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Setting up for gastrulation: *D. melanogaster*:

A gene regulatory program sets the stage

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Abstract

Drosophila melanogaster embryos develop initially as a syncytium of totipotent nuclei and subsequently, once cellularized, undergo morphogenetic movements associated with gastrulation to generate the three somatic germ layers of the embryo: mesoderm, ectoderm, and endoderm. In this chapter, we focus on the first phase of gastrulation in *Drosophila* involving patterning of early embryos when cells differentiate their gene expression programs. This patterning process requires coordination of multiple developmental processes including genome reprogramming at the maternal-to-zygotic transition, combinatorial action of transcription factors to support distinct gene expression, and dynamic feedback between this genetic patterning by transcription factors and changes in cell morphology. We discuss the gene regulatory programs acting during patterning to specify the three germ layers, which involve the regulation of spatiotemporal gene expression coupled to physical tissue morphogenesis.

Keywords

Drosophila melanogaster; gastrulation; embryonic development; syncytium; maternal-to-zygotic transition; anterior-posterior patterning; dorsal-ventral patterning; germ-band elongation; morphogen gradients; mesoderm; endoderm; ectoderm

I. Introduction

Gene regulatory programs drive developmental progression during early *Drosophila* embryo development (rev. in Briscoe & Small, 2015; Stathopoulos & Levine, 2005). Dynamic gene expression is initiated during the early syncytial stage, when nuclei are totipotent, and continues to the cellularized blastoderm stage at which point cells are thought to exhibit a stepwise restriction of developmental potential (Beer, Technau, & Campos-Ortega, 1987; rev. in G. M. Technau, 1987). While many of the genes responsible for this patterning are conserved in other animals, the *Drosophila* embryo exhibits several atypical mechanisms that support early patterning.

First, while mammals rely on a symmetry breaking event of sperm entry at fertilization to provide a polarity cue, embryonic polarity in *Drosophila* is established during oogenesis when the mother deposits mRNAs into the developing oocyte (Ajduk & Zernicka-Goetz,

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2016; Moussian & Roth, 2005; Fig. 1A). These maternally-provided transcripts encode transcription factors or signaling pathway components that influence gene expression in the early embryo. A subset provide positional information, by establishing gradients of maternal proteins in the embryo or within the extracellular space that impact spatial zygotic gene expression (rev. in Reeves & Stathopoulos, 2009; Wieschaus, 2016).

Second, the *Drosophila* embryo develops as a syncytium. The first 13 nuclear division cycles include a short DNA replication S phase and no G2 phase, and the nuclei are not enclosed in separate membrane compartments but instead present in a joint cytoplasm (Foe & Alberts, 1983). This streamlined division cycle likely relates to the speed at which *Drosophila* embryos develop as it permits a rapid increase in cell number before gastrulation. Indeed, short synchronous nuclear cycles are also employed by other fast-developing embryos such as amphibians (*Xenopus*) and teleost fish (zebrafish). Further, the *Drosophila* maternal-to-zygotic transition (MZT), the phase during which the zygotic genome is activated and maternal transcripts are degraded, occurs over the course of only a few hours, compared to days in pre-implantation mammalian embryos (Jukam, Shariati, & Skotheim, 2017). Once the length of the cell cycle increases, the *Drosophila* embryo switches from relying on maternal instructions to a program that is driven predominantly by the zygotic products (rev. in Blythe & Wieschaus, 2015a).

Lastly, it is only after the 14th nuclear division that the length of the cycle increases and cellularization occurs. After 14 nuclear cycles, the syncytial embryo is comprised of roughly 6000 nuclei within a common cytoplasm. During cellularization individual nuclei that had previously migrated to the embryo periphery become encased by individual membrane compartments. After this point, precise cell-to-cell signaling, which had been limited pre-cellularization, becomes widespread (rev. in Norbert Perrimon, Pitsouli, & Shilo, 2012; Stathopoulos & Levine, 2004).

Despite, but in many cases because of, these atypical properties of its early development, the *Drosophila* embryo offers an attractive system to study the mechanisms that govern embryonic patterning. Although early development is rapid, it also is tractable, occurring externally over the course of a few hours. Furthermore, *Drosophila* embryonic development has been well characterized over many decades, forming a strong basis for mechanistic studies; numerous genetic tools exist that have and will continue to catalyze progress. Lastly, precisely staged embryos can be obtained with relative ease to support a variety of methodological approaches including genomics, biochemistry, and *in vivo* imaging studies. In the following chapter, we discuss progress made towards understanding the gene regulatory program that establishes patterning of the embryo and prepares it to undergo cell movements associated with gastrulation.

II. Establishment of embryonic polarity occurs in oocytes

Gradients of maternal proteins are responsible for establishing polarity within the early embryo to specify its anterior-posterior (A-P) and dorsal-ventral (D-V) axes. A-P axis orientation relates to localization in the oocyte of transcripts, which provide the positional information necessary to specify anterior and posterior ends of the embryo. The localization

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of transcripts to the presumptive anterior and posterior poles within developing oocytes pre-fertilization depends on the RNA binding protein Staufen (Stau) (Johnston, St Johnston, Beuchle, & Nüsslein-Volhard, 1991). In particular, *bicoid* (*bcd*) and *oskar* (*osk*) mRNAs are localized to presumptive anterior and posterior poles, respectively, of the developing oocyte (Fig. 1A).

This localization of transcripts to the oocyte (i.e., *bcd*, *osk*) results in the production of at least 5 gradients of maternal factors in the early embryo that are important for patterning. The Bcd protein gradient is produced from a gradient of *bcd* mRNA in embryos, that stems from transcript localization to the anterior of oocytes (Ali-Murthy & Kornberg, 2016; Spirov et al., 2009). Bcd, which encodes a homeodomain protein, is pivotal for patterning along the A-P axis, but additional input also comes from from maternal Hunchback (Hb) and Caudal (Cad) transcription factor gradients (Driever & Nüsslein-Volhard, 1988; Ochoa-Espinosa, Yu, Tsirigos, Struffi, & Small, 2009; C. Schulz & Tautz, 1995). The posterior maternal system is specified from local translation of *osk* transcripts, producing Osk protein which acts to localize another posterior maternal factor, Nanos (Nos), to the posterior. *nos* transcript localization as well as its translation is Osk-dependent (Ephrussi, Dickinson, & Lehmann, 1991; C. Wang & Lehmann, 1991). Furthermore, the posterior gradient of Nos acts to establish an anterior gradient of maternal Hb through translational repression of the initially uniformly distributed *hb* mRNA in posterior regions of the embryo (Fig. 1B; V. Irish, Lehmann, & Akam, 1989; G. Struhl, 1989; Tautz, 1988). Both maternal and zygotic *hb* expression contribute to setting borders of the earliest zygotic genes expressed along the A-P axis, the so-called gap genes (Jaeger, 2011; A. Porcher et al., 2010). In addition to its role as a transcription factor in which it binds to DNA and regulates transcription (Niessing et al., 2000; Gary Struhl, Struhl, & Macdonald, 1989), Bcd also mediates translational repression using its same homedomain to bind RNA and regulate translation (Dubnau & Struhl, 1996; Niessing et al., 2000; Rivera-Pomar, Niessing, Schmidt-Ott, Gehring, & Jäckle, 1996). In this role, Bcd forms an AP-oriented concentration-gradient and acts to repress translation of *cad* mRNA in the anterior of the embryo, forming an inverse gradient of Cad protein (Macdonald & Struhl, 1986; Mlodzik & Gehring, 1987). Cad is important for specification of abdominal as well as posterior segments including the hindgut (Fig. 1C; C. Schulz & Tautz, 1995; Wu & Lengyel, 1998).

On the other hand, positional information that contributes to establishing the D-V axis and to patterning the embryonic anterior and posterior termini derives from signaling pathways active earlier in the oocyte. A transcriptional program in developing oocytes regulates the expression of two extracellular matrix proteins: Pipe and Torso-like (rev. in Johnson, Henstridge, & Warr, 2017; Moussian & Roth, 2005). *pipe* expression is influenced by the positioning of the nucleus within the oocyte. The oocyte nucleus expresses the ligand Gurken, which activates EGFR signaling in dorsal follicle cells and indirectly limits expression of *pipe* to the ventral-most follicle cells (Schüpbach, 1987; Sen, Goltz, Stevens, & Stein, 1998). *pipe* encodes a protein sharing homology with vertebrate heparan sulfate 2-O-sulfotransferases, but likely functions independent of heparan in the extracellular matrix on the ventral side of the egg and ultimately impacts signaling in the embryo (Zhu, Sen, Stevens, Goltz, & Stein, 2005). The *torso-like* (*tsl*) gene, encoding a secreted protein with homology to perforins, exhibits localized expression to a distinct subset of follicle cells at

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the anterior and posterior ends of the oocyte (Stevens, Beuchle, Jurcsak, Tong, & Stein, 2003). Tsl is incorporated into the vitelline membrane, the inner layer of the eggshell, and is subsequently transferred to the plasma membrane of the embryo thereby conveying spatial information from the follicle cells surrounding the oocyte to the developing embryo (A. Mineo, Furriols, & Casanova, 2015). Pipe and Tsl proteins serve as initial polarity cues for the embryo to define dorsal-ventral polarity and terminal ends, respectively.

Pipe and Tsl expression leads to spatial activation of signaling pathways within the early embryo. Ventrally-deposited Pipe protein leads to activation of Toll receptor signaling through the localized activation of a protease cascade in the fertilized egg (Cho, Stevens, & Stein, 2010; rev. in Moussian & Roth, 2005). Toll signals intracellularly through a conserved NF-kappaB pathway and establishes a ventral-to-dorsal nuclear gradient of the Rel transcription factor Dorsal (DL) (Fig. 1D; Roth, Stein, & Nüsslein-Volhard, 1989; C. A. Rushlow, Han, Manley, & Levine, 1989; Steward, 1989). The DL gradient directs D-V patterning and this transcription factor is thought to act as a morphogen, controlling gene expression along the D-V axis in a concentration-dependent fashion (rev. in Hong, Hendrix, Papatsenko, & Levine, 2008; Jiang & Levine, 1993; Reeves et al., 2012). On the other hand, extracellular Tsl facilitates spatially-localized activation of signaling through the Torso (Tor) receptor in the early embryo in cooperation with Torso's presumed ligand Trunk (Trk) (Amarnath, Stevens, & Stein, 2017; Alessandro Mineo, Furriols, & Casanova, 2018). Tor is activated at the embryonic poles by Trk, a broadly expressed-secreted molecule, which relies on terminally-localized Tsl for its activation (Casanova, Furriols, McCormick, & Struhl, 1995; Casanova & Struhl, 1989; Sprenger & Nüsslein-Volhard, 1992). Activation of Tor signaling supports expression of particular genes at the embryo termini responsible for the formation of terminal embryonic structures, the anterior acron and posterior telson (Fig. 1D; Weigel, Jurgens, Klingler, & Jackle, 1990). Torso transduces signals mainly by means of the Ras–extracellular signal regulated kinase (ERK) signaling cassette (Duffy & Perrimon, 1994; Lu, Perkins, & Perrimon, 1993; N. Perrimon et al., 1995), but a subset of Torso signaling utilizes the *Drosophila* STAT (DStat92E) instead (J. Li, Xia, & Li, 2003; W. X. Li, Agaisse, Mathey-Prevot, & Perrimon, 2002). In particular, MAPK signaling through Tor has been shown to support degradation of a maternally-provided ubiquitous repressor, Capicua (Cic), limiting its levels at the embryonic termini (Astigarraga et al., 2007; de las Heras & Casanova, 2006). The Cic protein gradient is important for patterning of the trunk regions of embryos, contributing both to A-P and D-V patterning (Jiménez, Guichet, Ephrussi, & Casanova, 2000).

In this manner, despite lack of cell membranes while the embryo is a syncytium, broad signaling domains are established by molecules spatially deposited to the eggshell during oogenesis. These domains permit the spatial activation of signaling pathways downstream of Toll and Torso receptors in the early embryo to establish D-V axis and terminal end positional information, respectively. In the fertilized embryo, gene expression programs require input both from maternal transcription factors including those that provide polarity cues, as discussed above, as well as additional ubiquitous maternal transcription factors that serve as timing factors that help to manage the MZT, discussed in the following section.

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III. Navigating the maternal-to-zygotic transition

Upon fertilization, the transition from dependence on maternal transcripts deposited into the egg to newly transcribed zygotic transcripts is carefully regulated to ensure proper development of early embryos. This maternal-to-zygotic transition, or MZT, requires reprogramming of the early embryonic genome by a network of maternal transcription factors, ultimately resulting in a hand off of the gene regulatory program to zygotic transcription factors. In *Drosophila*, this transition from dependence on maternal to zygotic products requires precise regulation of the division-cycle, DNA replication, initiation of zygotic transcription, maternal RNA clearance and chromatin remodeling (rev. in Blythe & Wieschaus, 2015a; Hamm & Harrison, 2018; Vastenhouw, Cao, & Lipshitz, 2019; Yuan, Seller, Shermoen, & O'Farrell, 2016).

In the *Drosophila* embryo, the division-cycles and DNA replication start off short and then increase in length (Fig. 2A). The first two hours of development comprise 13 rapid nuclear division cycles within a shared cytoplasm. Nuclei are located in the center within the yolk, but then migrate to the periphery during nuclear cycle 8–10. At this point, the yolk becomes localized in the center and cleavage occurs in the cortical area; but cleavage is superficial and does not bisect the yolk. The first 13 nuclear divisions occur rapidly, roughly every 8–10 minutes and without cytokinesis, within a shared cytoplasmic space (Hamm & Harrison, 2018). These mitoses lack gap phases and therefore involve repeating cycles of rate-limiting DNA synthesis (Foe, 1989; McCleland, Farrell, & O'Farrell, 2009; Shermoen, McCleland, & O'Farrell, 2010). However, after the 14th nuclear division, the cycle slows dramatically and nuclei become cellularized (Foe & Alberts, 1983; Shermoen et al., 2010). Cell membranes emerge to encapsulate nuclei, forming a single layered epithelium. In addition, at nuclear cycle 14, developmental changes relating to DNA replication occur; namely a lengthened S-phase and the introduction of G2 phase. This key milestone, the so-called midblastula transition (MBT) when embryonic programs of morphogenesis and differentiation initiate, is also associated with clearance of a subset of maternally provided mRNAs, large-scale transcriptional activation of the zygotic genome, and an increase in cell cycle length (rev. in Blythe & Wieschaus, 2015a; Yuan et al., 2016).

The MZT, on the other hand, encompasses the entire period during which maternal products are cleared and zygotic genome activation occurs, roughly from fertilization to the onset of gastrulation (Fig. 2). Furthermore, although widespread zygotic transcription is delayed until the 14th nuclear cycle when cellularization occurs, some zygotic transcripts are produced earlier and likely contribute to the gene regulatory decisions that influence patterning and development in concert with maternal products. Zygotic expression of a small number of genes has been observed as early as nuclear cycle 7, even before nuclei migrate to the cortex at nuclear cycle 9 (Ali-Murthy & Kornberg, 2016; Ali-Murthy, Lott, Eisen, & Kornberg, 2013). It is possible that these early zygotic transcripts contribute to patterning, but not much is known about their roles. However, transcripts expressed early are on average shorter in size and contain small introns if any, possibly constrained in length by the short interphase length; whereas transcripts present at nuclear cycle 14 on average are longer and contain larger introns (K. Chen et al., 2013; Kwasnieski, Orr-Weaver, & Bartel, 2019). A recent study found that truncated forms of long genes are expressed prior to nuclear cycle 14, and

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that these products act to regulate the timing of signal transduction either by generating dominant negative or constitutively active forms of signaling molecules (Sandler et al., 2018).

The exact molecular mechanism that coordinates division-cycle slowing and widespread activation of the zygotic genome at cycle 14 is not known, but several regulatory mechanisms have been proposed (Fig. 2C). One model suggests that a ‘maternal clock’ acts to track time elapsed since fertilization (Blythe & Wieschaus, 2015b; Tadros & Lipshitz, 2009). A set amount of developmental time following fertilization may be necessary for levels of maternal factors that trigger zygotic genome activation to accumulate. Furthermore, the concentration of soluble histones in the nucleus drops within embryos as they approach to zygotic genome activation (Shindo & Amodeo, 2019). Decreasing histone levels advances zygotic transcription, cell cycle elongation, cell cycle checkpoint kinase Chk1 activation, and gastrulation; whereas increasing histone levels has the opposite effect, causing delays in transcription and the addition of an extra cell cycle (Wilky, Chari, Govindan, & Amodeo, 2019). A drop in the concentration of soluble histones in the nucleus likely provides an opportunity for the pioneer factors that herald zygotic gene expression to successfully compete for DNA access and activate transcription. For example, during this time, the ubiquitous, maternal transcription factor Zelda is a pivotal activator as it directly binds to and opens chromatin at enhancers, facilitates binding of other transcriptional activators to these DNA sequences, and allows initiation of zygotic gene expression (Harrison, Li, Kaplan, Botchan, & Eisen, 2011; Liang et al., 2008; Nien et al., 2011). Zelda binds nucleosomes and is considered a pioneer factor (McDaniel et al., 2019). Loss of Zelda therefore leads to a large-scale, global decrease in zygotic gene expression as many enhancer regions remain inaccessible and thus non-functional (K. N. Schulz et al., 2015). As such, binding of Zelda to promoters and enhancers may be facilitated when histone levels decrease as chromatin may be more easily accessed.

Furthermore, it is likely not only the balance between histones and pioneering activators that is responsible for timing MZT, but levels of repressors that can impact timing as well. For instance, an alternative model to explain MZT timing posits the need to titrate away an additional repressor in order to allow zygotic transcription to initiate. Titration may be accomplished by increasing the nuclear to cytoplasmic ratio, which would decrease the effective concentration within individual nuclei. Some broadly-expressed repressors may fulfill this role and contribute to timing of the MZT. The transcription factors Tramtrack (Ttk), Suppressor of Hairless [Su(H)], and Runt are maternally loaded and have been shown to broadly repress the transcription of genes in the early embryo (Brown & Wu, 1993; Koromila & Stathopoulos, 2017; Read, Levine, & Manley, 1992). Reducing the amount of these factors or the number of binding sites within particular enhancers of Ttk and Runt target genes *fushi-tarazu* and *short-gastrulation*, respectively, results in earlier initiation of transcription, while increasing the amount of these proteins has the opposite effect - to delay transcription (Koromila & Stathopoulos, 2017; Pritchard & Schubiger, 1996). While the molecular mechanisms these repressors use to impact gene expression at MZT remain unknown, it has been proposed that they act as a counterbalance to Zelda to influence chromatin accessibility or other transcriptional activators (i.e. Bicoid or Dorsal) and

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consequently to influence levels of expression (Koromila & Stathopoulos, 2019; Ozdemir, Ma, White, & Stathopoulos, 2014).

Lastly, MZT also involves degradation of maternal transcripts and products once zygotic transcription has been initiated. At this time, the maternal instructions (i.e. mRNAs and proteins) are actively cleared from the embryo. mRNA degradation programs targeting maternal transcripts account for clearance of over half of all maternal mRNAs degraded during the MZT (Bashirullah et al., 1999; Lefebvre & Lécuyer, 2018; Tadros & Lipshitz, 2009). This process is regulated laregly by RNA binding proteins (RBPs) including Smaug (SMG), Brain tumour (BRAT), and Pumillion (PUM) that bind to *cis*-acting elements within the open reading frames or 3'-untranslated regions of maternal transcripts (Laver et al., 2015; Tadros et al., 2007). In addition, microRNAs also support maternal transcript degradation. A major player in this pathway is the zygotically-expressed miR-309 cluster that contains eight microRNA (miR) genes (Bushati, Stark, Brennecke, & Cohen, 2008) and is required for the degradation of mRNAs encoded by approximately 400 maternally expressed genes (Bushati et al., 2008). This clearance, through action of RBPs and microRNAs, allows the transition from dependence on the maternal program, which is erased, to dependence on the newly transcribed zygotic program.

IV. Gene expression patterns establish the prospective germ layers

By three hours of development, the MZT and cellularization of the blastoderm are complete, and have resulted in approximately 6000 cells forming a single-layered, tall columnar epithelium surrounding the yolk, with the apical surface of cells facing outward. Germ cells are set aside earlier, and lie at the posterior pole of the embryo on the surface of the blastoderm epithelium (Fig. 3A). By this stage, the embryo is already divided into territories with specified fates, judged by gene expression patterns and transplantation experiments (Campos-Ortega & Hartenstein, 1997; Leptin in Stern, 2004). The prospective mesoderm occupies, ventrally, approximately 20% of the circumference and 70% of the length of the embryo; whereas the endoderm anlagen invaginate from two distinct blastoderm regions, one anterior to the ventral furrow and the other at the posterior pole (Figs. 3 & 4B). These two endoderm anlagen develop independently of each other, and are joined by cell migration later in embryogenesis (Reuter, Grunewald, & Leptin, 1993). The ectodermal primordium is composed of several regions with different fates. Along the D-V axis and dorsal to the mesoderm primordia on each side of the embryo lie the mesectoderm, large regions of neuroectoderm, and the lateral ectoderm (Fig. 3). Neuroblasts will delaminate from the neuroectoderm, moving toward the inside of the embryo (Hartenstein & Wodarz, 2013), whereas the lateral and dorsal ectoderm will form the majority of the larval epidermis and the tracheal system (Ray, Arora, Nüsslein-Volhard, & Gelbart, 1991). The dorsal side of the embryo will develop into amnioserosa. The proctodeal ring, which lies just anterior to the posterior endoderm and is composed of ectodermal tissue, will form the hindgut (Kispert, Herrmann, Leptin, & Reuter, 1994), while the anterior tip of the blastoderm lying above the anterior endodermal primordium will form the foregut (Figs. 3B and 4B; Rehorn, Thelen, Michelson, & Reuter, 1996). Borders of the future germ layers correlate with the expression patterns of several genes at the cellular blastoderm stage. The focus of this section is a

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discussion of how the gene expression patterns that define these prospective germ layers arise.

The borders of the future mesoderm germ layer can be visualized by the expression pattern of cells located most ventrally on the dorsoventral axis, which express the important mesodermal determinant genes *snail* and *twist* (Alberga, Boulay, Kempe, Dennefeld, & Haenlin, 1991; Thisse, Perrin-Schmitt, Stoetzel, & Thisse, 1991). The presumptive mesodermal domain covers most, but not all, of the length of the embryo as it is excluded from both anterior and posterior ends. *twist* expression extends the length of the embryo including ends; whereas, *snail* expression extends the length of the embryo including the anterior end but is repressed at posterior end (Reuter & Leptin, 1994). The posterior border of *snail* expression coincides with the mesoderm primordium posterior border (Fig. 3).

The anterior and posterior terminal regions of the embryo contribute to endoderm as well as to ectodermal derivatives. Expression of the gap genes *tailless* (*tll*) and *huckebein* (*hkb*) mark the borders of germ layers along the A-P axis and are expressed both at the posterior and anterior caps of the embryo in response to Torso signaling (Klingler, Erdélyi, Szabad, & Nüsslein-Volhard, 1988; Strecker, Kongsuwan, Lengyel, & Merriam, 1986; Weigel et al., 1990). *tll* encodes a conserved, orphan nuclear receptor (Pignoni et al., 1990), whereas *hkb* encodes an Sp1/Egr-like Zn-finger protein (Brönner et al., 1994). The presumptive endoderm is also marked by *serpent* (*srp*), encoding a GATA transcription factor, which is expressed on the ventral side of the anterior-most part of the embryo and in a cap at the most posterior end of the embryo, specifying the anterior and posterior midgut primoridia, respectively (Fig. 3; Rehorn et al., 1996).

The ectoderm ventral boundary is positioned by *snail*, encoding a transcription factor that predominantly acts as a repressor (Kosman, Ip, Levine, & Arora, 1991). Immediately adjacent to the mesoderm lies the mesectoderm, which is defined by expression of the gene *single-minded* (*sim*) and subsequently will form the midline glia (Fig. 3B; Crews, Thomas, & Goodman, 1988). Notch signaling is important for constraining *sim* expression to only a single row of mesectodermal cells along the D-V axis (Morel & Schweisguth, 2000). Expression of *zerknüllt* (*zen*) marks a band of cells on the dorsal side of the embryo that will ultimately encompass the amnioserosa (C. Rushlow, Frasch, Doyle, & Levine, 1987). The presumptive neuroectoderm lies between the *sim* and *zen* expression domains and expresses genes including *SoxNeuro*, encoding a Sox HMG DNA binding domain, *short-gastrulation* (*sog*), encoding a secreted inhibitor of BMP signaling, and *brinker* (*brk*) encoding a transcriptional repressor (Fig. 3B; Biehs, François, & Bier, 1996; Crémazy, Berta, & Girard, 2000). Sog functions to antagonize the activity of the ligand Decapentaplegic (Dpp), limiting activation of BMP signaling to dorsal regions and inhibiting its activity locally within the presumptive neurogenic region (Ashe & Levine, 1999; Biehs et al., 1996). Brk represses expression of Dpp target genes, excluding them from ventrolateral regions (Ja wi ska, Kirov, Wieschaus, Roth, & Rushlow, 1999; Zhang, 2001). Limiting Dpp activity in ventral regions is necessary to support formation of the neurogenic ectoderm, which gives rise to both the ventral epidermis and the central nervous system.

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In the dorsal region lies the non-neurogenic ectoderm. Dpp signaling both suppresses neurogenesis and maintains expression of genes that promote dorsal cell fates (E. L. Ferguson & Anderson, 1992). Dpp is present in a gradient and acts as a morphogen, in that it specifies many different cell fates via intercellular signaling in a concentration-dependent fashion (Fig. 3B; Edwin L. Ferguson & Anderson, 1992; Ray et al., 1991). High levels of Dpp activity specify the amnioserosa, while progressively lower levels specify dorsal and lateral ectoderm (Christine Rushlow & Roth, 1996). Lower Dpp levels in the dorsolateral region specify the non-neurogenic ectoderm harboring peripheral nervous system precursors. This potential for Dpp to specify cell fate is highly dosage sensitive (Wharton, Ray, & Gelbart, 1993). In the wild-type embryo, increasing the gene dosage of Dpp can shift cell fates along the dorsal-ventral axis. Sog inhibitor and Brk repressor proteins work in concert to specify the dorsolateral limits of Dpp target genes (Ashe, Mannervik, & Levine, 2000; Ja wi ska, Rushlow, & Roth, 1999).

Thereby, the establishment of mesoderm and ectoderm relies predominantly on D-V axis positional information, whereas endoderm requires input from terminally-localized cell signaling along the A-P axis. It is unclear if the neurogenic ectoderm requires positive input to define this cell state or whether, rather, it represents a default state.

V. Gene regulatory interactions prepare cells for diverse cell movements at gastrulation

Germ layer formation begins in the cellularized blastoderm with two major cell movements (Campos-Ortega & Hartenstein, 1997). Ectoderm and mesoderm separate as a result of the ventral furrow formation and invagination of the presumptive mesoderm in ventral regions. The developing anterior and posterior endoderm anlagen grow towards the center, where they combine to form the midgut, the only endodermal derivative of the fly (Skaer in Bate & Arias, 1993). The cells that remain at the surface of the gastrulating embryo develop mainly epidermis and neural tissue (Campos-Ortega & Hartenstein, 1997). In addition to these mesodermal and endodermal invaginations, the morphogenetic movements of *Drosophila* gastrulation also include germ-band extension, cephalic furrow invagination, and formation of dorsal folds (Leptin & Grunewald, 1990; Spencer, Siddiqui, & Thomas, 2015; Sweeton, Parks, Costa, & Wieschaus, 1991). In this section and discussed in the subsequent chapters of this book (Chapters 6 and 7), we will focus on the genes and regulatory interactions known to support specification and differentiation of the cells that encompass germ layers, as well as changes in gene expression that occur concurrently with or just preceding major cell movements at gastrulation.

In the embryo, maternal polarity cues are used to establish two morphogen gradients of transcription factors that help to direct gene expression along the two axes. Bcd and DL maternal gradients regulate the expression of target genes along the A-P and D-V axes, respectively, in a concentration-dependent manner (rev. in Briscoe & Small, 2015; Hong et al., 2008; Aude Porcher & Dostatni, 2010; Fig. 1). These threshold outputs are also supported by input from ubiquitous, maternal activator Zelda and broadly-expressed repressors (Foo et al., 2014; Liberman & Stathopoulos, 2009; Löhr, Chung, Beller, & Jäckle,

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2009; Ozdemir et al., 2014; Xu et al., 2014). In addition, spatially localized repressors help define boundaries of gene expression patterns (H. Chen, Xu, Mei, Yu, & Small, 2012; e.g. Ip, Park, Kosman, Bier, & Levine, 1992). These broad domains established by the Bcd and DL morphogen gradients are subsequently refined by hierarchical zygotic gene-regulatory networks to produce differentiated tissues. Most target genes receive both A-P and D-V axis positional information, and contribute to cell specific gene expression profiles and changes in morphology.

The mesoderm layer is formed through invagination of ventral cells during gastrulation (Ip, Park, Kosman, Yazdanbakhsh, & Levine, 1992; Kosman et al., 1991; Thisse et al., 1991). The nuclear DL gradient along the D-V axis of the embryo (Fig. 1D) results in ventral expression of the genes *twist* and *snail*, which encode transcription factors important for the positioning and initiation of cell shape changes associated with ventral furrow formation (Leptin & Grunewald, 1990). Both Twist and Snail regulate gene expression in the mesoderm as well as in neighboring regions, but each employs a different mechanism (Leptin, 1991). Snail, a characterized transcriptional repressor, is expressed in the presumptive mesoderm but excluded from the posterior endoderm and inhibits mesodermal expression of genes that are destined to be active only in more lateral or dorsal regions. Conversely, Twist is required for the activation of downstream mesodermal genes (Ip, Park, Kosman, Yazdanbakhsh, et al., 1992; Kosman et al., 1991; Simpson, 1983). Only in the absence of both *twist* and *snail* are mesodermal characteristics lost. Genes activated as a result of Twist and Snail expression are important for modulating the activity or subcellular localization of components of cell-cell junctions and the contractile actomyosin network, thereby providing a link between patterns of upstream gene expression established in the blastoderm and proteins that produce mechanical forces necessary for tissue-specific cell shape changes and movement.

In the mesoderm, Snail represses expression of *neuralized* (*neur*), which encodes an E3 ubiquitin ligase needed to disassemble subapical adherens junctions (Chanet & Schweisguth, 2012). Proteins of the Bearded (Brd) family interact with Neur, providing spatial regulation of internalization of the Notch ligand Delta along the DV axis important for precise positioning of the mesectoderm (Bardin and Schweisguth 2006). Additionally, Snail together with Twist supports expression of three genes encoding key modulators of the contractile actomyosin network: the G-protein-coupled receptor, Mist, and its apically-secreted ligand, Folded Gastrulation (Fog) (Costa, Wilson, & Wieschaus, 1994; Manning, Peters, Peifer, & Rogers, 2013) as well as the transmembrane protein T48. Live imaging studies have shown that the transcriptional dynamics of *fog* and *T48* relate to spatial activation of myosin, foreshadowing mesoderm invagination, and suggest that this morphogenetic process of invagniation is controlled through the regulation of spatiotemporal gene expression (Kölsch, Seher, Fernandez-Ballester, Serrano, & Leptin, 2007; Lim, Levine, & Yamazaki, 2017). Together, Mist/Fog and T48 serve to recruit Ras-like GTP-binding protein (Rho) guanine nucleotide exchange factor (RhoGEF)2 to the apical cell cortex. Further, TNF-receptor-associated factor 4 (Traf4), also induced by Twist and Snail, is required for apical accumulation of adherens junctions (rev. in Gilmour, Rembold, & Leptin, 2017). Specifically, Snail induces stochastic fluctuation of mesoderm apical cell surface size through Fog and RhoGEF2 to promote medio-apical accumulation of Myosin-II, which,

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along with junctional repositioning, leads to apical constriction of the presumptive mesoderm cells (Fig. 4A; Martin, Kaschube, & Wieschaus, 2009; Mitrossilis et al., 2017; Pouille, Ahmadi, Brunet, & Farge, 2009). Mesoderm invagination is an instructive example of how broad domains established by morphogens (i.e. DL) permit subdivision of epithelial sheets into discrete domains of expression of tissue-specific transcription factors (i.e. Twist, Snail) that drive the regulation of cell machinery to modulate the activity of broadly expressed mechanosensory components. Coordinated arrangement of these components, including actomyosin and adherens junctions, permit stereotyped cell shape changes and mechanical forces that are translated across interconnected epithelial sheets to produce tissue-scale morphological outputs.

Whereas the entire mesoderm undergoes coordinate invagination regulated by a single set of inputs, the endoderm invaginates as two distinct parts (Fig. 4B) in striking difference to formation of this germ layer in most other animals (U. Technau & Scholz, 2003). The anterior and posterior endoderm primordia are not only specified separately but also utilize distinct morphogenetic mechanisms to invaginate. At the posterior of the embryo, *hkb*'s anterior-ventral boundary abuts the *sna* posterior boundary. In this posterior region, *tll* overlaps the *hkb* expression domain, but also extends further anteriorly (Fig. 4B). *hkb* is necessary for the expression of *srp*, which is able to not only specify endoderm from ectoderm and is also required for the morphogenesis and function of the endoderm (Reuter, 1994). Where *tll* overlaps with *sna*, and *hkb* is also absent, defining a region that expresses *brachyenteron* (*byn*) and that will invaginate together with the posterior midgut primordium to specify ectodermal hingut and anal pads, and caudal visceral mesoderm (Kispert et al., 1994; Kusch & Reuter, 1999; Singer, Harbecke, Kusch, Reuter, & Lengyel, 1996; Fig. 4C). At the opposite end of the embryo, the anterior midgut primordium is specified from a region in which *hkb, snail*, and *twist* are coexpressed, and which corresponds precisely to the anterior expression domain of *srp* (Rehorn et al., 1996). Endoderm morphogenesis emerges from a combination of local transcriptional initiation as well as mechanically-driven cell deformation resulting from either mesoderm invagination (posterior endoderm) or germ-band elongation (anterior endoderm), discussed below (Bailles et al., 2019; Mitrossilis et al., 2017). Notably, the posterior endoderm invaginates by apical constriction governed by a pathway highly similar to that employed during mesoderm invagination including recruitment of RhoGEF2 (Sweeton et al., 1991). Moreover, while Sna and Fog are required for apical accumulation of myosin to support invagination of the mesoderm, Fog, but not Snail, is expressed in the posterior endoderm. Stretching of the posterior endoderm cells, as a result of mesoderm invagination, activates Myosin-II in these cells about 80 seconds later (Mitrossilis et al., 2017). Alternatively, the anterior midgut is thought to invaginate using a different mechanism that depends instead on germ-band elongation-induced expression of *twist* (Farge, 2003).

A few minutes following mesoderm invagination, cell intercalation within the lateral epidermis initiates and leads to germ-band extension (GBE) (Fig. 4C). During this process following mesoderm invagination, the germ-band from the left and right side of the embryo join at the ventral midline. GBE is largely driven by cell intercalation and cell shape changes that are a passive response to extrinsic tensile forces that deform the germ band (Bertet, Sulak, & Lecuit, 2004; Blankenship, Backovic, Sanny, Weitz, & Zallen, 2006; Irvine &

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Wieschaus, 1994). In short, cells converge in the D-V direction while extending in the A-P direction causing overall lengthening of the germ band along the sides of the embryo (Fig. 4C). Cell intercalation supports GBE and is regulated by the pair-rule transcription factors Even-skipped (Eve) and Runt (Zallen & Wieschaus, 2004). Eve and Runt direct the spatial expression of three Toll-family receptors in partially overlapping patterns, creating asymmetry along the A-P axis. Heterophilic interactions between Toll receptors are necessary for the polarized distribution of Myosin to cell surfaces orthogonal to the plane of extension (Paré et al., 2014). Some evidence also supports a role for the classical Frizzled-based planar cell polarity pathways in cell polarity in the germ-band (Warrington, Strutt, & Strutt, 2013), but whether Toll and Frizzled pathways cooperate in generating planar polarity is unclear (Tepass, 2014). In addition, tissue-level pulling forces generated by invaginations of the posterior midgut contribute to GBE (Collinet, Rauzi, Lenne, & Lecuit, 2015; Lye et al., 2015), but GBE is thought to be independent of mesoderm invagination (Irvine & Wieschaus, 1994).

Additional cell movements during gastrulation contribute to the formation of folds along the A-P axis however these events differ in the mechanism of fold formation from either mesoderm or endoderm invagination (Fig. 4D). For example, Bcd regulates expression of the gap genes, *paired* and *buttonhead*, which subsequently interact to specify the anterior-most expression of the pair rule gene *eve* (Vincent, Blankenship, & Wieschaus, 1997). This particular domain of *eve* expression directs cell behavior at the cephalic furrow which involves actin- (but not myosin) dependent shortening of cells along their apical-basal axis (Spencer et al., 2015; Fig. 4). Further, dorsoventral differences in cephalic furrow depth depend on D-V axis positional information (e.g. V. F. Irish & Gelbart, 1987), providing an example of how cells at distinct positions along the A-P and D-V axes of the embryo come to acquire a specific fate and morphology determined by the complement of transcription factors they express.

The anterior and posterior dorsal transverse folds (ADF & PDF, respectively - Fig. 4D) are epithelial folds that form on the dorsal side of the gastrulating *Drosophila* embryo at stereotypical locations coincident with the second and fifth stripes of the Runt expression (Y.-C. Wang, Khan, Kaschube, & Wieschaus, 2012). These dorsal folds are formed via a myosin-independent mechanism (i.e. actin) to apically-constrict cells involving differential positioning of adherens junctions and controlled by modulation of epithelial apical–basal polarity. The dorsal folds do not appear to contribute to germ-band extension, but it is conceivable that D-V convergence of the extending germ-band mechanically pulls on the amnioserosa to support cell flattening (Lye et al., 2015; Pope & Harris, 2008). Finally, the dorsal-most ectoderm, which does not participate in GBE, develops into the extra-embryonic amnioserosa and non-neural ectoderm. The amnioserosa is a transient structure that is only present for half of embryogenesis and does not contribute to the larva, but has been shown to support the process of germ-band retraction later in embryogenesis following gastrulation (Lynch et al., 2013). In this way, broad domains established in the blastoderm adopt distinct programs of downstream gene expression to activate effectors of cellular machinery that subsequently produce cell movements and tissue morphologies resulting in a gastrulated embryo.

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VI. Dynamic feedback between genetic patterning and physical tissue morphogenesis

In this chapter, we have provided a framework for understanding patterning of the embryo as it relates to germ layer formation, focusing on the gene regulatory interactions that control specification of distinct cell types. However, studies of gene expression *in vivo* have provided more evidence that patterning is a dynamic process. Cell-fate decisions are likely not fixed, but evolving; able to refine as tissues physically take shape. Therefore, rather than patterning of embryo being organized in a strict hierarchical relationship, in which transcription factors work in series to change expression patterns, it is likely that genetic patterning involves significant feedback. Intercellular signaling pathways that control cell-fate decisions and tissue patterning are emerging as central mediators of morphogenetic feedback control (rev. in Gilmour et al., 2017).

Any morphogenetic event that changes the relative position of cells that produce or receive signals has the potential to provide feedback on the patterning process. This includes, but is not limited to, alterations in the number or arrangement of cells, such as proliferation or convergent extension; or changes in cell adhesion or polarity, that can influence the trafficking and presentation of ligands to impact cell signaling. In addition, mechanical forces have been shown to contribute to driving tissue-shaping processes, and provide another important channel for feedback between cell form and fate. For example, a mechanosensitive pathway, involving the Wnt–cadherin effector β-catenin, has been shown to regulate *Drosophila* mesodermal identity *in vivo* and has been found to work similarly in zebrafish (Brunet et al., 2013). Further, cell movements during gastrulation have been shown to be affected by the mechanical properties of neighboring germ layers suggesting complex interdependencies between tissues that likely depend on crosstalk between the gene networks that regulate cell tension throughout the embryo (Perez-Mockus et al., 2017; Rauzi et al., 2015). Mechanistic investigations into how tissue shaping is encoded genetically and how, conversely, tissue shaping feeds back into gene expression is an important area of future study.

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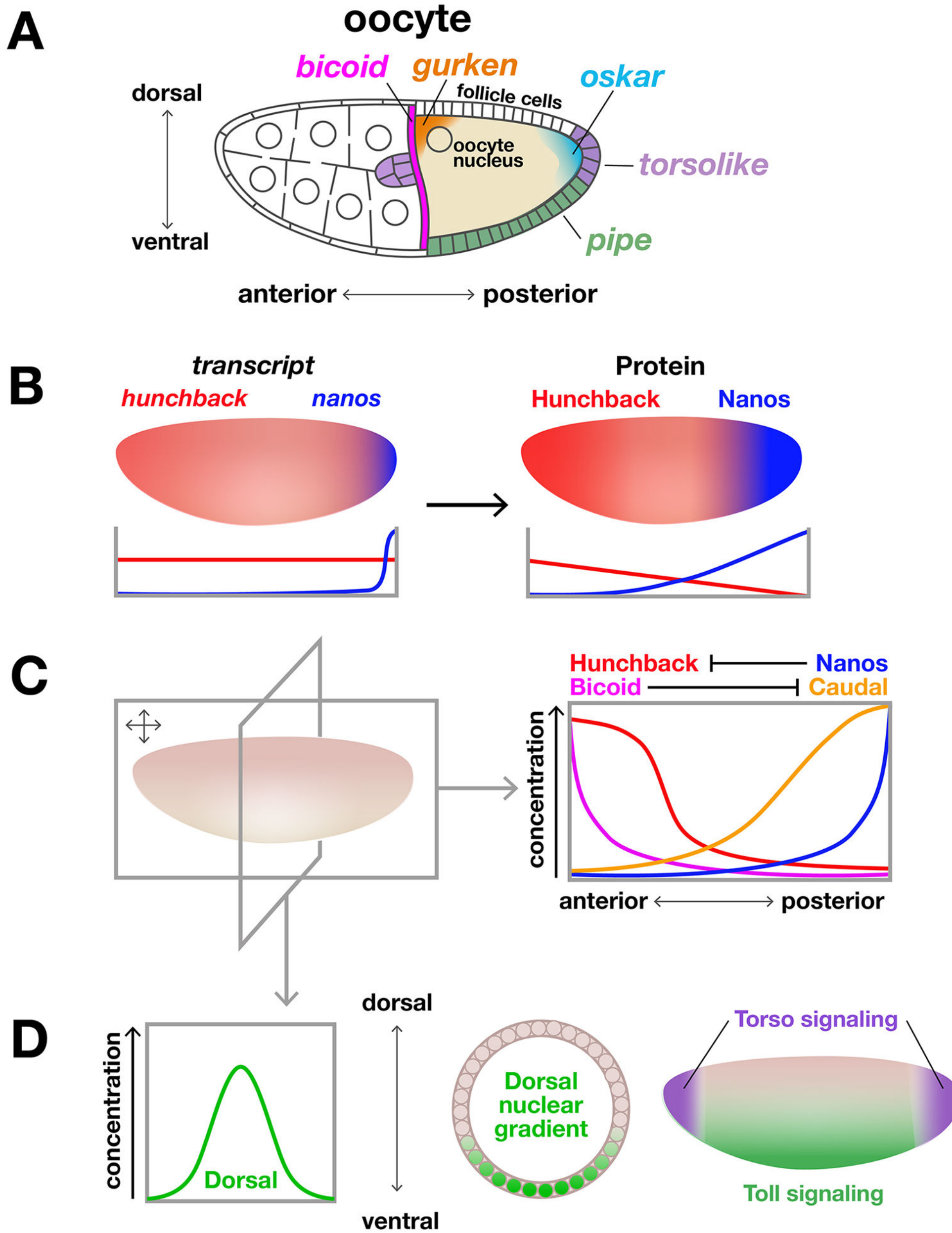

Figure 1 - Establishment of embryonic polarity

A) Key maternal transcripts are specifically localized within the oocyte by RNA-binding proteins establishing basic polarity that is transferred to the zygote. B) Oskar-dependent posterior localization of *nanos* transcript establishes an anterior-to-posterior gradient of Hunchback protein through translational repression of uniformly-distributed *hunchback* mRNA. C) Schematic of distribution of four key patterning factors along the A-P axis of the pre-blastoderm embryo. D) D-V biased Toll signaling resulting from Pipe localization in the ventral oocyte produces a nuclear gradient of the transcription factor Dorsal. *tsl* transcript deposited to the anterior and posterior of the oocyte ultimately activates Torso signaling in corresponding terminal domains in the early embryo.

Note: all follicle and embryo diagrams are oriented with anterior left and dorsal up unless otherwise specified.

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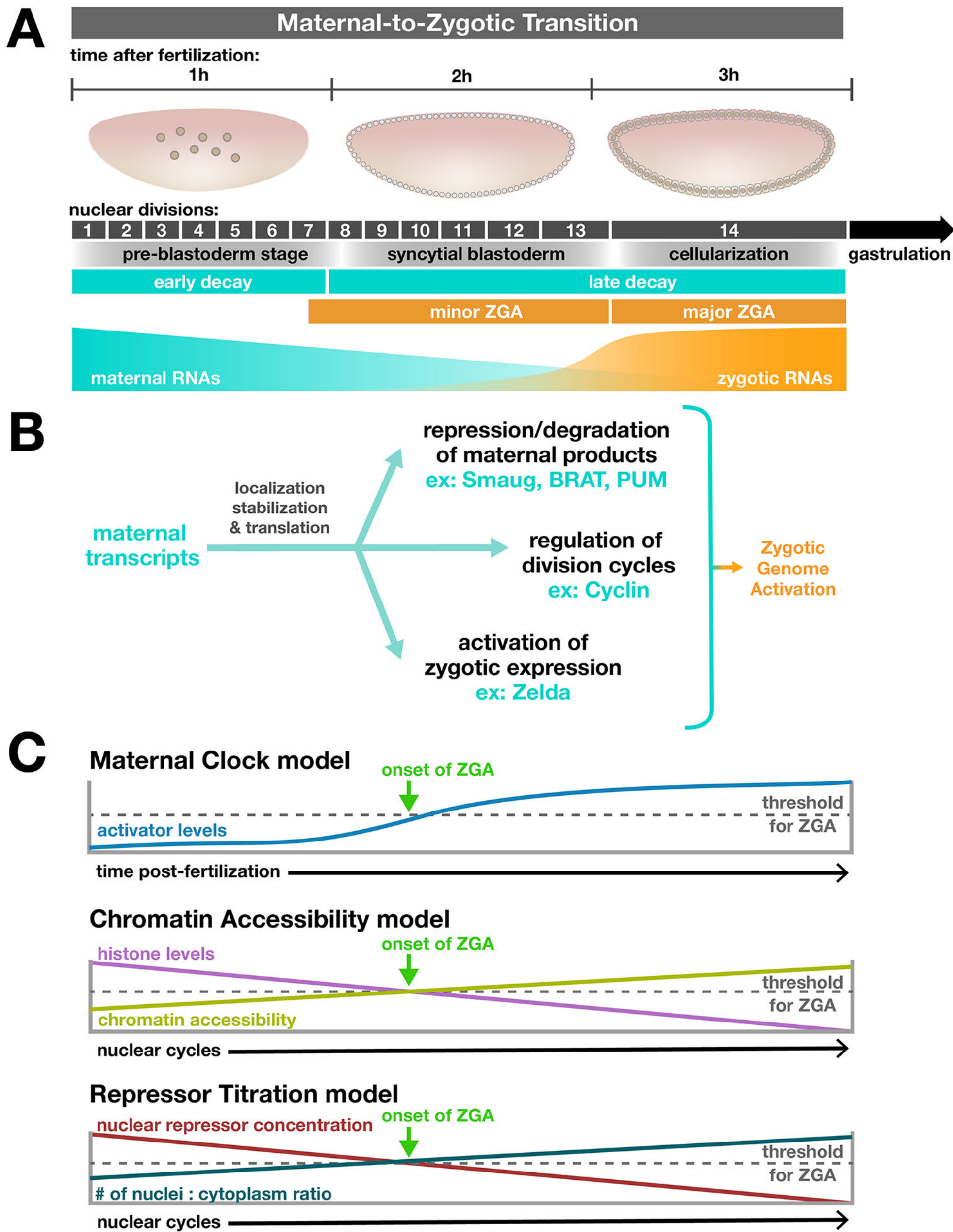

Figure 2 - Maternal to Zygotic Transition

A) Early zygotic development entails 13 rapid nuclear divisions within a common cytoplasm to produce a syncytium. Nuclei migrate to the embryo periphery and then cellularize forming a blastoderm of ~6000 cells surrounding a central yolk. In order to transition from control of development by maternal RNA and proteins to regulation driven by zytotic products, maternal factors are systematically cleared from the embryo in two phases. B) Maternal transcripts deposited into the zygote affect early development and the onset of Zygotic Genome Activation (ZGA) by their precise localization within the embryo, stabilization and translation of RNA into factors that regulate key phenomena: the repression and degradation of maternal factors, regulation of nuclear divisions as early blastoderm development proceeds, and activation of zygotic gene expression. C) Models for timing of

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ZGA. Top: “Maternal Clock” model - time required for buildup of key activators determines onset of ZGA. Middle: Chromatin Accessibility model - as nuclei divide, the concentration of soluble histones declines. Combined with other mechanisms, including activity of pioneer factors, chromatin accessibility (particularly at enhancers) increases and zygotic genes begin to be expressed. Bottom: Repressor Titration - as the ratio of nuclei to cytoplasm increases during early divisions, concentration of maternal repressors is diluted within individual nuclei; once below repression threshold, zygotic targets of repression begin to be expressed. Note: Models shown are conceptual, exact timing of ZGA and levels of relevant factors likely differ depending on context. Adapted from Hamm & Harrison, 2018.

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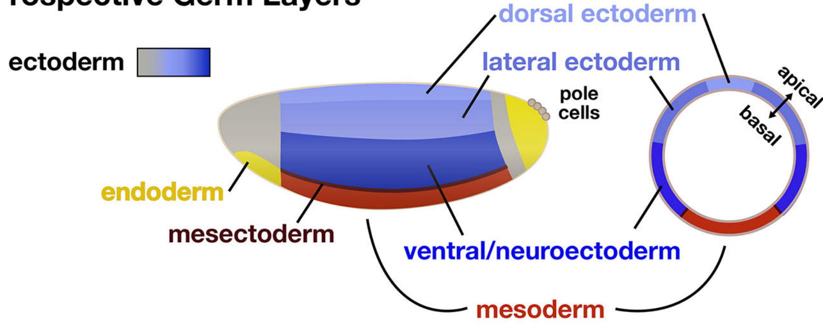

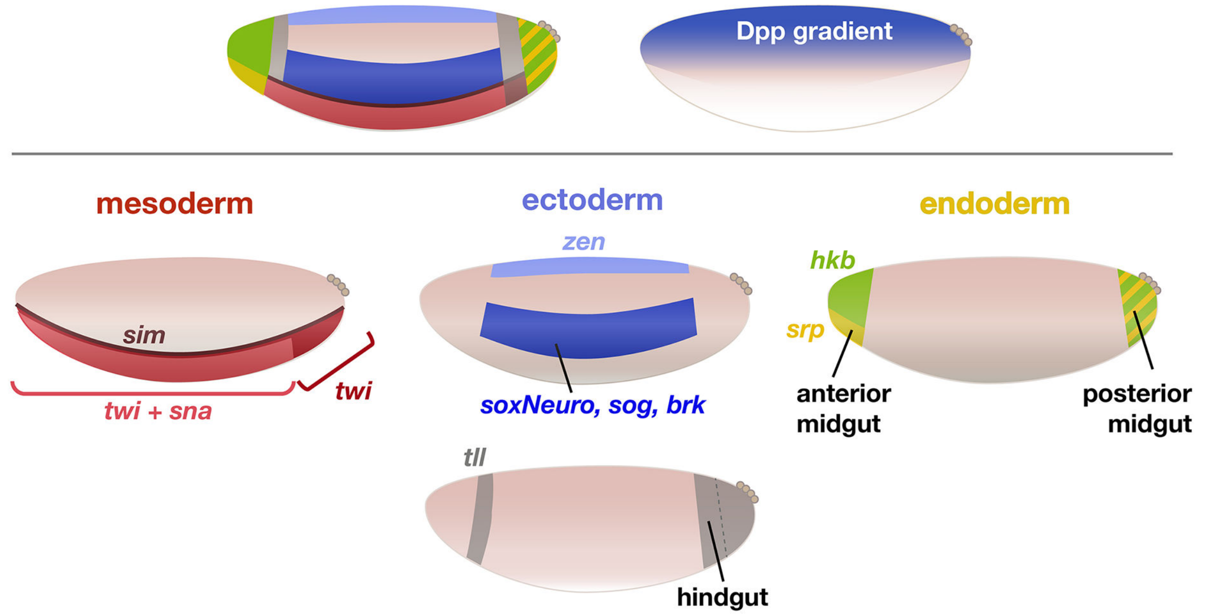

Figure 3 - Gene expression patterns establish prospective germ layers

A) Broad patterns of gene expression subdivide the embryo into domains that prefigure the germ layers. B) Ventral cells specified by expression of *twist* and *snail* and delineated by expression of *sim* will develop into the mesoderm. Domains along the trunk dorsal to *sim* comprise the future ectoderm. A dorsal gradient of Dpp signaling contributes to further subdivision of the ectodermal primordia into ventral/neuroectoderm and non-neuroectoderm (the lateral ectoderm and dorsal ectoderm/amnioserosa). Anterior and posterior ectodermal domains marked by *tll* will form the fore- and hindgut, respectively. Domains at the embryo termini marked by the expression of *hkb*, *tll*, and *srp* will form the endoderm.

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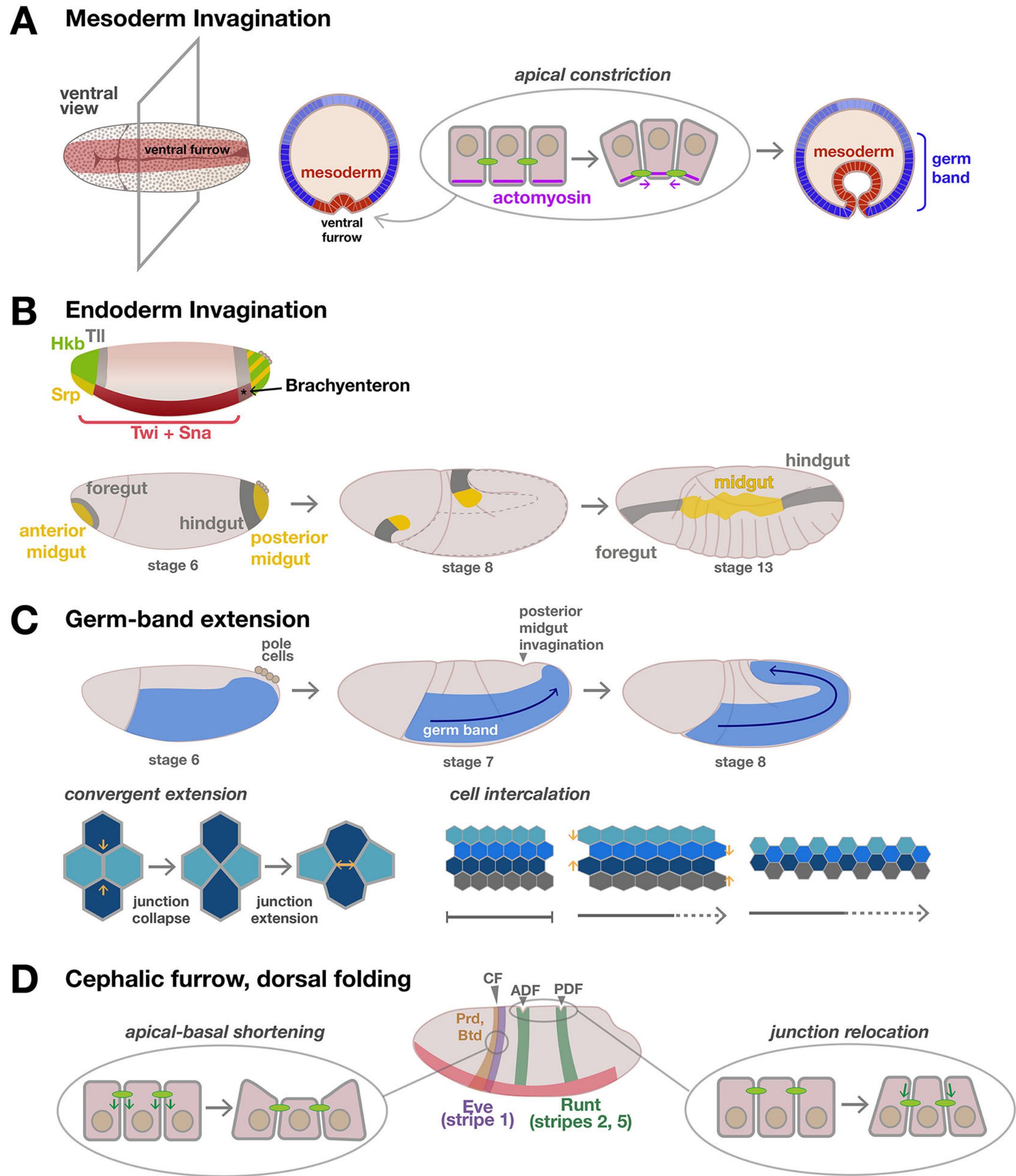

Figure 4 - Gene regulatory interactions prepare cells for diverse cell movements at gastrulation
(A) Mesoderm Invagination: Expression of Twist and Snail lead to apical constriction which generates a ventral furrow (B) Endoderm Invagination: The anterior and posterior endoderm are specified by distinct mechanisms and invaginate separately. They eventually merge to form the gut. (C) Germ-band Extension: GBE is largely driven by cell intercalation and cell shape changes that are a passive response to extrinsic tensile forces. Cells undergo convergent extension, shape changes and intercalation which leads to the overall lengthening of the ectoderm along the A-P axis, orthogonal to Toll gene expression (not shown). (D) Cephalic Furrow Formation: *eve* expression leads to myosin-independent cell shortening. Actomyosin-independent dorsal fold formation occurs at stripes of *runt* expression and

results from basal junctional repositioning. Note: For simplicity, only the relevant stripes of eve and runt expression are shown. Adapted from Gilmour, Rembold, & Leptin, 2017.

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