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# A positive role for Short gastrulation in modulating BMP signaling during dorsoventral patterning in the *Drosophila* embryo

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## SUMMARY

**Positional information in the dorsoventral axis of the *Drosophila* embryo is encoded by a BMP activity gradient formed by synergistic signaling between the BMP family members Decapentaplegic (DPP) and Screw (SCW). *short gastrulation* (*sog*), which is functionally homologous to *Xenopus* Chordin, is expressed in the ventrolateral regions of the embryo and has been shown to act as a local antagonist of BMP signaling. Here we demonstrate that SOG has a second function, which is to promote BMP signaling on the dorsal side of the embryo. We show that a weak, homozygous-viable *sog* mutant is enhanced to lethality by reduction in the activities of the Smad family members *Mad* or *Medea*, and that the lethality is caused by defects in the molecular specification and subsequent cellular differentiation of the dorsal-most cell type, the amnioserosa. While previous data had suggested that the negative function of SOG is directed against SCW, we present data that suggests that the positive activity of SOG is directed towards DPP. We demonstrate that Chordin shares the same apparent ligand specificity as does SOG, preferentially inhibiting SCW but not DPP activity. However, in *Drosophila* assays Chordin does not have the same capacity to elevate BMP signaling as does SOG, identifying a functional difference in the otherwise well conserved process of dorsoventral pattern formation in arthropods and chordates.**

Key words: *short gastrulation*, *decapentaplegic*, *screw*, BMP, Chordin, dorsoventral patterning, *Drosophila melanogaster*

## INTRODUCTION

Patterning along the dorsoventral (DV) axis in both arthropod and vertebrate embryos is established by activity gradients of Bone Morphogenetic Proteins (BMPs) (reviewed by Dale and Wardle, 1999; Holley and Ferguson, 1997). In *Drosophila*, high levels of BMP activity in the dorsal 10% of embryonic cells promote differentiation as extraembryonic amnioserosa, lower BMP levels cause dorsolateral cells to become dorsal epidermis, and the absence of BMP signaling allows ventrolateral cells to differentiate as neurogenic ectoderm (reviewed by Podos and Ferguson, 1999). While the biological output of the activity gradient is straightforward with respect to the tissue types it generates, the molecular complexities underlying the establishment and interpretation of the activity gradient are just beginning to be understood.

One of the unusual features of the BMP activity gradient is that it is generated by the combined action of two BMP ligands, Decapentaplegic (DPP) and Screw (SCW). DPP, which is expressed at uniform intensity throughout the dorsal 40% of the embryonic circumference, is absolutely necessary and under experimental conditions can be sufficient to generate all positional values within the ectoderm. However, in vivo SCW, which is uniformly expressed at the blastoderm stage, is necessary for production of the most dorsal cell fates (Arora et al., 1994). Moreover, functional data suggests that SCW acts through the type I receptor Saxophone (SAX), and that SAX signaling synergizes with signaling by the DPP type I receptor Thickveins (TKV) to promote dorsal cell fates (Neul and Ferguson, 1998; Nguyen et al., 1998).

Because both BMP ligands are expressed at uniform intensities over the regions in which they are required, the BMP activity gradient must be generated by post-transcriptional modification of ligand activity. The *short gastrulation* (*sog*) gene, which is expressed in the ventrolateral regions of the embryo, encodes a large cysteine-rich extracellular protein (François et al., 1994; Holley et al., 1995) that antagonizes BMP activity ventrally. There is a DPP-dependent expansion of dorsal cell types in *sog* mutants, and ectopic dorsal expression of SOG can lead to a partial ventralization of the embryo (Biehs et al., 1996; Ferguson and Anderson, 1992b; Holley et al., 1995). In *Xenopus*, the functional homologue of SOG, Chordin (Chd), inhibits BMP4 by binding to it and preventing BMP4 from activating its receptor (Piccolo et al., 1996). Overexpression of SOG can block SCW, but not DPP activity, suggesting that the inhibitory action of SOG is likely to be directed against the SCW protein (Neul and Ferguson, 1998; Nguyen et al., 1998).

The existence of a diffusible BMP inhibitor raised the possibility that a BMP activity gradient could be formed

through a gradient of the SOG inhibitor. Support for this hypothesis came from the characterization of a secreted zinc metalloprotease, *tolloid* (*tld*) in flies and Xolloid (Xld) in *Xenopus* (Piccolo et al., 1997; Shimell et al., 1991). *tolloid* is expressed dorsally in the same domain as *dpp* and is required for production of dorsal structures. In vitro, TLD cleaves SOG, and this cleavage is stimulated by the presence of DPP (Marqués et al., 1997). Similarly, Xld cleaves Chd and Chd/BMP4 complexes, releasing biologically active BMP (Piccolo et al., 1997). These results suggested that a ventral source of the SOG inhibitor coupled with a dorsal sink for SOG (the cleavage of SOG by TLD) could result in a ventral-to-dorsal gradient of SOG, causing a reciprocal dorsal-to-ventral gradient of DPP activity (Marqués et al., 1997).

The inhibitory function of SOG, however, is not sufficient to fully explain its mutant phenotype. Specifically, in addition to their ventral defects, *sog* mutants also lack amnioserosa, the most-dorsal tissue type (Zusman et al., 1988). Thus, SOG must function outside of its domain of expression to promote amnioserosa formation. All other mutants that lack amnioserosa have defects that reduce BMP signaling, and it is not immediately obvious how loss of a negative regulator of BMP signaling would disrupt amnioserosa formation. We had previously proposed a model to explain this phenotype in which binding of BMP ligand to SOG allowed for diffusion of the ligand-SOG complex to the dorsal region of the embryo where release of the ligand from the inhibitory complex, possibly by TLD cleavage of SOG, would lead to elevated ligand signaling (Holley et al., 1996). However, at that time this model lacked experimental proof.

Specification of amnioserosa is well correlated with the expression pattern of the direct DPP target gene *zerknüllt* (*zen*) at the cellular blastoderm stage (Ray et al., 1991; Rushlow et al., 2001). At this stage in wild-type embryos, the expression of *zen* is restricted to the dorsal 10% of embryonic nuclei that will become amnioserosa. In ventralized mutants that lack amnioserosa, such as *dpp*, *tld* and *scw*, transcription of *zen* disappears at this stage (Ray et al., 1991). In contrast, in *sog* null mutants, *zen* transcription encompasses the dorsal 40% of embryonic nuclei (Ray et al., 1991; Rushlow and Levine, 1990). This expanded pattern has been interpreted to suggest that SOG acts solely as a negative regulator of *zen* transcription. Recently, however, Rushlow et al. (Rushlow et al., 2001) demonstrated that loss of various subsets of MAD binding sites in the *zen* promoter also resulted in *zen* transcription in the dorsal 40% of the embryo, suggesting that the spatial extent of *zen* transcription may not be a linear readout of the strength of BMP signaling at the cellular blastoderm stage.

Another extracellular modulator of BMP signaling, *twisted gastrulation* (*tsg*), is expressed in the dorsal 40% of the embryo and encodes a secreted cysteine rich protein that has weak similarity to Connective Tissue Growth Factor (CTGF; Mason et al., 1994). *tsg* mutants also display a weakly ventralized phenotype, with loss of amnioserosa (Mason et al., 1994; Zusman and Wieschaus, 1985), suggesting that TSG is also required for maximal BMP signaling. However, *zen* transcription is also expanded in *tsg* mutants, in a pattern similar to that observed in *sog* mutants, although the intensity of *zen* protein level is decreased relative to that observed in *sog* mutants (Rushlow and Levine, 1990). A recent series of papers concerning the action of TSG and its vertebrate homologues have presented conflicting interpretations as to its function. These papers have shown that TSG physically interacts with SOG; however, different conclusions were reached as to the biological function of the complex. Oelgeschlager et al. (Oelgeschlager et al., 2000) suggested that TSG acts to potentiate BMP signaling, while others conclude that a TSG/SOG complex acts to inhibit BMP signaling (Chang et al., 2001; Ross et al., 2001; Scott et al., 2001; Yu et al., 2000). Although these different interpretations have not yet been resolved, the data are suggestive that both SOG and TSG may have multiple functions in vivo.

In this paper we provide direct genetic evidence that SOG has a dual function in modulating the BMP activity gradient. In addition to its previously described ability to locally inhibit BMP signaling ventrally, we unambiguously establish that SOG is required for maximal BMP activity dorsally. We show that a weak homozygous viable allele of *sog* is enhanced to lethality by reduction in the activity of the essential DPP signal transduction components, *Mad* or *Medea*, and that this enhancement can be observed as a reduction in the level of transcription of the direct DPP target *zen*. During the course of this work Ashe and Levine (Ashe and Levine, 1999) showed by gain-of-function experiments that SOG can inhibit the transcription of a dorsal-specific gene at the site of expression and can activate the transcription of the same gene in a long range fashion. We confirm their findings, and in addition, we present results that suggest that the positive activity of SOG is directed toward DPP rather than SCW. We demonstrate, in *Drosophila*, that *Xenopus* Chd has the same ligand specificity as SOG, but that it does not have the same ability to potentiate BMP activity. We discuss our data in the context of the recent findings concerning the activities of SOG and TSG, and we present a model that may help reconcile their mutant phenotypes and their demonstrated biological activities.

## MATERIALS AND METHODS

### *Drosophila* stocks and genetic constructions

Wild-type embryos were from a stock containing a *P[Kr-lacZ]* insertion on the third chromosome. $sog^{P129D}$, listed in FlyBase as $sog^{supp}$, was recovered as a dominant extragenic suppressor of $tld^{10E}$ in a P-element mutagenesis screen (Ferguson and Anderson, 1992b). $Mad^{12}$ (Sekelsky et al., 1995), $Med^{13}$ and $Med^{15}$ (Hudson et al., 1998), $zen^{1}$, $snk^{1}$ and $snk^{229}$ (FlyBase, 1999) have been described previously.

$zen^{1}$ is a weakly temperature-sensitive mutation. To determine the phenotype of $sog^{P129D}$; $zen^{1}$ embryos at 25°C, a $sog^{P129D}$/*FM7*; $zen^{1}$/*TM3* doubly balanced stock was grown at 18°C. Rare double mutant escapers of genotype $sog^{P129D}$; $zen^{1}$ were transferred to 25°C, and the phenotypes of the progeny embryos were examined.

To obtain *sog* null embryos with three copies of $dpp^{+}$, $sog^{YSO6}$/*FM7 P[ftz-lacZ]* females were crossed to *Dp(2;2)DTD48*/*CyO*; *P[Kr-lacZ]* males. Stage 13 *sog* embryos were identified as those that did not stain for the *P[ftz-lacZ]* insert. Stage 6a *sog* embryos were identified as those that did not express the *sog* transcript ($sog^{YSO6}$ is a transcript null allele (Francois et al., 1994)). In both cases, 1/2 of the *sog* embryos carried an extra copy of $dpp^{+}$.

Dorsalized embryos were obtained by mating $snk^{229}$/$snk^{1}$ females with wild-type males. Embryos lacking *scw* were obtained as the progeny of a *Df(2L)OD16 P[Kr-lacZ]*/*CyO P[ftz-lacZ]* stock that did not stain for the *P[ftz-lacZ]* insert.

### Recovery of dominant enhancers of *sog$^{P129D}$* to lethality

We performed a screen to identify maternal and X-linked zygotic mutations that dominantly enhanced the phenotype of *sog$^{P129D}$* to lethality. *sog$^{P129D}$*, *y cv v f car* males were starved for 8 hours, fed 25 mM EMS in 1% sucrose for 24 hours, and mated to *sog$^{YL26}$*, *w$^a$*/*FM7b*, *y w$^a$* females. $F_1$ *sog$^{P129D}$*, *y cv v f car* (*)/*FM7b*, *y w$^a$* females were individually mated both to *sog$^{P129D}$*, *f car* males (to test for the presence of an enhancer mutation), and to *FM7b* males (to recover the mutant chromosome). In the $F_2$ generation, we scored the ratio of *sog$^{P129D}$*, *y cv v f car* (*)/*sog$^{P129D}$*, *f car* females to *sog$^{P129D}$*, *f car*/*FM7b*, *y w$^a$* females to minimize the false positives that could arise from low progeny numbers. To distinguish X-linked zygotic from autosomal maternal enhancers, individual $F_3$ candidate females carrying the mutagenized chromosome in *trans* to *FM7b* were crossed to *sog$^{P129D}$*, *f car* males. An X-linked zygotic enhancer should be evident in the progeny of every female analyzed. However, only 50% of the $F_3$ females should carry a dominant maternal enhancer on either the second or third chromosome.

The maternal enhancer mutation on chromosome 2, *Mad$^{ES1}$*, was recovered through iterated matings of individual candidate females to *sog$^{P129D}$*, *f car*; *S cn bw*/*CyO*, *cn pr* males. Each female progeny that carried *CyO* was assessed for dominant enhancement of the *sog$^{P129D}$* mutation. Those that carried the enhancer mutation were mated again to the same males, and the enhancer mutation was observed to segregate away from the balancer chromosome. The two maternal enhancer mutations on chromosome 3, *Med$^{ES2}$* and *Med$^{ES3}$*, were recovered in *trans* to the balancer chromosome *TM3*, *Sb e* through a similar strategy of iterated matings of individual candidate females to *sog$^{P129D}$*, *f car*; *srp e*/*TM3*, *Sb e* males.

The genetic identities of the enhancer mutations were assessed both by multi-factorial recombinant mapping with respect to visible marker mutations and by complementation analysis. The enhancer mutation on chromosome 2 was localized to the chromosomal interval between the *aristaless* and *dumpy* loci and was shown to be an allele of *Mad* by non-complementation with the phenotypically null mutation *Mad$^{12}$*. The two enhancer mutations on chromosome 3 were mapped distal to the visible marker *claret* and were identified as alleles of *Med* by non-complementation with the null mutation *Med$^{13}$*.

The degree of enhancement of *sog$^{P129D}$* by various maternal-effect mutations was obtained by pooling viability data from at least 10 crosses of *sog$^{P129D}$* males to individual females doubly balanced for both mutations.

### Embryonic phenotypes

Embryonic cuticle preparations were performed as described previously (Wieschaus and Nüsslein-Volhard, 1980). β-galactosidase expression was assayed in stage 13 embryos as described by Ferguson and Anderson (Ferguson and Anderson, 1992a).

### RNA microinjections

Capped mRNAs were generated by in vitro transcription with SP6 mRNA polymerase from the Message Machine kit (Ambion). pSP35T plasmids containing *sog* or *chd* (Holley et al., 1995) were linearized using *Sac*I and *Xba*I respectively. *chd* mRNA encodes a *sog/chd* chimera that contains the N-terminal 89 amino acids of SOG, followed by two serine residues, followed by Chd starting at amino acid 35 (Holley et al., 1995). pSP35T-*scw* was linearized with *Pst*I. *dpp* mRNA was expressed from the pGEM-7zf(+)-*dpp* vector (Ferguson and Anderson, 1992a), which was linearized using *Xho*I.

mRNA was injected into preblastoderm embryos as described previously (Ferguson and Anderson, 1992a). Microinjection needles were pulled using standard microinjection parameters, broken to produce an opening of diameter less than or equal to 8.7 μm, and subsequently ground to a uniform bevel using a Narishige microgrinder. A Narishige picospritzer was used to deliver a constant bolus of approximately 20 pl for embryos in Figs 5 and 6, or of approximately 100 pl for embryos in Figs 8 and 9. The diameter of the injected bolus was measured by an eyepiece reticle.

### In situ hybridization of whole-mount embryos

In situ hybridization for the detection of *zen*, *sog*, *Race* (also known as *Ance*), and *chd* transcripts was performed as described previously (Patel, 1996). *zen*, *sog* and *chd* riboprobes were labeled with digoxigenin (DIG RNA Labeling Mix, Roche), and *Race* with fluorescein (Fluorescein RNA Labeling Mix, Roche). All embryos were histochemically stained with the appropriate antibody coupled to alkaline phosphatase (Roche). NBT/BCIP (Promega) was used as substrate for the AP reaction to give the 'blue' color. T-NBT/BCIP (Agarwala et al., 2001) was used as an AP substrate to give the 'brown' color.

Embryos injected with *sog* or *chd* mRNA were allowed to develop under oil, and before fixation eggs were gently punctured on the ventral side with an empty injection needle to allow the even penetration of fix into the embryo. Fixation was carried out on the coverslip after the oil had been removed by repeated rinsing with heptane. Embryos were fixed for 18 minutes in a 35 mm Petri dish containing 3.7% formaldehyde in fixation buffer (100 mM Hepes pH 6.9, 2 mM $MgSO_4$, 1 mM EGTA). The coverslip containing the fixed embryos was placed in methanol for 5 minutes and then rinsed with PBS pH 7.4 containing 0.1% Triton X-100. Embryos were hand-dissected in this solution and then subjected to hybridization as described above.

### Photomicroscopy

Photomicroscopy was performed with an Axiophot microscope (Zeiss) with DIC Nomarski or darkfield optics. Darkfield cuticle images were taken with TECHPAN film and digitized using a Polaroid Sprint Scan 35 slide scanner. DIC images were obtained with a Progres 3012 digital camera (Kontron Electroniks). Figures were assembled using Adobe PhotoShop.

## RESULTS

### *Mad* and *Medea* enhance a viable allele of *sog* to lethality

The homozygous viable *sog* mutation, *sog$^{P129D}$*, which is caused by insertion of a P element 106 bp 5′ to the beginning of the *sog* transcript (Holley, 1997), reduces the level of *sog* transcript relative to wild type (Fig. 1). The *sog$^{P129D}$* phenotype is very sensitive to changes in levels of BMP signaling: while 80% of *sog$^{P129D}$* embryos survive to adulthood, only 10% survive either in *trans* to a null allele of *sog* or with two extra copies of *dpp$^+$*. We thus reasoned that loss of one copy of a gene that cooperates with *sog* could enhance the *sog$^{P129D}$* mutant to lethality. Specifically, although loss of *sog* activity does not completely eliminate neurogenic ectoderm (Ferguson and Anderson, 1992b), ectopic dorsal localization of *sog* mRNA is sufficient to inhibit formation of dorsal tissues (Holley et al., 1995), suggesting that other genes act in concert with SOG to repress BMP signaling ventrally. We had originally planned to screen for such genes; however, the results of our experiments, as described below, instead gave us insight into a second function of SOG.

We conducted a screen of 3000 chromosomes from which we recovered three independent mutations that acted as dominant maternal-effect enhancers of *sog$^{P129D}$* (Materials and Methods). Females carrying one copy of any of the three mutations enhanced the *sog$^{P129D}$* phenotype such that only 2 to 4% of *sog$^{P129D}$* embryos survived to adulthood (Table 1). Genetic mapping and complementation analysis (Materials and Methods) indicated that one of these mutations (*ES1*) is an

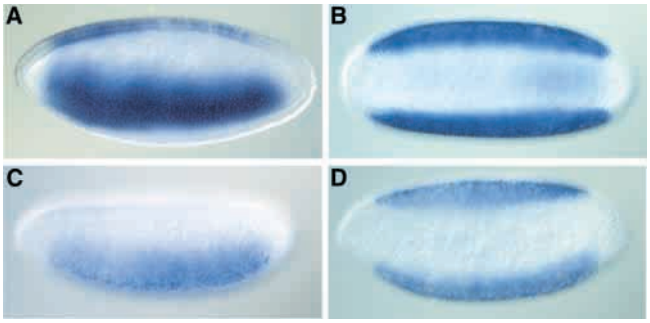


**Fig. 1.** *sog* transcription is reduced in $sog^{P129D}$ embryos. *sog* expression in wild-type (A,B) or $sog^{P129D}$ (C,D) cellular blastoderm embryos. Lateral views (A,C) and ventral views (B,D). To directly compare levels of *sog* expression, wild-type and $sog^{P129D}$ embryos were mixed in the same tube and assayed for both *sog* and *Race* transcription (both in blue). The lack of *Race* expression in the presumptive amnioserosa of $sog^{P129D}$ embryos was used to differentiate the two genotypes.

allele of *Mad* and that the other two (*ES2* and *ES3*) are alleles of *Medea*. *Mad* and *Medea* are essential components of the BMP signaling pathway (Das et al., 1998; Hudson et al., 1998; Newfeld et al., 1996; Wisotzkey et al., 1998), coupling DPP receptor activation to the control of target gene expression (reviewed by Massagué and Chen, 2000).

If SOG acts only to inhibit BMP signaling, loss-of-function mutations in essential components of the BMP signal transduction pathway should suppress rather than enhance the *sog* mutant phenotype. We therefore tested whether known loss-of-function alleles of *Mad* and *Medea* behaved in the same fashion. We observed that the null alleles $Mad^{12}$ and $Med^{13}$ enhanced $sog^{P129D}$ to an extent similar to that caused by the alleles obtained in our screen, while a hypomorphic allele of *Medea*, $Med^{15}$, acted as a less severe enhancer of the *sog* mutant (Table 1). Furthermore, this enhancement appears to be extremely sensitive to the level of *sog* activity, since loss of one copy of maternal *Mad* also caused some $sog^{P129D}$ heterozygotes to die as embryos (Table 1). These findings thus provided strong support for the previous suggestions that SOG plays a positive role in BMP signaling.

**Table 1. Maternal enhancement of a weak *sog* allele to lethality**

| Genotype | | | |
|---|---|---|---|
| Maternal | Zygotic | % Viability* | *n* |
| + | $sog^{P129D}$ | 80 | 191 |
| $Mad^{ES1}$/+ | $sog^{P129D}$ | 4 | 270 |
| $Mad^{ES1}$/+ | $sog^{P129D}$/+‡ | 39 | 177 |
| $Mad^{12}$/+ | $sog^{P129D}$ | 2 | 111 |
| $Med^{ES2}$/+§ | $sog^{P129D}$ | 2 | 354 |
| $Med^{ES3}$/+ | $sog^{P129D}$ | 3 | 196 |
| $Med^{13}$/+ | $sog^{P129D}$ | 5 | 193 |
| $Med^{15}$/+ | $sog^{P129D}$ | 22 | 60 |

*$sog^{P129D}$/*FM7* females heterozygous for the maternal genotype specified were crossed to $sog^{P129D}$ males. The percentage viability of $sog^{P129D}$ progeny was calculated as the ratio of homozygous $sog^{P129D}$ females to heterozygous female siblings. Crosses were performed at 18°C unless otherwise noted.

‡$Mad^{ES1}$/+ females were crossed to $sog^{P129D}$ males. The degree of lethality was calculated as the ratio of unhatched ventralized eggs to the expected number of female progeny (1/2 of the total of 355).

§$Med^{ES2}$ is a temperature-sensitive allele. Data is given for 25°C, the non-permissive temperature. At 18°C, the survival is 29% (*n*=228).

## *sog* is necessary for maximal BMP signal dorsally

In order to understand the biological basis underlying this genetic interaction, we characterized the lethal phenotype of the enhanced $sog^{P129D}$ embryos. The mutant embryos die with cuticular defects similar to those of weakly ventralized embryos, possibly indicative of a defect in amnioserosa specification (Fig. 2A,C). To directly assay the amount of differentiated amnioserosa, we analyzed the expression of β–galactosidase from a *P[Kr-lacZ]* construct, which is expressed in this tissue type (Fig. 2B). While embryos from $Mad^{ES1}$/+ females have a grossly normal amount of amnioserosa (not shown), $sog^{P129D}$ embryos laid by $Mad^{ES1}$/+ females have a reduced amount of amnioserosa (Fig. 2D).

These data focused our attention on the role of SOG in specifying amnioserosa. *sog* was originally isolated as a result of a failure in germ band elongation, indicative of the lack of amnioserosa (Zusman et al., 1988). Although in *sog* null mutants *Kr-lacZ* expression is greatly reduced or absent (Fig. 7E), the genetic basis for this defect has remained unclear. The

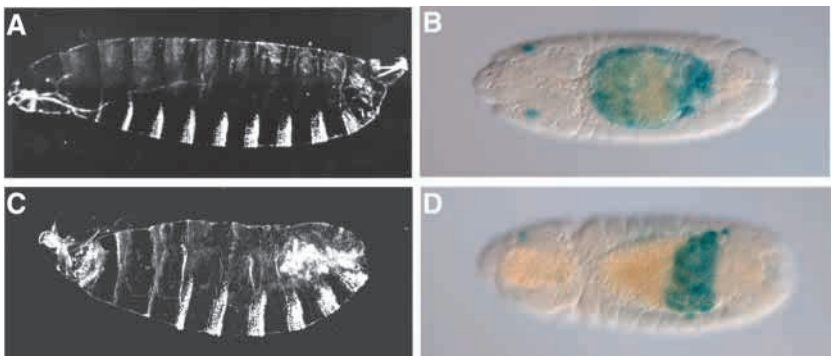


**Fig. 2.** The amnioserosa is not properly patterned in $sog^{P129D}$ embryos derived from $Mad^{ES1}$/+ females. (A) A darkfield photomicrograph of a cuticle of a $sog^{P129D}$ embryo (lateral view, dorsal up, anterior left). The ventral-most ectodermal cells form the neurogenic ectoderm and are characterized by a segmentally repeated pattern of denticle bands. Dorsolateral cells differentiate dorsal hairs, only faintly visible in the cuticle preparation. The filzkörper, respiratory structures of the tail, are derived from cells in a dorsolateral position in the blastoderm. The amnioserosa, which does not contribute to the embryonic cuticle, can be visualized in a dorsal view of a $sog^{P129D}$ stage 13 embryo (B) after staining for β-galactosidase activity from a *P[Kr-lacZ]* construct expressed in this tissue. (C) Lateral view of a cuticle from a $sog^{P129D}$ embryo derived from a $Mad^{ES1}$/+ female. The internalized filzkörper and the lack of head elements are indicative of a weakly ventralized embryo. The internalization of the filzkörper results from defects in germband extension in embryos that lack a fully functional amnioserosa. The embryo was raised at 25°C. (D) β-galactosidase activity from the *P[Kr-lacZ]* construct in a stage 13 $sog^{P129D}$ embryo derived from a $Mad^{ES1}$/+ female. This embryo shows a large reduction in the amount of amnioserosa. Embryos in B and D were raised at 29°C.

A

| genotype | % hatching at 25°C | n |
|---|---|---|
| $zen^{1}$ | 98 | 346 |
| $sog^{P129D}$ | 80 | 1022 |
| $sog^{P129D}$; $zen^{1}$ | 5 | 135 |

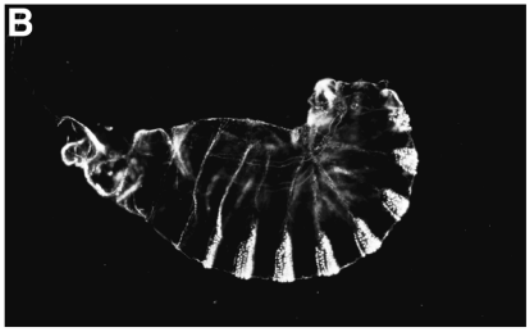


**Fig. 3.** Synergistic lethality between weak alleles of *zen* and *sog*. (A) At 25°C, $sog^{P129D}$ and $zen^{1}$ are both homozygous viable. Occasional $sog^{P129D}$; $zen^{1}$ double mutant flies arise from a $sog^{P129D}$/*FM7*; $zen^{1}$/*TM3* stock maintained at 18°C. When these flies are transferred to 25°C, they produce dead embryos with a partially ventralized phenotype (as seen in B).

fact that amnioserosa specification in a weak *sog* mutant is enhanced by reduction in the activity of *Mad* and *Medea* strongly suggests that the defect in amnioserosa formation in *sog* null embryos results from dorsal reduction of BMP signaling.

In the wild-type embryo, the pattern of *zen* transcription correlates with the future differentiation of amnioserosa. In *sog* mutant embryos, however, this correlation does not hold: the pattern of *zen* transcription is expanded to encompass the dorsal 40% of the embryo, but the amnioserosa does not differentiate. The pattern of *zen* transcription in *sog* null embryos therefore could reflect not only the loss of negative regulation of BMP signaling, possibly manifest as an expansion in the spatial extent of *zen* transcription, but also the elimination of an activity that promotes BMP signaling, possibly manifest as a decrease in the level of *zen* transcription. If this hypothesis is correct, a reduction in the level of *zen* transcription due to loss of the positive function of SOG might allow us to observe a synthetic interaction between weak alleles of the two genes. While $sog^{P129D}$ and $zen^{1}$ are each homozygous viable at 25°C (Fig. 3A), double mutant embryos die with a partially ventralized cuticular phenotype (Fig. 3A,B). Thus, reduction of SOG function can enhance a weak *zen* mutation, indicating that SOG is needed for maximal *zen* activity.

To determine whether this genetic enhancement could be observed at the level of *zen* transcription, we examined *zen* expression in $sog^{P129D}$ embryos laid by $Mad^{ES1}$/+ females. Because *zen* transcription is very dynamic, we confined our observations to a 5-minute period (stage 6a, according to Campos-Ortega and Hartenstein (Campos-Ortega and Hartenstein, 1985)) immediately after the onset of gastrulation. Compared to wild-type embryos (Fig. 4A), $sog^{P129D}$ embryos showed both a spatial expansion of *zen* expression and a slight reduction in intensity of expression (Fig. 4B). Embryos laid by $Mad^{ES1}$/+ females expressed *zen* in the dorsal 10% of nuclei but with a reduced intensity (Fig. 4C). In contrast, *zen* transcription in both heterozygous and homozygous *sog* embryos laid by $Mad^{ES1}$/+ females was greatly reduced compared to either parental strain and in some embryos was

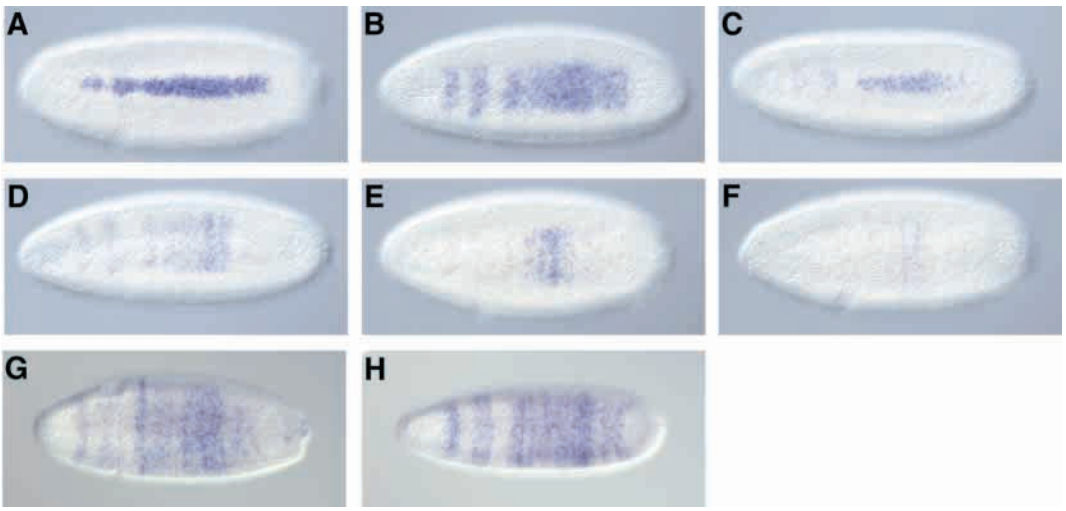


**Fig. 4.** *zen* expression is strongly reduced in $sog^{P129D}$ embryos derived from $Mad^{ES1}$/+ females. Because *zen* transcription is very dynamic, it was assayed in stage 6a embryos. At this stage, which lasts approximately 5 minutes, the cephalic furrow is evident but the invagination of the mesodermal primordium is not yet visible. All embryos were grown at 29°C. All in situ hybridizations in A-E were carried out in parallel, as were the hybridizations in G-H, with the same concentration of probe and were developed for exactly the same amount of time. (A) Dorsal view of the distribution of *zen* mRNA in a wild-type embryo. At this stage *zen* is expressed only in the 10% most dorsal tissue. (B) Expression of *zen* in a $sog^{P129D}$ embryo. Note a small reduction in the intensity of transcription, but a larger spatial domain of expression. (C) *zen* expression in an embryo derived from a $Mad^{ES1}$/+ female. Note that *zen* is expressed in the same spatial domain as in wild type, but with reduced intensity. (D-F) Reduction in *zen* expression in $sog^{P129D}$ heterozygous and homozygous embryos derived from $sog^{P129D}$/*FM7*; $Mad^{ES1}$/+ females crossed to $sog^{P129D}$ males. 66% ($n$=35) of the stage 6a embryos analyzed displayed a reduction or elimination of *zen* transcription; the remaining embryos, of putative genotype *FM7*, were identical to those in C. (G) *zen* expression in a $sog^{YSO6}$ embryo. (H) *zen* expression in a $sog^{YSO6}$ embryo derived from a $Mad^{12}$/+ female. The intensity of *zen* transcription was found to be variable to an equivalent degree in embryos of both genotypes. In both cases, the embryos shown have an intermediate intensity of *zen* transcription.

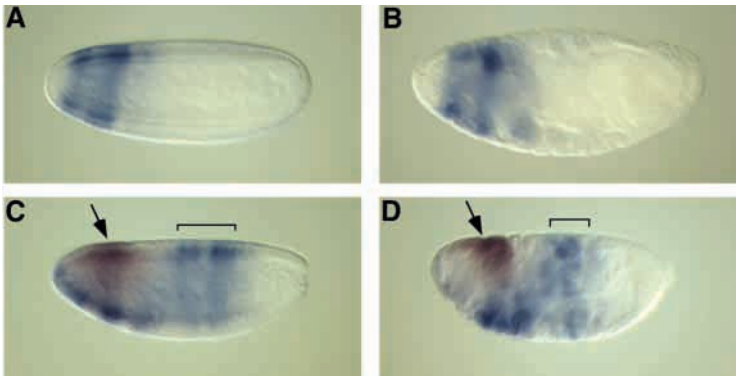


**Fig. 5.** Injection of *sog* mRNA locally inhibits the transcription of a dorsal-specific gene, but activates its transcription at a distance from the site of injection. Lateral views of *snake* (*snk*) mutant embryos hybridized with *Race* (blue) and *sog* (brown) riboprobes. Late cellularization (A,C) and post gastrulation (stage 7 by developmental time; B,D). (A,B) *Race* expression in uninjected embryos is restricted to the anterior domain, and no endogenous *sog* transcription is present. (C,D) Embryos injected in a dorsal anterior position (marked by arrow) with a small bolus of *sog* mRNA (brown). *Race* expression is inhibited by *sog* at the site of injection, and is activated at a distance from the source of *sog*, both more posteriorly (bracket) and at a ventral-anterior position. Note that the white 'halo' is more pronounced in the older embryo, corresponding to inhibition of endogenous *Race* expression outside the domain of detectable *sog* mRNA.

almost completely eliminated (Fig. 4D-F). These data directly demonstrate that SOG is required for maximal expression of a DPP target gene.

Interestingly, in the enhanced $sog^{P129D}$ embryos that retained some *zen* transcription, the spatial extent of *zen* transcription appeared to be broader than in the $sog^{P129D}$ embryos alone. This result, together with data from Rushlow et al. (Rushlow et al., 2001), suggests that refinement of *zen* transcription to the dorsal 10% of the embryonic nuclei may require maximal BMP signaling, and that lower levels of BMP signaling at the cellular blastoderm stage could permit *zen* to continue to be transcribed in more lateral regions of the embryo. Such a hypothesis could possibly explain the expanded pattern of *zen* transcription in *sog* null mutants without the necessity of invoking loss of negative regulation of BMP signaling as the sole cause for this defect.

We then wondered whether, in the background of a *sog* null mutation, reduction in *Mad* activity would result in a similar decrease in *zen* expression. We thus assayed *zen* transcription in both $sog^{YS06}$ embryos and in $sog^{YS06}$ embryos laid by $Mad^{12}$/+ females. We found that the level of *zen* transcription is variable in $sog^{YS06}$ embryos (Fig. 4G), and we observed approximately the same range of variability in $sog^{YS06}$ embryos from a $Mad^{12}$/+ female (Fig. 4H). Thus, in the absence of *sog* activity, *Mad* no longer displays a dosage-sensitive phenotype in this assay. Possibly, an overall elevation in the level or activity of MAD caused by loss of SOG eliminates its dosage dependence in this genetic background. We hypothesize that the complete lack of SOG in $sog^{YSO6}$ mutants may mask the decrease in *zen* transcription clearly seen in $sog^{P129D}$ embryos laid by *Mad* heterozygote mothers.

## Sog has a dual role upon BMP signaling

While SOG's negative activity has been shown to be exerted at the site of its transcription in the ventral regions of the embryo, its positive activity is required for amnioserosa formation in the dorsal-most cells, and thus must occur at a distance from its site of expression. To examine both activities simultaneously it is necessary to assess the effects of localized *sog* expression over a large field of equipotent cells such as is found in a dorsalized embryo. Embryos laid by females mutant for the *dorsal* group of genes (Anderson and Nüsslein-Volhard, 1984) express dorsal-specific genes, such as *dpp*, *zen* and *tolloid*, around the embryonic circumference and do not

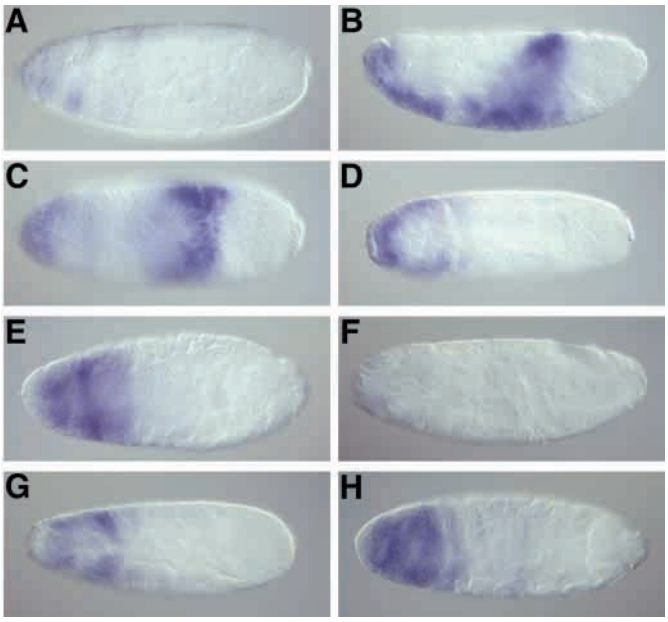


**Fig. 6.** *Race* expression in dorsalized embryos injected with different amounts of *sog* or *chordin* mRNAs. Embryos laid by *snk* females were injected with decreasing amounts of *sog* mRNA (A-E) or *chordin* mRNA (F-H) and stained after injection for *Race* expression (blue). Each embryo illustrates the most frequent outcome after the injection of a particular mRNA concentration, the total numbers are given in Table 2 for *sog* mRNA and Table 3 for *chd* mRNA. Injections were performed in an anterior dorsal position (similar to that in Fig. 5) and the size of the bolus of injected mRNA was held constant in all injections. (A) Lateral view of an embryo injected with 40 μg/μl of *sog* mRNA. The inhibition of endogenous *Race* expression is strong and no ectopic *Race* expression is observed. (B) Lateral view of an embryo injected with 0.8 μg/μl of *sog* mRNA. *Race* expression is inhibited at the site of injection and ectopic *Race* expression is detected at a distance from the site of injection. (C) Dorsal view of the embryo shown in B. (D) Dorsal view of an embryo injected with 0.2 μg/μl of *sog* mRNA. *Race* expression is inhibited locally but no ectopic expression is observed. (E) Dorsal view of an embryo injected with 0.08 μg/μl of *sog* mRNA. No inhibition of *Race* is observed. (F) Lateral view of an embryo injected with 4.5 μg/μl of *chd* mRNA showing complete inhibition of *Race* expression. (G) Dorsal view of an embryo injected with 2.25 μg/μl of *chd* mRNA showing local inhibition but no ectopic expression of *Race*. (H) Dorsal view of an embryo injected with 1.5 μg/μl of *chd* mRNA. No inhibition of *Race* expression is observed.

express ventral specific genes, such as *sog* (Ray et al., 1991; Rushlow et al., 1987). Although all cells in dorsalized embryos therefore have a pattern of gene expression similar to that of the most dorsal cells in the wild-type embryo, the great majority of embryonic cells in dorsalized embryos differentiate as dorsal ectoderm and not as amnioserosa (Wharton et al., 1993). We and others had previously speculated that the absence of amnioserosa in dorsalized embryos could be due to the lack of the 'potentiating' ability of SOG upon BMP signaling (Neul and Ferguson, 1998; Nguyen et al., 1998).

During the course of this work, Ashe and Levine (Ashe and Levine, 1999) presented data that directly verified this hypothesis. They showed that embryos laid by dorsalized mutant mothers express *Race*, a dorsal marker, in a circumferential domain that is restricted to the anterior region of the embryo (Fig. 5A,B). They misexpressed SOG in this dorsalized background from the *eve*-stripe2 enhancer in a narrow stripe around the circumference of the embryo and observed both local inhibition of the *Race* transcript in the *eve*-stripe2 domain and ectopic expression of *Race* at a distance from the site of SOG expression (Ashe and Levine, 1999).

We confirmed these results by injecting dorsalized *snk* mutant embryos with a small bolus of *sog* mRNA (4 μg/μl) in a dorsoanterior position at a site that overlapped the domain of *Race* expression. After doubly staining these embryos for *sog* and *Race* mRNA expression, we found that *sog* both inhibited the expression of *Race* locally at the site of dorsoanterior injection (98%; *n*=102) and ectopically activated *Race* expression at a distance from the site of injection (80%; *n*=102: Fig. 5C,D).

Given that our screen identified second site mutations that affected SOG's positive function, but none that affected its negative activity, we wondered whether the positive function of SOG was more sensitive to a reduction in the level of SOG protein. We tested this hypothesis by injecting a range of concentrations of *sog* mRNA into *snk* embryos (Table 2). At concentrations higher than in our original experiment (40 μg/μl), injected *snk* embryos did not express *Race* anywhere along the AP axis (Fig. 6A), suggesting that local inhibition of *Race* activity is dominant over long-range activation. However, at concentrations (0.4-0.2 μg/μl) lower than those that produced long-range activation, local inhibition of anterior *Race* transcription was still present (Fig. 6D). At even lower concentrations no inhibition was observed (Fig. 6E). These experiments indicate that the long-range positive activity of SOG requires a higher level of protein than does its local inhibition of BMP signaling.

## Amnioserosa is restored in *sog* null embryos by elevation of *dpp* dosage, but not by elevation of *screw* activity

Because the negative activity of SOG has been postulated to be directed toward the inhibition of SCW rather than DPP (Neul and Ferguson, 1998; Nguyen et al., 1998), we wished to determine whether the positive activity of SOG could be similarly ascribed to action on either ligand.

**Table 2. Quantitation of *Race* expression after injection of different concentrations of *sog* mRNA into dorsalized embryos**

| *sog* mRNA concentration* (μg/μl) | Global inhibition‡ (%) | Local inhibition and long-range activation§ (%) | Local inhibition¶ (%) | No effect (%) | *n* |
|---|---|---|---|---|---|
| 40 | 90 | 8 | 0 | 2 | 139 |
| 8 | 0 | 87 | 9 | 4 | 202 |
| 0.8 | 0 | 61 | 38 | 2 | 173 |
| 0.4 | 0 | 0 | 90 | 10 | 213 |
| 0.2 | 0 | 0 | 67 | 33 | 133 |
| 0.08 | 0 | 0 | 5 | 95 | 127 |

*Different concentrations of *sog* mRNA were injected as described in Fig. 6 and a single round of mRNA detection was performed to assess *Race* transcription. Each concentration was repeated at least twice, and results were averaged.

‡Global inhibition: embryos in which *Race* transcription was completely absent or only weakly present in an anterior-ventral location.

§Local inhibition and long-range activation: embryos in which there was a clear anterior-dorsal 'spot' that lacked endogenous *Race* expression and ectopic *Race* transcription at, or more posterior than, 50% egg length.

¶Local inhibition: embryos in which there was a clear anterior-dorsal 'spot' that lacked *Race* expression but there was remaining endogenous *Race* expression dorsally either anterior or posterior to the clear 'spot'.

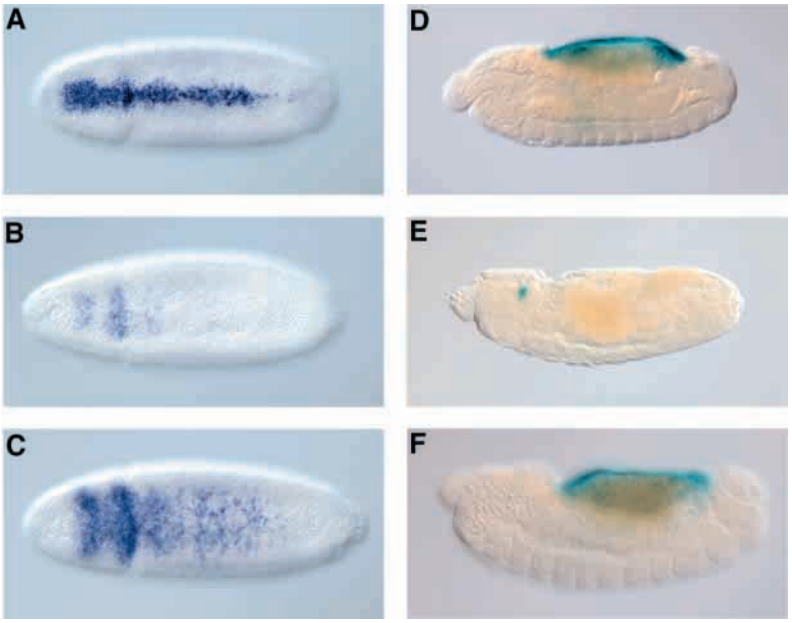


**Fig. 7.** Elevation of $dpp^+$ dosage restores amnioserosa in $sog^{YSO6}$ embryos. (A-C) Dorsal views of stage 6a embryos hybridized with digoxigenin-labeled *Race* riboprobes showing *Race* expression in the presumptive amnioserosa. (A) Distribution of *Race* mRNA in a wild-type embryo. At this stage *Race* mRNA is present only in the 10% dorsal-most cells. (B) *Race* mRNA is absent in the presumptive amnioserosa in a $sog^{YSO6}$ embryo. Weak staining is present in the anterior dorsal region of the embryo. (C) *Race* expression is partially restored along the AP axis in a $sog^{YSO6}$ embryo that carries an extra copy of $dpp^+$. *Race* expression is expanded compared to wild type in the dorsolateral regions of the embryo. From a cross of $sog^{YSO6}$/*FM7* females with *Dp(2;2)DTD48* males, 51% (*n*=78) of the $sog^{YSO6}$ embryos showed partially restored *Race* staining. (D-F) Lateral views of stage 13 embryos stained for β-galactosidase activity driven by a *P[Kr-lacZ]* construct expressed in the amnioserosa. (D) Wild-type embryo. (E) $sog^{YSO6}$ mutant embryos do not differentiate amnioserosa as evidenced by the absence of β-galactosidase activity. (F) Restoration of amnioserosa in a $sog^{YSO6}$ embryo by the presence of an extra copy of $dpp^+$. From the same cross as above, 51% (*n*=70) of the $sog^{YSO6}$ embryos showed restoration of β-galactosidase staining in the amnioserosa.

Previous results had demonstrated that injection of high concentrations of *dpp* mRNA is sufficient to restore amnioserosa in all zygotic ventralizing mutants (Neul, 1998). The phenotype of most of these mutants can also be weakly suppressed by a doubling of the $dpp^+$ gene dosage (Ferguson and Anderson, 1992b and E. Ferguson unpublished results). However, unlike all other mutants tested, we found that the addition of only one extra copy of the $dpp^+$ gene restored large amounts of amnioserosa to a *sog* null embryo (Fig. 7D-F). We also found that an extra copy of $dpp^+$ partially restored the expression of *Race* in a *sog* mutant (Fig. 7A-C). Thus, *sog* mutants appear uniquely sensitive to small elevations in $dpp^+$ gene dosage.

We then tested whether elevation of SCW activity was sufficient to rescue a *sog* phenotype. While a low concentration of *scw* mRNA (27ng/μl) is sufficient to rescue the amnioserosa defect of a *scw* mutant embryo, injection of *scw* mRNA at a hundred-fold greater concentration was not sufficient to restore amnioserosa in a *sog* mutant (Fig. 8A,B). The lack of response of *sog* embryos to elevation of SCW activity, coupled with the extreme sensitivity of the *sog* mutant to elevation of $dpp^+$ gene dosage, suggests that the positive activity of SOG is exerted mainly, if not exclusively, upon DPP.

### Limited conservation of Chd activity in flies

Chd and SOG are components of a conserved system for dorsoventral patterning in arthropods and vertebrates (Holley et al., 1995; Schmidt et al., 1995). Although the sequence similarity between SOG and *Xenopus* Chd is only 27% (François and Bier, 1995), the overall architecture of the two proteins is conserved. Both proteins contain four cysteine-rich domains (CR) that contain most of the sequence identity (e.g., 47% amino-acid identity in the first CR repeat). Because the CR repeats have been shown to bind BMPs (Larraín et al., 2000), we tested whether Chd showed a ligand specificity similar to that described for SOG (Neul and Ferguson, 1998; Nguyen et al., 1998). Using the assay developed by Neul and Ferguson (Neul and Ferguson, 1998), we co-injected *chd* mRNA with biologically equivalent concentrations of either *scw* or *dpp* mRNAs into *scw* mutant embryos and observed whether the co-injection of *chd* mRNA could block the activity of either ligand (i.e., restoration of amnioserosa in the *scw* mutant). We observed that injection of 4.5 μg/μl of *chd* mRNA completely abolished the activity of injected *scw* mRNA (Fig. 9A,C), but it did not block the activity of injected *dpp* mRNA (Fig. 9B,D). This result suggests that in *Drosophila*, Chd, like SOG, locally inhibits BMP signaling by abrogating the effects of the SCW ligand.

We then assayed whether Chd had a positive effect upon BMP signaling. We injected increasing amounts of *chd* mRNA (Table 3) into *snk* mutant embryos and stained for both *chd* and *Race* mRNAs (data not shown) or just for *Race* mRNA (Table 3). High amounts of *chd* mRNA (4.5 μg/μl) caused a strong inhibition of *Race* (Fig. 6F), abolishing expression throughout the dorsal side of the embryo; lower quantities (2.25 μg/μl) resulted in partial inhibition of dorsal *Race* staining (Fig. 6G), and concentrations below 1.5 μg/μl had no inhibitory effect (Fig. 6H). While the range of *chd* mRNA concentrations tested caused the same classes of inhibition of *Race* expression as did *sog* mRNA, we never observed ectopic *Race* at a distance from the site of injection in any *chd*-injected embryo. These results indicate that in this *Drosophila* assay Chd lacks the ability to act as a long-range enhancer of BMP signaling.

**Table 3. Quantitation of *Race* expression after injection of different concentrations of *chd* mRNA into dorsalized embryos**

| *chd* mRNA concentration* (μg/μl) | Global inhibition (%) | Local inhibition and long-range activation (%) | Local inhibition (%) | No effect (%) | *n* |
|---|---|---|---|---|---|
| 4.5 | 95 | 0 | 0 | 5 | 98 |
| 2.25 | 7 | 0 | 64 | 29 | 90 |
| 1.5 | 0 | 0 | 6 | 94 | 81 |

*All descriptions are similar to those in Table 2.

## DISCUSSION

Morphogen gradients, once a purely theoretical concept, are now viewed as central players in the establishment of cell identity in a broad range of developmental processes. However, the exact biological mechanisms used to establish and maintain a morphogen gradient vary depending on the biological context. In the *Drosophila* embryo, while DPP can act in a dose-dependent fashion to specify different cell fates along the DV axis, in vivo its activity is modulated spatially by other components of the patterning system. In particular, SOG, a diffusible BMP-binding protein, has been shown to inhibit BMP signaling ventrally by preventing ligand access to the BMP receptors. In this paper we characterize a novel aspect of SOG's function. Specifically, we demonstrate that SOG functions cell non-autonomously to elevate BMP signaling on the dorsal side of the embryo. Thus, the interpretation of any experiment to elucidate the role of SOG in the control of dorsoventral patterning must take into account the two apparently opposing functions of the protein.

We identified loss-of-function mutations in *Mad* or *Medea* as dominant enhancers of a weak homozygous-viable *sog* mutation, and showed that the enhanced embryos have defects in amnioserosa specification. Furthermore, we demonstrated synthetic lethality between weak homozygous-viable alleles of *sog* and *zen*, indicating that both are required for maximal production of amnioserosa. Lastly, we showed that there was a dramatic decrease in the level of *zen* transcription in $sog^{P129D}$ embryos that were derived from *Mad*/+ females, compared to the level of *zen* transcription in either genotype alone. Taken together, these results unambiguously demonstrate that the positive action of SOG is exerted before gastrulation to attain the maximal expression of a direct BMP target gene.

In a series of elegant experiments, Ashe and Levine (Ashe and Levine, 1999) showed that expression of SOG in a specific anterior-posterior position in dorsalized embryos causes the inhibition of the dorsal-specific gene *Race* locally, and the upregulation of *Race* transcription at a distance from the site of expression, formally demonstrating the dual action of SOG upon a field of equipotent cells. However, *Race* is not necessary for amnioserosa specification, since $sog^{P129D}$ embryos, which have functional amnioserosa, lack *Race* expression (Fig. 1C). Our results demonstrate that the positive activity of SOG is also

exerted upon expression of *zen*, which is known to be required for amnioserosa specification. These results provide a direct link between SOG's effect on *zen* transcription and loss of amnioserosa in *sog* mutants.

We confirmed the results of Ashe and Levine (1999) by mRNA injections, and used our ability to vary the amount of mRNA to demonstrate that the minimal amount of *sog* mRNA that must be injected to observe the ectopic transcription of *Race* is four-fold higher than the minimal amount necessary to locally inhibit *Race* transcription. Thus, a small decrease in the concentration of SOG affects the positive activity of SOG to a greater extent than it affects the negative function. This could be for any of a number of reasons, including a marked decrease in the concentration of SOG (or one of its proteolytic fragments) as it diffuses away from its site of synthesis.

These results also correlate well with our phenotypic and genetic analysis of $sog^{P129D}$, which causes a reduction in the level of *sog* transcription. Although this allele is homozygous viable, it appears to cause a preferential reduction in the positive activity of SOG, as evidenced by the loss of *Race* transcription in the amnioserosa. The preferential loss of positive activity in the $sog^{P129D}$ mutant could also explain why we isolated second site mutations that decreased the positive function of SOG, but did not recover mutations in genes such as *brinker* (Jazwinska et al., 1999) that cooperate with SOG to repress BMP signaling ventrally.

We and others had previously suggested that the inhibitory function of SOG is primarily directed against the SCW ligand. We now present data that suggest that the positive function of SOG may be directed towards DPP. In particular, we show that a 50% increase in *dpp* copy number is sufficient to restore amnioserosa to *sog* mutant embryos, indicating that *sog* is more sensitive than any other known ventralizing mutation to an increase in $dpp^{+}$ gene dosage. We also demonstrated that the lack of amnioserosa in *sog* embryos was not rescued by injection of an amount of *scw* mRNA far in excess of that required to rescue a *scw* mutant. These results are strongly suggestive that SOG's positive function may be directed against DPP, not against SCW.

These findings may allow us to clarify a series of recent results concerning the action of a second extracellular factor that modulates DPP activity, the product of the *twisted gastrulation* (*tsg*) gene. While the phenotype of *tsg* embryos, a partial ventralization caused by lack of amnioserosa, is suggestive of a positive activity of TSG upon BMP signaling, TSG has been shown to form a complex with SOG, and coexpression of TSG with SOG is sufficient to block DPP activity (Yu et al; 2000; Ross et al., 2001). Similar results have been demonstrated for the vertebrate homologs of the TSG and SOG proteins (Chang et al., 2001; Scott et al., 2001). These results have been primarily interpreted to suggest that, in vivo, SOG and TSG cooperate to block DPP signaling. In contrast, Oelgeschlager et al. (Oelgeschlager et al. 2000) suggest that the *Xenopus* homologue of TSG promotes BMP signaling primarily by antagonizing BMP binding to one of the cysteine rich domains (CR1) of Chd.

We would like to combine our data with the published results to reconcile the different interpretations of the biological functions of SOG and TSG. Specifically, we propose that the positive activity we have shown for SOG, which we have postulated is directed toward DPP, is in fact mediated by a tripartite complex composed of SOG, TSG and DPP. We suggest that this complex promotes BMP signaling by sequestering DPP from its receptor, thus allowing the dorsally directed diffusion of laterally produced DPP ligand. We suggest that the cleavage of SOG by TLD in the dorsal-most cells releases the DPP ligand from the tripartite complex, allowing it to signal. After cleavage of SOG by TLD, TSG could function in a manner similar to that described by Oelgeschlager et al. (Oelgeschlager et al., 2000), antagonizing the further binding of DPP by the proteolytic fragments of

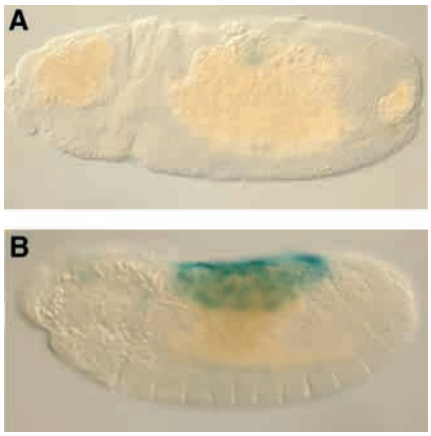


**Fig. 8.** Elevation of *screw* activity does not rescue a $sog^{YSO6}$ mutant. (A) Lateral view of a stage 13 $sog^{YSO6;}$ *P[Kr-lacZ]* embryo after dorsal injection of 2.7 μg/μl of *scw* mRNA embryo. No β-galactosidase staining is observed, indicating that the overexpression of *scw* mRNA does not restore amnioserosa in *sog* embryos (0%; *n*=38). (B) Lateral view of a *Df(2L)OD16 P[Kr-lacZ]* stage 13 embryo lacking *scw*, stained for β-galactosidase activity after injection of 27 ng/μl of *scw* mRNA. While uninjected *scw* mutants fail to develop amnioserosa, injection of 27 ng/μl of *scw* mRNA resulted in restoration of amnioserosa (83%; *n*=34).

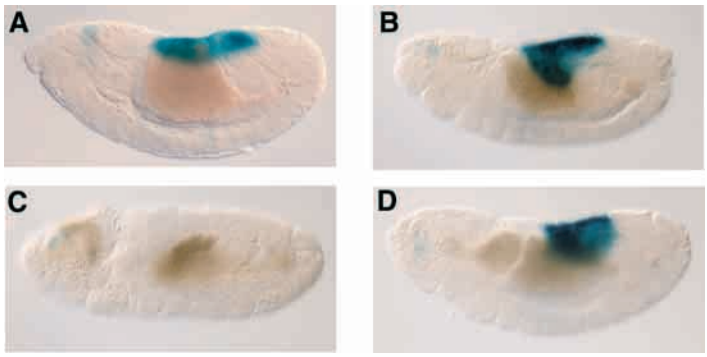


**Fig. 9.** *chordin* mRNA injection can block the activity of injected *scw* mRNA but not that of injected *dpp* mRNA. (A-D) Lateral views of stage 13 *Df(2L)OD16 P[Kr-lacZ]* embryos lacking *scw*. Each embryo was injected dorsally with a different combination of mRNAs before the blastoderm stage and stained for β-galactosidase activity. (A) Injection of 30 ng/μl of *scw* mRNA resulted in restoration of amnioserosa in 81% (*n*=114) of *scw* embryos. (C) Co-injection of 4.5 μg/μl of *chd* mRNA completely abolished the ability of injected *scw* mRNA to restore amnioserosa (0%; *n*=107). (B) Injection of 370 ng/μl of *dpp* mRNA resulted in restoration of amnioserosa in 83% (*n*=83) of *scw* embryos, however, co-injection of 4.5 μg/μl of *chd* mRNA with the *dpp* mRNA (D) was not sufficient to block amnioserosa formation (85%; *n*=68).

SOG. This model, and the one proposed by Ross et al. (Ross et al., 2001), are conceptually based on the model presented by Holley et al. (Holley et al., 1996), except that these models incorporate the recently described functions of TSG.

We propose that the antagonistic aspects of the complex toward DPP signaling that were observed through overexpression studies in various developmental contexts are due to the lack of, or improper stoichiometry of, a component present in the embryo that leads to disassociation of the tripartite complex. For example, if in these developmental contexts the TLD protease was not present in sufficient quantities to release the ligand from the tripartite complex, there would be continued DPP sequestration by the TSG/SOG complex leading to antagonism of BMP signaling. Alternatively, there may be one or more components present in the embryo whose function is to antagonize the stability of the tripartite complex, also potentiating ligand release. In that regard, it is interesting that mutations in the *shrew* gene cause a phenotype (lack of amnioserosa) that is similar to that caused by *sog* or *tsg* mutations. Possibly, *shrew*, the sequence of which has not been reported, could encode a component that aids in the formation or dissociation of the tripartite complex.

The conservation of the molecular mechanisms underlying the process of dorsoventral pattern formation between arthropods and chordates has allowed functional studies of vertebrate proteins to be carried out in *Drosophila*. SOG and Chd have been shown to be functionally interchangeable in their BMP inhibitory function (Holley et al., 1995). In this paper, we demonstrate that Chd, like SOG, preferentially inhibits SCW signaling, and under the assay conditions used is not capable of blocking DPP activity. This finding suggests that in vertebrates, as in flies, the activity of different ligands can have differential responsiveness to particular inhibitors. Although SCW does not have a known vertebrate ortholog, phylogenetic analyses have placed it in the 60A/BMP7 subgroup (Miya et al., 1997) or in a separate clade with mouse GDF3 (Newfeld et al., 1999). Furthermore, the likely receptor for SCW, SAX, has been placed in the same subfamily as the BMP7 receptor, ALK2, based in part on homology in the L45 loop that is critical in determining signaling specificity (Macias-Silva et al., 1998; Newfeld et al., 1999; Persson et al., 1998). Thus, we would propose that in vivo, Chd alone preferentially inhibits BMP7, not BMP2/4.

We wondered whether Chd, like SOG, would display a long-range positive activity. We found that although injection of *Chd* mRNA inhibits *Race* expression in the anterior domain of dorsalized embryos, it does not cause activation of *Race* transcription in the posterior domain. Thus, in *Drosophila*, Chd can inhibit BMP signals locally, but does not display long-range activation of BMP signaling.

If our model concerning the positive action of SOG is correct, Chd's inability to promote a long-range elevation of BMP signaling could be for any of a variety of reasons. For example, data from Ashe and Levine (Ashe and Levine, 1999) indicated that wild-type levels of the metalloprotease TLD are required for the positive activity of SOG. If the positive activity of SOG is mediated by a proteolytic fragment of SOG, the differences in the in vitro patterns of cleavage of SOG and Chd (Marqués et al., 1997; Piccolo et al., 1997) could be critical for determining biological function. Another possibility is that Chd is unable to form sufficiently stable tripartite complexes with DPP and TSG to permit long-range diffusion. Alternatively, if Chd displays a higher affinity for DPP than does SOG, possibly sufficient DPP remains associated with the proteolytic fragments of Chd to prohibit long-range signaling. In support of this, Oelgeschlager et al. (Oelgeschlager et al., 2000) have proposed that one function of TSG is to remove BMP ligands from the proteolytic fragments of Chd. If either of the last two explanations were correct, elevation of TSG levels in the *Drosophila* embryo might permit Chd to display a positive activity. We therefore tested whether coinjection of *tsg* and *chd* mRNAs would result in positive long-range signaling. Although we injected a ten-fold concentration range of *tsg* mRNA with the *chd* mRNA, none of the embryos in our injection experiments displayed long-range activation of *Race* transcription (data not shown).

Although we cannot yet ascertain the mechanistic difference between SOG and Chd function, our results do provide an opportunity to determine the domains of SOG that are important for its positive activity. Interestingly, all known zygotic-lethal *sog* mutations are defective in both activities of the gene (E. Ferguson, unpublished), even though absence of its positive activity alone would have been sufficient to confer embryonic lethality. More generally, up to now there has been striking conservation of function of each individual component of the dorsoventral patterning system in arthropods and chordates. The identification of the basis of functional differences in apparently homologous proteins could provide insights into the degree of evolutionary divergence that can exist within the constraints of a conserved signaling system.

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