biniou (*FoxF*), a central component in a regulatory network controlling visceral mesoderm development and midgut morphogenesis in *Drosophila*

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The subdivision of the lateral mesoderm into a visceral (splanchnic) and a somatic layer is a crucial event during early mesoderm development in both arthropod and vertebrate embryos. In *Drosophila*, this subdivision leads to the differential development of gut musculature versus body wall musculature. Here we report that *biniou*, the sole *Drosophila* representative of the FoxF subfamily of forkhead domain genes, has a key role in the development of the visceral mesoderm and the derived gut musculature. *biniou* expression is activated in the trunk visceral mesoderm primordia downstream of *dpp*, *tinman*, and *bagpipe* and is maintained in all types of developing gut muscles. We show that *biniou* activity is essential for maintaining the distinction between splanchnic and somatic mesoderm and for differentiation of the splanchnic mesoderm into midgut musculature. *biniou* is required not only for the activation of differentiation genes that are expressed ubiquitously in the trunk visceral mesoderm but also for the expression of *dpp* in parasegment 7, which governs proper midgut morphogenesis. Activation of *dpp* is mediated by specific Biniou binding sites in a *dpp* enhancer element, which suggests that Biniou serves as a tissue-specific cofactor of homeotic gene products in visceral mesoderm patterning. Based upon these and other data, we propose that the splanchnic mesoderm layers in *Drosophila* and vertebrate embryos are homologous structures whose development into gut musculature and other visceral organs is critically dependent on FoxF genes.

[*Key Words*: Visceral mesoderm; splanchnic mesoderm; midgut; mesoderm induction; forkhead/winged helix domain]

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The musculature of higher organisms consists of three major categories of muscle types: skeletal, cardiac, and visceral muscle. These types of muscle exhibit significant differences with respect to their developmental origins, ultrastructure, contractile properties, and physiological functions. Visceral muscles, which are the focus of the present study, are important components of many different internal organ systems, particularly the gastrointestinal and urogenital tracts, respiratory tract, and vascular system. In *Drosophila*, the visceral musculature is less diverse and primarily consists of the musculature of the digestive tract. Similar to vertebrates, the musculature of the *Drosophila* midgut is composed of an inner layer of circular muscles and an outer layer of longitudinal muscles (Campos-Ortega and Hartenstein 1997). Vertebrate and *Drosophila* visceral musculatures have additional similarities, including the features that the fibers are spindle-shaped and display slow, supercontracting properties. Although *Drosophila* visceral muscles are striated, their striation is atypical, and several of their ultrastructural features are reminiscent of vertebrate smooth muscles (Sandborn et al. 1967; Goldstein and Burdette 1971).

In both vertebrates and arthropods, the visceral mesoderm is largely derived from the lateral (splanchnic) mesoderm, which is under the inductive influence of BMP/Dpp signals (Holley and Ferguson 1997). Previous studies of *Drosophila* have shown that Dpp, which is secreted from the dorsal ectoderm, is critically required for the induction of visceral muscle in the underlying lateral (in insects dorsal) mesoderm (Frasch 1995). The mesodermal response to Dpp consists of the transcriptional activation of at least two important regulatory genes in the dorsal mesoderm, which are the NK homeobox genes *tinman* (*tin*) and *bagpipe* (*bap*) (Staehling-Hampton et al. 1994; Frasch 1995). *tin* is genetically required for the formation of all dorsal mesodermal derivatives (heart,

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dorsal body wall muscles, and midgut visceral muscles), whereas *bap* is required exclusively for the specification of the visceral (specifically the circular) muscles of the midgut and functions downstream and as a direct target of *tin* (Azpiazu and Frasch 1993; Bodmer 1993; Yin and Frasch 1998; H.-H. Lee and M. Frasch, unpubl.). *bap* is therefore the first example of a regulator that is uniquely involved in the specification of visceral muscles.

The functional differences between *tin* and *bap* are reflected in their patterns of expression in the dorsal mesoderm. *tin* is induced by Dpp in the entire dorsal mesoderm, whereas the induction of *bap* occurs in segmentally repeated clusters of cells within this area, which represent the primordia of the circular musculature of the midgut (Azpiazu and Frasch 1993). Subsequent cell shape changes lead to the anterior-posterior extension and coalescence of *bap*-expressing cell clusters into a continuous visceral mesoderm layer, the splanchnopleura, which ultimately surrounds the midgut to form the circular midgut musculature. *bap* is also expressed in primordial cells of the foregut and hindgut muscles, which form independently of Dpp near the anterior and posterior gut invagination sites, respectively. However, it is not expressed in longitudinal gut muscle precursors, which are specified in the caudal mesoderm, adjacent to the hindgut muscle progenitors. Longitudinal gut muscle formation requires anterior migration and dispersion of these cells along the entire midgut prior to their differentiation (Campos-Ortega and Hartenstein 1997).

Both *tin* and *bap* are expressed transiently in the dorsal and visceral mesoderm, and their protein products disappear upon the coalescence of the visceral mesoderm primordia. Because gut muscle morphogenesis and differentiation occur significantly later in development, it would appear that *tin* and *bap* are mainly required for visceral mesoderm specification and are not sufficient for the differentiation steps. It is likely that the combination of these two genes, or *bap* alone, activates the expression of downstream genes that are required to regulate various differentiation events. These events include the expression of adhesion/guidance molecules that direct the invagination of the anterior and posterior endoderm towards the center of the embryo, the subsequent migration and spreading of visceral mesoderm around the gut endoderm, the expression of contractile proteins, and the acquisition of differential properties along the anterior/posterior axis that determine the looping and overall morphogenesis of the midgut. This latter patterning process involves the spatially restricted expression of homeotic genes and signaling molecules, particularly Dpp and Wingless, within the developing visceral mesoderm (Bienz 1994).

In the present study, we describe a new gene, named *biniou* (*bin*), which functions as a critical regulator of visceral mesoderm development. *biniou* encodes a forkhead domain transcription factor of the FoxF subfamily and is expressed throughout embryonic development in all types of gut muscles and their progenitors. We show that the visceral mesoderm fails to differentiate in the absence of *biniou* activity and is partially transformed into somatic mesoderm. Conversely, ectopic expression of *biniou* in the somatic mesoderm results in the ectopic activation of visceral mesoderm markers within this layer. We further demonstrate that Biniou binds to a previously described enhancer of the *dpp* gene and is required in conjunction with the homeotic gene products Ubx and Exd to activate *dpp* expression in parasegment 7 of the visceral mesoderm, which in turn controls midgut morphogenesis.

Results

biniou *is the single* Drosophila *representative of the FoxF subfamily of forkhead domain genes*

biniou (*bin*) cDNA encodes a 676 amino acid protein which includes a forkhead (winged helix) domain in its central portion (Fig. 1; see Materials and Methods). Sequence comparisons show that Biniou (Bin) is the only fly representative of the FoxF subfamily of forkhead domain proteins and is equally related to FoxF1 and FoxF2. The sequence similarities between Bin and its vertebrate orthologs are confined to the forkhead domains (Fig. 1A).

The *bin* gene maps to 65DE on the third chromosome and consists of four exons (Fig. 1B). Among lethal mutations that were obtained in a saturation screen at this location (Anderson et al. 1995), we identified one complementation group with three EMS-induced alleles that correspond to *bin*. Sequencing of the *bin* loci from the respective mutant chromosomes confirmed that all three alleles, bin^{I1}, bin^{S4}, and bin^{R22}, carry mutations in the *bin* open reading frame (ORF). The mutations map to three different positions within the first exon and in each case introduce a transition from a CAG (Gln) to TAG (Stop) codon (Fig. 1B,C). Because the encoded polypeptides are truncated N-terminally to the forkhead domain and are expected to lack any DNA-binding activity, all three alleles are likely null alleles.

bin *is expressed in all types of visceral muscles and their progenitors*

Whole-mount stainings of stage 10 embryos with labeled *bin* cDNA probes and Bin antibodies revealed that *bin* is expressed in progenitors of all three types of visceral musculature (Fig. 2A,B). These include the 11 bilateral patches of circular midgut muscle progenitors in the dorsal mesoderm in parasegments (PS)2–12 (also known as trunk visceral mesoderm progenitors), the hindgut and foregut visceral mesoderm, as well as the caudal visceral mesoderm which is located just anteriorly to the hindgut visceral mesoderm (Fig. 2A). Double stainings of early embryos with Bin and Bap antibodies showed that the expression of Bin and Bap is activated almost simultaneously in the trunk visceral mesoderm progenitors (Fig. 2B, B′). However, at early stage 10, we occasionally detect cells that express Bap but not Bin (Fig. 2B′), indicating that *bap* expression initiates shortly before *bin*. We note that *bin* is expressed in an additional, smaller group

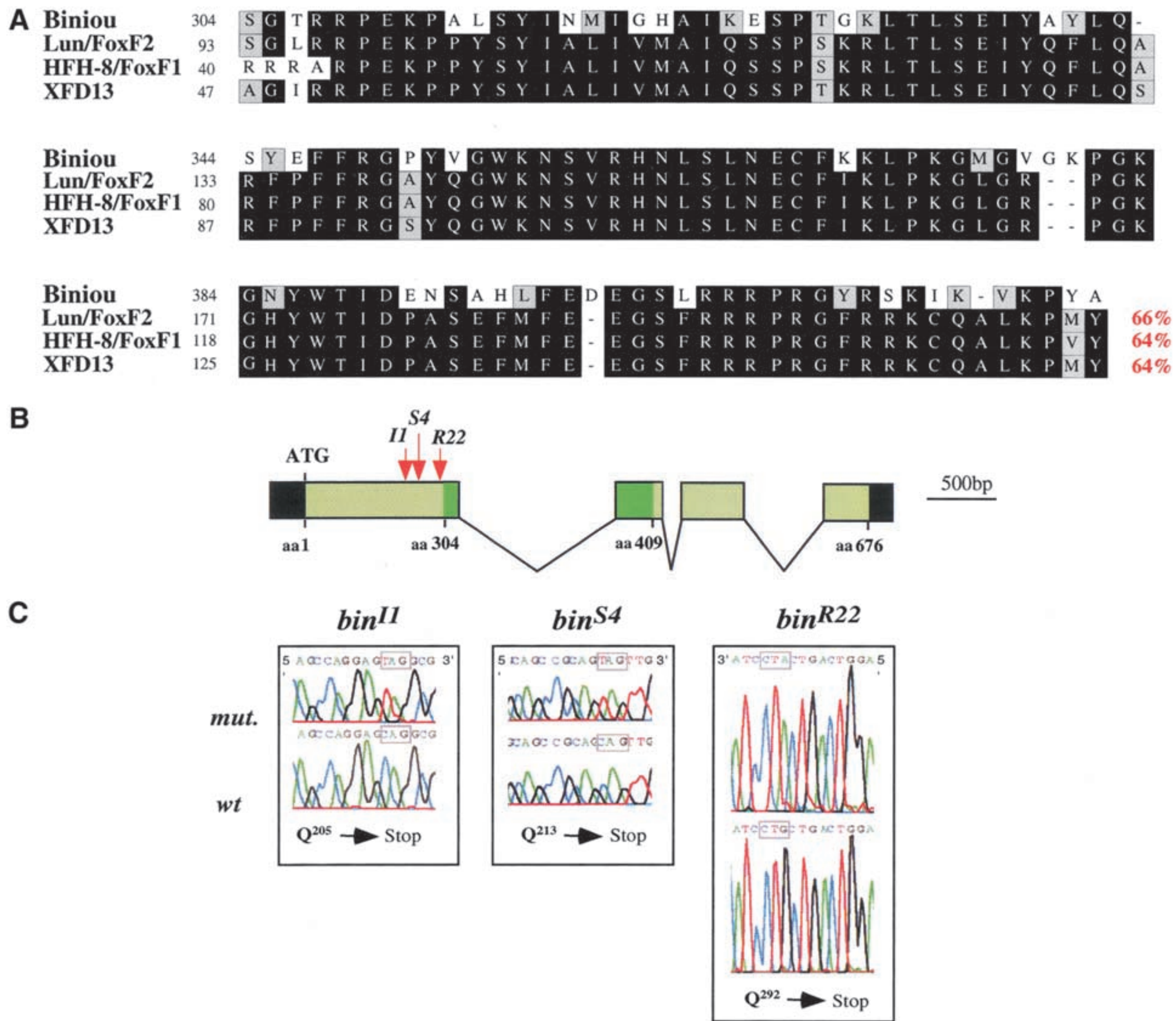

Figure 1. Molecular organization of the *biniou* locus and characterization of the mutant alleles. (*A*) Amino acid alignments of the forkhead domain of Biniou (GenBank AAK97051) with those of murine FoxF2 (Lun, amino acids 93–206; GenBank NM_010225), FoxF1 (HFH-8, amino acids 40–155; GenBank I49735), and *Xenopus* XFD13 (amino acids 47–161; GenBank CAB44732). Identical residues are boxed in black (see percent identity, in red), and conservative changes are shaded lightly. (*B*) Schematic representation of the genomic structure of the *bin* locus at 65D with intron–exon structure and ORF. Green and black boxes represent coding and noncoding sequences, respectively (dark green: forkhead domain). The locations of EMS mutations in *bin* (I1, S4, and R22 alleles) are indicated by arrows. (*C*) The sequence alterations in different *bin* alleles: bin^{I1}, CAG (Q^{205}) → TAG (stop); bin^{S4} CAG (Q^{213}) → TAG (stop); bin^{R22} CAG (Q^{292}) → TAG (stop).

of dorsal mesodermal cells in PS 1, which upon coalescence of the metameric cell clusters also becomes part of the trunk visceral mesoderm (Fig. 2A,C). An important difference between *bin* and *bap* is that after the coalescence and segregation of the trunk visceral mesoderm, *bap* expression becomes segmental and then disappears, whereas *bin* is maintained uniformly and at constant levels. The circular midgut muscle precursors, which at stage 14 form a dorsal row and a ventral row and then surround the midgut, as well as the foregut and hindgut muscles continue to express *bin* until late embryogenesis (Fig. 2D,E).

The early expression of *bin* in all visceral mesoderm derivatives allowed us to examine the dynamic morphogenetic changes and compare the gene activities in these developing tissues. Double stainings for Bin and Bap showed that, in contrast to the trunk visceral mesoderm, the hindgut visceral mesoderm expresses *bin* shortly before *bap*, and the caudal visceral mesoderm does not express Bap at any time (Fig. 2F–H). The caudal visceral mesoderm splits bilaterally and then each group of cells moves towards and onto the posteriormost patch of trunk visceral mesoderm progenitors (Fig. 2F–H). The nuclei of the caudal visceral mesoderm cells retain Bin

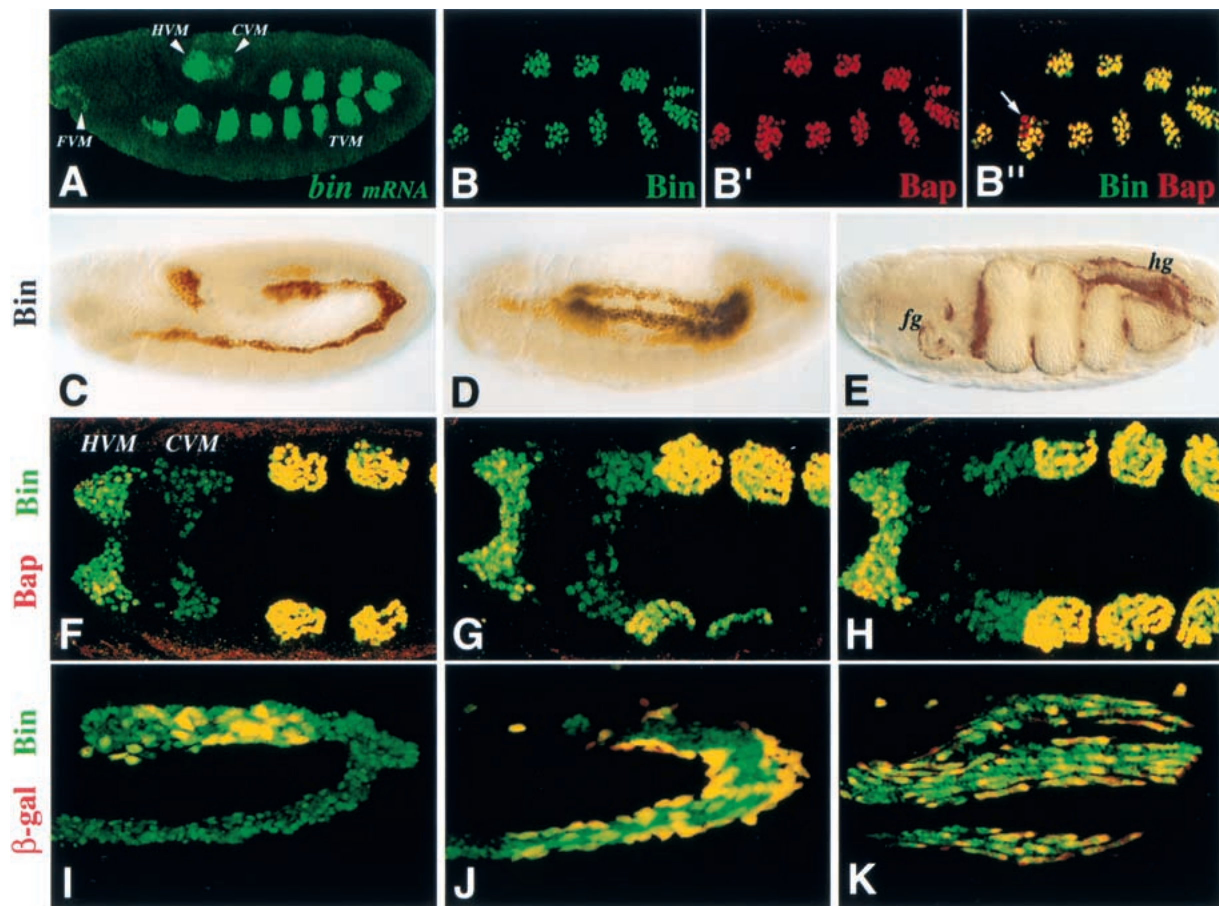

Figure 2. Expression of *biniou* in wild-type embryos and morphogenesis of caudal visceral mesoderm. Lateral (*A–E*,*I–K*) and dorsal (*F–H*) views of whole-mount embryos are shown. Embryo in *A* was hybridized with *bin* mRNA probe using TSA-FITC detection; embryos in *B–K* were stained with an anti-Bin antibody; embryos in *B′* and *F–H* were doubly labeled for Bin and Bap, and embryos in *I–K* for Bin and LM.m4-lacZ-driven β-gal (marks CVM; H.-H. Lee, unpubl.). (*A*) *bin* expression is initiated at stage 10 in 11 patches in the dorsal mesoderm (primordia of the circular midgut muscles), in the foregut and hindgut visceral mesoderm primordia and in the caudal visceral mesoderm (primordium of longitudinal visceral muscles). (*B–B′′*) Early stage 10 embryo showing colocalization of Bin protein (green in *B*) and Bap protein (red in *B′*). Merged image of *B* and *B′* (*B′′*) shows exclusive expression of Bap protein (red) in some cells (arrow). (*C*) Early stage 12 embryo with persistent Bin protein expression in the trunk and hindgut visceral mesoderm. (*D*) Stage 13 embryo; Bin is expressed in the circular as well as longitudinal precursors of midgut muscles. (*E*) Stage 16 embryo; Bin is expressed in foregut (fg), midgut and hindgut (hg) visceral muscles. (*F–H*) Colocalization (yellow signal) of Bap (red) and Bin (green) protein in dorsal mesoderm and hindgut mesoderm but not in the caudal visceral mesoderm (CVM). The three different caudal views illustrate the CVM movement relative to the other derivatives during stage 11. (*I–K*) High-magnification view of embryos at mid-stage 12, late-stage 12, and stage 15. Migrating longitudinal muscle precursors coexpress Bin and β-gal (yellow), whereas circular muscle precursors expresses only Bin (green). (CVM) caudal; (HVM) hindgut; (TVM) trunk visceral mesoderm.

during their migration and dispersion along the trunk visceral mesoderm until they differentiate into longitudinal midgut muscles (Fig. 2I–K).

bin *expression in the trunk visceral mesoderm primordia is controlled by* tin, bap, *and* dpp

Previous studies have shown that *bap*-expressing domains are defined by the intersecting dorsal activities of *dpp*/*tin*, which act positively, and segmentally modulated activities of *wg*/*slp*, which have repressing effects (Azpiazu and Frasch 1993; Staehling-Hampton et al. 1994; Frasch 1995; Azpiazu et al. 1996; Riechmann et al. 1997; Lee and Frasch 2000). As shown in Figure 3B (cf. Fig. 3A), *bin* also requires *tin* activity for normal expression in the trunk visceral mesoderm primordia. Whereas *bap* expression is virtually absent in these cells upon loss of *tin* activity (Azpiazu and Frasch 1993), residual *bin* expression is observed in small clusters of cells. To test the possibility that residual expression of *bin* in *tin* mutant embryos is due to direct inputs from Dpp, we examined *bin* expression in embryos in which *dpp* expression was induced ectopically in the entire mesoderm. Ectopic *dpp* in a wild-type background, which causes *tin* expression to be expanded ventrally (Frasch 1995), results in an analogous expansion of the *bin* domains (Fig.

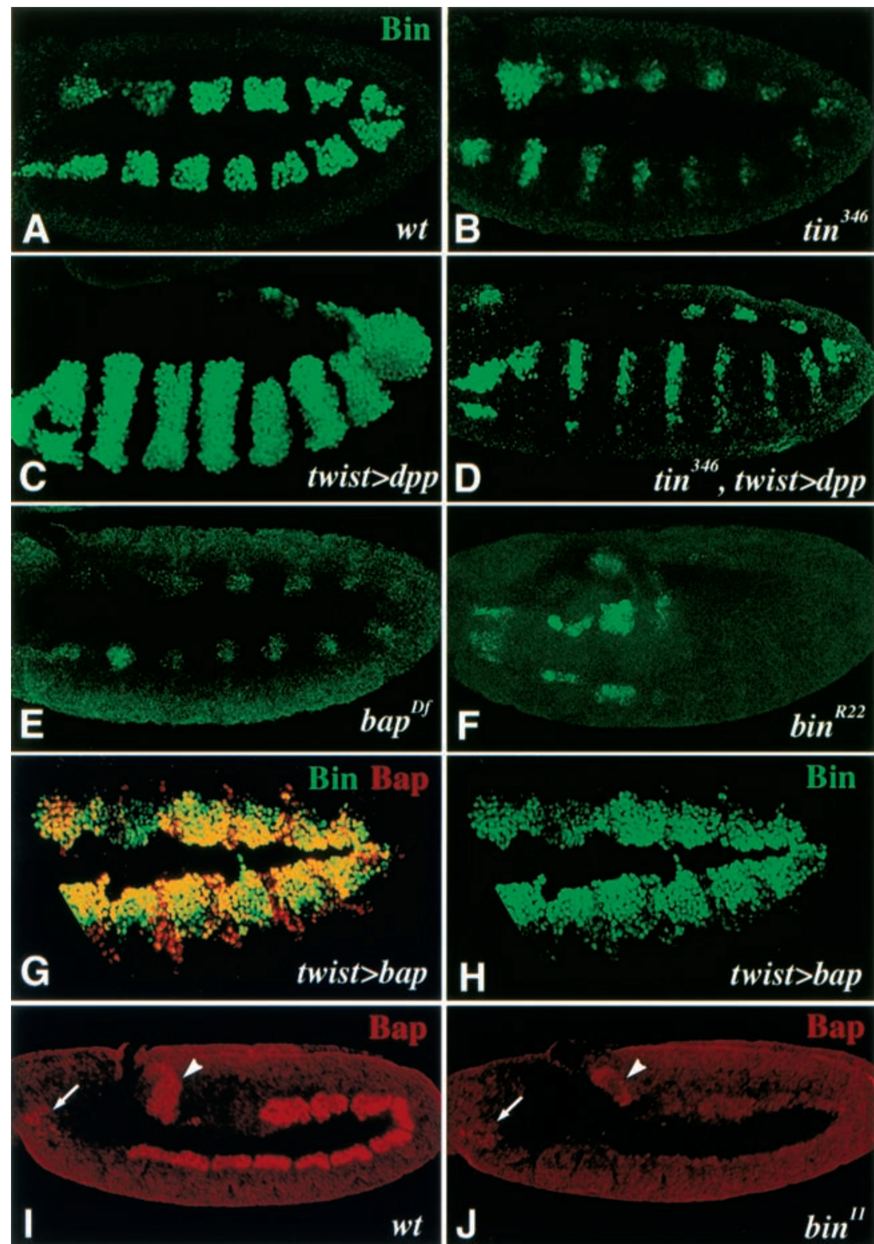

Figure 3. Regulation of *bin* and *bap* expression in the trunk visceral mesoderm (TVM) primordia. (*A*) Stage 10 wild-type embryo stained for Bin protein. (*B*) Stage 10 tin^{346} mutant embryo with strongly reduced Bin expression in the TVM primordia but normal expression in the hindgut visceral mesoderm. (*C*) Ventrolateral view of stage 10 embryo with ectopic mesodermal *dpp* expression (driven by *twist*–GAL4). The segmented Bin expression has expanded to the ventral midline (cf. *A*). (*D*) Ventrolateral view of stage 10 tin^{346} mutant embryo with ectopic mesodermal *dpp* expression. Stripes of Bin have expanded to the ventral domain but are much weaker than in a wild-type background (cf. *C*). (*E*) Stage 10 bap^{Df} mutant embryo with strongly reduced *bin* mRNA expression. (*F*) Ventrolateral view of stage 10 *bin* mutant embryo in which *bin* mRNA expression has disappeared in most of the TVM primordia, except for parasegments 1 and 2. (*G*,*H*) Stage 11 embryo with uniform *bap* expression driven by *twist*–GAL4 in mesoderm. Expansion of Bap expression (red) is accompanied by the expansion of Bin (green) (see *H*). (*I*) Stage 11 wild-type embryo stained for Bap protein (red). Bap is expressed in the forming midgut, hindgut (arrowhead), and foregut (arrow) visceral mesoderm. (*J*) Stage 11 *bin* embryo stained for Bap protein. Bap expression (red) is prematurely lost in the midgut mesoderm but not in foregut (arrow) and hindgut (arrowhead) mesoderm (cf. *I*).

3C). Notably, ventral expansion of the *bin* domains is also observed upon ectopic *dpp* expression in the absence of *tin* activity, although the domains are narrow (Fig. 3D, cf. B and C). Thus, Dpp is able to induce *bin* in the absence of *tin*, although *tin* activity is required for normal expression levels. The residual expression of *bin* in *tin* mutant embryos is unstable and not maintained in later stages of development (data not shown).

Similar to *tin*, *bap* activity is also required for normal *bin* expression (Fig. 3E). This result is in agreement with the temporal sequence of *bap* and *bin* expression (see above; Fig. 2B′) and with the observed expansion of *bin* throughout most of the dorsal mesoderm upon ectopic *bap* expression in the mesoderm (Fig. 3G,H). These data suggest that *bin* is furthest downstream within a mesoderm-intrinsic cascade of gene activation: *twist* → *tin* → *bap* → *bin*. Moreover, *bin* itself is required for normal *bin* expression. Although *bin* expression initiates normally in stage 10 *bin* mutant embryos, it disappears at early stage 11 in the trunk visceral mesoderm primordia of *bin* mutants, except for those in PS1 and 2 (Fig. 3F; data not shown). *bin* expression in these two parasegments is also less sensitive to the loss of *tin* and *bap* activity (Fig. 3B,E). Furthermore, the expression of *bin* in foregut, hindgut, and caudal visceral mesoderm does not depend on any of the genes examined in the present study.

Whereas the above data show that maintenance of *bin* expression in most of the presumptive trunk visceral mesoderm requires positive autoregulation, they do not

establish whether this autoregulatory loop is direct or indirect. Of note, maintenance of *bap* during stage 11 (but not its initiation during stage 10) also requires *bin* activity (Fig. 3, cf. J with I). Therefore, it is possible that, at least during stage 11, *bin* and *bap* maintain each other's expression through a cross-regulatory feedback loop.

bin *is required for differentiation of the circular and longitudinal midgut muscles*

The function of *bin* in visceral mesoderm development was studied by staining *bin* mutant embryos with a variety of markers including the Ig-domain adhesion molecule Fasciclin III (FasIII), the $G_o\alpha$ subunit Brokenheart (Bkh), and the Armadillo-repeat protein Vimar, which represent early and specific markers for the trunk visceral mesoderm and developing circular midgut musculature (Fig. 4A,C,E; Patel et al. 1987; Lo and Frasch 1998; Fremion et al. 1999). In *bin* mutant embryos, none of these three markers are expressed in the trunk visceral mesoderm at any stage of development (Fig. 4B,D,F). The specificity of this phenotype is underscored by the observation that the progenitors and differentiated cells of the heart, in which *bin* is not expressed, show normal expression of all tested markers in *bin* mutant embryos (see Fig. 4D for *bkh*; results with Tin and Eve are not shown). In addition, FasIII expression and tissue morphology can be restored in *bin* mutant embryos to almost normal levels by expressing wild-type *bin* in the trunk visceral mesoderm primordia under the control of a *bap* enhancer (Fig. 4K). The disruptions in the trunk visceral mesoderm in *bin* mutant embryos are slightly stronger than those found in *bap* null mutants, which show residual FasIII expression in thoracic segments and trace amounts in abdominal segments (coinciding with residual *bin* expression in these areas; see Fig. 3E) (Fig. 4M). The observed partial rescue of FasIII expression in abdominal segments of *bap* null mutants upon forced *bin* expression with a *bap* enhancer (Fig. 4N, arrows) suggests that *bin* is a major effector of *bap* activity in trunk visceral mesoderm development.

The development of the caudal visceral mesoderm in *bin* mutant embryos was examined using the expression of the FoxC gene *crocodile* (*croc*; Häcker et al. 1995) and the *bHLH54F* gene (Georgias et al. 1997) as markers. The normal expression of both genes in the early caudal visceral mesoderm in *bin* mutants shows that *bin* is not required for the formation of these cells. However, in *bin* mutants, anterior migration of these cells stalls during stage 11 (Fig. 4, cf. H and J with G and I). Because *bin* is expressed in the longitudinal gut muscle precursors (Fig. 2F–K), the observed disruption of the migration and differentiation of these cells could reflect a cell-autonomous function of *bin*. However, the requirement of *bin* for normal trunk visceral mesoderm differentiation and the known function of the trunk visceral mesoderm in guiding caudal visceral mesoderm migration (Kusch and Reuter 1999; M. Frasch, unpubl.) could also suggest that the observed phenotype is nonautonomous. To distinguish between these two possibilities, we examined *bHLH54F* expression in *bin* mutant embryos in which *bin* expression was brought back exclusively in the trunk visceral mesoderm via a *bap* enhancer. In these embryos, *bHLH54F* expression as well as the migration and morphology of the longitudinal muscle precursors appears normal, thus indicating that the disruption of these processes in the complete absence of *bin* activity is, at least predominantly, a nonautonomous effect (Fig. 4L). Presumably, the abnormal or absent trunk visceral mesoderm in *bin* mutants is unable to support proper guidance and differentiation of longitudinal gut muscle precursors. A similar effect is observed for the endoderm, which has also been shown to require normal trunk visceral mesoderm as a substrate for migration (data not shown; Azpiazu and Frasch 1993; Reuter et al. 1993). In summary, the *bin* phenotypes in the trunk and caudal visceral mesoderm as well as the endoderm are almost identical to the ones that we had observed in *tin* and *bap* null mutant embryos. In contrast to the midgut musculature, we did not detect any abnormalities in the morphology or gene expression patterns of the foregut and hindgut visceral mesoderm in either *bin* or *bap* mutants (Fig. 4F; data not shown).

Mutation and ectopic expression of bin *causes transformations between visceral and somatic mesodermal cell fates*

The fate of the primordial cells of the trunk visceral mesoderm in *bin* or *bap* mutant embryos was further studied with a *bap-lacZ* marker (*bap3-lacZ*; see Materials and Methods). In wild-type as well as *bin* and *bap* mutant backgrounds, *bap3-lacZ* expression initiates during stage 10 in a normal metameric pattern (H.H. Lee and M. Frasch, unpubl.), and β-gal protein perdures in descendants of these cells until late embryogenesis. Therefore, *bap*-driven β-gal marks the trunk visceral mesoderm and, at late stages, the circular gut muscle cells of wild-type embryos (Fig. 5A–C). In *bin* mutant embryos carrying *bap3-lacZ*, β-gal stainings show that the presumptive trunk visceral mesoderm primordia segregate towards the interior and coalesce into a band as in wild-type embryos (Fig. 5D). However, there are irregularities in the arrangement of the visceral mesoderm cells, which become much more pronounced after stage 11. At stage 13, these cells fail to become columnar, are not tightly attached to the endoderm, and become clustered segmentally instead of maintaining a continuous band (Fig. 5, cf. E with B). During stages 14–17, only a few of the *bap3-lacZ* expressing cells are attached to the endoderm, whereas the majority of them are scattered in areas within or underneath the somatic mesoderm (Fig. 5, cf. F with C). In addition, the midgut fails to undergo any constrictions. *bap* null mutants show a similar although more severe phenotype. More specifically, the presumptive visceral mesoderm cells internalize, but coalescence of the clusters during stage 11 is incomplete (Fig. 5G), and at later stages, very few of the *bap3-lacZ* expressing cells are attached to the endoderm (Fig. 5H,I).

Because of the apparent intermingling of *bap3-lacZ*-

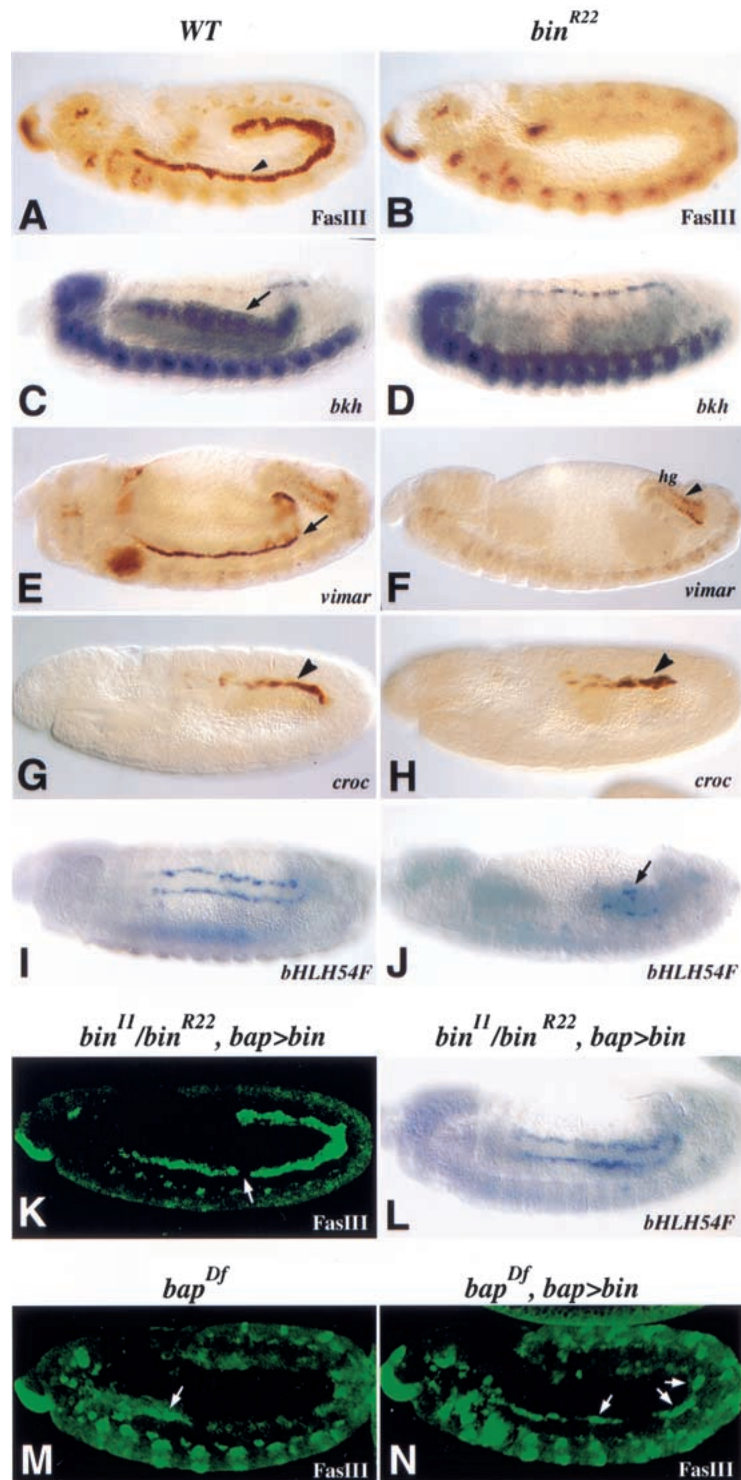

Figure 4. *bin* function is necessary for the development of the midgut visceral mesoderm. (*A*,*C*,*E*,*G*,*I*) Wild-type; (*B*,*D*,*F*,*H*,*J*) *bin* mutant embryos. (*K*,*L*,*N*) *bin* and *bap* mutant embryos, respectively, with *bin* expression in the visceral mesoderm provided via *bap3*–GAL4/UAS–*bin*. (*A*) Wild-type embryo stained for Fasciclin III, which marks trunk visceral mesoderm (TVM; arrowhead). (*B*) *bin* mutant embryo lacking Fasciclin III expression in the area of the TVM. (*C*) Stage 13 wild-type embryo hybridized with *bkh* probe. *bkh* mRNA is expressed in the dorsal vessel, ventral nerve cord, and midgut visceral mesoderm (arrow). (*D*) *bin* mutant embryo with *bkh* expression missing in the area where circular muscle precursors are normally found. (*E*) Wild-type embryo at late stage 13 stained for *vimar-lacZ*-driven β-gal expression in midgut (arrow) and hindgut visceral muscle precursors. (*F*) In *bin* mutant embryo carrying *vimar-lacZ*, β-gal expression is observed in hindgut but not midgut visceral muscles. (*G*) Late stage 11 embryo stained for *croc-lacZ* to visualize longitudinal gut muscle precursors (arrowhead). (*H*) In *bin* mutant background, *croc-lacZ*-expressing cells appear normal at stage 11 (arrowhead). (*I*) Stage 13 embryo hybridized with *bHLH54F* probe to visualize longitudinal muscle precursors after migration. (*J*) *bin* mutant embryo stained for *bHLH54F* reveals a strong reduction in the number of longitudinal muscle precursors (arrow, cf. *I*). (*K*) Stage 12 *bin* mutant embryo with *bap*-GAL4 driven *UAS-bin* and stained for Fasciclin III. The circular midgut muscle precursors are rescued, except for a few gaps (arrow). (*L*) *bHLH54F* expression in embryo of the same genetic background as in *K*, showing complete rescue of longitudinal gut muscle formation (cf. *J*). (*M*) Stage 12 *bap* null mutant embryo (*tin*Re28-58,*Df(3R)e*^{D7}) stained for FasIII (arrow, residual FasIII in anterior TVM). (*N*) *bap* null embryo (*tin*Re28-58,*Df(3R)e*^{D7}/Df(3R)e^{F1}) with *bap3*–Gal4 and UAS–*bin* (arrows, rescued FasIII in TVM).

positive cells with somatic mesoderm, we tested whether cells derived from the trunk visceral mesoderm primordia become incorporated into body wall muscle fibers in *bin* and *bap* mutant embryos. As shown in Figure 5L–O, syncytial muscle fibers stained for myosin heavy chain (MHC) are positive for β-gal, whereas in wild-type backgrounds, *bap3-lacZ* does not produce any significant signals in the somatic musculature (see Fig. 5J,K). Because the particular β-gal from this construct is both nuclear and cytoplasmic, we infer that the β-gal-positive nuclei seen in the syncytial muscle fibers in *bin* and *bap* mutant embryos (Fig. 5L–O, arrowheads) are the ones that are derived from cells that would normally form midgut muscles. In *bin* mutant embryos, the trans-

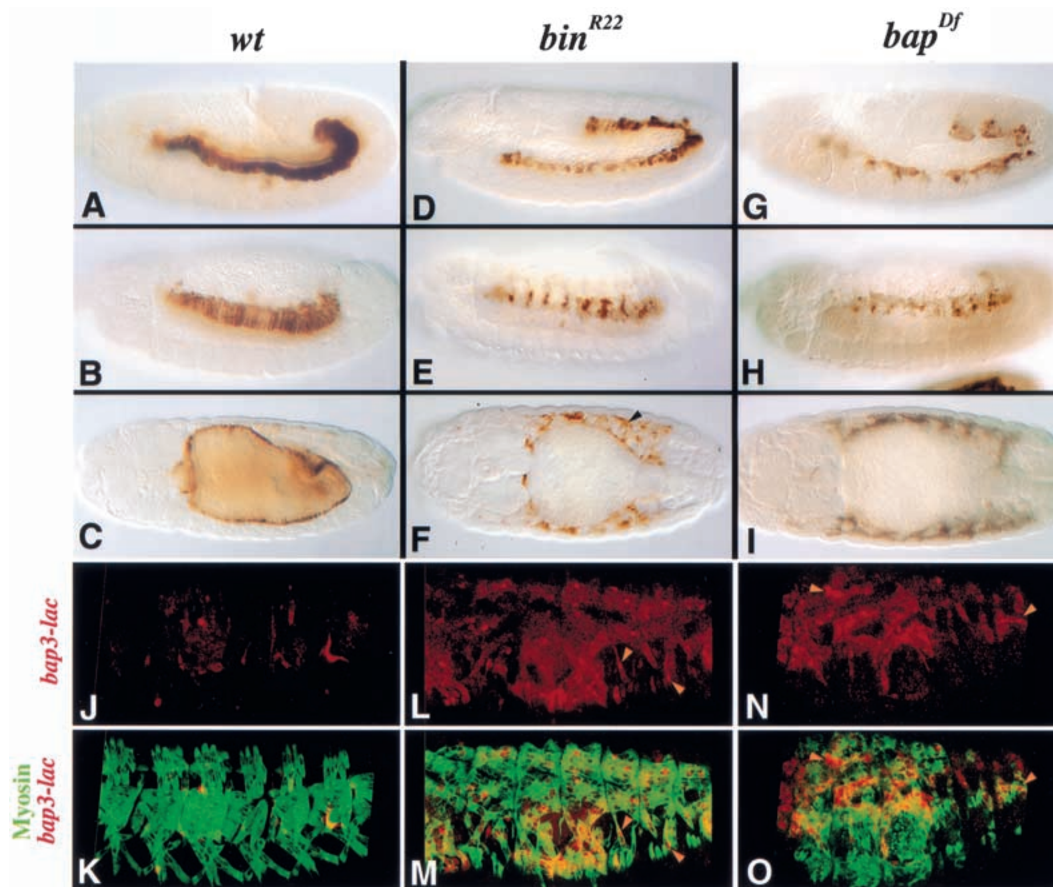

Figure 5. Functions of *bin* and *bap* in the maintenance of visceral mesodermal cell fates. Embryos with wild-type (*A–C*,*J*,*K*), *bin* mutant (*D–F*,*L*,*M*), and *bap* null mutant (*G–I*,*N*,*O*) backgrounds carrying a *bap3-lacZ* insertion. (*A–I*) Embryos were stained for β-gal to visualize circular midgut muscle precursors and follow their cell fate. (*J–O*) Embryos doubly stained for MHC (green) and β-gal (red) and scanned via confocal microscopy. (*A–C*) Stage 12, 13, and 15 wild-type embryos carrying *bap3-lacZ*. β-Gal from the trunk visceral mesoderm primordia perdures in developing circular midgut muscles. (*D*) Stage 12 *bin* mutant embryo in which *lacZ*-expressing precursors of circular midgut muscles in the dorsal mesoderm are slightly disorganized. (*E*) Stage 13 *bin* mutant embryo with segmentally arranged β-gal-expressing cells in the trunk region. (*F*) Dorsal view of stage 15 *bin* mutant embryo. Only a few β-gal-stained cells remain around the midgut, whereas the majority of them are localized within or near the body wall muscles layer (arrowhead). (*G*,*H*,*I*) Stage 12, stage 13, and stage 15 *bap* mutant embryos, respectively, carrying *bap3-lacZ*. β-Gal staining reveals visceral mesoderm abnormalities similar to those in *bin* mutants. (*J*,*K*) Stage 16 wild-type embryo with *bap-lacZ*, doubly stained for MHC (green) and β-gal (red) proteins. β-gal protein is rarely detected in somatic muscle cells. (*L*,*M*) In *bin* mutant embryo with *bap3-lacZ*, detection of MHC (green) and β-gal (red) proteins shows that cells originating from trunk visceral mesoderm primordia have fused into syncytia of somatic muscles (arrowheads). (*N*,*O*) In *bap* mutant embryos with *bap3-lacZ*, MHC (green) and β-gal (red) proteins are extensively colocalized (arrowheads).

formation from a visceral into a somatic muscle fate appears to affect a large proportion although not all of the *bap3-lacZ*-positive cells, whereas in *bap* null mutants, essentially all of them appear to be transformed.

To test whether *bin* has the potential to cause cell fate transformations from somatic to visceral mesoderm, we assessed the effects of ectopic expression of *bin* in the entire early mesoderm under the control of a *twist* enhancer. Ectopic *bin* causes ectopic expression of *bap*, which becomes expressed almost uniformly in the dorsal mesoderm (Fig. 6, cf. B with A). The expression of additional differentiation markers for trunk visceral mesoderm, FasIII (Fig. 6C,D), *vimar* mRNA (Fig. 6E,F), and *vimar-lacZ* (data not shown), is also expanded upon ectopic *bin* expression to include ventral areas that are normally occupied by the somatic mesoderm. Stainings for Even-skipped (Eve) demonstrate that ectopic *bin* interferes with the specification of dorsal somatic muscle and pericardial cell progenitors (Fig. 6, cf. H with G). Furthermore, stainings for β3-tubulin and muscle myosin show that ectopic *bin* disrupts somatic muscle differentiation, including myoblast fusion (Fig. 6, cf. J and L with I and K). Altogether, these observations strongly suggest that the presence of *bin* activity is able to force the development of somatic mesodermal cells towards the trunk visceral mesoderm pathway. However, the atypical morphology, the supernumerary cells which express visceral mesoderm markers, and the diminished ectopic marker expression at late stages (data not shown) indicate that, at least under these particular conditions

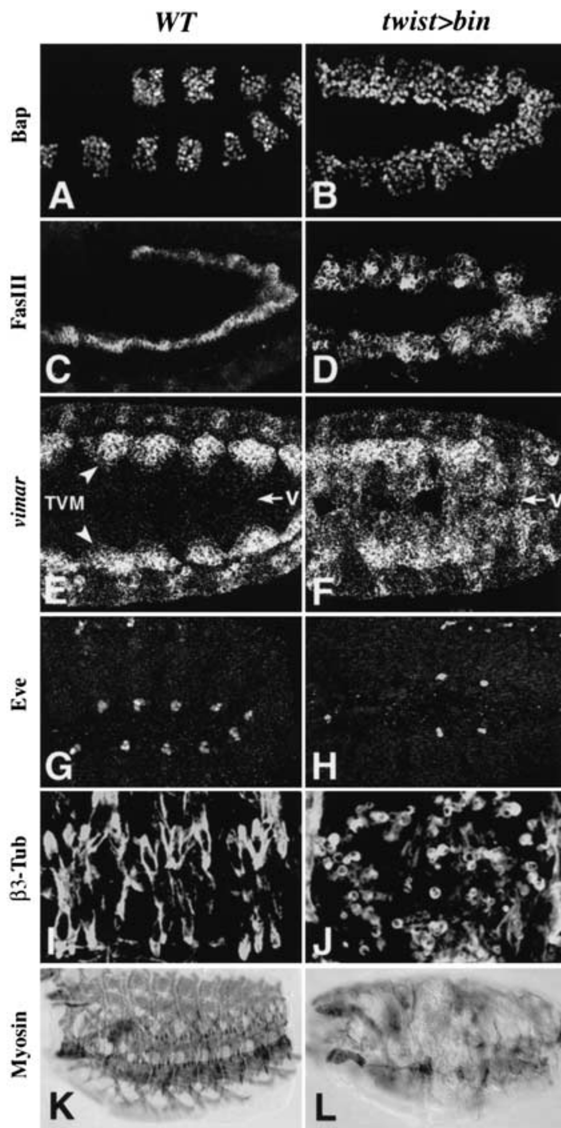

Figure 6. Expansion of visceral mesoderm and disruption of somatic mesoderm as a consequence of *bin* misexpression. High-magnification views of wild-type (*left*) and *twist*–GAL4/UAS–*bin* embryos (*right*). (*A*) Stage 10 embryo showing Bap protein expression. (*B*) Upon misexpression of *bin* in the entire mesoderm, Bap becomes uniformly expressed in the dorsal mesoderm. (*C*,*D*) Stage 12 embryos stained for Fasciclin III (FasIII) protein. The wild-type embryo shows FasIII in a narrow band of visceral mesoderm, while the embryo with ectopic *bin* shows a strong expansion of FasIII expression. (*E*,*F*) Early stage 12 embryos showing *vimar* mRNA in the trunk visceral mesoderm (TVM) and, upon ectopic expression of *bin*, also in ventral areas (v, arrows) normally occupied by somatic musculature. (*G*,*H*) Stage 12 embryos stained for Even-skipped (Eve) protein. The embryo with ectopic *bin* expression shows a reduction of Eve-positive pericardial and dorsal muscle progenitors. (*I*,*J*) Stage 16 embryos stained for β3-Tubulin (β3-Tub) protein, which is expressed in all myogenic cells. The wild-type embryo shows β3-Tub in multinucleate somatic muscle fibers, while the embryo with ectopic *bin* shows mostly unfused, mononucleated cells. (*K*,*L*) MHC antibody stainings show a severe reduction of somatic muscle fiber formation upon ectopic *bin* expression at earlier stages.

of ectopic *bin* expression, the cell fate transformation in this direction are not complete.

bin *is a direct upstream regulator of* dpp *within the visceral mesoderm*

Besides tissue-specific differentiation genes that are expressed throughout the trunk visceral mesoderm, several key regulators of midgut morphogenesis are known to be expressed in a spatially restricted manner within this tissue. This type of gene products includes the homeotic factor Ubx and the secreted factor Dpp, both of which are expressed in PS7 of the visceral mesoderm (see Fig. 8E for *dpp* mRNA, which has an additional expression domain in the prospective gastric caecae in PS3). Although it has been established that Ubx and Dpp maintain their expression in PS7 through a crossregulatory loop and the action of Wg from the adjacent PS8, there is evidence that their expression requires at least one additional, visceral mesoderm-specific cofactor, for which Bin may be a candidate (Panganiban et al. 1990; Reuter et al. 1990; Hursh et al. 1993; Thüringer et al. 1993; Capovilla et al. 1994; Manak et al. 1994; Sun et al. 1995; Yu et al. 1996). To test this possibility, we examined Ubx and *dpp* expression in *bin* mutant embryos, which carried *bap3-lacZ* to allow the unambiguous identification of the disrupted visceral mesoderm layer. Visceral mesoderm expression of Ubx in *bin* mutant embryos is similar to that of wild-type embryos until at least stage 13, although there is a low level of ectopic expression (Fig. 7A,B). Likewise, Ubx expression is also observed in β-gal-positive cells in *bap* mutant embryos, albeit with reduced levels and an expanded domain that are comparable to the somatic mesoderm (Fig. 7C). These data demonstrate that the establishment of Ubx expression in the visceral mesoderm requires neither *bin* nor *bap* activity. In contrast, *dpp* is not expressed at any stage in PS7 in the visceral mesoderm of *bin* mutant embryos, indicating that Bin may serve as a critical tissue-specific cofactor for the regulation of *dpp* expression (Fig. 7, cf. E with D). The expression of *wg* in PS8 is also absolutely dependent on *bin* activity (data not shown). The absence of these morphogenetic factors is likely to contribute to the defective midgut morphology in *bin* mutant embryos.

The identification of visceral mesoderm-specific enhancer elements of *dpp* (Hursh et al. 1993; Manak et al. 1994; Sun et al. 1995) allowed us to test the possibility that *bin* might be a direct upstream regulator of *dpp* in the visceral mesoderm. We focused our attention on two minimal enhancer elements, the 130 bp element BM (Sun et al. 1995) and the 231 bp element PB (a slightly shorter version of dpp261 (Manak et al. 1994; see Materials and Methods) (Fig. 8A). PB is able to drive reporter gene expression in PS3 and PS7 of the visceral mesoderm in a pattern that is similar to that of endogenous *dpp*, although *PB-lacZ* expression in PS7 is less robust (Fig. 8F; note that unlike the related *dpp261-lacZ*, our construct is not subject to derepression in PS4–6). In contrast to PB and as reported by Sun et al. (1995), BM is active in a broad region extending from PS7 to PS12 in

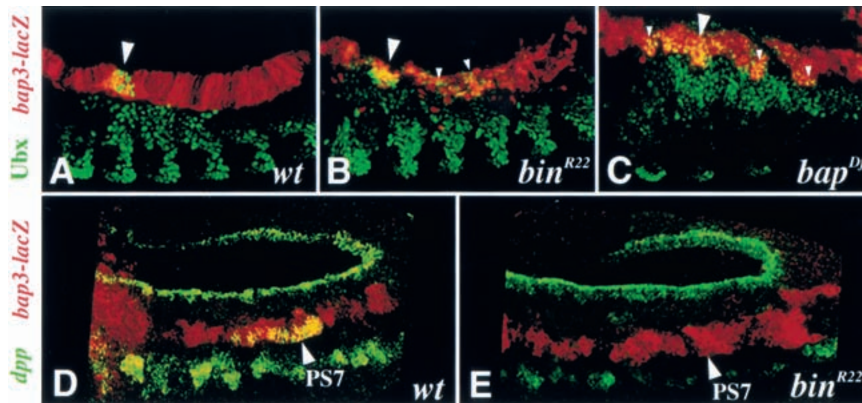

Figure 7. *biniou* is required for the activation of *dpp* but not Ubx in PS7 of the midgut visceral mesoderm. Shown are wild-type and mutant embryos carrying *bap3-lacZ* to identify the visceral mesoderm layer. (*A–C*) Stage 13–14 embryos double-stained for Ubx (green) and β-gal (red) proteins. Wild-type (*A*), *bin* mutant (*B*), and *bap* null mutant (*C*) embryos all show accumulation of Ubx in PS7 of the visceral mesoderm (large arrowheads; yellow signal). Small arrowheads denote ectopic expression. (*D*,*E*) Stage 12 embryos double labeled for *dpp* mRNA (green) and β-gal protein (red). In contrast to the wild-type embryo (*D*), the *bin* mutant embryo (*E*) shows a complete lack of *dpp* mRNA in parasegment 7 (PS7) (arrowheads).

the visceral mesoderm (Fig. 8H). In addition, the combination of BM and PB results in a significant enhancement of PS7 expression compared to PB alone (Fig. 8J; see also Sun et al. 1995). Because of the broad activity of BM in the visceral mesoderm and its enhancing effect on PB (or longer versions thereof), BM has been proposed to act as a general visceral mesoderm enhancer (GVME), whereas PB is predominantly targeted by spatially restricted activities that include Ubx and Exd (Fig. 8A; Manak et al. 1994; Sun et al. 1995).

We performed DNaseI protection assays with recombinant Bin protein to test for the presence of Bin binding sites within BM and PB. These experiments identified two protected regions within BM, termed Bin I and Bin II, which are about 50 bp apart from one another (Fig. 8B). PB contains a third strongly protected sequence, Bin III, and two minor binding sites which overlap with the previously described Exd binding sites e1 and e2 (Fig. 8C; see Sun et al. 1995). All three of the strongly protected sequences and the weaker e1 contain sequence motifs that perfectly match forkhead domain binding sites, including the optimal binding site of a vertebrate ortholog, HFH-8 (Fig. 8B–D; Kaufmann et al. 1995; Peterson et al. 1997). The presence of overlapping inverted and direct repeats of this sequence motif in Bin II and Bin III, respectively, may indicate that these two sites represent dimeric binding sites. Interestingly, the sequences of the three strong and two weak Bin binding sites within PB are highly conserved between *D. melanogaster* and *D. virilis* (Manak et al. 1994), suggesting that they are functionally important.

To test whether any of the strong Bin binding sites are required for enhancer activity in vivo, we introduced nucleotide exchanges that completely abolished in vitro binding of Bin (Fig. 8B,C). Mutation of Bin III results in an almost complete loss of PB enhancer activity in PS7 (Fig. 8, cf. G with F), suggesting that Bin binding to Bin III plays an important role for the activation of *dpp* in this parasegment. The presence of two weak Bin binding sites in the mutated PB derivative may allow residual expression in a few visceral mesoderm cells within PS7 (Fig. 8G, arrowhead). The fact that PS3 expression is not affected significantly upon Bin III mutation may be due to the activity of Exd binding sites, of which one was previously shown to regulate PS3 expression (Manak et al. 1994).

BM enhancer activity in the visceral mesoderm is completely lost when both Bin I and Bin II are mutated (Fig. 8, cf. I with H). When this mutated version of BM is combined with a wild-type version of PB, there is no enhancement of PS7 expression and the same pattern as with PB alone is observed (data not shown; see Fig. 8F). Finally, the combination of BM and PB with mutated Bin I, II, and III binding sites does not exhibit any significant enhancer activity in PS7 (Fig. 8, cf. K with the wild-type combination in J). These data suggest that both BM and PB contain functionally important Bin binding sites. Bin binding to Bin I and Bin II may be key to providing BM with its general visceral mesoderm enhancer activity, whereas binding to Bin III is required in concert with spatially restricted activities to provide the PB enhancer with a basal level of activity in PS7.

Discussion

Despite their diverse developmental origins, the different types of visceral muscles in *Drosophila* share a number of morphological and functional characteristics, which clearly distinguish them from the body wall and cardiac musculature. By analogy to the situation in vertebrate skeletal muscles, one might expect that these common features are due to the activity of one or several key regulatory factors that are shared among the developing circular and longitudinal midgut muscles as well as foregut and hindgut muscles. In the present study, we identified the forkhead domain gene *biniou* (*bin*) as a regulator of visceral muscle identities in *Drosophila*. *bin* represents the first example of a gene that is specifically expressed in all types of visceral muscles and their precursors. Our analysis focused on the regulation and function of *bin* in the developing trunk visceral (or splanchnic) mesoderm and the derived circular midgut musculature.

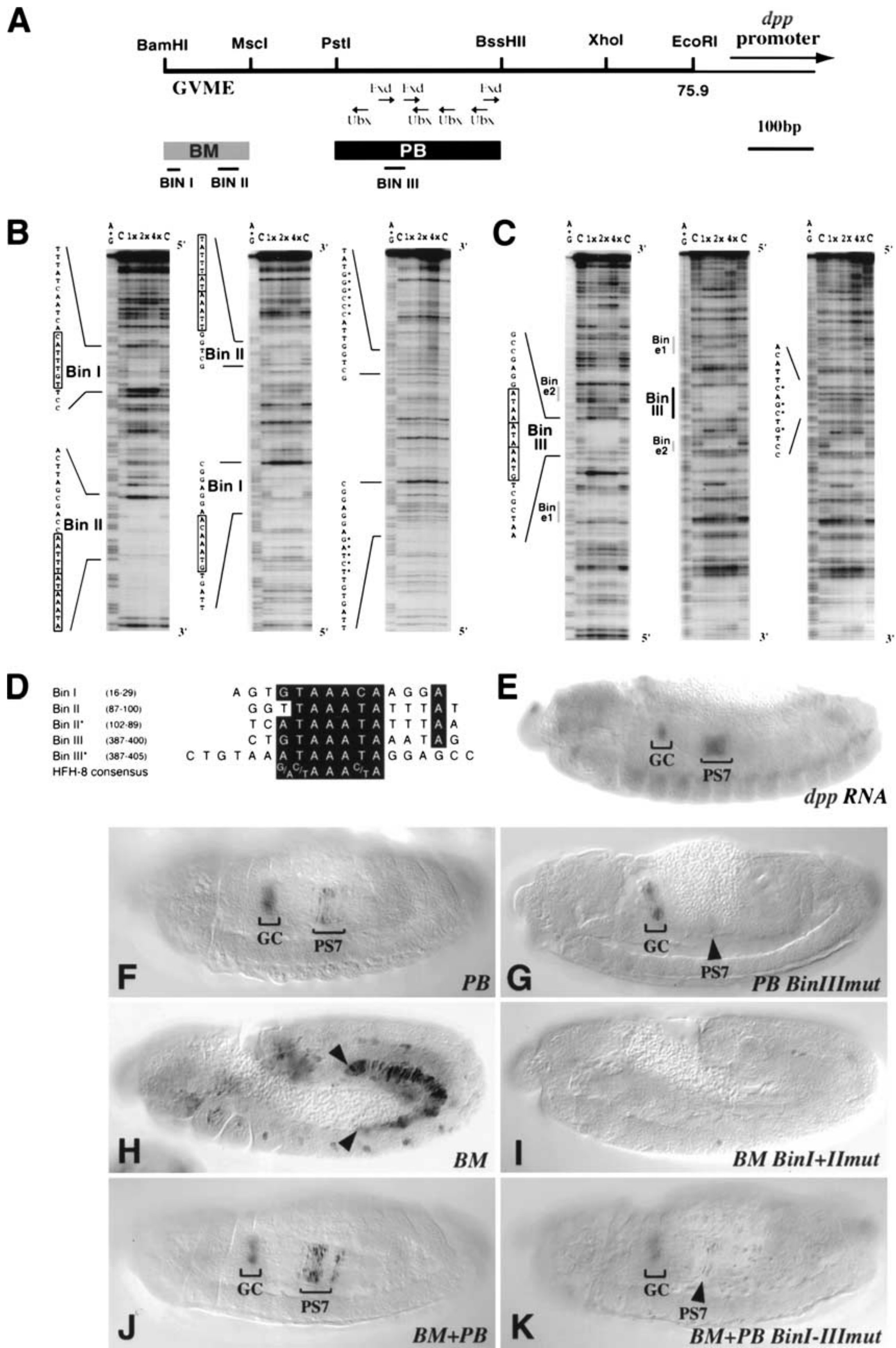

Figure 8. (*Legend on facing page*)

bin *as a component of a regulatory gene network during trunk visceral mesoderm development*

Previous studies established *bap* as a key regulator in the trunk visceral mesoderm primordia and showed that the spatial domains of *bap* expression are defined by intersecting dorsoventral and anteroposterior cues (Azpiazu and Frasch 1993; Azpiazu et al. 1996). It was determined that the combined activities of the homeobox gene *tin* and ectodermally derived Dpp activate *bap* in the dorsal mesoderm, while *wg* via its downstream gene *slp* causes an abrogation of this activation process (Staehling-Hampton et al. 1994; Frasch 1995; Riechmann et al. 1997; Lee and Frasch 2000). As a result, *bap* expression is turned on in a segmentally repeated pattern within the dorsal mesoderm. The data presented herein indicate that *bin* requires the combined activities of *bap* and *dpp* for its normal activation, which suggests that *bin* is positioned furthest downstream in the known regulatory hierarchy of transcription factors during visceral mesoderm development. Another important conclusion is that inductive Dpp signals are active not just once in the dorsal mesoderm but rather operate successively during each of the three known steps of gene activation that participate in the pathway leading to trunk visceral mesoderm (Fig. 9). These activation steps ($tin \rightarrow$ dorsal $tin \rightarrow bap \rightarrow bin$) occur in rapid succession, apparently within minutes after the mesoderm has spread beneath the dorsal ectodermal domain of Dpp expression.

We have shown previously that the induction of *tin* expression by Dpp in the dorsal mesoderm involves the combined binding of Smad proteins (Medea and Mad) and Tin itself to a Dpp-responsive enhancer of the *tin* gene (Xu et al. 1998). Our genetic data for *bin* suggest an analogous mechanism for *bin*, in which the combined binding of Smads and Bap (an NK homeodomain protein related to Tin) may activate a Dpp-responsive *bin* enhancer. However, in contrast to the induction of *tin* and *bap* by Dpp, which stringently requires *tin* activity, low levels of *bin* can be induced by Dpp even in the absence of *tin* and *bap*. This observation indicates that there is at least one other mesoderm-intrinsic factor, in addition to

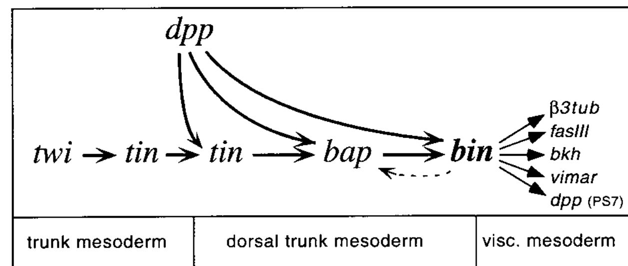

Figure 9. Regulatory interactions during specification and differentiation of the trunk visceral mesoderm. Only positive inputs are shown in the diagram.

Tin and Bap, which helps to provide the mesoderm with the competence to respond to Dpp and allow *bin* induction.

The relatively normal spatial pattern of residual *bin* expression in the absence of *bap* indicates that *bin* may also receive direct inputs from striped regulators. This regulation could be analogous to that of *bap* as well and involve the negative activities of *wg* and *slp*. Lastly, we have shown that there is feedback regulation in which *bin* regulates prolonged expression of *bap*. However, this indirect feedback loop is only operative until early stage 12, when *bap* expression ceases in the trunk visceral mesoderm. The maintenance of *bin* expression during later stages of visceral mesoderm development could involve direct autoregulation.

The roles of bin *and* bap *in visceral mesoderm specification and differentiation*

The similarities in the early expression patterns of *bin* and *bap* in the trunk visceral mesoderm primordia raise the question of the relative contribution of these two genes to visceral mesoderm development. Are all functions of *bap* in this developmental process mediated through *bin*, or does *bap* also fulfill *bin*-independent roles? We have shown that in the absence of either *bin* or

Figure 8. Bin binding sites in *dpp* enhancers are essential for enhancer activity in parasegment 7 of the visceral mesoderm. (*A*) *dpp* VM enhancer region upstream of *dpp* (from *Bam*HI to *Eco*RI; see Manak et al. 1994; Sun et al. 1995). (GVME) General visceral mesoderm enhancer (Sun et al. 1995). The *Pst*I/*Bss*HII (PB) fragment refers to the 261-bp enhancer of Manak et al. (1994). Previously identified Ubx and Exd binding sites within PB are shown as in Manak et al. (1994). (*B*) DNase I footprinting analysis with increasing amounts of Bin protein or no Bin (lane *C*) of ^{32}P-labeled *dpp*-BM and (*C*) of *dpp*-PB sequences, respectively. Binding experiments with wild-type sequences (sense and antisense) are shown to the *left* and experiments with mutated sequences to the *right* (see asterisks; antisense strand is shown for BM and sense strand for PB). The protected regions (boxed sequences) are named Bin I, II, or III. Bin/e1 and Bin/e2 are sites of weak protection with Bin that also bind Exd (Manak et al. 1994; Sun et al. 1995). (*D*) Sequence alignments of protected Bin binding sites within *dpp*-BM, *dpp*-PB, and the HFH-8 consensus sequence (Peterson et al. 1997). (Asterisks) Alignments of the second copies of tandemly arranged consensus motifs within Bin II and Bin III. Nucleotide numbers refer to GenBank entry X81977. (*E*) Stage 14 wild-type embryo stained for *dpp* mRNA expression for a comparison and (*F–H*) embryos carrying various *dpp-lacZ* constructs and stained for β-gal expression. (*F*) The 231-bp *dpp*-PB element drives expression in PS7 and the gastric caeca primordia (GC). (*G*) Mutation of the Bin III binding site within PB causes severe reduction of PS7 expression. (*H*) The 130-bp *dpp*-BM element drives uniform expression starting from stage 11 in the posterior portion (PS7–12) of the visceral mesoderm. (*I*) Mutation of both Bin I and Bin II binding sites within the *dpp*-BM element abolishes enhancer activity (cf. *H*). (*J*) Combination of BM with PB produces strongly enhanced PS7 expression (cf. *F*). (*K*) Mutation of all three major *bin* binding sites within the combined BM+PB elements leads to a near loss of PS7 expression (cf. *J*).

bap activity, a large portion of the cells that are normally destined to form visceral muscles become incorporated into body wall muscles. Likewise, the expression of several trunk visceral mesoderm markers is affected similarly in *bin* and *bap* mutants (Fig. 4; Lo and Frasch 1998). The similarities of these phenotypes as well as the partial rescue of *bap* phenotypes upon forced expression of *bin* suggest that most of the activities of *bap* in the trunk visceral mesoderm involve the activation and function of its downstream gene *bin*. Because of the temporal overlap of Bap and Bin expression during stage 10 to early stage 12, it is also possible that both proteins are required in combination to activate some target genes. Based upon our lineage tracing data, the transformation from visceral into somatic muscle fates appears to be more complete in *bap* mutants compared to *bin* mutants, and defects in the migration behavior and morphogenesis of the trunk visceral mesoderm are evident at an earlier stage in *bap* mutants. It is therefore likely that *bap* has some additional targets in the visceral mesoderm that do not require *bin*.

The lineage tracing experiments with the *bap-lacZ* marker also showed that the earliest steps of trunk visceral mesoderm, which consist of the segregation of primordial cells from the mesodermal monolayer towards the interior, do not require *bap* or *bin* activity. This behavior is unlikely to be due to functional redundancy between the two genes, because *bin* expression is largely missing in *bap* mutants. Rather, this observation points to the existence of regulatory gene(s) in addition to *bap* and *bin* that are present in the 11 clusters of dorsal mesodermal cells and control their segregation towards the interior to form visceral mesoderm. The emerging picture is that multiple regulatory genes are activated in metameric clusters of dorsal mesodermal cells that define the trunk visceral mesoderm primordia. Based on the available genetic data, it is likely that the induction of all of these regulators requires Dpp and is prevented by *slp*. In addition to Dpp, *bap* is strictly and *bin* largely dependent on *tin*, whereas induction of yet unknown genes that regulate visceral mesoderm segregation may be largely or fully independent of *tin* (Taylor 2000). While each of these regulatory genes appears to have some unique functions in visceral mesoderm development, we have shown herein that three of the Dpp-induced genes, *tin*, *bap*, and *bin*, are part of a mesoderm-intrinsic cascade of gene activations, which also involves feedback regulation. We propose that a major outcome of this regulatory cascade is the activation of *bin* in the primordial cells of the trunk visceral mesoderm and its maintenance in the developing circular midgut muscles. It appears that *bin* plays a key role in activating multiple, if not the majority, of patterning and differentiation genes which define the morphological and functional features of midgut muscles and prevent visceral mesodermal cells from fusing with somatic muscle precursors. Molecular and genetic studies show that at least two of these downstream genes, *dpp* (see below) and β-*3tubulin* (S. Zaffran and M. Frasch, in prep.), are direct targets of *bin*.

Bin as a tissue-specific cofactor controlling dpp *expression in the visceral mesoderm*

Spatially restricted expression of *dpp* in PS7 of the trunk visceral mesoderm plays an important role in normal midgut morphogenesis (Immerglück et al. 1990; Hursh et al. 1993; Masucci and Hoffmann 1993). Previous studies have identified Ubx and Exd as directly and positively acting upstream regulators of *dpp* in the visceral mesoderm (Reuter et al. 1990; Hursh et al. 1993; Capovilla et al. 1994; Chan et al. 1994; Manak et al. 1994; Sun et al. 1995). Because these regulators are present in other tissues where they do not activate *dpp* expression, it has been proposed that they require tissue-specific cofactor(s) for activating *dpp* in the visceral mesoderm (Hursh et al. 1993). Further evidence of the involvement of cofactor(s) that are predicted to be expressed uniformly in the visceral mesoderm came from the dissection of the visceral mesodermal *dpp* enhancer. In particular, truncations or specific mutations within this enhancer caused an expansion of enhancer activity beyond the Ubx domain and in some cases throughout the trunk visceral mesoderm (Manak et al. 1994; Sun et al. 1995; Yang et al. 2000). These data suggest that, in the absence of repressing activities, uniformly expressed visceral mesodermal factor(s) are able to activate the *dpp* enhancer without a requirement for Ubx and Exd.

The present study demonstrates that the FoxF protein Bin corresponds to such a visceral mesoderm-specific factor during the activation of *dpp* expression. Bin is expressed in the visceral mesoderm prior to *dpp*, is genetically required for *dpp* expression, and the Bin binding sites within the *dpp* enhancer are essential for enhancer activity in PS7 of the visceral mesoderm. Of note, our data suggest that Bin has a key role in the activation of a previously identified general visceral mesoderm enhancer of *dpp* (see Fig. 8; Sun et al. 1995), which is active throughout PS7 to PS12 of the visceral mesoderm. In the normal context, Bin binding to these sequences is required for the enhancement of *dpp* expression in PS7. However, the presence of additional functionally important Bin binding sites that are interdigitated with Ubx and Exd sites demonstrates that Bin is an integral component of PS7-specific enhancer activation as well and that a clean separation between general and PS7-specific enhancer elements does not exist. The available data suggest that PS7-specific expression of *dpp* in the visceral mesoderm is regulated by an exquisite balance between positive and negative activities. Negative regulators have been shown to include dTCF (or a factor with related binding specificity), which acts in a *wg*-independent manner in the entire trunk visceral mesoderm (Yang et al. 2000), and Abd-A, which is only active between PS8 and PS12 (Reuter et al. 1990). Based on these combined data, we propose a model in which the activator *bin* is neutralized by the negative factors to provide a sensitized equilibrium of gene activities, which is set below the threshold level of *dpp* activation. In this model, the role of Ubx and Exd would be to disrupt this equilibrium and shift it towards the active state in PS7.

Evolutionary conservation of FoxF *gene functions in the splanchnic mesoderm?*

An early binary decision during mesoderm development in both insect and vertebrate embryos results in the splitting of a single layer into the two separate layers of somatic and splanchnic mesoderm, which become associated with lateral ectoderm and endoderm, respectively. In *Drosophila* and other insects, the splanchnic mesoderm develops exclusively into gut musculature, whereas in vertebrates it contributes to many additional internal organs. Interestingly, the observations described in the present paper together with recently published data suggest that the overt morphological and developmental similarities in the splanchnic mesoderm of insects and vertebrates extend to the molecular level. Similar to *Drosophila bin*, the two vertebrate orthologs *FoxF1* and *FoxF2* (previously termed *FREAC1/2* [mouse, rat, human *Foxf1/2*], *HFH-8* [mouse *Foxf1*], *lun* [mouse *Foxf2*], and *XFD-13* [frog *FoxF1*]) are predominantly expressed in the splanchnic mesoderm and mesenchyme that line the digestive tract (Hellqvist et al. 1996; Peterson et al. 1997; Mahlapuu et al. 1998; Funayama et al. 1999; Köster et al. 1999; Aitola et al. 2000). At later stages, expression continues in various tissues that are derived from splanchnic mesoderm, including smooth muscles of the intestine, lung, and liver capsule. In addition to similarities in their expression patterns, the phenotype of mouse embryos with a targeted disruption of *Foxf1* suggests similarities in developmental functions of the *Drosophila* and vertebrate genes. In *Foxf1*$^{-/-}$ embryos, splitting of the lateral plate mesoderm into splanchnic and somatic layers is frequently incomplete or absent, and ectopic expression of the somatic mesoderm marker *Irx1* indicates that the splanchnic mesoderm assumes characteristics of somatic mesoderm (Mahlapuu et al. 2001). Both of these alterations are strongly reminiscent of the *bin* phenotype. Other striking similarities with *Drosophila bin* include the requirement of *Foxf1* for the activation of BMP4 expression in the lateral plate and allantois (Mahlapuu et al. 2001) as well as its coexpression with the *bap* ortholog *Bapx1* in the splanchnic mesoderm (Tribioli et al. 1997). Moreover, the demonstration that the lateral plate mesoderm is under the influence of BMP signaling (Tonegawa et al. 1997) could imply that, analogous to *bin*, which is initially a target of Dpp and at later stages activates *dpp* expression, *FoxF* genes are both downstream and upstream of BMPs. Collectively, these data suggest that the splanchnic mesoderm in insect and vertebrate embryos are homologous tissues and that their development is controlled by related genetic circuits in which members of the *FoxF* class of forkhead domain genes occupy a central position.

Materials and methods

Drosophila *strains and genetics*

The genotype termed *bap*^{Df}, which was obtained by crossing *Df(3R)e*^{F1} and a *Df(3R)e*^{D7} chromosome carrying the *tin* genomic rescue construct *tin* Re28–58 (Azpiazu and Frasch 1993), produces an amorphic *bap* phenotype in the visceral mesoderm that can be rescued by adding a *bap* Re3 genomic rescue construct (M. Frasch, unpubl.). The *bap3-lacZ* #6 line carries a *bap* enhancer/pCaSpeRhs43 β-gal construct on the second chromosome (H.-H. Lee and M. Frasch, unpubl.). The null allele *tin*^{346} and hypomorphic allele *bap*^{208} were described by Azpiazu and Frasch (1993), the *croc-lacZ* line *croc*^{5F59} by Häcker et al. (1995), and *B6-2-30* (*vimar-lacZ*) by Lo and Frasch (1998). The EMS alleles *bin*^{I1}, *bin*^{R22}, and *bin*^{S4} were isolated in an F_2 screen using the deficiency *Df(3L)W5.4* (Anderson et al. 1995); the collection of lethal mutations at this locus was kindly provided by Wayne Johnson. The phenotypes of homozygous *bin* alleles were identical to each other and to those of various allelic combinations in trans. *bin* mutations were balanced over either *TM3Sb P[ftz-lacZ]* or *TM3Sb P[eve-lacZ]* to identify homozygous mutant embryos.

Molecular techniques and point mutation detection

A PCR fragment covering the forkhead domain sequence of *bin* was isolated by using the P1 clone DS06194 (from BDGP) as a template. The fragment was used to screen a 4- to 8-hr embryonic cDNA library (Brown and Kafatos 1988), and the longest cDNA (2413 bp plus poly A) was sequenced. Genomic DNA was isolated from handpicked homozygous *bin* mutant embryos, which were stained with a β-galactosidase antibody to exclude embryos carrying *TM3-lacZ* chromosomes. DNA fragments corresponding to exons were amplified by PCR and cloned into pCRII-Topo (Invitrogen). All PCR amplifications were repeated several times, and the products were sequenced on both strands. The GST/Bin fusion protein was obtained by cloning a *Bam*HI/*Bam*HI *bin* fragment (amino acids 135–615) into pGEX-2T (Pharmacia), and DNaseI footprinting assays were performed as described by Lee and Frasch (2000).

Generation of UAS/GAL4 constructs

Full-length *bin* and *bap* cDNAs were cloned into pUAST (Brand and Perrimon 1993). A UAS-*dpp* line (Frasch 1995), *twist*-GAL4 (SG24; Greig and Akam 1993), and *bap3*-GAL4 were used for ectopic expression studies. For the latter, a 180-bp fragment corresponding to a minimal trunk visceral mesoderm enhancer of *bap* (H-H. Lee and M. Frasch, unpubl.) was cloned into *Eco*RI/*Not*I of the *pGAL4–221* vector (gift from Christian Klämbt, Münster University, Germany).

Construction of dpp-lacZ *transformation plasmids*

dpp upstream enhancer elements were obtained by PCR amplification with *Drosophila* genomic DNA and subcloned into pBluescript KS (Stratagene) or pCRII-Topo (Invitrogen). Based on published sequence information (Manak et al. 1994; Sun et al. 1995), the primers *dpp3′-Pst*I, 5′-GTTCTGTTGCGGGATCC GAAATAGTTAGT-3′ and *dpp3′-Not*I, 5′-ATTACTGTCAAG TGGCCAACGGAAGGGAGAGACATC-3′ were used to generate *dpp*-BM (+1/+149), and the primers *dpp5′dw*, 5′-GGC GATTACGTGGAGTACTAC-3′ and *dpp5′up*, 5′-CGATTCAA ATTTATTACTAATTGGGTG-3′ were used to generate the *dpp*-PB (+282/+513).

For site-directed in vitro mutagenesis of *dpp* elements, pBluescript KS *dpp*-BM and pCRII-Topo *dpp*-PB were used as templates for inverse PCR. Appropriate oligonucleotides were designed to introduce restriction sites replacing the forkhead domain binding sites. The primers 5′-AGTGTCTAGAGGAGG CACTCTTGAGAACG-3′ and 5′-TCCTCTAGAACACTAACT

ATTTCGGATCCC-3′, which introduce an *Xba*I site, and 5′-TGGTTCCCGGGTTATGAAATCATAAAATTTG-3′ and 5′-ATTTCATACCCGGGTAACCAGCGATTCAATC-3′, which introduce an *Sma*I site, were used to mutate Bin I and Bin II, respectively, in *dpp*-BM. The primers 5′-GTAAGTCGACAG GAGCCGCAGATCAAAGG-3′ and 5′-CCTGTCGACTTACG CGATTGTAAAACAA-3′ were used to mutate Bin III within *dpp*-PB. All constructs were sequenced, then cloned into pCaSpeRhs43 β-gal, and six transformant lines were analyzed for each construct.

Embryo staining

The following primary antibodies were used: rabbit anti-β-galactosidase (1:3000; Cappel), rabbit anti-Bap and rabbit anti-Eve (1:500 and 1:2000, respectively; Lee and Frasch 2000), rabbit anti-β3-Tubulin (1:1500; gift from R. Renkawitz-Pohl, Marburg University, Germany), mouse anti-Fasciclin III (7G10, 1:20; Developmental Studies Hybridoma Bank, Univ. of Iowa), mouse anti-Ubx (1:10; gift from Rob White, Cambridge University, England), mouse anti-myosin heavy chain (1:5; gift from D. Kiehart, Duke University, Durham, NC), and rat anti-Bin (1:500; this work). For Bap and Bin antibody fluorescent stainings, Tyramide Signal Amplification (NEN) in conjunction with the VectaStain ABC components and microscopy was done as in Zaffran and Frasch (2000). Bin antibodies were raised in rats against a His fusion protein containing amino acids 135–615 of Bin (*bin Bam*HI/*Bam*HI cDNA fragment) induced from a pET30a expression vector (Novagen).

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References

Aitola, M., Carlsson, P., Mahlapuu, M., Enerback, S., and Pelto-Huikko, M. 2000. Forkhead transcription factor FoxF2 is expressed in mesodermal tissues involved in epithelio–mesenchymal interactions. *Dev. Dyn.* **218:** 136–149.

Anderson, M., Perkins, G., Chittick, P., Shrigley, R., and Johnson, W. 1995. *drifter*, a *Drosophila* POU-domain transcription factor, is required for correct differentiation and migration of tracheal cells and midline glia. *Genes & Dev.* **9:** 123–137.

Azpiazu, N. and Frasch, M. 1993. *tinman* and *bagpipe*: Two homeo box genes that determine cell fates in the dorsal mesoderm of *Drosophila*. *Genes & Dev.* **7:** 1325–1340.

Azpiazu, N., Lawrence, P., Vincent, J.-P., and Frasch, M. 1996. Segmentation and specification of the *Drosophila* mesoderm. *Genes & Dev.* **10:** 3183–3194.

Bienz, M. 1994. Homeotic genes and positional signalling in the *Drosophila* viscera. *Trends Genet.* **10:** 22–26.

Bodmer, R. 1993. The gene *tinman* is required for specification of the heart and visceral muscles in *Drosophila*. *Development* **118:** 719–729.

Brand, A.H. and Perrimon, N. 1993. Targeted gene expression as a means of altering cell fates and generating dominant phenotypes. *Development* **118:** 401–415.

Brown, N.H. and Kafatos, F.C. 1988. Functional cDNA libraries from *Drosophila* embryos. *J. Mol. Biol.* **203:** 425–437.

Campos-Ortega, J.A. and Hartenstein, V. 1997. *The embryonic development of Drosophila melanogaster*. Springer Verlag, Berlin, Germany.

Capovilla, M., Brandt, M., and Botas, J. 1994. Direct regulation of *decapentaplegic* by *Ultrabithorax* and its role in *Drosophila* midgut morphogenesis. *Cell* **76:** 461–475.

Chan, S.K., Jaffe, L., Capovilla, M., Botas, J., and Mann, R.S. 1994. The DNA binding specificity of Ultrabithorax is modulated by cooperative interactions with extradenticle, another homeoprotein. *Cell* **78:** 603–615.

Frasch, M. 1995. Induction of visceral and cardiac mesoderm by ectodermal Dpp in the early *Drosophila* embryo. *Nature* **374:** 464–467.

Fremion, F., Astier, M., Zaffran, S., Guillen, A., Homburger, V., and Semeriva, M. 1999. The heterotrimeric protein Go is required for the formation of heart epithelium in *Drosophila*. *J. Cell Biol.* **145:** 1063–1076.

Funayama, N., Sato, Y., Matsumoto, K., Ogura, T., and Takahashi, Y. 1999. Coelom formation: Binary decision of the lateral plate mesoderm is controlled by the ectoderm. *Development* **126:** 4129–4138.

Georgias, C., Wasser, M., and Hinz, U. 1997. A basic-helix-loop-helix protein expressed in precursors of *Drosophila* longitudinal visceral muscles. *Mech. Dev.* **69:** 115–124.

Goldstein, M.A. and Burdette, W.J. 1971. Striated visceral muscle of *Drosophila melanogaster*. *J. Morphol.* **134:** 315–334.

Greig, S. and Akam, M. 1993. Homeotic genes autonomously specify one aspect of pattern in the *Drosophila* mesoderm. *Nature* **362:** 630–632.

Häcker, U., Kaufmann, E., Hartmann, C., Jürgens, G., Knöchel, W., and Jäckle, H. 1995. The *Drosophila* fork head domain protein *crocodile* is required for the establishment of head structures. *EMBO J.* **14:** 5306–5317.

Hellqvist, M., Mahlapuu, M., Samuelsson, L., Enerback, S., and Carlsson, P. 1996. Differential activation of lung-specific genes by two forkhead proteins, FREAC-1 and FREAC-2. *J. Biol. Chem.* **271:** 4482–4490.

Holley, S. and Ferguson, E. 1997. Fish are like flies are like frogs: Conservation of dorsal–ventral patterning mechanisms. *BioEssays* **19:** 281–284.

Hursh, D., Padgett, R., and Gelbart, W. 1993. Cross regulation of *decapentaplegic* and *Ultrabithorax* transcription in the embryonic visceral mesoderm of *Drosophila*. *Development* **117:** 1211–1222.

Immerglück, K., Lawrence, P., and Bienz, M. 1990. Induction across germ layers in *Drosophila* mediated by a genetic cascade. *Cell* **62:** 261–268.

Kaufmann, E., Müller, D., and Knöchel, W. 1995. DNA recognition site analysis of *Xenopus* winged helix proteins. *J. Mol. Biol.* 239–254.

Köster, M., Dillinger, K., and Knöchel, W. 1999. Genomic structure and embryonic expression of the *Xenopus* winged helix factors XFD-13/13′. *Mech. Dev.* **88:** 89–93.

Kusch, T. and Reuter, R. 1999. Functions for *Drosophila brachyenteron* and forkhead in mesoderm specification and cell signalling. *Development* **126:** 3991–4003.

Lee, H. and Frasch, M. 2000. Wingless effects mesoderm

patterning and ectoderm segmentation events via induction of its downstream target *sloppy paired*. *Development* **127:** 5497–5508.

Lo, P. and Frasch, M. 1998. *bagpipe*-dependent expression of *vimar*, a novel armadillo-repeats gene, in the *Drosophila* visceral mesoderm. *Mech. Dev.* **72:** 65–75.

Mahlapuu, M., Pelto-Huikko, M., Aitola, M., Enerback, S., and Carlsson, P. 1998. FREAC-1 contains a cell-type-specific transcriptional activation domain and is expressed in epithelial-mesenchymal interfaces. *Dev. Biol.* **202:** 183–195.

Mahlapuu, M., Ormestad, M., Enerback, S., and Carlsson, P. 2001. The forkhead transcription factor Foxf1 is required for differentiation of extra-embryonic and lateral plate mesoderm. *Development* **128:** 155–166.

Manak, J., Mathies, L., and Scott, M. 1994. Regulation of a *decapentaplegic* midgut enhancer by homeotic proteins. *Development* **120:** 3605–3619.

Masucci, J.D. and Hoffmann, F.M. 1993. Identification of two regions from the *Drosophila decapentaplegic* gene required for embryonic midgut development and larval viability. *Dev. Biol.* **159:** 276–287.

Panganiban, G.E.F., Reuter, R., Scott, M.P., and Hoffmann, F.M. 1990. A *Drosophila* growth factor homolog, *decapentaplegic*, regulates homeotic gene expression within and across germlayers during midgut morphogenesis. *Development* **110:** 1041–1050.

Patel, N.H., Snow, P.M., and Goodman, C.S. 1987. Characterization and cloning of fasciclin III: A glycoprotein expressed on a subset of neurons and axon pathways in *Drosophila*. *Cell* **48:** 975–988.

Peterson, R., Lim, L., Ye, H., Zhou, H., Overdier, D., and Costa, R. 1997. The winged helix transcriptional activator HFH-8 is expressed in the mesoderm of the primitive streak stage of mouse embryos and its cellular derivatives. *Mech. Dev.* **69:** 53–69.

Reuter, R., Panganiban, G.E.F., Hoffmann, F.M., and Scott, M.P. 1990. Homeotic genes regulate the spatial expression of putative growth factors in the visceral mesoderm of *Drosophila* embryos. *Development* **110:** 1031–1040.

Reuter, R., Grunewald, B., and Leptin, M. 1993. A role for the mesoderm in endodermal migration and morphogenesis in *Drosophila*. *Development* **119:** 1135–1145.

Riechmann, V., Irion, U., Wilson, R., Grosskortenhaus, A., and Leptin, M. 1997. Control of cell fates and segmentation in the *Drosophila* mesoderm. *Development* **124:** 2915–2922.

Sandborn, E., Duclos, S., Messier, P., and Roberge, J. 1967. Atypical intestinal striated muscle in *Drosophila melanogaster*. *J. Ultrastruct. Res.* **18:** 695–702.

Staehling-Hampton, K., Hoffmann, F.M., Baylies, M.K., Rushton, E., and Bate, M. 1994. *dpp* induces mesodermal gene expression in *Drosophila*. *Nature* **372:** 783–786.

Sun, B., Hursh, D., Jackson, D., and Beachy, P. 1995. Ultrabithorax protein is necessary but not sufficient for full activation of *decapentaplegic* expression in the visceral mesoderm. *EMBO J.* **14:** 520–535.

Taylor, M. 2000. A novel *Drosophila*, *mef2*-regulated muscle gene isolated in a subtractive hybridisation-based molecular screen using small amounts of zygotic mutant RNA. *Dev. Biol.* **220:** 37–52.

Thüringer, F., Cohen, S., and Bienz, M. 1993. Dissection of an indirect autoregulatory response of a homeotic *Drosophila* gene. *EMBO J.* **12:** 2419–2430.

Tonegawa, A., Funayama, N., Ueno, N., and Takahashi, Y. 1997. Mesodermal subdivision along the mediolateral axis in chicken controlled by different concentrations of BMP-4. *Development* **124:** 1975–1984.

Tribioli, C., Frasch, M., and Lufkin, T. 1997. *Bapx1*: An evolutionary conserved homologue of the *Drosophila bagpipe* homeobox gene is expressed in splanchnic mesoderm and the embryonic skeleton. *Mech. Dev.* **65:** 145–162.

Xu, X., Yin, Z., Hudson, J., Ferguson, E., and Frasch, M. 1998. Smad proteins act in combination with synergistic and antagonistic regulators to target Dpp responses to the *Drosophila* mesoderm. *Genes & Dev.* **12:** 2354–2370.

Yang, X., van Beest, M., Clevers, H., Jones, T., Hursh, D., and Mortin, M. 2000. *decapentaplegic* is a direct target of dTcf repression in the *Drosophila* visceral mesoderm. *Development* **127:** 3695–3702.

Yin, Z. and Frasch, M. 1998. Regulation and function of *tinman* during dorsal mesoderm induction and heart specification in *Drosophila*. *Dev. Genet.* **22:** 187–200.

Yu, X., Hoppler, S., Eresh, S., and Bienz, M. 1996. *decapentaplegic*, a target gene of the wingless signalling pathway in the *Drosophila* midgut. *Development* **122:** 849–858.

Zaffran, S. and Frasch, M. 2000. *Barbu*: An *E(spl) m4/m*(α)-related gene that antagonizes Notch signaling and is required for the establishment of ommatidial polarity. *Development* **127:** 1115–1130.