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Human BMP sequences can confer normal dorsal–ventral patterning in the *Drosophila* embryo

(decapentaplegic/human BMP4/embryonic rescue/chimeric genes)

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ABSTRACT **The type β transforming growth factor family is composed of a series of processed, secreted growth factors, several of which have been implicated in important regulatory roles in cell determination, inductive interactions, and tissue differentiation. Among these factors, the sequence of the DPP protein from *Drosophila* is most similar to two of the vertebrate bone morphogenetic proteins, BMP2 and BMP4. Here we report that the human BMP4 ligand sequences can function in lieu of DPP in *Drosophila* embryos. We introduced the ligand region from human BMP4 into a genomic fragment of the *dpp* gene in place of the *Drosophila* ligand sequences and recovered transgenic flies by *P*-element transformation. We find that this chimeric *dpp–BMP4* transgene can completely rescue the embryonic dorsal–ventral patterning defect of null *dpp* mutant genotypes. We infer that the chimeric DPP–BMP4 protein can be processed properly and, by analogy with the action of other family members, can activate the endogenous DPP receptor to carry out the events necessary for dorsal–ventral patterning. Our evidence suggests that the DPP–BMP4 signal transduction pathway has been functionally conserved for at least 600 million years.**

The type β transforming growth factor (TGF-β) family is composed of several members of a secreted family of polypeptides that have profound effects on cell growth and differentiation (1–6). Their effect on cells occurs through binding to specific serine/threonine kinase receptors that transduce signals that alter the expression of downstream genes. The developmental effects of this growth factor family cover a wide spectrum but are usually associated with negative growth control.

In *Drosophila*, there are two known TGF-β-like members, the *dpp* gene and the *60A* gene. The *dpp* ligand acts in a signal transduction pathway to establish the identity of dorsal ectoderm in the early embryo. Later in development, the DPP protein is involved in other morphological events, such as visceral mesoderm formation and disk development. Mutations in the *60A* gene have not been identified, but its expression pattern suggests a role in embryonic mesoderm and ectoderm determination.

All nascent polypeptides of the TGF-β family members that have been studied are proteolytically processed to produce a propeptide and a mature polypeptide. The C-terminal mature region (in dimeric form) is the bioactive part of the molecule that binds the appropriate cellular receptors. It is the mature region, typically 110–130 amino acids long, that contains the seven invariant cysteine residues characteristic of all family members. The similarity of *dpp* to other TGF-β-like molecules follows a continuum, with the human BMP2/BMP4 proteins being most similar. DPP and BMP2/BMP4 are 75% identical over the C-terminal 100 amino acids of the mature region (Fig. 1*A*) and ≈30% identical in the propeptide region (7). Besides these high levels of sequence conservation, the localization of three *dpp* point mutations that disrupt all phenotypes controlled by *dpp* indicates that this C-terminal 100-amino acid domain is required for all *dpp* functions (K. Wharton, R. Ray, and W.M.G., unpublished results).

We have begun to examine whether the extensive structural similarity reflects functional conservation of DPP to BMP2/BMP4. We are defining functional conservation to mean a mechanistic conservation rather than a common set of cells or tissues affected by these protein factors. Presumably, the developmental potential of the cell will determine the consequences of signaling by these growth factor ligands, as is true for the effects *dpp* has on the developing *Drosophila*. For this purpose, we have used the earliest requirement for the *dpp* gene, determination of embryonic dorsal ectoderm (8–10), as a bioassay. We have constructed chimeric molecules and asked whether they are able to rescue the dorsal ectoderm of animals lacking *dpp*.

MATERIALS AND METHODS

Generation of Molecular Constructs. To facilitate construction of the chimeric genes, restriction sites were introduced into the appropriate clones. *Nar* I and *Sca* I restriction sites were introduced into *dpp* or *BMP4* by site-directed mutagenesis using mutant oligonucleotides (11). A *Nar* I site was introduced after the first conserved cysteine in the C terminus of the mature ligand region. The *Sca* I site was introduced after the termination codon of the protein. The introduction of new restriction sites did not change any of the amino acids encoded by either gene. The entire mutagenized insert was sequenced to verify that no unwanted mutations were introduced during the *in vitro* manipulations. The remainder of the coding region outside the *Nar* I and *Sca* I sites is derived from the *Hin* region of the *Drosophila dpp* gene. An 8-kb fragment containing the *dpp–BMP4* chimeric gene was cloned into the *P*-element transformation vector CaSpeR (12).

***Drosophila* Strains and Manipulations.** *dppH61* is a small deficiency that removes most of the 3′ coding exon of *dpp* (13) and *Df(2L)DTD48* is a deficiency for all of *dpp* (14). *SM6a* is a balancer for the second chromosome (15). Strains containing multiple copies of the chimeric transposon were generated by transposing the construct onto the desired chromosome with a strain containing an active transposase (16) or by recombining multiple copies onto a single chromosome. Germ-line transformants were obtained by standard techniques (17). Cuticle preparations were done on embryos as described (18).

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Abbreviation: TGF-β, type β transforming growth factor.

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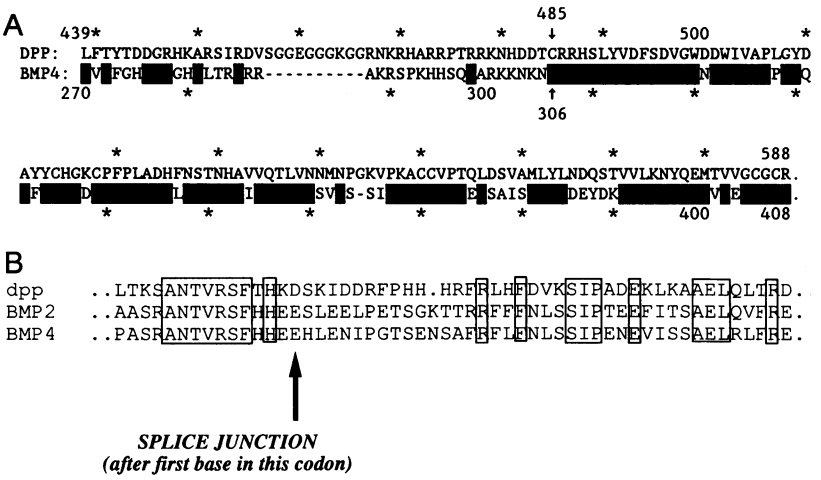
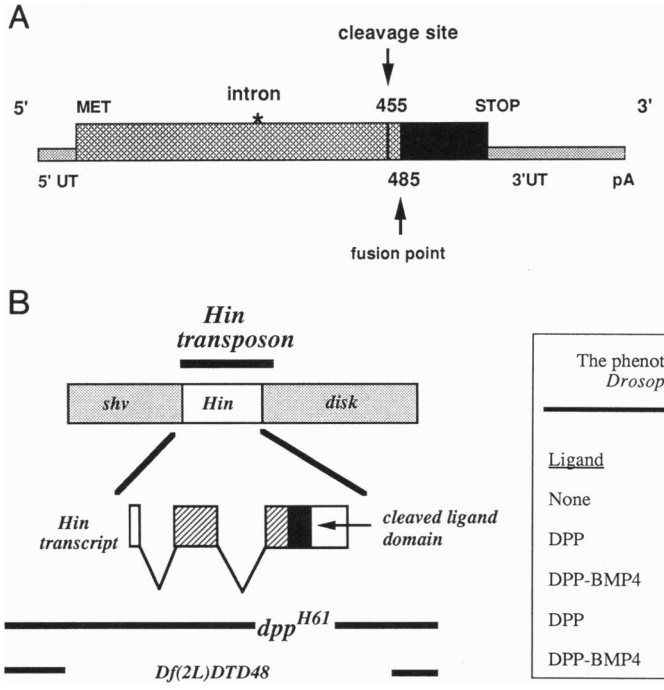


FIG. 1. Relationship of *dpp* and *BMP4*. (*A*) C-terminal alignment of DPP and BMP4 polypeptides. Solid boxes in place of amino acid sequences indicate perfect matches between the two factors. Asterisks denote increments of 10 amino acids in the sequence. (*B*) Alignment of amino acid sequences around the common intervening sequence. Shown are sequences on both sides of the introns of the *dpp* and *BMP4* genes, which allow for unambiguous placement of the intron.

RESULTS AND DISCUSSION

Conservation of Gene Structure. In addition to the sequence similarities of the DPP and BMP2/BMP4 polypeptides, there are considerable parallels between their transcripts. While the *dpp* gene (>55 kb) produces several transcripts, each has a similar structure (8). Each transcript consists of a unique 5′ untranslated exon together with common middle and 3′ exons. Because the open reading frame is entirely contained within the middle and 3′ exons, all *dpp* transcripts encode the identical polypeptide (R.W.P. and W.M.G., unpublished results). BMP2 and BMP4 have 5′ noncoding exons as well. Furthermore, like *dpp*, the coding regions of BMP2 and BMP4 are contained in two exons (J.M.W., unpublished results). The position of the intron between the two coding exons is in exactly the same position for all three genes. This is apparent by examining the alignment of protein sequences around this intron of the three genes (Fig. 1*B*). Sequences just upstream (ANTVRSF) and downstream (AELQ/R) of the splice sites for *dpp* and BMP2/BMP4 permit an exact alignment of the proteins in this region. Strikingly, the splice sites are at identical positions, after the first base in the fourth



The phenotypes of *Df(2L)DTD48/ dpp^{H61}* embryos carrying *Drosophila dpp* or human BMP4 chimera transgenes.

Ligand	Transgene copy number	Embryonic phenotype
None	0	strong ventralization
DPP	1	weak ventralization
DPP-BMP4	2	weak ventralization
DPP	2	normal
DPP-BMP4	3	normal

FIG. 2. *dpp–BMP4* chimera rescue experiments. (*A*) Structure of the mRNA derived from the *dpp–BMP4* chimeric transgene. Solid box represents human BMP4 ligand sequences that were introduced into the chimeric construct as a replacement for comparable sequences of the DPP ligand. Fusion site of the DPP and human BMP4 polypeptides is at the first conserved cysteine in the C terminus (amino acid 485 in the DPP sequence and 306 in BMP4) (see arrows in Fig. 1*A*), 30 amino acids from the putative proteolytic cleavage site (20, 21). Other than this inclusion of DPP sequences, the ligand is totally derived from BMP4. Asterisk indicates position of the common intron between the middle and 3′ exons. *dpp* genomic sequences extend for 1 kb on the 5′ and 1.5 kb on the 3′ side of the *Hin* transcription unit. UT, untranslated region. (*B*) Summary of chimera rescue experiments. (*Left*) Position in the *dpp* gene where the sequences for the rescue are derived. Transposons that contain the entire *Hin* region of *dpp* are capable of rescuing animals to hatching (19). (*Right*) Mutant phenotypes obtained by different doses of wild-type *dpp* and chimeric transposons.

codon C-terminal to the ANTVRSF motif. The structural parallels between their processed transcripts further strengthen the argument that *dpp* and BMP2/BMP4 have a common evolutionary origin.

The Chimeric Gene Can Rescue *Drosophila* Embryos. To test whether the human BMP ligand sequences could substitute for *dpp* in early development, we chose to replace the *Drosophila dpp* sequences with the human counterpart. In *Drosophila*, the formation of the dorsal ectoderm offers a sensitive bioassay for function. The dorsal–ventral patterning function of *dpp* in embryonic development is contained within an 8-kb fragment spanning the centrally located *Hin* region of the *dpp* gene (19). This region includes the most abundant *dpp* embryonic transcription unit (6, 8) together with sufficient cis-regulatory sequences to confer expression of *dpp* in the dorsal ectoderm during blastoderm and germband extension stages (R.W.P. and W.M.G., unpublished data). If transgenic constructs produce sufficient amounts of correctly regulated *dpp* activity, animals completely lacking endogenous *dpp* gene activity will hatch to larvae and display a normal larval cuticular pattern. For the 8-kb *Drosophila Hin* region construct, two copies of the transgene typically fully rescue dorsal–ventral patterning in a *dpp* null genetic background. We chose to attempt the rescue of a *dpp* null animal with our chimeric transgene rather than to attempt to create gain-of-function phenotypes by ectopic expression, since rescue directly and stringently tests whether the transgene can fulfill the normal requirement for *dpp* activity in its signal transduction pathway.

We have introduced a chimeric *Hin* region rescue transgene, in which the bulk of the ligand region (amino acids 485–588) was derived from the human *BMP4* gene (Fig. 2*A*), using standard *P*-element-mediated germ-line transformation (17). In these experiments, we did not want to determine whether the C-terminal proteolytic processing was conserved between *Drosophila* and humans. For this reason, we selected amino acids 485–588 encoded by the human gene for the *dpp–BMP4* swap to ensure that we were not replacing the C-terminal sequences necessary for protease recognition and cleavage of the mature ligand from the propeptide region. If the chimeric protein was not properly processed, then we would not be able to test whether it was able to function in place of the endogenous *dpp* gene. The phenotypic effects of this chimeric transgene in dpp mutant backgrounds were compared to those of a *Hin* transgene composed solely of *Drosophila dpp* sequences.

We have observed functional dpp^+ activity of the chimeric constructs in several genotypes with reduced or no endogenous *dpp* activity. For example, a single copy of the chimeric transgene is sufficient to rescue fully the otherwise haplolethal $dpp^+/Df(2L)dpp$ genotype (data not shown). The most striking demonstration of the activity of the chimeric transposon comes from genotypes totally lacking endogenous *dpp* activity (Figs. 2*B* and 3; Table 1). This genotype is heterozygous for two *dpp* deletions: a large one in which the entire chromosomal region surrounding *dpp* has been removed [*Df(2L)DTD48*] and a small one in which most or all of the 3′ exon, including the ligand coding region, is missing (dpp^{H61}). In such a *dpp* null background, partial and full rescue of

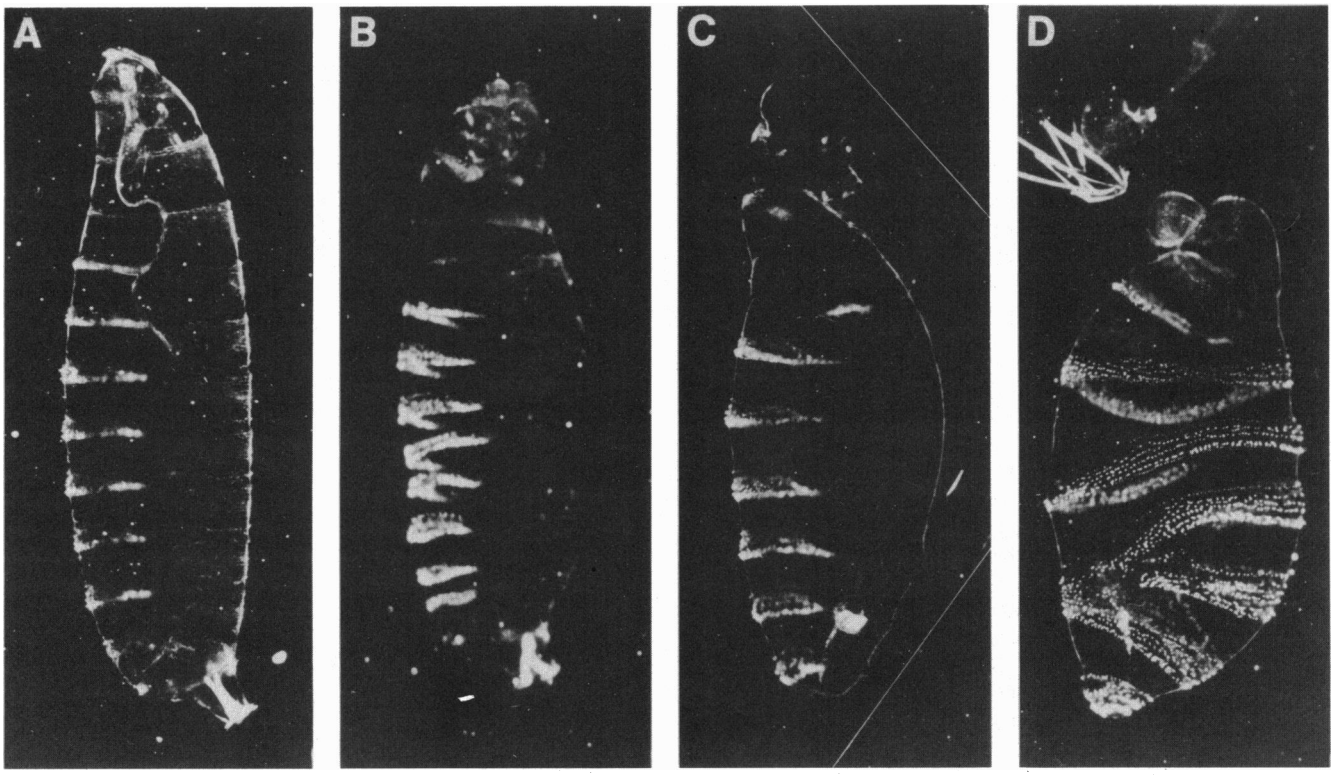


FIG. 3. Dark-field micrographs of cuticle preparations of wild-type and transgenic embryos. Dorsal is on top and anterior is on the left. All animals were derived from crosses that resulted in offspring containing the dpp^{H61} and *Df(2L)DTD48* null *dpp* alleles and different numbers of transposons as indicated. Rescuing activity can be observed by comparing the mutant phenotype of a *dpp* null allele (*D*) with different doses of the transposon (*A–C*). (*A*) Phenotype of a wild-type larva. The cuticle of this larva is indistinguishable from one mutant for dpp^{H61}/*Df(2L)DTD48* and harboring three or four copies of the chimeric transgene. (*B*) Mutant phenotype of a dpp^{H61}/*Df(2L)DTD48* embryo containing two copies of the transgene. Note that the primary defects of these embryos reside in the head and terminal region, reminiscent of mutant phenotypes seen in weak *dpp* alleles. (*C*) Mutant phenotype of an embryo containing one copy of the transposon. Note the more severe reductions in cephalic structures and the posterior filzkörper relative to *B*. (*D*) Mutant phenotype of a *dpp* null embryo. This dpp^{H61}/*Df(2L)DTD48* embryo lacks all endogenous *dpp* function and contains no transgene. This results in the complete absence of dorsal epidermis; there is a concomitant expansion of the ventral epidermal domain around the entire circumference of the embryo.

Table 1. Crosses to determine effects of the chimeric transgene on rescue of *dpp* null genotypes

Cross	Cross to generate test embryos		Resulting progeny*	
	♀ parent	♂ parent	Total fertilized eggs	% dead eggs
1	dpp^{H61}/*CyO-P23*†	*Df(2L)DTD48/CyO-P23*	378	46‡
2	dpp^{H61} *TnA*§/*SM6a*	*Df(2L)DTD48 TnB*/+	1480	24¶
3	*Df(2L)DTD48 TnC/SM6a*	dpp^{H61} *TnA*/+	621	29
4	dpp^{H61} *TnA TnD/SM6a*	*Df(2L)DTD48 TnB*/+	304	1
5	dpp^{H61} *TnA TnD/SM6a*	*Df(2L)DTD48 TnC TnD*/+	504	4
6	dpp^{H61} *TnA TnE/SM6a*	*Df(2L)DTD48 TnC TnD*/+	1379	6

*Embryos from brief egg lay collections were placed on medium-containing grids. Unfertilized eggs (white eggs) and dead embryos (discolored eggs) were counted 24–36 hr later.

†dpp^{H61} and *Df(2L)DTD48* are haplolethal. To create viable balanced strains containing these mutations, a transgene containing a second copy of the *dpp Hin* region was transposed onto the standard *CyO* balancer. This derived balancer, *CyO-P23*, contributes sufficient dpp^+ activity to rescue balanced *dpp* null alleles.

‡Approximately one-half of the dead embryos from cross 1 are dpp^{H61}/*Df(2L)DTD48*. The others are presumably *CyO-P23* homozygotes.

§In crosses 2–6, each *dpp* null-bearing second chromosome also contains one or more copies of the chimeric transgene. Five different independent insertions of the transgene were used (designated *TnA–TnE*).

¶In crosses 2–6, the male parent was heterozygous for a wild-type (i.e., dpp^+) chromosome. Thus, the only progeny class that could potentially give rise to a large proportion of inviable eggs would be the dpp^{H61} *Tn*/*Df(2L)DTD48 Tn* offspring. Hence, if this genotype were totally inviable in one of the crosses, 25% dead embryos are expected; this result obtains in crosses 2 and 3. If the genotype is totally viable, essentially no dead embryos are expected; this result obtains in crosses 4–6.

embryonic dorsal–ventral pattern is achieved by one and two copies, respectively, of the *Drosophila* transgene (19). In contrast, the chimeric *dpp–BMP4* construct is less efficient at rescuing this genotype. One or two copies of the chimeric *dpp–BMP4* transgene only achieves partial rescue. To determine whether this partial rescue were simply a quantitative effect or an intrinsic limitation of the chimeric transgene, genotypes containing three or four copies were then tested. Full rescue of the embryonic dorsal–ventral pattern is achieved with both three and four copies of the chimeric transgene as indicated by the proportion of dpp^- individuals that hatch (Table 1; Fig. 3). Thus, we conclude that, in the complete absence of endogenous dpp activity, a sufficient level of active chimeric DPP–BMP4 ligand can be generated to lead to a wild-type level of activation of the DPP receptor.

Why is the response of a given dosage of the chimeric transposon less robust than the comparable dosage of the *Drosophila dpp* transgene? Our preferred model is that the human ligand may have a lower affinity for the DPP receptor. The residues that bind to the receptor have not been positively identified so we cannot examine this possibility. One-fourth of the amino acids in the chimera are different from those of DPP and we know that flies are sensitive to the dose and activity of the *dpp* gene. There are other reasonable possibilities to account for the reduced activity of the chimeric gene. For example, it may be that we have altered the rate of proteolytic processing (in spite of our choice for the fusion site between the two genes) since these sites are not well defined. It may be that the DPP propeptide region or some other factor [e.g., tolloid (22)] does not interact with the BMP4 ligand region as efficiently, thus reducing the amount of active protein (23). Our present assay systems are incapable of distinguishing among these possibilities.

We have made a similar chimera between *dpp* and *BMP2*. While not yet tested for its ability to rescue homozygous null *dpp* genotypes, we have found that the one copy of the *dpp–BMP2* chimera fully rescues *dpp* monosomics (that are otherwise inviable), just as the *dpp–BMP4* chimera does (data not shown).

CONCLUDING REMARKS

The results of these studies are significant for several reasons. A secreted protein from humans has been shown to function in invertebrates. Most protein swapping experiments have shown conserved function of transcription factors in heterologous systems (24, 25). Furthermore, our experiments required that normal levels of the protein rescue a mutant phenotype. Most other gene replacement experiments involve overexpression of the heterologous genes to achieve a mutant phenotype, a less stringent criterion of conserved function. The similar structures of the *dpp* and *BMP2/BMP4* transcription units, in conjunction with functional conservation, add strength to the argument that these genes are true evolutionary homologs in the arthropod and vertebrate lineages, respectively.

Given strong arguments for evolutionary and functional conservation of these genes, it may be that they have retained common developmental roles (4, 5, 13, 26, 27). At present, this is difficult to determine. Since it is hard to equate the embryological events between *Drosophila* and vertebrates with our current knowledge, it is difficult to assign a similar developmental function to *dpp*, *BMP2*, and *BMP4*. Furthermore, both of these related human genes are involved in several uncharacterized developmental events, adding further difficulty to correlating developmental functions. However, it is clear from our study that these growth factors do not function to produce one kind of developmental event but rather send cellular signals that are interpreted in the context of the developmental state of the cell. This aspect of this growth factor family has been conserved for at least 600 million years.

The secreted proteins in the TGF-β family are involved in many protein–protein interactions prior to binding their respective cellular receptors (28–31). These protein interactions may be important for processing and/or altering the activity of the growth factor proteins, such as the role postulated for the tolloid/BMP1 proteins (7, 22). Receptors for DPP or any BMP have not been identified to date. However, given the nature of the structural conservations between all TGF-β family members, it is most plausible to expect that DPP and the BMPs act by binding to a transmembrane receptor, thereby initiating a signal transduction cascade. Since the chimeric DPP–BMP4 protein functions in *Drosophila*, then it must be folded properly, processed properly, bound in protein complexes, and presented to the receptor in a manner very similar to the endogenous *Drosophila* protein. Because this experiment was successful, it seems likely that many of the components of the signal transduction pathway have been conserved between *Drosophila* and vertebrates. These studies indicate that insights

we gain by studying these secreted factors in one system can likely be applied to other systems.

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