Multistep molecular mechanism for Bone morphogenetic protein extracellular transport in the Drosophila embryo

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In the Drosophila embryo, formation of a bone morphogenetic protein (BMP) morphogen gradient requires transport of a heterodimer of the BMPs Decapentaplegic (Dpp) and Screw (Scw) in a protein shuttling complex. Although the core components of the shuttling complex—Short Gastrulation (Sog) and Twisted Gastrulation (Tsg)—have been identified, key aspects of this shuttling system remain mechanistically unresolved. Recently, we discovered that the extracellular matrix protein collagen IV is important for BMP gradient formation. Here, we formulate a molecular mechanism of BMP shuttling that is catalyzed by collagen IV. We show that Dpp is the only BMP ligand in Drosophila that binds collagen IV. A collagen IV binding–deficient Dpp mutant signals at longer range in vivo, indicating that collagen IV functions to immobilize free Dpp in the embryo. We also provide in vivo evidence that collagen IV functions as a scaffold to promote the shuttling complex assembly in a multistep process. After binding of Dpp/Scw and Sog to collagen IV, protein interactions are remodeled, generating an intermediate complex in which Dpp/Scw-Sog is poised for release by Tsg through specific disruption of a collagen IV–Sog interaction. Because all components are evolutionarily conserved, we propose that regulation of BMP shuttling and immobilization through extracellular matrix interactions is widely used, both during development and in tissue homeostasis, to achieve a precise extracellular BMP distribution.

Bone morphogenetic proteins (BMPs) form a conserved family of signaling proteins with important functions during development and disease. BMP activity is regulated by a large number of extracellular proteins that modulate BMP receptor binding, stability, or distribution (1, 2). The early Drosophila embryo serves as an excellent model system to study extracellular mechanisms of BMP regulation, because a gradient of BMP activity specifies cell fates along the dorsoventral axis (3). Although the two BMP ligands, Dpp and Scw, are broadly expressed, a conserved set of extracellular regulators mediate the redistribution of the Dpp/Scw heterodimer to the dorsal midline, where peak signaling occurs (4). In dorsal regions, Dpp and Scw are bound by Sog and Tsg into an inhibitory shuttling complex that is unable to bind to receptors but is capable of moving (5–9). Dpp/Scw is released from the shuttling complex by the protease Tolloid, which cleaves and inactivates Sog (10). If Tolloid cleavage of Sog within the complex occurs in dorsolateral regions near the Sog source, Dpp/Scw is re-bound by Sog/Tsg. Multiple cycles of complex cleavage and reformation can occur, until Tolloid cleaves the complex in dorsal-most regions where the concentration of Sog is low, allowing Dpp/Scw to bind its receptors and activate high levels of signaling (5).

We have shown that the extracellular matrix protein collagen IV is important for BMP gradient formation (11). In embryos mutant for the collagen IV genes viking or Dcg1, peak signaling is lost and Dpp accumulation at the dorsal midline is reduced. In vitro experiments revealed that the C-terminal NC1 domain of collagen IV can bind to both Dpp and Sog. Based on these and additional findings, we proposed that collagen IV enhances BMP gradient formation by facilitating the assembly of the Dpp/Scw-Sog-Tsg shuttling complex, thereby promoting the long-range movement of BMPs (11). Although this model is supported by recent quantitative modeling of the BMP gradient in the Drosophila embryo (12), the molecular mechanism of shuttling complex assembly on collagen IV, and whether it occurs in vivo to regulate BMP distribution, is unknown.

Here, we have mapped the collagen IV binding sites on both Dpp and Sog. Based on these and previous interaction studies, we formulate a multistep, molecular model for the assembly of the Dpp/Scw-Sog-Tsg shuttling complex on collagen IV, which we test in vivo by using a collagen IV binding deficient mutant of Dpp. These experiments also provide in vivo evidence that collagen IV restricts movement of free Dpp ligands. We propose that collagen IV may regulate short- and long-range signaling of BMPs in diverse contexts.

Results

Mapping the Collagen IV Binding Site in Dpp. We previously showed that the NC1 domain of the Drosophila collagen IV proteins Viking and Dcg1 (named VkgC and Dcg1C, respectively) can directly interact with an HA-tagged form of Dpp (11). Mature Dpp is secreted from Drosophila S2 cells in two forms resulting from alternative proprotein cleavage at the S1 or S3 site (13). In GST-pulldown (GST-PD) experiments with bacterially purified GST-VkgC, we found that the larger S1 form binds better to collagen IV than the N-terminally truncated S3 form (Fig. 1A and B; compare ratio of S1 to S3 forms in bound vs. input FL fractions). This result suggested that the N-terminus of mature Dpp is important for binding to collagen IV. The S3 site and downstream residues form a highly basic stretch (Fig. L1). Partial or complete deletion of this basic region in Dpp-ΔA and Dpp-Δc, respectively, strongly reduced binding of Dpp to collagen IV (Fig. 1B), demonstrating that the basic motif is important for its binding to collagen IV. These data also suggest that the observed binding of the S3 form to VkgC (Fig. 1B, lane 2) is likely to be due to its dimerization with the S1 form.

Collagen IV Binding Site Is Not Conserved in Other Drosophila BMPs. We recently determined the N-termini of the other two Drosophila BMP ligands, Scw and Glass bottom boat (Gbb) (14). Sequence alignment reveals that the Scw and Gbb ligand domains lack the basic stretch found in the S1 form of Dpp (Fig. 2A).

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validate the Dpp basic motif as a collagen IV binding site and show that the only Drosophila BMP protein that can interact with the collagen IV NC1 domain is Dpp.

In the early embryo, the Dpp/Scw heterodimer is proposed to be both the most potent signaling species and the best substrate for Sog/Tsg-mediated shuttling to the dorsal midline (9). As shown in Fig. 2C, the Dpp/Scw heterodimer displays similar, if not better, binding to VkgC as the Dpp homodimer, again showing relatively more binding of the Dpp S1 form. Consistent with Scw being unable to bind the collagen IV NC1 domain, the collagen IV binding activity of the Dpp/Scw heterodimer is mediated by the basic motif in Dpp, because the DppΔαa/Scw heterodimer is unable to interact with VkgC (Fig. 2C).

**Mapping the Collagen IV Binding Site in Sog.** We next mapped the collagen IV binding sites on Sog. Sog is a large glycoprotein consisting of four cysteine rich (CR) domains separated by a long “stem” region between CR1 and CR2 (10) (Fig. 3A). To identify the regions of Sog important for interaction with the collagen IV NC1 domain, we tested binding of a panel of deletion constructs (Fig. 3A and B). Overall, these experiments suggest that (i) the CR domains mediate binding to collagen IV in a partially redundant fashion (e.g., compare FL vs. Δ3 vs. Δ4), and (ii) the stem region has an inhibitory effect on binding of Sog to collagen IV (compare FL vs. Δ5). We next tested the binding of each CR domain individually. As shown in Fig. 3C, CR1 and CR4 bound efficiently to VkgC, whereas no binding was detected for CR2 and CR3. An alignment of the Sog CR domains revealed that both CR1 and CR4 contain basic motifs (named B1, B2, B3, and B4) not found in CR2 or CR3 (Fig. 3D). For Sog CR1, mutation of B1 greatly reduced binding to VkgC (Fig. 3E). For Sog CR4, B2, B3, or B4 single or double mutants caused a partial loss of binding to VkgC, and the B2/B3/B4 triple mutant further attenuated the interaction (Fig. 3F), suggesting that all three basic motifs in CR4 function with partial redundancy in binding collagen IV. Mutation of either B1 or B2/B3/B4 in the context of full-length Sog also partially blocked binding to VkgC, and binding was very weak when basic motifs were mutated in both CR1 and CR4 (Fig. 3G).

**Molecular Model for Shuttling Complex Assembly on Collagen IV.** We previously presented evidence that collagen IV enhances BMP transport dorsally and proposed that it acts as a scaffold to enhance shuttling complex formation (11). The identification of the collagen IV binding sites on Sog and Dpp, combined with pre-existing data, allows us to formulate a molecular model for the assembly of the Dpp/Scw-Sog-Tsg shuttling complex on collagen IV. Our previous data showed that the Dpp/Scw heterodimer and Sog together can bind VkgC, but the addition of Tsg releases Dpp/Scw and Sog from collagen IV into the Dpp/Scw-Sog-Tsg shuttling complex (11). Here, we show that the Dpp/Scw heterodimer has only one binding site for collagen IV and that Sog has two binding domains, CR1 and CR4. These findings suggest a probable mechanism of release of Dpp/Scw and Sog from collagen IV into the shuttling complex. The Dpp/Scw heterodimer and Tsg together, but neither of them alone, can release Sog from VkgC (11), suggesting that each—Dpp/Scw and Tsg—competes with one of the two collagen IV binding sites on Sog. In vertebrates, the Dpp ortholog BMP2 and Tsg both bind strongly to CR1 and CR3 (15, 16), whereas the Scw-related protein BMP7 interacts preferentially with CR1 and CR4 (14) (Fig. 4A). Because only Sog binds to CR4 with high affinity, we propose that Dpp/Scw interferes with the Sog-CR4/VkgC interaction (via Scw binding to CR4), whereas Tsg competes for binding of collagen IV to Sog-CR1. Furthermore, because neither Sog nor Tsg alone can release Dpp from VkgC (11), we suggest a multistep model for shuttling complex assembly by collagen IV, in which release of Dpp/Scw is coupled to its transfer onto Sog. In the first step, Sog and Dpp/Scw both bind

Fig. 2. Dpp is the only BMP ligand in Drosophila to bind collagen IV. (A) Alignment of Dpp, Gbb, and Scw proteins at the N-terminus of the mature ligand region. Main (S1 for Dpp) and Shadow (S3 for Dpp) processing sites are highlighted. The basic sequence in Dpp required for collagen IV is not conserved in Gbb or Scw. (B) GST-PD between GST-VkgC and Dpp-HA, Scw-FLAG, or Gbb-FLAG (Left); Scw-FLAG and Gbb-FLAG show very weak binding to GST-VkgC (Right). (C) GST-PD between GST-VkgC and either Dpp-FL monomer, or Dpp-FL/Scw or Dpp-ΔαaScw heterodimer. Dpp-FL/Scw shows strong binding to VkgC, which depends on the collagen IV binding site in Dpp, because Dpp-ΔαaScw cannot bind to VkgC.

Consistent with the absence of a basic motif, Scw and Gbb show little or no binding to VkgC (Fig. 2B). These results further validate the Dpp basic motif as a collagen IV binding site and

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to the NC1 domain of collagen IV within the same NC1 trimer (Fig. 4B, step 1). This binding restricts Dpp and Sog diffusion, but it also promotes the transfer of Dpp/Scw onto Sog, through interactions of Dpp with Sog-CR3 and of Scw with Sog-CR4, which leads to disruption of both Dpp/collagen IV and Sog-CR4/collagen IV interactions (Fig. 4B, step 2). The resulting Dpp/Scw-Sog complex is bound to collagen IV by the Sog-CR inputs (20%). The resulting Dpp/collagen IV interactions (Fig. 4B, step 2). The resulting Dpp/Scw-Sog complex is bound to collagen IV by the Sog-CR inputs (20%).

Consistent with the formation of a Dpp/Scw-Sog “poised” intermediate on collagen IV, we show that Dpp-Delta/Scw, which lacks the collagen IV binding site, can associate with GST-VkgC in the presence Sog (Fig. 4C).

We used the collagen IV binding site mutant of Dpp (Dpp-Delta) to test our model in the context of the early embryo. First, we made use of a system for monitoring Dpp-Delta movement and signaling, based on ectopic expression from a stripe that runs perpendicular to the normal dpp expression domain (Fig. 4 D–F) (17). Activity of the peak threshold BMP target gene Race was visualized, which is normally expressed in a narrow stripe along the dorsal midline (presumptive amnioserosa) (Fig. 4G). As shown (7, 17), misexpression of wild-type Dpp under the even-skipped stripe 2(st2)-enhancer activates BMP signaling in cells within and outside the stripe, leading to expanded Race expression (Fig. 4H), consistent with the ability of the Dpp/Scw heterodimer to move long-range in the early embryo. In st2-dpp-Delta embryos, Race expression is also broadened and extends more posteriorly than in st2-dpp embryos (Fig. 4I), suggesting that Dpp-Delta can signal normally and may be more mobile than wild-type Dpp. Additionally, evidence is presented that the Delta mutation does not affect Dpp protein levels (Fig. S1).

We next used this expression system to test key aspects of our model of shuttling complex assembly by examining the behavior of Dpp-Delta in sog+ and sog- backgrounds. In both mutants, Race expression is lost in the presumptive amnioserosa but expanded in the anterior head region (Fig. 4J and M), reflecting the loss of peak signaling and uniform intermediate signaling, due to the loss of dorsal BMP shuttling (6). In our model, collagen IV restricts movement of free Dpp (i.e., when not bound to Sog); thus, one prediction is that Dpp-Delta, unlike wild-type Dpp, can move and signal long range in a sog+ background (Fig. 4K and L). When st2-dpp-Delta is expressed in sog+ embryos, it induces ectopic Race expression outside of its expression domain (Fig. 4L),
Fig. 4. Molecular model for Dpp/Scw-Sog-Tsg shuttling complex assembly on collagen IV. (A) Binding domains on Sog for collagen IV (VkgC), Dpp (BMP2), Scw (BMP7), and Tsg. (B) Molecular model for BMP shuttling complex assembly on collagen IV. (C) GST-PD between GST-VkgC or VkgC-Sog complex and Dpp, Dpp-Δa, or Dpp-Δa/Scw. (D–O) RNA in situ hybridizations for dpp (D–F; lateral views, dorsal up) or the BMP target gene Race (G–O; dorsal views) in control embryos and those expressing st2-dpp or st2-dpp-Δa transgenes. Embryos are wild-type (D–F), sog−/+ (J–L) or sog− (M–O). Brackets mark ectopic Race induction posterior of the st2 expression domain (arrows). Anterior is left in all images. (K–O) Cartoon depicting Dpp/Scw interactions as predicted by our molecular model. Intermediate-range signaling is attributed to a small number of Dpp/Scw molecules undergoing long-range signaling. Signaling in the st2 domain is at least in part attributed to Dpp homodimers (omitted from cartoons for simplicity). Thus, it is capable of long-range movement. This property is in stark contrast to that of ectopically expressed wild-type Dpp, whose effect is restricted to the st2 expression domain (7) (Fig. 4K). A second feature of our model is that Tsg acts to release the Dpp/Scw-Sog complex from collagen IV by disrupting the Sog-collagen IV interaction. Therefore, we predict Dpp-Δa would, in general, be unable to move and signal long range in a tsγ background, where most Dpp-Δa/Scw-Sog should remain trapped on collagen IV (Fig. 4O). Indeed, in the absence of Tsg, the effect of Dpp-Δa is largely restricted to its expression stripe (Fig. 4O). Finally, our model proposes that collagen IV enhances the association of Dpp/Scw, suggesting that formation of the Dpp/Scw-Sog complex should be less efficient for Dpp-Δa.

In the tsγ mutant, where assembly of the Dpp/Scw-Sog complex immobilizes BMPs on collagen IV, we therefore predict that some Dpp-Δa/Scw can move out of its expression stripe, whereas all wild type Dpp/Scw is trapped by collagen IV-Sog (Fig. 4 N′ and O′). Indeed, compared with wild-type Dpp, which is entirely restricted to its expression domain in tsγ embryos (Fig. 4N), some Dpp-Δa can activate signaling in cells away from its source. This intermediate-range signaling is observed as Race induction in a limited number of cells posterior to the st2 domain in the majority (∼70%) of embryos (Fig. 4O). In summary, our in vivo data support three key predictions from our model of shuttling complex assembly.

Discussion

There is ample experimental and theoretical support for the notion that BMP gradient formation in the early embryo involves the concentration of the most potent signaling species, the Dpp/Scw heterodimer, at the dorsal midline in a process involving Sog and Tsg (4). Here, we present in vivo evidence for a role of collagen IV in two key aspects of this shuttling model, which have remained mechanistically unresolved. First, collagen IV functions to immobilize free Dpp, explaining why Sog and Tsg are needed for Dpp movement. Second, collagen IV acts as a scaffold for assembly of the Dpp/Scw-Sog-Tsg shuttling complex. The advantage to BMP gradient formation of assembling the shuttling complex on collagen IV has been suggested by analysis of organism-scale mathematical models (12). These models reveal that the in vitro binding affinity between BMPs and Sog is too low to account for the rate of shuttling complex formation required in vivo. However, by acting as a scaffold, collagen IV would increase complex formation by locally concentrating Dpp/Scw and Sog. Models with a 10–20% reduction in diffusion rates for Dpp/Scw and Sog and an increased apparent affinity of Dpp/Scw for Sog show the best fit to vivo data (12).

Our molecular model of shuttling complex assembly occurs in three steps. The first step involves independent binding of Dpp/Scw and Sog to collagen IV. The ability of Dpp-Δa to signal long range in sog− embryos, where wild type Dpp is trapped in its expression stripe, provides in vivo evidence that the Dpp-collagen IV interaction restricts movement of free Dpp ligands. The result also demonstrates that Sog and Tsg promote long-range movement of Dpp because they release Dpp from collagen IV, and not simply because they prevent Dpp–receptor interactions (8, 18). Restriction of Dpp diffusion by collagen IV may stabilize the gradient by preventing ventral movement of Dpp/Scw after release from Sog/Tsg and promoting Dpp/Scw–receptor inter-
actions at the dorsal midline. It will be interesting, ultimately, to directly visualize Dpp and Dpp-ΔA directly in sog and tsq mutant embryos. Although current methods allow detection of high levels of receptor-bound Dpp (8, 9), there are technical limitations associated with specifically detecting the pools of Dpp that would be informative here, i.e., Dpp/Scq heterodimer within the shuttling complex or Dpp-ΔA/Scq diffusing between cells. Our data show that Sog is unable to bind the NC1 domain of collagen IV. This lack of collagen IV-dependent immobilization can explain why Sog, unlike Dpp, is capable of long-range signaling in the absence of Sog (8).

Step 2 of shuttling complex assembly involves remodeling of the protein interactions to generate a poised intermediate. Specifically, step 2 is driven by Sog-mediated disruption of the Sog CR4–collagen IV interaction, so that Dpp/Sog is transferred from collagen IV to the Sog CR3-CR4 domains. Sog displacement of the Sog CR4 domain from collagen IV provides molecular insight as to why Sog is needed for Dpp transport (9). In addition to the binding preference of Sog and Tsg for the Dpp/Sog heterodimer (9), only Sog has a high affinity for the Sog CR4 domain. Therefore, Dpp/Sog can be released from collagen IV into the shuttling complex, whereas the Dpp homodimer remains trapped on collagen IV.

In the final step of our model, Tsg mobilizes the shuttling complex by disrupting the Sog CR1–collagen IV interaction. It has been noted that tsq mutants display a more severe reduction in BMP signaling than sog and sog tsq double mutants (8) (also see Race expression in Fig. 4J and M). This observation has been attributed to a potential Sog-independent pro-BMP activity of Tsg at the level of receptor binding (8). A second contributing factor is suggested by our model, where Sog and Tsg act at distinct steps to allow formation of the shuttling complex. In tsq mutants, Dpp/Sog is loaded onto Sog by collagen IV, but remains locked in this inhibitory poised complex, so that the only BMPs capable of signaling are Dpp and Sog homodimers, which are less potent than the Dpp/Sog heterodimer (9). By contrast, in sog or sog tsq mutants, Dpp/Sog is not shuttled dorsally but is still capable of signaling locally, adding to signaling by Dpp and Sog homodimers. The weaker level of Dpp/Sog signaling in tsq mutants also provides support for our proposed order of steps 2 and 3 in the assembly process, because this order gives rise to the inhibitory intermediate of Dpp/Sog. Previously it was shown that an N-terminal fragment of Sog, called Supersog, which contains the CR1 domain and a portion of the stem, can partially rescue the loss of peak Dpp/Sog signaling in sog mutants (11). Indeed, we note that an N-terminal fragment of Sog, called Supersog, which contains the CR1 domain and a portion of the stem, can partially rescue the loss of peak Dpp/Sog signaling in sog mutants (11).

BMP movement will impact on a number of other contexts in both flies and vertebrates.

Materials and Methods

DNA Constructs. Cu-inducible Sog and Gbb constructs (pMT-Sog-1xFLAG and pMT-Gbb-3xFLAG) (14), DppΔN-HA (21), and pGEX4T1-VkgC (11) have been described. The coding sequences of Dpp-HA (10) and Sog-Myc (19) were inserted into pMT-5/HisA (Life Technologies) and then modified by PCR to introduce deletions or mutations (details available on request). To express individual Sog CR domains, regions encoding amino acids 95–186 (CR1), 737–814 (CR2), 825–909 (CR3), or 935–1031 (CR4) were cloned into pMT-Bip/VS/His-A (Life Technologies) in frame with the C-terminal VS/His tag. For transgenesis, the DppΔa deletion was introduced into Sk-Asc2-Dpp (17) by PCR and transfected as an AscI fragment into the 22FPE vector (30).

Protein Expression and Purification. Scw-FLAG, Gbb-FLAG, Dpp-HA, and Sog-Myc proteins were produced in Drosophila 2SR cells by effectence-mediated transient transfection followed by Cu-induction as described (14). Sog-CR4 was expressed in S2 cells, because secretion from 2SR cells was inefficient. For Dpp/Sog heterodimers, we cotransfected 3.5 μg of pMT-Dpp-HA and 1.5 μg of pMT-Sog-1xFLAG. Heterodimers were purified from 2 ml of medium by incubation with 50 μl of anti-FLAG M2 matrix (Sigma) according to manufacturer’s instructions. GST and GST-VkgC were expressed in Escherichia coli BL21 cells and purified on GSH-Sepharose beads (GE Healthcare) (details of purification available on request).

GST-Pulldown Experiments and Western Blotting. Equal amounts of GST-fusion proteins bound to GSH beads were incubated with 20–200 μl of Dpp-HA, Sog-FLAG, Sog-Myc, or Sog-CR-domain-V5-Transfected medium or 100 μl of affinity-purified Scw-FLAG/Dpp-HA complexes in pulldown buffer (20 mM at Tris pH 8.0, 100 mM NaCl, 1 mM EDTA) at 4 °C for 1–2 h. Beads were washed three times in 1 ml of pulldown buffer + 0.1% Nonidet P-40, and eluted in Laemmli buffer. Samples were separated by reducing SDS-PAGE followed by Western blotting or Coomassie blue staining.

Fly Strains. Fly stocks used were yw, sogS6/FM7c, tsq2/ifM7c, and both from Bloomington Drosophila Stock Center), st2-dppΔa (17). st2-dppΔa lines were generated at Bestgene by injecting 22FPE-dppΔa into yw fly embryos. Transgene expression was activated as described (6).

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Supporting Information

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**Materials and Methods**

**dpp mRNA Quantification in Transfected S2R\(^+\) cells and in st-dpp Embryos.**

RNA was extracted from S2R\(^+\) cells transfected with pMT-Dpp-HA or pMT-Dpp-Δ\(\alpha\)-HA (see Materials and Methods for transfection methods) and from embryos derived from crossing y\(^67c23\) w\(^118\) fe-males to st2-dpp or st2-dpp-Δ\(\alpha\) homozygous males using TRIzol (Life Technologies) according to the manufacturer’s instructions. For RNA extraction from S2R\(^+\) cells, two additional rounds of acid phenol extraction were included before RNA precipitation. RNA was treated with DNase I (Roche) for 30 min at room temperature, followed by DNase I inactivation by heat for 45 min at 75 °C. RNA extractions were performed in triplicate for each set of experiments.

From each RNA sample, cDNA was generated in duplicate (plus one no-reverse transcriptase control) by using eAMV reverse transcriptase (Sigma), oligo-dT\(_{23}\) primers (Sigma), and 1 μg of total RNA. Quantitative PCR (qPCR) analysis was performed by using SYBR green on a Chromo 4 real-time PCR machine (MJ Research). Raw dpp or st2-dpp levels were normalized to the levels of the reference gene rp49 measured in the same cDNA sample. The following primers were used for qPCR amplification:

- **RP49:**
  5′-CCCAAGGGTATCGACAACAGA-3′ and 5′-CGATGTGTGCGAAGGCGTG-3′

- **Dpp (S2R\(^+\) cells):**
  5′-GGCAAGGTGCCGAAGGCGTG-3′ and 5′-GTCATCTCCTGGTAGTTCTTCAG-3′

- **st2-dpp transgenes (embryos):**
  5′-GGGAATCGGAGCGAGAGCA-3′ and 5′-ACAACTTCTGAGG-3′

Fig. S1. Quantification of Dpp-Δ\(\alpha\) levels in tissue culture and 2- to 4-h embryos. (A and B) Expression of the same levels of dpp-HA and dpp-Δ\(\alpha\)-HA mRNA in S2R\(^+\) cells results in equal extracellular accumulation of Dpp-HA and Dpp-Δ\(\alpha\)-HA protein in the supernatant. S2R\(^+\) cells were transiently transfected with Copper-inducible vectors encoding Dpp-HA and Dpp-Δ\(\alpha\)-HA. The levels of secreted HA-tagged forms of Dpp-HA and Dpp-Δ\(\alpha\)-HA were quantified by Western blotting, with one representative blot shown in A. The level of dpp mRNA in transfected S2R\(^+\) cells was quantified by qPCR and correlated with the resultant level of extracellular Dpp protein (B). The results indicate that expression levels of wild-type Dpp and Dpp-Δ\(\alpha\) are equivalent. Thus, the Δ\(\alpha\) mutation in dpp does not intrinsically affect any of the various stages of gene expression involved in production of Dpp protein. Results represent the mean of three independent experiments; error bar shows SEM. (C) Quantification of mRNA levels from st2-dpp and st2-dpp-Δ\(\alpha\) transgenes in 2- to 4-h embryos carrying one copy of the transgene in a wild-type background. mRNA levels were assessed by qPCR analysis by using primers specific for the st2-dpp transgenes. Graph shows the mean of three independent experiments; error bar is SEM. The results demonstrate that st2-dpp and st2-dpp-Δ\(\alpha\) embryos analyzed in Fig. 4 express similar levels of dpp mRNA from the transgene. In conjunction with the tissue culture data (A and B), these results suggest that Dpp and Dpp-Δ\(\alpha\) extracellular protein levels are similar in st2-dpp and st2-dpp-Δ\(\alpha\) embryos.
Fig. S2. The collagen IV and heparan sulfate proteoglycan binding sites on Dpp overlap. (A) Mutagenesis of the S1 and S3 processing sites in the previously generated heparan-sulfate proteoglycan binding site mutant, Dpp-ΔN (1), demonstrates that mature Dpp-ΔN arises from cleavage at the S3 site. Secreted HA-tagged forms were analyzed by Western blotting. Mutation of S3 (S3*) causes a size shift, whereas mutation of S1 (S1*) has no effect. (B) Amino acid sequence of Dpp in the region of the furin processing sites (S1, S2, S3) directly upstream of the HA-tagged ligand domain. The sequences of S1/S3 forms of mature wild-type Dpp, the collagen IV binding site mutants, Dpp-Δa and Dpp-Δc, and the HSPG binding site mutant, Dpp-ΔN, are shown below. Note that, because of its processing at the S3 site, Dpp-ΔN is very similar in sequence to Dpp-Δa and Dpp-Δc. Note that Dpp-ΔN also carries amino acids HA instead of QP at the positions directly following the S3 cleavage site, as shown. (C) GST-pulldown between GST-VkgC and wild-type or deletion mutants of Dpp-HA. The Western blot shown is the full version of the result presented in Fig. 1B. Dpp-ΔN shows a similar reduction in binding to the collagen IV NC1 domain as Dpp-Δa and Dpp-Δc.