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A distinct set of founders and fusion-competent myoblasts make visceral muscles in the *Drosophila* embryo

Beatriz San Martin*, Mar Ruiz-Gómez*,‡, Matthias Landgraf and Michael Bate§

Department of Zoology, University of Cambridge, Downing Street, Cambridge CB2 3EJ, UK
*Both authors contributed equally to the work
‡Present address: Centro de Biología Molecular 'Severo Ochoa', UAM-CSIC, Madrid, Spain
§Author for correspondence (e-mail: cmb16@cus.cam.ac.uk)

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SUMMARY

The embryonic *Drosophila* midgut is enclosed by a latticework of longitudinal and circular visceral muscles. We find that these muscles are syncytial. Like the somatic muscles they are generated by the prior segregation of two populations of cells: fusion-competent myoblasts and founder myoblasts specialised to seed the formation of particular muscles. Visceral muscle founders are of two classes: those that seed circular muscles and those that seed longitudinal muscles. These specialisations are revealed in mutant embryos where myoblast fusion fails. In the absence of fusion, founders make mononucleate circular or longitudinal fibres, while their fusion-competent neighbours remain undifferentiated.

Key words: *Drosophila melanogaster*, Founder, Myoblast, Embryo, Visceral muscle

INTRODUCTION

In *Drosophila* the body wall (somatic) muscles of the larva are syncytial fibres generated by the fusion of embryonic myoblasts (Bate, 1993). Fusion is a highly regulated process that leads to the formation of an intricate pattern of myotubes in every segment. The key to this process is the prior segregation of two classes of myoblasts: founder myoblasts and their partners in fusion, the fusion-competent cells (Baylies et al., 1998). Founders arise at specific locations in the somatic mesoderm and are programmed to seed the formation of particular myotubes. Each founder attracts, and fuses with, a cluster of neighbouring fusion-competent cells, thus forming the syncytial precursor of a specific muscle fibre at that location. Founders express an Ig domain protein, Dumbfounded (Duf) which acts as a myoblast attractant (Ruiz-Gómez et al., 2000), while fusion-competent cells express a second Ig domain protein, Sticks and stones (Sns) (Bour et al., 2000). Both proteins are required for myoblast fusion and it is possible that they act as ligand and receptor in the fusion process. In any event, since *duf* is expressed exclusively by founders and *sns* only by fusion-competent cells, they serve as useful markers for the two populations of cells.

We were interested to discover whether regulation of the fusion process by the subdivision of myoblasts into founders and fusion-competent cells is a unique adaptation only required to generate the thirty different myotubes that are present in each segment of the body wall or whether it is a general requirement for many different kinds of myogenesis.

Apart from the muscles of the body wall, a second set of fibres, the visceral muscles, provide the contractile machinery of the larval gut (Bate, 1993). On the endodermal tube of the midgut, the visceral muscles form an orthogonal array of longitudinal and circular fibres. It has been assumed that, unlike the somatic muscles, these visceral muscles are not syncytial, but formed from single, mononucleated fibres (Bate, 1993; Campos-Ortega and Hartenstein, 1997; Elder, 1975). However, we find that both sets of midgut visceral muscles are syncytial and that they form from populations of myoblasts that can be readily identified in the embryo. Furthermore, as in somatic myogenesis, these myoblasts are divided into separate populations of fusion-competent cells and founders. While there appears to be a common pool of fusion-competent visceral myoblasts, visceral founders are divided into two classes: those that seed the circular muscles and a second, migratory set that form the precursors of the longitudinal muscles.

MATERIALS AND METHODS

Fly stocks

The following stocks were used: Oregon R as wild type, *Df(1)w^{67k30}*, a deficiency that removes both *roughest* and *dumbfounded* (Ruiz-Gómez et al., 2000), an amorph allele of *even-skipped*, *eve^{R13}* (Tremml and Bienz, 1989a), an amorph allele of *sticks and stones*, *sns$^{ZN1.4}$*, kindly provided by Susan Abmayr (Bour et al., 2000), an amorph allele of *myoblast city*, *mbc1* (Rushton et al., 1995) and a P-element insertion in the 5′ region of the *hairy* gene, *hairy^{L43a}*, kindly provided by David Ish-Horowicz (Riddihough et al., 1991).

In situ hybridisations and immunocytochemistry

Whole-mount in situ hybridisations with digoxigenin-labeled DNA

probes were performed according to the method of Tautz and Pfeifle (Tautz and Pfeifle, 1989) as modified by Ruiz-Gómez and Ghysen (Ruiz-Gómez and Ghysen, 1993). Duf expression was monitored by means of whole-mount in situ hybridisations with a digoxigenin-labeled RNA probe as described previously (Taylor, 2000). Immunocytochemistry was as described by Ruiz-Gómez et al. (Ruiz-Gómez et al., 1997). The following antibodies were used: anti-Fasciclin III and anti-Wingless (available from Developmental Studies Hybridoma Bank from C. Goodman and S. Cohen, respectively), monoclonal anti-Hairy 24.1 kindly provided by D. Ish-Horowicz, anti-Connectin (Meadows et al., 1994), anti-β-galactosidase (Cappel), anti-MEF2 (1:200) (Bour et al., 1995), anti-CascadeBlue (Molecular Probes), anti-rabbit Alexa-Fluor-568 (Molecular Probes), anti-mouse Alexa-Fluor-488 (Molecular Probes).

Muscle cell fills

Guts were dissected out of staged embryos under saline, spread out on polylysine-coated coverslips and fixed for 20 minutes with 3.7% formaldehyde. Muscle cells were identified under Nomarski optics using a 60× water immersion lens on an Olympus BX50WI fixed stage compound microscope. Microelectrodes (80-100 MΩ resistance) pulled on a Brown-Flaming horizontal puller (Sutter Instruments Co.) were backfilled with 5% Cascade Blue hydrazide (Molecular Probes) and the electrode shanks were backfilled with 0.2 M LiCl. The dye was iontophoretically injected into muscle cells with a 2 nA hyperpolarising current passed for 30-60 seconds. Subsequently, preparations were fixed for a further 20 minutes in 3.7% formaldehyde and then immunohistochemically stained using standard procedures (Ruiz-Gómez et al., 1997). Nuclei were visualised with TOTO-3 iodide (Molecular Probes; 1:1000) after RNAseA incubation (0.1 mg/ml, 1 hour incubation). Cell fills were analysed using a Leica TCS SP confocal microscope.

RESULTS

Visceral muscles of the midgut are syncytial

Earlier descriptions of the midgut visceral muscles in flies reported that the fibres are mononucleate rather than syncytial (Bate, 1993; Campos-Ortega and Hartenstein, 1997; Elder, 1975). However, it has now been shown that *duf* and *sns*, genes characteristically required for myoblast fusion, are both expressed in the visceral mesoderm of the midgut (Bour et al., 2000; Ruiz-Gómez et al., 2000). In addition, the morphology of the midgut is abnormal in embryos mutant for genes that are required for myoblast fusion, including *duf* and *sns*. In such embryos the fore and hind gut appear relatively normal but the usual constrictions of the midgut fail to develop properly. Because of the apparent inconsistency between these findings and the earlier reports, we decided to investigate the roles of *duf* and *sns* in the development of the midgut. An obvious possibility was that the earlier reports of mononucleated visceral muscles were wrong and that the muscles of the midgut, like the somatic muscles, are multinucleate fibres. To show whether or not this is the case, we used a technique of dye injection into single cells, in combination with nuclear labelling to determine whether the filled cells contain more than one nucleus (Fig. 1). Fills were

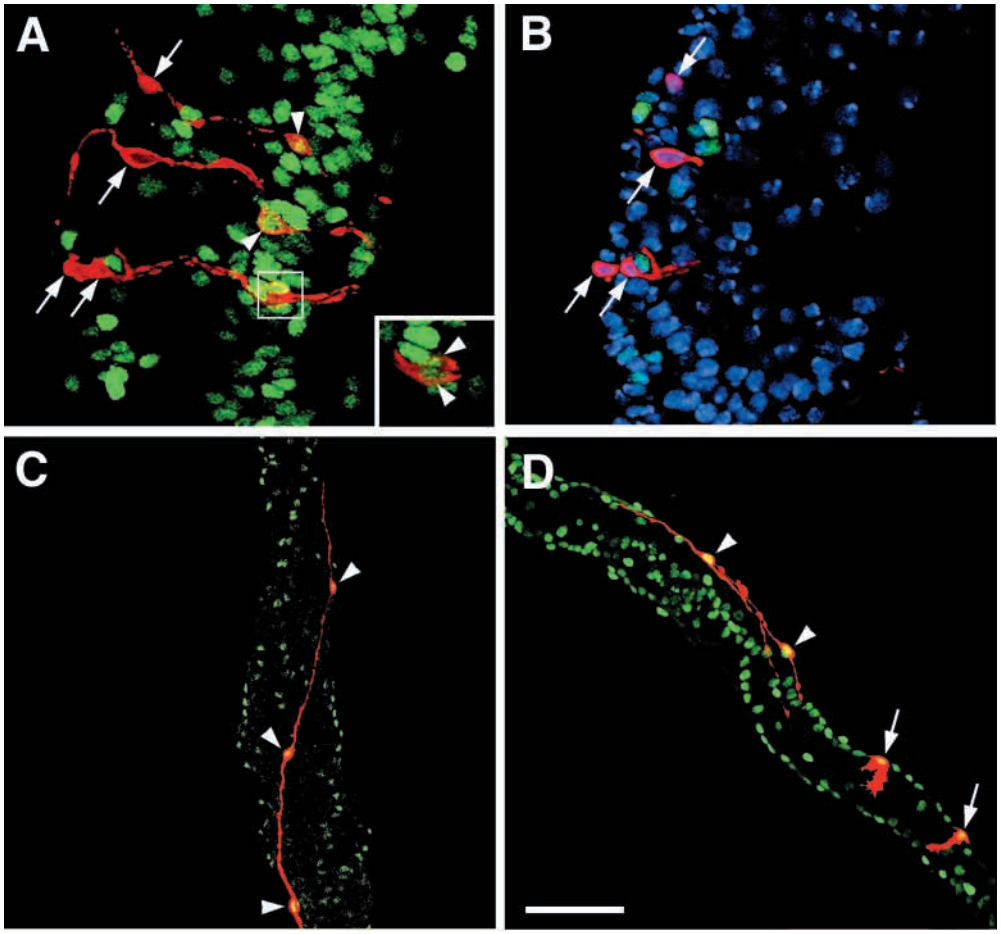

Fig. 1. Single cell dye fills (red) of midgut circular and longitudinal muscles. (A) Projection of a confocal Z-series showing four midgut circular muscles (red) in an early stage 14 embryo. The lower two muscles are adjacent – see inset. The embryo carries the RP298 enhancer trap (Nose et al., 1998) which marks founder cell nuclei (green) but is otherwise wild type. All four midgut circular muscles are binucleate and contain one RP298-positive (arrowheads in A) and one RP298-negative nucleus (arrows in A and B), thus demonstrating that these muscles are the product of a fusion between a founder cell and a fusion-competent non-founder myoblast. (B) A montage of single confocal sections (2 μm thickness) of the same preparation as shown in A. The nuclear label TOTO-3 shows nuclei of the midgut circular muscles. Arrows indicate RP298-negative nuclei of fibres seen in A. (C) Part of a wild-type midgut longitudinal muscle filled at late stage 16. Anti-MEF2 staining (green) reveals three nuclei in the syncytium (arrowheads). (D) Midgut muscle fills of a late stage 16 *mbc* mutant embryo (no myoblast fusion) carrying the RP298 enhancer trap. Midgut longitudinal (arrowheads) and circular (arrows) muscles form in non-fusion mutants, but are always mononucleate and positive for the founder cell marker RP298 (green). $n \geq 10$. Scale bar, A,B, 20 μm; C,D, 50 μm; inset in A, 10 μm.

made in embryos and larvae and they consistently revealed two classes of fibres, both of which are syncytial: smaller, circular fibres that contain two or three nuclei (Fig. 1A,B) and very elongated longitudinal fibres extending along the long axis of the gut, which contain three to five nuclei (Fig. 1C).

In non fusion mutants such as *myoblast city* (*mbc*; Rushton et al., 1995), dye fills reveal no syncytia in the midgut musculature (Fig. 1D), but two classes of fibres are still apparent: elongated, longitudinal cells and smaller circular fibres at right angles to them. In all cases (Fig. 1D) these two kinds of cells are mononucleated in the mutant embryos.

The expression patterns of *duf* and *sns* define complementary populations of visceral myoblasts

In the somatic mesoderm *duf* (also known as *kirre*) is expressed exclusively in founder myoblasts, while *sns* is expressed only in fusion-competent cells (Bour et al., 2000; Ruiz-Gómez et al., 2000). In the light of our finding that the visceral muscles are syncytial, we decided to compare the expression patterns of the two genes in the visceral mesoderm of the midgut (Fig. 2). We find that the two genes are expressed in distinct, non-overlapping populations of cells. We detect the following patterns of expression. (i) A conspicuous population of cells migrates to the midgut from the region of the forming hindgut visceral mesoderm. These are the cells of the caudal visceral mesoderm that have previously been shown to express *bHLH54F* and to form the longitudinal visceral muscles of the midgut (Georgias et al., 1997; Kusch and Reuter, 1999) (Fig. 2A). At late stage 12, when these cells are distributed along the midgut primordium, they begin to express *duf* (Fig. 2B). (ii) *duf* is also expressed in a continuous, undulating line of cells in the trunk mesoderm that have been described as the progenitors of the circular visceral muscles (Bate, 1993; Fig. 2C). (iii) *sns* is expressed in a more extensive population abutting this line of *duf*-expressing cells (Fig. 2D). The ventrolateral margin of the *sns* domain is delimited by the continuous file of *duf*-expressing cells (arrow in Fig. 2D).

Populations ii and iii constitute the trunk visceral mesoderm (Kusch and Reuter, 1999; Nguyen and Xu, 1998) and are derived from the clusters of dorsolateral *bagpipe* (*bap*)-expressing cells (Fig. 2E) that move internally at stage 10 (Azpiazu and Frasch, 1993). Initially these clusters appear uniform but by early stage 11 *bap* expression is downregulated in the more ventral marginal cells (Fig. 2F), and these cells become conspicuous by their orderly arrangement and their columnar morphology (Fig. 2F, arrow; Azpiazu and Frasch, 1993). These orderly marginal cells (the future *duf*-expressing cells) form a series of arches that border the looser aggregation of cells (the *sns*-expressing cells) that constitute the remainder of the original *bap* domain (Fig. 2F arrowhead). Both populations of cells in the trunk visceral mesoderm are labelled by the expression of Fasciclin III (Fas III; Patel et al., 1987), with stronger expression in the columnar, *duf*-expressing cells of the margin (Fig. 3A). Later the marginal cells divide once in a predominantly dorsoventral orientation to produce a double file of *duf*-expressing cells delimiting the domain of their *sns*-expressing neighbours (Fig. 3B). This lineage for the columnar *duf*-expressing cells resembles the origin of sibling founders for somatic muscles by the division of muscle progenitor cells (Carmena et al., 1995).

Taken together, these observations suggest that the columnar *duf*-expressing cells of the trunk visceral mesoderm and the

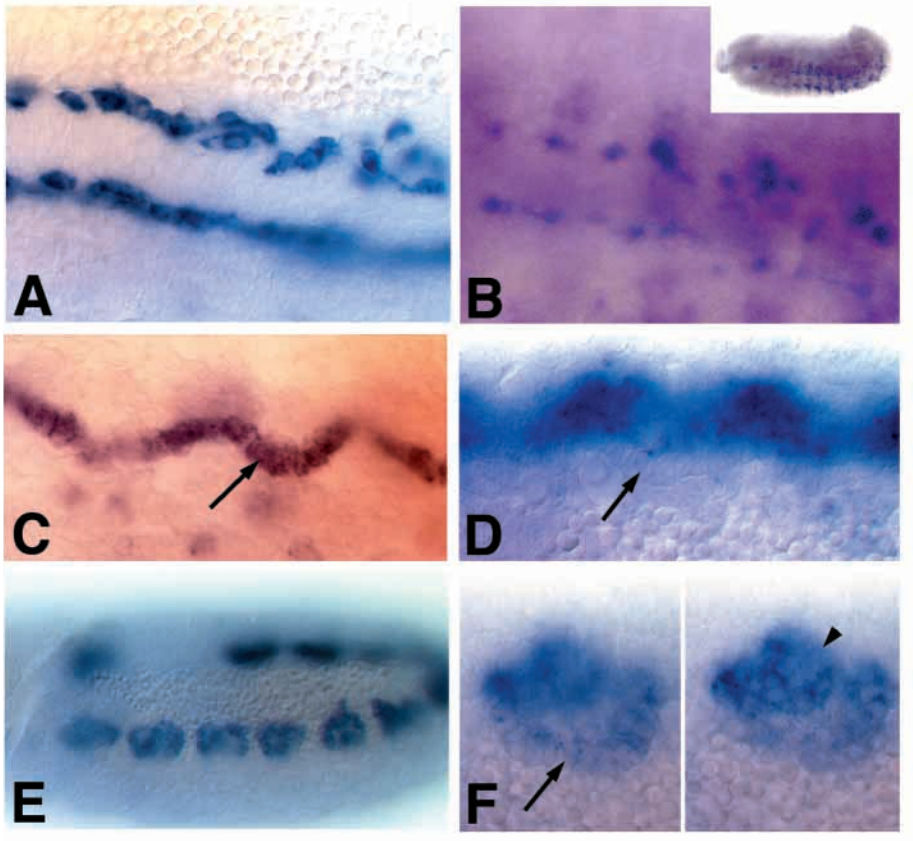

Fig. 2. Cell populations in the midgut visceral mesoderm.A,B embryos at late stage 12 (see inset B) stained to reveal migratory longitudinal muscle precursors. RNA in situ hybridizations show *bHLH54F* expression (A) and *duf* expression (B) on the midgut primordium. Note distribution of cells along palisade formed by immature circular muscles. (C,D) Dorsolateral views of part of stage 11 embryos to show differentiating midgut visceral mesoderm. RNA in situ hybridizations show *duf* expression in columnar marginal cells (C) and *sns* expression (D) in distinct but adjacent population of cells. Arrows indicate marginal columnar cells in these figures. (E) Early stage 11 embryo; DNA in situ hybridization to show *bap* expression in the invaginated primordia of the midgut visceral mesoderm. (F) Two different focal planes of these *bap*-expressing cells at later stage 11 showing the orderly arrangement of columnar cells with low expression (arrow, left) and relatively higher expression in adjoining cells (arrowhead, right).

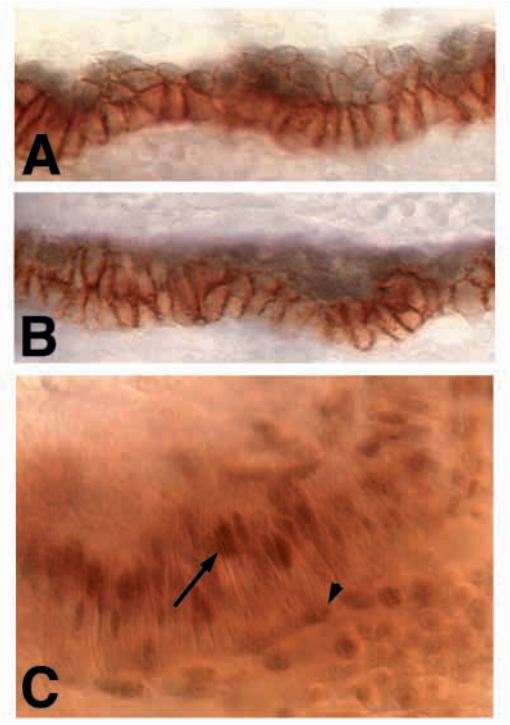

Fig. 3. Fusion-competent visceral myoblasts contribute to circular and longitudinal muscles. (A,B) Embryos (stage 11 A; early stage 12 B) double stained for antibodies against Fas III (brown) and Hairy (gray). (A) Hairy expression is not present in the marginal columnar cells but is present in adjoining aggregates of Fas III-expressing cells. (B) The marginal cells have divided to produce two rows of adjacent, columnar cells. Expression of Hairy remains confined to adjacent cells. (C) Embryo from strain Hairy^{L43} stained with anti-β-gal antibody. A posterior region of the forming midgut is shown with persistent β-gal expression in circular (arrow) and longitudinal muscle (arrowhead) precursors.

migratory *duf*-expressing cells of the caudal visceral mesoderm are founders for circular and longitudinal muscles respectively, while the *sns*-expressing cells are a population of fusion-competent myoblasts contributing to both sets of muscles. To substantiate this idea we analysed the expression of *hairy*, a gene whose expression in the somatic mesoderm is limited to fusion-competent myoblasts (M. R.-G. and M. B., unpublished). In the visceral mesoderm we find that Hairy is confined to the loose aggregates of Fas III-expressing cells in the trunk visceral mesoderm, (Fig. 3A,B). These are the cells that express *sns*. Hairy is not present in the immediately adjacent line of columnar, *duf*-expressing cells, nor is it in the migratory *duf*-expressing population of the caudal visceral mesoderm. To show that the *hairy*-expressing cells actually contribute to circular and longitudinal visceral muscles, we took advantage of the perdurance of β-gal expression driven by a *hairy*-β-gal construct (Riddihough and Ish-Horowicz, 1991). This persistent expression confirms that *hairy*-positive nuclei are incorporated into both kinds of visceral muscles (Fig. 3C). We conclude that the *sns*-expressing cells of the trunk visceral mesoderm are a population of fusion-competent myoblasts that contribute to both longitudinal and circular visceral muscle, while the *duf*-expressing cells of the trunk and caudal visceral mesoderm are two distinct populations of muscle founders, that initiate the formation of circular and longitudinal muscles respectively.

Localised patterns of gene expression are initiated in visceral progenitors and founders

In the somatic mesoderm, founder myoblasts express particular combinations of genes that endow these cells and the muscles they give rise to with unique characteristics (Keller et al., 1998; Knirr et al., 1999; Ruiz-Gómez et al., 1997). If our view that the *duf*-expressing cells are founder myoblasts for visceral muscle is correct, then we would expect that localised patterns of gene expression in the visceral mesoderm would be initiated

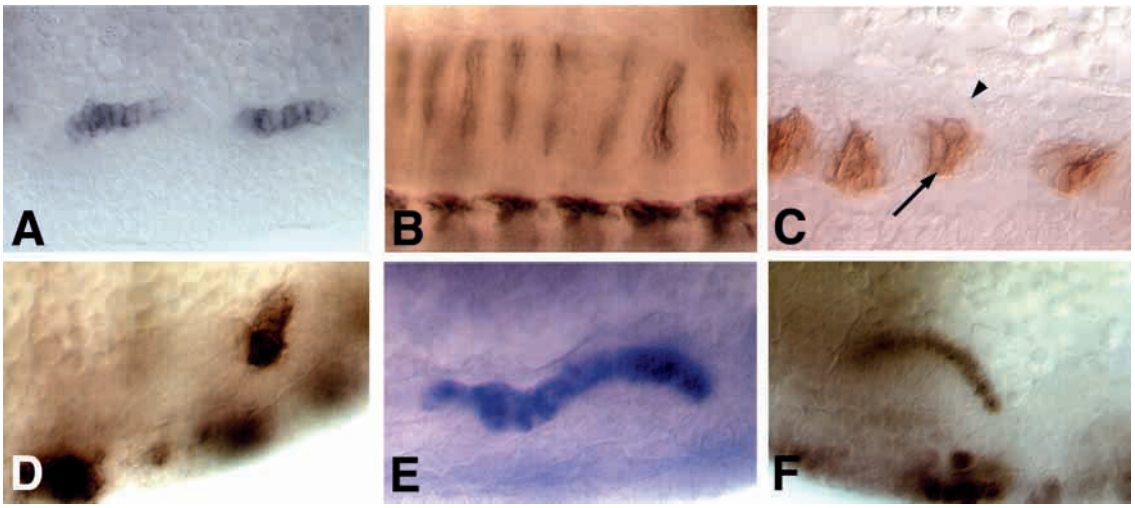

Fig. 4. Localised patterns of gene expression in visceral muscle founders. (A-C) Embryos stained with antibodies against Connectin. (A) Dorsolateral view of stage 11 embryo showing consecutive sets of Connectin-expressing visceral founders in adjacent segments. (B) Stage 13 embryo showing the dorsoventrally expanded domain of Connectin expression. (C) *sns* mutant embryo at stage 13 showing restriction of Connectin expression to the circular founders (arrow). Note the adjacent population of fusion-competent myoblasts (arrowhead) that are not recruited to expression in the absence of fusion. (D-F) Stage 11 embryos stained to reveal expression of (D) *wg* (anti-Wg), (E) *dpp* (in situ hybridisation), (F) *abd-A* (anti-Abd-A) in subsets of circular visceral muscle founder cells.

in these cells, and later spread to the syncytia that these cells seed. To test this idea we re-examined the expression patterns of several genes known to be expressed in restricted domains of the circular midgut musculature. Without exception we find that expression for each of the genes we analysed (*connectin* (Gould and White, 1992; Nose et al., 1992), *wingless* (van den Heuvel et al., 1989), *decapentaplegic* (Panganiban et al., 1990), *abdominal-A* (Tremml and Bienz, 1989b) is initiated in a subset of the marginal columnar cells of the trunk visceral mesoderm (Fig. 4A,D,E,F). Expression (*connectin*, Fig. 4B) later spreads to the full dorsoventral extent of the visceral muscles that these cells give rise to by fusion. In mutants where fusion fails (Fig. 4C) expression remains confined to the columnar founder cells.

Formation of visceral muscle is deranged in non-fusion mutants

As the germ band retracts, the band of Fas III-expressing visceral myoblasts in the trunk visceral mesoderm shortens and begins to adopt the characteristic palisade-like morphology of the mature circular visceral muscles. The loose aggregates of Fas III/Hairy-expressing cells become closely apposed to their columnar neighbours which begin to elongate in a dorsoventral direction. Gradually cells are incorporated into the forming palisade of the circular visceral muscles. Fas III expression now reveals a single cell type, namely the elongating columns of immature circular visceral muscles (Fig. 5A). In mutants where myoblast fusion fails, these events are disrupted in a characteristic and informative way. Mutations in *mbc* appear to block fusion by interfering with events subsequent to the aggregation of fusion-competent myoblasts on founder cells. In the somatic mesoderm this is revealed as a phenotype where clusters of unfused myoblasts accumulate at sites where muscles would normally form (Rushton et al., 1995). In the visceral mesoderm, the loose aggregates of Fas III-expressing myoblasts and their *duf*-expressing neighbours become closely apposed, but then fail to fuse (Fig. 5B). Mutations in *duf* and *sns*, however, interfere with myoblast aggregation and in the case of *duf* it is known that the protein acts as an attractant for fusion-competent myoblasts (Ruiz-Gómez et al., 2000). In the visceral mesoderm of such mutants, both populations of Fas III-expressing myoblasts form, but they remain distinct as the germ band retracts and show no sign of the close adhesion between them that develops in wild-type embryos and in mutants of *mbc* (Fig. 5C,D, *duf*; E,F, *sns*). From this we infer that, as in the somatic mesoderm, *duf* expression in the columnar cells serves to attract the associated clusters of *sns*-expressing myoblasts prior to fusion occurring. Thus it appears that an exactly analogous sequence of events unfolds in the somatic and visceral mesoderm as a prelude to the formation of syncytial muscles. In both, two populations of myoblasts are formed: *duf*-expressing founders and *sns*-expressing fusion-competent cells. *duf*-expressing founders attract to them *sns*-expressing myoblasts, and if either gene is mutated this attractive mechanism fails. In mutants where later events in fusion are blocked, the two populations adhere tightly but do not fuse: this adhesion is evident in the somatic mesoderm as clusters of aggregated myoblasts and in the visceral mesoderm as the two closely apposed populations of cells that normally give rise to the palisade of circular visceral muscles. In addition, in non-fusion mutants, the *duf*-expressing visceral founders, like their somatic counterparts (Rushton et al., 1995), continue to show specific patterns of gene expression and grow out with the appropriate orientations for the circular and longitudinal visceral muscles (Fig. 4C arrow and Fig. 1D). In contrast their *sns*, *hairy*-expressing neighbours do not elongate and do not manifest specific patterns of gene expression (Fig. 4C arrowhead).

DISCUSSION

The visceral muscles of the midgut form a latticework of circular and longitudinal fibres that encloses the endodermal

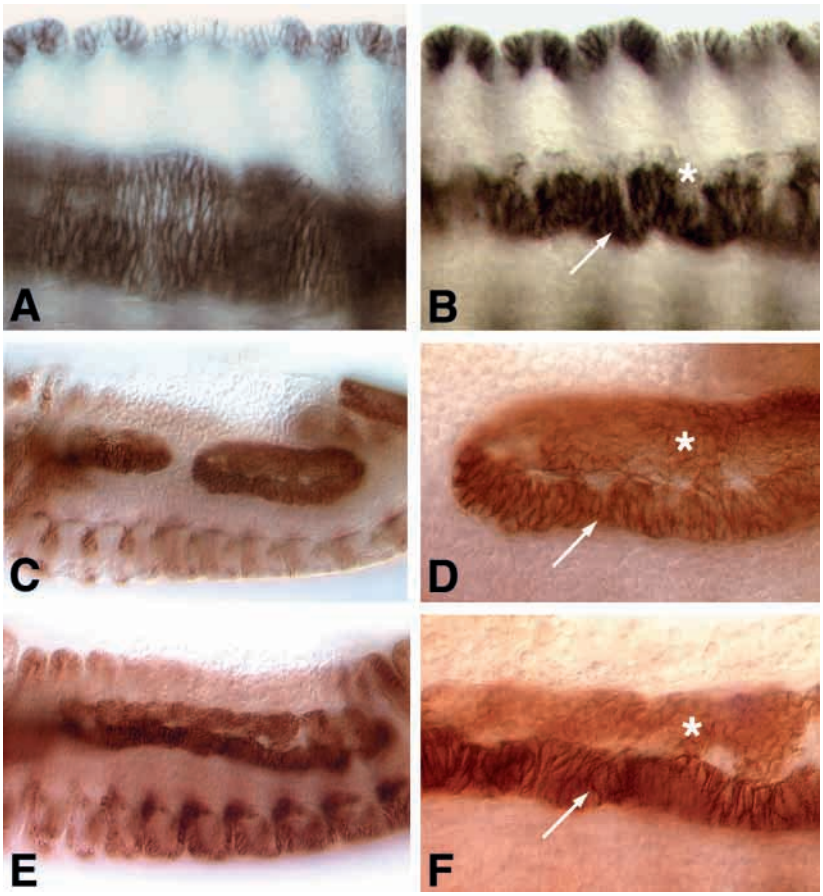

Fig. 5. Midgut phenotypes in wild-type and non fusion mutant embryos revealed by Fas III expression. (A-F) Midgut region of stage 13 embryos stained with antibody to Fas III to show, (A) palisade of forming circular visceral muscles in wild type; (B) elongated founders (arrow) and closely adherent but unfused myoblasts (asterisk) in an *mbc* mutant embryo; (C-F) Low and higher power views of (C,D) $Df(1)w^{67k30}$ and (E,F) $sns^{ZF1.4}$ embryos showing palisade of circular muscle founders (arrow) distinctly separated from adjacent population of fusion-competent cells (asterisk). Note similarity of the phenotypes in C,D and E,F.

tube of the gut itself. The longitudinal muscles form extended myotubes, each of which spans a considerable extent of the gut (Fig. 1C). The circular muscles are shorter and span the gut from ventral to dorsal (Fig. 1A,C). Our first finding is that both kinds of muscles are syncytial. Presumably an important feature of syncytial myotubes is that each fibre can increase in size by fusing with additional cells and thus the circular and longitudinal muscles of the midgut are sufficiently substantial to generate the peristaltic pumping movements of the larval digestive system. In addition, these fibres have to be sufficient to drive the constrictions of the midgut by their contraction during embryogenesis (Reuter and Scott, 1990), and in mutants where myoblast fusion fails, these constrictions are either absent or incomplete, while the morphogenesis of the fore and hindgut appears to be unaffected. It seems therefore that myoblast fusion is essential for the normal development of the midgut.

However, the real interest from our point of view is that these syncytial fibres represent a second population of muscles formed by myoblast fusion and therefore provide a test of the generality of the model for myotube formation that we have put forward for the somatic musculature. The somatic muscles consist of a remarkably diverse population of 30 different myotubes in each hemisegment of the body wall (Bate, 1993). Each myotube has its own specific set of characteristics, including its size, shape, sites of insertion on the body wall, and innervation by particular motorneurons. These properties of the syncytial myotubes seem to be largely dictated by the particular set of transcription factors expressed in each group of fused myoblasts as it differentiates to form a mature muscle fibre (Keller et al., 1998; Knirr et al., 1999; Ruiz-Gómez et al., 1997). These expression patterns derive from the specification of a single founder myoblast for each myotube. The specified founder attracts neighbouring fusion-competent myoblasts to fuse with it and at the same time recruits these cells to its own characteristic patterns of gene expression.

One possibility is that this mechanism for myoblast fusion applies to all processes of myogenesis, and that in every case some myoblasts will be found to act as seeds and others as feeders that fuse with them. Alternatively, the seeding model may be a special case uniquely adapted to the situation in which many different kinds of myotubes have to be rapidly and reliably generated in a complex pattern. If the model is general, then we would at least expect it to apply to all cases of syncytial myogenesis in the fly.

In the case of the visceral muscles we find that the same general mechanism operates, albeit to generate a less complex muscle pattern consisting of two main classes of myotubes: longitudinal and circular (Fig. 6). Myoblasts are recruited from a common pool of fusion-competent cells to take part in the assembly of one or other of the two alternative classes of muscle fibres that run at right angles to each other on the surface of the gut. The fate of each myoblast is determined by the nature of the founder with which it fuses. Thus in this case the muscle pattern is generated by the prior specification of two sets of founder cells with distinct properties and the presentation of these cells to a common pool of fusion-competent myoblasts.

The founders for the longitudinal muscles originate in the region of the hindgut visceral mesoderm and migrate anteriorly (Georgias et al., 1997; Kusch and Reuter, 1999). As they migrate they become oriented at right angles to the palisade of the circular muscles of the midgut and maintain this orientation as they begin to fuse, thus generating syncytial precursors of longitudinal muscles that are spread out over the entire territory of the forming midgut depending on the distance that each precursor has covered in its migration. By contrast, the founders of the circular muscles arise in situ, first as cells at the margin of the *bap*-expressing clusters that move inwards at stage 10 and later joining the marginal cells from neighbouring clusters to form a continuous line of founders along the length of the future midgut. From this line of founders the palisade of circular muscles will arise as each syncytium grows out by extending in the dorsoventral axis. Because these cells arise along the length of the anteroposterior axis of the trunk, they potentially retain information as to their position in this axis and they acquire locally distinct patterns of gene expression. Some of these are repetitive, eg *connectin*, while others such as *wg*, *dpp* and *abd-A* are unique to particular regions of the embryo and endow the cells at these points, and the muscles they give rise to, with locally specific properties. Thus the overall class of circular founders and muscles is refined by the expression of such genes to produce a regionally variegated set of fibres whose distinctive characteristics are required for the proper patterning and morphogenesis of the midgut (Skaer, 1993).

Are there distinct myoblast populations for somatic and visceral muscles?

We have identified what appear to be distinct populations of founders and fusion-competent cells for the somatic and visceral muscles. However, this distinction is based solely on the locations of the cells and their patterns of gene expression. What evidence is there that the two populations are really

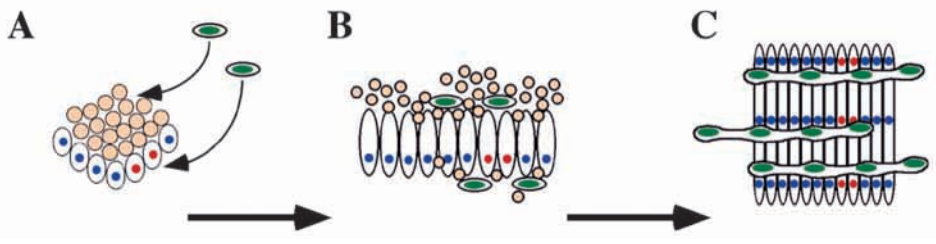

Fig. 6. Diagram to illustrate the formation of longitudinal and circular visceral muscles from founders and fusion-competent cells. (A) Visceral mesoderm of late stage 11 embryos with fusion-competent cells (orange) and associated circular founders (blue and red nuclei) together with inwardly migrating longitudinal founders (green nuclei). Local patterns of gene expression in circular founders indicated by red and blue nuclei. (B) Stage 12 embryo showing forming palisade of circular muscle precursors (red and blue nuclei) and associated longitudinal precursors (green nuclei) together with fusion-competent myoblasts (orange) which contribute to both kinds of muscles. (C) The completed lattice of circular and longitudinal visceral muscles, with localised patterns of gene expression as indicated.

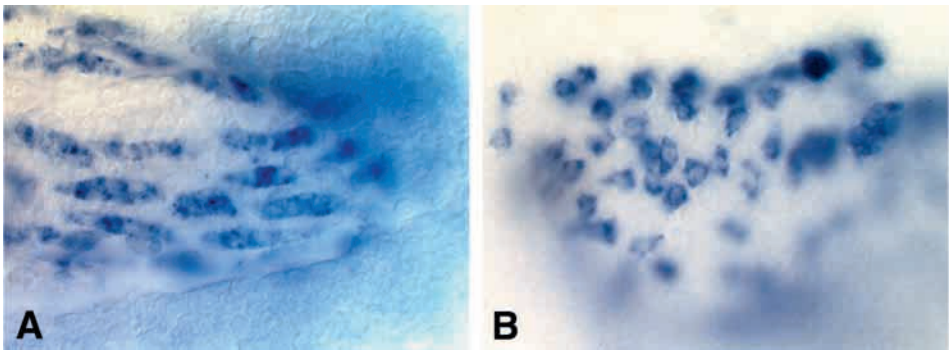

Fig. 7. Longitudinal visceral founders require visceral myoblasts to complete muscle formation. (A,B) DNA in situ hybridisation with probe for *bHLH54F* in stage 13/14 embryos showing longitudinal muscle precursors in (A) wild-type and (B) eve^{R13} mutant embryos. Note that the precursors are syncytial in A but that fusion fails to occur in the absence of trunk visceral mesoderm in the *eve* mutant embryo (B).

separate and that mixing does not occur? As far as we can see, mixing could only occur if somatic and visceral founders were able to recruit cells from each other's pools of fusion-competent myoblasts. We think it unlikely that such mixing would occur in normal development because founders and fusion-competent cells arise in close proximity to each other, or in the case of the founders for longitudinal visceral muscles, migrate into the region of the visceral fusion-competent cells. Thus local interactions would ensure that fusion-competent myoblasts would only fuse with their immediately adjacent founders. However, we know that the myoblast attractant Duf acts over distances of several cell diameters (Ruiz-Gómez et al., 2000), thus in abnormal circumstances it might be possible for visceral myoblasts to be attracted to somatic founders, or vice versa. It could be that the expression of Fas III in the midgut visceral mesoderm tends to hold this population of cells together so that the likelihood of cells migrating away from the region of forming visceral muscles is reduced. At the same time we observe that in *even-skipped* mutant embryos (Fig. 7) where the midgut visceral mesoderm of the trunk is absent, the *bHLH54F*-expressing founders of longitudinal muscles still develop and migrate into the region where the midgut would normally form. These cells however, remain mononucleate (Fig. 7B), despite the fact that they are adjacent to the pool of fusion-competent myoblasts from which somatic muscles will form. Thus in this instance at least there seems to be a block to the intermingling and fusion of cells across the two populations. Interestingly, the adhesive properties of the longitudinal visceral muscle founders seem to depend on their origin from a *brachyenteron* (*byn*)-expressing subset of the mesoderm (Kusch and Reuter, 1999). If *byn* is misexpressed throughout the mesoderm, the migration of the longitudinal founders is deranged and there is a generalised adhesion between somatic and visceral mesoderm cells, which makes them difficult to separate (Kusch and Reuter, 1999). Whether there is fusion between somatic and visceral myoblasts under these conditions is not clear. In general we think it is likely that there is a real barrier to mixing between the two cell populations, reflecting their very different origins in the embryo. Such barriers might be an important mechanism for partitioning the available population of myogenic cells between different muscle-forming tasks in the embryo.

In the two myogenic pathways, visceral and somatic, that we have investigated, myotubes form as a result of the prior segregation of seed myoblasts and others that act as feeders. In such a myogenic pathway, the properties of each syncytium can be dictated autonomously by the patterns of gene expression specific to particular classes of founder cells. At the same time, the number of myotubes and the locations where they will be formed are set by the local specification of founder myoblasts. We do not know how general this model for myotube formation may be. The weight of evidence in vertebrates (where myotubes are far less diverse; Hughes and Salinas, 1999) might be taken to indicate that in these organisms myoblasts simply align together and fuse to form myotubes. One question that this model leaves unresolved is how the number and location of forming myotubes would be regulated in such a system. Control could be exerted if the initial step in the formation of a myotube were a seeding event analogous to those we have described in *Drosophila*. It would not be essential for such seeding events to be so tightly controlled as in the fly, nor would the initiating event necessarily be in a participating myoblast. In other organisms it could either be that the environment provides local cues for the initiation of fusion, or that contact with such a local cue renders one myoblast capable of seeding fusion with its neighbours. Our view that such seeding events may be widespread and important is strengthened by the discovery of another such system in the fly.

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