Ectopic expression screen identifies genes affecting *Drosophila* mesoderm development

including the HSPG Trol

Nathanie Trisnadi¹ and Angelike Stathopoulos¹,*

¹ Division of Biology and Biological Engineering, California Institute of Technology, 1200 East California Boulevard, MC 114-96, Pasadena, CA 91125, USA

*Corresponding author: angelike@caltech.edu; phone: 001-626-395-5855
ABSTRACT

Gastrulation of the embryo involves coordinate cell movements likely supported by multiple signaling pathways, adhesion molecules, and extracellular matrix components. Fibroblast growth factors (FGFs) have a major role in Drosophila melanogaster mesoderm migration, however few other inputs are known and the mechanism supporting cell movement is unclear. To provide insight, we carried out an ectopic expression screen to identify secreted or membrane-associated molecules that act to support mesoderm migration. Twenty-four UAS insertions were identified that cause lethality when expressed in either the mesoderm (Twi-Gal4) or ectoderm (69B-Gal4). The list was narrowed to a subset of ten genes that were shown to exhibit loss-of-function mutant phenotypes specifically affecting mesoderm migration. These include the FGF ligand Pyramus, α-integrins, E-cadherin, Cueball, EGFR, JAK/STAT signaling components, as well as the heparan sulfate proteoglycan (HSPG) Terribly reduced optic lobes (Trol). Trol encodes the ortholog of mammalian HSPG Perlecan, a demonstrated FGF signaling cofactor. Here we examine the role of Trol in Drosophila mesoderm migration and compare and contrast its role with that of Syndecan (Sdc), another HSPG, previously implicated in this process. Embryos mutant for Trol or Sdc were obtained and analyzed. Our data support the view that both HSPGs function to support FGF-dependent processes in the early embryo as they share phenotypes with FGF mutants: Trol in terms of effects on mesoderm migration and caudal visceral mesoderm (CVM) migration, and Sdc in terms of dorsal mesoderm specification. The differential roles uncovered for these two HSPGs suggest that HSPG cofactor choice may modify FGF-signaling outputs.
INTRODUCTION

Embryonic development requires integration of multiple complex processes such as cell movement, proliferation, and differentiation, all of which are regulated by signaling pathways. Therefore, to ensure proper execution of the first movements during embryonic development that encompass the process of gastrulation, for instance, it is important that signaling pathway activation is tightly regulated (SOLNICA-KREZEL and SEPICH 2012). In Drosophila, the embryo undergoes extensive cell movements during gastrulation that support its lengthening through the process of germ-band elongation, as well as the establishment of a multilayered state through invagination of the mesoderm in ventral regions and its subsequent migration, internally, along the inner side of the ectoderm.

Fibroblast growth factor (FGF) signaling is important in supporting mesoderm migration during gastrulation of the Drosophila embryo. The Drosophila FGFs Pyramus (Pyr) and Thisbe (Ths) and their receptor Heartless (Htl) have been previously shown to function in supporting this process (rev. in WINKLBAUER and MULLER 2011; BAE et al. 2012). FGF signaling regulates the collective migration of the mesoderm as in mutants two populations of cells can be defined: cells in contact with the ectoderm move in a uniformly directional manner, whereas those located at a distance move aberrantly without apparent direction. FGF’s roles in this process include guiding symmetrical collapse of the invaginated tube of mesoderm cells as well as supporting formation of a monolayer of cells at the end of the migration process. Both these movements guide cells in the radial direction, and similar phenotypes (at least in part) were identified for the Rap1 GTPase and β-PS integrin, Myospheroid (Mys) (MCMAHON et al. 2008; MCMAHON et al. 2010). Rap1 mutants exhibit collapse defects, whereas in both Rap1 and Mys mutants cells fail to intercalate and do not form a monolayer. Since a subset of mesoderm cells is able to spread
dorsally in these mutants (MCMAHON et al. 2008), other inputs besides FGF, Rap1, and Mys are also likely important for guiding directional movement of mesoderm cells during gastrulation.

Specifically, we hypothesized that additional signaling pathways and/or regulators of cell adhesion may act to support mesoderm migration at gastrulation. To investigate how cells were able to migrate, nonetheless, in the absence of FGF signaling and also to discover additional components in the FGF pathway, we conducted a screen of a collection of UAS insertions located near cell-surface or secreted (CSS) proteins, first used in a neuronal pathfinding screen (KURUSU et al. 2008). The UAS/GAL4 system was used to ectopically express candidate genes in either the presumptive mesoderm or ectodermal tissues. We postulated that important signals guiding this process normally would be differentially expressed in tissues in the embryo, either in the mesoderm or ectoderm, in order to provide positional information to guide mesoderm cell movements. In this way, using this CSS collection, we identified 24 genes, of 311 tested, that impact Drosophila development when ectopically-expressed; ten of which were subsequently shown to specifically affect Drosophila gastrulation when mutated. We focused analysis on one gene isolated in this screen encoding a heparan sulfate proteoglycan (HSPG), Terribly reduced optic lobes (Trol), due to previous research linking HSPGs to FGF signaling. Crystal structures have revealed that HSPGs bind to the FGF ligand and receptor as a heterotrimeric complex (i.e., FGF-HSPG-FGFR) (PELLEGRINI et al. 2000). It has been proposed that HSPGs facilitate ligand-receptor interaction and/or stabilize the FGF-FGFR dimer complex (ORNITZ 2000).

HSPGs are comprised of a core protein attached with highly modified heparan sulfate glycosaminoglycan side chains that provide specificity to regulate multiple signaling pathways during development (LIN 2004). There are only four known core proteins in Drosophila: transmembrane Syndecan (Sdc), two membrane-anchored glypicans Dally and Dally-like (Dlp),
and the extracellular matrix protein Trol. Trol is the homolog of mammalian Perlecan (Pcan), and several lines of evidence support the view that Pcan promotes multiple pathways including FGF signaling in vertebrates (Farach-Carson et al. 2014). For instance, in vitro experiments measured a gradient of FGF-2 and correlated its levels with Pcan and pERK, a signal measuring activation of the Ras intracellular signaling pathway downstream of FGFR activation (Wu et al. 2014). Studies in the developing mouse heart show specific Pcan modifications (i.e. sulfations) are required for binding of different FGF-FGFR complexes (Allen and Rapraeger 2003). In Drosophila, studies of Trol in the larval lymph gland have suggested that this HSPG sequesters FGF ligands to downregulate FGF signaling within this tissue (Dragojlovic-Munther and Martinez-Agosto 2013). However, trol mutant phenotypes in the Drosophila early embryo had not previously been investigated. HSPG Sdc function was studied in late embryogenesis to examine its role in supporting cardiogenesis, and it was noted that mutants exhibit mesoderm spreading defects earlier (Knox et al. 2011). Here, we compare and contrast the roles of Trol and Sdc during several FGF-dependent processes in early development of the Drosophila embryo.

**METHODS & MATERIALS**

*Fly strains and genetic crosses*

\[P\{GAL4-twi.G\}, \text{ w}^1 \text{ (BDSC \#914)} \text{ and w}; \ P\{GawB\}69B \text{ (#1774)} \text{ lines were used in experiments analyzing mesoderm spreading. For screening, females from “virginator" y}^1\text{ w/Dp(2;Y)G, }P\{hs-hid\}Y \text{ (#8846)} \text{ versions of these Gal4 stocks were crossed with males from the UAS insertion collection. Wildtype refers to yw or Gal4 lines. Mutant strains were crossed with balancers containing a lacZ marker to identify homozygous embryos: Sp}^1/CyO, P\{wingless-lacZ\} \text{ (Kadam et al. 2009) or Dr}^{Mio/TM3}, P\{ftz-lacZ\} \text{ (#3218).} \]
For germline clones, trof\textsuperscript{G011}, FRT.19A/FM7 were crossed with P\textit{ovoD1-18}\texttildelow P4.1, P\textit{hsFLP}\text{12}, y\textsuperscript{1} w\textsuperscript{1118} sn\textsuperscript{3} P\textit{neoFRT}\textsuperscript{19A/C(1)DX}, y\textsuperscript{1} w\textsuperscript{1} f\textsuperscript{1} (\#23880) and allowed to lay for approximately 12 hours at 25°C. A 2-hour heat shock at 37°C was performed on days 2, 3, and 4. Non-Bar females were then crossed with y\textsuperscript{1} arm\textsuperscript{d} w/FM7c, P\{ftz/lacC\}YH1 males (\#616) and collected embryos were analyzed. The zygotic lethality exhibited by trof\textsuperscript{G011} can be rescued by a Trol duplication on the Y chromosome (\#4284; data not shown). A similar protocol was used with sdc\textsuperscript{2639}, FRT42B/CyO (M. Freeman, UMass Medical School; STORK \textit{et al.} 2014) and \textit{hsFLP/Y; ovoD 42B/CyO} (#1929 x #4434) to generate sdc germline clones (maternal loss-of-function), but then crossed to males of the same genotype (i.e. sdc\textsuperscript{2639}, FRT42B/CyO) to generate embryos (~half) devoid of zygotic sdc (CHOU and PERRIMON 1996).

The 5053-Gal4 driver \textit{w; P\{GawB\}tey5053A/TM6B, Tb+} (\#2702) was used for ectopic expression in the CVM cells (REIM \textit{et al.} 2012). bHLH-gap-Venus (Y-K. Bae et al., in review) is a transgene used as a reporter to detect CVM cells with a GFP antibody; the same enhancer has been shown previously to support expression within CVM cells (KADAM \textit{et al.} 2012). Additional stocks, including the lines from the CSS collection (KURUSU \textit{et al.} 2008), are listed in Table S1.

UAS insertions for all genes were confirmed. Sim-Gal4 (S. Crews, UNC School of Medicine, USA; XIAO \textit{et al.} 1996) or ZenKr-Gal4 (M. Frasch, Univ. of Erlangen, Germany; FRASCH 1995), which support ectopic expression at the embryonic midline or trunk region respectively, were used to drive expression from the insertions and in situ hybridization experiments confirmed ectopic expression of genes (data not shown).
In situ hybridization and immunohistochemistry

Embryos were collected and aged at 25°C or 18°C to obtain embryos of stages of interest, and standard protocols for fixing and staining were used. Antisense RNA probes were made to detect in vivo gene expression using in situ hybridization. For antibody stainings, primary antibodies used were the following: rat anti-Twist (1:200; this study), rabbit anti-Eve (1:100; M. Frasch, Univ. of Erlangen, Germany), mouse anti-dpERK (1:150; Sigma), rabbit anti-β-galactosidase (1:200; Molecular Probes), mouse anti-αPS2 (1:10; Developmental Studies Hybridoma Bank), and rabbit anti-GFP (1:2000; Life Technologies).

Sample preparations and imaging

For cross-sections, stained embryos were embedded in araldite (Electron Microscopy Sciences). 10 or 20 µm slices were sectioned using the LKB Bromma 2218 Historange Microtome and mounted in 1:1 araldite:acetone solution. For cuticle preparations, 24-hour old embryos were dechorionated in bleach, devitilllinized in 1:1 MeOH:heptane, and mounted in lactic acid. Slides were incubated at 55°C overnight. All images were collected using a Zeiss Axioplan microscope.

RESULTS

Ectopic expression screen identifies genes in multiple pathways affecting mesoderm development

Presumptive mesoderm cells are initially specified prior to gastrulation in ventral regions of the embryo (REEVES and STATHOPOULOS 2009; SOLNICA-KREZEL and SEPICH 2012). These ventral cells undergo shape changes that cause a furrow to form comprising the presumptive mesodermal domain. Apical constriction of cells drives their invagination during which time a
tube is formed on the inside of the embryo. Cells undergo epithelial-mesenchymal transition (EMT) and, subsequently, the invaginated tube collapses upon the inner surface of ectodermal cells. These presumptive mesoderm cells then migrate in the dorsal direction, and at the end of the process undergo small movements (intercalations) toward the ectoderm to establish a single layer of mesoderm cells on the inside of the embryo (Figure 1A).

In order to elucidate potential signaling pathways and adhesion molecules that influence mesoderm migration, we conducted a screen of a library comprised of 311 insertions at the presumed 5’ end of genes encoding cell surface or secreted (CSS) factors (Figure 1B). These lines were previously selected to help with identification of extracellular-acting signaling molecules and used in a screen of neuronal targeting (KURUSU et al. 2008). Using these fly stocks in the current study, we aimed to identify novel regulators of mesoderm spreading during gastrulation. To this end, genes were overexpressed using Gal4 drivers that support expression in the mesoderm (Twi-Gal4; KUSCH and REUTER 1999) or ectoderm substratum (68B-Gal4; BRAND and PERRIMON 1993) (Figure 1B). Twenty-four insertions were identified that caused lethality upon ectopic expression in the mesoderm and/or ectoderm (see Table 1).

Next, we screened these candidates in order to determine if lethality was caused by defects in mesoderm migration. Lethality could also relate, instead, to a dominant negative effect where ectopic expression of genes, even if not normally acting to affect mesoderm migration, may compete with normal processes. Therefore, candidate genes were selected that were expressed within embryonic domains that could impact mesoderm migration; meaning (i) genes were expressed within the period of stage 5-10 that encompasses invagination of the mesoderm through monolayer formation; and (ii) genes were expressed within the migrating mesoderm and/or in proximity to the mesoderm within the ectoderm. We then examined embryo cross-
sections for spreading defects in loss-of-function mutant backgrounds for this set of genes (Figure 1B). Single null mutants were examined if available and, if not, deficiency chromosomes deleting the gene in question (along with many others) were assayed. We reasoned that genes normally acting to support the mesoderm spreading processes would exhibit mutant phenotypes.

These phenotypes were classed into three different levels of severity (Figure 2A-D). Mild indicates only a few cells did not intercalate creating an uneven layer. Mesoderm defects of moderate phenotype present multilayered cells, which nevertheless evenly spread upon the ectoderm. Severe phenotypes include both uneven spreading of cells upon the ectoderm (i.e. not centered at the midline) as well as multilayered/clumping of cells, as in the case with htl mutants, which exhibit defects in mesoderm collapse, spreading, and intercalation (McMAHON et al. 2008). Also, we have observed that htl phenotypes are variable ranging from mild to severe (Table 2, see htl). To quantify phenotypes that could be variable, at least 7 and as many as 23 embryos were examined for mutants assayed. Furthermore, a score was calculated based on frequency of phenotypes observed (see Table 2). In a recent study, cadherin mutants were found to exhibit non-monolayer phenotypes, but a role for these molecules in supporting mesoderm formation was dismissed since germ layers were specified (SCHAFER et al. 2014). Since the goal of our screen was to uncover signals guiding proper mesoderm migration, lack of a monolayer is relevant and indicates defects in effective mesoderm migration. For this reason, we considered mutant phenotypes that span the range of mild to severe.

Screening in this manner identified ten genes of interest that include the FGF ligand Pyramus (Table 1, footnote ‘a’). These ten genes had both relevant expression patterns (i.e. endogenous mesoderm and/or ectoderm expression) and mutant phenotypes relating to mesoderm migration. Spreading defects for these ten genes as well as a number of controls,
genes previously implicated in mesoderm migration (i.e. \textit{htl^{AB42}}, \textit{pyr^{02915}/BSC25}, and \textit{pyr^{18}/BSC25}), were scored and quantified into the different levels of severity: normal, mild, moderate, or severe (see Figure 2A-D, Table 2).

\textit{Classes of signaling components and adhesion molecules known to be regulators of mesoderm migration during gastrulation were identified}

Genes encoding one FGF ligand, two integrins, and one cadherin were identified by the screen; these genes were expected and support previous roles in facilitating mesoderm migration during gastrulation (MCMAHON \textit{et al.} 2010; CLARK \textit{et al.} 2011). An insertion upstream of the FGF ligand Pyr (GS22603) resulted in embryonic lethality upon ectopic expression with the 69B-Gal4 driver (data not shown). A previous study characterized the role of the FGF ligand Pyr in supporting monolayer formation (KADAM \textit{et al.} 2009).

In addition, prior studies also identified a role for the $\beta$-PS integrin Mys in this process, demonstrating that it is required solely for monolayer formation at the end of the process following spreading of cells on the ectoderm (MCMAHON \textit{et al.} 2010). In the current screen, two alpha integrins, $\alpha$-PS3 (Scab; EP2591) and $\alpha$-PS5 (GS12413), were identified, which may act with the $\beta$-PS integrin Mys. Integrins function in tetramers with the binding of two $\alpha$- and two $\beta$-integrins (BULGAKOVA \textit{et al.} 2012). Ectopic expression of $\alpha$-PS3, using the mesoderm driver, or $\alpha$PS5, using the ectoderm driver, was lethal; whereas expression of each in the alternate tissue was not (Table 1). Both genes, $\alpha$-PS3 and $\alpha$-PS5, are expressed in the mesoderm, and single mutants affecting each of these integrins show mild spreading defects (Figure 2E,F,I,J) supporting the view that both act to support mesoderm spreading. The tissue-specific effects observed for ectopic expression suggest that the balance of integrin subunits is important. For
instance, it is possible that multiple integrins, including Mys, α-PS3, and α-PS5 as well as others, act collectively or redundantly to support mesoderm migration during gastrulation through effects on regulation of adhesion and/or signaling state. *Drosophila* contains three additional alpha-integrins, all of which are present during early mesoderm development (Supporting Information, Figure S1A-C).

Lastly, E-cadherin (Ecad, *Drosophila* Shotgun) was isolated. Cadherins play pivotal roles in controlling adhesion and epithelial-to-mesenchymal transition (EMT) (ODA and TSUKITA 1999). Ecad is expressed in the ectoderm at gastrulation when mesoderm migration occurs (ODA *et al.* 1998), and mutants exhibit a severe mesodermal phenotype (Figure 2G,K and Table 2).

*Newly identified regulators of mesoderm migration include signaling components, adhesion molecules, and modifying enzymes*

Because these genes had already been implicated in controlling cell movements during gastrulation, we focused our analyses on other genes that might provide novel insights into this process.

Only two genes induced embryonic lethality when overexpressed in either the mesoderm or ectoderm. Both of these genes encode secreted factors and ligands influencing signaling pathways: Unpaired (Upd; G17133, Figure S1D-E) regulates the JAK/STAT pathway, whereas Vein (Vn; GS12044) regulates EGFR signaling. While previous studies have focused on *upd* during heart diversification (JOHNSON *et al.* 2011), a role in the early mesoderm at gastrulation had not been identified. Upd is expressed in ectodermal stripes and mutant embryos result in a moderate multilayer phenotype (Figure 2M,Q). Modulation of other JAK/STAT signaling components had mild to moderate effects on mesoderm migration (Figure S1F-H). However,
while the receptor Domeless (Dome) is expressed in the mesoderm, dome mutants do not result in any spreading defects (Figure S1I-L). It is possible that Dome and EGFR signaling is required later in the mesoderm after migration is complete.

The second secreted molecule that resulted in embryonic lethality when ectopically expressed in either the mesoderm or ectoderm was Vein, an epidermal growth factor receptor (EGFR) ligand (Figure S1M,N). Normally, vn is expressed in the ectoderm, and vn mutants have a moderate mesoderm phenotype (Figure 2N,R). Another EGF pathway component, Argos (Aos), was also identified in the screen. Aos is expressed in the mesoderm and the deficiency lacking aos also presented a moderate mesoderm spreading phenotype (Figure 2O,S). Since two components of the EGFR pathway were identified in the screen, we also examined the phenotype associated with the receptor itself (SHILO 2005). EGFR is upregulated in the mesoderm when spreading is complete, and expressing its dominant negative form in the mesoderm resulted in a mild phenotype (Figure 2P,T). However, egfr mutants and ectopic expression of a EGFR dominant negative in the ectoderm had little to no effect on the mesoderm even though EGFR is present in the ectoderm at earlier stages (Figure S2O-T).

It is possible that the JAK/STAT and EGFR signaling pathways are active in the mesoderm during migration. Future studies may distinguish direct from indirect roles; for instance, these pathways may regulate gene expression and/or protein distributions of other genes within the ectoderm required to instruct mesoderm migration.

We identified an insertion (EY1263) near the cueball (cue) gene, which encodes a membrane-bound protein that is EGF-like and contains LDLR repeats. It is expressed in the mesoderm, and embryos lacking cue exhibit a mild phenotype (Figure 2H,L). It is possible that
Cue supports localization of membrane proteins as previous studies suggest it impacts vesicle trafficking (Hirst and Carmichael 2011).

Our screen also isolated additional genes that were either previously uncharacterized and/or had unknown functions (Table 1 and Figure S2). Two are predicted enzymes, a sulfotransferase CG9550 (GS18034), and a galactosyltransferase CG34056 (GS11028). Analyses of these two genes show weak mesoderm expression and spreading defects when analyzed in the context of deficiency chromosomes (Figure S2A,B). However, more than twenty genes were uncovered by these large deletions; therefore, it is unclear whether these phenotypes directly relate to the genes in question. However, expression of RNAi targeting these genes and/or ectopic expression results in moderate defects providing additional support for a role for these genes in supporting mesoderm migration (Figure S1U-Y). These enzymes could potentially function in the synthesis and/or modification of proteoglycans, which were also found in the screen (see below). In addition, two genes from the Toll family of receptors, which can spatially influence heterophilic cell-cell interactions (Pare et al. 2014), were also identified. However, these genes and the others identified require further characterization to identify whether they impact mesoderm spreading directly (see Table 1 and Figure S2).

Comparing proteoglycans in the Drosophila embryo

Proteoglycans have a variety of activities that directly and indirectly modulate signaling, including the FGF pathway; however, their role in mesoderm migration has not been fully investigated. The Drosophila genome contains four HSPGs: Trol, Sdc, Dally, and Dlp (Lin 2004). Trol was identified in our screen, and Sdc was previously reported to play a role in mesoderm development in the embryo (Knox et al. 2011). In addition, there are two predicted
chondroitin sulfate proteoglycans (CSPGs) based on sequence homology: PTP99A and Kon-tiki (Kon) (Song et al. 2012). Our screen also isolated Ptp99A and, although it is unclear if Ptp99a is a true CSPG (see Discussion), we proceeded to investigate both these families of proteoglycans more closely for their embryonic expression. All genes, except kon, are maternally deposited and are expressed during mesoderm migration (Figure 3). In addition, Trol, and Kon are expressed in what appears to be the caudal visceral mesoderm (CVM), another group of FGF-dependent migrating cells that undergo migration at later stages following gastrulation (Kadam et al. 2012).

The trol locus spans ~75 kB and includes as many as 58 exons encoding 22 unique polypeptides (Figure 4A). Ectopic-expression of UAS-TrolGE10067 or trol RNAi (Grigorian et al. 2013) constructs in either the ectoderm or mesoderm results in mild to moderate spreading defects (Figure 4B-E). Germline clones devoid of both maternal and zygotic (m-z-) trol transcripts exhibit mesoderm tube collapse defects (compare Figure 1A with 4F,G) that result in a severe multilayer mesoderm phenotype (Figure 4H). Furthermore, we show that maternal Trol contribution is sufficient to rescue the collapse defect and partially rescues the spreading phenotype to mild (Figure 4I). These results suggest that maternal Trol contribution supports early mesoderm migration, namely tube collapse, whereas zygotic Trol influences monolayer formation (and likely additional subsequent functions). It is possible that localized expression and/or increased levels of Trol, supported by zygotic transcription is necessary to support proper monolayer formation (see Discussion).

Importantly, the phenotype in trol germline clones is similar to that found in embryos lacking FGF signaling (McMahon et al. 2008). We therefore investigated whether FGFR receptor activation was possible in the absence of Trol by assaying for dpERK expression in the mesoderm. dpERK is a measure of RTK intracellular signaling activation. At the end of
gastrulation, dpERK staining is present within a subset of mesoderm cells that have migrated to the dorsal-most position (Figure 4J, J' arrow) as well as in patches within the ectoderm; mesodermal and ectodermal dpERK staining has been demonstrated to relate to FGFR versus EGFR RTK-activation, respectively (GABAY et al. 1997b). We found that dpERK is absent from the mesoderm in embryos from trol germline clones (Figure 4K). Furthermore, when trol is overexpressed in the ectoderm or mesoderm, dpERK is expanded or ectopically expressed, respectively (Figure 4L,M). Trol may also support other signaling pathways as embryos lacking trol had an overall reduction of EGFR-dependent dpERK in ectodermal cells (Figure 4K). In addition, trol germline clones exhibit a “tail-up” phenotype suggesting an additional role related to TGF-β signaling possibly at a later stage (Figure 4N,O, see Discussion) (FERGUSON and ANDERSON 1992).

Trol and Sdc have different roles in embryonic development

Because both Trol (Figure 4) and Sdc (KNOX et al. 2011) mutants exhibit phenotypes that affect the mesoderm of early embryos, we investigated their expression patterns during early mesoderm development to provide more specific insights into their functions. Both genes are maternally-expressed and present ubiquitously at low levels; however, at two stages, localized expression was detected. Once the furrow is formed, trol is upregulated in the ventral-most cells where the mesoderm will collapse onto the ectoderm; in contrast, Sdc at this stage remains ubiquitously diffuse (compare Figure 5A,C). Conversely, sdc becomes localized to the ectoderm later when the mesoderm intercalates to form a single layer of cells (Figure 5D arrow); in contrast, trol at this later stage is no longer spatially upregulated and instead is present
everywhere at low levels (Figure 5B). The dynamics of sdc expression suggest that Sdc, like zygotic Trol, may be required only for monolayer formation at later stages.

In accordance with the sdc expression pattern, sdc^{2639} germline clones (STORK et al. 2014) exhibit normal collapse during early mesoderm migration (compare Figure 1A with 5E). However, at later stages, these embryos have mild spreading defects often seen when cells are unable to intercalate to form a monolayer (Figure 5F; KNOX et al. 2011). Nevertheless, FGF-dependent dpERK staining within the mesoderm is present in sdc germline clones (Figure 5J). Ectopic expression of sdc in the mesoderm results in a moderate phenotype and leads to dpERK presence throughout the mesoderm (Figure 5G,K). In contrast, increasing sdc in the ectoderm where it is already expressed has little to no effect on mesoderm spreading or on dpERK activation (Figure 5H,L). Unlike with trol, EGFR-dependent dpERK expression in the ectoderm does not appear to change in either sdc germline clones or overexpression of Sdc (Figure 5J-L). However, sdc germline clones do have severe cuticle phenotypes similar to trol mutants (Figure 4J,M), indicative of TGF-β signaling defects.

Furthermore, ectopic expression of other HSPGs Dally and Dally-like display mild or no mesoderm spreading defects (Figure S3A-H). While overexpression of Ptp99a in the mesoderm resulted in a moderate spreading phenotype, removing ptp99a in the embryo had little effect (Figure S3I-K). Kon was not examined since this gene is not expressed until later embryonic stages, and thus does not regulate mesoderm migration (Figure 3F). Therefore, the roles of Trol and Sdc in supporting mesoderm migration are specific and not shared by other HSPGs. In addition, both FGF and EGFR signaling, as assayed by dpERK activation, appear to be affected by Trol and Sdc in different ways.
Pericardial and dorsal somatic muscle cells derived from the dorsal mesoderm are known to express Even-skipped (Eve) (Frasch 1999) and require proper migration of the mesoderm at an earlier stage prior to their specification. Once mesoderm cells migrate to dorsal regions of the ectoderm (Figure 2A arrowhead; Figure 6A arrow), they are induced by signals originating from the ectoderm to express Eve within ten clusters, of three cells each, spanning the trunk of the embryo (Figure 6B,C). These differentiation cues include FGF, Wg, and Dpp – all of which have the ability to cooperate with HSPGs in the context of receptor activation (Lin 2004). In both trol and sdc mutant embryos, mesoderm cells reach the dorsal ectoderm as a result of their migration despite their non-monolayered spreading (Figures 4H and 5F). We examined if Trol is required for the subsequent patterning of dorsal somatic lineages, but no measurable defect in Eve-specification was observed in embryos derived from trol germline clones (Figure 6D). Previous studies, however, have shown that sdc zygotic mutants, in contrast, do exhibit defects in Eve-induction and linked this to effects on FGF signaling through genetic interaction (Knox et al. 2011). Likewise, we found that in embryos obtained from sdc germline clone a significant reduction of Eve+ cells was observed (Figure 6F). These results reinforce the view that Sdc is required to support the FGF’s role in differentiation of dorsal somatic mesoderm lineages. Overexpression of either Trol or Sdc in the ectoderm results in increased Eve+ cell number. However, overexpressing Sdc had a stronger phenotype than Trol with multiple clusters containing 5+ cells (compare Figure 6E with G). Though Trol is not required to support differentiation of dorsal somatic lineages, it can potentially substitute for Sdc when ectopically expressed (compare Figure 6D with E).
FGF signaling is also known to function during development of longitudinal muscle fibers (Kadam et al. 2012). Of the proteoglycans examined by expression analysis, we found Trol and Kon are present in a migrating population of cells originating from the CVM (Figure 3A,F). At stages 11-13, the CVM forms two clusters of cells that migrate on the trunk visceral mesoderm (TVM) substratum. Similar to the arrangement in mesoderm migration, migrating CVM cells express the FGFR Htl, while the TVM substratum expresses the FGF ligands Pyr and Ths (Figure 7A) (Kadam et al. 2012). Both trol germline clones and trol RNAi in the CVM cells resulted in a migration defect in which cells from each of the two migrating collectives merge at the midline (compare Figure 7B with C,D arrows), similar to the phenotype caused by removing FGF signaling (Kadam et al. 2012). These trol mutants, along with kon RNAi, also exhibited increased apoptosis of CVM cells as indicated by the punctate spots at the posterior of the embryo (compare Figure 7G with H,I,K arrows). Whether this is due to a role for Trol in supporting cell survival and/or mis-migration is unclear as either could account for the phenotype. Lastly, introducing sdc RNAi into CVM cells had no effect (Figure 7E,J), further supporting the view that Trol and Kon, but not Sdc, are required in the migrating CVM.

**DISCUSSION**

Previously, a limited number of extracellular effectors were shown to be important for mesoderm migration during gastrulation, including the FGF receptor Htl, its two FGF ligands (Pyr and Ths), and the β-PS integrin Mys (Baē et al. 2012). In our screen of cell surface and secreted proteins, we identified 10 additional effectors, based on mutant phenotype, as well as highlight 14 other genes that may also play a role in supporting mesoderm migration. Some results were expected and others provide novel insight into this process. Several α-integrin genes
were isolated, some or all of which may bind to known player β-integrin Mys to form tetramers. This result suggests that cell adhesion has a role in mesoderm development. Our screen also detected E-cadherin, which regulates adhesion between cells. While other studies have suggested E-cadherin is necessary for EMT at the onset of mesoderm migration (ODA and TSUKITA 1999) or for differentiation of dorsal somatic lineages rather than for supporting the subsequent process of mesoderm migration (SCHAFER et al. 2014), our results suggest E-cadherin does impacts the mesoderm spreading process as mutants display a moderate to severe phenotype that is similar to FGF mutants. Recent studies have also shown that cadherins may influence the cell’s ability to support cell signaling through modification of adhesion states (CAI et al. 2014). Therefore E-cadherin may affect mesoderm migration through modulation of FGF signaling and/or impairing the tissue’s mobility due to levels of adhesiveness.

Adhesion may also be impacted by CSPGs (PEREZ-MORENO et al. 2014). Our screen identified PTP99A, which is predicted to be a CSPG; the only other in Drosophila being Kon (SONG et al. 2012). These CSPGs may regulate adhesion, like integrins, and/or FGF ligand-receptor interactions, like HSPGs. While Kon is an ortholog of mammalian CSPG4 (PEREZ-MORENO et al. 2014) and shows defects in CVM migration (Figure 7K), Ptp99a shares sequence homology with the CSPG Phosphocan only across their cytoplasmic phosphatase regions. Ptp99a does not contain homology to the extracellular domain of Phosphocan, which comprises the CSPG. Nevertheless, overexpression of Ptp99a resulted in a moderate mesoderm phenotype (Figure S3K); whether this relates to CSPG activity is thus unclear but possible. In addition, identification of Cue through the screen is suggestive of the importance of trafficking of signaling components and/or adhesion molecules towards regulation of mesoderm development.
The signaling pathways JAK/STAT and EGFR may also function in parallel with FGF to guide the spreading process.

Fourteen additional genes were identified (Table 1 and Figure S2). While their weak endogenous expression and/or mild to no mutant spreading phenotype led us to conduct only a preliminary characterization, several genes are of note. Our screen isolated Toll-8, a receptor that has been reported to provide spatially-localized heterophilic associations within the ectoderm necessary for supporting germband elongation (Pare et al. 2014). We also identified Toll-9, which is expressed in the mesoderm, and thus we hypothesize this Toll receptor may support a similar role in mesoderm development. Two enzymes were also uncovered, CG9550 and CG34056, which have the potential to function in the biosynthesis of heparan sulfate (HS) side chains found on HSPGs. Other enzymes of this class were previously found to impact mesoderm migration as they genetically interacted with FGFR Htl in this process (Lin et al. 1999). To address how HSPGs impact FGF signaling, in this study, we decided to characterize the role of proteoglycans in supporting mesoderm migration as only limited information was available previously.

**Trol requirement in multiple pathways in Drosophila**

Several studies have linked Trol with FGF signaling as well as other signaling pathways. While we highlight the role of Trol and Sdc in FGF signaling, our data also suggest that these HSPGs can modulate EGFR signaling as indicated by the decrease of dpERK in the tracheal pits of the ectoderm in mutant embryos (Figure 4K and 5K; Gabay et al. 1997a) and also TGF-β signaling as revealed by cuticle defects (Figure 4O and 5I,M; Ferguson and Anderson 1992). One of the earlier reports in *Drosophila* demonstrated that Trol is required for FGF signaling
through the FGFR Breathless and FGF Branchless to support neuroblast proliferation (Park et al. 2003). They also showed that vertebrate Perlecan co-immunoprecipitated with vertebrate FGF-2 and that this interaction can be outcompeted upon addition of heparin. In addition, trol mutants displayed higher levels of Hedgehog (Hh), another morphogen, nearer to its source of expression suggesting that Trol is required for diffusion of Hh (Park et al. 2003). Another study yielded similar results in the neuromuscular junction by examining Wingless (Wg)-GFP of the Wnt pathway (Kamimura et al. 2013). Total Wg levels were not affected in trol mutants, however Wg appeared to remain near the presynaptic membranes where it is secreted while the postsynaptic bouton acquired defects analogous to inhibition of Wnt signaling. These reports support the view that a general function for Trol is to effect ligand distribution.

\textit{HSPG specificity in modulating different FGF-dependent processes}

Our screen isolated the HSPG Trol, a secreted protein, but another HSPG, Sdc which contains a transmembrane domain, was reported previously to work with FGF during mesoderm development in the early embryo (Knox et al. 2011). Comparing Trol and Sdc revealed spatiotemporal differences in their expression (Figures 3A,B and 5A-D) and non-overlapping phenotypes relating to several FGF-dependent processes (Figure 4-7). FGF signaling regulates a variety of activities that include communication between both distant cells and adjacent cells. However, their ability to modulate the range of FGF signaling is undetermined. Both Trol and Sdc are expressed in the ventral ectoderm during mesoderm migration (Figure 5A,D arrows), and their expression patterns overlap with that of the FGF ligand Ths at these stages (Kadam et al. 2009). However, localized Trol is expressed earlier than Sdc. Furthermore, ectopic expression of Sdc in the mesoderm (both broadly and earlier than normal) results in a moderate spreading
phenotype (Figure 5G), which we suggest is due to its sequestration of Ths ligand from Trol. Trol normally supports tube collapse, based on our genetic analysis, and likely is the only HSPG that normally supports this process. Based on these data, we propose the model that Trol, a component of the extracellular matrix (ECM), is able to promote FGF-FGFR interactions such as tube collapse in which mesoderm cells at a distance from the ectoderm respond to activation (“long-range” action). On the other hand, cell membrane-associated Sdc likely works locally to regulate FGF-FGFR interactions between neighboring cells (“short-range” action) as, for example, in the induction of dorsal somatic lineages (e.g. Eve). As Trol is secreted, it may be better suited to support long- or at least longer-range diffusion of the ligand relative to Sdc, which contains a transmembrane domain. For instance, during the FGF-dependent collapse of the invaginated tube of cells following EMT, Trol may aid in delivering FGF ligand to the receptor present in mesoderm cells located initially (before collapse) at a distance from the ectoderm (Figure 8A-1). On the other hand, the fact that Sdc is membrane-associated suggests the model that Sdc, and not Trol, functions to support short-range FGF signaling in adjacent cells to support the processes of cell intercalation (Figure 8B) and cell differentiation (Figure 8C).

Another alternative hypothesis to that of diffusion is that Trol stabilizes FGF and allows presentation of the ligand to be taken up by cells expressing the receptor through cell protrusions such as cytonemes (Figure 8A-2) (ROY et al. 2014). These mechanisms may also play a role during dorsal mesoderm differentiation and CVM migration, both FGF-dependent processes, as Sdc is required for Eve specification while Trol is required in the CVM. Our model, along with other studies, implies direct interaction between HSPGs and the FGF-FGFR complex (PELLEGRINI et al. 2000).
**HSPGs in ECM architecture**

Alternatively, or in addition, it is possible that HSPGs affect receptor-ligand interactions indirectly by influencing distribution of the ligand through changes to organization of the basement membrane and ECM, which can result in positive or negative effects on signaling pathways (Kim et al. 2011). For example, S2R+ cell culture studies with the HSPG Dlp revealed that it can both enhance and inhibit Wnt signaling, depending on the context (Baeg et al. 2004). Recently, genetic interactions suggest that Trol sequesters the Ths ligand and prevents FGF-dependent differentiation in the larval lymph gland, thus serving an inhibitory role towards FGF signaling (Dragojlovic-Munther and Martinez-Agosto 2013). However, secreted HSPGs, such as Trol, are also components of the basement membrane and can modify organization of the ECM (Sarrazin et al. 2011). Perhaps in these lymph glands, Trol negatively regulates FGF signaling through changes to the ECM structure as the surrounding basement membrane was shown to also have defects that affected Hh distribution (Grigorian et al. 2013). Additionally, the ECM receptor Dystroglycan (Dg) has been shown to bind Trol and is found between the mesoderm-ectoderm interface (Schneider and Baumgartner 2008), thus potentially influencing Trol function during mesoderm migration. Therefore, trol mutants could also indirectly contribute to altered signaling activities, such as FGF distribution, at gastrulation due to changes in the ECM structure within these mutants.

**Extracellular vs. membrane-tethered HSPGs**

In addition to Sdc function in late mesoderm specification (this study; Knox et al. 2011), several other reports implicate membrane-bound HSPGs’ involvement in short-range signaling. Axon guidance by Slit/Robo signaling in Drosophila embryos requires two HSPGs, Dlp and Sdc.
The distribution and concentration of Dlp and Sdc are discrete in order to generate a distinct spatial field able to direct axonal growth (Smart et al. 2011). Another HSPG, Dally, is necessary in conjunction with BMP signaling for germline stem cell maintenance in Drosophila ovaries (Guo and Wang 2009). This requirement of Dally is limited to the germline only and not the nearby somatic cells, revealing its short range of action. In the vertebrate system, membrane-tethered HS chains are required for FGF signaling in adjacent cells during mouse embryogenesis (Shimokawa et al. 2011). All of these reports emphasize the importance of membrane-bound HSPGs in regulating ligand distribution and limiting signaling activity within a short distance. Alternatively, the property of Trol to be secreted is unique among Drosophila HSPGs. Our comparison of HSPGs Trol and Sdc in supporting FGF-dependent processes in the Drosophila early embryo has revealed that they support different signaling outputs. A future direction would be to examine whether their differential roles relate to how each affects ligand distribution.

ACKNOWLEDGMENTS

We are grateful to the Zinn lab (Caltech) for sharing their CSS insertion fly stock collection and Marc Freeman, Stephen Crews, and Manfred Frasch for sharing additional fly stocks and antibodies. We also would like to thank Kai Zinn for helpful discussions, Young-Kyung Bae for sharing unpublished results, and Man Ho Wong and Molly Lichten for their invaluable help with the screen. This work was funded by a grant to A.S. from the NIH/NIGMS R01GM104838.
LITERATURE CITED


Shilo, B. Z., 2005 Regulating the dynamics of EGF receptor signaling in space and time. Development 132: 4017-4027.


FIGURES AND TABLES

FIGURE 1

Figure 1. Ten genes identified by the ectopic expression screen confer mesoderm migration defects.

(A) Here and in following figures unless otherwise noted: cross-section of Drosophila embryos stained with Twist antibody to mark mesoderm cells during development; ventral side down. In stages 5-10, the mesoderm invaginates to form the ventral furrow, which subsequently collapses onto the ectoderm. Lateral migration follows and the process is complete after intercalation helps to specify a monolayer.
(B) Workflow of ectopic expression screen. Cell-surface and secreted (CSS) proteins were overexpressed in the mesoderm using Twi-Gal4 and in the ectoderm using 69B-Gal4. Candidates were narrowed to ten genes based on their RNA expression and mutant phenotypes: *pyramus* (blue), which previously had been characterized for its role in mesoderm migration (McMAHON *et al.* 2010); and nine novel genes (red). Genes comprise three different classes: Adhesion, Signaling Components, and Proteoglycans. Here, and in all following figures, lateral views of whole mount embryos are positioned with anterior facing left and ventral side facing down. Cross-section depicting Gal4 lines taken from (KADAM *et al.* 2009).
Figure 2. Expression and mutant phenotypes of adhesion molecules and signaling components isolated from the screen.

Here and in subsequent figures, cross-sectioned embryos are of stage 9-10 when mesoderm cells are at the end of their migration; unless otherwise noted.

(A-D) A comparison of wildtype with mild, moderate, and severe mesoderm spreading phenotypes. (A) Wildtype embryos have a monolayer of mesoderm cells. The arrowhead marks where the mesoderm cells have reached the dorsal region of the embryo, where cells receive additional differentiation signals. (B) pyr\(^{18}\)/Df BSC25 trans-heterozygous mutant embryos have a mild phenotype marked by regions where mesoderm cells are multilayered (arrow). However
some cells intercalate into a single layer (arrowhead). (C) \textit{pyr}^{e02915}/\textit{Df BSC25} embryos have a moderate phenotype where the mesoderm is uniformly multilayered. \textit{Df BSC25} is a deficiency that encompasses both Pyr and Ths, FGF ligands for the FGFR Htl. (D) \textit{htl}^{AB42} mutants have severe defects such that the mesoderm forms lumps of cells.

(E-H,M-P) Preliminary expression and mutant analysis of genes isolated in the screen. RNA expression patterns in wildtype embryos of stage 8-9 (lateral views: E-H, M-P) and cross-section of zygotic mutant embryos showing \(\alpha\)-Twi expression to mark mesoderm (cross-sections: I-L,Q-T). Single mutants were assayed if available (I,J,K,L,Q,R) for genes isolated from the ectopic expression screen otherwise data for deficiencies is shown (\textit{aos}: S). For assay of \textit{egfr}, the dominant negative (DN) form of \textit{egfr} was overexpressed using the Twi-Gal4 driver (T). In situ hybridization was performed using riboprobes specified for the indicated genes. Genes in red denote those isolated from this screen.
Figure 3. Embryonic expression of *Drosophila* proteoglycans is dynamic.

Wildtype expression patterns for HSPGs detected using in situ hybridization and specific riboprobes for: (A) *trol*, (B) *sdc*, (C) *dally*, (D) *dally-like*, and putative CSPGs (E) *ptp99a* and (F) *kon*. Lateral views of embryos staged at pre-cellularization (left column), gastrulation (stage 8; middle), and in germ-band elongated embryos (stage 12; right).
Figure 4. *trol* germline clones exhibit defects in mesoderm migration similar to FGF mutants.
(A) Image of *trol* locus obtained from Flybase GBrowse depicting location of the reagents used in this study: GE10067 is a UAS insertion and G0211 is a lacZ insertion that was recombined with FRT 19A in order to support generation of germline clones.

(B-E) Mesoderm migration phenotypes in embryo cross-sections (stage 9-10) detected using α-Twi antibody resulting from *trol* ectopic expression and tissue-specific downregulation. Ectopic expression of *trol* in the ectoderm (B) or mesoderm (C) results in mesoderm spreading defects. Downregulation of *trol* levels by tissue-specific RNAi in the ectoderm (D) or mesoderm (E) also yields spreading defects.

(F-I) *trol* maternal + zygotic mutant phenotypes in a time-course of embryo cross-sections of stage 6 (F), stage 7 (G), and stage 9-10 (H) detected using α-Twi antibody. While invagination is normal (F), defects in EMT are observed as tube collapse is non-symmetrical (G) and the mesoderm remains multilayered even at stage 10 (H). Zygotic mutants have normal spreading (I) thus maternal *trol* contributes to the spreading defects observed.

(J-M) α-dpERK stainings in cross-sections of stage 10 embryos also showing magnified views (J’-M’). In wildtype embryos (J), FGF-dependent dpERK is found in only 2-3 of the dorsal most mesoderm cells (J’, arrows). This staining is absent in *trol* germline clones (K, arrow). In embryos where *trol* is ectopically-expressed in the ectoderm with the 69B-Gal4 driver, dpERK staining has expanded to 5+ cells (L, arrow). Embryo ectopically expressing *trol* in the mesoderm with Twi-Gal4 shows ectopic dpERK throughout the mesoderm (M, arrow).

(N,O) Cuticles associated with wildtype (N) or *trol* germline clones, which display a “tail-up” phenotype (O).
Figure 5. *sdc* mutant embryos exhibit mild defects in mesoderm migration.

(A-D) In situ hybridization of (A,B) *trol* and (C,D) *sdc* in wildtype embryos. At stage 6, *trol* is upregulated in the ventral-most ectoderm cells surrounding the invaginated furrow (A, arrow). In contrast, *sdc* is localized to the same position but at a later stage when mesoderm cells intercalate to form a monolayer (D, arrow).
(E) sdc germline clones have normal mesoderm collapse (i.e. symmetrical). (F) sdc germline clones have mild spreading defects as mesoderm cells form a non-monolayer (arrows). (G) Ectopically expressing sdc in the mesoderm results in a multilayered mesoderm (arrow). (H) Overexpressing sdc in the ectoderm has little effect as mesoderm spreading appears normal (i.e. monolayer).

(I,M) sdc germline clones exhibit a range of cuticular phenotypes that range from ‘tail-up’ to twisted/loss-of-head.

(J-K) α-dpERK staining is detected in cross-sections of embryos from sdc germline clones within dorsal-most mesoderm cells (J; magnified view: J’, arrows), possibly at a reduced level compared to wildtype (see Figure 4J). Ectopic expression of sdc within the mesoderm results in ectopic dpERK throughout the tissue (K; K’, arrow); whereas overexpression of Sdc in the ectoderm has little effect (L; L’, arrow).
Figure 6. Embryos obtained from sdc, but not trol, mutant germline clones exhibit defects in Eve specification.

(A) Schematic cross-section of the ventral half of an embryo at stage 11 during Eve specification. Mesoderm cells that reach the dorsal regions of the embryo (arrows) are able to receive signals, including FGF, from the ectoderm (dark orange) and undergo cell differentiation (dark blue).

(B-G) Dorsal somatic mesoderm cell differentiation at stage 11 embryos is marked by Eve expression. (B) Wildtype whole embryo stained using anti-Eve antibody includes red box
showing region of magnification in subsequent panels. (C) Wildtype embryos have 10 clusters of 3 Eve+ cells. (D) *trol* germline clones show normal number of Eve+ cells. (E) *69B>trol* embryos occasionally have clusters with 4 Eve+ cells as indicated by the arrow. (F) *sdc* germline clones are missing clusters (arrows) and/or have reduced number of Eve+ cells within a cluster (arrowhead). (G) *69B>sdc* embryos have multiple clusters with 5 or more Eve+ cells (arrows).
Figure 7. Embryos with reduced trol but not sdc levels exhibit defects in CVM migration.

(A) Schematic depicting CVM migration. Red cells are the two migrating CVM clusters that express the FGFR Htl FGFR as well as the HSPG Trol (Figure 3A, right). The TVM substratum is shown in green and this tissue expresses both FGF ligands Pyr and Ths. (A, left; B-F) Dorsal view of stage 11 embryos. (A, right; G-K) Lateral view of stage 13 embryos.

(B-K) Embryos containing the CVM-specific driver 5053-Gal4 and CVM marker transgene bHLH-gap-Venus. Anti-GFP staining marks the CVM in control embryos (B,G), trol germline clones (C,H), and in embryos expressing RNAi hairpin constructs directed against trol (D,I), sdc
(E,J), and *kon* (F,K) in CVM cells. (G'-K’) Magnified view of CVM cells to show ectopic cell death. Arrows point to merging phenotype (C,D) or ectopic cell death (H,I,K).

Schematic in (A) reprinted with permission from Bae *et al.* (2012).
Figure 8. Model for differential action of Trol and Sdc HSPG-mediated activation of FGF signaling: hypothesized differences in signaling range.

The mesoderm (blue) expresses the FGF receptor (FGFR) while the ectoderm (orange) expresses the FGF ligands. In mesoderm development, we show in this study that FGF signaling is modulated by Trol during the early steps of its migration (A) and by Sdc at later stages (B,C). Trol is secreted into the ECM and has the potential to signal to non-neighboring cells. This may occur through diffusion to target cells (#1) and/or the ability to be taken up by target cells via cytonemes (#2). Sdc is bound at the membrane and can thus only signal to adjacent cells to support small movements such as intercalation of the mesoderm (#3). Once migration is complete, Sdc can continue to act in neighboring FGF-producing cells (C, dark orange) for differentiation of cells at the dorsal mesoderm (C, dark blue) (#4).
Table 1. Twenty-four genes found to confer embryonic lethality when ectopically expressed in the ectoderm or mesoderm.

<table>
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<tr>
<th>Lethality</th>
<th>Gene ID</th>
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<th>Endogenous expression, stg 5-10</th>
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<td>Twi-Gal4</td>
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<td>Ana</td>
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<td>Lambik</td>
<td>Lbk</td>
<td>GS 17119 ectoderm</td>
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<tr>
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<td>CG7476</td>
<td>Methuselah-like 7</td>
<td>Mthl7</td>
<td>GS 21256 weak mesoderm&lt;sup&gt;c&lt;/sup&gt;</td>
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<td></td>
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<td>Microsomal triacylglycerol transfer protein</td>
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<sup>a</sup>Total of ten genes were identified that had relevant (i.e. mesoderm or ectoderm) endogenous RNA expression and had mutant mesoderm defects: nine novel genes plus the FGF ligand Pyramus, which has previously been shown to function in mesoderm migration (KADAM et al. 2009).

<sup>b</sup>Berkeley Drosophila Genome Project (BDGP) reports zero expression of CG9550 at embryonic stages 5-10.

<sup>c</sup>No developmental timecourse of expression data available for mthl7 at BDGP.

<sup>d</sup>Low to moderate expression for mtd is reported at embryonic stages 5-10 at BDGP.
## Table 2

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<th>Severe 4</th>
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<th>Avg. Score</th>
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<td>8</td>
<td>2.5</td>
<td>moderate</td>
</tr>
<tr>
<td>ptp99a\textsuperscript{1}</td>
<td>3</td>
<td>3</td>
<td>1</td>
<td></td>
<td>7</td>
<td>1.7</td>
<td>mild</td>
</tr>
<tr>
<td>tro\textsuperscript{G011} m-z-</td>
<td>2</td>
<td>5</td>
<td>6</td>
<td>9</td>
<td>22</td>
<td>3.0</td>
<td>moderate/severe</td>
</tr>
<tr>
<td>sdc\textsuperscript{CM33} m-z- and m-z+</td>
<td>9</td>
<td>8</td>
<td>6</td>
<td></td>
<td>23</td>
<td>1.9</td>
<td>mild</td>
</tr>
</tbody>
</table>

Table 2. Quantification of mesoderm spreading phenotypes in mutant embryos.

Stage 9-10 embryos were stained with Twist antibody to mark the mesoderm and cross-sectioned to determine any mesoderm spreading defects (Figure 2). Embryos mutant for genes identified from the screen, in addition to embryos mutant for the FGF pathway as controls, were tallied. A weight was given for each phenotypic class and averaged. Only three genes, \textit{htl}, \textit{shg}, and \textit{trol}, had a score of 3 or above and, therefore, their mesoderm migration phenotypes were classified as moderate/severe. “Moderate” phenotypes had a score of 2.5-2.9 and “mild” phenotypes had a score of 1.5-2.4. A score below 1.4 was classified as “normal” mesoderm spreading. \textit{Df BSC25} uncovers both genes \textit{pyr} and \textit{ths}, encoding ligands for the FGFR Htl.