

JB Review

New insights into extracellular and post-translational regulation of TGF- β family signalling pathways

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Members of the transforming growth factor- β (TGF- β) family of secreted proteins are present in all multicellular animals. TGF- β proteins are versatile intercellular signalling molecules that orchestrate cell fate decisions during development and maintain homeostasis in adults. The Smad family of signal transducers implements TGF- β signals in responsive cells. Given the ability of TGF- β ligands to induce dramatic responses in target cells, numerous regulatory mechanisms exist to prevent unintended consequences. Here we review new reports of extracellular and post-translational regulation in *Drosophila* and vertebrates. Extracellular topics include the regulation of TGF- β signalling range and the coordination between tissue morphogenesis and TGF- β signalling. Post-translational topics include the regulation of TGF- β signal transduction by Gsk3- β phosphorylation of Smads and by cycles of Smad mono- and deubiquitylation. Extension of the ubiquitylation data to the Hippo pathway is also discussed.

Keywords: BMP/facilitated transport/phosphorylation/Smad/ubiquitylation.

Abbreviations: BMP, bone morphogenetic protein; Dpp, decapentaplegic; Gbb, glass bottom boat; GFP, green fluorescent protein; Gsk3, glycogen synthase kinase3; Sog, short gastrulation; TGF- β , transforming growth factor- β ; Tsg, twisted gastrulation; Zw3, zeste white3.

Intercellular signalling is essential for proper development of all multicellular organisms. In animals, developmental signalling proteins in the transforming growth factor- β (TGF- β) family perform a multitude of tasks. The family contains two large subfamilies: the decapentaplegic/bone morphogenetic protein (Dpp/BMP) and TGF- β /activin/nodal. Mutations disrupting signal transduction pathways downstream of TGF- β proteins are often found in tumours.

The multifunctional Smad family of signal transducers contains three subfamilies: R-Smads are dedicated to one of the two TGF- β subfamilies, Co-Smads (Smad4 in vertebrates or Medea in flies) facilitate signalling for all TGF- β superfamily members and I-Smads antagonize TGF- β superfamily signals (1).

A general model for TGF- β signal transduction begins with a complex of transmembrane receptor serine–threonine kinases. The Type II receptor is a constitutively active kinase that binds the TGF- β ligand. Subsequently the Type II receptor recruits and phosphorylates the Type I receptor. This stimulates the Type I receptor to phosphorylate its cognate R-Smad. Upon phosphorylation, an R-Smad seeks out and binds the Co-Smad and then translocates to the nucleus. The R-Smad:Co-Smad complex then participates in the regulation of target genes (2). This basic scenario is shared by the TGF- β /Activin/Nodal and Dpp/BMP subfamilies, although each uses a different set of R-Smads (Smad2/3 or Smad1/5/8, respectively, in vertebrates).

Here we review new reports of extracellular and post-translational mechanisms that modulate the pleiotropic functions of TGF- β family members. From the extracellular perspective, studies in *Drosophila* describe an experimental system where functional Dpp ligands can be visualized, allowing researchers to conduct quantitative analyses of Dpp movement. From the post-translational perspective, studies using a variety of model systems describe two highly conserved mechanisms: phosphorylation of R-Smads by the Wnt-associated kinase Gsk3- β and cycles of mono- and deubiquitylation affecting Co-Smads.

Extracellular Regulation of Dpp Distribution

The spatial distribution of a Dpp/BMP ligand after secretion is regulated extracellularly such that concentration gradients can be created within a developing tissue. Cells then respond differently depending on the concentration of the ligand in their immediate environment. Ligands that specify cell fate in a concentration-dependent manner are termed morphogens. One of the best-studied examples of a morphogen gradient is that of Dpp (homolog of vertebrate BMP2/4) in *Drosophila* wing development in a tissue known as the wing imaginal disc.

Dpp long-range signalling in the wing imaginal disc

During larval stages, *dpp* is transcribed along the anterior–posterior compartment boundary of the wing disc. When secreted from this central location Dpp protein then forms a long-range morphogen gradient

in both directions (Fig. 1A). This gradient organizes pattern and growth of the larval disc (3). Analyses using functional GFP-tagged Dpp in wing discs provided direct evidence that Dpp ligands move through the extracellular space to form a concentration gradient (4, 5).

Several transmembrane proteins and secreted proteins cooperate in the extracellular regulation of Dpp's spatial distribution and thus in the formation of the concentration gradient in the wing disc. For

example, the Dpp Type I receptor Thickveins plays a critical role in regulating Dpp distribution (6). *thickveins* is transcribed at low levels in the centre of the wing disc but is highly transcribed in lateral regions. The low level of Thickveins in the centre, where *dpp* is transcribed and secreted, allows Dpp to diffuse easily. The high level of Thickveins at the periphery, where Dpp concentration is low, increases the sensitivity of cells to Dpp therefore contributing to long-range signalling (Fig. 1A). In the *Drosophila* haltere, a

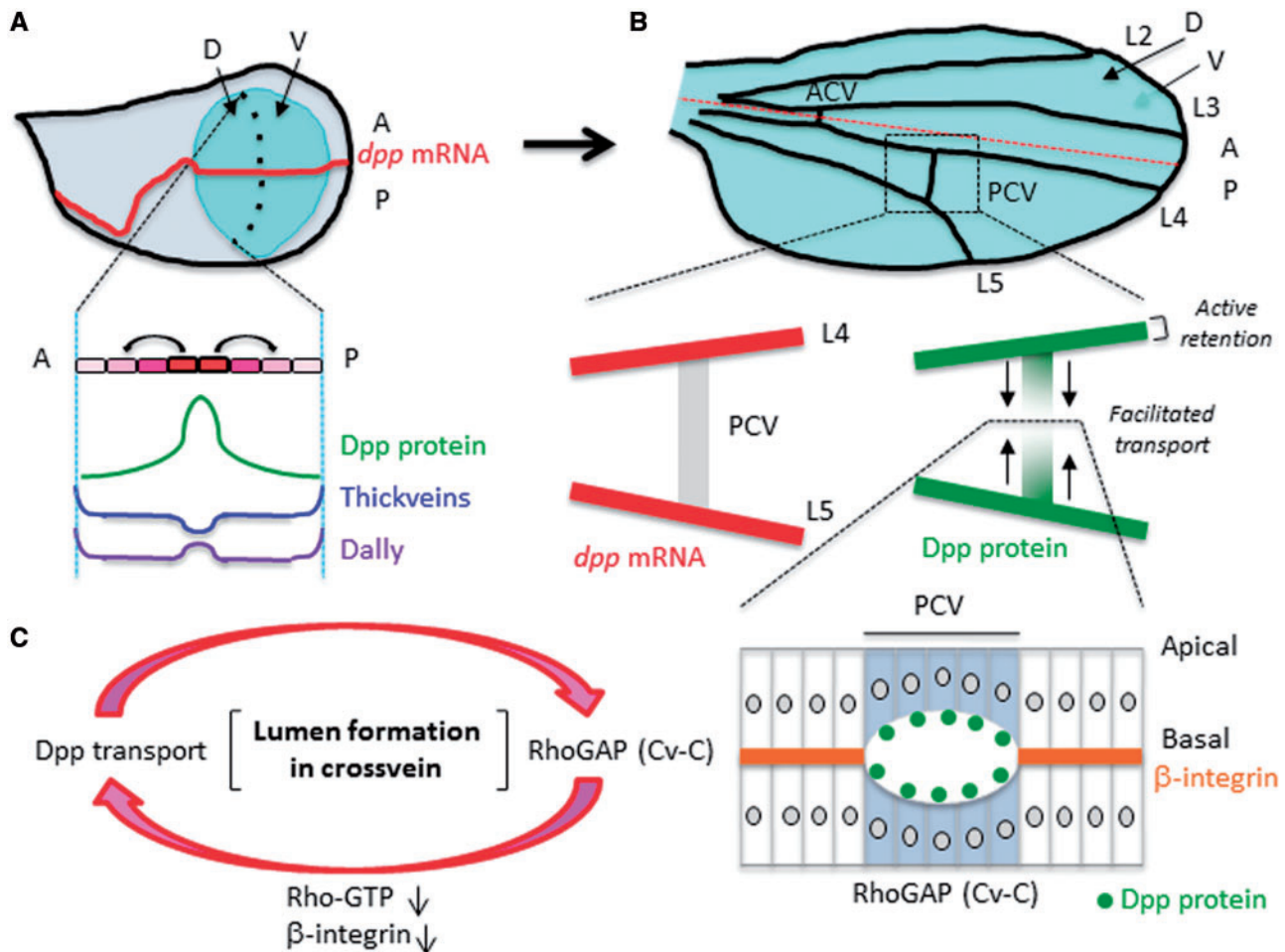


Fig. 1 Spatial distribution of Dpp is regulated by distinct extracellular mechanisms. (A) Long-range Dpp gradient in the *Drosophila* larval wing imaginal disc. Top: schematic model of the third-instar wing imaginal disc. The boundary between dorsal (D) and ventral (V) compartments of wing pouch (light blue) is shown by dashed line (black). *dpp* mRNA is expressed at the anterior (A)–posterior (P) compartment boundary (red stripe). Bottom: wing disc is composed of single-layered epithelial cells. Dpp is secreted from the cells at the anterior–posterior boundary (marked by bold lines), moves outward in both directions (black arrows) and its movement is regulated by extracellular and transmembrane proteins. Schematic view of protein distribution of Dpp (green), Thickveins (blue) and Dally (purple) from anterior to posterior in the wing pouch. Interactions between these three proteins result in a bilaterally symmetrical gradient of Dpp concentration (5). (B) BMP signalling in the *Drosophila* pupal wing is achieved by a blend of long- and short-range signalling. Top: schematic figure of the adult fly wing which is composed of two cell layers—dorsal (D) and ventral (V) epithelial cells (shown by arrows). The boundary between anterior (A) and posterior (P) compartments is shown by dashed line (red). L2–L5, ACV and PCV denote longitudinal veins 2–5, anterior and posterior crossveins, respectively. Bottom: schematic model of Dpp signalling for posterior crossvein formation during pupal stages. Left: *dpp* mRNA (red) is expressed in longitudinal veins (LVs) but not in the presumptive posterior crossvein region (PCV, light grey) during early pupal stages. Right: short-range Dpp (green) movement in longitudinal veins is restricted by active retention. Dpp movement into the presumptive posterior crossvein region requires long-range facilitated transport (arrows) (16). (C) Dpp signalling and posterior crossvein morphogenesis in pupal wings. Left: model of a feed-forward loop coupling Dpp transport and posterior crossvein formation. Upper arrow shows that RhoGAP Crossveinless-C (Cv-C) expression is upregulated by Dpp signalling in the presumptive posterior crossvein region. Lower arrow shows that Crossveinless-C then downregulates integrin accumulation on the basal surface of the epithelial bilayer, through inactivation of the Rho GTPases. Loss of integrin provides an optimal extracellular environment for facilitated Dpp transport (22). Right: cross-section through the presumptive posterior crossvein region viewed in the direction of the arrows in the bottom right section of B. Dpp (green) accumulates on the basal surface of wing epithelial bilayer cells that have lost integrin (orange) expression via facilitated transport. Posterior crossvein formation proceeds in parallel as cells without integrins lose their cell adhesion allowing a lumen to form along their basal surface.

flight appendage with a similar structure to the wing, *thickveins* is highly transcribed in the centre of the haltere disc. This serves to keep the ligand from diffusing very far and results in smaller size (7).

In addition to the Type I receptor, the cell-surface heparan sulfate proteoglycan Dally regulates Dpp distribution and signalling in the larval wing disc (8). Dally fosters the spatial expansion of Dpp by stabilizing it and regulating its diffusion. Dally also functions to facilitate long-range Dpp signalling by sensitizing signal transduction as a co-receptor. Recent studies suggest that Pentagone, a novel secreted protein, plays a critical role in the maintenance of long-range Dpp signalling in the wing disc (9). *pentagone* is a transcriptional target of BMP signalling and is highly expressed in lateral regions of the wing disc. Pentagone physically interacts with Dally to promote the spread of Dpp ligands.

Dpp gradient formation in the early embryo

Dpp also serves as a morphogen during dorsal–ventral axis formation in *Drosophila* embryonic development. Genetic analysis revealed that seven genes are required

for proper cell fate determination in the dorsal half of the *Drosophila* embryo. These are *dpp*, *screw*, *short gastrulation*, *twisted gastrulation*, *tolloid*, *shrew* and *zerknüllt* (10). Five of these genes encode secreted proteins: two Dpp/BMP subfamily ligands (Dpp and Screw), one protease (Tolloid) and two Dpp/BMP subfamily-binding proteins [short gastrulation (Sog; homolog of vertebrate Chordin) and twisted gastrulation (Tsg)]. Dorsal-most and dorsal–lateral cells decide their fate based on a concentration gradient of secreted Dpp and Screw proteins. Since both *dpp* and *screw* are ubiquitously expressed in the dorsal half of the embryo, the spatial distribution of these ligands must be regulated extracellularly. Biochemical studies have shown that Dpp and Screw form a heterodimer that is the primary morphogen and that this heterodimer is transported by a Sog:Tsg complex from lateral regions to the dorsal-most region (Fig. 2). In the dorsal-most region, the protease Tolloid cleaves and inactivates Sog in a Dpp:Screw-dependent manner. This frees the ligands, concentrated in this region by Sog:Tsg facilitated transport, to bind to receptors for maximal signalling (11). Sog is transcribed and

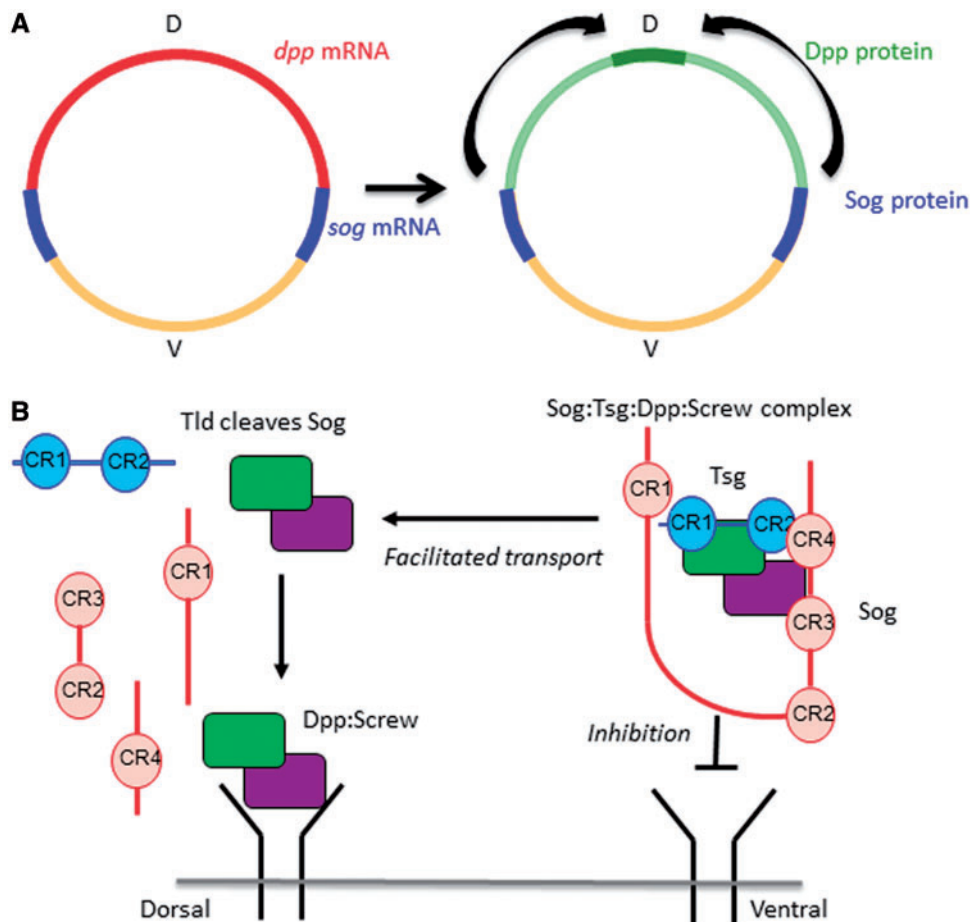


Fig. 2 Dpp gradient in the *Drosophila* early embryo. (A) Schematic cross-section of an early embryo. Dorsal (D) is up and ventral (V) is down. Left: *dpp* mRNA is expressed in the entire dorsal half of the embryo (red), while *sog* mRNA is expressed only in ventral lateral cells (blue). Right: diffusion of Sog (blue) towards the dorsal-most region (arrows) facilitates the transport of Dpp protein (green) leading to a graded distribution of Dpp. (B) Schematic model of facilitated transport. The solid grey line at the bottom represents the cell membrane with the angled region of the Dpp receptors (black lines) outside the cell. A Dpp:Screw heterodimer (green, purple) is transported by the Sog:Tsg complex (red, blue; CR: cysteine-rich domain from ventral regions to the dorsal-most region where the Sog:Tsg complex is destroyed by Tolloid). This allows the Dpp:Screw heterodimer to bind to its receptor complex (11).

secreted from the ventral–lateral region and then diffuses dorsally providing directionality for Sog:Tsg transport of Dpp:Screw. Recent studies showed that type IV collagen also plays a critical role in the formation of this morphogen gradient via interactions with Dpp:Screw (12).

Intriguingly, the regulation of Dpp/BMP signalling by Dpp/BMP binding proteins is widely observed in metazoans. Animals from simple sea anemones to humans use highly conserved signalling pathways containing Dpp and Sog or BMP2/4 and Chordin. In addition to *Drosophila*, this conserved facilitated transport machinery has been studied extensively during dorsal–ventral axis formation in the frog *Xenopus laevis* and in the beetle *Tribolium castaneum* (13, 14). These studies clearly showed that homologous transportation mechanisms for Dpp/BMP ligands facilitate long-range signalling during development in evolutionarily diverse animals.

In contrast to the long-range morphogen gradients described in the two examples above, Dpp can also act in a short-range manner. For example, Dpp signals maintain *Drosophila* germline stem cells in a pluripotent state. Movement away from the germline niche cells that secrete Dpp is limited to one to two cell diameters, just enough to reach the adjacent germline stem cells. Restriction of Dpp range involves extracellular interactions with collagen IV and heparan sulfate proteoglycans (12, 15).

Dpp signalling in wing vein development

Recent studies show that *Drosophila* wing vein development is also an excellent model for analyzing the spatial distribution of Dpp ligands. After its roles in patterning and growth of the larval wing disc, Dpp functions as a wing vein determinant in early pupal development. During pupal stages Dpp is required to maintain the fate of longitudinal veins and to induce crossvein formation. Although *dpp* is only transcribed in longitudinal veins, Dpp activity is detected in all primordial veins including crossveins. Visualizing GFP-Dpp in the pupal wing demonstrated that GFP-Dpp moves from longitudinal veins into the region where the posterior crossvein will develop. In contrast to Dpp long-range movement into the presumptive posterior crossvein region, the majority of secreted Dpp is immobilized near the longitudinal veins where they function in short-range signalling.

The immobilization mechanism, designated ‘active retention’, is critical for establishing longitudinal vein width. Dpp mobility is tightly restricted in longitudinal veins by its Type I receptor Thickveins and further restricted via a positive feedback loop using Dpp target genes (16). Thus, short-range Dpp signalling in longitudinal veins and long-range Dpp movement and signalling in crossveins are required to form the normal wing vein pattern (Fig. 1B). Genetic analyses identified several components of the molecular machinery facilitating Dpp movement into the posterior crossvein region: Glass bottom boat (Gbb; a Dpp/BMP subfamily member), the protease Tolloid-related and the Dpp/BMP binding proteins Sog and Crossveinless. The latter is a paralog of Tsg (17, 18).

Similar to the early embryo, posterior crossvein formation requires the facilitated transport of ligands. Here a Dpp:Gbb heterodimer produced in longitudinal veins is moved into the posterior crossvein region by a Sog:Crossveinless complex. Then Dpp:Gbb is released from the Sog:Crossveinless complex by the activity of the Tolloid-related protease. The direction of Dpp:Gbb transport appears to be, at least partially, based on the direction of movement of Sog (16). The extracellular protein Crossveinless-2 contains an N-terminal cysteine-rich domain and a C-terminal von Willebrand Factor domain. This protein is also required for Dpp movement into the posterior crossvein region and to sustain Dpp short-range signalling (19). *crossveinless-2* is transcriptionally regulated by Dpp in the posterior crossvein region and thus functions as part of a feedback loop that is critical for posterior crossvein formation. Crossveinless-D encodes a vitellogenin-like lipoprotein that also regulates posterior crossvein formation by modulating Dpp movement as part of a lipid–Dpp–lipoprotein complex (20).

Taken together, these findings clearly show that the range of Dpp movement is tightly regulated to maximize either long- or short-range signalling depending on the cellular context. They also indicate that highly conserved, extracellular mechanisms of facilitated transport are among the primary processes governing the spatial distribution of Dpp/BMP ligands.

Coordination between Dpp Signalling and Tissue Morphogenesis

Tissue morphogenesis often coincides with arrival of extracellular signals. However, little is known about the mechanisms that coordinate extracellular signalling and the dynamic process of morphogenesis. In one example, an investigation of *Drosophila* renal tubule morphogenesis in the embryo found that hemocytes secrete basement membrane components including type IV collagen around the growing tubules (21). As the tubules elongate, type IV collagen is critical for the sensitivity of tubule cells to Dpp signals that act as guidance factors to promote proper anterior projection. In the absence of hemocytes or collagen IV, Dpp signalling fails and tubules do not project anteriorly. In this case, Dpp signalling modulates morphogenesis.

In other contexts, morphogenesis modulates the spatial distribution of Dpp proteins. Analyses of posterior crossvein development in *Drosophila* revealed that the spatial distribution of Dpp is coordinated with wing morphogenesis. All wing veins in *Drosophila* are formed by deformation of the basal surfaces of two apposed epithelial cells with different adhesive properties than their neighbours. As noted above Dpp moves outward from its source in the longitudinal veins and accumulates in the posterior crossvein region. Detailed studies showed that in this region Dpp is located preferentially along the basal surfaces of the epithelial bilayer that constitutes a wing disc (Fig. 1C), suggesting that Dpp transport to this specific location is associated with crossvein morphogenesis (22).

The RhoGAP protein encoded by *crossveinless-C* was found to be the key molecule that couples Dpp transport and crossvein morphogenesis. Crossveinless-C is induced in posterior crossvein precursor cells by Dpp signalling where it cell-autonomously inactivates signalling from several Rho-type small GTPases. This leads to the cell-autonomous downregulation of Rho GTPase targets such as integrins (cell adhesion proteins). Intriguingly, Crossveinless-C is also required non-cell-autonomously for Dpp transport into the posterior crossvein region. The cellular distribution of integrins appears to be essential for Dpp transport, since downregulation of integrins on the basal side of posterior crossvein epithelial cells provides an optimal extracellular environment for facilitated Dpp transport. These data indicate the presence of a feed-forward loop through which posterior crossvein morphogenesis and Dpp transport are coupled (Fig. 1C).

This coupling mechanism allows the precise distribution of extracellular ligands along dynamic morphogenesis without restricting signal reception by cells. Simultaneously, the positive feedback mechanism facilitates continuous signalling to the target cells. Similar mechanisms using Dpp/BMP subfamily ligands are likely to play a significant role in coordinating signalling and tissue morphogenesis during development in many species.

Multiple Outcomes Downstream of Smad Linker Phosphorylation by Gsk3- β

The role of phosphorylation in TGF- β signal transduction is well known. The first report of non-C-terminal R-Smad phosphorylation noted that Erk-MAP kinase phosphorylation in the linker region antagonizes C-terminal activation (23). Subsequent studies showed that additional kinases phosphorylate this region with the same outcome. These include Gsk3- β in the Wnt pathway (24, 25) and the Cdk8/Cdk9 cyclin-dependent kinases (26). Follow-up data indicate that Cdk8/Cdk9 linker phosphorylation signals for Nedd4L ubiquitin ligase stimulated degradation (27). Here we describe studies associated with linker phosphorylation by Gsk3- β , including a recent report suggesting that there are outcomes besides degradation.

The first hint that R-Smad linker phosphorylation might have an association with the Wnt pathway is found in a phylogenetic analysis of the linker region (1) that identified conserved Gsk3- β (serine-threonine kinase) sites in all R-Smads belonging to the Smad1/5/8 subfamily (dedicated to BMP signalling). Gsk3- β is a well-known kinase participating in the Wnt pathway where it antagonizes the transcriptional activity of the transcription factor β -catenin by targeting it for polyubiquitin-mediated degradation. The analysis ended with a prediction that Smad1/5/8 linker phosphorylation by Gsk3- β was a mechanism of Smad–Wnt interaction, a prediction that was soon experimentally confirmed.

In mammalian cells and *Xenopus* embryos, two studies showed that Wnt signalling inhibited Gsk3- β phosphorylation of Smad1 leading to Smad1

stabilization. In the absence of Wnt, Gsk3- β phosphorylation of Smad1 resulted in degradation and the termination of TGF- β signalling (24, 25). At the biochemical level, the transcriptional activity of C-terminally phosphorylated Smad1 was antagonized by subsequent Gsk3- β phosphorylation in the Smad1 linker region and that linker phosphorylation lead to polyubiquitinylation.

Several studies in *Drosophila* of Mad (Smad1/5/8 homolog) linker phosphorylation by Zw3 (Gsk3- β homolog) were published. First, investigators developed a phospho-specific antibody recognizing Zw3 phosphorylated Mad and a Mad transgene with its Zw3 phosphorylation sites mutated (28). Data gathered with these reagents suggested that Mad is required for Wingless (Wnt homolog) signalling during embryonic segmentation and larval wing disc development. Experiments in *Xenopus* embryos suggested that Wnt-dependent segment border formation was disrupted by Smad8 depletion. The authors conclude that Mad performs a conserved role in Wingless signalling that is also terminated by Zw3 linker phosphorylation. In a new report, these authors noted that as a general rule Mad has distinct roles in the Dpp (TGF- β family) and Wingless (Wnt family) pathways that depend on Mad's phosphorylation state (29). They propose that unphosphorylated Mad participates in canonical Wingless signalling by interacting with the transcription factors Armadillo and Pangolin (homologs of β -catenin and TCF, respectively) and that C-terminal phosphorylation of Mad draws it to canonical Dpp signalling (Mad's established role). They claim that both Wingless and Dpp functions of Mad are terminated by Zw3 linker phosphorylation leading to degradation.

Most recently an exhaustive analysis of Wingless, Dpp and Notch signalling in the developing wing examined the relationship between Wingless, Mad and Zw3 (30). They used the same Mad mutant transgene and phospho-specific antibody as the previous studies (28, 29). The antibody studies showed that Zw3 linker phosphorylation of Mad is Wingless-dependent but that this event is highly restricted. In the wing it occurs only in actively dividing cells of the lineage leading to sensory organs and only within one quadrant of the wing blade. Expressing the Mad linker mutant transgene, or Mad-RNAi, in sensory organ precursor cells that are Wingless but not TGF- β responsive generated ectopic sensory organs. These results, together with others, lead the authors to suggest that non-phosphorylated Mad responds to Zw3 linker phosphorylation by performing a function (restriction of self-renewing mitosis) distinct from the function of C-terminally phosphorylated Mad (regulation of TGF- β target genes) and distinct from the outcome of Zw3 plus C-terminal Mad phosphorylation (degradation). The three outcomes of Mad differential phosphorylation are modelled in Fig. 3. The conservation of Zw3/Gsk3- β phosphorylation sites in vertebrate Smad1/5/8 suggests that this triphasic response to Wingless- and TGF- β -dependent Smad phosphorylation will also be a feature of embryonic development in these species.

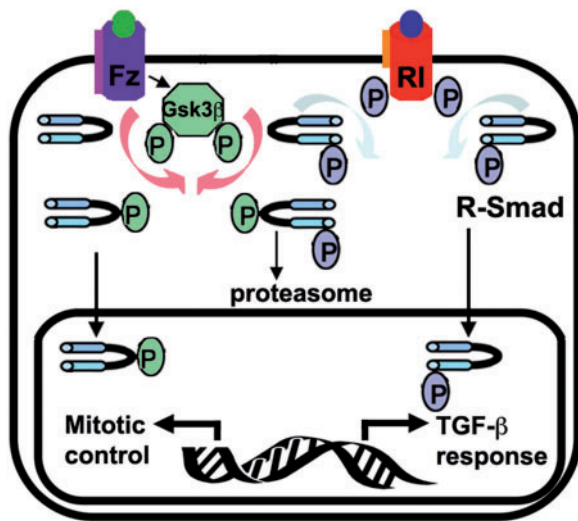


Fig. 3 Model for three distinct outcomes resulting from R-Smad C-terminal and linker phosphorylation by Gsk3- β . Schematic of potential outcomes within a cell stimulated by TGF- β activating the canonical Type I receptor kinase (RI) pathway and Wnt activating an unconventional Frizzled/Dishevelled (Fz) pathway. Both pathways lead to R-Smad phosphorylation; RI phosphorylates R-Smad directly on its C-Terminus (purple P), while the Fz pathway stimulates the Gsk3- β kinase to phosphorylate R-Smad on its linker region (green P). Red and blue arrows indicate the direction of phosphate transfer from kinase to R-Smad. Right side: when phosphorylated by RI only, R-Smads move to the nucleus (black arrow) and elicit an expected outcome—TGF- β dependent responses. Middle: when phosphorylated by RI and Gsk3- β , R-Smads move to the proteasome for degradation (black arrow), an outcome that terminates TGF- β signalling. Left side: when phosphorylated by Gsk3- β only, R-Smads move to the nucleus and induce a novel outcome—the restriction of self-renewing mitosis as seen in *Drosophila* sensory organ precursor cells (30).

TGF- β Signal Transduction Is Regulated by Cycles of Mono- and Deubiquitylation

Ubiquitylation is the covalent attachment of an ubiquitin polypeptide to a target protein, typically on a lysine residue. Polyubiquitylation, a chain of four or more ubiquitin molecules on a single lysine, leads to protein degradation and the recycling of its amino acids—an essential aspect of eukaryotic homeostasis. Polyubiquitylation-mediated degradation is also a regulatory mechanism affecting a wide variety of cellular processes including signal transduction. Monoubiquitylation is a modulator of protein function comparable to phosphorylation; it regulates protein activity and can be reversed by the activity of deubiquitylases.

Here we describe studies associated with mono- and deubiquitylation in the TGF- β pathway. One group of papers described the Smad4 ubiquitin ligase Ectoderm/Trim33/Tif1- γ and its counterpart, the deubiquitylase Fam/Usp9X. The first reported the cloning of Tif1- γ and its characterization as a TGF- β antagonist. This paper also showed that Tif1- γ antagonizes both TGF- β and BMP signals by binding to Smad4 and promoting its ubiquitylation (31). Independently, a bioinformatics analysis of lysine conservation in the Smad family of proteins was reported (32). In this paper the hypothesis was that a lysine

conserved across nematodes, flies and mice (1.1 billion years of divergence) that was not necessary for protein function (as shown by crystal structure) was a candidate for conservation as a regulatory ubiquitylation target. The study showed that Smad4 K507 is the only lysine that is universally conserved at the homologous position in all Smads. The hypothesis was supported by reports in the literature that Smad4 K507 was a site of ubiquitylation (33, 34). Then the authors identified Smad4 K519 as a Co-Smad subfamily-specific conserved lysine. Based on the data for Smad4 K507, the authors predicted that Smad4 K519 would also be ubiquitylated.

Subsequently the deubiquitylase Usp9X was identified as a TGF- β pathway component required for Smad4 activity (35). This study found that Usp9X sustains both Nodal (TGF- β subfamily) and BMP signals by deubiquitylating Smad4 and that it does so by counteracting the inhibitory activity of Tif1- γ . They mapped the target lysine to Smad4 K519, validating the prediction and then documented the underlying mechanism. They showed that Smad4 K519 is located near a surface that binds to R-Smads such that K519-monoubiquitylated Smad4 is unable to form a complex with phosphorylated Smad2. Studies in the fly wing further demonstrated the epistatic relationship between Usp9X and Tif1- γ in the competition for Smad4. The authors conclude that the complementary activities of Tif1- γ and Usp9x constitute 'ubiquitylation cycles' that regulate the ability of Smad4 to participate in Smad complex formation and that these cycles therefore modulate the cell's ability to respond to TGF- β signals.

Next addressed was the issue of where Smad4 monoubiquitylation takes place within the cell (36). Smad4 ubiquitylation was regulated via an association with chromatin and the authors proposed that this interaction allows Smad transcriptional complexes to limit their own activity. The logic was that when a TGF- β -responsive Smad complex translocates to the nucleus and binds to a promoter it carries inactive Tif1- γ . Once bound to chromatin, Tif1- γ is locally activated (possibly by chromatin modifications induced by Smad binding), ubiquitylates Smad4 and destabilizes the complex, thus ending the TGF- β response.

Loss of function studies in mice for *Tif1- γ* extended the scope of Smad4 ubiquitylation cycling to new developmental roles. First there was an investigation of early embryogenesis in *Tif1- γ* homozygous knockout mice (37). These embryos have anterior visceral endoderm phenotypes that are the opposite of those caused by the loss of Smad4 or R-Smads but similar to those generated by Nodal overexpression. These results are consistent with data from *Xenopus* (31) indicating that negative regulation of Smad4 by Tif1- γ is a conserved mechanism essential for regulating Nodal signals in early embryos. Analyses of Nodal-responsive tissues in *Tif1- γ* mutants revealed that cellular responses depended on an interaction between Nodal extracellular concentration and the amplitude of Smad signal transduction.

An analysis of mice in which the Cre/LoxP system was used to delete *Tif1- γ* solely in mouse mammary

gland epithelial cells (38) showed no effect on mammary development or on virgin mice, but that lactation defects were observed in mothers. Detailed investigation showed that the inability to lactate was due to the loss of Smad4 antagonism in these cells. This loss resulted in ectopic, TGF- β -dependent, Smad4-mediated repression of Prolactin pathways and their milk protein target genes. The authors propose that Tif1- γ facilitates lactation by inhibiting Smad4, a conclusion consistent with the *Xenopus*, knockout and cell culture studies.

Examining the other half of the Smad4 ubiquitylation cycle, a study in *Drosophila* used both maternal and zygotic loss of function mutants for *fat facets* (Usp9X homolog). They reported that the *fat facets* deubiquitylase is essential for proper interpretation of the Dpp morphogen gradient that patterns the embryonic dorsal–ventral axis (39). The mechanism underlying this requirement is that the loss of the Fat facets reduces the activity of Medea (homolog of Smad4) below the minimum necessary for adequate Dpp signalling. The reduction in activity is due to excessive ubiquitylation on Medea K738, the lysine homologous to Smad4 K519. The analysis rigorously demonstrates that the control of cellular responsiveness to TGF- β signals requires modulation of Smad4 activity by deubiquitylation (Fig. 4). Taken together, the studies described here indicate that ubiquitin cycling of Co-Smads is a highly conserved mechanism, and one

of equal importance to the regulation of extracellular ligand concentration, required for proper implementation of morphogen gradients.

Recently, studies of deubiquitylation have been reported for R-Smads and TGF- β Type I receptors. First, Usp15 was identified as a deubiquitylase for Smad1 and Smad3 (40). Even after activation by C-terminal phosphorylation, these Smads remain monoubiquitylated on lysines essential for DNA recognition (Smad3 K33 and K81) such that monoubiquitylation prevents DNA binding. Usp15 removes these monoubiquitins facilitating DNA-binding activity. The authors conclude that monoubiquitylation of R-Smads is independent of their phosphorylation state, takes place in the nucleus in conjunction with transcriptional activity and could function as a termination mechanism for signal activation. Usp15 was also identified as a deubiquitylase for the Type I receptor (41). An examination of Usp15 in tumours influenced by TGF- β activity revealed that *Usp15* is amplified in glioblastoma, breast and ovarian cancers. Studies in an orthotopic mouse model of glioblastoma showed that knockdown of Usp15 decreases the oncogenic capacity of glioma cells due to a reduction in TGF- β signal transduction. Most recently, Usp4 is shown to deubiquitylate the Type I receptor (42). Akt (Protein kinase B) phosphorylation leads to the relocation of normally nuclear Usp4 to the membrane where it encounters and deubiquitylates the Type I receptor.

As a group, studies of TGF- β pathway regulation by mono- and deubiquitylation reveal that ubiquitin cycling is a highly efficient ‘off-on switch’ for this signal transduction pathway.

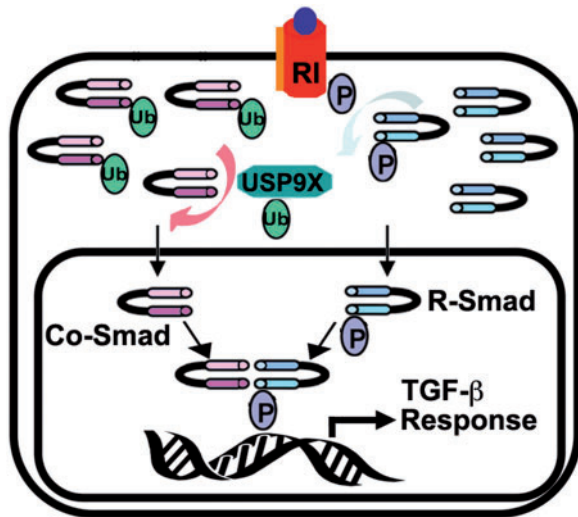


Fig. 4 Model for the impact of deubiquitylation on TGF- β responsiveness. Schematic of events within a cell stimulated by a TGF- β ligand activating the canonical Type I receptor kinase (RI) pathway. RI activation leads to phosphorylation of R-Smad (blue) directly on its C-Terminus (purple P). A blue arrow indicates the direction of phosphate transfer from RI to R-Smad. C-terminal phosphorylated R-Smad moves into the nucleus (rightmost black arrow). Cells normally contain pools of monoubiquitylated Co-Smads (pink with green ubiquitin) and one or more deubiquitylases (in this example USP9X in dark green). A subset of Co-Smads are deubiquitylated by USP9X (red arrow) allowing them to move into the nucleus (leftmost black arrow). In the nucleus the deubiquitylated Co-Smad and the phosphorylated R-Smad form an activated complex (middle arrows) that drives TGF- β responses. The magnitude of the response is dictated by the relative frequency of R-Smad phosphorylation and Co-Smad deubiquitylation—as seen in *Drosophila* embryonic dorsal ectoderm cells (39).

Future Directions

Regarding these topics, here we note a few of our thoughts for where they are headed. A study of wing vein morphogenesis in the four-winged hymenopteran *Athalia rosae* (sawfly) has extended our understanding of how Dpp/BMP signalling generates species-specific patterns. The analysis revealed that the Dpp transport system, identified in the two-winged dipteran *Drosophila*, is conserved and functions to specify the highly distinct fore- and hindwing vein patterns in sawfly. The ability of the Dpp signalling pathway to specify multiple wing vein patterns suggests the hypothesis that vein patterns are a reflection of the direction of Dpp transport in wing precursors (43). Additional tests of this hypothesis may shed new light on a longstanding question—how do conserved signalling systems generate diversified morphologies?

Regarding Smad linker phosphorylation, *Drosophila* data suggesting that unphosphorylated Mad has a role in Wg signalling (28, 29) or that Zw3 phosphorylated Mad not also C-terminally phosphorylated has a role in mitotic control (30) have yet to be corroborated in vertebrates. One hurdle for the latter is that cell type specificity will likely complicate the identification of an appropriate neural precursor cell type in which to conduct the requisite experiments. Studies of Co-Smad and R-Smad ubiquitylation in mammalian cells revealed that monoubiquitylation appears to be their

default state. Thus, the analysis of mechanisms regulating the activity of Smad deubiquitylases is a new frontier. To date, Akt phosphorylation of Usp4 is the only identified upstream component.

From a larger perspective, the value of ubiquitin cycles as a means of pathway regulation is likely not restricted to TGF- β proteins. Using a comparative phylogenetics approach, a candidate 'monoubiquitylation signature' was recently identified (44). If the signature is experimentally validated, it would streamline the identification of monoubiquitylated lysines considerably. The authors also predicted that two Hippo pathway signal transducers, Salvador and Merlin/Nf2 (the latter a well-known tumour suppressor), are regulated by monoubiquitylation. While this also awaits validation, these results suggest that the creative application of phylogenetics can predict new mechanisms for regulating signal transduction in any pathway.

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Conflict of Interest

None declared.

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