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Biphasic activation of the BMP pathway patterns the *Drosophila* embryonic dorsal region

Ruslan Dorfman and Ben-Zion Shilo*

Department of Molecular Genetics, Weizmann Institute of Science, Rehovot 76100, Israel
*Author for correspondence (e mail: Benny.Shilo@Weizmann.ac.il)

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SUMMARY

The BMP pathway patterns the dorsal region of the *Drosophila* embryo. Using an antibody recognizing phosphorylated Mad (pMad), we followed signaling directly. In wild-type embryos, a biphasic activation pattern is observed. At the cellular blastoderm stage high pMad levels are detected only in the dorsal-most cell rows that give rise to amnioserosa. This accumulation of pMad requires the ligand Screw (Scw), the Short gastrulation (Sog) protein, and cleavage of their complex by Tolloid (Tld). When the inhibitory activity of Sog is removed, Mad phosphorylation is expanded. In spite of the uniform expression of Scw, pMad expansion is restricted to the dorsal domain of the embryo where Dpp is expressed. This demonstrates that Mad phosphorylation requires simultaneous activation by Scw and Dpp. Indeed, the early pMad pattern is abolished when either the Scw receptor Saxophone (Sax), the Dpp receptor Thickveins (Tkv), or Dpp are removed. After germ band extension, a uniform accumulation of pMad is observed in the entire dorsal domain of the embryo, with a sharp border at the junction with the neuroectoderm. From this stage onward, activation by Scw is no longer required, and Dpp suffices to induce high levels of pMad. In these subsequent phases pMad accumulates normally in the presence of ectopic Sog, in contrast to the early phase, indicating that Sog is only capable of blocking activation by Scw and not by Dpp.

Key words: BMP, Dpp, Screw, Smad, Mad, Embryonic patterning, *Drosophila melanogaster*

INTRODUCTION

The initial subdivision of the *Drosophila* embryo along its dorsoventral axis is executed by the maternal Dorsal pathway, culminating in a gradient of nuclear localization of the Dorsal protein (Roth et al., 1989; Rushlow et al., 1989; Steward, 1989). This single gradient subdivides the embryo into three distinct domains of zygotic gene expression. The ventral-most region giving rise to mesoderm, the neuroectoderm, and the dorsal part of the embryo (Jiang and Levine, 1993). At least two distinct cell fates are eventually generated within this dorsal region. The dorsal-most cell rows will give rise to amnioserosa, while dorsolateral cells will form the dorsal ectoderm. Recent evidence even suggests a sudivision to three distinct fates within the dorsal domain (Ashe et al., 2000).

Graded activation of the Decapentaplegic (Dpp) pathway was implicated in patterning the dorsal ectoderm. Injection of large doses of Dpp into unpatterned embryos induced amnioserosa, while lower levels generated dorsal ectoderm (Ferguson and Anderson, 1992). However, it was not clear from the expression pattern of the key players how this graded activation is achieved. The BMP-like molecule Dpp is expressed uniformly in the dorsal ectoderm. Its type I and type II receptors, Thickveins (Tkv) and Punt (Put), have a uniform maternal component (Brummel et al., 1994; Nellen et al., 1994; Penton et al., 1994; Letsou et al., 1995; Ruberte et al., 1995). Another BMP family ligand, Screw (Scw), is uniformly expressed in the embryo (Arora et al., 1994), and its distinct type I receptor, Saxophone (Sax), and common type II receptor Put, are also uniformly expressed in the early embryo (Xie et al., 1994).

The key to graded activation in the dorsal ectoderm is the *short gastrulation* (*sog*) gene, which is expressed only in the neuroectoderm. It encodes a membrane-tethered protein which becomes secreted, and is predicted to form a complex with BMP family members (Francois et al., 1994; Biehs et al., 1996). Sog is capable of diffusing to the dorsal ectoderm. This non-autonomous activity is evident from the dorsal defects of *sog* mutant embryos (Zusman et al., 1988), and from ectopic expression studies (Ashe and Levine, 1999).

Examination of the biological activity of Sog in the embryo and wing disc indicated that it is capable of inhibiting signaling by Scw, but not by Dpp (Neul and Ferguson, 1998; Nguyen et al., 1998; Yu et al., 2000). Sog diffuses to the dorsal region, where the secreted metalloprotease Tolloid (Tld) is expressed (Shimell et al., 1991). The Sog/ligand complex is cleaved by Tld, to release the active ligand (Marques et al., 1997). This cleavage generates a “sink”, leading to local elevation in the level of the activating ligand. The combined activity of Tld and Sog is thus responsible for generating the maximal level of signaling, inducing amnioserosa cell fates.

Studies carried out to date dissected the involvement of the different BMP signaling components, by monitoring expression of target genes. Since this is an indirect assay for signaling, the precise timing and profile of activation could not be determined. All BMP type I receptors signal by phosphorylation of a Smad protein, which then complexes with a second Smad protein and translocates to the nucleus to induce gene expression (reviewed in Massague and Chen, 2000; Raftery and Sutherland, 1999). An antibody which is specific to the phosphorylated C-terminal domain of Smad1 has been generated (Persson et al., 1998). This antibody was shown to recognize also the phosphorylated form of the *Drosophila* Mad protein, and could detect graded activation by Dpp in the wing imaginal disc (Tanimoto et al., 2000).

We have used this antibody to follow patterning of the embryonic dorsal domain by Dpp and Scw. Surprisingly, activation in the dorsal region is biphasic. In the cellular blastoderm embryo, the antibody detects pMad only in the dorsal-most cells that will give rise to amnioserosa. This pattern requires simultaneous activation by both Scw and Dpp. After germ band extension, activation by Dpp suffices to induce uniform accumulation of pMad in the entire dorsal domain, with no detectable activation in the neuroectoderm.

MATERIALS AND METHODS

Fly strains

The following strains were used: FRT 40 *mad*^{12} (provided by R. Padgett) *screw*^{l2}, *tolloid*^{2}, *tolloid*^{7}, Matα4-Gal4 VP16 (provided by D. St Johnston), UAS-*sog* 21A and UAS-*dpp* (provided by M. Hoffmann), *sog*^{6}, FRT 42B G13 *sax*^{4}, UAS-*activated tkv* (provided by S. Cohen), FRT 40 *tkv*^{strII}(provided by K. Basler). Germline clones of *sax* and *tkv* were induced by standard procedures with lines carrying *hs-flp*; FRT *Ovo*^{D}/FRT *sax* or *tkv*. For altering the number of *dpp* copies we used the strain *dpp*^{1846} *sp cn bw/CyO 23P[dpp*$^{+}$*]* (provided by S. Roth), crossed either to itself or to wild-type flies.

Antibodies and staining

Rabbit anti pMad antibody was kindly provided by P. ten Dijke. Freshly collected embryos were fixed in 7% formaldehyde/PBS. Primary antibodies were used at a dilution of 1:150. The remaining steps of staining were according to standard procedures. Cy2-conjugated anti-rabbit secondary antibodies (Jackson ImmunoResearch) were used. Embyros were examined using a Biorad 1024 confocal microscope, and viewed either as single optical sections or as a Z series.

RESULTS

pMAD in wild-type embryos

The distribution of pMad was monitored in wild-type embryos, by staining with an anti-pMad antibody. In all cases staining appears nuclear, consistent with the translocation of pMAD to the nucleus. The earliest pattern is observed in cellular blastoderm embryos (stage 5). A prominent stripe is formed in the dorsal-most 8-10 rows of cells (Fig. 1A). Within this region pMad levels are graded peaking in the dorsal midline. The sensitivity of detection does not allow reliable visualization of pMad beyond these rows. The observed pattern is highly reproducible. Quantitative analysis of a normalized pattern monitored in over 50 wild-type embryos from different genetic backgrounds, shows that all curves are virtually superimposable (Eldar et al., unpublished).

Noticeable pMad staining is also observed in all pole cells (Fig. 1B). pMad is retained by persistent signaling during the one hour period between stages 5 and 8 (Fig. 1C,D). Generation of folds in the dorsal ectoderm at stage 7 results in a less organized pMad pattern around the folds, while the head region maintains the same distribution.

By stage 9, pMad in the dorsal part of the head is diminished. A prominent pattern appears in the cephalic furrow and in the dorsal epidermis, encompassing approximately 10 cell rows on each side of the embryo (Fig. 1E). In contrast to the earlier stage, the distribution of pMad is uniform, covering the entire dorsal epidermis. A sharp boundary of activation at the junction with the neuroectoderm is notable. By this stage, pMad is also observed in the large amnioserosa cells which have formed. At stage 10/11 this pattern disappears, and a striped form of activation is observed, corresponding to the new ventrolateral and dorsal stripes of *dpp* expression (Fig. 1F). In the dorsal-most cells *dpp* is expressed in a single row (Riesgo-Escovar and Hafen, 1997). The pattern of pMad emanating from that column extends over approximately five cell rows, marking the diffusion of Dpp at this stage.

To verify that the above staining patterns represent exclusively phosphorylated Mad, we stained embryos obtained from germ-line clones, in which both maternal and zygotic Mad has been eliminated. Indeed, no staining was observed in these mutant embryos at stage 5 or stage 9 (Fig. 1G,H).

Regulation of the early pMad phase

The position of the early pMad phase in the dorsal-most cells corresponds to the region that will give rise to amnioserosa. To test if this pattern is induced by Scw, pMAD was monitored in *scw* homozygous mutant embryos. Indeed, the early stage 5 pattern in the dorsal ectoderm is completely eliminated, while a low residual pMad is retained in the pole cells (Fig. 2A). By stage 8/9, the second phase of pMad is normally observed in these embryos (Fig. 2B), indicating that this aspect does not depend on Scw. Induction of amnioserosa was previously shown to require the activity of Tld. In *tld* mutant embryos, the early pMad staining is diminished (Fig. 2C), while the later aspect is retained (Fig. 2D).

Tld expression in the dorsal region is uniform, while the distribution of Sog emanating from the neuroectoderm may be graded, such that lower levels of Sog are present in the dorsal-most region. To test the contribution of graded Sog distribution, we expressed Sog uniformly by a maternal Gal4 driver. The observed pMad pattern was remarkably similar to *scw* or *tld* mutants. Staining was eliminated in most of the dorsal cells, and retained only in the pole cells (Fig. 3A). This result indicates that the normal distribution of Sog is instructive for early patterning. Staining with anti-Kr (Kruppel) antibodies, which mark amnioserosa differentiation, indeed demonstrate the absence of amnioserosa cells following Sog misexpression (Fig. 3C,D). The second and third phases of pMad, which do not rely on Scw, are normal in embryos overexpressing Sog (Fig. 3B).

In *sog* mutant embryos, a dramatic expansion of the early pMad pattern is observed, such that up to 25 cell rows display high levels of pMad (Fig. 3E,F). In contrast, at stage 9 a normal pMad pattern is observed, maintaining the sharp boundary with

the neuroectoderm (Fig. 3G). The expanded early pattern agrees with earlier experiments which detected expansion of target-gene expression under the same conditions (Nguyen et al., 1998).

In spite of the expanded pMad pattern, a reduced number of amnioserosa cells is detected in *sog* mutants (Fig. 3H). The Sog protein or one of its cleavage products may thus provide an additional input to signaling, which is not reflected in the pMad levels.

Screw/Dpp cooperativity

Scw is uniformly expressed in the embryo, while Dpp is restricted to the dorsal region. If Scw is a cardinal ligand in the early phase, why is pMad seen in *sog* mutant embryos only in the dorsal domain (Fig. 3E), in the same region where *dpp* is expressed? The most likely explanation is employment of a cooperative signaling mechanism by Scw and Dpp. Thus, in the absence of the inhibitory effects of Sog, signaling by Scw can only expand within the boundaries of *dpp* expression.

Cooperative interactions between Scw and Dpp signaling have been proposed previously, and the option of activation by Scw/Dpp heterodimers was ruled out (Neul and Ferguson, 1998; Nguyen et al., 1998; Haerry et al., 1998). Since the assays relied on the induction of gene expression or the resulting cuticular structures, it was not possible to distinguish between cooperativity at the level of receptor activation, or in the actual induction of target genes. The ability to monitor pMad directly, provides a means to distinguish between these options.

Germline clones were generated for *sax* and *tkv* mutants. Elimination of maternal and zygotic *sax* leads to removal of the early dorsal pMad, while retaining it in the pole cells (Fig. 4A). In the second phase, these embryos, which can be distinguished by the reduced number of amnioserosa cells, display normal pMad distribution (Fig. 4B). Embryos lacking maternal *sax* but carrying a normal zygotic *sax* allele, showed normal pMad staining in both early and late stages (Fig. 4C,D). The zygotic contribution of Sax is therefore introduced early in embryogenesis.

Absence of maternal and zygotic *tkv* gives rise to complete elimination of the early pMad pattern, including the pole cells (Fig. 4E). In the second phase, pMad is also completely missing in all cells (Fig. 4F). Again, the pattern can be rescued by providing a normal zygotic *tkv* allele (Fig. 4G,H). Similarly, in homozygous *dpp* mutant embryos, pMad was not detected, at any stage (Fig. 4I,J). We can thus conclude that cooperativity between Scw and Dpp occurs at the level of receptor activation, leading to Mad phosphorylation.

Contribution of Dpp to early pMad pattern

It has been shown that embryos containing only one functional *dpp* allele show a haplo-insufficient phenotype and lack amnioserosa (Wharton et al., 1993). Therefore, it was interesting to monitor the early pMad patterns in embryos containing a different copy number of *dpp*. Embryos with a single *dpp* allele show a dramatically reduced early pMad pattern, with residual signaling observed only in the anterior and posterior parts of the embryo (Fig. 5A). In embryos containing three *dpp* copies, the early pMad pattern is

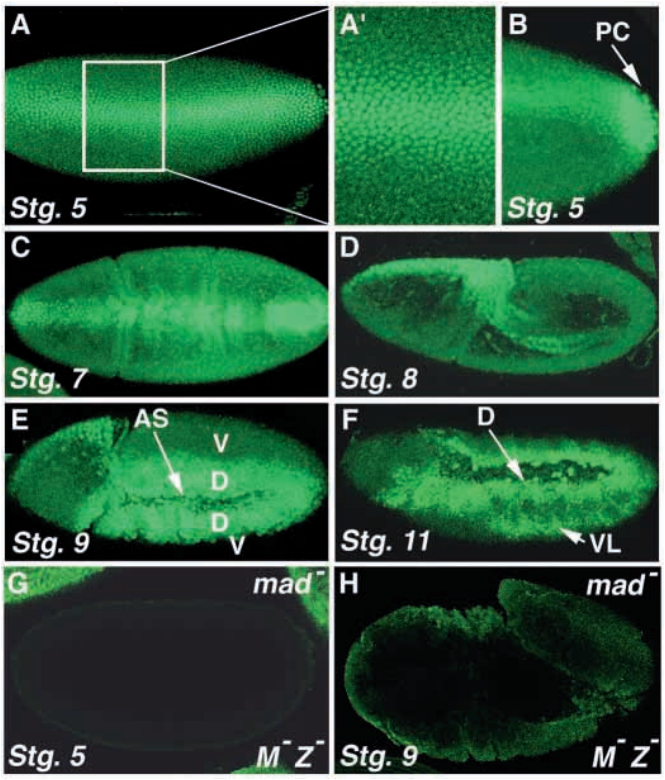

Fig. 1. pMad patterns during embryogenesis. (A) pMad pattern first appears at stage 5, in the dorsal-most nuclei of the cellular blastoderm embryo. (A′) Enlargement of boxed area in A. (B) Staining is also detected in the pole cells (PC). (C) The early pattern persists through to stage 7. (D) At stage 8, staining in the head region is still observed, while staining in the trunk is faint. (E) At stage 9 a new pattern appears, encompassing the entire dorsal ectoderm (D). Staining in the large amnioserosa (AS) nuclei is also detected. There is a sharp boundary with the adjacent ventral ectoderm (V) domain. Note that owing to the process of germ band extension, the embryo is folded such that the dorsal domain is duplicated at the center, while the ventral domain is facing outside. (F) By stage 11, a new pMad pattern is observed, corresponding to the two stripes of Dpp expression (dorsal-D and ventro lateral-VL). (G,H) Germ-line clones were used to generate *mad* mutant embryos lacking both maternal and zygotic Mad. No staining is seen at stages 5 or 9.

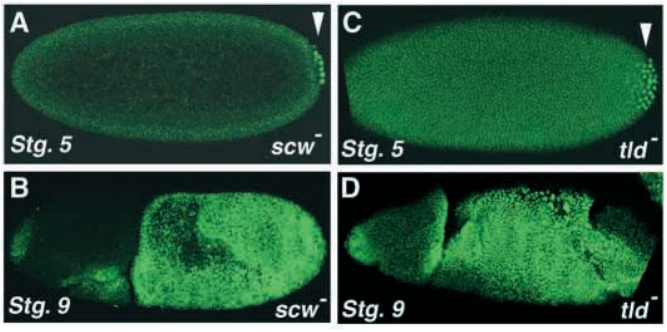

Fig. 2. pMad in *screw* and *tolloid* mutant embryos. Identical patterns of pMad staining are observed in *scw* and *tld* mutant embryos. (A) Stage 5 *scw* mutant embryos lack pMad in the dorsal region, but retain low levels in the pole cells (arrowhead). (B) At stage 9, a normal pMad pattern is observed in all dorsal cells. (C) In stage 5/6 *tld* embryos pMad is found only in the pole cells (arrowhead). (D) In stage 9 *tld* embryos pMad is present in all dorsal cells.

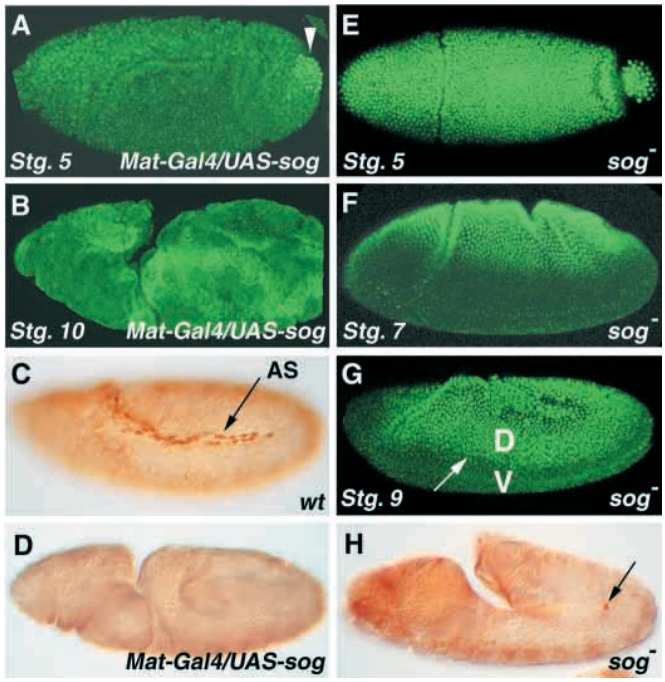

Fig. 3. Sog represses signaling by Screw. (A) Sog misexpression by Matα4-Gal4 VP16 eliminates pMad in the dorsal region at stage 5, while retaining it in the pole cells (arrowhead). (B) pMad at stages 9 and 10/11 is normal in these embryos. (C) Wild-type embryo showing Kr staining in the amnioserosa cells (AS) at stage 10. (D) Following Sog misexpression, Kr expression is eliminated. (E,F) In *sog* mutant embryos at stages 5-7, expansion of pMad is observed within the entire dorsal region. (G) At stage 9, normal pMad expression is detected in the dorsal domain of *sog* mutants. Note the retention of the sharp boundary (arrow) between the dorsal (D) and ventral (V) ectoderm. (H) *sog* mutant embryos display Kr staining in the amnioserosa in very few cells (arrow).

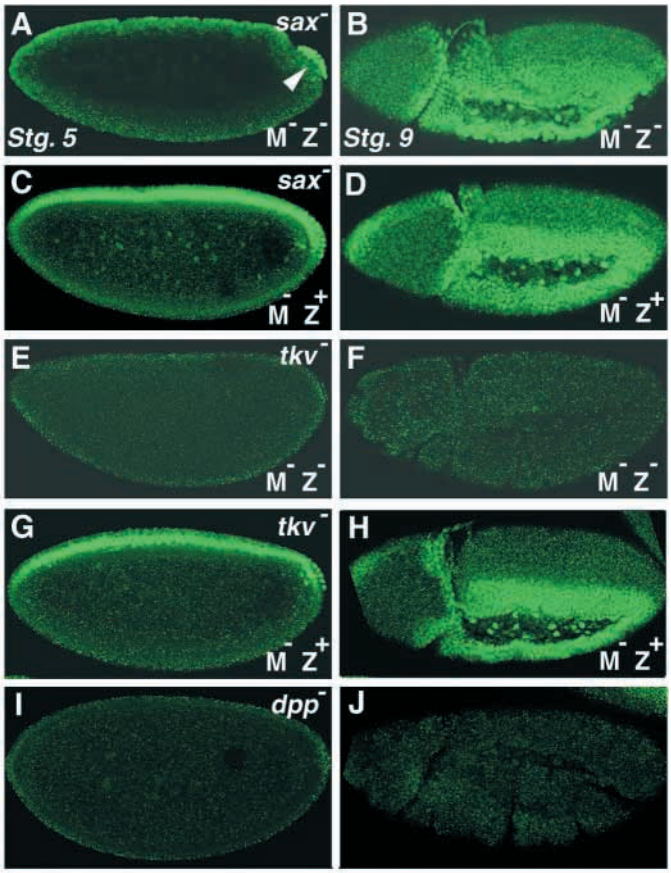

Fig. 4. pMad in *sax* and *tkv* germline clones. (A) Elimination of *sax* maternal and zygotic contribution abolished the early pMad pattern at stage 5 in the dorsal cells, while retaining it in the pole cells (arrowhead). (B) By stage 9, a normal pMad distribution is observed in the dorsal region in these embryos. (C,D) Embryos lacking maternal *sax* but carrying one normal zygotic *sax* allele, exhibit normal pMad patterns at stage 5 and 9. (E,F) Elimination of *tkv* maternal and zygotic contributions leads to absence of all pMad staining at stage 5, and (F) stage 9. (G,H) Embryos lacking maternal *tkv* but carrying a normal zygotic allele show normal pMad patterns at stage 5 and 9. (I,J) Homozygous *dpp* mutant embryos show no pMad staining at stage 5 and 9.

expanded and covers approximately 20 cell rows (Fig. 5C). These results show that in the early phase, signaling by Dpp contributes not only to the capacity of Scw to signal and induce pMad accumulation, but also to the actual level of signaling. In wild-type embryos carrying two functional *dpp* alleles, the level of Dpp signaling is precisely poised to induce, in collaboration with graded Scw signaling, the proper signaling profile (Fig. 5B). Any alteration in the copy number of *dpp* leads to a dramatic increase or decrease in the overall signaling profile. The pMad patterns in embryos carrying different copy numbers of *dpp* are schematized in Fig. 5D-F.

In contrast to the early phase, the second phase of signaling takes place normally in embryos containing one and up to four *dpp* copies (Fig. 5G-I). At this stage *dpp* expression is autoregulated (Jazwinska et al., 1999), and even one functional allele of *dpp* suffices to induce high and uniform levels of pMad in the entire dorsal domain.

Second pMad phase

The second phase of BMP signaling at stage 9 requires only Dpp/Tkv activation. Distinct borders of pMad are observed at this stage, with no apparent activation in the adjacent neuroectoderm cells (Fig. 1E). In accordance with the limited effects of Sog on Dpp signaling (Neul and Ferguson, 1998; Nguyen et al., 1998; Yu et al., 2000, Fig. 3B), no apparent expansion of pMad is observed in *sog* mutant embryos at stage 9 (Fig. 3G). Another unknown mechanism may therefore be operating in the neuroectoderm at this stage, to restrict the response to activation by Dpp or to limit the diffusion of Dpp. In contrast, the pMad pattern observed at stage 11 (Fig. 1F), indicates that Dpp, emanating from the dorsal cell stripe, can diffuse and induce pMad over 5 cell rows away.

A sharp boundary of pMad accumulation is observed at the junction between the dorsal ectoderm and the neuroectoderm at stage 9. Does this restriction extend to the entire neuroectoderm? By using the *paired*-Gal4 driver to express Dpp orthogonally in stage 9/10 embryos, it is possible to examine the response to Dpp in the circumference of the embryo. At 18°C where the level of induction by Gal4 is lower, the ectopic Dpp stripes induce pMad at a level comparable to the endogenous one (in the dorsal region). Under these conditions activation does not extend beyond the stripes of *paired* expression, and is similar to the cell autonomous

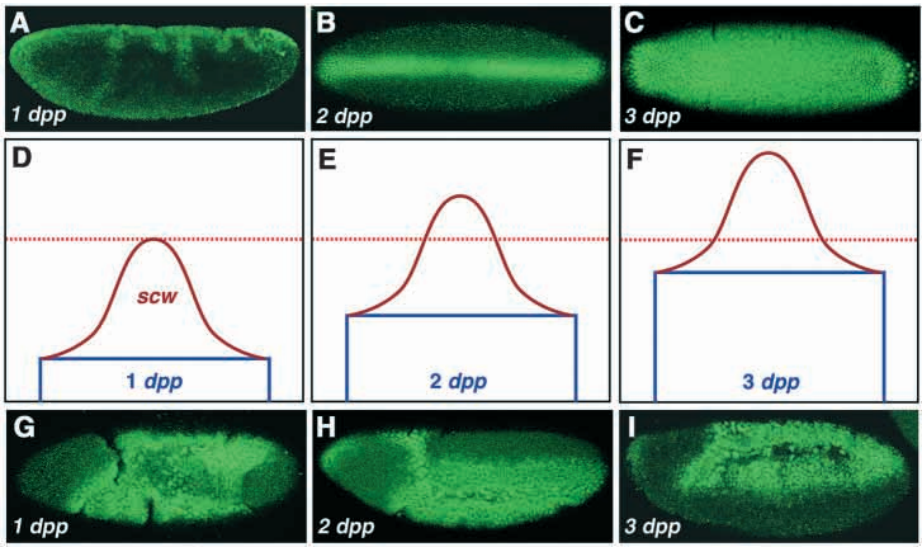

Fig. 5. *dpp* copy number affects early pMad pattern. **(**A) Embryos containing a single copy of *dpp* show only low, barely detectable levels of pMad at stage 5/6. (B,C) Embryos containing three copies of *dpp* exhibit an expansion of the early pMad pattern, compared to embryos with two alleles of *dpp*. (D-F) Schematic representations of the contribution of Dpp levels to the overall early pMad activation profile. The dashed red line shows the detection limits with anti-pMad antibody. (G) At stage 9, embryos containing a single *dpp* copy show a normal pMad pattern. The overall morphology of these embryos is deranged as a result of defects in germ band extension at earlier stages. (H,I) Stage 9 embryos containing three *dpp* copies show a pMad pattern similar to embryos with two *dpp* alleles.

activation observed by expression of activated Tkv (Fig. 6A,C,D). Higher levels of Dpp expression (at 25°C) were capable of extending beyond the *paired* expression domains and induced pMad uniformly in the embryo (Fig. 6B).

DISCUSSION

The capacity to follow signaling by the BMP pathway directly, has provided several novel insights to patterning of the dorsal ectoderm (schematized in Fig. 7). Two distinct phases of activation were identified (Fig. 1). The early phase requires activation by both Scw and Dpp ligands, while the second phase depends only on Dpp.

Scw, Tld and Sog establish the early pMad pattern

Signaling is first detected in the cellular blastoderm embryo. While activation is observed within the dorsal-most 8-10 cell rows, the sensitivity of the detection method fails to monitor signaling in the rest of the dorsal domain. High signaling levels are induced by Scw, and give rise to amnioserosa. Within the domain where pMad is observed, we detected graded activation, which may have the capacity to induce more than one cell fate in the region.

The cardinal players in the generation of the early pMad gradient are Scw, Tld and Sog. Tld was suggested to generate a sink for the active ligand, by cleaving the Sog/ligand complex (Marques et al., 1997). The similarity between the pMad pattern of *scw* and *tld* mutants suggests that Tld is primarily involved in the release of Scw from the complex with Sog.

Absence of Scw, Tld or Sax abolished the early pMad pattern while retaining the second phase, indicating that the second phase relies only on Dpp signaling (Figs 2, 4). Similarly, overexpression of Sog eliminated only the early but not the subsequent pMad patterns (Fig. 3). This suggests that Sog preferentially associates with Scw, in agreement with previous biological assays of Sog activity (Neul and Ferguson, 1998; Nguyen et al., 1998; Yu et al., 2000).

Generation of graded patterning in the dorsal region does not rely on restricted gene expression within this domain. Rather, expression of genes confined to the neuroectoderm may lead to graded distribution of their gene products within the dorsal domain. The essential component for generation of graded patterning appears to be Sog, which is produced only in the neuroectoderm, but is capable of diffusing to the dorsal region. Disruption of the normal distribution of Sog by uniform misexpression, abolished the early pMad activation profile. This suggests that normally Sog may form a graded distribution in the dorsal region, which is essential for patterning. When the Sog/Scw complex is cleaved by Tld, Scw is released and can bind either Sog or Sax. Our data suggests that in regions closer to the neuroectoderm, the levels of Sog are high and titrate the free ligand. In the dorsal-most region however, where Sog levels are low, the released Scw has a greater probability of binding and activating the Sax receptor, rather than being trapped again by Sog. Thus, the graded distribution of Sog is critical for generating the reciprocal distribution of Scw, and the ensuing activation profile.

In *sog* mutant embryos we observed an expansion of the early pMad pattern (Fig. 3). In the absence of Sog, a uniform distribution of Scw is expected, and hence the activation level should be lower than the maximal level in wild-type embryos.

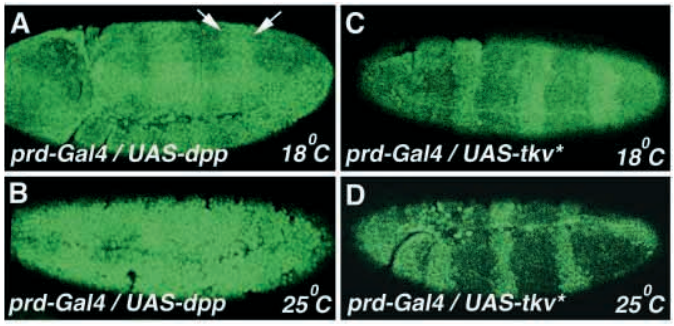

Fig. 6. Restricted activation by Dpp at stage 9. Ectopic expression of UAS-*dpp* by *paired*-Gal4 at 18°C induces pMad at a level comparable to the endogenous level (in the dorsal domain). Under these conditions, pMad is confined to the *paired* stripes (arrows). (B) At 25°C when the level of Gal4 induction is higher, the effect of Dpp extends beyond the *paired* stripes to induce pMad uniformly. (C,D) At 18°C or 25°C, expression of activated Tkv induces pMad in the *paired* stripes in a cell autonomous response.

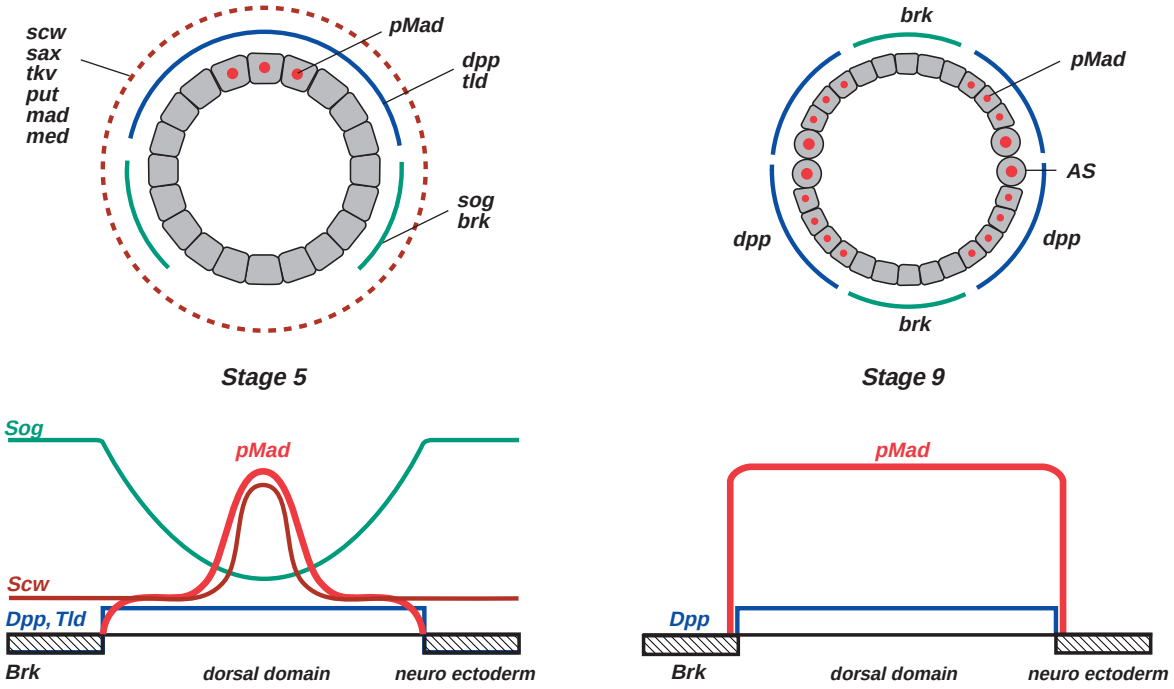

Fig. 7. Biphasic BMP activation in the embryonic dorsal region. pMad is generated in two phases to pattern the dorsal region. At stage 5, Scw cooperates with Dpp signaling, and the generation of high pMad levels is restricted to the dorsal-most cells by a graded distribution of Sog. This leads to induction of amnioserosa cell fates. At stage 9, activation by Dpp accounts for all pMad patterns. Dpp is expressed in the entire dorsal region, leading to uniform appearance of pMad in the same domain, and expression of dorsal ectoderm genes.

We have quantitated the staining levels in wild-type and *sog* mutant embryos. While the pattern of staining is reproducible in all wild-type embryos, variations in the absolute levels of up to threefold between embryos were observed in any given staining reaction. It is thus difficult to compare reliably the wild-type level to the absolute staining levels of *sog* mutants. Nevertheless, the impression is that the expanded pMad in *sog* mutant embryos is comparable in levels to the maximal signaling levels in wild-type embryos. In spite of this expanded pMad activation pattern, amnioserosa cell fates are abolished in *sog* mutants (Fig. 3; Jazwinska et al., 1999). This result suggests that in addition to the role of Sog in determining the graded distribution of Scw, Sog or its cleavage products may provide an additional signal facilitating the induction of amnioserosa cell fates.

Contribution of Dpp to the early pMad pattern

Activation of Tkv by Dpp is essential for the appearance of the early pMad pattern, corresponding to the future amnioserosa cells. At this stage, distinct cell fates are also induced in the dorsolateral cells, as reflected by expression of *pnr* and repression of *msh* expression (Jazwinska et al., 1999; Winick et al., 1993; D'Alessio and Frasch, 1996). We assume that low levels of activation that may be induced by Dpp alone, but not detected by pMad antibodies, are responsible for these fates.

Elimination of Dpp or Tkv leads to complete absence of early, as well as late, pMad patterns (Fig. 4). Thus, Scw is not sufficient for the early activation phase, and the presence of Dpp is crucial. Cooperativity between Scw and Dpp occurs at the level of receptor activation. One possibility is that the observed pMad levels reflect only an additive effect of Scw and Dpp signaling. Indeed, we show that the number of *dpp* copies has a profound effect on signaling levels and the shape of the early pMad distribution (Fig. 5). Alternatively, it is possible that there is a synergistic interaction between Scw and Dpp signaling. In this case, the requirement of both ligands for the production of the early pMad pattern may indicate that synergy occurs at the level of receptor activation. Phosphorylation of Mad may require the formation of heterotetrameric receptors, containing both Sax/Put and Tkv/Put pairs. Cross linking experiments of the vertebrate receptors support this model (Yamashita et al., 1994).

Scw is required for generating the pMad pattern only in the early phase. All subsequent patterns rely only on Dpp. This feature may be explained differently by each of the above two models. If Scw and Dpp are required additively in the early phase, higher levels of Dpp may suffice to induce the pMad pattern at later stages. The autoregulatory effects of Dpp on its transcription (Jazwinska et al., 1999) may account for the elevation in Dpp levels. Alternatively, if Scw and Dpp signaling is synergistic, we have to ask why such a synergism is necessary only in the early phase. In the early embryo, a maternal transcript encoding an inhibitor of BMP signaling may be translated, to block signaling by Sax/Put or Tkv/Put dimers. Such inhibitor(s) may be displaced only in ligand-bound heterotetrameric receptor complexes. The maternal transcripts of the inhibitor(s) may diminish by stage 9, to allow pMad production by activation of Tkv/Put alone.

Dpp controls the second pMad phase

By stage 8/9, Dpp/Tkv activation is sufficient to induce detectable levels of phosphorylated Mad. The second phase of

activation does not rely on execution of the early phase, and is detected in *scw*, *tld* or *sax* mutants. A uniform pattern of pMad is observed at this stage within the entire dorsal domain, in accordance with the pattern of autoregulated *dpp* expression. In the neuroectoderm, *brinker* (*brk*) is expressed to suppress Dpp autoregulation (Jazwinska et al., 1999). The uniform pMad pattern corresponds to the resulting expression pattern of genes like *pannier* (*pnr*) at stage 9 (Heitzler et al., 1996), indicating that this second phase of activation is indeed instructive for induction of target genes in the entire dorsal domain. Once cell intercalation leading to germ band extension has been completed, it may be necessary to induce, within the dorsal region, such a uniform activation of Dpp target genes.

In the second phase, sharp borders of pMad localization are observed, with no detectable activation in the neuroectoderm (Fig. 1E). Dpp is a diffusible ligand, as indicated by the induction of pMad several cell rows away from the dorsal row of cells expressing Dpp at stage 11 (Fig. 1F). Direct visualization of Dpp in the wing disc also demonstrated its diffusion capacity over many cell rows (Teleman and Cohen, 2000; Entchev et al., 2000). How are the sharp pMad borders generated at stage 9, in view of the diffusability of Dpp? We suggest that the neuroectoderm cells may produce an inhibitor that prevents activation of the pathway by Dpp molecules that could diffuse from the adjacent dorsal region. Alternatively, the neuroectoderm cells may express cell surface proteins that would block the diffusion of Dpp into the neuroectoderm. We show that when Dpp is expressed ectopically at physiological levels in perpendicular stripes, no pMad activation is observed in the neuroectoderm outside the stripes of Dpp expression (Fig. 6). Thus, lower levels of Dpp are not capable of activating the pathway in the neuroectoderm at stage 9.

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