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EXTRA VIEW

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Jun N-terminal kinase signaling makes a face

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ABSTRACT

decapentaplegic (*dpp*), the *Drosophila* ortholog of BMP 2/4, directs ventral adult head morphogenesis through expression in the peripodial epithelium of the eye-antennal disc. This *dpp* expressing domain exerts effects both on the peripodial epithelium, and the underlying disc proper epithelium. We have uncovered a role for the Jun N-terminal kinase (JNK) pathway in *dpp*-mediated ventral head development. JNK activity is required for *dpp's* action on the disc proper, but in the absence of *dpp* expression, excessive JNK activity is produced, leading to specific loss of maxillary palps. In this review we outline our hypotheses on how *dpp* acts by both short range and longer range mechanisms to direct head morphogenesis and speculate on the dual role of JNK signaling in this process. Finally, we describe the regulatory control of *dpp* expression in the eye-antennal disc, and pose the problem of how the various expression domains of a secreted protein can be targeted to their specific functions.

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Bone Morphogenetic Proteins (BMPs) are powerful secreted signaling molecules that regulate pattern formation, differentiation, morphogenesis and proliferation. A common theme for these proteins is their highly regulated spatial and temporal expression, which serves as part of the control of their context specific functions. The major *Drosophila* BMP, *decapentaplegic* (*dpp*), has been studied for over 30 years, but how spatially specific Dpp is delivered to target cells to carry out distinct actions is still an unresolved and active area of study. We are investigating this question using the role of *dpp* in ventral adult head morphogenesis, where a single source of *dpp* expression has both autocrine, short range, and paracrine, longer range effects. The genetic behavior of this system suggests that secreted Dpp must be partitioned correctly to long range and short range targets, and acts with different protein partners in these activities.

The adult *Drosophila* head is constructed largely from paired eye-antennal imaginal discs, sac-like structures comprising a columnar disc proper epithelium and a mostly squamous peripodial epithelium, separated by a lumen. The eye-antennal disc gives rise to 4 morphologically distinct organs (eye, antenna, ocelli, and maxillary palps) as well as the external cuticle (head capsule), and is subdivided during development into distinct morphological fields by the expression of transcription factors and signaling molecules. At third instar, *dpp* is expressed in a wedge on the lateral side of the antennal disc proper. Expression in the disc proper epithelium is also seen in the eye disc, within the morphogenetic furrow that will form the retina, and along the lateral and medial edges of that disc (Fig. 1A). In the peripodial layer, *dpp* is expressed in a diffuse area on the medial side of the eye-antennal disc, and in 2 stripes on the lateral side, along the future ventral side of the head (Fig. 1B). We focus on this lateral peripodial *dpp* expression, which arises in late 2nd instar eye-antennal discs and persists throughout the pupal period. Lateral peripodial expression is controlled by the homeotic transcription factor *labial* (*lab*)[1] and the pair-rule gene *odd-paired*[2,3]

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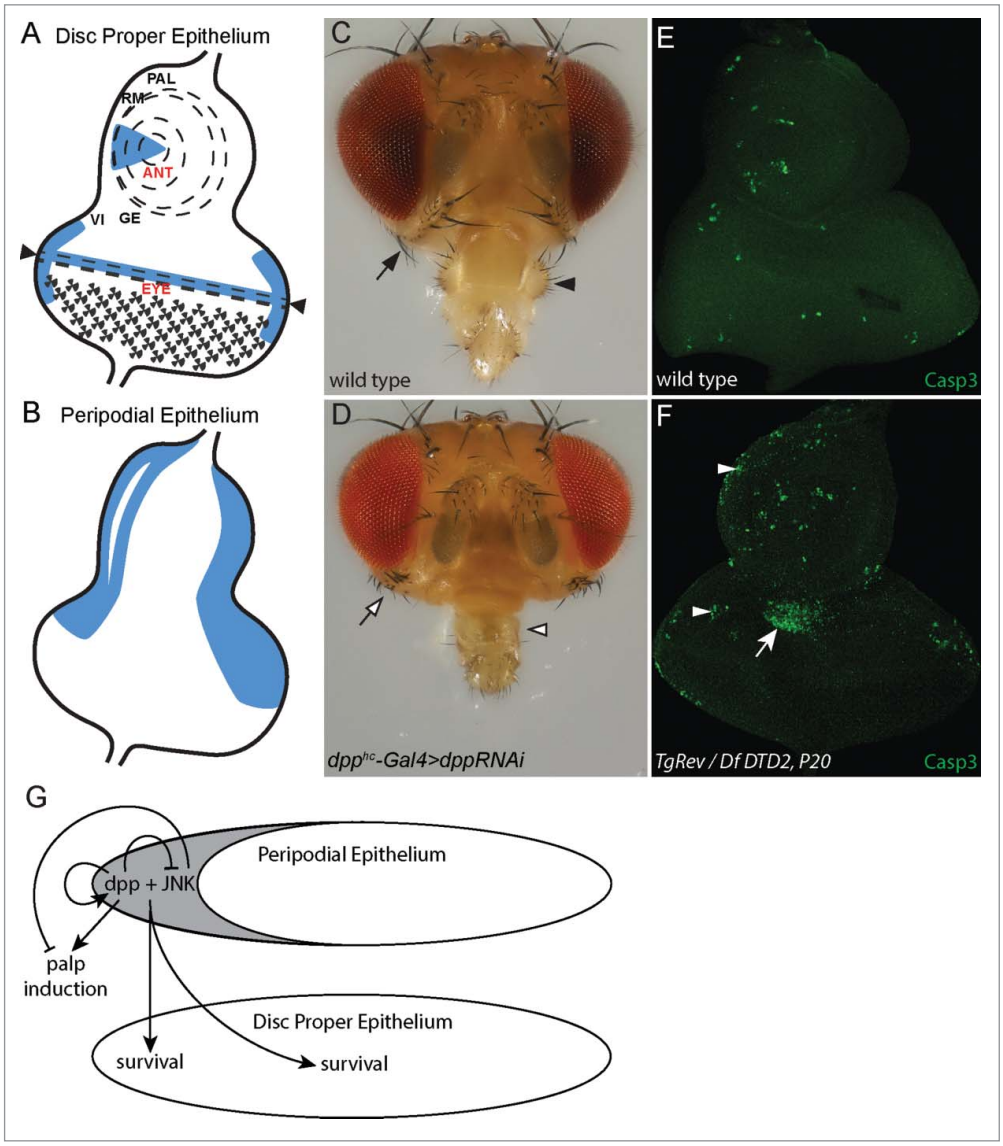

Figure 1. Schematic depiction of *dpp* expression in the (A) disc proper epithelium and (B) peripodial epithelium of third instar eye-antennal discs. The position of mapped primordia within the disc proper epithelium is summarized[4,8,38] and indicated by: PAL - maxillary palps, RM - rostral membrane, VI - vibrissae, GE - gena, ANT - antennal field, and EYE - eye field. The position of the morphogenetic furrow is marked with arrowheads. *dpp* expression domains are depicted in blue. (C) Wild-type adult head compared to a (D) dpp head capsule mutant head induced by expression of *dppRNAi*^{33767} in the lateral peripodial domain using *dpp*^{hc}*-Gal4*. Note the disruption of ventral head structures including a smaller eye, loss of rostral membrane tissue, disordered sensory vibrissae, and missing maxillary palps. Solid arrow indicates wild-type vibrissae and solid arrowheads indicate wild-type palps. Open arrows indicate mutant vibrissae, and open arrowheads indicate missing palps. Third instar imaginal discs were stained with antibody to activated cleaved Caspase-3 to visualize apoptotic cells in (E) wild type and (F) a strong *dpp* head capsule mutant generated by the transheterozygous combination of *TgRev*$^{46.1}$*/ Df DTD2, P20. TgRev*$^{46.1}$ is a complex rearrangement with an inversion breakpoint within the *dpp* head capsule enhancer. *Df DTD2, P20* is a large deficiency that removes *dpp*. Haplolethality is covered by a rescue construct that does not contain the *dpp* head capsule enhancer.[5] Arrowheads indicate small apoptotic cell cluster, and arrow indicates anterior eye disc cell death aggregate. In all discs lateral is oriented to the left and medial to the right. (G) Model of signaling between the peripodial epithelium and the disc proper epithelium. Dpp plus JNK are required for Dpp to support cell survival in the disc proper. Dpp is also required for palp formation. In the absence of Dpp, high JNK activity is seen, which inhibits palp formation.

through an enhancer located in the 5′ end of the *dpp* gene.[4] Like most post-embryonic *dpp* functions, the role of lateral peripodial *dpp* in ventral head morphogenesis was discovered through the recovery of cis-regulatory mutations that specifically disrupt this tissue specific expression.[5] *dpp* adult head mutations, referred to as head capsule mutations, cause defects of the ventral head. The eye is round instead of oval, due to loss of ventral ommatidia. Sensory vibrissae along the ventral margin of the eye are eliminated, or shifted

and bunched. The "cheek" area: gena and rostral membrane tissue, which lies adjacent to the vibrissae, is malformed and reduced (Fig. 1D).[5-7] Reduction, loss, or duplication of maxillary palps is also sometimes observed. The third instar imaginal discs appear grossly normal in size and morphology, but increased cell death is seen in the disc proper, and to a lesser extent in the peripodial epithelium in mutant discs.[4] A fate map of adult cuticular structures within the disc, constructed by transplantation of imaginal disc fragments into adult abdomens, places the primordia of the majority of adult head structures, including those disrupted in ventral head mutants, within the disc proper.[8] The observed cell death in the disc proper coupled with the placement of affected primordia within this tissue layer, suggest that peripodial *dpp* contributes to the morphogenesis of head structures in the underlying disc proper by supporting cell viability. However, this disc proper cell death is not correlated with any effects of Dpp on its known transcriptional targets. *dpp* target genes such as *Daughters against dpp* (*Dad*), an inhibitory factor induced by *dpp* signaling, and *brinker (brk)*, a transcriptional repressor of *dpp* targets that is repressed by *dpp* signaling, display both peripodial and disc proper expression, but only peripodial expression of these genes is disrupted in ventral head mutations.[4] Phosphorylation of the SMAD transcription factor, *Mothers' against dpp* (Mad) is another marker of canonical *dpp* signal transduction. Alterations in p-Mad expression are seen in the peripodial layer in mutant discs, but no clear spatial alterations are observed in disc proper p-Mad expression.

The lack of a Dpp-induced transcriptional response in the disc proper could be due to a secondary factor induced by peripodial Dpp that carries out the communication between the peripodial and disc proper layers. To test this possibility we took advantage of a constitutively active form of the BMP receptor Thickveins (Tkv^{QD}) which would restore Dpp-type signaling without producing Dpp. Unlike peripodially expressed exogenous Dpp, which rescues the ventral head mutant phenotype, Tkv^{QD} expressed in the same domain is unable to rescue, indicating that secreted Dpp is required in cells beyond the lateral peripodial epithelium to support head morphogenesis.[7] This suggests that peripodial Dpp is actively moving from that tissue layer, and its function cannot be substituted by Dpp diffusing from elsewhere (i.e. the disc proper), supporting the hypothesis that peripodially produced Dpp is contributing directly to cell viability in the disc proper, but not through conventional targets. Intriguingly, we found that we could discriminate between different components of the mutant phenotype of *dpp* ventral head mutants by their relationship with the Jun N-terminal kinase (JNK) pathway. Dpp's direct role in supporting cell viability in the disc proper requires peripodial JNK pathway activity. In contrast, disruption of maxillary palp morphogenesis is a consequence of excessive peripodial JNK signaling, and subsequent apoptosis, occurring in the absence of *dpp* (Fig. 1G).

JNK plays positive and negative roles in BMP-mediated head morphogenesis

The role of Jun N-terminal kinase (JNK) pathway in head morphogenesis was uncovered through a dominant interaction screen for genes that collaborate with *dpp* in head formation. The penetrance of *dpp* head mutations is a function of BMP pathway signaling strength. We demonstrate that reduced function (hypomorphic) mutations in the *dpp* protein (*hin-r* alleles) that weakly compromise *dpp* activity have low penetrance of head defects in trans to *dpp* head capsule mutations, while strong *dpp* hypomorphic alleles in trans to *dpp* head capsule mutations show high penetrance. In addition, dominant genetic interactions are seen with loss of function mutations in other Dpp pathway members, such as *tkv* and the negative regulator of *dpp* targets, *schnurri* (*shn*).[7] These observations suggest that the ventral head defect is highly sensitive to Dpp signaling strength and can serve as a sensitized background to screen for genes that collaborate with *dpp* in head morphogenesis. In such a dominant interaction screen, we identified members of the JNK pathway.

The JNK pathway is an intracellular kinase cascade that acts in many developmental events in *Drosophila* and plays roles in cell adhesion, morphogenesis and cell death.[9,10] We found that loss of function alleles of JNK pathway components such as *connector of kinase to AP1* (*cka*), *Jun-related antigen* (*Jra*), and *basket* (*bsk*) enhance *dpp* head capsule mutants, suggesting that JNK plays a positive, collaborative role with *dpp* in head morphogenesis. However, we saw very little JNK activity in wild-type discs, as measured by a *puckered-β-galactosidase* (*puc-LacZ*) enhancer trap in this

target gene, but strong induction of *puc-LacZ* expression in the lateral peripodial domain in *dpp* head capsule mutants.[7] This suggests that loss of *dpp* triggers increased JNK activity, which contributes to the mutant phenotype. This observation was hard to reconcile with the apparent requirement for JNK in normal *dpp*-mediated head morphogenesis as indicated by the genetic interaction data. To resolve this apparent discrepancy, we examined an allelic series of head capsule mutations and found that mutations whose defects are limited to the vibrissae, eye, gena and rostral membrane do not induce JNK in the peripodial epithelium, and only show increased cell death in the disc proper. However, mutations that also disrupted maxillary palps exhibited clearly elevated JNK activity in the lateral peripodial epithelium. This implies that JNK is used in 2 different pathways during ventral head morphogenesis: 1) low-level/normal peripodial JNK signaling required for *dpp* action on the disc proper and the formation of vibrissae, gena and rostral membrane; and 2) high-level/abnormal peripodial JNK signaling resulting in loss of maxillary palps.

We tested this by varying the activity of JNK signaling within the peripodial epithelium, using a lateral peripodial-specific *Gal4* driver, and UAS driven JNK pathway components in sensitized genetic backgrounds (i.e., that displayed only vibrissae, gena and rostral membrane defects). Increasing JNK activity rescued all these defects. Strong reduction of JNK activity in the same region in wild type flies via RNAi knock down or expression of dominant negative transgenes caused ventral cuticle defects identical to those seen in *dpp* mutations. This further supports a positive requirement for JNK activity in the peripodial epithelium for ventral head morphogenesis. In contrast, in mutants that had missing maxillary palps in addition to these ventral cuticle defects, reduction of JNK activity in the lateral peripodial epithelium significantly rescued the palp defect, while increasing the severity of the ventral cuticle defects. Thus, in the same flies, JNK activity appears to suppress vibrissae defects while enhancing palp defects.

It is important to note that in *dpp* alleles, palp defects are always accompanied by ventral cuticle defects. However, manipulation of JNK signaling uncoupled these defects, further supporting their distinct causality. For example, increased JNK signaling in the lateral peripodial epithelium, either by co-expression of the Jun kinase *bsk* and the upstream JNKKK, *slipper* (*slpr*) or expression of the upstream JNK activator *eiger* (*egr*), caused ablation of the maxillary palps with no other head defects. Taken together, these data indicate that peripodial JNK signaling is required for the *dpp* activity responsible for correct formation of vibrissae, gena and rostral membrane, but that excessive peripodial JNK signaling, seen in strong head capsule mutants, specifically causes loss of maxillary palps. Below we outline remaining prominent questions.

Peripodially expressed dpp: Different roles in different tissue layers?

How is Dpp, synthesized and secreted by peripodial cells, directed to the disc proper and what are the targets for its activity? In our current model, Dpp, synthesized in peripodial cells that have active, but limited JNK activity, is transported to the disc proper where it functions to support cell viability (Fig. 1G). The role of *dpp* in cell viability is unclear. Clonal analysis indicates that cells with compromised Dpp signaling proliferate slowly and undergo apoptosis.[11-14] Cells compete for survival factors, and cells with insufficient factors are eliminated by cell competition or morphogenetic apoptosis.[15,16] However, specific targets for *dpp* in its role as a growth and survival factor, distinct from its role in pattern formation, have not been identified. This has led to complex models about how a gradient of *dpp* activity can also control homogeneous cell proliferation.[17,18] Recent experiments have called such models into question. In the wing imaginal disc, the stripe of *dpp* expression along the anterior-posterior compartment boundary is the source of ligand that triggers both p-Mad phosphorylation and target gene expression in a graded fashion. While compartment boundary Dpp directs pattern formation in the disc, this expression turns out to be either largely, or entirely dispensable for growth of the wing disc.[19,20] Dpp from another source has been implicated as providing a growth stimulatory signal.[19] This leaves open the possibility that peripodial sources of Dpp may contribute to this stimulatory signal, but the identity of their targets remains elusive.

The cell death observed within the disc proper in head capsule mutants appears as both small scattered clusters on the ventral side of the disc (Fig. 1F, arrowhead), and an aggregate of apoptotic cells in the middle of the anterior eye disc, adjacent to the antennal

disc (Fig. 1F, arrow).[4] The small clusters differ from disc to disc, and none of the observed cell death exactly overlaps the mapped primordia of structures altered in the mutations. The larger anterior aggregate is seen more consistently and overlaps a region identified as a signaling center for growth promotion in the eye disc.[21,22] Within this region, activated Notch signaling initiates a genetic cascade leading to cell proliferation. Determining how cell death impinges on the expression of genes triggered by this growth promoting center, such as *eyegone* and *4-jointed*, may shed light on how peripodial *dpp* causes ventral tissue disruption.

What is the contribution of peripodial *dpp* to palp morphogenesis? Palp defects only occur in *dpp* head capsule mutant backgrounds with highly penetrant vibrissae, gena and rostral membrane defects. This suggests that palp defects arise only when peripodial Dpp signaling is compromised beyond a certain threshold. Strongly reduced Dpp is correlated with strong induction of peripodial JNK signaling, peripodial cell death and loss of palps. Canonical *dpp* transcriptional targets such as *Dad* and *brk* are disrupted in this region in mutant discs, so Dpp may play a role in the patterning of the primordia of the maxillary palps. However, according to the fate map, the palps arise from the disc proper epithelium. Furthermore, the expression driven by the *dpp* head capsule enhancer at the pupal stage is adjacent, but not in the formed maxillary palps.[7] These data suggest that the lateral peripodial cells expressing *dpp* do not contribute to the adult maxillary palp. We speculate that *dpp* plays an inductive role on the development of maxillary palps, rather than patterning cells that contribute to that organ directly.

The maxillary palp arises from a primordium in the ventral anterior-most region of the antennal disc as an outgrowth that emerges at the transition between the third instar and pupal periods. Patterning genes involved in antennal formation are also expressed in this outgrowth, in a regulatory program that is temporally offset from that specifying the antennae.[23] In addition, the homeotic genes *lab* and *Deformed* (*Dfd*) are required for the formation of maxillary palps.[1,24,25] *lab* and *Dfd* are expressed exclusively in peripodial epithelium of the eye-antennal disc: *lab* on the lateral edge, where it directly activates *dpp* expression via the head capsule enhancer.[1,26] *Dfd* is expressed in an adjacent, more medial domain and negatively regulates the expression of *lab*.[1,26] Cells of the lateral edge of the peripodial epithelium have a distinct morphology; they are cuboidal rather than squamous, and are often referred to as margin cells. Recent work indicates *Dfd* expression in these margin cells causes a reorganization of the subcellular localization of the adhesion protein DE-cadherin. This reorganization produces a fold that is hypothesized to create a distinct maxillary field within the *Dfd* expressing domain that is required for maxillary palp morphogenesis.[27] It will be of interest to determine how the cells of this *Dfd* expressing region contribute to the final differentiated palp, and how *lab* and *dpp* play a role in this process.

Multiple roles for JNK in eye-antennal disc development

Our genetic data indicate that the JNK pathway is used for at least 2 different functions during head development. Disruption of each function results in a distinct mutant phenotype, suggesting a role for JNK signaling in the morphogenesis of those structures, rather than a more general role in the synthesis or processing of Dpp. The loss of maxillary palps seen in strong head capsule mutants is associated with high JNK activity and induction of apoptosis in the peripodial layer. JNK activity is also often seen within the large aggregate of apoptotic cells (Fig. 1F), and in these contexts is presumed to be part of a quality control mechanism that removes developmentally abnormal cells. This mechanism is employed when growth factor signaling is compromised, and/or when apical-basal polarity is disrupted. These two circumstances may be connected, as disruption of Dpp signaling compromises cytoskeletal organization and causes extrusion of affected cells from epithelial sheets.[28,29] In the wing disc, Dpp signaling increases the activity of the small GTPase Rho1, which results in changes in cell shape.[30] Rho1 also interacts with members of the JNK pathway to stimulate apoptosis.[31] While the molecular details are unclear, this suggests that JNK mediated apoptosis could be directly connected to cytoskeletal disruption caused by alterations in Dpp signaling. This connection of *dpp* to cellular architecture may also be relevant to peripodial margin cell morphology, and formation of a distinct maxillary field in the antennal disc.

A confounding factor is the observation that loss of function of JNK pathway components, such as *slpr*

and *hemipterous* (*hep*), cause loss of maxillary palps,[32] indicating a positive requirement for JNK in palp formation. Such loss of function mutations would affect JNK activity over the entire disc. Our experiments only address JNK activity in the peripodial epithelium, so the discrepancy might reflect a requirement for JNK activity in the disc proper during palp morphogenesis. It could also be that JNK activity is required at a later time. During metamorphosis, JNK and Dpp activities contribute to thorax closure by wing discs.[33-35] JNK activity in peripodial wing disc cells is required for disc eversion.[36] JNK signaling could be deployed at multiple times during eye-antennal disc development, including times during metamorphosis.

What is the contribution of JNK signaling to the normal morphogenesis of vibrissae, rostral membrane and gena tissue? JNK signaling contributes to the function of peripodial Dpp to support cell viability in the disc proper. We speculate that Dpp is transported from the apical surface of the peripodial cells to the disc proper, by secretion or some other facilitated transport mechanism, such as exosomes, cytonemes or other cellular protrusions. JNK signaling could be required for such polarized secretion, or for the formation of peripodial cell extensions.

To tease apart these functions, we must determine what parts of the JNK pathway are used in common between them. The basic JNK signaling module, consisting of Hep, Bsk, Kay, and Jra, can be activated by many upstream modifiers (Fig. 2). These inputs from different modifiers impart specificity to the basic signaling module. In addition, JNK signal transduction can target multiple downstream effectors. All this explains the wide variety of cellular activities controlled by JNK signaling. Our observation that targeted peripodial gene expression can produce flies with only ventral cuticle and vibrissae defects, versus flies that are missing only maxillary palps, suggests that we may be able to discriminate among these varied JNK functions with a genetic screen of available RNAi and protein reagents.

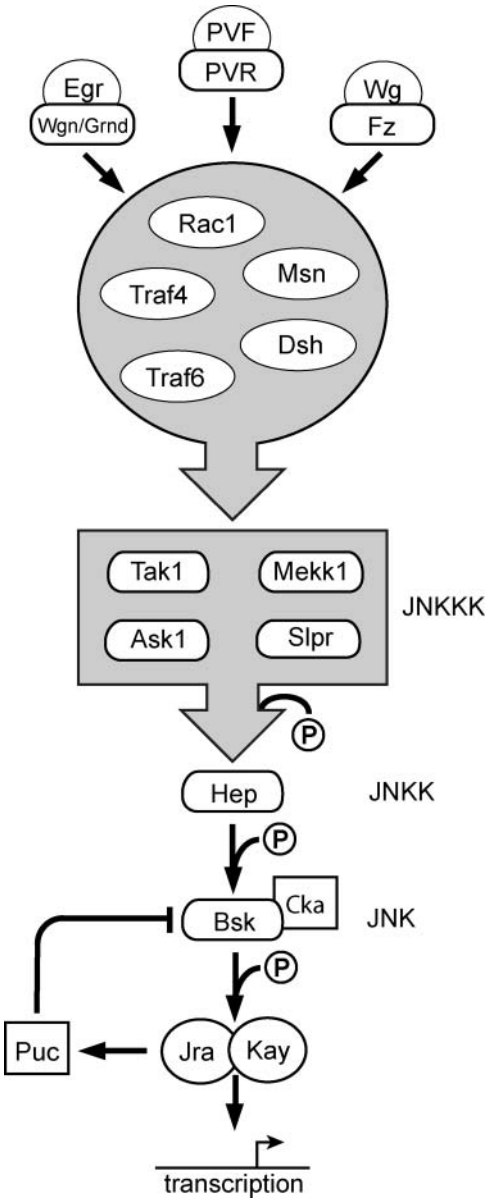

Figure 2. The *Drosophila* JNK pathway. The core signaling module is comprised of a JNKK: *Hemipterous*, JNK: *Basket, Jun: Jun related antigen*, and Fos: *Kayak (Kay)*. Upstream JNKKKs and further upstream inputs diversify, and lend specificity to the signaling pathway. Other pathway members include: *Wengen (Wgn), Grindelwald (Grnd), PDGF- and VEGF-related factor (PVF), PDGF- and VEGF-receptor related (PVR), Wingless (Wg), Frizzled (Fz), TNF-receptor-associated factor 4 (Traf4), TNF-receptor-associated factor 6 (Traf6), Misshapen (Msn), Dishevelled (Dsh), TGF-β activated kinase 1 (Tak1), and Apoptotic signal-regulating kinase 1 (Ask1)*.

Why is there so much dpp?

In all imaginal discs *dpp* expression is discrete and has a highly dynamic temporal and spatial pattern. *dpp* is a large gene, with greater than 10 kb in the 5′ cis-regulatory region (shortvein, shv) and 25 kb of cis-regulatory information in the 3′ noncoding region (disk), as well as several large introns.[37] All these different enhancers and numerous promoters regulate production of a single Dpp protein (Fig. 3). The disc proper expression of *dpp* seen at third instar is controlled by enhancer elements within the 3′ cis-regulatory region.[38,39] These enhancers are poorly delineated, but appear to be both redundant, and not disc specific.

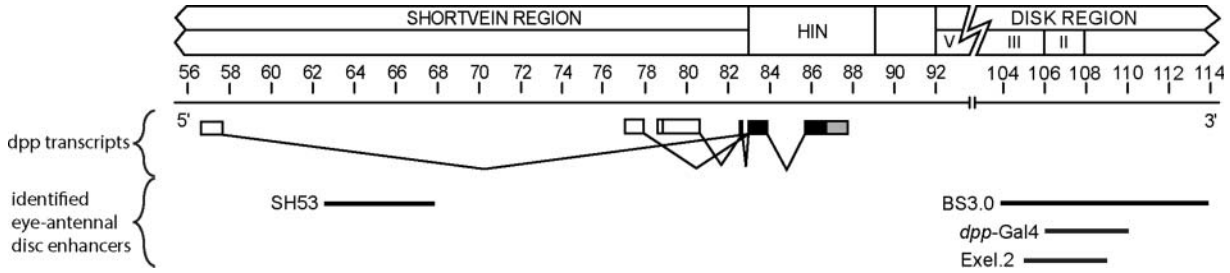

Figure 3. Schematic diagram of the *dpp* gene. Coordinates from St. Johnston et al.[37] are adjusted to reflect the actual genome sequence. The location of the exons for the 5 *dpp* transcripts are immediately below. Black boxes represent protein coding exons, white boxes represent non-coding exons. The disc region has been truncated for space considerations. The genomic positions of enhancer regions whose eye-antennal disc expression has been reported from transgenic constructs are indicated below. Enhancers include: the head capsule enhancer (SH53),[4] *dpp-lacZ* (BS3.0),[38] the "blink" driver (*dpp-Gal4*),[54] and Exelixis *dpp-lacZ* (Exel.2).[39] An additional construct, *dpp-Gal4*.PS (85.8MX),[55] located in an intron within the coding region, has been reported to express in the eye-antennal disc,[56] but we are unable to replicate this result, and believe this construct has been confused with *dpp.blink-Gal4*.

The two known shv enhancer regions that control imaginal disc expression, the head capsule enhancer and the shortvein enhancer,[5,40] are associated with *dpp* mutant phenotypes and *dpp* expression limited to specific discs. The majority of known *dpp* mutations disrupt the 5′ shv and 3′ disk cis-regulatory units, as *dpp* is haplo-insufficient, and true loss of function mutations are dominant embryonic lethals. Mutations that disrupt the 5′ cis-regulatory region do not interact genetically with those that disrupt the 3′ cis-regulatory region (an exception is 5′ mutations that knock out the 3 proximal promoters located in the shv region). At face value, this suggests that each pool of expressed, secreted Dpp acts separately from the others, even within the same disc. Many genetic analyses, demonstrating unique functions associated with each specific *dpp* expression domain, support this hypothesis. This is hard to reconcile with the observation that Dpp, localized by antibody, is broadly distributed and concentrates within the lumen that separates the peripodial and disc proper epithelia in both the eye-antennal and wing discs.[41] How Dpp undergoes targeted dispersion remains a major question. Dpp dispersion has been studied most extensively in the wing disc. Planar dispersion has been modeled as passive extracellular diffusion, facilitated extracellular transport, transcytosis, and targeted delivery by cellular extensions.[42-44] In addition to our work, others have documented evidence of vertical signaling from the peripodial layer to the disc proper, or disc proper to peripodial layer at different times during imaginal disc development.[41,45-49] Mechanisms for such signaling may include protein-facilitated transport, vesicles, or cellular extensions.[50,51] In addition, Dpp has been found in migratory cells such as hemocytes.[52] Other *Drosophila* BMP members have been reported to be secreted into the general circulation by neuroendocrine cells.[53] There is therefore an abundance of potential sources of Dpp for many tissues, which must be able to isolate and extract their specific BMP signal. In addition to facilitated transport, other mechanisms such as ligand processing, receptor accessibility, and various co-ligand/receptor availabilities and binding affinities may contribute to a cell's ability to decipher the Dpp signal intended uniquely for it. Establishing how this level of discrimination is achieved remains a major question in BMP signal transduction.

Disclosure of potential conflicts of interest

No potential conflicts of interest were disclosed.

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