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Fine-tuned Shuttles for Bone Morphogenetic Proteins

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Abstract

Bone morphogenetic proteins (BMPs) are potent secreted signaling factors that trigger phosphorylation of Smad transcriptional regulators through receptor complex binding at the cell-surface. Resulting changes in target gene expression impact critical cellular responses during development and tissue homeostasis. BMP activity is tightly regulated in time and space by secreted modulators that control BMPs extracellular distribution and availability for receptor binding. Such extracellular regulation is key for BMPs to function as morphogens and/or in the formation of morphogen activity gradients. Here, we review shuttling systems utilized to control the distribution of BMP ligands in tissue of various geometries, developing under different temporal constraints. We discuss the biological advantages for employing specific strategies for BMP shuttling and roles of varied ligand forms.

Introduction

BMPs are a functionally diverse group of secreted signaling factors belonging to the TGF-β superfamily. Originally identified in bone extracts as important inducers of bone deposition [1], BMPs are now recognized to mediate cellular communication between adjacent cells (short range) or cells far apart (long range) [2,3]. Misregulation of BMP signaling is associated with many developmental abnormalities and disease states highlighting the need for this pathway to be tightly regulated, especially within the extracellular space where ligands bind their receptors to initiate signaling.

Active BMP ligands are processed from a proprotein [4]. Secreted dimeric ligands bind to a multi-component signaling complex composed of at least two different types of transmembrane Ser/Thr kinase (Type I and Type II receptors). Different combinations of ligand homo- and heterodimers associate with different combinations of receptors to generate the signaling complex [4,5]. Following ligand binding, the Type I receptor kinase, activated by Type II transphosphorylation, phosphorylates the intracellular R-Smad transducer (Smad1, 5, or 8 in vertebrates, and Mad in *Drosophila*) [6,7]. The association of pR-Smad with other proteins including the related co-Smad allows accumulation in the nucleus and a direct transcriptional response.

BMPs are notoriously ‘sticky’ molecules [8], they bind their receptors with slow kinetics (reviewed in [9]), and their signal is short lived [3,10,11]. Yet in a number of instances

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BMPs have been shown to travel over multiple cell diameters to generate morphogen gradients with signaling maintained over long periods. How, then, do BMPs reach and signal to cells afar? The answer appears to be in the many molecules that bind BMPs in the extracellular space and prevent them from receptor-mediated internalization and degradation, increasing their lifetime and activity range. Here, we discuss the importance of extracellular factors and their ability to control the distribution of BMP ligands and their availability for receptor binding. We first review the Short gastrulation (Sog)/Chordin multi-component shuttling system and discuss the evolution of its components. Then, we briefly review other 'shuttling' mechanisms that involve different extracellular modulators as well as varied ligand forms.

Shuttling BMPs via Sog/Chordin

In the early *Drosophila* embryo, high levels of BMP signaling specify amnioserosa fates at the dorsal midline, lower levels specify dorsal ectoderm in the lateral regions, while neuronal cell fates arise in the absence of signaling [12–16]. Similarly, specification along the D/V axis in frog (*Xenopus laevis*) and zebrafish (*Danio rerio*) embryos depends on a gradient of BMP signaling [17–19]. In all cases, positive intracellular feedback sharpens the boundaries of distinct BMP signaling domains and ensures reproducible cell fate allocation and tissue size [16,20].

In the early *Drosophila* embryo, *decapentaplegic* (*dpp*), encoding a BMP2/4 homologue, is uniformly transcribed throughout the dorsal domain, yet a narrow domain of high BMP signaling is seen along the dorsal midline. Dpp secreted into the perivitelline space is rapidly (30 min) concentrated extracellularly at the dorsal midline where a peak of phosphorylated Mad (pMad) is generated [20,21]. This rapid concentration of BMP signaling relies on the spatial redistribution of ligands by several extracellular modulators (Figure 1). The dorsal domain of *dpp* expression is flanked laterally by ventrally expressed *short gastrulation* (*sog*), which encodes a Dpp binding protein [22]. Sog binds Dpp via its Cysteine-rich (CR) von Willebrand factor type C domains to locally inhibit signaling and to prevent movement ventrally. At the same time, this complex protects Dpp from degradation and receptor-mediated internalization, thus, facilitating long-range ligand shuttling of Dpp from the lateral and dorsal domains to the dorsal midline [20–22]. Sog has a short-range negative role on BMP signaling since it inhibits the access of ligands to receptors; at the same time Sog has a long-range positive effect on BMP signaling as it facilitates Dpp movement. In the absence of Sog, BMP signaling levels remain uniformly low with a failure to specify the amnioserosa. Similarly, in the pupal wing Sog abuts and limits the region of BMP signaling where it is also required to create a domain of high BMP signaling in the posterior crossvein (PCV)[23–25]. Dpp is produced by the longitudinal proveins but is moved into the PCV competent zone to create a corridor of high signaling.

In both instances two BMP family members are required for normal patterning raising the possibility that BMP heterodimers are involved. Heterodimers have been reported to exhibit increased signaling ability [21,26–29]. The mechanism underlying these differences in activity is currently under intense investigation [30]. In the early fly embryo a combination of Dpp, Screw (Scw) homodimers and Dpp:Scw heterodimers are thought to pattern the dorsal domain [20,21,31], while in the pupal wing, Dpp and Glass bottom boat (Gbb) are each essential for PCV formation [32,33]. Interestingly, BMP heterodimers have a higher affinity for Sog than the homodimers and thus, are favored for the long-range transport. In fact, Gbb appears required for the movement of Dpp into the PCV competent zone [24]* where BMP signaling induces *dpp* transcription [25]. Given the widespread expression of *gbb*, this activation of *dpp* expression could then trigger the secretion of Dpp:Gbb to enhance of signaling in these cells.

The Sog-BMP shuttling complex requires at least one additional molecule, Twisted Gastrulation (Tsg) in the early embryo, and a related molecule Tsg2/Cv in the pupal wing [21,34–36]. Tsg proteins form complexes with Sog that are more efficient for BMP binding and long-range shuttling. A molecule resembling half-Tsg, Shrew (Srw), is also required for high levels BMP signaling in the early embryo [37]. The assembly of the shuttling complex is aided by collagen IV, which functions as a scaffold for BMP-Sog binding [38]. Binding of Tsg appears to release the BMP-Sog complex from collagen IV and promote its movement [39]**.

Strikingly, BMP shuttling as a strategy is highly conserved across the phyla suggesting an ancient origin for BMP signal modulation using opposing BMP and BMP antagonist gradients. In zebrafish and frog embryos, BMP4 and 7 are expressed and secreted from ventral tissue whereas a specialized dorsal tissue known as Spemann's organizer secretes the Sog homologue Chordin together with other BMP-binding proteins such as Follistatin, Noggin and Cerberus to modulate BMP activity (reviewed in [17,18]).

Tolloids release BMPs from the shuttle complexes

A key component that helps create the flux is the processing of Sog by BMP-1 family metalloproteases, Tolloid (Tld) and Tolloid-related (Tlr) or Tolkin [23,40–45]. Upon cleavage of Sog, the released BMP ligands can bind receptors, or be re-captured by Sog. Sog concentrations are high in lateral regions of the early embryo and the probability of BMP re-capture is high, whereas in the dorsal most region where Sog levels are low, released ligands are more likely to find receptors. Reiterated cycles of complex assembly, diffusion and destruction produce a physical re-distribution of the BMP ligands with an increase in the domains of high BMP signaling [21,24]. The embryonic and pupal BMP signaling gradients differ in their spatial and temporal constraints, yet in both cases a balance between the inhibitory and positive activities of Sog remains crucial for proper patterning. This balance is kept by Tld in the early embryo and Tlr in the pupal wing. Tld and Tlr cannot substitute for each other and appear to have matched their catalytic properties to the temporal requirements of their respective developmental windows [23]. For example, Tlr processes Sog with at least one order of magnitude slower kinetics than Tld, while the more rapid kinetics of Tld are matched to the rapid development of the embryo.

Mathematical models indicate that lower rates of Tld processing result in sharper signaling distributions and with a greater net transport of BMP ligands away from the Sog/Chordin source [46]. Furthermore, in order for the shuttling model to meet the requirements for scale invariance of *Xenopus* embryos or robustness of *Drosophila* embryos, Sog/Chordin must be bound by a BMP ligand for Tlds processing [15,47,48]. In fact, *Drosophila* Sog cannot be cleaved by Tlds in the absence of BMP, an obligatory co-substrate [23,41]. In contrast, vertebrate Chordin can be processed in the absence of BMPs, albeit the processing rate is enhanced in their presence [42]. An obligatory co-substrate for Sog processing is thought to reflect a BMP-induced conformational modification allowing Sog-BMP, but not Sog alone, to fit into the Tlds catalytic pocket. Identification of Sog processing sites and their comparison with Chordin revealed the molecular basis for BMP-dependent cleavage by Tlds [49]** (Figure 2). Interestingly, aromatic substitutions at Sog processing sites produced a "Chordin-like" Sog that was processed by Tld independently of the BMP co-substrate, and was more prone to Tld-destruction at lower BMP levels. Whether aromatic residues in the Tlds catalytic domain confer substrate selectivity remains to be demonstrated, although high conservation is observed of such residues across phylogenetic lineages (Figure 2).

The impact of the Tld-mediated Sog processing on the profile of BMP gradients in early *Drosophila* embryos was demonstrated by replacement of endogenous Sog with "Chordin-

like" variants [49]. The normal steep BMP signaling gradient in early *Drosophila* embryos changed to a shallower profile, analogous to that observed in some vertebrate embryos, with accompanying changes in cell fate and tissue size. The resulting expansion of dorsal-most amnioserosa cell fates could be partially rescued with multiple copies of "Chordin-like" Sog that increase BMP transport, but the boundary of the high BMP signaling domain remain diffuse and variable between individuals of the same genotype. Mathematical simulations showed that only a model with a modest increase in "BMP-free-Sog" processing by Tld could fit these experimental data. In contrast, embryos with only one copy of *sog* have a broader domain of high BMP signaling and expanded amnioserosa, but their BMP signaling gradients remain sharp, step-like. Thus, the acquisition of BMP-dependent Sog processing during evolution appears to facilitate long-range ligand diffusion and formation of robust BMP morphogen gradients required for early *Drosophila* patterning. Furthermore, *Drosophila* Sog has evolved to include a third processing site, right before its second CR domain (Figure 2), also BMP-dependent. This additional site appears to be a bottleneck for Tlds-mediated Sog destruction and its processing is highly enhanced by addition of BMPs and Tsgs [49,50].

Sog/Chordin – and evolution of shuttling mechanisms

An intriguing question is why only *Drosophila* has evolved such an intricate mechanism to ensure formation of BMP gradients while the other phylogenetic lineages did not. A possible explanation lies in the features and the constraints of this system. The *Drosophila* embryo has already attained its anterior-posterior and D/V axes during oogenesis. The early stages proceed in a syncytium of rapidly dividing nuclei with cells forming only during the last division before the immediate onset of gastrulation. In vertebrate embryos and even in other arthropods, such as spiders (*Achaearanea*), these early events are more protracted, and formation of BMP gradients occurs over longer time frames and relies on cell-cell communication, as opposed to the limited cellular input and shorter developmental window in early *Drosophila* embryos. As such, a set of genes arising by duplication and subsequent divergence has been proposed to have enabled BMP-mediated patterning to occur given the cellular constraints imposed by the syncytial nature and rapidity of early development in higher Diptera [51]**. None of these "dedicated" genes are found in the genome of short band insect *Tribolium castaneum* (Coleoptera) or in *Apis mellifera* (Hymenoptera). *Tribolium* has only a Tlr-like protease and lacks the faster acting protease Tld, required in early *Drosophila* patterning. Similarly *Tribolium*, as well as frog and fish, have one Tsg, whereas two Tsg-like molecules are required in *Drosophila*, one for early patterning, one for pupal development, and an additional half-Tsg, Srw, with no known homologue in other systems, also required for early *Drosophila* patterning. Finally Scw, a Gbb/BMP7-like ligand with no homologue in *Tribolium* and *Apis*, fulfills new signaling modes in the higher Diptera while retaining the ability to function in various Gbb-dependent processes in *Drosophila* [51,52]. Interestingly, the gain of "dedicated" genes in higher Diptera was accompanied by loss of several other BMP modulators including BAMPI and DAN [51].

Phylogenetic analyses have placed the origin of Scw between mosquitos (Culicomorpha) and the higher Diptera, suggesting that the origin of Scw coincides with a reduction in the extraembryonic membranes observed in higher Diptera [51,53]. In lower Diptera and other insects the dorsal ectoderm is expanded and the dorsal midline is subdivided into amnion and serosa lineages, where a broadened domain of higher BMP signaling is observed (reviewed in [54]). Nonetheless, Sog plays a role in dorsal enrichment of Dpp in *Tribolium* and in aspects of the Dpp signaling profile in *Anopheles* [55,56], but not in Dpp transport during axis specification in the spider *Achaearanea* [57]. A preliminary inspection reveals that the putative proximal processing sites in *Tribolium* and *Anopheles* Sog are more similar to the *Drosophila* Sog consensus than to that of vertebrates Chordin (Figure 2). Further

biochemical analysis is needed to determine if and in what lineages Tld-mediated processing of Sog requires an obligatory BMP co-substrate. Nonetheless, these comparisons raise the possibility that the BMP-dependence of Sog processing preceded the reduction of extraembryonic membranes observed in higher Diptera and arose earlier as an efficient BMP-mediated transport required for robust patterning. A 'Chordin-like' Sog cannot ensure robust *Drosophila* patterning [49]. Also, Gbb or Tlr, the ancestral BMP pathway components, cannot replace Scw or Tld during early patterning [23,58]*. Thus, acquisition of BMP-dependence for Sog destruction by Tlds may represent a process of canalized development.

Cv-2, an ancient player that helps refine BMP morphogen gradients

If BMP shuttling in the *Drosophila* PCV competent zone during pupal development constitutes the ancestral function, we expect that some conserved modulators would be used at this stage but not during early development. One such example is Crossveinless-2 (Cv-2) or BMPER in vertebrates, conserved from fly to humans (reviewed in [9]). Named for its role in modulating BMP signaling during PCV formation, Cv-2 is not required for embryonic patterning [25,33,59]. Like Sog, Cv-2 binds BMPs via CR domains (von Willebrandt factor C) and exhibits both BMP agonistic and antagonist activities. Unlike Sog, Cv-2 does not mediate long-range shuttling of BMP ligands and acts locally within the crossvein [9,59]. Cv-2 binds to cell surfaces via heparan sulfate proteoglycans (HSPGs) (Dally) and by associating with the Type I BMP receptor [59,60]. The binding of Cv-2 to Type I receptors can increase the flow of BMPs to the receptors, or sequester them into inactive BMP/Cv-2/receptor complexes depending on BMP-receptor binding affinities. BMP ligands with high receptor affinity (such as Dpp) will be pulled into inactive complexes, while low affinity ligands (such as Gbb) will be recruited into close proximity to the receptors [59]. Since Cv-2 also binds to Sog/Chordin-Tsg complexes, BMPs released from a Sog/Chordin shuttle could be concentrated at the cell surface by binding to Cv-2 (then available for exchange with the receptors) [60]. In an alternative model, the "sink" model, Cv-2 can locally reduce the concentration of soluble complexes by binding to Sog/Chordin with or without BMPs, acting as a sink to increase flow towards Cv-2 expressing cells [61,62]*.

Cv-2 is also a transcriptional target of BMP signaling that helps refine the BMP morphogen gradient in the developing pupal wing and could provide the positive feedback/spatial bi-stability required for sharp, step gradients [59,63]. Molecules like Cv-2 could also buffer signaling noise and attenuate stochastic fluctuations in models of BMP-mediated cell signaling [64]*.

It could all begin with a diverse pool of ligands

In many cases BMPs do not need to be moved over long distances but instead act locally [3]. In the numerous roles for Dpp and Gbb throughout Drosophila development, both local or restricted signaling and long range signaling has been observed [65–73] and our understanding of the relative contributions of each ligand is still evolving. In the wing imaginal disc where Dpp has long been thought to act as the quintessential diffusible morphogen, in fact in the absence of Gbb, Dpp is unable to elicit a response far beyond the cells from which it is produced [74,75]**. Furthermore, the range may depend on particular ligand variants.

In actuality BMP signaling begins with the production of the dimeric ligand (Figure 3). New studies have revealed that this initial step is likely to be a key point in the regulation of BMP signaling. Sequential proconvertase processing of BMP4 within the linker between prodomain and C-terminal ligand domain influences signaling activity [76,77]. Dpp follows

a similar pattern of cleavage as BMP4. A Dpp variant produced by only one cleavage rescues loss of *dpp* signaling between adjacent cell layers in the embryonic midgut but not in imaginal discs where Dpp is thought to act over longer distances [78,79]*. In the case of *Drosophila* BMPs, Scw and Gbb, processing at alternative furin cleavage sites also influences activity, but here, one site (NS) resides within the less conserved prodomain [58,75]. Active Gbb ligands significantly different in size (Gbb38 and Gbb15) are produced by cleavage at either the NS site or the conventional S1 site, but this does not hold true for Scw. In vivo analyses indicate that that the large variant, Gbb38, exhibits a long range while Gbb15 does not, and Gbb15 is enriched in tissues where Gbb is known to act at short range [73,75]. Biochemical and structural studies have identified a core or 'arm' domain with a conserved fold that is contributed by the prodomain [80]*. NS processing would leave this 'prodomain core' or arm domain associated with the cysteine knot ligand domain but remove the 'strait jacket' and lasso that interfere with receptor binding and confer latency in proTGF-β [81]**. Significantly, mutations in an NS consensus sequence in human BMP4, BMP15 and AMH are associated with specific developmental abnormalities, cleft lip/palate (CL/P, premature oovarian failure (POF) and persistent Müllerian duct syndrome (PMDS), respectively [75]** (Figure 3).

What function could the added prodomain core serve in a large BMP variant not seen in the conventional small BMP? *In vivo* studies suggest a difference in signaling range [75]**. This prodomain core could influence binding of antagonists, such as Noggin/Chordin/Sog, and thus, indirectly impact ligand range. Or it could more directly mediate ligand distribution by interacting with extracellular factors. The differential interaction of BMP-5, 7 and GDF-8 prodomains with fibrillin and perlecan has been reported [82]*, although the consequences of such interactions on ligand distribution and activity remain to be investigated. Alternatively, the prodomain core could influence receptor complex formation as the BMP7 prodomain has been shown to impact Type II and Type I receptors interactions [83].

Other modes of moving BMP ligands in the extracellular space

The distribution of BMP ligands is not only affected by the constellation of BMP variants but also by an increasing number of extracellular modulators distinct from the Sog/Chordin family. Of particular interest in the wing primordium are the GPI-linked *Drosophila* glypicans, Dally and Dally-like protein (Dlp), and two secreted molecules, Pentagone (Pent) and Larval Translucida (Ltl) that appear to provide a 'BMP shuttle' function while ensuring tight feedback controls (reviewed in [84]) (Figure 4). The ability of Dally to stabilize Dpp at the cell surface in *trans*, has led to a model by which BMPs are handed off from one cell to another in a series of glypican-BMP association-dissociations [85]**. Dally and Dlp exhibit different specificities for Dpp and Gbb. With their different spatial distributions one can envision that these glypicans could act to corral different dimer types. Interestingly, HSPGs also function in more confined settings to regulate BMP signaling, such as in synapse development at the larval NMJ, where Dlp and Syndecan both influence the accumulation and distribution of Gbb [86]*.

In contrast to cell-bound Dally and Dlp, Pent and Ltl are secreted. Their expression is regulated by BMP signaling, *pent,* negatively, and *ltl*, positively. Pent acts to broaden the domain of BMP signaling and Dally is the likely mediator of Pent's influence on BMP signaling [87]*. Ltl, on the other hand is expressed in the medial domain of the wing disc and acts to limit signaling [88]*. Ltl acts similarly to Cv-2 and it too, may act through glypicans, as *ltl* has been shown to genetically interact with *dlp*. While the dynamics of these interactions are not yet known, it is clear that this extracellular control of ligand distribution and availability is reinforced by feedback controls, a reoccurring theme in many BMP-

controlled processes [84,89]**. Another completely different mode of regulation occurring during PCV formation involves a vitellogenin-like protein, Cv-d, that makes its way from the fat body through the hemolymph to the pupal wing to bind BMPs and enable signaling [90]*.

Perspectives

Many factors that regulate the availability of BMP ligands in the extracellular space have being identified. Some of them work through complex mechanisms such as Sog/Chordin-mediated shuttling, others through mechanisms that appear less elaborate but serve the same general purpose to protect BMPs from receptor-mediated internalization and turnover, and allow them to reach the cells in need of signal. Do the different molecules and mechanisms co-opted for 'shuttling' BMPs in the early *Drosophila* embryo vs the developing wing reflect fundamental differences in the cellular environment and time over which a BMP signaling gradient is built and maintained to accomplish its task in development? Sog-mediated BMP shuttling may be necessary for the rapid generation of a steep gradient with a high amplitude while a 'capture and release' mechanism with multiple feedback controls is better suited for coordinating tissue growth and longer term maintenance of graded signaling [89,91]**. In light of the role of Dally and Dlp as modulators of BMP signaling in the wing disc, it is interesting that in the early embryo a translational block is in place that prevents HSPG synthesis and thus, eliminates this point of regulation from the mix of molecules available for interaction during early BMP shuttling [92].

As the molecular details of the various BMP shuttling systems emerge we are beginning to understand how they have been exploited for diversified patterning during evolution. However, a number of issues remain unresolved. For example when did signaling by BMP heterodimers appear during evolution? Heterodimers seem to pattern early embryos in insects such as *Drosophila*, but not *Tribolium* or *Apis mellifera*. More importantly, how do heterodimers signal synergistically? Are two Type I BMP receptors needed? Two Type I receptors are also required at the *Drosophila* NMJ even though only one ligand, Gbb, mediates signaling [72,93]. How do Sog and BMPs have access to collagen IV scaffolding surfaces? The sites for Dpp binding were mapped to residues within NC1 domain, in a region involved in multimerization and formation of the characteristic chicken-wire structure [38]. Such residues would be presumably inaccessible unless some NC1 domain(s) are removed to expose the scaffolding surfaces. Also, Tlds are secreted enzymes yet Tld acts cell autonomously in the early *Drosophila* embryos [20]. Moving Tld enzymatic activity from a soluble phase to the cell surfaces is expected to greatly enhance the efficiency of proteolytic activity, but the way this is achieved remains a mystery. The early *Drosophila* embryos utilizes positive feedback for sharpening BMP morphogen gradients [20]. What are the molecular determinants for such positive feedback? How does a system achieve a fine balance between perfecting elaborate BMP shuttle complexes and recruiting multiple feedback mechanisms to modulate BMP signaling in time and space? Mathematical models have been instrumental in describing the dynamics of BMP tissue distribution as well as the dynamics of BMP reception at the level of a single cell. As mathematical models become more comprehensive, the field will increasingly engage them to describe and predict the many layers of regulation of BMP signaling.

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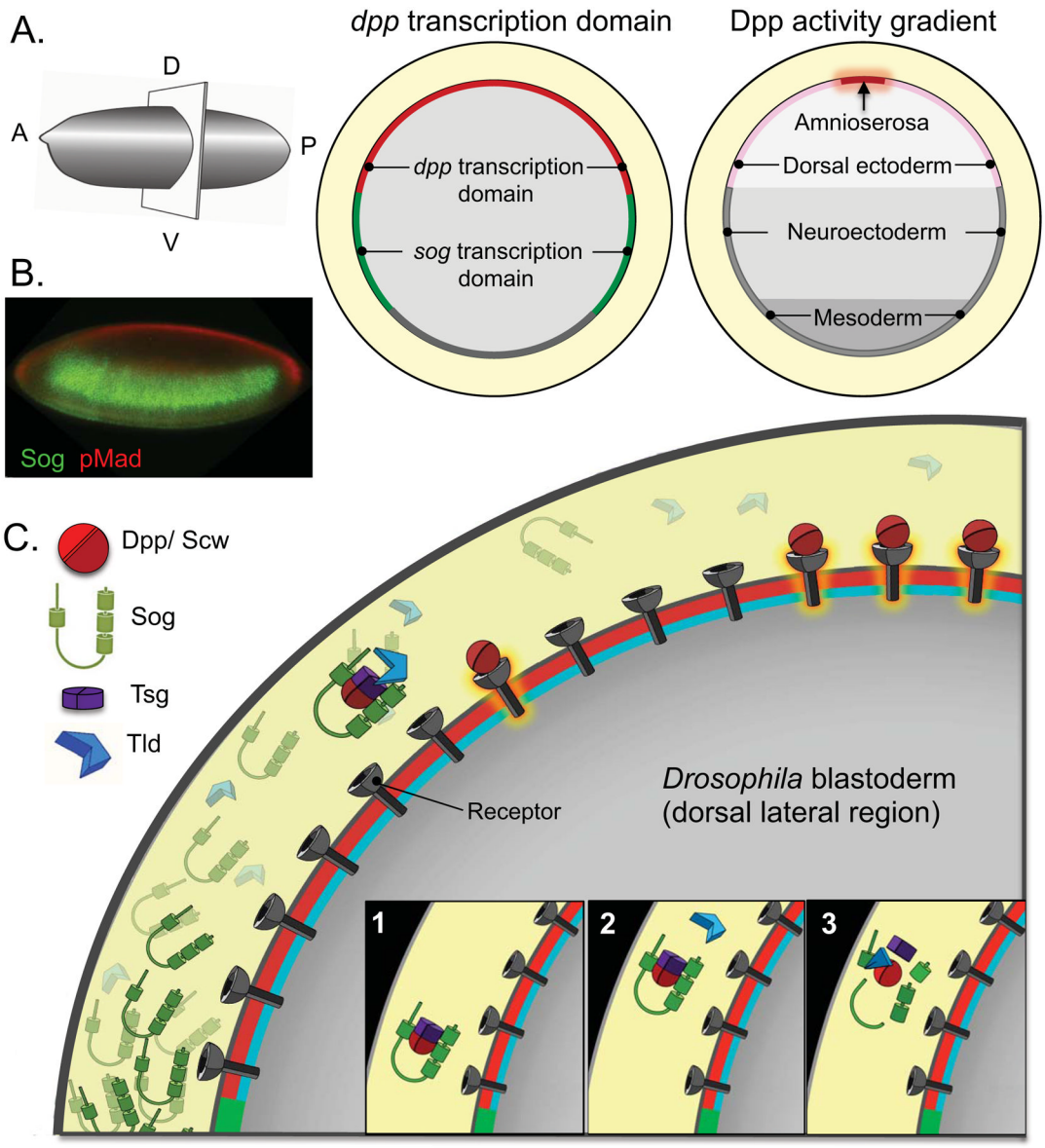

Figure 1. Sog-mediated BMP shuttling in the early *Drosophila* embryos requires several secreted modulators

(A) Diagrams of *dpp* and *sog* transcription domains and cell fate maps are shown in cross-sections of an early *Drosophila* embryo, oriented with dorsal side up and anterior to the left. (B) Confocal image of a blastoderm stage *Drosophila* embryo immunostained against pMad (red) and Sog (green). pMad signals are high in the dorsal-most domain (with high level of BMP signaling) but drop sharply below the limit of detection in the dorsal-lateral regions (with low levels of BMP signaling). Sog is produced in ventral-lateral regions and diffuses dorsally in the perivitelline space to form an extracellular gradient with a range controlled by Tlds activities [94]. (C) Cartoon representation of the steps of BMP gradient formation in the early *Drosophila* embryos. Ventrally secreted Sog makes a complex with BMPs that inhibits BMP signaling from spreading into the lateral domain. This complex also protects BMPs from degradation and receptor binding and internalization and allows them to diffuse. The assembly of Sog-BMPs complex uses collagen IV as a scaffold [38]; binding of Tsg releases the complex from collagen IV and promotes its movement [39]. In the dorsal

domain, the complexes will encounter Tld, which cleaves Sog releasing the ligands. Released BMPs have 2 possible fates: they can bind to receptors and signal, or they can be captured by another complex. When the Sog levels are high, as in the lateral domain, the probability of recapture is high, whereas at the midline, BMPs are more likely to bind to receptors and signal. Multiple cycles of complex formation (1), diffusion (2) and destruction by Tld (3) generate a net movement of BMPs from the lateral domain towards the midline. An animated description of this shuttling process is available [49].

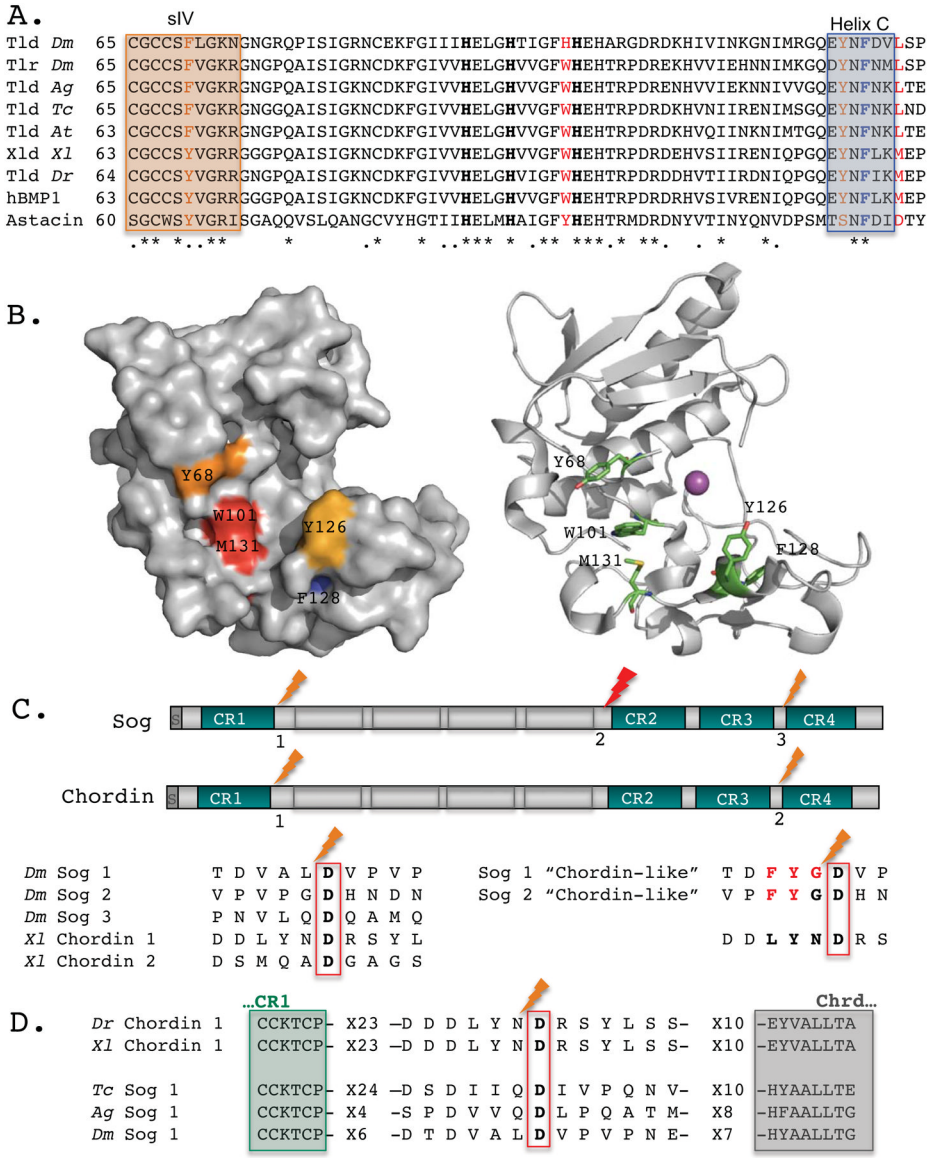

Figure 2. Tlds catalytic domains and their processing sites in Sog/Chordin

(A) ClustalW alignment reveals highly conserved aromatic residues within the catalytic domain of Tld-type enzymes in *Drosophila melanogaster* (*Dm*), *Anopheles gambiae* (*Ag*), *Tribolium castaneum* (*Tc*), *Achaearanea tepidariorum* (*At*), *Xenopus laevis* (*Xl*), *Danio rerio* (*Dr*), and humans (hBMP-1) as compared with the crayfish astacin. Three zinc-binding His residues are shown in bold. Crystal structure of hBMP-1 catalytic domain indicates that P2 residue of the substrate extends towards Trp101 and Met131, shown in red [95]. Boxes mark the β-sheet strand (sIV) on the upper side of the catalytic pocket and Helix C on the lower side. These motifs contain additional aromatic residues that may come in close proximity to P3 (brown). Helix C also includes F128 (blue), a residue mutated in autosomal recessive osteogenesis imperfecta [96]. (B) A surface diagram of hBMP-1 catalytic domain (left) and a ribbon diagram (right) capture the cavity where P3 and P2 residues bind. The conserved residues discussed are color coded (left) or shown as sticks colored by elements (right) (C, green; O, red; N, blue). The catalytic zinc is shown as a magenta sphere. (C) *Drosophila* Sog and *Xenopus* Chordin shared similar organization, with four Cysteine-rich von Willenbrandt

factor C domains (CR) separated by four “Chordin-like” (Chrd) motifs. The Tld-processing sites in Sog and Chordin show little conservation besides the S1′ Asp residue, a hallmark of this family of protease. Several residues (P1–P3) are responsible for making Sog dependent on BMP for Tld processing, while Chordin is not. Changes at these positions (shown in red) make Sog a BMP-independent substrate for Tld, “Chordin-like” [49]. (D) Comparison of Tld processing sites, residing between CR1 and Chrd repeats, suggests that *Tribolium* and *Anopheles* Sog are BMP-dependent for their processing, resembling the *Drosophila* Sog.

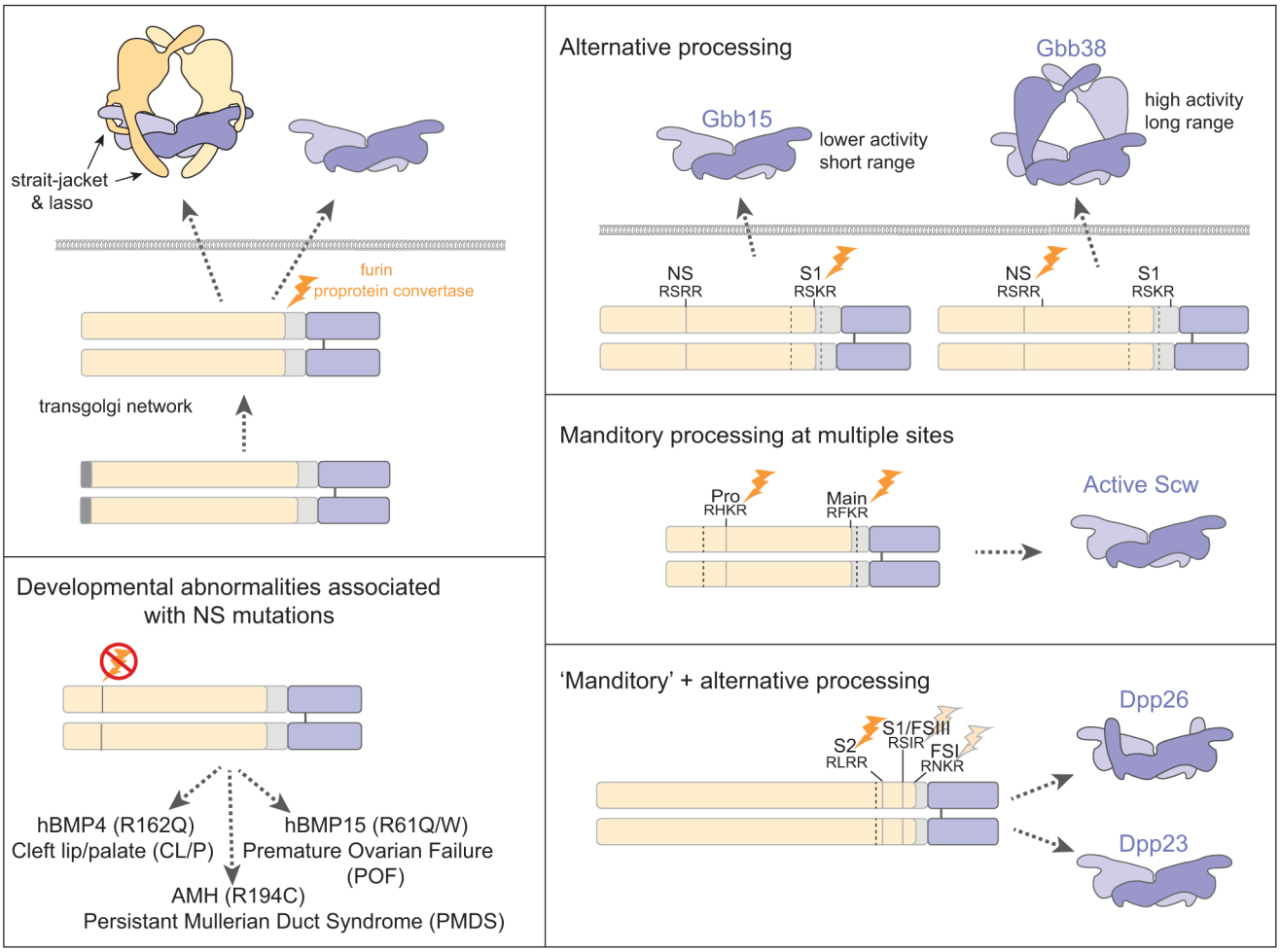

Figure 3. Alternative furin proconvertase processing generates BMP variants

(A) TGF-β/BMP superfamily ligands are synthesized as preproproteins and processed by furin proprotein convertases after dimerization. The highly conserved C-terminal domain (blue) defining the family are secreted alone or in complex with the less conserved prodomain (yellow). In many cases prodomain association renders the ligand inactive, as suggested by the ability of the strait jacket and lasso motifs of the TGF-β latent protein structure to interfere with receptor binding [81]. (B, C) An alternative cleavage site within the prodomain of Gbb (NS) and Scw (Pro) is critical for full ligand activity. Differential cleavage of Gbb proprotein at the NS and the conventional S1 site gives rise to two ligand variants with varying activities *in vivo* (B) [75]. In contrast production of an active Scw ligand requires cleavage at both sites (pro and main) [58]. (D) Point mutations with the putative NS site of hBMP4, hBMP15 and AMH are associated with human developmental abnormalities [75]. (E) Of three furin cleavage sites identified in Dpp, cleavage at S2 is essential with alternative processing that gives rise to two Dpp variants with differentially signaling abilities [78,79].

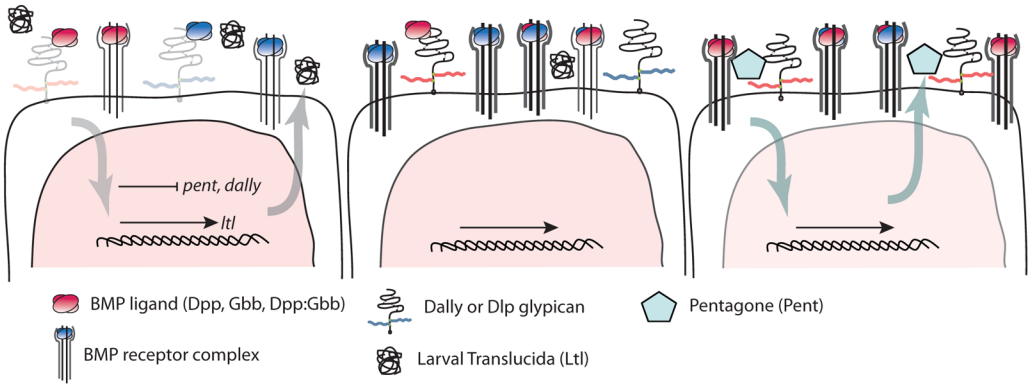

Figure 4. Extracellular factors + feedback regulate BMPs in the wing disc

A gradient of BMP signaling (pink nucleus) is established in the medial (left) to lateral (right) wing imaginal disc. Both Dpp and Gbb are essential for gradient formation and wing patterning [74]. Visualizing the spatial distribution of homodimers and putative heterodimers has been impeded by the recognition that tagging either ligand with fluorescence proteins impacts activity most likely due to its effect on protein-ligand interactions that could alter movement [75]. Nevertheless differential interactions between glypicans (Dally and Dlp) and BMPs (Dpp and Gbb) could affect their spatial distribution. Ltl is abundant in the medial disc to facilitates signaling while Pent is present in the lateral region and is necessary in those cells for the breadth of signaling, while it hinders signaling in the medial cells [87,88]. BMP signaling-dependent transcriptional regulation is responsible for the spatial distribution of these BMP modulators. High levels of BMP signaling in the medial cells (left) result in a down regulation of *dally*, *pent* and the type I receptor *tkv* but an up regulation of *ltl* (see [84]). Low levels of signaling in the lateral cells enable high levels of Pent and the glypicans.