Developmental Cell
Review

TGF-β Superfamily Signaling in Embryonic Development and Homeostasis

Mary Y. Wu[1] and Caroline S. Hill[1,*]
[1]Laboratory of Developmental Signalling, Cancer Research UK London Research Institute, 44 Lincoln's Inn Fields, London WC2A 3PX, UK
*Correspondence: caroline.hill@cancer.org.uk
DOI 10.1016/j.devcel.2009.02.012

TGF-β superfamily signaling pathways emerged with the evolution of multicellular animals, suggesting that these pathways contribute to the increased diversity and complexity required for the development and homeostasis of these organisms. In this review we begin by exploring some key developmental and disease processes requiring TGF-β ligands to underscore the fundamental importance of these pathways before delving into the molecular mechanism of signal transduction, focusing on recent findings. Finally, we discuss how these ligands act as morphogens, how their activity and signaling range is regulated, and how they interact with other signaling pathways to achieve their specific and varied functional roles.

Introduction

The transforming growth factor β (TGF-β) superfamily of growth factors, which contains over 30 members including TGF-βs, bone morphogenetic proteins (BMPs), growth and differentiation factors (GDFs), Activins, and Nodal, is vital for the development and homeostasis of metazoans (Feng and Derynck, 2005). The ligands and their downstream pathway components are extremely well conserved during evolution, and they regulate diverse cellular functions such as growth, adhesion, migration, apoptosis, and differentiation. Moreover, their actions are modulated in time and space during embryonic development, leading to a diversity of cellular responses that is staggering. The best understood signal transduction pathway utilized by these growth factors is seemingly simple and linear. Ligand dimers bind and activate heteromeric complexes of type I and type II transmembrane receptors. Activated receptors then phosphorylate the intracellular mediators (Smads), which form complexes with each other and other proteins to modulate transcription of target genes in the nucleus. However, in order to perform their varied roles throughout animal life, there exists a level of complexity we are only just beginning to understand. In this review we begin by describing some key events in the development of both invertebrates and vertebrates that require TGF-β superfamily signaling, and some human diseases that result from deregulated signaling. We then look at the pathway in greater molecular detail, concentrating on recent published studies. Finally, we tie together the functions of these ligands with the mechanisms by which they signal to explain how the TGF-β superfamily signaling pathways achieve their complex in vivo roles.

Functions of TGF-β Superfamily Members in Embryonic Development and Disease

Early Development, Axis Formation, and Patterning

Members of the Nodal/Activin and BMP subfamilies are key players in the generation of axes and in the subsequent patterning of tissues across these axes during embryogenesis. They are morphogens that form concentration gradients and signal in a dose-dependent manner, thereby providing positional information to a field of cells and initiating diverse downstream molecular programs. Whereas the first asymmetry in the embryo is often determined by localized maternal factors, sperm entry, or both, zygotic activation of Nodal and BMPs initiates complex circulatory loops of signaling to define and pattern the first embryonic axis.

In *Drosophila* embryos, the BMP orthologs Decapentaplegic (Dpp) and Screw (Scw) are required for dorsal-ventral (D/V) axis specification and patterning. Prior to cellularization of the blastoderm, *dpp* is expressed in the dorsalmost 40% of the embryo, limited ventrally by a maternal NF-κB ortholog, Dorsal (Dl) (Morisato and Anderson, 1995). After cellularization, a gradient of Dpp signaling is established with highest activity at the dorsal midline, lower activity at the lateral regions, and no activity ventrally (O'Connor et al., 2006). Ubiquitously expressed Scw synergizes with Dpp to attain the highest levels of BMP signaling (O'Connor et al., 2006). Cells along this D/V axis respond to different levels of Dpp signaling and undergo different programs of differentiation. Highest levels of Dpp specify the amnioserosa, lower levels specify dorsal ectoderm, and lack of Dpp signaling allows the formation of neural ectoderm (O'Connor et al., 2006).

While vertebrate and arthropod embryos share a similar body plan, their D/V axes are inverted. In arthropods, neural tissue arises from ventral lateral ectoderm, while in vertebrates, it is derived from dorsal ectoderm. Despite this difference, molecular requirements for defining and patterning the D/V axis in vertebrates and *Drosophila* are highly conserved (Little and Mullins, 2006). In *Xenopus* and zebrafish, an early zygotic manifestation of the D/V axis is the dorsal organizer, which was originally identified in a salamander gastrula embryo and is sufficient to induce ectopic dorsal tissue (Spemann and Mangold, 1924). It is known as the Spemann organizer in *Xenopus* and the shield in zebrafish. In both systems formation of the dorsal organizer requires Nodal signaling (De Robertis and Kuroda, 2004; Feldman et al., 1998) (Figure 1A). Studies have revealed that many of the factors secreted from the organizer are BMP antagonists (De Robertis and Kuroda, 2004). From the onset of zygotic transcription to the end of gastrulation, *Bmp4* and *Bmp7* expression become gradually limited to the ventral side of embryos in *Xenopus*, with *Bmp2b* (*swirl*) and *Bmp7* (*snailhouse*) undergoing similar temporal and spatial regulation in zebrafish (Little and Mullins,

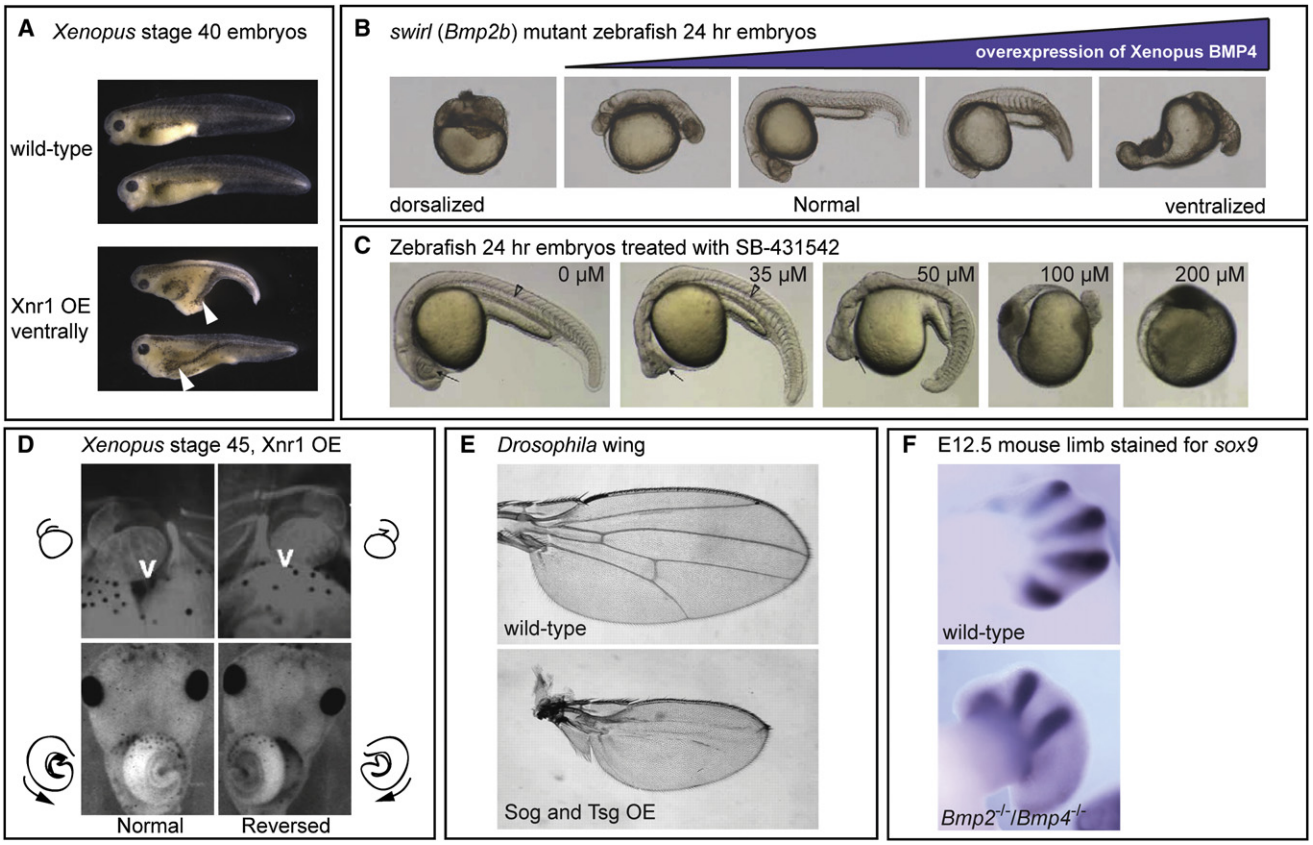


Figure 1. Disrupting TGF-β Family Signaling Produces Specific and Striking Effects during Early Development

(A) Overexpression (OE) of the Nodal ligand Xnr1 ventrally induces an ectopic organizer, resulting in a second axis, indicated by the white arrowheads.

(B) A ventral to dorsal gradient of BMP signaling patterns tissues in the early embryo. *Bmp2b* mutant fish are completely dorsalized, lacking ventrally derived structures. Increasing expression of *Xenopus* BMP4 rescues the phenotype, and excess BMP signaling gradually ventralizes embryos.

(C) Inhibiting Nodal signaling in zebrafish with the type I ALK4/5/7-specific inhibitor SB-431542 results in gradual loss of mesodermal and endodermal tissue and induces cyclopia and loss of anterior dorsal cell types. The arrow and the arrowhead indicate the eyes and notochord, respectively.

(D) Overexpression of Xnr1 in *Xenopus* embryos results in randomization of the L/R axis, which manifests as reversed heart and gut looping. v, ventricle.

(E) Overexpression of BMP antagonists Sog and Tsg in the *Drosophila* wing inhibits BMP signaling, resulting in lack of vein formation and a decrease in proliferation.

(F) Loss of BMP2 and BMP4 in the mouse affects posterior digit development during limb morphogenesis as seen by *Sox9* chondrogenic staining of digits.

The images were reproduced courtesy of Kishimoto et al. (1997) (B); Sun et al. (2006) (C); Sampath et al. (1997) (D); Shimmi and O'Connor (2003) (E); and Bandyopadhyay et al. (2006) (F).

2006). Higher BMP activity then induces other secreted factors on the ventral side (De Robertis and Kuroda, 2004). In addition, another BMP family member, *Admp*, is expressed in the Spemann organizer, being inhibited by high BMP levels on the ventral side (Reversade and De Robertis, 2005). The concerted actions of these molecules set up a ventral to dorsal gradient of BMP, capable of patterning tissues (Little and Mullins, 2006) (Figure 1B). In the vertebrate ectoderm, high BMP activity induces epidermis and low activity specifies neural tissue. Neural crest cells are specified at the border between these tissues in cells exhibiting intermediate BMP signaling (Sauka-Spengler and Bronner-Fraser, 2008).

In contrast, the first zygotically induced axis in mice is the anterior-posterior (A/P) axis, but again, Nodal is a key player. Nodal is initially expressed throughout the epiblast, the radially symmetrical cup-shaped embryo proper, and its involvement can be summarized in three steps (Schier, 2003; Yamamoto et al., 2004). First, Nodal is required for the induction of the distal visceral endoderm (DVE), part of the extraembryonic tissue. Second, in response to Nodal, some cells of the visceral endoderm (VE) secrete Nodal antagonists, and inhibition of Nodal in the vicinity of these cells prevents Nodal-induced proliferation in a portion of the VE, hence providing asymmetry and positional cues for determining anterior versus posterior. Third, Nodal induces cells of the DVE to migrate toward the anterior (becoming the anterior VE [AVE]), thus setting up the A/P axis.

Germ-Layer Specification, Patterning, and Gastrulation

In addition to its roles in axis specification, Nodal is also required in vertebrates for the induction of the three germ layers: endoderm, mesoderm, and indirectly, ectoderm. It first induces mesendoderm, and then different levels of Nodal signaling are required for further patterning and refinement of domains, with high levels inducing endoderm and lower levels inducing mesoderm (Zorn and Wells, 2007). In mice, Nodal antagonism by secreted factors from the AVE, along with complex positive feedback circuits from the extraembryonic ectoderm (ExE), induces

a gradual localization of Nodal expression to the proximal posterior region of the epiblast during A/P axis formation, thus setting up the Nodal gradient required for induction of endoderm and mesoderm (Zorn and Wells, 2007). In *Xenopus*, the maternal vegetally localized Nodal-related ligand Vg1 (Birsoy et al., 2006), in conjunction with zygotically expressed Nodal ligands, creates the required Nodal gradient in the vegetal pole, which is high in the dorsal vegetal region and fades ventrally and toward the animal pole (Schier, 2003). In zebrafish, a similar enrichment of Nodal exists dorsally, proposed to result from the accumulation of *Squint* (*sqt*) transcripts on the dorsal side of the embryo at the four-cell stage (Gore et al., 2005). In fish, a mutant lacking both the short and long-range ligands, Cyclops (Cyc) and Sqt respectively, develops with no endoderm and almost no mesoderm (Feldman et al., 1998), and a similar phenotype is seen in embryos treated with an inhibitor of Nodal receptors (Sun et al., 2006) (Figure 1C).

The third germ layer, ectoderm, is often considered a default tissue type, since tissues removed from the effects of Nodal signaling become ectoderm (Zhang et al., 1998). However, in the embryo, active inhibition of Nodal signaling is required (Schier, 2003). Moreover, normal gastrulation cannot occur without the appropriate specification of the three germ layers, and loss-of-function mutations in Nodal signaling lead to severe gastrulation and primitive streak defects (Zorn and Wells, 2007).

Left-Right Asymmetry

The left-right (L/R) axis is specified after the A/P and D/V axes and is important for the future position of the organs and the directional looping of tubules in the body. Here again, Nodal plays a crucial role (Figure 1D). Nodal signaling during L/R specification is dynamic, both spatially and temporally. In mice, *Nodal* is initially expressed symmetrically at E7.0 at the lateral edges of the node. By E7.5, its expression shifts, with higher levels found in the left perinodal region (Marques et al., 2004). Shortly after at E8.0, *Nodal* expression is found in the left lateral plate mesoderm (LPM). Genetic ablation of *Nodal* expression in the node has demonstrated that Nodal is required for its own asymmetrical expression later in the LPM, but how the signal is transduced to manifest the later event is unclear (Brennan et al., 2002). In *Xenopus* and zebrafish, where there are multiple Nodal ligands, *Xnr1* and *Southpaw*, respectively, are expressed asymmetrically in the LPM (Long et al., 2003; Sampath et al., 1997).

BMP signaling is also involved in L/R patterning. Evidence from mouse and chick reveal different roles. In mouse, BMP4 represses *Nodal* expression in the right LPM (Mine et al., 2008). In the chick, BMP2 induces *Nodal* expression on the left side (Schlange et al., 2002). Experiments in zebrafish suggest that active BMP signaling can do both, but these are separate events, regulated temporally. In this case, BMP4 is required at early stages to suppress *Nodal* in the right LPM, but later, it is required for left-side-specific gene expression (Chocron et al., 2007).

Organogenesis and Developmental Disease

So far, we have discussed a few key players, mainly Nodal and several BMPs, that are required for early embryogenesis. These ligands continue to be deployed for later developmental processes, along with other superfamily members that have extensive and specialized roles in tissue morphogenesis and homeostasis. A comprehensive and detailed listing of all TGF-β superfamily ligand functions is beyond the scope of this review, so we will just highlight a few instructive examples.

In *Drosophila*, Dpp signaling is vital for the morphogenesis and development of the imaginal discs, which give rise to the external appendages and organs of the adult fly (O'Connor et al., 2006). For example, in the early wing disc a Dpp gradient patterns the A/P axis, providing accurate positional cues for longitudinal vein (LV) formation (O'Connor et al., 2006) (Figure 1E). This initial expression of *dpp* at the compartment boundary is also required for proliferation of cells in the imaginal disc. Later during early pupal development, *dpp* expression is refined to areas where LVs have been specified. This maintains LV fate and contributes to the induction of cross veins, which additionally requires another *Drosophila* BMP ligand, Glass bottom boat (Gbb) (O'Connor et al., 2006).

In vertebrates, one or more TGF-β superfamily ligands play roles in the morphogenesis of most organs, and defects in signaling in this context can lead to serious human diseases. Anti-müllerian hormone (AMH) is a highly specialized member of the TGF-β family restricted in its expression both spatially and temporally. This is in contrast to other family members previously discussed, such as BMP4 and Nodal, which are expressed in many different tissues to elicit diverse responses. AMH is required for the regression of müllerian ducts in male fetuses and deficiencies in AMH or the AMH receptor (AMHR) result in Persistent Müllerian Duct Syndrome (PMDS), where fetuses that are genetically male develop with rudimentary female organs. Usually, functional testes develop but do not descend, and obstructions or other defects which occur during the formation of the secretory ducts can render individuals infertile (Josso et al., 2006). AMH is also required for follicular development in females. Its expression is used as a marker for follicular reserve in adult women, and excessive AMH can cause polycystic ovary syndrome (Wang et al., 2007).

TGF-β family members are well known for their ability to induce epithelial-mesenchymal transition (EMT) (Yang and Weinberg, 2008). This process allows polarized cells of an epithelial sheet to delaminate, assume a spindle-like mesenchymal shape, migrate from their site of origin, and invade surrounding tissue (Yang and Weinberg, 2008). EMT is essential for a variety of developmental processes, a prominent example being the invasion of the heart cushion by endocardial cells from the atrioventricular (AV) canals, which eventually gives rise to heart valves (Mercado-Pimentel and Runyan, 2007). In mice, TGF-β1, 2, and 3 are expressed in the developing heart in temporally distinct phases. While mice null for each of the three TGF-β ligands have been generated, only the *TGF-β2* null mouse exhibited EMT-specific phenotypes in the heart. However, in chicken AV explants, use of neutralizing antibodies has demonstrated that TGF-β2 is required for initiation of EMT, while TGF-β3 affects invasion/migration in a sequential manner, consistent with the order of expression of these ligands. Since the expression of the three TGF-βs is also temporally controlled in the mouse heart, they may all contribute to EMT, but at different steps of this process.

BMPs and GDFs make up the majority of the TGF-β superfamily members, and BMP2, 4, and 7, as well as GDF5 and 6, have all been implicated in limb development (Bandyopadhyay et al., 2006; Settle et al., 2003) (Figure 1F). The BMPs are redeployed

at various stages, including apical ectodermal ridge (AER) formation, AER regression, cartilage and bone differentiation, and interdigital webbing regression, and their temporal coexpression can play synergistic as well as antagonistic roles depending on the stage (Robert, 2007). In addition, TGF-β2 and 3 are also required for inducing programmed cell death during interdigital webbing regression (Dunker et al., 2002). Mutations in TGF-β superfamily signaling components, such as the ligands *GDF5* and *CDMP1*, the type I BMP receptor *BMPR1B*, and the antagonist *Noggin*, can lead to severe defects resulting in shortening of limbs and loss of joints and/or digits as seen in diseases such as brachydactyly (Lehmann et al., 2006, 2007; Seemann et al., 2005) and chondrodysplasia (Faiyaz-Ul-Haque et al., 2008; Thomas et al., 1997).

Homeostasis and Disease

TGF-β superfamily signaling continues to function in fully developed organisms where it is required for tissue homeostasis. A well-studied example is the maintenance of the vasculature, and relevant to this, mutations in a TGF-β receptor, Activin receptor-like kinase 1 (*ALK1*), and a coreceptor, *Endoglin*, have been linked to the disease Hereditary Hemorrhagic Telangiectasia (HHT) or Rendu-Oslr-Weber syndrome (ten Dijke and Arthur, 2007). Besides telangiectasis (formation of dilated blood vessels at the surface of the skin and mucous membranes), patients often have arteriovenous malformations, nosebleeds, and gastrointestinal bleeding. Mutations in *Endoglin* are associated with HHT1 while mutations in *ALK1* are linked to HHT2 with the clinical diagnosis differing at the level of penetrance and localization of malformations. In most cases, the mutations are thought to inactivate receptor function and disease occurs due to haploinsufficiency (ten Dijke and Arthur, 2007).

Interestingly, a subset of patients with HHT often also suffers from juvenile polyposis (JP). JP is diagnosed when five or more hamartomatous gastrointestinal polyps are found, usually in the colon at a young age. Mutations in *Smad4*, a signal transducer downstream of TGF-β superfamily signaling, and a BMP type I receptor, *BMPR1A* (*ALK3*), have been linked to the disease (Levy and Hill, 2006). Interestingly, combined JP-HHT is linked to mutations in *Smad4* (Gallione et al., 2004). Intuitively, this makes sense due to the role of Smad4 downstream of BMPR1A/ALK3, ALK1, and Endoglin (see below). A study recently investigated HHT patients that do not carry mutations in *Endoglin* or *ALK1* and have not been diagnosed with combined JP and found that 10% of these cases have mutations in *Smad4* (Gallione et al., 2006). Because JP is associated with a high risk of developing gastrointestinal cancers, HHT patients who have not been diagnosed with JP may indeed be afflicted by the combined syndrome and harbor this risk.

Another disease associated with the maintenance of the vasculature is primary pulmonary arterial hypertension (PAH). This results from remodeling of pulmonary arteries leading to a constriction of the vessels that increases blood pressure and decreases the efficiency of the heart in distributing oxygen-rich blood to tissues around the body (ten Dijke and Arthur, 2007). Prolonged stress to the heart due to PAH often leads to heart failure and premature death. Germline mutations in the BMP type II receptor, *BMPR2*, have been isolated in patients with PAH. The changes in BMP signaling associated with these mutations may contribute to an increased susceptibility of endothelial cells to apoptosis, leading to damage of vessels. The same changes in signaling can also increase myofibroblast and smooth muscle cell proliferation, leading to abnormal repair of vessels (Eickelberg and Morty, 2007; Morrell, 2006).

Myofibroblasts are key players in wound-healing and tissue repair. They respond to cues from TGF-β in order to carry out this function. Therefore, disturbances in TGF-β signaling can lead to fibrotic diseases, which are a result of excessive scarring due to increased extracellular matrix (ECM) deposition by overactive myofibroblasts. This abnormal scarring can eventually interfere with organ function and lead to organ failure. Excessive TGF-β signaling has been linked to fibrotic diseases and is thought to function by promoting the production of ECM components, in particular type I collagen (Gordon and Blobe, 2008), and in renal fibrosis, by inducing EMT in renal epithelial cells, converting them to myofibroblasts, leading to tubular atrophy (Zeisberg and Kalluri, 2004).

The importance of TGF-β's involvement in the maintenance of ECM is also evident in Marfan syndrome (MFS). This is a hereditary congenital disorder resulting from connective tissue defects that manifest in symptoms such as scoliosis (curved spine), arachnodactyly (long spidery fingers), dolichostenomelia (long slender limbs), pectus excavatum (sunken chest), or carinatum (protrusion of chest), and sometimes ectopia lentis (mispositioning of the crystalline lens of the eyes) (Robinson et al., 2006). A large percentage of patients diagnosed with MFS harbor mutations in the gene *Fibrillin-1*. Initially, this finding attributed the disorder to problems with the structural roles of Fibrillins in the ECM (Robinson et al., 2006). However, *Fibrillin-1* is a member of the Fibrillin/Latent TGF-β binding protein superfamily and members of this family play an important role in regulating TGF-β ligands. Indeed, MFS type II is caused by mutations in *TGFB2* and a related disease, Loeys-Dietz syndrome (LDS), which has overlapping symptoms with MFS, has been shown to be associated with mutations in either *TGFB1* or *TGFB2* (Gordon and Blobe, 2008).

Due to the pleiotropic roles of TGF-β superfamily members, it is not surprising that deregulation of TGF-β superfamily signals can lead to the progression of many cancers. It was the discovery in 1996 that TGF-β exhibited a biphasic action during skin carcinogenesis, inhibiting the formation of benign tumors, but enhancing the progression to invasive spindle tumors (Cui et al., 1996), that crystalized the view that TGF-β plays both tumor suppressive and tumor promoting roles in human cancer. Since that time these roles have been dissected in detail, but as this has been extensively reviewed recently (Massague, 2008), it will not be discussed here.

The Molecular Mechanism of TGF-β Superfamily Signaling

From the discussion above it is evident that TGF-β superfamily members play critical and diverse roles throughout embryonic development in vertebrates and invertebrates, and that deregulated signaling contributes to many human diseases. These ligands function by initiating new programs of gene expression in responding cells and in this section we explain how this is achieved. The best understood signaling pathway downstream of the receptors is relatively simple, but a considerable amount of combinatorial mixing and matching at the level of the ligands, receptors, and Smads produces a large diversity in transcriptional outputs.

The Ligands

The TGF-β superfamily ligands are secreted as precursors comprising a large prodomain and a C-terminal mature polypeptide. Dimerization, which is stabilized by intermolecular disulphide bonds, requires the prodomains and thus occurs intracellularly. The mature ligands are cleaved from the prodomain by furin-like enzymes (Feng and Derynck, 2005). For the most part, the ligands homodimerize, but heterodimerization also occurs between Nodal and BMP4 or BMP7 (Yeo and Whitman, 2001) or, in *Drosophila*, between Dpp and Scw (O'Connor et al., 2006). A major regulatory step in TGF-β superfamily signaling is the regulation of ligand accessibility by extracellular diffusible ligand-binding proteins. This is particularly important for the creation of gradients, such as the D/V BMP gradient as discussed in more detail below. Examples of such ligand-sequestering molecules are the BMP antagonists Chordin/Short gastrulation (Sog), Noggin, Twisted gastrulation (Tsg), Crossveinless-2 (Cv-2), Sclerostin, members of the DAN family, and Follistatin, an Activin inhibitor that also interacts with BMPs and GDF8 (De Robertis and Kuroda, 2004).

The Receptors

TGF-β superfamily members require two different serine/threonine kinase receptors to signal, a type I and a type II. There are seven type I receptors (ALKs 1–7) and five type II receptors in the human genome (for phylogenetic trees, see Schmierer and Hill, 2007). The ligand brings the receptors together in a heterotetrameric complex in which the type II receptors phosphorylate and activate the type I receptors (Figure 2A). For some ligand-receptor interactions, coreceptors, such as Betaglycan, Endoglin, and members of the EGF-CFC family, are also required (Feng and Derynck, 2005). In the case of the TGF-β receptors, the ligand-receptor interaction is highly cooperative. The ligand-receptor complexes assemble through the recruitment of the low-affinity type I receptor by the ligand-bound high-affinity type II receptor, facilitated by direct type I-type II interactions at the composite ligand-type II interface (Groppe et al., 2008). In contrast, the BMP receptor complexes do not assemble cooperatively and the type I and type II extracellular domains do not interact directly. Instead they are linked via the ligand, and membrane localization may also promote ligand-receptor assembly (Groppe et al., 2008).

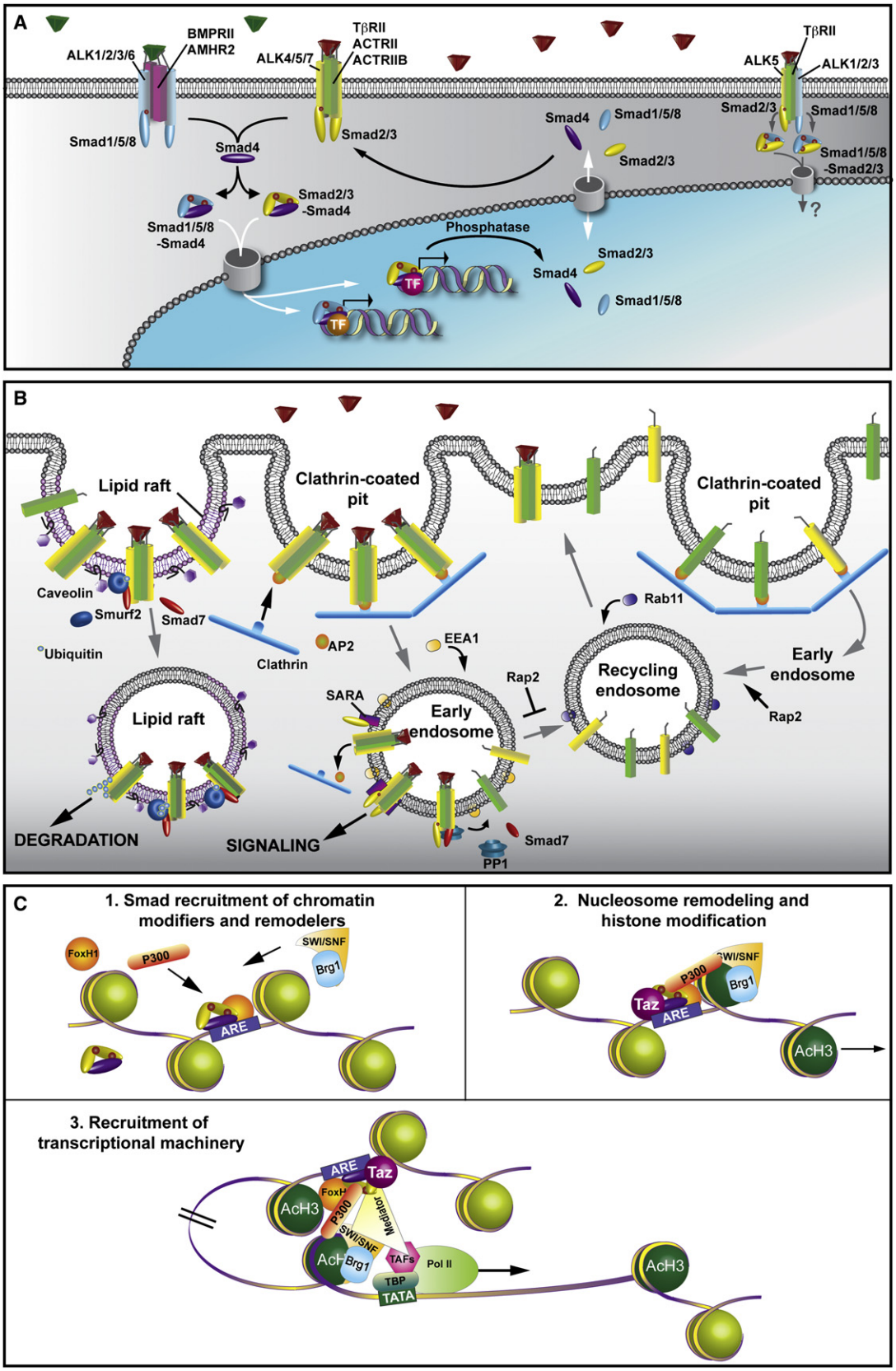
It is becoming apparent that many different combinations of ligand-receptor interactions and type I and type II receptor pairings can occur, creating a huge potential diversity in the outputs of the signaling pathways (Feng and Derynck, 2005). If the ligands have different affinities for distinct receptor combinations, then it is easy to see how ligand dose-dependent responses may be generated. For the most part, the type II and type I receptor dimers in a ligand-receptor complex are assumed to be homodimers. However, in endothelial cells TGF-β activates both ALK1 and ALK5, and a complex has been proposed comprising these two distinct type I receptors along with the TGF-β type II receptor TβRII (Goumans et al., 2003). Similarly, mixed receptor complexes containing TβRII, ALK5, and either ALK2 or ALK3 have been proposed to mediate a novel branch of TGF-β signaling in epithelial cells (Daly et al., 2008). In this case, higher concentrations of TGF-β are required to activate the putative heteromeric TβRII-ALK5-ALK2/3 receptor complex, compared with the canonical TβRII-ALK5 receptor complex. In *Drosophila*, a receptor complex comprising the two type I receptors Thickveins (Tkv) and Saxophone (Sax) and the type II receptor Punt appears to be costimulated by a ligand heterodimer comprising Dpp and Scw to elicit highest levels of signaling (O'Connor et al., 2006).

The TGF-β receptors are internalized constitutively in a clathrin-dependent manner into EEA1-positive early endosomes and in a non-clathrin-dependent manner via caveolin-positive lipid rafts (Di Guglielmo et al., 2003) (Figure 2B). Receptor signaling and degradation are thought to occur in different cellular compartments. Receptor degradation occurs via the lipid raft-caveolar internalization pathway. It is mediated via Smad7, which is a member of the Smad family (see below), which recruits the HECT domain E3 ubiquitin ligases Smurf1/2 to the activated type I receptors to degrade them (Feng and Derynck, 2005). Receptor dephosphorylation also occurs, mediated via the phosphatase PP1, which is targeted to the active type I receptors with its regulatory subunit GADD34, again by Smad7 (Shi et al., 2004). It is not clear in which cellular compartment this occurs, although it is tempting to speculate that it might be part of the receptor recycling pathway (Figure 2B). Active signaling is thought to occur in the endosomes (Di Guglielmo et al., 2003). Work on the Nodal/Activin pathway in *Xenopus* indicates that receptor trafficking is controlled by the Ras GTPase family member Rap2 (Choi et al., 2008). In the absence of signal, Rap2 directs the receptors into a recycling pathway via early sorting endosomes, which prevents their degradation and maintains their levels at the plasma membrane. Upon Nodal/Activin stimulation, Rap2 delays receptor turnover to promote receptor activity (Choi et al., 2008) (Figure 2B).

The Smads

The most studied signaling pathway downstream of TGF-β superfamily receptors is the Smad pathway. The Smads are a group of intracellular signaling molecules comprising the receptor-regulated Smads (R-Smads) Smad1, 2, 3, 5, and 8, the co-Smad Smad4, and the inhibitory Smads Smad6 and 7. Upon ligand stimulation, the R-Smads are phosphorylated by the type I receptors at two serines in an S-M/V-S motif at their extreme C termini (Massague et al., 2005). They then form both homomeric and heteromeric complexes with Smad4 that accumulate in the nucleus and directly regulate the transcription of target genes (Figure 2A). The original view of TGF-β superfamily signaling pathways were that there were two branches: a BMP/GDF branch signaling through ALKs 2, 3, and 6 and R-Smads 1, 5, and 8; and a TGF-β/Nodal/Activin branch signaling through ALKs 4, 5, and 7 and R-Smads 2 and 3 (Massague et al., 2005). However, this is an oversimplification, since some GDFs, for example GDF8, 9, and 11, signal through ALKs 4, 5, and 7 (Schmierer and Hill, 2007). Moreover, TGF-β activates both Smad2/3 and Smad1/5/8 in a variety of endothelial, epithelial, fibroblast, and tumor cells (Bharathy et al., 2008; Daly et al., 2008; Goumans et al., 2003; Liu et al., 2009). In epithelial cells this results in the formation of mixed R-Smad complexes, containing, for example, activated Smad1 and Smad2 in addition to the canonical Smad2/3-Smad4 complexes (Daly et al., 2008) (Figure 2A).

The mechanism whereby ligand stimulation leads to nuclear accumulation of active Smad complexes is now well understood. The R-Smads and Smad4 constantly shuttle between the cytoplasm and nucleus, both in unstimulated and ligand-stimulated cells, and nuclear accumulation of active Smad complexes is


A
BMPRII
AMHR2
ALK1/2/3/6
TβRII
ACTRII
ACTRIIB
ALK4/5/7
TβRII
ALK5
ALK1/2/3
Smad1/5/8
Smad4
Smad2/3
Smad4
Smad1/5/8
Smad2/3
Smad2/3
Smad1/5/8
Smad1/5/8
-Smad2/3
?
Smad2/3
-Smad4
Smad1/5/8
-Smad4
Phosphatase
Smad2/3
Smad4
Smad1/5/8
TF
TF
B
Lipid raft
Clathrin-coated
pit
Clathrin-coated
pit
Caveolin
Smurf2
Smad7
Ubiquitin
Clathrin
AP2
EEA1
Rab11
Early
endosome
Rap2
Recycling
endosome
Rap2
SARA
Lipid raft
Early
endosome
DEGRADATION
SIGNALING
Smad7
PP1
C
1. Smad recruitment of chromatin
modifiers and remodelers
FoxH1
P300
SWI/SNF
Brg1
ARE
2. Nucleosome remodeling and
histone modification
P300
SWI/SNF
Brg1
Taz
ARE
AcH3
3. Recruitment of
transcriptional machinery
ARE
Taz
FoxH1
AcH3
P300
Mediator
SWI/SNF
AcH3
Brg1
TAFs
Pol II
TBP
TATA
AcH3

achieved by a profound decrease in Smad nuclear export rate and an increase in import rate compared with monomeric Smads (Schmierer et al., 2008). The failure of Smad complexes to be exported may result from their active retention in the nucleus. Consistent with this the transcriptional regulator TAZ has been demonstrated to bind nuclear Smad2/3-Smad4 complexes and is recruited with them to TGF-β-responsive promoter elements (Varelas et al., 2008). If TAZ is knocked down, Smad2 and 3 do not accumulate in the nucleus upon TGF-β stimulation. A prerequisite for Smad nuclear export during signaling is Smad dephosphorylation, and several Smad phosphatases have been identified. Smad1 phosphatases include pyruvate dehydrogenase phosphatase (PDP) and small C-terminal phosphatases (SCP1, 2 and 3), while PPM1A can dephosphorylate both subsets of R-Smad (Chen et al., 2006; Knockaert et al., 2006; Lin et al., 2006). In none of these cases does knockdown of the phosphatase completely abolish Smad dephosphorylation when the receptors are inactivated, leaving open the possibility that other Smad C-terminal phosphatases remain to be discovered.

Activated Smad complexes bind to promoter sequences and regulate transcription both positively and negatively (Figure 2C). This has recently been extensively reviewed (Ross and Hill, 2008), so we will just summarize the salient points here. Different activated Smad complexes have distinct DNA sequence specificities, explaining the diverse transcriptional responses of different TGF-β superfamily members. The N-terminal MH1 domains of Smad3 and 4 recognize the sequence 5′-GTCT-3′ or its reverse complement, 5′-AGAC-3′, and complexes of Smad3 and Smad4 thus bind direct or inverted repeats of these Smad binding elements (SBEs) (Dennler et al., 1998). Smad1/5 bind the GC-rich consensus 5′-GRCGNC-3′ and when complexed with Smad4, bind a combinatorial site comprising the GC-rich element and an SBE spaced five base pairs apart (Pyrowolakis et al., 2004). These complexes are stabilized by the transcriptional regulator Schnurri, which contacts both Smads. These Smad1/5-Smad4-Schnurri complexes are inhibitory in *Drosophila*, but activatory in mammalian tissue culture cells (Yao et al., 2006). In fact in *Drosophila*, many Dpp-induced target genes are not directly regulated by an activatory Smad1-Smad4 complex as they are for vertebrate BMP target genes, but are indirectly induced through repression of a transcriptional repressor Brinker (Brk). *brk* is repressed by Dpp signaling via a Schnurri-Smad1-Smad4 complex, and loss of Brk then derepresses the Dpp target genes (Pyrowolakis et al., 2004). In the case of Smad2, although a spliced variant exists that binds DNA indistinguishably from Smad3, the major Smad2 isoform does not bind DNA directly and thus most Smad2-Smad4 complexes are recruited to DNA through interactions with other transcription factors (for reviews see Feng and Derynck, 2005; Ross and Hill, 2008). Well-characterized examples of Smad2-interacting transcription factors are the FoxH1 family of winged-helix transcription factors and members of the Mix family of homeobox proteins, Mixer, Milk, and Bix3, which recruit Smad2-Smad4 complexes to target gene promoters in response to Nodal signaling (Kunwar et al., 2003; ten Dijke and Hill, 2004). Importantly, these two families of transcription factors exhibit different expression patterns and have different DNA-binding specificities (Hill, 2001), and thus they dictate cell-type-specific responses, mediating transcription of different sets of genes in distinct cell types in response to Nodal signaling (Hoodless et al., 2001; Kunwar et al., 2003; ten Dijke and Hill, 2004).

All Smad-DNA binding interactions have been studied to date in the context of R-Smad-Smad4 complexes, but a considerable number of TGF-β-induced genes do not require Smad4 for their regulation (Levy and Hill, 2005). It is possible that complexes containing only R-Smads, which form in the absence of Smad4, may be recruited to promoters of such genes. Indeed, Smad2/3 has been shown to interact with the nuclear IκB kinase α (IKKα) in the absence of Smad4 to regulate transcription of the Myc antagonist *Mad1* (Descargues et al., 2008). Furthermore, the transcriptional regulator TRIM33/Tif1γ/Ectodermin has been suggested to act as an alternative Smad4 to promote TGF-β-induced erythroid differentiation (He et al., 2006), although this is controversial as TRIM33/Tif1γ/Ectodermin, which is a RING-finger-containing E3 ubiquitin ligase, was previously identified as a negative regulator of Smad4 (Dupont et al., 2005). The very recent discovery that activated R-Smad complexes act in the absence of Smad4 to regulate the processing of a microRNA, miR-21, raises the very interesting possibility that the Smads can additionally regulate gene expression without directly affecting transcription (Davis et al., 2008).

The Smads absolutely require chromatin to assemble the basal transcription machinery and activate transcription, and so predominantly act through chromatin remodeling. This is in contrast to most transcription factors, which can directly recruit the basal machinery to proximal promoters (Ross et al., 2006) (Figure 2C). Smad-induced chromatin remodeling requires the histone acetylase p300, which specifically acetylates histone H3 on lysines 9 and 18, and also the SWI/SNF component Brg1 (Ross et al., 2006; Xi et al., 2008). Undoubtedly other chromatin remodeling and histone modifying enzymes are also involved.

Non-Smad Signaling

Although the Smads are the best understood signal transducers downstream of TGF-β superfamily receptors, other signaling pathways can also be activated directly in response to TGF-β.

Figure 2. Summary of the Molecular Mechanism of TGF-β Superfamily Signaling

(A) Heterotetrameric receptor complexes are activated by ligand dimers (red and green triangles) and phosphorylate downstream effectors, the R-Smads (Smad1/5/8/2/3). Phosphorylated R-Smads then form complexes with each other (right-hand side of figure) and/or the co-Smad, Smad4 (left-hand side of figure), and accumulate in the nucleus to regulate target gene transcription. The function of the mixed R-Smad complexes in the nucleus is not yet clear (Daly et al., 2008). Transcription factors (TF) cooperate with Smad complexes on DNA. Nonphosphorylated monomeric Smads shuttle in and out of the nucleus. Multimeric complexes dissociate after R-Smad dephosphorylation in the nucleus and shuttle back to the cytoplasm. This is a receptor activity monitoring system.

(B) Receptors are internalized constitutively via clathrin-mediated endocytosis and caveolin-positive lipid rafts. Smad7/Smurf2-mediated degradation occurs in lipid rafts while receptors are recycled to the plasma membrane through endosomes. Active signaling occurs in EEA1-positive early ensodomes where SARA presents R-Smads to activated receptor complexes. PP1 dephosphorylates receptors in a Smad7-dependent manner. The Ras GTPase Rap2 promotes recycling in the absence of signal and delays recycling in presence of signal.

(C) Smad-dependent transcription requires chromatin remodeling. Smads recruit the histone deacetylase p300 and Brg1-SWI/SNF complex. Nucleosome remodeling and histone modification, for example histone H3 acetylation (AcH3), then allows the recruitment of the basal transcriptional machinery.

The type II receptor phosphorylates PAR6, a regulator of epithelial polarity, leading to the dissolution of tight junctions, an initiating step in EMT (Ozdamar et al., 2005). TGF-β can also activate the ERK MAP kinase (MAPK) signaling pathway through the ability of ALK5 to phosphorylate the scaffold protein ShcA, which recruits Grb and Sos, thereby activating Ras and downstream MAPKs (Lee et al., 2007). Moreover, two very recent papers have described the mechanism whereby TGF-β induces JNK and p38 MAPK signaling (Sorrentino et al., 2008; Yamashita et al., 2008). In this case, TRAF6 interacts with the TGF-β receptors and TGF-β induction leads to K63-linked ubiquitination of TRAF6. This in turn leads to activation of the MAPKKK Tak1, an upstream activator of JNK and p38. Finally, in mesenchymal cells, but not epithelial cells, TGF-β activates the kinase PAK2 and this occurs via the activation of the small GTPases Rac1 and Cdc42 (Wilkes et al., 2003). The basis of this cell type specificity is now explained in a paper in this issue (Wilkes et al., 2009). The authors show that in fact in both cell types TGF-β can activate PAK2, but in epithelial cells activated PAK2 is bound and inhibited by a complex of Erbin and the tumor suppressor Merlin. This does not occur in mesenchymal cells as they express very low levels of Erbin.

How Do TGF-β Superfamily Members Achieve Their In Vivo Roles?

To achieve the in vivo roles in embryonic development summarized in the first section, TGF-β superfamily ligands must be capable of functioning in a graded fashion as morphogens (for example BMPs during D/V patterning in *Drosophila* and vertebrates) and/or be able to signal in a highly dynamic manner both spatially and temporally (for example Nodal in L/R patterning and BMPs/GDFs during vertebrate limb development). Having outlined the intracellular signaling pathways in the second section, we now highlight the known mechanisms that regulate the formation of ligand gradients and dynamic signals, and those that regulate the downstream pathway to interpret the signals and determine the specificity of the responses. We discuss how BMPs are able to form gradients of ligand activity, which, over time, frequently sharpen to stepwise signals that ultimately define different tissue types (Sutherland et al., 2003) (Figure 3). We examine how graded signals are sensed by receiving cells, leading to differential transcriptional responses according to ligand dose. In both of these areas, mathematical modeling has proved to be a very powerful tool and this will be highlighted. We also discuss how the range of signaling is determined, newly discovered mechanisms whereby signal strength, duration, or both are modulated, and how other signal transduction pathways modulate TGF-β superfamily signaling. All of these regulatory mechanisms provide qualitative and quantitative differences in signaling that are capable of increasing the diversity of functional outputs.

Generating Gradients of TGF-β Superfamily Ligand Activity

Our current understanding of gradient formation derives mostly from studies in *Drosophila*, and these studies will thus be a focal point for our discussion. The traditional view of morphogen gradients is that the ligand is secreted from a localized source, diffuses across a field of cells, and forms a concentration gradient in the target tissue (reviewed in Kicheva and Gonzalez-Gaitan, 2008). The Dpp gradient that specifies A/P patterning of the *Drosophila* wing obeys this paradigm. During larval development, Dpp is secreted in the wing imaginal disc from a central stripe of cells and forms a concentration gradient both anteriorly and posteriorly. This gradient has an approximately exponential shape, governed by the rates of ligand diffusion, production, and degradation, and the presence of an immobile fraction (Kicheva and Gonzalez-Gaitan, 2008). The exact mechanism whereby the ligand spreads is still a matter of debate. One proposed mechanism involves transcytosis (Kicheva et al., 2007), where Dpp is secreted from the producing cells and then endocytosed and secreted by neighboring cells. An alternative mechanism is that Dpp simply diffuses through the target tissue, with its diffusion rate being restricted by heparan sulfate proteoglycans (Kicheva and Gonzalez-Gaitan, 2008). In reality both mechanisms may be relevant (Affolter and Basler, 2007). Interestingly, the staining pattern of phosphorylated Mad (P-Mad), the downstream signal transducer, in the wing disc does not correlate with Dpp ligand concentration, suggesting that additional BMP ligands are involved. Indeed Gbb, which is broadly expressed in the wing, also signals in the wing disc, acting over long distances, while Dpp acts over a shorter range (Bangi and Wharton, 2006).

In contrast to the wing disc, a gradient of Dpp activity forms in the early *Drosophila* embryo within a domain of uniform Dpp mRNA expression in the dorsalmost 40% of the embryo. This means that gradient formation cannot simply involve diffusion

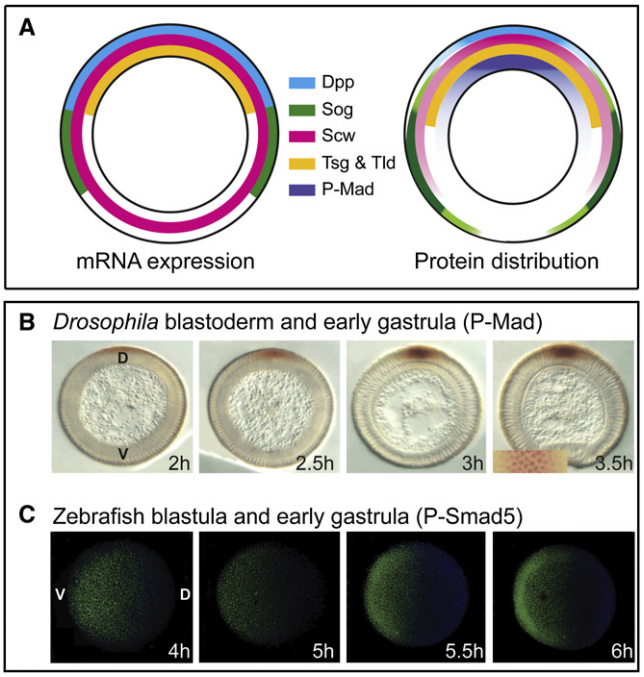


Figure 3. BMP Signaling Gradients in Early Embryos

(A) mRNA and protein distributions in the *Drosophila* blastoderm and early gastrula.

(B and C) BMP signal activity as detected by phospho-R-Smads in *Drosophila* (P-Mad) (B) and zebrafish (P-Smad5) (C). In both cases, BMP signaling activity is weak and diffuse over a broad area to begin with, but becomes increasingly localized, at the dorsal midline for *Drosophila*, and on the ventral side for zebrafish, with a gradient of activity diminishing toward the lateral regions.

These images are taken from Rushlow et al. (2001) (B) and Tucker et al. (2008) (C) with permission.

of ligand away from a localized source, but instead involves redistribution of ligand within the expression domain (Figure 3A). An important feature of the underlying mechanism is that Dpp acts together with another ligand, Scw, expressed uniformly in the blastoderm. Homomeric and heteromeric dimers of Dpp and Scw form and bind with different affinities to the ligand antagonists Sog and Tsg (O'Connor et al., 2006). *sog* is expressed in ventral lateral regions, while *tsg* is expressed in the dorsal half of the embryo (O'Connor et al., 2006) (Figure 3A). Dpp-Scw heterodimers bind with high affinity to Sog and Tsg, forming a complex incapable of binding receptors, and are transported away from the source of Sog to the dorsal midline. There, they are released from Sog and Tsg as a result of Sog cleavage by a dorsally expressed metalloprotease, Tolloid (Tld), allowing the ligand dimer to activate receptor complexes containing the type I receptors Tkv and Sax (O'Connor et al., 2006). Tld-induced cleavage of Sog in more lateral regions results in rebinding of Dpp-Scw to other molecules of Sog and Tsg, due in part to higher levels of Sog in these regions. Homodimers of Dpp and Scw have lower affinity for Sog and Tsg and thus are not transported as far and are thought to elicit the weaker BMP signal in dorsal lateral regions of the embryo. Mathematical modeling suggests that this mechanism can account for a BMP ligand concentration gradient. However, it predicts that over time the domain of P-Mad would broaden and P-Mad levels would increase in intensity, whereas in reality, the dorsalmost region of high P-Mad activity actually contracts, giving a step gradient (O'Connor et al., 2006) (Figure 3B). The most likely explanation is that there is positive feedback in the system such that the initial Dpp-Scw signal induces an as yet unidentified component that either reduces the interaction of receptors with an inhibitory factor or promotes ligand binding to receptors (Wang and Ferguson, 2005). Very recently, type IV collagens have been shown to play a role in formation of the Dpp D/V gradient, promoting assembly of the Dpp/Scw-Sog-Tsg complex, and in the absence of Sog, facilitating Dpp/Scw-receptor interactions, thus amplifying the signal at the dorsal midline (Wang et al., 2008).

This mechanism for gradient formation might not be unique to the early *Drosophila* embryo, but may also function in early *Xenopus* embryos, where the BMP ligands are also expressed in a very broad domain, but nevertheless develop into a ventral-dorsal activity gradient (Little and Mullins, 2006). *Xenopus* orthologs of *sog* (known as *Chordin* in vertebrates), *tsg*, and *tolloid* have all been identified and biochemically the proteins have been shown to act in the same way as in *Drosophila* (Oelgeschlager et al., 2000; Piccolo et al., 1996, 1997; Sasai et al., 1995). Mathematical modeling has been used to compare two possible mechanisms of BMP gradient formation in *Xenopus* embryos (Ben-Zvi et al., 2008). One model assumes an inhibition-based mechanism whereby a gradient of BMP antagonists is created over a uniform field of BMP ligands, and the other assumes a shuttling-based mechanism where the ligands are transported by inhibitors as described above for Dpp-Scw. The authors found that while both mechanisms can generate a BMP activity gradient, the shuttling-based mechanism gives a sharper and more robust gradient. These models were tested experimentally and the evidence suggested that the shuttling-based mechanism could be involved in BMP gradient formation in *Xenopus* embryos.

Very recently another molecule involved in D/V axis formation in *Xenopus* embryos has been identified. ONT1, a member of the Olfactomedin family of secreted proteins, has been shown to maintain appropriate Chordin levels in dorsal regions by acting as a scaffold between Chordin and BMP1/Tld-class proteases, promoting Chordin cleavage and degradation (Inomata et al., 2008). This is required to prevent uncontrolled increases in Chordin levels that could shift the BMP gradient and lead to expansion of the dorsal domain.

How Gradients Are Sensed and Interpreted

Ligand gradients must be quantitatively sensed and interpreted, and several features of the TGF-β superfamily signaling pathway have been uncovered that help explain how this is accomplished. The Smad pathway is ideal for interpreting graded signals, as it has no amplification steps (Schmierer and Hill, 2007). Moreover, continuous shuttling of the Smads between the cytoplasm and nucleus during signaling, mediated by cycles of receptor-induced Smad phosphorylation in the cytoplasm and constitutive Smad dephosphorylation in the nucleus, allows the Smads to constantly monitor receptor activity (Schmierer and Hill, 2007). This concept has been explored by mathematical modeling, which has confirmed that the levels of active Smad2-Smad4 complexes in the nucleus directly reflect the activation level of the receptors at all times during signaling (Schmierer et al., 2008) (Figure 4). In this way both the strength and duration of signaling is constantly monitored. The computational model was used to identify individual steps in the signaling cascade that, when modulated, can affect system output most significantly. The most important step for positively influencing the peak concentration of nuclear active Smad2-Smad4 complexes is Smad2 phosphorylation, while Smad2 dephosphorylation, dissociation of active Smad complexes, and Smad4 nuclear export are the steps that have the most negative influence (Schmierer et al., 2008). Consistent with this, studies in *Drosophila* have shown that the nuclear export rate of the *Drosophila* Smad4 Medea is promoted by SUMOylation and, as a consequence, failure to SUMOylate Medea results in increased Dpp signaling range (Miles et al., 2008).

Just as the levels of receptor activity are continuously monitored, so the ligand levels must be sensed accurately and dynamically by the receptors. This is currently not well understood. It is tempting to speculate that constitutive receptor recycling through the endocytic pathway (Di Guglielmo et al., 2003; Figure 2B) may act to monitor ligand levels in an analogous fashion to the Smads' monitoring of receptor activity. A similar mechanism is known to be important for controlling the duration of EGF signaling (Sigismund et al., 2008).

Smad nucleocytoplasmic shuttling results in levels of activated nuclear Smad complexes that reflect the extent of receptor activation, but how is signal intensity "translated" into differential gene expression programs? An important determinant is the affinity of Smad-binding sites for activated Smad complexes, and this has been directly demonstrated in the early *Drosophila* embryo for the Dpp target gene *race*. Increasing the affinity of the Smad-binding sites in its enhancer placed upstream of a reporter gene broadened the reporter expression pattern (Wharton et al., 2004). However, as discussed above, many Dpp target genes in the *Drosophila* embryo are not activated by Dpp per se, but are derepressed as a result of Dpp-induced repression of the transcriptional repressor Brk. Multiple

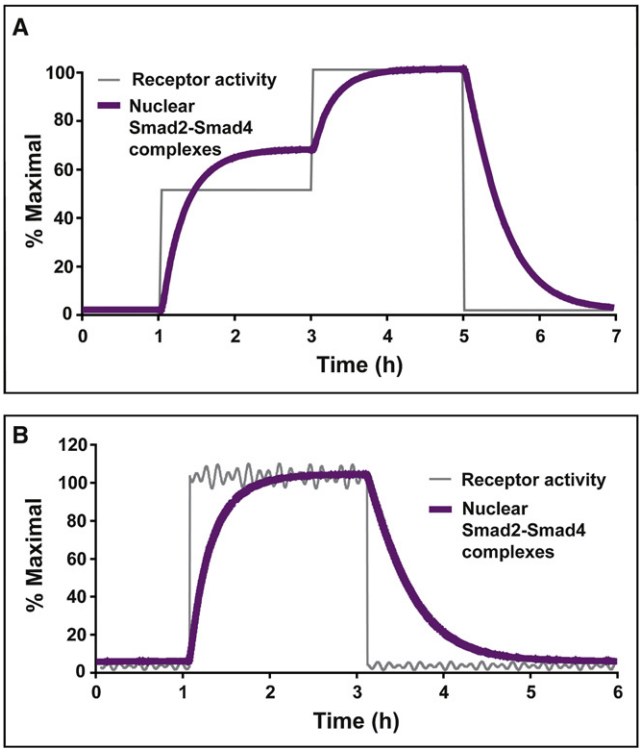


Figure 4. Nucleocytoplasmic Shuttling of the Smads Acts as a Signal Interpretation System

(A) Using the mathematical model of nucleocytoplasmic shuttling, receptor levels were altered in a step-wise fashion as shown. The simulation demonstrates that Smad nucleocytoplasmic shuttling couples the amount of nuclear Smad2-Smad4 complexes to receptor activity, although with a time delay.

(B) Because of this delay, fluctuations in receptor activity are strongly dampened and do not cause corresponding fluctuations in the concentration of nuclear Smad2-Smad4 complexes.

This image was taken from Schmierer et al. (2008) with permission.

regulatory modules in the enhancer of *brk* bind repressive Schnurri-Mad-Medea complexes, resulting in an inverse expression gradient relative to the Dpp activity gradient (Yao et al., 2008). Some Dpp concentration-dependent transcription is then explained by the fact that Dpp-responsive genes have Brk binding sites with different affinity in their enhancers. In addition, although loss of Brk-induced repression is sufficient for activation of some genes, others additionally require binding of activatory Mad-Medea complexes (Affolter and Basler, 2007). For Smad2-dependent responses in vertebrates, the affinity of activated Smad2 for the DNA-bound Smad2-interacting transcription factors may play a role in interpreting the level of signal intensity (Randall et al., 2004).

We have discussed mechanisms that allow cells to interpret a signaling gradient. However, cells also need to be competent to respond to the signals. In early zebrafish embryos, this seems to be regulated in a temporal fashion (Tucker et al., 2008). The developing embryo may be thought of as a four-dimensional system, since besides acting in space, gradients may achieve functional diversity by acting over time as well. Using heat shock induction of Chordin to inhibit BMP signaling at different times during zebrafish development, Tucker et al. (2008) tested how time is involved in the response of tissues to the BMP gradient. Interestingly, cells along the A/P axis become competent to respond to BMP signals in a temporal fashion such that as gastrulation proceeds, progressively more caudal cells respond to the BMP gradient and adopt their D/V identity. This temporal regulation thus coordinates D/V with A/P patterning.

Determinants of Signaling Range

Several studies have revealed that distinct TGF-β family members have very different signaling ranges in vivo (Chen and Schier, 2001; Jones et al., 1996), and this seems to be determined by the ligand prodomain, which is the least conserved part of the protein. Prodomain swapping and mutation experiments have demonstrated that these domains can affect the stability of these proteins (Cui et al., 2001; Le Good et al., 2005). In most cases, stability is affected during secretion of the ligands, and limiting the concentration of the secreted product limits their range. Indeed, the prodomain of the short-range zebrafish Nodal ligand Cyc contains a lysosome-targeting region that destabilizes the precursor and thus restricts Cyc activity (Tian et al., 2008). Additionally, BMP4 precursors are cleaved sequentially at two sites in the prodomain. The first cleavage site activates the ligand, while the second cleavage site affects stability and range (Cui et al., 2001; Sopory et al., 2006). Posttranslational modifications of domains, such as glycosylation, in addition to cleavage, also contribute to ligand regulation (Le Good et al., 2005).

Signal range can also be influenced by the interaction of ligands with secreted binding partners, and a good example of this is Cv-2. Unlike Chordin, Cv-2 is not diffusible, but is tethered to the membrane by heparan sulfate proteoglycans and thus modulates BMP signals over a short range (Serpe et al., 2008). Studies focusing on the role of Cv-2 in the *Drosophila* wing indicate that Cv-2 activates BMP signaling at low concentrations, but antagonizes it at higher concentrations (Serpe et al., 2008). Moreover, Cv-2 binds BMP type I receptors as well as ligands. As with other aspects of TGF-β superfamily signaling, mathematical modeling has been used to gain insights into the mechanism (Serpe et al., 2008). The model suggests that a transient low-affinity tripartite complex forms and facilitates ligand-receptor binding, but at high Cv-2 concentrations, this is compromised and it instead sequesters ligands to inhibit signaling. A crystal structure of the Cv-2 N-terminal Von Willebrand factor type C domain 1 (VWC1) bound to BMP2 reveals that Cv-2 sequesters ligand by blocking receptor binding sites (Zhang et al., 2008). Since *Cv-2* is itself induced by BMP activity, Cv-2 expression not only serves as a direct measure of BMP activity, but functions as part of a very effective feedback loop capable of maintaining signaling activity at an appropriate level and preventing runaway signaling.

As in *Drosophila*, *Cv-2* is expressed in regions of high BMP signaling in both *Xenopus* and zebrafish embryos (Ambrosio et al., 2008; Rentzsch et al., 2006). The zebrafish Cv-2 also has pro- and anti-BMP effects, and loss-of-function experiments reveal that Cv-2 has mainly a signal-promoting function, since Cv-2 morphants are moderately dorsalized (Rentzsch et al., 2006). However, in *Xenopus*, Cv-2 has a predominantly inhibitory role in BMP signaling, and may be part of a complex molecular circuit through its interactions with other secreted modulators of BMP signaling (Ambrosio et al., 2008). Cv-2 is capable of synergizing with the BMP-induced modulator Tsg to act as a local feedback inhibitor on the ventral side of the embryo. It also

interacts with Chordin, and may exert a pro-BMP signaling influence by concentrating diffusable Chordin-BMP-Tsg in ventral regions of the embryo (Ambrosio et al., 2008).

Modulation of TGF-β Superfamily Signaling Activity and Dynamics

Spatial and temporal control of Nodal signaling was recently shown to be regulated by microRNAs in zebrafish and *Xenopus* (Choi et al., 2007; Martello et al., 2007). In zebrafish, miR-430 has been shown to target both a Nodal ligand and Nodal antagonists, *sqt* and *Lefty1/2*, respectively, with its effects strongest on *Lefty2* (Choi et al., 2007). miR-430 functions by balancing the expression of these positive and negative players to achieve optimal signal levels required for organizer formation and germ layer specification (Choi et al., 2007). Nodal induces itself as well as *Lefty*, and this auto-activatory-inhibitory loop limits signal range, duration, and level, thus creating borders, patterns, or asymmetries (Solnica-Krezel, 2003). The Nodal-Lefty mechanism fits what is known as the Turing reaction-diffusion model (reviewed by Solnica-Krezel, 2003). This model explains how noise in an otherwise uniform diffusion system can become amplified by the coexpression of a ligand and its antagonist. For this system to work, the antagonist must be able to diffuse faster than the ligand, which thus creates short-range activation and long-range inhibition. Changes in expression levels of either component, through, for example, the action of miR-430, would influence signal range, duration, and level. It will be interesting to see if other microRNAs are involved in Nodal-Lefty regulation at later stages, such as during L/R specification.

In *Xenopus*, the Nodal signaling pathway has also been shown to be modulated by the action of a microRNA, miR-15/16, the target of which is the type II Nodal receptor *ACVR2A* (Martello et al., 2007). While the expression of the miR-15/16 primary transcript is ubiquitous, processing of this transcript into mature functional miR-15 is inhibited by the Wnt/β-catenin pathway. Since β-catenin accumulates on the dorsal side of the embryo after fertilization, mature miR-15 is found in a gradient, with the highest levels ventrally. This feature of miR-15 creates a reverse expression gradient of ACVR2A, and hence restricts responsiveness to Nodal ligands in a spatial manner and controls the size of the organizer.

In both studies, whether balancing the levels of secreted agonists and antagonists or spatially restricting responsive components in the pathway, microRNAs seem to provide robustness to the signaling and can prevent transcriptional misregulation of signaling components from causing catastrophic changes during early development.

Modulation of TGF-β Superfamily Signaling by Other Signal Transduction Pathways

Finally, it is important to consider that TGF-β superfamily signaling in any given cell does not occur in isolation, but is subject to modulation by other signaling pathways. The duration of TGF-β superfamily signaling can be regulated in this way, and in addition, transcriptional responses to TGF-β superfamily signals can be influenced due to the integration of multiple signals at the promoters of target genes.

It is well established that receptor tyrosine kinase (RTK) signaling can have an antagonistic effect on BMP signaling in many developmental contexts, such as during neural induction, limb development, lung morphogenesis, cranial suture fusion, and tooth development (De Robertis and Kuroda, 2004). It now turns out that the activity of the Smads themselves is directly influenced by RTK signaling. R-Smads and co-Smads have a proline-rich linker that connects the MH1 and MH2 domains. MAPKs activated downstream of RTKs phosphorylate several serines and threonines in the Smad1 linker, which promotes binding of the E3 ubiquitin ligase Smurf1. This in turn enhances degradation of Smad1, which reduces BMP signal transduction, and curtails responses (Sapkota et al., 2007). The corollary of this is that any signal that inhibits Smad1 linker phosphorylation would prolong the duration of BMP signaling. Interestingly, the MAPK sites in the Smad1 linker act as priming sites for glycogen synthase kinase 3 (GSK3), which is inhibited by Wnt signaling (Fuentealba et al., 2007). GSK3 phosphorylation of linker serines and threonines close to the MAPK sites also destabilizes Smad1, and thus it has been suggested that Wnt signaling synergizes with BMP signaling by stabilizing the Smads and increasing the duration of BMP signaling (Fuentealba et al., 2007).

It is not only the Smads that can integrate signals from other pathways; Smad-interacting transcription factors can also perform this function. RTK signaling via Ras has long been established as a requirement for Nodal/Activin-induced mesoderm formation in *Xenopus* embryos, although it was unclear exactly how it modulated the Nodal signaling pathway. Recent work has demonstrated that the target of RTK signaling in this case is the transcription factor p53 (Cordenonsi et al., 2007). p53 is required for induction of a subset of Nodal target genes and functions by interacting directly with Smad2 at the promoters of these genes. RTK signaling via Ras leads to phosphorylation of p53 via the kinase CK1ε/δ. This increases the association of p53 with activated Smad2, and thus promotes the transcription of mesodermal target genes.

A further informative example of signal integration at the level of enhancer or promoter elements is the integration of Wnt and Dpp signals at the Leg Trigger (LT) enhancer of the *Distalless* (*Dll*) gene in *Drosophila,* which is involved in leg development (Estella et al., 2008). The LT enhancer contains binding sites for TCF (which binds an activatory complex of β-catenin, Legless, and Pygopus in response to Wnt signaling), Mad, and Brk, and active transcription occurs only when both Wnt and Dpp signaling is high. This can be explained as follows. When both signaling pathways are operating, the TCF and Mad sites bind their respective activatory complexes and the Brk site is unoccupied; thus, transcription is active. In cells exhibiting low Dpp signaling, but high Wnt signaling, the binding of Brk to the enhancer inhibits transcription. Conversely, in cells with high Dpp signaling, but no Wnt signaling, the binding of activated Mad complexes is not sufficient for transcriptional activation. In this way, the spatial domain of *Dll* transcription is defined by cells exhibiting simultaneously high Wnt and Dpp signaling.

Perspectives

Our view of TGF-β superfamily signaling pathways has evolved significantly since a decade ago, when the pathway was perceived to be a three-step system of receptor activation by ligands followed by effector molecule activation through phosphorylation, resulting in transcriptional activation or repression. The tremendous effort by scientists around the world using a combination of developmental, cellular, and molecular systems

has contributed to our current understanding of the modulatory mechanisms that provide both robustness and diversity to this signaling system.

It is evident from our discussion that the model organisms and systems used have different strengths, and thus have been exploited to address different questions. While playing to the strengths of specific models is the most efficient way to gain understanding, integration and consolidation of data between systems is now essential. For example, transport of BMPs within their expression domain is established as a mechanism for gradient formation in the *Drosophila* blastoderm, and recent evidence suggests that the same may occur in *Xenopus* embryos. However, while Activin dose-dependent responses are well documented (Green and Smith, 1990), whether Nodal and Activin utilize similar mechanisms for gradient formation requires further investigation. Studies in *Drosophila* have also revealed an elegant mechanism whereby dose-dependent transcription is controlled by multiple repressive and/or activatory elements in the promoters of various Dpp target genes. A parallel mechanism for regulating vertebrate BMP- and Nodal-induced target gene transcription has not yet been demonstrated, because the relevant promoter elements have not been studied to such depth. Finally, it is becoming apparent that in addition to their complex roles in early development, TGF-β superfamily signaling pathways are involved in many human diseases as a result of mutations in components of the pathways or deregulation of signaling. We anticipate that the more we learn about the functions of these pathways and their mechanisms of action in embryonic development, the greater will be our ability to understand and treat these diseases.

ACKNOWLEDGMENTS

We thank all the members of the Hill lab, Megan Cully, Mike Howell, Erik Sahai, Jim Smith, and Nic Tapon for very useful comments on the manuscript. The work in the Hill lab is funded by Cancer Research UK and the EU (MRTN-CT-2004-005428). M.Y.W. acknowledges an ORS award.

REFERENCES

Affolter, M., and Basler, K. (2007). The Decapentaplegic morphogen gradient: from pattern formation to growth regulation. Nat. Rev. Genet. *8*, 663–674.

Ambrosio, A.L., Taelman, V.F., Lee, H.X., Metzinger, C.A., Coffinier, C., and De Robertis, E.M. (2008). Crossveinless-2 Is a BMP feedback inhibitor that binds Chordin/BMP to regulate Xenopus embryonic patterning. Dev. Cell *15*, 248–260.

Bandyopadhyay, A., Tsuji, K., Cox, K., Harfe, B.D., Rosen, V., and Tabin, C.J. (2006). Genetic analysis of the roles of BMP2, BMP4, and BMP7 in limb patterning and skeletogenesis. PLoS Genet. *2*, e216.

Bangi, E., and Wharton, K. (2006). Dpp and Gbb exhibit different effective ranges in the establishment of the BMP activity gradient critical for Drosophila wing patterning. Dev. Biol. *295*, 178–193.

Ben-Zvi, D., Shilo, B.Z., Fainsod, A., and Barkai, N. (2008). Scaling of the BMP activation gradient in Xenopus embryos. Nature *453*, 1205–1211.

Bharathy, S., Xie, W., Yingling, J.M., and Reiss, M. (2008). Cancer-associated transforming growth factor β type II receptor gene mutant causes activation of bone morphogenic protein-Smads and invasive phenotype. Cancer Res. *68*, 1656–1666.

Birsoy, B., Kofron, M., Schaible, K., Wylie, C., and Heasman, J. (2006). Vg 1 is an essential signaling molecule in Xenopus development. Development *133*, 15–20.

Brennan, J., Norris, D.P., and Robertson, E.J. (2002). Nodal activity in the node governs left-right asymmetry. Genes Dev. *16*, 2339–2344.

Chen, Y., and Schier, A.F. (2001). The zebrafish Nodal signal Squint functions as a morphogen. Nature *411*, 607–610.

Chen, H.B., Shen, J., Ip, Y.T., and Xu, L. (2006). Identification of phosphatases for Smad in the BMP/DPP pathway. Genes Dev. *20*, 648–653.

Chocron, S., Verhoeven, M.C., Rentzsch, F., Hammerschmidt, M., and Bakkers, J. (2007). Zebrafish Bmp4 regulates left-right asymmetry at two distinct developmental time points. Dev. Biol. *305*, 577–588.

Choi, W.Y., Giraldez, A.J., and Schier, A.F. (2007). Target protectors reveal dampening and balancing of Nodal agonist and antagonist by miR-430. Science *318*, 271–274.

Choi, S.C., Kim, G.H., Lee, S.J., Park, E., Yeo, C.Y., and Han, J.K. (2008). Regulation of activin/nodal signaling by Rap2-directed receptor trafficking. Dev. Cell *15*, 49–61.

Cordenonsi, M., Montagner, M., Adorno, M., Zacchigna, L., Martello, G., Mamidi, A., Soligo, S., Dupont, S., and Piccolo, S. (2007). Integration of TGF-β and Ras/MAPK signaling through p53 phosphorylation. Science *315*, 840–843.

Cui, W., Fowlis, D.J., Bryson, S., Duffie, E., Ireland, H., Balmain, A., and Akhurst, R.J. (1996). TGFβ1 inhibits the formation of benign skin tumors, but enhances progression to invasive spindle carcinomas in transgenic mice. Cell *86*, 531–542.

Cui, Y., Hackenmiller, R., Berg, L., Jean, F., Nakayama, T., Thomas, G., and Christian, J.L. (2001). The activity and signaling range of mature BMP-4 is regulated by sequential cleavage at two sites within the prodomain of the precursor. Genes Dev. *15*, 2797–2802.

Daly, A.C., Randall, R.A., and Hill, C.S. (2008). Transforming growth factor β-induced Smad1/5 phosphorylation in epithelial cells is mediated by novel receptor complexes and is essential for anchorage-independent growth. Mol. Cell. Biol. *28*, 6889–6902.

Davis, B.N., Hilyard, A.C., Lagna, G., and Hata, A. (2008). SMAD proteins control DROSHA-mediated microRNA maturation. Nature *454*, 56–61.

De Robertis, E.M., and Kuroda, H. (2004). Dorsal-ventral patterning and neural induction in Xenopus embryos. Annu. Rev. Cell Dev. Biol. *20*, 285–308.

Dennler, S., Itoh, S., Vivien, D., ten Dijke, P., Huet, S., and Gauthier, J.M. (1998). Direct binding of Smad3 and Smad4 to critical TGF β-inducible elements in the promoter of human plasminogen activator inhibitor-type 1 gene. EMBO J. *17*, 3091–3100.

Descargues, P., Sil, A.K., Sano, Y., Korchynskyi, O., Han, G., Owens, P., Wang, X.J., and Karin, M. (2008). IKKα is a critical coregulator of a Smad4-independent TGFβ-Smad2/3 signaling pathway that controls keratinocyte differentiation. Proc. Natl. Acad. Sci. USA *105*, 2487–2492.

Di Guglielmo, G.M., Le Roy, C., Goodfellow, A.F., and Wrana, J.L. (2003). Distinct endocytic pathways regulate TGF-β receptor signalling and turnover. Nat. Cell Biol. *5*, 410–421.

Dunker, N., Schmitt, K., and Krieglstein, K. (2002). TGF-β is required for programmed cell death in interdigital webs of the developing mouse limb. Mech. Dev. *113*, 111–120.

Dupont, S., Zacchigna, L., Cordenonsi, M., Soligo, S., Adorno, M., Rugge, M., and Piccolo, S. (2005). Germ-layer specification and control of cell growth by Ectodermin, a Smad4 ubiquitin ligase. Cell *121*, 87–99.

Eickelberg, O., and Morty, R.E. (2007). Transforming growth factor β/bone morphogenic protein signaling in pulmonary arterial hypertension: remodeling revisited. Trends Cardiovasc. Med. *17*, 263–269.

Estella, C., McKay, D.J., and Mann, R.S. (2008). Molecular integration of wingless, decapentaplegic, and autoregulatory inputs into Distalless during Drosophila leg development. Dev. Cell *14*, 86–96.

Faiyaz-Ul-Haque, M., Faqeih, E.A., Al-Zaidan, H., Al-Shammary, A., and Zaidi, S.H. (2008). Grebe-type chondrodysplasia: a novel missense mutation in a conserved cysteine of the growth differentiation factor 5. J. Bone Miner. Metab. *26*, 648–652.

Feldman, B., Gates, M.A., Egan, E.S., Dougan, S.T., Rennebeck, G., Sirotkin, H.I., Schier, A.F., and Talbot, W.S. (1998). Zebrafish organizer development and germ-layer formation require nodal-related signals. Nature *395*, 181–185.

Feng, X.H., and Derynck, R. (2005). Specificity and versatility in TGF-β signaling through Smads. Annu. Rev. Cell Dev. Biol. *21*, 659–693.

Fuentealba, L.C., Eivers, E., Ikeda, A., Hurtado, C., Kuroda, H., Pera, E.M., and De Robertis, E.M. (2007). Integrating patterning signals: Wnt/GSK3 regulates the duration of the BMP/Smad1 signal. Cell *131*, 980–993.

Gallione, C.J., Repetto, G.M., Legius, E., Rustgi, A.K., Schelley, S.L., Tejpar, S., Mitchell, G., Drouin, E., Westermann, C.J., and Marchuk, D.A. (2004). A combined syndrome of juvenile polyposis and hereditary haemorrhagic telangiectasia associated with mutations in MADH4 (SMAD4). Lancet *363*, 852–859.

Gallione, C.J., Richards, J.A., Letteboer, T.G., Rushlow, D., Prigoda, N.L., Leedom, T.P., Ganguly, A., Castells, A., Ploos van Amstel, J.K., Westermann, C.J., et al. (2006). SMAD4 mutations found in unselected HHT patients. J. Med. Genet. *43*, 793–797.

Gordon, K.J., and Blobe, G.C. (2008). Role of transforming growth factor-β superfamily signaling pathways in human disease. Biochim. Biophys. Acta *1782*, 197–228.

Gore, A.V., Maegawa, S., Cheong, A., Gilligan, P.C., Weinberg, E.S., and Sampath, K. (2005). The zebrafish dorsal axis is apparent at the four-cell stage. Nature *438*, 1030–1035.

Goumans, M.J., Valdimarsdottir, G., Itoh, S., Lebrin, F., Larsson, J., Mummery, C., Karlsson, S., and ten Dijke, P. (2003). Activin receptor-like kinase (ALK)1 is an antagonistic mediator of lateral TGFβ/ALK5 signaling. Mol. Cell *12*, 817–828.

Green, J.B., and Smith, J.C. (1990). Graded changes in dose of a *Xenopus* activin A homologue elicit stepwise transitions in embryonic cell fate. Nature *347*, 391–394.

Groppe, J., Hinck, C.S., Samavarchi-Tehrani, P., Zubieta, C., Schuermann, J.P., Taylor, A.B., Schwarz, P.M., Wrana, J.L., and Hinck, A.P. (2008). Cooperative assembly of TGF-β superfamily signaling complexes is mediated by two disparate mechanisms and distinct modes of receptor binding. Mol. Cell *29*, 157–168.

He, W., Dorn, D.C., Erdjument-Bromage, H., Tempst, P., Moore, M.A., and Massague, J. (2006). Hematopoiesis controlled by distinct TIF1γ and Smad4 branches of the TGFβ pathway. Cell *125*, 929–941.

Hill, C.S. (2001). TGF-β signalling pathways in early Xenopus development. Curr. Opin. Genet. Dev. *11*, 533–540.

Hoodless, P.A., Pye, M., Chazaud, C., Labbe, E., Attisano, L., Rossant, J., and Wrana, J.L. (2001). FoxH1 (Fast) functions to specify the anterior primitive streak in the mouse. Genes Dev. *15*, 1257–1271.

Inomata, H., Haraguchi, T., and Sasai, Y. (2008). Robust stability of the embryonic axial pattern requires a secreted scaffold for chordin degradation. Cell *134*, 854–865.

Jones, C.M., Armes, N., and Smith, J.C. (1996). Signalling by TGF-β family members: short-range effects of Xnr-2 and BMP-4 contrast with the long-range effects of activin. Curr. Biol. *6*, 1468–1475.

Josso, N., Picard, J.Y., Rey, R., and di Clemente, N. (2006). Testicular anti-Mullerian hormone: history, genetics, regulation and clinical applications. Pediatr. Endocrinol. Rev. *3*, 347–358.

Kicheva, A., and Gonzalez-Gaitan, M. (2008). The Decapentaplegic morphogen gradient: a precise definition. Curr. Opin. Cell Biol. *20*, 137–143.

Kicheva, A., Pantazis, P., Bollenbach, T., Kalaidzidis, Y., Bittig, T., Julicher, F., and Gonzalez-Gaitan, M. (2007). Kinetics of morphogen gradient formation. Science *315*, 521–525.

Kishimoto, Y., Lee, K.H., Zon, L., Hammerschmidt, M., and Schulte-Merker, S. (1997). The molecular nature of zebrafish swirl: BMP2 function is essential during early dorsoventral patterning. Development *124*, 4457–4466.

Knockaert, M., Sapkota, G., Alarcon, C., Massague, J., and Brivanlou, A.H. (2006). Unique players in the BMP pathway: small C-terminal domain phosphatases dephosphorylate Smad1 to attenuate BMP signaling. Proc. Natl. Acad. Sci. USA *103*, 11940–11945.

Kunwar, P.S., Zimmerman, S., Bennett, J.T., Chen, Y., Whitman, M., and Schier, A.F. (2003). Mixer/Bon and FoxH1/Sur have overlapping and divergent roles in Nodal signaling and mesendoderm induction. Development *130*, 5589–5599.

Le Good, J.A., Joubin, K., Giraldez, A.J., Ben-Haim, N., Beck, S., Chen, Y., Schier, A.F., and Constam, D.B. (2005). Nodal stability determines signaling range. Curr. Biol. *15*, 31–36.

Lee, M.K., Pardoux, C., Hall, M.C., Lee, P.S., Warburton, D., Qing, J., Smith, S.M., and Derynck, R. (2007). TGF-β activates Erk MAP kinase signalling through direct phosphorylation of ShcA. EMBO J. *26*, 3957–3967.

Lehmann, K., Seemann, P., Boergermann, J., Morin, G., Reif, S., Knaus, P., and Mundlos, S. (2006). A novel R486Q mutation in BMPR1B resulting in either a brachydactyly type C/symphalangism-like phenotype or brachydactyly type A2. Eur. J. Hum. Genet. *14*, 1248–1254.

Lehmann, K., Seemann, P., Silan, F., Goecke, T.O., Irgang, S., Kjaer, K.W., Kjaergaard, S., Mahoney, M.J., Morlot, S., Reissner, C., et al. (2007). A new subtype of brachydactyly type B caused by point mutations in the bone morphogenetic protein antagonist NOGGIN. Am. J. Hum. Genet. *81*, 388–396.

Levy, L., and Hill, C.S. (2005). Smad4 dependency defines two classes of transforming growth factor β (TGF-β) target genes and distinguishes TGF-β-induced epithelial-mesenchymal transition from its antiproliferative and migratory responses. Mol. Cell. Biol. *25*, 8108–8125.

Levy, L., and Hill, C.S. (2006). Alterations in components of the TGF-β superfamily signaling pathways in human cancer. Cytokine Growth Factor Rev. *17*, 41–58.

Lin, X., Duan, X., Liang, Y.Y., Su, Y., Wrighton, K.H., Long, J., Hu, M., Davis, C.M., Wang, J., Brunicardi, F.C., et al. (2006). PPM1A functions as a Smad phosphatase to terminate TGFβ signaling. Cell *125*, 915–928.

Little, S.C., and Mullins, M.C. (2006). Extracellular modulation of BMP activity in patterning the dorsoventral axis. Birth Defects Res. C. Embryo Today *78*, 224–242.

Liu, I.M., Schilling, S.H., Knouse, K.A., Choy, L., Derynck, R., and Wang, X.F. (2009). TGFβ-stimulated Smad1/5 phosphorylation requires the ALK5 L45 loop and mediates the pro-migratory TGFβ switch. EMBO J. *28*, 88–98.

Long, S., Ahmad, N., and Rebagliati, M. (2003). The zebrafish nodal-related gene southpaw is required for visceral and diencephalic left-right asymmetry. Development *130*, 2303–2316.

Marques, S., Borges, A.C., Silva, A.C., Freitas, S., Cordenonsi, M., and Belo, J.A. (2004). The activity of the Nodal antagonist Cerl-2 in the mouse node is required for correct L/R body axis. Genes Dev. *18*, 2342–2347.

Martello, G., Zacchigna, L., Inui, M., Montagner, M., Adorno, M., Mamidi, A., Morsut, L., Soligo, S., Tran, U., Dupont, S., et al. (2007). MicroRNA control of Nodal signalling. Nature *449*, 183–188.

Massague, J. (2008). TGFβ in Cancer. Cell *134*, 215–230.

Massague, J., Seoane, J., and Wotton, D. (2005). Smad transcription factors. Genes Dev. *19*, 2783–2810.

Mercado-Pimentel, M.E., and Runyan, R.B. (2007). Multiple transforming growth factor-β isoforms and receptors function during epithelial-mesenchymal cell transformation in the embryonic heart. Cells Tissues Organs *185*, 146–156.

Miles, W.O., Jaffray, E., Campbell, S.G., Takeda, S., Bayston, L.J., Basu, S.P., Li, M., Raftery, L.A., Ashe, M.P., Hay, R.T., and Ashe, H.L. (2008). Medea SUMOylation restricts the signaling range of the Dpp morphogen in the Drosophila embryo. Genes Dev. *22*, 2578–2590.

Mine, N., Anderson, R.M., and Klingensmith, J. (2008). BMP antagonism is required in both the node and lateral plate mesoderm for mammalian left-right axis establishment. Development *135*, 2425–2434.

Morisato, D., and Anderson, K.V. (1995). Signaling pathways that establish the dorsal-ventral pattern of the Drosophila embryo. Annu. Rev. Genet. *29*, 371–399.

Morrell, N.W. (2006). Pulmonary hypertension due to BMPR2 mutation: a new paradigm for tissue remodeling? Proc. Am. Thorac. Soc. *3*, 680–686.

O'Connor, M.B., Umulis, D., Othmer, H.G., and Blair, S.S. (2006). Shaping BMP morphogen gradients in the Drosophila embryo and pupal wing. Development *133*, 183–193.

Oelgeschlager, M., Larrain, J., Geissert, D., and De Robertis, E.M. (2000). The evolutionarily conserved BMP-binding protein Twisted gastrulation promotes BMP signalling. Nature *405*, 757–763.

Ozdamar, B., Bose, R., Barrios-Rodiles, M., Wang, H.R., Zhang, Y., and Wrana, J.L. (2005). Regulation of the polarity protein Par6 by TGFβ receptors controls epithelial cell plasticity. Science *307*, 1603–1609.

Piccolo, S., Sasai, Y., Lu, B., and De Robertis, E.M. (1996). Dorsoventral patterning in Xenopus: inhibition of ventral signals by direct binding of chordin to BMP-4. Cell *86*, 589–598.

Piccolo, S., Agius, E., Lu, B., Goodman, S., Dale, L., and De Robertis, E.M. (1997). Cleavage of Chordin by Xolloid metalloprotease suggests a role for proteolytic processing in the regulation of Spemann organizer activity. Cell *91*, 407–416.

Pyrowolakis, G., Hartmann, B., Muller, B., Basler, K., and Affolter, M. (2004). A simple molecular complex mediates widespread BMP-induced repression during Drosophila development. Dev. Cell *7*, 229–240.

Randall, R.A., Howell, M., Page, C.S., Daly, A., Bates, P.A., and Hill, C.S. (2004). Recognition of phosphorylated-Smad2-containing complexes by a novel Smad interaction motif. Mol. Cell. Biol. *24*, 1106–1121.

Rentzsch, F., Zhang, J., Kramer, C., Sebald, W., and Hammerschmidt, M. (2006). Crossveinless 2 is an essential positive feedback regulator of Bmp signaling during zebrafish gastrulation. Development *133*, 801–811.

Reversade, B., and De Robertis, E.M. (2005). Regulation of ADMP and BMP2/4/7 at opposite embryonic poles generates a self-regulating morphogenetic field. Cell *123*, 1147–1160.

Robert, B. (2007). Bone morphogenetic protein signaling in limb outgrowth and patterning. Dev. Growth Differ. *49*, 455–468.

Robinson, P.N., Arteaga-Solis, E., Baldock, C., Collod-Beroud, G., Booms, P., De Paepe, A., Dietz, H.C., Guo, G., Handford, P.A., Judge, D.P., et al. (2006). The molecular genetics of Marfan syndrome and related disorders. J. Med. Genet. *43*, 769–787.

Ross, S., and Hill, C.S. (2008). How the Smads regulate transcription. Int. J. Biochem. Cell Biol. *40*, 383–408.

Ross, S., Cheung, E., Petrakis, T.G., Howell, M., Kraus, W.L., and Hill, C.S. (2006). Smads orchestrate specific histone modifications and chromatin remodeling to activate transcription. EMBO J. *25*, 4490–4502.

Rushlow, C., Colosimo, P.F., Lin, M.C., Xu, M., and Kirov, N. (2001). Transcriptional regulation of the Drosophila gene zen by competing Smad and Brinker inputs. Genes Dev. *15*, 340–351.

Sampath, K., Cheng, A.M., Frisch, A., and Wright, C.V. (1997). Functional differences among Xenopus nodal-related genes in left-right axis determination. Development *124*, 3293–3302.

Sapkota, G., Alarcon, C., Spagnoli, F.M., Brivanlou, A.H., and Massague, J. (2007). Balancing BMP signaling through integrated inputs into the Smad1 linker. Mol. Cell *25*, 441–454.

Sasai, Y., Lu, B., Steinbeisser, H., and De Robertis, E.M. (1995). Regulation of neural induction by the Chd and Bmp-4 antagonistic patterning signals in Xenopus. Nature *376*, 333–336.

Sauka-Spengler, T., and Bronner-Fraser, M. (2008). A gene regulatory network orchestrates neural crest formation. Nat. Rev. Mol. Cell Biol. *9*, 557–568.

Schier, A.F. (2003). Nodal signaling in vertebrate development. Annu. Rev. Cell Dev. Biol. *19*, 589–621.

Schlange, T., Arnold, H.H., and Brand, T. (2002). BMP2 is a positive regulator of Nodal signaling during left-right axis formation in the chicken embryo. Development *129*, 3421–3429.

Schmierer, B., and Hill, C.S. (2007). TGFβ-SMAD signal transduction: molecular specificity and functional flexibility. Nat. Rev. Mol. Cell Biol. *8*, 970–982.

Schmierer, B., Tournier, A.L., Bates, P.A., and Hill, C.S. (2008). Mathematical modeling identifies Smad nucleocytoplasmic shuttling as a dynamic signal-interpreting system. Proc. Natl. Acad. Sci. USA *105*, 6608–6613.

Seemann, P., Schwappacher, R., Kjaer, K.W., Krakow, D., Lehmann, K., Dawson, K., Stricker, S., Pohl, J., Ploger, F., Staub, E., et al. (2005). Activating and deactivating mutations in the receptor interaction site of GDF5 cause symphalangism or brachydactyly type A2. J. Clin. Invest. *115*, 2373–2381.

Serpe, M., Umulis, D., Ralston, A., Chen, J., Olson, D.J., Avanesov, A., Othmer, H., O'Connor, M.B., and Blair, S.S. (2008). The BMP-binding protein Crossveinless 2 is a short-range, concentration-dependent, biphasic modulator of BMP signaling in Drosophila. Dev. Cell *14*, 940–953.

Settle, S.H., Jr., Rountree, R.B., Sinha, A., Thacker, A., Higgins, K., and Kingsley, D.M. (2003). Multiple joint and skeletal patterning defects caused by single and double mutations in the mouse Gdf6 and Gdf5 genes. Dev. Biol. *254*, 116–130.

Shi, W., Sun, C., He, B., Xiong, W., Shi, X., Yao, D., and Cao, X. (2004). GADD34-PP1c recruited by Smad7 dephosphorylates TGFβ type I receptor. J. Cell Biol. *164*, 291–300.

Shimmi, O., and O'Connor, M.B. (2003). Physical properties of Tld, Sog, Tsg and Dpp protein interactions are predicted to help create a sharp boundary in Bmp signals during dorsoventral patterning of the Drosophila embryo. Development *130*, 4673–4682.

Sigismund, S., Argenzio, E., Tosoni, D., Cavallaro, E., Polo, S., and Di Fiore, P.P. (2008). Clathrin-mediated internalization is essential for sustained EGFR signaling but dispensable for degradation. Dev. Cell *15*, 209–219.

Solnica-Krezel, L. (2003). Vertebrate development: taming the nodal waves. Curr. Biol. *13*, R7–R9.

Sopory, S., Nelsen, S.M., Degnin, C., Wong, C., and Christian, J.L. (2006). Regulation of bone morphogenetic protein-4 activity by sequence elements within the prodomain. J. Biol. Chem. *281*, 34021–34031.

Sorrentino, A., Thakur, N., Grimsby, S., Marcusson, A., von Bulow, V., Schuster, N., Zhang, S., Heldin, C.H., and Landstrom, M. (2008). The type I TGF-β receptor engages TRAF6 to activate TAK1 in a receptor kinase-independent manner. Nat. Cell Biol. *10*, 1199–1207.

Spemann, H., and Mangold, E. (1924). Uber Induktion von Embryo- nanlagen durch Implantation artfremder Organisatoren. Arch. mikr. Anat. EntwMech. *100*, 599–638.

Sun, Z., Jin, P., Tian, T., Gu, Y., Chen, Y.G., and Meng, A. (2006). Activation and roles of ALK4/ALK7-mediated maternal TGFβ signals in zebrafish embryo. Biochem. Biophys. Res. Commun. *345*, 694–703.

Sutherland, D.J., Li, M., Liu, X.Q., Stefancsik, R., and Raftery, L.A. (2003). Stepwise formation of a SMAD activity gradient during dorsal-ventral patterning of the Drosophila embryo. Development *130*, 5705–5716.

ten Dijke, P., and Hill, C.S. (2004). New insights into TGF-β-Smad signalling. Trends Biochem. Sci. *29*, 265–273.

ten Dijke, P., and Arthur, H.M. (2007). Extracellular control of TGFβ signalling in vascular development and disease. Nat. Rev. Mol. Cell Biol. *8*, 857–869.

Thomas, J.T., Kilpatrick, M.W., Lin, K., Erlacher, L., Lembessis, P., Costa, T., Tsipouras, P., and Luyten, F.P. (1997). Disruption of human limb morphogenesis by a dominant negative mutation in CDMP1. Nat. Genet. *17*, 58–64.

Tian, J., Andree, B., Jones, C.M., and Sampath, K. (2008). The pro-domain of the zebrafish Nodal-related protein Cyclops regulates its signaling activities. Development *135*, 2649–2658.

Tucker, J.A., Mintzer, K.A., and Mullins, M.C. (2008). The BMP signaling gradient patterns dorsoventral tissues in a temporally progressive manner along the anteroposterior axis. Dev. Cell *14*, 108–119.

Varelas, X., Sakuma, R., Samavarchi-Tehrani, P., Peerani, R., Rao, B.M., Dembowy, J., Yaffe, M.B., Zandstra, P.W., and Wrana, J.L. (2008). TAZ controls Smad nucleocytoplasmic shuttling and regulates human embryonic stem-cell self-renewal. Nat. Cell Biol. *10*, 837–848.

Wang, Y.C., and Ferguson, E.L. (2005). Spatial bistability of Dpp-receptor interactions during Drosophila dorsal-ventral patterning. Nature *434*, 229–234.

Wang, J.G., Nakhuda, G.S., Guarnaccia, M.M., Sauer, M.V., and Lobo, R.A. (2007). Mullerian inhibiting substance and disrupted folliculogenesis in polycystic ovary syndrome. Am. J. Obstet. Gynecol. *196*, 77.e1-5.

Wang, X., Harris, R.E., Bayston, L.J., and Ashe, H.L. (2008). Type IV collagens regulate BMP signalling in Drosophila. Nature *455*, 72–77.

Wharton, S.J., Basu, S.P., and Ashe, H.L. (2004). Smad affinity can direct distinct readouts of the embryonic extracellular Dpp gradient in Drosophila. Curr. Biol. *14*, 1550–1558.

Wilkes, M.C., Murphy, S.J., Garamszegi, N., and Leof, E.B. (2003). Cell-type-specific activation of PAK2 by transforming growth factor β independent of Smad2 and Smad3. Mol. Cell. Biol. *23*, 8878–8889.

Wilkes, M., Repellin, C., Hong, M., Bracamonte, M., Penheiter, S., Borg, J., and Leof, E. (2009). Erbin and the NF2 tumor suppressor Merlin cooperatively regulate cell type-specific activation of PAK2 by TGF-β. Dev. Cell *16*, this issue, 433–444.

Xi, Q., He, W., Zhang, X.H., Le, H.V., and Massague, J. (2008). Genome-wide impact of the BRG1 SWI/SNF chromatin remodeler on the transforming growth factor β transcriptional program. J. Biol. Chem. *283*, 1146–1155.

Yamamoto, M., Saijoh, Y., Perea-Gomez, A., Shawlot, W., Behringer, R.R., Ang, S.L., Hamada, H., and Meno, C. (2004). Nodal antagonists regulate formation of the anteroposterior axis of the mouse embryo. Nature *428*, 387–392.

Yamashita, M., Fatyol, K., Jin, C., Wang, X., Liu, Z., and Zhang, Y.E. (2008). TRAF6 mediates Smad-independent activation of JNK and p38 by TGF-β. Mol. Cell *31*, 918–924.

Yang, J., and Weinberg, R.A. (2008). Epithelial-mesenchymal transition: at the crossroads of development and tumor metastasis. Dev. Cell *14*, 818–829.

Yao, L.C., Blitz, I.L., Peiffer, D.A., Phin, S., Wang, Y., Ogata, S., Cho, K.W., Arora, K., and Warrior, R. (2006). Schnurri transcription factors from Drosophila and vertebrates can mediate Bmp signaling through a phylogenetically conserved mechanism. Development *133*, 4025–4034.

Yao, L.C., Phin, S., Cho, J., Rushlow, C., Arora, K., and Warrior, R. (2008). Multiple modular promoter elements drive graded brinker expression in response to the Dpp morphogen gradient. Development *135*, 2183–2192.

Yeo, C., and Whitman, M. (2001). Nodal signals to Smads through Cripto-dependent and Cripto-independent mechanisms. Mol. Cell *7*, 949–957.

Zeisberg, M., and Kalluri, R. (2004). The role of epithelial-to-mesenchymal transition in renal fibrosis. J. Mol. Med. *82*, 175–181.

Zhang, J., Houston, D.W., King, M.L., Payne, C., Wylie, C., and Heasman, J. (1998). The role of maternal VegT in establishing the primary germ layers in Xenopus embryos. Cell *94*, 515–524.

Zhang, J.L., Qiu, L.Y., Kotzsch, A., Weidauer, S., Patterson, L., Hammerschmidt, M., Sebald, W., and Mueller, T.D. (2008). Crystal structure analysis reveals how the Chordin family member crossveinless 2 blocks BMP-2 receptor binding. Dev. Cell *14*, 739–750.

Zorn, A.M., and Wells, J.M. (2007). Molecular basis of vertebrate endoderm development. Int. Rev. Cytol. *259*, 49–111.