FGF ligands in *Drosophila* have distinct activities required to support cell migration and differentiation

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Fibroblast growth factor (FGF) signaling controls a vast array of biological processes including cell differentiation and migration, wound healing and malignancy. In vertebrates, FGF signaling is complex, with over 100 predicted FGF ligand-receptor combinations. *Drosophila melanogaster* presents a simpler model system in which to study FGF signaling, with only three ligands and two FGF receptors (FGFRs) identified. Here we analyze the specificity of FGFR [Heartless (Htl) and Breathless (Btl)] activation by each of the FGF ligands [Pyramus (Pyr), Thor (Ths) and Branchless (Bnl)] in *Drosophila*. We confirm that both Pyr and Ths can activate Htl, and that only Bnl can activate Btl. To examine the role of each ligand in supporting activation of the Htl FGFR, we utilize genetic approaches that focus on the earliest stages of embryonic development. When pyr and ths are equivalently expressed using the Gal4 system, these ligands support qualitatively different FGFR signaling responses. Both Pyr and Ths function in a non-autonomous fashion to support mesoderm spreading during gastrulation, but Pyr exhibits a longer functional range. pyr and ths single mutants exhibit defects in mesoderm spreading during gastrulation, yet only pyr mutants exhibit severe defects in dorsal mesoderm specification. We demonstrate that the *Drosophila* FGFs have different activities and that cell migration and differentiation have different ligand requirements. Furthermore, these FGF ligands are not regulated solely by differential expression, but the sequences of these linked genes have evolved to serve different functions. We contend that inherent properties of FGF ligands make them suitable to support specific FGF-dependent processes, and that FGF ligands are not always interchangeable.

**KEY WORDS:** Cell migration and differentiation, *Drosophila*, FGF signaling, Ligand-receptor interactions

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**INTRODUCTION**

Most Fibroblast growth factors (FGFs) have N-terminal signal peptides and are secreted from cells, allowing binding to and activation of their cell surface receptors (FGFRs). Studies in vertebrates, as well as simpler invertebrate model systems, have yielded valuable insights into the functions of this important gene family in multiple biological processes (Huang and Stern, 2005; Rottinger et al., 2008; Thiss and Thiss, 2005). In embryos, these functions include mesoderm induction and patterning, cell growth, migration and differentiation. Later functions include organ formation and maintenance, neuronal differentiation and survival, wound healing and malignant transformation (Chen and Deng, 2005; Coumoul and Deng, 2003; Eswarakumar et al., 2005). FGFs often signal directionally across epithelial-mesenchymal boundaries and exhibit diverse and dynamic patterns of expression that are both spatially and temporally restricted. Tightly regulated expression is one mechanism that helps to ensure the specificity of FGF signaling through FGFRs. An additional level of specificity is most likely imparted by their divergent amino acid sequences, resulting in differential binding affinity for the FGF, heparan sulfate proteoglycan (HSPG)-interaction specificity (a co-factor involved in FGF-FGFR interaction), and/or FGF ligand range of action (e.g. diffusibility).

FGF and FGFR genes have been identified in organisms ranging from the nematode and fly to mouse and human. There are two FGFs and one FGFR in *Caenorhabditis elegans*, and three FGFs and two FGFRs in *Drosophila melanogaster*, as compared with 24 FGFs and four FGFRs (three of which exhibit alternative splicing) in humans and mice (Birnbaum et al., 2005; Huang and Stern, 2005). In vertebrates, over 100 potential FGF-FGFR complexes are predicted (Zhang et al., 2006). Thus, the expectation is that with so many combinations possible, tight regulation of FGF activity and receptor specificity must exist to regulate signaling. Many FGF-FGFR interactions have been studied in the vertebrate system, but the genetic redundancy can make dissection of the functional contribution of particular FGF-FGFR interactions challenging (e.g. Mariani et al., 2008). Often, more than four ligands interact with a particular FGFR isoform at any one time.

*Drosophila* is an excellent model system for studying FGF signaling, especially now that it appears that the full repertoire of FGF ligands and receptors has been identified (reviewed by Huang and Stern, 2005). Relatively few FGF-FGFR interactions are possible, with only three FGF ligands [Pyramus (Pyr), Thor (Ths) and Branchless (Bnl)] and two FGF receptors [Heartless (Htl) and Breathless (Btl)] (reviewed by Ornitz and Itoh, 2001; Szebenyi and Fallon, 1999). The FGFR Btl, and its FGF ligand Bnl, control tracheal branching in the embryo, mesoderm migration over male genital discs, and air sac formation in the larva (Ahmad and Baker, 2002; Sato and Kornberg, 2002; Sutherland et al., 1996). The preliminary function of the Htl FGFR is to control mesoderm migration during gastrulation (Beiman et al., 1996; Gisselbrecht et al., 1996; Shishido et al., 1997). Later in development, among other functions, Htl is also required for the differentiation of cells that form the heart and hindgut musculature (Michelson et al., 1998; San Martin and Bate, 2001). The ligands for the Htl FGFR in *Drosophila* had remained elusive, until pyr and ths were identified by genomic screens (Stathopoulos et al., 2004; Gryzik and Muller, 2004). Pyr and Ths share homology with the FGF family of vertebrate FGFs. Genetic evidence suggests that this pair of invertebrate FGFs functions through a single FGFR, Htl, to control cell migration and differentiation, as a deficiency mutant that removes both ligands phenocopies the htl mutant (Stathopoulos et al., 2004; Gryzik and...
Muller, 2004). Thus, Drosophila melanogaster provides a unique opportunity within an invertebrate model system, amenable to genetic approaches, to gain insights into why multiple FGF ligands are utilized to activate the same receptor isoform, as is typically the case in vertebrates.

A question in the FGF field is whether the specificity of receptor-ligand interactions is accomplished through the differential expression of ligands or through differences in the signaling properties of the ligand proteins themselves. An impressive analysis of all vertebrate FGF-FGFR interactions was recently completed, in which the binding specificities of ligand-receptor interactions were examined in tissue culture (Ormitz et al., 1996; Zhang et al., 2006). Specificity of receptor-ligand interactions was demonstrated in this system; however, how this relates to in vivo processes is, for the most part, undetermined. Only a limited number of in vivo studies have been conducted to analyze the function of particular FGF-FGFR interactions (e.g. Rentzsch et al., 2008; Yang et al., 2002).

In this study, we examine the individual functions of the FGF ligands Pyr and Ths in Drosophila in order to define whether these ligands have distinct functions and/or function redundantly, and to gain insights into why multiple ligands are typically involved in activating a particular FGFR coordinately. We find that the regulated expression of ligands and their divergent protein sequence both contribute to the specificity of FGF-FGFR interactions.

MATERIALS AND METHODS

Fly stocks and genetic approaches

Unless otherwise noted, fly stocks were obtained from the Bloomington Stock Center and were reared under standard conditions at 25°C. The genotype ‘wild type’ refers to the yw genetic background. Df(2R)BSC25 has been described (Stathopoulos et al., 2004). The Gal4 drivers zenVRE.Kr-Gal4 and sim-Gal4 were obtained from M. Frasch (University of Erlangen-Nuremberg, Germany) and Stephen Crews (University of North Carolina, Chapel Hill, NC, USA), respectively. Two PiggyBac insertion stocks were obtained from the Exelixis Collection Stock Center at Harvard Medical School (Thibault et al., 2004); we determined that P02915 is located in the first intron of pyr, and confirmed that P02026 is located in the second intron of ths.

Df(2R)ths238 is a deletion of the ths gene that was created in the course of this study through a genetic screen for male-specific recombination (see Preston et al., 1996) using P9.1.2, a viable P-element insertion located ~400 bp upstream from the ths transcription start site, as mapped by RACE (see Fig. 2A). Line P9.1.2 resulted from mobilization of P10004, a viable P-element insertion located (Stathopoulos et al., 2004). The lines chosen [pyr line1: UAS-pyr::AMS330(III) and ths line2 and ths line1: UAS-ths::AMS289.22(III)] exhibit comparable levels of ectopic expression as assayed by qPCR (see Fig. S4 in the supplementary material) and support equivalent Eve+ expression when driven by 69B-Gal4 (see Fig. S3D,E in the supplementary material). In another set of insertions, approximately twice as much ectopic ths was supported, relative to the levels of pyr (see Fig. S4 in the supplementary material, ths line2 and pyr line2); nevertheless, the same results were obtained with this other set of lines in the zenVRE.Kr-Gal4-driven rescue experiments (data not shown). Therefore, we contend that the ability of pyr alone to support the migration of mesodermal cells at a distance is not due to differences in expression levels resulting from positional effects on transgene expression.

Immunohistochemistry, in situ hybridization and sectioning

The following antibodies were used: guinea pig anti-Twist (1:200; from M. Levine, UC Berkeley, CA, USA), mouse anti-dpERK (1:200; Sigma), rabbit anti-β-galactosidase (1:200; Molecular Probes), rabbit anti-Eve (1:400; from M. Frasch) and monoclonal 2A12 (1:3, Developmental Studies Hybridoma Bank). In situ hybridization (Kosman et al., 2004; Lehmann and Tautz, 1994), double antibody plus in situ hybridization detection of protein and transcript levels (Frasch, 1995), and staining of tracheal branches using 2A12 antibody (Sutherland et al., 1996) were conducted as previously described.

For sectioning, embryos were sorted for appropriate stage and genotype (i.e. using balancer chromosomes containing reporters), and embedded in acetone-Araldite (Electron Microscopy Sciences) as described (Leptin and Grunewald, 1990). Sections (10 μm) were cut using a Historange 2218 microtome.

Because variability is present in the Df(2R)BSC25 mutant background, a system of quantitation of mutant phenotype was devised. We scored mesoderm spreading phenotypes in the deficiency and the ectopically
expressing lines (see Figs S5 and S6 in the supplementary material). Clumping was used as a measure of the ability of ligands to attract cells to the ventral midline; the multilayer phenotype was scored by assessing the ability of ligands to attract cells to the dorsal-most ectoderm.

**RESULTS**

**pyr and ths are dynamically expressed within Drosophila embryos**

It is likely that Pyr and Ths are ligands for the Htl FGF receptor, as these genes are expressed near sites of Htl expression (Stathopoulos et al., 2004). During gastrulation, pyr and ths are expressed in distinct domains within the ectoderm at the surface of the embryo (see Fig. 1A-C). Early in development, ths is expressed in ventral regions (Fig. 1F,G) and pyr is expressed in dorsal regions (Fig. 1D,E). Once invagination is complete and all mesoderm cells have entered the interior of the embryo, these cells proceed to migrate along the inner ectodermal surface, forming a monolayer. Activation of the FGFR Htl is thought to trigger the spreading of the mesoderm across the underlying ectoderm (Fig. 1A). The dynamic expression domains of the ligands suggested a possible mechanism by which directional information could be imparted to the mesoderm cells moving beneath, causing these cells to move in the dorsal direction.

**Ligand choice and expression domain are important for FGF signaling**

To determine whether the distinct domains of ligand expression are important for proper mesoderm migration, we altered their expression domains and assayed effects on mesoderm spreading. Using the 69B-Gal4 driver, the bnl, pyr and ths FGF-encoding genes were ectopically expressed throughout the entire ectoderm (Fig. 1H-M). For pyr and ths, several transgenic lines were first compared with the control for positional effects that might influence the levels of gene expression, and a set of lines was chosen that exhibited comparable levels of ectopic expression by Gal4 as assayed by qPCR (see Fig. S4 in the supplementary material). Ectopic expression of Pyr throughout the ectoderm resulted in mesoderm migration defects (Fig. 1J,K; n=9). The pyr ectopic expression defect was specific; mesoderm spreading occurred, but a cell monolayer was not formed (Fig. 1K). By contrast, ectopic expression of Bnl or Ths in the same manner had little to no effect on mesoderm monolayer formation (Fig. 1G,I and Fig. 1L,M; n=6 and n=10, respectively).

The MAPK pathway is activated in migrating mesodermal cells as a result of FGFR receptor tyrosine kinase activation, and leads to dual phosphorylation of MAPK [also known as ERK (Gabay et al., 1997) and Rolled – FlyBase]. A monoclonal antibody that recognizes this activated form of MAPK (dpERK) can be used to monitor activation of this pathway within cells. dpERK staining is observed within gastrulating embryos at the leading edge of the migrating mesoderm cells (see Fig. S1A in the supplementary material), but is lost in the absence of Htl activation (Michelson et al., 1998; Stathopoulos et al., 2004). In embryos ectopically expressing pyr or ths via twist-Gal4, which supports their expression throughout the mesoderm, dpERK is ectopically expressed throughout all mesoderm cells (see Fig. S1C,E in the supplementary material) (Stathopoulos et al., 2004). However, when the 69B-Gal4 driver was used to ectopically express pyr or ths, only the ectopic expression of pyr supported ectopic activation of ERK throughout the entire mesoderm (compare Fig. S1D with Fig. S1F in the supplementary material). Ectopic expression of bnl had no effect; dpERK remained localized at the leading edge of the migrating mesoderm [compare Fig. S1B with Fig. S1A (see arrowheads) in the supplementary material]. Depending on where the ligands were expressed, differences in the capacity of Pyr versus Ths to activate signaling were observed, whereas Bnl had no effect. Furthermore, the ability of these ligands to support ectopic dpERK expression was found to correlate with the inhibition of mesoderm migration (see Fig. 1; data not shown).

We then assayed the ability of Pyr and Ths to influence trachea formation, a function that is supported by Bnl-Btl. Ectopic expression of bnl via 69B-Gal4 leads to a substantial increase in tracheal branching (compare Fig. S2B with Fig. S2A in the supplementary material) (Sutherland et al., 1996). However, no such effect on the trachea was observed upon ectopic expression of pyr or ths (see Fig. S2C,D in the supplementary material), suggesting that neither Pyr nor Ths can activate the Btl FGFR.

**Isolation and initial characterization of pyr and ths single mutants**

Mesoderm spreading defects are observed in htl mutants and in a deficiency mutant, Df(2R)BSC25, that removes both pyr and ths (Fig. 2A) (Beiman et al., 1996; Gisselbrecht et al., 1996; Stathopoulos et al., 2004). We sought to test further the requirement for Pyr or Ths in activation of the Htl FGFR by analysis of single-
mutant phenotypes. First, we identified two PiggyBac insertions located in proximity to pyr and ths (see Fig. 2A). The line containing the pyr insertion, pyr02915, is zygotically lethal, whereas the line containing the ths insertion, ths02026, is only semi-lethal [i.e. homozygous flies are obtained at very low frequencies (~0.5%) and are short-lived]. Each insertion disrupts expression of the respective gene, and we detect no effect on expression of the other ligand (see Fig. 2E,G, compare with Fig. 2A-C). As phenotypes associated with insertions can vary, we chose to identify true null alleles.

Deletions of the entire pyr or ths coding sequences were created (see Fig. 2A and Materials and methods). Because pyr and ths are linked, deletions resulting from mobilization of the insertion P10004 had the potential to remove either ths or pyr sequences. Through several rounds of male-specific recombination screening, we were able to identify a deletion that removes the ths gene in its entirety, which we named Df(2R)ths238. The entire ths coding sequence, as well as ten proximal genes, are absent, yet the entire pyr locus remains intact. Using excision-based screening with the same P9.1.2 insertion, we obtained a deletion that removes sequence upstream of ths, including the entire pyr gene and five proximal genes, which we named Df(2R)pyr36.

Complementation tests were conducted to determine genetic interactions between our allelic series of pyr and ths mutants. We believe that the pyr02915 allele represents a pyr null, as no expression of pyr was detected at any time-point examined. It is unclear whether the ths02026 allele represents a ths null, as expression could be detected in the visceral mesoderm at later stages (data not shown). Df(2R)pyr36 and Df(2R)ths238 represent null alleles of pyr and ths, respectively, because the entire coding region of each gene is absent; however, of note is the fact that other genes are also removed by these ~100 kb deletions (see Materials and methods for details). This allelic series (i.e. PiggyBac insertions: weak alleles; Df(2R)pyr36 and Df(2R)ths238 deficiencies: strong alleles; Df(2R)BSC25 deficiency: double mutant; see Fig. 2A) provided the first opportunity to analyze transheterozygous combinations in order to gain insights into pyr and ths single-mutant phenotypes. In both pyr and ths single mutants, the mesoderm spreading was aberrant (Fig. 3G-I, compare with Fig. 3A,B). The mesoderm was multilayered and a monolayer rarely formed. However, neither mutant exhibited a phenotype as severe as that of the Df(2R)BSC25 or htl mutants (Fig. 3C-F), suggesting a role for both Pyr and Ths in guiding mesoderm spreading.

**Importance of positional information in the function of ligands during mesoderm spreading**

To determine whether the localized expression of each ligand is important for its function, we took an ectopic expression approach using Gal4 to promote expression of a given ligand in the visceral mesoderm within the lower half of gastrulating embryos (see Fig. 4K) (Frasch, 1995). The sim-Gal4 driver can be used to deliver ligands to the dorsal domain of the ectoderm within the lower half of gastrulating embryos (see Fig. 4K) (Frasch, 1995).

When the sim-Gal4 driver was used to express either pyr or ths in the Df(2R)BSC25 mutant background (Fig. 4F), no rescue of mesoderm spreading was observed (Fig. 4G,H). In fact, clumps of mesoderm cells were observed overlying the site of Pyr ectopic expression, suggesting that this ligand had attracted mesoderm cells to this position (Fig. 4G). This phenotype appeared to be more severe than that of Df(2R)BSC25 mutants (Fig. 4G,H, compare with Fig. 4B,C; see Fig. S5 in the supplementary material; P<0.01). Ectopic expression of ths in this manner also had a weak effect, as
cells tended to pool at the ventral midline (Fig. 4H), but this effect was not supported by statistical significance as the Df(2R)BSC25 mutants themselves exhibit variability (Fig. 4B,C and see Fig. S5 in the supplementary material).

Different results were obtained using the zenVRE.Kr-Gal4 driver. In this case, ectopic expression of either pyr or ths did appear sufficient to direct mesoderm cells toward dorsal regions of the ectoderm in the lower ventral half of Df(2R)BSC25 mutant embryos (Fig. 4L,M; n=17 and n=19, respectively), within the domain of ectopic expression supported by the driver (see Fig. 4K). In Df(2R)BSC25 mutant embryos, some spreading toward dorsal regions was observed in only 28% of the embryos examined (n=10 of 36 embryos scored in total) (Fig. 4B and see Fig. S5 in the supplementary material); therefore, this ‘rescue’ in the lower half of the embryos by pyr and ths is significant (see Figs S5 and S6 in the supplementary material). Surprisingly, however, ectopic expression of pyr in this manner was also capable of directing the movement of mesoderm cells in the top half of embryos, at a distance from the source of pyr expression supported by zenVRE.Kr-Gal4. For ths ectopic expression, the effect on the top half was not as strong and exhibited more variability (see Figs S5 and S6 in the supplementary material).
The strongest alleles of Df(2R)pyr36/Df(2R)pyr36 on Eve+ cell specification (see inset; often two Eve+ cells are present instead of three). At later stages, defects are more apparent (I).

Drosophila mutant embryos, which lack both pyr and ths genes, at stage 11 (D), and stage 14 (F); and ths single mutants at stage 11 (G,H) and stage 14 (I). The insets display Eve+ cell clusters at 10× magnification. (A,C) In wild-type embryos at stage 11 (A), there are 11 independent clusters of three Eve+ cells each within the dorsal somatic mesoderm. At stage 14 (C), these join to form a continuous row of heart progenitors. (B) Eve+ clusters are absent from the dorsal mesoderm of Df(2R)BSC25 mutant embryos. (D-F) In both the weakest (pyr02915/ths02026) and the strongest [Df(2R)pyr36/Df(2R)pyr36] alleles of pyr single mutants (D and E, respectively), the number of Eve+ clusters is significantly reduced, resulting in gaps within the row of heart progenitors at stage 14 (F). (G-I) By contrast, within ths mutant embryos at stage 11, when either a weak mutant [Df(2R)ths238/ths02026] or the strongest alleles [Df(2R)ths238/Df(2R)ths238] are examined (G and H, respectively), there are only subtle effects on Eve+ cell specification (see inset; often two Eve+ cells are present instead of three). At later stages, defects are more apparent (I).

Collectively, these results suggest that localized pyr expression is important to support mesoderm spreading, as zen/RE.Kr-Gal4 is able to promote spreading, whereas sim-Gal4 is not, and also suggest the hypothesis that Pyr protein can function at a distance, whereas Ths functions more locally. To test this, embryos were assayed for dpERK activity (Fig. 4D-O). Both pyr and ths expression through sim-Gal4 was able to support dpERK activation (Fig. 4F,J). Therefore, the weaker effect of ths is unlikely to be due to an inability to activate the receptor in this domain. Moreover, pyr expression through zen/RE.Kr-Gal4 supported strong activation of dpERK throughout the lower half of the embryos (Fig. 4N), whereas the effect of ectopic expression of ths in this same domain was more limited (Fig. 4O). Both pyr and ths expression supported dpERK activation at the leading edge of mesodermal cells in the top half of embryos (Fig. 4N,O). Mesoderm cells in the top half of the embryo are able to receive both Pyr and Ths signals, but only a subset of the cells migrates toward the dorsal ectoderm in response to Ths (see Discussion).

**FGF ligand specificity during mesoderm differentiation**

After mesoderm cell spreading has been completed, both Pyr and Ths are expressed in regions of the dorsal ectoderm that abut a subset of the mesoderm cells (Fig. 2D and see Fig. S3A,B in the supplementary material). pyr is present in more-dorsal regions of the ectoderm, overlying sites of dorsal mesoderm specification; limited expression of ths is also seen in this same region (Fig. 2D). The mesoderm cells that come into contact with the dorsal ectoderm receive an inductive signal from Decapentaplegic (Dpp), a Drosophila TGFβ homolog (reviewed by Frasch, 1999). Thereby, expression of genes such as even skipped (eve) and tinman, which are required for the differentiation of cardiac and pericardial tissues, depend on Dpp as well as on Htl-dependent FGF signaling.

We sought to define the role of each ligand in the specification of dorsal mesoderm cell lineages within germ-band elongated embryos. Normally, Eve is expressed within 12 hemisegments. In pyr single mutants, often only a few hemisegments exhibited Eve expression (Fig. 5D,E, compare with Fig. 5A), whereas within ths single-mutant embryos, only subtle defects in Eve+ cell specification were observed (Fig. 5G,H, compare with Fig. 5A). The pyr mutant phenotype was much stronger than the phenotype exhibited by ths single mutants at all stages examined, and even when comparisons were made between the weakest pyr allele (pyr02915) and the strongest ths allele [Df(2R)ths238] (Fig. 5C,F, compare with Fig. 5I). Moreover, the phenotype of Df(2R)pyr36 was consistently stronger than that of pyr02915, suggesting that either pyr02915 is not a null allele or that ths gene function also supports Eve+ cell specification. Although Pyr has the more dominant role, Ths probably also plays a role in supporting Eve expression because the Df(2R)BSC25 mutant phenotype is more severe than that of Df(2R)pyr36 mutants. No Eve+ cells are present within the dorsal mesoderm of Df(2R)BSC25 mutants, a phenotype similar to that of htl mutants (Fig. 6B) (Stathopoulos et al., 2004).

To investigate the roles of Pyr and Ths in controlling cell differentiation, which might differ from their roles during cell migration, we focused on how ectopic expression of these ligands affects specification of Eve+ cells within the dorsal mesoderm of the embryo. We have demonstrated previously that when Ths is ectopically expressed in the ectoderm using the 69B-Gal4 driver, ectopic Eve+ cells result; this is similar to the phenotype observed when constitutively activated Htl is ectopically expressed in the same manner (see Fig. S3E in the supplementary material) (Stathopoulos et al., 2004). We found that ectopic expression of Pyr driven by 69B-Gal4 also increases the number of Eve+ cells (see Fig. S3D in the supplementary material). Normally, three to four Eve+ cells are specified in 12 hemisegments (see Fig. S3C in the
Drosophila FGFs have distinct activities

DISCUSSION

The experiments outlined above demonstrate that the Drosophila FGFs Pyr, Ths and Bnl have different functions and that the activation of FGF receptors by specific ligands affects particular biological processes. Examination of an allelic series of pyr and ths mutants suggests that pyr and ths are not redundant in function; both influence mesoderm spreading, whereas pyr is the dominant player controlling Eve+ cell specification within the dorsal mesoderm (Figs 3 and 5). We have demonstrated previously that ectopic expression of ths by twist-Gal4 and 69B-Gal4 in the Df(2R)BSC25 mutant background can support Htl FGFR activation (Stathopoulos et al., 2004). However, in this study, we assayed whether the expression supported in distinct domains would support Htl activation. By a series of ‘rescue’ experiments, through ectopic expression of one ligand in the Df(2R)BSC25 mutant background, we obtained evidence that localized expression of the ligands is important for proper mesoderm spreading. We find, surprisingly, that the ligands exhibit differences in their functional range of action (Fig. 4). In addition, using this same approach, we find that either Pyr or Ths can support Eve+ cell specification within the dorsal mesoderm, but that Bnl cannot (Fig. 6). Collectively, these data suggest that the Pyr and Ths FGFs function as ligands for the Htl FGFR and that specificity of FGF-FGFR interactions exists in Drosophila.

Fig. 6. Pyr and Ths, but not Bnl, can support Htl activation to effect expression of Eve within dorsal mesoderm lineages.

Drosophila embryos oriented with anterior to the left and dorsal up. Ectopic expression of UAS-pyr, UAS-ths or UAS-bnl in a Df(2R)BSC25 mutant background, which lacks the endogenous pyr and ths genes, was achieved using various Gal4 drivers that support expression in different domains of the ectoderm. Depicted are lateral views of stage 11 embryos stained using an anti-Eve antibody (green) and by in situ hybridization with riboprobes to detect pyr (D,G), ths (A-C,E,H) or bnl (F,I) transcripts (red). (A) Endogenous expression of ths in wild-type embryos. Eve+ cells are present in 12 hemisegments. (B) ths expression in Df(2R)BSC25 mutant embryos. No dorsal mesoderm-derived Eve+ cells are present. Eve staining is detected only in the central nervous system, expression that is FGF-signaling independent. (C) Expression of ths in the ventral midline using sim-Gal4 does not support expression of Eve in a homozygous Df(2R)BSC25 mutant background. (D-F) Ectopic expression of pyr (D) or ths (E) in the ectoderm using 69B-Gal4 does support expression of Eve+ in a homozygous Df(2R)BSC25 mutant background, whereas bnl (F) does not. (G-I) Ectopic expression of pyr (G) or ths (H) in the ectoderm using zenVRE.Kr-Gal4 also supports expression of Eve+ in a homozygous Df(2R)BSC25 mutant background, whereas again bnl (I) does not.

Specificity of FGFR activation: only three FGF-FGFR combinations function in Drosophila

Our results demonstrate that both Pyr and Ths FGF ligands can activate the Htl FGFR, whereas only the Bnl FGF ligand can activate the Btl FGFR (Fig. 6 and see Fig. S2 in the supplementary material). Specificity of FGFR activation was observed: pyr or ths, but not bnl, expression is able to activate Htl to affect expression of Eve, and bnl, but neither pyr nor ths, is able to support tracheal specification. No evidence was obtained that other cross-interactions occur (i.e. Pyr-Btl, Ths-Btl or Bnl-Htl), which demonstrates that Gal4-mediated ectopic expression does not simply ‘swamp the system’. This experimental approach also ‘levels the playing field’, as expression of each ligand is driven at the same time and place and presumably at similar levels. We conclude that only three FGF-FGFR combinations function in Drosophila (i.e. Pyr-Htl, Ths-Htl and Bnl-Btl), which supports the idea that FGFRs exhibit ligand-binding preferences. Previous studies have investigated FGF signaling specificity by analyzing the ability of other receptor tyrosine kinases to support cell migration or by activating particular intracellular signaling pathways to examine which are required to effect FGFR-dependent cell migration versus cell differentiation (Dossehbach et al., 2001; Wilson et al., 2005). In this work, we analyzed the specificity of FGF ligand-receptor interactions and how they contribute to particular developmental processes.

Different FGF ligand activities regulate particular developmental processes

When ligand expression is supported by twist-Gal4, Htl FGFRs presumably become saturated because dpERK is ectopically activated in all cells and spreading is negatively affected (see Fig. S1 in the supplementary material). One explanation for why this might affect mesoderm cell spreading is that these FGF-saturated...
Ths is sufficient to support Eve+ cell specification (Fig. 6D,E). The ‘rescue’ experiments suggest that ectopic expression of either Pyr or Ths; we suggest that in vivo imaging and quantitative analysis (McMahon et al., 2008) of single-mutant phenotypes will provide insights.

Additional ligands activate the receptor with different affinities. Additional diffusion capabilities, but an alternative explanation is that the ligands differ in their range of diffusion, or the proteins themselves may have different stabilities. Future experiments will also define the FGF ligand preferences that exist to support FGF signaling at later stages of development.

Fig. 7. A model for FGF signaling through Heartless. (A) The location of the Ths and Pyr expression domains is important for the proper regulation of mesoderm migration. Both ligands are required, presumably because they have different activities. (B) During specification of dorsal mesoderm lineages, including specification of Eve+ cells (orange), FGFs feed into an array of signaling molecules [Wingless (Wg) and Dpp] necessary to specify dorsal mesoderm lineages. We suggest that any FGF ligand (red), expressed in the region of Dpp and Wg overlap and able to activate the respective FGFRR, would suffice to support cell differentiation. Wg, Wingless.

We propose that Pyr and Ths have different activities that fulfill aspects of FGF activation required to support cell migration (Fig. 7A). Ectopic expression of Pyr within the ectoderm negatively affects mesoderm spreading (Fig. 1H-M), which suggests that the refined expression domain of pyr within the cells of the dorsal ectoderm is normally required to guide the mesoderm cells toward dorsal regions. However, even though ectopic expression of ths in the ectoderm has no effect on mesoderm spreading, ths mutants also exhibit defects in mesoderm spreading, demonstrating that both genes are required, perhaps to control different aspects of the migration. Our ‘rescue’ experiments using the zenVRE.Kr-Gal4 driver support the view that Pyr has a longer functional range than Ths (Fig. 4N,O). These differences in range of function might correlate with different diffusion capabilities, but an alternative explanation is that the ligands activate the receptor with different affinities. Additional experiments will be necessary to distinguish their exact functions and to uncover the molecular basis for the differential functions of Pyr and Ths; we suggest that in vivo imaging and quantitative analysis (McMahon et al., 2008) of single-mutant phenotypes will provide insights.

With regard to the FGF-dependent cell differentiation, our ‘rescue’ experiments suggest that ectopic expression of either Pyr or Ths is sufficient to support Eve+ cell specification (Fig. 6D,E). The reason why loss of ths has less of an effect on Eve+ cell specification is most likely because pyr is prominently expressed in the vicinity of the future Eve+ cells; normally, Pyr supports this function, but Ths can support this activity if presented at sufficient levels within the correct domain. Furthermore, we propose that FGF signaling might not play an instructive role in supporting eve expression (Fig. 7B). Other signaling pathways already provide positional information required for the specification of Eve+ cells; FGF signaling pathway activation might simply serve a permissive role, and in this context either ligand would suffice.

Conclusion: implications for vertebrate biology? We have used Drosophila to study FGF signaling and determine why multiple ligands are utilized to activate the same receptor. It will be informative to obtain additional insights into how these ligands differ in their activities. The expression domains of the ligands do confer information that is important for controlling developmental processes, but their individual protein sequences also impart differential functionality. For instance, the ligands might exhibit different affinities for specific HSPGs that influence their range of diffusion, or the proteins themselves may have different stabilities. Future experiments will also define the FGF ligand preferences that exist to support FGF signaling at later stages of development.

At least 15 human genetic diseases result from mutations within FGFR genes and each disease is caused by a different mutation shown to affect receptor activation (Chen and Deng, 2005). Several mutations in FGFR lead to an expansion of FGF ligand-binding preference (Ornitz, 2005); however, it is still not clear why different mutations yield different syndromes. Continuing this work in order to understand how different FGF ligands activate the same receptor to effect different outcomes is an important goal, as this may provide insights into why different mutations in the same FGFR lead to various dysplasias and diseases (Wilkie, 2005).

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Supplementary material
Supplementary material for this article is available at http://dev.biologists.org/cgi/content/full/136/5/739/DC1

References
Drosophila FGFs have distinct activities

RESEARCH ARTICLE


