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Control of epithelial morphogenesis by cell signaling and integrin molecules in the *Drosophila* foregut

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SUMMARY

Coordinated cell movements are critical for tissue and organ morphogenesis in animal development. We show that the *Drosophila* genes *hedgehog* and *wingless*, which encode signaling molecules, and the gene *myospheroid*, which encodes a β subunit of the integrins, are required for epithelial morphogenesis during proventriculus development. In contrast, this morphogenetic process is suppressed by the *decapentaplegic* gene, which encodes a member of the TGFβ family of growth factors. These results identify a novel cell signaling center in the foregut that directs the formation of a multiply folded organ from a simple epithelial tube.

Key words: *hedgehog*, *wingless*, *decapentaplegic*, integrins, gut, morphogenesis

INTRODUCTION

Pattern formation and morphogenesis are two interconnected processes in animal development (Gurdon, 1992). In *Drosophila*, great progress has been made on the genetic and molecular interactions that establish the body pattern in the early embryo (St Johnston and Nüsslein-Volhard, 1992; Hoch and Jäckle, 1993; Pankratz and Jäckle, 1993). Much less is known on the morphogenetic mechanisms that bring about the diverse tissues and organs of the body. In contrast to the early pattern forming processes that occur in a syncitium, these later developmental events involve interactions of cells with each other and with their extracellular environment.

One class of molecules important for such interactions are secreted molecules involved in intercellular signaling, and several conserved families of secreted growth and differentiation factors have been identified in different organisms (Jessell and Melton, 1992; Greenwald and Rubin, 1992, for reviews). The *Drosophila* gene *hedgehog* (*hh*), which encodes a secreted protein, and the genes *wingless* (*wg*) and *decapentaplegic* (*dpp*), which encode members of the Wnt and TGFβ families of growth factors, respectively, have been shown to act in assorted combinations in a variety of developmental contexts: *hh* and *wg* are required for epidermal segment patterning (Nüsslein-Volhard and Wieschaus, 1980); *hh* and *dpp* are required for the progression of the morphogenetic furrow in the eye (Ma et al., 1993; Heberlein et al., 1993); *wg* and *dpp* are required for the second constriction in the midgut (Immerglück et al., 1990; Panganiban et al., 1990; Reuter et al., 1990); and all three are required for limb patterning (Basler and Struhl, 1994; Diaz-Benjumea et al., 1994). Their vertebrate homologues have also been shown to be involved in patterning and induction during embryonic development (Kessler and Melton, 1994, for review).

Parallel studies have shown that cell adhesion molecules are also important mediators of cell interactions. One of the major classes of molecules modulating cell adhesion are the integrins, originally identified in vertebrates. Integrins belong to a family of cell surface adhesion receptors that mediate cell-cell and cell-extracellular matrix interactions (Hynes, 1992, for review). All integrins are αβ heterodimers, and an individual β subunit can associate with several different α subunits to form functional receptors with varying ligand and adhesive specificities. To date, 14 α and 8 β subunits are known in vertebrates. Integrins have also been identified in *Drosophila*, where they have been shown to be required for adhesion between different cell types and layers. These include attachments between muscles and epidermis, the visceral mesoderm and the adhering endoderm, and the dorsal and ventral parts of the wing blades (Newman and Wright, 1981; Wilcox et al., 1989; Leptin et al., 1989; Zusman et al., 1990, 1993; Brown, 1994).

In this paper, we describe a cellular system in which the roles of the cell signaling and cell adhesion molecules can be studied in a single developmental context. We show that epithelial morphogenesis during proventriculus organ development requires the activities of *wg*, *hh* and *dpp*, as well as the integrin class of cell surface adhesion receptors. We further provide evidence that cell signaling in the foregut operates through a distinct genetic circuitry as that in the midgut.

MATERIALS AND METHODS

Drosophila stocks

We used the following stocks: Oregon R, wg^{IG22}, wg^{IL114}, arm^{XK}, hh^{IJ35}, ci^{D}, dpp^{48}, ptc^{IN108}, en^{IIB86}, en^{IK57} (provided by the Tübingen and Umea stock centers), HS-*dpp* (a gift from S. Cohen), HS-*hh* (a gift of P. Ingham), HS-*wg* (a gift of S. Cohen), HS-*ptc* (a gift of I. Guerrero), and mys^{XG43} and if^{K27e} (gifts of M. Affolter). The flies were maintained and embryo collections made according to standard procedures.

Immunocytochemistry and in situ hybridization

BrdU (Sigma) labeling was performed, with modifications for embryos, essentially as described (Truman and Bate, 1988). The embryos were incubated for 30 minutes with BrdU prior to fixation.

Antibody staining of whole-mount embryos was carried out as described previously (Macdonald and Struhl, 1986), using the Vectastain ABC Elite-horseradish peroxidase system. $NiCl_2$ or $Ni/CoCl_2$ enhancement was used where necessary. The stained embryos were embedded in Araldite in capillaries according to the procedure of Schmidt-Ott and Technau (1992).

We used the following antibodies at the dilutions indicated in parenthesis: mAb22C10 (Zipursky et al., 1984; 1:20), anti-β-galactosidase (Cappel; 1:10000), anti-armadillo (Riggleman et al., 1990; 1:100), anti-MHC (Kiehart and Feghali, 1986; 1:1000), anti-forkhead (Weigel et al., 1989a; 1:150), and anti-crumbs (Tepass et al., 1990; 1:50). All antibodies were preabsorbed against wild-type embryos before use.

In situ hybridization was performed essentially as described in Tautz and Pfeifle (1989). The probes used were: *dpp* (a gift of S. Cohen), *hh* (a gift of P. Ingham), *wg* (a gift of S. Cohen), *ptc* (a gift of I. Guerrero), *en* (a gift of S. Cohen), *ci* (a gift of R. Holmgren), α1, α2 and β-integrins (gifts of T. Bunch and D. Brower).

Heat-shock protocols

HS-*dpp:* 0-20 hour embryo collections at 18°C were placed at 37°C for 45 minutes two times with 3 hours at 18°C between each heat shock, allowed to recover for 3 more hours at 25°C, then fixed as described for immunohistochemical staining. The same protocol with wild type, HS-*ptc*, HS-*hh* or HS-*wg* harboring transgenic strains did not produce proventricular defects.

wg^{IL114}: 0-20 hour collections were taken at 18°C and then the embryos were transferred to 29°C for 12-16 hours and fixed as above. For the feeding assay, 0-24 hour embryos were placed at 29°C for 1 hour and then returned to 18°C until hatching.

Feeding assay

The larvae were allowed to grow on applejuice plates containing yeast that had been dyed with Carmine red (Sigma). Mutant feeding phenotypes were scored at various times under the dissecting microscope.

RESULTS

Morphogenesis of the proventricular epithelium

The foregut of the *Drosophila* larva is functionally and structurally subdivided into the pharynx, the esophagus and the proventriculus (Fig. 1A,B). The proventriculus is located at the caudal end of the esophagus and serves as a valve in regulating food passage into the midgut (Strasburger, 1932; Graham-Smith, 1934; Rizki, 1956). It is composed of two tissue layers, the ectodermal epithelial layer and the ensheathing visceral mesoderm (Fig. 1C,D; Skaer, 1993, for review). An exception is the area that will form the inside portion of the proventriculus, which is completely free of mesodermal tissues (Fig. 1D; Tepass and Hartenstein, 1994). This internal portion, called the cardiac valve (the proventriculus is also referred to as the cardia, Snodgrass, 1935; King, 1988), is innervated by three axons from the proventricular ganglion (Fig. 1F), one of four major interconnected ganglia that constitute the stomatogastric nervous system (Poulson, 1950; Willey, 1961; Schoeller, 1964; Penzlin, 1985; Campos-Ortega and Hartenstein, 1985; Hartenstein et al., 1994).

The proventriculus develops at the junction of the foregut and the midgut (Fig. 2A; Poulson, 1950; Campos-Ortega and Hartenstein, 1985). There is initially an outward buckling of the foregut tube, in a region that is free of visceral mesoderm, to form what we refer to as the 'keyhole' structure (Fig. 2B). This area will undergo further outward movement, then fold back on itself and move inwards to form the mature, multilayered proventriculus (Fig. 2C). The cells moving inwards assume a stretched appearance with long cytoplasmic extensions (Fig. 1E). These late steps in proventriculus morphogenesis are due to migration of cells and are not accompanied by cell proliferation, as assayed by BrdU incorporation experiments (data not shown; Hartenstein and Campos-Ortega, 1985). A major advantage of the proventriculus for studying morphogenesis is the relative ease with which one can monitor the movement of the epithelial cells at all stages of development.

Control of proventriculus morphogenesis by *hh* and *wg*

Both *hh* and *wg* are expressed in spatially restricted domains in the developing proventricular epithelium. *hh* is expressed in the keyhole region (Fig. 3A,C) and persists until the late stages of proventriculus formation (Fig. 3B,D). The expression pattern of *wg* is more dynamic. It is initially expressed as a contiguous band spanning the keyhole (Fig. 3E); this domain then splits in the middle to form two narrow bands that now flank the keyhole (Fig. 3G). The two expression bands remain until the final stages of proventriculus development (Fig. 3F,H). The *hh* and *wg* expression domains show striking spatial correspondence with the mesoderm-free area: the posterior border of *hh* corresponds with one of the mesoderm borders while the two *wg* domains flank both mesodermal borders (Fig. 3C,G).

To correlate these gene expression patterns with morphogenetic function, we examined the proventriculus phenotypes of the corresponding mutant embryos using anti-forkhead (nuclear marker for the foregut epithelial cells), anti-myosin heavy chain (MHC) (mesodermal muscle marker) or anti-crumbs (epithelial apical membrane marker) antibodies (see Materials and Methods). In *hh* mutants, the keyhole structure develops normally, but the internal cardiac valve is not formed; the outer wall of the proventriculus is still present but is narrow and hollow as compared to the wild-type morphology (Fig. 4A,B). There are clearly foregut cells on top of this hollow proventriculus, but they are clustered and crowded, suggesting that the defect observed in *hh* mutants is due to the failure of the cells to move into the internal region of the proventriculus. For descriptive purposes, we use the term 'cardiac arrest' phenotype to describe this specific type of proventriculus defect. In some mutant embryos, the foregut tube succeeds in inserting into the proventriculus but does not complete the full range of movement into the midgut (data not shown).

Interpreting the phenotype of *wg* mutants through analysis of null alleles is problematic since most of the esophagus and the proventriculus are missing due to failure of the stomodeum to invaginate properly (Skaer, 1993; see also Fig. 4C). However, the analysis of a temperature-sensitive allele (wg^{ts}) demonstrates that *wg* activity is required for morphogenetic movements during proventriculus development (Fig. 4D). This phenotype is very similar to what is observed in *hh* mutants.

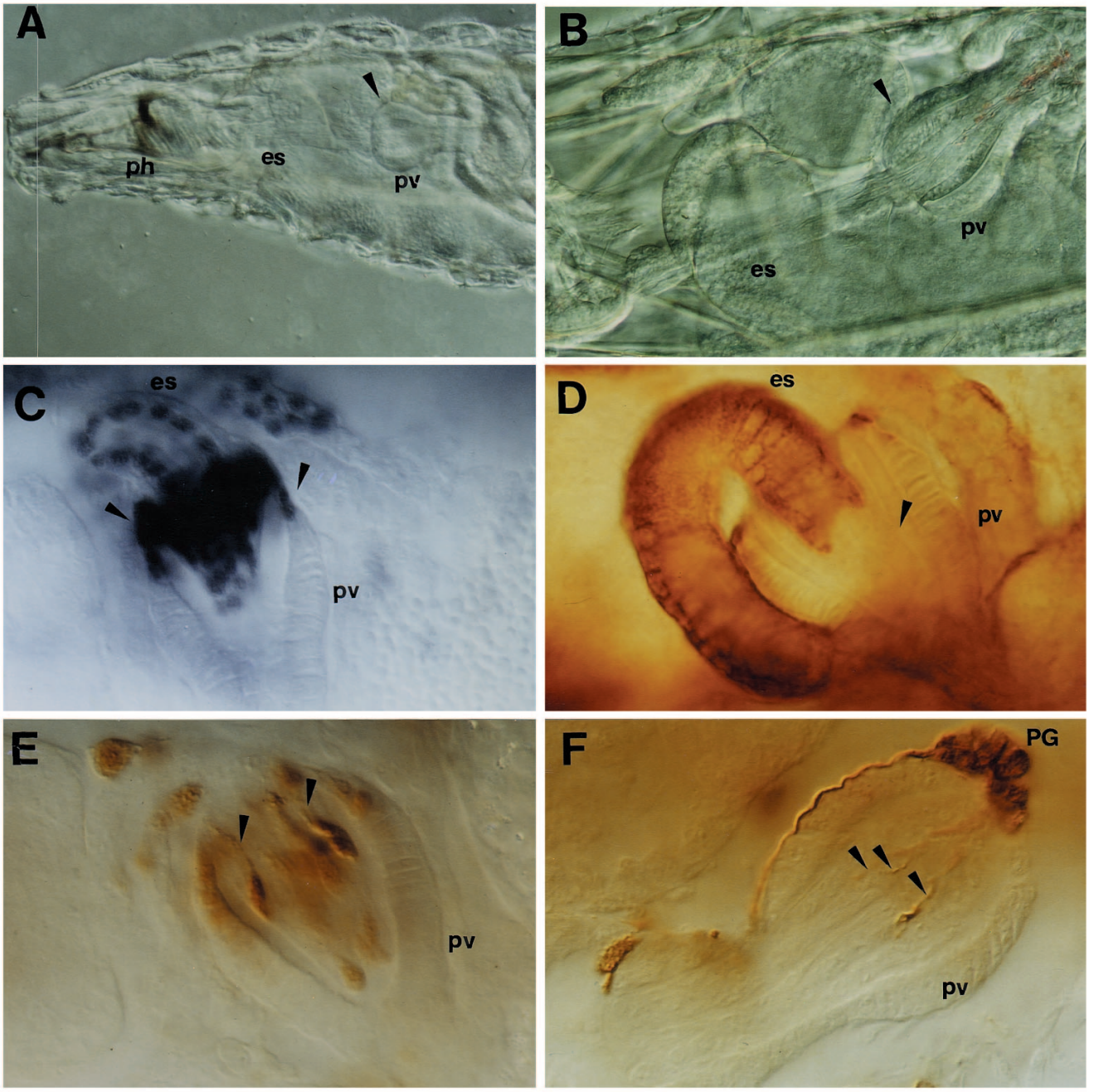

Fig. 1. Structure of the proventriculus. (A) A live larva showing the three major subdivisions of the foregut: the pharynx (ph), the esophagus (es), and the proventriculus (pv; arrowhead). (B) Higher magnification of the proventriculus (arrowhead) of a live larva. This larva has been feeding on yeast dyed with Carmine red. Note the red material at the posterior part of the proventriculus, reflecting the passage of dyed yeast into the midgut. (C) Proventriculus of late embryo (stage 17) stained with anti-fkh antibody. The fkh staining cells in the region between the two arrowheads will move inwards. Note that the nuclei in the esophagus are widely spaced apart, whereas those in the proventriculus are tightly packed. The altered distance between these nuclei most likely reflects alterations in cell shape as they move. (D) Proventriculus of a slightly older embyo than in C, in which the cells have moved down further, stained with anti-MHC antibody. Arrowhead points to the internal epithelial region which is not ensheathed by visceral muscles. (E) Proventriculus of late embryo (stage 17) harboring an enhancer trap construct driving expression of*lacZ* in the proventricular cells (Hoch and Jäckle, unpublished data), stained with anti-β-galactosidase antibody. Note long processes of the cell cytoplasm (arrowheads). (F) Three axons (arrowheads) from the proventricular ganglion (PG) innervating the cardiac valve of the proventriculus, stained with anti-22C10 antibody. This embryo is just before hatching; note that the internal portion of the proventriculus has extended much further down as compared to the embryos shown above. Embryonic stages according to Campos-Ortega and Hartenstein (1985).

There is a wide range of severity with regard to esophageal and proventricular defects, which most likely reflects the precise time at which the embryo has experienced the temperature shift. To see whether we could obtain proventricular defects in the absence of any other morphological foregut defects, we performed pulsed temperature-shift experiments and tested the larvae for their ability to feed (see Materials and Methods), the reasoning being that if the proventriculus does not function properly due to specific structural defects, the larvae should also not be able to feed normally. Using this assay we could, remarkably, recover larvae that move about normally but which cannot feed due to a specific defect in the inward movement of the proventricular cells (Fig. 4E,F); consequently, the food cannot be efficiently transported into the midgut, resulting in an engorged esophagus (Fig. 4E).

The above results indicate that both *hh* and *wg* activities are required for proper epithelial morphogenesis of the proventriculus. *hh* and *wg* are members of the segment polarity class of genes, which constitute a cell signaling network in epidermal patterning (Martinez Arias, 1993; Perrimon, 1994, for reviews). The gene *cubitus interruptus* (*ci*), which encodes a zinc-finger transcription factor, is another member of the segment polarity genes and is expressed in the keyhole region (Fig. 4G); unlike *hh* and *wg*, this expression domain disappears soon afterwards (data not shown). In *ci* mutant embryos, the keyhole structure does not form (Fig. 4H). Thus, *ci* could be involved in the specification and/or outgrowth of the foregut region to form the keyhole structure. However, not all of the segment polarity genes are involved in proventriculus morphogenesis. For example, the gene *patched* (*ptc*), which encodes a transmembrane protein (Hooper and Scott, 1989; Ingham et al., 1991), is expressed in the foregut but *ptc* mutants do not show the cardiac arrest proventricular phenotype (data not shown). Most notably, *engrailed* (*en*), which plays a key role in epidermal as well as limb patterning, is not expressed in the foregut, and *en* mutants show no proventricular defects (not shown).

Control of proventriculus morphogenesis by Armadillo and integrins

The range of movements that the cells of the keyhole region undergo in forming the proventriculus suggested that factors involved in cell-cell or cell-extracellular matrix adhesions may also be important for this process. In addition, it has been documented that the region inside the proventriculus is rich in extracellular matrix components (King, 1988). We therefore investigated the role of genes involved in cell adhesion: *armadillo* (*arm*), encoding a homolog of the vertebrate β-catenin which is a component of the adherens junctions (Peifer and Wieschaus, 1990; Peifer, 1993; Peifer et al., 1993), and genes encoding the integrin subunits, which form heterodimeric cell surface receptors involved in cell-extracellular matrix interactions (Hynes, 1992; Brown, 1993, for reviews).

As *arm* has been shown to be post-transcriptionally regulated (Riggleman et al., 1990), we used, in the case of *arm*, antibodies to monitor the distribution of the gene product. Armadillo is distributed throughout the foregut but is concentrated in specific areas of the developing proventriculus. At early stages, it is concentrated near the keyhole region (Fig. 5A); at later stages, Armadillo becomes highly concentrated in areas undergoing the most extensive cell movements (Fig. 5B).

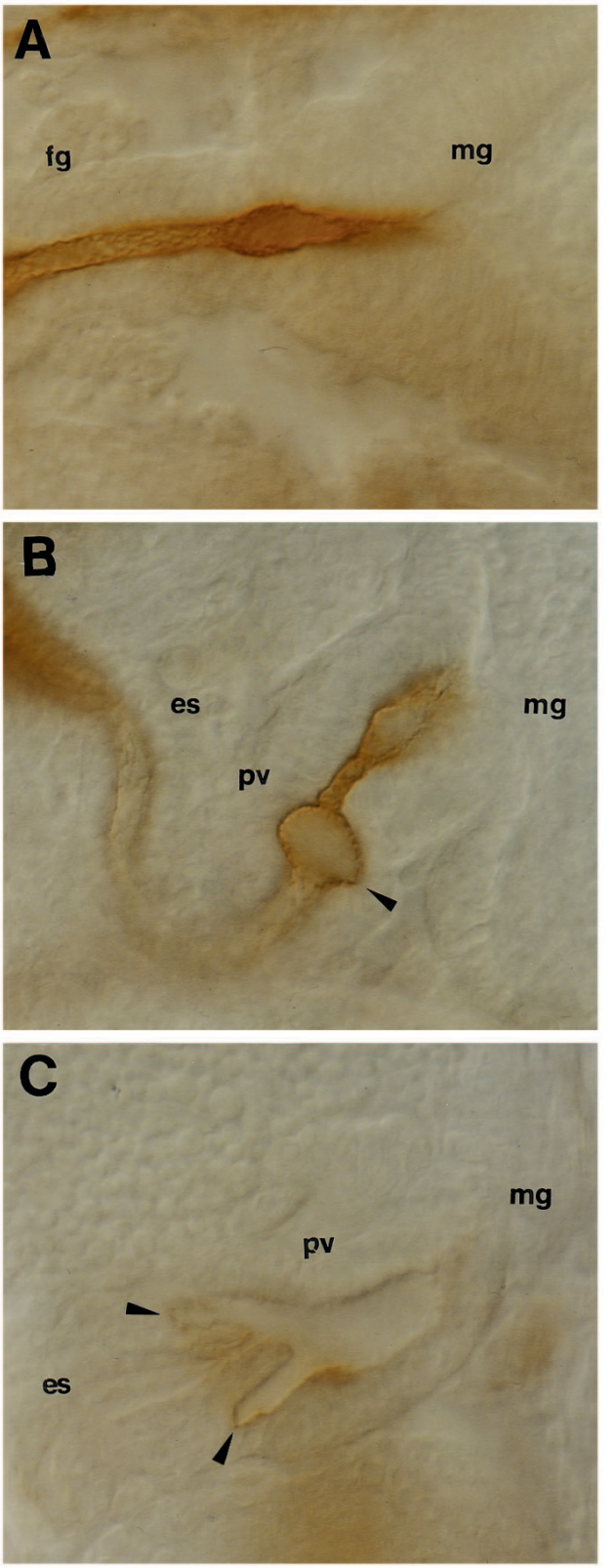

Fig. 2. Cell movements during proventriculus morphogenesis illustrated through anti-crb-antibody staining. (A) Stage 13 embryo showing the foregut (fg) tube abutting the midgut (mg). (B) Stage 15 embryo showing the keyhole region (arrowhead). (C) Stage 17 embryo showing the inward movement (arrowheads) of cells in the proventriculus (pv).

In *arm* mutant embryos, the proventriculus phenotype is similar to that of *wg* null embryos in that the entire foregut region is affected (Fig. 6A,B). The phenotype can vary, and we observe embryos in which the foregut tube has formed to a greater degree; a comparable temperature-sensitive allele as that of *wg* is not available for *arm* at this point, so we do not

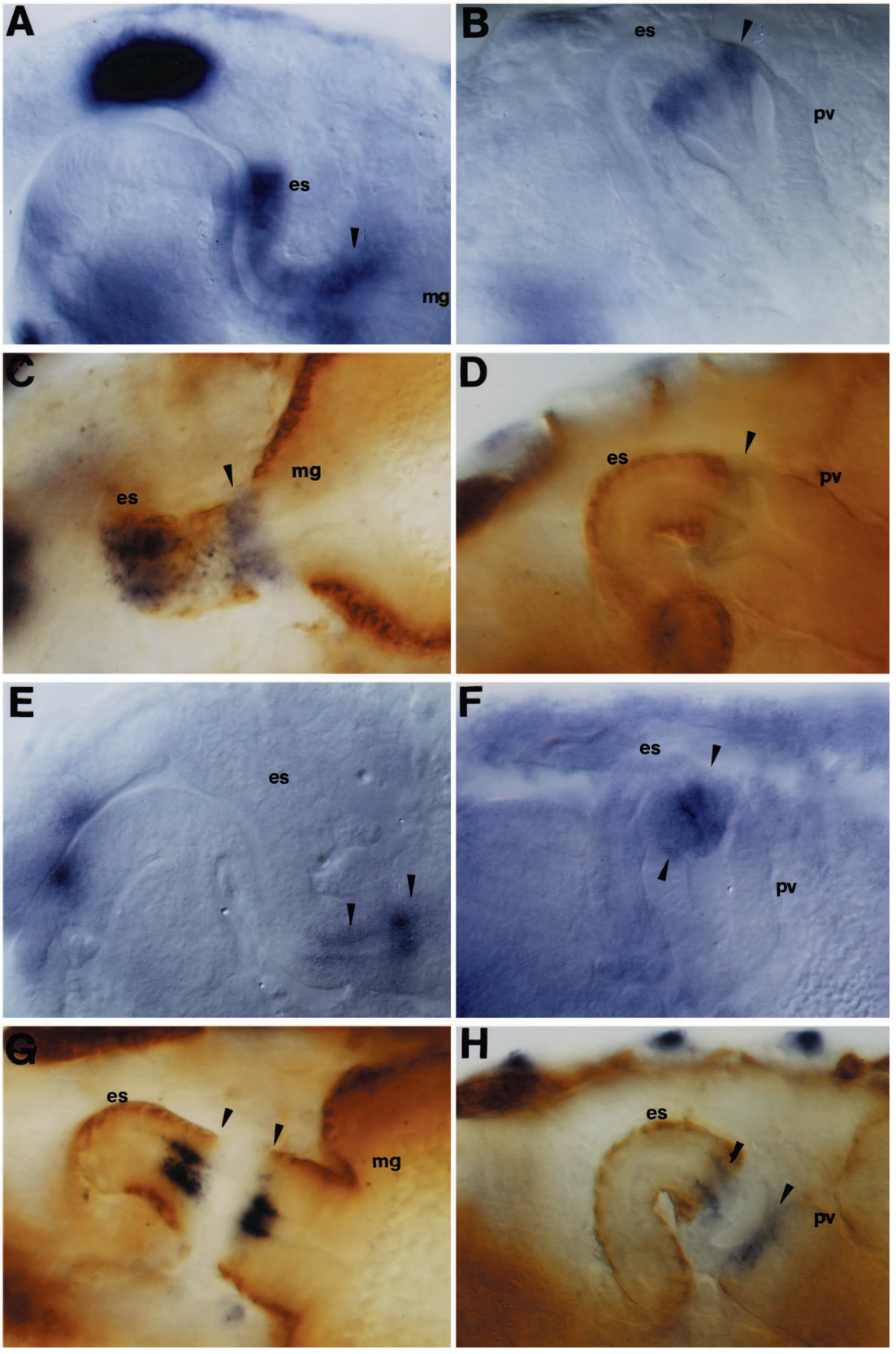

Fig. 3. Whole-mount in situ hybridization patterns of hh and wg in the proventriculus. (A) Stage 15 embryo showing *hh* expression in the keyhole region (arrowhead), which will later form the internal portion of the proventriculus. (B) Stage 17 embryo showing *hh* expression in the region that will move inwards (arrowhead). (C) Double labeling with anti-MHC antibody (brown) and *hh* (blue) probe of stage 15 embryo; arrowhead denotes the mesoderm free keyhole region. (D) Double labeling with anti-MHC and *hh* probe of stage 16 embryo; the relative position of MHC and *hh* stainings (arrowhead) is maintained. (E) Stage 15 embryo showing *wg* expression in the keyhole region, just before the expression domain splits (region between arrowheads). Note that *wg* transcripts are localized to the apical side of the cells. (F) Stage 16 embryo showing *wg* expression in cells that will move inwards (the two arrowheads). (G) Double labeling with anti-MHC antibody (brown) and *wg* (blue) probe of stage 15 embryo; the two arrowheads denote borders of the mesoderm-free zone. By this stage, the *wg* expression domain has split. (H) Double labeling with anti-MHC antibody and *wg* probe of stage 16 embryo; the relative position of MHC and *wg* stainings is maintained (the two arrowheads). Abbreviations: es, esophagus; mg, midgut; pv, proventriculus.

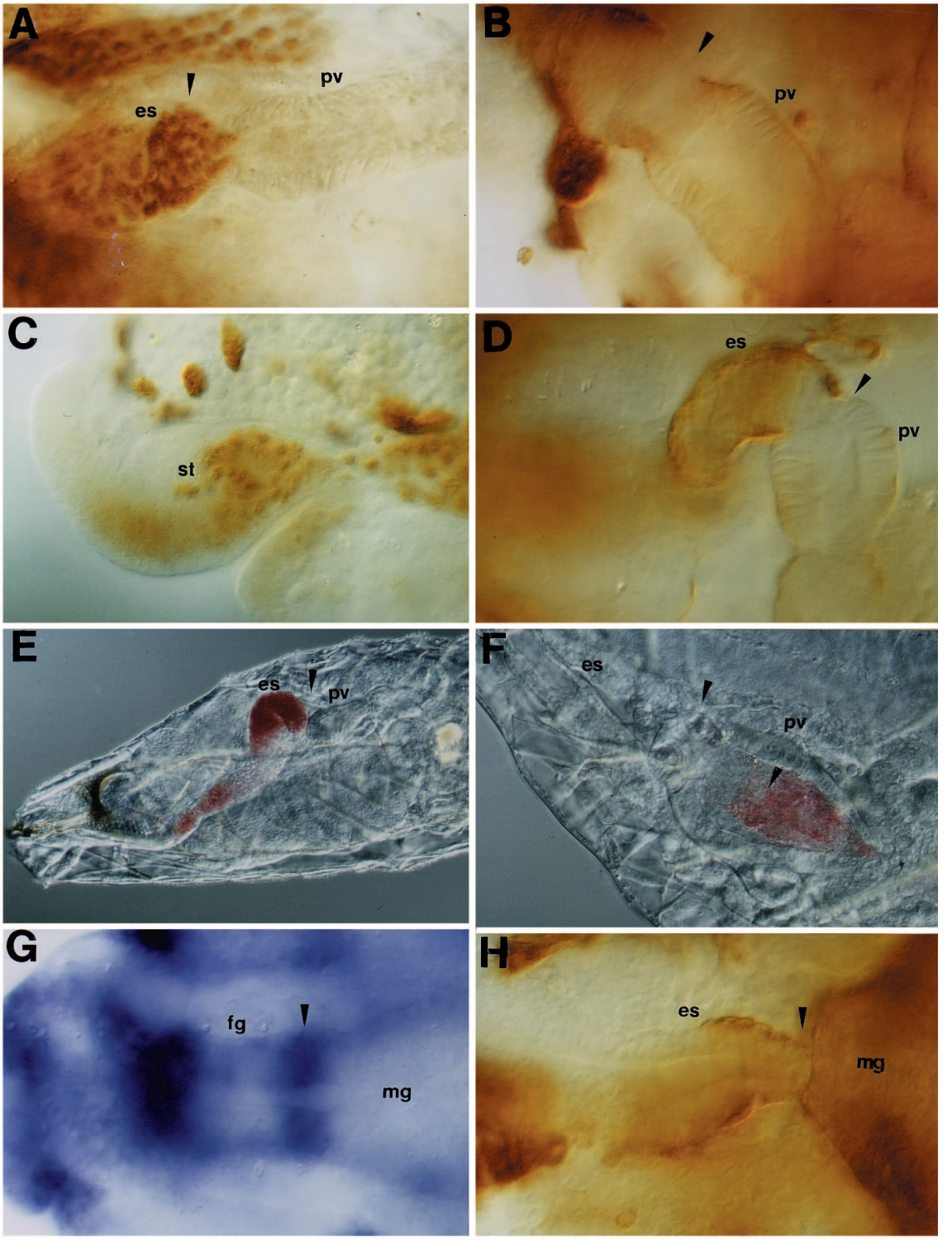

Fig. 4. Proventricular phenotypes of hh and wg mutants. (A) *hh* mutants stained with anti-fkh antibody, showing the cluster of foregut cells (arrowhead) that do not migrate inwards. We refer to this as the 'cardiac arrest ' phenotype. Note that the proventriculus has a much narrower appearence due to the absence of the internal cardiac valve portion (stage 17 embryo). (B) *hh* mutants stained with anti-MHC antibody, marking the point where the cells fail to move inwards (arrowhead). (C) wg^{ts} mutants raised at non-permissive temperature stained with anti-fkh antibody, showing the null phenotype; the stomodeum (st) fails to invaginate properly. (D-F) wg^{ts} mutants shifted to non-permissive temperatures at various times after egg laying (see Materials and Methods). (D) The outer portion of the proventriculus as well as the esophagus have formed but the proventricular cells do not move inwards (arrowhead), resulting in a very similar proventricular phenotype as in *hh* mutants (stage 17 embryo, stained with anti-MHC antibody). (E) Larva derived from wg^{ts} embryos exposed to a one hour pulse of non-permissive temperature, fed with coloured yeast. The specific proventricular defect in which the foregut cells fail to move into the proventriculus (arrowhead) prevents food from entering into the midgut, resulting in an engorged esophagus. Compare this larva with the wild type which has also been fed with red yeast (Fig. 1B). (F) Another larva from the same experiment as in E, showing a slight variation in the phenotype. In this case, the foregut cells have partly moved inwards but do not complete their migration. The lower arrow indicates the limit of the cell migration; the upper arrow indicates the cells that have failed to move inwards and remain looped out on top of the proventriculus. This defect causes the red food material to fill the proventriculus, rather than emptying into the midgut. (G) *ci* expression in stage 12 embryo showing expression in the keyhole region (arrowhead). (H) *ci* mutants stained with anti-MHC antibody, showing the lack of keyhole structure (arrowhead) at the junction of foregut and midgut (stage 14 embryo). Abbreviations: es, esophagus; mg, midgut; fg, foregut.

have unequivocal evidence as to how *arm* acts at later stages of proventriculus development. However, the overlap of the expression patterns between *arm* and *wg* through the final stages of proventriculus formation, the similarities of the foregut phenotypes in the mutants of the two genes, and the demonstration that *wg* activity affects the level of Armadillo in tissue culture cells (van Leeuwen et al., 1994), suggest the requirement of *arm* during proventriculus morphogenesis.

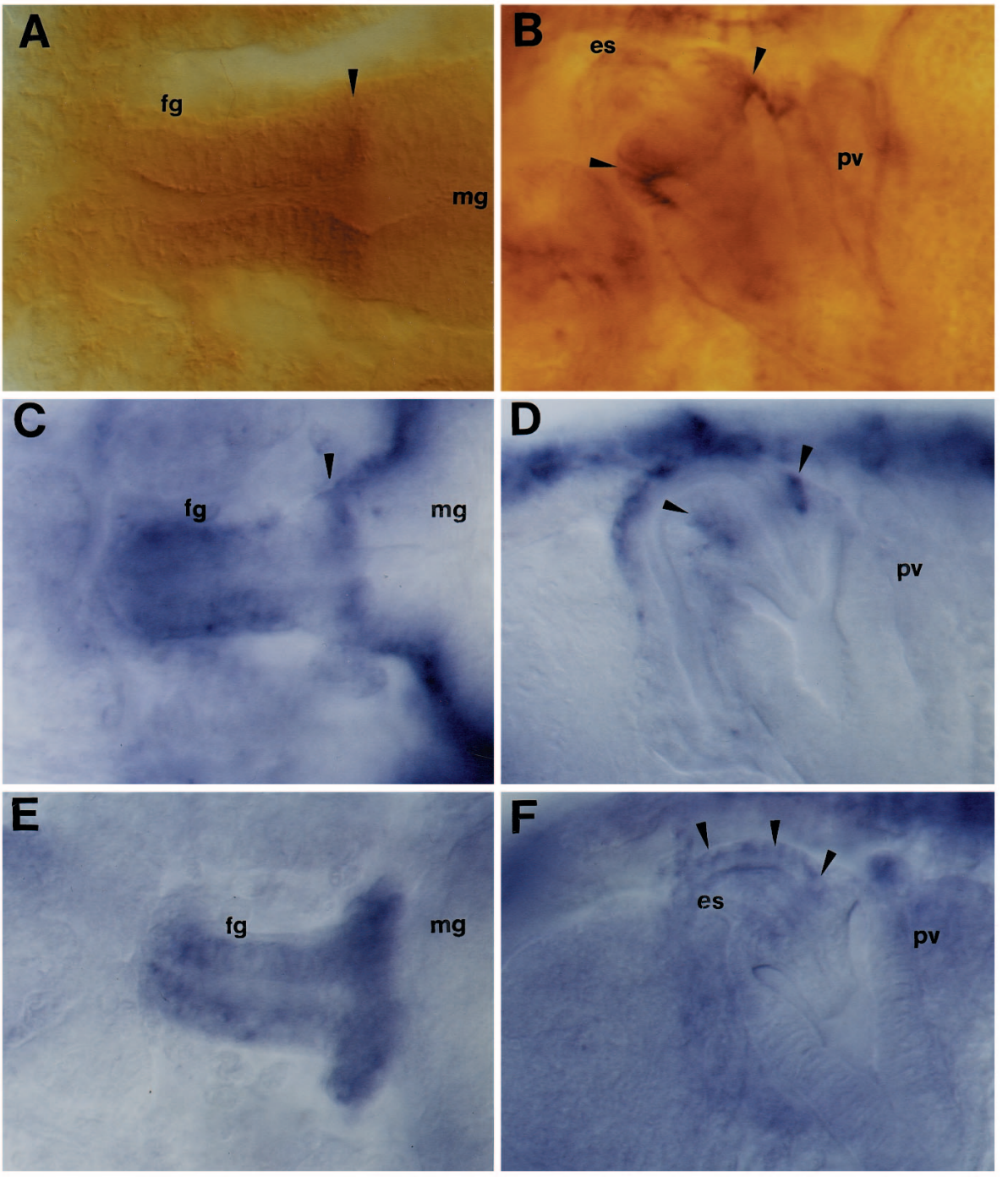

Fig. 5. Localization of *arm* and integrin gene products. (A) Stage 13 embryo stained with anti-arm-antibody; there is a concentrated staining at the anterior side of the keyhole region (arrowhead). (B) Stage 16 embryo stained with anti-arm-antibody; there is a concentrated staining at the region that has folded back on itself and which is about to move inwards (arrowhead). (C,D) In situ hybridization with a probe for the integrin α2 subunit at stages 14 and 17, respectively, showing a band of expression in the keyhole area (C; arrowhead) and expression as the cells are migrating inwards (D; the two arrowheads). (E) In situ hybridization with a probe for the integrin α1 subunit at stage 14, showing staining in the foregut ectoderm. The strong staining at the junction between the foregut and the midgut marks cells that will eventually move on top of the midgut. (F) In situ hybridization with a probe for the integrin β subunit, showing patches of stainings (arrowheads) in the esophagus and the proventriculus. Abbreviations: es, esophagus; mg, midgut; pv, proventriculus; fg, foregut.

The different subunits of the integrins (PS1α, PS2α and PSβ, collectively known as the position-specific integrins) have been shown to be distributed differentially in the developing embryo (Bogaert et al., 1987; Leptin et al., 1989; Zusman et al., 1990; Wehrli et al., 1993). In the foregut region, the α2 subunit is found in the mesodermal layer and as a narrow band in the ectoderm in the keyhole region (Fig. 5C); the expression pattern persists into the late stages of proventriculus development (Fig. 5D). The α1 subunit is found in the foregut ectoderm (Fig. 5E) and remains at low levels at later stages (not shown). The β subunit is uniformly distributed at low levels throughout most of the foregut (Fig. 5F).

The β integrin subunit is encoded by the *myospheroid* (*mys*) gene (MacKrell et al., 1988; Leptin et al., 1989). In *mys* mutant embryos, we observe a proventricular phenotype which is very similar to that of *hh* and wg^{ts} mutant embryos: the cells are clustered at the top of the esophagus, but cannot migrate inside to form the cardiac valve (Fig. 6C,D). The α 2 subunit is encoded by the *inflated* gene (Brower and Jaffe, 1989; Wilcox et al., 1989; Brown, 1994); we did not observe proventricular cell migration defects in *inflated* mutant embryos (data not shown). For the α1 subunit, a mutant for the corresponding gene does not yet exist. Taken together, these results indicate that the integrin β subunit molecules, which can heterodimerize with either of the two α subunits (Brower et al., 1984; Wilcox et al., 1984; Leptin et al., 1987), are required for epithelial morphogenesis in proventriculus development.

Suppression of proventriculus morphogenesis by *dpp*

dpp is expressed only in the anterior region of the foregut tube and not in the keyhole region (Fig. 7A,B). In *dpp* mutants, despite drastic effects on the global morphology of the embryo, much of the proventriculus still forms. However, more cells move into the proventriculus as compared to wild-type embryos (Fig. 7C). In certain embryos, one observes an extra outbudding zone in the foregut epithelium near the proventriculus (Fig. 7D). Although it is not clear whether this is a primary effect of *dpp* mutations or a secondary consequence of the torsional stress brought about by the twisting of the esophagus, it is possible that *dpp* functions to suppress cell movements in more anterior regions of the foregut, thereby allowing only the keyhole region to move outwards. In this case, the extra outbudding zone in the esophagus could

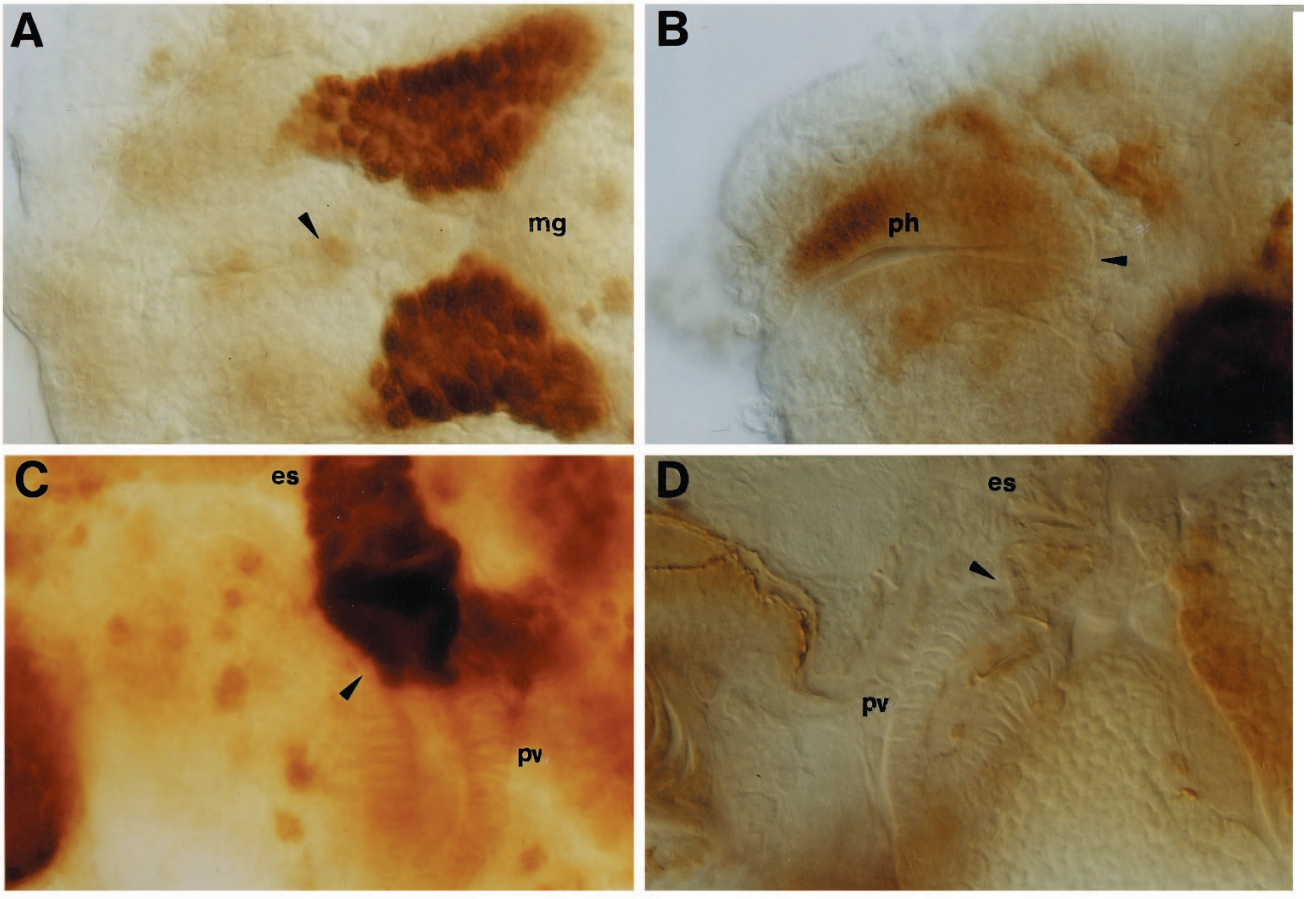

Fig. 6. Proventricular phenotypes of *arm* and *mys* mutants. (A) Anti-fkh antibody staining of an *arm* mutant embryo (dorsal view), showing lack of esophagus and proventriculus (arrowhead denotes where they should normally form). The heavily stained fkh-positive cells at each side of the arrowhead are the salivary glands (stage 17 embryo). (B) Anti-fkh antibody staining of an *arm* mutant, showing stomodeal invagination defect (arrowhead). (C) Anti-fkh antibody staining of a *mys* mutant (stage 17), showing foregut cells that are clustered on top of the proventriculus and fail to move inwards (arrowhead). (D) Anti-crb antibody staining of a *mys* mutant (stage 17), showing the lack of internal portion of the proventriculus due to lack of cells entering inwards (arrowhead points to the junction between esophagus and proventriculus; compare this with the wild-type structure in Fig. 2C. Abbreviations: es, esophagus; mg, midgut; pv, proventriculus; ph, pharynx.

represent attempts to form an ectopic proventriculus due to the lack of repressing activity by *dpp*. To explore this possibility, we examined embryos in which the *dpp* gene was ectopically expressed under heat-shock control (see Materials and Methods). In these embryos the development of the proventriculus can be suppressed, resulting in the cardiac arrest phenotype (Fig. 7E,F). Thus, *dpp* activity imposes an opposite effect on proventriculus morphogenesis as *wg*, *hh* and the integrins.

Genetic interactions in the foregut

The foregut arises from an anterior region of the blastoderm embryo; the anterior portion of the invaginating foregut will form the esophagus while the posterior portion (the 'keyhole') will give rise to the proventriculus. *ci*, *hh* and *wg* are all expressed in the keyhole region, whereas *dpp* is expressed only in the anterior part of the foregut. As a first step towards examining the potential genetic interactions between the genes

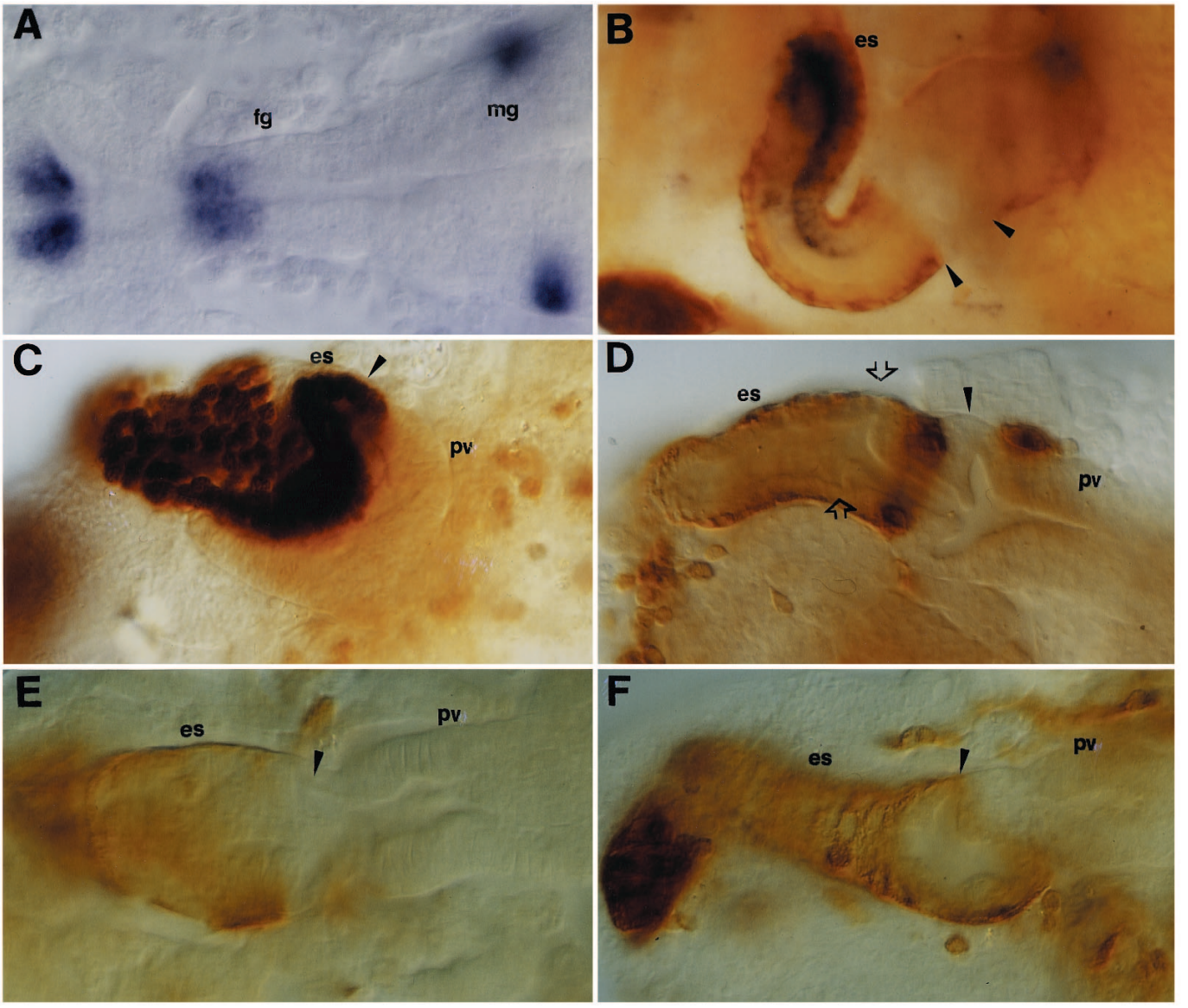

Fig. 7. Role of *dpp* in proventriculus development. (A) Whole-mount in situ hybridization with a *dpp* probe in a stage 13 wild-type embryo, showing *dpp* expression in the anterior part of the foregut. (B) Double staining with anti-MHC antibody and *dpp* in situ hybridization probe, showing *dpp* expression (blue) in the foregut at the border to the keyhole region (delimited by the two arrowheads). (C) A *dpp* mutant embryo stained with anti-fkh antibody, showing more cells in the inner portion of the proventriculus (arrowhead). (D) Another *dpp* mutant embryo stained with anti-MHC antibody, showing a variation in the proventricular phenotype. The inward cell movement has begun in the normal position of the foregut (arrowhead), but an extra outbudding zone (open arrows) now appears in the esophagus opposite to the developing proventriculus. The embryos shown in C through F are all late stage embryos, most likely corresponding to stage 16/17; due to drastic alterations in gross morphology of these mutant embryos, precise staging by morphological landmarks is not feasible. (E) A HS-*dpp* embryo (for heat-shock protocol, see Materials and Methods), stained with anti-MHC antibody, showing suppression of proventricular cell migration. Note that there are some residual signs of cell movement in the foregut (arrowhead). (F) Another HS-*dpp* embryo stained with anti-MHC antibody, where the inward proventricular cell migration does not occur. There are residual signs of cell movement in this embryo as well (arrowhead). Abbreviations: es, esophagus; mg, midgut; pv, proventriculus; fg, foregut.

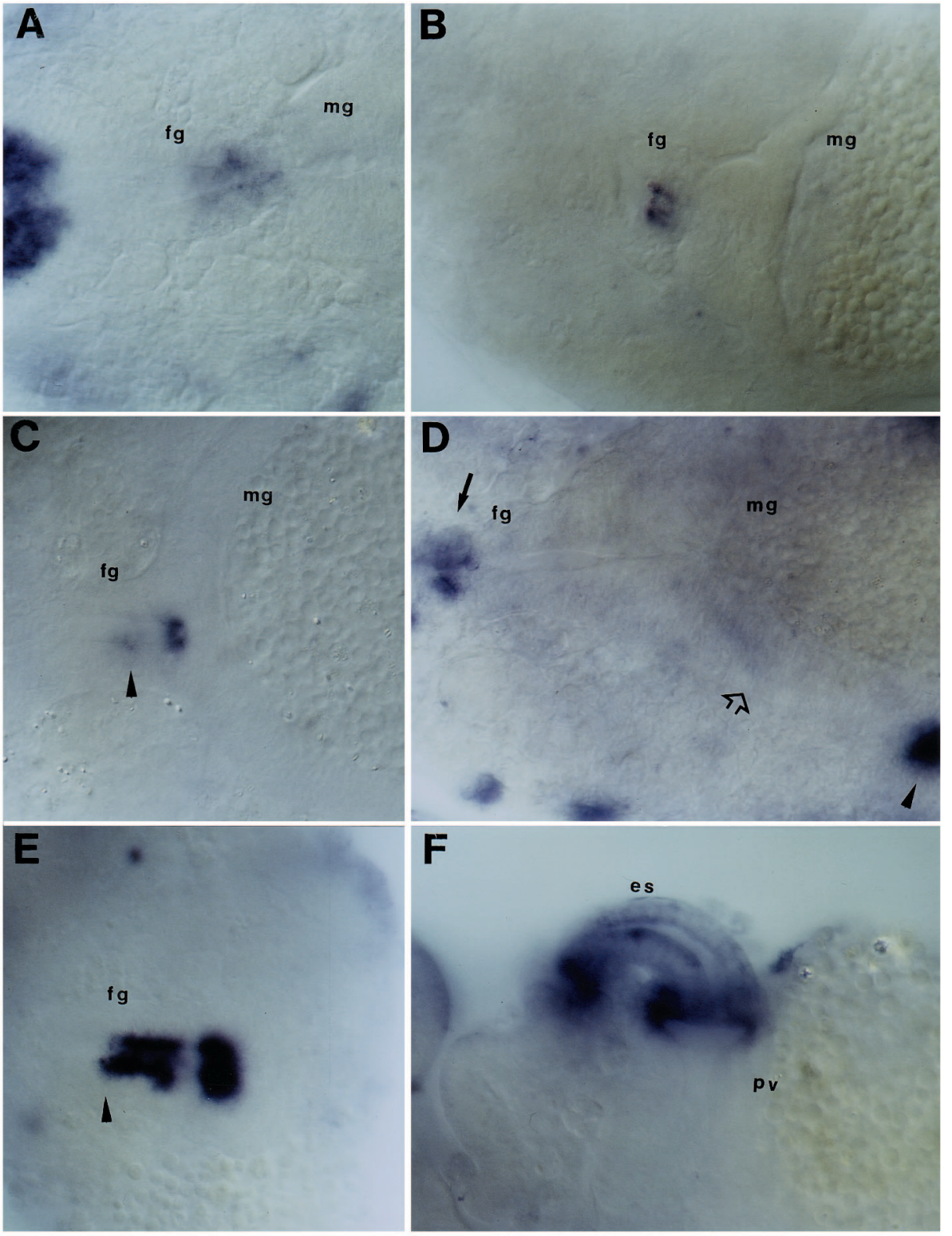

Fig. 8. Genetic interactions in the foregut. (A) *hh* expression in *ci* mutant embryo; the expression is decreased as compared to wild type. (B) *wg* expression in *ci* mutant embryo; the expression is decreased. (C) *wg* expression in *hh* mutant embryo; the expression is decreased (arrowhead). (D) *dpp* expression in *hh* mutant embryo; the foregut domain of *dpp* is still present and is relatively unaffected (arrow on the left). *dpp* expression in the gastric caecae is completely missing (open arrow in the middle), while the domain in the central midgut is also unaffected (arrowhead on the right). The embryos in A-D are at approximately stage 13. (E) *wg* expression in *dpp* mutant embryo; the staining of the anteriorly split *wg* domain in the keyhole region is quite strong (arrowhead). (F) *hh* expression in *dpp* mutant embryo; the expression is relatively unaffected. This embryo is at a later stage as the one in E, since the proventricular structure is easier to identify. Abbreviations: es, esophagus; mg, midgut; pv, proventriculus; fg, foregut.

involved in proventriculus development, we monitored the expression of *hh*, *wg* and *dpp* in various mutant backgrounds. In *ci* mutants, both *hh* and *wg* expression are normal at an earlier stage of proventriculus development, but decrease at a later stage (Fig. 8A,B), suggesting that *ci* is required to maintain the expression of *hh* and *wg*. In *hh* mutants, *wg* is expressed normally at early stages but is decreased at later stage, indicating that maintenance of *wg* expression also requires *hh* activity (Fig. 8C). We do not observe a significant change in *dpp* expression in the foregut of *hh* mutants, although the *dpp* expression in the gastric caecae of the midgut is completely lost (Fig. 8D). Taken together, these studies indicate that the genetic interactions are mostly modulatory in nature, rather than one gene being strictly dependent on another for expression. The initial activation of *wg* and *hh* is most likely carried out by other genes controlling foregut development, e.g., *forkhead* (*fkh*) is required for the expression of both *wg* and *hh* (Pankratz and Hoch, unpublished; Mohler et al., 1995).

In *dpp* mutants, *wg* expression remains quite strong in the keyhole region; in fact, the level of expression is consistently stronger than in wild-type embryos (Fig. 8E). Thus, *dpp* could be involved in repressing *wg* activity in the proventriculus. In the absence of internal staining control, we cannot estimate to what extent *wg* expression is derepressed in *dpp* mutants. However, the genetic interaction that we observe is clearly different from what is found during the formation of the second midgut constriction, where *dpp* activates *wg* expression (Immerglück et al., 1990). We did not observe any major change of *hh* expression in *dpp* mutants (Fig. 8F).

DISCUSSION

We have described a model system where the process of epithelial morphogenesis is accessible to genetic analysis. We discuss below how signaling molecules and integrins may function to coordinate this morphogenetic process in proventriculus development (Fig. 9).

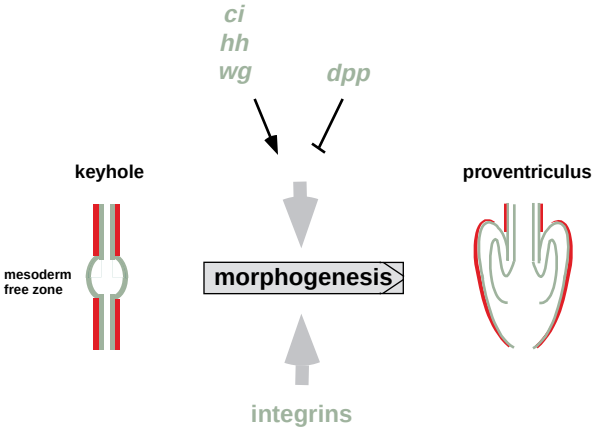

Fig. 9. A model of the genetic requirements for proventriculus morphogenesis. The arrows represent positive regulatory interactions; the bar represents negative ones. The green layer in the keyhole and the proventriculus represent ectodermal epithelium; the red layer represents the visceral mesoderm. See text for details.

Role of *wg*, *hh* and *dpp*

During late stages of embryogenesis, a specific region of the foregut epithelium moves outwards, folds back on itself and moves inwards, thereby transforming an epithelial tube into a multiply folded organ, the proventriculus. The genes *wg*, *hh* and *dpp* are involved in controlling these morphogenetic events. *hh* and *wg* are both expressed in the foregut epithelium that moves inside during proventriculus formation, and the corresponding mutant embryos display a very similar mutant phenotype, namely, the failure of the final inward movement to occur. This results in a proventriculus that is hollow inside and where cells are clustered on top (the 'cardiac arrest' phenotype), rather than the normal multiply folded structure.

Several cellular processes could underlie this failure in epithelial morphogenesis. One is a defect in cell proliferation which results in the absence of the cells that normally move inwards. Another is a defect in coordinating cell movements or shape changes, whereby the cells cannot properly migrate into the proventriculus. It has been shown that *wg* is involved in cell proliferation at early stages of gut development (Skaer and Martinez Arias, 1992; Skaer, 1993). In *hh* mutants, the esophagus is shortened, suggesting that *hh* may also be involved in cell proliferation at the early stages of foregut development. For the proventricular 'cardiac arrest' phenotype at the later stages of embryogenesis, however, we favor the view that a failure to coordinate cell movements is responsible. This is based on several considerations. First, in both *hh* and wg^{ts} mutant embryos, many cells are clustered on top of the proventriculus, indicating that cells are in fact available for moving inwards. Second, we can obtain, from experiments with the wg^{ts} allele, crawling larvae with a specific proventricular defect in which cells have not moved inwards but remain looped out. Third, embryos mutant for the gene encoding the integrin β subunit, a molecule involved in cell adhesion, show a very similar proventricular defect as wg^{ts} and *hh* mutants (see below).

dpp mutants show an opposite phenotype as *hh* and wg^{ts} mutants, namely, the uncontrolled movement of cells in the proventriculus and in parts of the esophagus. As *dpp* expression is restricted to the esophagus, and ectopic *dpp* can shut down proventriculus morphogenesis, *dpp* formally suppresses proventriculus development, perhaps through delimiting the area within the foregut tube where the proventriculus can form. The mechanism by which *dpp* performs this function is unclear. *wg* expression appears to be negatively regulated by *dpp* as assayed in *dpp* mutant embryos, but we do not observe a significant repression of *wg* in heat-shock *dpp* transgenic embryos (data not shown). However, this may be due to the lack of sensitivity of our assay system. An alternative possibility is that *dpp* acts on the downstream effectors of *wg* and *hh* activities through a parallel pathway.

Cell signaling in the gut

hh is required to maintain *wg* expression in the proventriculus but not for the initial activation. *hh* and *wg* are in turn dependent on *ci* for maintenance of their expression pattern, but again, not for initial activation. A potential activator of both *wg* and *hh* is *fkh*, since both *hh* and *wg* expression in the foregut is gone in *fkh* mutants (Pankratz and Hoch, unpublished; Mohler et al., 1995). *fkh* is required for the development of the

esophagus and the proventriculus, and is expressed throughout the foregut region at high levels from the blastoderm stage to the end of embryogenesis (Weigel et al., 1989a,b). As *fkh* encodes a putative transcription factor containing a HNF3/forkhead DNA-binding domain (Weigel and Jäckle, 1990), it is possible that *fkh* directly regulates the transcription of *hh* and *wg*.

The most intriguing aspect of the spatial control of gene expression in the foregut is the splitting of the *wg* domain such that the gap between the two resulting *wg* domains coincide with the mesoderm-free zone. As *wg* is initially expressed as a contiguous band, there must be another factor that is responsible for repressing *wg* expression in the keyhole region.

The signaling mechanisms underlying proventricular cell movements in the foregut differ from that of another morphogenetic process in the gut, the formation of the second midgut constriction. For example, *dpp* acts to prevent proventricular cell movement, whereas in the midgut it acts to promote cells movements forming the second constriction. In addition, *dpp* activates *wg* in the midgut (Immerglück et al., 1990), whereas we do not observe activation of *wg* by *dpp* in the foregut. Recently several receptors for *dpp* have been identified that are differentially expressed in the embryo (Affolter et al., 1994; Nellen et al., 1994; Penton et al., 1994; Brummel et al., 1994). The different effects of *dpp* in the two developmental contexts could thus be mediated through different receptors.

A further difference is reflected in the differing roles that the various tissue layers play in the morphogenetic processes in the foregut and midgut. The second midgut constriction depends on cell signaling across germ layers from the visceral mesoderm to the underlaying epithelial endoderm: *dpp* and *wg* are expressed in a specific region of the midgut mesoderm and their gene products interact with cells across the germ layer to induce expression of various target genes in the adhering endoderm (Panganiban et al., 1990; Reuter et al., 1990; Reuter and Scott, 1990; Immerglück et al., 1990; Mathies et al., 1994). In striking contrast, the visceral mesoderm is completely absent in the keyhole region that will form the proventriculus. In addition, *dpp*, *wg* and *hh* are expressed only in the ectodermal layer of the foregut, even in regions outside the keyhole that are ensheathed in mesoderm. Furthemore, in embryos where the visceral mesoderm is missing due to specific mutations (*twist* and *snail* double mutants), the proventriculus develops normally and the inward cell migration takes place (Hartenstein et al., 1992; our unpublished observations). These observations indicate that the ectodermal cells of the proventriculus do not take cues from the mesoderm for their morphogenetic movements.

Integrins as potential effectors for *hh*, *wg* and *dpp* signaling activities

The observation that embryos lacking β integrin activity have very similar proventricular phenotypes to those of *hh* and wg^{ts} mutants suggests that integrins may be involved in mediating some of the activities of these secreted molecules. In cases where the mutant phenotypes of the *Drosophila* integrin genes have been analysed at the cellular level, a common theme has been the failure of different cell layers to attach or remain attached (Newman and Wright, 1981; Wilcox et al., 1989; Leptin et al., 1989; Zusman et al., 1990, 1993; Brown, 1994). By contrast, the proventricular phenotype that we observe in embryos that lack the PSβ subunit most likely arises from defects in cell migration: the cells are clustered on top of the proventriculus and do not move inwards (see Fig. 6C).

It is known that many types of cell movements require interactions with the extracellular matrix. The inside of the proventriculus is rich with extracellular matrix material (King, 1988), and it has been shown that there is an alteration in the accumulation of extracellular matrix material in *mys* mutants (Wright, 1960; Newman and Wright, 1981). It has also been demonstrated that the *Drosophila* integrins can interact with vertebrate extracellular components (Hirano et al., 1991: Bunch and Brower, 1992). Therefore, the proventricular cell migration defect in *mys* embryos is consistent with studies in vertebrate systems demonstrating the biochemical function of integrins in mediating cell-extracellular matrix interactions.

We have not observed proventricular defects in embryos lacking the α2 subunit. However, since different α subunits can form heterodimers with the same β subunit (Brower et al., 1984; Wilcox et al., 1984; Leptin et al., 1987), this could be due to the fact that one type of α subunit can substitute for another.

As *mys* is expressed uniformly in the entire foregut, *hh* and *wg* may provide the spatial cues for regulating the activity of the integrins. We do not know the mechanism by which this could occur, and we do not observe any differences in *mys* expression in *hh* mutants (data not shown). However, there exists a link between *wg* and another class of cell surface adhesion receptors, the cadherens. This link is provided by Armadillo, one of the components that transduce the *wg* signal: Armadillo is a cytosolic component of the adherens junction multiprotein complex which is associated intracellularly with the actin cytoskeleton (see Peifer et al., 1993 and references therein). The integrins are also associated with the cytoskeleton network through several cytosolic components (Luna and Hitt, 1992; Stossel, 1993, for reviews). Therefore, *hh* and/or *wg* may function through modulating the activities of various, as yet unidentified, cytosolic components that interact with the integrins.

Evolutionary considerations

The primitive gut of animals is essentially a closed sac, which has invaginated from one side of the body during gastrulation; the stomodeal and proctodeal openings were later evolutionary additions that facilitated ingestion and egestion. Wolpert (1994) has suggested that the openings to the primitive gut 'could originally have resulted from the invaginating gut making contact with the ectoderm and thus providing a signal for making these cells different – possibly the first inductive event in development'. *wg*, *hh* and *dpp*, in addition to being expressed in the proventriculus, which forms at the junction between foregut and midgut, are also expressed at the junction between midgut and hindgut. Furthermore, the mesoderm-free zone that is found at the foregut/midgut junction is also found at the midgut/hindgut junction (Tepass and Hartenstein, 1994), suggesting that signaling mechanisms in the foregut and hindgut may be quite analogous. It is also noteworthy that the basic tubular structure of the gut extending from the mouth to the anus is found in almost all metazoans outside of Porifera, Plathelminthes and Cnidaria (see Remane et al., 1981). Recently, it has been shown that a mouse homologue of the *fkh*

gene, HNF3β, is expressed in the mouse embryonic gut (Ang et al., 1993; Monaghan et al., 1993); a mutation in HNF3β affects the expression of Sonic hedgehog (Shh), a homologue of the *Drosophila hh* gene, and results in gut defects (Ang and Rossant, 1994; Weinstein et al., 1994). Therefore, it will be interesting to determine whether genetic interactions controlling gut development have been conserved between *Drosophila* and vertebrates.

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