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Distinct functional specificities are associated with protein isoforms encoded by the *Drosophila* dorsal-ventral patterning gene *pipe*

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Spatially regulated transcription of the *pipe* gene in ventral cells of the *Drosophila* ovary follicle cell epithelium is a key event that specifies progeny embryo dorsal-ventral (DV) polarity. *pipe* encodes ten putative protein isoforms, all of which exhibit similarity to vertebrate glycosaminoglycan-modifying enzymes. Expression of one of the isoforms, Pipe-ST2, in follicle cells has previously been shown to be essential for DV patterning. *pipe* is also expressed in the embryonic salivary gland and its expression there is required for normal viability. Here, we show that in addition to Pipe-ST2, seven of the other Pipe isoforms are expressed in the ovary, whereas all Pipe isoforms are abundantly expressed in the embryo. Of the ten isoforms, only Pipe-ST2 can restore ventral and lateral pattern elements to the progeny of otherwise *pipe*-null mutant females. By contrast, three Pipe isoforms, but not Pipe-ST2, support the production of a novel *pipe*-dependent epitope present in the embryonic salivary gland. These data indicate that differences in functional specificity, and presumably enzymatic specificity, are associated with several of the Pipe isoforms. In addition, we show that uniform expression of the Pipe-ST2 isoform in the follicle cell layer of females otherwise lacking *pipe* expression leads to the formation of embryos with a DV axis that is appropriately oriented with respect to the intrinsic polarity of the eggshell. This suggests the existence of a second mechanism that polarizes the *Drosophila* embryo, in addition to the ventrally restricted transcription of the *pipe* gene.

KEY WORDS: Dorsoventral, Sulfotransferase, Sulfyltransferase, Sulfation, Sulfonation, PAPS, Oogenesis, Embryogenesis

INTRODUCTION

The determination of *Drosophila* embryonic dorsal-ventral (DV) polarity is mediated by the ventral activation within the embryo of a receptor, Toll, that is uniformly distributed in the embryonic membrane (Anderson and Nüsslein-Volhard, 1986; Govind and Steward, 1991; Morisato and Anderson, 1995; Schüpbach and Wieschaus, 1989). Gastrulation-defective (Gd) (Konrad et al., 1998), Snake (DeLotto and Spierer, 1986) and Easter (Chasan and Anderson, 1989) are secreted serine proteases present in the perivitelline space between the eggshell and the embryonic membrane. Gd cleaves and activates Snake, which then cleaves and activates Easter (Dissing et al., 2001; LeMosy et al., 2001). Easter then processes an inactive precursor form of Spätzle, another perivitelline space protein. One of the cleavage products of Spätzle is the activating ligand for Toll (Morisato and Anderson, 1994; Schneider et al., 1994). It has been suggested that a ventral cue, produced during oogenesis, is deposited ventrally within the egg, where it leads to ventral activation of the protease cascade (Anderson, 1998; Sen et al., 1998). Activated Toll transmits the ventralizing signal into the embryonic cytoplasm, leading to the formation of a nuclear gradient of the transcription factor Dorsal (Roth et al., 1989; Rushlow et al., 1989; Steward, 1989) that is responsible for differential transcription, along the DV axis, of zygotic genes responsible for the formation of correct pattern (Stathopoulos and Levine, 2002).

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The expression of *pipe* in the ventral cells of the follicle layer is responsible for the localized activity of the dorsal group serine protease cascade (Sen et al., 1998). *pipe* encodes ten distinct protein isoforms, generated by alternatively spliced mRNAs, all of which exhibit significant sequence homology with the vertebrate enzymes heparan sulfate 2-O-sulfotransferase (HS2ST) and dermatan/chondroitin sulfate 2-O-sulfotransferase (D/CS2ST), two Golgi resident proteins that mediate the sulfation of glycosaminoglycan (GAG) side chains of proteoglycans (Kobayashi et al., 1997; Kobayashi et al., 1999; Sen et al., 1998). HS2ST and D/CS2ST mediate the transfer of a sulfate group to the 2-O position of the uronic acid residues in their respective GAG substrates (Kobayashi et al., 1996; Kobayashi et al., 1999). The similarity between the Pipe isoforms and HS2ST and D/CS2ST initially suggested that Pipe-ST2 might act in follicle cells to sulfate a proteoglycan carrying HS or D/CS side chains. Although subsequent studies have confirmed that Pipe-ST2 acts as a sulfotransferase in ventral follicle cells (Zhu et al., 2005), the nature of the putative carbohydrates modified by Pipe-ST2 remain unknown. Genes involved in GAG synthesis need not be expressed in the follicle cell layer of female flies in order for progeny embryos with normal DV polarity to be produced (Zhu et al., 2005; Zhu et al., 2007). Similarly, the production of a *pipe*-dependent sulfated molecule in the embryonic salivary gland does not require the expression of genes needed for GAG synthesis. Moreover, purified Pipe-ST2 fails to exhibit sulfotransferase activity on HS, CS or DS in vitro (Xu et al., 2007) (A. Amiri and D.S., unpublished). In contrast to these data suggesting that GAGs do not represent the carbohydrate substrate of Pipe, a recent report indicates that HS exhibits an altered pattern of sulfation in *pipe* mutant ovaries (Park et al., 2008). Regardless of the specific structure of its target, the current model for Pipe protein action suggests the existence of a glycoprotein-associated carbohydrate molecule that is synthesized in all follicle cells and sulfated by Pipe protein(s) in ventral cells of the epithelium. Following modification,

this molecule is secreted, becoming associated with either the oocyte membrane or the vitelline membrane of the eggshell, where it remains until embryogenesis, when it somehow activates the serine protease cascade ventrally in the perivitelline space.

Among the Pipe isoforms, only Pipe-ST2 has been shown to be essential for embryonic DV polarity (Zhu et al., 2005) or to be capable of directing the formation of lateral and ventral pattern elements in the progeny of transgene-expressing females (Sen et al., 1998). Nevertheless, the complex pattern of splicing of the *pipe* primary transcript has led us to examine the functional basis for the existence of the multiple protein isoforms encoded by the locus. In one potential scenario, the various Pipe protein isoforms could be associated with distinct enzymatic activities, with a subset of the proteins being required in the ovary for the generation of the progeny embryo DV pattern, and other isoforms functioning in the embryonic salivary gland or in other tissues. Alternatively, all isoforms might share a common activity despite their distinct amino acid sequences. To distinguish between these possibilities, we have examined the expression of the individual isoforms and have observed that all are highly expressed in the embryo, whereas several isoforms, in addition to Pipe-ST2, are expressed in the ovary. Among the various isoforms, only the expression of Pipe-ST2 in the ovary is required for the production of embryos with DV polarity. None of the other isoforms expressed transgenically in the follicle cell layer can substitute for Pipe-ST2. These observations suggest that Pipe-ST2 provides a unique enzymatic specificity that is distinct from those associated with the other isoforms.

We have extended the investigations of Pipe isoform functional specificity using a fortuitously identified antibody that detects a sulfated, *pipe* gene-dependent epitope that is present in the lumen of embryonic salivary glands. By individually expressing Pipe isoforms in the salivary glands of embryos otherwise lacking all Pipe activity, we have observed that three of the Pipe isoforms are capable of restoring the presence of the antigen, whereas Pipe-ST2 cannot. This provides further evidence that proteins encoded by the *pipe* locus differ in their enzymatic and functional specificities.

Finally, in a separate set of experiments, we have observed that uniform follicle cell expression of Pipe-ST2 in females that otherwise completely lack Pipe protein expression leads to the production of embryos with residual DV polarity that is appropriately oriented with respect to the eggshell. This suggests the existence of a second polarizing input, in addition to the ventral transcription of the *pipe* gene, that influences the orientation of the embryonic DV axis.

MATERIALS AND METHODS

Fly stocks, crosses and maintenance

All stocks were maintained employing standard conditions and procedures. The wild-type *D. melanogaster* stock used was Oregon R. Mutant alleles of *pipe* have been described as follows: $pipe^{1}$ and $pipe^{2}$ (formerly $pipe^{386}$ and $pipe^{664}$) (Anderson et al., 1985); $pipe^{9}$, $pipe^{3}$ and $pipe^{C14}$ (Zhu et al., 2005); $pipe^{ZH1}$ (Sergeev et al., 2001); $Df(3L)pipe^{A13}$ (Sen et al., 1998). Stocks carrying the following mutations have been described previously: $Papss^{2}$ (Zhu et al., 2005); sll^{7E18} (Luders et al., 2003); $wind^{M88}$ (Konsolaki and Schupbach, 1998). The following transgenic P-element insertions used in this study have been described previously: UAS-*pipe-ST2* (Sen et al., 1998); *55B-Gal4* (Brand and Perrimon, 1994); *T155-Gal4*, *CY2-Gal4* and UAS-λtop (Queenan et al., 1997); *fkh-Gal4* (Henderson and Andrew, 2000). Laboratory strains of the *Drosophila* species *D. ananassae*, *D. erecta*, *D. immigrans*, *D. pseudoobscura*, *D. sechellia*, *D. simulans*, *D. virilis*, *D. willistoni* and *D. yakuba* were obtained from the *Drosophila* Species Stock Center at the University of Arizona in Tucson (current location, University of California at San Diego).

Analyses of *pipe* expression in ovaries and embryos

Total RNA was isolated from embryos or dissected ovaries using Trizol reagent (Invitrogen, Carlsbad, CA, USA). cDNA for RT-PCR was prepared using the SuperScript III First-Strand Synthesis System (Invitrogen).

Thirty cycles of PCR amplification (94°C for 30 seconds, 59°C for 30 seconds, 72°C for 60 seconds) were carried out for the detection of reverse-transcribed cDNAs. The primer 5′-GGATGTCTCTGAACGCCGAGCG-3′, which starts at the –2 position within the 5′ UTR of all spliced *pipe* mRNA isoforms, served as the 5′ oligo in all polymerization reactions. The 3′-end primers specific to each isoform were derived from sequences within the unique fourth exon; for Boxes 1 through 10, respectively, they were (5′ to 3′): GAGCAACTCCATCATCTTCTC, CATCAGCTCCATCA-GCGATTG, CATCAACTCGATCAGCGTTTC, GATGAGTCGCGTCAT-TGACTG, CATAAGCTCAATCAGCTGCAT, AAAGAGCTCCAGCAT-AGATTC, CATAAACTCCGTAAGCGATTC, GGAGAGTTCCATTAG-AGCTTC, GAAAAGTGGGACCATTTGCTC and AAGGAGCTCCAT-AAACGTCTG. As a control for the quality of the mRNA and reverse transcription, the same cDNA preparations were used to amplify a 797 bp fragment of the *windbeutel* transcript using the 5′-end primer 5′-AGTCTCTGGTTTTTGCCACG-3′, which corresponds to nucleotides +42 to +61 of the transcript, and 5′-TCACAGTTCCTCCTTTTCCG-3′, which corresponds to nucleotides +819 to +838.

Transgenic constructs

pipe isoforms were cloned following 30 cycles of PCR amplification as above with high-fidelity Pfu DNA polymerase (Stratagene, La Jolla, CA, USA) using cDNA produced with mRNA from wild-type embryos as the amplification template. The 5′-end primer 5′-CCGAGCGC-GGCCGCAAAATGAAACTACGCGATGTGG-3′ starts at the –4 position of the 5′ untranslated region shared by all spliced isoforms, with the addition of a *Not*I restriction site upstream of the initiation codon. For Boxes 1 through 10, respectively (Sergeev et al., 2001), the isoform-specific 3′ primers used for amplification were (5′ to 3′): GGATCCT-CTAGACTAATCCCACAGCTTGGGATC, GGATCCTCTAGACTAATA-GTCCTCATTGTATTC, GGATCCTCTAGATTAGAAGTGCAATTG-CTTATTTAT, GTACTATCTAGACTAAAATTCGTTCTTATGGAC, GGA-TCCTCTAGACTAGTTGTTGATGGCCATTTC, GTACTATCTAGACT-AGTTTAACAGACCATGCAC, GGATCCTCTAGATTAGGAAAAAT-TCAGTCGTTCCAA, GGATCCTCTAGATTAAGTAGTCCCTGAA-ACATTTTT, GGATCCTCTAGATTAGTGAAGATTGTTCTCGAG and GTGAAGTCTAGACTTCAATTGCCAATCAGGCCG. The 3′ oligonucleotides contained the stop codon for each particular isoform, as well as an *Xba*I restriction site downstream of the stop codon. PCR products corresponding to isoform cDNAs were digested with *Not*I and *Xba*I and ligated to similarly digested pUAST (Brand and Perrimon, 1993).

For the construction of pHS-CaSpeR-*pipe-ST2*, pBluescriptSK-*pipe-ST2* (Sen et al., 1998), which contains a cDNA corresponding to *pipe-ST2*, was digested at the *Apa*I site 3′ of the cDNA insert. This site was then made blunt-ended using Klenow enzyme and an *Xba*I linker was added. The *pipe-ST2* cDNA was then excised as an *Xba*I fragment and ligated to *Xba*I-digested phs-CaSpeR (Bang and Posakony, 1992). A plasmid carrying the *pipe-ST2* cDNA downstream of the *hsp70* promoter in the sense orientation was identified and designated pHS-CaSpeR-*pipe-ST2*.

For expression studies, a transgenic insertion of pHS-CaSpeR-*pipe-ST2* was introduced into $pipe^{1}/pipe^{2}$ mutant females. These females were subjected to two rounds of heat shock (1.5 hours at 37°C, 3 hours apart). Embryos were collected at 6-hour intervals. Heat-shocked females produced few eggs, most of which were collapsed and failed to develop until 18 hours after heat shock. Embryos undergoing development, many of which exhibited restoration of lateral and ventral pattern elements, were first observed during the interval 18-24 hours after heat shock. The number of embryos exhibiting phenotypic rescue decreased dramatically in the 30-36 hour interval, with most embryos exhibiting a completely dorsalized phenotype. Based on these results, subsequent analysis of *hsp70* promoter-directed expression of Pipe-ST2 was carried out using embryos collected 18-30 hours after heat shock of transgene-bearing mutant females.

Immunostaining of embryos and egg chambers

Whole-mount staining of embryos and egg chambers was carried out according to Macdonald and Struhl (Macdonald and Struhl, 1986). The rabbit polyclonal anti-Twist antiserum (Roth et al., 1991) was used at a dilution of 1:2000, and the affinity-purified rabbit polyclonal anti-Engrailed antiserum (Myat and Andrew, 2002) was used at 1:1000. The monoclonal anti-Engrailed antibody (4D9, Developmental Studies Hybridoma Bank) was used at 1:25. The rabbit polyclonal antibody directed against an N-terminal peptide present in all Pipe protein isoforms (Zhu et al., 2005) was pre-absorbed against ovaries from $pipe^{C14}/Df(3L)pipe^{A13}$ females at a dilution of 1:100, and used at a final dilution of 1:2000. Primary antibodies were used in conjunction with biotinylated goat anti-rabbit or anti-mouse secondary antibodies (pre-absorbed against wild-type embryos, final dilution 1:500 or 1:2000, respectively) and visualized with avidin-HRP complex (Vector Laboratories, Burlingame, CA, USA).

Collection of embryos for staining with Alcian Blue or with Engrailed* antiserum

Embryos from stocks carrying mutations balanced over either CyO, *Krüppel-Gal4* UAS-GFP or TM3, *Sb Krüppel-Gal4* UAS-GFP were collected on apple juice agar plates overnight, dechorionated in 50% sodium hypochlorite, then transferred to a glass plate and covered with hydrocarbon 27 oil (Sigma). Stage 12-16 embryos were separated into groups containing fluorescent wild-type or non-fluorescent homozygous mutant embryos using a Leica MZFLIII dissecting microscope equipped for detection of GFP. Embryos were stained with Alcian Blue according to Zhu et al. (Zhu et al., 2005), or were processed for whole-mount immunohistochemical staining using the Engrailed* antibody as outlined above.

To examine the function of the various Pipe protein isoforms in embryos, stocks that carry the $pipe^{3}$ allele together with each Pipe protein isoform cloned into pUAST were individually crossed to the stock $pipe^{C14}$ *fkh-Gal4*/TM3, *Sb Krüppel-Gal4* UAS-GFP, and progeny embryos were collected overnight on apple juice agar plates. As described above, stage 12-16 embryos were separated into fluorescent and non-fluorescent populations. Non-fluorescent embryos expressed an individual Pipe protein isoform in the salivary glands of embryos that otherwise lacked *pipe* expression. Embryos were stained with Alcian Blue or subjected to whole-mount staining with the Engrailed* antibody as described above.

Examination of gastrulation patterns and cuticular phenotypes

Gastrulation patterns were examined under halocarbon 27 oil by conventional light microscopy (Wieschaus and Nüsslein-Volhard, 1986). Larval cuticles were prepared according to van der Meer (van der Meer, 1977). DV phenotypes of embryonic cuticles were classified according to Roth et al. (Roth et al., 1991) with the modifications described in the text.

RESULTS

Multiple Pipe isoforms are expressed in *Drosophila* ovaries and embryos

Ten distinct Pipe protein isoforms can be generated through differential splicing of the primary *pipe* transcript (Sergeev et al., 2001) (http://flybase.org/reports/FBgn0003089.html). All mature mRNAs share the first three exons, which encode the N-terminal 95 amino acids of the protein including the putative membrane-spanning, Golgi-targeting determinant (Sen et al., 2000). The putative sulfotransferase domain of each isoform is encoded by a distinct set of isoform-specific exons. The regions encoding the isoform-specific exons were initially referred to as Box 1 through 10, based on their proximity to the common 5′ exons, with the Box 1 exons proximal and the Box 10 (Pipe-ST2) exons distal to the 5′-end of the gene (Sergeev et al., 2001). Outside of the common region, the ten ORFs share 37-50% amino acid sequence identity with one another, and 21-32% identity with the orthologous regions of the vertebrate enzymes HS2ST and D/CS2ST. An alternate nomenclature has been adopted by FlyBase based on the annotation of the sequenced *Drosophila* genome, with the predicted *pipe* mRNA isoforms referred to as *pipe-RK*, *RL*, *RC*, *RF*, *RD*, *RG*, *RH*, *RI*, *RJ* and *RA* (*pipe-ST2*), which correspond to the three common exons joined to the exons represented by Boxes 1 through 10, respectively. Corresponding proteins are referred to as Pipe-PK through PA. According to the FlyBase annotation, Box 2 forms a portion of *pipe-RL* and of a second predicted mRNA, denoted *pipe-RE*. We have never detected expression of the predicted *pipe-RE* isoform, suggesting that it does not represent a bona fide mRNA. Similarly, an additional isoform is predicted by FlyBase, *pipe-RM*, that carries segments of both Box 9 and Box 10 and generates a protein that corresponds to the first 292 amino acid residues of Pipe-PJ. As with *pipe-RE*, we have never detected cDNA corresponding to *pipe-RM*, suggesting that it is not expressed in vivo.

The results of RT-PCR using isoform-specific oligonucleotide primers indicated that all predicted Pipe isoforms (except *pipe-RE* and *pipe-RM*, see above) are abundantly expressed in wild-type embryos (Fig. 1A), presumably in the salivary glands. In addition to *pipe-ST2* (Box 10), we found that the mRNAs corresponding to Boxes 1 through 7 are also expressed in wild-type ovaries, although most of them, with the exception of Box 4, are expressed at low levels (Fig. 1B) as compared with their level of expression in embryos. As a control for the sensitivity of this assay, RT-PCR analysis of ovarian mRNA from mutant females of the RNA-null mutant genotype $pipe^{C14}/Df(3L)pipe^{A13}$ showed a complete absence of *pipe* mRNA expression (Fig. 1C).

Only the Pipe-ST2 isoform is capable of directing ventralization of progeny embryos

Among 11 available EMS-derived mutant alleles, ten are associated with lesions affecting *pipe-ST2*-specific exons (Zhu et al., 2005). One exception, $pipe^{3}$, carries a stop codon at the end of the common

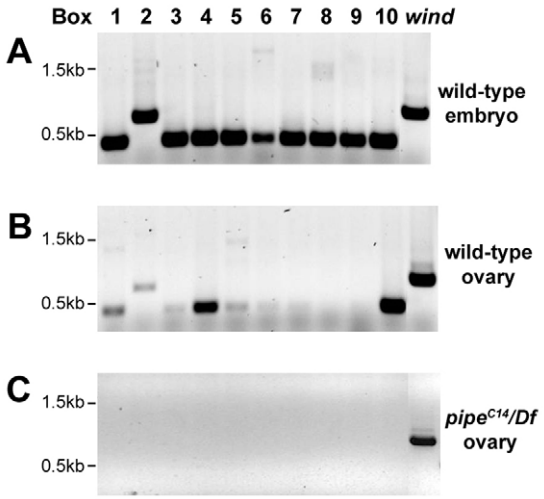

Fig. 1. Expression of *pipe* mRNA isoforms in *Drosophila* embryos and ovaries. mRNA isolated from wild-type embryos (**A**) and ovaries (**B**) and from $pipe^{C14}/Df(3L)pipe^{A13}$ mutant ovaries (**C**) was reverse transcribed and subjected to PCR amplification with isoform-specific oligonucleotide primers, followed by agarose gel electrophoresis. Lanes are labeled according to the Box1-10 nomenclature (Sergeev et al., 2001). A 797 bp RT-PCR product of the *windbeutel* (*wind*) cDNA derived from the same mRNA is shown in each gel. For each of the three panels, samples and controls were electrophoresed simultaneously and in tandem on a single gel. In preparation of the figure, extraneous lanes between the Box 10 sample and the *windbeutel* control have been deleted in order to align the corresponding control samples for each of the three sources of mRNA.

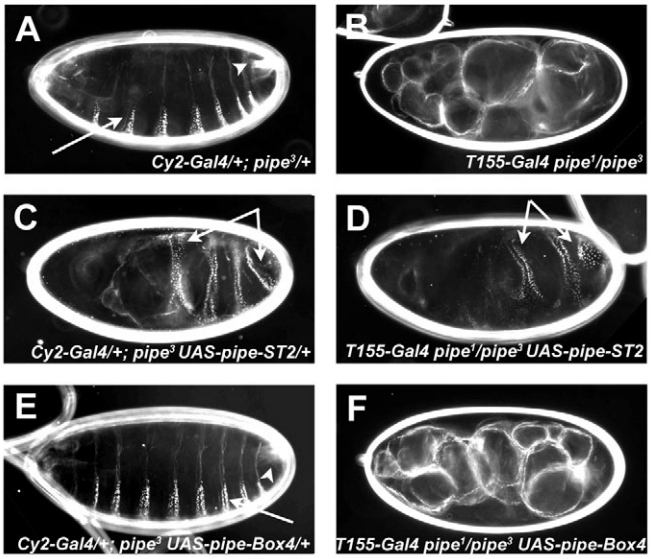

Fig. 2. Pipe-ST2 protein is uniquely capable of directing ventralization of progeny embryos. cDNAs corresponding to each of the ten Pipe protein isoforms were expressed in ovarian follicle cells of *pipe*^{3}/+ and *pipe*^{1}/*pipe*^{3} females under the control of the *CY2-Gal4* and *T155-Gal4* enhancer-trap drivers. Results of the expression of Box 10 (Pipe-ST2) (**C**,**D**) and Box 4 (**E**,**F**) are shown versus no-cDNA controls (**A**,**B**). cDNAs corresponding to Boxes 1-3 and Boxes 5-9 provided identical results to those observed for Box 4. The arrows indicate ventral denticles. Arrowheads indicate Filzkörper material. Maternal genotypes are shown at the bottom of each panel.

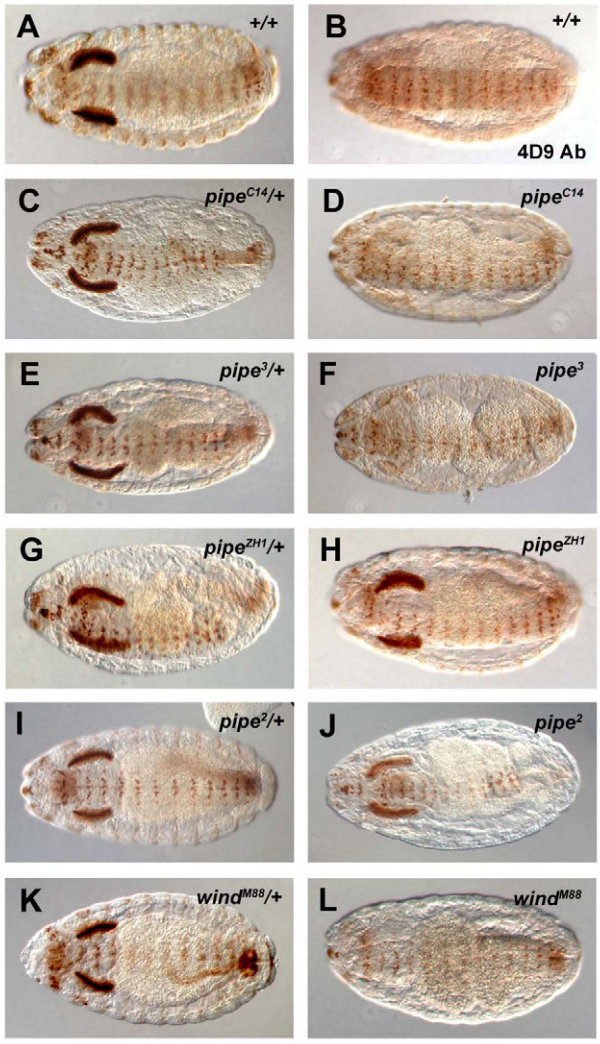

Fig. 3. *pipe* and *wind* are required for the formation of the En* epitope. *Drosophila* embryos derived from wild-type females and from females homozygous (**D**,**F**,**H**,**J**) or heterozygous (**C**,**E**,**G**,**I**) for various *pipe* alleles (maternal genotypes labeled at top right of each panel) stained with the polyclonal anti-En* antiserum. Anti-En*-stained embryos from females homozygous (**L**) or heterozygous (**K**) for *wind*^{M88}. Wild-type embryos stained with the anti-En* antiserum (**A**) or anti-Engrailed monoclonal antibody 4D9 (**B**).

third exon and leads to the formation of a truncated protein of 94 amino acids by all mRNAs. No mutations associated with exons specific to any of the other Pipe protein isoforms have been identified. The detection of multiple *pipe* mRNA isoforms in the ovary led us to revisit the question of whether any of the other isoforms participate in embryonic DV patterning. Overexpression of other isoforms that participate in DV patterning might be expected to lead to the production of ventralized embryos. Similarly, isoforms that are not normally expressed in the ovary, but which have the same enzymatic activity as Pipe-ST2, should to be able to functionally substitute for Pipe-ST2. To test these possibilities we individually expressed cDNAs corresponding to each of the Pipe isoforms throughout the follicle cell layer of *pipe*^{3}/+ and *pipe*^{1}/*pipe*^{3} females, under the control of the follicle cell-specific Gal4 enhancer-trap elements *CY2-Gal4* and *T155-Gal4* (Queenan et al., 1997), respectively. As negative controls, *CY2-Gal4*/+; *pipe*^{3}/+ females produced normal embryos (Fig. 2A), whereas embryos from *T155-Gal4 pipe*^{1}/*pipe*^{3} females were completely dorsalized (Fig. 2B). Whereas expression of the Pipe-ST2 isoform was capable of directing the formation of ventralized embryos by *pipe*^{3}/+ females (Fig. 2C), none of the other isoforms was capable of doing so (Fig. 2E). Similarly, whereas expression of Pipe-ST2 led to the formation of ventral pattern elements in embryos from *pipe*^{1}/*pipe*^{3} females (Fig. 2D), the progeny of *pipe*^{1}/*pipe*^{3} females expressing any of the other isoforms were dorsalized (Fig. 2F). Thus, despite the expression of multiple *pipe* mRNA isoforms in the wild-type ovary, only Pipe-ST2 is associated with an activity that is necessary and sufficient for the production of ventral and lateral pattern elements along the embryonic DV axis.

An epitope present in embryonic salivary glands is dependent on *pipe* gene expression

Myat and Andrew (Myat and Andrew, 2002) described an embryonic salivary gland luminal epitope that is detected by an antiserum (raised in the laboratory of Dr Patrick O'Farrell) against *Drosophila* Engrailed protein. In addition to the conventional segmental staining pattern exhibited by Engrailed, this polyclonal antiserum detects the salivary gland antigen, which we refer to as En* (Fig. 3A). This antigen does not correspond to Engrailed protein, as the anti-Engrailed monoclonal antibody 4D9 (Patel et al., 1989) does not stain embryonic salivary glands (Fig. 3B). We tested for the presence of En* staining in embryos homozygous for several *pipe* alleles. The En* antibody did not detect the salivary gland antigen in embryos homozygous for either *pipe*^{C14} or *pipe*^{3} (Fig. 3D,F), both of which lead to the elimination of all Pipe protein isoforms. By contrast, En* antigen was detected in embryos

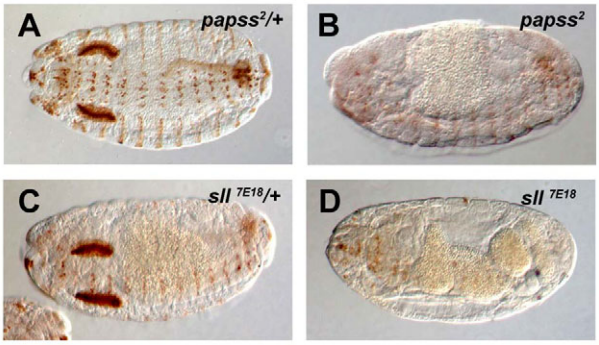

Fig. 4. En* epitope formation requires the activity of genes involved in sulfation. *Drosophila* embryos homozygous for either *Papss*^{2} (**B**) or *sll*^{7E18} (**D**) failed to stain for the En* epitope, whereas their corresponding heterozygous siblings were stained (**A**,**C**).

homozygous for the alleles *pipe*^{ZH1} and *pipe*^{2} (Fig. 3H,J). *pipe*^{ZH1} is a deletion mutant that eliminates the Box 9 and Box 10/*pipe-ST2*-specific exons as well as part of the Box 8-specific region, but leaves intact the exons specific to Box 1 through 7. *pipe*^{2} is a mutation that interferes with the correct splicing of the mRNA encoding Pipe-ST2. These findings indicate that the presence of the En* epitope in the embryonic salivary gland is dependent on the expression of the *pipe* gene and that Pipe isoforms distinct from Pipe-ST2 are capable of supporting its production.

Embryos homozygous for mutations affecting the *windbeutel* (*wind*; *wbl*) gene also lacked En* staining (Fig. 3L). *wind* encodes a homolog of the vertebrate endoplasmic reticulum protein Erp29 (Konsolaki and Schupbach, 1998), and we have previously shown that Wind protein is required for the correct subcellular localization and function of Pipe-ST2 and probably of all other Pipe isoforms as well (Sen et al., 2000; Zhu et al., 2005).

To confirm that the En* epitope represents a sulfated species, we stained for its presence in the salivary glands of embryos homozygous for mutations in *Paps synthetase* (*Papss*) (Jullien et al., 1997; Zhu et al., 2005), which generates the universal sulfate donor PAPS, and in *slalom* (*sll*), which encodes the PAPS Golgi transporter (Kamiyama et al., 2003; Luders et al., 2003). Embryos homozygous for mutations in either of these genes lacked En* staining (Fig. 4B,D). Collectively, our results are consistent with the premise that the En* epitope is sulfated in the Golgi of embryonic salivary gland cells by one or more of the Pipe protein isoforms, but that Pipe-ST2 is dispensable for its formation. The En* epitope exhibits characteristics similar to those of sulfated material present in the lumen of embryonic salivary glands that we have previously detected using the histochemical stain Alcian Blue (Zhu et al., 2005).

Generation of the En* epitope reveals differences in functional specificity among the different Pipe protein isoforms

Determining whether individual Pipe protein isoforms are capable of producing the En* epitope provides another way of distinguishing their functional specificities. To test this, we used the embryonic salivary gland-specific Gal4 enhancer-trap line *fkh-Gal4* (Henderson and Andrew, 2000) to direct expression of each Pipe isoform individually in the salivary glands of *pipe*^{3}/*pipe*^{C14} mutant embryos and stained for the presence of En*. *pipe*^{3}/*pipe*^{C14} mutant embryos do not produce functional versions of any of the Pipe isoforms and lack En* antigen (Fig. 5B). However, the three Pipe isoforms Box 2

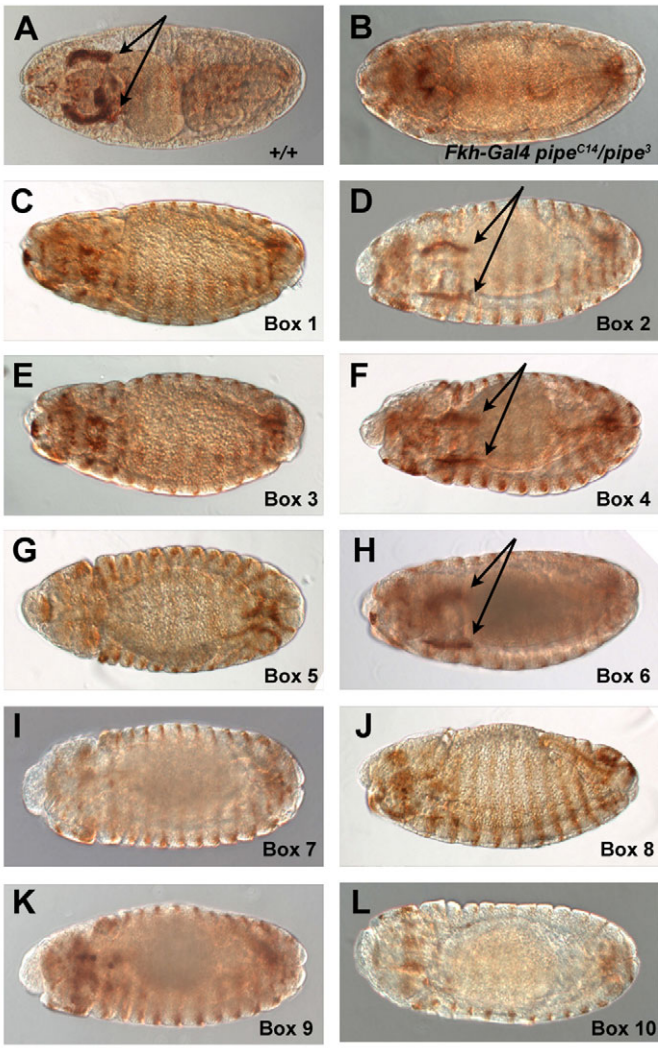

Fig. 5. Pipe protein isoforms Box 2, 4 and 6 can mediate the formation of the En* epitope. *Drosophila* embryos individually expressing each of the Pipe protein isoforms (**C-L**, as listed bottom right in each panel) in salivary glands were immunostained using the anti-En* antiserum. The presence of the En* epitope is indicated by arrows. Staining in wild-type (**A**) and negative control (**B**) embryos is shown for comparison.

(Fig. 5D), Box 4 (Fig. 5F) and Box 6 (Fig. 5H), individually supported the production of the En* epitope. Pipe-ST2, by contrast, was not capable of producing the En* epitope (Fig. 5L).

We also tested whether the Pipe isoforms were capable of individually restoring Alcian Blue staining to otherwise *pipe*-null mutant embryos. None of the Pipe protein isoforms was capable of doing so (data not shown). Based on this result, we suspect that Alcian Blue staining requires wild-type levels of *pipe* expression owing to the low sensitivity of the stain. Presumably, the En* antibody can detect its sulfated target at concentrations that are too low for detection by Alcian Blue.

Based on these results, the various Pipe protein isoforms can be distinguished into three functional classes. Pipe-ST2 (Box 10) expression in follicle cells can rescue the *pipe* mutant phenotype and mediate the formation of lateralized/ventralized embryos, but its expression in embryonic salivary glands cannot generate the En*

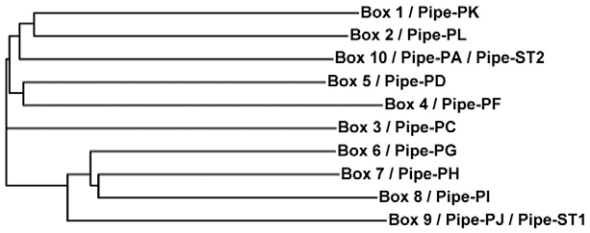

Fig. 6. Phylogenetic relationship between the Pipe protein isoforms. Amino acids corresponding to each of the ten *D. melanogaster* Pipe protein isoforms, excluding the first three common exons, were aligned using ClustalW from EMBL-EBI (www.ebi.ac.uk) (Larkin et al., 2007). Lengths of lines are proportional to the inferred evolutionary distance.

epitope. Boxes 2, 4 and 6 cannot direct the formation of ventral or lateral pattern elements in embryos, but they can facilitate the formation of the En* epitope. Boxes 1, 3, 5, 7, 8 and 9 can neither ventralize/lateralize embryos nor produce the En* epitope (Fig. 5C,E,G,I-K). Interestingly, despite the fact that Boxes 2, 4 and 6 share the ability to generate the En* epitope, they do not represent members of a phylogenetically related subgroup among the Pipe protein isoforms (Fig. 6).

Our suggestion that the En* antigen represents a specific structural component of the Alcian Blue-staining material is supported by tests for its presence in a number of other species of *Drosophila*, all of which are predicted to express multiple Pipe protein isoforms based on an examination of genomic sequence. We found that Alcian Blue-staining material was present in all of the additional *Drosophila* species that we tested (data not shown). By contrast, En* epitope was present in species of the *melanogaster* group (*D. melanogaster*, *D. simulans*, *D. sechellia*, *D. yakuba*, *D. erecta*, *D. ananassae*) (Fig. 7A-F), but was not present in the salivary glands of the more evolutionarily distant species *D. pseudoobscura*, *D. willistoni*, *D. virilis* and *D. immigrans* (Fig. 7G-J). The most parsimonious explanation for these findings is that the Alcian Blue-staining material represents a heterogeneous population of epitopes, generated through the action of multiple Pipe isoforms, which is required for the correct development or function of the embryonic salivary gland. En* represents a specific structural component of that material. Differences in the structure of the Alcian Blue-staining material generated over the course of evolution of the genus *Drosophila* have led to the presence of the En* antigen in species of the *melanogaster* group and its absence from more distantly related species.

A second polarizing input influences the formation of the embryonic DV axis

The ventrally restricted expression of *pipe* in the follicle cell layer, and the ability of ectopic expression of Pipe-ST2 in dorsal follicle cells to invert the polarity of progeny embryos, led to the initial assumption that ventral expression of Pipe-ST2 in the follicular epithelium is the primary, and perhaps only, determinant of embryonic DV polarity (Sen et al., 1998). However, when the Gal4/UAS system was used to direct uniformly high levels of Pipe-ST2 expression throughout the follicular epithelium of $pipe^{1}/pipe^{2}$ mutant females, the progeny embryos exhibited some residual polarity that could be detected in the pattern of gastrulation movements (D.S., unpublished). If the ventral transcription of Pipe-ST*2* in the follicle cell layer were the only polarizing factor

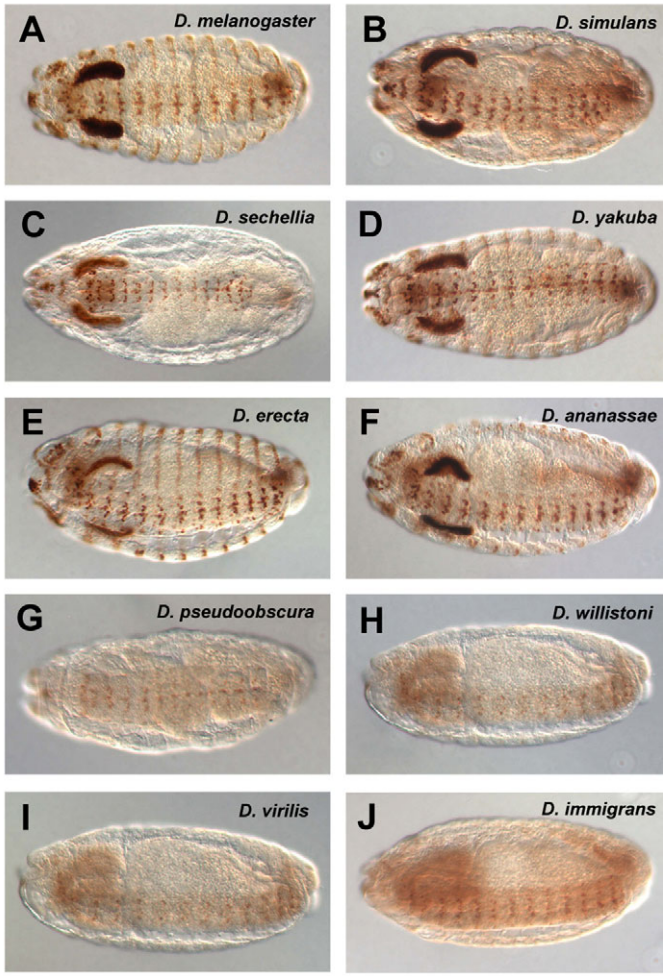

Fig. 7. Members of the '*melanogaster* group' of *Drosophila* species express the En* antigen. (**A-J**) Whole-mount immunostaining of embryos from various *Drosophila* species using the anti-En* antiserum.

influencing the embryonic DV axis, then the progeny of females experiencing uniform follicle cell expression of Pipe-ST2 would be expected to exhibit apolar gastrulation movements. These results suggest the existence of a second polarizing input, the influence of which can be observed when Pipe-ST2 expression in the follicle cell layer is uniform.

The $pipe^{1}$ and $pipe^{2}$ mutant lesions specifically affect the Pipe-ST2 isoform (Zhu et al., 2005). The polarity observed when Pipe-ST2 is expressed uniformly in this mutant background could result from residual Pipe-ST2 enzymatic activity associated with the expression of one of these alleles in ventral follicle cells. Alternatively, other Pipe protein isoforms that are expressed ventrally in the $pipe^{1}/pipe^{2}$ mutant background could partially circumvent the effects of uniform Pipe-ST2 expression. Although the results reported above indicate that only Pipe-ST2 is essential for the production of lateral and ventral embryonic pattern elements, those results do not rule out the possibility that other Pipe isoforms influence the amount, subcellular distribution or activity of Pipe-ST2. If any of those mechanisms do operate, uniform expression of Pipe-ST2 in females otherwise completely lacking *pipe* mRNA or protein would lead to the formation of apolar lateralized or ventralized embryos. By contrast, the production by these females of embryos with residual polarity would

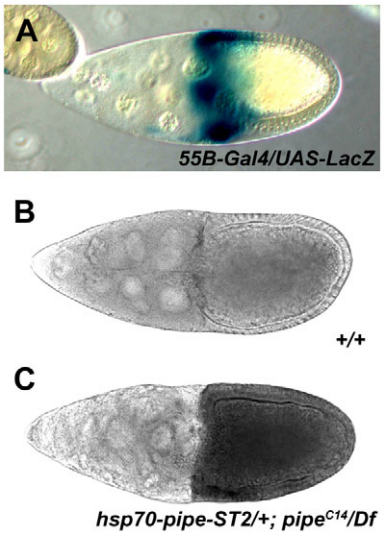

Fig. 8. Uniform expression of the *55B-Gal4* enhancer trap and of *hsp70* promoter-driven Pipe-ST2 along the DV axis of the follicular epithelium. (**A**) *55B-Gal4*-driven expression of UAS-*lacZ* in the stage 10 egg chamber as detected by X-Gal cleavage (blue). (**B**,**C**) Egg chambers dissected from heat-shocked wild-type (B) and *hsp70-pipe-ST2/+*; $pipe^{C14}/Df(3L)pipe^{A13}$ females 2-3 hours after heat shock (C) stained with an antiserum that detects all Pipe protein isoforms (Zhu et al., 2005).

be evidence of the existence of another factor that, in addition to ventral transcription of the *pipe* gene, contributes to the asymmetry of the DV signal transduction pathway.

To distinguish between the possibilities outlined above, we expressed Pipe-ST2 uniformly along the DV circumference of the follicle cell layers in egg chambers from several types of *pipe* mutant females and systematically examined the phenotypes of the progeny embryos, focusing on the extent of rescue of the lateral and ventral pattern elements, and on whether the rescued embryos exhibited appropriately polarized gastrulation movements. We expressed Pipe-ST2 either under the control of the heat-inducible *hsp70* promoter or under the control of the *55B-Gal4* enhancer-trap insertion, which expresses Gal4 in a band of follicle cells surrounding the anterior end of the developing oocyte (Goentoro et al., 2006; Mantrova and Hsu, 1998) (Fig. 8A). Although the anti-Pipe antibody that we have generated is not sufficiently sensitive to detect endogenous Pipe expression in wild-type ovaries (Fig. 8B) or Pipe-ST2 expressed under the control of *55B-Gal4* (data not shown), we were able to confirm that *hsp70* promoter-mediated expression of Pipe-ST2 in otherwise *pipe*-null mutant [$pipe^{C14}/Df(3L)pipe^{A13}$] females leads to high and apparently homogeneous levels of protein expression throughout the follicle cell layer of heat-shocked females (Fig. 8C).

The following *pipe* mutations were used in testing the ability of Pipe-ST2 to restore DV pattern elements: $pipe^{1}$ (V123D, a missense mutation affecting Pipe-ST2), $pipe^{9}$ (R123Stop, affecting Pipe-ST2), $pipe^{ZH1}$ (a deletion that eliminates Pipe-ST2, Box 9 and a portion of Box 8), $pipe^{3}$ (Q95Stop, affecting all Pipe isoforms), $pipe^{C14}$ (*pipe* RNA-null mutation) and $Df(3L)pipe^{A13}$ (a large deficiency that uncovers the *pipe* locus).

Following *hsp70*- or *55B-Gal4*-directed expression of Pipe-ST2, the patterns of gastrulation movements and the cuticles of progeny embryos were examined. The classification of embryonic cuticular phenotypes was carried out according to Roth et al. (Roth et al., 1991) with some modifications. Depending on the severity of the phenotype, dorsalized embryos were classified into one of four groups: completely dorsalized (D0, dorsal epidermis and amnioserosa only), strongly dorsalized (D1, Filzkörper or Filzkörper material present, but lacking ventral denticles), moderately dorsalized (D2, Filzkörper and ventral denticle bands of narrowed width present) and weakly dorsalized (D3, twisted or tail-up phenotype or head defects observed). Ventralized (V) embryos were classified into two groups: weakly ventralized (V1, ventral denticle bands of normal or slightly larger than normal width present, together with a reduction or loss of Filzkörper material) and strongly ventralized (V2, exhibit an expansion of non-cuticular tissue that corresponds to mesoderm, together with patches of ventral denticles compressed to one side of the embryo). Based on the presence of pattern elements derived from different regions of the DV circumference, the D1, D2, D3, V1 and V2 embryos were polar. In addition to D0 embryos, two classes of lateralized embryos exhibited apolar phenotypes in which Dorsal group signaling appeared to have occurred uniformly all around the DV circumference of the embryos. L1 embryos were encircled by bands of small denticles, like those found laterally in wild-type larvae, and L2 embryos were encircled by bands of larger denticles, like those produced ventrally by wild-type larvae.

Expression of Pipe-ST2 under the control of *hsp70* or *55B-Gal4* led to the production of embryos that exhibited a range of DV phenotypes in each of the *pipe* mutant backgrounds tested (Table 1; data not shown). The variation in the extent of phenotypic rescue observed upon expression of Pipe-ST2 in the various *pipe* mutant backgrounds precludes useful deductions as to how the presence of the ventrally expressed endogenous *pipe* transcript, or of the other Pipe isoforms, might influence the action of the uniformly expressed Pipe-ST2. However, the ability of Pipe-ST2 expressed uniformly in $pipe^{C14}/Df(3L)pipe^{A13}$ RNA/protein-null egg chambers to direct the formation of normally polarized embryos, as assessed by cuticular pattern elements (Fig. 9G,J), gastrulation movements (Fig. 9H,K), or expression of the ventral marker Twist (Fig. 9I), provides strong evidence that another polarizing input, in addition to the ventral transcription of the *pipe* gene, contributes to the formation of the embryonic DV axis.

The restriction of Pipe-ST2 to ventral cells of the follicle cell layer in wild-type egg chambers is mediated by the repression of *pipe* transcription in dorsal follicle cells as a result of activation of the receptor tyrosine kinase Torpedo (Egfr – FlyBase) (Sen et al., 1998). In order to determine whether the second polarizing input is, like *pipe* transcription, controlled by signaling through the Torpedo receptor, we simultaneously expressed both λtop (Queenan et al., 1997), a constitutively active version of Torpedo, and Pipe-ST2 throughout the follicle cell layer, under the control of the *CY2-Gal4* enhancer-trap line. The majority of embryos produced by $pipe^{ZH1}/pipe^{C14}$ females expressing Pipe-ST2 under the control of *CY2-Gal4* exhibited a polar ventralized phenotype (V2) in which there was a massive expansion of mesoderm as well as a small region of cuticular material bearing ventral denticles that was often pushed to a position adjacent to the dorsal side of the egg (Fig. 9L). By contrast, expression of λtop under the control of *CY2-Gal4* led to the formation of dorsalized (D0) progeny contained within eggshells that were also dorsalized (Fig. 9M). When the *CY2-Gal4* driver was used to direct uniform transgenic expression of Pipe-ST2 in follicle cell layers in which Torpedo signaling was also uniformly active, the resultant progeny embryos, also contained within dorsalized eggshells, produced cuticle that was encircled by faint bands of lateral denticles (L1) (Fig. 9N). Thus, the constitutive activation of Torpedo had two effects upon the consequences of uniformly expressed Pipe-ST2 that were not the

Table 1. Cuticular phenotypes of embryos produced 18 to 30 hours after heat shock of adult females carrying *hsp70-pipe-ST2* in various *pipe* mutant backgrounds

Maternal *pipe* mutant background expressing Pipe-ST2	Proportion [*n* (%)] of embryos exhibiting denoted DV phenotypes*							
	D0	D1	D2	D3	V1	V2	L1	L2
$pipe^{1}/Df(3L)pipe^{A13}$ (*n*=138)	3 (2)	14 (10)	40 (29)	14 (10)	19 (14)	33 (24)	11 (8)	4 (3)
$pipe^{9}/Df(3L)pipe^{A13}$ (*n*=131)	13 (10)	12 (9)	26 (20)	34 (26)	9 (7)	11 (8)	14 (11)	12 (9)
$pipe^{ZH1}/Df(3L)pipe^{A13}$ (*n*=125)	23 (18)	9 (7)	24 (19)	42 (34)	6 (5)	6 (5)	12 (10)	3 (2)
$pipe^{3}/Df(3L)pipe^{A13}$ (*n*=63)	3 (5)	16 (25)	12 (19)	19 (30)	0	5 (8)	5 (8)	3 (5)
$pipe^{C14}/Df(3L)pipe^{A13}$ (*n*=89)	42 (47)	24 (27)	10 (11)	0	1 (1)	1 (1)	9 (10)	2 (2)

*Embryonic phenotypes are scored according to Roth et al. (Roth et al., 1991), with modifications as described in the text.

result of a decrease in *pipe* transcription. First, constitutive Torpedo activation led to a decrease in the ventral character of the most ventral pattern elements produced, leading to the formation of lateral epidermis rather than mesoderm. Second, it led to the loss of polarity. From these results we can conclude that the second polarizing input that we have observed depends upon the pre-existing polarity imposed by the Gurken/Torpedo signaling cassette and that Torpedo signaling in the dorsal follicle cells of wild-type egg chambers has a dorsalizing effect that is distinct from its repressive effects upon the transcription of the *pipe* gene.

DISCUSSION

Distinct functional specificities are associated with various Pipe protein isoforms

pipe is a highly conserved gene in the insects. In insects other than the cyclorrhaphan (true) flies, only a single Pipe isoform is normally present. Moreover, the individual Pipe isoforms that are predicted to be expressed in mosquito (*Anopheles gambiae*), silkworm (*Bombyx mori*), honeybee (*Apis mellifera*) and flour beetle (*Tribolium casteneum*) exhibit greatest sequence similarity to the Pipe-ST2 isoform, among the multiple isoforms expressed by *Drosophila melanogaster*. By contrast, the genomes of all *Drosophila* species that have been sequenced (*D. ananassae*, *D. erecta*, *D. grimshawi*, *D. mojavensis*, *D. persimilis*, *D. pseudoobscura*, *D. sechellia*, *D. simulans*, *D. virilis*, *D. willistoni* and *D. yakuba*) predict multiple Pipe protein isoforms. Taken together, these observations suggest that an insect ancestral to the holometabolous Diptera, Coleoptera, Hymenoptera and Leptidoptera expressed a single Pipe isoform, the role of which was to regulate the formation of the embryonic DV axis. Pipe protein may also regulate DV axis formation in more basal genera of insects and in other arthropods, as the genome of the water flea (*Daphnia pulex*), a crustacean, apparently encodes an orthologous protein that is more similar to Pipe-ST2 than to HS2ST or D/C2ST (http:/wfleabase.org).

The existence of multiple protein isoforms in the genomes of the various *Drosophila* species indicates that several duplications of *pipe* exons have occurred during the evolution of the members of the dipteran suborder Brachycera, perhaps in the line leading to the family Drosophilidae. Some of the isoforms might have arisen as a means of modulating the effects of the essential Pipe-ST2 isoform during oogenesis, perhaps by modifying the carbohydrate target of Pipe-ST2 to make it a more avid substrate. This pre-modification of the Pipe target would not be essential for Pipe-ST2 action, but would make the developmental process more robust and reproducible from egg chamber to egg chamber, an adaptation to the rapid oogenesis in the Drosophilid species. Alternatively, the multiple Pipe protein isoforms in the Drosophilids might have arisen to provide a function that is totally unrelated to embryonic DV patterning. We have previously shown that the absence of expression of all isoforms of Pipe leads to the formation of structurally aberrant salivary glands and to a decrease in fly viability and growth (Zhu et al., 2005). Although these effects are correlative, it is reasonable to assume that the decrease in viability and growth results from an alteration in feeding behavior that is attributable to a perturbation of the development or function of the salivary gland owing to the absence of all Pipe catalytic activity. As such, the presence of multiple isoforms of the Pipe protein in the Drosophilids might represent an adaptation that is specifically related to the feeding behavior and physiology of those species. Ultimately, an understanding of the basis for the multiple Pipe proteins and their functions will require a precise determination of the distinct catalytic activities of the various proteins, a goal that will first require the identification of the class of molecule(s) on which these enzymes act.

A second signal, in addition to the ventrally restricted transcription of *pipe*, influences the polarity of the embryonic DV axis

The production of appropriately polarized, partially rescued embryos by females expressing Pipe-ST2 in an otherwise *pipe*-null background strongly suggests the existence of a second signal that can impart polarity, even in a situation in which Pipe-ST2 activity is not ventrally restricted. Carneiro et al. (Carneiro et al., 2006) have proposed a model in which maternally directed activation of the Decapentaplegic (Dpp) pathway facilitates degradation of the Cactus protein on the dorsal side of embryos in a Toll-independent manner, thus representing a second mechanism for polarizing the DV axis of the embryo. Although this pathway could, in principle, correspond to the second polarizing input, we do not favor this explanation. The findings of Carneiro et al. (Carneiro et al., 2006) are difficult to reconcile with the existence of mutant gain-of-function alleles of *easter* and *Toll* that cause uniform activation of Toll around the DV circumference of the embryo, and the consequent production of apolar lateralized or ventralized embryos. The production of such apolar embryos would not be expected were the Dpp pathway acting independently of Toll signaling to establish embryonic DV axis formation. Based on the existence of these unusual apolar alleles of *easter* and *Toll*, we favor

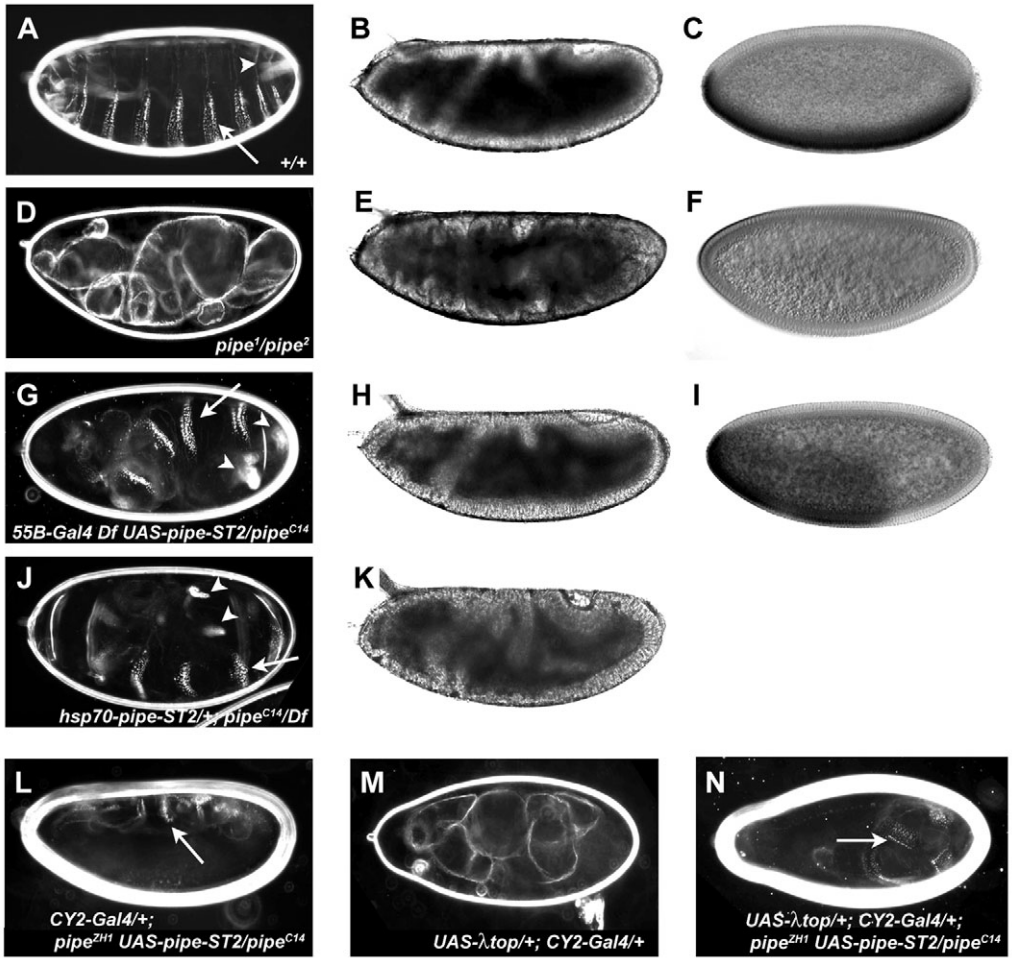

Fig. 9. Residual DV polarity in embryos from females expressing Pipe-ST2 uniformly around the DV circumference of the follicular epithelium. (**A-N**) Larval cuticles (A,D,G,J,L,M,N), embryonic gastrulation movements (B,E,H,K) and Twist expression patterns (C,F,I) of progeny from wild-type (A-C), *pipe*^{1}*/pipe*^{2} (D-F), *55B-Gal4 Df(3L)pipe*^{A13} *UAS-pipe-ST2; pipe*^{C14} (G-I) and heat-shocked *hsp70-pipe-ST2/+; pipe*^{C14}*/Df(3L)pipe*^{A13} females (J,K). Arrows indicate ventral denticles and arrowheads indicate Filzkörper material. Note the presence of both ventral and lateral pattern elements (G,J), the appropriately polarized gastrulation movements (H,K) and Twist expression pattern in otherwise *pipe*-null mutant females expressing Pipe-ST2 uniformly under the control of the *55B-Gal4* enhancer-trap insertion (G-I) or the *hsp70* promoter (J,K). (L,M,N) Cuticles produced by females expressing either Pipe-ST2 (L, maternal genotype *CY2-Gal4/+; pipe*^{ZH1} UAS-*pipe-ST2/pipe*^{C14}), λtop (M, maternal genotype UAS-λtop/+; *CY2-Gal4/+*) or both (N, maternal genotype UAS-λtop/+; *CY2-Gal4/+; pipe*^{ZH1} UAS-*pipe-ST2/pipe*^{C14}) throughout the follicle cell layer under the control of the strong *CY2-Gal4* enhancer-trap element. Note that in L and N, no Pipe-ST2 is expressed from the endogenous *pipe* locus.

a model in which the *pipe*-independent signal that we have observed contributes to the polar activation at a step upstream of Easter-mediated cleavage of Spätzle.

We propose two possible explanations for the second polarizing influence. According to the current model, Pipe protein action in ventral follicle cells leads to the sulfation of a secreted glycoprotein that is deposited ventrally in the egg, where it mediates the activation of the dorsal group serine protease cascade. If any of the genes that encode proteins involved in the synthesis of the direct carbohydrate target of Pipe or its predicted carrier protein were expressed preferentially in ventral follicle cells, then the ventrally enriched production of the Pipe substrate could account for the residual polarity of progeny embryos under conditions of uniform Pipe-ST2 activity. The ability of Pipe-ST2 to invert the polarity of progeny embryos when expressed ectopically in dorsal follicle cells would result from the ability of Pipe-mediated sulfation to overcome the polarizing effects of ventral enrichment of the Pipe target.

An alternative basis for residual polarity in the presence of uniform Pipe-ST2 activity might be the existence of an inhibitor of Gd or Snake that is enriched dorsally within the perivitelline space, or of a ventrally enriched, positively acting regulator of the processing or activity of Gd, Snake or Easter, distinct from Pipe-ST2. Although a serpin inhibitor of Easter, Spn27A, has been shown to operate in the perivitelline space, this molecule is uniformly distributed (Hashimoto et al., 2003; Ligoxygakis et al., 2003). Moreover, available data suggest that Spn27A acts to restrict Easter activity to the ventral side of the embryo following its activation, and does not have any intrinsic ability to orient the activity of Easter.

The geometry of egg formation might also introduce an asymmetry that could influence the effects of Pipe-ST2 along the embryonic DV axis. Over the course of oogenesis, the egg chamber and egg assume characteristic shapes that are polarized along the DV axis. The morphogenetic processes that govern egg formation and the physical properties of the mature egg might influence the

distribution of the modified target of Pipe or the spatial constraints of serine protease activation and in this way impart a weakly polarizing influence that is detectable when *pipe* expression is uniform in the developing egg chamber.

The extent to which this second polarizing input is required for the formation of embryonic DV polarity in wild-type embryos is difficult to assess. The second input cannot impart DV polarity to embryos in the absence of Pipe-ST2 activity. This suggests that the role of the second input might be to buffer the effects of rare perturbations in the normal ventral pattern of *pipe* expression. The power of this second input to influence the DV axis is demonstrated by our generation of viable stocks in which Pipe-ST2 is expressed uniformly along the DV axis under the control of *55B-Gal4* in otherwise *pipe*-null mutant backgrounds that lack endogenous ventral expression of Pipe-ST2.

In conclusion, our investigations of the functions of the various Pipe protein isoforms has demonstrated the existence of additional layers of complexity in the mechanism generating embryonic DV polarity, including the presence of a previously unappreciated mechanism that reinforces the effects of the ventral transcription of the *pipe* gene. The elucidation of the structure of the substrate of Pipe-ST2 and its function in regulating the dorsal group serine protease cascade is likely to provide insights into this alternate polarizing input and into the specific roles of the sulfotransferases encoded by the *pipe* locus.

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