FGF signalling and the mechanism of mesoderm spreading in *Drosophila* embryos

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Summary

FGF signalling is needed for the proper establishment of the mesodermal cell layer in *Drosophila* embryos. The activation of the FGF receptor Heartless triggers the di-phosphorylation of MAPK in the mesoderm, which accumulates in a graded fashion with the highest levels seen at the dorsal edge of the mesoderm. We have examined the specific requirement for FGF signalling in the spreading process. We show that only the initial step of spreading, specifically the establishment of contact between the ectoderm and the mesoderm, depends upon FGF signalling, and that unlike the role of FGF signalling in the differentiation of heart precursors this function cannot be replaced by other receptor tyrosine kinases. The initiation of mesoderm spreading requires the FGF receptor to possess a functional kinase domain, but does not depend upon the activation of MAPK. Thus, the dispersal of the mesoderm at early stages is regulated by pathways downstream of the FGF receptor that are independent of the MAPK cascade. Furthermore, we demonstrate that the activation of MAPK by Heartless needs additional cues from the ectoderm. We propose that FGF signalling is required during the initial stages of mesoderm spreading to promote the efficient interaction of the mesoderm with the ectoderm rather than having a long range chemotactic function, and we discuss this in relation to the cellular mechanism of mesoderm spreading.

Key words: Signalling, FGF, Mesoderm, *Drosophila*

Introduction

The establishment of the mesodermal germ layer from the invaginated mesodermal primordium in the *Drosophila* embryo involves the transition of the epithelium to a mesenchymal state and the migration of cells away from the site of invagination. The cells spread over the ectoderm until the entire surface is covered. Subpopulations then receive differentiation signals from specific regions of the ectoderm and develop into precursors of heart, muscle, fat body, somatic gonad and other tissues. Proper spreading of the mesoderm depends on FGF signalling; a signal from the ectoderm (Gryzik and Müller, 2004; Stathopoulos et al., 2004) triggers the activity of the FGF receptor Heartless (Htl) in the mesoderm, resulting in the activation of MAPK (Gabay et al., 1997b). A second role for FGF in the mesoderm is to provide a differentiation signal for the heart cell precursors located in the population of cells that become apposed to the dorsal edge of the ectoderm (Michelson et al., 1998b).

The transmission of the signal from an activated FGF receptor to intracellular targets within cells is only partly understood. One protein that is essential for signal transduction by the *Drosophila* FGF receptors is the intracellular protein known as Dof, Heartbroken or Stumps (Imam et al., 1999; Michelson et al., 1998a; Vincent et al., 1998), which can physically interact with the receptor (Battersby et al., 2003; Petit et al., 2004; Wilson et al., 2004). Mutations in *dof* specifically affect processes that depend upon FGF signalling, and the protein is only present in cells that express FGF receptors. A number of components shared with other receptor tyrosine kinase (RTK) signalling pathways have also been shown to have a role in FGF signalling (Casci et al., 1999; Gabay et al., 1997a; Gabay et al., 1997b; Gisselbrecht et al., 1996; Hacohen et al., 1998; Johnson Hamlet and Perkins, 2001; Kramer et al., 1999; Perkins et al., 1996; Reich et al., 1999; Reichman-Fried et al., 1994). For example, Corkscrew (Csw) is important for the FGF-dependent formation of heart precursors and the development of the tracheal system (Gabay et al., 1997a; Johnson Hamlet and Perkins, 2001; Perkins et al., 1996). One function of Dof may be to recruit Csw to the signalling complex (Petit et al., 2004; Wilson et al., 2004). Other proteins that associate with Dof have been identified (Battersby et al., 2003), but their physiological significance in the activation of MAPK and the morphogenesis of the mesoderm and the tracheae remains to be established. Recently the Rac GTPases have been shown to be required for the development of the tracheae, but it is not clear whether they act in the FGF pathway, as the dynamic filopodia, which are promoted by FGF-signalling (Ribeiro et al., 2002; Sato and Kornberg, 2002), are still present at the tips of tracheal branches with reduced Rac activity (Chihara et al., 2003).

FGF signalling is implicated in cell migration and morphogenetic movements during gastrulation in many organisms, but the mechanisms and biochemical pathways responsible for these cellular movements are poorly understood. In *Xenopus* embryos Sprouty2 is induced in response to MAPK activation by FGF signalling. Unlike

Drosophila Sprouty, the *Xenopus* protein does not block Ras/MAPK signalling, but regulates a process that is not well defined to inhibit convergent extension movements (Nutt et al., 2001). Thus, in vertebrates the FGF-dependent pathways that regulate differentiation and morphogenesis can be distinguished by their sensitivity to Sprouty2. FGF signalling need not directly regulate the ability of cells to migrate. During gastrulation in mouse embryos, FGF signalling has been shown to allow cells to escape from the mass of the invaginated mesodermal primordium, by promoting the downregulation of E-Cadherin during the epithelial to mesenchymal transition (EMT), rather than by affecting the migratory properties of the cells (Ciruna and Rossant, 2001; Ciruna et al., 1997). In *Drosophila*, the mechanisms by which FGF signalling leads to the dispersal of mesodermal cells over the ectoderm are not known, nor is it known what additional cellular mechanisms participate in this process. Integrins and cadherins are both expressed at this stage of development, but surprisingly neither appears to be involved in the dispersal of the mesoderm (Leptin et al., 1989; Oda et al., 1998). FGF signalling alone cannot be responsible for this process, as in embryos mutant for components of the signalling system, a considerable amount of spreading still occurs. To understand mesoderm spreading, the potential roles for adhesion, intercalation, FGF-independent migration or chemotactic behaviour need to be dissected. It has been proposed that FGF signalling could be instructive, and direct migration of the mesodermal cells dorsally (Gabay et al., 1997b; Gryzik and Müller, 2004; Stathopoulos et al., 2004), but it is equally possible that the dispersal of the mesoderm depends upon the induction of motility by FGF and the preferential adhesion between mesoderm and ectodermal cells, rather than on a specific directional cue (Stathopoulos et al., 2004; Wilson and Leptin, 2000). In this paper, we investigate the specific requirement for FGF signalling in the dispersal of the mesoderm cells over the ectoderm, and assess the significance of the activation of MAPK within the mesoderm following the invagination of the mesoderm anlage.

Materials and methods

Fly stocks and genetic techniques

The following mutant alleles and transgene insertions were used in this study: *top*^{18A}, *brk*^{M68}, *sog*^{YS06}, *Star*$^{L2\text{-}20}$, *htl*^{AB42}, *dof*^{1}, *stg*^{7M53}, *Raf*^{EA75}, *Raf*$^{1(1)11\text{-}29}$, *DSor1*^{LH110}, *Ras85D*^{c40B}, *drk*$^{\Delta P24}$, *hep*^{r75}, *csw*$^{E(sev)1A\text{-}eOP}$, *dshc*^{13G}, *dshc*$^{111\text{-}40}$, *Rac1*^{J11}, *mbc*^{c2}, *mbc*$^{D11.2}$, *Rac1*^{EY05848}, *Rac2*$^{\Delta}$, *Mtl*$^{\Delta}$, *RhoA*^{72O}, *dock*^{P1}, *trio*^{1}, *trio*^{4}, *Pak*^{14}, *Pak*^{6}, *pbl*^{3}, *P[w*$^{+}$, *UAS-Htl]* (Michelson et al., 1998b), *P[w*$^{+}$, *UAS-λHtl]* (Michelson et al., 1998a), *P[w*$^{+}$, *UAS-λBtl]* (Lee et al., 1996), *P[w*$^{+}$, *UAS-λEGFR]* (Queenan et al., 1997), *P[w*$^{+}$, *UAS-λPVR]* (M. Y. Zhu, R.W. and M.L., unpublished), *P[w*$^{+}$, *UAS-Ras85D*^{Act}*]* (Gisselbrecht et al., 1996), *P[w*$^{+}$, *UAS-Draf*^{F20}*]* (Martin-Blanco et al., 1999), *P[w*$^{+}$, *UAS-Rac1*^{V12}*]* (Luo et al., 1994), *P[w*$^{+}$, *UAS-Cdc42*^{V12}*]* (Luo et al., 1994), and *P[w*$^{+}$, *sqh-E20, E21]* (Winter et al., 2001). The *rho* and *spi* stocks were gifts from the laboratory of C. Klämbt. They carry strong alleles of these genes, but the allele designations were unfortunately no longer traceable at the time of writing. The transgenes encoding receptors containing the dimerisation domain of the lambda repressor (λ) instead of their extracellular domains produce proteins that dimerise spontaneously and become autophosphorylated in a ligand-independent fashion (see Lee et al., 1996). To remove the maternal contribution of a gene the FLP-DFS technique was used (Chou et al., 1993). If an allele was not already linked to an FRT site, we used standard genetic recombination and selection techniques to combine the allele with the appropriate FRT site (Roberts, 1998; Theodosiou and Xu, 1998). Embryos that lacked the maternal and zygotic contribution were identified by using balancer chromosomes carrying *P[ry*$^{+}$*, ftz-lacZ]* insertions. Insertions of a *P[w*$^{+}$*, twi-Gal4]* element on a *htl*^{AB42} chromosome were generated as previously described for the *P[w+, twi-Gal4], dof*^{1} chromosome (Wilson et al., 2004).

Identification of transgenic and homozygous mutant embryos

Unless stated otherwise, all crosses used stable lines with Balancer chromosomes that carried a *lacZ*-transgene. The expression of *lacZ* was controlled by the ftz-promoter, and can be detected in early embryos from the stage of gastrulation. Embryos were double stained for β-Gal and the indicated markers, embedded in approximately 1 ml unpolymerised araldite in 1 cm diameter dishes and sorted under a dissecting microscope according to their β-galactosidase staining pattern. *lacZ*-positive and *lacZ*-negative embryos were matched in age by various criteria. In the case of MAPK staining, the highly dynamic staining pattern (in regions not affected by the mutations) provides an excellent additional frame of reference. In the case of Twist-staining, we have a great deal of experience in using the degree of germ band extension, the depth of the dorsal folds and the appearance of the head fold as age markers (especially as germ band extension is in its most rapid phase at the time that is relevant for our analysis). In the case of the Eve-staining, the Eve stripes provide a further marker that is very useful for early stages.

Age-matched embryos were then selected for sectioning, transferred to polymerisation moulds, photographed and sectioned. This allowed us to go back after sectioning and compare the phenotype in the sections with the age of the embryos. Several embryos covering various points during the time-span of interest were analysed in each case.

For rescue experiments, one parent usually carried a recombinant third chromosome with the *htl* mutation and the Twi-GAL4 transgene, the other parent carried a recombinant third chromosome with the *htl* mutation and the UAS-transgene. Thus, all homozygous mutant progeny expressed the transgene. Alternatively, one parent was homozygous for one of the transgenes (e.g. in cases where no insertions were available on the third chromosome), so that, again, all homozygous mutant progeny expressed the transgene. In each case, the recombinant chromosomes or the *htl* mutant chromosomes were kept over one of the marked balancers so that the mutant embryos could be identified by the absence of β-Gal staining.

The ‘activated’ kinase dead Htl transgene

A *Bgl*II-*Bam*HI fragment encoding Htl was isolated from a pSP73 cDNA clone, kindly provided by A. Michelson, and ligated into pAlter that had been digested with *Bam*HI. A recombinant pAlter.htl clone was chosen with the 5′ *htl* coding sequence juxtaposed to the ampicillin resistance gene of the vector. A mutagenesis reaction was performed with single-stranded DNA (ssDNA) derived from this clone with the following oligonucleotides: 5′-GAAGTCTTCAGA-TCTTGGAAG-3′, 5′-TGTCGCCGTGCGAATGGTGAAGGAAG-3′ and 5′-GTGGTGTAATTACCTAGGCTAAAGAAATCAGGAT-3′. The resulting clone pAlter.htl.BglII.K443A.AvrII was sequenced in its entirety. Subsequently, a *Bgl*II-*Avr*II fragment encoding the trans-membrane and cytoplasmic domains of Htl was ligated in a three-way reaction to a *Not*I-*Bgl*II fragment from pKS+-λbtl (M. Y. Zhu, R.W. and M.L., unpublished) encoding the lambda *cI* dimerisation motif, and a *Not*I-*Avr*II fragment of Not-5′Flag-Dof-AvrII-2xHA-Asc.pNB40 (details available on request) to provide HA epitope tags and a bacterial replication origin. Finally, a *Not*I-*Asc*I fragment of the resulting clone pNB40.λhtl.K443A-2xHA was ligated into a *Not*I-*Asc*I vector prepared from pUAST-λbtl.3′Asc (Wilson et al., 2004) to create pUAST-.λhtl.K443A-2xHA. Transgenic flies were produced according to standard procedures. The specific chromosome carrying

an insertion was identified by the segregation of the insertion from dominant markers on second and third chromosomes, and sex linkage in the case of the X chromosome. To confirm that the K443A mutation had abolished the kinase function of the receptor, the receptor was expressed in S2 cells and whole cell extracts were analysed for the presence of phosphotyrosine by western blotting (details available on request).

Immunohistochemistry and microscopy

Standard procedures were followed to collect embryos, which were fixed over 30 minutes at 37°C using a phosphate-buffered saline solution containing 3.7% formaldehyde, or in the case of anti-diphospho-MAPK staining, 8% formaldehyde. For immunohistochemistry, we used antibodies directed against Twist (kindly provided by S. Roth, Cologne), Eve (courtesy of M. Frasch, Mount Sinai), diphospho-MAPK (Sigma M8159), and β-Galactosidase (Sigma G4644). Proteins were detected in situ by using the coupled-peroxidase system (Vectastain ABC Kit, Vector Laboratories). Two-colour staining was accomplished in sequential steps, with the addition of Ni and Co in the second detection reaction to produce a dark precipitate. Embryos were sorted and embedded in Araldite, as previously described (Leptin and Grunewald, 1990); sections were cut at 8 μm using a Leica RM 2065 microtome. Photographs were taken on a Zeiss Axioplan microscope at 400× using a Kontron ProgRes 3008 digital camera, and the images were processed using Adobe Photoshop 5.5. To analyse the data, a web page of the pictures was produced with the help of Quicknailer 1.7.1 and Adobe PageMill 3.0, and a QuickTime movie of the images from each embryo was created with GraphicConverter 4.4. Transmission electron microscopy was performed essentially according to Tepass and Hartenstein (Tepass and Hartenstein, 1994), except that embryos were fixed in a 0.1 M phosphate (pH 7.2) buffer containing 1% osmiumtetraoxide and 2% glutaraldehyde.

Results

FGF-dependent and -independent events of mesodermal morphogenesis

In the studies presented here we have examined several steps of mesodermal morphogenesis and differentiation that occur after invagination of the mesoderm primordium. Initially, the cells of the invaginated epithelium closest to the ectoderm (arrowheads in Fig. 1A) can be observed to extend cytoplasmic extensions towards the inner surface of the ectoderm (Fig. 1C-E). The invaginated epithelial tube then flattens symmetrically against the ectoderm (movement indicated by arrows in Fig. 1A), and the cells make intimate contact with the ectoderm and activate MAPK (shading in Fig. 1A). The mesoderm subsequently becomes mesenchymal, and cells divide and disperse from the site of invagination towards the dorsal edge of the ectoderm to form a single cell layer on the ectoderm (arrows in Fig. 1B). At this stage, extracellular signals trigger the development of the heart precursors from segmentally repeated subpopulations of the most dorsally located cells, an event also associated with the activation of MAPK. FGF signalling is required for the establishment of contact between the mesoderm and the ectoderm, as well as for MAPK activation within the mesoderm (Beiman et al., 1996; Gabay et al., 1997b; Gisselbrecht et al., 1996; Imam et al., 1999; Michelson et al., 1998a; Shishido et al., 1997; Vincent et al., 1998). However, even in the absence of FGF signalling, the mesoderm eventually collapses onto the surface of the ectoderm, often very asymmetrically, and cells still have the ability to migrate towards the dorsal edge of the ectoderm. Hence, cell migration as such does not depend on the activation of the FGF receptor. In summary, there are three distinct aspects to the dispersal of the mesoderm: the FGF-dependent establishment of contact between the mesoderm and the ectoderm, which brings the epithelial tube into close apposition with the ectoderm; the transition to a mesenchymal state; and, finally, the migration or spreading of the individual cells on the ectoderm.

During mesoderm ingression in mouse embryos, FGF signalling is necessary for cell migration away from the site of ingression, not because it provides a chemotactic signal but because it enables cells to undergo an epithelial to mesenchymal transition (EMT) and escape from the site of ingression (Ciruna and Rossant, 2001; Ciruna et al., 1997). We wondered whether the ability of the *Drosophila* mesodermal

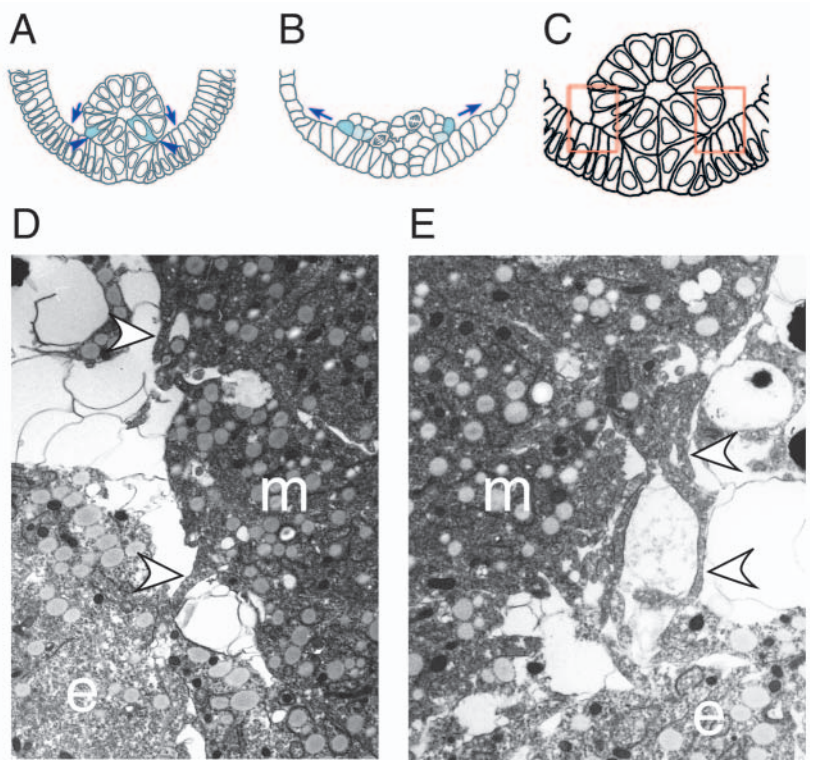

Fig. 1. Events during mesoderm spreading. (A,B) The events that occur during the dispersal of the mesoderm. (A) Following invagination, the mesoderm flattens down onto the surface of the ectoderm (arrows). The first cells make contact with the ectoderm symmetrically on either side of the midline (arrowheads) and accumulate high levels of the activated form of MAPK (cells marked in blue). (B) The mesodermal cells subsequently undergo an epithelial to mesenchymal transition, divide and spread out away from the site of invagination to cover the surface of the ectoderm (arrows). A gradient of activated MAPK is observed in the mesoderm with the highest levels at the edge of the mesodermal sheet (cells shaded from light to dark blue). (C) A drawing of a cross section through an embryo at an early stage of mesodermal spreading to illustrate the regions shown in D and E (red boxes). (D,E) Electron micrographs of mesodermal cells (m) showing cytoplasmic extensions (arrowheads) protuding towards the ectoderm (e).

cells to escape from the invaginated mesodermal mass in the absence of FGF signalling was aided by the rounding up of cells and loss of cell contact caused by cell division. To test this, we examined *dof* mutants that were also mutant for the cell-cycle regulator *stg*, which is essential for post-blastoderm cell division (Edgar and O'Farrell, 1989) but normally has no role in early mesoderm morphogenesis (Leptin and Grunewald, 1990). We found that mesodermal cells in *dof, stg* double mutants were able to disperse (Fig. 2G). If anything, the reduction in the number of cells improved spreading rather than enhancing the retention of mesodermal cells near the site of invagination. We conclude that mesodermal EMT in *Drosophila* can occur in the absence of FGF signalling.

The role of the ectoderm in mesodermal morphogenesis

We have previously shown that changes in the fates of subpopulations of ectodermal cells do not affect the behaviour of the mesoderm (Wilson and Leptin, 2000). These experiments were conducted to test the hypothesis that mesodermal migration is guided by a chemotactic signal produced by dorsally located cells (e.g. cells at the dorsal edge of the neural ectoderm, or the amnioserosa). We found that the most extreme change in dorsal cell fates, in *dpp* mutants, in which all ectodermal cells assume the fate of the ventral neuroectoderm, did not disrupt the dorsal migration of mesodermal cells. We have now extended our studies to include mutations in genes that affect the ventral ectoderm. This region is patterned by EGF-signalling and by repression of Dpp function under the control of *brinker* and *sog*. In *brinker, sog* double mutants (Fig. 2A,B), in mutants in genes required for EGF signalling (*Egfr*, *spitz*, *Star* and *rhomboid*) (Fig. 2C,D), and in *brinker, sog; Egfr* triple mutants (Fig. 2E,F) the mesoderm was able to make contact with the ectoderm. High levels of activated MAPK accumulated in these mutants, and the mesoderm spread out over the ectoderm, indicating that the FGF-receptor was properly activated and the cells received all the signals necessary for migration. Together these results show that the early morphogenesis of the mesoderm does not depend upon the zygotically defined subdivision of the ectoderm, and make a model in which a spatially restricted ectodermal ligand acts as a long-range chemotactic signal almost impossible to maintain. This is confirmed by the expression pattern of the recently identified ligands for Htl, the FGF8-homologues expressed throughout the ventral and dorsal ectoderm anlage (Gryzik and Müller, 2004; Stathopoulos et al., 2004).

A further experiment suggests that long-range chemotaxis is not responsible for the dispersal of the mesoderm. A constitutively active form of Htl expressed in the mesoderm of *htl* mutants rescues the ability of all cells to move efficiently away from the site of invagination, although there is no directional information in this situation (Fig. 2H) (Michelson et al., 1998a). Thus, the role of FGF signalling in early mesoderm spreading appears to be permissive, promoting the attachment and dispersal of the mesodermal cells over the surface of the ectoderm, rather than being instructive or having a chemotactic function that directs mesodermal cells towards a particular location.

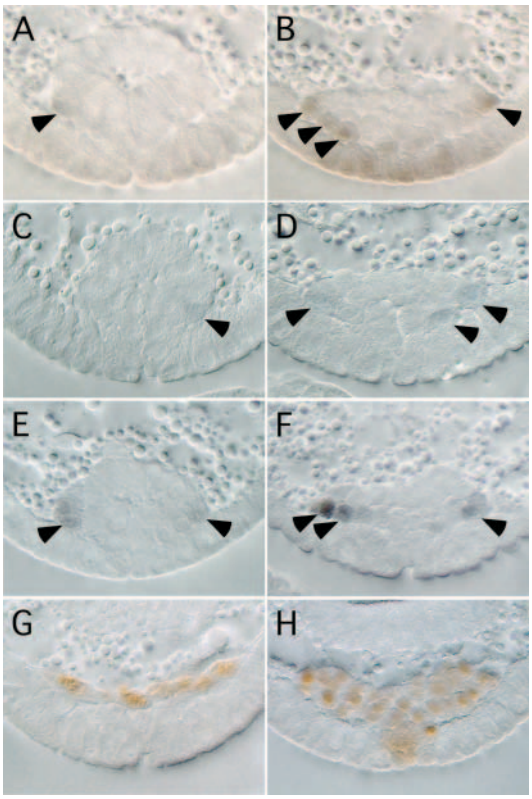

Fig. 2. Mesoderm spreading in mutant embryos. (A-F) Ectodermal cell fate and mesoderm morphogenesis. Cross sections of embryos showing the development of the mesoderm in embryos with altered ectodermal cell fates. The embryos have been stained to reveal the di-phosphorylated form of the MAP kinase ERK. Each set of panels show an early and late timepoint during the flattening of the mesoderm. (A,B) Sections of *brk, sog* mutant embryos. (C,D) Sections of *Egfr* (*top*^{18A}) mutant embryos. (E,F) Sections of *brk, sog; Egfr* (*top*^{18A}) triple mutant embryos. Arrowheads indicate mesodermal cells in which high levels of the di-phosphorylated form of ERK are present. (G,H) Sections of embryos stained with antibodies directed against Twist to reveal mesodermal cells. (G) A section of a late *dof, stg* double mutant embryo. (H) Mesoderm development in a late *htl* mutant embryo expressing an activated form of Htl throughout the mesoderm.

Specificity of receptor tyrosine kinase signalling during mesodermal spreading

Receptor tyrosine kinases (RTKs) share many downstream signalling elements, and can replace each other in many assays. For example, the functions of the FGF receptor Breathless during tracheal morphogenesis can be replaced in part by constitutively active forms of other RTKs (Lee et al., 1996; Reichman-Fried et al., 1994). We examined whether this was also the case in the mesoderm by expressing constitutively active forms (see Materials and methods) of Heartless, Breathless, the PDGF and VEGF-related receptor (PVR), and the EGFR in *htl* mutants under the control of Twist-Gal4. We initially tested the function of these constructs in the mesoderm by scoring their effects on the activation of MAPK in the primordia of the Eve-expressing heart precursors, which have been shown to depend upon Htl activity (Beiman et al., 1996; Gisselbrecht et al., 1996; Shishido et al., 1997). All constructs lead to the phosphorylation of MAPK in these cell clusters, although there were differences in the extent of Eve expression

(Fig. 3). Similarly, all constructs were able to activate Eve transcription in heart precursors, albeit to different levels, and very inefficiently in the case of Breathless and PVR (Fig. 3). These differences mirrored the efficiency of the constructs in other assays for RTK activity (e.g. inducing roughening of the eye, rescue of tracheal defects in *btl* mutants (M. Y. Zhu, R.W. and M.L., unpublished) (Freeman, 1996; Reichman-Fried et al., 1994). We assume this reflects differences in the signalling strength of the receptors.

Having established that the constructs function in the mesoderm, we tested their ability to rescue the early morphogenetic events in *htl* mutants (Fig. 4). We found that the wild-type Heartless FGF-receptor, as well as the lambda-Htl and lambda-Btl constructs, was able to induce bilaterally symmetrical flattening of the mesodermal tube onto the ectoderm. In the case of lambda-Htl and wild-type Htl, we observed very early establishment of contact with the ectoderm, indicating that twi-Gal4 is able to induce sufficiently early expression of the transgenes. The effect of lambda-Btl was delayed and we assume that this can be ascribed to the generally weaker activity of this protein. Lambda-EGFR and lambda-PVR were unable to rescue either early contact or symmetric flattening of the mesodermal tube. These results demonstrate that FGF signalling rather than a generic RTK signal is necessary to initiate mesoderm spreading.

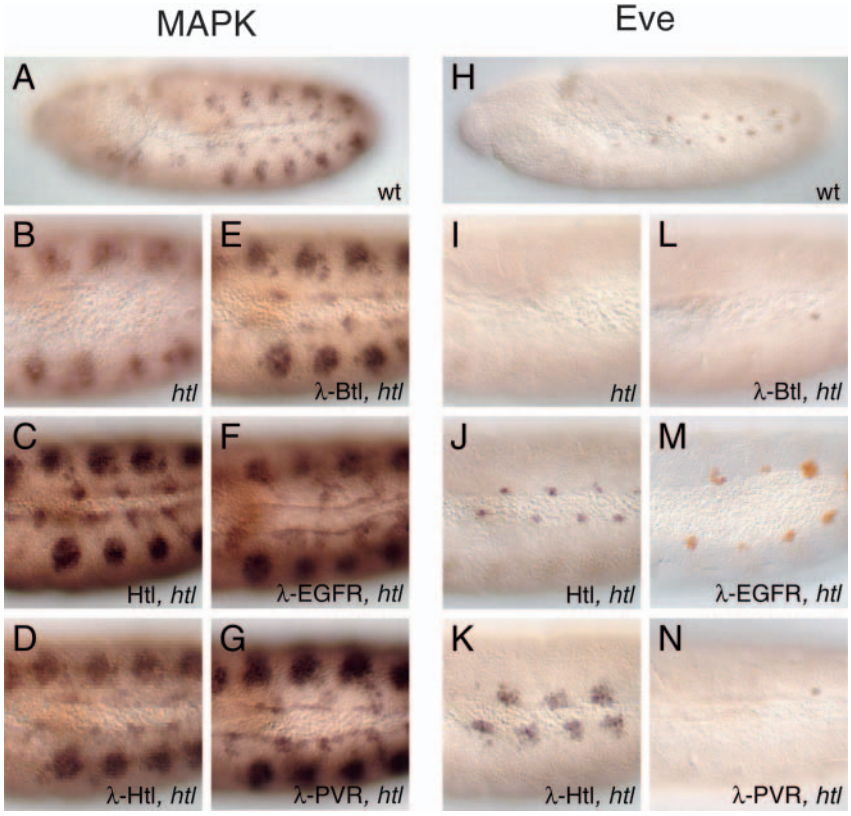

Fig. 3. Activation of MAPK and rescue of Eve-positive cells in the mesoderm of *htl* mutants. Embryos were stained with antibodies directed against dp-ERK (left), or Even-skipped (right). (A,H) Whole-mounts of wild-type embryos; (B-G,I-N) enlarged views of the trunk regions of *htl* mutant embryos. (B,I) The homozygous *htl* mutant phenotype. (C-G,J-N) The effect of expressing transgenes in the mesoderm of *htl* mutant embryos. (C,J) Htl; (D,K) an activated form of Htl; (E,L) an activated form of Btl; (F,M) an activated form of EGFR; (G,N) an activated form of PVR.

When we tested whether the transgenes were able to trigger the first phase of MAPK phosphorylation, we found that Htl and lambda-Htl did so efficiently and early, lambda-Btl had an effect slightly later, whereas lambda-EGFR and lambda-PVR did not lead to activation of MAPK at this stage (Fig. 4 and data not shown). Thus, although all four RTKs are able to induce MAPK activation and heart cell differentiation once morphogenesis of the mesoderm is complete, at early stages only the FGF-receptors are able to transmit signals to the MAPK cascade and trigger the morphogenetic activities of mesodermal cells.

It was particularly surprising that the constitutively active EGFR was unable to stimulate MAPK phosphorylation, as it is a very potent RTK in *Drosophila*, and all the components necessary for signal transmission are present in the early embryo. Indeed the EGFR shows high levels of activity in the cells neighbouring the mesoderm (Gabay et al., 1997a; Gabay et al., 1997b). This suggests that MAPK activation by RTKs other than FGFRs is blocked in the mesoderm. This inhibition appears to act on the MAPK cascade itself, because expression of activated Raf or Ras in *dof* mutants did not result in phosphorylation of MAPK (Fig. 4C and data not shown), even though at later stages activated Ras is able to trigger the accumulation of diphospho-ERK (Michelson et al., 1998a). More importantly, we also observed that even the constitutively active FGF-receptors were not able to lead to early MAPK activation in all of the invaginated mesodermal cells, although they are expressed in all cells. In *htl* embryos expressing lambda-Htl, MAPK phosphorylation was evident solely in the mesodermal cells that were in contact with the ectoderm, just as observed in wild-type embryos. This indicates that activation of the FGF receptor alone is not sufficient to promote MAPK activation within the mesoderm at early stages of mesoderm spreading.

Our observation is in apparent contradiction to recent results showing that expression of an Htl ligand throughout the mesoderm can trigger MAPK activation in all cells (Stathopoulos et al., 2004). We suspect that the activation of the wild-type receptor by high levels of its ligand produces a stronger signal than the constitutively active constructs, and that this high level of activity is capable of overriding the requirement for contact with the ectoderm. In the tracheal system, it is notable that there is a marked difference between the effect of overexpressing the FGF ligand Branchless, and a constitutively activated FGF receptor (R.W., E.V. and M.L., unpublished) (Imam et al., 1999; Michelson et al., 1998; Sato and Kornberg, 2002; Vincent et al., 1998). Nevertheless, the expression of lambda-Htl throughout the mesoderm of *htl* mutant embryos reveals a difference in the requirement for MAPK activation between those cells that make contact with the ectoderm and those that do not. We suggest that contact with the ectoderm, which in itself depends on FGF signalling,

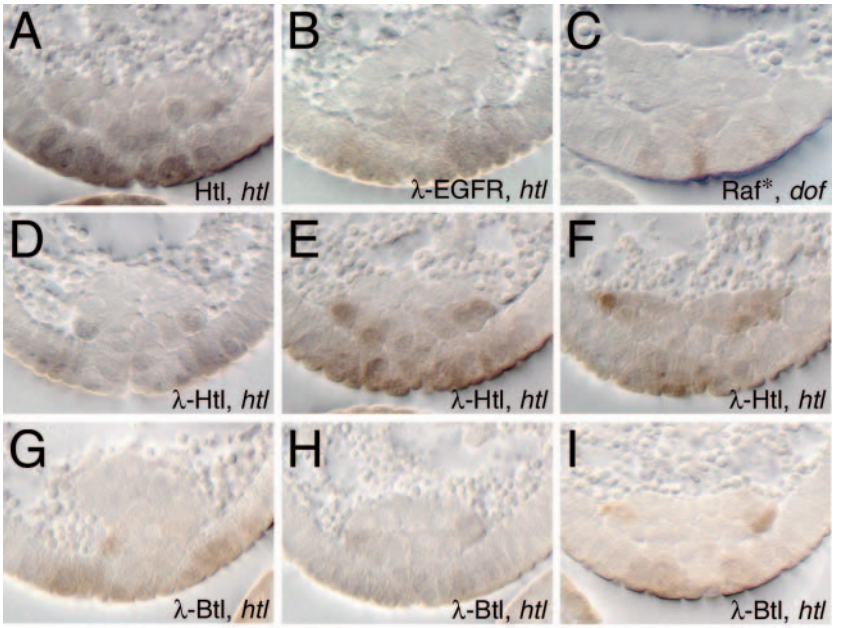

Fig. 4. Rescue of MAPK activation and spreading at early stages of mesoderm morphogenesis. Sections of mutants expressing transgenes in the mesoderm stained to reveal activated MAPK. (A) Expression of Htl in the mesoderm of an *htl* mutant embryo completely rescues mesoderm spreading. (B) An activated form of the EGF receptor expressed in an *htl* mutant fails to promote interaction of the mesoderm with the ectoderm and does not stimulate activation of MAPK within the mesoderm at this developmental stage. (C) An activated form of Raf does not induce mesoderm spreading or the activation of MAPK in a *dof* mutant embryo. Note that we chose a slightly older embryo, showing that even at a stage when the mesoderm collapses onto the ectoderm no MAPK activation is detectable. (D-I) Sections from a young embryo are shown on the left of each row, with older embryos towards the right. (D-F) Expression of an activated form of Htl rescues both early contact of the mesoderm with the ectoderm and the activation of MAPK, notably at early stages this is only evident in the mesodermal cells that make contact with the ectoderm. (G-I) Expression of an activated form of Btl also rescues mesoderm spreading, but the onset of spreading occurs slightly later than in wild-type embryos. Activated MAPK is only observed in mesodermal cells that are in contact with the ectoderm.

must provide an additional signal that acts in conjunction with the activation of Heartless to trigger MAPK phosphorylation. This is consistent with the fact that in *pebble* mutant embryos, in which early contact of the mesoderm with the ectoderm does not occur (Schumacher et al., 2004; Smallhorn et al., 2004), there is no MAPK activation at early stages (Fig. 6F), although FGF signal transduction as such at later stages is functional (Schumacher et al., 2004; Smallhorn et al., 2004). One possible explanation for this might be that, because of the lack of proximity of the mesoderm to the ectoderm, the FGF ligand cannot reach the mesodermal cells. However, even when this potential problem is bypassed by expressing an activated form of Htl in the early mesoderm of *pebble* mutants, no activation of MAPK can be seen (data not shown). These results suggest that the ectoderm has an important role in triggering the accumulation of activated MAPK within the mesoderm, in addition to providing the Htl ligands.

Importance of MAPK function

We decided to examine whether the pathway downstream of the FGF receptor that leads to MAPK activation is required to establish contact between mesodermal and ectodermal cells. The components of the MAPK cascade are all provided maternally, so we examined embryos derived from germ line mutant clones that were devoid of the maternal and zygotic functions of Draf (MAPKKK) or Dsor (MAPKK). The activation of ERK is completely abolished in these embryos, as is evident from the absence of ppERK staining (data not shown) (Ghiglione et al., 1999). However, sections of the embryos from Draf and Dsor germ line clones showed that the mesoderm was nevertheless able to make contact with and flatten against the ectoderm in a symmetric fashion (Fig. 5C and data not shown). Removal of the function of the p38 MAPK-kinase *hep*, which is important for the formation of actin-based cellular protrusion during dorsal closure (Jacinto et al., 2000; Martin-Blanco et al., 2000), also had no effect upon the initiation of mesoderm spreading (not shown). Hence, the ability of the FGF-receptor to promote contact between the mesoderm and the ectoderm is not mediated by the activation of these MAPK signalling pathways.

The activities of both Heartless and Dof are required to trigger the activation of MAPK and to promote contact of the mesoderm with the ectoderm. A kinase-dead version of lambda-Heartless (lambda-Htl^{K443A}) was unable to rescue mesodermal spreading in *htl* mutants (data not shown). Thus, while the events that lead to the establishment of contact with the ectoderm do not depend upon the activation of MAPK, they are strictly dependent upon the kinase function of the receptor. To establish at which point downstream of Heartless and Dof the pathway responsible for the activation of MAPK diverges from the pathway that promotes contact between the mesoderm and the ectoderm, we examined embryos lacking components that connect RTKs to MAPK and other targets within the cell. Two molecules that mediate receptor tyrosine kinase signalling are Ras and Drk/Grb2. When we examined *ras85D* and *drk* mutant germ line clones, we found that the initiation of mesoderm spreading was unaffected (Fig. 5D and data not shown). FGF signalling in the tracheal system and in the activation of Eve in heart precursors depends upon the function of Csw (Gabay et al., 1997a; Johnson Hamlet and Perkins, 2001; Perkins et al., 1996), but the mesoderm of embryos derived from homozygous *csw$^{E(sev)1A\text{-}eOP}$* germ line clones still established contact with the ectoderm (Fig. 5E). In this case however, the overall appearance of the invaginated mesodermal tube appeared to be abnormal. Finally, we tested the contribution of D-Shc to mesoderm spreading. The phenotype of embryos derived from *D-Shc* mutant germ line clones is less pronounced than that of *htl* and *dof* mutants, but the mutants show clear defects in their ability to establish contact between the mesoderm and ectoderm (Fig. 5F). In summary, the

pathways that initiate mesoderm spreading and the activation of MAPK appear to diverge from one another just downstream of, or at the level of, D-Shc.

The role of Rho GTPases and their effectors

We tested whether molecules known to regulate the shape and migration of cells in other contexts would influence mesoderm spreading. The generation of protrusive force required for cell migration is thought to be due to the activity of the small Rho family GTPases and their regulators and downstream effectors, such as those involved in axon guidance or border cell migration during oogenesis (Awasaki et al., 2000; Bateman et al., 2000; Duchek et al., 2001; Garrity et al., 1996; Hing et al., 1999; Liebl et al., 2000; Newsome et al., 2000). The initial stages of mesoderm spreading were normal in embryos lacking the function of *Pak*, *dock*, *trio* and *myoblast city* (Fig. 6E, and data not shown), although in embryos lacking the function of *Pak* we observed that the adhesion of the mesoderm to the ectoderm was affected at later stages (data not shown). An activated form of the *Drosophila* myosin regulatory light chain, Spaghetti squash, which is one of the major targets of *Drosophila* Rho-associated kinase (Winter et al., 2001), was unable to rescue the defects in the mesoderm of *dof* mutant embryos (not shown). These observations suggest that either these particular regulatory molecules are not needed for the dispersal of the mesoderm, or that they act together with other pathways in a redundant fashion. It is not possible to generate embryos that completely lack RhoA function (Magie et al., 1999). When we examined embryos with a reduced maternal dose of RhoA, we observed that the shape of the invaginated mesoderm was abnormal, although it did not resemble *htl* or *dof* mutants (Fig. 6B). By contrast, we observed strong defects in mesoderm spreading when the function of the Rac GTPases was compromised. In *Drosophila* there are three Rac-like proteins, Rac1, Rac2 and Mtl. All are provided maternally, and single mutants in these genes are viable and fertile (Hakeda-Suzuki et al., 2002; Ng et al., 2002), and show little or no defects in mesoderm spreading (Fig. 6A and data not shown). When we generated Rac1, Rac2 double mutant germ line clones we did not obtain any eggs. However, when we analysed embryos derived from mothers with a reduction in the maternal dose of Rac function, we found that the ability of the mesoderm to spread was compromised. Defects became visible when the dose of Rac1 and Rac2 was reduced (Fig. 6C), and were severe when the function of all three Rac-like proteins was reduced simultaneously (Fig. 6D). Thus, a normal level of Rac activity appears to be important for the mesoderm to be able to make contact with the ectoderm. To test whether Rho family GTPases could act in a linear pathway downstream of FGF signalling, we expressed activated forms of Rac1 and Cdc42 within the mesoderm of *htl* and *dof* mutant embryos. Mesoderm spreading was not rescued in these embryos (data not shown), suggesting that the *htl* and *dof* mutant phenotype is unlikely to be due only to a lack of Rac function per se, and

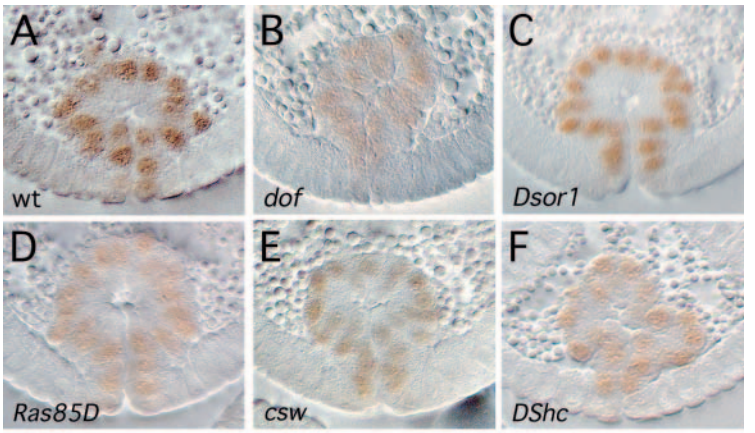

Fig. 5. Mesoderm spreading is initiated in the absence of MAPK activation. Cross sections of embryos stained for Twist to reveal the development of the mesoderm (brown). (A) A wild-type embryo. (B) A homozygous *dof*^{1} mutant embryo. (C-F) Mesoderm development in embryos derived from germline mutant clones lacking both the maternal and zygotic function of (C) *Dsor1*^{LH110}, (D) *Ras85D*^{c40B}, (E) *csw*$^{E(sev)1A\text{-}eOP}$ and (F) *dShc*^{13G}.

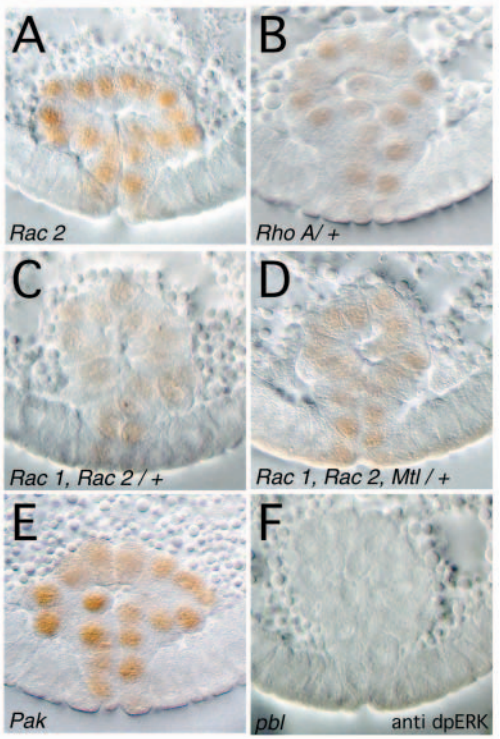

Fig. 6. The role of Rho-family GTPases in mesoderm spreading. (A) A section through an embryo that lacks the function of *Rac2*. The mesoderm flattens down onto the ectoderm as in wild type, but does not adhere to the ectoderm properly. (B) The effect of reducing the maternal dose of *RhoA*. The interaction of the mesoderm with the ectoderm is not affected, but the shape of the cells in the mesoderm is abnormal. (C,D) Embryos derived from mothers with reduced dose of (C) *Rac1* and *Rac2*, or (D) *Rac1, Rac2* and *Mtl*. In these mutants, contact between the mesoderm and the ectoderm fails to be established properly. (E) Embryos that lack the materal and zygotic function of the Rac effector *Pak* show no effect upon the initation of mesodermal spreading. (F) A section of a homozygous *pebble* mutant embryo. Contact of the mesoderm with the ectoderm does not occur, and MAPK activation is not observed within the mesoderm at this stage of development. Embryos shown in A-E were stained with Twist and the embryo in Fwas stained with anti-dp-ERK.

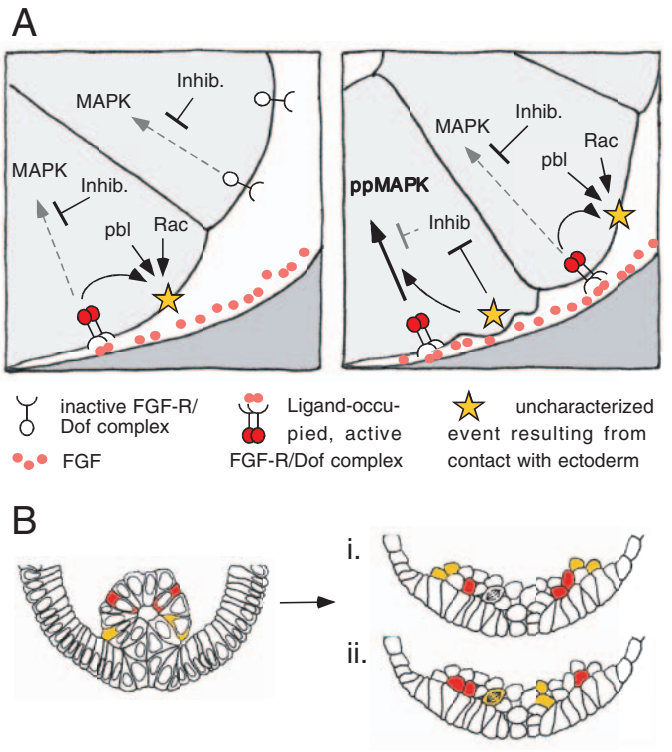

Fig. 7. Models for the signalling events and cell movements during early mesoderm spreading. (A) Diagram of part of the invaginated mesodermal tube (light grey) and the underlying ectoderm (dark grey) showing a model of events during the establishment of contact between mesoderm and ectoderm. Left panel: FGF receptors on the mesodermal cell close to the ectoderm bind FGF secreted by the ectoderm (red dots), dimerise, undergo autophosphorylation and phosphorylate Dof (shown here as part of the receptor complex). However, FGF receptor activation alone is not sufficient to activate MAPK (dashed grey arrow) or lead to cell shape changes, either because it does not deliver a sufficiently strong signal or because of the presence of an inhibitor (Inhib.). It is postulated that an event at the cell surface (yellow star) is also needed that can only occur in the vicinity of the ectoderm and depends on FGF signalling, the Rho exchange factor Pebble and Rac GTPases. As a result of this event (right panel), the activation of MAPK becomes possible, either by suppression of the postulated inhibitor, or by strengthening the MAPK signalling pathway (large black arrow). Cell-shape changes ensue that bring the next cell into the vicinity of the ectoderm and allow it to undergo the same process. The requirement for the membrane event can be overridden by overexpression of FGF. (B) Diagram of the invaginated mesoderm before (left) and after (right) it has begun to spread. Depending on the mechanism by which cells move away from the site of invagination, the cells marked in red and yellow end up in different positions (right): (i) if the first mesodermal cells to make contact with the ectoderm (yellow) become the leading edge of a migrating cell sheet, these cells would end up in a more dorsal position than the cells starting at a position more distant from the ectoderm (red); and (ii) if cells that make contact with the ectoderm become stationary and other cells crawl over them, then the first cells to make contact with the ectoderm will remain near the site of invagination, while other mesodermal cells will end up in more dorsal positions.

that the FGF receptor and the Rac proteins do not necessarily function exclusively in the same biochemical pathway.

Discussion

Distinct and independent phases of FGF signalling during mesoderm development

Morphogenesis of the mesodermal cell layer has been considered to depend entirely on FGF signalling, but in fact, FGF signalling is essential only for the initial establishment of contact between mesoderm and ectoderm, and for the late heart-differentiation signal, and these two processes are independent and experimentally separable. Dominant-negative FGF-receptor constructs disrupt differentiation, but do not affect spreading when expressed after the initial contact has been made (Michelson et al., 1998b). Conversely, constitutively active tyrosine kinases other than FGF receptors expressed in *htl* mutants rescue late differentiation, but not early spreading. Similarly, in *pbl* mutants, no early contact is made, and spreading is therefore inefficient, but cells that reach the dorsal region of the mesoderm are able to respond to FGF, activate MAPK, and differentiate into heart precursors (Schumacher et al., 2004; Smallhorn et al., 2004).

The activation of MAPK within the mesoderm

As the mesoderm spreads out over the surface of the ectoderm, the mesodermal cells that are in contact with the ectoderm accumulate high levels of the active form of MAPK. The fact that this accumulation of active MAPK is seen only in embryos with a functional FGF-signalling system in the mesoderm, but not in *htl* or *dof* mutant embryos, indicates that it is triggered by the FGF receptor. Htl and Dof are expressed throughout the mesoderm, which suggests that the local activation of MAPK is induced by the local availability of a ligand, consistent with the expression pattern of the recently discovered ligands for Htl in the ectoderm (Gryzik and Müller, 2004; Stathopoulos et al., 2004). However, even a constitutively active form of Heartless expressed throughout the mesoderm, which is able to rescue spreading in *htl* mutants, only mediates MAPK activation at early stages in the cells directly apposed to the ectoderm. We conclude that the presence of an activated form of the FGF receptor is not sufficient to trigger MAPK activation in mesodermal cells.

This result may appear to contradict earlier studies showing the ability of activated FGF-receptors to trigger MAPK activation throughout the mesoderm (Michelson et al., 1998a), but the embryos in these studies were not analysed during the phase of the earliest contact of the mesoderm with the ectoderm, but rather at later stages, just before the time when MAPK activation normally occurs in the heart precursors in the dorsal region of the mesoderm. This phase of FGF-dependent MAPK activation in the mesoderm clearly has different requirements from the early phase, as is also shown by our results using other RTKs or downstream effectors of the RTK signalling pathway. These experiments demonstrate that signals from activated Raf cannot be transduced to MAPK in the cells during the early phase, except in the presence of an activated FGF receptor. We conclude that, in addition to the signal from an activated RTK via Raf, a second event is necessary for MAPK to become phosphorylated. This event could either generate a second positive signal, or it could lead to the release of a negative, inhibitory signal (see Fig. 7A).

Two points suggest that the event depends on contact of the

mesodermal cells with the ectoderm. First, as mentioned above, Lambda-htl induces MAPK phosphorylation only in mesodermal cells contacting the ectoderm, although it is expressed at uniform levels in all mesodermal cells. Second, the phenotype of *pbl* mutants supports this view. As in *htl* and *dof* mutants, the early contact of the mesoderm with the ectoderm fails to be made in *pbl* mutants, and mesoderm spreading is impaired. At later stages, Htl is able to trigger MAPK phosphorylation in the dorsal part of the mesoderm of *pbl* mutants, showing that FGF signalling in the mesoderm as such does not depend on *pbl* (Schumacher et al., 2004; Smallhorn et al., 2004). By contrast, the early activation of MAPK is abolished. We therefore argue that contact is a prerequisite for early FGF-receptor induced MAPK activation (see Fig. 7A).

Both the establishment of mesoderm-ectodermal cell contact and the activation of MAPK require the kinase domain of the FGF receptor to be intact, which suggests that these events depend upon a substrate of the FGF receptors not recognised by other activated receptor tyrosine kinases. One possibility is that this substrate is Dof, which is specifically phosphorylated by an activated FGF receptor. In this situation, Dof would provide a unique function that cannot be substituted by other activated receptor tyrosine kinases. Alternatively, this substrate could be a second receptor that is activated upon contact of the mesoderm with the ectoderm, or a component that acts in, or on, a pathway triggered by the engagement of the mesoderm with the ectoderm (see Fig. 7A).

Several pathways promote contact between mesodermal and ectodermal cells

It seemed likely that the early morphogenetic activity might require changes in subcellular architecture involving cytoskeletal regulators. Indeed, the establishment of contact between the mesoderm and the ectoderm is affected by mutations in the gene encoding the RhoGEF Pebble (Schumacher et al., 2004; Smallhorn et al., 2004), and, as shown here, a reduction in the level of Rho and Rac proteins within the embryo. We do not know whether the Rho-family GTPases act downstream of or in parallel with FGF signalling. The defects of *htl* mutants cannot be rescued by the expression of an activated form of Rac or Cdc42. Thus, if Rac acts downstream of the FGF receptor, it is not in a simple epistatic pathway but requires the activation of other pathways as well. Alternatively, FGF signalling may act in conjunction with a separate pathway that directs the activity of the Rac proteins to promote contact between the mesoderm and the ectoderm (see Fig. 7A).

FGF signalling is not essential for migration of mesodermal cells

Spreading of the mesoderm on the ectoderm leads to a redistribution of mesodermal cells away from the site of invagination towards the dorsal edge of the ectoderm. This is often considered to be a process of directed cell migration. In this view, the graded distribution of activated MAPK levels in the nuclei of the mesodermal cells is suggestive of a response to a chemotactic signal originating from the target region (Gabay et al., 1997b). Both the expression pattern of the Htl ligands (Gryzik and Müller, 2004; Stathopoulos et al., 2004) and the phenotypes of mutants in which the fate of the target region has been changed (Wilson and Leptin, 2000) are inconsistent with this view. The activation of Heartless appears to be permissive for mesoderm spreading and we suggest that FGF signalling functions primarily to promote the efficient interaction of the entire mesodermal primordium with the surface of the ectoderm and that this could act to impose order during the transition from an epithelial to a mesenchymal state. Simple spatial constraints could lead to an apparently directed migration. With the mass of mesodermal cells initially concentrated near the site of invagination, the only direction available for migration is away from this site. Hence, a signal-inducing motility would automatically promote directional movement. The dispersal of the mesoderm mass in *dof* mutants is noticeably improved by blocking cell division, and we believe that this might be due to the smaller number of cells in the mesodermal primordium having greater access to the surface of the ectoderm.

Potential cellular mechanisms involved in dispersal of the mesoderm

These observations raise the questions of how mesodermal cells spread over the surface of the ectoderm, and how activated MAPK accumulates in a graded fashion. A number of possibilities can be envisioned to account for the migration of the mesoderm. For example, the first cell that makes contact with the ectoderm could crawl over the ectoderm and function as the ‘leading’ cell of the mesodermal sheet. The other cells of the mesoderm tube would make contact with the ectoderm sequentially to follow the leading cell as it migrates dorsally (Fig. 7B, part i). In this case, the MAPK gradient would be explained by the accumulation of the highest levels of activated MAPK in the cells that had been in contact with the ectoderm for the longest period of time. Alternatively, the cell that makes the initial contact with the ectoderm could remain largely stationary, and other mesodermal cells would reach the ectoderm by crawling over that cell (Fig. 7B, part ii). Once a mesodermal cell is in contact with the ectoderm, motility of the cell would cease, as in the process of ‘boundary capture’ described for mesodermal cells in *Xenopus* reaching the notochord during convergence movements (Keller et al., 2000). In this model, contact between the ectoderm and mesoderm would have an important role in establishing the single cell layer of mesoderm that covers the surface of the ectoderm at later stages. The MAPK gradient can be explained in this case by a transient activation of MAPK, which is downregulated once motility ceases, a model that is more consistent with known feedback mechanisms that operate during signal transduction (Freeman, 2000; Freeman and Gurdon, 2002; Perrimon and McMahon, 1999; Rebay, 2002). This model implies that the cells with the highest level of MAPK at the edge of the mesoderm would have only just come into contact with the ectoderm (see Fig. 7B, part ii). In order to distinguish between the two mechanisms, cell labelling experiments will be required.

FGF signalling is only one of many mechanisms that contribute to the establishment of the mesodermal cell layer. It is not essential for migration as such, but is clearly important for the orderly dispersal of mesodermal cells away from their site of invagination. Our results suggest that FGF-signalling facilitates cell spreading by promoting the apposition of the invaginated mesodermal epithelium against the ectoderm.

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