# Collective Migrations of *Drosophila* Embryonic Trunk and Caudal Mesoderm-Derived Muscle Precursor Cells

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**ABSTRACT** Mesoderm migration in the *Drosophila* embryo is a highly conserved, complex process that is required for the formation of specialized tissues and organs, including the somatic and visceral musculature. In this FlyBook chapter, we will compare and contrast the specification and migration of cells originating from the trunk and caudal mesoderm. Both cell types engage in collective migrations that enable cells to achieve new positions within developing embryos and form distinct tissues. To start, we will discuss specification and early morphogenetic movements of the presumptive mesoderm, then focus on the coordinate movements of the two subtypes trunk mesoderm and caudal visceral mesoderm, ending with a comparison of these processes including general insights gained through study.

**KEYWORDS** adhesion; apicobasal polarity; caudal visceral mesoderm (CVM); collective cell migration; *Drosophila melanogaster*; FGF signaling; mesoderm migration; trunk visceral mesoderm (TVM); FlyBook

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IN bilaterian organisms, an important hallmark of embryonic development is the specification of three germ layers: ectoderm, mesoderm, and endoderm (Solnica-Krezel and Sepich 2012; Stathopoulos and Newcomb 2020). This nomenclature is descriptive of corresponding positions in the developing embryo: ectoderm generally refers to the outermost layer of tissue, mesoderm refers to the middle layer, and endoderm refers to the innermost layer. These three germinal layers give rise to different cell and tissue lineages, with nervous system and epidermal tissues arising from ectoderm, and gastrointestinal and other organ epithelia arising from endoderm. The mesoderm, which gives rise to muscle, circulatory, connective, and several other more specialized cell types in higher organisms will be the focus of this review. In particular, we will be focusing on specification and early morphogenetic movements of the presumptive mesoderm, namely invagination and epithelial-to-mesenchymal transition (EMT), followed by focused overviews of the migrations of two mesodermal cell types in the *Drosophila* embryo, which has long been established as a tractable model system for obtaining general insights into development.

Mesoderm cells are specified at the ventral side of cellular blastoderm embryos downstream of dorsal-ventral (DV) patterning (Stathopoulos and Newcomb 2020). The subsequent apical constriction and internalization of those cells results in the formation of a tube-shaped structure accomplished at stage 7. Migration takes place at the beginning of stage 8, after the mesoderm tube collapses through making contacts with the ectodermal layer, and ends at stage 10 by forming a monolayer of cells in between the ectoderm and yolk. It is important that spreading of the mesoderm cells is uniform on both sides of the ventral midline and that a monolayer is achieved, as the proper subdivision and differentiation of distinct mesoderm lineage requires correct number of precursor cells be in position to receive appropriate differentiating signals (Figure 1; Maggert *et al.* 1995). Ligands of FGF, Wnt, and BMP signaling pathways are synthesized in distinct domains in the ectoderm (Stathopoulos and Levine 2004). Depending on which particular ectoderm cells they ultimately contact as a result of migration, mesoderm cells receive different combinations of those instructive signals, and later give rise to cardiac, pericardial, visceral, or somatic cell types.

Therefore, proper specification and completion of mesoderm migration during gastrulation is crucial to the proper positioning and specification of distinct muscle cell types, in particular, the visceral muscle precursors that, once specified, also migrate. The *Drosophila* embryonic visceral musculature, which is responsible for the peristaltic movements required for the passage of food through the gut tube, originates from two different cell types: longitudinal visceral muscles, which are seeded by cells of the caudal visceral mesoderm (CVM); and circular visceral muscle, which arise from transverse visceral mesoderm (TVM) (Figure 1E, red and cyan, respectively; Azpiazu and Frasch 1993; Martin *et al.* 2001). These mesodermal lineages vary in their form and function, and accordingly display distinct migratory behaviors. CVM cells arise from a strip of cells at the ventral-posterior border of the

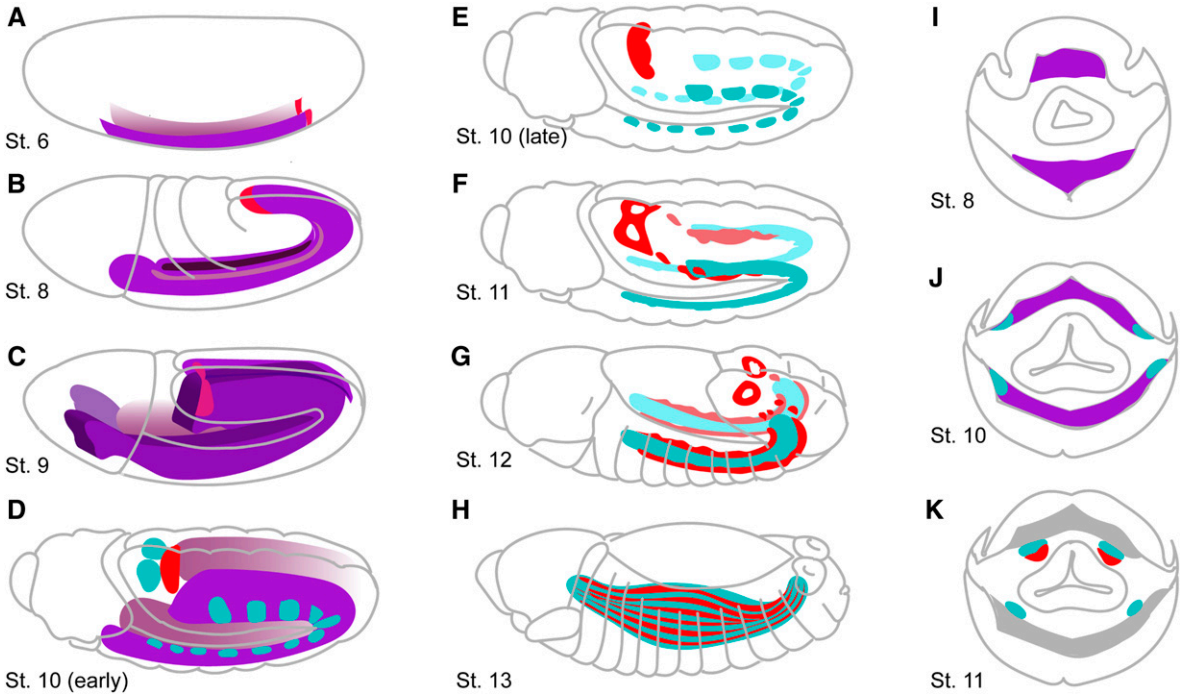


**Figure 1** Domains in the *Drosophila* embryo relating to trunk and caudal mesoderm cell lineages. (A–H) Steps of presumptive mesoderm (purple) specification at gastrulation, ventral furrow formation, and dorsolateral spreading concomitant to germband extension in stage 6–13 embryos (Hartenstein 1993). CVM cells (red) are specified as a stripe at the ventroposterior edge of the presumptive mesoderm (A–D), before becoming migratory (E–G), eventual fusion with TVM (cyan), to facilitate ensheathment of the entire length of the gut (H). (I–K) Transverse cross sections of embryos showing mesoderm spreading (purple) and subsequent differentiation into distinct subtypes, focusing on arrangement of TVM (cyan) and CVM (red) relative to somatic mesoderm (gray) at three representative stages. Somatic mesoderm was omitted in E–H, for clarity.

trunk mesoderm before germband extension (GBE), a rapid, posteriorly directed movement of both ectoderm and mesoderm cells (Irvine and Wieschaus 1994), during the cellular blastoderm stage (Figure 1A, red); whereas, after mesoderm spreading, the most-dorsally positioned mesoderm cells specify the TVM cell fate (Figure 1, B–D, J, cyan; Lee and Frasch 2005). Subsequent to specification, the CVM cells arrange into two bilaterally symmetric, migrating cohorts that synchronously move along the TVM before undergoing cell division about halfway through their anteriorly directed movement (Figure 1, E–G, K). CVM cells then fuse with fusion-competent myoblasts to form the complete gut musculature (Figure 1H). This highly stereotyped and ordered migration of CVM cells along the TVM, the longest migration of any cell type in the developing *Drosophila* embryo, is necessary to properly position longitudinal visceral muscles along the entire length of the gut. To accomplish this long-distance migration, CVM cells must integrate extracellular signaling cues as well as modulate intercellular interactions.

In the next sections, we will discuss what is known about the migration of two of these cell types, trunk mesoderm migration at gastrulation and CVM migration in later-staged embryos (Table 1).

## Mesoderm Specification, Invagination, and EMT

### *Mesoderm specification*

The mesoderm of the *Drosophila* embryo is specified by input from maternal and zygotic transcription factors at the blastoderm stage, in embryos containing a single layer of ~5000 cells (Zalokar and Erk 1976). The maternal transcription factor *dorsal (dl)* is present in a nucleocytoplasmic gradient that spans the entire DV axis, with highest nuclear levels present ventrally [reviewed by Reeves and Stathopoulos (2009)]. Early patterning through Dl along the DV axis establishes the domains of presumptive mesoderm, neurogenic ectoderm, dorsal ectoderm, and amnioserosa. Dl activates the gene *twist (twi)*, encoding a transcription factor, in a ventral domain of 16–20 cells in width (Figure 2A, D; Jiang *et al.* 1991; Ray *et al.* 1991). Twi and Dl coordinately activate the expression of another transcription factor, *snail* (*sna*) (Ip *et al.* 1992). Both *twi* and *sna* genes are necessary for the specification of mesodermal cell fate; however, they generally play complementary roles (Figure 2A). Twi activates mesodermal target genes in the ventral presumptive mesoderm, whereas Sna acts predominantly to repress other genes and limit their expression to the more dorsal ectodermal regions (Leptin 1991; Thisse *et al.* 1991).

**Table 1 Key genes involved in trunk and caudal visceral mesoderm migration**

| Symbol | Name | Additional names | Functions | References |
|---|---|---|---|---|
| *arm* | *Armadillo* | β-*Catenin* | Key component of adherens junctions and Wnt signaling pathway | Müller and Wieschaus (1996), Martin *et al.* (2010) |
| *baz* | *Bazooka* | *Par3* | Apical polarity regulator, PDZ domain-containing scaffold protein | Tepass (2012), Weng and Wieschaus (2017), Sun and Stathopoulos (2018) |
| *bin* | *Biniou* | | Transcription factor, mesoderm specification | Zaffran *et al.* (2001), Jakobsen *et al.* (2007), Ismat *et al.* (2010) |
| *Cdc42* | | | Rho family small GTPase, key regulator of actin cytoskeleton | Clark *et al.* (2011) |
| *dl* | *Dorsal* | *NFκB* | Transcription factor, functions downstream of Toll signaling pathway in DV patterning | Reeves and Stathopoulos (2009) |
| *dof* | *Downstream of fgf* | *Stumps*, *heartbroken* | Scaffold protein for FGF signaling through the Ras-MAPK pathway | Michelson *et al.* (1998), Vincent *et al.* (1998), Imam *et al.* (1999) |
| *E-cad* | *E-Cadherin* | *DE-Cadherin*, *shotgun* | Mediate cell-cell adhesion, key component of adherens junctions | Oda *et al.* (1994), (1998), Clark *et al.* (2011); Schäfer *et al.* (2014) |
| *eve* | *Even skipped* | | Transcription factor, AP patterning, mesoderm and CNS development | Azpiazu and Frasch (1993), Carmena *et al.* (1998) |
| *HLH54F* | | *bHLH54F* | Transcription factor, CVM specification | Georgias *et al.* (1997), Ismat *et al.* (2010) |
| *htl* | *Heartless* | | Tyrosine kinase receptor for FGF ligands, signals through Ras-MAPK pathway | Michelson *et al.* (1998), Wilson *et al.* (2005), McMahon *et al.* (2008) |
| *mys* | *Myospheroid* | β*PS1*, β*1-integrin* | Mediate cell-extracellular matrix adhesion, bidirectional signaling | Leptin *et al.* (1989), McMahon *et al.* (2010), Sun and Stathopoulos (2018) |
| *N-cad* | *N-Cadherin* | *DN-Cadherin*, *CadherinN (CadN)* | Mediate cell-cell adhesion, axon patterning | Iwai *et al.* (1997), Oda *et al.* (1998), Schäfer *et al.* (2014) |
| *pbl* | *Pebble* | | Rho GEF, cytokinesis | Schumacher *et al.* (2004), Smallhorn *et al.* (2004), van Impel *et al.* (2009) |
| *pyr* | *Pyramus* | | FGF8-like ligand for receptor Htl | Stathopoulos *et al.* (2004), Kadam *et al.* (2009), Klingseisen *et al.* (2009), Clark *et al.* (2011) |
| *Rac* | *Rac GTPase* | *Rac1*, *Rac2*, *Mig-2-like (Mtl)* | Rho family GTPases, regulator of actin cytoskeleton, axon development, epithelial morphogenesis | Wilson *et al.* (2005), van Impel *et al.* (2009) |
| *Rap1* | *Rap1 GTPase* | *Ras-related protein 1* | Ras family small GTPase, diverse roles during morphogenesis, cell polarity and migration | McMahon *et al.* (2010), Spahn *et al.* (2012), Choi *et al.* (2013) |
| *Sdc* | *Syndecan* | | Transmembrane heparan sulfate proteoglycan, axon guidance | Knox *et al.* (2011), Trisnadi and Stathopoulos (2015) |
| *sna* | *Snail* | | Transcription factor | Ip *et al.* (1992), Weng and Wieschaus (2016) |
| *stg* | *String* | *Cdc25* | Phosphatase to activate Cyclin-dependent kinase 1, promotes cell cycle progression | O'Farrell (2001), Clark *et al.* (2011), Sun and Stathopoulos (2018) |
| *ths* | *Thisbe* | | FGF8-like ligand for receptor Htl | Stathopoulos *et al.* (2004), Kadam *et al.* (2009), Klingseisen *et al.* (2009) |
| *trbl* | *Tribbles* | *Trb* | Induces Cdc25 degradation, delays cell cycle progression | Grosshans and Wieschaus (2000), Mata *et al.* (2000), Seher and Leptin (2000) |
| *trol* | *Terribly reduced optic lobes* | *Perlecan*, *zw1* | Secreted heparan sulfate proteoglycan, regulates signaling pathways including FGF | Trisnadi and Stathopoulos (2015) |
| *twi* | *Twist* | | Transcription factor, mesoderm specification | Jiang *et al.* (1991), Ray *et al.* (1991), Farge (2003) |
| *zfh1* | *Zn finger homeodomain 1* | | Transcription factor, mesoderm specification, mesoderm migration | Kusch and Reuter (1999), Ismat *et al.* (2010) |

### *Invagination*

Dynamic gene expression in the mesoderm cells is the driving force of the morphogenetic processes that cells initiate following specification. Most genes expressed in the ventral embryo, corresponding to the presumptive mesoderm, exhibit similar expression dynamics; for example, representative genes *twi* and *sna* exhibit a monotonic increase in expression levels followed by a plateau at about the time of cellularization (Sandler and Stathopoulos 2016). However, some other genes expressed in the region, such as *Transcript 48* and *folded gastrulation* (*fog*), exhibit spatiotemporal differences in expression that are thought to be essential for driving cell shape changes (*i.e.*, apical constriction) that lead to mesoderm

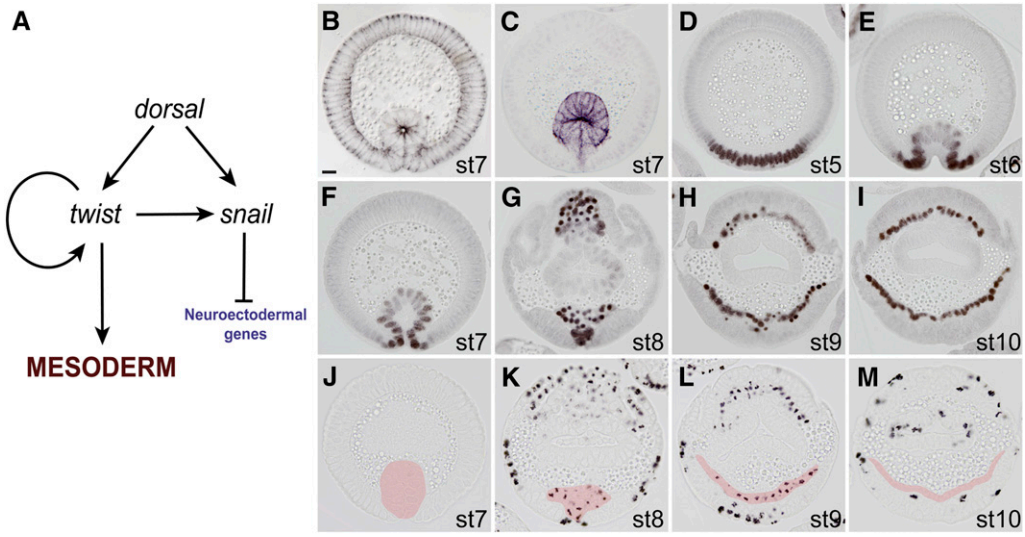


**Figure 2** Mesoderm specification and cell divisions during gastrulation. (A) Gene regulatory network showing specification of the mesoderm. Dorsal and Twist transcription factors work together to specify the mesoderm cell fate in ventral regions of the embryo (see also D). Snail is also expressed in ventral regions but acts as a repressor of neuroectodermal genes, limiting their expression to more dorsal regions. (B–M) Cross sections of stage 5–10 embryos stained for E-Cadherin and Heartless, both enriched in the apical center of the mesoderm tube (B and C, respectively); as well as Twist (D–I), marking mesoderm cell nuclei, and Phosphohistone H3 (PH3) (J–M), a marker of cell division. Anti-PH3 staining demonstrates two rounds of cell division occur during gastrulation, at stage 8 while the tube collapses, and again at stage 9, just before monolayer formation. Mesoderm cells on the bottom half of the embryos are pseudocolored red. Developmental stages are as indicated. Scale bar, 10 μm.

invagination (Figure 2E; Morize *et al.* 1998; Lim *et al.* 2017). Intriguingly, as mesoderm invagination also generates mechanical stress on the cortical membrane and *twi* gene expression is mechanosensitive, it has been proposed that this morphogenetic process could further contribute to *twi* and its target gene expression (Farge 2003; Brouzés *et al.* 2004).

Before invagination, the presumptive mesoderm cells in the ventral blastoderm exhibit apicobasal polarity and are morphologically indistinguishable from their surrounding ectodermal neighbors. Identified through genetic screens in *Caenorhabditis elegans* and *Drosophila*, three evolutionarily conserved protein complexes have been shown to be crucial for apicobasal polarity formation: namely, the Scribble/Discs large/Lethal giant larvae (Scrib/Dlg/Lgl) complex, localized in the lateral membrane basal to the adherens junctions (AJs); the Crumbs/Stardust/PATj (Crb/Sdt/PATj) complex apical to the AJs; and the Partitioning defective-homolog/Atypical protein kinase C/Cdc42 (Par/aPKC/Cdc42) complex, which exhibits context-dependent localizations (Mellman and Nelson 2008). While *crb* is repressed by *sna* and absent from the presumptive mesoderm cells in ventral regions (Leptin 1991), members of the Par complex are expressed. Besides supporting apicobasal polarity, Bazooka (Baz), the *Drosophila* Par3 homolog, also associates with AJs and regulates cell-cell adhesion (Müller and Wieschaus 1996; Laprise and Tepass 2011; Tepass 2012).

*baz* encodes a PDZ domain-containing scaffold protein that is regulated through phosphorylation by aPKC and Par1 (Laprise and Tepass 2011). Baz can function independently of aPKC and Cdc42 to regulate cell polarity and adhesion, and is targeted to distinct membrane domains through interaction with different binding partners (Laprise and Tepass 2011; Tepass 2012). Baz acts upstream of Cadherin-Catenin and Crb and Dlg complexes in the embryonic ectoderm to establish apicobasal polarity (Harris and Peifer 2004, 2005; Laprise and Tepass 2011). It recruits kinases aPKC/Par6 and Sdt to the apical surface while associating with Phosphatase and tensin homolog (Pten) at the AJs, enriching phosphoinositide Phosphatidylinositol-4,5-diphosphate [PtdIns(4,5)P2, PIP2] at the apical membrane (Harris and Peifer 2005; von Stein *et al.* 2005; Mavrakis *et al.* 2009; Krahn *et al.* 2010).

Following their specification at the ventral most part of the embryo, mesoderm cells undergo apical constriction, invaginate to form a furrow, and ultimately become a tube-shaped structure (*e.g.*, Figure 2C, F). This process is discussed in more detail within another FlyBook chapter (Adam Martin, FlyBook). Briefly, downstream of both Twi and Sna, the initial cell shape change and nuclear shifting in the mesoderm is driven by pulsed contraction of the actomyosin network and ratchet-like constriction of the cell apex (Martin *et al.* 2009, 2010). Meanwhile, small GTPases, RhoGEFs, cell polarity regulators, and components of AJs all play crucial parts in the process to facilitate the tissue-scale cell shape changes/morphogenesis while maintaining tissue integrity (Martin *et al.* 2010; Weng and Wieschaus 2017). For example, maternally supplied RhoGEF2 is required for the proper formation of the furrow in ventral regions (Grosshans *et al.* 2005). RhoGEF2 acts downstream of Fog, a ligand for G protein–coupled receptors including Mist and Smog, the α-subunit of G-protein Concertina (Cta) and transmembrane anchor protein Transcript 48 to activate Rho1 and drive mesoderm cell shape change/apical constriction through regulation of the actin cytoskeleton and AJs (Barrett *et al.* 1997; Hacker and Perrimon 1998; Kölsch *et al.* 2007; Manning *et al.* 2013; Kerridge *et al.* 2016). Furthermore, epithelial tension along the AP axis is generated as a result of mesoderm invagination, and loss of function of AJ components [*e.g.*, E-Cadherin (E-

cad), α-Catenin, and β-Catenin (β-cat)] results in tears in the ventral furrow (Martin *et al.* 2010). Recent studies have also shed light on the interaction between Baz and AJs during apical constriction (Weng and Wieschaus 2016, 2017). The initial downregulation of Baz by Sna causes a specific decrease in AJ levels in the presumptive mesoderm; however, Baz reaccumulates and follows AJs as they are strengthened and shift apically in response to force generated by contractile actomyosin at the site of apical constriction (Weng and Wieschaus 2016, 2017). Such bidirectional interplay between Baz and AJs highlights the highly cooperative regulation between cell polarity and adhesion during mesoderm invagination, which is accomplished as cells in the ventral furrow are fully internalized and form a tube. The center of this tube represents the apical side of the mesoderm cells where both Baz and junction proteins (*e.g.*, E-cad) are enriched (Figure 2B).

### *EMT and cell division*

Completion of trunk mesoderm invagination is followed by symmetric tube collapse as cells initiate an EMT, which also marks the starting point of the mesoderm migration process associated with gastrulation (Figure 2G). Twi target genes, including the FGF receptor *heartless* (*htl*) and FGF scaffold protein *downstream of fgf* (*dof/stumps/heartbroken*), are expressed in mesoderm cells (Figure 2C; Michelson *et al.* 1998; Vincent *et al.* 1998; Imam *et al.* 1999). These genes function together with regulators of cell polarity, cell adhesion, cell division, and the cytoskeleton to ensure the fidelity of the EMT process by coordinating the collective cell movement and the two, synchronized cell divisions in the mesoderm during gastrulation (Wilson *et al.* 2005; Sun and Stathopoulos 2018).

The mesodermal tube collapses through a prolonged EMT, in which cell-cell attachments are decreased, but not eliminated (Sun and Stathopoulos 2018). The attachment between cells is mediated by homotypic interactions between the extracellular domain of cadherin molecules, which are most enriched at the AJs (Nagafuchi *et al.* 1987; Oda *et al.* 1994). Two members of the class I subtype of cadherins are expressed in the early *Drosophila* embryo: E-cad (encoded by *shotgun*) and N-Cadherin (N-cad) (Oda *et al.* 1994; Iwai *et al.* 1997). Similar to classical EMT, mesoderm cells also lose their apicobasal polarity and adopt mesenchymal morphology, a process accompanied by decreases in AJ number and E-cad levels, and an increase in N-cad levels (Oda *et al.* 1998; Schäfer *et al.* 2014). However, while cells that have undergone a complete EMT process effectively migrate as individuals (Nieto 2011), mesoderm cells continue to maintain cell-cell contacts and move as a collective presumably because they retain transient AJs (Sun and Stathopoulos 2018).

Unlike the classical EMT model in which expression of Sna leads to AJ disassembly, the strength of AJs as indicated by levels of E-cad/β-cat [encoded by *armadillo* (*arm*)] complex increases during invagination (Lamouille *et al.* 2014; Nieto *et al.* 2016). This is due to actomyosin contraction at the apical cortex countering Sna function and increasing E-cad levels at AJs, presumably through the regulation of endocytic vesicle trafficking (Levayer *et al.* 2011; Weng and Wieschaus 2016, 2017). As the tube collapses, mesoderm cells undergo EMT. Tension is released as myosin expression diminishes. Baz levels and the number of AJs decrease; however, both remain detectable throughout the subsequent process of mesoderm migration (Weng and Wieschaus 2016; Sun and Stathopoulos 2018), supporting the view that EMT is progressive. Surprisingly, overexpression of E-cad or N-cad in the mesoderm zygotically, or ubiquitously maternally, does not perturb invagination or EMT. This lack of phenotype upon increase in cadherin levels has several possible explanations, including (1) the cortical localization of E-cad or N-cad is under strict regulatory control; or (2) the normal downregulation of E-cad is not the only mechanism toward EMT, and may be working together with several other pathways including FGF signaling and mitosis (Schäfer *et al.* 2014). The fact that mesoderm cells in the tube still disperse (*i.e.*, collapse occurs) when E-cad is overexpressed in a mutant background blocking both FGF signaling and mitosis, suggests the presence of additional players in promoting EMT (Clark *et al.* 2011; Sun and Stathopoulos 2018).

Symmetric collapse of the ventral tube is crucial for normal mesoderm development, in part because it helps to distribute the mesoderm equally to both left and right sides of the embryo. Although the exact mechanism that ensures this symmetry remains unknown, it likely depends on the cell shape change of mesoderm cells in the mediolateral position of the tube and their connections with the ectoderm through cytoplasmic extensions (Schumacher *et al.* 2004; Wilson *et al.* 2005). These protrusions or cytoplasmic extensions, resembling filopodia, extend from the mesoderm toward ectoderm cells and are absent in *htl* mutants, which exhibit asymmetric tube collapse. FGF signaling is therefore hypothesized to induce the chemoattractive movement of mesoderm cells evenly toward the ectoderm, such that cells disperse from the ventral tube in a symmetrical manner (Bae *et al.* 2012).

The number of mesoderm cells increases through tightly regulated processes of mitotic cell division during gastrulation (Figure 2, J–M). It is thought that cell division helps to decrease mesoderm cell-cell attachment, thus supporting tube collapse and EMT (Clark *et al.* 2011; Sun and Stathopoulos 2018). However, successful tube formation necessitates the presumptive mesoderm cells ingressing in a synchronous manner and requires cell cycle progression to be arrested. The derepression of cell division coincides with the initiation of EMT (Figure 2K). Specifically, Twi activates the dual-specificity phosphatase Cdc25 [encoded by *string* (*stg*)], which dephosphorylates Cyclin-dependent kinase 1 (Cdk1) and induces proliferation (O'Farrell 2001). The role of Cdc25 is actively countered in mesoderm cells by zygotically expressed Tribbles (Trbl), Frühstart (Frs/Z600), and maternally supplied Held Out Wing (How) (Grosshans and Wieschaus 2000; Nabel-Rosen *et al.* 2005). Trbl is a serine-threonine pseudokinase

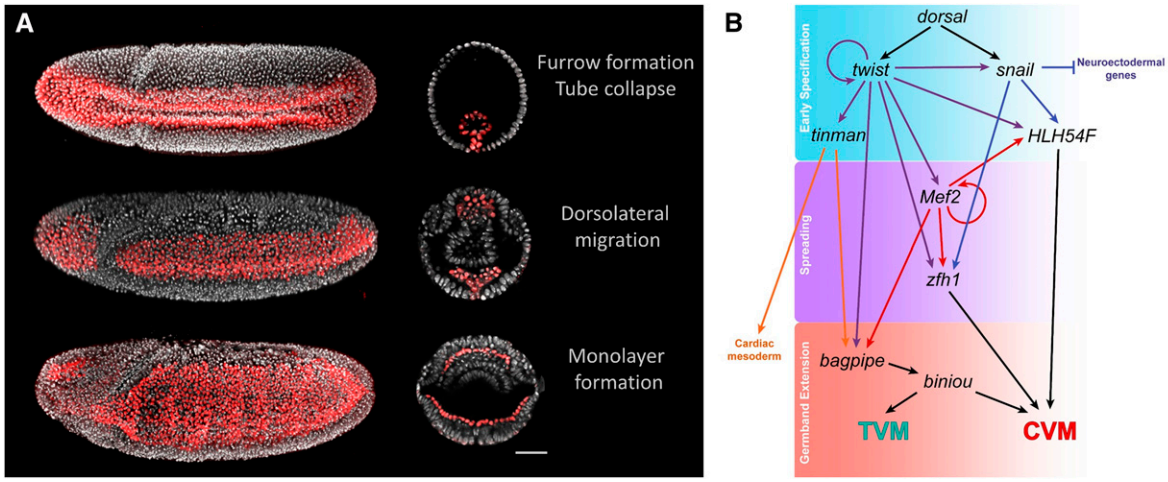


**Figure 3** Stages of early mesoderm spreading. (A) Mesoderm (red) shown in whole-mount (left) and sectioned (right) embryos to illustrate the different stages of mesoderm spreading from furrow formation (top) until monolayer formation (bottom). Embryos were stained with anti-Twist antibody (red) to label mesoderm and with DAPI (gray) to label all nuclei. (B) Gene regulatory network diagram demonstrating how presumptive mesoderm cells specified in the course of dorsal-ventral patterning early (stage 5, blue box; see also Figure 2A) support at later stages (*i.e.*, stages 6–10: purple and red boxes) expression of distinct subtypes of mesoderm cells including cardiac, trunk visceral mesoderm (TVM), and caudal visceral mesoderm (CVM), depending on combinatorial input from a number of different genes encoding transcription factors: *tinman*, *Mef2*, *bagpipe*, *zfh1*, *HLH54F*, and *biniou*. Scale bar, 50 μm.

domain-containing protein that functions to control the timing of cell division by promoting Cdc25 degradation in the mesoderm (Mata *et al.* 2000; Seher and Leptin 2000). Small basic protein Frs was also found to delay cycle progression, possibly by binding to cyclins and interfering with Cdk1 function (Grosshans and Wieschaus 2000; Gawliński *et al.* 2007). The RNA binding protein How acts to post-transcriptionally downregulate activity of *cdc25* and inhibit premature mesoderm cell division. Mutants depleted of both maternal and zygotic *how* exhibit severely delayed invagination (Nabel-Rosen *et al.* 2005). It is thought that these factors act to prevent precocious mitosis in cells of the ventral furrow region while they are undergoing morphogenetic changes, thus supporting invagination (Grosshans and Wieschaus 2000). Therefore, the cell cycle has to be strictly controlled during EMT.

## Mesoderm Spreading

### *Dorsolateral migration followed by monolayer formation*

***Dorsolateral migration:*** Dorsal migration follows the collapse of the invaginated tube, as mesoderm cells crawl over the ectoderm and symmetrically move away from the midline of the embryo (Figure 2F–H and Figure 3A). As mentioned above, EMT is prolonged, continuing throughout mesoderm migration and likely supports collective movement. Cells gradually downregulate their intercellular adhesive contacts, while retaining interactions needed to ensure coordinated movement of the migrating collective (Sun and Stathopoulos 2018). As a result, migrating mesoderm cells, although mesenchymal in nature, are also well connected through transient AJs.

Mesoderm cell migration takes place in a spatially constrained environment between the ectoderm and the yolk ~4 hr after egg laying (stages 8 and 9), and cells require ~1 hr to reach the dorsal ectoderm before intercalation ensues (Figure 2, H and I; McMahon *et al.* 2010). There is no evidence supporting the existence of an extracellular matrix (ECM) between the ectoderm and mesoderm as mesoderm cells interact directly with ectoderm cells. As they migrate, mesoderm cells send radial protrusions toward the ectoderm as well as protrusions oriented dorsolaterally in the direction of their migration (McMahon *et al.* 2010; Clark *et al.* 2011).

Furthermore, concomitant with the processes of tube collapse and the onset of dorsal migration, the embryo undergoes GBE. Individual cell tracking of live *in vivo* imaging data (Figure 4) has revealed that mesoderm cells move in a posteriorly oriented fashion aided by the movement of the ectoderm substrate during GBE, while simultaneously migrating in a dorsolaterally oriented (*i.e.*, azimuthal) direction away from the midline (Murray and Saint 2007; McMahon *et al.* 2008). This migration strategy can be likened to moving sideways on a moving walkway, and ultimately allows the mesoderm cells to make direct contact with the dorsal ectoderm. Cell-tracking experiments have also shown that this migration process is collective and coordinated, as mesoderm cells generally maintain their relative positions from the tube stage (through retention of local cell-cell contacts) during dorsolateral migration (before intercalation) (Murray and Saint 2007; McMahon *et al.* 2008). Additionally, cells do not cross the midline, further supporting the view that their movement is directional (Figure 4C).

As discussed above, each mesoderm cell undergoes two rounds of cell division, with the first division initiating during tube collapse (*e.g.*, Figure 2J, K; Borkowski *et al.* 1995). Tracking analysis of individual mesoderm cells (*i.e.*, both migration trajectory and mitosis) has revealed that cells that

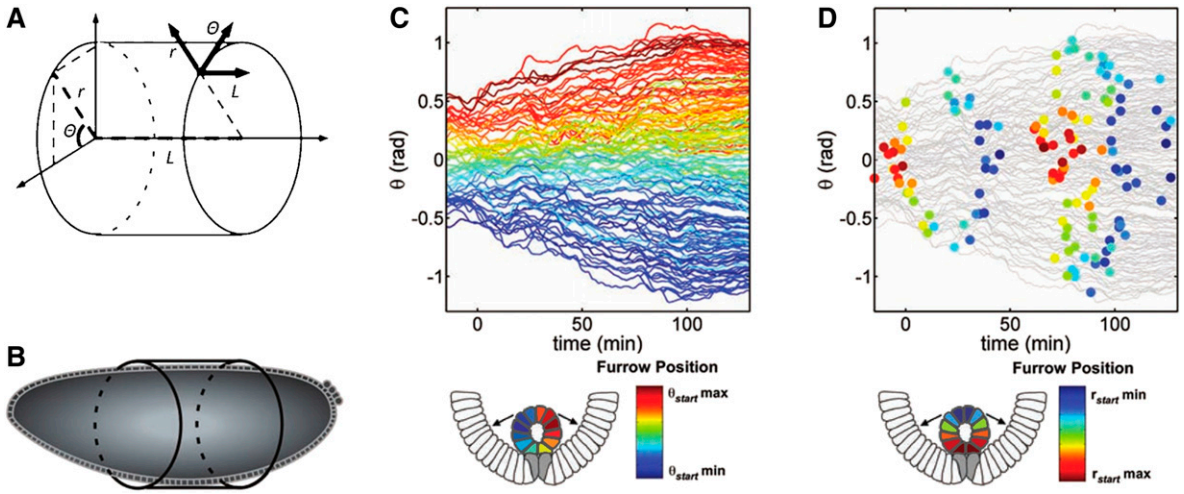


**Figure 4** Mesoderm spreading. (A–B) The position of each individual cell in the middle portion of a stage 5 embryo can be defined using cylindrical coordinates. In this system, each axis corresponds to a morphogenetic movement. Mesodermal cell movements directed toward the underlying ectoderm, such as tube collapse and intercalation, are occurring in the radial (*r*) direction; dorsolateral migration occurs in the angular (*i.e.*, azimuthal, *θ*) direction; and anterior-posterior movements, like germband extension, occur in the longitudinal (*L*) direction. (C) Spatial organization of cells in the azimuthal (*θ*) direction is preserved over time. The color code marks the angular position of cells within the invaginated tube at stage 7 and shows the spatial organization as cells move over time. Each line represents the trajectory of one cell. (D) Position and timing of cell divisions (colored circles) during spreading. The color code represents the radial position in the invagined tube at stage 7. Cells positioned at the ventral part of the invaginated tube divide first, followed by those cells originally located in more dorsal positions. Figure adapted from McMahon *et al.* (2008), with permission.

originate in the ventral-most position of the invaginated tube (*i.e.*, closest to ectoderm) divide first, followed by those located in successively more dorsal positions (Figure 4D; McMahon *et al.* 2008). The second mesoderm cell division occurs at the end of spreading approximately an hour after tube collapse and is synchronous with radially oriented cell intercalation (McMahon *et al.* 2010); additionally, the order of cell divisions is maintained according to their original position in the tube.

While it remains unclear exactly how mesoderm cell migration is regulated, a number of mechanisms have been proposed. Spreading may be mediated by the physical constraints of the environment, differential adhesion, or external cues (*e.g.*, chemoattractants). More specifically, the physical constraints of the environment (*i.e.*, being surrounded by ectoderm and yolk) may bias mesoderm cells toward dorsolaterally oriented movement in the direction of least resistance. Alternatively, in models that invoke differential adhesion (Murray and Saint 2007), mesoderm cells may migrate directionally and ultimately reach the dorsal ectoderm due to preferential interaction with ectoderm cells and/or the yolk. In support of this model, AJs are observed between yolk membrane and mesoderm cells, and are also enriched at the mesoderm-ectoderm interface (Tepass and Hartenstein 1994; Sun and Stathopoulos 2018), supporting the view that mesoderm cells continue to attach to ectoderm cells after collapse. Additionally, E-cad was identified via a genetic screen to affect mesoderm spreading upon ectopic expression (Trisnadi and Stathopoulos 2015), suggesting that regulation of cell-cell adhesion levels is important. Lastly, dorsal migration is thought to be guided by external cues, such as chemoattractants originated from the dorsal ectoderm that instruct the dorsolaterally directed movement of mesoderm cells.

This chemoattractant model has received the most attention, likely because mesoderm cells at the dorsal edge of the migration front contain high concentration of di-phosphorylated ERK (dpERK), a kinase activated downstream of receptor tyrosine kinases (RTKs) in response to MAPK (ERK) signaling (Gabay *et al.* 1997; Wilson *et al.* 2005) (Figure 5A, dark red cells). Similarly, activation of the ERK intracellular signaling cascade (*i.e.*, dpERK) by chemoattractant cues, particularly in the migratory leading cells, has been reported in border cells (Duchek and Rørth 2001; Poukkula *et al.* 2011; Pocha and Montell 2014). This observation supports the view that FGFR Htl, an RTK, serves as a guidance receptor to regulate the directionality of mesoderm migration, and dpERK-enriched mesoderm cells at the dorsal edge may also serve as leaders (Figure 5A, red cells), while cells located at the back are followers (Figure 5A, blue and pink cells).

***Monolayer formation:*** Mesoderm spreading is finalized when a monolayer of cells forms, immediately after the second wave of mesoderm cell mitosis (Stathopoulos *et al.* 2004; McMahon *et al.* 2008). During this step, the multilayered mesoderm stops migrating and cells rearrange into a single layer through intercalation. Surprisingly, the intercalation of mesodermal cells in the radial direction does not lead to further dorsal spreading (McMahon *et al.* 2008). Therefore, this process is more similar to zippering than a convergent extension. Integrins, specifically the β1-integrin subunit (βPS1) Myospheroid (Mys; discussed below in more detail), are found to be enriched at the mesoderm-ectoderm

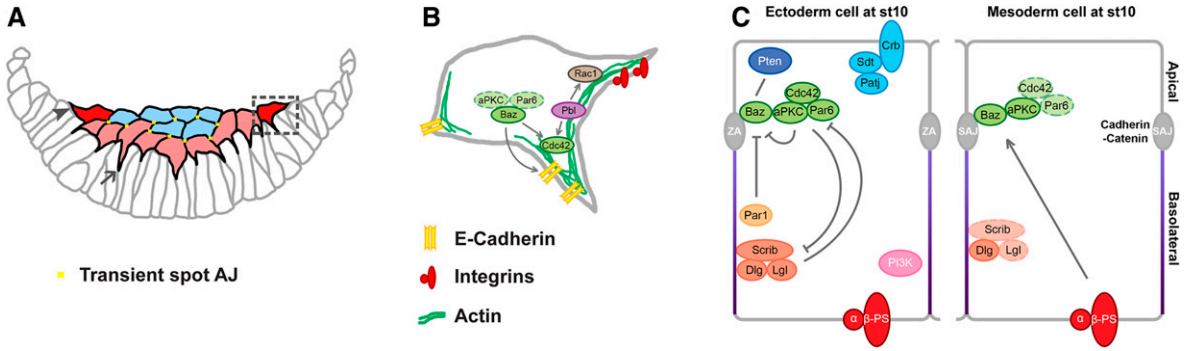


**Figure 5** Polarity in mesoderm cells. (A) Diagram of a transverse section from the bottom half of a stage 8 embryo, showing front–back polarity present in migrating mesoderm cells. Two types of protrusions are present: radial protrusions into the ectoderm (arrow) and dorsolateral protrusions in line with the direction of movement (arrowhead) associated with cells at the front (red). Connections with the neighboring mesoderm cells, present at the back (blue) are supported by transient spot AJs (yellow dots), to ensure the collectiveness of mesoderm migration. Cells at the dorsal edge (darker red) are dpERK-positive. (B) Magnified view of a polarized mesoderm cell from region of dashed box in A, showing molecular players. E-Cadherin (yellow) is enriched at the basal interface and integrins (red) are localized to the dorsal portion of the basal membrane (may be ectoderm-originated). Adhesion molecules are connected through the actin cytoskeleton (green). Baz can associate with Cadherin-Catenin complex but also promotes the localization of Cdc42 to the membrane, where Pbl activates Cdc42 to control actin dynamics and the formation of radial protrusions. Dorsal protrusions, on the other hand, require Pbl-activated Rac GTPases. (C) Schematic of *Drosophila* ectoderm cell *vs.* mesoderm cell at stage 10. In epithelial cells, Baz is enriched at AJs [zonula adherens (ZAs)] together with Cadherin-Catenin complexes. The establishment and maintenance of the apical-basal axis in the epithelium depends on the competitive interactions between apical *vs.* basolateral complexes (left). Mesoderm cells at stage 10 with integrins localized to the basal membrane and Baz associated with Cadherin-Catenin complex at spot AJs (SAJs; right). Localization of Baz in mesoderm cells depends on the function of integrins. Proteins encircled by dashed lines indicate suggested interactions. Basolateral membrane marked in purple shows domain of Neurotactin localization.

interphase during this final stage of mesoderm spreading (Figure 5C; MacKrell *et al.* 1988; Leptin *et al.* 1989; McMahon *et al.* 2010). Mutant embryos devoid of maternal and zygotic Mys present a multilayered phenotype, demonstrating that integrins are critical for intercalation (McMahon *et al.* 2010).

Mesoderm cells also undergo a mesenchymal-to-epithelial transition (MET) at this stage (Kadam *et al.* 2009; Sun and Stathopoulos 2018). As a result, Mys is localized to the basal side of cells, whereas the apical polarity regulator Baz localizes to the presumptive apical side (Figure 5C; Macara 2004; Sun and Stathopoulos 2018). Presumably, monolayer formation facilitates even distribution of mesoderm cells in the space between the ectoderm and the yolk to ensure the consistency of their subsequent differentiation.

Previous studies have suggested that apicobasal polarity is completely lost upon collapse of the invaginated tube (Oda *et al.* 1998); however, the process of EMT is progressive and mesoderm cells exhibit differences in polarity within the population. Indeed, mesoderm cells become spherical during collapse as mitosis also happens at this time, in contrast to their earlier wedge-shape when present within the invaginated tube of the gastrulating embryo. However, as these cells respond to cues from the ectoderm substratum and possibly also from the yolk and move in a directional manner, it is likely that some degree of polarity is restored subsequent to tube collapse, especially during the dorsolaterally directed migratory phase. This view is also supported by the observation that actively migrating mesoderm cells send E-cad-enriched protrusions into the ectoderm, interacting with the substrate from the basal side (McMahon *et al.* 2010; Clark *et al.* 2011).

### *Protrusive activity and cell polarity*

***Protrusive activity and front–back polarity:*** Formation of membrane protrusions is a common feature of migrating cells. Protrusions are extensions of the plasma membrane usually generated in response to external stimuli, such as growth factors or cytokines, the concentrations of which change spatiotemporally as cells migrate and often activate the Ras-ERK/MAPK intracellular signaling cascade (Huang *et al.* 2004). Migrating mesoderm cells are also front–back polarized as they actively send out protrusions in radial and dorsolateral directions (Figure 5A; Clark *et al.* 2011). The migration front attaches to the ectoderm cells, while the rear contacts the neighboring mesoderm cells (Schumacher *et al.* 2004). The protrusions at the front are driven by small GTPases and the actin cytoskeleton (Figure 5B). Presumably, the directional cue provided by localized activation of FGFR Htl is responsible for the enrichment of the larger and more persistent protrusions at the migration front, where mesoderm cells interact with the ectoderm (Gryzik and Müller 2004; Schumacher *et al.* 2004; McMahon *et al.* 2010).

FGFRs, which are RTKs, also signal through the Ras-ERK/MAPK branch in *Drosophila* (Muha and Müller 2013; Shilo 2014). Membrane protrusions and integrin-based adhesion both could be regulated by FGF signaling pathway, as MAPK activation results in cytoskeleton changes and focal adhesion turnover (Huang *et al.* 2004). During gastrulation activation of the MAPK pathway, resulting in dpERK, is first detected in the mesoderm tube in an FGF-dependent manner, most

noticeable in the cells at the mediolateral position that form long, cytoplasmic extensions toward the ectoderm (Wilson *et al.* 2005). Active migrating cells at the dorsal edge also accumulate dpERK (Figure 5A), which is *htl*-dependent as well (Michelson *et al.* 1998). Therefore, it appears that downstream of FGFR, mesoderm cells that establish initial contacts with the ectoderm experience the highest MAPK signaling pathway activation.

Protrusions take many forms, including filopodia, lamellipodia, lobopodia, invadopodia, and blebbing (Ridley 2011). The requisite cell shape changes that give rise to these varied forms are the subject of extensive study; for instance, several members of the Rho family have been shown to contribute to the cell shape change and the formation of the cytoplasmic extensions of mesoderm cells (Wilson *et al.* 2005; Clark *et al.* 2011), expanded upon in the sections below.

*Cdc42 and protrusions:* Cdc42, a small GTPase of the Rho family, plays an evolutionarily conserved role in regulating cell polarity (Etienne-Manneville 2004). Cdc42 cycles between a GDP-bound inactive state and a GTP-bound active state, whereupon it can signal to its downstream effectors. Cdc42-GTP levels are regulated positively by guanine nucleotide exchange factors (GEFs) and negatively by GTPase-activating proteins (Etienne-Manneville and Hall 2002). Additionally, Cdc42 can be activated through junction proteins at cell-cell contacts as well as by integrin, Phosphatidylinositol-3-kinase (PI3K), heterotrimeric G protein, or RTK signaling (Liu and Burridge 2000; Etienne-Manneville and Hall 2001; Chou *et al.* 2003; Li *et al.* 2003; Merlot and Firtel 2003). Besides controlling actomyosin and adhesion dynamics in protrusions, Cdc42 also regulates microtubule organization, vesicle trafficking, and apicobasal polarity (Figure 5A; Hutterer *et al.* 2004).

In *Drosophila* embryos, GTP-bound Cdc42 binds to and activates the Wiskott–Aldrich Syndrome proteins, which in turn bind and activate the Arp2/3 complex to induce actin polymerization and protrusion formation (Etienne-Manneville 2004). During early embryogenesis, Cdc42 is required for the proper function of the actomyosin cytoskeleton in furrow canal formation and nuclei positioning; disrupting Cdc42 function results in developmental arrest at cellularization (Crawford *et al.* 1998). During both tube collapse and dorsolateral spreading, mesoderm cells actively send out actin-rich radial protrusions into the ectodermal layer, and expressing a dominant negative form of Cdc42 significantly reduces this protrusive activity and E-cad accumulation at the mesoderm-ectoderm interface (Figure 5, A and B; Clark *et al.* 2011). The dorsolaterally directed protrusions formed at the leading edge of the migrating mesoderm cell collective, on the other hand, are independent of Cdc42 but require Rac activity, which is discussed in the following paragraphs along with RhoGEFs. It has been suggested that different intracellular signaling responses downstream of FGF control the formation of the two types of protrusions (Clark *et al.* 2011).

*RhoGEF Pebble:* Pebble (Pbl), the *Drosophila* ortholog of the human proto-oncogene Ect2, has also been implicated in mesoderm migration (Schumacher *et al.* 2004; Smallhorn *et al.* 2004; van Impel *et al.* 2009). In *pbl* mutants, EMT is stalled as the cytoplasmic extensions from the base of the mesodermal tube are not induced, neither does dpERK enrichment occur (Schumacher *et al.* 2004; Wilson *et al.* 2005). As cells move dorsolaterally, the dorsal protrusions are dramatically reduced and mesoderm cells appear much more tightly associated with each other (Schumacher *et al.* 2004; Smallhorn *et al.* 2004). Pbl's conserved role in supporting cytokinesis relates to its substrate Rho1 and can be separated from the regulation of mesoderm migration (Prokopenko *et al.* 1999; Schumacher *et al.* 2004). As the requirement for Pbl in cytokinesis is suppressed by blocking mitosis (*i.e.*, in *pbl*, *stg* double mutants); nevertheless, defects in mesoderm migration remain evident (Schumacher *et al.* 2004). Furthermore, expression of a mutant form of Pbl that lacks its N-terminal BRCT (BRCA1 C-terminal) domains rescues the mesoderm phenotype but not the cytokinetic phenotype (Smallhorn *et al.* 2004). Therefore, Pbl supports a role in mesoderm migration that is independent of its function in cytokinesis.

Two studies have associated Pbl with Rac GTPases (Wilson *et al.* 2005; van Impel *et al.* 2009). Embryos derived from mothers with reduced maternal *Rac* function show similar phenotypes to *pbl* mutants, and EMT is compromised as mesoderm cells fail to establish contact with the ectoderm due to the lack of protrusions (Wilson *et al.* 2005; van Impel *et al.* 2009). Furthermore, Rac1 and Rac2 interact with Pbl biochemically (*in vitro*) and genetically (*in vivo*), and also are essential for mesoderm migration (van Impel *et al.* 2009). Pbl is localized to both the nucleus and the cell cortex of mesoderm cells, but it is Pbl's cortical localization, mediated by its Pleckstrin homology (PH) domain and a conserved C-terminal tail, that is essential for Rac activation to support protrusion formation and mesoderm migration (van Impel *et al.* 2009). A screen aimed at identifying new genes that genetically interact with *pbl* uncovered phosphatidylinositol phosphate regulators (Murray *et al.* 2012). This interaction likely relates to the ability of [PtdIns(4,5)P2, PIP2] to recruit Pbl to the plasma membrane through its PH domain and, ultimately, to influence Rac activity.

***Apicobasal polarity:*** *Baz, aPKC, and the AJs:* During mesoderm spreading, polarity regulator Baz and AJs (the latter assayed through colocalization of α- and β-cats) remains detectable in the mesoderm cells throughout the entire process of EMT and dorsolateral migration, until the monolayer forms (Sun and Stathopoulos 2018). Cell adhesion and cell polarity are tightly regulated, and it is possible that Baz and other polarity regulators support the collective movement of mesoderm cells during gastrulation through regulation of cell-cell adhesion. Therefore, understanding how mesoderm migration is coordinated requires a focus on genes involved in both polarity and adhesion, detailed below.

During dorsolateral migration, AJs are formed transiently between highly motile mesoderm cells and do not necessarily

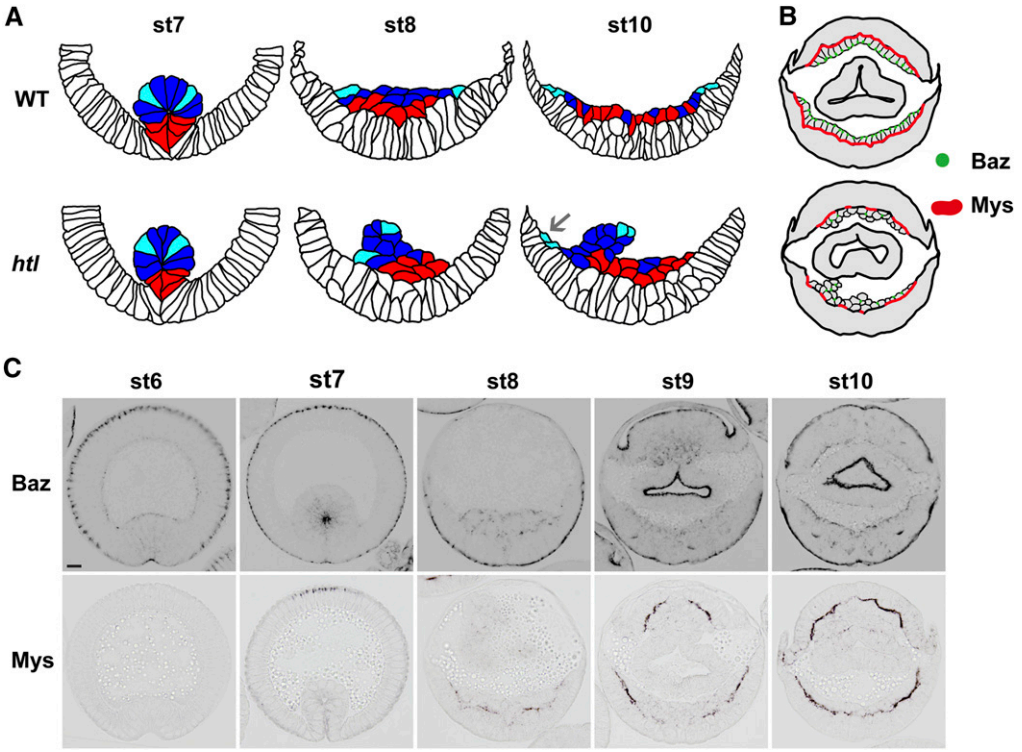


**Figure 6** Comparison of mesoderm cell movement between wild-type and *htl* mutant embryos and expression of Baz and Mys in wild-type embryos during gastrulation. (A) Schematic model of mesoderm migration based on McMahon *et al.* (2008) and Murray and Saint (2007), reproduced from Bae *et al.* (2012), with permission. In wild-type (WT) embryos, mesoderm cells at mediolateral position in the tube (cyan) migrate over the outer cells (blue) and reach the dorsal-most ectoderm region at stage 10. In *htl* mutants, only cells contacting the ectoderm undergo directed movement (arrow). Cells at the bottom of the tube (red) are in contact with the ectoderm. (B) Diagram showing stage 10 WT and *htl* mutant embryos with expression of Mys (red) and Baz (green). Mys is localized to the interface between the mesoderm and ectoderm, while Baz is localized to the apical side of mesoderm cells in the WT embryo but fail to do so in the mutant embryo. (C) Cross-sections of stage 6–10 WT embryos stained with antibodies against Baz and Mys. Scale bar, 10 μm.

colocalize with Baz as found in the embryonic ectoderm (Harris and Peifer 2005). These AJs are likely transient, spot AJs in contrast to the belt-like zonula adherens in mature epithelial cells, and may be composed of different protein complexes. At stage 10, Baz as well as AJs are repolarized and enriched to the apical side of mesoderm monolayer facing the yolk (Figure 6C), marking the earliest MET process observed during *Drosophila* embryogenesis. aPKC is localized to the same membrane domain in a Baz-dependent manner (Sun and Stathopoulos 2018). Loss of either Baz or aPKC in the mesoderm also leads to defects in spreading (Sun and Stathopoulos 2018).

It remains unclear how Baz interacts with aPKC and/or AJs in the mesoderm; however, Mys placement on the opposite side (*i.e.*, the presumptive basal side) of the cells at the monolayer stage is necessary to localize Baz apically. Removing both maternal and zygotic contribution of Mys from the embryo also completely abolishes Baz expression (Sun and Stathopoulos 2018). Cell-tracking experiments revealed that *baz*, like *mys*, is required for monolayer formation (McMahon *et al.* 2010; Sun and Stathopoulos 2018). These results collectively support the view that Baz and Mys function cooperatively to control MET.

The transiently epithelialized mesodermal cell sheet, associated with monolayer formation at stage 10, lacks the Crb complex. However, Crb is apparently not always required for the establishment or maintenance of apicobasal polarity, and Baz has been shown to compensate for the lack of Crb (Tepass *et al.* 1990; Tanentzapf and Tepass 2003; Campbell *et al.* 2009). Furthermore, basolateral markers Neurotactin (Nrt) and Dlg are excluded from the apical membrane (Sun and Stathopoulos 2018), suggesting that mesoderm cells are polarized independent of Crb.

In summary, polarity regulators (*e.g.*, Baz, aPKC) play a central role in managing the behavior of AJs. By doing so, they function to maintain the collectiveness of the mesoderm cells as they migrate and contribute to monolayer formation, which ensures the proper distribution of differentiated mesoderm cell types.

*E-cad and N-cad dynamics during mesoderm spreading:* The early requirement of maternal E-cad for oogenesis and embryogenesis preclude analysis of its role in gastrulation (Tepass *et al.* 1996). It is gradually degraded in the mesoderm through a Sna-driven, post-transcriptional mechanism that requires Neuralized (an E3 ubiquitin ligase) and its inhibitors, the Bearded family proteins (Chanet and Schweisguth 2012). Zygotic E-cad on the other hand, although expressed at much lower levels as compared to in the ectoderm, accumulates at the mesoderm-ectoderm interface and becomes enriched in the mesodermal protrusions in a manner dependent on Cdc42 activity (Clark *et al.* 2011). RNA interference (RNAi)-mediated knockdown of E-cad in the mesoderm specifically reduces this basal localization (Sun and Stathopoulos 2018), suggesting that the homophilic interactions between E-cad molecules mediate the attachment of mesoderm cells to the ectoderm.

Transcription of *N-cad* is activated by Twi in the mesoderm anlage before apical constriction, and the protein becomes apparent by stage 9. However, the zygotic products of both Cadherins appear to be nonessential for mesoderm spreading. Intriguingly, the lower levels of E-cad in the mesoderm, appears to be important for monolayer formation and to enable proper levels of Wingless signaling during differentiation (Schäfer *et al.* 2014; Trisnadi and Stathopoulos 2015).

Consistent with the fact that N-cad and E-cad behave differently in recruiting Arm (Loureiro and Peifer 1998; Schäfer *et al.* 2014), they are preferentially enriched at distinct membrane surfaces upon MET. In the subapical domain of the monolayer, N-cad likely replaces E-cad in the Cadherin-Catenin clusters present to mediate cell-cell adhesion, whereas E-cad predominantly localizes to the basal side of mesoderm cells (Clark *et al.* 2011; Sun and Stathopoulos 2018).

*Phosphoinositide signaling and mesoderm cell polarity:* Phosphoinositides are lipids that mediate a wide variety of conserved cellular processes in both mammals and *Drosophila*, including the establishment of apicobasal polarity. In short, PIP2 promotes the growth of apical membrane while PtdIns(3,4,5)P3 (PIP3) promotes basolateral identity (Gassama-Diagne *et al.* 2006; Kierbel *et al.* 2007; Claret *et al.* 2014). Accumulation of PI3Ks and their catalytic product PIP3 is characteristic of basolateral identity (Pinal *et al.* 2006). PI3K and Rac GTPases have been shown to cooperatively antagonize the activity of apical regulator Crb in the embryonic epithelium (Chartier *et al.* 2011). *Drosophila* Pten, the phosphatase that negatively regulates PI3K pathway and dephosphorylates PIP3 to produce PIP2, associates with Baz at the subapical junction and enriches PIP2 to define the apical membrane domain (von Stein *et al.* 2005; Pinal *et al.* 2006). PIP2 has also been shown to tether Baz to the AJs (Claret *et al.* 2014), reinforcing this polarized distribution of phosphoinositides.

Phosphoinositides also play a role in mesoderm migration by contributing to the cortical localization of RhoGEF Pbl. Increasing levels of PIP2, normally enriched in the leading front of migrating cells, can compensate for a decrease in *pbl* (Murray *et al.* 2012). Similarly, overexpression of Pten rescues mesoderm migration in *pbl* mutants, while loss of Pten exacerbates their phenotype (Murray *et al.* 2012). It remains unclear how and which particular species of phosphoinositides are involved in controlling this process. Furthermore, enrichment of PIP2 rather than PIP3 is present at the leading front of mesoderm cells, which is clearly disparate from other models of cell migration (Insall and Weiner 2001; Pickering *et al.* 2013).

*Integrin βPS1 Mys, Rap1, and the differential affinity model:* Integrins comprise a diverse class of cell adhesion molecules that are required throughout development (Brown 2000; Campbell and Humphries 2011). Composed of α- and β-subunits, these molecules require interaction with ECM components to mediate their function, distinguishing them from other cell adhesion molecules like cadherins, which are capable of forming direct interactions. While 18 α-subunits and 8 β-subunits have been characterized for integrins in mammals, there are 5 α-subunits (α1–α5) and 2 β-subunits (β-PS and β*ν*) in *Drosophila*. In addition to conferring a structural function by serving as a tether between the actin cytoskeleton and the ECM, essentially allowing a migrating cell to move along a substratum, integrins can mediate downstream signaling such as activation of Rho GTPases, focal adhesion kinase, and the ERK and JNK pathways that support migration. Additionally, integrins can also mediate migration by priming/assembling ECM components as a substrate scaffold.

Mys is the only known *Drosophila* β-integrin expressed during gastrulation (Leptin *et al.* 1989). It is maternally supplied and also plays an early role in stimulating the BMP signaling pathway to help establish patterning of the DV axis (Sawala *et al.* 2015). At stage 8, Mys protein begins to localize to the mesoderm-ectoderm interface, representing the basal sides of both tissues (Figure 6C; McMahon *et al.* 2010; Sun and Stathopoulos 2018). By the end of stage 10, expression of laminin can be seen in the same region (Jingjing Sun and Angelike Stathopoulos, unpublished data), suggesting that an integrin-ECM interaction is engaged to anchor the mesoderm cells. Early morphogenesis is not affected in general, and mesoderm cells exhibit normal dorsolateral migration upon removal of both the maternal and zygotic product of the gene (Leptin *et al.* 1989). However, embryos derived from *mysXG43* germline clones exhibit defects in intercalation and monolayer formation, suggesting a requirement for *mys* at the final stage of this migration during MET (Sun and Stathopoulos 2018).

Furthermore, integrin function is commonly associated with basal identity and is capable of orienting cells into apicobasal polarity through activating Rac to direct ECM deposition (Yu *et al.* 2005; Fernandes *et al.* 2014). This is consistent with localization of Mys at the basal interface of the mesoderm monolayer, as well as its role in placing apical Baz and aPKC to establish cell polarity in these cells (Sun and Stathopoulos 2018). The correct localization of Mys depends on the expression of Inflated (α2-integrin), the *Drosophila* FGFR Htl and, most importantly, Rap1 (Ras-related protein 1) GTPase.

Rap1 is a Ras superfamily GTPase that functions independently from Ras1 to support morphogenesis in *Drosophila* embryos (Asha *et al.* 1999). Rap1 is activated by *Drosophila* PDZ-GEF Dizzy (Dzy) (Lee *et al.* 2002; Spahn *et al.* 2012). Both maternally loaded, Rap1 and Dzy play diverse roles that are critical during embryogenesis. For instance, Rap1 is known to be essential for the correct localization of Baz and AJs; and like other Rho GTPases, it regulates the cytoskeleton and supports cell shape change and polarity formation in the epithelium (Knox and Brown 2002; Spahn *et al.* 2012; Choi *et al.* 2013). Notably, a Dzy-Rap1-Mys pathway has been shown to promote directional migration of macrophages by increasing adhesion to stabilize cellular protrusions (Huelsmann

*et al.* 2006). Indeed, expression of Mys at stage 10 is completely lost in embryos derived from *rap1* germline clones; however, they exhibit far more severe mesoderm phenotypes compared to those of embryos derived from $mys^{XG43}$ germline clones (McMahon *et al.* 2010), including much diminished and mislocalized Baz. Therefore, the mesoderm defect of *Rap1* mutant embryos is likely due to a combination of dysregulated, Mys-mediated adhesion as well as mislocalized Baz, AJs, and associated cytoskeleton components.

### Signaling pathway inputs

***FGF mutant phenotypes and roles:*** The functional requirement of FGF signaling in mesoderm development in *Drosophila* is well documented (Beiman *et al.* 1996; Gisselbrecht *et al.* 1996). In zygotic *htl* loss-of-function mutants, mesoderm cells fail to evenly spread over the ectoderm, leading to failure in differentiation of multiple muscle lineages, including the dorsal somatic muscle, the visceral mesoderm, and the heart (Beiman *et al.* 1996; Gisselbrecht *et al.* 1996; Shishido *et al.* 1997). Mutations in both FGF ligand-encoding genes *pyramus* (*pyr*) and *thisbe* (*ths*), as well as mutation of the gene *dof/stumps/heartbroken*, cause generally similar defects in mesoderm cell migration and differentiation, suggesting that these genes act through Htl FGF signaling (Michelson *et al.* 1998; Vincent *et al.* 1998; Stathopoulos *et al.* 2004; Kadam *et al.* 2009; Klingseisen *et al.* 2009).

Numerous attempts have been made to understand the migration phenotypes associated with FGF mutants (McMahon *et al.* 2010; Clark *et al.* 2011; Sun and Stathopoulos 2018), including a cell-tracking study discussed above (*e.g.*, Figure 4; McMahon *et al.* 2008). Tracking live mesodermal cells revealed that in *htl* mutant embryos furrow collapse is defective, frequently positioning cells to one side of the embryo, and spreading of the whole group was found to be less cohesive. Surprisingly, mesoderm cells in contact with the ectoderm are able to migrate laterally even in *htl* zygotic mutants, while mesoderm cells positioned at a distance from the ectoderm are not able to migrate directionally and result in a multilayered, "lumpy" mesoderm phenotype (Figure 6A). Although much is still unknown about the intracellular pathways downstream of Htl that act to control the behavior of mesoderm cells during migration, recent findings indicate that Htl-dependent FGF signaling clearly plays multiple roles contributing to both tube collapse/EMT and mesoderm spreading (Wilson *et al.* 2005; McMahon *et al.* 2010). More specifically, FGF signaling is required for (1) the support of symmetrical tube collapse [reviewed in Bae *et al.* (2012)], (2) the formation of protrusions in support of active migration (Schumacher *et al.* 2004; Klingseisen *et al.* 2009), (3) the synchronization of mesoderm cell divisions during EMT (McMahon *et al.* 2008), and (4) the increase in cell-cell adhesion/AJ numbers and polarity formation during MET (Sun and Stathopoulos 2018).

Monolayer formation at stage 10 represents an MET. This is based on the observation that (1) the AJs, Baz, and aPKC are repositioned to the subapical membrane domain (*e.g.*, Figure 5B), while Nrt is localized to the basolateral membrane; (2) expression of Mys and Laminin is present at the mesoderm-ectoderm interface in the presumptive basal domain; and (3) N-cad and E-cad are differentially localized to the apical and basal sides of mesoderm cell membranes, respectively (Sun and Stathopoulos 2018). Loss of function in *htl* severely disrupts MET and a monolayer is only achieved in scattered positions (Figure 6B). This random monolayer formation is not sufficient to polarize mesoderm cells; Baz and AJs fail to localize to the apical membrane domain, while Mys expression at the basal side is significantly reduced (Sun and Stathopoulos 2018). These results suggest that FGF signaling functions upstream of Mys to regulate the localization of Baz and polarity formation during MET.

While the FGFR Htl works cell autonomously to control apicobasal polarity, paracrine signals from FGF ligands expressed elsewhere are required to mediate this and other Htl-dependent functions. The genes encoding the two Htl FGF ligands, *ths* and *pyr*, were identified and found to be expressed during mesoderm spreading in the ectoderm in a dynamic manner (Stathopoulos *et al.* 2004). Double mutants for both *ths* and *pyr* have mesoderm spreading defects resembling the *htl* mutant phenotypes. Single mutants have variable mesodermal spreading defects, often exhibiting a nonmonolayer phenotype (Kadam *et al.* 2009; Klingseisen *et al.* 2009, 2010). Normal collapse of the furrow is likely dependent on correct *ths* expression pattern (McMahon *et al.* 2010), with influence of *pyr* also found to support symmetric collapse (Klingseisen *et al.* 2009). Whether the subsequent dorsal migration itself is FGF-mediated or abnormal spreading is a secondary effect of abnormal furrow collapse is still debated.

The more localized *pyr* pattern within the ectoderm at stages 8–10 (Figure 7A, A') and diminished dpERK signal in *pyr* mutants support an attractive *pyr*-dependent chemotactic model for dorsal spreading (Clark *et al.* 2011). However, two previous studies drew different conclusions regarding the role of Pyr ligand through *in vivo* live imaging (McMahon *et al.* 2010; Clark *et al.* 2011). It remains unclear whether Pyr serves as a chemoattractant cue to guide mesoderm migration by supporting MAPK activation at the leading edge of the migrating collective, or whether the dpERK signal present at the leading front instead serves to prepare cells for subsequent differentiation [*e.g.*, inducing *eve-skipped* (*eve*)] once they reach their final position in association with the dorsal ectoderm (Figure 7B, B'; Azpiazu and Frasch 1993; Carmena *et al.* 1998). The observation that cells contacting the ectoderm are still able to engage in lateral spreading in *htl* mutants (*e.g.*, Figure 6A, arrow; McMahon *et al.* 2008) suggests that a mechanism distinct from chemoattraction (*e.g.*, differential adhesion) supports spreading.

***Intracellular signaling downstream of FGFR activation:*** FGFs signal through RTKs, therefore the intracellular signaling responses share many components with other RTK signaling cascades. Genes that function commonly downstream of RTK activation, *e.g.*, Corkscrew (Csw), Downstream of

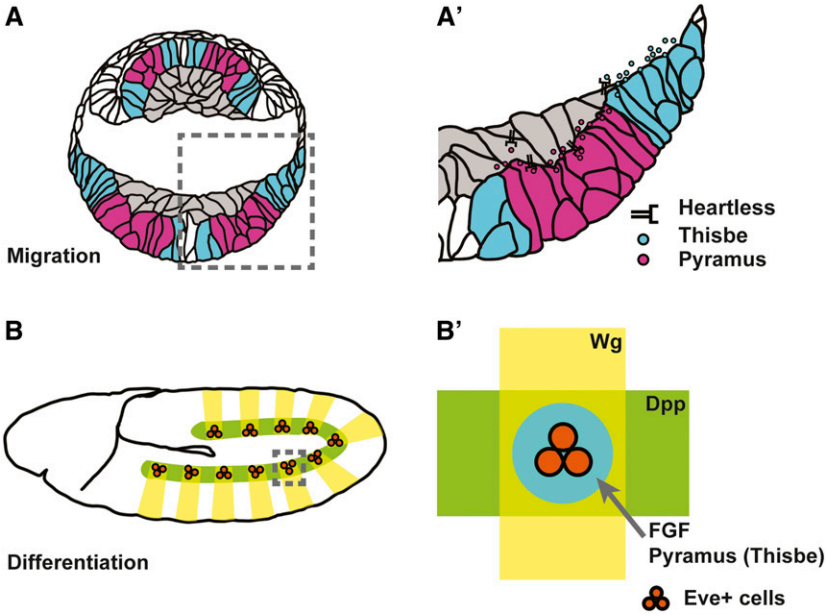


**Figure 7** A model for FGF signaling through Heartless. (A and A') The location of the Ths (pink) and Pyr (cyan) expression domains is important for the proper regulation of mesoderm migration. Both ligands are required, presumably, because they have different activities that possibly relate either to their range of action or differential activation of Htl-receptor intracellular signaling. Htl-expressing mesoderm cells are in gray. (B and B') During specification of dorsal mesoderm lineages, including specification of 2–4 Eve+ cells (orange), FGFs feed into an array of signaling molecules [Wingless (Wg; yellow) and Dpp (green)] necessary to specify dorsal mesoderm lineages. We suggest that any FGF ligand (cyan), expressed in the region of Dpp and Wg overlap and able to activate the respective FGFR, would suffice to support cell differentiation. Pyr is normally expressed in this domain (cyan) within the overlying ectoderm, but Ths may also contribute as it is diffusible. Figure adapted from Kadam *et al.* (2009), with permission.

receptor kinase (Drk, Grb2), Son of sevenless (Sos), *etc*., have been covered in a recent review focused on FGF signaling in *Drosophila* and will not be described here (Figure 8; Muha and Müller 2013). In brief, FGFs bind to their receptors and this interaction is stabilized by association with heparan sulfate proteoglycans (HSPGs). Formation of a FGF:FGFR:HSPG ternary complex is required to support signaling (Muha and Müller 2013; Trisnadi and Stathopoulos 2015). Downstream of receptor activation, Ras is activated and thought to increase motility of the mesodermal cells (Gisselbrecht *et al.* 1996; Imam *et al.* 1999).

Dof, also known as Heartbroken/Hbr or Stumps, was identified by several groups as a regulator of both mesoderm and tracheal cell migration (Michelson *et al.* 1998; Vincent *et al.* 1998; Imam *et al.* 1999); the latter is a process also regulated by FGF signaling, but through a different receptor encoded by the gene *breathless* (*btl*) (Sutherland *et al.* 1996). *dof* is only present in FGFR-expressing cells, and mutants exhibit defects in migration specifically dependent on the FGF-Ras-MAPK signaling branch, but not other RTKs (Vincent *et al.* 1998). Therefore, it is unlikely to be a general regulator of cell migration, but rather acts specifically in the FGF pathway. In *dof* mutants, MAPK is not activated during mesoderm spreading and later dorsal mesoderm structures are almost entirely absent. Expression of activated Htl fails to improve the mesoderm phenotype in *dof* mutants, while activated Ras results in a partial rescue, indicating Dof acts downstream of FGFR but upstream of, or in parallel with, Ras (Michelson *et al.* 1998; Imam *et al.* 1999). Dof serves as a modular scaffold protein to support intracellular signal transduction (Figure 8; Petit *et al.* 2004). In addition, cytosolic UDP-N-Acetylglucosamine (UDP-GlcNAc) levels are important for FGF signaling in *Drosophila*, and it has been shown that UDP-GlcNAc is required downstream of the receptor at the level of Dof (Mariappa *et al.* 2011). Dof was shown to be O-GlcNAcylated in an O-GlcNAc transferase-dependent fashion, indicating that post-translational modification of Dof by O-GlcNAcylation is required for its function in the pathway. The exact function of this modification on Dof's activity remains to be determined.

FGF signaling could also activate the PI3K pathway to regulate protrusive activity and cell polarity in the mesoderm (Muha and Müller 2013). This branch is thought to also require the presence of Dof, as this adaptor protein contains potential binding sites for PI3K and motifs similar to a vertebrate protein (BCAP) that is known to regulate PI3K activation (Battersby *et al.* 2003). However, whether Dof binds PI3K *in vivo* has not been confirmed, and it remains unclear how the PI3K pathway is involved in mesoderm migration.

Furthermore, Pbl plays a general role in regulating the actin cytoskeleton and promoting protrusion formation. It has been proposed that Pbl functions downstream of FGFR Htl but in parallel to the Ras-MAPK pathway, to induce mesoderm cell shape changes, as loss of function in *pbl* results in a grossly similar mesoderm phenotype to that of *htl* mutants while not affecting dpERK enrichment (Schumacher *et al.* 2004). Therefore, FGF signaling could act on Rac GTPases (Pbl substrate) and actin cytoskeleton through Pbl to induce protrusive activity among mesoderm cells during migration.

***Role of HSPGs in supporting FGF signaling:*** HSPGs comprise a core protein attached with highly modified heparan sulfate glycosaminoglycan side chains that are thought to provide specificity toward the regulation of multiple signaling pathways during development (Lin 2004). There are four known core proteins in *Drosophila*: transmembrane Syndecan (Sdc); two membrane-anchored glypicans Dally and Dally-like (Dlp); and the ECM protein Terribly reduced optic lobes (Trol) (Lin 2004). Trol is the homolog of mammalian HSPG Perlecan (Pcan), and several lines of evidence suggest

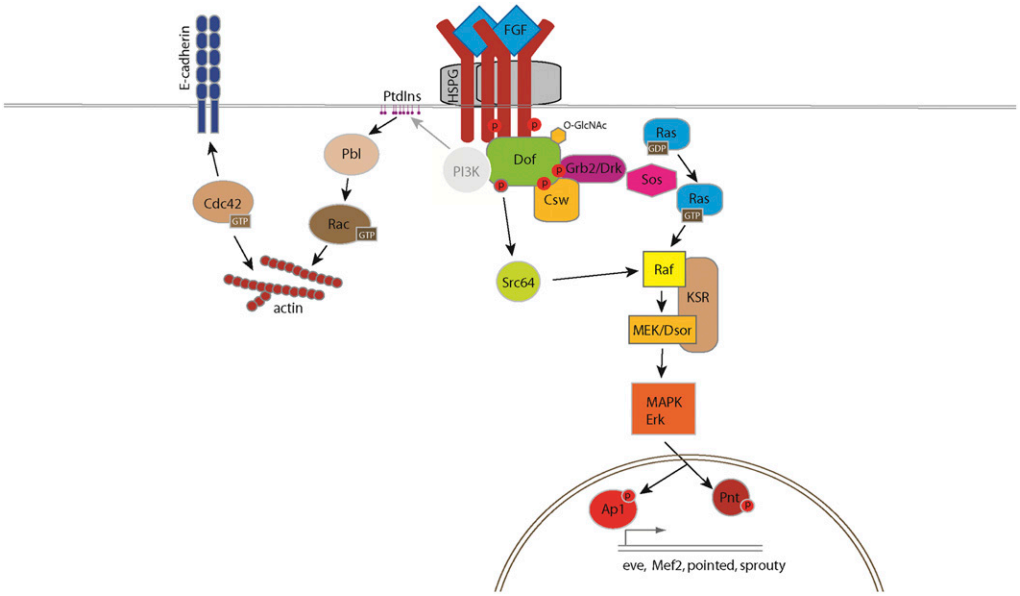


**Figure 8** Schematic model of the FGF signaling cascade in *Drosophila melanogaster*. FGFs bind to their FGFRs and this interaction is stabilized by heparan sulfate proteoglycans (HSPG). Activation of the FGFR leads to auto- and *trans*-phosphorylation of their tyrosine kinase domains and to phosphorylation of its adaptor protein Dof. Dof protein is O-GlcNAcylated (O-GlcNAc) and possesses multiple clusters of tyrosine residues directing the signal toward various cascades, three of which—the Csw/Shp2, Grb2/Drk, and Src64B pathways—have been proposed to contribute to MAPK activation. This route of FGF signaling is responsible for inducing gene transcription, and executing proliferative and antiapoptotic responses. Dof also contains a putative binding site for PI3K that could locally modify the phosphatidylinositol (PtdIns) composition of the plasma membrane, and thus recruit downstream signaling components for example the RhoGEF Pbl. Pbl acts on the Rac pathway to promote the formation of actin rich protrusions in the mesoderm. Actin polymerization is required for protrusion formation in a Rac-, Cdc42-, and FGF-dependent fashion. Putative interactions are indicated as gray arrows and the putative component of the *Drosophila* FGF pathway, PI3K, is represented as a circle with no border. Figure reproduced from Muha and Müller (2013), with permission.

that Pcan promotes FGF signaling in vertebrates (Farach-Carson *et al.* 2014). 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). Trol was isolated in an ectopic expression-based screen focused on identifying genes that affect mesoderm spreading at gastrulation (Trisnadi and Stathopoulos 2015). Mutants for transmembrane protein Sdc also were found to exhibit defects in mesoderm spreading at gastrulation as well as to affect cardiogenesis in late-stage embryos (Knox *et al.* 2011). Dally and Dlp have not been linked to FGF signaling. In a recent study, we compared and contrasted the roles of the two HSPGs Trol and Sdc, which had been linked to FGF signaling, to characterize their roles in supporting FGF-dependent processes in *Drosophila* embryos.

Comparing Trol and Sdc revealed spatiotemporal differences in their expression within early *Drosophila* embryos and distinct phenotypes relating to several FGF-dependent processes (Trisnadi and Stathopoulos 2015). Both Trol and Sdc are expressed in a pattern overlapping with FGF ligand Ths during mesoderm migration. However, Trol is localized to the ventral ectoderm earlier than Sdc, and likely the only HSPG to support FGF-dependent mesoderm tube collapse (Trisnadi and Stathopoulos 2015). As a secreted protein, Trol may be better suited to support developmental processes like tube collapse that require a longer range of action of FGF ligands (or require FGF ligands to act from a distance). In contrast, Sdc contains a transmembrane domain that might make it better suited toward controlling a local process such as mesoderm cell intercalation (*i.e.*, monolayer formation) or differentiation (*i.e.*, Eve expression). Consistent with this view, *trol* mutants exhibit a mesoderm tube collapse phenotype similar to *ths* mutants, whereas the Sdc mutant was found to exhibit defects in differentiation of dorsal somatic mesoderm lineages similar to the *pyr* mutant.

## CVM Cell Migration

### *Specification of visceral mesoderm lineages*

While the majority of the presumptive mesoderm requires robust inputs from both *sna* and *twi* for specification, a small population of cells at the very posterior tip of the mesoderm appears to require *sna*, but only minimally requires *twi*. These cells eventually form the longitudinal muscles enclosing the embryonic midgut and are called the CVM primordium (Nguyen and Xu 1998; Kusch and Reuter 1999). The specification of the CVM before GBE requires a gene regulatory network (Figure 3B) that first involves restricted expression of *sna* by *huckebein* and coordinated expression of the genes *brachyenteron* (*byn*) and *forkhead* (*fkh*). Together, *byn* and *fkh* are responsible for specification of both the posterior hindgut and the CVM (Kusch and Reuter 1999; Ismat *et al.* 2010). The loss of either *byn* or *fkh* results in fewer CVM cells being specified, and *byn*, *fkh* double mutants present a complete loss of CVM cells. Furthermore, the transcription factor *zn finger homeodomain 1* (*zfh1*) was also found to be involved in CVM specification (Kusch and Reuter 1999; Ismat *et al.* 2010). While expressed in the central nervous system as well as multiple differentiated mesodermal tissues (including the CVM), the highest levels of *zfh1* mRNA expression are observed at the anterior and posterior embryonic poles. Indeed,

pan-mesodermal expression of both *byn* and *fkh* in a *byn*, *fkh* double mutant background only results in ectopic CVM formation at the anterior- and posterior-most ends of the mesoderm, where *zfh1* levels are highest (Kusch and Reuter 1999). Loss of Zfh1 function also results in impaired migration of the CVM cells (Ismat *et al.* 2010), suggesting that this factor also is an important regulator of this process.

Additionally, the basic helix-loop-helix transcription factor HLH54F was found to play a crucial role in specifying the CVM (Ismat *et al.* 2010). Its expression is dependent on the combined activities of *byn* and *fkh* and is the earliest known marker of the CVM primordia, maintaining its expression throughout CVM migration (Georgias *et al.* 1997; Ismat *et al.* 2010). Loss of *HLH54F* similarly results in a complete absence of CVM cells. Analysis of a CVM-specific enhancer for the *Dorsocross* family of homeobox transcription factors uncovered a combinatorial role for *HLH54F* in conjunction with the forkhead family transcription factor *biniou* (*bin*), in supporting gene expression within CVM cells. Ectopic expression of either *HLH54F* or *bin* is sufficient to expand activity of this enhancer within the migrating CVM cohorts and coexpression of both transcription factors has a significantly stronger effect on expanding enhancer activity, suggesting that, together, these transcription factors promote expression of *HLH54F* in a feed-forward mechanism (Ismat *et al.* 2010).

The transcription factor *bin* is the only *Drosophila* ortholog of the FoxF subfamily of Forkhead (winged helix) domain proteins, and is expressed in all visceral muscle types in the developing embryo, including TVM, CVM, as well as hindgut visceral mesoderm (HVM) (Zaffran *et al.* 2001; Jakobsen *et al.* 2007). *bap* is required for proper *bin* expression in the presumptive TVM via a possible cross-regulatory positive feedback loop (as maintenance of *bap* expression is affected in *bin* mutants). Alternatively, *bin* expression in the CVM and HVM does not appear to be dependent on either *bap*, *dpp*, or *tin*, but CVM-specific expression requires *HLH54F* (Ismat *et al.* 2010).

### Migration of the CVM

Shortly after specification, the CVM cells undergo proliferation before arranging themselves into bilateral cohorts (Figure 9A). A population of nonmigratory *HLH54F*-positive cells remain at the origin proximal to the HVM (Ismat *et al.* 2010). The two groups of migratory CVM cells then migrate along the posterior midgut (PMG) before contacting the TVM. The migratory behavior of the CVM can best be described as cell streaming, as opposed to a tightly associated collective; the cells move in a more loosely associated conformation compared to other examples of collective cell migration, such as *Drosophila* border cells and zebrafish lateral line primordia, but are more tightly associated than individually co-migrating cells like the *Drosophila* primordial germ cells (PGCs), which are generally not considered an example of collective migration (Aman and Piotrowski 2010; rev. in; Pocha and Montell (2014)).

### Signaling

Migrating cells often form dynamic interactions with their extracellular milieu to accomplish forward movement, which may involve coupling extracellular cues with remodeling of adhesion and polarity complexes. In this section, we summarize what is currently known about the cell autonomous and cell-nonautonomous signaling pathways that regulate CVM migration, and describe the heterotypic interactions with other tissues that facilitate their forward movement.

***Integrin signaling:*** In addition to a requirement for integrin in mediating mesoderm spreading (see *Invagination* above), a role for integrin in migration has previously been characterized in three other tissue types in the *Drosophila* embryo: the midgut endoderm, the tracheal visceral branches, and the salivary glands (Roote and Zusman 1995; Martin-Bermudo *et al.* 1999; Bradley *et al.* 2003; Devenport and Brown 2004). As different tissues can express different integrin subunits, coordinated migration of these tissues (which all migrate along the trunk visceral mesoderm) requires heterotypic, combinatorial expression of α- and β-subunits (Reuter *et al.* 1993; Tepass and Hartenstein 1994). As discussed above, *mys* encodes the βPS1 and is widely expressed; expression of the β$\nu$-subunit is primarily confined to the midgut endoderm (Devenport and Brown 2004). βPS1 is able to form heterodimers with all five α-subunits. The TVM expresses αPS2, which is required for migration of the visceral branches and endoderm and is encoded by the gene *inflated* (Martin-Bermudo *et al.* 1999; Boube 2001). The CVM, on the other hand, expresses αPS1, which is encoded by the gene *multiple edematous wings*.

As the midgut–foregut transition corresponds to thoracic segments T1 and T2, these serve as convenient landmarks for assessing migration rate defects; the anterior-most CVM cells are able to reach T1 and T2 at the conclusion of stage 13 in normal development (Figure 9B). Additionally, CVM cells are able to arrange into bilateral groups and contact the midgut in embryos lacking TVM, such as *twi* and *bin* mutants (Kusch and Reuter 1999; Zaffran *et al.* 2001). As these CVM present arrested migration at abdominal segment A4, this segment can serve as a landmark for scoring a severe to complete loss of forward migration along the TVM. A loss of both maternal and zygotic expression of βPS1 results in CVM migration defects (Urbano *et al.* 2011). While the initial migration of the CVM from the PMG to the TVM appears to be unaffected in βPS1 mutants, only a small percentage of embryos present complete migration (up to T1 and T2) of the CVM; the majority display a gamut of mild-to-severe migratory delays. These migration defects are phenocopied in maternal and zygotic mutants for Talin, which is the first integrin-binding protein identified (Horwitz *et al.* 1986; Urbano *et al.* 2011).

***FGF signaling is required for directional CVM migration and survival:*** To accomplish their unique migratory behavior, CVM cells integrate extracellular signaling cues from surrounding tissues. A central role for FGF signaling via Pyr and Ths in supporting CVM migration and survival has been

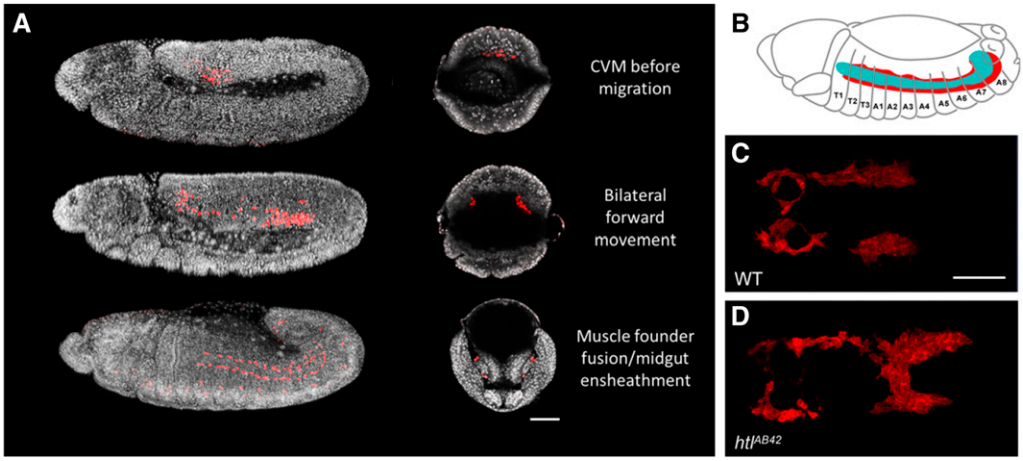


**Figure 9** Stages of CVM migration, embryonic segmentation guide, and comparison of normal and midline crossing phenotypes. (A) CVM cells (red) in whole-mount lateral view (left) and transverse cross section (right). Before initiating migration CVM cells begin as a single cohort of cells before splitting into two cohorts, before eventually dividing and arranging into longitudinal rows that sheath the midgut concomitant to germband retraction. Embryos were stained with an antibody against Teyrha-meyrha (Tey) protein (red), a marker used to visualize the CVM (Macabenta and Stathopoulos 2019a), and DAPI (gray) to label all nuclei. (B) Lateral view of stage 13 embryo showing three thoracic (T1–T3) and eight abdominal (A1–A8) segments. The CVM cells (red) normally reach T1–T2 at the conclusion of migration along the TVM (blue). (C and D) Dorsal view of embryos expressing a CVM-specific GFP reporter (Trisnadi and Stathopoulos 2015; Stepanik *et al.* 2016). During normal development, the CVM cells undergo symmetric migration as two cohorts (C); however, in *htl* mutants, the CVM cohorts fail to maintain bilateral migration and cross at the midline (D) (Kadam *et al.* 2012). Scale bar, 50 μm.

characterized (Kadam *et al.* 2012; Reim *et al.* 2012). At the onset of CVM migration at stage 11, *pyr* is initially expressed in the TVM and endoderm; as CVM migration progresses along the TVM, expression of *pyr* in the TVM is lost, while endodermal expression is maintained (Reim *et al.* 2012). Similar to *pyr*, *ths* is expressed in the TVM at stage 11, but is not expressed in the endoderm at any point. Additionally, TVM-specific expression of *ths* persists beyond that of *pyr*, well past stage 12.

Mutation of both FGFs or the *htl* FGF receptor results in significant migration defects in the CVM, including asynchrony and midline crossing of the bilateral cohorts (Figure 9, C and D; Kadam *et al.* 2012; Reim *et al.* 2012). Furthermore, FGF mutants present extensive cell death defects at stage 13, with very few CVM cells successfully migrating along the TVM postgermband retraction. Tissue-specific rescue of the cell death phenotype via GAL4/UAS-mediated expression of the antiapoptotic p35 protein in the CVM does not attenuate the cell migration defects, suggesting that additional functions for FGF are required to promote proper CVM pathfinding and migration (Reim *et al.* 2012).

***ECM proteins mediate CVM migration:*** As integrins both require ECM interactions for signaling and can reciprocally regulate ECM protein expression, researchers have investigated whether the integrins expressed in the CVM and TVM can modify the ECM. The ECM protein Nidogen (Ndg) is strongly expressed at the interface between the CVM and TVM from stage 12 onward, and the lamininW trimer is also expressed at the visceral mesoderm interface, starting at stage 13. Embryos that are mutant for the αPS2-integrin subunit not only present TVM morphology defects, but additionally present abnormal accumulation of Ndg at the visceral mesoderm interface (Urbano *et al.* 2011). This phenotype is not observed in αPS1 mutant embryos, suggesting that αPS2 in the TVM is primarily responsible for proper Ndg accumulation at the visceral mesoderm interface; however, αPS1 can sufficiently substitute for αPS2 in regulating Ndg expression.

Investigation of the potential roles of ECM proteins has revealed a requirement for laminin proteins in promoting forward migration of CVM cells; embryos that are mutant for the Laminin β-chain present significantly impaired anterior migration of the CVM cohorts without affecting either TVM morphology or CVM differentiation (Urbano *et al.* 2011). As there are two laminin trimers in *Drosophila* (lamininA, which is composed of the α3,5-, β1-, and γ1-subunits, and lamininW, which is composed of the α1,2-, β1-, and γ1-subunits), it was of interest to researchers to determine if one or both trimers are required for CVM migration. Because no apparent migration defects are observed in mutants for the α3,5-subunits, while mutants for α1,2 phenocopy the defects seen in Laminin β-chain mutants, it is likely that the lamininW trimer is specifically required for normal CVM migration (Urbano *et al.* 2011).

An ectopic expression screen identified multiple genes that encode proteins that have previously been implicated in mediating cell migration, including two classes of proteoglycans: the HSPG *trol*, described in a previous section, and the chondroitin sulfate proteoglycan *kon-tiki* (*kon*) (Trisnadi and Stathopoulos 2015). Both maternal mutants and CVM-specific RNAi knockdown mutants for *trol* present midline crossing of the CVM cohorts in a manner reminiscent of embryos lacking normal FGF signaling. Additionally, apoptosis of CVM cells was observed in both *trol* and *kon* RNAi mutants, further providing evidence that multiple ECM components are required for supporting CVM migration. It is possible that HSPGs like Trol are required for mediating FGF signaling in the CVM, perhaps by regulating ligand distribution, as a role in enhancing FGF signaling to support neuroblast

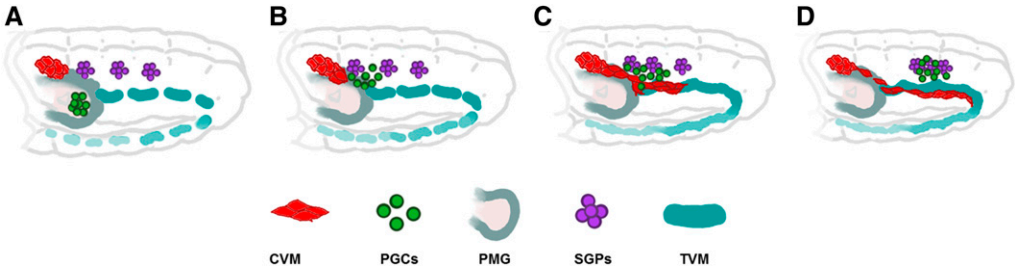


**Figure 10** Schematic illustrating steps of CVM migration before germband retraction and interaction with multiple tissues. (A) CVM cells begin their migration above and the primordial germ cells (PGCs) within the posterior midgut (PMG) epithelium. TVM muscle founder cells have not yet completed their fusion before start of migration. (B) As the PGCs exit the PMG, the CVM cells move forward and interact with the PGCs, which move toward the somatic gonadal precursors (SGPs). (C) CVM cells eventually migrate along the TVM muscle founders as they complete their fusion, while the PGCs comingle and continue migrating toward the SGPs, which (D) concomitantly migrate and coalesce with the PGCs to eventually form the gonads. Figure adapted from Stepanik *et al.* (2016), with permission.

proliferation via FGFR Btl and FGF ligand Branchless was previously described (Park *et al.* 2003).

***CVM and PGC migration is interdependent:*** FGF signaling clearly plays a significant role in promoting CVM pathfinding, migration, and survival; however, mutations in FGF do not completely ablate forward migration, suggesting that additional cues are required in this process, potentially involving interactions with multiple tissues (Figure 10). While the FGF- and integrin-mediated interactions between the CVM and TVM are better characterized, research into the relationship of the CVM with other tissues that occupy the extracellular milieu remains limited.

Analysis of *zfh1* mutants uncovered a role for the CVM in mediating PGC migration (Broihier *et al.* 1998; Stepanik *et al.* 2016). The CVM moves in close proximity to the PGCs at stage 10, with the CVM contacting the PGCs at the end of the PMG before migrating onto the TVM (Broihier *et al.* 1998; Stepanik *et al.* 2016). In addition to being expressed in the CVM, *zfh1* is also expressed in the presumptive mesoderm and the somatic gonadal precursors (SGPs) (Lai *et al.* 1991). PGCs comigrate with CVM cells toward the SGPs, which initiate as three bilateral clusters immediately ventral to TVM precursors in parasegments 10–12 (Boyle *et al.* 1997). At stage 12, the SGP clusters migrate toward each other until they coalesce with the PGCs in parasegment 10 at stage 14. In *zfh1* mutants, PGCs fail to migrate effectively to the SGPs, with a number of PGCs remaining in the gut endoderm (Broihier *et al.* 1998). A loss of *zfh1* function also results in impaired migration of the CVM cells, with the cohorts failing to interact with the PGCs at the end of the PMG. Furthermore, analysis of *byn* mutant embryos (which results in the formation of fewer CVM cells) presented defects in PGC migration, with many PGCs remaining attached to the PMG and failing to migrate along the TVM (Broihier *et al.* 1998). These results first suggested that the CVM normally mediates PGC migration, particularly their movement from the PMG to the TVM (*e.g.*, Figure 10C).

The finding that PGC migration is dependent on CVM migration raised the question of whether the CVM and PGCs share similar guidance cues, and whether the PGCs can reciprocally influence CVM migration. Analysis of maternal and zygotic mutants for *wunen 1* (*wun1*) and *wunen 2* (*wun2*), which encode lipid phosphate phosphatases that have previously been described as repellent cues and survival factors that support directional PGC migration (Zhang *et al.* 1996, 1997; Montell 2006), uncovered a striking correlation between PGC and CVM migration defects (Stepanik *et al.* 2016). Both *wun1* and *wun2* are expressed in identical patterns in PGCs and the posterior-most end of the PMG epithelium (Starz-Gaiano *et al.* 2001; Renault *et al.* 2002), and PGCs in both maternal and zygotic *wun1*,*wun2* mutants fail to migrate and exit the PMG properly. More recently, it was observed that CVM cells mismigrate in both maternal and zygotic *wun1*,*wun2* mutants, with CVM cells becoming more attracted to PGCs stuck in the PMG epithelium, stalling in their anterior-ward migration and resulting in detachment of CVM from the TVM, resulting in fewer longitudinal muscle fibers later on (Stepanik *et al.* 2016). This phenotype was most pronounced in *wun1 wun2* maternal mutants, which removed PGC-specific expression, suggesting that the CVM cells are responding to a putative PGC-derived attractant with Wuns acting as a repellent counterbalance (Stepanik *et al.* 2016). Furthermore, in embryos that are mutant for the G protein–coupled receptor *Trapped in endoderm 1*, PGCs are unable to exit the PMG epithelium and become immobilized (Kunwar *et al.* 2003); in this mutant background, CVM cells actively invade the PMG epithelium and make contact with the immobilized PGCs (Stepanik *et al.* 2016). PGCs likely express a chemoattractant that attracts CVM cells. While the identity of this factor is undefined, Wun appears to attenuate its activity during comigration of the CVM and PGCs.

Live imaging of *germ cell-less* (*gcl*) maternal mutant embryos, which present a complete loss of PGCs, revealed further insights into the interaction between PGCs and CVM (Broihier *et al.* 1998). In wild-type embryos, the CVM cells normally migrate as cohesive, yet somewhat loosely associated streams. In contrast, CVM cells in *gcl* maternal mutants migrate in a highly compact, undeviating fashion (Stepanik *et al.* 2016). A morphogenetic consequence of this altered migration profile is the presence of extra longitudinal muscle fibers in the midgut musculature, suggesting that the interdependent migration of PGCs and CVM is critical to ensuring

an orderly arrangement of muscle fibers at the completion of muscle fusion.

***Additional signaling pathways:*** Current research has focused on identifying the molecular cues that are responsible for the unique behavior exhibited by CVM cells. Recently, a gene expression profiling study aimed at identifying genes that are enriched in the CVM uncovered multiple cell migration regulators that are common in several migrating cell types, including *Drosophila* border cells (BCs) and hemocytes (HCs), and *Gallus* (chick) neural crest (Bae *et al.* 2017). BCs are found in *Drosophila* ovarioles and comprise a small cluster of 10 cells that undergo directional migration from the ovariole stalk to the developing oocyte (Prasad *et al.* 2015). The mechanism for this migration continues to be extensively studied considering their unique behavior and environment, which involves two nonmotile polar cells enclosed by eight motile cells migrating as a tight collective through a highly constrained space between much larger nurse cells (Montell *et al.* 2012). Considering the highly divergent migratory behaviors exhibited by CVM (which engage in a more loose, streaming migration along multiple tissues) and BCs, it was of interest to researchers to identify potential common regulators that may hint at common signaling pathways supporting migration. Through a comparative gene expression profiling analysis, the gene *neyo* (*neo*) was identified as expressed by both CVM cell and BCs (Bae *et al.* 2017).

*neo* encodes a zona pellucida domain protein that was previously characterized for its role in modifying epithelial morphogenesis by remodeling the ECM (Fernandes *et al.* 2010). RNAi-mediated knockdown of *neo* in either the CVM or the BCs resulted in significant cell migration defects, including asynchronous migration between the two CVM cohorts and severe migratory delays in BC migration toward the oocyte, which hints at a potential role for *neo* in regulating in two seemingly disparate cell migration models (Bae *et al.* 2017).

A similar comparison was made between CVM cells and HCs, which are akin to vertebrate immune cells and whose migration was covered in detail in another FlyBook chapter (see Figure 2 in Banerjee *et al.* 2019). HCs are derived from head mesoderm and follow several avenues of migration throughout the embryo, including toward the tail and along the amnioserosa and ventral nerve cord (Tepass *et al.* 1994). HCs additionally mediate the proper development of the ventral nerve cord through deposition of laminin to properly assemble the surrounding ECM (Sánchez-Sánchez *et al.* 2017), and also comigrate with CVM cells at stage 11; however, there is currently no evidence that HC migration influences CVM migration, as arresting HC migration (or removing HCs entirely) did not have any apparent consequence on anterior-ward movement of the CVM (Urbano *et al.* 2011).

Lastly, a comparison of the *Drosophila* CVM and HC RNA-sequencing data sets with that of chick neural crest uncovered seven orthologous genes that are related to several functional processes, with ECM-related functions being most highly represented. One of these was *matrix metalloprotease 2*, which was previously characterized for its role in regulating motor axon guidance and fasciculation in *Drosophila* (Miller *et al.* 2008); likewise in vertebrates, matrix metalloproteases are essential in promoting proper neural crest migration (Leigh *et al.* 2013). Another, ADAM-TS, the Drosophila ortholog of human ADAMTS 9 and ADAMTS 20, is important for detaching migrating salivary gland cells from the apical ECM, to support cell movement (Ismat *et al.* 2013). As ADAM-TS is expressed also by CVM cells, HCs, and PGCs, it is likely that this protease plays a general role in supporting cell migration. These insights incentivize further investigation of the role of the ECM in promoting CVM cell migration, and such studies are likely broadly applicable to other model systems.

## Linking Trunk Mesoderm to CVM Streaming

### *Cell collective-substrate relations in mesoderm cell migration*

Cell movement can only be interpreted relative to its environment. To move forward, most cells need a substrate to crawl on (Paluch *et al.* 2016). During morphogenetic movement, it is often assumed that cells are migrating over a static substrate. However, that is not true in many cases and, instead, a collectively migrating group of cells has been shown to greatly influence the motion of its neighboring cell layer even if not tightly attached, by rearranging cell positions (Smutny *et al.* 2017; Labernadie and Trepat 2018).

Instead of being a static substrate, the *Drosophila* embryonic ectoderm also undergoes significant changes during the course of mesoderm spreading. For example, neuroblasts ingress from the ventral ectoderm, resulting in a multilayered ectodermal structure, as mesoderm cells continue to migrate dorsolaterally upon this substrate (Figure 5A). Meanwhile, along the AP axis, the ectoderm goes through convergent extension and cells divide to extend the germband posteriorly by two and a half times its original length. Mesoderm cells have to keep up with this posterior extension in addition to spreading dorsolaterally. Unlike in zebrafish and *Xenopus*, where mesoderm cells undergo convergent extension to increase tissue length (Tada and Heisenberg 2012; Huebner and Wallingford 2018), the elongation of this germ layer in *Drosophila* is instead facilitated by the increase in mesoderm cell number through mitotic cell divisions. It is known that ectodermal cell movements help orient the posterior-ward migration of the mesoderm (McMahon *et al.* 2008; Winklbauer and Müller 2011). However, it remains largely unknown how these two different tissues coordinate their growth and movements while being simultaneously subjected to significant changes at the cellular level.

More recently, migratory CVM cells were found to influence their migration substrate, the TVM (Macabenta and Stathopoulos 2019a). In *htl* FGF mutant embryos, CVM mismigration in the midline correlates strongly with

contralateral merging of the TVM tracks. Two roles for FGF signaling were identified: FGF supports morphogenesis and stability of the TVM tracks by controlling integrin expression, and additionally acts within the CVM cells to support their directional movement. Contralateral merging of the two CVM clusters occurs in *htl* mutants because both the TVM is destabilized and the CVM cells go off track, supporting remodeling of the TVM (their substrate). Taken together, this represents an example of how migratory cell-substrate interactions can be more interdependent than previously believed (Macabenta and Stathopoulos 2019b).

We anticipate that future research investigating collective cell movement would further elucidate mechanisms that describe how migratory behavior is influenced by the environment, in addition to how migratory cohorts might in turn affect their environment. As novel examples of interdependent migration between two or more migrating cell types have shifted the paradigm of intercellular leaders and followers in collective movement, it is likely that prevailing models that posit the idea of a static substrate may change in different systems (Macabenta and Stathopoulos 2019b).

### *Reciprocal shifts between EMT and MET drive mesoderm migration in Drosophila embryos*

Prolonged EMT of the mesoderm ensures that cell movement is collective and directional (Clark *et al.* 2011; Schäfer *et al.* 2014; Sun and Stathopoulos 2018). The process requires both disassembly and reassembly of the transient spot AJs between the migratory collective (*i.e.*, mesoderm cells) and the substrate (*i.e.*, ectoderm cells). A reverse process, MET, takes place as a monolayer forms, when mesoderm cells lose migratory freedom but regain apicobasal polarity and AJs—both characteristics of the epithelial tissue.

These reciprocal transitions between epithelial and mesenchymal states of mesoderm cells are regulated by FGF signaling and, in contrast to vertebrates, not dependent on Sna. FGF signaling plays a general role to induce protrusive activity and cytoskeletal changes in migrating cells (Schumacher *et al.* 2004; Wilson *et al.* 2005). Additionally, cell division is an important contributing factor for EMT in *Drosophila*. The mesoderm defect in *htl*, *stg* (*cdc25*) double mutants is much more severe compared to single mutants, suggesting that FGF signaling functions redundantly or in parallel with *stg* to initiate EMT (Clark *et al.* 2011; Sun and Stathopoulos 2018).

Similar to EMT, MET is progressive and intermediate states can exist (Pei *et al.* 2019). Both processes are repeatedly employed during embryogenesis to generate different structures, facilitating the morphogenetic movement of cells and their differentiation. MET often requires interaction with surrounding tissues, discussed above (*Mesoderm Spreading*) regarding trunk mesoderm migration but relating also to TVM and CVM tissues. TVM cells have been shown to be required for midgut endoderm MET; in the absence of TVM, endoderm MET fails completely (Wolfstetter *et al.* 2009). In addition, the posterior-most CVM cells do not contribute to longitudinal muscles of the gut, but instead migrate toward ectodermal epithelial buds. In turn, these epithelial cells provide a polarity cue that causes the posterior CVM cells to undergo an MET and integrate into the epithelium, ultimately differentiating into secretory cells, the stellate cells of *Drosophila* renal tubules (Denholm *et al.* 2003; Campbell *et al.* 2010). These examples illustrate how cells can go through sequential EMT and MET cycles during development, and such transitions are supported locally by their neighbors.

***Final words:*** Collective cell migration has relevance not only in development, but also in wound healing, cancer metastasis, and other biological processes. Importantly, the movement of the whole group is not predicted by the movement of its individual cells, since the interactions between cells plays a key role in influencing the movement characteristic of a migrating collective. Frequently, cells are better at navigating as a group, as migration is more efficient than would be accomplished by individual cell movements (Vicsek and Zafeiris 2012; Szabó *et al.* 2016; Paul *et al.* 2017).

Mesoderm and CVM represent two different collectively migrating systems in the developing *Drosophila* embryo. Mesoderm cells represent a more cohesive system, where cells align themselves through adhesion molecules E-cad/AJs/N-cad (Schäfer *et al.* 2014; Sun and Stathopoulos 2018) and seem to polarize both in the radial direction toward the ectoderm, as well as at the leading edge in the direction of motion (Clark *et al.* 2011). Since they spread out symmetrically, there are two leading edges, which can be described as pulling the group in two opposite directions. In comparison, CVM cells represent a somewhat less cohesive system of collective migration, with cohorts moving as loosely associated streams reminiscent of models of endothelial cell migration, such as vertebrate neural crest cells (Szabó and Mayor 2016). Furthermore, CVM cells exhibit several unique characteristics that makes their study fertile ground for novel insights into the role of collective cell migration in organogenesis. These cells undergo bilateral migration that is symmetric, interact with multiple surrounding tissues (including TVM, PMG, and PGCs), as well as exhibit dynamic shifts in cell state and polarity, before they ultimately fuse with fusion-competent myoblasts of the TVM.

Embryonic development and organ formation in all higher metazoans requires the coordinated migration of progenitor cells to give rise to a plethora of functional biological structures. Through the study of model organisms such as *Drosophila*, developmental biologists have identified conserved mechanisms that drive cell migration across different taxa and cell types, reaffirming the enduring necessity of these models for understanding more complex phenomena. Even within the *Drosophila* embryo, cell types of common lineage, such as the trunk and caudal mesoderm, adopt more nuanced migration strategies to account for extracellular environment and divergence of function. Nevertheless, similarities in signaling pathways and cytoskeletal factors that drive

directional movement and the spatial organization of cells within migrating cohorts demonstrate basic principles that serve as foundations for current models of collective cell behavior, which were outlined and contextualized in this review. Much remains to be learned from *Drosophila* mesoderm migration, and we foresee many future insights of interdisciplinary relevance through continued study.

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