Agonists and Antagonists of TGF-β Family Ligands

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The discovery of the transforming growth factor β (TGF-β) family ligands and the realization that their bioactivities need to be tightly controlled temporally and spatially led to intensive research that has identified a multitude of extracellular modulators of TGF-β family ligands, uncovered their functions in developmental and pathophysiological processes, defined the mechanisms of their activities, and explored potential modulator-based therapeutic applications in treating human diseases. These studies revealed a diverse repertoire of extracellular and membrane-associated molecules that are capable of modulating TGF-β family signals via control of ligand availability, processing, ligand–receptor interaction, and receptor activation. These molecules include not only soluble ligand-binding proteins that were conventionally considered as agonists and antagonists of TGF-β family of growth factors, but also extracellular matrix (ECM) proteins and proteoglycans that can serve as "sink" and control storage and release of both the TGF-β family ligands and their regulators. This extensive network of soluble and ECM modulators helps to ensure dynamic and cell-specific control of TGF-β family signals. This article reviews our knowledge of extracellular modulation of TGF-β growth factors by diverse proteins and their molecular mechanisms to regulate TGF-β family signaling.

Transforming growth factor β (TGF-β) family signaling uses a large number of secreted growth factors that engage a limited number of cell-surface receptors, and regulate diverse processes, such as embryonic induction and patterning, tissue maintenance and repair, stem cell renewal and differentiation, and organism growth and regeneration. The prevalence of TGF-β family signaling in almost all metazoan cell types, the overlapping and distinct functions of many related ligands, and the strict temporal and spatial requirement for suitable signaling levels necessitate stringent control of TGF-β family signaling. A prominent strategy used by cells to regulate TGF-β family signaling is through the use of extracellular agonists and antagonists of TGF-β family ligands. An impressive array of such regulatory molecules has been identified and they associate with TGF-β family ligands directly or indirectly to modulate their processing, secretion, stability, diffusion, and presentation. Collectively, extracellular agonists and antagonists play crucial roles in determining TGF-β family signaling strength, range, timing, and duration, and serve as nodes for signal cross talk with other growth factor

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pathways. Here, I combine the TGF-β family modulators into two groups, soluble regulators versus extracellular matrix (ECM) residents and proteoglycans. My main emphasis is on the various modes to regulate ligand availability and activity, with omission of many additional functions because of limited space.

SECRETED REGULATORS OF TGF-β FAMILY LIGANDS

Extracellular modulation of TGF-β family signals is achieved by structurally diverse soluble TGF-β-binding proteins (Table 1). These secreted regulators have overlapping and distinct substrate specificities, bind to ligands with different affinities, and show differential interactions with ECM components or cell-surface molecules. Their combined actions are often crucial elements in defining the outcome of TGF-β family signals (Figs. 1 and 2).

Follistatin Domain Proteins

Follistatin

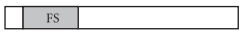
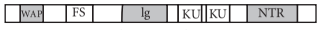
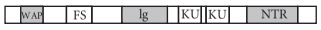
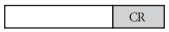
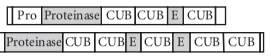
The follistatin (FS) domain is defined by 10 spatially conserved cysteines in a ~70 amino acid stretch. Follistatin (FST), the prototype protein, contains three FS modules and is a monomeric glycoprotein that binds activin with high affinity (K_D 46 to 900 pM) (Nakamura et al. 1990; Sugino et al. 1993; Schneyer et al. 1994; Hashimoto et al. 2000; Sidis et al. 2001; Keutmann et al. 2004). Interaction with follistatin masks the receptor binding sites in activin, resulting in inhibition of activin signaling (de Winter et al. 1996; Thompson et al. 2005; Harrington et al. 2006). Of the three follistatin isoforms, the short form, Fst288, has a 6- to 10-fold higher affinity for activin than the long form, Fst315, and associates uniquely with cell-surface heparan sulfate proteoglycan (HSPG) to promote endocytosis and subsequent lysosomal degradation of activin in pituitary cells (Inouye et al. 1991; Nakamura et al. 1991; Sugino et al. 1993; Hashimoto et al. 1997). The antagonistic interaction between activin and follistatin modulates a variety of cellular processes in many tissues, such as gonads, pituitary gland, vasculature, and liver (reviewed by Phillips and de Kretser 1998; Bilezikjian et al. 2012).

Besides activin, follistatin also interacts functionally and biochemically with other TGF-β family members. Follistatin binds directly to bone morphogenetic protein 2 (BMP-2), BMP-4, and BMP-7, but with much lower affinities (K_D ~5.29 nM to 80 nM) than to activin (Fainsod et al. 1997; Iemura et al. 1998; Amthor et al. 2002). Follistatin does not block BMP-4 from binding to its type I BMP receptor. Instead, it forms a nonfunctional ternary complex with BMP and its receptor (Iemura et al. 1998) and inhibits BMP signaling. For example, follistatin regulates BMP activities during dorsoventral patterning of early *Xenopus* embryos (Hemmati-Brivanlou et al. 1994; Fainsod et al. 1997; Iemura et al. 1998; Yamamoto et al. 2000), BMP-7 function in muscle growth (Amthor et al. 2002) and blocks the growth-inhibitory activity of BMP-7 in mammalian cell culture (Yamashita et al. 1995). Different from what is expected from its in vitro measured affinity, follistatin blocks BMP-7 more efficiently than BMP-4 in functional assays (Liem et al. 1997). Follistatin also binds myostatin (growth and differentiation factor 8 [GDF-8]) with high affinity (K_D 584 pM) (Amthor et al. 2004), blocks association of myostatin with its type II receptor, ActRIIB, and interferes with the function of myostatin to inhibit muscle growth (Lee and McPherron 2001; Zimmers et al. 2002; Amthor et al. 2004). Additionally, follistatin can form an inactive complex with BMP-15 and prevents it from regulating proliferation and differentiation of granulosa cells in the ovary (Otsuka et al. 2001). Furthermore, follistatin antagonizes the activities of BMP-11 and anti-dorsalizing morphogenetic protein (ADMP) during early *Xenopus* development, although biochemical interactions of follistatin with BMP-11 or ADMP have not been shown (Gamer et al. 1999; Dosch and Niehrs 2000).

FSTL1 and FSTL3

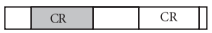
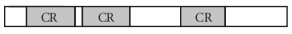
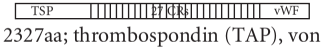
Follistatin-like 1 (FSTL1, also known as FRP, Flik, or TSC36) is a secreted glycoprotein with

Table 1. Agonists and antagonists of TGF-β family growth factors

Gene	Official symbol (human/ *Drosophila*)	Chromosomal location (human)	Structure	Ligands[a]	Comments
Follistatin	Follistatin (FST)	5q11.2	 288–315aa; cysteine-rich follistatin modules (FS)	**Activin** >> **BMP-2, -4, -7**[b]**, -15, GDF-8,** BMP-11/GDF-11, ADMP	Blocks activin–receptor interaction; forms inactive complex with BMP and BMPRI
FRP, FLIK, TSC-36, FSTL1	FSTL1	3q13.33	 306aa	**BMP-4**, BMP-2, TGF-β1, activin-A	Binds multiple receptors too to modulate ligand–receptor interaction
FSRP, FLRG, FSTL3	FSTL3	19p13	 256aa	**Activin-A, GDF-8** >> **BMP-2, -6, -7**	
WFIKKN1, GASP-2	WFIKKN1	16p13.3	 548aa; whey acidic protein (WAP), immunoglobin (Ig), Kunitz (KU) family protease inhibitor	**GDF-8, GDF-11** >> TGF-β1, BMP-2, -4	Binds to propeptide of GDF-8 and mature GDF-8 using different domains
WFIKKN2, GASP-1	WFIKKN2	17q21.33	 576aa; whey acidic protein (WAP), immunoglobin (Ig), Kunitz (KU) family protease inhibitor	**GDF-8, GDF-11** >> TGF-β1, BMP-2, -4	
Tomoregulin1, TMEFF1	TMEFF1	9q31	 ~370aa; epidermal growth factor (EGF) domain, transmembrane (TM)	Nodal, Vg1, BMP-4	Binds Nodal coreceptor Cripto to block assembly of Nodal/Vg1-R complex
Noggin	NOG	17q21 -q22	 210–240aa; cysteine-rich (CR)	**BMP-2, -4>>BMP-5, -6, -7, GDF-5, -6**	Blocks binding of BMPs to their receptors
Chordin	CHRD/sog	3q27	 941–956aa; cysteine-rich (CR) VWC domain	**BMP-2, -4, -7, ADMP**[c]	Blocks binding of BMPs to their receptors
BMP-1 and Tolloid-like proteins	BMP1, TLL1, TLL2	BMP1, 8p21 TLL1, 4q32-q33 TLL2, 10q23-q24	 BMP1 (top) and TLLs; EGF domain (E), complement-uegf-BMP1 domain (CUB)	**BMPs (enhance) GDF-8, -11 (activate)**	Cleaves chordin to enhance BMP signals; process propeptides of GDF-8 and -11 to activate

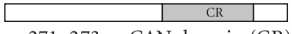
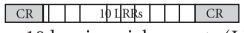
Continued

Table 1. *Continued*

Gene	Official symbol (human/ *Drosophila*)	Chromosomal location (human)	Structure	Ligands[a]	Comments
Ogon/Sizzled			 281aa; frizzled-like cysteine-rich domain [CRD(Fz)]	BMPs	Binds BMP-1/Tolloid proteases to prevent chordin association, thus stabilizing chordin
Olfactomedin 1/ ONT1	OLFM1	9q34.3	 458–485aa; coiled-coil domain (CC), olfactomedin domain (OLF)	BMPs	Binds BMP-1/Tolloid and Chordin, dose-dependent enhancement or inhibition of BMPs
Twisted Gastrulation	TWSG1/tsg	18p11.3	 222–260aa; cysteine-rich (CR)	**BMPs (dual)**	Both promote and inhibit BMPs; can form ternary complex with BMP and chordin/CHRDL to block BMP-BMPR interaction
CHL, Neuralin, Ventroptin	CHRDL1	Xq22.3	 333–451aa; chordin-like VWC (CR)	**BMP-4**>>BMP-5,-6, >>TGF-β2	Blocks binding of BMP-4 to BMPRIB
CHL2	CHRDL2	11q14	 ~430aa; chordin-like VWC (CR)	**BMP-2**[c]**, -4**[c]**, -6**[c]**,-7**, BMP-5, GDF-5, activin-A[c]	Blocks binding of BMP-4 to BMPRIB
Brorin	VWC2	7p12.2	 325aa; cysteine-rich VWC (CR)	BMP-2, -6	
Crossveinless-2, Bmper, CRIM3	BMPER	7p14.3	 678–751aa; von Willebrand factor D domain (VWD), trypsin inhibitor-like cysteine-rich domain (TIL), VWC domain (CR)	**BMP-2, -4**, BMP-6, -7, GDF-5 **(dual)**	Cleavage, chordin-, and ECM-association alters its anti- and pro-BMP activities
CRIM-1	CRIM1	2p21	 1029–1036aa; insulin-like growth factor–binding protein (IGFBP), chordin-like (CR)	**BMP-4, -7**	Reduces processing and retains surface-bound BMP precursors
Kielin			 2327aa; thrombospondin (TAP), von Willebrand factor (vWF), 27 CRs	BMP?	

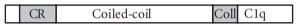
Continued

Table 1. *Continued*

Gene	Official symbol (human/ *Drosophila*)	Chromosomal location (human)	Structure	Ligands[a]	Comments
KCP1	CRIM2	7q32.1	 1254aa; 18 CRs	**BMP-7 (enhance) Activin A, TGF-β1**	Enhances binding of BMP-7 to BMPRIA
DAN	NBL1	1p36.13-p36.11	 175–181aa; CAN domain (CR)	**GDF-5 > BMP-2, BMP-4**	
Cerberus, Cer-l, Caronte, Charon	CER1	9p23-p22	 271–273aa; CAN domain (CR)	**Nodal/Xnr1, BMP-4, -7**	Blocks binding of ligands to their receptors
Coco, Dante, Cer2, GREM3	DAND5	19p13.13	 185–217aa; CAN domain (CR)	**Xnr1, BMP-4**	
Drm, Gremlin, DAND2	GREM1	15q13-q15	 184aa; CAN domain (CR)	**BMP-2 > BMP-4 > BMP-7**	Can bind to BMP-4 precursor to prevent its processing and secretion
PRDC, DAND3	GREM2	1q43	 168aa; CAN domain (CR)	**BMP-2, -4** >> BMP-6, -7	
Sclerostin, SOST	SOST	17q11.2	 211–213aa	**BMP-5, -6, -7 >> BMP-2, -4**	Blocks binding of BMPs to BMPRIA/II; promotes retention and degradation of BMP-7; binds Lrp5/6 to inhibit Wnt in bone
Ectodin, USAG-1, Wise, Sostl	SOSTDC1	7p21.1	 206aa	**BMP-2, -4, -6, -7**	Inhibits both BMP and Wnt signals
CTGF	CTGF	6q23.1	 349aa; von Willebrand factor type C (VWC/CR), thrombospondin (TSP), carboxy-terminal cystine knot (CT)	**TGF-β1 (enhance) BMP-4**	Promotes TGF-β binding to its receptors; inhibits BMP-4-BMPRIA interaction
Norrie	NDP	Xp11.4	 133aa; carboxy-terminal cysteine-knot-like domain (CTCK)	**Xnr1,** Activin**, BMP-4**	
Decorin	DCN	12q21.33	 360aa, 10 leucine-rich repeats (LRRs)	**TGF-β1, 2, 3 (dual), GDF-8,** activin-C	Matrix-dependent inhibition or enhancement of TGF-βs

Continued

Table 1. *Continued*

Gene	Official symbol (human/ *Drosophila*)	Chromosomal location (human)	Structure	Ligands[a]	Comments
Biglycan	BGN	Xq28	 369aa; 10 leucine-rich repeats (LRRs)	TGF-β1, 2, 3 **BMP-4 (dual)**	Forms ternary complex with BMP and chordin to block BMPs; may also enhance BMPs in other contexts
Tsukushi	TSKU	11q13.5	 351–355aa; 12 leucine-rich repeats (LRRs)	**BMP-4, Vg1 (enhance)**	Forms ternary complex with BMP and chordin to block BMPs
Glypican 3	GPC3	Xq26.1	GPI-anchored proteoglycan	**BMP/Dpp (dual)**	Controls ligand diffusion as well as cellular responses to BMPs
Syndecan-2, fibroglycan	SDC2	8q22-q23	Transmembrane proteoglycan	**TGF-β1, Vg1 (enhance)**	Possible coreceptor for Vg1 in *Xenopus*
α2-macroglobulin	A2M	12p13.3-p12.3	Homotetrameric glycoprotein	**TGF-β**	Matrix-dependent of retention and release of TGF-βs
Procollagen IIA			Structural matrix protein	**BMP-2, -4,** TGF-β1	Controls ligand availability
Emilin	EMILIN1	2p23.3-p23.2	 1016aa; cysteine-rich EMI domain (CR), collagenous domain (Coll)	**pro-TGF-β1**	Prevents processing of pro TGF-β1 by Furin-like pro protein convertases

TGF, Transforming growth factor β; ADMP, antidorsalizing morphogenetic protein; VWC, Von Willebrand factor type C; BMP, bone morphogenetic protein; GPI, glycosylphosphatidylinositol.

[a]The ligands in bold are tested biochemically and functionally (in cell culture or in model organisms); the ones in regular font bind to agonists/antagonists biochemically; and the ligands underlined are examined functionally only. Unless indicated in parentheses in the table, the ligands are inhibited by genes shown.

[b]Biochemical data suggest that follistatin binds to BMP-2 and -4 (5.29 nM and 23 nM, respectively) with higher affinity than BMP-7 (80 nM), but functional data indicate that follistatin may inhibit BMP-7 more efficiently in vivo.

[c]Conflicting results have been reported on these molecules.

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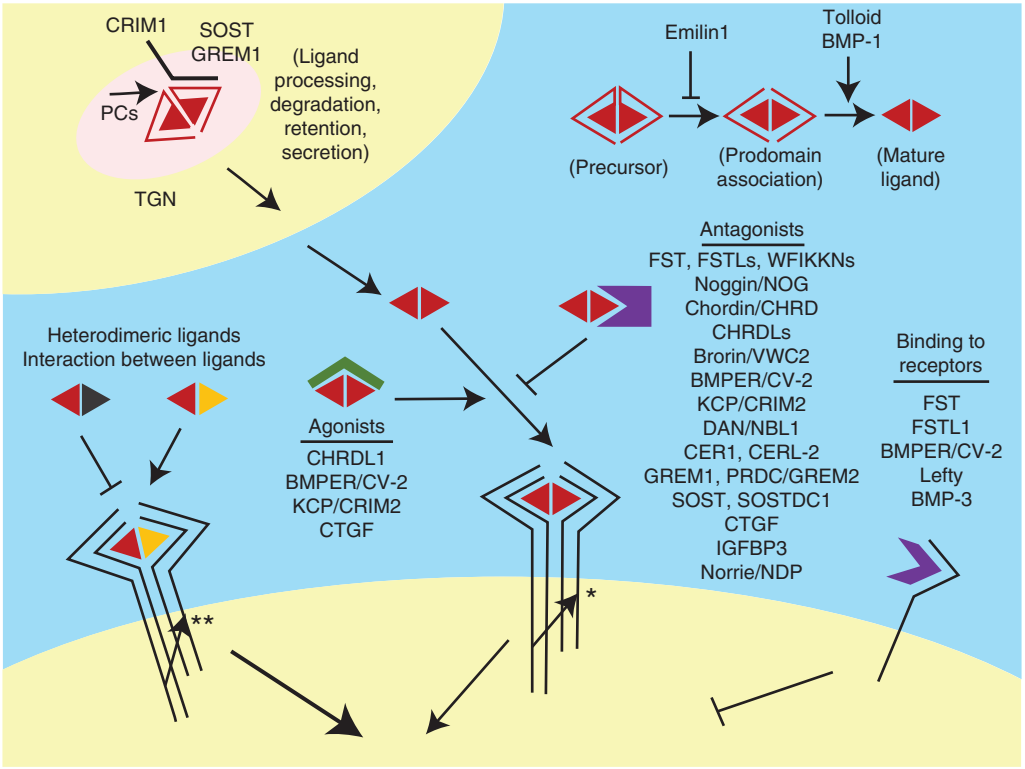

Figure 1. Regulation of transforming growth factor β (TGF-β) family signals by extracellular agonists and antagonists. Most extracellular agonists and antagonists act to facilitate or prevent binding of mature TGF-β family ligands to their receptor complexes, respectively. The secreted proteins CHRDL1, BMPER/CV-2, KCP/CRIM2, and connective tissue growth factor (CTGF) act both as agonists and antagonists depending on the particular ligands they regulate and the presence or absence of other factors in cell-type-specific microenvironments they encounter. Certain soluble modulators, including follistatin (FST), FSTL1, BMPER/CV-2, Lefty, and bone morphogenetic protein 3 (BMP-3), can also bind to type I and/or type II receptors to form a nonsignaling complex. Regulation of ligand processing, secretion, activation, and/or stability by CRIM1, SOST, GREM1, and the propeptides in the ligand-producing cells can control ligand availability. Extracellular regulation of ligand processing by Emilin1 and ligand release by Tolloid/BMP-1 family proteinases also control ligand bioactivity. Furthermore, TGF-β family ligands can form heterodimers or interact with each other, which leads to either blocking or enhancing TGF-β family signaling depending on the particular ligands involved. TGN, *trans*-Golgi network.

a single FS module. Biochemical analyses reveal some contradictory results on FSTL1 binding to multiple TGF-β family ligands, possibly because of different cell types and methods used (Tanaka et al. 2010; Geng et al. 2011; Xu et al. 2012a). FSTL1 binds to BMP-2, BMP-4 (K_D 0.19 nM to 7.2 nM), TGF-β1 (K_D 12 pM to 36 nM), and possibly activin A (K_D 1.43 nM). FSTL1 is also reported to bind several type II receptors (BMPRII, ActR-IIB, TβRII), as well as type I BMP receptors (ALK-3/BMPRIA and ALK-6/BMPRIB), to modulate TGF-β family signaling. FSTL1 regulates many developmental processes in vertebrates, such as dorsoventral patterning, lung, skeletal, and ureter development, and altered FSTL1 levels are associated with many diseases, including inflammation, cardiac diseases, and cancer (reviewed by Sylva et al. 2013). A related protein, Follistatin-like 3 (FSTL3, also known as FSRP or FLRG), is a glycoprotein with two FS modules and binds activin A with an affinity of 40–850 pM and to

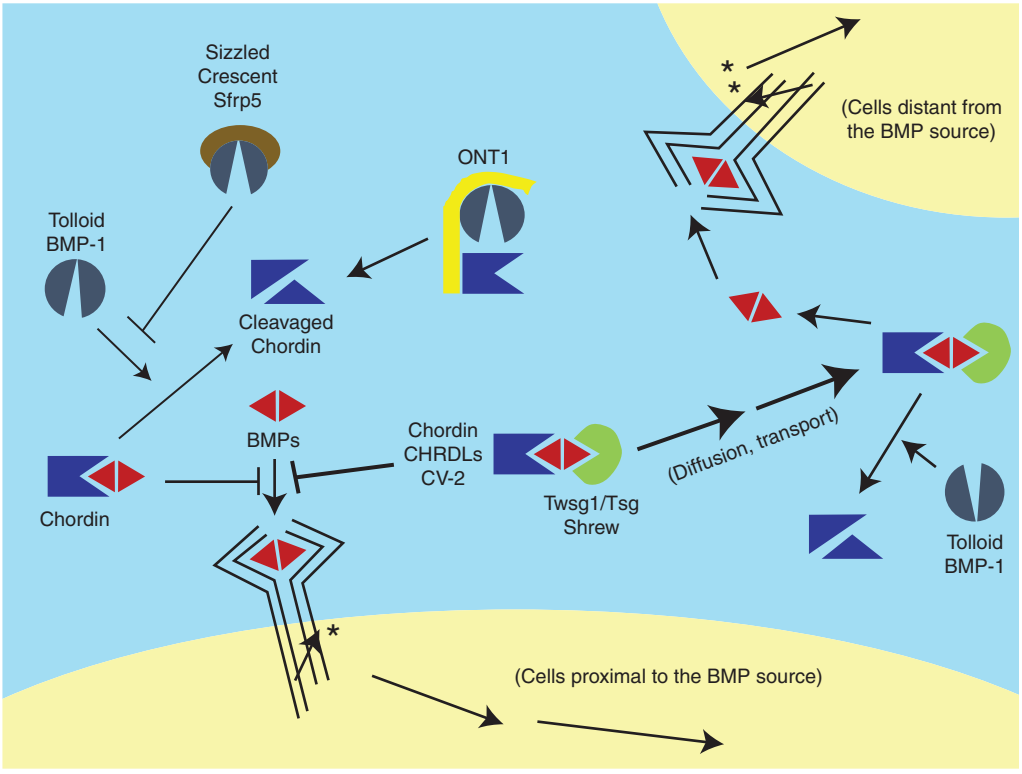

Figure 2. Regulation of bone morphogenetic protein (BMP) signaling by chordin-dependent extracellular regulatory network. Inhibition of BMP signaling by chordin can be enhanced by formation of a ternary complex with Twsg1/Tsg. At the same time, this complex promotes transport of the BMP ligands to a distant site to allow formation of a sharp high BMP activity center. Inactivation of chordin function is achieved by Tolloid/BMP-1-dependent proteolytic processing to release the associated BMP ligands. Cleavage of chordin is prevented by Sizzled, Crescent, or Sfrp5, which titrate Tolloid/BMP-1 away from chordin. The scaffolding protein ONT1 binds both Tolloid/BMP-1 and chordin to facilitate chordin processing. The proteins containing chordin-like cysteine-rich (CR) domains, including CHRDLs and CV-2, can form a similar ternary complex with BMP and Twsg1 to have stronger BMP-inhibitory activity.

BMP-2, -6, and -7 with low affinity. Like follistatin, FSTL3 inhibits the activities of activin and BMPs in cell culture (Tsuchida et al. 2000; Schneyer et al. 2001; Sidis et al. 2002, 2006). FSTL3 also forms a tight complex with myostatin in serum, and can inhibit myostatin function (Hill et al. 2002). Crystal structure reveals a unique association of an amino-terminal domain of FSTL3 with myostatin, but not activin A (Cash et al. 2012). Mice with targeted deletion of *Fstl3* are viable and fertile with defects in glucose and lipid homeostasis (Mukherjee et al. 2007). Other follistatin-like proteins also exist. FSTL2, also known as IGFBP7, may act in the insulin-like growth factor pathway (Evdokimova et al. 2012), whereas the biological activities of FSTL4 and FSTL5 are not well understood.

WFIKKN1 and WFIKKN2

WFIKKN1 and WFIKKN2 (also known as GASP-2 and GASP-1 for "growth and differentiation factor-associated serum protein" -2 and -1) are secreted factors that contain a single FS module in addition to a WAP, an immunoglobulin, two Kunitz-type protease inhibitors, and an NTR domain. Both proteins bind with high affinity to mature myostatin (GDF-8, K_D 33.5 nM and 286 pM, respectively) as well as GDF-11 (K_D 2.25 nM and 164 pM, respectively),

and they also associate with the propeptide of myostatin. Domain analysis shows that WFIKKN1 binds myostatin primarily through its FS module, whereas it binds the myostatin propeptide with its NTR domain. WFIKKN binding inhibits the activity of myostatin to regulate muscle development (Hill et al. 2003; Kondas et al. 2008; Szlama et al. 2013). WFIKKN1 and WFIKKN2 also bind TGF-β1, BMP-2, and BMP-4 with affinities in the micromolar range. However, unlike GDF-8 and GDF-11, WFIKKN binding to these growth factors does not block their signaling in reporter assays (Szlama et al. 2010).

TMEFFs

TMEFFs (or tomoregulins) are transmembrane proteins with two FS modules in their ectodomains (Eib and Martens 1996; Horie et al. 2000). One of these, TMEFF1, inhibits nodal, Vg1, and BMP signaling in *Xenopus* without affecting activin signaling (Chang et al. 2003). Unlike most FS domain proteins, TMEFF1 does not bind nodal. Instead, it interacts with the nodal coreceptor Cripto to prevent assembly of a signaling ligand–receptor complex. This represents a novel mechanism for regulation of TGF-β signaling by FS module–containing proteins (Harms and Chang 2003).

Other FS domain–containing proteins include ECM proteins like SPARC and agrin, but their functions in regulating TGF-β family growth factors have not been shown, although they may affect ligand expression at the transcriptional level (Schneyer et al. 2001).

Noggin

Noggin is a small soluble protein of 210–240 amino acids. It contains a carboxy-terminal cysteine-rich domain and is secreted as a homodimeric glycoprotein. Noggin was originally identified in *Xenopus* embryos as a dorsalizing factor (Smith and Harland 1992; Lamb et al. 1993; Smith et al. 1993). Subsequently, four noggin homologs were identified in zebrafish, Fugu, *Xenopus tropicalis, Xenopus laevis*, and chick, but only one noggin gene exists in mammals (Furthauer et al. 1999; Fletcher et al. 2004; Eroshkin et al. 2006). Noggin binds directly to BMP-2 and BMP-4 with high affinity (K_D of 19 pM), and less efficiently to BMP-7, and prevents ligand interaction with their cognate BMP receptors (Zimmerman et al. 1996). Noggin also interacts biochemically and functionally with several other BMP ligands, including GDF-5 (Merino et al. 1999a), GDF-6 (Chang and Hemmati-Brivanlou 1999), BMP-5, and BMP-6 (Aspenberg et al. 2001; Beck et al. 2001; Haudenschild et al. 2004). Noggin2 in *Xenopus* has also been shown to associate with activin B, nodal/Xnrs, and Wnt8 to inhibit their signaling (Bayramov et al. 2011). The sequences of BMP-6 and GDF-5 mutants that are not inhibited by noggin identify key residues that mediate BMP inhibition by noggin (Seemann et al. 2009; Song et al. 2010). The crystal structure of noggin bound to BMP-7 reveals that the noggin dimer assumes a cystine-knot structure similar to that of BMP-7, and that a "back-to-back" arrangement of noggin and BMP-7 dimers results in masking of a hydrophobic patch in BMP-7 that is required for contact with type II receptors (Groppe et al. 2002). Besides BMPs, noggin also binds heparan sulfate and is retained at the cell surface by HSPGs. Heparan sulfate-bound noggin remains functional and binds BMP-4 at the plasma membrane. Binding of noggin to cell-surface HSPGs may limit the diffusion, and thus the action range, of this antagonist (Paine-Saunders et al. 2002), and may also contribute to noggin's ability to promote BMP-2 internalization (Alborzinia et al. 2012). A developmentally regulated endosulfatase Qsulf1 can release noggin from HSPGs and may regulate noggin distribution (Viviano et al. 2004). Inhibition of BMP signaling by noggin plays important roles in many processes during embryogenesis and adult homeostasis, such as regulation of neural induction, patterning of the neural tube and somites, guidance of dorsal root ganglion axons, joint formation and skeletal development, fusion of cranial sutures, and hair follicle development (Brunet et al. 1998; McMahon et al. 1998; Botchkarev et al. 1999; Bachiller et al. 2000; Anderson et al. 2002; Dionne et al. 2002; Warren et al. 2003; Khokha et al. 2005). Hypomorphic mutations in the

human *NOGGIN* gene associate with skeletal dysplasia syndromes, such as proximal symphalangism (SYM1), multiple synostoses syndrome (SYNS1), and tarsal/carpal coalition syndrome (TCC) (Balemans and Van Hul 2002), illustrating the importance of noggin in joint and skeletal development.

Besides noggin, several genes in planarians encode noggin-like proteins (nlgs) that have a sequence insertion between the fifth and sixth cysteine residues. Functional assays indicate that planarian nlg8 and a similar *Xenopus* Xl-nlg ventralize embryos and suppress neurogenesis, partially mimicking effects of ectopic BMP signals. However, nlg8 and Xl-nlg do not significantly affect Smad1 or 5-phosphorylation, suggesting that they may regulate patterning independent of Smad activation (Molina et al. 2009, 2011). The functions of these nlg genes are distinct from that of a noggin-like gene in Hydra, which encodes canonical BMP-inhibitory activities when expressed in *Xenopus* (Chandramore et al. 2010).

Chordin and Its Regulators: BMP-1/Tolloid Proteases, Ogon/Sizzled, Crescent/Frzb2-Sfrp5, Olfactomedin 1, and Twisted Gastrulation

The chordin-dependent BMP regulatory network is complex and extensive, and best characterized in *Xenopus* and *Drosophila* in the context of early dorsoventral patterning. It utilizes multiple extracellular factors (Fig. 2) to regulate the interaction between BMPs and chordin by controlling the stability of chordin, the transport of the BMP–chordin complex to a distant site, and the transcription of the network components. Together, they constitute a reaction–diffusion feedback regulatory loop to finely tune BMP signaling levels for robust embryonic patterning along the dorsoventral axis.

Chordin

Chordin was originally identified as the product of a dorsally expressed gene in *Xenopus* gastrulae that showed dorsalizing activities (Sasai et al. 1994, 1995). This gene encodes a secreted protein of 941 amino acids that contains four cysteine-rich (CR) repeats with a long linker sequence between the first and second CR domain. Like noggin, chordin antagonizes BMP function through direct binding to BMP-2, -4, and -7, thus preventing the ligands from interacting with the BMP receptors. The affinity of chordin for BMP-4 (K_D 300 pM) is ~10-fold lower than that of noggin (Piccolo et al. 1996), and the first and third CR domains can individually bind BMP-4 with a K_D of 2 nM and inhibit BMP signals less efficiently than full-length chordin (Larrain et al. 2000). Differentially spliced variants of human chordin *(CHRD)* mRNA encode isoforms with different numbers of CR domains and distinct abilities to block BMP signaling (Millet et al. 2001). Besides BMPs, chordin may bind and block ADMP, albeit with conflicting results (Joubin and Stern 1999; Dosch and Niehrs 2000; Reversade and De Robertis 2005). In vertebrate development, chordin, either alone or in cooperation with noggin and follistatin, regulates early dorsal patterning, forebrain formation, mandibular outgrowth, pharyngeal development, septation of the cardiac outflow tract, chondrocyte maturation, and axial skeleton development, among other processes (Hammerschmidt et al. 1996a,b; Schulte-Merker et al. 1997; Streit et al. 1998; Streit and Stern 1999; Bachiller et al. 2000, 2003; Stottmann et al. 2001; Anderson et al. 2002; Zhang et al. 2002; Oelgeschlager et al. 2003a; Khokha et al. 2005). Some phenotypes in $Chrd^{-/-}$ mice resemble those in patients with DiGeorge syndrome, implying that *CHRD* mutations may contribute to the etiology of this disease (Bachiller et al. 2003).

The antagonism between chordin and BMPs may have arisen early during metazoan evolution (Rentzsch et al. 2007). Comparative studies in *Drosophila* and vertebrates yield insight into the conservation and divergence of chordin function in animals. The *Drosophila* homolog of chordin, short gastrulation (Sog), binds and inhibits the BMP ligands Screw (Scw), Glass-bottom boat (Gbb), and, with less efficiency, Decapentaplegic (Dpp) to regulate dorsoventral patterning of early embryos and pattern formation in imaginal discs (Francois et al. 1994; Francois and Bier 1995; Holley

et al. 1995; Schmidt et al. 1995; Biehs et al. 1996; Neul and Ferguson 1998; Nguyen et al. 1998). Interestingly, although Sog blocks BMPs locally, it can enhance signaling by Dpp/Scw dimer in cells that are distant from the source of Sog production by facilitating transport and presentation of BMP ligands. This role is essential for establishing a sharp domain of peak levels of Dpp/Scw activity in the dorsal region of early fly embryos (Ashe and Levine 1999; Decotto and Ferguson 2001; Eldar et al. 2002; Shimmi and O'Connor 2003; Shimmi et al. 2005a; Wang and Ferguson 2005). Vertebrate chordin cannot replace Sog to promote Dpp signaling in *Drosophila* (DeCotto and Ferguson 2001), although some evidence suggests that chordin may collaborate with BMP-2b in early dorsoventral patterning of the zebrafish tail (Wagner and Mullins 2002), and help shuttle BMP ligands from the intervertebral disc to the vertebral body during mouse skeletal development (Zakin et al. 2010). It is therefore possible that vertebrate chordin maintains its ability to transport BMP in certain tissue contexts.

BMP-1/Tolloid Family of Metalloproteinases

Tolloid, a zygotic gene in the *Drosophila* dorsal developmental regulatory network, encodes a protein homologous to human BMP-1 in that both contain an amino-terminal zinc metalloproteinase domain (Shimell et al. 1991). Genes for a second *Drosophila* homolog, Tolkin/Tlr-1, and four genes encoding vertebrate BMP-1/Tolloid-like (BMP-1/TLL) proteins were subsequently identified (Maeno et al. 1993; Nguyen et al. 1994; Finelli et al. 1995; Scott et al. 1999). Biochemical, genetic, and embryological studies in *Drosophila*, *Xenopus,* and mammalian cells show that BMP-1/TLL family members cleave Sog/chordin at several sites to inactivate or attenuate this BMP antagonist, and thus promote BMP signaling (Blader et al. 1997; Marques et al. 1997; Piccolo et al. 1997; Scott et al. 1999; Serpe et al. 2005). However, some forms of partially cleaved Sog/chordin may display stronger BMP-inhibitory activity or an altered spectrum of preferred BMP ligands (Yu et al. 2000; Troilo et al. 2014, 2015). Although cleavage of Sog in *Drosophila* requires the presence of Dpp, vertebrate BMP-1/TLLs can process chordin in the absence of BMPs (Peluso et al. 2011). BMP-4 can bind to the CUB domain of BMP-1/TLL to inhibit its proteinase activity, and this may provide concentration-dependent feedback modulation of BMP signaling (Lee et al. 2009). A further difference between *Drosophila* and vertebrates lies in the number and locations of the processing sites in Sog and chordin (Marques et al. 1997; Piccolo et al. 1997; Scott et al. 1999). In addition, the four vertebrate BMP-1/TLL members have differential abilities to cleave chordin (Scott et al. 1999; Pappano et al. 2003; Berry et al. 2010). Inactivation of Sog by Tolloid in *Drosophila* generates a ventral-high to dorsal-low gradient of Sog protein (Srinivasan et al. 2002), and is essential to establish a reverse dorsal-high, ventral-low Dpp/Scw activity gradient in the dorsal region of early embryos (Marques et al. 1997; Shimmi and O'Connor 2003; Peluso et al. 2011). In vertebrates, BMP-1/TLLs modulate chordin distribution along the dorsoventral axis and are required for ventral tissue development in *Xenopus* and zebrafish (Piccolo et al. 1997; Goodman et al. 1998; Connors et al. 1999, 2006; Wardle et al. 1999; Blitz et al. 2000; Jasuja et al. 2006; Plouhinec et al. 2013). The activity of Tolloid/BMP-1 is positively modulated by the ECM proteins fibronectin and collagen IV, as these proteins bind Tolloid/BMP-1 and enhance its processing of chordin (Huang et al. 2009; Winstanley et al. 2015). Besides chordin, BMP-1 family proteases target other substrates in the extracellular milieu, including latent TGF-β-binding protein 1 (LTBP1), and this cleavage contributes to activation of TGF-β ligands (Pappano et al. 2003; Ge and Greenspan 2006a,b).

Sizzled/Ogon, Crescent, and Sfrp5

Sizzled (also known as mercedes or short tail) was first identified as the zebrafish Ogon mutant that caused ventralization of embryos similar to chordin mutants. Genetic interactions with other dorsoventral patterning factors in zebrafish suggest that the gene product is a BMP antagonist that acts upstream of BMP-2b and

the type I BMP receptor ALK-8 (Miller-Bertoglio et al. 1999; Wagner and Mullins 2002). The encoded protein, Sizzled, inhibits BMP signals in *Xenopus* and zebrafish, and regulates dorsoventral patterning (Bradley et al. 2000; Collavin and Kirschner 2003; Martyn and Schulte-Merker 2003; Yabe et al. 2003). Inhibition of BMPs by Sizzled is chordin-dependent and uses a novel mechanism. Sizzled competes with chordin to bind BMP-1 and Tolloid-like 1, but cannot be cleaved by these metalloproteinases. In this way, Sizzled prevents BMP-1 proteins from processing chordin, and thus helps to stabilize chordin and enhance its antagonistic effect on BMP in vivo (Fig. 2) (Lee et al. 2006; Muraoka et al. 2006). Counterintuitively, Sizzled is expressed in the ventral regions and its expression is stimulated by BMP signaling. Sizzled thus acts as a negative feedback regulator of BMPs that diffuses over a long distance to finely tune the BMP signaling levels along the dorsoventral axis in a chordin- and Tolloid-dependent manner (Inomata et al. 2013). No Sizzled-like molecule has been found in *Drosophila* to regulate signals by the BMP-like ligands Dpp, Scw, or Gbb, implying that this mechanism arose later in evolution.

Crescent and Sfrp5 are secreted Frizzled-related proteins (sFRP's) that are closely related to Sizzled. Similar to Sizzled, Crescent (K_D ~11 nM) and Sfrp5 bind BMP-1/TLL and block their proteinase activity. They thus protect chordin from degradation and participate in an extracellular BMP regulatory network to modulate dorsoventral patterning of early *Xenopus* and zebrafish embryos. Unlike Sizzled, Crescent is expressed in the dorsal region of *Xenopus* gastrulae, and both *Xenopus* Crescent and zebrafish Sfrp5 are expressed in embryonic endoderm and additionally inhibit canonical and noncanonical Wnt signals (Pera and De Robertis 2000; Schneider and Mercola 2001; Shibata et al. 2005; Ploper et al. 2011; Stuckenholz et al. 2013). Interestingly, mammalian sFRP-2 enhances, rather than inhibits, BMP-1 in processing procollagen C, but has no effect on chordin processing (Kobayashi et al. 2009). Studies of other mammalian sFRPs show that they lack the ability to modify BMP-1 proteinase activity, suggesting that specific residues in the frizzled domain may be crucial for sFRP to inhibit BMP-1/TLL proteinases (Bijakowski et al. 2012).

Olfactomedin 1 (ONT1)

ONT1, a member of olfactomedin family of secreted proteins, acts as an extracellular scaffold to facilitate association of chordin and BMP-1. ONT1 has a coiled-coil domain near its amino terminus and a conserved olfactomedin domain in its carboxy-terminal half. These domains bind BMP-1/Tolloid and chordin, respectively. By bringing the enzyme and its substrate together, ONT1 promotes chordin degradation (Fig. 2). This pro-BMP action of ONT1 is concentration-dependent, as high levels of ONT1 permit binding of chordin and BMP-1 in distinct complexes and thus separate the two proteins. ONT1 is expressed in the dorsal organizer of early *Xenopus* embryos; thus, its expression and activity are opposite to those of Sizzled. Both ONT1 and Sizzled are critical in the robust dorsoventral self-regulating patterning system in *Xenopus* (Inomata et al. 2008, 2013).

Twisted Gastrulation (TWSG1/tsg)

Twisted gastrulation (tsg) was first identified in *Drosophila* as a mutation that affects the development of the dorsal midline amnioserosa (Zusman and Wieschaus 1985). The gene encodes a small secreted protein with two cysteine-rich domains and is required for peak Dpp activity in early *Drosophila* embryos (Mason et al. 1994, 1997; Ross et al. 2001). Tsg enhances Sog to bind and inhibit Dpp in the dorsolateral region. However, at the same time Tsg promotes the transport of the Sog–Dpp complex to the dorsal midline, and facilitates the processing and alternative cleavage of Sog by Tolloid once it reaches the most dorsal region (Yu et al. 2000; Shimmi and O'Connor 2003). This Tsg–Sog–Dpp–Tolloid interaction network ensures the establishment of a sharp dorsal boundary between the peak and immediate BMP activity levels so that cells adopt either an amnioserosa or dorsal ectodermal fate (Eldar et al. 2002; Shimmi and O'Connor 2003;

Shimmi et al. 2005a; Wang and Ferguson 2005). Another twisted gastrulation-like gene, *shrew*, performs a similar function to promote peak BMP signaling at the aminoserosa (Bonds et al. 2007). In addition, a comparable Dpp transport and release mechanism may operate during wing development in *Drosophila*, in which a Tsg homolog, Tsg2/Crossveinless (Cv), cooperates with Sog to create a spatially restricted Dpp/Gbb signaling center in the posterior crossvein of the developing wing (Shimmi et al. 2005b; Vilmos et al. 2005). Tsg can also alter the processing of Sog by Tolloid to create a novel Sog cleavage product, called Supersog, which has a broader spectrum of BMP inhibition than full-length Sog (Yu et al. 2000). Tsg and Tsg2/Cv therefore exert positive as well as negative effects on BMP signaling in *Drosophila* via regulation of Sog and Tolloid activities, and thus helps to establish a sharp high BMP signaling boundary by local inhibition and long distance enhancement of BMP signals.

Vertebrate Twisted gastrulation, abbreviated as Twsg1 and not as Tsg as in *Drosophila*, has also been shown to act as both agonist and antagonist of BMPs. Twsg1 binds BMP-2, -4, and -7 and forms a ternary complex with chordin and BMPs to enhance the inhibitory activity of chordin on BMP (Oelgeschlager et al. 2000; Chang et al. 2001; Larrain et al. 2001; Ross et al. 2001; Scott et al. 2001; Zakin et al. 2005). In cell culture, Twsg1, either alone or in combination with chordin, blocks BMP-mediated effects on proliferation and/or differentiation of osteoblasts, osteoclasts, and thymocytes (Graf et al. 2002; Gazzerro et al. 2005; Petryk et al. 2005; Pham et al. 2011). However, Twsg1 may also promote chordin degradation by BMP-1 family metalloproteinases, and thus promote BMP signaling (Oelgeschlager et al. 2000; Larrain et al. 2001). Twsg1 increases the rate of chordin processing by Tolloid-like 1 and alters the processing site of mouse chordin, but not zebrafish or *Xenopus* chordin. In *Xenopus*, a dominant negative Tolloid-like 1 (Xolloid) blocks the BMP stimulatory activity of Twsg1 (Larrain et al. 2001; Scott et al. 2001; Xie and Fisher 2005). In addition, Twsg1 may have a chordin-independent, BMP-enhancing activity (Oelgeschlager et al. 2003a, 2004; Little and Mullins 2004; Xie and Fisher 2005), indicating that other factors also interact with Twsg1 to modulate BMP signaling. Although it is tempting to speculate that Twsg1 may help present BMP ligands to their receptors when stimulating BMP signaling, binding of BMPs per se is not required for this stimulatory activity of Twsg1 (Oelgeschlager et al. 2003b). In osteoclast cells, mutations that abolish Twsg1 binding to BMP change its activity from BMP-inhibitory to BMP-enhancing, arguing for a ligand-binding-independent mechanism for Twsg1 to promote BMP signaling (Huntley et al. 2015).

The in vivo functions of vertebrate Twsg1, as assessed by loss-of-function studies, reveal an equally complex story. Depletion of endogenous Twsg1 expression using morpholino antisense oligonucleotides shows that Twsg1 and chordin coordinate in regulating dorsoanterior development of early *Xenopus* embryos (Blitz et al. 2003; Wills et al. 2006), supporting the critical role of Twsg1 as a BMP antagonist in *Xenopus*. Twsg1 and BMP-7, however, also act together in controlling the posteroventral mesoderm and ventral tail fin formation in *X. laevis*, suggesting stimulation of BMP activity by Twsg1 (Zakin et al. 2005). In zebrafish, antisense oligonucleotide-mediated knockdown experiments show that Twsg1 may promote rather than inhibit BMP signaling (Little and Mullins 2004; Xie and Fisher 2005). In mammals, *Twsg1*$^{-/-}$ mice show defects in axial skeleton, thymocyte development, and craniofacial structures (Graf et al. 2001; Nosaka et al. 2003; Petryk et al. 2004; Zakin and De Robertis 2004). Twsg1 may function as a BMP antagonist in axial skeletal and T-cell development (Nosaka et al. 2003; Ikeya et al. 2008; Zakin et al. 2008) and neutralizes the protective activity of BMP-7 in podocyte injury (Yamada et al. 2014). However, Twsg1 may enhance BMP signaling during forebrain development and postnatal mammary gland morphogenesis (Zakin and De Robertis 2004; Forsman et al. 2013). Thus, Twsg1 has context-dependent pro- or anti-BMP activities. A second twisted gastrulation gene has also been identified in *Xenopus*, and shows a temporal expression pattern that differs from *Twsg1*

expression (Oelgeschlager et al. 2004). In addition, Twsg1 may regulate TGF-β signaling in T lymphocytes by binding to TGF-β proteins (Tzachanis et al. 2007), implying a broader spectrum of TGF-β ligand regulation.

Other Modulators of Chordin

The activities of chordin are regulated by additional factors, such as proteoglycans and other cell-surface molecules. Two secreted small leucine-rich proteoglycans, biglycan and Tsukushi, can each form a ternary complex with chordin and BMP-4 and enhance the inhibitory function of chordin on BMP (Ohta et al. 2004; Moreno et al. 2005). Chordin also binds HSPGs, such as syndecans, but does not associate with the basement membrane HSPG perlecan. Binding of chordin to cell-surface HSPGs potentiates the BMP antagonistic function of chordin, facilitates chordin retention and uptake by cells, and limits diffusion of chordin over a long distance in tissues (Jasuja et al. 2004). Interaction of chordin with HSPGs hence regulates the local concentration and gradient formation of chordin and its ability to block BMP signaling. The distribution of chordin may also be affected by its interaction with integrins that act as receptors for ECM proteins. Chordin binds α_3-integrin in vertebrate cells, and this binding facilitates endocytosis and uptake of chordin into cells (Larrain et al. 2003). In *Drosophila*, Sog interacts genetically with several integrins, including βPS, αPS1, and αPS3 (PS stands for position-specific antigen), during wing vein specification. Sog and a truncated Sog directly bind αPS1, and the function of integrins is required for Sog transport from the intervein to pro-vein regions (Araujo et al. 2003). Integrins may thus control the range and the availability of different forms of Sog and chordin proteins to regulate BMP signals.

Chordin-Like Cysteine-Rich (CR) Domain-Containing Proteins

CHL/Neuralin/Ventroptin and CHL2 (CHRDL1 and 2)

Chordin has four CR modules (also known as von Willebrand factor type C, or VWC domains) that are defined by characteristic spacing of 10 conserved cysteines within a stretch of 60 to 80 amino acids (Garcia Abreu et al. 2002). Chordin-like CR domains are found in several extracellular proteins, including matrix proteins such as procollagen (see below) as well as soluble factors. Chordin-like (CHL/CHL1, CHRDL1) is a secreted molecule with three CR modules and is also known as neuralin in the mouse, and ventroptin in the chick (Coffinier et al. 2001; Nakayama et al. 2001; Sakuta et al. 2001). It has an expression pattern complementary to that of chordin during early mouse embryogenesis. CHRDL1 binds BMP-4, -5, and -6, but not activin A, and blocks binding of BMPs to their receptors (Nakayama et al. 2001; Sakuta et al. 2001). Unlike chordin, CHRDL1 also binds TGF-β2 (Nakayama et al. 2001). When overexpressed, CHRDL1 induces secondary axis formation in early *Xenopus* embryos, dorsalizes zebrafish embryos, represses BMP-4 activity during dorsoventral patterning of chick retina, impairs distal digit formation in chick limbs, and promotes neuronal differentiation of adult neural stem cells (Coffinier et al. 2001; Nakayama et al. 2001; Sakuta et al. 2001; Branam et al. 2010; Allen et al. 2013; Gao et al. 2013). Interestingly, CHRDL1 enhances BMP-4 and BMP-7 signaling in several cell lines when expressed alone, but switches into a selective BMP-7 antagonist when in complex with Twsg1. The BMP-inhibitory function of Chrdl1 and Twsg1 may regulate injury repair and homeostasis of mammalian kidney (Larman et al. 2009). Mutations in CHRDL1 are associated with X-linked megalocornea disorder in human patients (Webb et al. 2012). Another closely related and secreted factor with 3 chordin-type CR domains is CHRDL2 (CHL2), which binds BMP-2, -4, -5, -6, -7, and GDF-5, but not activin A or TGF-βs. Overexpression of CHRDL2 blocks BMP-mediated differentiation of C2C12 cells (Nakayama et al. 2004), and a ternary complex with Twsg1 and BMP-2 enhances the BMP-inhibitory activity of CHRDL2 (Zhang et al. 2007). Recombinant human CHRDL2, however, interacts with activin A, but not BMP-2, -4, or -6. The functional significance of these differences in interactions is

unknown (Oren et al. 2004). The discrepancy between the two studies may be because of the complex alternative splicing patterns of CHRDL2 in different tissues, as spliced variants may have different functions and specificities in the regulation of distinct TGF-β family ligands (Oren et al. 2004). Like chordin, zebrafish CHRDL can be processed by BMP-1, which blocks its dorsalizing activity (Branam et al. 2010).

Brorin/Vwc2 and Brorin-Like (Vwc2l)

Brorin (also known as Vwc2) was isolated from mouse as a brain-specific secreted protein with two chordin-like CR domains. Brorin inhibits BMP-2 and -6 activities in cultured preosteoblastic cells and promotes neurogenesis in neural precursor cells (Koike et al. 2007). A similar protein, Brorin-like (Vwc2-like or Vwc2l), was isolated from mouse, human, and zebrafish. It has an expression profile that partially overlaps with that of Brorin, but has a weaker BMP-inhibitory activity (Miwa et al. 2009).

Crossveinless-2 (BMPER/cv-2)

Crossveinless-2 (cv-2) is a gene mutation in *Drosophila* that leads to a loss of crossveins in the wing. The gene encodes a secreted protein with five chordin-type CR domains followed by a partial von Willebrand factor (vWF) domain. Genetic studies suggest that Crossveinless-2 enhances BMP signals in the crossveins, which require a high level of Dpp/Gbb signaling, but antagonizes BMP signaling during early embryogenesis (Conley et al. 2000; Gavin-Smyth et al. 2013). Crossveinless-2 binds the type I BMP receptor Thickveins and shows a concentration-dependent biphasic modulation toward BMPs (Serpe et al. 2008). Vertebrate Crossveinless-2, also known as Bmper, has been identified in zebrafish, chick, mouse, and human, and contains an additional carboxy-terminal trypsin-inhibitor-like cysteine-rich domain that is not found in *Drosophila* Crossveinless-2. *Cv2/Bmper* is expressed in chick and mouse at sites that require elevated BMP signals, such as the posterior primitive streak and ventral tail bud, whereas functional assays suggest BMP agonist and antagonist activities of Cv2/Bmper in vertebrates. (Coffinier et al. 2002; Coles et al. 2004; Kamimura et al. 2004). Cv2/Bmper binds BMP-2, -4, -6, -7, -9 (K_D ~1.4–3.5 nM for BMP-2, -4, -7) and GDF-5 (K_D ~ 34 nM). Structural analysis shows that Cv2/Bmper binds BMP-2 via the amino-terminal Clip segment and subdomain 1 in the first CR module (VWC1 domain), resulting in masking of the type I and type II receptor-binding interfaces in BMP-2 (Zhang et al. 2008; Fiebig et al. 2013). As a BMP inhibitor, Cv2/Bmper induces a secondary axis in *Xenopus*, blocks BMP-responsive gene expression in 293T cells, and interferes with BMP-dependent differentiation of embryonic stem cells into the endothelial lineage and BMP-induced chondrogenic and osteogenic differentiation. However, Cv2/Bmper also enhances Smad1 phosphorylation in COS7 cells and promotes premature neural crest cell migration in chick. The latter results are consistent with its function in elevating BMP signaling (Moser et al. 2003; Binnerts et al. 2004; Coles et al. 2004; Kamimura et al. 2004; Ambrosio et al. 2008; Yao et al. 2012). Gene silencing in zebrafish and mice suggest that Cv2/Bmper enhances BMP signaling during gastrulation, neural crest specification, nephrogenesis, cardiovascular development, and axial skeletal formation, although it may block BMP-9 signaling in vascular endothelium, whereas Cv2/Bmper overexpression shows activities that are consistent with functions in both enhancing and inhibiting BMP signaling (Ikeya et al. 2006, 2008, 2010; Rentzsch et al. 2006; Moser et al. 2007; Zakin et al. 2008; Yao et al. 2012; Reichert et al. 2013; Dyer et al. 2014). Several mechanisms, including differential activities of full-length and processed Cv2/Bmper and differential association with other BMP modulators and/or ECM components, may account for the switch between the two opposing activities of Cv2/Bmper. Full-length Cv2/Bmper is a BMP antagonist and binds to HSPG-containing ECM with high affinity. The cleaved product of Cv2/Bmper that contains only five CR domains acts as BMP agonist and does not associate efficiently with the ECM. Processing of Cv2/Bmper may thus alter its regulation of

BMP signaling (Rentzsch et al. 2006). Cv2/Bmper also interacts genetically and biochemically with other BMP modulators to affect BMP signaling. Cv2 binds chordin using sequences distinct from those for BMP binding and is required for relocalization of chordin from intervertebral disc to vertebral body during axial skeletal development. Binding of chordin, rather than BMP, is essential for Cv2's pro-BMP effect (Ambrosio et al. 2008; Zakin et al. 2010; Zhang et al. 2010). Cv2 also complexes with Twsg1 and BMP and genetically acts with *Twsg1* to regulate axial skeleton and embryonic nephron development (Ambrosio et al. 2008; Ikeya et al. 2008, 2010; Zakin et al. 2008). Furthermore, Cv2/Bmper binds LRP1 (low density lipoprotein receptor-related protein 1) and may contribute to endocytic regulation of BMP availability and signaling (Kelley et al. 2009; Pi et al. 2012). Cross talk with various BMP modulators, ECM components, and cell-surface proteins may thus provide a basis for the activities of Cv2/Bmper as agonist or antagonist of BMP ligands. Mutations in BMPER have been linked to an autosomal recessive perinatal lethal skeletal disorder, diaphanospondylodysostosis, in humans (Funari et al. 2010; Ben-Neriah et al. 2011; Zong et al. 2015).

CRIM1

CRIM1 (cysteine-rich motor neuron 1) is a glycosylated type I transmembrane protein with six chordin-type CR repeats and an amino-terminal insulin-like growth factor–binding protein (IGFBP)-like motif in its extracellular domain. CRIM1 can be cleaved to release a soluble ectodomain. Although soluble CRIM1 does not bind BMPs in solution, CRIM1 interacts with BMP-4 and BMP-7 in the Golgi compartments when coexpressed in the same cell. This association is mediated by the CR domains and can lead to reduced processing and secretion of BMPs (Fig. 1) (Wilkinson et al. 2003). CRIM1 thus antagonizes BMP activity using a unique mechanism in that it regulates the production and release of mature BMPs cell-autonomously in BMP-expressing cells. Consistent with this idea, the *Drosophila* CRIM1 homolog, Crimpy, inhibits the BMP ligand Gbb cell-autonomously in motorneurons (James and Briohier 2011). However, the *Caenorhabditis elegans* CRIM1 homolog, Crm-1, facilitates BMP presentation and promotes BMP signaling to control body size (Fung et al. 2007). In vertebrates, CRIM1 is expressed in the notochord, somites, limb, floor plate, motor neurons, and sensory organs, but CRIM1 overexpression in chick does not affect neural patterning in the spinal cord. Instead, loss-of-function studies in chick, frog, zebrafish, and mouse suggest that CRIM1 modulates the formation of renal vasculature, neural tube morphogenesis, limb patterning, and eye development (Kolle et al. 2000, 2003; Glienke et al. 2002; Kinna et al. 2006; Pennisi et al. 2007; Ponferrada et al. 2012; Fan et al. 2014). CRIM1 may control some of these processes by acting through extracellular signals other than BMPs such as vascular endothelial growth factor (VEGF)-A and cadherins (Ponferrada et al. 2012; Fan et al. 2014).

Kielin and KCP (CRIM2)

Kielin is a 2327 amino acid protein, containing 27 chordin-like CR modules, an amino-terminal thrombospondin homology region and a carboxy-terminal vWF type D domain-like sequence. Kielin was identified by screening for secreted molecules in *Xenopus* and is expressed in dorsal midline structures during early frog development. Overexpression of Kielin dorsalizes ventral mesodermal explants; however, Kielin is not sufficient to induce secondary axis formation or neural induction in *Xenopus*, suggesting that, unlike noggin or chordin, Kielin may not efficiently inhibit BMP signaling (Matsui et al. 2000). No interaction of Kielin with TGF-β ligands has been reported and it is unclear whether Kielin regulates TGF-β signals directly or through other signaling pathways. A kielin/chordin-like protein, KCP (CRIM2), has also been isolated in the mouse, and contains 18 CR motifs and a carboxy-terminal vWF type D domain. KCP binds BMP-7, but intriguingly increases BMP-7 binding to BMPRIA/ALK-3 instead of blocking this interaction, and may form a ternary complex with them.

KCP enhances Smad1 activation and BMP-responsive gene expression, and promotes BMP signaling to attenuate renal interstitial fibrosis (Lin et al. 2005). KCP also binds activin A and TGF-β1, and blocks Smad2/3 activation and Smad2/3-mediated transcription (Lin et al. 2006). KCP hence functions in opposite ways to regulate activin/TGF-β and BMP signals. In adult mouse kidney, KCP attenuates acute and chronic renal injury (Soofi et al. 2013).

Amnionless

Amnionless was discovered as a recessive insertional gene mutation in the mouse that interferes with the development of the primitive streak that gives rise to trunk mesoderm (Wang et al. 1996). The gene encodes a type I transmembrane protein with a single CR module in its extracellular region and is therefore thought to regulate BMP signals (Kalantry et al. 2001). However, Amnionless acts with cubilin, a multiligand scavenger receptor, to regulate vitamin B12 uptake and absorption of low molecular weight proteins in visceral endoderm and embryonic kidney (Strope et al. 2004; Pedersen et al. 2010; Zhang et al. 2013). Therefore, although Amnionless contains a CR domain homologous to chordin, it may not participate in the regulation of BMP signals.

DAN/Cerberus/Gremlin Family Members

The DAN/Cerberus/Gremlin family includes several small soluble proteins with a characteristic cysteine-rich domain (CAN domain) with the consensus sequence $CX_6QX_6CX_6NX_2$-$CXGXCXSX_3PX_{(8-13)}CX_2CXPX_8TLXCX_{(15-18)}$-CXC (Avsian-Kretchmer and Hsueh 2004). All family members are secreted as glycosylated monomers or dimers that bind and inhibit BMPs.

DAN/NBL1

DAN (differential screening-selected gene aberrative in neuroblastoma, also known as NBL1) is the founding member of the family, and was first identified as the product of a gene that is down-regulated in oncogene-transformed rat fibroblasts (Ozaki and Sakiyama 1993). Dan encodes a secreted molecule that forms a noncovalent homodimer, and has dynamic expression patterns in mouse, chick, and frog (Hsu et al. 1998; Stanley et al. 1998; Pearce et al. 1999; Eimon and Harland 2001; Ogita et al. 2001; Gerlach-Bank et al. 2002; Kattamuri et al. 2012; Nolan et al. 2015). Dan binds and antagonizes BMP-2, BMP-4, BMP-7, and GDF-5, but does not block nodal-like signaling in *Xenopus* or chick (Hsu et al. 1998; Stanley et al. 1998; Dionne et al. 2001; Katsu et al. 2012). In the chick, DAN controls left–right patterning and inner ear development (Yamanishi et al. 2007; Katsu et al. 2012). In the mouse, Dan is expressed in the somites, cranial and facial mesenchyme, and axonal processes. However, $Dan^{-/-}$ mice do not display obvious abnormalities, suggesting compensation for loss-of-function in Dan activities (Dionne et al. 2001).

Cerberus (CER1)

Cerberus/Cer1 was originally isolated in *Xenopus* by screening for dorsally enriched genes (Bouwmeester et al. 1996). It encodes a small secreted protein localized in the anterior organizer of *Xenopus* gastrulae. Cerberus binds Xnr1, Wnt8, and BMP-4 through distinct domains and blocks signal transduction of all three pathways. By simultaneously inhibiting these signals, Cerberus promotes the head structure formation in *Xenopus* (Glinka et al. 1997; Hsu et al. 1998; Piccolo et al. 1999). Loss-of-function studies indicate that Cerberus regulates head and heart development in *Xenopus* (Schneider and Mercola 1999; Silva et al. 2003; Foley et al. 2007). In zebrafish, Cerberus, also known as Charon, inhibits nodal activity and is required for left–right patterning of the body axis (Hashimoto et al. 2004). In chick, Cerberus (i.e., Caronte) binds BMP-4, -7, and nodal, but not BMP-5, GDF-5, or activin. Cerberus is expressed in the left lateral plate mesoderm to antagonize BMP signals on the left side, and participates in left–right axis determination (Rodriguez Esteban et al. 1999; Yokouchi et al. 1999; Zhu et al. 1999a; Tavares et al. 2007).

Chick Cerberus is also expressed in the hypoblast cell layer in early embryos and regulates formation of the primitive streak by antagonizing nodal signaling (Bertocchini and Stern 2002; Chapman et al. 2002).

In mouse, Cerberus (also known as Cerberus-like/Cer-l, Cerberus-related gene/Cerr1) is expressed during early gastrulation in anterior visceral endoderm, a region equivalent to the *Xenopus* Cerberus expression domain in the deep anterior endoderm of the organizer (Belo et al. 1997; Biben et al. 1998; Shawlot et al. 1998; Pearce et al. 1999). Like its *Xenopus* homolog, mouse Cerberus binds and inhibits BMP-4 and Xnr1, but unlike the *Xenopus* gene, mouse Cerberus does not block Wnt signaling and does not induce a secondary head in *Xenopus* (Belo et al. 1997, 2000; Pearce et al. 1999). Mice with targeted disruption of *Cerberus/Cer1* do not show morphological or molecular defects in the head or other structures, suggesting compensation for loss of Cerberus (Simpson et al. 1999; Belo et al. 2000; Shawlot et al. 2000; Stanley et al. 2000). Indeed, compound mutants lacking functional expression of *Cerl* and *Lefty1* develop ectopic primitive streaks and patterning defects of the streaks, which are rescued by eliminating one copy of the *Nodal* gene. The results thus suggest that Cerberus plays a role in spatially restricting primitive streak formation by antagonizing nodal signaling in mouse (Perea-Gomez et al. 2002). Human Cerberus also binds and inhibits nodal to suppress aggressive phenotypes of breast cancer cells (Aykul et al. 2015).

Coco/Dante/Cerl-2

Coco is a *Xenopus* homolog of Cerberus, and, like Cerberus, binds Xnr1 and BMP-4, blocks signaling by Xnr, BMP, and Wnt, and induces neural markers directly in ectodermal explants. However, Coco is not expressed in the organizer, but shows maternal expression in an animal-to-vegetal gradient with the highest level in ectodermal cells, and may regulate cell fate specification and competence before onset of gastrulation (Bell et al. 2003). A second gene encoding Cerberus-like 2 (Cerl-2, previously known as Dante) was shown to modulate mouse left–right laterality. Cerl-2 is first expressed in a symmetric pattern around the mouse node, but its expression level on the left side gradually diminishes, whereas the right side expression remains strong (Pearce et al. 1999; Marques et al. 2004). This positions Cerl-2 as a unique patterning molecule in left–right axis formation. *Cerl2* knockout mice show laterality defects as well as left ventricular cardiac hyperplasia (Araujo et al. 2014). Like other members of the Cerberus family, Cerl-2 binds and inhibits Xnr1 and BMP-4, and balanced regulation of the activities of these TGF-β ligands may explain its function in left–right axis specification (Marques et al. 2004).

Drm/Gremlin (GREM1)

Drm (down-regulated in mos-transformed cells) was first identified in the rat as the product of a gene down-regulated in oncogene-transformed fibroblasts. Like Dan, Drm is a secreted glycoprotein with growth-inhibitory activities in cultured cells (Topol et al. 1997, 2000). The *Xenopus* Drm homolog, Gremlin (Grem1), was identified in a screen for genes with dorsalizing ability (Hsu et al. 1998). Gremlin binds and inhibits BMP-2, -4, and -7, but does not block nodal, Vg1, or activin (Hsu et al. 1998; Church et al. 2015). Although Gremlin prevents BMPs from interacting with their receptors, an intracellular inhibitory mechanism may also operate, whereby Gremlin binds the BMP-4 precursor and blocks processing and secretion of mature BMP-4 (Fig. 1) (Sun et al. 2006). Gremlin is expressed in posterior limb mesenchyme in vertebrate embryos, and is the principal BMP antagonist in the developing limb bud. It acts to maintain a Sonic hedgehog (Shh)/fibroblast growth factor (FGF) feedback regulatory loop to control limb outgrowth (Capdevila et al. 1999; Merino et al. 1999b; Zuniga et al. 1999; Khokha et al. 2003; Verheyden and Sun 2008). Gremlin also regulates myogenesis in the head, lung morphogenesis, kidney development, angiogenesis, and bone formation during vertebrate embryogenesis (Lu et al. 2001; Tzahor et al. 2003; Michos et al. 2004, 2007; Gazzerro et al. 2007; Stabile et al. 2007; Stafford et al.

2011; Canalis et al. 2012). The Gremlin–BMP axis is involved in pathogenesis of several diseases, including diabetic nephropathy, pulmonary hypertension, and cancer progression (Zhang and Zhang 2009; Cahill et al. 2012; Karagiannis et al. 2015). Besides BMPs, Gremlin interacts with and activates VEGF receptor 2 to stimulate angiogenesis (Mitola et al. 2010), and binds and inhibits macrophage migration inhibitory factor (MIF) to attenuate atherosclerosis (Muller et al. 2013).

PRDC (GREM2)

PRDC (protein related to DAN and Cerberus) was first identified by gene trapping in embryonic stem cells (Minabe-Saegusa et al. 1998). It encodes a small secreted glycoprotein that forms noncovalent, hydrogen-bonded homodimers that assume a TGF-β-like two-finger-wrist conformation (Kattamuri et al. 2012; Nolan et al. 2013). PRDC binds and inhibits BMP-2 and BMP-4 efficiently. PRDC and Gremlin also weakly inhibit BMP-6 and BMP-7 signaling, but do not affect the activities of activin, TGF-β, GDF-5, or GDF-9 (Sudo et al. 2004). Heparin binding by PRDC interferes with its ability to block BMP signaling (Nolan et al. 2013). PRDC is widely distributed, with high-level expression in ovary, brain, and spleen. PRDC regulates BMP signaling to control placode neurogenesis during cranial nerve formation in chick and to modulate follicle development in ovary (Sudo et al. 2004; Kriebitz et al. 2009). PRDC also influences osteoblast differentiation during osteogenesis (Ideno et al. 2009). Mice deficient for the *Prdc* gene have smaller incisors, implying a role of the gene in tooth morphogenesis (Vogel et al. 2015).

Sclerostin (SOST)

Sclerostin/SOST was first identified as the product of a gene whose mutations are responsible for sclerostosis, a recessive autosomal sclerosing bone dysplasia characterized by progressive skeletal overgrowth (Balemans et al. 2001; Brunkow et al. 2001). Sclerostin is a small secreted factor with a cystine-knot structure similar to that in Dan, Cerberus, and Gremlin, and was therefore proposed to be a BMP antagonist. Sclerostin binds BMP-5, -6, and -7, but not TGF-βs, and prevents binding of BMPs to their cognate receptors. It is expressed in osteocytes and inhibits BMP-5- or -6-stimulated bone differentiation, but has less effect on BMP-2 and -4 (Kusu et al. 2003; Winkler et al. 2003; van Bezooijen et al. 2004). In contrast to the loss-of-function phenotype leading to skeletal overgrowth, transgenic mice overexpressing sclerostin show reduced bone mass and bone strength (Winkler et al. 2003). Subsequently, sclerostin was shown to inhibit BMP-7 activity only in a cell-autonomous fashion, when coexpressed, because of direct binding of sclerostin to both the pro- and mature peptides of BMP-7 in ligand-expressing cells, thus promoting intracellular retention and proteasomal degradation of BMP-7 (Fig. 1) (Krause et al. 2010). The catabolic effect of sclerostin on bone growth is now mainly attributed to its ability to inhibit Wnt signaling by direct binding to the Wnt coreceptor LRP5/LRP6 (Li et al. 2005; Semenov et al. 2005; van Benzooijen et al. 2007; Kamiya et al. 2008).

Ectodin/Wise/USAG-1 (SOSTDC1)

SOSTDC1 was isolated as USAG-1 (uterine sensitization-associated gene 1) from sensitized endometrium of rat uterus, as ectodin from mouse, and Wise (Wnt modulator in surface ectoderm) from *Xenopus* (Simmons and Kennedy 2002; Itasaki et al. 2003; Laurikkala et al. 2003). It is a small secreted factor that is closely related to sclerostin, and binds and inhibits BMP-2, -4, -6, and -7 (Laurikkala et al. 2003; Yanagita et al. 2004). Functional studies with SOSTDC1-deficient mice reveal its role in regulation of BMP-7 signaling during renal injury as well as in teeth patterning during development (Kassai et al. 2005; Yanagita et al. 2006; Murashima-Suginami et al. 2008; Kiso et al. 2014). SOSTDC1/Wise also selectively inhibits BMP-7, but not BMP-2 or Wnt-3a, in breast cancer cells (Clausen et al. 2011), and suppresses both BMP-7 and Wnt-3a in renal cancer cells (Blish et al. 2008). Similarly to sclerostin,

SOSTDC1 binds LRP6 to modulate Wnt signaling, and regulates early *Xenopus* patterning and development of bone, teeth, mammary, and skin appendage placodes in mice (Itasaki et al. 2003; Lintern et al. 2009; Ahn et al. 2010, 2013; Ellies et al. 2014).

The CCN Family Proteins

The CCN family of growth factors includes six members, including CYR61 (CCN1), connective tissue growth factor (CTGF, CCN2), nephroblastoma overexpressed (NOV, CCN3), and WISP1, 2, and 3 (CCN4, 5, 6). These proteins show similar sequence configurations with four conserved domains, an amino-terminal domain similar to that in IGFBP, a CR module found in chordin and vWF, a thrombospondin type I repeat (TSP-1)-like sequence, and a carboxy-terminal motif with the cystine-knot structure (reviewed in Brigstock 2003; Perbal 2004; Katsube et al. 2009). Several CCN members regulate TGF-β signals. CTGF binds BMP-2 (K_D 0.77 nM), -4, -7 (K_D 14 nM) and TGF-β1 through its CR domain. By doing so, CTGF inhibits BMP-4 binding to its type I receptor but enhances TGF-β1 binding to its receptor complex. CTGF thus blocks BMP and promotes TGF-β signaling in cultured cells (Abreu et al. 2002; Nguyen et al. 2008; Maeda et al. 2009). Disruption of its gene in mouse reveals that CTGF is required for coordination of chondrogenesis and angiogenesis during skeletal development (Ivkovic et al. 2003), and this may depend on the activity of CTGF to modulate BMP signaling during chondrocyte differentiation (Maeda et al. 2009; Mundy et al. 2014). CTGF also inhibits BMP-7 in the diabetic kidney and contributes to diabetic nephropathy (Nguyen et al. 2008). CCN3 blocks BMP-2 function during osteoblast differentiation and modulates bone regeneration as an inhibitor of BMP-induced Smad signaling (Minamizato et al. 2007; Rydziel et al. 2007; Matsushita et al. 2013). Targeted disruption of the *Ccn3* gene results in abnormal skeletal and cardiac development (Heath et al. 2008). In contrast, CCN4 binds BMP-2 and enhances its signaling during osteogenic differentiation (Ono et al. 2011). Besides TGF-β family ligands, CCN proteins also interact with many other signaling proteins, including integrins, LRP, Wnt, VEGF-A, and Notch, to control multiple developmental and physiological processes (Babic et al. 1999; Segarini et al. 2001; Mercurio et al. 2004; Perbal 2013).

Insulin-Like Growth Factor–Binding Protein 3 (IGFBP3)

IGFBP3 was originally identified as the main carrier of IGF1 in serum, and acts as reservoir and modulator of IGF1 (Baxter 2014). However, IGFBP3 directly binds and antagonizes BMP-2 and -4 in zebrafish, and induces chordin expression to further inhibit BMP signaling (Zhong et al. 2011). IGFBP3 thus exerts an IGF1-independent function in modulating early zebrafish development. In mammals, IGFBP3 also enhances TGF-β1 activity and opposes BMP-7 signaling in glomerular podocytes to control cell survival or apoptosis, although direct association of IGFBP3 with these TGF-β family ligands has not been reported in this context (Peters et al. 2006).

Norrie (NDP)

Norrie is a secreted cysteine-rich protein encoded by the *NDP* gene whose mutations are associated with Norrie disease and familial exudative vitreoretinopathy (FEVR), two X-linked recessive disorders that affect neuroretina degeneration or result in incomplete development of retinal vasculature (Berger et al. 1992; Chen et al. 1993). Norrie stimulates the canonical Wnt-β-catenin pathway to control retinal vasculature development (Xu et al. 2004). However, Norrie also binds directly to nodal-related growth factors and BMP-4, and inhibits their signaling to promote anterior neural patterning in *Xenopus*. Norrie blocks BMP-2 and BMP-4 signaling in reporter assays in mammalian cells. Some Norrie mutants responsible for FEVR and Norrie disease show normal Wnt-enhancing ability, but impaired BMP-inhibitory function, suggesting that control of BMP signaling contributes to the activity of Norrie in eye development (Xu et al. 2012b; Deng et al. 2013).

Propeptides of TGF-β Family Ligands

Proteolytic cleavage to release TGF-β family proteins from their precursors is mediated by subtilisin/kexin-like proprotein convertases (PCs) (reviewed by Nakayama 1997; Taylor et al. 2003; Constam 2014). Although most of these enzymes are located in the *trans*-Golgi network and act in this compartment to process ligands, secreted convertases may additionally function in a non-cell-autonomous fashion to cleave soluble precursors in vivo (Beck et al. 2002; Birsoy et al. 2005). With the exception of the nodal-related Xnr2, which has diminished signaling capacity as precursor (Beck et al. 2002; Eimon and Harland 2002), other TGF-β family proteins are not active when the cleavage sites are mutated, and many such mutants act as dominant-negative ligands (Hawley et al. 1995; Joseph and Melton 1998; Sun et al. 1999; Eimon and Harland 2002). Regulation of ligand processing, by intracellular PCs or secreted factors, thus constitutes one of the first steps in the regulation of TGF-β family signaling (Constam 2014). Subsequent to ligand processing, cleaved propeptide products regulate the folding, sorting, stability, and/or activity of the mature TGF-β family proteins.

The classical example is TGF-β, which is in a latent state by noncovalent association with its precursor polypeptide latency-associated peptide (LAP) (reviewed in Harrison et al. 2011). Similarly, myostatin (GDF-8), its close relative GDF-11 (BMP-11), and BMP-10 all form latent, noncovalent complexes with their respective propeptides. Activation of these ligands is achieved by cleavage of the propeptides by BMP-1/Tolloid metalloproteinases, and/or by integrin-mediated interaction with the latent complex, leading to its conformational changes and release of the mature ligands (Hill et al. 2002; Wolfman et al. 2003; Ge et al. 2005; Sengle et al. 2011). The prodomain of the BMP-like ligand dorsalin also associates with the mature protein, although the functional consequence of this interaction is unclear (Constam and Robertson 1999). Besides regulating the bioactivity of mature ligands, propeptides also control the deposition of the ligands in the ECM, and stability and secretion of TGF-β family proteins. The prodomain of BMP-4, for example, associates with mature BMP-4 and directs it to lysosome- and proteasome-mediated degradation. A second cleavage inside the prodomain is required to release mature BMP-4, thereby stabilizing it, enhancing its activation, and allowing long-range signaling by this growth factor (Cui et al. 2001; Degnin et al. 2004). The prodomain of BMP-7 may similarly regulate the secretion and stability of mature BMP-7, because a single amino acid mutation in the proregion leads to reduced BMP-7 activity in zebrafish without affecting its processing (Dick et al. 2000). The prodomain of nodal facilitates degradation of mature nodal after cleavage (Constam and Robertson 1999), which may promote autocrine signaling and restrict its signaling range. Because uncleaved nodal precursor is detected in cell culture medium, long-range nodal signaling may be regulated by nodal transport in its precursor form to desired sites before processing into mature growth factor for signaling (Le Good et al. 2005). The proregions of Xnr3 and Xnr5, two nodal-related ligands in *Xenopus*, also bind and inhibit mature BMP-4, providing a mechanism for propeptides to regulate TGF-β family signaling in *trans* (Haramoto et al. 2004).

TGF-β Family Ligands as Agonists/Antagonists of TGF-β Family Signaling

TGF-β family signaling is not only regulated by soluble agonists and antagonists, but often modulated by heterodimerization or interaction with other TGF-β family polypeptides. An example is the inhibition of activin homodimer signaling by heterodimeric inhibin (reviewed by Bilezikjian et al. 2012). Other ligands that also modulate TGF-β family signaling include lefty, Xnr3, GDF-3, and BMP-3.

Lefty

Lefty (also known as antivin) is a divergent TGF-β family member that is asymmetrically expressed along the left–right axis (Meno et al. 1996). It lacks the cysteine involved in disulfide bond formation between dimer sub-

units, and thus exists as a monomer. Two lefty genes are found in vertebrates and play important roles in negative feedback regulation of left-sided nodal signaling during left–right axis determination (Meno et al. 1997, 1998, 1999; Cheng et al. 2000; Ishimaru et al. 2000). In addition, lefty expression during gastrulation limits nodal signaling in mesendoderm formation (Bisgrove et al. 1999; Thisse and Thisse 1999; Tanegashima et al. 2000; Agathon et al. 2001; Branford and Yost 2002; Chen and Schier 2002; Feldman et al. 2002; Cha et al. 2006). Besides nodal, lefty also antagonizes Vg1, GDF-1, and GDF-3, but has no effect on activin or TGF-β1 signaling. Lefty binds to the Cripto/Cryptic coreceptor for nodal and Vg1, GDF-1 and GDF-3, and blocks the formation of a functional ligand–receptor complex (Chen and Shen 2004; Cheng et al. 2004; Tanegashima et al. 2004). Lefty also binds directly to mature nodal and GDF-3 and prevents them from activating activin receptors (Chen and Shen 2004; Chen et al. 2006). In addition, lefty has been implicated in interference with BMP and Wnt signaling; however, the mechanisms involved in the regulation of these signals have not been defined (Meno et al. 1997; Branford et al. 2000; Ulloa and Tabibzadeh 2001; Branford and Yost 2002). In zebrafish and *Xenopus* embryos, it is proposed that nodal-lefty forms a reaction–diffusion system to pattern embryonic tissues, as lefty is induced by nodal and diffuses faster than nodal (Marjoram and Wright 2011; Muller et al. 2012). However, this view is challenged by the observation that nodal may only signal over a short range, and the gradient of nodal signaling readout may rely on differential temporal regulation of nodal and lefty expression (van Boxtel et al. 2015).

Xnr3

Xnr3 is a divergent nodal-related factor in *Xenopus* that lacks the carboxy-terminal cysteine (Cys-7) that is conserved among all other TGF-β family members, and functions as a monomer (Smith et al. 1995; Haramoto et al. 2007). Unlike other nodal-related proteins, Xnr3 does not specify mesendodermal cell fate; instead, it induces neural marker expression and blocks mesodermal induction by BMP-4 and activin, but not by Xnr2, in *Xenopus* (Hansen et al. 1997; Haramoto et al. 2004). Studies of Xnr2 and Xnr3 chimeras suggest that amino- and carboxy-terminal segments of mature Xnr3 are required for neural inducing activity, whereas the central region can be replaced with that of mature Xnr2 to induce dorsal mesoderm (Ezal et al. 2000). The prodomain of Xnr3 alone binds and inhibits BMP-4 and is both necessary and sufficient for Xnr3 to block BMP-4 activities (Haramoto et al. 2004, 2006). A role of Xnr3 in blocking ligand–receptor assembly has also been proposed (Ezal et al. 2000). Loss-of-function studies reveal that Xnr3 regulates convergent extension movements during gastrulation in *Xenopus*, and may do so by interacting with the divergent Cripto family protein FRL1, which binds and activates the FGF receptor 1. The prodomain of Xnr3 may be important for activation of FGF signaling in this process (Glinka et al. 1996; Yokota et al. 2003).

GDF-3

GDF-3 is closely related to Vg1, but lacks the conserved cysteine used in dimer formation of TGF-β family ligands (McPherron and Lee 1993). GDF-3 signals through the nodal pathway, using ALK-4 and ALK-7 receptors and Cripto coreceptor, during early mouse development (Chen et al. 2006; Andersson et al. 2007, 2008). In addition, GDF-3, both as precursor and as mature peptide, inhibits BMP signaling through direct interaction with mature BMPs (Levine and Brivanlou 2006; Levine et al. 2009). GDF-3 expression is high in pluripotent cell types, including human embryonic stem cells, and may regulate their maintenance and differentiation (Levine and Brivanlou 2006; Peerani et al. 2007).

BMP-3

BMP-3 and *Xenopus* BMP-3b are closely related BMP-like ligands. Unlike BMPs, however, BMP-3 and BMP-3b dorsalize *Xenopus* embryos, implying that they can act as BMP antagonists

(Hino et al. 2003; Gamer et al. 2005). BMP-3 and BMP-3b block BMP-2 and ADMP signaling in *Xenopus* ectodermal explants. BMP-3 also inhibits activin but not Xnr1 or a nodal-like ligand Derriere, whereas BMP-3b blocks both Xnr1 and Derriere. In addition, BMP-3 inhibits BMP-2-induced differentiation of osteoprogenitor cells and opposes TGF-β1 actions in bone marrow stromal cells (Faucheux et al. 1997; Daluiski et al. 2001). BMP-3 and BMP-3b form heterodimers with BMP-2, ADMP, and Derriere, suggesting that BMP-3 or BMP-3b may exert its effects through formation of inactive ligand dimers (Hino et al. 2003). BMP-3b also binds Xnr1 noncovalently and may prevent Xnr1 from associating with its receptors (Hino et al. 2003). BMP-3 further interacts with the type II receptor ActRIIB without inducing Smad activation, and this provides another means for the ligand to inhibit activin and BMP signaling (Gamer et al. 2005; Kokabu et al. 2012). In myoblastic C2C12 cells, however, BMP-3b can stimulate Smad2/3 signaling and activin-responsive reporter expression. It also inhibits BMP-2-induced osteoblastic differentiation in this system, apparently through competing for the common Smad4 by activated Smad2/3 (Matsumoto et al. 2012).

Heterodimers

Formation of heterodimers among TGF-β family proteins can regulate the activities of the ligands (Guo and Wu 2012). As mentioned, BMP-3 and BMP-3b can form heterodimers with BMP-2, ADMP, and Derriere, and inhibit the activities of these factors (Hino et al. 2003). Nodal also forms heterodimers with BMPs to antagonize BMP signaling (Yeo and Whitman 2001). Nodal heterodimers with GDF-1, in contrast, have higher activity than nodal homodimers (Fuerer et al. 2014). Similarly, heterodimers of BMP-2 and -7 or BMP-4 and -7 are more active in mesoderm induction or patterning in *Xenopus* and zebrafish and in inducing bone formation in osteoprogenitor cells than their homodimers (Aono et al. 1995; Israel et al. 1996; Suzuki et al. 1997; Nishimatsu and Thomsen 1998; Little and Mullins 2009). Heterodimers, but not homodimers, of BMP-2 and BMP-7 engage simultaneously two different type I BMP receptors (i.e., ALK-3 or ALK-6 and ALK-2 or ALK-8) in the receptor complex to activate BMP signaling in zebrafish (Little and Mullins 2009). BMP-7 and GDF-7 also form heterodimers in vitro that have a higher axon-orienting activity than BMP-7 or GDF-7 homodimers (Butler and Dodd 2003). Heterodimers of GDF-9 and BMP-15 are more potent regulators of ovarian granulosa and cumulus cells than their corresponding homodimers (Peng et al. 2013; Mottershead et al. 2015). BMP-2 and BMP-6 heterodimers are also more effective than the corresponding homodimers to induce differentiation of human embryonic stem cells (Valera et al. 2010). The formation of heterodimers between *Drosophila* Dpp and Scw also facilitates transport of the ligands in early *Drosophila* embryos and promotes threshold readout of the BMP morphogen gradient (Shimmi et al. 2005a).

PROTEOGLYCANS AND EXTRACELLULAR MATRIX PROTEINS

Secreted TGF-β family ligands encounter not only soluble regulatory factors, as discussed, but also various ECM proteins. The complex interplay between TGF-β family proteins, their soluble regulators and ECM proteins controls the diffusion of the ligands among target cells, and ligand availability for receptor binding and activation. The ECM-associated proteins are often not dedicated TGF-β modulators. The HSPG glypicans, for example, regulate signals from FGF, Wingless/Wnt, and hedgehog family proteins in addition to TGF-β/BMPs (Lin and Perrimon 2000). Many ECM molecules can also act as both agonists and antagonists. A balance in actions between sequestration, storage, and presentation of ligands may account for the dual functions of ECM proteins (Fig. 3).

Small Leucine-Rich Proteoglycans (SLRPs)

The SLRP family consists of 17 members with similar domain organization (reviewed in Hocking et al. 1998; Iozzo 1999; Young et al.

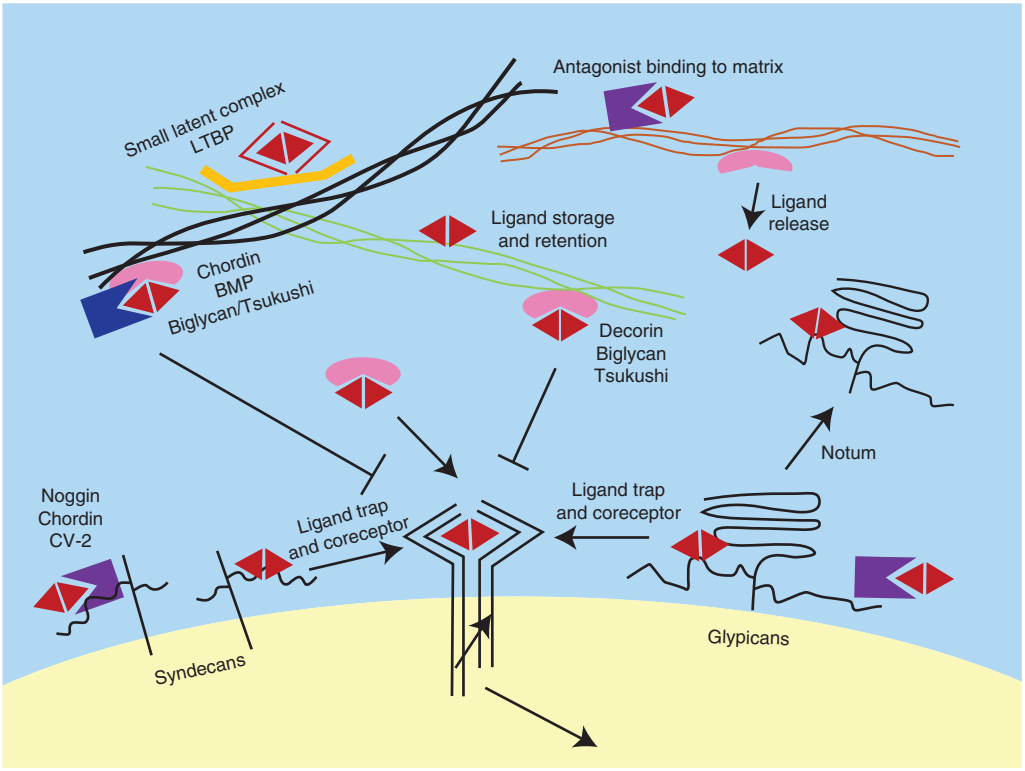

Figure 3. Regulation of transforming growth factor β (TGF-β) family signals by proteoglycans and extracellular matrix proteins. Small leucine-rich proteoglycans, such as decorin, biglycan, and Tsukushi, interact with both TGF-β family ligands and extracellular matrix (ECM) proteins. Depending on the composition of ECM proteins, they may tether the ligands in the ECM to prevent signaling, or release the ligands to create a local pool of cytokines to enhance signaling. Biglycan and Tsukushi also form a ternary complex with chordin and bone morphogenetic protein (BMP) to block BMP signaling. Cell-surface heparan sulfate proteoglycans (HSPGs), including glypicans and syndecans, interact with both TGF-β family proteins and their secreted modulators. They can serve as ligand coreceptors to enhance TGF-β family signaling, but they can also trap and facilitate internalization of the ligands and their soluble regulators to modulate signal duration and range. They, therefore, have cell-context-dependent positive or negative roles in regulation of TGF-β signals. The *Drosophila* protein Notum facilitates release of glypicans from the cell surface and can convert glypicans from ligand-presenting factors to ligand-binding antagonists. Several ECM proteins bind TGF-β family ligands directly and influence storage, activation, and diffusion of these proteins to control signaling strength and range. Secreted TGF-β family regulators can also associate with ECM proteins to affect ligand availability. LTBPs (latent TGF-β-binding proteins) interact with ECM proteins to control TGF-β activation from the small latent complex (SLC).

2003; Schaefer and Iozzo 2008). The core proteins contain six to twelve 24-amino-acid leucine-rich repeats and a characteristic amino-terminal cysteine-rich region. Chondroitin/dermatan sulfate and keratin sulfate glycosaminoglycan (GAG) chains are attached to the core proteins. Several SLRP proteins are shown to regulate TGF-β signaling.

Decorin (DCN)

Decorin is a small ECM proteoglycan that is expressed in connective tissues. It binds to mature, but not latent, TGF-β1, -β2, and -β3 through its polypeptide core, with GAG modifications reducing this binding. Decorin reduces the interaction of TGF-β1 with its type I and

type III receptors, and may sequester the ligand in the ECM (Hildebrand et al. 1994). Cells deficient in the *Dcn* gene show enhanced TGF-β binding to its receptors (Droguett et al. 2006). Expression of decorin in CHO cells blocks TGF-β-induced cell proliferation (Yamaguchi et al. 1990), and administration of decorin in a mouse kidney disease model can attenuate the disease manifestation, similarly to TGF-β neutralization with antibody (Border et al. 1992). Although these data suggest that decorin inhibits TGF-β function, other studies imply that decorin enhances the bioactivity of TGF-β. Addition of decorin promotes TGF-β1 binding to the TGF-β receptors and betaglycan, and strengthens the inhibitory effect of TGF-β1 on osteoblast cell proliferation, whereas depletion of decorin reduces myoblast responsiveness to TGF-β-mediated inhibition of skeletal muscle differentiation (Takeuchi et al. 1994; Riquelme et al. 2001). Several explanations may account for the dual functions of decorin in regulating TGF-β activities. Decorin binds TGF-β1 at two interfaces with high and low affinity, respectively (Hildebrand et al. 1994; Takeuchi et al. 1994). Association of TGF-β1 with different sites may affect the conformation and/or stability of decorin-TGF-β1 complex, thus affecting retention of TGF-β1 inside the ECM or ligand presentation to its receptors. In addition, decorin interacts with many ECM components, such as collagens and fibronectin. Differential association of decorin with distinct ECM proteins may control the function of decorin (Kresse and Schonherr 2001). ECM-immobilized decorin sequesters TGF-β and prevents it from signaling, whereas soluble decorin does not control TGF-β activity (Markmann et al. 2000). In addition, dermatopontin, a small ECM component, can interact with both decorin and TGF-β1. Whereas free dermatopontin competes with decorin for TGF-β1 and decreases the formation of the decorin–TGF-β1 complex, a dermatopontin–decorin complex has enhanced binding to TGF-β1. Thus, dermatopontin may differentially influence the activity of decorin depending on whether it forms a ternary complex with decorin and TGF-β1, or interacts with the two proteins separately (Okamoto et al. 1999). Besides TGF-βs, decorin binds myostatin and reverses its inhibition of myoblast proliferation in vitro (Miura et al. 2006). It also associates with activin C and may modulate its activity in stimulating cell growth and migration of colorectal cancer cells (Bi et al. 2015).

Biglycan (BGN)

Biglycan is closely related to decorin in structure and has similar and distinct functions in binding and regulating TGF-β ligands (Hildebrand et al. 1994; Droguett et al. 2006; Wu et al. 2014). One distinct activity of biglycan is its role in modulating BMP signals. Targeted disruption of the *Bgn* gene leads to age-related osteoporosis (Xu et al. 1998), and reduced osteoblast responsiveness to BMP-4-induced differentiation may at least partially account for this defect (Chen et al. 2004b). Although the GAG chains enhance biglycan's ability to promote BMP-4 signaling in osteoblasts, they reduce BMP-2 signaling in myogenic C2C12 cells (Miguez et al. 2011; Ye et al. 2012). The conflicting results may be because of cell-type-specific ECM proteins that can modify the activity of biglycan. Although these findings indicate a role of biglycan in promoting BMP signaling in skeletal development, biglycan can inhibit BMPs in early *Xenopus* development. Biglycan binds BMP-4 directly and blocks its function upstream of the BMP receptors. It can also form a ternary complex with BMP and chordin, and enhance the BMP-inhibitory activity of chordin (Moreno et al. 2005). Similarly to decorin, the dual activities of biglycan may depend on the microenvironment and the presence or absence of other factors, such as chordin, BMP-1/Tolloid metalloproteinases, or other ECM proteins (Wadhwa et al. 2004).

Tsukushi (TSK)

Tsukushi, a unique SLRP protein, was first discovered in the chick, and vertebrate homologs were identified in *Xenopus*, zebrafish, mouse, and human. Although structurally different from biglycan (e.g., Tsukushi has 12 leucine-rich repeats instead of 10 in biglycan), Tsukushi has similar activities as biglycan in that it binds

directly to BMP-4 and blocks BMP signaling in chick and frog. Tsukushi also forms a ternary complex with BMP-4 and chordin and enhances the BMP-inhibitory activity of chordin (Ohta et al. 2004). Differently from biglycan, Tsukushi associates with Xnr-2 and Vg1, and enhances nodal-like signaling during germ layer and primitive streak formation in *Xenopus* and chick, respectively (Ohta et al. 2006; Morris et al. 2007). Besides TGF-β family ligands, Tsukushi can bind and inhibit FGF-8b, interact with Delta to regulate Notch signaling, and bind Frizzled 4 (Fzd4) to antagonize Wnt signaling (Kuriyama et al. 2006; Morris et al. 2007; Ohta et al. 2011). Loss-of-function studies indicate that Tsukushi regulates neural crest formation during early *Xenopus* development and controls anterior commissure formation in mouse brain (Kuriyama et al. 2006; Ito et al. 2010).

Fibromodulin (FMOD) and Lumican (LUM)

Fibromodulin and lumican are both SLRPs with keratin sulfate GAG chains, in contrast to chondroitin/dermatan sulfate found in decorin and biglycan. Like decorin and biglycan, fibromodulin binds TGF-β1 and may inhibit its signaling through ligand sequestration in the ECM (Hildebrand et al. 1994; Embree et al. 2010). Physical interaction between lumican and TGF-β ligands has not been reported, but lumican may repress TGF-β2 signaling in osteosarcoma cells (Nikitovic et al. 2011). Lumican also binds to the type I TGF-β receptor TβRI/ALK-5 to regulate epithelial wound healing (Yamanaka et al. 2013).

Heparan Sulfate Proteoglycans

HSPGs are proteins with heparan sulfate glycosaminoglycan chains attached. They regulate diverse cellular processes ranging from adhesion and migration to proliferation, differentiation, and morphogenesis (Bernfield et al. 1999). Two families of surface HSPGs exist, glypicans, which are linked to the plasma membrane via glycosylphosphatidylinositol (GPI) anchor, and syndecans, which are transmembrane HSPGs. Four syndecans and six glypicans exist in mammals, whereas a single syndecan and two glypicans are found in *Drosophila*. Several HSPG family proteins modulate TGF-β signaling.

Glypicans

The two *Drosophila* glypican homologs, Dally and Dally-like, do not regulate signaling from Dpp/BMP during early embryogenesis. However, they are critically required at the pupal stages when imaginal discs, such as eye, antenna, genitalia, and wing discs, develop (Jackson et al. 1997; Tsuda et al. 1999; Fujise et al. 2003; Belenkaya et al. 2004). Mosaic mutant clone studies indicate that Dally and Dally-like control cell-autonomous responses to Dpp as well as cell-non-autonomous movement of Dpp to form a Dpp morphogen gradient in the wing disc. In addition, Dally can act as both positive and negative regulator of Dpp. Low doses of Dally are required for Dpp signaling, whereas high doses of Dally reduce Dpp responses. Dally may thus serve as a coreceptor for Dpp to enhance its signaling but, at the same time, limits Dpp diffusion to restrict its distribution (Fujise et al. 2003; Belenkaya et al. 2004). Another secreted molecule, Pentagon, interacts with Dally to facilitate long-range transport of Dpp in the wing disc (Vuilleumier et al. 2010). Dally-like can be modified by the secreted enzyme Notum, which facilitates its cleavage at the GPI anchor. Notum thus converts this glypican from a membrane-tethered coreceptor to a soluble, ligand-binding antagonist (Kreuger et al. 2004). Interestingly, in addition to acting as a coreceptor for Dpp, Dally can also promote Dpp signaling in *trans*, in neighboring cells in the *Drosophila* ovary, to maintain germline stem cells. Stabilization of Dpp at the cell surface may contribute to this cell-contact-dependent, *trans*-Dpp stimulatory function of Dally (Akiyama et al. 2008; Guo and Wang 2009; Hayashi et al. 2009; Dejima et al. 2011). A role of heparan sulfate in glypican-dependent modulation of Dpp is implied by the observation that mutations in heparan sulfate biosynthesis enzymes in *Drosophila* attenuate Dpp signals in the wing imaginal disc (Bornemann et al. 2004; Han et al. 2004; Takei et al. 2004). In *C. elegans*, the glypican LON-2 binds

BMP-2 to inhibit its signaling in body length control (Gumienny et al. 2007; Taneja-Bageshwar and Gumienny 2012). In mammals, glypican-3 enhances BMP-4 signaling during limb patterning and skeletal development (Paine-Saunders et al. 2000), and participates in BMP-regulated renal branching morphogenesis (Grisaru et al. 2001). However, increased glypican-3 expression in hepatocellular carcinoma inhibits BMP-7 signaling (Midorikawa et al. 2003). Glypican-1 is required for optimal TGF-β1 signaling in pancreatic cancer cells (Li et al. 2004), but inhibits BMP-4 signaling in lymphoblastoid cells (O'Connell et al. 2007). Both glypican-1 and -3 also bind BMP-2 and block BMP-2 signaling in cranial osteogenesis (Dwivedi et al. 2013). In zebrafish, glypican-4 attenuates BMP signaling to regulate cardiac development (Strate et al. 2015). As glypicans also regulate Wnt, hedgehog, and FGF signaling (Bernfield et al. 1999; Lin and Perrimon 2000; Fico et al. 2011), the six vertebrate glypicans may differentially modulate overlapping and distinct signaling pathways during vertebrate embryogenesis and homeostasis.

Syndecans

The role of syndecan in signal transduction of TGF-β family ligands is less understood. Among four vertebrate homologs, syndecan-2 binds TGF-β1 and Vg1, and less efficiently activin, through its ectodomain polypeptide backbone (Kramer and Yost 2002; Chen et al. 2004a). A mutant syndecan-2 inhibits signaling by Vg1, but not activin or nodal, in *Xenopus* ectodermal explants. Syndecan-2 may act as a coreceptor for Vg1 during left–right patterning in *Xenopus* (Kramer and Yost 2002). Syndecan-2 also increases TGF-β1-induced fibronectin expression in renal papillary fibroblasts (Chen et al. 2004a) and promotes TGF-β2-enhanced fibrosarcoma cell adhesion (Mytilinaiou et al. 2013). Syndecan-4 also binds TGF-β1, but does so through its heparan sulfate chains instead of the core polypeptide. Syndecan-4-deficient mice show impaired inhibition of interleukin-1β production by TGF-β1 in macrophages (Ishiguro et al. 2001) and reduced induction of dermal fibroblast contraction by TGF-β1 (Chen et al. 2005). Enhanced expression of syndecan-4 in cutaneous T-cell lymphomas traps TGF-β1 at the cell surface and facilitates its inhibition of T-cell activation (Chung et al. 2011). Unlike syndecan-2 and -4, syndecan-3 may block, rather than promote, BMP-2 signaling during cartilage differentiation of limb mesenchyme, although no interaction of syndecan-3 with BMP-2 or BMP receptors has been shown (Fisher et al. 2006). Syndecan-1 has a biphasic effect on BMP signaling. It enhances BMP signaling at low doses and inhibits it at high doses. Endogenous syndecan-1 regulates BMP signaling during dorsoventral patterning of embryonic ectoderm in *Xenopus* (Olivares et al. 2009).

In addition to binding TGF-β family ligands and thus directly regulating their activities, HSPGs also associate with soluble BMP regulators such as noggin, chordin, and Crossveinless-2 (Paine-Saunders et al. 2002; Jasuja et al. 2004; Viviano et al. 2004; Rentzsch et al. 2006). These interactions may restrict their diffusion and help define their activities (see above). HSPGs thus regulate TGF-β family signaling by controlling the activities of both the TGF-β family ligands and their soluble modulators.

Extracellular Matrix Proteins

α_2-Macroglobulin

α_2-Macroglobulin (α_2M) is a large homotetrameric plasma glycoprotein that functions as an extracellular inhibitor of all four classes of proteases, that is, serine, cysteine, aspartate, and metalloproteases. Cleavage of α_2M by bound proteases results in a conformational change that traps and inhibits the proteases in nondissociable complexes. Protease-activated α_2-macroglobulin (α_2M^*) then loses protease-inhibitory activity and is recognized by the cell-surface receptor LRP. The role of α_2M in controlling TGF-β activity was first revealed by the observation that TGF-β forms an inactive complex with α_2M in serum (O'Connor-McCourt and Wakefield 1987; Huang et al. 1988). In contrast, latent TGF-β does not bind α_2M (Wakefield et al.

1988). α_2M binds TGF-β2 with higher affinity (K_D ~14 nM) than TGF-β1 (K_D 320 nM), and, accordingly, inhibits TGF-β2 more efficiently (Danielpour and Sporn 1990; Webb et al. 1996). Protease activation modulates the affinity of α_2M for TGF-βs, as trypsin and thrombin decrease and plasmin and methylamine enhance α_2M^* binding to TGF-β (LaMarre et al. 1991; Webb et al. 1996). Methylamine-α_2M^* promotes TGF-β1-induced smooth muscle proliferation using the LRP receptor (Stouffer et al. 1993). The interfaces that mediate TGF-β interaction with α_2M are mapped to hydrophobic regions in both proteins, and may mimic the hydrophobic interface between TGF-β and TβRII, which may explain α_2M's ability to block TGF-β binding to TβRII (Liu et al. 2001; Arandjelovic et al. 2003). Heparin and heparan sulfate reverse the inhibition of TGF-β by α_2M by promoting the dissociation of TGF-β from α_2M, and does so more efficiently for TGF-β1 than TGF-β2 (McCaffrey et al. 1989; Lyon et al. 1997). α_2M may function with other tissue-specific ECM components to control TGF-β ligand availability.

Collagens

Collagens have long been shown to bind TGF-β family ligands, including TGF-βs, BMPs, and activin (Paralkar et al. 1992), and are used as collagen sponges to protect and deliver BMPs during interventions aimed at bone and cartilage repair (Geiger et al. 2003). Type IV collagen directly controls BMP signaling in *Drosophila* (Wang et al. 2008; Bunt et al. 2010; Sawala et al. 2012). The carboxy-terminal region of collagen IV binds the BMP ligand Dpp, but not Scw or Gbb, and serves as a scaffold to facilitate formation of the Dpp shuttling complex with Sog/chordin and Tsg, thus augmenting long-range Dpp signaling by promoting Dpp gradient formation. Collagen IV may use a similar mechanism to enhance BMP signaling in *Drosophila* renal tubule morphogenesis (Bunt et al. 2010). However, collagen IV sequesters Dpp and restricts its signaling range in the *Drosophila* ovary (Wang et al. 2008), suggesting that the function of collagen IV in BMP signal regulation depends on cellular context.

Type II procollagen also modulates BMP signaling. Type IIA procollagen contains an amino-terminal 69-amino acid CR sequence, which is spliced out in the type IIB isoform that is expressed in mature chondrocytes. The CR domain is homologous to that in chordin and mediates type IIA procollagen binding to BMP-2 and -4 and TGF-β1. This domain does not bind activin or epidermal growth factor (EGF), and the type IIB form that lacks this domain does not interact with BMP or TGF-β (Zhu et al. 1999b; Larrain et al. 2000). Binding of procollagen IIA to BMPs can inhibit BMP function in *Xenopus* and induce a secondary axis (Larrain et al. 2000). Procollagen IIA may control storage and release of BMPs and TGF-βs and, thus, the availability of active TGF-β family ligands for cell differentiation and tissue homeostasis.

Matrilin-3 (MATN3)

Matrilins are a family of four ECM proteins that share structural features that consist of vWF A (vWFA) domains, EGF-like repeats, and a carboxy-terminal coiled-coil module. Matrilins form oligomers and can bind to other ECM components such as collagens and aggrecan (Deak et al. 1999). Both matrilin (MATN)-1 and -3 regulate cartilage homeostasis. MATN3, but not MATN1, inhibits BMP-dependent collagen X expression in hypertrophic chondrocytes via its EGF repeats and coiled-coil domain. MATN3 directly binds BMP-2 with a K_D of 217 nM, suggesting that binding of BMP ligands may explain its role in blocking BMP signaling in chondrocytes (Yang et al. 2014). Mutations in MATN3 result in several skeletal diseases, such as multiple epiphyseal dysplasia and age-related osteoarthritis (Mostert et al. 2003; Stefansson et al. 2003).

Cartilage Oligomeric Matrix Protein (COMP)

COMP (also known as thrombospondin-5, or TSP-5) is a large pentameric extracellular glycoprotein that interacts with many ECM proteins, such as fibronectin, collagens, matrilins, and aggrecan, and plays an important role in ECM assembly (Acharya et al. 2014). Each

COMP monomer consists of an amino-terminal coiled-coil domain, four EGF repeats, eight calcium-binding type III TSP repeats, and a carboxy-terminal globular domain. COMP binds TGF-β1, BMP-2, -4, and -7 using its carboxy-terminal domain. This binding enhances TGF-β1 and BMP-2 signaling in human bone mesenchymal stem cells (Haudenschild et al. 2011; Ishida et al. 2013), but inhibits BMP-2 signaling in vascular smooth muscle cells by preventing BMP-2 binding to its receptors (Du et al. 2011). COMP thus regulates TGF-β signaling in a cell-type-specific fashion. Mutations in human COMP are linked to the skeletal diseases pseudoachondroplasia (PSACH) and multiple epiphyseal dysplasia (MED) (Acharya et al. 2014).

Tenascin-X

Tenascin-X (TNX) is a member of tenascin family of ECM glycoproteins. Like other tenascin proteins, it has many EGF and fibronectin modules and a carboxy-terminal fibrinogen-like (FBG) domain. It binds several ECM components, such as collagen and decorin, and regulates the three-dimensional ECM organization. TNX binds the small latent TGF-β complex through its FBG domain and activates TGF-β ligands, most likely by inducing a conformational change in the complex. This activity requires the cell-surface integrin α11β1, and may enable TNX to promote epithelial-to-mesenchymal transition in mammary epithelial cells (Alcaraz et al. 2014).

Matrix GLA Protein (MGP)

MGP is a small (10 kDa) ECM-associated protein that binds BMP-2 and -4, and regulates osteogenesis, vascular endothelial cell proliferation and migration, and sympathetic neuron growth (Bostrom et al. 2001, 2004; Moon and Birren 2008; Yao et al. 2010). The interaction of MGP with BMP enhances or attenuates BMP signaling, which may relate to the opposite effects of the amino- and the carboxy-terminal domains of MGP on BMP signaling (Bostrom et al. 2001; Zebboudj et al. 2002). MGP also enhances TGF-β1 activity in cultured endothelial cells (Bostrom et al. 2004). Mice deficient in MGP show arteriovenous malformations in lungs and kidneys and vascular calcification (Yao et al. 2010, 2011).

Emilin1

Emilin1 was first isolated from chick aorta as the secreted glycoprotein gp115 (Bressan et al. 1983) and contains an amino-terminal CR domain, followed by coiled-coil, collagen, and C1q domains (Doliana et al. 1999). Emilin1 binds pro-TGF-β1, but not mature TGF-β1 or LAP, through its CR motif. In this way, it blocks pro-TGF-β1 processing by furin-like proprotein convertases in the extracellular space and inhibits TGF-β signaling. This represents a unique mechanism for an ECM protein to modulate TGF-β availability. Emilin1 also inhibits Xnr1 and TGF-β3 activities. Lack of emilin1 expression in mice leads to elastic fiber defects in aorta and skin and increased blood pressure as a result of elevated TGF-β signaling in the vascular wall (Zanetti et al. 2004; Zacchigna et al. 2006).

Fibulins (FBLNs)

The fibulin family of extracellular glycoproteins contains seven members with multiple calcium-binding EGF motifs and a carboxy-terminal fibulin-type module. They bind diverse ECM proteins and proteoglycans and regulate tissue structures during development and in disease processes (Timpl et al. 2003; de Vega et al. 2009). Fibulin-1 (Fbln1) binds BMP-2 and is required for BMP-2-mediated induction of Osterix expression during bone formation. Fbln1-deficient mice show defects in membranous and endochondral bone formation in the skull (Cooley et al. 2014). Fibulin-3 inhibits TGF-β signaling in breast cancer and endothelial cells to prevent cancer progression. Interestingly, fibulin-3 does not bind TGF-β1 appreciably, but instead interacts with the type I receptor TβRI/ALK-5 and blocks formation of a functional ligand–receptor complex (Tian et al. 2015). Other fibulins may also regulate TGF-β signaling (Renard et al. 2010; Radice et al. 2015), but

the underlying molecular mechanism has not been elucidated.

Latent TGF-β-Binding Proteins (LTBPs) and Fibrillins (FBNs)

The LTBPs and FBNs are related ECM proteins with multiple calcium-binding EGF domains and characteristic eight-cysteine-containing TGF-β-binding (TB) modules (reviewed in Olivieri et al. 2010; Davis and Summers 2012; Robertson et al. 2015). LTBPs form a covalent bond with the TGF-β prodomain in the latent TGF-β complex and facilitate secretion and folding of TGF-β ligands, target latent TGF-β complexes to ECM, and control activation of TGF-βs. LTBPs bind the amino-terminal region of fibrillins and target the large latent TGF-β complex to ECM microfibrils to control TGF-β availability (Massam-Wu et al. 2010). Fbn-1, best known as encoded by the gene whose mutations lead to type I Marfan syndrome, and Fbn-2 both regulate TGF-β signaling through LTBP-mediated sequestration (Neptune et al. 2003; Chaudhry et al. 2007; Nistala et al. 2010). Fibrillins also interact through their amino-terminal regions with the propeptide of BMP-2, -4, -5, -7, -10, and GDF-5 and target these cytokines to the ECM. *Fbn1*$^{-}$, but not *Fbn2*$^{-}$, deficient mice show enhanced BMP signaling (Gregory et al. 2005; Sengle et al. 2008, 2011; Nistala et al. 2010). LTBPs and fibrillins thus serve both as structural components of ECM to maintain tissue architecture and to control the bioavailability of TGF-β family ligands.

SUMMARY AND PERSPECTIVES

Since the discovery of TGF-β ligands in latent forms and the realization that extracellular factors regulate the bioactivity of TGF-βs, an escalating number of secreted modulators of TGF-β family proteins has been identified, and the mechanisms underlying their functions are increasingly clear. One insight gained from the studies is that agonists and antagonists originate very early during evolution, probably around the time that TGF-β family ligands arose. Noggin, for example, is found in sponges, the most basal metazoans (phylum Porifera) (Muller et al. 2003); and, although lost in some clades (e.g., Ctenophore) (Pang et al. 2011), noggin is expressed in a wide range of metazoans, including Hydra (Cnidaria) (Chandramore et al. 2010), Planaria (Platyhelminthes) (Molina et al. 2009, 2011), sea urchin (Echinodermata) (Lapraz et al. 2006), and all vertebrates. Similarly, chordin is expressed in Hydra and sea anemone (Cnidaria) (Matus et al. 2006; Rentzsch et al. 2007), sea urchin (Echinodermata) (Lapraz et al. 2006), and ascidians (Urochordata) (Darras and Nishida 2001). The appearance of multiple agonists and antagonists in metazoans is accompanied by extensively overlapping functions in development. This is shown, for instance, by the absence of a severe phenotype in mice with inactivated expression of individual regulators (e.g., Dan, Cerberus), and the dramatic disruption of embryonic axes and tissue development only when multiple regulators are silenced (De Robertis and Kuroda 2004; Kuroda et al. 2004; Khokha et al. 2005; Vonica and Brivanlou 2006; Stafford et al. 2011). Conversely, proteins with similar biochemical properties and expression patterns can have distinct activities. This is evident by differential effects of noggin and chordin in mesodermal patterning and somite formation during early chick development (Streit and Stern 1999). It is also increasingly appreciated that endogenous agonists and antagonists interact extensively with other extracellular molecules. Tissue-specific cellular landscapes can influence the actions of soluble modulators of TGF-β family ligands, resulting in distinct signal outcomes. Thus, in contemplating how agonists and antagonists control TGF-β family signaling, one needs to determine not only which regulators are present in which isoforms at which places, but also their specificities and affinities toward ligands, and to also integrate information on the ECM proteins and cell-surface molecules in their environments. The following points should be considered in building a holistic picture (Figs. 1–3).

First, although most abundant agonists and antagonists are soluble or membrane-associated ligand-binding factors, some do not directly

interact with ligands and nonetheless control TGF-β family signals by modifying ligand-binding proteins. Examples include BMP-1/Tolloid metalloproteinases, Sizzled, and the glypican-modifying enzyme Notum. Such non-dedicated regulators can both promote and inhibit TGF-β family signaling depending on the cellular environment.

Second, although most agonists and antagonists enhance or block TGF-β family signals by regulating ligand–receptor interactions, others control secretion, storage, maturation, stabilization, transport, and release of TGF-β family ligands in a spatiotemporal manner. The actions of CRIM1 and emilin1 illustrate this point nicely. These modulators help shape the patterns of ligand distribution in tissues and determine the range and the duration of TGF-β family signals.

Third, agonists and antagonists can interact with each other in competitive or cooperative modes, and collectively modulate the outcome of TGF-β family signaling in different contexts. The BMP antagonists noggin and sclerostin, for example, show reduced BMP-inhibitory activity when associated with each other (Winkler et al. 2004). In contrast, Twsg, biglycan, and Tsukushi all interact with chordin to enhance inhibition of BMPs. Association of soluble agonists and antagonists with ECM proteins and cell-surface proteoglycans can also change the properties of these modifiers and the tissue responses to TGF-β family signals. An example is the influence of HSPGs on retention, diffusion and activities of the BMP regulators noggin, chordin, and Crossveinless-2.

Fourth, the functions of agonists and antagonists are not absolute. Depending on cellular localization (soluble or membrane-associated), and presence or absence of interacting factors in the tissue, an agonist may be converted into an antagonist, and vice versa. Proteins that act as both agonists and antagonists in different cellular contexts include decorin, biglycan, glypican, betaglycan, Crossveinless-2, Twsg, chordin/Sog, and COMP. A regulator can also act as agonist of one ligand and antagonist of another; CTGF is one example. The detailed mechanisms responsible for functional switch of these molecules may vary, and all of them are not equally understood. These examples highlight the importance of integrating all tissue-specific extracellular components to fully understand the actions of agonists and antagonists.

Fifth, agonists and antagonists often act in feedback control through transcriptional regulation by TGF-β family proteins. TGF-β modulates the expression of its regulators decorin and α_2-macroglobulin, whereas BMP-2 induces the expression of its antagonists noggin and Gremlin in osteoblasts (Shi et al. 1990; Heimer et al. 1995; Gazzerro et al. 1998; Pereira et al. 2000). Noggin expression is also induced by BMP-4 in chick somites, and GDF-11 induces follistatin expression in chick limb bud (Amthor et al. 1999; Gamer et al. 2001). Stimulation of TGF-β antagonists by ligands often serves to restrict signaling range, strength, and duration. A consequence of such negative feedback regulation is the generation of stable patterns during development (Turing 1952; Meinhardt and Gierer 2000). Nodal-lefty feedback regulation establishes a nodal activity gradient in zebrafish (Chen and Schier 2001, 2002), whereas BMP-Sizzled and noggin/chordin-ADMP feedback loops regulate dorsoventral patterning in *Xenopus* (Collavin and Kirschner 2003; Reversade and De Robertis 2005; De Robertis 2009; Inomata et al. 2013).

Sixth, agonists and antagonists of TGF-β family proteins can be regulated by other signaling pathways and often cross-modulate other signals as well. This is illustrated with the regulation of Wnt signal transduction by CTGF, sclerostin, and ectodin/Wise/USAG-1. Cerberus coregulates nodal, BMP, and Wnt signals by associating with these ligands. Tsukushi modulates the Delta-Notch pathway in addition to BMPs, and IGFBP3 controls IGF1 signaling. Cross-regulation among signals is a general rule for cells to integrate different stimuli, and agonists and antagonists of TGF-β ligands represent a node for integrating the ever-expanding extracellular signaling networks.

In summary, although our knowledge on individual agonists and antagonists of TGF-β family ligands has increased dramatically, further studies need to reveal dynamic interactions of these molecules with tissue-specific extracel-

lular components. It is reasonable to expect that we will continue to uncover new agonists and antagonists of TGF-β family ligands, novel activities of these regulators in development and diseases, and unsuspected links of these proteins with other signaling pathways. Additional directions will likely include detailed characterization of biochemical interactions of these factors with relevant ligands, other modulators, ECM components, and secreted protein modification enzymes. The research will not only investigate the strength, kinetics, and feedback regulation of physical associations among these molecules, but also analyze the roles of these interactions in protein localization, stability, processing, bioavailability, and local and long-range signaling. These studies will lead to more in-depth understanding of the mechanisms used by agonists and antagonists of TGF-β family ligands to control TGF-β signaling in various tissue contexts in normal and diseased conditions.

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