PHYSIOLOGY

Matters of context guide future research in TGFβ superfamily signaling

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The highly conserved wiring of the SMAD-dependent transforming growth factor β (TGFβ) superfamily signaling pathway has been mapped over the last 20 years after molecular discovery of its component parts. Numerous alternative TGFβ-activated signaling pathways that elicit SMAD-independent biological responses also exist. However, the molecular mechanisms responsible for the renowned context dependency of TGFβ signaling output remains an active and often confounding area of research, providing a prototype relevant to regulation of other signaling pathways. Highlighting discoveries presented at the 9th FASEB meeting, *The TGFβ Superfamily: Signaling in Development and Disease* (July 12–17th 2015 in Snowmass, Colorado), this Review outlines research into the rich contextual nature of TGFβ signaling output and offers clues for therapeutic advances.

Introduction

The transforming growth factor β (TGFβ) family of about 33 related ligands in vertebrates (7 in *Drosophila*, 5 in *Caenorhabditis elegans*) participates in many developmental processes and in adult homeostasis. As might be expected of potent growth factors, misregulation or mutation in family signaling components contributes to a variety of diseases and oncogenic growth. TGFβ appears to be an animal invention, being found in the most primitive animals, sponges, and trichoplax (*1*–*3*), but not in yeast or plants. Signaling is initiated with ligand binding to two related dual specificity kinase transmembrane receptors (types I and II; see Table 1). Because these receptors are unique in animals, their discovery suggested that the downstream signaling components might be different from known signal transducers, which turned out to be true. SMAD-dependent signaling involves serine phosphorylation of receptor-regulated SMADs (R-SMADs; SMADs 1, 2, 3, 5, or 8/9) by a type I receptor. Four R-SMADs together form a heterohexameric complex with two units of the common-mediator SMAD (co-SMAD; SMAD4); this R-SMAD/co-SMAD complex then translocates to the nucleus where it interacts with transcription factors and binds gene promoters to change the transcription of target genes (Fig. 1). Output from TGFβ receptors also emanates through SMAD-independent pathways, including extracellular signal–regulated kinase (ERK), the mitogen-activated pathway kinase (MAPK) p38, the transcription factor c-Jun, and/or the kinase TAK1. At each step of these pathways, many different accessory proteins influence the signaling output.

Diminishing complexity of TGFβ signaling components from multiple ligands outside the cell, through reduced numbers of receptors and only five R-SMADs that each binds and normally acts via a single common co-SMAD4, provides a tantalizing puzzle for biologists regarding specificity (Table 1). Approaches to disentangling contextual readout, including genetic dissection in model organisms, structural biology and biochemistry, and mammalian cell and tissue biology, are being used to elucidate how small molecular perturbations in TGFβ signaling can markedly alter body plan, stem cell fate, or human disease pathology. Detailed molecular mechanisms were presented for control of TGFβ latency, cell surface ligand activation by tension, regulation of TGFβ receptor and R-SMAD action, and turnover by a variety of post-translational modifications and by the differential compartmentalization of TGFβ receptor activities within endocytic compartments of the cell (Fig. 2). A number of strikingly novel findings were reported, which could open the floodgates to conceptually new areas of research, including regulation of circular RNA biogenesis during TGFβ-induced epithelial-mesenchymal transition (EMT) (*4*); discovery of the transcriptionally active nuclear intracellular domain of TGFβ type I receptor (TGFβRI) and its regulation of tumor cell invasive character (*5*–*7*); and observation of cytoneme-mediated signaling in *Drosophila*, which conducts transport of Dpp and establishes direct contact and ligand delivery between producing and receiving cells, therefore contributing to establishment of long-range morphogen gradients (*8*, *9*). These basic science studies from *C. elegans*, *Drosophila*, fish, mice, and humans have synergistically led to novel drug targets, and new drugs and therapeutic approaches for the common killers, cancer and fibrosis.

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Current and Emerging Concepts in TGFβ Signaling

Extracellular morphogen gradients in establishment of body plan

Gradients play important roles in distributing morphogens to receiving cells and have been studied for many years. Distinct concentrations of active ligand have been shown to induce different sets of genes, thereby establishing alternative cell fates (*10*, *11*). Recent results from several laboratories are helping define controls of TGFβ itself and offering insights into how TGFβ gradients are established. Groundbreaking work from Kornberg's laboratory showed the role that specialized filopodia (cytonemes) play in transporting *decapentaplegic* [*dpp*; a *Drosophila* bone morphogenetic protein (BMP) ortholog] across cells to locations where it is needed for proper development of the air sac primordium within the wing disc (*8*, *9*, *12*, *13*). A series of elegant imaging experiments revealed details of this process and define some key mechanistic steps. In the wing disc, proper trachea development requires *dpp*, which is only expressed in a narrow stripe along the anterior/posterior (A/P) compartment boundary. Transport of *dpp* from expressing cells of the A/P boundary is accomplished by cytonemes that extend out from recipient air sac primordia making contact with *dpp*-expressing cells. Cytonemes avoid delivery of ligand to intervening cells as Dpp is transported in a conduit, surpassing intervening cells, to the primordia where it functions. Where signaling occurs is not clear, the Dpp-containing cytonemes also house Tkv (a type I receptor for Dpp), which they transport as well. Transport by cytonemes is not restricted

to TGFβ family members but has been shown for fibroblast growth factor (FGF), Wingless, and Hedgehog—presumably this system is in play for many other signaling molecules (*14*, *15*). However, cytonemes do not seem to mix different signaling pathways in a single protrusion—there are cytonemes specific for Dpp and for FGF, but not both. They are not restricted to *Drosophila*, but are known to function in zebrafish (*16*), so they are likely conserved in most multicellular animals.

Other factors affect peak levels of ligand as the gradient is established. Work in *Drosophila* had previously shown that *short gastrulation* (*sog*), the Chordin homolog, transports Dpp to the dorsal area of the developing embryo to achieve a high level of ligand (*17*). Sog binds Dpp, and mutants in *sog* allow Dpp to be distributed, rather than concentrated at the dorsal apex. Chordin was known to be involved in vertebrate BMP signaling, but whether it functioned like its fly counterpart was not known (*18*, *19*). Mullins and colleagues reported on advances in understanding the role of Chordin in shaping the vertebrate BMP gradient in comparison to that in *Drosophila* by *sog*. Using a quantitative assay of a direct BMP signaling readout, they found both conserved and nonconserved functions for Chordin in regulating the signaling gradient. Hill and colleagues are questioning the dogma with regard to formation of Nodal signaling gradients in early zebrafish embryos and find no evidence for a reaction diffusion model.

Table 1. General specificity of ligands and receptors in the TGFβ superfamily. Brackets and arrows indicate binding partners. The type I receptors show more promiscuity for ligand binding than do the type II receptors.

Ligand class	Type II receptor	Type I receptor	R-SMAD	co-SMAD
TGFβ	TGFβRII	TGFβRI (ALK-5) → SMAD2/SMAD3 ACVRL1 (ALK-1) → SMAD1/SMAD5/SMAD8		SMAD4
Nodal GDF8(myostatin)/ GDF11	ACVR2B	ACVR1C (ALK-7) ACVR1B (ALK-4)	SMAD2/SMAD3	
Activins/inhibins	ACVR2A ACVR2B	ACVR1B (ALK-4) ACVR1C (ALK-7)	SMAD2/SMAD3	
BMP	BMPR2	BMPR1A (ALK-3) BMPR1B (ALK-6) ACVR1 (ALK-2) ACVRL1 (ALK-1)	SMAD1/SMAD5/SMAD8	

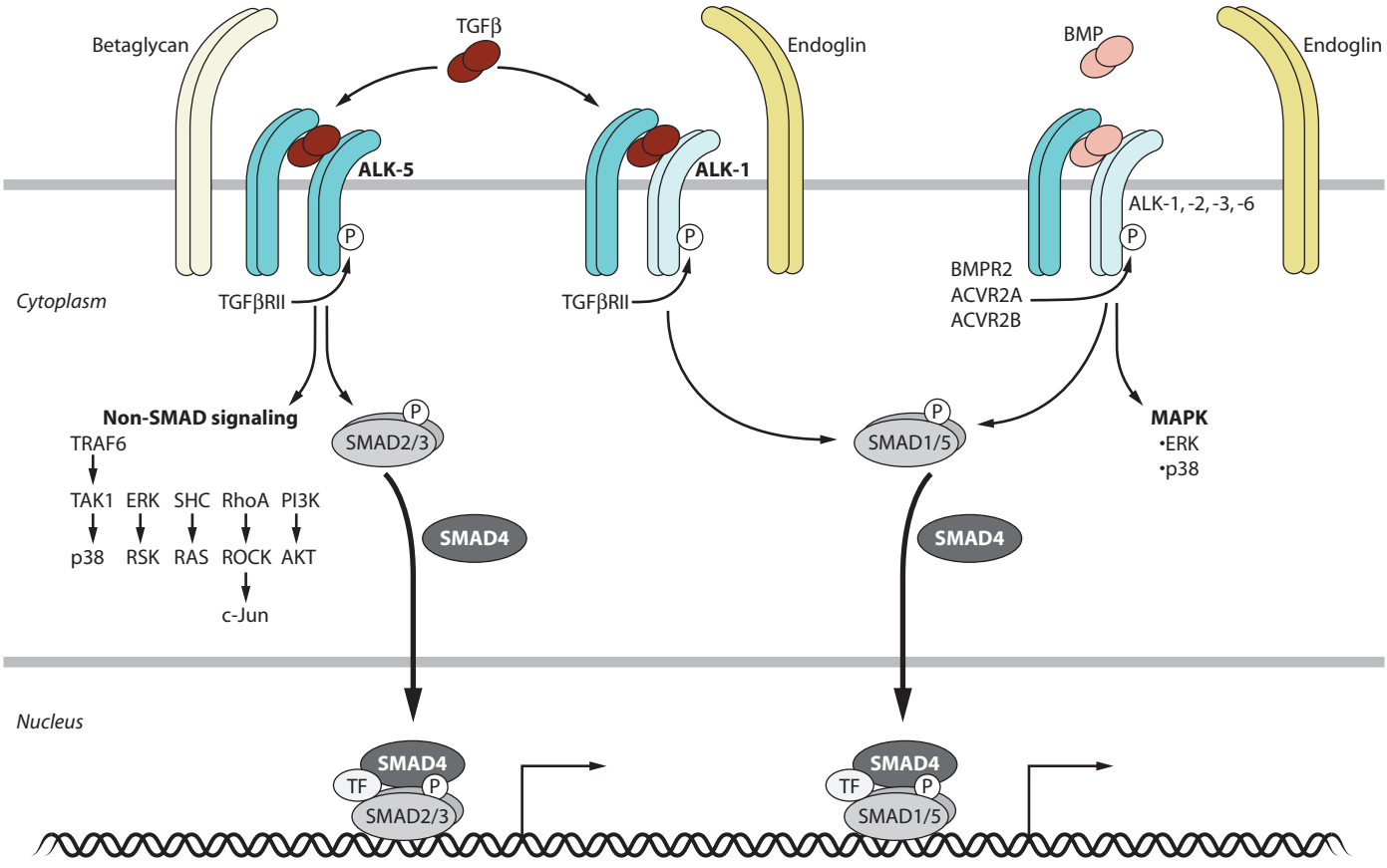

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Fig. 1. TGFβ and BMP signaling pathways. Endothelial, immune, and stem cells express the co-receptor endoglin, whereas most other cell types express the co-receptor betaglycan. Note that ACVRL1/ALK-1 can be triggered by TGFβ or by BMP9 to activate the SMAD1/5/8 pathway. Both receptors can also signal to non-SMAD pathways indicated in green shaded boxes.

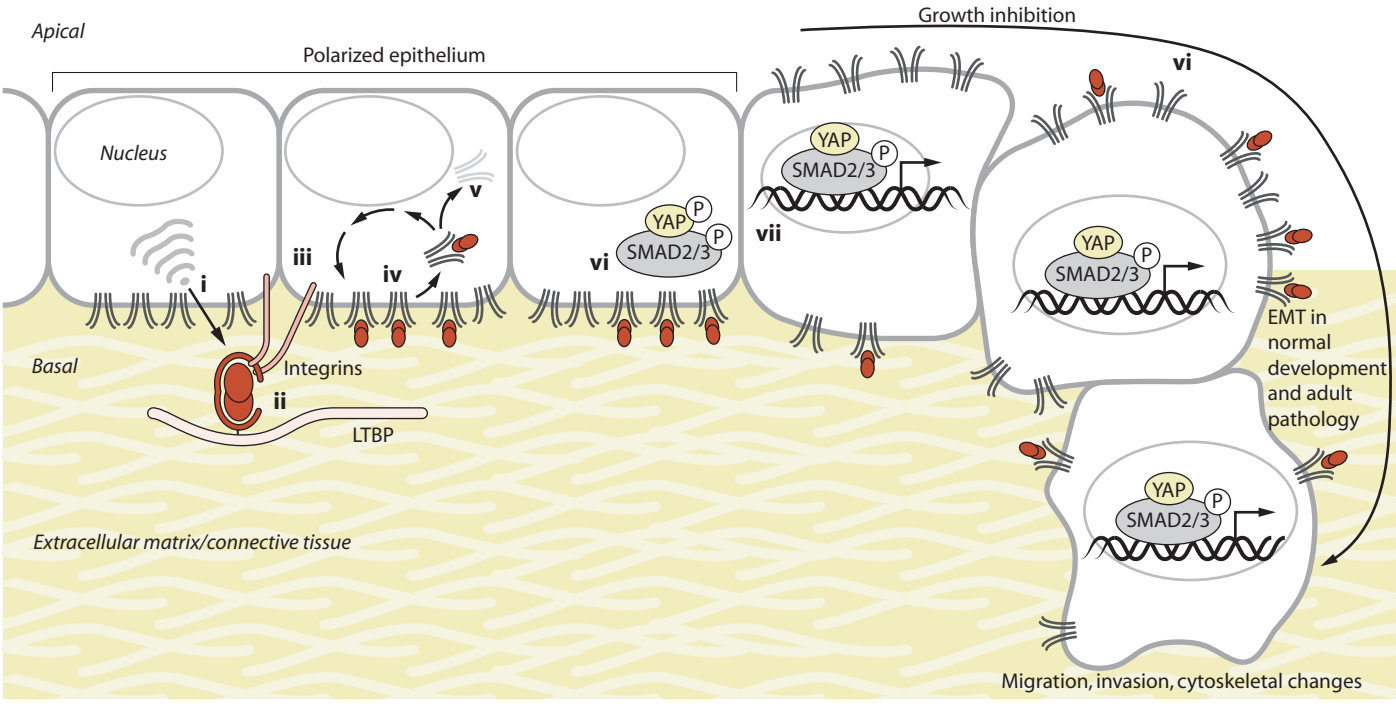

Fig. 2. Epithelial homeostasis, EMT, and nodes of TGFβ signaling regulation. Cartoon shows differential regulation of TGFβ signaling at the levels of (i) ligand synthesis from the Golgi and secretion from the cell; (ii) integrin-mediated TGFβ activation from the extracellular matrix (ECM)–bound latent complex; (iii) restriction of TGFβ receptors to basolateral surface of epithelial cells, preventing activation by apically applied TGFβ; (iv) receptor recycling or (v) degradation, presumably together with ligand, through the retromer, canonical endocytic pathway, and/or clathrin-coated pits; (vi) cytoplasmic sequestration of phosphorylated SMAD2 (pSMAD2) by binding pYAP/TAZ; (vii) context-dependent transcriptional activation by pSMAD2/3, independent or dependent on YAP/TAZ (see Fig. 4). TGFβ inhibits growth in normal epithelia (left) but induces EMT during normal development and in adults during fibrosis, cancer, and in other pathological contexts.

As might be expected, other signaling components can be regulated to alter the signaling strength of the BMP gradient. Eivers and colleagues examined phosphorylation of the linker region of Mad (a *Drosophila* BMP-activated R-SMAD) (*20*). After the initial studies of phosphorylation of the C termini of R-SMADs years ago, attention was given to other phosphorylation sites on SMADs. These studies have sometimes painted a cloudy picture, partly because the linker region is not well conserved and several molecules may modify this domain depending on spatial or temporal constraints (*21*–*26*). But a universal finding of most studies was that lack of linker phosphorylation resulted in prolonged BMP signaling. However, most studies did not address its effect on the BMP gradient. Using *Drosophila*, Eivers showed that cyclin-dependent kinase 8 (CDK8) and Shaggy [glycogen synthase kinase 3 (GSK3)] phosphorylate three serines in the Mad linker. These modifications control the peak intensity and range of the BMP signal in *Drosophila*, and add another layer of control on BMP gradients (*20*).

Pioneering work by Christian's laboratory in vertebrates established multiple proteolytic processing sites in the prodomain of TGFβ family members and showed that differential cleavage affected the signaling range of the mature ligand (*27*–*29*). Wharton and colleagues examined processing of the prodomain of Gbb in *Drosophila* (BMP5 to BMP7) and found an additional previously unknown N-terminal proconvertase processing site (*30*). Mutating the canonical S1 site reduced signaling causing expected defects, but mutating the N-terminal site results in more profound phenotypic effects. Removing both sites was additive and abolished most ligand activity. These different processing sites result in different mature ligand sizes and levels in different tissues as well as affecting the gradient with Dpp. Alternative furin cleavage sites are an evolutionarily conserved feature of ligand maturation because they are also observed in human TGFβ family members, including BMP4, BMP15, and anti-Müllerian hormone/Müllerian-inhibiting substance. Human mutations and/or genetic variants in this N-terminal cleavage site are associated with cleft palate, premature ovarian failure, and persistent Müllerian duct syndrome, respectively (*30*).

Intracellular trafficking of ligands and receptors

Basic principles of receptor trafficking have not been as clearly elucidated for TGFβ family members as they have in other signaling pathways, but recent progress has revealed interesting aspects of TGFβ receptor recycling. Early work by the laboratories of Leof, Chen, Henis, Wrana, and others established some of the fundamental principles in the TGFβ endocytosis pathway (*31*–*37*). Given that TGFβ signals through two related receptors, the question arises whether the two receptors are trafficked and recycled in a similar manner. The laboratory of Padgett examined this issue using well-developed molecular tools available in *C. elegans*, and showed that the type I and type II BMP receptors are trafficked differently from each other (*38*). The type I receptor is recycled through the retromer and sorting nexin-3 (SNX-3), whereas the type II receptor is recycled through a pathway using ARF-6 and the recycling endosome. The retromer is an ancient invention, being discovered first in yeast and is important for recycling several proteins. This difference in how the two BMP receptors recycle may provide an additional layer of regulatory complexity to the TGFβ pathway by controlling each receptor separately. It will be important to see how widespread the use of the retromer is in recycling other TGFβ family

members. Evidence supporting disparate recycling of type I and type II receptors in vertebrates comes from a proteomics study showing that the abundance of type I receptors BMPR1A, activin receptor type 1B, and TGFβRI was decreased in HeLa cells in which both SNX-27 and vacuolar protein sorting–associated protein 35 (VPS-35) were depleted, but the type II receptors were unchanged (*39*). Data show that some of the signaling components (SMAD3, SMAD7, Smurf2, and SARA) are found in a novel vesicle resulting from the fusion of clathrin- and caveolin-positive vesicles, presumably targeted to the early endosome (*40*).

Other proteins that affect receptor trafficking have been identified from genetic screens for modifiers of *C. elegans* TGFβ signaling by the laboratories of Padgett and Liu (*41*, *42*). These include *SMA-10*/leucine-rich repeats and immunoglobulin-like domains 1 (LRIG1), *DRAG-1*/repulsive guidance molecule (RGM), *UNC-40*/neogenin/deleted in colon cancer, and the tetraspanins (*41*, *43*–*45*). Their roles are diverse and not yet well defined, but mutations in modifier genes either block the pathway or strongly attenuate it. They are likely to add important insights into how decisions to recycle or degrade TGFβ receptors (and other signaling molecules) are made. In addition to these proteins, the laboratories of Heldin and Landström have shown that the Cbl-interacting protein CIN85 interacts with TGFβRI to promote recycling of the receptors in a TRAF6-dependent way (*46*). With respect to metabolic control of TGFβ signaling, Derynck's laboratory had previously shown that high glucose levels induce a rapid increase in TGFβ receptor presentation at the cell surface, thus increasing TGFβ responsiveness (*47*). He now demonstrated that insulin can do this, too (*48*). The rapid increase in surface receptors is mediated by insulin-induced Akt/PKB activation that then phosphorylates AS160 (*49*), thus derepressing the intracellular retention of AS160-positive vesicles that contain TβRII and TβRI, enabling the receptors to be presented at the cell surface.

BMP ligands are also trafficked at synapses. BMP directs growth and neurotransmitter release at the neuromuscular junction (NMJ) in flies, as reported by Broihier and Serpe. The fly ligand Gbb is released from nearby muscles to promote NMJ growth, but Gbb is also synthesized on the presynaptic side, but there it does not regulate growth but instead drives neurotransmission. How these differential functions are regulated is becoming clearer. Mutations in *Crimpy* cause excessive NMJ growth and impaired neurotransmitter release (*50*, *51*). Crimpy traffics Gbb into dense core vesicles in neurons, where it functions to promote transmitter release. As expected, Crimpy has a vertebrate homolog, cysteine-rich transmembrane regulator 1 (Crim1) (*52*), and it will be interesting to determine whether this protein functions similarly. Further complexities in the regulation of BMP in the NMJ were presented by Serpe and colleagues (*53*).

Tension and stress as regulators of TGFβ signaling

The importance of TGFβ latency in providing an inactive pool of ligand poised for rapid release and the pivotal role that activation serves in determining downstream signaling were established in the 1980s (*54*–*57*). Decades later, detailed mechanisms that regulate latent TGFβ activation are being revealed at submolecular resolution. Springer and colleagues presented elegant molecular structure models of both TGFβ and BMP9 bound to their respective latency peptides, as well as presenting a molecular mechanism of latent ligand activation instigated by inter- and intramolecular forces (Fig. 3) (*58*, *59*).

Latent TGFβ is generated intracellularly by furin cleavage of the full-length TGFβ propeptide into a cysteine-linked dimeric mature ligand encapsulated within a "straitjacket" composed of a disulfide-linked dimer of latency-associated peptides (LAPs), each derived from the N-terminal cleavage product of pro-TGFβ (Fig. 3A) (*58*). The two LAP arms cross, embracing the mature dimer such that each ligand monomer makes intimate contact with its partner's LAP. This straitjacket holds the active dimer in a distinct conformation from that of the free dimer and physically shields active TGFβ from contact with its receptors. The latent complex is tethered to elastin-rich fibrils of the ECM by cysteine crosslinking of one molecule of fibrillar latent TGFβ binding protein (LTBP) to one of the LAP arms of the complex. This unusual 1:2 molecular stoichiometry or the more conventional 2:2 ratio may be observed in binding between the other LAP arm and its integrin partner (β_6, β_8, or β_1) via RGD sites in the TGFβ1 and TGFβ3 LAPs (*60*). Tension is generated by tethering between ECM on the one hand and the cellular cytoskeleton, via integrin linkage and focal adhesion complexes, on the other (Fig. 2). Cellular contraction or other tissue forces distort the latency cage, releasing mature dimer able to activate the TGFβRII-TGFβRI complex. Notably, various pathological situations can also activate latent TGFβ by other mechanisms, such as pH, radiation, and extracellular proteases (*61*–*63*).

The primary structures of prodomains of TGFβ superfamily members are highly divergent, although interspecies ortholog conservation is high.

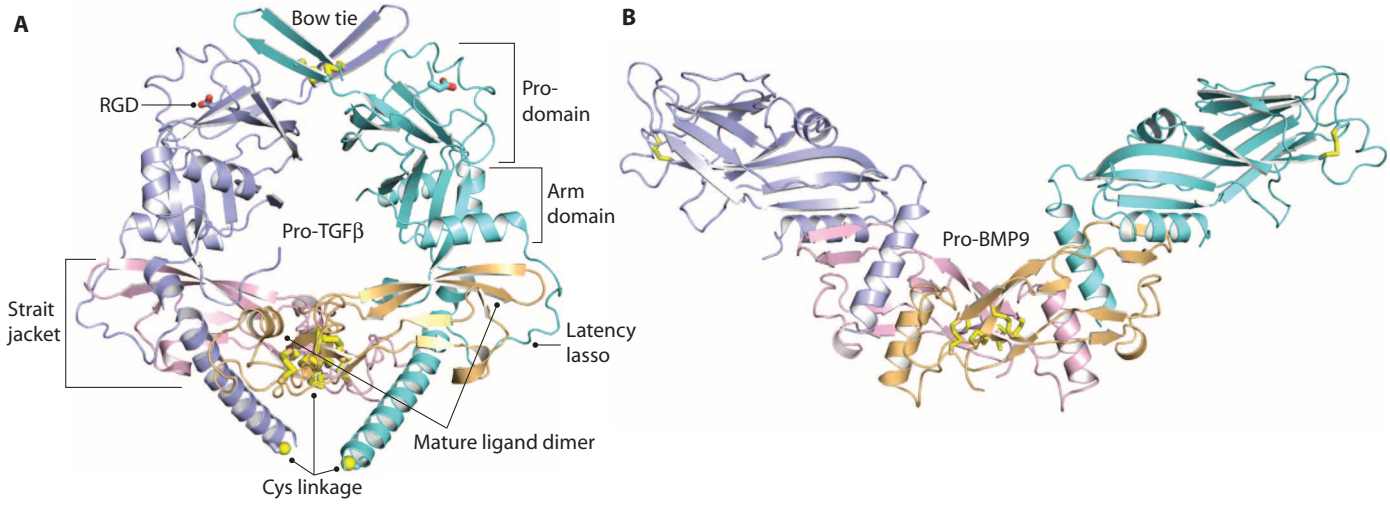

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Fig. 3. Molecular structure of TGFβ and BMP9 in complex with their LAPs. (**A** and **B**) The tightly closed structure of latent TGFβ (A) contrasts with the open structure of dimeric "latent" BMP (B). The associated movie (movie S1) shows the capability of latent BMP9 to exist in either open or closed conformations. The latter may be fixed by coupling to the ECM. Figure and movie provided with permission by Timothy Springer, adapted from (*59*).

The potential for diverse roles of member prodomains was explored by the laboratory of Springer by structural analysis of pro-BMP9, an important player in angiogenesis and vascular integrity. In contrast to the compact cross-armed and cysteine cross-linked "straightjacket" of latent TGFβ, BMP9 has an open armed, presumably nonlatent conformation unconstrained by disulfide bridges (Fig. 3B). Nevertheless, evolutionary polypeptide sequence alignments and structural modeling studies suggest that, under certain circumstances that occur in vivo but not in a test tube, pro-BMP9 and probably most proligands can take on a latent closed arm conformation, stabilized by association with ECM components, providing contextual complexity to sites of signaling activity (movie S1) (*59*).

Although tension and stress regulate the regional activation of TGFβ ligands outside the cell, SMADs interact with components of downstream Hippo signaling within both cytoplasm and nucleus to modulate TGFβ biological readouts in response to cell density and mechanosignal transduction (Figs. 2 and 4). The role of YAP/TAZ/SMAD interaction in cell density–dependent TGFβ signaling in polarized epithelial cells was debated. Wrana and colleagues previously showed that TGFβ responses depend on repression of Hippo kinase activity in polarized epithelial cells, namely, EpH4 cells and preimplantation mouse embryos (*64*). When Hippo signaling is active, triggered by an intact Crumbs complex on the apical side of polarized cells at high cell density, TGFβ-induced pSMAD2 is cytoplasmically sequestered by binding inactive cytoplasmic pYAP/TAZ. At low cell density, YAP/TAZ is unphosphorylated and localizes to the nucleus, releasing the tethering of pSMAD2 in the cytoplasm and allowing its nuclear accumulation and transcriptional responses (Fig. 2) (*64*). Consistent with this, Siggia and colleagues reported cell density–dependent changes in YAP/TAZ and SMAD nuclear localization in human embryonic stem cells, wherein at low density SMAD2 and YAP/TAZ are both nuclear, whereas at high density they are both cytoplasmic (*65*). Mauviel and colleagues questioned this mechanism and presented extensive new data from their laboratories demonstrating YAP/TAZ-independent regulation of pSMAD2/3 nuclear translocation in many cell lines, including polarized EpH4 cells (*66*). Rather, their data suggest that in polarized epithelial monolayers at high cell density, R-SMADs are not phosphorylated by TGFβ because the receptors are inaccessible to ligand by virtue of their basolateral location below tight junctions (Fig. 2). Wrana and colleagues alternatively suggested

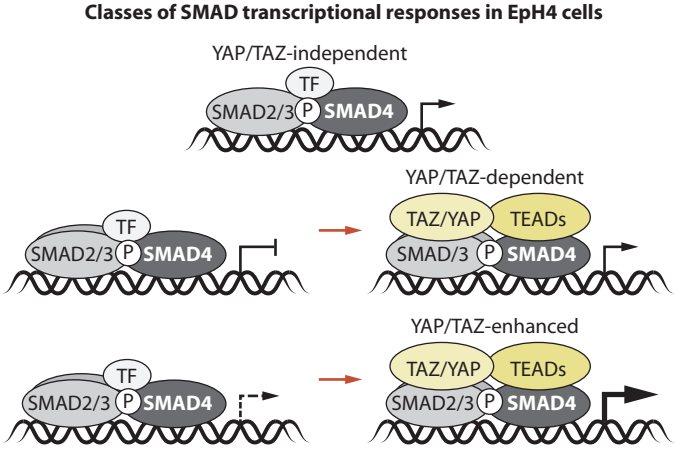

Fig. 4. pSMAD signaling can be dependent or independent of YAP/TAZ/TEAD activation. Schematic denotes possible modes of interaction between YAP/TAZ/TEAD and pSMAD2 at the level of transcription within EpH4 cells, as described by Attisano (with permission to quote unpublished concepts).

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that regulation of pSMAD3 nucleocytoplasmic distribution was biphasic. Early and transiently during the establishment of cell polarity, the Crumbs complex activates Hippo signaling to phosphorylate YAP/TAZ, forming a cytoplasmically restricted pYAP/pSMAD2 complex (*64*). Subsequently, relocalization of TGFBRs to basolateral surfaces prevents SMAD2/3 phosphorylation by apically applied ligand (*67*). An additional, and more widely accepted, model of YAP/TAZ regulation of TGFβ signaling output occurs at the transcriptional level (*68*). Taking an omics approach, Attisano further addressed this issue by analysis of SMAD2/3 transcriptional dependency on YAP/TAZ signaling in EpH4 cells genome-wide. She found that Hippo signaling differentially regulates expression of distinct subsets of SMAD target genes. Some genes show complete dependence on active YAP/TAZ for TGFβ transcriptional responses, others show TAZ-independent TGFβ responses, and, in a third category of genes, TGFβ-dependent effects are enhanced by active YAP/TAZ (Fig. 4). The rich contextuality and diversity of TGFβ signaling outputs are therefore demonstrated within a single polarized cell line in culture.

YAP/TAZ interaction with pSMADs in a transcriptional complex not only is limited to polarized epithelial cells but also occurs in nonpolarized epithelial cells and fibroblasts. In fibroblasts, Wrana and colleagues showed that matrix stiffness increases nuclear YAP/TAZ and promotes TGFβ signaling. Verteporfin, a destabilizer of YAP/TAZ, inhibited formation of both TEAD- and SMAD2/3-containing YAP/TAZ complexes, and decreased nuclear SMAD2 localization and transcriptional output. Moreover, in a unilateral uretral obstruction-induced kidney fibrosis model in vivo, YAP/TAZ forms a feed-forward loop with TGFβ/SMAD2/3 signaling to reinforce fibrosis, which can be attenuated by verteporfin. In pancreatic, ovarian, and mammary tumor models, Mythreye revealed that BMP4-SMAD1–driven EMT is dependent on YAP and matrix rigidity. Consistent with earlier reports on the role of CDK8 in mediating YAP/TAZ recruitment to SMADs (*21*), CDK8 and CDK19 are both required for BMP4-induced EMT because blocking CDK8/CDK19 kinase activities using Senexin B (*69*) abrogates BMP4-induced EMT. CDK8 and CDK19 appear to play specific roles only in mediating BMP-driven EMT responses because inhibition of cell proliferation by BMP4 is independent of these two kinases. Finally, Kapus and colleagues showed that myofibroblast differentiation, a major contributor to fibrosis and cancer progression, is regulated by interaction between TGFβ signaling and mechanosignal transduction. The prototypical myofibroblast gene α*SMA* is transcriptionally activated by myocardin-related transcription factor (MRTF). However, the gene promoter also harbors closely apposed recognition sites for TEAD and SMAD3/4. During wound healing or physical stretching, TAZ, MRTF, and TGFβ signaling all co-regulate αSMA expression, with TGFβ effects being dependent on TAZ signaling (*70*). In particular, TAZ and MRTF exhibit multilevel crosstalk, with TAZ having opposing effects on αSMA transcription under basal mechanosignaling conditions versus mechanosignaling in the presence of TGFβ. TAZ inhibits αSMA expression under the former condition but is indispensable for αSMA induction in the presence of TGFβ.

TGFβ signaling in human disease

As an endogenous regulator of tissue homeostasis, TGFβ maintains and/or reestablishes tissue integrity after damage or stress. TGFβs suppress epithelial, endothelial, and stem/progenitor cell proliferation and can induce differentiation and apoptosis. It stimulates epithelial migration around wound margins and limits inflammatory responses. During normal development and in pathological situations, growth inhibitory responses to TGFβ can be subverted toward an epithelial- or endothelial-to-mesenchymal transformation (EMT or End-MT) that contributes to disease progression (Fig. 2). The molecular mechanisms of EMT and End-MT have been examined in depth because they might be targets for drug development to treat

the common killers, fibrosis and cancer. Bailly and keynote speaker Dejana also showed that End-MT contributes to less common diseases that involve tissue remodeling, such as development of patent ductus arteriosus of the heart (*71*) and cavernous vascular malformations in the brain (*72*). Christofori and colleagues showed that during TGFβ-induced EMT of mammary epithelial cells and murine breast cancer cells, increased expression of the Hippo transcription factor TEAD2 caused increased nuclear TEAD2/YAP/TAZ complex formation that drove an EMT transcription program to stimulate invasion and migration (*73*). Their genome-wide ChIP-seq analysis revealed Zyxin, a mechanosensor, as a positive target of the TEAD2 transcription factor during EMT. Increased Zyxin expression contributed to cell invasion and migration. Moreover, Luo's laboratory showed that SKI antagonizes TGFβ signaling and EMT by acting on multiple components of the Hippo signaling pathway to suppress YAP/TAZ and TEAD signaling (*74*).

A number of molecular changes have been reported to flip the molecular switch between TGFβ-induced cytostasis and EMT. Both Massagué and Christofori and their colleagues reported the pivotal role of the homeodomain transcription factor Sox4 in supporting tumor progression through context-specific action on EMT (*75*). In premalignant pancreatic progenitor cells that express activated KrasG12D, Massagué and colleagues showed that activation of both Snail and Sox4 by TGFβ is incompatible with life, resulting in apoptosis because of the competing actions of Snail in driving EMT versus Sox4, which supports maintenance of an epithelial transcriptional program. In this cancer type, tumor progression requires genetic loss of either *SMAD4* or *SOX4* to suppress apoptosis (*76*). In contrast, Christofori and colleagues showed that in mammary epithelial cells, TGFβ-induced Sox4 is indispensable for EMT and cell survival, because this transcription factor directly regulates Ezh2 to control global epigenetic silencing of a network of genes necessary for EMT. Accordingly, combined high expression of Sox4 and Ezh2 correlates with metastatic progression and poor clinical outcome in breast cancer patients. Conversely, the loss of *KLF4* expression, which normally drives an epithelial cell transcriptional profile and suppresses JNK1 (MAPK8), is required for EMT and metastasis (*77*). Khew-Goodall and colleagues described a balance between epithelial and mesenchymal phenotypes of mammary epithelial cells by negative feedback between the miRNA miR-200 and TGFβ-Zeb transcription that mediate epithelial and EMT programs, respectively (*78*, *79*). Knockdown of miR-200 in HMLE cells reduced EMT, stem cell markers, and mammosphere formation in vitro. Human breast cancer cells isolated from patient biopsies and fluorescence-activated cell sorting–enriched for stem cell markers were relatively depleted in miR-200. miR-200 targets a network of genes that promote EMT, and TGFβ induces epigenetic changes in the two genomic loci encoding miR-200 clusters; repressive H3K27me3 histone methylation marks were induced within the chromosome 1 miR-200 locus, whereas the H3K4me4 activation tag was suppressed within the miR-200 cluster on chromosome 12. In another cancer model, Miyazono and colleagues demonstrated that TTF-1 suppresses EMT in lung cancer by competing with SMAD4 for SMAD3 binding, thus inhibiting SMAD3/4 transcriptional responses, with genome-wide effects on SMAD3 targets (*80*).

It is well established that several cancer types demonstrate preponderance for somatic genetic loss of TGFβ signaling components, such as *DPC4/SMAD4* in pancreatic cancer and *TGFBR2* in colon tumors with microsatellite instability (*81*). But how common TGFβ signaling loss is in other tumor types, whether by genetic or epigenetic means, is debatable and has implications for TGFβ-induced EMT as a driver of tumorigenesis. Inman and colleagues presented evidence for somatic mutation and consequent loss of SMAD signaling by *TGFBR1* and/or *TGFBR2* as a common event in human cutaneous squamous cell carcinoma (cSCC), a tumor type with a heavy ultraviolet-induced mutational load. About 70% of *TGFBR1/TGFBR2* mutations were nonsynonymous point mutations that were predicted and/or demonstrated to show loss of function. It remains to be determined if any retain some activity or even gain new activities [see later about fibrodysplasia ossificans progressiva (FOP)]. In a *Hras*-driven mouse skin tumor model, Oshimori and colleagues used a lentiviral SBE-reporter and found heterogeneous expression of TGFβ signaling within cSCCs in vivo. Tumor cells that exhibited high TGFβ signaling were enriched for stem cell properties including $CD34^+$ expression, and they exhibited slower growth but higher tumor-initiating ability. These "stem cells" were resistant to cisplatin treatment because of an NRF2-mediated elevation in enzymes of glutathione metabolism, revealing a novel mechanism of TGFβ-mediated cancer cell drug resistance, independent of TGFβ effects on cytostasis (*82*). In an emerging research area, Aguirre-Ghiso and colleagues examined mechanisms of tumor dormancy of disseminated tumor cells (DTCs), with the goals of understanding how DTCs survive to successfully eradicate them and/or to induce malignant cells to acquire an extended dormant state. They showed that TGFβ2 and Coup-TFI, which are both activated by all-trans retinoic acid, work through independent but cooperative pathways to enhance dormancy. They suggest that therapies that activate the TGFβ2 isoform and/or Coup-TFI, such as retinoic acid and 5′-azacytidine, might extend life by inducing therapeutic tumor cell dormancy (*83*, *84*).

Studying model organisms can offer insights into human biology and molecular pathology of human disease, as shown by Wharton and colleagues (*30*). Conversely, dissecting the molecular pathological causes of rare human diseases not only has potential for remediating symptoms for these few patients but also may reveal novel molecular mechanisms of signal transduction and possible relevance to more common diseases. Both Bullock and Economides and their respective colleagues undertook elegant molecular studies into the molecular pathology of FOP (OMIM 135100). This progressive, morbid, and lethal disease leads to widespread irreversible heterotopic ossification (HO) of muscle throughout the body, as a result of a recurrent R206H mutation within the cytosolic GS domain of ACVR1/ALK-2, as well as other less prevalent mutations in this protein. ALK-2 is a BMP receptor, and structural biology studies used by the Bullock laboratory show that these mutations would break open the inhibitory conformation of the cytosolic domain, allowing hyperactivation of the BMP/ALK-2 pathway (*85*). He noted that identical *ACVR1* somatic mutations were recently identified in diffuse intrinsic pontine glioma (DIPG), a lethal cancer of young children (*86*). Economides and colleagues pushed studies on the R206H FOP mutation one step further, showing that this mutation enables ALK-2 to use activin A as an agonistic ligand. They [and Olsen and colleagues (*87*)] showed that activin A normally antagonizes BMP signaling through ALK-2. In contrast, mutant ALK-2.R206H perceives activin A as an agonist that, in BMP-like fashion but requiring ACVR2A/2B type II receptors, drives R-SMAD1/5 signaling. Blocking antibodies against activin A blocked HO in an inducible mouse knock-in model of FOP, mimicking the effects of a small-molecule ALK-2 kinase inhibitor, LDN-212854, and indicating that the acquired activin A responsiveness via this normally activin A–unresponsive receptor is necessary and sufficient to drive HO in FOP (*88*). Anti–activin A antibodies might therefore be used to treat FOP or DIPG patients carrying ALK-2.R206H mutations. These findings highlight the concept of neofunction of mutant receptors driving disease processes and raise the question of how an intracellular mutation in the receptor can qualitatively alter the response to ligand.

Therapeutic horizons

Showcasing the challenges and context specificity of TGFβ inhibitor drug use for oncology, Wakefield and colleagues demonstrated that a pan-TGFβ ligand antibody decreased tumor metastasis in 3 of 12 distinct immunocompetent mouse models of breast cancer after orthotopic implantation,

but enhanced metastasis in a further 3 of the 12. The challenge will therefore be to identify and select patients who would benefit from TGFβ antagonism, avoiding those with potential prometastatic responses (*89*). Wakefield and colleagues showed that a variety of classical TGFβ signaling "biomarkers," such as ligand or pSMAD2 levels, were uninformative as response predictors. However, transcriptomic analysis of tumors before treatment revealed a gene expression signature that showed potential as an indicator of drug responses. Mirza presented the development of a novel panel of humanized anti-ligand blocking antibodies with differential ligand specificities and strong target binding affinities. He demonstrated that an anti–pan-TGFβ antibody was efficacious when tested in the immunodeficient Detroit 562 xenograft model of pharyngeal carcinoma. Similar findings were made by Meyer, Akhurst, and colleagues using the same drug in an immunocompetent cSCC isograft model. TGFβ1 is clearly a major driver of protumorigenic signaling activities, but whether it is necessary or desirable to target TGFβ1, TGFβ2, and/or TGFβ3 in some tumor types or stages remains to be thoroughly assessed. Blocking TGFβ2 in breast cancer patients in remission, for example, would be counterindicated by Aguirre-Ghiso and colleagues' findings on tumor dormancy (*84*).

Blobe and Henis and their colleagues showed that levels of the TGFβ type III receptor, betaglycan, decrease during tumor progression, leading to reduced extracellular release of soluble betaglycan, an endogenous TGFβ inhibitor (*90*–*92*). This elevation in active TGFβ levels and consequent enhanced local immunosuppression (*92*) suggest that TGFβ blockade may complement tumor vaccines or other immunotherapies. This notion was also supported by data from Akhurst's laboratory using a chemically induced SCC model.

Clinical progress with TGFβ antagonism for oncology has been hampered by the fear of blocking a tumor suppressor or reactivating dormant cells and by the pleiotropic activity of this signaling pathway. However, phase 1 clinical trials of anti-ligand antibodies and small-molecule receptor kinase inhibitors have not shown severe adverse effects (*93*, *94*). Meyer and colleagues noted that when used in the right combinations with other anticancer agents, these drugs may show powerful anticancer effects. Personalized therapy will be essential to find patients likely to benefit from such therapy, but this remains to be comprehensively investigated. Although most companies focus on blocking TGFβ signaling, there is interest in activating TGFβ ligands for the treatment of autoimmune diseases or for the suppression of organ transplant rejection or graft-versus-host disease. Schürpf presented studies revealing the first TGFβ1-activating drug to be developed on the basis of elucidation of the molecular structure of TGFβ latency and activation (Fig. 3). This drug, an antibody, is specific for latent TGFβ1, not affecting activation of TGFβ2 or TGFβ3.

With the hope of more specific and predictable drug responses, drugs are in development that target less pleiotropic molecules and/or cellular or molecular processes required for TGFβ signaling. Sheppard and colleagues demonstrated that integrins $\alpha_v\beta_6$, $\alpha_v\beta_8$, and $\alpha_v\beta_1$ are functionally dedicated to activation of latent TGFβ1 and TGFβ3. Integrin $\alpha_v\beta_6$ expression is restricted to epithelial cells, whereas $\alpha_v\beta_1$ is the major integrin on lung fibroblasts that activates TGFβ in the setting of tissue fibrosis. $\alpha_v\beta_6$ expression is increased on epithelial cells of patients with idiopathic pulmonary fibrosis (IPF), a disease that kills as many people per year as breast cancer. On the basis of this knowledge, Sheppard and colleagues developed both large- and small-molecule drugs targeting TGFβ-activating integrins. STX-100, an anti-$\alpha_v\beta_6$ integrin antibody, was shown to reduce bleomycin-induced lung fibrosis (*95*) and kidney fibrosis in a mouse model of Alport's syndrome (*96*). A small-molecule $\alpha_v\beta_1$ inhibitor potently inhibited fibroblast-derived TGFβ activation and reduced chloroform-induced liver fibrosis and bleomycin-induced lung fibrosis in mice (*97*). $\alpha_v\beta_8$ integrin is preferentially expressed on immune dendritic cells and T cells, especially regulatory T cells (T_{regs}), where it may regulate immune tolerance through autocrine activation of latent TGFβ1, which in T_{regs} is covalently bound to glycoprotein A repetitions predominant (GARP) rather than LTBP in a large latent complex (*98*, *99*). Stockis and colleagues presented data showing that anti-GARP blocking antibodies could attenuate TGFβ activation and thus the immunosuppressive effects of TGFβ1 on T_{regs}, with potential use as immunotherapy in oncology (*100*).

On the basis of Leof's fundamental studies on regulation of SMAD signaling via the early endosome (*33*, *37*), his laboratory developed an ingenious tool to specifically target pSMAD3 but spare pSMAD2 signaling. Such targeting could provide an opportunity to develop intervention strategies to enhance or dampen specific aspects of the cellular response to TGFβ. It was previously found, for example, that SMAD3 and SMAD2 have opposing effects in models of skin cancer and kidney fibrosis, with SMAD3 being the protumorigenic, profibrotic partner (*101*, *102*). To this end, he reported that sorting nexin 9 (SNX9) bound pSMAD3 (but no other phosphorylated co-receptor SMADs) via the SNX9 SH3 domain. This interaction, together with C-terminal binding of SNX9 to importin 8 (IMP8), was essential for pSMAD3 nuclear import with no effect on pSMAD2. They subsequently developed a TAT-SH3 domain peptide that competes with pSMAD3 for SNX9 binding. Addition of this peptide to cultured cells prevents nuclear import of SMAD3 (but not SMAD2), expression of SMAD3-dependent target genes, and TGFβ-induced cell migration in a two-dimensional wound in vitro assay. Current studies are testing whether similar efficacy can be obtained in vivo.

Drug development is also under way to target other components of the TGFβ signaling family, with applications in various diseases. Blobe and Bocci and their colleagues described the efficacy of soluble anti–ACVRL1/ALK-1 (dalantercept) to inhibit tumor angiogenesis and metastatic seeding in vivo (*103*, *104*), whereas Kumar and colleagues reported on the development of luspatercept for the treatment of anemia consequent to thalassemia or myelodysplastic syndrome (*105*). The latter drug, a soluble activin type 2B receptor fused to human IgG1 Fc, blocks activin B, GDF8 (myostatin), and GDF11 action on blood cells to reduce SMAD2/3 signaling and rectify ineffective erythropoiesis. The robust pro-erythropoietic effects of luspatercept could not be achieved by inhibition of any one of the aforementioned ligands but only by combined inhibition of all three. Intriguingly, Lee and colleagues from Harvard reported an alternative positive role for GDF8 and/or GDF11 in protection from cardiac aging, a major factor in systolic heart failure for which there is no current treatment. They found that old mice develop left ventricular hypertrophy that can be reversed by parabiosis with young mouse blood or by exogenous GDF11 (*106*). Lee's collaborators at the University of California at San Francisco found that in humans, GDF8/11 levels fall during aging. Over 10 years, they followed GDF8/GDF11 levels in >850 aging patients with cardiac issues and found that low circulating GDF8/11 levels at study entry was a powerful predictor of heart disease mortality within the ensuing 8 years (*107*). GDF11 may have relevance for other organs, for example, it is active within the aging brain (*108*). The reports of Kumar and Lee and their colleagues illustrate the varied biology that may arise from the manipulation of one or more ligands of the TGFβ superfamily, and raise the issue of which ligand or ligands should be targeted or infused to treat anemia or cardiac/cognitive health in geriatrics (*109*).

Emerging Areas and Future Directions

Much has been learned since the discovery of TGFβ in the early 1980s, but plenty remains open to further exploration, including the role of TGFβ in regulation of metabolism, prevention of aging, stem cell technologies, regenerative medicine, and epigenetics. There is considerable knowledge of

the role of TGFβ within the immune system, but the impact of TGFβ- targeted immunomodulation to treat autoimmune diseases, prevent transplant rejection, enhance stem cell therapies, or potentiate cancer immunotherapy will require more detailed understanding of cellular and molecular mechanisms—an important and emerging area of investigation. Given the plethora of ligands widely expressed in human tissues, and that these and their signaling components are either the root cause or at least contribute significantly to many disease states, it is important to learn how to regulate their signaling pathways. To date, most studies have focused on the eponymous TGFβ ligands in disease (TGFβ1, TGFβ2, and TGFβ3), but there is emerging interest in the role of BMPs and other superfamily ligands in fibrosis and cancer (*110*–*113*). One important challenge is to modulate one aspect of TGFβ signaling while leaving other signaling components unaffected. As evidenced by this meeting, scientists are beginning to find ways to achieve this goal. This will require a deeper understanding of the nuances of this signaling pathway and how pathological processes might perturb signaling in unexpected ways (*7*, *88*, *114*). In cancer, the ability to target TGFβ tumor-promoting activity but spare effects on tumor suppression has been an area of intense investigation, and one that will depend on tumor type, stage, tumor genomics, and features of the tumor microenvironment. In oncology, it is accepted that combinations with other drugs will be essential for successful anti-TGFβ therapy (*115*), and optimizing the best combinations is under way preclinically. TGFβ signaling represents the archetypical context-dependent signaling pathway; thus, development of drugs that target TGFβ pathways will not only require detailed understanding of molecular mechanisms of regulation but also depend heavily on the application of personalized medicine, including development of biomarkers and predictive signatures (*116*–*118*).

SUPPLEMENTARY MATERIALS

www.sciencesignaling.org/cgi/content/full/8/399/re10/DC1
Movie S1. Dynamics of latent BMP9 conformation.

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