heartless encodes a fibroblast growth factor receptor (DFR1/DFGF-R2) involved in the directional migration of early mesodermal cells in the *Drosophila* embryo

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After invagination of the mesodermal primordium in the gastrulating *Drosophila* embryo, the internalized cells migrate in a dorsolateral direction along the overlying ectoderm. This movement generates a stereotyped arrangement of mesodermal cells that is essential for their correct patterning by later position-specific inductive signals. We now report that proper mesodermal cell migration is dependent on the function of a fibroblast growth factor (FGF) receptor encoded by *heartless* (*htl*). In *htl* mutant embryos, the mesoderm forms normally but fails to undergo its usual dorsolateral migration. As a result, cardiac, visceral, and dorsal somatic muscle fates are not induced by Decapentaplegic (Dpp), a transforming growth factor β family member that is derived from the dorsal ectoderm. Visceral mesoderm can nevertheless be induced by Dpp in the absence of *htl* function. Ras1 is an important downstream effector of Htl signaling because an activated form of Ras1 partially rescues the *htl* mutant phenotype. The evolutionary conservation of *htl* function is suggested by the strikingly similar mesodermal migration and patterning phenotypes associated with FGF receptor mutations in species as diverse as nematode and mouse. These studies establish that Htl signaling provides a vital connection between initial formation of the embryonic mesoderm in *Drosophila* and subsequent cell-fate specification within this germ layer.

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The mesoderm in *Drosophila* originates from the ventral-most cells of the blastoderm embryo. This germ layer is established by the function of an intricate hierarchy of maternal gene products whose interactions culminate in the graded nuclear expression of the transcription factor encoded by *dorsal* (*dl*) along the dorsoventral axis of the embryo (Morisato and Anderson 1995). High nuclear concentrations of Dl activate the transcription of two zygotic genes, *twist* (*twi*) and *snail* (*sna*), in cells of the mesodermal primordium (Jiang et al. 1991; Pan et al. 1991; Thisse et al. 1991; Ip et al. 1992). The early activities of these latter genes are essential for the specification of general mesodermal cell fate (Simpson 1983), whereas additional factors both intrinsic and extrinsic to this germ layer influence the subsequent differentiation of particular cells (Beer et al. 1987; Greig and Akam 1993; Michelson 1994; Staehling-Hampton et al. 1994;

Baker and Schubiger 1995; Baylies et al. 1995; Frasch 1995; Wu et al. 1995; Baylies and Bate 1996; Ranganayakulu et al. 1996).

Twf/Sna-expressing mesodermal cells invaginate through the ventral furrow at gastrulation, an event that is accompanied by characteristic changes in cellular morphology that drive these cell movements (Leptin and Grunewald 1990; Kam et al. 1991; Sweeton et al. 1991; Leptin et al. 1992). Although it initially forms an epithelial tube, the mesoderm subsequently loses this appearance as the invaginated cells flatten, disperse, and undergo a series of mitotic divisions (Leptin and Grunewald 1990; Bate 1993). Simultaneously, the mesodermal cells begin to migrate in a dorsolateral direction beneath the overlying ectoderm until they attain a uniform monolayer that reaches as far as the dorsal margin of the epidermis. After an additional cell division, the mesoderm is subdivided into an inner or visceral layer and an outer or somatic layer (Bate 1993; Dunin Borkowski et al. 1995).

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The visceral mesoderm is demarcated by the expression domains of two homeo domain-encoding genes, *tinman* (*tin*) and *bagpipe* (*bap*), the activities of which are required for the formation of this dorsal derivative (Azipazu and Frasch 1993; Bodmer 1993). *tin* function is also essential for differentiation of the heart, another derivative of the dorsal mesoderm. Furthermore, the dorsal subdivision of the mesoderm is dependent on proximity of this germ layer to cells of the dorsal ectoderm that supply a diffusible, inductive signal in the form of a transforming growth factor β (TGF β) family member encoded by the *decapentaplegic* (*dpp*) gene (Staehling-Hampton et al. 1994; Frasch 1995). Thus, dorsolateral migration of mesodermal cells is absolutely essential for the acquisition of particular cell fates through induction by the adjacent ectoderm. Modulation of the level of *Tw* expression along the anteroposterior axis also is critical to the partitioning of the embryonic mesoderm into its various components (Dunin Borkowski et al. 1995; Baylies and Bate 1996).

Although the early steps in mesoderm formation are well delineated, the genetic and molecular control of later morphogenetic events within this germ layer remain poorly understood. We now demonstrate that the activity of a fibroblast growth factor (FGF) receptor is essential for the proper directional migration of mesodermal cells following their invagination at the onset of gastrulation. In the absence of this receptor tyrosine kinase (RTK) signaling pathway, the invaginated mesodermal cells initially do not dissociate from each other nor do they spread correctly beneath the ectoderm. Subsequent patterning of this germ layer is grossly disrupted as specific mesodermal cells fail to acquire proper positional cues for their later commitment and differentiation. This is particularly evident in the dorsal region of the mesoderm where the induction of cardiac, somatic, and visceral muscle fates is dependent on a growth factor signal produced by the dorsal ectoderm (Staehling-Hampton et al. 1994; Frasch 1995). As a consequence, one of the most prominent features of FGF receptor-deficient embryos is the absence of the heart or dorsal vessel, a phenotype which has led us to name this gene *heartless* (*htl*). Next to *twi* and *sna*, *htl* has the earliest characterized function of a zygotically expressed mesoderm-specific gene.

Results

Isolation of mesodermal FGF receptor mutants

The pair-rule gene, *even-skipped* (*eve*), is expressed in a small number of dorsal mesodermal cells following its segmentation function in the early *Drosophila* embryo (Frasch et al. 1987). As development proceeds, *Eve* persists in two mesodermal derivatives, a subset of pericardial cells, and a single dorsal somatic muscle in each hemisegment (Fig. 1A). We have found that the specification of the *Eve*-positive muscle but not the pericardial cells is dependent on the activity of the *Drosophila* epidermal growth factor (EGF) receptor (E. Buff and A.M.

Figure 1. Mesodermal *Eve* expression in homozygous *Sr*⁶ and *htl*^{AB42} embryos. Dorsal views of stage 16 wild-type (WT; A), *Sr*⁶ (B), and *htl*^{AB42} (C) embryos stained with an antibody directed against *Eve*. Anterior is to the left. *Eve* is normally expressed in a pair of pericardial cells (PC) and a single dorsal somatic muscle (dorsal acute 1 or DA1; muscle nomenclature after Bate 1993) in each hemisegment. In embryos homozygous for the *Sr*⁶ deficiency in which the *DFR1/htl* gene is deleted (Shishido et al. 1993), none of the *Eve*-expressing mesodermal cells is present (B). A similar phenotype is seen in a null allele of *htl* (C).

Michelson, unpubl.). In contrast, loss of maternal and zygotic function of *Ras1*, an important downstream effector of RTK signal transduction pathways (Lowy and Willumsen 1993; van der Geer and Hunter 1994), leads to loss of both *Eve*-expressing mesodermal cell types (A.M. Michelson, unpubl.). The latter finding suggested that an additional RTK might be involved in the formation of at least these dorsal mesodermal derivatives. One candidate for this RTK is a mesoderm-specific FGF receptor, designated DFR1 (Shishido et al. 1993) or DFGF-R2 (cited in Reichman-Fried et al. 1994). This *Drosophila* gene was cloned on the basis of its homology to vertebrate FGF receptors, and was mapped to the right arm of the third chromosome in a region that is uncovered by the *Sr*⁶ deficiency (DeLaPompa et al. 1989; Bellen et al. 1992; Shishido et al. 1993). Consistent with the possible requirement of DFR1/DFGF-R2 for the development of *Eve*-positive pericardial and somatic muscle cells, *Sr*⁶

homozygous embryos entirely lack Eve expression in the dorsal mesoderm (Fig. 1B).

Because several genes are known to fall within the *Sr¹⁶* deficiency (DeLaPompa et al. 1989; Bellen et al. 1992; Shishido et al. 1993; Volk and VijayRaghavan 1994), mutations specific to the *DFR1/DFGF-R2* gene are required to confirm a role for this RTK in mesodermal pattern formation. We have identified several such alleles in a screen of third chromosome embryonic lethal mutations induced by the chemical mutagen ethylmethanesulfonate (EMS). A collection of ~4000 independently generated lethal lines was screened for aberrant Eve expression in both the mesoderm and the central nervous system (J.B. Skeath and C.Q. Doe, unpubl.). From this screen, 49 lines were identified in which the mesodermal Eve pattern was altered but the CNS Eve pattern was not appreciably affected. Of these, seven lines failed to complement *Sr¹⁶*. Furthermore, these seven mutants were found to constitute a single complementation group. As was the case with *Sr¹⁶*, neither pericardial nor somatic muscle cells expressing Eve are found in an embryo homozygous for a representative allele (Fig. 1C). Definitive evidence that this phenotype is attributable to loss of mesodermal FGF receptor function was obtained by sequence analysis of the *DFR1/DFGF-R2* genes cloned from six members of this complementation group (see below).

Cardiac, somatic, and visceral mesodermal derivatives are all dependent on FGF receptor function

Eve is expressed in only a small subset of embryonic mesodermal cells. To further characterize the phenotypes associated with loss of mesodermal FGF receptor function, we examined the development of the heart, somatic, and visceral muscles in one mutant line, designated *AB42*. The phenotypes observed in homozygous *AB42* embryos are equivalent to those of *AB42/Sr¹⁶*, suggesting that *AB42* is a null allele. As described below, molecular characterization of the *DFR1/DFGF-R2* gene in *AB42* confirms that it represents complete loss of function of mesodermal FGF receptor activity.

A monoclonal antibody that reacts with an antigen expressed on all pericardial cells of the normal embryonic heart (T. Volk, pers. comm.) detects only a few such cells in the *AB42* mutant (Fig. 2A, F). When comparing multiple mutant embryos of the same genotype, the locations of these residual pericardial cells appear to be randomly distributed (data not shown). Similarly, there is a marked reduction in the number of cardiac cells as revealed by expression of *tin* transcripts (Fig. 2B, G) as well as myosin heavy chain (Fig. 2C, H) and D-MEF2 (Fig. 7B, C) proteins. Based on this striking defect in cardiac development, we have named this gene *heartless* (*htl*). However, the developmental defects in *htl* mutant embryos are not restricted to this mesodermal derivative. Most of the dorsal somatic muscles are entirely missing (Fig. 2C, H), and gaps are seen in the lateral and ventral muscle groups as well (Fig. 2D, I). Again, there is considerable variability among embryos in the precise muscles

Figure 2. Cardiac, visceral, and somatic muscle defects in *htl* mutant embryos. Stage 16 wild-type (WT; A–E) and *htl^{AB42}* mutant (F–J) embryos were examined for expression of a number of markers of mature mesodermal derivatives. Dorsal views are shown in A–C and F–H; anterior is to the left. Lateral views are shown in D, E, I and J; anterior is to the left and dorsal is up. (A, F) Pericardial cells of the embryonic heart were visualized using a specific monoclonal antibody (T. Volk, pers. comm.). Only a few scattered pericardial cells remain in the dorsal mesoderm of the *htl* mutant. Independent mutant embryos show varying numbers of residual pericardial cells in different locations along the anteroposterior axis (data not shown). (B, G) Cardiac cells were revealed by in situ hybridization with a *tin* probe. Only a few randomly distributed *tin*-expressing cells are seen in the mutant, although these are confined to the dorsal midline as in wild type. (C, H) Myosin heavy chain expression is greatly reduced in the dorsal mesoderm of the *htl* mutant embryo, indicating that the majority of dorsal somatic muscles and cardiac cells are missing. (D, I) Numerous somatic muscles are also absent from the dorsal and lateral groups in the *htl* mutant, as indicated by staining for myosin heavy chain expression. (E, J) Myosin heavy chain expression in the visceral mesoderm. The normal midgut develops three constrictions at this stage (arrows in E). These constrictions fail to form in the absence of *htl* function, and the midgut retains a bloated appearance.

that are missing in the latter locations. Of further note, a few unfused myoblasts expressing myosin heavy chain can be seen in *htl* mutant embryos, and the myofibers that do develop in these embryos form a relatively normal residual pattern (Fig. 2I).

In addition, the visceral musculature fails to undergo its normal morphogenesis in the absence of *htl* function. Whereas the midgut acquires three constrictions in normal embryos (Fig. 2E), this fails to occur in *htl* mutants (Fig. 2J). This gut phenotype resembles that associated with mutations in *bap* (Azpiazu and Frasch 1993) and *D-mef2* (Bour et al. 1995; Lilly et al. 1995; Ranganayakulu et al. 1995). Other mesodermal tissues, including the fat body and hemocytes, appear largely normal in the absence of *htl* function, as do ectodermal derivatives such as the cuticle and central nervous system (data not shown). The latter finding is of particular significance because the Htl FGF receptor is expressed in a small number of cells in the developing nervous system (Shishido et al. 1993).

Early progenitors of each of the affected mesodermal derivatives fail to form in htl mutant embryos

Given the defects observed for various differentiated mesodermal derivatives, we used a series of markers for the corresponding progenitors to identify when in development the FGF receptor is required. Eve-expressing precursors of both the pericardial cells and dorsal somatic muscle were absent from stage 13 mutant embryos (Fig. 3A,E). These cells also were missing at stage 10 when they first appear in normal embryos (data not shown). Specific muscle precursors expressing *nautilus* (*nau*), the *Drosophila* MyoD homolog (Michelson et al. 1990; Paterson et al. 1991), and *Kruppel* (*Kr*; Gaul et al. 1987) were also missing from *htl* embryos (Fig. 3B,C,F,G). In both cases, there was considerable variation in the pattern defects among different segments of the same or different embryos, and the dorsal precursors were more strongly reduced in number than those in the lateral and ventral groups.

The variability of the somatic muscle defects associated with *htl*^{AB42} was quantitated for Kr-positive dorsal, lateral, and ventral abdominal muscle precursors (see Materials and Methods). As illustrated in Figure 3C, Kr normally is expressed in two dorsal muscles (DA1 and DO1), several lateral muscles (LL1 and two LT muscles), and a ventral acute muscle (VA2; additional ventral muscles expressing Kr are out of the plane of focus in Fig. 3C). Whereas an average of only 10.8±7.7% of DA1 or DO1 muscle precursors was present per embryo, 56.9±13.7% of abdominal hemisegments contained LL1 and 98.0±3.7% contained VA2 (n=28 embryos or 392 abdominal hemisegments). Thus, there is both an increasing severity and variability in muscle loss from ventral to dorsal positions of *htl* mutant embryos.

The findings with somatic muscle precursor markers parallel the defects in the mature myofiber pattern, as described above (Fig. 2H,I). In addition to the loss of specific precursors revealed by the Eve, Nau, and Kr patterns, there was a generalized reduction in muscle precursor number as indicated by D-MEF2 expression in the somatic mesoderm, with the most marked effect again evident in the dorsal region (Fig. 3D,H). The latter marker also demonstrated that cardiac cell precursors

Figure 3. Loss of *htl* function affects the development of somatic muscle precursors. Lateral views of wild-type (WT; A–D) and *htl*^{AB42} (E–H) stage 13 embryos stained with α-Eve (A,E), α-Nau (B,F), α-Kr (C,G), and α-DMEF2 (D,H). Anterior is to the left and dorsal is up. In each case, multiple muscle precursors are missing from the *htl* mutant. This reduction is more severe in dorsal than in lateral and ventral regions. Note that there is considerable variability among segments in the specific muscle precursors that are absent, and additional variability is seen in comparing different mutant embryos (see text for quantitative assessment of this phenotype). Arrows in C point to Kr-expressing muscle precursors that were quantitated in *htl*^{AB42} mutant embryos, and include dorsal acute muscle 1 (DA1), dorsal oblique muscle 1 (DO1), lateral longitudinal muscle 1 (LL1), and ventral acute muscle 2 (VA2; muscle nomenclature is after Bate 1993). Arrowhead in C indicates two additional Kr-expressing lateral transverse muscles. Other ventral Kr-positive muscle precursors are located at more internal positions within the mesoderm and are out of the plane of focus in C.

were largely absent at stage 13 (Fig. 3D,H), and even at earlier developmental times (data not shown).

tin initially is expressed throughout the mesoderm but then becomes restricted to the dorsal region in wild-type embryos (Azpiazu and Frasch 1993; Bodmer 1993) (Fig. 4A). As early as stage 10, this dorsal domain of *tin* expression is significantly reduced in *htl* mutants (Fig. 4E). Slightly later, the *tin* pattern normally is subdivided into two parts, an outer cardiac and an inner visceral component (Fig. 4B). There is an overall reduction of both regions in *htl* mutant embryos, but the cardiac domain is much more severely affected than the visceral (Fig. 4F). As predicted by this abnormal expression of *tin*, the numbers of *bap*- and Fasciclin III-expressing cells in the visceral mesoderm are severely diminished (Fig. 4C–H).

Figure 4. *htl* is required for proper specification of the cardiac and visceral mesoderm. Lateral views of wild-type (WT; A–D) and *htl*^{AB42} (E–H) embryos are shown with anterior to the left in each case. (A,E) *tin* transcripts normally are expressed at stage 10 in a broad but dorsally restricted domain that is markedly reduced in the absence of *htl* function. (B,F) By stage 11, dorsal *tin* expression resolves into visceral (VM) and cardiac (CM) domains. Both the VM and CM are significantly diminished in the *htl* mutant, but note that the CM is more severely affected. (C,G) *bap* transcripts are expressed in the visceral mesoderm of a stage 10 wild-type embryo in a series of segmentally repeated patches along the anteroposterior axis. This pattern is greatly reduced in the *htl* mutant, with some segments showing little or no *bap* expression in the visceral mesoderm while others contain small clusters of *bap*-positive cells. (D,H) Fasciclin III protein is found in a continuous ribbon of visceral mesodermal cells in a stage 11 wild-type embryo. Significantly fewer Fasciclin III-expressing cells are found in the visceral mesoderm of an age-matched *htl* mutant embryo.

Thus, in all cases where mature mesodermal tissues are dependent on *htl* function, the defect (and its relative severity) can be traced to an early developmental stage.

htl function is essential for the normal dorsolateral migration of mesodermal cells

Because the dorsally restricted domain of *tin* expression was abnormal in *htl* embryos, we examined the earlier, panmesodermal *tin* pattern. As shown in Figure 5A and D, the complement of *tin*-expressing cells in stage 9 *htl* mutant embryos does not encompass its normal dorsal limit. An identical phenotype is seen with both D-MEF2 and *Twist* expression at the same stage of development (Fig. 5B,C,E,F). With each of these three markers, the

dorsolateral edge of the mesoderm in *htl* embryos has an irregular margin that falls significantly short of the dorsal ectoderm when compared with age-matched wild-type controls.

After invagination of the mesoderm at gastrulation, further differentiation involves loss of the epithelial character of the internalized cells, several rounds of mitosis, and migration along the overlying ectoderm. Together these processes generate an inner layer of mesoderm that spans the entire dorsoventral axis of the embryo (Leptin and Grunewald 1990; Bate 1993). The abnormal appearance of the mesoderm in stage 9 *htl* mutant embryos could be attributable to a defect in any one or a combination of these processes. That is, an inadequate number of cells could invaginate, the cells could fail to proliferate, or they could remain aggregated and fail to migrate toward the dorsal ectoderm. To distinguish among these possibilities, transverse sections of *Twist*-stained wild-type and mutant embryos were compared at various stages of development. This analysis revealed that there is no reduction in the initial number of invaginated *Twist*-positive mesodermal cells in *htl* embryos (Fig. 6A,B). Furthermore, the population of mutant mesodermal cells expands appropriately by cell division (Fig. 6C–H). However, in mutant embryos, the mesodermal cell mass initially remains aggregated and does not undergo the full extent of dorsal migration characteristic

Figure 5. Early mesodermal phenotype associated with loss of *htl* function. Ventral views of stage 9 wild-type (WT; A–C) and *htl*^{AB42} (D–F) embryos are shown stained for expression of *tin* (A,D), DMEF2 (B,E), and *Twist* (C,F). By this stage, mesodermal cells uniformly expressing all three markers have migrated to form a monolayer that spans the full extent of the dorsoventral axis in wild-type embryos (also see Fig. 6E). In contrast, the mesoderm does not reach the dorsal ectoderm in the absence of *htl* function, and the dorsolateral edge of the mutant mesoderm forms an irregular margin along the anteroposterior axis.

Figure 6. *htl* is required for the proper dorsolateral migration of the embryonic mesoderm. Transverse sections (5 μ m-thick) of wild type (WT; A,C,E,G) and *htl*^{AB42} (B,D,F,H–K) Twist-stained embryos are shown at four different developmental stages. (A,B) Stage 8: The mesodermal primordium has invaginated and begun to disaggregate and spread laterally along the inner surface of the ectoderm in the wild-type embryo. In contrast, in the *htl* mutant, the mesodermal cells remain clustered close to the ventral midline (ventral is toward the bottom of the panels; these sections are anterior to the extending germ band so that amnioserosa is visible at the top). Note that a normal number of mesodermal cells is present in the mutant. (C,D) Stage 9: The number of mesodermal cells increases in both wild-type and mutant embryos, but only in the former do they continue to migrate in a dorsolateral direction on both sides of the extended germ band. (E,F) Early stage 10: Whereas the wild-type mesoderm has formed a monolayer that spans the complete dorsoventral axis of the embryo, the *htl* mutant mesodermal cells remain in a multilayered collection that has undergone incomplete lateral spreading. Note that mutant cells even appear deep within the central yolk cavity. (G,H–K) Mid- to late stage 10: Further cell division has caused an expansion of the mesoderm in both wild-type and mutant embryos. The sections in H–K are from the same *htl*^{AB42} embryo and are spaced 20 μ m apart, thus representing two adjacent segments on either side of the extended germ band. There is considerable variability in the shape of the mesodermal cell mass in these serial sections, as well as in the number of cells that are in proximity to the ectoderm at any given level along the dorsoventral axis. For example, on one side of the ventral midline in the lower part of panel I, there are no Twist-expressing nuclei near the lateral ectoderm, whereas on the other side several mesodermal cells extend to a mid-lateral position.

of its wild-type counterparts. By early stage 10, the mutant mesoderm appears as a multilayered collection of cells on either side of the extended germ band instead of a monolayer beneath the ectoderm (Fig. 6E,F). Mesodermal cells are even found in the middle of the central yolk cavity, suggesting that they are capable of migrating but not in the proper direction (Fig. 6F). Further proliferation occurs in the mesoderm of later stage 10 wild-type and *htl* mutant embryos (Fig. 6G–K).

The randomness of the cell migration that occurs in the absence of *htl* function is more clearly revealed by the serial sections shown in Figure 6H–K. These 5- μ m thick sections from the same mutant embryo are spaced 20 μ m apart along the anteroposterior axis and reveal a striking variation in the arrangement of mesodermal cells beneath the ectoderm. This variability is not seen in comparable wild-type embryos (data not shown). Although there consistently is a complete absence of mesodermal cells in the most dorsal positions, there is also considerable variability in the number of these cells at more ventral and lateral levels. The non-

uniform appearance of the mutant mesoderm is particularly impressive when considering that these four sections span two adjacent segments on either side of the extended germ band. This early defect prefigures the graded phenotypic severity along the dorsoventral axis that has already been described for the mature mesodermal derivatives and their precursors. These findings indicate that Htl is essential for the normal dorsolateral migration of the embryonic mesoderm.

An allelic series of *htl* mutations

To obtain definitive evidence that *htl* encodes DFR1/DFGF-R2, the previously described mesoderm-specific FGF receptor (Shishido et al. 1993), we identified molecular lesions in the DFR1/DFGF-R2 coding region for six of our seven *htl* alleles. The analysis was begun by PCR amplification of the DFR1/DFGF-R2 genomic region from wild-type and mutant strains. Because the DFR1/DFGF-R2 coding sequence lacks introns (Shishido et al.

1993), primers in the 5'- and 3'-untranslated regions were used to amplify this entire sequence as a single 2.3-kb fragment. This approach immediately revealed an internal deletion of ~200 bp that mapped to the 3'-end of the DFR1/DFGF-R2 coding sequence in one of the mutant lines, *htl*^{ZZ81} (data not shown). Sequencing of this segment in *htl*^{ZZ81} precisely mapped the deletion breakpoints to nucleotides 1901–2087 (nucleotide numbers are in accord with Shishido et al. 1993). In addition, two novel nucleotides were inserted in the deleted region, thus introducing a frameshift and 11 novel amino acids followed by a premature stop codon (Fig. 7A). The net result is that the carboxyl terminus of the tyrosine kinase domain of the mesodermal FGF receptor is truncated in *htl*^{ZZ81}.

The remaining six *htl* alleles yielded full-length coding regions by PCR amplification. To facilitate the rapid identification of additional molecular lesions in the FGF receptors encoded by these mutants, we scanned their coding regions using the single-strand conformation

polymorphism (SSCP) technique (Orita et al. 1989). This approach localized sequence variations to single, ~200-bp segments for each allele (data not shown). These target regions were sequenced from at least two independent PCR reactions for five of the remaining *htl* mutant lines. The FGF receptor/*htl* gene from the parental isogenic *red e* chromosome used in the original mutagenesis was sequenced in its entirety as a wild-type reference. The latter analysis revealed several differences from the previously reported DFR1 sequence (Fig. 7A). All of these nucleotide variations were confirmed in an additional normal *htl* gene, this one isolated from another lethal-bearing *red e* chromosome that was obtained from the screen and which complemented the *Sr*¹⁶ deficiency as well as an actual *htl* allele (data not shown). The differences between our wild-type *htl* sequence and that published for DFR1 may represent strain-specific polymorphisms. However, these sequence variations have no bearing on our *htl* alleles because in each case the mutant FGF receptor differs from the parental version at a

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1  MTSGSVVVLV LLLTIWQPAV QVEGRRQMAN SQEMIKDHLG ARSQNKTPAI
51  TNNANQSSTS SADLDDGAAD DDDNKADLPV NVSSKPYWRN PKKMSFLQTR
    (V)
101 PSGSLLTLNC HALGNPEPNI TWYRNGTVDW TRGYGSLKRN RWTLTMEDLV
151 PGDCGNYTCK VCNSLGCIHR DTQVIVSDRV NHKPILMTGP LNLTLVVNST
    (A)
201 GSHCKYKLSL LTSKKAWIFV PCHGMTNCSN NRSIIAEDKD QLDFVNVNME
251 QEGWYTCVES NSLGQSNSTA YLRVVRSLHV LEAGVASGSL HSTS FVYIFV
301 FGGLIFIFMT TLFVFYAIRK MKHEKVLKQR IETVHQWTKK VIIFKPEGGG
351 DSSGSMDTMI MPVVRIOKQR TTVLQNGNEP APFNEYEFPL DSNWELPRSH
401 LVLGATLGEG AFGRVVMAEV NNAIVAVKMY KEGHTDDIA SLVREMEVMK
    (M)
451 IIGRHINIIN LLGCCSONGP LYVIVEYAPH GNLKDFLYKN RPEGRDQDRD
    (R) (RS)
501 SSQPPSPPPA HVITEKDLIK FAHQIARGMD YLASRRCIHR DLAARNVLVS
    (L)
551 DDYVLKIADF GLARDIOSTD YRKNTNGRL PIKWMAPESL OEKFYDSKSD
601 VWSYGILLWE IMTYGOQYYP TIMSAEELYT YLMSGORMEK PAKCSMIYI
651 LMROCWHFNA DRRPPFTEIV EYMDKLLQTK EDYLDVDIAN LDTFPSTSDE
701 EEDETDNLQK WCNY

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Figure 7. Molecular lesions and variable phenotypes of a series of *htl* alleles. (A) The amino acid sequence of the Htl/DFR1 protein is shown in the one-letter code. The signal sequence is italicized, the transmembrane domain is boxed, and the kinase domain is underlined (the break in the line represents the kinase insertion). Where our results differ from the published sequence, the published amino acid is indicated in parentheses. Bold letters below the wild-type sequence indicate positions at which missense mutations were identified in *htl* alleles: *htl*^{AB111} has a glycine to glutamate change at position 408 in the canonical glycine-rich loop, and both *htl*^{YY262} and *htl*^{AD332} have a glutamate to lysine substitution at position 588. Dots (·) below the sequence indicate residues that are changed to stop codons. In *htl*^{AB42} a nonsense codon occurs at position 276, whereas in *htl*^{AD326} there is a nonsense codon at position 524. The asterisk indicates the last wild-type amino acid present in *htl*^{ZZ81}; a 187-bp deletion (with insertion of two novel nucleotides) replaces the remainder of the protein with the sequence DRRSATFYGNRStop. (B–G) D-MEF2 expression in wild-type (WT) and five *htl* alleles, as indicated by the label in each panel. A dorsal view of a stage 16 embryo is shown in each case. D-MEF2 is normally expressed in the cardiac cells of the heart and in all of the somatic muscles. In *htl*^{AB42}, *htl*^{AB111}, and *htl*^{AD326} there is an equally strong reduction in the number of D-MEF2-expressing mesodermal cells in the dorsal region. In contrast, a significant number of both dorsal muscles and cardiac cells develop in *htl*^{YY262}. The phenotype of *htl*^{ZZ81} is intermediate between that of the strong group and that of *htl*^{YY262}.

single nucleotide that can be interpreted as having a deleterious effect on FGF receptor activity.

In *htl*^{AB42}, cytosine 935 is mutated to thymine, thus introducing a premature nonsense codon. Because this site lies just amino-terminal to the transmembrane domain of the FGF receptor (Fig. 7A), no functional receptor should be produced by this mutant gene, and the homozygous phenotype should be that of a null allele. As discussed previously, this allele behaves genetically as an amorph and, along with two other alleles, exhibits the strongest phenotype of our *htl* mutants (see below).

The FGF receptor gene in *htl*^{AB111} contains a guanine to adenine transition at nucleotide 1332, that changes a glycine codon to glutamate (Fig. 7A). This corresponds to the first invariant glycine in the canonical glycine-rich loop of protein kinases (Bossemeyer 1994; Hanks and Hunter 1995). A bulky amino acid side chain such as that of glutamate in this position is predicted to interfere significantly with ATP binding, thus severely compromising RTK function (Bossemeyer 1994; Hanks and Hunter 1995; Mohammadi et al. 1996). The *htl*^{AD326} mutation is a cytosine to thymine transition at nucleotide 1679. This creates a nonsense codon just carboxy-terminal to the insert in the tyrosine kinase domain, thus deleting nearly 200 amino acids of the intracellular domain of the receptor (Fig. 7A). In addition to the potential adverse effect of this large deletion on folding of the kinase domain, catalytic function may be severely compromised because the deleted segment includes the critical aspartate residue that is thought to deprotonate the phosphate acceptor on the peptide substrate (Taylor et al. 1992; Hanks and Hunter 1995). Finally, two independent alleles, *htl*^{AD332} and *htl*^{YY262}, had the identical mutation, a guanine to adenine transition at position 1871 that changes a glutamate codon to lysine (Fig. 7A). Based on homology among different classes of protein kinases (Taylor et al. 1992; Hanks and Hunter 1995), and in particular to human FGFR1 for which the crystal structure has been determined (Mohammadi et al. 1996), the affected glutamate in Htl should normally form a stabilizing salt bridge with arginine 663. This structure would be disrupted by the glutamate to lysine mutation in *htl*^{AD332} and *htl*^{YY262} and lead to at least a partial reduction in kinase activity (see below).

Given the established structures of the FGF receptors encoded by our *htl* alleles, we have attempted to make structure–function correlations by ordering the mutants into a series based on phenotypic severity. Because dorsal mesodermal derivatives are most sensitive to partial loss of Htl activity, we used the expression of D-MEF2 in the heart and dorsal somatic muscles of stage 16 embryos as an indicator of phenotypic strength (Fig. 7B–G). Three alleles exhibit equally severe reductions in dorsal mesodermal D-MEF2 expression, including *htl*^{AB42} (Fig. 7C), *htl*^{AB111} (Fig. 7D), and *htl*^{AD326} (Fig. 7E). This strong phenotype can be understood in terms of the predicted severities of the corresponding molecular lesions, as described above. However, it is noteworthy that even in these mutants, there are a few D-MEF2-expressing dorsal mesodermal cells. This finding is consistent with the

previous indication that, in the absence of Htl activity, cells may be competent to migrate but not in a directed manner.

In contrast to the previous group of *htl* mutants, *htl*^{ZZ81} has relatively greater expression of D-MEF2 in cardiac and dorsal somatic muscle cells (Fig. 7F). This observation suggests that this mutant retains some residual receptor function but is nevertheless a strong hypomorph. This conclusion is consistent with the carboxy-terminal truncation of the protein encoded by *htl*^{ZZ81}. *htl*^{YY262} is the weakest allele, exhibiting only moderate loss of cardiac cells and dorsal group myofibers (Fig. 7G). This weak, hypomorphic phenotype can be explained by the likely modest destabilizing effect of the *htl*^{YY262} mutation on FGF receptor structure.

The htl mutant phenotype is partially rescued by targeted mesodermal expression of an activated form of Ras1

RTKs, of which Htl is a subfamily member, transduce extracellular signals via a highly conserved pathway involving the small GTP-binding protein, Ras (Lowy and Willumsen 1993; van der Geer et al. 1994). In *Drosophila*, the *Ras1* gene has been implicated in signaling by several different RTKs, including Sevenless (Simon et al. 1991; Fortini et al. 1992), Torso (Doyle and Bishop 1993; Lu et al. 1993), the EGF receptor (Diaz-Benjumea and Hafen 1994), and the Breathless FGF receptor (Reichman-Fried et al. 1994). We were interested in assessing whether Ras1 also acts downstream of Htl in mediating early mesodermal cell migration. To this end, we employed the targeted ectopic expression system developed by Brand and Perrimon (1993) to express a constitutively active form of Ras1 in the mesoderm of *htl* mutant embryos.

Two transgenes were separately introduced into a *htl*^{ZZ81} mutant background (see Materials and Methods). One corresponds to a cDNA encoding an activated form of Ras1 cloned downstream of Gal4-binding sites (X. Lu and N. Perrimon, unpubl.), and the other contains a Gal4 gene under control of the *twi* promoter (Greig and Akam 1993). When these two lines are crossed together, activated Ras1 is expressed in the early mesoderm of both wild-type and *htl* mutant embryos. If Ras1 normally functions downstream of Htl, then activated Ras1 should be capable of bypassing the requirement for the FGF receptor. This result would be manifest by the development of dorsal mesodermal derivatives as a measure of the successful migration of mesodermal cells to the dorsal ectoderm that supplies a necessary inductive signal (Staehling-Hampton et al. 1994; Frasch 1995). The most reliable marker for this experiment proved to be Eve because it has a simple, dorsally restricted expression pattern that is totally absent from homozygous *htl*^{ZZ81} embryos (Fig. 8A,C). Ectopic expression of activated Ras1 in a wild-type genetic background led to the marked overproduction of the segmentally repeated Eve-expressing cells (Fig. 8B). This effect is a manifestation of the involvement of Ras1 in signaling by another RTK,

Figure 8. Partial rescue of the *htl* mutant phenotype by activated Ras1. Lateral views of stage 11 embryos are shown stained with an antibody directed against Eve. (A) Wild type: Eve is normally expressed in the dorsal mesoderm at this stage in small, segmentally repeated clusters of cells. (B) Expression of a constitutively active form of Ras1 was targeted to the embryonic mesoderm of an otherwise wild-type embryo using the Gal4/UAS system (see Materials and Methods for details of the genetics). The Eve clusters are markedly enlarged but still retain their normal positions along the anteroposterior and dorsoventral axes. This effect is unrelated to the FGF receptor but is attributable to the involvement of another Ras-dependent pathway in mesodermal Eve cell fate specification (E. Buff, S. Gisselbrecht, and A.M. Michelson, unpubl.; see text). (C) In a homozygous *htl*^{ZZ81} embryo, no Eve-expressing mesodermal cells develop. (D) Ectopic expression of activated Ras1 in a homozygous *htl*^{ZZ81} embryo generates a significant number of Eve-positive dorsal mesodermal cells. Some segments show no Eve expression, others have a wild-type number of Eve-positive cells, and others have expanded Eve clusters typical of the effect of activated Ras1 in a wild-type genetic background. (E–G) Transverse sections of Twist-stained embryos of the indicated genotypes. Arrowheads point to the dorsalmost ectoderm in each embryo. (E) Activated Ras1 does not grossly affect mesodermal cell migration in a wild-type background. (F) Twist-expressing cells migrate to the dorsal-most ectoderm under the influence of activated Ras1 in one region of a *htl* mutant embryo. (G) In another region of the same mutant embryo, there is no apparent rescue of the mesodermal migration defect. The embryo in E is slightly younger than that in F and G. The latter is comparable in age to those shown in Fig. 6G–K.

the *Drosophila* EGF receptor, which is also essential for mesodermal Eve expression (E. Buff, S. Gisselbrecht, and A.M. Michelson, unpubl.). When activated Ras1 was expressed in the absence of *htl* function, partial rescue of the *htl* phenotype was observed (Fig. 8D). Some segments of the rescued embryos exhibit a nearly normal number of Eve-positive cells, some appear fully mutant, and others have an increased amount of Eve expression

approaching that seen with activated Ras1 in a wild-type background.

Most significantly, the rescued Eve-expressing cells are confined entirely to the dorsal mesoderm, implying that activated Ras1 is capable of at least partially inducing proper dorsolateral migration of the mesoderm in the absence of normal Htl FGF receptor function. This was assessed directly by examining the arrangement of Twist-positive cells in transverse sections of *htl* mutant embryos expressing activated Ras1. In some regions of such embryos, the mesodermal cell mass undergoes considerable spreading such that many cells reach all the way to the dorsal ectoderm (Fig. 8F). In other regions of the same embryo, the mesoderm shows minimal dorsolateral migration and appears indistinguishable from a *htl* mutant not expressing activated Ras1 (Fig. 8G; cf. Fig. 6H–K). These findings are entirely consistent with the variability seen with the rescue of Eve expression (Fig. 6D). Of further interest, mesodermal expression of activated Ras1 in an otherwise wild-type background did not grossly alter the overall arrangement of Twi-positive mesodermal cells (Fig. 8E). Collectively, the Eve and Twist expression data indicate that activated Ras1 is capable of partially rescuing the mesodermal migration defect associated with loss of *htl* function.

Rescue of visceral mesoderm formation in htl mutant embryos by ectopic expression of Dpp

Failure of mesodermal cells to migrate toward the dorsal ectoderm in the absence of *htl* function prevents these cells from acquiring visceral and cardiac fates under the inductive influence of Dpp (Staehling-Hampton et al. 1994; Frasch 1995; see Discussion). To determine whether mesodermal cells deficient in Htl activity are nevertheless competent to form dorsal mesodermal derivatives, we ectopically expressed Dpp throughout the mesoderm in a *htl* mutant background. In wild-type embryos, such ectopic Dpp expression dorsalizes the mesoderm (Staehling-Hampton et al. 1994; Frasch 1995). This is manifest, for example, by the induction of *bap* expression at ventral and lateral levels of the mesoderm where this visceral mesodermal marker is never normally expressed (Staehling-Hampton et al. 1994; Fig. 9A,B). When Dpp was provided to the entire mesoderm of *htl* mutant embryos, *bap* transcription was activated all along the dorsoventral axis where mesodermal cells are found, including in the most ventral positions (Fig. 9D). However, the dorsal edge of this mesodermal *bap* domain had the typical irregular appearance of *htl* embryos secondary to incomplete dorsolateral migration of the mesoderm (cf. Figs. 5A–F and Figs. 9B,D). These findings demonstrate that the Htl-deficient mesoderm is competent to respond to Dpp in the specification of visceral mesodermal fates.

As described previously (Staehling-Hampton et al. 1994), ectopic Dpp induces *bap* expression in segmentally repeated stripes in otherwise wild-type embryos (Fig. 9B). This presumably reflects the input of segmentation genes to early mesodermal pattern formation. It is significant that in a *htl* mutant background these meso-

Figure 9. Induction of *bap* expression by Dpp in the absence of *htl* function. Ventral views of early stage 10 embryos stained for expression of *bap* transcripts. (A) *bap* is expressed solely in segmentally repeated patches of dorsal mesodermal cells in wild-type embryos at this developmental stage (cf. Fig. 4C). (B) When Dpp is expressed throughout the mesoderm of an otherwise wild-type embryo under the influence of *twi*-Gal4, *bap* transcripts are induced along the entire dorsoventral axis but are restricted to segmentally repeated stripes. (C) Dorsally restricted *bap* expression is markedly reduced in the absence of *htl* function (cf. Fig. 4G). (D) Ectopic Dpp induces *bap* expression at all dorsoventral levels in mesoderm that has undergone incomplete migration in the absence of *htl* function. However, the normally sharp anteroposterior boundaries of these *bap* stripes are lost during the stages of mesodermal spreading.

dermal stripes initiate correctly during gastrulation (data not shown), but then lose their characteristically sharp anteroposterior borders during the stages of mesodermal cell spreading (Fig. 9D). Thus, *htl* appears to be required for the correct positioning of mesodermal cells along both the anteroposterior and dorsoventral embryonic axes.

Discussion

Htl FGF receptor activity is involved in early mesodermal cell migration

The present work establishes that *htl* encodes an FGF receptor that is essential in the germ band extending *Drosophila* embryo for the proper directed migration of mesodermal cells along the inner surface of the ectoderm. Formation of the mesoderm by the inward movement of ventral blastoderm cells is not dependent on *htl* function, nor does the subsequent proliferation of these cells require *Htl* activity. The most striking feature of the *htl* mutant phenotype is that the invaginated mesodermal cells initially remain aggregated and fail to spread correctly in a dorsolateral direction. There is also an associated loss of correct positioning of mesodermal cells along the anteroposterior axis of the embryo. These early migration defects lead to subsequent failure to form the heart, visceral mesoderm, and the complete complement of body wall muscles.

Mesodermal cells in *htl* mutant embryos are not completely immobile because they can be found at various positions along the lateral ectoderm and even deep

within the yolk cavity (Fig. 6). In addition, occasional somatic muscle and cardiac cells are present in the dorsal mesoderm of null *htl* alleles (Figs. 2 and 7), consistent with random movement allowing rare cells to reach the dorsal ectoderm. These findings suggest that *Htl* specifically contributes to directional cell migration and not to a more general aspect of cell motility. A similar function has been described for a *C. elegans* FGF receptor that is involved in sex myoblast migration (Devore et al. 1995).

There are several possible mechanisms whereby a RTK could provide guidance for cell movements. In one model, the receptor might respond directly to a chemotactic signal emanating from neighboring cells. This could represent a graded concentration of ligand secreted by the overlying ectoderm and toward which mesodermal cells would be attracted. Such a mechanism is operative in vertebrates in the migration of myogenic precursors from the somites to the limbs (Bladt et al. 1995). The c-kit and PDGF β RTKs also mediate directed cell migration by a chemotactic mechanism (Blume-Jensen et al. 1991; Kundra et al. 1994).

One prediction of the chemoattractant gradient hypothesis is that a constitutively active receptor or intermediate signal transducer should eliminate the critical graded output by bypassing the involvement of the ligand. However, when an activated form of Ras1 was expressed uniformly throughout the mesoderm of *htl* mutant embryos, dorsal mesodermal cell migration was partially rescued without an overall disruption of this process. Furthermore, forced expression of activated Ras1 in the mesoderm of wild-type embryos did not induce the aberrant movement of early ventral mesodermal cells. This suggests that a graded response is not essential for *Htl* function, although subtle disturbances of such a mechanism may not have been detected in our phenotypic analyses. It is also possible that additional migratory effects of *Htl* could be mediated by signal transducers other than Ras1. Similar observations have been made for deregulated activity of Breathless, the other known *Drosophila* FGF receptor, which is involved in tracheal, glial, and follicle cell migration (Klambt et al. 1992; Reichman-Fried et al. 1994; Murphy et al. 1995).

Integration between adhesion and growth factor receptor signaling pathways plays a critical role in the regulation of many cellular processes, including directed cell migration (Huttenlocher et al. 1995; Schwartz et al. 1995). Thus, another way in which RTK activity might control cell movement is to alter the adhesion of motile cells to spatially restricted external guidance cues. Axonal pathfinding in the nervous system relies on such a mechanism (Goodman and Shatz 1993), and at least one RTK has been shown to play a role in such events (Calahan et al. 1995). Alternatively, FGF receptors might mediate signals provided directly or indirectly by cell adhesion molecules (Green et al. 1996).

A RTK also could affect cell movement by controlling the self-adhesive properties of migratory cells. For example, by promoting the dissociation of such cells, the RTK could indirectly influence their capacity to move in re-

sponse to another stimulus. In this context, it is noteworthy that mesodermal cells deficient in Htl activity appear to remain aggregated at a time when their wild-type counterparts already have begun to spread along the ectoderm. Finally, it is possible that Htl could induce changes in the transcription of those genes whose products are involved in cell migration. These various modes of action are not mutually exclusive, and it is conceivable that some combination of mechanisms may mediate the mesoderm-specific effects of Htl.

Ras1 acts downstream of htl

An activated form of Ras1 partially rescues the mesoderm migration defect associated with loss of *htl* function. As a result of the partially restored mesodermal spreading stimulated by activated Ras1, Eve is induced in the dorsal mesoderm of *htl* mutant embryos by ectodermally derived Dpp. Thus, Ras1 appears to be an important downstream mediator of Htl signaling. This conclusion is consistent with a more general involvement of Ras in signal transduction by a wide variety of RTKs (Lowy and Willumsen 1993; van der Geer et al. 1994), including many found in *Drosophila* (Simon et al. 1991; Fortini et al. 1992; Doyle and Bishop 1993; Lu et al. 1993; Diaz-Benjumea and Hafen 1994; Reichman-Fried et al. 1994) and those mediating cell migration in other systems (Sosnowski et al. 1993; Kundra et al. 1994; Reichman-Fried et al. 1994; Devore et al. 1995; Lee et al. 1996). It is not yet clear whether additional members of the signaling cascade that commonly act in concert with Ras also are used by Htl. For example, both Raf-dependent and Raf-independent mechanisms function downstream of Ras in different instances of RTK-mediated cell migration in *Drosophila* (Reichman-Fried et al. 1994; Lee et al. 1996).

The ability of Ras1 to only partially substitute for Htl in facilitating dorsal mesodermal cell migration can be explained in two ways. First, it is possible that our targeted ectopic expression system fails to completely mimic the endogenous activation of Ras1 by Htl. This could be attributable to a suboptimal level of activated Ras1 expression, an improper spatial expression pattern of this factor, and/or a failure to duplicate the correct temporal occurrence of this signal. Second, Ras1 may not be the sole transducer of the migration signal mediated by Htl. Branching signal transduction pathways have been described for other RTKs (van der Geer et al. 1994), including those that mediate cell migration (Reichman-Fried et al. 1994; DeVore et al. 1995).

Role of the ectoderm in inducing mesodermal cell fates

Htl is required for the normal dorsolateral migration of the earliest population of mesodermal cells. Failure of these cells to reach the dorsal ectoderm can explain most of the later defects seen in *htl* mutant embryos. Induction of cardiac and visceral mesodermal fates is dependent on Dpp, a TGF β family member that diffuses to the

mesoderm from the dorsal ectoderm (Staehling-Hampton et al. 1994; Frasch 1995). Dorsal somatic muscle fate also requires the activity of Dpp (A.M. Michelson and S. Gisselbrecht, unpubl.). Thus, if mesodermal cells do not reach the dorsally restricted domain of Dpp expression, all of these derivatives will fail to be induced in *htl* mutants. Similar observations were made in two other experimental paradigms in which contact between the mesoderm and dorsal ectoderm was limited (Baker and Schubiger 1995; Maggert et al. 1995). It is also noteworthy that our ectopic Dpp expression experiment revealed that mesodermal cells are competent to respond to this growth factor even in the absence of Htl function; that is, Htl itself is not involved directly in the induction of visceral mesodermal cell fate.

A prominent feature of the *htl* mutant phenotype is the loss of somatic muscles in ventral and lateral as well as dorsal regions of the embryo. Even this defect can be ascribed to a cell migration problem because additional abnormalities of mesodermal cell positioning along the anteroposterior embryonic axis were identified at all dorsoventral levels (Fig. 9). Thus, failure of prospective muscle progenitors to acquire position-specific inductive cues could account for muscle loss not only from dorsal but also from ventral and lateral groups. The apparent randomness of mesodermal cell movements also can explain the variable distribution of missing muscles in all regions of *htl* mutant embryos.

Dpp acts as a morphogen in patterning the dorsoventral axis of the *Drosophila* embryo (Ferguson and Anderson 1992; Wharton et al. 1993). Thus, amnioserosa and specific ectodermal cell fates are established at different threshold levels of a Dpp activity gradient. Because Dpp also induces visceral and cardiac mesodermal fates by acting across germ layers (Staehling-Hampton et al. 1994; Frasch 1995), does the ectodermal Dpp activity gradient influence mesoderm development? One aspect of the *htl* mutant phenotype suggests that this may be the case. Loss of *htl* function causes a greater reduction in cardiac than in visceral mesodermal cells. Given that the dorsal defects in *htl* mutants can be attributed to a failure of proper mesodermal cell migration into the Dpp domain, the more severe loss of heart precursors suggests that a higher level of Dpp activity may be required for cardiogenesis. This hypothesis is consistent with the observation that cardiac cells derive from the most dorsal region of the mesoderm (Dunin-Borkowski et al. 1995) where Dpp activity is highest (Ferguson and Anderson 1992; Wharton et al. 1993). Similarly, if a lower Dpp threshold specifies visceral mesodermal fates, significantly more cells would be expected to reach this more lateral level of the Dpp activity gradient in *htl* mutant embryos.

FGF receptor signaling in mesoderm development

FGF receptors have evolved multiple functions in the development of the mesoderm and its derivatives. In *Xenopus*, FGF signaling plays a critical early role in mesoderm induction (Amaya et al. 1991; Cornell et al. 1995),

whereas mouse FGFR-1 is essential for mesodermal patterning but not for the formation of this germ layer (Deng et al. 1994; Yamaguchi et al. 1994). Interestingly, some aspects of the *fgfr-1* mutant phenotype can be explained by a defect in mesodermal cell migration from the primitive streak (Yamaguchi et al. 1994). Involvement of FGF receptor signaling in cell migration is emerging as a recurring theme, particularly in mesodermal development (Deng et al. 1994; Yamaguchi et al. 1994; DeVore et al. 1995; Itoh et al. 1996; this work).

As is the case for murine FGFR-1, the *Drosophila* Htl FGF receptor is involved in patterning but not in early specification of the embryonic mesoderm. In the absence of *htl* function, mesodermal cells fail to migrate to their proper positions, and, as a result, do not receive the later inductive signals that are essential for their commitment to somatic, visceral, and cardiac fates. FGF receptor signaling in *Drosophila* thus provides a crucial link between the establishment of general mesodermal cell fate at gastrulation and the subsequent induction of lineage-specific identities by other signaling pathways.

Materials and methods

Fly strains and genetic techniques

A genetic screen employing EMS mutagenesis of an isogenic third chromosome marked with *red* and *ebony* (*e*) was undertaken essentially as described by Seeger and coworkers (Seeger et al. 1993). Lethal third chromosome mutations were recovered over a TM3 balancer containing a *fushi tarazu* (*ftz*)–*LacZ* transgene. Initial phenotypic screening was for lines in which Eve expression was aberrant in the central nervous system and/or the mesoderm.

Targeted ectopic expression of an activated form of Ras1 (containing a glycine to glutamine change at amino acid position 13 [X. Lu, pers. comm.]) was achieved using the Gal4/UAS system (Brand and Perrimon 1993). A UAS–*Ras1^{Act}* transgene inserted on the second chromosome (generously provided by X. Lu and N. Perrimon, Harvard Medical School, Boston, MA) was introduced into a *htl^{ZZ81}* mutant background by standard genetic crosses to yield a line of the genotype, UAS–*Ras1^{Act}*/CyO,*ftz*–*LacZ*; *htl^{ZZ81}*/TM6B,*abdA*–*LacZ*. Similarly, a *twi*–*Gal4* insertion on the second chromosome (Greig and Akam 1993) was combined with a *htl^{ZZ81}* third chromosome to yield a stock of the genotype, *twi*–*Gal4*; *htl^{ZZ81}*/TM6B,*abdA*–*LacZ*. When these two lines are crossed together, one eighth of the embryos are homozygous for the *htl* mutation and express *Ras1^{Act}* throughout the mesoderm. These embryos can be identified by the absence of *LacZ* expression directed by the balancer chromosomes. An identical strategy using a UAS–*dpp* transgene insertion on the second chromosome (Staehling-Hampton et al. 1994) was used to target expression of Dpp to the mesoderm of *htl^{ZZ81}* mutant embryos. Oregon R was used as the reference wild-type stock. All flies were grown on standard yeast–cornmeal agar medium at 25°C.

Immunohistochemical staining and in situ hybridization of embryos

Embryos were collected on molasses agar plates at 25°C, aged for the requisite times, and fixed with 4% formaldehyde, as described previously (Michelson et al. 1990; Michelson 1994). Antibody staining was carried out as described (Michelson

1994), using antibodies at the following dilutions: rabbit polyclonal α -Eve (1:5000), α -myosin heavy chain (1:500), α -Kruppel (1:1500), α -DMEF2 (1:1500), α - β -galactosidase (Cappel, 1:500), and α -Twist (1:10,000); rat polyclonal, affinity-purified α -Nau (1:50); mouse monoclonal α -Fasciclin III (1:3), α - β -galactosidase (Promega; 1:500), and α -pericardial cell antigen (1:3). Markers used to analyze the central nervous system in *htl* mutant embryos included mouse mAb 22C10 (1:10), mouse α -RK2 (1:5), mouse α -Engrailed (1:5), and rabbit α -Eve (as above). Biotinylated secondary antibodies (Vector Laboratories) were preadsorbed against an overnight collection of embryos and used at a dilution of 1:500. In situ hybridization of embryos was carried out using digoxigenin-labeled DNA probes (Tautz and Pfeifle 1989), as modified by Michelson et al. (1990). Alternatively, RNA probes were used for embryo in situ hybridizations, as described (O'Neill and Bier 1994).

Embryos were photographed under Nomarski optics on a Zeiss Axiophot microscope using Kodak EPT160 slide film. Slides (35-mm) were scanned and only the contrast, brightness, and color balance of these digital images subsequently were manipulated using Adobe Photoshop.

Quantitation of muscle defects

The numbers of Kr-expressing dorsal (DA1 or DO1), lateral (LL1), and ventral (VA2) muscle precursors were counted in 28 individual stage 13–14 *htl^{AB42}* mutant embryos. Abdominal segments 1–7 were examined on both sides of each embryo ($n = 392$ hemisegments). The mean percentage ($\pm$ S.D.) of muscle precursors present per embryo was calculated (number observed/number expected).

Embryo sectioning

Embryos were embedded in Spurr resin (Spurr Low Viscosity Embedding Medium, Polysciences) prior to sectioning. In brief, darkly stained embryos were dehydrated through an ethanol series, washed twice in 100% ethanol, infiltrated overnight with a mixture of Spurr medium and propylene oxide (1:1), and finally incubated for several hours in 100% Spurr medium at room temperature. Embryos were then aligned in embedding molds in an orientation suitable for transverse sectioning and the resin was allowed to harden overnight at 65°C. Serial 5- μ m sections were cut from the resulting blocks using a Leica microtome fitted with a tungsten carbide blade (Energy Beam Sciences), adhered to coated microscope slides (Fisher Superfrost Plus), cleared by application of immersion oil and examined under Nomarski optics, as described above.

Preparation of embryo DNA

Flies bearing balanced *htl* alleles were outcrossed to an Oregon R stock and embryos were collected from matings between the resulting *htl*/+ adults. These embryos were incubated until all viable progeny had hatched (~24 hr at 25°C). Fifty of the unhatched homozygous *htl* mutant embryos were crushed in 200 μ l extraction buffer (10 mM Tris at pH 8.2, 1 mM EDTA, 25 mM NaCl, 200 μ g/ml proteinase K freshly diluted from a frozen 20 mg/ml stock), incubated at 37°C for 30 min, and boiled for 10 min. Particulate material was removed from the crude extract by spinning for 5 min at top speed in a microcentrifuge. The supernatant was used as a source of genomic DNA for polymerase chain reactions without further processing.

SSCP analysis

The approximate site of each of the *htl* mutations was determined by SSCP analysis of genomic DNA isolated from the mutant lines (Orita et al. 1989). Fifteen pairs of oligonucleotide primers were used to generate overlapping PCR products of 250 bp or less that span the entire *DFR1/htl* coding sequence (Shishido et al. 1993; primer sequences available on request). Genomic DNAs from three different strains were used as controls: Oregon R, *red e* (the parental strain employed in the EMS mutagenesis screen), and another lethal-bearing mutant line from the screen that complemented the *htl* alleles. Primer pairs were 5' end-labeled with T4 polynucleotide kinase and [γ -³²P]ATP (6000 Ci/mmol), and then were supplemented with a 2.5-fold excess of the same unlabeled primers. Hot-start PCR reactions were performed in 12 μ l total volume containing 1 μ l of denatured embryonic genomic DNA as template (see above), 200 nM each primer, 200 μ M each dNTP, and 0.2 μ l of Expand High Fidelity thermostable DNA polymerase mixture (Boehringer Mannheim). PCR amplification was carried out for 28 cycles, as follows: 1 min at 94°C, 1 min at 55°C, and 1 min at 72°C. A 2- μ l aliquot of each PCR reaction was combined with 4 μ l of denaturation buffer (95% formamide, 0.05% bromophenol blue, 0.05% xylene cyanol, 20 mM EDTA), incubated at 95°C for 5 min and then chilled on ice. Samples were loaded on 5% nondenaturing polyacrylamide gels and run in 0.5 $\times$ Peacock's buffer at 4°C for 2 hr at 40 W constant power. Gels were dried onto filter paper without fixation and exposed to X-ray film.

Cloning and sequence analysis

The complete *htl* coding region was PCR-amplified from the genomic DNA of all *htl* mutant alleles and from the *red e* parental strain using the following oligonucleotides as primers: 5'-GGGAATTCATATACCAAAAATGGCTGCCGCCTG-3' and 5'-ACGTCTCGAGACCAAGTGTGGTCCGAACCTTACAGGAC-3'. These primers correspond to nucleotides 54–78 and 2309–2280 of the published *DFR1* sequence, respectively; the italicized nucleotides were added to introduce *Eco*RI and *Xho*I restriction sites. PCR amplification was for 30–40 cycles, as follows: 1 min at 94°C, 1 min at 60°C, and 3 min at 72°C. The amplified DNAs were digested with *Eco*RI plus *Xho*I and cloned into the polylinker of pSP73 (Promega). Sequences of the cloned inserts were determined on both strands using an ABI automated sequencer (primer sequences available on request). The complete wild-type coding region was sequenced from three independent PCR reactions, whereas for each of the *htl* alleles (with the exception of *htl*^{AB42} which was sequenced in its entirety), only the region that demonstrated variation by SSCP analysis was sequenced. All mutations were confirmed by sequencing the mutant *htl* genes cloned from at least two independent PCR amplifications.

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