF-β family signaling initiated from constitutively active TGF-β Type I receptors, i.e. ALK1, ALK2, ALK3, ALK4, ALK5, ALK6 and ALK7, the authors concluded that BAMBI must indeed inhibit TGF-β receptor-mediated signaling in a ligand-independent manner. Consistent with this

mechanism, Onichtchouk *et al.* [27] showed that the extracellular domain of BAMBI did not directly bind to radiolabeled BMP2 or TGF-β1. Thus, while BAMBI contains an extracellular domain similar to TGF-β/BMP Type I receptors, its mode of action seems to be connected through an interaction with the receptors rather than through the ligands. This somewhat unexpected finding was confirmed by co-immunoprecipitation, which showed that BAMBI binds all TGF-β/BMP Type I receptors except for ALK2. However, the direct interaction of BAMBI with TGF-β/BMP Type I receptors does not readily yield a mechanism explaining its non-discriminatory negative regulatory action on TGF-β-, activin- and BMP signaling. When further analyzing receptor activation in the presence of BAMBI, Onichtchouk *et al.* found that for TGF-β1, BAMBI was recruited into a heteromeric Type I:Type II receptor complex, which resulted in lower phosphorylation of the Type I receptor and hence attenuated TGF-β signaling. Thereby the BAMBI-mediated inhibition of TGF-βs, activins and BMPs seems to be due to the ligand-independent formation of Type I receptor:BAMBI assemblies. When these are recruited into ternary complexes with Type II receptors via ligand stimulation, the heterodimeric Type I receptor:BAMBI complexes prevent the formation of signaling-competent Type I:Type II receptor assemblies and seemingly shutdown all signaling from the TGF-β ligands. Based on these data, BAMBI seems to act therefore as a non-specific (with respect to the nature of the TGF-β ligand) decoy receptor rather than as a ligand-interacting TGF-β/BMP co-receptor.

Our current view of BAMBI acting solely as a membrane-located pan-TGF-β antagonist could be however biased, as only the limited, negatively regulatory effects of BAMBI on TGF-β/activin/BMP-mediated SMAD signaling or its BMP-characteristic functions during early embryonic development were analyzed [27,226,227]. The intracellular domain, which is highly conserved among different species, might exert additional functions other than only facilitating the direct interactions between BAMBI and the Type I receptors, as suggested by Onichtchouk *et al.* [27]. Another group, Yan *et al.* [228] showed that BAMBI's intracellular domain binds to SMAD7 (or in context of BMPs, it binds to SMAD6) and forms a ternary complex with ALK5, which increases the inhibitory activities of SMAD7 and BAMBI in a synergistic manner. Other interaction partners for BAMBI's cytoplasmic domain aside from SMAD6 and 7 possibly exist, and they might differently alter the signaling outcome of these BAMBI:TGF-β/BMP receptor assemblies. Conformingly, a link between BAMBI and the β-catenin/Wnt pathway has been described indicating that the intracellular domain of BAMBI interacts with the Wnt co-receptor LRP6 to form a ternary complex BAMBI:frizzled:LRP6 that positively regulates Wnt signaling [229]. From the data of this study, it is yet unclear whether BAMBI exerts its effects on Wnt signaling in a TGF-β/activin/BMP-independent manner or whether BAMBI's interaction with components of the Wnt receptor form the basis of TGF-β-Wnt crosstalk. To understand the significance of BAMBI as a TGF-β/BMP co-receptor, it is however also important to note that genomic deletion of BAMBI in mice did not yield a strong phenotype; $BAMBI^{-/-}$ mice were viable and showed no developmental defects, indicating no essential role for BAMBI in limb and craniofacial development [230], which is surprising considering that BAMBI seems to be co-expressed with BMP4 and supposedly acts as a negative feedback loop to control BMP activity [231]. Similarly, another study also identified only a mild endothelial dysfunction in $BAMBI^{-/-}$ mice, although this mutation in diabetic mice resulted in glomerular abnormalities, thereby impairing kidney function [232]. On the other hand, BAMBI expression levels seem to correlate with the aggressiveness and invasiveness of tumor cells in various cancer types, possibly by interfering with apoptotic/growth arrest-inducing signals from TGF-β and/or BMPs [233–237]. Hence the main function of TGF-β/BMP co-receptor BAMBI *in vivo* might exist more in a pathophysiological setting, such as cancer rather than in regulating TGF-β/BMP signaling during development.

Repulsive guidance molecules (RGMs): a BMP-stimulatory BMP-specific co-receptor acting on its own or bridging BMP and neogenin pathways?

In 2005, the so-called repulsive guidance molecule proteins were identified as the first co-receptors specific for SMAD1/5/8-activating BMPs and GDFs [238–241]. DRAGON (also known as RGMb), hemojuvelin (also known as RGMc), and RGMa are highly related members of the repulsive guidance molecule family in mammals, which share a common architecture with an N-terminal signal peptide for secretion and a modular structure comprising two domains (deduced from protein expression studies that revealed two fragments resulting from proteolytic processing at an acid-labile sequence motif) [242,243] (**Fig. 4**). RGM proteins lack a transmembrane domain, but they can localize with the cell membrane through post-translational attachment of a glycosylphosphatidylinositol (GPI)-moiety at their C-terminal region. In mice, RGMa and RGMb were found to be mainly expressed in the central nervous system, but were also detected at lower levels in the developing heart, lung, pancreas, liver, and limb cartilage [243,246,247]. A third isoform, RGMc/hemojuvelin exhibits a vastly different expression pattern and was found almost exclusively in skeletal muscle, heart, and liver [248]. The latter expression site links RGMc to its name hemojuvelin, as RGMc regulates the expression of the small peptide hormone hepcidin in the liver, which controls the absorption of iron from the gut (for reviews: [249,250]). Loss-of-function mutations in either hepcidin or RGMc/hemojuvelin are the molecular cause of the disease juvenile hemochromatosis, which leads to iron overload in the liver, cardiomyopathy, and diabetes (for reviews: [249,251]). In chicken, RGM, the ortholog of the mammalian RGMa and RGMb, is involved in guiding temporal retinal axons to their correct position in the optical tectum [242]. This led to the name repulsive guidance molecules and this activity seems to be unrelated to its function as a BMP co-receptor, as in this case RGM proteins seem to solely function as ligands for the netrin receptor neogenin [252,253]. While RGMa and RGMb are also expressed in neuronal tissues (similar to chicken RGM), both mammalian RGMs serve different functions in the CNS, likely due to their distinct non-overlapping expression pattern in neuronal tissues: e.g. RGMa is involved in neural tube closure and RGMb was shown to inhibit neurite outgrowth after injury [243,254]. *In vitro* experiments suggested that RGMb furthermore might have adhesive properties and can additionally promote adhesion between neuronal cells via homophilic interactions [246].

All three mammalian RGM isoforms were shown to enhance BMP but not TGF-β, signaling *in vitro*, and this BMP-sensitizing effect was most prominent at BMP concentrations too low to directly initiate BMP signaling [238,239,241]. However, the BMP signaling enhancing activity strictly required membrane-located RGMa, RGMb or RGMc/hemojuvelin and was due to a direct interaction with mature BMP and GDF ligands [239]. BMP:RGM interaction occurs with high affinities, but the three isoforms exhibit differential binding kinetics and affinities to various BMP and GDF ligands [255]. In contrast to the membrane-located RGMs, soluble RGM proteins inhibited BMP signaling in a dose-dependent manner

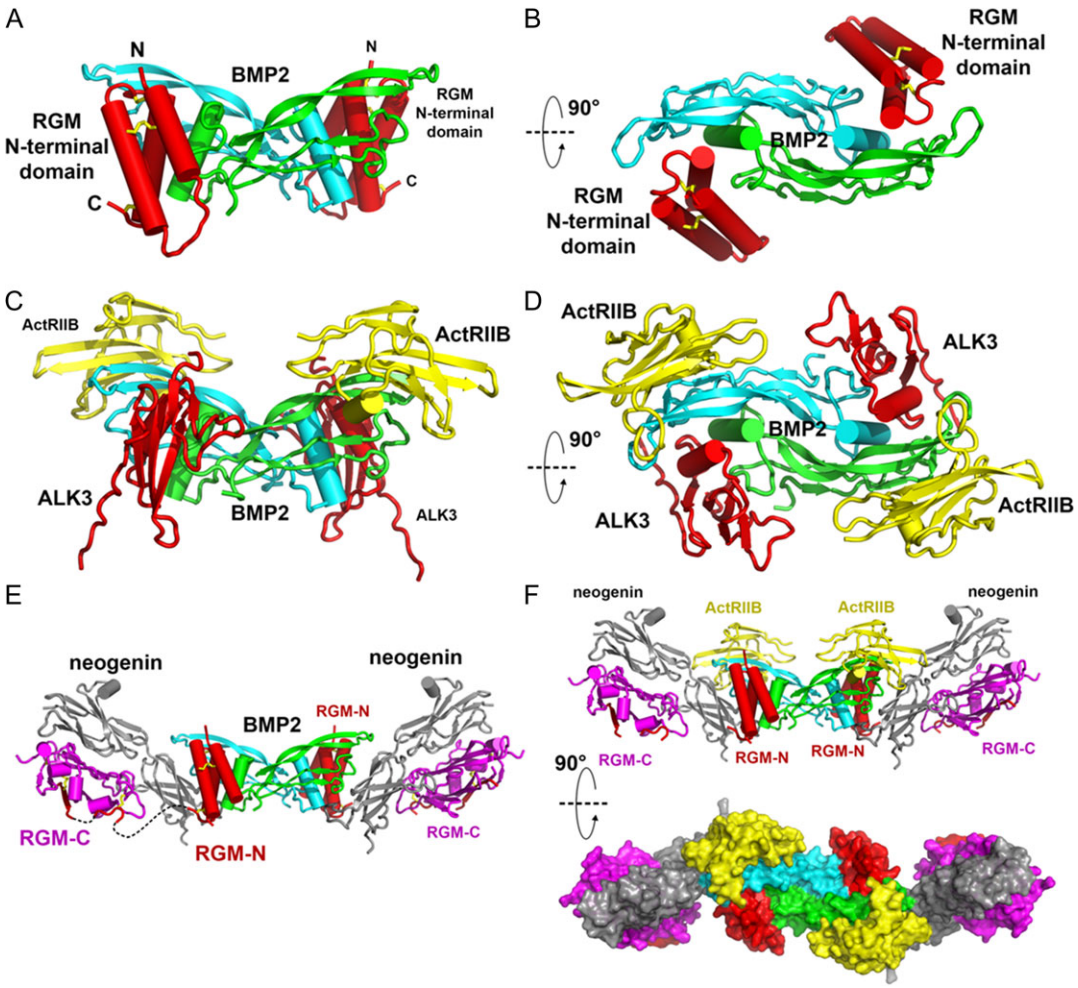

Figure 4. Structural determinants of the interaction between BMP and RGMs (A) The N-terminal domain of RGMs form a disulfide bond stabilized, three-helical bundle, which binds BMPs in their Type I receptor epitope [244]. (B) As in (A), but rotated by 90° counterclockwise around the *x*-axis. (C,D) A comparison of the BMP ligand:RGM co-receptor complex with the structure of the ternary complex of BMP2 bound to Type I receptor ALK3 and Type II receptor ActRIIB [245] clearly indicates that BMP:RGM interaction will block Type I receptor binding very likely explaining the inhibitory activity of soluble RGM proteins on BMP signaling. (E) Structure analysis have shown that BMPs can form ternary complexes with RGMs and the RGM receptor neogenin, where the N-terminal domain of RGMs (RGM-N shown in red) facilitates binding to BMPs (cyan/green), while the C-terminal RGM domain (RGMC marked in magenta) interacts with neogenin (shown in gray). Despite the pH-dependent proteolytic processing of RGM, the N-terminal and C-terminal subdomains remain covalently associated (indicated by stippled lines) by two intramolecular disulfide bonds (indicated as yellow sticks). This complex formation indicates that BMP and/or neogenin signaling can possibly affect/modulate each other [244]. (F) In the ternary BMP2:RGM:neogenin complex (see E) neogenin does not directly interact with BMP2 and hence does not block Type II receptor access to BMP2. This allows to build a theoretical model of a quaternary complex of BMP bound to RGM, neogenin and a Type II receptor, which would either link BMP and neogenin signaling or allow neogenin to modulate BMP signaling by altering receptor complex formation.

reminiscent of the inhibition of BMPs by BMP antagonists, such as noggin [256–259]. Although various studies used recombinantly produced soluble RGM forms and therefore their BMP inhibition might be of limited significance to RGM function *in vivo*, other groups reported endogenous soluble forms of RGMc/hemojuvelin in the serum that also clearly inhibited BMP activity in contrast to membrane-bound RGMc/hemojuvelin [257,260,261]. It has been suggested that the existence of soluble and membrane-bound RGMc/hemojuvelin *in vivo* might serve to tightly control BMP signaling via their opposing activities, and soluble RGMc/hemojuvelin production was shown to be indeed regulated by iron levels *in vitro* and *in vivo* [257,261,262]. While RGMs as GPI-anchored proteins could principally be converted to soluble proteins by shedding employing phospholipases, as reported for cripto-1 and other receptors (e.g. [170,263]), RGMc/hemojuvelin shedding was shown to be due to proteolytic processing by a proprotein convertase, such as furin, at a polybasic site highly conserved in RGMc/hemojuvelin from different species [264–266]. From mutational data of patients suffering from iron-refractory iron-deficient anemia, Silvestri *et al.* [267] identified the serine protease matriptase-2 (TMPRSS6) as one protease candidate responsible for generating soluble RGMc/hemojuvelin *in vivo*. Although the polybasic site for proteolytic processing present in the C-terminus of RGMc/hemojuvelin is absent in the two other RGM isoforms, shedding by different proteolytic processing or via removal of the glycosylphosphatidylinositol moiety by phospholipases might still be possible for RGMa and RGMb. So far, *in vivo* evidence for soluble RGMa or RGMb is sparse, and only soluble fragments of RGMa resulting from processing by the proprotein convertases SK-1 and furin have been reported [268]. RGMc/hemojuvelin might be therefore the only RGM protein that

exists in soluble and membrane-bound form, thereby exhibiting inhibitory or stimulatory activities.

The simplest way to antagonize BMP signaling is to block binding of the ligand to its receptors, which is the working paradigm of all BMP antagonists (for reviews: [1,222]). Consistent with such a mechanism, crystal structure analyses of RGM:BMP complexes by the group of Siebold *et al.* revealed that RGM binds and blocks the Type I receptor interaction site in BMP ligands [244]. The analysis also showed that RGMs are composed of two domains, an N-terminal α-helical BMP-binding domain and a C-terminal β-strand rich domain, the latter of which was shown to independently bind to the RGM receptor neogenin [269]. The acid-labile proteolysis site conserved in all three RGM isoforms is located between the two domains, which nevertheless remain covalently linked via two disulfide bonds after processing. The tight interaction between the N-terminal domain of RGMs and BMPs somewhat resembles that of BMP Type I receptor complexes, with similar interactions on the amino acid level, although the three-helical bundle of the RGM N-terminal domain shares little to no structural similarity with the three-finger toxin fold of the BMP Type I receptors [244] (**Fig. 4**). While full overlap of the binding sites for RGM and the Type I receptors on BMPs explains how soluble RGM proteins can effectively antagonize canonical BMP signaling, the RGM:BMP assembly raises questions about how membrane-located RGM proteins, on the other hand, can enhance BMP signaling. Since it is highly unlikely that the epitope on BMP differs between the membrane-located and soluble form of RGM, binding of membrane-located RGM to BMP should result in the same blockage of receptor binding and hence lead to inhibition of BMP signaling. These two opposing activities argue for a release mechanism by which the co-receptor is discarded from the ligand to make way for binding of the ligand to its respective TGF-β/BMP receptors. A similar release or replacement mechanism might be claimed to explain the endoglin-mediated enhancement of BMP9/10 signaling. Here, endoglin occupies the Type II receptor-binding site of BMP9/10, explaining how soluble endoglin can interfere with BMP9/10 signaling, while on the contrary, membrane-located endoglin, despite its binding ultimately blocks Type II receptor binding, promotes BMP9/10 signaling [33].

For RGMs, it is unclear whether neogenin might be involved in such a release mechanism, but structure analysis of a ternary complex of BMP2 bound to RGMb and a fragment of neogenin has revealed that RGMs can bind simultaneously to BMPs and neogenin, with both the N-terminal and C-terminal domains acting seemingly independently [244,269]. While the structural interactions of RGMs with either BMPs or neogenin seem to be independent, several functional studies showed that neogenin can be involved in BMP signaling ([270–273], for a review: [274]) (**Fig. 4**). To test the significance of the ternary BMP:RGM:neogenin complex formation found in crystal structure analyses, Healey *et al.* employed super-resolution microscopy, finding that BMP-mediated neogenin cluster formation occurred at the cell surface in the presence of membrane-bound RGM [244]. As clustering of receptors at the cell surface was proposed as a mechanism to augment signaling [275,276], this result might provide a hint as to how BMP signaling might be enhanced; however, it also indicates that RGMs and neogenin potentially together form a two-component BMP co-receptor system, raising the question as to whether RGMs also enhance BMP signaling in the absence of neogenin and if so, by which mechanism. While this explanation connects RGMs, neogenin, and BMPs, it still does not provide a fix to the mechanistic issue that interaction of RGMs with BMPs ultimately leads to the obstruction of BMP Type I receptor binding, the latter of which is however a prerequisite to initiate canonical SMAD signaling. In their binding analysis, Healey *et al.* observed a possible solution to this issue by showing that BMP binding of all three RGM isoforms is pH-dependent, whereas the binding of Type I receptors to BMPs is not [244]. The dissociation of RGMs from BMPs at a pH below 6.5 led the authors to suggest a mechanism by which RGMs first recruit BMPs to the cells surface and possibly into defined membrane microcompartments also known as lipid rafts. Here, these RGM:BMP complexes then possibly assemble into ternary complexes with BMP Type II receptors, because Type II receptor binding is not blocked by RGM-BMP interaction [244]. Upon constitutive clathrin-mediated endocytosis, which has been described for BMP Type I and Type II receptors ([277], for a review: [278]), these RGM:BMP:Type II receptor assemblies might be enriched together with non-occupied BMP Type I receptors in endosomes. Here, RGMs would then dissociate from the BMP:Type II receptor complex due to endosomal acidification to give way to binding of Type I receptors, leading to the subsequent formation of a signaling-competent heteromeric BMP ligand:receptor complex. As the endosome presents a 'protected' environment, endosomal capture and localization of such an RGM-mediated ternary BMP ligand:receptor complex might then confer enhanced SMAD signaling from this compartment [244]. However, this elegant mechanism is unfortunately not fully consistent with the observation that BMP2, which is covalently immobilized on macroscopic surfaces and therefore clearly excludes an endocytosis of intermediate RGM:BMP complexes prior to receptor and SMAD activation, still exerts full signaling capacities ([279–281], for a review [282]). Hence, either two SMAD activation schemes exist, which differ with respect to their subcellular localization; one, which is not facilitated by RGMs and emanates at the plasma membrane and another RGM-mediated scheme initiated from endosomal compartments; alternatively, another yet unknown mechanisms must explain the RGM-mediated enhancement of BMP-induced SMAD signaling.

One important, but often neglected, aspect here might be the fact that BMP receptors and downstream SMAD signaling do not require formation of a (symmetrical) tetrameric receptor assembly as (artificially) suggested by structural analyses (see also [1,11,12]). Studies using heterodimeric BMP2 variants with Type I or the Type II receptor-binding sites that were individually destroyed showed that loss of a single Type II receptor epitope already fully abrogates BMP-mediated signaling [16]. This result shows that two Type II receptors are required to initiate SMAD and p38/MAP-kinase signaling, which were proposed to be induced by different receptor assemblies, either by binding of the ligand to a preformed (receptor) complex (PFC, which induces SMAD) or by the BMP-induced signaling complex (BISC, which results in p38/MAP-kinase activation) [283]. In contrast, heterodimeric BMP2 variants that lack one Type I receptor sites could still activate SMAD signaling with almost no difference from wild-type BMP2, and p38/MAP-kinase activation (determined via induction of alkaline phosphatase expression) was attenuated only to a minor extent [10,16]. Hence, one possible alternative mechanism that explains how binding of membrane-located RGMs to BMPs might not directly lead to inhibition of BMP signaling could thus be due to formation of 'quaternary' complexes comprised of the BMP ligand, two Type II receptors, and one RGM and one Type I receptor. While such complexes would allow BMP signaling, it is unclear how, on the one hand, such complexes could be formed in a defined/steered fashion and why, on the other hand, such RGM/Type I receptor mixed assemblies then result in enhanced BMP signaling.

MuSK: a tyrosine kinase co-receptor for BMPs and a new era of growth factor crosstalk?

Numerous past reports have indicated the existence of an interaction/crosstalk between the TGF-β/BMP signaling pathway initiated by its serine/threonine receptor kinases and members of the more 'classical' receptor tyrosine kinases (RTKs) or pathway components of the latter [284–287]. In many reports, the crosstalk was considered to occur in the cytoplasm at the level of R-SMAD phosphorylation, thereby altering the transcriptional activity of the SMAD protein. Other connections for crosstalk were due to the binding of RTKs or their signaling components to the cytoplasmic domain of BMP receptors, e.g. interaction of Src kinase with the C-terminus of BMP Type II receptor BMPRII or receptor tyrosine kinase TrkC, which negatively regulates BMP signaling by binding to BMPRII and blocking the interaction of BMPRII with BMP Type I receptors [288–290]. Similar to these reports, another study showed a highly specific interaction of orphan tyrosine kinase receptor ROR2 with BMP Type I receptor ALK6 to modulate GDF5 signaling [291]. Here, the interaction was supposedly facilitated by the extracellular domain of ALK6 and the frizzled-CRD domain of ROR2, but it occurred independently of the ligand GDF5. According to the authors, complex formation then led to transphosphorylation of ROR2's cytoplasmic domain, resulting in inhibition of SMAD1/5/8 signaling and activation of SMAD-independent pathways [291]. While these reports suggest a potential involvement of tyrosine kinase receptors in BMP receptor activation and signaling under certain conditions, none showed that these interactions were dependent on, required, or involved the TGF-β ligand, and hence, the RTKs mentioned above might not be considered co-receptors like RGM or endoglin. However, in 2016, Fallon's group showed that the receptor tyrosine kinase MuSK (muscle-specific kinase) is a direct co-receptor for BMPs [21]. The RTK MuSK comprises an almost 500 aa large modular extracellular domain, a single-span transmembrane segment and a cytoplasmic region harboring the tyrosine kinase domain, a membrane-proximal motif for binding to phospho-tyrosine-binding (PTB) domain-containing proteins and a C-terminal sequence for interaction with PDZ-domain-containing proteins (for a review: [292]). The extracellular domain of MuSK comprises three immunoglobulin-like domains (Ig1 to -3), followed by a frizzled-like cysteine-rich domain (previously classified as C6 and a separate fourth Ig-like domain) [293]. Notably, MuSK shares an overall architectural similarity with a number of other RTKs, including members of the ROR family [294]. Before its discovery as a BMP co-receptor, the RTK MuSK was known for its involvement in neuromuscular synapse formation (for reviews: [292,295]). In this context, MuSK forms a complex with agrin, a large secreted proteoglycan produced in motor neurons and concentrated in the synaptic basal lamina, and low-density lipoprotein receptor-related protein 4 (LRP4), which is also involved in Wnt signaling. Upon assembly of these three components, MuSK becomes phosphorylated, which subsequently initiates synapse-specific transcription, thereby leading to accumulation and stabilization of postsynaptic proteins, which most importantly includes the acetylcholine receptor (AChR) [296,297]. Due to its impact on neuromuscular synapse formation, mutations in MuSK (or one of the other above-mentioned components) lead to failure in synapse formation and result in presynaptic defects known as congenital myasthenia (e.g. [298]; for a review: [299]). Moreover, autoantibodies against MuSK are found in a subfraction of patients suffering from the auto-immune disease myasthenia gravis, which, like congenital myasthenia, is a severe muscle-wasting disease (for a review: [300]). Interestingly, the dual (possibly unrelated) functions of MuSK, i.e. as a receptor for agrin:LRP4 in neuromuscular synapse formation and as a co-receptor to modulate BMP signaling in muscle cells, share some similarities with the dual functionalities of the repulsive guidance molecules, which also function as BMP co-receptors and paracrine ligands for the receptor neogenin to encode neuronal activities (see above). However, while biochemical and functional data for RGMs suggest that the two functions might not be fully independent (as highlighted by the finding of quaternary interactions between BMPs, BMP receptors, RGMs and neogenin), a crosstalk or connection between two functionalities of MuSK has not yet been reported.

In their study, Yilmaz *et al.* [21] showed that MuSK binds via its extracellular domain BMP4 and related SMAD1/5/8-activating BMPs with very high affinities. Employing gene expression analysis using either wild-type ($MuSK^{+/+}$) or $MuSK^{-/-}$ myoblasts, they showed that the presence of MuSK upregulates the expression of a distinct set of genes. Which genes are differentially affected seems to depend on the differentiation status, as gene expression differed between myoblasts and myotubes. The authors furthermore showed that BMP-mediated canonical SMAD signaling is enhanced by MuSK, which was similarly found for RGMs. Importantly, the upregulation of MuSK-dependent transcripts seems to depend on BMP canonical SMAD signaling, indicating that MuSK is part of the BMP ligand-receptor complex and likely does not form an alternative BMP receptor complex. Corroborating this hypothesis, Yilmaz *et al.* found that the tyrosine kinase activity of MuSK is not required for modulating BMP signaling and BMP4-stimulation did not result in phosphorylation of MuSK [21]. While kinase activity of MuSK seems therefore dispensable, membrane localization was identified as a prerequisite for its BMP-stimulatory activity, as soluble MuSK (similar to RGMs) inhibited BMP activity in a dose-dependent manner. This inhibitory activity of soluble MuSK points towards a similar co-receptor-ligand interaction mechanism to that observed for RGMs or endoglin, with MuSK possibly binding to and thereby blocking either the Type I or the Type II receptor-binding epitope. However, in contrast to RGMs, MuSK was found to bind to BMP Type I receptors directly in a ligand-independent manner, which indicates that the interaction mechanisms of MuSK and RGMs might differ at least in the details, although on the basis of the current data, no defined MuSK-facilitated activation mechanism can be drawn.

In spite of the fact that MuSK-mediated enhancement of BMP signaling does not involve its tyrosine kinase activity, which was long awaited in order to find a link between the observed effects addressed by the TGF-β/BMP receptor serine/threonine kinases and the observed involvement of components/pathways of receptor tyrosine kinases, other interactions might interconnect both signaling pathways and might point towards a direct crosstalk not depending on transcriptional regulation as an intermediate step. The similarity of MuSK with other RTKs furthermore allows us to speculate that in the near future, other RTKs might be identified to act as BMP co-receptors, thereby providing new possibilities to explain the high functional diversity of TGF-β ligands, which has not been possible so far with the very limited set of classical TGF-β/BMP receptors.

Conclusion

In our review we decided to analyze co-receptor functionalities and their impact on the signaling pathway(s) of individual TGF-β members by separating the co-receptors into two classes: co-receptors that interact with SMAD2/3-activating TGF-β ligands and co-receptors involved in modulation of SMAD1/5/8-activating BMPs and GDFs. This classification is based on the observation that the TGF-βs, the activins and inhibins as well as SMAD2/3-activating GDFs can potentially exert

also long-range activities besides their usual autocrine and paracrine functions (for reviews: [301–303]). In contrast, the majority of the SMAD1/5/8-activating TGF-β ligands from the BMP/GDF subgroup (exceptions are BMP9 and 10) seem to function in a strict paracrine manner and in many cases act as classical morphogens (for reviews: [196,304]). This assignment is corroborated by the higher serum levels found for TGF-βs, activins and GDF8 than for BMP2, BMP4 and BMP7, which differ by two to three orders of magnitude [305–310]. Growth factor processing and activation also differ significantly between these two classes: The proprotein complexes of TGF-βs and the SMAD2/3-activating GDF8 and -11 are stored in a latent form and require active, ECM-mediated release of the mature growth factor domain (for a review: [311,312]). In contrast, BMP/GDF proprotein complexes are usually not latent [216] and hence ECM interactions play a major role in regulating the active concentration of the mature growth factor in interstitial fluids and in controlling its distribution throughout the tissues (for a review: [196,313]). Also receptor activation differs between these two classes. The specific activities of TGF-βs and activins are often found to exceed the binding affinities to their cognate receptors, raising the question how these factors can activate their receptors so effectively [314–317]. For most BMPs (except for BMP9 and 10, see [318]), the effective half-maximal concentration required to trigger receptor activation is usually in the range of their receptor-binding affinities [10]. It seems therefore reasonable to assume that co-receptor functionality likely differs for the members of either TGF-β growth factor branch. For instance, TGF-βs, activins and inhibins often utilize co-receptors to activate signaling capacities of a particular TGF-β ligand, e.g. ligand presentation by betaglycan enables effective TGF-β2-signaling [25,36], cripto-1 mediates activation of nodal signaling (for a review: [175]). Co-receptors acting on TGF-βs and activins/inhibins are also often involved in switching signaling between two TGF-β growth factors: For instance, betaglycan can switch-on inhibin-mediated antagonism of activins through competing-off activins from their activin Type II receptors shared with inhibins, whose receptor-binding affinities are however too low in the absence of betaglycan [73,76]. Similarly, the presence of cripto-1 activates nodal signaling, while in the absence of cripto-1, activin signaling would constitute the default [188,189]. In contrast, BMP-specific co-receptors such as RGMs or MuSK seem to rather modulate than to activate BMP signaling, e.g. by slightly enhancing the signaling capacity thereby lowering the required BMP concentration for receptor activation or by slightly altering the gene expression profile [21,238,241]. It is of particular interest that RGMs and MuSK also act in other signaling pathways unrelated to BMPs and thus have both dual signaling capabilities: RGMs exert neuronal functions via the netrin receptor neogenin and MuSK together with LRP4 act as receptors for the proteoglycan agrin in the formation of neuromuscular synapses (for reviews: [295,319]). It is unclear so far, whether these alternative functions of RGMs and MuSK are 'autonomous' and independent of their BMP co-receptor function, like dual functionalities in the so-called moonlighting proteins (for reviews: [320–322]), or whether both functionalities cooperate and result in direct crosstalk. Although quaternary interactions between BMPs, their receptors, RGMs and neogenin have been confirmed biochemically, reports about functional dependencies of BMP and neogenin signaling are conflicting [273,323–328]. For MuSK, a direct involvement of MuSK RTK components in BMP signaling needs to be validated first and it is also not clear yet, as to whether interaction of BMP with MuSK will influence agrin:LRP4:MuSK signaling, although a functional connection between BMP4 and agrin has been described recently [329]. However, mapping of the interaction sites does not exclude quaternary interactions for BMPs with the MuSK:agrin:LRP4 complex [21].

One common, mechanistically important feature of various co-receptors for TGF-β/BMP ligands is their dual, opposing activity when acting in membrane-bound form compared to their soluble form. For RGMs and endoglin, the inhibitory activity of the soluble forms could be explained with the overlap between the binding epitopes for the co-receptors and either a Type I or a Type II receptor on the respective BMP ligand, hence allowing exclusive binding only of either the co-receptor or the BMP receptor. For other co-receptors of TGF-β ligands that similarly exhibit inhibitory activity in their soluble form a similar receptor-blocking mechanism seems likely. This however creates a dilemma, if the membrane-bound co-receptor instead exerts an activating function. Since the binding epitope of the co-receptor certainly does not differ between the soluble and membrane-located form, the expectable blockage of the receptor-binding site(s) by the membrane-bound co-receptor inevitably requires that also this form must act as an inhibitor of the respective TGF-β ligand contrary to what is observed. However, the switch between an inhibitory and a promoting ligand activity is somewhat reminiscent to the opposing *in vitro* and *in vivo* function of the BMP inhibitors chordin, crossveinless-2 (BMPER) and twisted gastrulation (Tsg), that all act as BMP antagonists when applied in soluble form in cell-based assays, but *in vivo* were shown to rather act as local BMP agonists ([330–332], for a review: [333]). While all three components act individually as BMP antagonist when applied in cell-based assays, *in vivo* they cooperate and upon proteolytic processing by specific metalloproteases, these antagonists are locally converted into BMP-promoting transport or storage molecules, which release BMPs possibly in high concentration in spatially confined areas [333]. The possibility of a proteolytic processing as potential switch between inhibitory and promoting activity of co-receptors for TGF-β ligands seems not very likely given the diversity of the various TGF-β co-receptors. Other mechanisms such as an intermediate endocytosis step to disrupt co-receptor blockage in endosomal compartments, as proposed for RGMs, also have their limitations, requiring a pH-dependent binding of the co-receptor and contradict activity of modified BMP factors, which cannot be internalized. What has not yet considered as potential mechanism is the membrane localization of the co-receptor itself. Our assumption that soluble and membrane-located forms of these co-receptors must similarly interfere with the assembly of a signaling-competent hetero-tetrameric TGF-β/BMP receptor complex is however based on *in vitro* binding affinity data obtained solely with soluble proteins. It hence completely disregards the effect that a confinement of interaction partners in a two-dimensional plane might have on the underlying interaction [334,335]. Furthermore, interactions between the transmembrane segments or the intracellular domains of the canonical TGF-β/BMP receptors might implement a sequential binding mechanism with an intermediate, transient complex involving the co-receptor, which, however, then fosters the formation of the known TGF-β/BMP receptor assembly. Hence the blocking of a receptor epitope on a TGF-β/BMP ligand by a co-receptor, while seemingly being incompatible with an activity-promoting functionality, might not impede complex formation and receptor activation in all contexts.

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