The *Drosophila decapentaplegic* and *short gastrulation* genes function antagonistically during adult wing vein development

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SUMMARY

TGF-β-related signaling pathways play diverse roles during vertebrate and invertebrate development. A common mechanism for regulating the activity of TGF-β family members is inhibition by extracellular antagonists. Recently, the *Drosophila short gastrulation* (*sog*) gene was shown to encode a predicted diffusible factor which antagonizes signaling mediated by the TGF-β-like Decapentaplegic (Dpp) pathway in the early blastoderm embryo. *sog* and *dpp*, which are among the earliest zygotic genes to be activated, are expressed in complementary dorsal-ventral domains. The opposing actions of *sog* and *dpp* in the early embryo have been highly conserved during evolution as their vertebrate counterparts, *chordin* and *BMP-4*, function homologously to define neural versus non-neural ectoderm in *Xenopus*. Here we exploit the genetically sensitive adult wing vein pattern to investigate the generality of the antagonistic relationship between *sog* and *dpp*. We show that *dpp* is expressed in vein primordia during pupal wing development and functions to promote vein formation. In contrast, *sog* is expressed in complementary intervein cells and suppresses vein formation. *sog* and *dpp* function during the same phenocritical periods (i.e. 16-28 hours after pupariation) to influence the vein versus intervein cell fate choice. The conflicting activities of *dpp* and *sog* are also revealed by antagonistic dosage-sensitive interactions between these two genes during vein development. Analysis of vein and intervein marker expression in *dpp* and *sog* mutant wings suggests that *dpp* promotes vein fates indirectly by activating the vein gene *rhomboid* (*rho*), and that *sog* functions by blocking an autoactivating Dpp feedback loop. These data support the view that Sog is a dedicated Dpp antagonist.

Key words: *decapentaplegic* (*dpp*), *short gastrulation* (*sog*), antagonism, wing vein development, *Drosophila*

INTRODUCTION

The *decapentaplegic* (*dpp*) gene encodes a member of the TGF-β superfamily (Padgett et al., 1987), which is most related to the vertebrate BMP-4 and BMP-2 proteins (Kingsley, 1994). *dpp* is expressed in the dorsal-most 40% of the early blastoderm embryo (St. Johnston and Gelbart, 1987) where it plays a key role in establishing dorsal cell fates (Irish and Gelbart, 1987; Wharton et al., 1993). Another gene required for patterning the dorsal region of the blastoderm embryo, *short gastrulation* (*sog*), is expressed in broad lateral stripes constituting the neuroectoderm (François et al., 1994). Expression of *sog* in lateral stripes adjacent to the dorsal domain is consistent with genetic evidence that *sog* functions non-autonomously to influence dorsal cell fates (Zusman and Wieschaus, 1988).

*dpp* and *sog* exert opposing influences in patterning the dorsal region of the blastoderm embryo. For example, expression of the *rhomboid* (*rho*) gene in dorsal-most presumptive amnioserosal cells is abolished in *dpp*^{-} mutants, but expands ventrally in *sog*^{-} mutants (François et al., 1994). Genetic evidence further supports the view that *sog* opposes *dpp* activity as reducing the gene dose of *sog* rescues lethality resulting from weak mutations in the Dpp pathway (Ferguson and Anderson, 1992; Wharton et al., 1993; François et al., 1994).

During other stages of embryogenesis (François et al., 1994), *dpp* and *sog* are also expressed in adjacent sets of cells. For example, *dpp* and *sog* are expressed in alternating longitudinal stripes during germ-band extension and retraction, and in a series of parallel non-overlapping rings during gut formation. Such correlated expression patterns are consistent...
with the possibility that dpp and sog interact during later stages of development.

To investigate this possibility in detail, we examined the role of dpp and sog in the simple context of adult wing vein development. During pupal stages, several forms of cell-cell communication contribute to the ultimate differentiation of continuous and straight veins (García-Bellido, 1977; García-Bellido and de Celis, 1992; Sturtevant and Bier, 1995). These cell-cell interactions include: (1) dorsal-to-ventral (D→V) signal(s) required for maintaining vein fates in cells on the ventral surface of the wing (García-Bellido, 1977; Sturtevant and Bier, 1995), (2) lateral inhibitory signal(s) emanating from vein primordia, which limit the width of veins differentiating within broad vein-competent regions (García-Bellido, 1977; Shellenbarger and Mohler, 1978, Sturtevant et al., 1993; Sturtevant and Bier, 1995), and (3) vein continuity signal(s) promoting vein formation in straight lines along the axis of vein extension (García-Bellido, 1977; Sturtevant and Bier, 1995).

Here, we show that, during early pupal development, dpp is expressed in vein primordia and sog is expressed in complementary intervein cells. dpp and sog function contemporaneously to exert opposing influences on the vein versus intervein cell fate choice: dpp promotes vein formation while sog suppresses vein development. Analysis of vein and intervein marker gene expression in sog and dpp mutant backgrounds suggests that Sog blocks an autoactivating function of Dpp and thereby channels Dpp activity along the vein axis. These data support models in which sog functions as a dedicated antagonist of Dpp signaling. We discuss the possible role of Dpp autoactivation in assuring vein continuity during pupal wing development.

**MATERIALS AND METHODS**

**Fly stocks**
All genetic markers and chromosome balancers are described in Lindsey and Grell (1968) and Lindsey and Zimm (1992). Construction of the 8XP[hs-dpp] stock is described in Twombly et al. (1996). Other stocks were obtained from the Bloomington, Indiana and Bowling Green, Ohio Drosophila Stock Centers.

**Construction of the HS-sog and UAS-sog vectors**
A Norl-HindIII fragment of a sog cDNA (17.12) containing the full predicted Sog protein coding sequence (François et al., 1994) was excised from the pNB40 vector and subcloned into a BlueScript vector. The HindIII and Norl sites were sequentially changed to Xbal sites by linker insertion. The resulting Xbal sog cDNA fragment was then subcloned into the Xbal site of the hs-CaSpeR heat-shock P-element vector (Bang and Posakony, 1992) and the Xbal site of the pUAST-P-element vector (Brand and Perrimon, 1993). Subclones were checked for correct insertional orientation and injected into white (w) embryos. Transformant flies were identified by screening for the linked mini-white (w<sup>+</sup>) marker (Rubin and Spradling, 1982).

**Enhancer piracy**
Enhancer piracy using a HS-sog P-element insertion was carried out as described in Noll et al. (1994). Briefly, females homozygous for a w<sup>+</sup> HS-sog P-element insertion on the X chromosome (abbreviated EP1-sog) were crossed to yw; Δ2-3 Sb v<sup>y+</sup>/TM6Bx males. EP1-sog; Δ2-3 Sb v<sup>y+</sup>/+ male progeny were collected and crossed to w females. Transposition of the w<sup>+</sup> HS-sog P-element from the X chromosome to autosomes generates w<sup>+</sup> male progeny. Over 1,000 independent male transposants were induced in individual bottle crosses of this kind, each of which was examined carefully for dominant phenotypes. Males with visible phenotypes were back-crossed to w females to determine whether the original phenotype was dominant and heritable. Balanced stocks were made from lines with highly penetrant dominant enhancer piracy phenotypes. 12 enhancer piracy lines (denoted EP2-EP13) were isolated in which various sections of veins were missing (see legend to Table 3). Other than venation defects, we did not recover any other dominant phenotypes in this screen.

**Mosaic analysis**
Female flies of the genotype y<sup>f6a</sup> sog<sup>6</sup> FRT<sup>18A</sup>neo/FTM7c or f<sup>6b</sup> sog<sup>6</sup> FRT<sup>18A</sup>neo/FTM7c were crossed to males of the genotype f<sup>+</sup> FRT<sup>18A</sup>neo/Y; MKRS,FLP3/TM6B, and the progeny were heat shocked at first and second instar larvae to generate mosaic adults containing homozygous sog<sup>−</sup> clones marked with y and f (sog<sup>−</sup>) or with f alone (sog<sup>−</sup>)

**Heat inductions**
Larvae or pupae carrying 8 copies of a HS-dpp construct (8XP[hs-dpp], Twombly et al., 1996), which we refer to as 8XP-HS-dpp in this study, 8 copies of the HS-sog construct described above (8XP-HS-sog), or 4 copies of the HS-sog construct in a dpp<sup>80b</sup> mutant background (4XS-HS-sog; dpp<sup>80b</sup>) were heat shocked according to the following regimen, which was repeated for a total of four cycles: 30 minutes of heat shock in a 37-38°C water bath followed by a 30 minute period of recovery at room temperature. Animals were staged at 25°C with respect to formation of white prepupa. Larvae were heat shocked in submerged glass vials, while prepupae and pupae were heat shocked on wet filter paper in a Petri dish. A minimum of 20 flies was scored for each time interval.

**Mounting fly wings**
Wings from adult flies were dissected in isopropanol and mounted in Canadian Balsam mounting medium (Gary’s magic mountant) following the protocol of Lawrence et al. (in Roberts, 1986). Mouted wings were photographed under a compound microscope using Nomarski optics for high magnification exposures (i.e. 20x or 40x lens) and without the condenser or Nomarski optics for low magnification photographs of complete wings (i.e. 4x lens).

**In situ hybridization to whole-mount embryos or discs**
In situ hybridization to whole-mount wings was performed using digoxigenin (Boehringer-Mannheim, 1093 657) or biotin-labeled RNA probes (O’Neill and Bier, 1994) as previously described (e.g. Sturtevant et al., 1993; O’Neill and Bier, 1994).

**RESULTS**

**dpp and sog are expressed in complementary patterns during pupal wing development**
To determine whether the theme of correlated sog and dpp expression observed during embryogenesis (François et al., 1994) continues into adult development, we examined the pattern of sog expression in imaginal discs and pupal wings. In wing and leg imaginal discs, sog is expressed in stripes running parallel to the dpp stripe along the compartment border (data not shown). In early prepupae, dpp expression along the A/P boundary disappears. In late prepupae, 6-9 hours after pupariation (AP), dpp expression is initiated in a series of stripes corresponding to vein primordia (data not shown). Following a cycle of separation and reposition of the two wing surfaces (Fristrom et al., 1994), expression of dpp in vein primordia re-emerges in early P1 stage pupae (18-20 hours AP; Fig. 1A). dpp
expression remains restricted to vein primordia in later stage P2 pupae (25-30 hours AP; Fig. 1C). In P1 pupae, sog expression is initiated in the center of intervein domains (Fig. 1B) and then expands in P2 pupae to include nearly all intervein cells (Fig. 1D). Double-label in situ hybridization with a digoxigenin-labeled dpp probe and a biotin-labeled sog probe reveals that the dpp and sog expression domains are strictly complementary throughout most of the wing except in the proximal region of the L5 vein, where there is a gap approximately one cell wide between dpp and sog expressing cells (data not shown). No wing cells express both dpp and sog, however. Thus, as in the early embryo, sog and dpp are expressed in abutting domains during pupal wing development.

**dpp promotes vein development during pupariation**

A class of loss-of-function dpp alleles (Fig. 2B; Segal and Gelbart, 1985; St Johnston et al., 1990; Posakony et al., 1990), certain combinations of Dpp receptor mutants (Nellen et al., 1994; Penton et al., 1994; Brummel et al., 1994; Terracol and Lengyel, 1994; Ruberte et al., 1995; Letsou et al., 1995) and loss-of-function clones of *schmurr*, which encodes a transcription factor likely to propagate a portion of the dpp signal (Arora et al., 1995; Grieder et al., 1995; Staehling-Hampton et al., 1995), lead to vein-loss phenotypes. Other combinations of Dpp receptor alleles, however, generate thick-vein phenotypes (Penton et al., 1994; Terracol and Lengyel, 1994). In addition, small clones of cells mis-expressing dpp have been observed to form localized patches of ectopic vein material (Zecca et al., 1995). These observations implicate dpp signaling in vein development. That dpp plays a role in vein formation per se remains unclear, however, since wing vein phenotypes associated with Dpp pathway mutants include thick veins as well as loss of veins. An additional complication is that dpp also functions earlier during larval development to supply anterior-posterior positional information and to direct imaginal disc outgrowth. The early function of dpp in anterior-posterior patterning indirectly affects vein formation (Segal and Gelbart, 1985; Sturtevant and Bier, 1995; Zecca et al., 1995; M. A. Sturtevant and E. Bier, unpublished observations).

To assess whether Dpp signaling plays a specific role in vein formation, we mis-expressed dpp during larval and pupal development in short pulses using the 8·HS-dpp stock (Twombly et al., 1996), which contains eight copies of a HS-dpp transgene (Table 1). Heat induction of 8·HS-dpp flies during larval or prepupal stages, when endogenous dpp is expressed in a stripe along the A/P compartment boundary, had

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**Table 1. Temporal profile of HS-dpp ectopic vein phenotypes**

<table>
<thead>
<tr>
<th>Stagea</th>
<th>Phenotype</th>
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<tbody>
<tr>
<td>3rd Instarb</td>
<td>high mortalityb, escapers and dissected pharate adults: &lt; 50% mild broad wing, &gt; 75% extra dorso-central macrochaetaeab</td>
</tr>
<tr>
<td>0h APc</td>
<td>&gt; 75%: mild wide wing</td>
</tr>
<tr>
<td>0-4h APd</td>
<td>&gt; 50%: mild broad wing, &gt; 75%: twigged posterior cross vein</td>
</tr>
<tr>
<td>4-8h APd</td>
<td>&gt; 75%: twigged posterior cross vein and distal tips of L4, L5</td>
</tr>
<tr>
<td>8-12h APd</td>
<td>&gt; 75%: twigged posterior cross vein and distal tips of L4, L5</td>
</tr>
<tr>
<td>12-16h APd</td>
<td>&gt; 75%: twigged veins and short ectopic veins (most branching anteriorly from L3)</td>
</tr>
<tr>
<td>16-20h APd</td>
<td>&gt; 90%: thick and long ectopic veins</td>
</tr>
<tr>
<td>20-40h APd</td>
<td>&gt; 90%: thick and long ectopic veins</td>
</tr>
<tr>
<td>24-28h APd</td>
<td>&gt; 90%: thick and long ectopic veins</td>
</tr>
<tr>
<td>25-32h APd</td>
<td>&gt; 90%: massive central blister, occasional ectopic veins</td>
</tr>
<tr>
<td>32-36h APd</td>
<td>&gt; 90%: thick veins, 50%: mild blisters at post. cross vein and/or short ectopic veins</td>
</tr>
<tr>
<td>36-40h APd</td>
<td>&gt; 75%: thick posterior cross vein, &lt; 50%: mild blisters and/or short ectopic veins</td>
</tr>
<tr>
<td>40-44h APd</td>
<td>&gt; 90%: wild type</td>
</tr>
</tbody>
</table>

(a) Stage when heat induction was begun. The heat-shock protocol consisted of four cycles of 30 minute heat shocks at 38°C separated by 30 minutes of recovery (see Materials and Methods for details). (b) feeding and wandering third instar larvae, (c) white prepupae were collected over a brief period of time and then heat shocked, (d) white prepupae were collected during 4 hour time intervals and then subjected to the heat-shock regimen, (e) 29/44 wandering third instar larvae and 20/22 feeding third instars failed to eclose, and (f) extra dorso-central macrochaetae were found spaced between the normal dorsocentrals and were usually shorter than the normal dorsocentrals (ectopic spaced scutellar macrochaetae were also occasionally observed, but the pattern of macrochaetae in other positions was normal). The only phenotype observed in flies heat shocked in 4 hour intervals between 44 and 60 hours AP was a short ectopic vein extending from the posterior cross vein. This latter phenotype is likely to be a heat-shock artifact, however, as it was also observed in heat-shocked wild-type flies.
little effect on venation. In contrast, heat shocks delivered between 16 and 40 hours after pupariation (AP) caused severe ectopic vein and blister phenotypes. Strong penetrant production of ectopic veins dominated in flies heat shocked 16-28 hours AP (Fig. 2D). Blistering, another phenotype commonly associated with widespread ectopic venation (Sturtevant et al., 1993; Noll et al., 1994; Fristrom et al., 1994; Sturtevant and Bier, 1995), was the most frequent phenotype at later times (28-40 hours AP). Heat shocks administered after 44 hours AP had no effect (Table 1). As the critical period during which dpp mis-expression generates ectopic veins coincides with restricted dpp expression in vein primordia (Fig. 1A,B), it is likely that Dpp acts locally to promote vein development. Genes such as rho (Sturtevant and Bier, 1995) and Notch (Shellenbarger and Mohler, 1978) also function during this period to influence the vein-versus-intervein cell-fate choice.

sog suppresses vein development during pupariation

To determine whether sog plays a role in vein formation, we first generated homozygous loss-of-function sog mutant clones in the wing using the FLP-FRT somatic recombination system (Golic, 1991). While the vein pattern is grossly normal in mosaic wings containing sog- clones, we consistently observed

<table>
<thead>
<tr>
<th>Stagea</th>
<th>Phenotype</th>
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<tbody>
<tr>
<td>0-4h AP</td>
<td>&gt;25%: truncated L4</td>
</tr>
<tr>
<td>4-8h AP</td>
<td>&lt;10%: truncated L4</td>
</tr>
<tr>
<td>8-12h AP</td>
<td>&lt;10%: truncated L4</td>
</tr>
<tr>
<td>12-16h AP</td>
<td>&lt;10%: truncated L4</td>
</tr>
<tr>
<td>16-20h AP</td>
<td>&gt;40%: truncated L4 and/or posterior cross vein</td>
</tr>
<tr>
<td>20-24h AP</td>
<td>&gt;60%: truncated L4, L5, and/or posterior cross vein</td>
</tr>
<tr>
<td>24-28h AP</td>
<td>&gt;40%: truncated L4, L5, and/or posterior cross vein</td>
</tr>
<tr>
<td>28-32h AP</td>
<td>&lt;10%: truncated L4</td>
</tr>
<tr>
<td>32-36h AP</td>
<td>&lt;10%: truncated L4</td>
</tr>
<tr>
<td>36-40h AP</td>
<td>&lt;10%: truncated L4</td>
</tr>
<tr>
<td>40-44h AP</td>
<td>&gt;90% wild type</td>
</tr>
</tbody>
</table>

(a) Stage when heat induction protocol was started, (b) wandering third instar larvae, (c) white prepupae were collected over a brief period of time and then heat shocked, (d) white prepupae were collected during 4 hour time intervals and then subjected to the heat-shock regimen, (e) 20/22 wandering third instar larvae failed to eclose and (f) short perpendicular vein segments (<20%) branching from L2 or L3 (0 hours and 32-40 hours AP) or long perpendicular vein segments (>30%) which occasionally formed cross veins (44-52 hours AP) were observed in heat-shocked 8xHS-sog wings. Similar ectopic vein phenotypes were observed sporadically in various combinations of UAS-sog x GAL 4 lines. The basis for this poorly penetrant phenotype, which occurs outside of the peak period for vein-loss phenotypes, remains to be determined. p. cross vein = posterior cross vein.
Table 3. Vein-loss phenotypes resulting from sog mis-expression

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Vein loss phenotype</th>
</tr>
</thead>
<tbody>
<tr>
<td>sog/dpp</td>
<td>GAl4</td>
</tr>
<tr>
<td></td>
<td>Ant. cross vein</td>
</tr>
<tr>
<td>UAS1/+</td>
<td>32B+</td>
</tr>
<tr>
<td>*UAS2/+</td>
<td>32B+</td>
</tr>
<tr>
<td>UAS2 EP1/+</td>
<td>32B+</td>
</tr>
<tr>
<td>UAS2 ++/+ sog-</td>
<td>32B+</td>
</tr>
<tr>
<td>*UAS2/+; Dppdp/+</td>
<td>32B+</td>
</tr>
<tr>
<td>UAS3/+</td>
<td>32B+</td>
</tr>
</tbody>
</table>

* UAS1/+ 69B/+ 0 0 ++ (L2,L3,L4,L5) |
* UAS2/+ 69B/+ 0 ++++ 0 |
* UAS2/UAS2 69B/+ ++++ ++++ (L2,L3,L4,L5) |
* UAS2 EP1/+ 69B/+ 0 ++++ (L4,L5) |
* UAS2/dpp (Y)sog’ 69B/+ 0 ++++ +++ (L3,L4,L5) |
* UAS2 ++/+ sog- | 69B/+ 0 0 0 |
* UAS2; Dppdp/+ | 69B/+ 0 0 0 |
* UAS3/+ 69B/+ 0 ++ (L4,L5) |
* UAS1/+ ptc/+ | + | 0 | 0 |
* UAS2/+ ptc/+ | ++ | 0 | 0 |
* EP1 | - | 0 | 0 | 0 |
* EP2;3,4;5,6/+ | - | 0 | ++ | 0 |
* EP2;3,4;5,6 homozygous | - | 0 | +++ | 0 |
* EP7,8,9;10,11/+ | - | 0 | 0 | ++ (L4,L5) |
* EP7 homozygous | - | 0 | 0 | ++ (L3,L4,L5) |
* EP12,13/+ | - | 0 | 0 | ++ (L5) |
* EP12 homozygous | - | 0 | 0 | ++ (L4,L5) |
* EP7/dppdp | - | 0 | 0 | 0 |
* dppdp+/+ | - | 0 | 0 | ++ (L3,L4,L5) |
* dppdp+/+;EP2;3/+ | - | 0 | 0 | +++ (L2,L3,L4,L5) |
* dppdp+/+;EP2;3/dppdp | - | 0 | 0 | ++++ (L2,L3,L4,L5) |
* tkv/tkv | - | 0 | 0 | 0 |
* Ep2;3/tkv | - | 0 | 0 | 0 |
* det/det | - | 0 | 0 | 0 |
* dppdp+/+;det/det | - | 0 | 0 | 0 |

Symbol key: Ant. cross vein, anterior cross vein; Post. cross vein, posterior cross vein; 0, wild-type vein pattern; +, slight and partially penetrant (>50% of wings) vein truncation; ++, moderate and frequent (>50% of wings) vein truncation; ++++, strongly and highly penetrant (>80% of wings) vein truncation; a, entire cross vein missing; parentheses indicate which of wings) vein truncation; a, entire cross vein missing; parentheses indicate which longitudinal veins are affected; UAS, UAS-sog lines (UAS1 is a weaker responder than UAS2 or UAS3); EP, enhancer piracy lines recovered in an enhancer piracy screen (Noûl et al., 1994) of more than 1,000 independent jumps of a HS-sog p-element vector (EP1) from the X-chromosome to the autosomes (see Materials and Methods). EP1 has no phenotype in a wild-type background, while EP2, EP3, EP4, EP5 and EP6 lack the anterior half of the posterior cross vein with high penetrance (>70% of wings). EP7, EP9, EP10 and EP11 lack the distal tip of L4 and L5 (>70% of wings), and EP12 and EP13 lack the distal tip of L5 (>70% of wings). EP2 and EP3 express the sog transgene in the intervein regions of third instar larval discs and pupal wings at high levels, suggesting their vein-loss phenotypes result from a non-autonomous action of the overexpressed sog transgene; GAL4-32B and GAL4-69B express GAL-4 throughout most of the wing disc (Brand and Perrimon, 1993), and GAL4-14tct expresses GAL4 in a narrow stripe running anterior to the anterior-posterior compartment boundary that intersects the future anterior cross vein; *, data shown in Fig. 2; †, construction of Dp(1;Y)sog; * Bx sog* by L. Deutsch and D. L. Lindsay will be reported in the Drosophila Information Service; bold entries indicate genetic interactions.

that veins meander about their normal trajectories and vary in thickness in clones covering veins (Fig. 2E, bottom panel). These irregularities contrast with the straight and uniform diameter veins typical of wild-type wings (Fig. 2E, top panel).

The interven pattern of sog expression and the irregular course of veins in sog- clones suggest that sog normally plays a role in restricting vein formation to the center of broad vein-competent regions.

Another way that we examined the function of sog during vein development is by mis-expressing sog ubiquitously at various developmental stages (Table 2) in 8xHS-sog flies, which carry eight copies of a HS-sog transgene. We also
ectopically expressed sog using the GAL4-UAS system (Brand and Perrimon, 1993) and enhancer piracy (Noll et al., 1994) (Table 3). Mis-expression of sog by each of these methods leads to various vein-loss phenotypes (Table 3; Fig. 2F,H), the most extreme of which (Fig. 2H) is very similar to the cumulative pattern of vein-loss resulting from various combinations of Dpp signaling mutants (Nellen et al., 1994; Penton et al., 1994; Brummel et al., 1994; Terracol and Lengyel, 1994; Ruberte et al., 1995; Letsou et al., 1995). The gain-of-function sog phenotype observed in UAS2-sog; GAL4-69B flies is due to a combination of endogenous and transgene encoded sog, since reducing the dose of endogenous sog normalized the vein pattern, whereas increasing the dose of endogenous sog aggravated the vein-loss phenotype (Table 3).

We determined the phenocritical period for HS-sog-induced vein-loss from a series of staged 8·HS-sog heat inductions (16-28 hours AP, Table 2) and found that it coincides with the period for generating ectopic veins in 8×HS-dpp pupae (Table 1). Thus, sog and dpp function during the same developmental interval to influence the vein-versus-intervein cell-fate choice.

dpp and sog mutations interact antagonistically during vein development

Consistent with the opposite vein phenotypes resulting from dpp versus sog mis-expression, we observed strong antagonistic genetic interactions between these two genes during vein development. For example, gain-of-function EP-sog alleles, which on their own generate only a partial truncation of the posterior cross vein (Table 3), greatly enhance the loss of longitudinal veins associated with the dpp<sup>shv</sup> allele (Fig. 2C). Enhancement of the dpp<sup>shv</sup> vein-loss phenotype, which is also observed as a result of heat shocking 4×HS-sog: dpp<sup>shv</sup> pupae,
**Table 4. Temporal profile for the dpp<sup>shv</sup> ↔ HS-sog interaction**

<table>
<thead>
<tr>
<th>Test Genotype</th>
<th>Effect on gain-of-function rho phenotype</th>
</tr>
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<tbody>
<tr>
<td>rho&lt;sup&gt;HS-Wk&lt;/sup&gt;/+</td>
<td>⬆️</td>
</tr>
<tr>
<td>rho&lt;sup&gt;HS-Mod&lt;/sup&gt;+</td>
<td>⬆️ ⬆️</td>
</tr>
<tr>
<td>rho&lt;sup&gt;HS-Stg&lt;/sup&gt;+</td>
<td>⬆️ ⬆️ ⬆️</td>
</tr>
</tbody>
</table>

Symbol key: ⬆️, strongly enhanced rho<sup>HS</sup> phenotype; ⬆️, enhanced rho<sup>HS</sup> phenotype; ⬆️ ⬆️, strongly suppressed rho<sup>HS</sup> phenotype; ⬆️ ⬆️ ⬆️, suppressed rho<sup>HS</sup> phenotype; ⬇️, weak incompletely penetrant modification of rho<sup>HS</sup> phenotype; ⬇️ ⬆️, rho<sup>HS</sup>-Stg/+ wings have slight ectopic vein phenotypes that are too subtle to score reliably for suppression, rho<sup>HS-Mod</sup>+ wings (Table 3A) have a mild ectopic vein phenotype typified by a short vein segment between L3 and L4 near the margin, rho<sup>HS-Stg</sup>+ wings have a large amount of ectopic vein material, which often results in separation of the dorsal and ventral surfaces in blisters (75% of wings have blisters, n=28; Fig. 3B). The frequency of rho<sup>HS-Stg</sup>+ blistering was strongly suppressed by sog<sup>EP2;3</sup> (+10% of wings have blisters, n=80) and by Dpsp<sup>10</sup> (10% of wings have blisters, n=80). dpp<sup>+</sup>; rho<sup>HS-Stg</sup> escapers were only rarely recovered due to rho<sup>HS</sup> haplolethality, but all (n=8) showed the same extreme suppression of the rho<sup>HS-Stg</sup> phenotype (Fig. 3F). See Sturtevant et al. (1993) and Sturtevant and Bier (1995) for examples of genetic interactions between other mutants and these three rho<sup>HS</sup> lines.

**Table 5. Opposite effects of sog versus dpp gene dosage on ectopic vein phenotypes**

<table>
<thead>
<tr>
<th>Stage&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Unmodified&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Moderately enhanced&lt;sup&gt;b&lt;/sup&gt; dpp&lt;sup&gt;shv&lt;/sup&gt;</th>
<th>Strongly enhanced&lt;sup&gt;b&lt;/sup&gt; dpp&lt;sup&gt;shv&lt;/sup&gt;</th>
</tr>
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<tbody>
<tr>
<td>no heat shock</td>
<td>50%</td>
<td>50%</td>
<td>0%</td>
</tr>
<tr>
<td>3&lt;sup&gt;d&lt;/sup&gt; Install&lt;sup&gt;b&lt;/sup&gt;</td>
<td>50%</td>
<td>50%</td>
<td>0%</td>
</tr>
<tr>
<td>4-16 AP&lt;sup&gt;c&lt;/sup&gt;</td>
<td>30%</td>
<td>70%</td>
<td>0%</td>
</tr>
<tr>
<td>16-28 AP&lt;sup&gt;d&lt;/sup&gt;</td>
<td>0%</td>
<td>50%</td>
<td>50%</td>
</tr>
<tr>
<td>28-40h AP&lt;sup&gt;e&lt;/sup&gt;</td>
<td>50%</td>
<td>50%</td>
<td>0%</td>
</tr>
</tbody>
</table>

(a) Stage when heat induction protocol was started, (b) wandering third instar larvae, (c) white prepupae were collected over a brief period of time and then heat shocked, (d) unmodified dpp<sup>shv</sup> phenotype (i.e. truncated L4 only), (e) moderately enhanced dpp<sup>shv</sup> phenotype (i.e. truncated L2 and L4), and (f) strongly enhanced dpp<sup>shv</sup> phenotype (i.e. truncated L2, L3 and L4).

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**Fig. 4.** Regulatory relationships between dpp, sog and rho in pupal wings. (A) rho expression in a wild-type P1 (18-20 hours AP) pupal wing is restricted to longitudinal veins. No cross veins have formed at this stage. L1-L5 veins are labeled 1-5. (B) rho expression in a 8xHS-dpp P1 pupal wing derived from an individual that was heat shocked for 30 minutes and allowed 30 minutes of recovery prior to fixation for in situ hybridization, rho expression is ectopically induced throughout intervein regions, except in narrow strips of cells running along veins (arrowheads). There is a wedge of elevated staining in the region between L4 and L5 in which the posterior cross vein will ultimately form (see E). rho expression is normal in a parallel cohort of heat-shocked wild-type wings (data not shown). (C) sog expression in a 8xHS-dpp P1 pupal wing derived from an individual that was heat shocked for 30 minutes and allowed 30 minutes of recovery prior to fixation for in situ hybridization, sog expression is globally reduced in distal regions of the wing (compare with Fig. 1B). Arrowheads indicate proximal regions of the wing in which sog expression is nearly normal. This labeling serves as an internal positive control for staining efficiency. sog expression is normal in a parallel cohort of heat-shocked wild-type wings (data not shown). (D) rho expression in a wild-type P2 pupal wing (25-30 hours AP) is observed in both longitudinal veins and cross veins. (E) rho expression in an 8xHS-dpp P2 pupal wing derived from an individual that was heat shocked for 30 minutes and aged 5 hours prior to fixation for in situ hybridization. Note the prominent restricted domains of ectopic rho expression near the posterior cross vein (solid arrow), anterior to L2 (arrowhead) and at the distal tip of the wing near L3 (open arrow). The P1 pupal wing shown in B would develop into a wing such as this. (F) sog expression in a 8xHS-dpp P2 pupal wing derived from an individual that was heat shocked for 30 minutes and aged 5 hours prior to fixation for in situ hybridization. The regions of strong down-regulation (compare with Fig. 1D) coincide with those ectopically expressing rho (indicated as in E of this figure). The P1 pupal wing shown in C would develop into a wing such as this. (G) dpp expression in longitudinal veins in a dpp<sup>0</sup>/dpp<sup>shv</sup> P2 pupal wing is virtually abolished (compare with Fig. 1C). Staining in the proximal (arrowhead points to L1 and L6 is indicated by 6) and hinge (out-of-focus) regions of the wing is relatively normal and there is low-level residual dpp expression in the L5 vein of this wing (indicated by 5). (H) rho expression in longitudinal veins is normal in a dpp<sup>0</sup>/dpp<sup>shv</sup> P2 pupal wing except in the distal region of L4 (arrow), which is missing in dpp<sup>0</sup>/dpp<sup>shv</sup> mutant wings (see Fig. 2B). Another anomaly typical of the dpp<sup>0</sup>/dpp<sup>shv</sup> mutation in certain genetic backgrounds (Diaz-Benjumea and Garcia-Bellido, 1990) is a small ectopic vein segment between L2 and L3 (arrowhead). In our dpp<sup>shv</sup> stock, however, this ectopic vein is found only occasionally in adult wings, although it is present in most or all pupal wings. (I) sog expression in longitudinal veins is normal in a dpp<sup>0</sup>/dpp<sup>shv</sup> P2 pupal wing (compare with Fig. 1D) except in the distal region of L4 (arrow), where rho expression is lost (see H). Also note that the small hole in sog expression between L2 and L3 (arrowhead) is in the same location as the ectopic island of rho expression shown in panel H. (J) dpp expression in a 8xHS-sog P1 pupal wing is strongly suppressed. Some residual labeling is observed in proximal (arrowhead) and extreme distal regions of the wing (compare with Fig. 1A). Similar heat-shock treatments of wild-type pupae also generate sporadic loss of dpp expression, particularly in the L2 promidium. This effect of heat shocking wild-type wings is significantly less severe than that observed in heat-shocked rho<sup>HS</sup>-sog wings such as the one shown in this panel, and may reflect interruption of the Dpp autoregulatory loop during the heat-shock treatment. (K) rho expression in a 8xHS-sog P1 pupal wing is indistinguishable from wild-type (e.g. A of this figure). (L) dpp expression in a UAS-sog; GALA-69B P2 pupal wing is strongly suppressed (compare with Fig. 1D). Some residual intermittent labeling is observed. Typically, expression in L2 and the dorsal surface of L3 is most strongly affected. The arrow points to the absence of a posterior cross vein, which would be present in a corresponding wild-type P2 wing, but is missing with high penetrance in UAS-sog; GALA-69B wings. In contrast, rho expression is normal in UAS-sog; GALA-69B pupal wings except in the regions where veins are missing from adult wings (e.g. the posterior cross vein).
reducing the level of sog by 50% strongly enhances rho\(^{HS}\) ectopic vein phenotypes (Fig. 3C), while elevated sog expression markedly suppresses the formation of rho\(^{HS}\) veins (Fig. 3D; Table 5).

**Ectopic dpp activates rho expression and suppresses sog expression**

To further analyze the role of dpp in promoting vein formation, we examined expression of several vein and intervein markers in 8xHS-dpp \\
8xHS-dpp expression, we observed widespread ectopic expression of the vein gene rho (Fig. 4B) and generalized suppression of sog expression in intervein regions (Fig. 4C).

Diego et al., 1990). Consistent with this possibility, we observed a strong enhancement effect on vein formation per se since rho is expressed normally in dpp\(^{HS}\) pupal veins except at the tip of L4 (Fig. 4H), which is missing in adult dpp\(^{HS}\) wings (Fig. 2B). sog expression in dpp\(^{HS}\) pupal wings is excluded from vein cells expressing rho (Fig. 4I). As dpp expression is globally compromised in dpp\(^{HS}\) wings, it seems unlikely that sog is a direct target of the Dpp pathway. On the other hand, the correlated expression of rho and suppression of sog in HS-dpp and dpp\(^{HS}\) wings suggests that rho may be more directly involved in excluding sog from vein primordia than is Dpp. In addition, these experiments reveal that rho expression is not dependent on high levels of dpp activity throughout most of the wing.

**sog may function by blocking Dpp autoactivation**

One important function of Dpp signaling during embryogenesis is to activate expression of dpp itself (autoactivation). To determine whether Dpp might play a similar autoactivating role during vein development, we examined dpp expression in HS-sog wings since the only known function of sog is to block Dpp signaling (B. Bietsch et al., unpublished data). A pulse of ectopic sog expression in 8xHS-sog pupal wings mimics the dpp\(^{HS}\) phenotype in that dpp expression is rapidly lost (Fig. 4I) without affecting the pattern of rho expression (Fig. 4K). A similar reduction in dpp, but not rho, expression was observed in pupal wings mis-expressing sog via the GAL4-UAS system (Fig. 4L).

**DISCUSSION**

**dpp and sog exert opposite influences on vein development**

The data presented above demonstrate that dpp promotes vein development during pupal stages and that sog antagonizes Dpp signaling in intervein regions. The vein-promoting activity of dpp appears to be entirely separate from its earlier role in establishing anterior-posterior polarity during larval development of the wing imaginal disc, although defects in this earlier function may also lead to venation defects (Segal and Gelbart, 1985; Johnston et al., 1990; Posakony et al., 1990; Sturtevant and Bier, 1995). As sog antagonizes dpp function in dorsal (Ferguson and Anderson, 1992; Wharton et al. 1993; François et al., 1994) and lateral (Bietsch et al., 1996) regions of the blastoderm embryo, it may be a general rule that the Sog product interferes with Dpp signaling. Preliminary data indicate that sog also blocks dpp activity during later stages of embryogenesis (B. Bietsch and E. Bier, unpublished observations). Further analysis of sog versus dpp function in other developmental contexts will be required to address the generality of dpp and sog constituting a signal-and-inhibitor genetic cassette.

**A network of gene interactions promotes vein fates**

The diagram in Fig. 5A summarizes the two basic results of our analysis of vein and intervein gene expression in various mutant backgrounds. The first of the proposed gene interactions is that dpp promotes vein development indirectly, possibly by maintaining rho expression in veins, which plays a key role in defining the vein fate by hyperactivating EGFR signaling (Sturtevant et al., 1993; Noll et al., 1994; Sturtevant and Bier, 1995). Thus, when dpp is ectopically expressed, rho expression is rapidly induced in intervein regions, except in narrow strips of cells flanking veins (i.e. near-vein regions), which appear to be refractory to the effects of Dpp. Ectopic rho expression in intervein cells presumably reflects a Dpp activity which
normally functions to maintain rho expression in veins. The basis for the refractory behavior of near-vein cells is not known; however, we note that these cells express enhanced levels of various intervein markers including tkv (B. Biels and E. Bier, unpublished data) which encodes a type I Dpp receptor (Nellen et al., 1994; Penton et al., 1994; Brummel et al., 1994). When heat-induced HS-dpp wings are allowed to develop, ectopic rho expression becomes restricted to a specific pattern. While the basis for this spatial bias is unknown, it provides an insight into the mechanism by which Dpp suppresses sog expression since the pattern of sog down-regulation in these aged wings correlates with that of ectopic rho expression. Another indication that rho is likely to play a more direct role than dpp in regulating sog is that sog expression is excluded from veins in dpp<sup>shv</sup> mutant wings. These vein primordia express normal levels of rho but do not accumulate appreciable levels of dpp. Thus, the pattern of sog expression in dpp<sup>shv</sup> mutant wings is complementary to that of rho, not dpp, which is nearly absent in this regulatory mutant. Finally, ectopic rho suppresses sog expression in a similar pattern to that of induced endogenous rho expression (data not shown).

The uncoupling of dpp and rho expression in HS-sog and dpp<sup>shv</sup> mutant wings suggests that there are other pathways maintaining rho expression, which function in parallel with Dpp. The EGFr-R pathway is a candidate for such a parallel genetic function since ectopic expression of a rho transgene induces endogenous rho expression. Furthermore, rho and Egr-R interact throughout the course of vein development to promote vein development (Sturtevant et al., 1993; Noll et al., 1994; Sturtevant and Bier, 1995).

The second major feature of the model depicted in Fig. 5A is that Sog antagonizes Dpp signaling by blocking Dpp autoactivation. Thus, ectopic sog, produced by either heat shock or the GAL4-UAS system, leads to dramatic reduction in dpp expression without affecting rho expression. Since available data support the view that Sog functions as a dedicated Dpp antagonist (this study and Biels et al., 1996), the most plausible explanation for the loss of dpp expression in HS-sog wings is that maintenance of Dpp expression requires an autoactivation loop, which can be broken by Sog. It remains possible, however, that Sog acts through some unknown independent pathway to antagonize dpp expression.

Potential functions for dpp and sog during vein development

Several types of cell-cell communication have been described during the latter stages of pupal wing vein development including: (1) lateral inhibitory signal(s) elaborated by presumptive vein cells restricting vein formation to the center of broad vein-competent domains, (2) dorsal-to-ventral signal(s) required by ventral vein cells to maintain their vein identity, and (3) vein continuity signals promoting vein formation along the axis of vein extension (García-Bellido, 1977; Díaz-Benjumeda and García-Bellido, 1990; García-Bellido and De Celis, 1992; Sturtevant and Bier, 1995). These various signals presumably collaborate to insure that the dorsal and ventral components of veins are strictly aligned and uninterrupted.

It is not certain whether the dpp and sog activities described in this study correspond to any of these known signaling functions or represent novel genetic functions for channelling veins along straight trajectories. We briefly consider whether the interaction between dpp and sog could be involved in each of the three types of cell-cell interaction mediating vein development. As discussed below, we favor the possibility that Dpp functions as a vein continuity signal.

A potential role for Dpp signaling in lateral inhibition is suggested by the thick-veins phenotype that is associated with various loss-of-function alleles of genes encoding components Dpp pathway (Penton et al., 1994; Terracol and Lengyel, 1994). Also, as described in this study, we observed irregularly thickened veins in HS-dpp and sog<sup>-</sup> mosaic wings. However, we believe that the vein phenotypes that we observed are unlikely to result from defects in lateral inhibition. The thickened veins typical of lateral inhibition mutants such as Notch and Delta are more solid and uniformly broad than those that we observed. In addition, we would expect that Dpp misexpression would generate a vein-loss phenotype not an ectopic vein phenotype if Dpp were functioning as a lateral inhibitory signal. It is possible, however, that Dpp plays a lateral inhibitory role during another distinct developmental stage. We tested for potential HS-dpp-induced vein-loss phenotypes over a broad window of pupal development and found no evidence for such an activity (Table 1). Furthermore, certain specific regions of veins are more affected than others in lateral inhibition mutants (e.g. the entire length of L3), while the vein thickening that we observe in HS-dpp or sog<sup>-</sup> mosaic wings is chaotic. Finally, we attempted to reveal a role for sog in regulating lateral inhibition by mis-expressing sog in a tkv mutant background. We expected that the EP-sog; tkv combination (Table 3) might aggravate the thick vein phenotype if sog further reduced the level of Dpp signaling mediated by the tkv receptor. Such enhancement of the tkv phenotype has been observed by lowering the level of schnurri (Staehling-Hampton et al., 1995), which propagates part of the Dpp signal. Surprisingly, however, we observed a loss-of-vein phenotype in EP-sog; tkv wings, which was much more severe than the mild EP-sog phenotype alone (Table 3). Thus, while tkv may mediate both lateral inhibitory and vein-promoting signals, it appears that sog can oppose only the vein-promoting activity of Dpp. It is possible that dpp functions through a sog-independent pathway to influence lateral inhibition or that another TGF-β family member serves as the lateral inhibitory ligand.

A role for Dpp as a dorsal-to-ventral signal is an intriguing possibility. If this were the case, one would expect to observe strong asymmetric effects resulting from eliminating or ectopically inducing dpp activity on either the dorsal or ventral surfaces. For example, ventral clones of loss-of-function Dpp receptor mutants should have stronger phenotypes than similar dorsal clones. One would also expect that ectopic expression of dpp dorsally should induce ectopic veins with dorsal and ventral components while ventral mis-expression either should have no effect or only generate a ventral vein component. These types of asymmetries have not been noted to date (Posakony et al., 1990; Grieder et al., 1995; Zecca et al., 1995; E. Bier, unpublished observations), but more in depth analyses of such experiments with these predictions in mind are necessary to resolve this point.

We believe that Dpp signaling is most likely to be to provide a vein continuity function (Fig. 5B). The meandering vein phenotype observed in large sog<sup>-</sup> patches is consistent with disruption of a mechanism constraining vein formation to the center of vein-competent domains (Fig. 5C). Expression of sog
in intervein regions could contribute to establishing the directionality of vein extension by providing straight narrow channels of Dpp-responsive cells (Fig. 5B). A role for Dpp in promoting vein continuity is also apparent in the light of the autoactivating function of Dpp in veins (see above). Thus, sog may provide a conduit within which Dpp can diffuse and autoactivate, thereby spreading a signal to all cells along the vein primordium to retain the vein fate. This mechanism for achieving a uniform cell fate along the axis of the vein is ideally suited for preventing any interruption of vein continuity. Another indication that dpp and sog may contribute to vein extension is that mis-expression of sog leads to detached cross veins in weakly affected individuals of various genotypes (more strongly affected individuals of these same genotypes typically lack the affected cross veins altogether, see Table 3). A similar floating cross vein phenotype is observed in detached (det) mutants and ectopic floating veins are common in net; det double mutants (Sturtevant and Bier, 1995). These phenotypes have been interpreted tentatively as disruption of a vein continuity function (Sturtevant and Bier, 1995). Consistent with this view, det is a potent enhancer of dpp function (Table 3). Further genetic and mosaic analysis will be required to distinguish between the various possible roles for Dpp and Sog during vein development.
Generality of dpp and sog antagonism

Antagonism of dpp activity by sog appears to be a phylogenetically conserved mechanism for subdividing the ectoderm into neural versus non-neural domains. In Xenopus, BMP-4 (a dpp homologue) and chordin (a sog homologue) are expressed in complementary dorsal-ventral domains (Fainsod et al., 1994; Sasai et al., 1994; Schmidt et al., 1995a). While the relative dorsal-ventral order of abutting BMP-4 and chordin expression domains is reversed in Xenopus relative to that of dpp and sog in Drosophila (Arendt and Nüblerjung, 1994; François and Bier, 1995), the developmental potential of cells arising from these juxtaposed domains are similar (i.e. neuroectoderm forms from sog or chordin-expressing cells while non-neural ectoderm derives from dpp or BMP-4-expressing regions). In addition, BMP-4 and dpp are functionally equivalent in both Drosophila and Xenopus embryos (Padgett et al. 1993: Holley et al., 1995) and chordin and sog have similar functions in both organisms (Holley et al., 1995; Schmidt et al., 1995b). The mechanism of action of this pair of genes may even be conserved. In Xenopus, BMP-4 suppresses the default program of neurogenesis and Chordin alleviates this repression to define a BMP-4-free zone in which neurogenesis is permitted (Wilson and Hemmati-Brevanlou; Sasai et al., 1995). We have recently obtained evidence that dpp plays an analogous role in suppressing neurogenesis at a very early stage of Drosophila embryoogenesis and that sog blocks this action of dpp in the neuroectoderm (Biels et al., 1996). As BMP-4 and chordin are both expressed during subsequent stages of vertebrate development, such as formation of the neural tube, it will be interesting to determine whether they also function antagonistically in these developmental settings.

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