Myoblast determination in the somatic and visceral mesoderm depends on Notch signalling as well as on *milliways* (*mili*^{Alk}) as receptor for Jeb signalling

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Summary

The visceral muscles of the *Drosophila* midgut consist of syncytia and arise by fusion of founder and fusion-competent myoblasts, as described for the somatic muscles. A single-step fusion results in the formation of binucleate circular midgut muscles, whereas a multiple-step fusion process produces the longitudinal muscles. A prerequisite for muscle fusion is the establishment of myoblast diversity in the mesoderm prior to the fusion process itself. We provide evidence for a role of *Notch* signalling during establishment of the different cell types in the visceral mesoderm, demonstrating that the basic mechanism underlying the segregation of somatic muscle founder cells is also conserved during visceral founder cell determination.

Searching for genes involved in the determination and differentiation of the different visceral cell types, we identified two independent mutations causing loss of visceral midgut muscles. In both of these mutants visceral muscle founder cells are missing and the visceral mesoderm consists of fusion-competent myoblasts only. Thus, no fusion occurs resulting in a complete disruption of visceral myogenesis. Subsequent characterisation of the mutations revealed that they are novel alleles of *jelly belly* (*jeb*) and the *Drosophila* Alk homologue named *milliways* (*mili*^{Alk}). We show that the process of founder cell determination in the visceral mesoderm depends on Jeb signalling via the Milliways/Alk receptor.

Moreover, we demonstrate that in the somatic mesoderm determination of the opposite cell type, the fusion-competent myoblasts, also depends on Jeb and Alk, revealing different roles for Jeb signalling in specifying myoblast diversity. This novel mechanism uncovers a crosstalk between somatic and visceral mesoderm leading not only to the determination of different cell types but also maintains the separation of mesodermal tissues, the somatic and splanchnic mesoderm.

Key words: *Drosophila*, Myogenesis, Visceral and Somatic muscles, Founder cells, Fusion-competent cells, Myoblast fusion, *Notch*, *Delta*, *lethal of scute*, *jelly belly*, Alk, RTK signalling

Introduction

The early events of mesoderm formation and specification have been studied extensively (reviewed by Anderson, 1998), whereas the molecular mechanisms leading to different mesodermal cell types are less understood. The primordia of visceral, somatic and cardiac tissues are established at defined positions in each segment (Borkowski et al., 1995; Azpiazu et al., 1996; Riechmann et al., 1997; Hosono et al., 2003). The subdivision of mesodermal cells during gastrulation is the basis for the constitution of the main types of musculature in *Drosophila*: the somatic and visceral muscles. The formation of visceral midgut muscles depends on two homeobox genes, *tinman* (*tin*) and *bagpipe* (*bap*) (Bodmer, 1993; Azpiazu and Frasch, 1993). Expression of *bap*, which is required for the determination of the midgut visceral mesodermal anlage, is activated by Tin in the dorsal mesoderm. *bap* induction occurs in segmental clusters that migrate in anterioposterior direction forming a continuous band of visceral progenitor cells, which finally surrounds the midgut. Whereas *bap* is expressed transiently in the visceral precursor cells to determine the circular midgut muscles, the forkhead domain factor *binou* (*bin*) is activated downstream of *bap* and is necessary for the differentiation of all visceral derivatives. Hence, in *bap* and *bin* mutant embryos, the visceral mesoderm is partially transformed into somatic mesoderm (Azpiazu and Frasch, 1993; Zaffran et al., 2001).

Recently, Weiss et al. (Weiss et al., 2001) identified a novel signalling process that induces visceral cell identities. The *jelly belly* (*jeb*) gene encodes a secreted protein that is produced from somatic mesodermal cells and taken up by visceral cells. In *jeb* mutants, no visceral muscles develop due to a failure

of differentiation in the existing Bap-expressing visceral precursors. The emerging picture shows, on the one hand, visceral determination genes such as *tin*, *bap* and *bin*, which are responsible for subdividing the mesoderm into different tissues and, on the other hand, inductive signals like Jeb, which promote interactions between these tissues.

The differentiated visceral musculature of the *Drosophila* midgut consists of an inner layer of circular muscles and an outer layer of longitudinal muscles (Campos-Ortega and Hartenstein, 1997), the latter persisting throughout metamorphosis (Klapper, 2000). The founder cells of the longitudinal muscles have a distinct primordium at the posteriormost part of the mesoderm (Tepass and Hartenstein, 1994; Georgias et al., 1997; Kusch and Reuter, 1999), whereas the circular visceral founder cells and all fusion-competent myoblasts (fcms) for both visceral muscle types originate from the trunk mesoderm (Klapper et al., 2002). Recently, it has been shown that both types of visceral muscles are syncytial and arise by fusion of founders and fcms (Klapper et al., 2001; San Martin et al., 2001; Klapper et al., 2002). These two classes of myoblasts are closely associated in a band of visceral precursors, which express markers such as Fasciclin III (Patel et al., 1987) and are characterised by *binou* expression (Zaffran et al., 2001). The ventralmost row of these visceral myoblasts consists of *dumbfounded/kin of irre* (*duf/kirre*)-expressing founder cells with a characteristic columnar shape. The more dorsally located fcms are characterised as such by a more globular morphology and by expression of *sticks and stones* (*sns*). During stage 12, binucleate circular muscles are built via fusion of these founders and fcms. This fusion process is disturbed in *myoblast city* (*mbc*), *duf/kirre* and *sns* mutants, which are also known to be defective in somatic muscle fusion, indicating that the founder cell hypothesis applies both to somatic and visceral myogenesis. Thus, several of the known genetic components are common between somatic as well as visceral myoblast fusion (Klapper et al., 2001; San Martin et al., 2001; Klapper et al., 2002).

Differentiation of the syncytial somatic muscles depends on the determination of founder and fusion-competent myoblasts (Bate, 1990; Dohrmann et al., 1990). These two classes of myoblasts are specified by lateral inhibition by the neurogenic genes *Notch* and *Delta* from a group of equivalent somatic mesodermal cells (Carmena et al., 2002). As a consequence, expression of the proneural gene *lethal of scute* (*l'sc*) is restricted to muscle progenitor cells. These progenitors divide asymmetrically and give rise to muscle founder cells (Carmena et al., 1995), which are characterised by differential expression of myogenic genes (reviewed by Baylies et al., 1998; Paululat et al., 1999). After establishment of myoblast diversity, the fusion process starts, leading in a first fusion step to muscle precursor cells and in a second fusion step to formation of mature myotubes (Doberstein et al., 1997; Rau et al., 2001). This process can be disturbed at distinct levels (reviewed by Dworak and Sink, 2002; Taylor, 2002). Besides lateral inhibition and determination through distinct transcription factors cell-cell signalling is an important mechanism in myogenesis.

Receptor tyrosine kinases (RTKs) are involved in intercellular communication in a wide range of processes. RTKs are composed of three domains: an extracellular ligand-binding domain, a single membrane-spanning domain and a cytoplasmic catalytic domain (Yarden and Ullrich, 1988). Ligand binding to the extracellular domain induces activation of the kinase on the cytoplasmic side, which initiates the intracellular signalling. The activated RTKs phosphorylate themselves and cytoplasmic substrates, leading to activation of a number of downstream signalling molecules, and ultimately induce changes in gene expression and the phenotypic state of the cell (Fantl et al., 1993; van der Geer et al., 1994). RTKs thus play important roles in cellular proliferation and differentiation. The role of RTKs during embryonic development and especially during the determination of distinct cell types has been studied in detail in *Drosophila* (reviewed by Rebay, 2002). During specification of muscle progenitor cells from equivalent cell clusters, the Ras/MAPK pathway functions as an inductive cellular determination signal. This pathway is activated by both epidermal and fibroblast growth factor receptors in the dorsal embryonic mesoderm [(Gabay et al., 1997); DER (Buff et al., 1998) and *htl* (Michelson et al., 1998)], while Notch antagonises this activity by lateral inhibition (Carmena et al., 2002). Recently, a novel RTK named DAlk (*Drosophila* Anaplastic Lymphoma Kinase; Alk – FlyBase) was described which is expressed specifically in the developing visceral mesoderm and CNS of *Drosophila* (Lorén et al., 2001). Furthermore, activation of Alk mediated signalling is required for embryonic gut development and more specifically for the activation of MAP kinase in the visceral mesoderm (Lorén et al., 2003). As Alk drives MAPK activation in the visceral mesoderm it is an obvious candidate to be involved in determination of distinct visceral cell types.

In a search for genes involved in the determination of visceral cell types, we screened a collection of EMS-induced lethal mutations established previously (Hummel et al., 1999a; Hummel et al., 1999b) and identified two independent mutations with a nearly identical phenotype. Both exhibit a loss of circular visceral founder cells at early stages of visceral development, whereas fusion-competent visceral myoblasts are specified correctly and express markers like Fas3 and Sns. This results in a complete absence of visceral midgut muscles. Complementation analysis revealed that one mutation belongs to the previously described *jelly belly* (*jeb*) gene, while the second mutation named *milliways* (*mili^{Alk}*) represents a Alk allele. We describe the crucial role of these genes in the process of visceral founder cell determination and analyse the role of *Notch* and *Delta* during the distinction between visceral cell types. Moreover, we uncover Jeb signalling via the Alk RTK as a new determination step for the fusion-competent cells of the somatic mesoderm.

Materials and methods

Flystocks

To find out whether *Notch* and *Delta* mutants have defects in founder cell determination in the visceral mesoderm, we used *N^{55e11}* and *Dl^{B2}* mutants from the Bloomington *Drosophila* Stock Center. For analysis of the involvement of *Notch* and *Delta* in the founder determination process we used a dominant-negative form of *Notch* [*UAS-dnNotch* (Rebay et al., 1993; Go et al., 1998)], *UAS-Notch+Delta* (Bloomington *Drosophila* Stock Center, M. Muskavitch, unpublished) and *UAS-Notch^{intra}* flies (Lieber et al., 1993). *UAS-Notch^{intra}* allows expression of a constitutive active form of the Notch receptor.

The collection of EMS mutagenised flies was obtained from Christian Klämbt (Hummel et al., 1999a; Hummel et al., 1999b). To

screen for mutants with defects in the determination of founder cells in the visceral mesoderm, we stained 180 lines on the second and 270 lines on the third chromosome, with anti-Fas 3 antibodies (Patel et al., 1987). We isolated a new *jeb* allele, jeb^{weli} and a new *Alk* allele, which we named $mili^{Alk}$. These two mutants were used for all experiments described herein.

For complementation tests we used jeb^{k05644} (Weiss et al., 2001) from the Bloomington *Drosophila* Stock Center, and *Df(2R)AlkΔ21* which is a deficiency covering 53C (Lorén et al., 2003). β-galactosidase-expression in founder cells was achieved using the enhancer trap line *rP298-lacZ* (Nose et al., 1998; Klapper et al., 2002) and in the entire mesoderm using *bap-lacZ* (Azpiazu and Frasch, 1993; Zaffran et al., 2001). Overexpression studies were carried out using a *bap-GAL4* driver line (Zaffran et al., 2001), which drives expression in the circular visceral mesoderm from stage 10 onwards or a *twist-GAL4* driverline (*SG24-GAL4*), which drives expression in the entire mesoderm (gift from A. Michelson). As *UAS* lines we used *UAS-Alk* (Lorén et al., 2001) and *UAS-jeb* flies (Weiss et al., 2001). All overexpression studies were carried out at 25°C.

Immunohistochemical staining

Immunostaining was performed as described previously (Knirr et al., 1999; Klapper et al., 2002). The mouse Fas3 antiserum (Patel et al., 1987) was used for the visualisation of the visceral mesoderm cells (diluted 1:5, gift from C. Klämbt). A rabbit anti-β-galactosidase antibody (Biotrend, diluted 1:2500) was used to visualise muscle founder cells in the enhancer trap line *rP298-lacZ* and a mouse anti-β-galactosidase antibody (Promega, diluted 1:500) was used to visualise *bap-lacZ* expression. For analysis of the somatic mesoderm, we used rabbit anti-β3tubulin antibodies at a dilution of 1:2500 (Leiss et al., 1988). The rabbit anti-Alk (diluted 1:500) (Lorén et al., 2003), rabbit anti-Jeb (diluted 1:100) (Weiss et al., 2001), rabbit anti-Lmd (diluted 1:1000) (Duan et al., 2001), mouse anti-Notch (diluted 1:10) (Fuß and Hoch, 2002) and mouse anti-Delta antiserum (diluted 1:50, Developmental Studies Hybridoma Bank) were used in combination with the TSA signal amplification kit (NEN). For secondary antibodies, we used Cy2- and Cy3-labelled antibodies made in goat against rabbit and mouse from Dianova (diluted 1:40 and 1:100). The embryos were embedded in Fluoromount G (Southern Biotechnology Associates) and photos were taken under Nomarski optics with a Zeiss Axiophot microscope or a Leitz confocal microscope and processed with Adobe Photoshop 6.0 (Adobe Systems).

In situ hybridisation

In order to visualise fcms in the mesoderm, whole-mount fluorescent DNA hybridisation was performed with random digoxigenin-labelled *sticks and stones* cDNA probes according to Knirr et al. (Knirr et al., 1999). The *sns* cDNA was a gift from S. Abmayr (Bour et al., 2000). For the combination with antibody stainings to visualise β-galactosidase expression we used anti-β-galactosidase antiserum (Cappel) at a dilution of 1:3000.

Lethal phase analysis

To test the lethality of the progeny from *UAS-GAL4* crosses we mated homozygous lines carrying the founder cell marker *rP298-lacZ*, collected eggs for 24 hours and allowed further development for another 48 hours at 25°C. Afterwards, we counted at least 1000 progeny of each cross.

Results and discussion

Founder cell determination in the visceral mesoderm depends upon *Notch*

The process of lateral inhibition involving *Notch* and its ligand *Delta*, which was first discovered in the nervous system, also plays a role in determining the founder and fusion-competent myoblasts (fcms) of the somatic musculature (Carmena et al., 2002). As many of the processes involved in the development of the somatic musculature also seem to affect the development of the visceral muscles, we investigated whether the mechanism of determination of founder and fcms is also conserved.

Notch and *Delta* play important roles in various developmental processes and mutations in either gene lead to strong developmental defects during embryogenesis. Thus, it is difficult to analyse whether the visible defects in the visceral mesoderm are due to defects in the determination of the founder cells or are a result of secondary effects. In *Notch* mutant embryos more founder cells appear to be present in the visceral mesoderm (Fig. 1A,B). The visceral fcms seem to be reduced compared with the wild-type expression of *sticks and stones* (*sns*) as a marker for these cells (Fig. 1D,E). This reduction is not as severe as in the somatic mesoderm but still quite obvious. In *Delta* mutants, the number of founder cells also seems to be increased in comparison with the wild type and the fcms are reduced in mutant embryos (Fig. 1C,F).

These observations cannot exclude the possibility that the observed phenotypes are induced by secondary effects from defects in other tissues, among others the lack of fcms in the somatic mesoderm. We therefore decided to perform overexpression studies using the *UAS-GAL4* system (Brand and Perrimon, 1993). The *GAL4* and *UAS* lines employed here also carry *rP298-lacZ* (Nose et al., 1998), which serves to mark the founder cells. As a driver line we used *bap-GAL4* (Zaffran et al., 2001) to drive expression in the entire trunk visceral mesoderm. Expression of *UAS-Notch+Delta*, which contains the entire coding regions of both genes (M. Muskavitch, unpublished) or $UAS\text{-}Notch^{intra}$, which represents a constitutively active form of *Notch* (Lieber et al., 1993), in the visceral mesoderm, both result in a distinct phenotype. In midgut preparations of these embryos the founder cells of the circular visceral mesoderm are strongly reduced (Fig. 1K,L; data not shown for *UAS-Notch+Delta)* and later on, no functional visceral mesoderm can be observed (data not shown). By contrast, the founder cells of the longitudinal visceral muscles, which have a different origin at the posterior tip of the embryo are still present (arrowheads in Fig. 1K,L). Interestingly, *bap-GAL4*-driven expression of the *Notch* ligand *Delta* does not result in fewer founder cells in the visceral mesoderm (data not shown).

To exclude the possibility that the described defects are due to non-endogenous effects induced by the overexpression of the examined genes in the wrong tissue, we analysed wild-type Notch expression and found that it is indeed expressed in the visceral mesoderm. Notch is localised at cell membranes in the entire visceral mesoderm during stage 11, with expression becoming weaker in the fcms of the visceral mesoderm, which continue to express *bap-lacZ* after the determination process is finished (Fig. 1G,H, arrowhead in G). This reduction of Notch expression in the fcms after the establishment of the founder cells is similar to its expression in the somatic mesoderm, where Notch expression is also highest in the progenitor cell after the determination process is completed (Carmena et al., 2002). Surprisingly, the analysis of Delta expression exhibits that this Notch ligand is not expressed in the visceral mesoderm during founder cell formation. Delta expression was found in adjacent, probably somatic cells (Fig. 1I,J) and might be

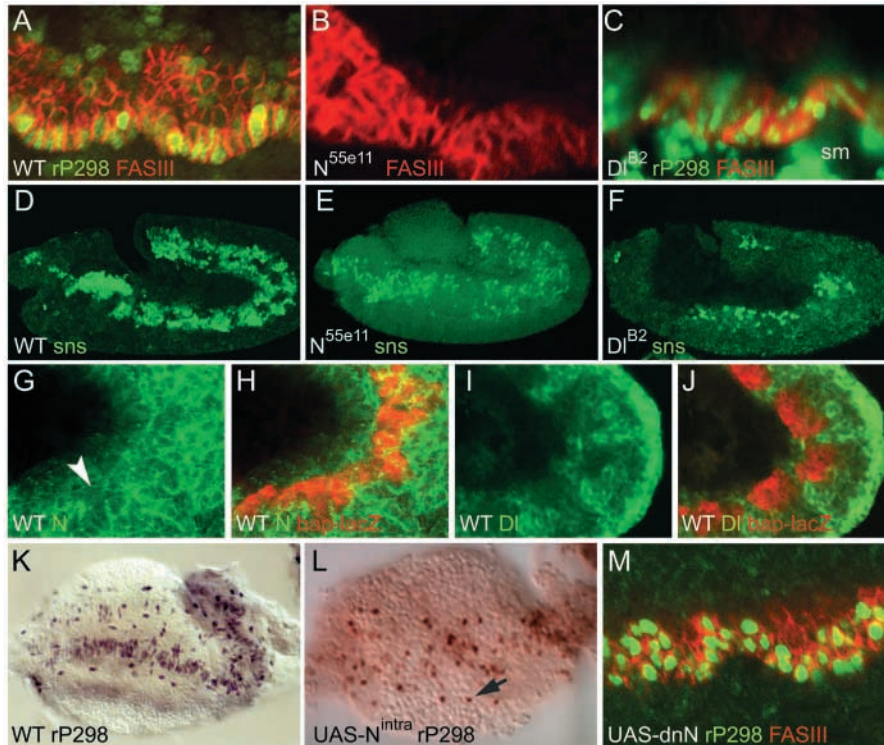

Fig. 1. *Notch* signalling is involved in founder cell determination in the visceral mesoderm. The visceral mesoderm is visualised by Fas3 (red in A,B,C,M), β-galactosidase expression from *bap-lacZ* (red in H,J) and founder cells are marked by *rP298-lacZ* expression (green in A,C,M brown in K,L). (A) Visceral mesoderm (vm) of a stage 11 wild-type embryo. (B) In Fas3 staining of *Notch*^{55e11} embryos, it seems as if most cells of the vm are converted into founder cells as indicated by the stronger Fas3 expression and the more rectangular shape. (C) In stage 11 *Delta*^{B2} mutants, the number of founder cells also seems to be increased in the visceral mesoderm and fewer fcms are visible (compare C with A). The clusters of *rP298-lacZ*-positive cells ventral to the visceral mesoderm belong to the somatic mesoderm (sm). (D-F) *sns* in situ hybridisation comparing the wild type (D) where *sns* is expressed in two bands with the *sns* expression in *N*^{55e11} (E) and *Dl*^{B2} (F) embryos where it is reduced. This reduction is more severe in the ventral band of the fcms of the somatic mesoderm but also visible in the dorsal band of the fcms of the visceral mesoderm. (G,H) Notch expression in the visceral mesoderm in stage 11 embryos. (G) Notch is expressed ubiquitously at the membrane of all cells of the visceral mesoderm. (H) Some cells seem to have a lower expression level of Notch (arrowhead in G). These cells also express *bap-lacZ*, which at this stage is mainly restricted to the fcms (H). (I,J) In contrast to the N expression Delta is expressed in the cells adjacent to the *bap-lacZ* positive cell clusters of the visceral mesoderm. (K) *rP298-lacZ* expression in the gut of a stage 14 wild-type embryo. (L) In gut preparations of stage 14 embryos with ectopic expression of *UAS-N*^{intra} in the visceral mesoderm, the number of *rP298-lacZ* positive founder cells of the circular visceral musculature is decreased. Founder cells of the longitudinal visceral muscles are not affected and migrate normally in anterior direction (arrow in L). (M) In stage 11 embryos overexpressing a dominant-negative form of Notch (*UAS-dnN*) with a *bap-GAL4* driverline, the number of *rP298-lacZ* positive founder cells is increased compared with the wild type (A) and also some cells which are not marked by *lacZ* expression exhibit a stronger Fas3 expression, which is characteristic for the founder cells in the visceral mesoderm.

needed there to participate in the visceral determination process, as indicated by the increased number of founder cells and reduced number of fcms in *Delta* mutants. Even though Dl is expressed in the cells surrounding the visceral mesoderm, ectopic expression of *UAS-Dl* in these cells with a *twi-GAL4* driver line does not result in an obvious phenotype (data not shown), which might be due to the fact that the amount of Delta in this tissue is not the limiting factor that restricts *Notch* signalling. Another explanation for a missing Delta expression in the visceral mesoderm might be that a different factor acts as a ligand for Notch in the visceral mesoderm and that the observed phenotype in *Delta* mutants is due to secondary effects.

As the ectopic expression causes such a severe phenotype we also tested the lethality of these embryos (Fig. 2). Most of the progeny of the cross between the *bap-GAL4* driver line and *UAS-N+Dl* or *UAS-N*^{intra} develop and hatch but die as first larvae (78% or 70%), presumably owing to the fact that they cannot ingest any food. Ectopic expression of *UAS-Dl* alone also increased lethality compared with the *UAS* and *GAL4* lines alone (data not shown), but still ~65% of the larvae survive.

To confirm these results, we overexpressed a dominant-negative form of *Notch* [*UAS-dnN* (Rebay et al., 1993; Go et al., 1998)] specifically in the visceral mesoderm with a *bap-GAL4* driverline. The embryos exhibit an obvious duplication of most visceral founder cells but still some fcms remain (Fig. 1M).

From these results, we conclude that *Notch* plays a role in the determination of the founder cells and fcms in the visceral mesoderm. *Delta*, which is expressed in the cells surrounding the visceral mesoderm, might serve as the ligand in this process but it is also possible that another factor takes over this role. Hence, not only is the fusion mechanism between the founder cells and the fcms in the somatic and visceral mesoderm conserved (San Martin et al., 2001; Klapper et al., 2002), but so is the initial mechanism of determination of these two cell types.

A screen for genes involved in visceral mesoderm development

To find out more about the mechanisms involved in the formation of the visceral muscles, we decided to screen a collection of EMS mutagenised flies (Hummel et al., 1999a; Hummel et al., 1999b) in order to search for genes involved in the determination of the two visceral cell types as well as in other aspects of visceral mesoderm differentiation.

Mutant embryos were stained and analysed with Fasciclin 3 (Fas3) (Patel et al., 1987), which marks the complete visceral mesoderm and allowed us to distinguish between the two cell types. Founder cells show a strong Fas3 expression and are characterised by a more columnar shape, while the more globular fcms show a clearly weaker Fas3 expression (Klapper et al., 2002). Using this approach, we identified several mutants with various defects in the development of the visceral musculature. These novel mutants can be summed up in four subgroups (Fig. 3).

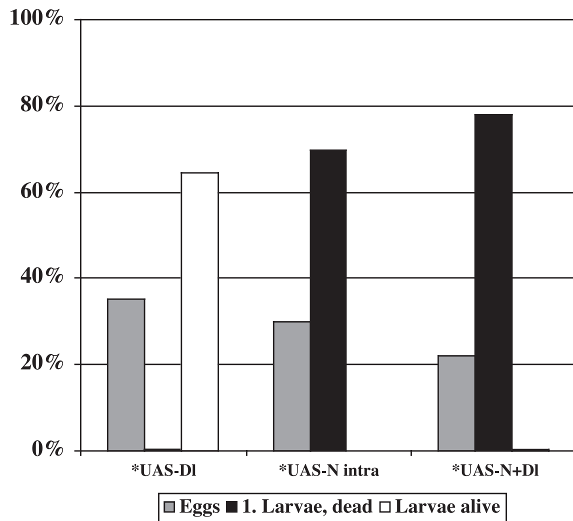

Fig. 2. Ectopic expression of *Dl*, N^{intra} and *N+Dl* in the visceral mesoderm results in increased lethality. Lethality and survival rate of the progeny of the *UAS-GAL4* crosses (n≈1000). All crosses were carried out with a *bap-GAL4* line (asterisk indicates crossed to *bap-GAL4*) carrying *rP298-lacZ* as a founder cell marker.

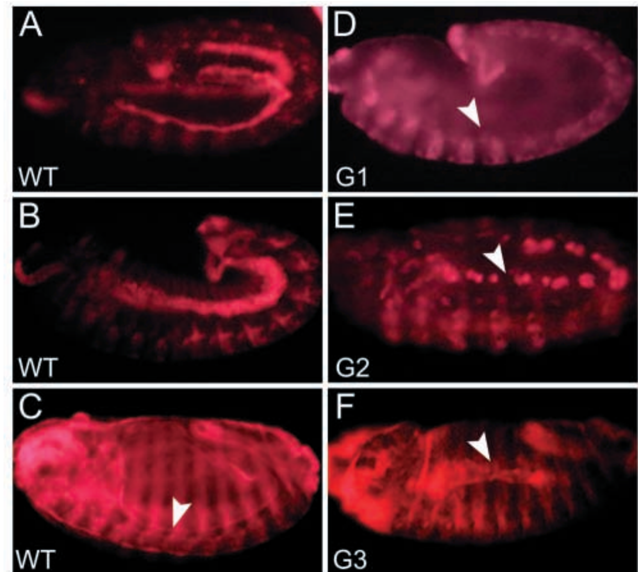

Fig. 3. An EMS screening approach identifies novel mutations, which display severe defects during visceral mesoderm development. The development of the visceral mesoderm is visualised by Fas3 expression. (A-C) Wild-type development at stages 11 (A), 12 (B) and stage 15 (C, arrowhead shows the midgut encircled by the visceral mesoderm). (D-F) Examples for the different subgroups of mutations are shown. (D) In embryos of the first subgroup (G1), no visceral mesoderm can be detected at stage 11 (arrowhead). (E) In the second group (G2), embryos develop the initial patches of the circular visceral muscles, but these patches subsequently fail to form a continuous band (arrowhead). (F) In the third group (G3), the continuous band is formed but the cells of the visceral mesoderm do not migrate dorsally and ventrally to encircle the entire midgut (arrowhead in F compare with arrowhead in C).

The first group (G1) shows no Fas3 staining in the visceral mesoderm at any stage of embryonic development when compared with the wild type (Fig. 3A,D). In these G1 mutants, the initial subdivision of the mesoderm in precursors of the somatic and visceral mesoderm might be defective. The second group (G2) shows Fas3 staining in the visceral mesoderm, but the cells fail to form a continuous band (Fig. 3E) as is observed in wild type from stage 11 onwards (Fig. 3B). The third group (G3) shows defects at even later stages. Here, the continuous band of the visceral mesoderm forms, but the cells do not migrate dorsally or ventrally (Fig. 3F) and thus do not encircle the midgut as they do in wild-type embryos (arrowhead in Fig. 3C). It remains unclear whether this is due to a failure of myoblast fusion or to other reasons. The analysis of these mutants will be described elsewhere, while the fourth group (G4) is described here.

Identification of *jelly belly* (jeb^{weli}) and the receptor for Jeb, *milliways* ($mili^{Alk}$) the *Drosophila Alk* homologue

In the same screen, we found a fourth subgroup (G4) consisting of two independent mutations, *wellville* (*weli*) and *milliways* (*mili*), with the same, distinct phenotype (Fig. 4). In these two mutants, the continuous band of the visceral mesoderm in stage 11 is formed, but when stained with Fas3, the more columnar shaped founder cells with the stronger Fas3 expression are absent (Fig. 4B,C compare with A). Thus, it appears that the founder cells of the circular visceral muscles are not determined in either of these mutants. Using the enhancer trap line *rP298-lacZ*, which shows a β-galactosidase pattern reflecting the expression of Duf/Kirre (Nose et al., 1998; Ruiz-Gómez et al., 2000), we could indeed show that in both mutants this founder cell marker is not expressed in the visceral mesoderm (Fig. 4E,F, compare with D). In contrast to these observations, the determination of founder cells in the somatic mesoderm is not affected (Fig. 4J-L), and the somatic muscle pattern shows only mild fusion defects, which are especially obvious in the dorsal and ventral muscles (Fig. 6A-C). At later stages no visceral mesoderm is present in either mutant (Fig. 4H,I, compare with G).

Both mutations, *weli* and *mili*, are located on the second chromosome. Complementation tests were subsequently performed with mutants on the second chromosome, which are known to affect visceral mesoderm development. Surprisingly, this analysis revealed that *weli* is a new *jelly belly* (jeb^{weli}) allele. *jeb* encodes a secreted protein that is produced in the somatic mesoderm but is needed for proper visceral mesoderm formation and has been proposed to be essential for the migration and differentiation of the visceral mesoderm (Weiss et al., 2001). The phenotype of the specific loss of founder cells of the circular visceral muscles has not been described.

mili displays the same distinct phenotype as *jeb* and we reasoned that it is likely that both genes are involved in the same pathway. As Jeb is a secreted protein the most promising candidate for *mili* was *Drosophila Alk*, a member of the Alk/Ltk family of receptor tyrosine kinases (RTKs), which is expressed in the nervous system and the visceral mesoderm (Lorén et al., 2001). Alk is considered to be a possible receptor for *jeb* signalling (Lorén et al., 2003).

In order to further analyse whether *mili* is indeed an allele

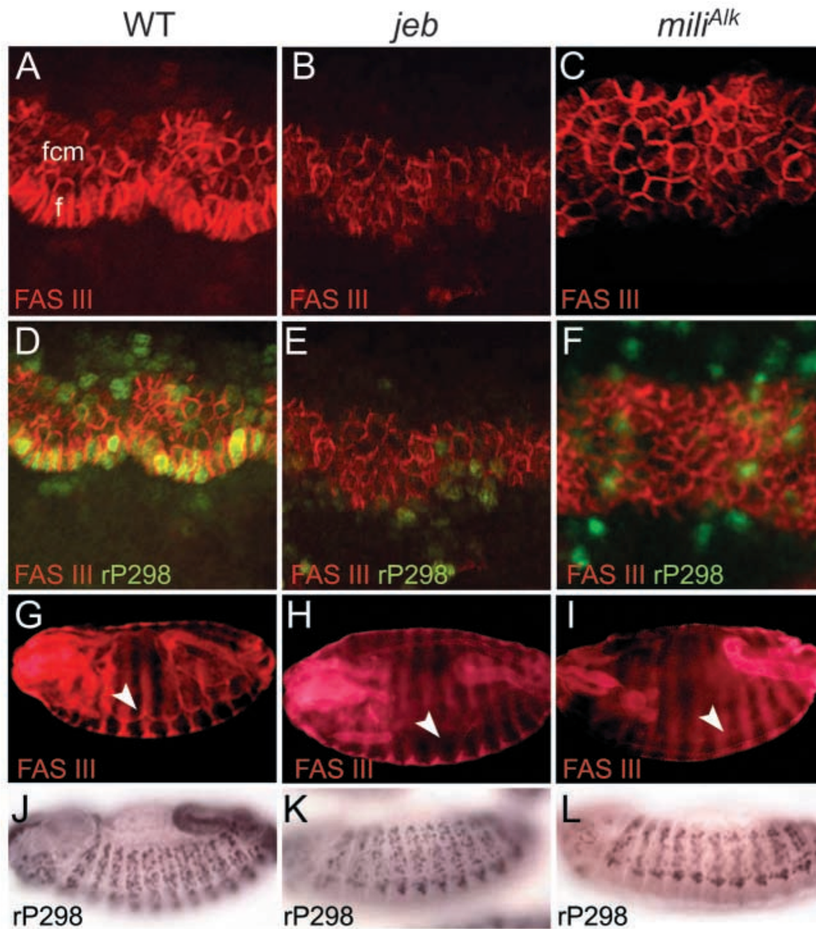

Fig. 4. In *jeb*^{weli} and *mili*^{Alk} mutants the founder cells of the circular visceral musculature are not determined. The development of the visceral mesoderm in wild-type (WT), *jeb*^{weli} and *mili*^{Alk} mutants as visualised by Fas3 expression (red in A-I). (A-F) Stage 11, (G-I) stage 15-16 and (J-L) stage 14. The nuclei of the founder cells are marked by *rP298-lacZ* expression (green in D-F, brown in J-L). In *jeb*^{weli} and *mili*^{Alk} mutants at stage 11, the more columnar-shaped founder cells are missing. Only cells that display the more globular shape of the fusion-competent myoblasts (fcm) are present (B,C,E,F). Additionally, the founder cell marker *rP298-lacZ* is absent in the visceral mesoderm in these mutants (E,F). The *rP298-lacZ*-positive cells, which can be observed over the fcms of the visceral mesoderm in D-F belong to the somatic mesoderm. At later stages, there are no signs of visceral musculature in either mutant (stage 16, arrowheads in H-I). (J-L) The founder cells of the somatic mesoderm are not affected in these mutants as visualised by *rP298-lacZ* expression (stage 14, K,L compare with J).

of *Alk*, we tested a newly created deficiency in the region (*Df(2R)Alk*Δ*21*) in which *Drosophila* Alk has been removed (Lorén et al., 2003). Indeed, *mili* is allelic to *Df(2R)Alk*Δ*21*, and furthermore, embryos transheterozygous for *Df(2R)Alk*Δ*21* and *mili* show the same phenotype as *mili* mutant embryos on Fas3 analysis (data not shown). We then tested *mili* directly against the newly generated *Alk*^{1} allele (Lorén et al., 2003) and could indeed confirm that *mili* is a new *Alk* allele, which we now named *mili*^{Alk}. The analysis of *mili*^{Alk} mutants with the help of Alk antibodies (Lorén et al., 2001) reveals that the mutant Alk protein is found in the cytoplasm instead of its normal localisation at the cell membrane. Therefore we conclude that the mutation is a phenotypic null allele. Furthermore, we could rescue the specific loss of founder cells in the visceral mesoderm by ectopic expression of *UAS-Alk* in the *mili*^{Alk} mutant background using *bap-GAL4* as driver (data not shown).

Thus, the two newly identified mutants, both of which display the same, very distinct, phenotype of loss of founder cells in the visceral mesoderm, turn out to be novel *jelly belly* and *Alk* alleles.

The remaining cells of the visceral mesoderm in *jeb*^{weli} and *mili*^{Alk} mutants differentiate to fusion-competent myoblasts

We have shown that the cells of the visceral mesoderm in *jeb*^{weli} and *mili*^{Alk} mutants do not express the founder cell marker *rP298-lacZ* and exhibit exclusively a globular shape upon Fas3 staining, which is characteristic for fcms. This raised the question of whether the cells indeed are determined to become fcms or remain undifferentiated. To clarify this question, we performed in situ hybridisation with *sns* as probe (Fig. 5). *sns* is expressed in all fcms, both in the somatic and in the visceral mesoderm (Bour et al., 2000; San Martin et al., 2001; Klapper et al., 2002). In the wild type during stage 11, two bands of *sns*-expressing cells can be observed in the mesoderm, which are connected in a ladder like pattern and represent the fcms of the somatic and visceral mesoderm (Fig. 5A). In *jeb*^{weli} and *mili*^{Alk} mutants (Fig. 5B,C), only one band is present whereas the other band is missing. As indicated by the location of the connecting cells ventral of the present band, the dorsal band consisting of the fcms of the visceral mesoderm is still present (arrowheads in Fig. 5B,C). Thus, the remaining cells in the visceral mesoderm differentiate as fcms and express genes that are characteristic for this differentiated cell type.

Jeb signalling and Alk expression are also responsible for fusion-competent myoblast differentiation in the somatic mesoderm

The finding that in *jeb*^{weli} and *mili*^{Alk} mutants in addition to the lack of founder cells in the visceral mesoderm also the fcms of the somatic mesoderm do not express fcm specific genes like *sns* was interesting as only mild defects in the somatic muscles are observed (Fig. 6B,C). To explain this phenotype, we took a closer look at the Alk expression in wild-type embryos. In addition to the expression of Alk in the cells of the visceral mesoderm, additional patches can be found in the neuroectoderm and the somatic mesoderm during stages 10 and 11 (Fig. 5D-F). We conclude that these patches of Alk expression in the somatic mesoderm are essential for the development of the somatic fcms because in Alk mutant embryos, which are unable to activate the RTK pathway, these cells do not express fcms-specific genes (Fig. 5C). Furthermore, *jeb* signalling is also required for this process, because the same phenotype can be observed in *jeb*

mutants (Fig. 5B). Therefore the RTK signalling pathway involving Jeb and Alk is not only needed for founder cell specification of the visceral mesoderm but also for the differentiation of the fcms in the somatic mesoderm.

Having found that *sns* is no longer expressed in the fcms of the somatic mesoderm, we decided to look at the transcription factor *lame duck/myoblast incompetent/gleeful* (*lmd/minc/glee*), which is expressed in the somatic and visceral fcms and is responsible for their determination (Duan et al., 2001; Furlong et al., 2001; Ruiz-Gómez et al., 2002). The expression pattern of Lmd in the wild type is similar to that of *sns* and the protein is present in two bands in stage 11-12 (Fig. 5G). In both jeb^{weli} and $mili^{Alk}$ mutants, the Lmd expression pattern is present (Fig. 5H,I) not only in the fcms of the visceral mesoderm but also in the somatic ones, even though it seems as if it is slightly weaker in the ventral part in the mutants than in the wild type. These data suggest that in jeb^{weli} and $mili^{Alk}$ mutants the initial determination of the fcms in the somatic mesoderm takes place, but the subsequent differentiation is blocked. Therefore, the Alk-RTK signalling pathway in the somatic mesoderm seems to be essential for the differentiation of the fcms but not for the initial determination.

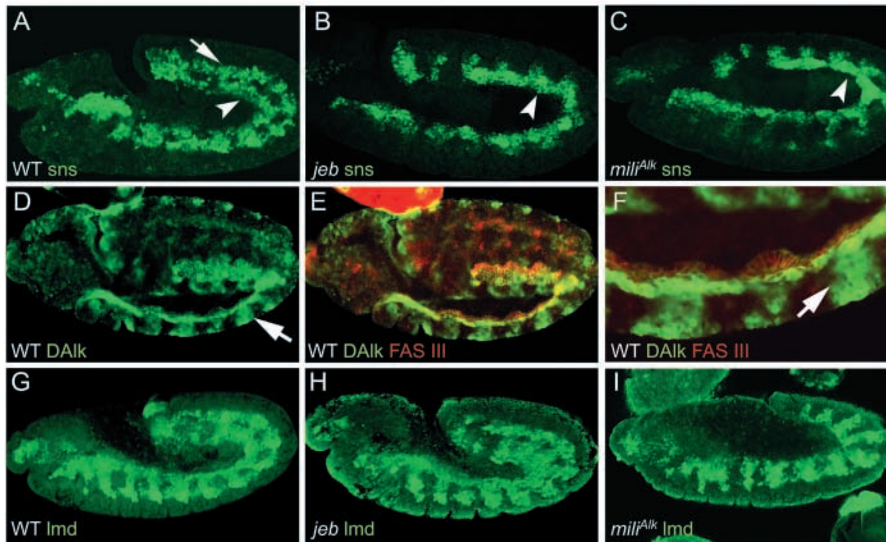

Fig. 5. The fusion-competent myoblasts of the somatic mesoderm do not differentiate in jeb^{weli} and $mili^{Alk}$ mutants. (A-C) *sns* in situ hybridisation of stage 11 embryos marks fcms in the visceral and somatic mesoderm. In wild-type embryos, *sns* is expressed in two bands along the entire length of the embryo (A). These bands are connected in a ladder-like pattern, where the ventral band represents the fcms of the somatic mesoderm (arrow in A), and the dorsal band consists of the fcms of the visceral mesoderm (arrowhead in A). In jeb^{weli} (B) and $mili^{Alk}$ (C) mutant embryos, the fcms of the visceral mesoderm and some cells that connect the two bands are present (arrowheads); however, the ventral band of fcms of the somatic mesoderm shows no *sns* expression. (D-F) Alk (green) and Fas3 expression (red) in stage 11 wild-type embryos. In addition to expression in the visceral mesoderm, Alk is also transiently expressed in some cells in the neuroectoderm and in patches in the somatic mesoderm (arrows in D,F). (G-I) Lmd expression of stage 12 embryos. In the wild type, Lmd is expressed in two bands in the fcms of the somatic and visceral mesoderm (G). In jeb^{weli} (H) and $mili^{Alk}$ (I) mutants, expression in both mesodermal cell types can be observed.

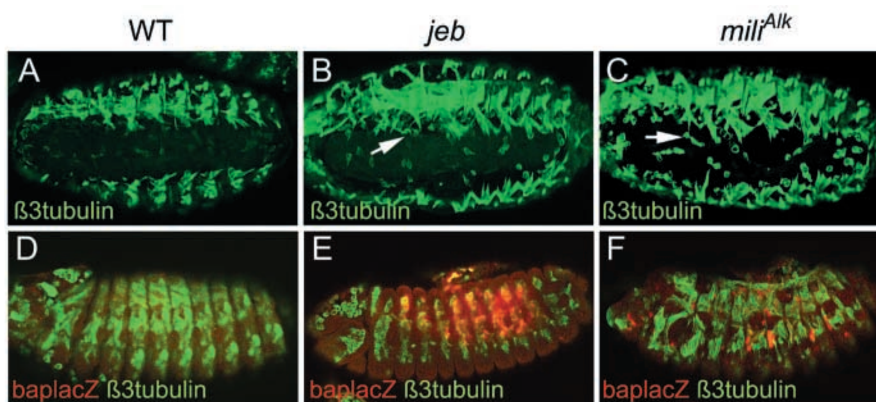

Fig. 6. The cells of the visceral mesoderm of jeb^{weli} and $mili^{Alk}$ mutants become incorporated in the somatic musculature. Ventrolateral (A,B,C) muscles of stage 16 embryos as visualised by β3tubulin expression (green in A-F). Cells of the visceral mesoderm are marked by *bap-lacZ* expression (red in D-F). Compared with the wild type (A) the somatic muscles in both jeb^{weli} and $mili^{Alk}$ mutants are thinner and show long thin projections (B,C; arrow in B). Several unfused myoblasts are visible (arrow in C). (D-F) In wild-type embryos, no *bap-lacZ* expression is found in the somatic mesoderm during all stages of development (stage 14, D). By contrast, jeb^{weli} and $mili^{Alk}$ mutant embryos exhibit *bap-lacZ* expression in the lateral muscles of the somatic mesoderm (stage 14, E,F).

The fusion-competent myoblasts of the visceral mesoderm become incorporated into the somatic mesoderm

Because most of the fcms of the somatic mesoderm are not expressing *sns* in jeb^{weli} and $mili^{Alk}$ mutants, we had a closer look at defects in this tissue. β-galactosidase antibody staining in mutants carrying the founder cell marker *rP298-lacZ* show a regular pattern of somatic founder cells compared with the wild type in the somatic mesoderm (Fig. 4J-L). Only in some of the mutant embryos could we detect local distortions because the defects in the visceral mesoderm (data not shown). β3tubulin antibody staining (Leiss et al., 1988) shows some mild fusion defects in the dorsal and ventral muscles in jeb^{weli} and $mili^{Alk}$ mutants indicated by unfused myoblasts in this region and long thin projections of the muscles (Fig. 6B,C, compare with A).

The development of the visceral mesodermal cells cannot be followed with Fas3 staining because it disappears in the mutants after stage 11. Therefore, we visualised the fate of the fcms using the visceral mesoderm marker *bap-lacZ*, which normally is expressed exclusively in the visceral mesoderm throughout embryonic development (Azpiazu and Frasch, 1993; Zaffran et al., 2001) (Fig. 6D). We observe that jeb^{weli} and $mili^{Alk}$

mutants carrying this marker show β-galactosidase expression in the somatic mesoderm from late stage 12 onwards (Fig. 6E,F).

From previous studies, it is known that a lack of *sns* expression in fcms in the somatic mesoderm results in strong defects in the somatic musculature where the founder cells become blocked at the point of myoblast fusion (Bour et al., 2000). Because we are not able to detect such a strong phenotype in $mili^{Alk}$ and jeb^{weli} mutants, and together with the fact that *bap-lacZ*-expressing cells are present in the somatic mesoderm, we conclude that the *sns*-expressing cells from the visceral mesoderm now become incorporated into the somatic mesoderm and replace at least a fraction of the somatic fcms.

Alk protein is mislocalised in *milliways*^{Alk} mutant embryos and co-localises with Jeb at the membranes of visceral founder cells

As *jeb* is a secreted protein we were interested whether the localisation of Alk controls the specification for the more ventral cells of the visceral mesoderm to become founder cells whereas the others develop into fcms. Staining with anti-Alk antibodies (Lorén et al., 2003) show that in the wild type the protein is localised at the cell membranes in the visceral mesoderm (Fig. 7A). Surprisingly, Alk can be found in the founder cells of the circular visceral muscles and in the fusion-competent myoblasts (Fig. 7A-C), which are not obviously affected in $mili^{Alk}$ mutants (Fig. 4C,F).

In jeb^{weli} mutants, the localisation of Alk is not affected. Identical to the wild type, it localises at the cell membranes and is also present in all cells of the visceral mesoderm, which persist in these mutants (Fig. 7D-F). In $mili^{Alk}$ mutant embryos, however, the Alk protein is still detectable in all cells of the visceral mesoderm, but it is not correctly localised at the cell membrane and is instead found in the cytoplasm (Fig. 7G-I). Because of this mislocalisation and the fact that the embryos transheterozygous for *Df(2R)Alk*Δ*21* and $mili^{Alk}$ display the same phenotype in the visceral mesoderm as the $mili^{Alk}$ mutant embryos alone (data not shown), we conclude that the mutation is a phenotypic null allele even though at least the N-terminal part of the protein, against which the antibody is raised, is still present. As the Alk receptor is not properly localised, the founder cells cannot receive the Jeb signal and thus the signal transduction pathway leading to the activation of *duf/kirre* in the visceral founders is disturbed.

As Alk is localised at the membranes of all visceral cells and not only in the founder cells, we reasoned that the localisation of the Jeb protein must be responsible for the activation of the RTK pathway only in visceral founder cells. Therefore we postulate a co-localisation of both proteins only at the prospective founder cells. The double immunolabelling with Jeb (Weiss et al., 2001) and Alk (Loren et al., 2003) antibodies demonstrates that Jeb protein only co-localises at the membranes of the visceral founder cells with the Alk protein (Fig. 7J,K). Moreover, this specific interaction cannot be found in $mili^{Alk}$ mutants where, owing to the mis-localisation of the receptor protein, no Jeb uptake takes place (Fig. 7L,M). Therefore these mutants display an inactive RTK pathway.

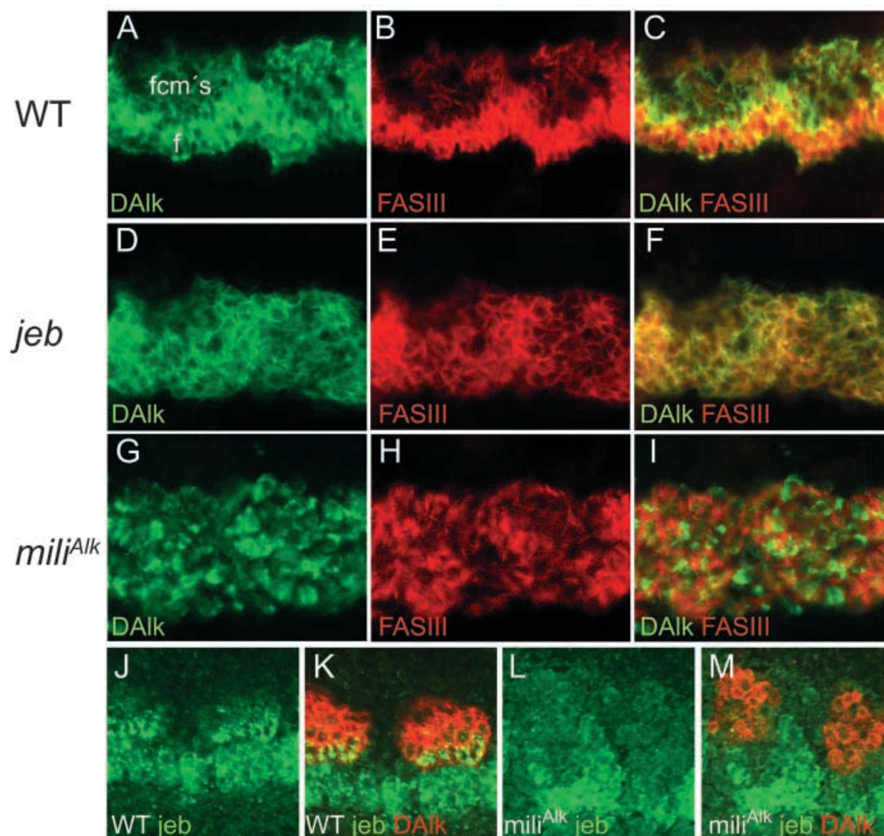

Fig. 7. Alk protein is mislocalised in $mili^{Alk}$ mutant embryos. Stage 11 embryos stained with Fas3 (B,E,H) to mark all cells of the visceral mesoderm or Alk antibodies (green in A,D,G). The stainings are merged in C,F,I. In wild-type embryos (A-C), Alk is expressed in both the founder cells (f) and fusion-competent myoblasts (fcms) of the visceral mesoderm and localises at the cell membrane. (A-C). In *jeb* mutant embryos (D-F), Alk is also expressed at the membranes of all visceral cells. In $mili^{Alk}$ mutants, the majority of the protein does not localise at the cell membranes but instead can be found in the cytoplasm (G-I). (J-M) In stage 11 wild-type embryos (J,K) Jeb (green in J-M) is taken up by the Alk-expressing cells of the visceral mesoderm (red in K,M), whereas in $mili^{Alk}$ mutants (L,M) where the Alk protein is present but non-functional there is no co-localisation of the two proteins.

Ectopic expression of *UAS-jeb* results in an increased number of founder cells in the visceral mesoderm

Previous work has shown that Jeb is secreted from the ventromedial cells of the somatic mesoderm, which are close to the visceral mesoderm (Weiss et al., 2001) (Fig. 9). Because all cells of the visceral mesoderm express the Alk RTK, it is theoretically possible that all are able to respond to *jeb* signalling. The fact that only the most ventral cells of the visceral

mesoderm display an active RTK pathway as a result of this interaction and later become the founders of the visceral mesoderm could be explained by the fact that these cells are closest to the cells that secrete the Jeb signal (Fig. 7K), which we suggest is the limiting factor. We therefore set out to test whether increased levels of Jeb can change the fate of the more dorsally located visceral fcms, which also express the receptor Alk, to become founder cells. We again used the *UAS-GAL4* system and expressed *UAS-jeb* in the entire mesoderm with a *twi-GAL4* driver. As expected, nearly all cells of the visceral mesoderm are now converted to founder cells (Fig. 8A,B). Even though fcms are missing, the founders are able to form visceral muscles that later on encircle the midgut. This ability to form muscles is one of the characteristics of founder cells. From *sns* and *mbc* mutants, it is known that even though no fusions take place, mini-muscles are formed in the somatic mesoderm that display the right orientation and attachment sites (Rushton et al., 1995; Bour et al., 2000). This has also been shown for the founder cells of the visceral mesoderm. In *sns* mutants, apart from the first gut constriction the visceral mesoderm develops normally (Bour et al., 2000). On closer inspection just the founder cells differentiate, whereas the fcms remain undifferentiated (Klapper et al., 2002). Thus, apart from the increased number of founder cells no defects are visible. The same phenotype can be observed if *UAS-jeb* is ectopically expressed only in the visceral mesoderm (data not shown).

sns in situ hybridisation confirms that through the overexpression of *UAS-jeb* in either the entire or just the visceral mesoderm, the fate of the fcms is changed so that they no longer express fcms-specific gene products such as SNS. This seems to be the reason why the band of fcms of the visceral mesoderm (Fig. 8D) is missing in these embryos (Fig. 8E). The somatic mesoderm (Fig. 8G) shows no defects as indicated by an anti-β3tubulin staining (Fig. 8H). Overexpression of *UAS-jeb* in a *Alk* mutant background shows that the *UAS-jeb* overexpression phenotype is suppressed in the Alk mutants (data not shown). Therefore *jeb* is dependent upon *Alk* as a receptor to activate the downstream signalling pathway.

In the wild type, the limited amount of the Jeb signal appears to restrict founder cell determination to the most ventral cells of the visceral mesoderm (Fig. 9). However, these findings prove that in principle all cells of the visceral mesoderm are able to respond to *jeb* signalling. Furthermore, we found no difference when the signal is produced from the somatic or the visceral mesoderm.

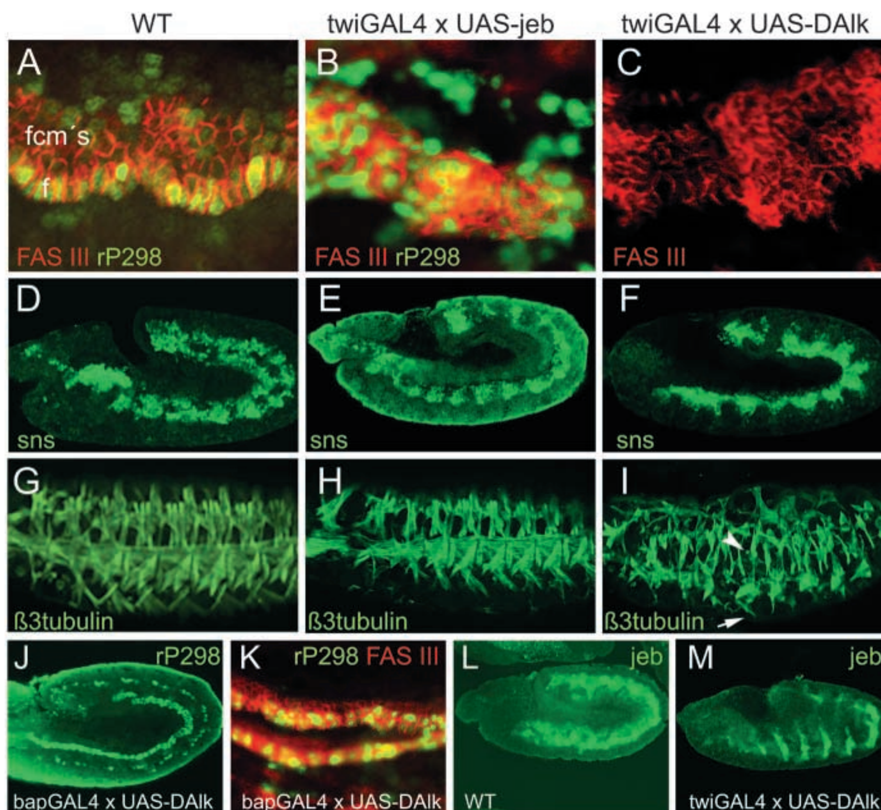

Fig. 8. Ectopic expression of *UAS-Alk* and *UAS-jeb* in the mesoderm gives rise to different phenotypes. Wild-type embryos are shown in A,D,G and L. (B,E,H) *UAS-jeb* is ectopically expressed with a *twi-GAL4* driverline. (C,F,I,J,K,M) *UAS-Alk* is ectopically expressed with a *twi-GAL4* (C,F,I,M) or *bap-GAL4* driver line (J,K). Founder cells (f) are marked by *rP298-lacZ* expression (green in A,B,J,K). (D-F) Fusion-competent myoblasts (fcms) are visualised by *sns* in situ hybridisation. (B) Ectopic expression of *UAS-jeb* converts all cells of the visceral mesoderm to founder cells. (E) *sns* expression is absent in these cells of the visceral mesoderm. (H) The somatic mesoderm shows no defects, as indicated by β3tubulin antibody staining. (C) No visceral founder cells can be detected by Fas3 staining of stage 11 embryos in which *UAS-Alk* is ectopically expressed in the entire mesoderm. (F) *sns* in situ hybridisation indicates that through the overexpression of *UAS-Alk* the fcms of the somatic mesoderm are missing in stage 11 and only the band of the fcms of the visceral mesoderm remains. (I) The dorsal and ventral somatic muscles in stage 16 show a fusion defect phenotype with thin projections (arrow) and unfused myoblasts (arrowhead) as indicated by β3tubulin staining. When *UAS-Alk* is expressed only in the visceral mesoderm with a *bap-GAL4* driverline, which also carries *rP298-lacZ* as founder cell marker, the founder cells of the visceral mesoderm are still present and seem to be doubled in number (J,K). In contrast to a single row in the wild type (A), a second row of founder cells is present in these stage 11 embryos. The bands of both halves of the embryo are shown (J,K). In the wild type, *jeb* is expressed in a continuous band in the mesoderm (L), whereas upon overexpression of *UAS-Alk* in the entire mesoderm the signal is reduced (M).

Ectopic expression of *UAS-Alk* gives a similar phenotype as in *mili*^{Alk} and *jeb*^{weli} mutants

Anti-Alk stainings on embryos carrying the visceral mesoderm marker *bap-lacZ* show that Alk is expressed in all cells of the visceral mesoderm, some neuroectodermal cells and transiently in stage 10 to 11 in cell clusters in the somatic mesoderm (Fig. 5D-F, Fig. 9). We were interested in the consequences of ectopic expression of *UAS-Alk* in the entire mesoderm. Surprisingly, the overexpression of *UAS-Alk* by a *twi-GAL4* driver produces a similar phenotype to

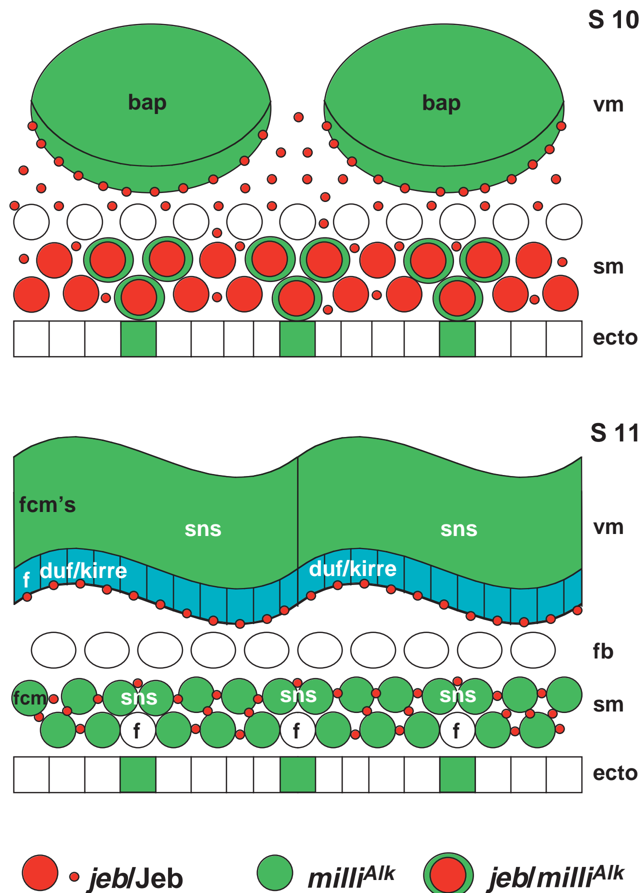

Fig. 9. The mode of action of Mili/Alk-mediated Jeb signalling during the determination and differentiation within the visceral and somatic mesoderm. At stage 10 (S 10) ventromedial somatic precursor cells start to express *jelly belly* (*jeb*, red cells) and secrete Jeb protein (small red dots) (Weiss et al., 2001). Some of these cells (red with green crescents) also express $mili^{Alk}$ (green) and all visceral precursor cells express $mili^{Alk}$ and *bagpipe* (*bap*) at this time. We conclude that Jeb signalling, especially the RTK pathway activation in visceral founder cells, depends on the level of Jeb protein reaching these cells. This activation leads in stage 11 (S 11) in the visceral mesoderm (vm) to the expression of *dumbfounded/kin of irre* (*duf/kirre*, blue) in the visceral founder cells (f), whereas the fusion-competent myoblasts (fcms) without an active pathway are characterised by *sticks and stones* (*sns*) expression. We further suggest that initial *jeb* expression in the somatic mesoderm (sm) is maintained mainly in *lame duck/myoblast incompetent/gleeful* (*lmd/minc/glee*)-expressing fcms responding on RTK pathway activation with differentiation into *sns*-expressing fcms. Nothing is known so far about the role of Jeb and Mili/Alk signalling in the ectoderm (ecto) and fat body (fb).

that in $mili^{Alk}$ or jeb^{weli} mutant embryos. In early stage 11 only fcms are visible in Fas3 stainings (Fig. 8C) and later on there is no evidence of the presence of visceral mesoderm any more. In the somatic mesoderm, defects can be seen by an anti-β3tubulin antibody staining (Fig. 8I). Several muscles are small and display a spindle-like shape with long and thin projections, indicating that only few myoblasts fuse to form the muscles. In comparison with jeb^{weli} and $mili^{Alk}$ mutants (Fig. 6B,C), in the Alk overexpressing embryos the muscle defects are stronger. Another surprising finding was that in this overexpression situation the *sns*-expressing cells of the somatic mesoderm are again missing (Fig. 8F).

It remains an unanswered question why the overexpression of *Alk* in the entire mesoderm results in a similar phenotype to that in jeb^{weli} and $mili^{Alk}$ mutants. One possible explanation for the visceral phenotype is that because of the absence of *sns*-expressing cells in the somatic mesoderm, *jeb* is not secreted anymore, which results in the absence of an active RTK pathway in the visceral founder cells. Therefore, we carried out anti-Jeb antibody stainings on these embryos. In stage 10 wild-type embryos, *jeb* is expressed in two bands in the somatic mesoderm and disappears in stage 12 from all mesodermal derivatives (Weiss et al., 2001) (Fig. 8L). In embryos overexpressing Alk in the entire mesoderm, we could observe only one small group of *jeb*-expressing cells per hemisegment (Fig. 8M). The reduced amount of the ligand Jeb thus phenocopies a *jeb* mutant situation where the visceral founders are not determined.

A distinct difference between the founder cells and the fcms in the somatic mesoderm is the expression of *lmd/minc/glee* in the fcms (Duan et al., 2001; Furlong et al., 2001; Ruiz-Gómez et al., 2002). We assume that in the wild type, only the fcms, which are characterised by this expression, are able to respond to the Jeb/Alk signalling pathway, which promotes the further differentiation of the somatic fcms. These in turn continue to secrete Jeb, which is required for the induction of the signalling pathway in the visceral mesoderm.

We assume that in the somatic mesoderm it is mainly the fcms that express Alk and suggest that the overexpression of Alk in the entire somatic mesoderm enables all cells of the mesoderm to take up the Jeb signal. Therefore, the signal necessary for the further differentiation of the fcms in the somatic mesoderm is downregulated through the increased Jeb uptake of the cells now ectopically expressing Alk. Another possibility to explain the visceral phenotype by overexpressing *UAS-Alk* in the whole mesoderm is that the overexpression of Alk itself leads to a strong downregulation of Jeb. As a consequence, the visceral founder cells are not specified, again owing to the lack of Jeb signal.

A further indication for the relevance of these changes in the somatic mesoderm for the visceral phenotype arises from the overexpression of *Alk* just in the visceral mesoderm with a *bap-GAL4* driverline. This does not result in the phenotype described above. In this case, the founder cells in the visceral mesoderm are present and seem to be even increased in number (Fig. 8J-K). We assume that due to the Alk overexpression additional cells of the visceral mesoderm are now able to take up some of the limited amount of Jeb signal from the somatic mesoderm and thus become founder cells. In this case, Jeb expression in the somatic mesoderm is not affected.

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