Tipping the scales: how *Drosophila* border cells balance cellular requirements for a successful collective migration

by

Emily Burghardt

B.A., Northern Michigan University, 2018

AN ABSTRACT OF A DISSERTATION

submitted in partial fulfillment of the requirements for the degree

DOCTOR OF PHILOSOPHY

Division of Biology
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2024

Abstract

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Approved by:

Major Professor
Dr. Jocelyn A. McDonald

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Abstract

Throughout the life of an organism, collective migrations underlie development and homeostasis, including wound healing and cancer metastasis. *Drosophila* border cells form a small collective and undergo a dynamic migration during normal egg development. During migration, the downstream activity of several signaling pathways directs collective behavior, including cell movements and adhesion remodeling, while maintaining cell-cell adhesion between border cells and responding to the dense surrounding environment. Eventually, the border cells cease migration and return to a stationary, epithelial fate. To understand how border cells balance these developmental roles, we performed RNA sequencing on border cells isolated at pre-, mid-, and late migration stages. Downstream gene ontology and protein-protein interaction network analyses identified differentially expressed genes expected to function in collective migration, but also revealed temporal regulation of ribosome biogenesis genes. Expression, interaction, and functional data obtained in these experiments can be used to identify new candidate gene groups with potential molecular roles in border cell migration. Surprisingly, activating transcription factor 4 (ATF4), a downstream effector of the integrated stress response (ISR), was among the most highly expressed genes in border cells during their migration. ATF4 overexpression and knockdown both resulted in migration defects in the border cell cluster, including significant changes in border cell number and overall cluster shape. Finally, we demonstrate that ATF4 overexpression prevents border cell motility, restricting border cells with elevated levels of ATF4 to the rear of the cluster during migration. Our transcriptome analysis and functional studies identified dynamic expression and a requirement for migration-related genes, but also ribosome biogenesis and stress response genes. Together, our data indicate a

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Dedication

I would like to dedicate this work to my parents, whose hard work and sacrifices set me up for future success.

Chapter 1 - Introduction

Collective cell migration underlies development, disease, and homeostasis throughout the life of an organism. These dynamic cell movements help to form organs, contribute to the spread of cancer, and heal wounds. Although collectively migrating cell cohorts may each accomplish different goals, defining properties are shared among them. First, cell collectives move into new tissue environments. To begin migration, the leading cells extend themselves into a new environment to explore the terrain and to pave the way for the cells following behind. Invasion of the collective can be accomplished with one or several leader cells forming protrusions, or a bud-like tip made up of several cells at the leading front (Figure 1.1; Friedl and Alexander, 2011). In a larger collective, many cells positioned at the leading edge of an epithelial sheet extend protrusions (Ridley, 2011). In some cases, invasion is facilitated by a complete or partial epithelial-to-mesenchymal transition (EMT), an induced switch in cellular characteristics commonly seen in many invasive cancers (Yang et al., 2020). Second, the invasions and ensuing migrations of collectives are coordinated by a suite of internal and external cues. Migration promoting signals, including the binding of chemokines or hormones, coordinate initial cell movement (Figure 1.1). Signaling pathways induced downstream of their receptors translate timing and physical environment into dynamic collective behavior and signaling within the cell cohort (Friedl and Alexander, 2011). Finally, collectives, especially in developmental contexts, are often marked by a return to a stationary, epithelial fate. Cells that undergo these rapid and dynamic changes retain some, or all, of their epithelial traits during migration (Friedl and Wolf, 2010; Friedl and Alexander, 2017). Through their roles in the formation of developing organs or wound healing, collective migrations return the migrating cells, and sometimes the entire organism, to homeostasis.

Collective migrations during development deliver cells to the proper locations for formation of new tissues or organs. Therefore, migration defects can result in a number of birth defects (Cordero et al., 2011). However, up-regulation of molecular mechanisms critical for collective migration drive over-proliferation and metastasis in invasive cancers (Friedl and Gilmour, 2009). Characterization of the invasion, signaling, and dynamic cell behaviors within the collective are critical for our understanding of development, disease, and homeostasis. This introductory chapter will define shared characteristics of collectives using four examples of migration during development. In the first section, the chick neural crest is used to demonstrate invasion of a collective into a new tissue environment. The second section focuses on dynamic cell behaviors in a collective in response to the environment during posterior lateral line formation in zebrafish. The third section highlights an example of continuous epithelial integrity during migration in *Drosophila* tracheal branching. Finally, sections four and five introduce the invasion, collective behavior, and return to epithelial fate of border cell migration in *Drosophila* and the work done in this system that will be presented in Chapters 2 and 3.

1.1 A brave new world: collective leaders enter diverse tissue environments

Collective migrations during development contribute to organogenesis, whether through guiding network formation in blood vessel or tracheal branching, or coiling and shaping the *Drosophila* testes myotube for proper organ shape and function (Bischoff and Bogdan, 2023; Kotini et al., 2019). Collectives also spread organ precursors throughout the developing organism, as in neural crest migration and posterior lateral line formation in vertebrates, including developing chick and zebrafish embryos, respectively (Olson and Nechiporuk, 2018; Szabó and Mayor, 2018). Each migration requires leader cells to guide the collective into a new environment. This section will use neural crest migration in the developing chick embryo to

examine how these collective leaders invade into their new environment through changes in cellular characteristics and behavior, including new transcriptomics research on defining a leader from the followers.

1.1.1 An epithelial-to-mesenchymal transition drives neural crest invasion

Neural crest cells migrate collectively during vertebrate embryogenesis and are widely studied in chick, zebrafish, and *Xenopus* embryos (Barriga et al., 2015). A hallmark of their migration is the complete or partial epithelial to mesenchymal transition (EMT) required for delamination and invasion into the surrounding tissue (Theveneau and Mayor, 2012). As cells of the collective leave the dorsal neural tube and move throughout the embryo, individual neural crest cells are deposited and differentiate at multiple sites (Figure 1.2A-B; Olson and Nechiporuk, 2018). Deposition of neural crest cells is essential for proper formation of the peripheral nervous system, pigment cells, craniofacial bone and cartilage structures, and muscle, including cardiac structures, smooth muscle cells, and tendons (Berndt et al., 2008; Kulesa and Gammill, 2010; Theveneau and Mayor, 2012). Thus, improper migration can result in a variety of neural crest disorders, or neurocristopathies, including common birth defects like cleft lip or palate and tube closure defects (Kulesa and McLennan, 2015; Ross and Zarbalis, 2014).

During embryogenesis, neural crest cells arise at the neural plate border between the developing neuroepithelium and surface ectoderm (Kulesa et al., 2004). Here, cues from the surrounding neural ectoderm, mesoderm, and neuroepithelium initiate signaling cascades in the pre-migratory neural crest cells to begin the cellular architecture remodeling and changes in adhesion associated with the EMT (Kulesa and Gammill, 2010). In the chick trunk neural crest, these inductive cues include BMP, Wnt, fibroblast growth factor (FGF), retinoic acid, and Notch signals (Figure 1.2C; Milet and Monsoro-Burq, 2012; Theveneau and Mayor, 2012). In neural

crest cells, the EMT encompasses a shift towards the mesenchymal state in leader cells, including modifications to cell-cell adhesion and polarity, and an increase in migratory and invasive behavior (Figure 1.2B-C; Nieto et al., 2016). A complete or partial EMT is required for the exit of neural crest cells from the dorsal neural tube and initiation of migration (Mayor and Theveneau, 2013). Despite the wide variety of species in which neural crest cell migration occurs, the signaling cascade initiated in pre-migratory neural crest cells is remarkably conserved (Theveneau and Mayor, 2012). Signals from the surrounding tissue activate expression of transcription factors Snail/Slug, Foxd3, and SoxE family members (Figure 1.2C; Berndt et al., 2008; Mayor and Theveneau, 2013). Each transcription factor then induces changes associated with an EMT, including changes in cadherin expression and activity, cell-cell adhesion, and the local extracellular matrix (ECM). The EMT transcription factor Slug is required as a specific delamination cue to finally initiate migration (Kulesa and Gammill, 2010).

Changes in cell-cell adhesions underlie the switch from stationary cells to a migratory collective in the neural crest. Prior to migration, the neural crest cells and surrounding neuroepithelium express high levels of N-cadherin, a component of the strong cell-cell adhesions between them (Theveneau and Mayor, 2012). In the trunk neural crest cells, transcription factors Snail/Slug, Foxd3, and Sox9/10 control a switch from high to low levels of N-cadherin expression, as well as expression of weaker type II cadherins (Figure 1.2B; Mayor and Theveneau, 2013; Theveneau and Mayor, 2012). Type II cadherins, including cadherins 6, 6B, 7, and 11, form weaker cell-cell adhesions and are associated with migratory and mesenchymal phenotypes, including in invasive cancer (Chalpe et al., 2010; Van Roy, 2014). As migration progresses, the initial continuous wave of neural crest cells exiting the dorsal neural tube splits into smaller, discrete streams (Theveneau and Mayor, 2012). The cells that comprise the streams

remain adhered to one another, but in a chain-like manner with more transient adhesions (Figure 1.2B; Olson and Nechiporuk, 2018). Neural crest cells also express proteases, including ADAM10, capable of cleaving cadherins. ADAM-10-dependent cleavage has been shown to both decrease cell-cell adhesion among the pre-migratory neural crest cells and loosen neural crest cells from neighboring neuroepithelial cells (Mayor and Theveneau, 2013; Theveneau and Mayor, 2012). Downstream transcription factor activity further promotes delamination and protrusion formation through basal lamina degradation and activation of RhoB and β-integrin expression (Theveneau and Mayor, 2012).

As in other collective migrations, cells at the leading front of the collective extend dynamic protrusions to sense the surrounding environment and move the collective forward. In a chick neural crest EMT, cells exhibit a sequence of protrusive activity: first, upon loss of contact with the surrounding tissue, gaps between cells are visible (Berndt et al., 2008). Then, membrane blebs are seen protruding into the gaps before cells exit the neural tube. Finally, upon exit from the tube, longer membrane extensions like lamellipodia and filopodia are formed by cells (Berndt et al., 2008; Kulesa and Gammill, 2010). Neural crest cells migrate in multicellular streams with distinct front, leading, and trail subregions (Morrison et al., 2017; Theveneau and Mayor, 2012). Cells at the front of the stream exhibit protrusive activity in multiple directions to sense the environment as they move forwards (Figure 1.2B; Kulesa et al., 2004; Kulesa and Gammill, 2010).

1.1.2 Invasion profiles separate the leaders from the followers

Migratory behaviors are initiated and directed in leader cells of a collective by cell signaling, often triggered by external cues. In the neural crest cell collective, migratory mesenchymal characteristics, including protrusion formation and changes in cell-cell adhesions,

are triggered in leader cells by EMT-associated transcription factors (Mayor and Theveneau, 2013). Recently, transcriptomics research has contributed to our understanding of gene-specific expression profiles in both developmental and disease contexts. In the neural crest cell population, cells migrate in one of three subregions: at the invasive front region, the immediately following lead region, or the trailing region (Morrison et al., 2017). Single cell RNA sequencing of neural crest cells identified differential gene expression in the invasive front compared to the lead and trail regions of the stream (McLennan et al., 2015; Morrison et al., 2017). Depending on their position within the stream, neural crest cells can differ in speed, direction, proliferation, and cell morphology (McLennan et al., 2012). Further, differences in gene expression separate the cell leaders, especially in the invasive front, from the trailing cells (McLennan et al., 2012). Neural crest cells at the invasive front region extend protrusions in multiple directions to sense the microenvironment as they move (Figure 1.2B; Kulesa et al., 2004; Kulesa and Gammill, 2010). Specifically, the expression profile of these cells at the invasive front differed the most from any other stream position, and included genes associated with Wnt and β-catenin signaling pathways, the EMT, tight and gap junctions, Rho family GTPases, and integrins (Figure 1.2C; Morrison et al., 2017). Genes consistently expressed by neural crest cells at the invasive front, termed “trailblazer” genes, contribute to the ability of the collective to move into a new environment (Figure 1.2C; McLennan et al., 2015; Morrison et al., 2017). Knockdown of several trailblazer genes results in a decreased invasive ability and distance migrated for neural crest cells (McLennan et al., 2015). A rapid change in gene expression profile also accompanies neural crest cell exit from the dorsal neural tube, as cells transition from the EMT to directed motility towards their target (Morrison et al., 2017).

Recent transcriptomics data has further underlined the similarities between collectively migrating cells and cancer metastasis, both in gene expression and cell behavior. Highly invasive cancer cells can utilize collective migration to metastasize throughout the body, allowing colonization of further tissue and negatively affecting patient prognosis (Stuelten et al., 2018). Both neural crest cells and cancer utilize an EMT, highly invasive cell behaviors, and long distance migrations to new tissue environments to spread throughout an organism (Mayor and Theveneau, 2013). Changes in cadherin expression are common during embryogenesis, including prior to neural crest cell delamination. Expression of N-cadherin is associated with an EMT, both in cancer and neural crest cell delamination (Nieto et al., 2016; Theveneau and Mayor, 2012). Snail, Twist, SoxE, and FoxD are among many transcription factors activated by a suite of signaling pathways in cancer, but are also required for the initiation of neural crest cell migration (Theveneau and Mayor, 2012). The tumor suppressor p53 and transcription factor Slug are expressed prior to and required during delamination for neural crest cells and are associated with the EMT during cancer metastasis (Campbell et al., 2018; Kulesa and Gammill, 2010; Nieto et al., 2016; Theveneau and Mayor, 2012). Just as neural crest cells express proteases like ADAMs to alter cell-cell adhesions with neighboring cells and local extracellular matrix, tumor cell expression of matrix metalloproteases and ADAMs is often associated with a high invasive potential (Herrlich and Herrlich, 2017; Mayor and Theveneau, 2013; Theveneau and Mayor, 2012). Additionally, a subset of the invasive gene signature identified in neural crest cells is shared with cancer, including neuroblastoma cells. Up-regulation of these invasive genes in neuroblastoma cells is correlated with their ability to invade into the chick embryo microenvironment in transplantation experiments (Morrison et al., 2021).

1.2 Answering the call: collective cell behaviors are shaped by dynamic cell signaling and microenvironment

Collective cell migrations are often initiated and driven by external cues from the surrounding tissue, including chemokine and ligand binding, signaling pathways, and hormones. Because cells in a collective do not migrate in free space, these initiators trigger downstream signaling pathways to direct behavior in response to other cells and the surrounding environment. This section details a collective migration required for zebrafish posterior lateral line formation to demonstrate the effects of external cues from the microenvironment, cell-cell signaling within the cluster, and the surrounding environment, on cell fate and collective cell behaviors.

1.2.1 External and internal signaling initiate migration and determine cell fate

External cues from the surrounding tissue typically drive migration initiation in the collective. These cues trigger downstream signaling pathways to shape the cell-cell communication critical for a successful migration. The zebrafish posterior lateral line primordium (PLLp) is an example of such downstream signaling that triggers cell fate and migration in a collective. The PLLp is a cluster of about 100 cells which migrates at around 22 hours post-fertilization along the lateral aspect of the trunk of the developing embryo (Olson and Nechiporuk, 2018). Throughout their roughly 24 hour migration to the tip of the developing tail, cells remain tightly connected to one another via adherens junctions (Olson and Nechiporuk, 2018). The PLLp cluster is composed of four regions: tip cells, at the very front of the cluster, the leading region, immediately behind, the trailing region (also known as the “follower” region), and finally, the depositing neuromast (Figure 1.3A; Haas and Gilmour, 2006; Olson and Nechiporuk, 2018). The tip cells have a more mesenchymal morphology, including a flatter shape, than the remaining cells (Chitnis et al., 2012; Olson and Nechiporuk, 2018). Cells of the

leading region display more epithelial characteristics, but are less differentiated than cells of the trailing, or "follower" region (Olson and Nechiporuk, 2018). Cells of this region are the most epithelial, with a more columnar morphology (Chitnis et al., 2012). During migration, these cells form polarized rosettes. Clusters of 20-30 cells, including the rosettes, are deposited from the trailing end during migration, leaving behind proto-neuromasts that will develop into the lateral line system (Figure 1.3A; Colombi et al., 2020; Olson and Nechiporuk, 2018). The lateral line is a network of mechanosensory organs innervated by afferent neurons to allow the fish to sense minute vibrations in the water (Colombi et al., 2020).

As in neural crest cell migration in vertebrate embryonic development, cells of the PLLp migrate in response to an external cue, specifically *stromal derived factor 1* (SDF1a/Cxcl12A in zebrafish), but hereafter referred to as Cxcl12A (Colombi et al., 2020). Cxcl12a, along with its chemokine receptor Cxcr4b, has been shown to direct the migration of leukocytes, neurons, and primordial germ cells, among others, during development (Haas and Gilmour, 2006). In zebrafish, this chemokine is expressed uniformly in cells forming the myoseptum, a developing tissue that the PLLp will migrate along (Figure 1.3B; Colombi et al., 2020; Olson and Nechiporuk, 2018). Cxcl12A is required for the initiation of PLLp migration- loss of the cue results in a failure to migrate, and ectopic expression of Cxcl12A causes PLLp migration towards the new source (Haas and Gilmour, 2006; Olson and Nechiporuk, 2018). Because Cxcl12A is uniformly expressed by the cells of the myoseptum, this external cue allows bidirectional migration along the developing lateral line (Haas and Gilmour, 2006). Therefore, an internal cluster gradient must be created within the PLLp cluster via differential expression of two chemokine receptors.

Cells in the leading region of the PLLp express the chemokine receptor Cxcr4b, while trailing region cells express receptor Cxcr7b (Figure 1.3B; Olson and Nechiporuk, 2018). Cxcr4b in the leading region, especially in the tip cells, triggers chemotaxis in response to Cxcl12A (Xu et al., 2014). In the trailing region, Cxcr7b acts as a ligand sink, internalizing and degrading Cxcl12A to create an area of lower concentration (Figure 1.3B; Dalle Nogare et al., 2020; Donà et al., 2013; Venkiteswaran et al., 2013). This gradient creates directionality for the cluster in response to Cxcl12A expression. Self-generated gradients promote migration of other collectives, including border cell migration (reviewed in section five) and tumor metastasis (Muinonen-Martin et al., 2014).

Within the migratory cluster, signaling pathway activity further defines leading and trailing regions. The canonical Wnt pathway, active in the leading region, is required for proper proliferation, migration, and deposition of proto-neuromasts (Olson and Nechiporuk, 2018). In the trailing region, fibroblast growth factor (FGF) signaling is required for proper proto-neuromast formation in the apical constriction of polarized cellular rosettes, specifically the induction of cell shape changes by Ras/MAPk signaling and hair cell specification (Chitnis et al., 2012). Wnt signaling in the leading region initiates expression of Fgf3/10A ligands in leading cells, which activate the downstream FGF signaling pathway in the trailing region (Figure 1.3C) (Olson and Nechiporuk, 2018). Here, activity of each signaling pathway activity defines its own cluster region while restricting activity of the other. In the leading region, Wnt further initiates expression of FGF inhibitors, including *dusp6* and *sef* (Figure 1.3C; Olson and Nechiporuk, 2018). FGF ligands expressed downstream of Wnt in the leading region cannot localize to activate FGF signaling in the presence of the inhibitors and thus diffuse to the adjacent trailing

end, activating FGF receptor signaling (Chitnis et al., 2012). FGF signaling at the trailing end then initiates expression of Wnt inhbitors *dkk1/2* (Figure 1.3C; Olson and Nechiporuk, 2018).

1.2.2 Physical interactions with the developing tissue guide dynamic cell behaviors

Expression of the chemokine Cxcl12A in the migration substrate, the developing myoseptum, directs the initiation of migration and direction, with a self-generated gradient within the PLLp. The tip cells at the very front of the PLLp, exposed to extracellular signals including Cxcl12A, actively migrate and show protrusive behavior (Figure 1.3B; Olson and Nechiporuk, 2018). Throughout migration, dynamic filopodia and pseudopodia are most apparent in the tip cells, the most mesenchymal and migratory cells of the cluster (Haas and Gilmour, 2006; Xu et al., 2014). Cxcr4b, the chemokine receptor expressed in the leading region and the tip cells, is a member of the GPCR family (Xu et al., 2014). Binding of Cxl12A to its receptor Cxr4b initiates GPCR signaling, promoting directed protrusions and changes in cell shape for forward migration (Chitnis et al., 2012; Xu et al., 2014). Specifically, the GB1 subunit of Cxcr4b has been shown to be required in the tip cells for migration and has a role in regulating F actin dynamics and protrusion formation (Xu et al., 2014). Although these dynamic processes are most apparent in the tip cells, protrusions are also visible in cells along the sides of the migratory PLLp that are also exposed to the chemokine Cxl12A (Haas and Gilmour, 2006). Recently, even the most epithelial cells of the cluster, found in the trailing region, have been shown to extend cryptic lamellipodia during migration (Figure 1.3B; Dalle Nogare et al., 2014, 2020). Even as the apical ends of these cells constrict to form rosettes, lamellipodia are still extended from the basal ends (Chitnis et al., 2012; Dalle Nogare et al., 2020).

Throughout migration, collectives form dynamic protrusions and adhesions to actively pull on, and generate traction with, the underlying substrate (Haeger et al., 2015). Recent work

has identified the overlying skin of the embryo as a new substrate in addition to the basement membrane substrate the PLLp migrates on (Dalle Nogare et al., 2020; Yamaguchi et al., 2022). The PLLp cluster makes significant contact with the skin as it migrates between the underlying somites and overlying skin of the embryo (Dalle Nogare et al., 2020). The presence of the skin is required for proper formation of the polarized, actin-rich protrusions, formed by the tip cells, that guide migration of the cluster (Dalle Nogare et al., 2014). Interestingly, the overlying skin is also required for formation of the smaller, basal protrusions seen in all cell regions of the cluster, even though not all of these cells experience contact with the skin during migration (Dalle Nogare et al., 2014). These smaller protrusions from non-leader cells may also be used to generate traction with the substrate like cryptic lamellipodia (Haeger et al., 2015).

1.3 Self-preservation: collectively migrating cells retain or return to their epithelial identity

Collective cell migration involves rapid, dynamic changes in cell shape and movement. Often, some loss of epithelial characteristics accompanies those changes, especially in leaders of the cell cohort. In contrast to the gain of mesenchymal characteristics in leading cells of the neural crest and lateral line primordium, some migrating collectives retain epithelial integrity, including cell-cell adhesions and apicobasal polarity. This section will highlight *Drosophila* tracheal branching, an example of epithelial integrity throughout collective migration, to detail first, how signaling pathways direct cell fate and remodeling in an epithelial sheet, and second, the incredible maintenance of epithelial characteristics during tube formation.

1.3.1 Cell signaling directs remodeling of an epithelial sheet

The building of complex organ structures from epithelial sheets requires extensive cell remodeling. To form blood vessels, airways of the lung, and the tracheal system, epithelial sheets

reorganize into tube-like structures in a process termed branching morphogenesis (Goodwin and Nelson, 2020). Although the molecular signals that initiate branching morphogenesis are remarkably conserved across organisms, the physical mechanisms of branching are varied (Varner and Nelson, 2014). Branch types, branching structures formed, and the degree of adhesion maintained between branching cells differs among systems (Goodwin and Nelson, 2020). In vertebrate blood vessel and *Drosophila* tracheal tube formation, collective migration drives branching morphogenesis (Scarpa and Mayor, 2016). Here, a subset of leading cells guides the extension of new branches while remaining adhered to the follower cells that lag behind (Olson and Nechiporuk, 2018; Varner and Nelson, 2014). The attached cell collective then undergoes critical aspects of cellular remodeling, including neighbor cell exchange and cell fate and shape changes, all while maintaining the epithelial integrity of the sheet (Spurlin and Nelson, 2017). This process results in highly complex branched structures formed by collective migration without alterations to cell-cell adhesions.

In the *Drosophila* embryo, collective migration drives branching morphogenesis to transform simple epithelial sheets into a highly branched and complex tracheal network. The tracheal system consists of a large dorsal trunk tubule, which connects openings in the *Drosophila* body wall known as spiracles to a larger tracheal network for airflow throughout the body (Hayashi and Kondo, 2018). Collective migration and epithelial integrity are critical for the primary branching process, in which 10 early tracheal branches form, invaginate into the body cavity, and rearrange into tubules without proliferation or loss of cell-cell contact (Affolter and Caussinus, 2008; Hayashi and Kondo, 2018). Primary branching is initiated by stage 11 of embryogenesis, when epithelial sacs form along the sides of the embryo. Epidermal cells of each sac, determined to become tracheal cells via specific transcription factor expression, invaginate

to form the tracheal pit while remaining attached to one another and the neighboring, non-tracheal cells (Affolter and Caussinus, 2008). Upon invagination, fibroblast growth factor (FGF) signaling begins to direct migration of the cell cohort (Lebreton and Casanova, 2013; Spurlin and Nelson, 2017).

In the newly formed primary branch, FGF and downstream signaling define leader and follower cell fate in the collectively migrating, branching cell population. FGF signaling selects a population of cells within the branch to become migratory (Spurlin and Nelson, 2017). Tracheal cells express breathless, an FGF receptor, and *dof*, an FGF-specific signaling adaptor (Figure 1.4A; Affolter and Caussinus, 2008). Migration occurs towards areas of high *branchless* (the *breathless* ligand, or FGF) concentration to extend the primary branches of the tracheal network outward into the body cavity (Affolter and Caussinus, 2008). Tip cells, the leaders of the migratory collective, compete for the position surrounded by the highest concentration of *branchless* (Spurlin and Nelson, 2017). In response to *branchless*, tip cells begin to display migratory characteristics. Tip cells have a longer shape than other cells in the collective and extend dynamic filopodia and lamellipodia towards the regions of high *branchless* expression (Figure 1.4A; Affolter and Caussinus, 2008; Lebreton and Casanova, 2013). Downstream signaling further defines the leaders from the followers in the collective. High levels of FGF signaling in the tip cells activate the *Notch* ligand, Delta, in the follower cells, also known as the stalk region of the collective (Affolter and Caussinus, 2008). *Notch* signaling in the stalk cells decreases FGF signaling, further defining their non-migratory fate (Figure 1.4A; Schottenfeld et al., 2010). Tip cells also enforce stalk cell fate via secretion of MMP2 to decrease the amount of active FGF ligand available to stalk cells (Schottenfeld et al., 2010).

After primary branch formation, signaling directs specification of tip cells as terminal or fusion cell types to continue tracheal network formation (Affolter and Caussinus, 2008). Fusion cell fate is specified by *Notch* signaling (Spurlin and Nelson, 2017). Each fusion cell deposits E-cadherin, triggering the formation of actin and microtubule-rich filopodia (Spurlin and Nelson, 2017). A fusion cell from each branch then extends filopodia towards the other, eventually connecting to link extending branches of the network together (Figure 1.4A; Kotini et al., 2019; Schottenfeld et al., 2010). Fusion of these cells produces a continuous tube (Schottenfeld et al., 2010). Tip cells can also be specified as terminal cells, from which further branching occurs during larval stages of development (Schottenfeld et al., 2010).

1.3.2 Keeping it together: remodeling of cell-cell contacts maintains epithelial integrity

Epithelial integrity is continuously maintained during the collective cell migration and remodeling of *Drosophila* tracheal branching. During the entire process, all tracheal cells remain connected as an epithelial sheet via adherens junctions (Kotini et al., 2019; Ribeiro et al., 2004). Tracheal cell proliferation stops as primary branching begins; therefore, cell remodeling is critical to achieve the final network (Lebreton and Casanova, 2013). To form the epithelial sheet into a longer, thinner tube, the stalk (follower) cells exchange neighbors and intercalate (Schottenfeld et al., 2010). Stalk cell intercalation occurs in all primary branches except for branches that form the large dorsal trunk (Caussinus et al., 2008). Along the outgrowing branch, individual stalk cells are arranged side to side and connected by adherens junctions (Figure 1.4B; Caussinus et al., 2008). The apical surfaces of each cell contribute to the lumen of the developing tube. Here, intercalation begins with a unique cellular behavior termed “reaching around” (Ribeiro et al., 2004). Individual cells reach around the lumen to make self-contact and establish

autocellular junctions (a junction formed as the cell makes self-contact by wrapping around the lumen) at the opposite side of the forming tube (Figure 1.4B; Kotini et al., 2019; Ribeiro et al., 2004; Tepass and Hartenstein, 1994). The autocellular junctions extend as paired stalk cells continue intercalation by moving past one another, a process termed “zipping up” (Figure 1.4B; Affolter and Caussinus, 2008; Ribeiro et al., 2004). To maintain epithelial integrity as the process continues, stalk cells must stop intercalation before all connections with neighboring cells are lost. Thus, the paired stalk cells stop rearranging once a chain-like, head to tail formation is reached from the initial side to side cell arrangement (Ribeiro et al., 2004). Stalk cells forming the tube have autocellular junctions along the outgrowing branch but remain connected to the more proximal and distal cells via small remaining intercellular junctions (Figure 1.4B; Ribeiro et al., 2004). Stalk cell intercalation is required for tube formation but may also occur in response to dorsal branch elongation. As tip cells migrate forward, tensile stress is created in the extending dorsal branch (Caussinus et al., 2008). To mediate this stress, stalk cells form a longer chain instead of remaining adjacent to one another. In this way, remodeling of cell-cell contacts shapes a sheet into a growing tube with careful maintenance of epithelial integrity. As in other collectives, a subset of leader cells guides migration, and downstream signaling defines and maintains the fate of the leaders and follower cells behind. However, the branching tracheal network is formed without the loss or significant changes to cell-cell adhesions between neighboring cells required in other collectives for delamination and invasion. Here, cells remain epithelial for their contribution to a complex epithelial structure.

1.4 *Drosophila* border cell migration: a model with definitive collective characteristics

Collective migrations throughout development are defined by shared characteristics: invasion and migration into a new environment, shaped by signaling and interaction with the surrounding tissue. Cells remain adhered to one another throughout migration, but in some collectives, epithelial integrity is carefully maintained. *Drosophila* border cells, which migrate collectively during normal egg development, are a genetically tractable *in vivo* model with which to investigate the molecular mechanisms of collective migration. The border cell collective must detach from the underlying tissue to migrate into and through a dense cellular environment. Similar to other collective models highlighted in this introductory chapter, both the initiation and direction of border cell migration is shaped by signaling and interaction with the large nurse cells, their migratory substrate. Finally, border cells also retain epithelial characteristics, including polarity and cell-cell adhesions, throughout their migration. After their migration, border cells return to a stationary, epithelial fate to contribute to the final maturation of the *Drosophila* egg. This section will further define border cell migration through introduction of the molecular players required for each shared characteristic of their collective migration.

1.4.1 How border cells cross the road: a collective migration through the egg chamber

Border cell migration takes place in the *Drosophila* ovary. Each ovary is made up of approximately 18 ovarioles of connected egg chambers progressing through the 14 developmental stages of oogenesis (Figure 1.5A; Bastock and St Johnston, 2008; Spradling, 1993). At the anterior end of each ovariole lies the germarium, which contains stem cells for germline and somatic tissue. Individual egg chambers begin as 16 cell cysts that bud from the

germarium. Each cyst, and eventual egg chamber, is comprised of 15 germline cells, termed nurse cells, and one oocyte (Figure 1.5A). The entire egg chamber is surrounded by a continuous follicular epithelium (Bastock and St Johnston, 2008; Spradling, 1993). Oogenesis itself is shaped by several individual and collective cell movements. Follicle cells migrate to shape the developing egg chamber from round to a more ovoid shape and to enclose the oocyte (Berg et al., 2023). Convergent extension movements contribute to the formation of two dorsal appendages, critical for gas exchange in the mature egg, and a small population of follicle cells, known as the border cells, migrate collectively through the larger nurse cells that fill the egg chamber (Berg et al., 2023).

Similar to other migrating collectives, border cells are specified in response to an external cue. During stage 8 of oogenesis, the border cell cluster is organized from the follicular epithelium by two polar cells at the anterior end of the egg chamber (Figure 1.5A). Polar cells secrete *Unpaired*, a JAK/STAT ligand, to establish a gradient of JAK/STAT activation in the adjacent follicle cells (Beccari et al., 2002; Ghiglione et al., 2002; Silver and Montell, 2001). *Unpaired* binds *Domeless*, the *Drosophila* JAK/STAT receptor, which activates JAK and allows for binding of STAT to initiate downstream signaling in the follicle cells (Silver et al., 2005). In the four to eight follicle cells with the highest levels of JAK/STAT signaling, expression of *slow border cells* (Slbo), a border cell fate determinant, is activated. These cells form the migratory cluster, in which Slbo further activates cascades of genes required for migration (Borghese et al., 2006; Silver and Montell, 2001; Wang et al., 2006). JAK/ STAT signaling in required throughout border cell migration; however, the correct level of STAT must be maintained in the cluster (Silver et al., 2005; Starz-Gaiano et al., 2008). STAT activity activates the transcription factor *apontic*. Both *apontic* and the small noncoding RNA *mir-279* function as STAT feedback

inhibitors to define migratory and non-migratory cells. Through transcriptional regulation, *apontic* and *mir-279* maintain high STAT in the migratory border cells and restrict activity in the follicle cells that remain behind (Starz-Gaiano et al., 2008; Yoon et al., 2011).

After specification, the border cells then round up into a cluster and begin their migration at early stage 9 of oogenesis (Figure 1.5B). Detachment, or delamination, of the cluster from the underlying epithelium is the result of remodeling adhesions with neighboring follicle cells and the formation of cellular extensions at the cluster front. Live imaging studies have shown that delamination often occurs slowly- from initial rounding of the cluster to final detachment from the underlying epithelium and basal lamina can take 2 hours (Chen et al., 2023; Prasad and Montell, 2007). Initially, one or two border cells extend and retract protrusions towards the oocyte, the direction of migration (Figure 1.5B; Fulga and Rørth, 2002; Mishra et al., 2019). A single leader cell eventually forms a large actin-rich protrusion, a hallmark of delamination, between the nurse cells (Figure 1.5C). As this leader cell finally moves forward, the remaining border cells delaminate from the epithelium (Mishra et al., 2019). Cytoskeletal dynamics, including protrusion formation and retraction, are critical for delamination and initiation of migration. Attachment of actin-rich protrusions to the substrate generates force to move the cluster forward, while myosin contractility retracts the lagging edge (Friedl and Gilmour, 2009; Plutoni et al., 2019). F-actin accumulates on the apical side of border cells prior to their detachment (McDonald et al., 2008). Both the actin bundling protein Fascin (*singed* in *Drosophila*) and the serine-threonine kinase *par-1* promote proper protrusion length and localization (Lamb et al., 2020; McDonald et al., 2008). *Par-1* additionally localizes to and increases active Myosin II at the cluster rear to promote contractility and detachment (Majumder et al., 2012).

After delamination, the border cell cluster moves rapidly through the egg chamber. The front, outer cell of the cluster is highly polarized and forms dynamic forward protrusions (Figure 1.5B; Prasad and Montell, 2007). These actin-rich protrusions probe and form adhesions with the microenvironment, as well as establish directionality (Montell et al., 2012). As the leading edge of collectives, including border cells, moves forward, the lagging edge retracts via actomyosin contraction (Vicente-Manzanares et al., 2009). The two non-motile polar cells are carried along in the center of the cluster (Figure 1.5B; Niewiadomska et al., 1999). Midway towards the oocyte, the border cell cluster moves more slowly (Bianco et al., 2007). Cells often appear more rounded, but continue to probe the surrounding environment with shorter extensions. During this second half of migration, the cluster can rotate or "tumble" more slowly, still moving towards the oocyte (Bianco et al., 2007). By stage 10 of oogenesis, border cells complete their roughly 150 μm migration and stop at the oocyte boundary (Figure 1.5B; Silver and Montell, 2001). The cluster flattens along the oocyte surface, turns, and migrates a much shorter distance, finally coming to rest near the oocyte nucleus (Bastock and St Johnston, 2008). The developmental goal achieved through border cell migration is the formation of the micropyle, a sperm entry port critical for fertilization of the mature egg (Figure 1.5A; Montell et al., 1992).

1.4.2 Inside and out: cluster signaling and interaction with the environment direct border cell migration

From initiation to completion of migration, the border cell cluster is directed by external cues, internal cluster signaling, and the surrounding environment. Secretion of a cytokine from the polar cells specifies border cells, but the timing of their migration, as in other collective examples, is controlled by an extracellular signal. Border cell migration is initiated by a pulse of the steroid hormone ecdysone (Figure 1.5C; Jang et al., 2009). Levels of ecdysone begin to rise

in the egg chamber during stage 8 (Montell et al., 2012). At stage 9, ecdysone levels are highest in anterior follicle cells, including the border cells (Jang et al., 2009). In border cells, ecdysone activates the ecdysone receptor, which forms a heterodimer with *Ultraspiracle* (Usp) to bind DNA and the ecdysone coactivator *Taiman* (Tai) (Bai et al., 2000). All three proteins are required for migration. *Abrupt*, expressed throughout the egg chamber, directly binds Tai to negatively regulate ecdysone signaling (Jang et al., 2009). JAK/STAT signaling activity in the border cells antagonizes *Abrupt* to promote the ecdysone response (Jang et al., 2009). Thus, external specification and timing cues are integrated in the border cell cluster.

Upon initiation of migration, guidance signaling from the oocyte directs border cells to move forward. Two receptor tyrosine kinases, PVR and EGFR, function in border cells to guide their migration (Figure 1.5D) (Duchek and Rørth, 2001; McDonald et al., 2003). Chemoattractant ligands for PVR (*PDGF and VEGF related factor 1*, PVF1) and EGFR (*Spitz*, *Keren*, and *Gurken*) are secreted by the oocyte (Duchek et al., 2001; Duchek and Rørth, 2001; McDonald et al., 2006). *Spitz* and *Keren* function with PVF1 to guide the initial phase of border cell migration towards the oocyte (McDonald et al., 2006). Near the end of migration, binding of *Gurken* to EGFR specifically guides border cells to migrate dorsally along the oocyte surface towards the oocyte nucleus (Duchek and Rørth, 2001). Both PVR and EGFR are important not only for guidance, but to ensure forward movement through regulation of front protrusions in the cluster (Figure 1.5C; Poukkula et al., 2011).

To gain net forward movement, cell signaling in the border cells restricts protrusion formation to the cluster leader. The small GTPase Rac1 is activated downstream of receptor tyrosine kinases PVR and EGFR in border cells (Figure 1.5C; Ramel et al., 2013; Wang et al., 2010). Rac is the main driving force for protrusion formation in border cells and has been

implicated in nearly every studied instance of cell migration (Montell et al., 2012; Ramel et al., 2013; Ridley, 2015). Rac activity is required in every cell of the cluster for migration, but the highest level of Rac activation, as a result of PVR and EGFR binding in the lead cell, determines the direction of movement (Wang et al., 2010). Additionally, Rac activity in the lead border cell suppresses protrusions from the followers (Wang et al., 2010) Although non-leader cells still form protrusions throughout migration, they are fewer in number and shorter in both length and persistence (Prasad and Montell, 2007). During early border cell migration, Rac activity is regulated by the GTPase exchange factor *Myoblast city* (Mbc) and its binding partner *Engulment and cell motility* (ELMO) (Bianco et al., 2007; Geisbrecht et al., 2008). In a non-canonical role, *Death-associated inhibitor of apoptosis 1* (DIAP1) regulates Rac-dependent actin polymerization along with profilin, an actin regulator (Geisbrecht and Montell, 2004). Downstream of PVR and EGFR, the adherens junction component E-cadherin connects border cells and communicates localized Rac activity (Figure 1.5C; Cai et al., 2014). In this way, PVR and EGFR guidance signaling establishes front-back polarity in the migrating border cell cluster.

Extensive remodeling of cell-cell adhesions is associated with the transition from stationary epithelia to a migratory collective (Friedl and Mayor, 2017). Throughout migration, adhesion remodeling allows for border cell cluster delamination, cluster integrity, and interaction with the substrate (Cai et al., 2014; Niewiadomska et al., 1999). Both border cell-border cell and border cell-nurse cell adhesion are promoted by the Jun-kinase (JNK) pathway (Figure 1.5C; Llense and Martín-Blanco, 2008). Armadillo and E-cadherin are components of the adherens junctions that keep border cells interconnected during their migration, while integrins are critical for both adhesion and mechanotransduction during cell migration (Llense and Martín-Blanco, 2008; Niewiadomska et al., 1999; Peifer et al., 1993). E-cadherin, the major *Drosophila*

cadherin, is localized at all cluster interfaces: between border cells, the two polar cells, between border and polar cells, and between the border and nurse cells (Figure 1.5C; Niewiadomska et al., 1999). Proper expression and distribution of E-cadherin is regulated in border cells by the ecdysone pathway and are required for cluster integrity throughout migration (Bai et al., 2000; Bhattacharya and Starz-Gaiano, 2024; Niewiadomska et al., 1999). Integrins connect to cytoskeletal adaptor proteins, like paxillin, to generate traction by cells against the substrate during migration (Friedl and Alexander, 2011). Paxillin is downregulated in JNK-negative border cell clusters (Llense and Martín-Blanco, 2008). Border cells lacking β-integrin still migrate; however, when combined with a JNK-deficient background, mutant border cell clusters dissociate and form abnormally short protrusions (Llense and Martín-Blanco, 2008). Like integrins, E-cadherin is also critical for border cell interaction with the substrate, providing traction for border cells to migrate upon the nurse cells (Figure 1.5C; Niewiadomska et al., 1999).

Collectively migrating cells *in vivo* often move through challenging spaces within tissues or organs to reach their destination. A migratory path filled with dense surrounding cells or extracellular matrix (ECM) can hinder migration (Ventura and Sedzinski, 2022). In response to physical properties such as confinement or substrate stiffness, cells *in vitro* can alter their speed, focal adhesions with the substrate, or protrusive structures to adapt and migrate forward (Charras and Sahai, 2014; Ventura and Sedzinski, 2022). Border cells migrate on and between the large nurse cells that fill the developing egg chamber (Figure 1.5B). The cluster preferentially follows a central migratory path where 3 or more nurse cells contact each other, creating a more permissible space for migration (Dai et al., 2020). Although the border cells remain tightly adhered to one another, the formation of transient adhesions with the substrate allows for

generation of traction to move forward (Montell et al., 2012). Continual remodeling of the E-cadherin-mediated adhesions between the border cell cluster and the nurse cell substrate is required for migration (Niewiadomska et al., 1999). Additionally, proper E-cadherin expression in nurse cells is important- when decreased, border cells "wander" off the central path (Cai et al., 2014). When E-cadherin levels are increased in the nurse cells, the border cell cluster migrates slowly and is hyperpolarized, forming more, and longer, protrusions (Cai et al., 2014). Within the cluster, E-cadherin mediated adhesion between nurse cells and the lead border cell creates a positive feedback loop with guidance receptor signaling and the small GTPase Rac to generate and maintain a single front protrusion (Figure 1.5C; Mishra et al., 2019).

Cell membrane stiffness balances contractile forces between migratory cells and substrate to allow for collective migration (Friedl and Wolf, 2010). During migration, border cells pull on nurse cell membranes, which visibly deform as the cluster passes through (Aranjuez et al., 2016; Dai et al., 2020). Non muscle myosin II (MyoII) regulates cortical tension in migrating cells through interaction with the F-actin cytoskeleton to maintain cluster shape (Aranjuez et al., 2016). In border cells, MyoII also maintains protrusion dynamics through Rac activation in the lead cell and retraction of protrusions (Mishra et al., 2019). Increased contraction via MyoII, and therefore increased stiffness, of the nurse cells impairs border cell migration (Aranjuez et al., 2016). When compressed by the stiffer nurse cell membranes, the border cell cluster becomes elongated and myosin levels are increased (Aranjuez et al., 2016). Throughout migration, MyoII is localized at the cluster periphery in "flashes" of dynamic activation (Aranjuez et al., 2016). Recent work has shown that the F-actin bundling protein Fascin limits myosin activity in both border cell and nurse cell membranes during migration (Lamb et al., 2021). Decreased Fascin

expression in border cells increases the stiffness of both border cell and nurse cell membranes, resulting in impaired migration (Lamb et al., 2021).

1.4.3 Border cells retain and require epithelial characteristics throughout migration

In addition to the front-back polarity established by protrusion restriction to the leader cell, the border cell cluster retains apicobasal polarity, a hallmark of epithelial tissue. Both individual polarity proteins and maintenance of polarity within the cluster are required for migration. Before initiation of migration, apical polarity markers of the Par complex (aPKC/Par-6/Par-3/Cdc42) and the Crumbs (Crb) complex (Crb/Stardust/PatJ) are localized at the apical surface of both border and polar cells (Montell et al., 2012; Wang et al., 2018). Basal markers *Lethal giant larvae* (Lgl), *Scribble* (Scrib), and *Discs large* (Dlg) localize at basolateral junctions in the follicular epithelium (Montell et al., 2012). Prior to migration, the apical surfaces of the border cells face the nurse cells that fill the central region of the egg chamber. As migration begins, the cluster rotates so the apical surface is roughly perpendicular to the direction of migration towards the oocyte (Pinheiro and Montell, 2004). Protrusions then guide the cluster forward from the basolateral side of the border cells (Montell et al., 2012). Several polarity markers and signaling pathways coordinate cellular and cluster behavior throughout migration. The serine/threonine-kinase and basal marker *Par-1* regulates both detachment and protrusion formation by activating Myosin II through myosin phosphatase (Majumder et al., 2012; McDonald et al., 2008). Proper distribution and activation of Par-3 (*Bazooka* in *Drosophila)* and *Par-1*, which phosphorylates and inhibits *Bazooka*, are required for cluster detachment at early stage 9 (McDonald et al., 2008). Localization of *Bazooka* in the border cells is mediated by Jun-kinase (JNK) signaling (Llense and Martín-Blanco, 2008). JNK signaling maintains cluster integrity through regulation of both polarity components and E-cadherin expression (Figure

1.5C; Llense and Martín-Blanco, 2008). Both Par-6 and *Bazooka* are required for proper E-cadherin localization within the cluster (Pinheiro and Montell, 2004). JNK signaling in the cluster is potentially activated by Rho family GTPase Cdc42 and regulated by the transcription factor *hindsight* (Hnt) (Llense and Martín-Blanco, 2008; Melani et al., 2008).

During migration, the border cell cluster also exhibits an inside-out polarity, with respect to the outer surface of the border cells (Montell et al., 2012; Wang et al., 2018). Par and Crb complex members primarily localize to border cell-border cell contacts during migration (Lucas et al., 2013; Wang et al., 2018). *Kibra*, *Expanded* (Ex), and *Merlin*, upstream components of the Hippo signaling pathway, localize with markers aPKC and Crb to the adherens junctions between border cells (Lucas et al., 2013). Downstream signaling via Hippo and Warts then inhibits *Enabled* (Ena) and promotes *Capping protein b* (Cpb) to restrict F-actin polymerization to the outer rim of the migrating border cell cluster (Figure 1.5C; Lin et al., 2014; Lucas et al., 2013). The Crb complex may also contribute by restricting aPKC, which promotes Rac-dependent protrusions, to the outer membranes of border cells (Wang et al., 2018). Both Scrib and aPKC function in Rac localization and activation, respectively, to induce protrusion formation (Campanale et al., 2022; Wang et al., 2018). This inside-out signaling mechanism is reminiscent of contact inhibition of protrusion important in other migrating collectives. In this way, polarity is preserved in border cells and mediates cluster cohesion and overall collective migration.

Collective migration can contribute to the spread of disease, as in metastatic cancer. Migratory cancer cells often undergo an epithelial to mesenchymal transition (EMT), in which loss of epithelial adhesions or polarity and the gain of mesenchymal and migratory characteristics facilitates invasion away from the tissue of origin (Nieto et al., 2016). In

developmental collective migrations such as neural crest migration, a partial or hybrid EMT functions to induce more mesenchymal characteristics in a subset of leader cells to guide collective migration. These include a switch from E-cadherin, a classical component of epithelial adhesions, to N-cadherin expression, as well as invasive activity in the leaders (Friedl and Gilmour, 2009). Border cells differ from these types of migratory cells in their maintenance of epithelial integrity, including adhesions and polarity, and the equality of the cells that comprise the cluster during migration. Throughout their migration, border cells highly express E-cadherin, and N-cadherin expression is not detectable in the cluster (Montell et al., 2012; Niewiadomska et al., 1999). Additionally, although the cluster has a front-back, leading to lagging edge polarity seen in individually migrating cells, the general apicobasal polarity characteristic of epithelial cells is maintained during migration. One or two cells form invasive protrusions and lead the cluster at a time; however, no one cell appears to acquire more migratory behavior than the other. There is no fated front cell. Instead, border cells change position during migration in a poorly understood process (Bianco et al., 2007; Cliffe et al., 2017). All outer border cells are capable of leading the cluster and forming dynamic protrusions (Prasad and Montell, 2007). Live imaging has demonstrated that in the surrounding border cells, the lead cell can be exchanged every 18 minutes (Bianco et al., 2007). It has been proposed that the cell with the highest levels of receptor tyrosine kinase (RTK) signaling is selected as the cluster leader, and may be exchanged once receptors become saturated (Bianco et al., 2007; Inaki et al., 2012).

As the border cell cluster approaches the oocyte and the end of their migration, the cluster turns once again, now to migrate over the oocyte surface. Finally, the border cells stop near the oocyte nucleus and flatten, with the apical polar cell surface facing the oocyte (Figure 1.5B; Bastock and St Johnston, 2008; Duchek and Rørth, 2001; Niewiadomska et al., 1999). Recent

work has shown that the border cell cluster actively "docks" to the oocyte surface in a process termed neolamination (Miao et al., 2020). First, a single border cell protrudes to make contact, then each border cell contacts the oocyte (Miao et al., 2020). Gap junction-forming proteins *Innexin 2* and *3* (Inx2/Inx3) are required for the "docking" process (Miao et al., 2020). After border cells stop at the oocyte at stage 10 of oogenesis, the centripetal follicle cells, which surround the oocyte, begin to migrate inward, or "centripetally" (Figure 1.5B; Montell et al., 1992). The flattened border cell cluster, including the polar cells, connect with the centripetal follicle cells to form a continuous epithelium covering the oocyte surface (Horne-Badovinac, 2020). Micropyle formation requires all three cell types (Horne-Badovinac, 2020). Border cells secrete the lamellar portion of the eggshell vitelline membrane, while polar cells extend long cytoplasmic protrusions to form the channel for sperm entry (Horne-Badovinac, 2020).

1.5 Using *Drosophila* border cell migration to understand dynamic balance in collectives

Border cell migration, like other developmental collective migrations, is shaped by dynamic signaling, from specification of the border cells to initiation of migration and invasion into the egg chamber. As the collective continues forward, feedback from the environment, challenges along the migratory path, and developmental trajectory require adaptive changes in cell behavior. Regulatory mechanisms critical for delamination and initiation may be less important during later phases of migration. In addition, specific changes in behavior may regulate a return to the previously epithelial fate, such as the requirement for gap junction-forming proteins at the end of border cell migration (Miao et al., 2020). As a small group of cells that undergo a relatively short migration in a small space, the border cells provide an excellent model to study the adaptation and dynamic balance of cellular requirements throughout

collective migration. How do border cells balance the intense demands of delamination and invasion processes with the additional challenges encountered later at mid-migration? What changes are required for border cells to slow down their migration and stop at the oocyte boundary? Recently, transcriptomics data in both single and collective modes of migration have begun to characterize the gene expression changes underlying cell fate and behavior in collectives (Borghese et al., 2006; Costa et al., 2023; McLennan et al., 2015; Morrison et al., 2017; Schwarz et al., 2012; Wang et al., 2006). This section will review previous work on border cell expression profiles, highlighting three transcription factors required to define and regulate border cell migration and behavior through downstream targets. Finally, this section reviews the integrated stress response (ISR) as a critical method of adaptation in cells under stress conditions, including evidence for an active stress response during border cell migration.

1.5.1 *Slow border cells*: a master regulator of border cell migration

In border cells, expression of key transcription factors, including *slow border cells* (Slbo), is required to promote collective behavior and migration. Slbo is the *Drosophila* homolog of the CCAAT enhancer binding protein, C/EBP, and a master regulator of border cell migration (Montell et al., 1992). In response to JAK/STAT signaling, expression of Slbo is activated in the border cells, which begin their migration just a few hours afterwards (Silver and Montell, 2001). Notch signaling, important for early follicle cell fate, is also required for Slbo expression (Beccari et al., 2002). Thus, the migrating border cells express Slbo from stage 8 of oogenesis onwards. Centripetal cells also express Slbo, although Slbo is not required for their migration in stage 10 (Montell et al., 1992). Precise levels of Slbo are required in the border cells for proper migration- misexpression causes defects in cell motility, protrusion formation, and a delay of 15 hours or longer in delamination and initiation (Inaki et al., 2022; Liu and Montell, 2001; Montell

et al., 1992; Rørth et al., 2000). During migration, a precise level of Slbo protein is regulated in border cells by *Tribbles*, a negative regulator that targets Slbo for degradation (Rørth et al., 2000).

Changes in cellular adhesion, shape, and behavior are required for otherwise stationary cells to begin their migration. Slbo, itself a target of the JAK/STAT pathway in border cells, upregulates downstream genes critical for migration (Borghese et al., 2006; Silver and Montell, 2001; Wang et al., 2006). In total, roughly a quarter of all genes enriched in border cells are regulated by Slbo (Borghese et al., 2006; Wang et al., 2006). Comparison of wild type and Slbo mutant border cells identified regulation of cytoskeletal regulators, enzymes, and additional transcription factors and nucleic acid binding proteins (Borghese et al., 2006; Wang et al., 2006). Slbo mutant border cells display defects in protrusion formation, which requires dynamic actin cytoskeleton remodeling (Inaki et al., 2022). Interestingly, Slbo also appears to target a group of genes involved in muscle development and structure, which may play a non-canonical role in cytoskeleton dynamics (Borghese et al., 2006). Additional transcription factors required for border cell migration, including STAT92E, Six4, and *anterior open* (aop) are upregulated by Slbo (Borghese et al., 2006). Six4 functions with *Eyes absent* (Eya) to control polar cell fate prior to migration (Borghese et al., 2006). *Anterior open*, also known as Yan, regulates E-cadherin expression in border cells during migration (Schober et al., 2005). Because border cells express Slbo throughout migration, its expression can be used as a reliable marker of border cell fate. Recent single cell RNA sequencing used Slbo expression to pool border and centripetal cells from the entire ovary population and characterize the border cell expression profile (Jevitt et al., 2020). These and other studies have identified expression of genes involved in adhesion, vitelline membrane formation, and regulation of endocytosis and the actin cytoskeleton in border

cells (Borghese et al., 2006; Jevitt et al., 2020; Rust et al., 2020; Wang et al., 2006). Markers of border cell fate, including previously identified genes like *Jaguar* and *Singed*, but also novel markers, have been identified using Slbo expression (Rust et al., 2020).

1.5.2 Transcription factors and downstream signaling balance cellular behavior and response to environment

A suite of signaling pathways, including JAK/STAT and ecdysone signaling, work in concert to ensure proper border cell migration. Their downstream targets direct cellular behavior, but also feed back to precisely control pathway activity. STAT92E, the only *Signal transducer and activator of transcription* (STAT) in *Drosophila*, is required in border cells throughout their migration (Silver et al., 2005). After border cells are specified, STAT activity in the cells activates expression of downstream targets, including Slbo, E-cadherin, and transcription factor *Jing* (Liu and Montell, 2001; Silver and Montell, 2001). Delamination is regulated indirectly by STAT through transcription regulation by targets *apontic* and *Notch* (Starz-Gaiano et al., 2008; Wang et al., 2007). Because Slbo is downstream of STAT, their activity in border cells is linked. The transcription factor *Traffic jam* (Tj) activates Slbo expression downstream of STAT, but at high levels functions in a feedback loop with Socs36E to repress STAT activity and reduce Slbo mRNA to normal levels. Therefore, Tj expression is downregulated early in border cells to allow for correct levels of both Slbo and STAT (Gunawan et al., 2013). The transcription factor *Apontic* functions to limit STAT signaling, and therefore Slbo expression, to border cells (Starz-Gaiano et al., 2008). Throughout migration, proper levels of STAT are further regulated in border cells by *big bang*, *brahma*, and *hindsight* (Aranjuez et al., 2012; Melani et al., 2008; Saadin and Starz-Gaiano, 2016). *Hindsight* (Hnt), a JNK signaling transcription factor, can downregulate both STAT and Slbo activity (Melani et al., 2008; Saadin

and Starz-Gaiano, 2016). Additional transcription factors, including *Jing*, Six4, *sima*, and *Jun-related antigen* (Jra), among others, are activated downstream of both STAT and the ecdysone receptor (Borghese et al., 2006; Liu and Montell, 2001; Wang et al., 2006).

The steroid hormone ecdysone regulates developmental transitions in *Drosophila*, including the initiation of border cell migration. Although ecdysone biosynthesis, and therefore titer, rises in the entire egg chamber between stages 8 and 10 of oogenesis, the response to ecdysone is specifically highest in anterior follicle cells, including the border cells (Montell et al., 2012). In border cells, ecdysone binds to a heterodimer, formed by the ecdysone receptor (EcR) and *Ultraspiracle* (Usp), which translocates to the nucleus to regulate transcription (Bai et al., 2000; Domanitskaya et al., 2014). A robust transcriptional response is then activated in border cells in response to ecdysone. Expression of some 400 genes is altered in EcR or Tai (*taiman,* an ecdysone coactivator) mutant border cells (Manning et al., 2017). Known regulators of border cell migration, chorion protein genes, cytoskeletal regulators, and extracellular matrix (ECM) structural components are regulated by ecdysone signaling and Tai in border cells (Manning et al., 2017).

Transcription factors and downstream signaling cascades regulate cellular response and adaptation as migratory cells move through, and are challenged by, their environment. Physical stressors can include confinement or "squeezing" by neighboring cells or extracellular matrix (ECM), a lack of available space to migrate through, or substrate stiffness (Ventura and Sedzinski, 2022). As border cells migrate, the transcription factor complex formed by *Myocardin-related transcription factor* (Mrtf) and *Serum response factor* (SRF, also known as *blistered* in *Drosophila*) senses tension from the surrounding environment (Salvany et al., 2014; Somogyi and Rørth, 2004). During migration, Mrtf accumulates in border cell nuclei. This

accumulation is more likely to occur in cells experiencing tension, with a stretched or elongated shape, than more round cells (Somogyi and Rørth, 2004). In response to the physical pulling force and tension associated with migration, the Mrtf/SRF transcription factor complex positively regulates genes associated with the F-actin cytoskeleton. Actin5C is the main target bound by Mrtf in border cells. However, Mrtf also binds a subset of heat shock proteins and genes enriched for stress response functions, potentially indicating a non-canonical role for these genes in response to physical stressors (Salvany et al., 2014). Actin cytoskeleton regulators, including *stathmin*, an effector of microtubule depolymerization, the actin bundler Fascin, and Profilin (*singed* and *chickadee* in *Drosophila*, respectively) are also upregulated by Slbo and ecdysone signaling to drive cytoskeletal dynamics (Borghese et al., 2006; Manning et al., 2017). When tension is experienced by the border cell cluster during migration, the mechanosensory information is translated into gene expression changes to compensate and continue migration (Somogyi and Rørth, 2004).

In addition to physical stress, migratory cells are challenged by, and must adapt to, physiological stressors. Changes in metabolism, whether through glycolysis or oxidative phosphorylation, can be a source of oxidative stress. Recent work has shown that these changes in metabolism frequently accompany cell migration or an epithelial to mesenchymal transition (EMT) (DeWane et al., 2021). Protein folding is an oxidative process in the cell. Rapid protein folding can alter endoplasmic reticulum (ER) homeostasis and induce oxidative stress (Tu and Weissman, 2004). Additionally, an accumulation of unfolded or misfolded proteins triggers endoplasmic reticulum (ER) stress, including a specific unfolded protein response (UPR) (Ryoo, 2015). Diverse cellular stresses, including ER stress, hypoxia, starvation, and viral threats trigger a common downstream signaling pathway, termed the integrated stress response (ISR) (Costa-

Mattioli and Walter, 2020). Individual kinases for each stressor phosphorylate the general translation initiation factor eIF2α to simultaneously inhibit global protein translation and upregulate specific transcription factors (Figure 1.6A; Ryoo and Vasudevan, 2017). In this way, the cell reduces amino acid consumption and protein folding while specific expression changes are triggered to aid cell recovery. *Activating transcription factor 4* (ATF4), a key transcription factor upregulated by the ISR in response to stress, in turn upregulates genes involved in amino acid and protein synthesis, metabolism, and glycolysis (Figure 1.6A; Han et al., 2013; Harding et al., 2003; Lee et al., 2015; Tameire et al., 2019). Recent work has demonstrated a role for ATF4 in cell survival under oxidative stress, including in tumor adaptation (Lee et al., 2015; Wortel et al., 2017). ATF4 expression is associated with cell invasion and metastasis in esophageal squamous cell and breast cell carcinomas (Limia et al., 2019). During normal development, ATF4 is required for osteoblast differentiation (Xiao et al., 2023). ATF4 expression also promotes invasion and migration of bone mesenchymal stem cells (prior to differentiation) and brain vascular smooth muscle cells *in vitro* (M. Feng et al., 2022; Y. Feng et al., 2022). In border cells, ATF4 overexpression impairs cluster delamination and migration (Chen and McDonald, 2024).

Additional ISR pathway components regulate border cell migration *in vivo*. Cells return to homeostasis after stress when translation recovers to normal levels. To achieve this, a protein phosphatase, composed of a catalytic subunit (Pp1C) and one of two regulatory subunits, PPP1R15A or PP1R15B, acts in a negative feedback loop to de-phosphorylate eIF2α (Costa-Mattioli and Walter, 2020). *Drosophila* only have one regulatory subunit, known as PPP1R15, or GADD34 (Figure 1.6A; Malzer et al., 2013). In border cells, both protein phosphatase 1 (Pp1) and PPP1R15 activity are required for migration (Chen et al., 2020; Chen and McDonald, 2024).

Pp1 functions in at least two ways to promote cluster cohesion. First, Pp1 is required to maintain proper levels of cadherin-catenin complex components, especially E-cadherin and Beta catenin, between border cells (Chen et al., 2020). Second, Pp1, in a complex with myosin phosphatase, controls myosin contraction at the collective level (Chen et al., 2020). PPP1R15 is required for proper coordination of protrusion formation from the lead border cell (Chen and McDonald, 2024). In addition, PPP1R15 regulates eIF2α phosphorylation and antagonizes expression of PERK, an ISR kinase, in border cells during migration (Chen and McDonald, 2024). The kinase PERK specifically responds to unfolded or misfolded proteins to trigger the endoplasmic reticulum (ER) stress, and specifically, the unfolded protein response (UPR) in cells (Figure 1.6A-B; Costa-Mattioli and Walter, 2020). This indicates a balance in components not only of the general ISR, like ATF4 and PPP1R15, but also the ER stress response in border cells during their migration.

1.5.3 *Drosophila* border cells: a model to investigate collective balance during migration

In this introductory chapter, I have outlined the internal and external mechanisms that drive collective behavior in a variety of developmental examples. Each of these models provides opportunity to study the cell signaling, behavior, and gene and protein expression changes required for collective migration. *Drosophila* border cells undergo a unique collective migration during oogenesis; ideally suited to investigate dynamic balance during migration. Their initial migration is marked by an active delamination and invasion into the egg chamber. As border cells migrate on and between the nurse cell substrate, they utilize specific transcription factors and downstream signaling to adapt their behavior to the migratory path (Somogyi and Rørth, 2004). Because components of the integrated stress response (ISR) and, specifically, the

endoplasmic reticulum (ER) stress response are required in border cells, active adaptation to physiological stressors may also promote collective migration. Finally, border cells stop migration and contribute to epithelia formation in the mature egg. The goal of this thesis is to investigate how balance of cellular requirements in border cells, through precise gene expression, contributes to their collective migration.

In this work, I used a transcriptome analysis of border cells to identify changes in gene expression during their migration. By comparing expression at different stages of migration, we identified temporally regulated gene networks in border cells. A combination of bioinformatic and database analyses uncovered genes previously uncharacterized in border cell migration, including genes involved in ribosome biogenesis. Here, I report a requirement for 27 dynamically expressed genes in border cell migration, including six genes with roles in ribosome biogenesis. One of the most highly expressed genes in border cells during migration was *activating transcription factor 4* (ATF4). ATF4 is critical for maintenance of cellular homeostasis through the integrated stress response (ISR) (Costa-Mattioli and Walter, 2020). Either overexpression or knockdown of ATF4 resulted in border cell migration defects, including a significant decrease in border cell number and cluster aspect ratio. Finally, ATF4-overexpressing border cells are improperly motile and restricted to the cluster back during migration.

Overall, these studies enhance our understanding of the dynamic balance in cellular requirements during border cell collective migration. As migration-related classes of genes, including cytoskeletal and adhesion regulators, are up-regulated, expression of other transcriptional programs, including ribosome biogenesis, are simultaneously down-regulated. In addition, a balance in expression of the stress response transcription factor, ATF4, is required for

border cell migration. ATF4 also appears to regulate either cluster cohesion or border cell recruitment, cluster shape, and proper motility. Our transcriptome analysis in border cells provides a wealth of cell processes and gene candidates to understand how cellular behaviors are balanced during migration. Further work will be required to fully define the role of ATF4 in border cells, including downstream gene targets in border cells. Taken together, our data indicate a balance in gene expression and regulation of transcriptional networks that promote cellular requirements for border cell invasion, adaptation, and migration.

1.6 Figures

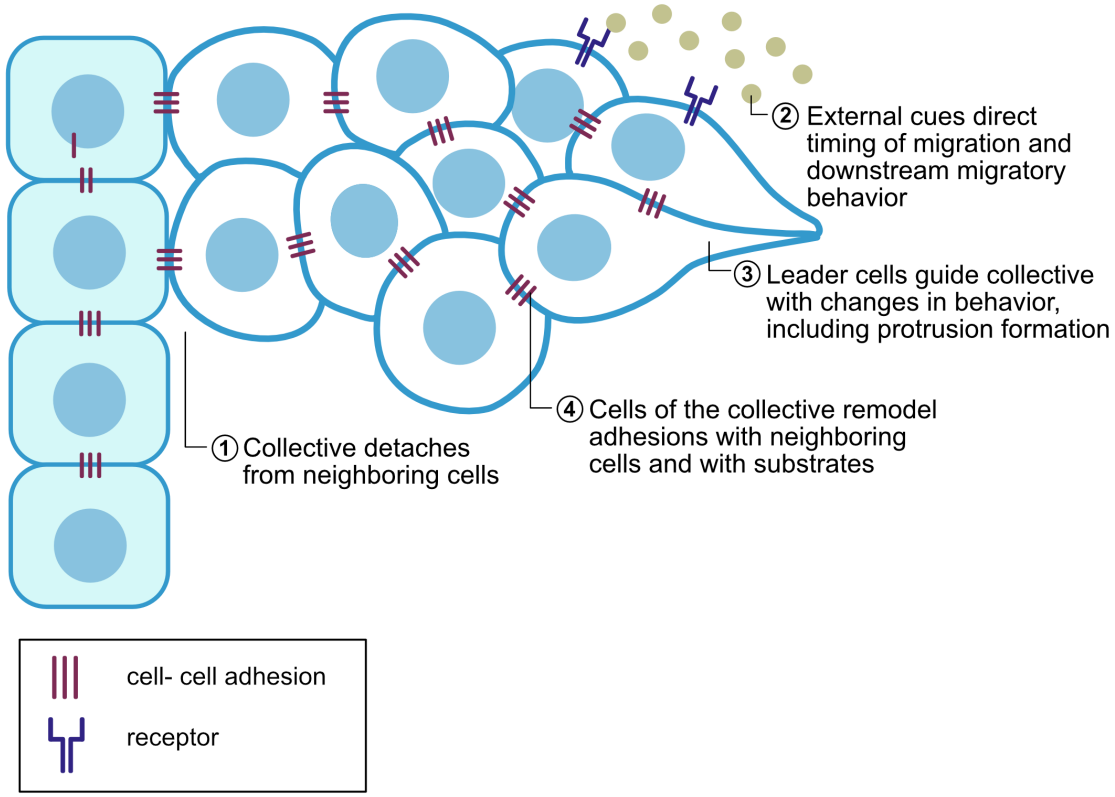


Figure 1.1 Shared characteristics of collective cell migrations.

Schematic demonstrating the shared characteristics of cell collectives in developmental migrations. The cell collective detaches from the stationary cells (colored light blue) to begin migration. In response to external cues (top right, in gold) leader cells guide the collective forward into a new environment. Although adhesion remodeling between cells is often required throughout migration, many collectives retain some or all cell-cell adhesions during migration.

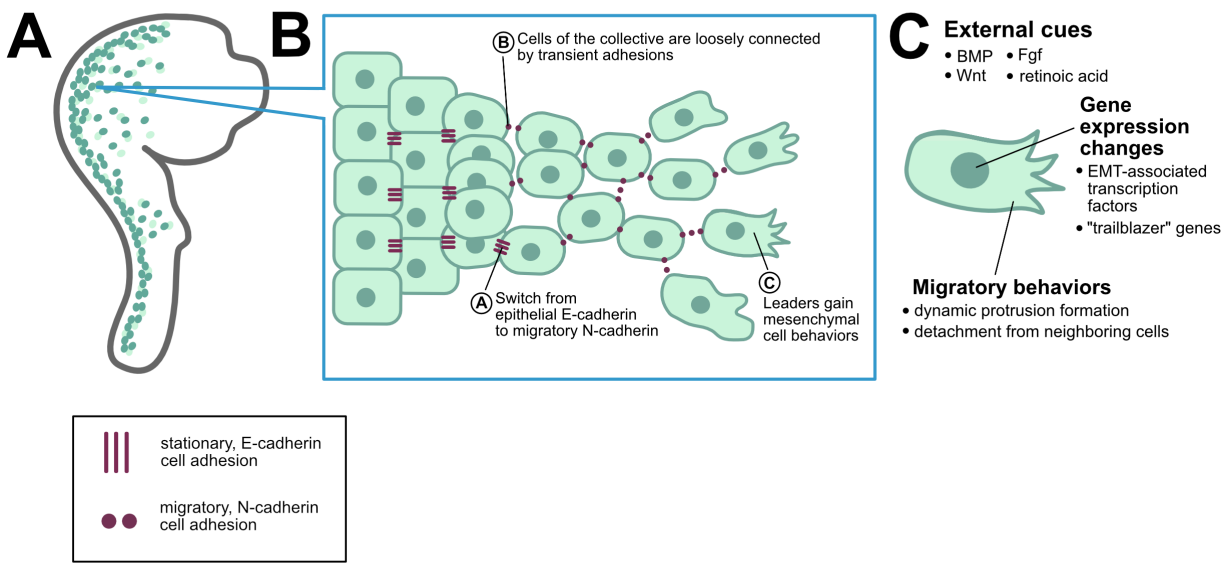


Figure 1.2 External cues and downstream gene expression drive neural crest cell delamination and migratory behavior.

A) Neural crest cells exit from the dorsal neural tube during chick embryogenesis. Their migration in discrete streams throughout the embryo ensures proper deposition and development into nervous system, craniofacial, and muscle structures. **B)** The complete or partial epithelial to mesenchymal transition required for neural crest cell delamination involves a cadherin "switch" and remodeling of cell-cell adhesions. **C)** In leader cells, external cues from the surrounding tissue activate downstream gene expression. This leader cell expression profile drives migratory behaviors, including dynamic protrusion formation.

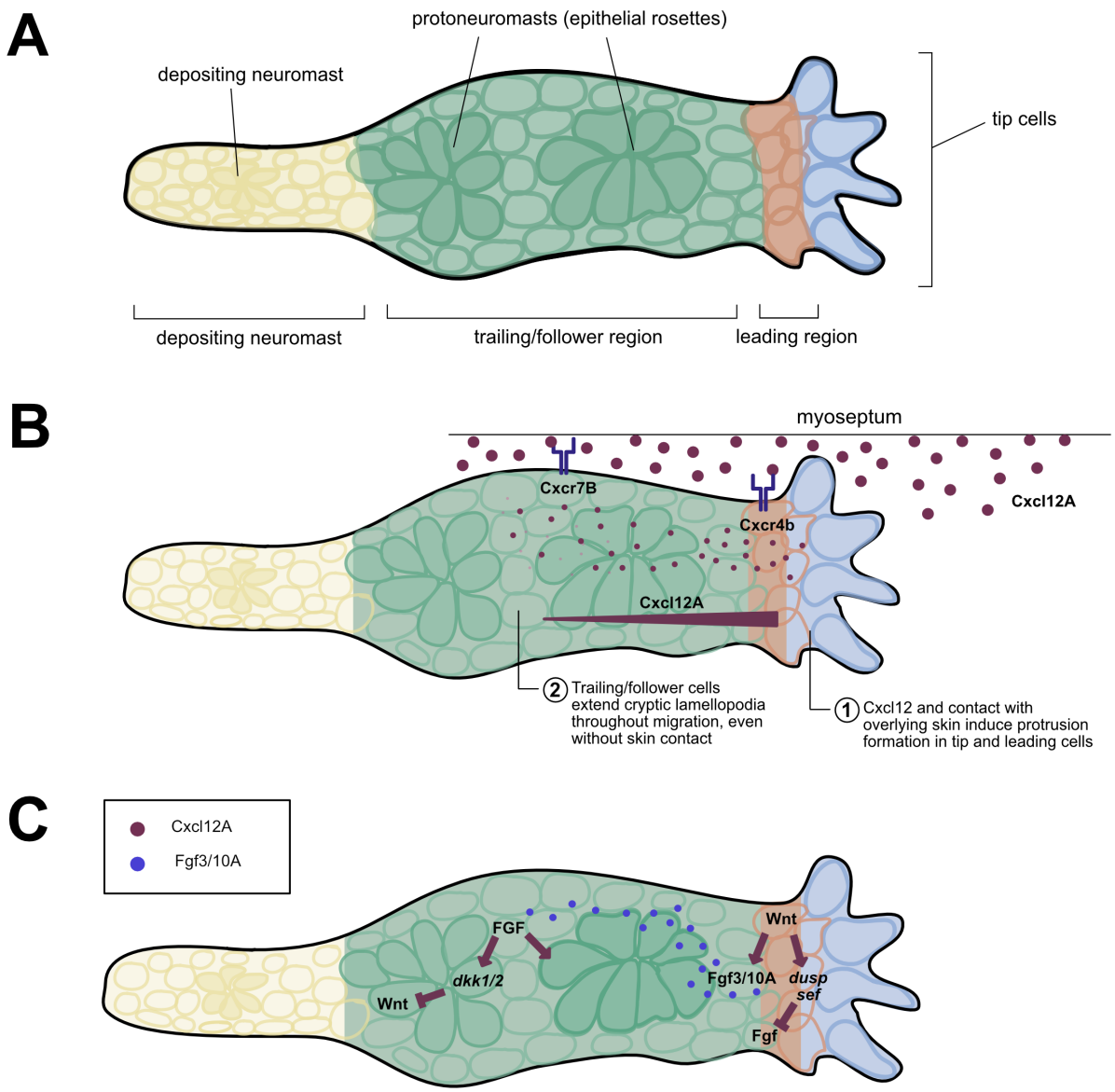


Figure 1.3 Interaction with the microenvironment shapes zebrafish posterior lateral line primordium migration.

A) The zebrafish posterior lateral line primordium (pLLP) is defined by four regions: tip cells (light blue), the leading region (light orange), the trailing/follower region (green), and the depositing neuromast (yellow). The tip cells at the invasive front are the most mesenchymal, with a flatter morphology and active protrusion extension. Apical constriction forms proto-neuromasts, also known as epithelial rosettes, in the trailing/follower region. Proto-neuromasts are deposited from the depositing neuromast region at the collective rear throughout migration to

develop into the lateral line system. **B)** In response to the Cxcl12A expression in the myoseptum, receptor internalization (Cxcr4b) or receptor degradation (Cxcr7B) of Cxcl12A in the leading and trailing/follower regions, respectively, establishes an internal gradient of Cxcl12A. This induces protrusion formation in the tip cells at the front of the collective. Contact with the overlying skin is also required for protrusion formation. However, cells of the trailing/follower regions also extend cryptic lamellipodia through migration, even without (or with less of) these inputs. **C)** Signaling pathways in the leading and trailing/follower regions important for proto-neuromast activity each restrict the activity of the other to their respective regions. Downstream activity of Wnt in the leading region restricts FGF to the trailing/follower region. Wnt activity also produces Fgf3/10A, which must localize to the trailing/follower region to be active. FGF signaling is critical for proto-neuromast formation in the trailing/follower region, where it inhibits Wnt activity.

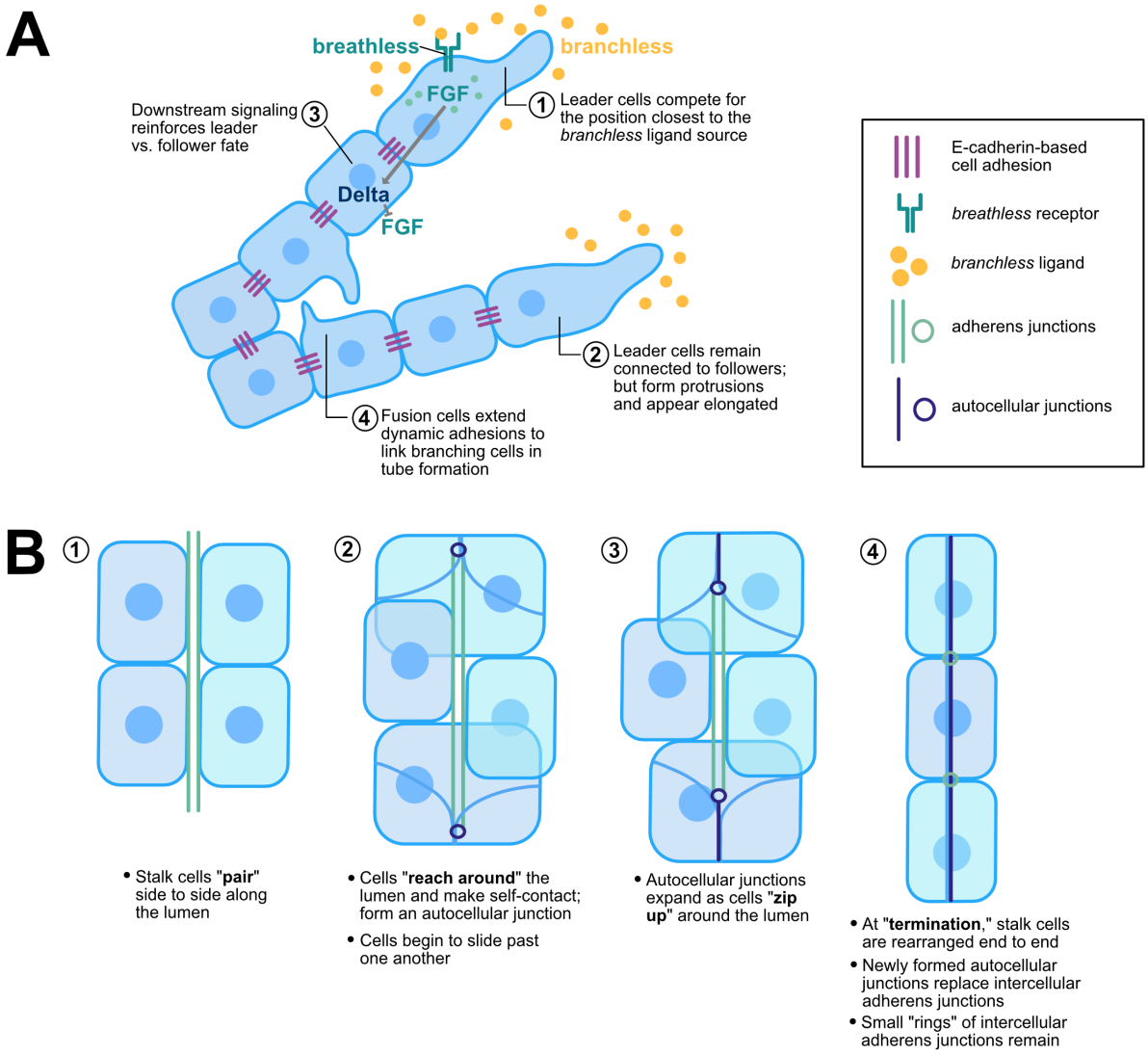


Figure 1.4 Epithelial maintenance is a critical driver of *Drosophila* tracheal branching. **A)** *Drosophila* tracheal cells migrate as a collective to form the branching tracheal network during embryogenesis. At the tip of each branch, a leader cell expresses *breathless,* an FGF receptor, and guide migration towards the source of FGF (*branchless* in *Drosophila*). Leader cells form protrusions and appear more elongated than other cells in the branch. FGF signaling in the leader cells activates the Notch ligand, Delta, in the follower cells. Follower cells decrease FGF signaling, further defining their non-migratory fate. As branching progresses, fusion cells are specified and extend filopodia to link extending branches together. Throughout migration,

epithelial integrity is maintained throughout the branch. **B)** Stalk, or follower, cells exchange neighbors and intercalate to form the long, thin tubes of the tracheal network. The process begins with individual stalk cells pairing along an outgrowing branch. They are connected to one another by adherens junctions (shown in green) and their apical surfaces contribute to the lumen of the developing tube. As cells begin to slide past one another, each cell "reaches around" the lumen to make self-contact on the other side. Here, each cell forms an autocellular junction (shown in dark blue). As cells continue to move past one another, autocellular junctions "zip up" as more of the cell surface contacts itself. Finally, stalk cells are arranged end-to-end at the "termination" stage. Each cell is wrapped around the lumen of the tube and forms a long autocellular junction with itself. Because epithelial integrity is carefully maintained and cell-cell contact is never fully lost, the remaining adherens junctions proteins are visible as "rings" between each cell of the branch.

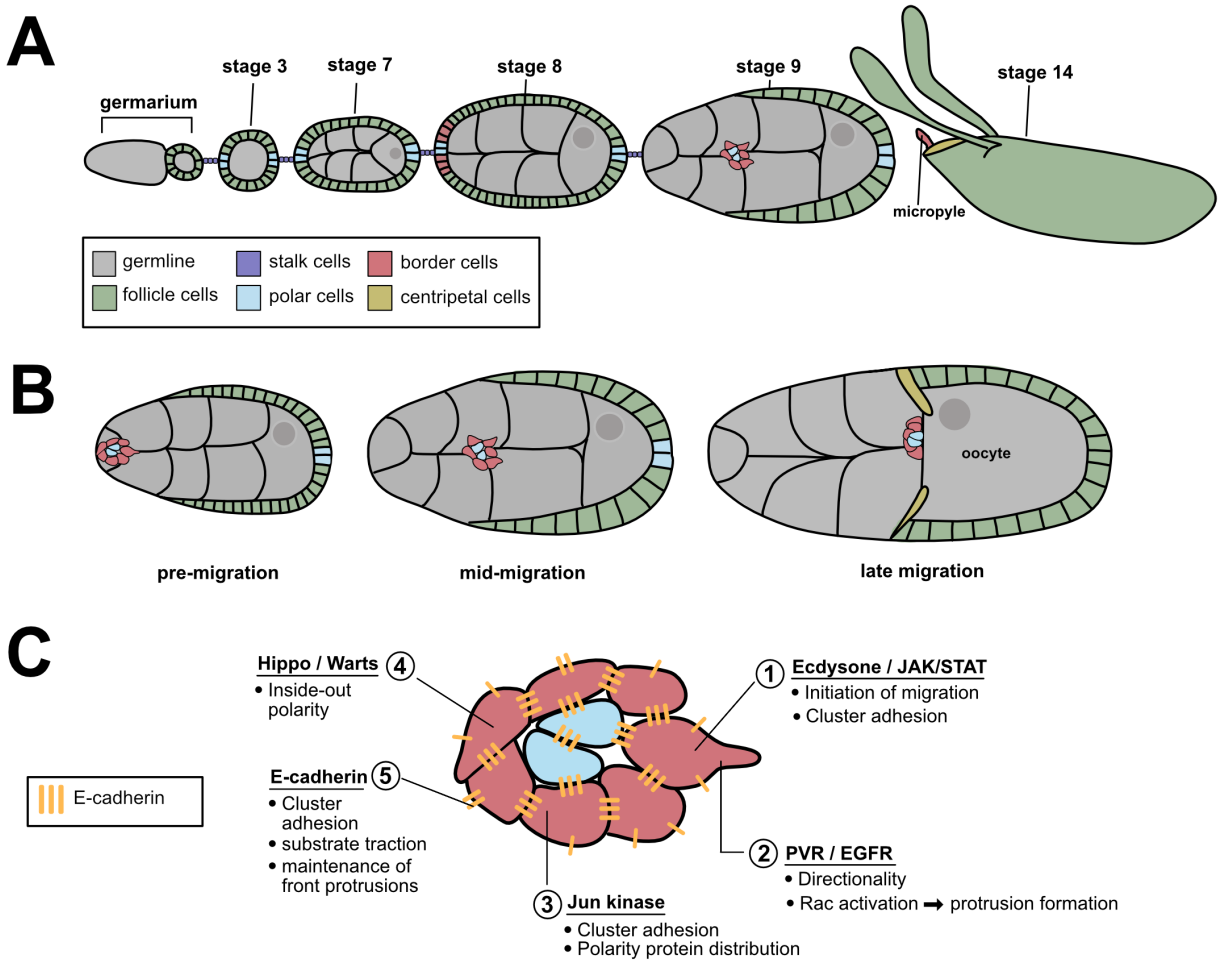


Figure 1.5 Border cell collective migration is coordinated by multiple signaling pathways during *Drosophila* oogenesis.

A) *Drosophila melanogaster* ovaries are comprised of ovarioles, each made up progressively more developed egg chambers connected by stalk cells (shown in purple). 16 cell cysts bud from the germarium (left) and progress through the 14 developmental stages of oogenesis. Each cyst, and eventual egg chamber, contains 15 germline cells (also known as nurse cells, shown in gray) and one oocyte (gray), and is surrounded by a continuous follicle cell epithelium (green). In stage 8, the polar cells (light blue) specify the migratory border cells (shown in pink). Border cells migrate during stage 9 of oogenesis and contribute to the micropyle, a sperm entry port in the mature egg (stage 14). **B)** In early stage 9, the migratory border cells and two nonmotile polar cells round into a cluster. One to two leader cells extend actin-rich protrusions between the nurse

cells. The border cell cluster eventually delaminates from the follicle epithelium and migrates through the nurse cells, reaching the oocyte boundary by stage 10. The border cell cluster makes contact with the oocyte and flattens, turning to migrate dorsally to finally stop near the oocyte nucleus (dark gray). Centripetal follicle cells (gold) migrate inward to form a continuous epithelium around the oocyte with the border cell cluster. **C)** Schematic of the border cell cluster at mid-migration and a subset of the multiple signaling pathways, signals, and proteins which promote border cell collective migration. E-cadherin (shown in orange) is highly expressed at the border cell-polar cell and border cell-border cell interfaces within the cluster. Although less E-cadherin is expressed at the border cell-nurse cell interface, E-cadherin-mediated adhesions with the nurse cell substrate are required for migration.

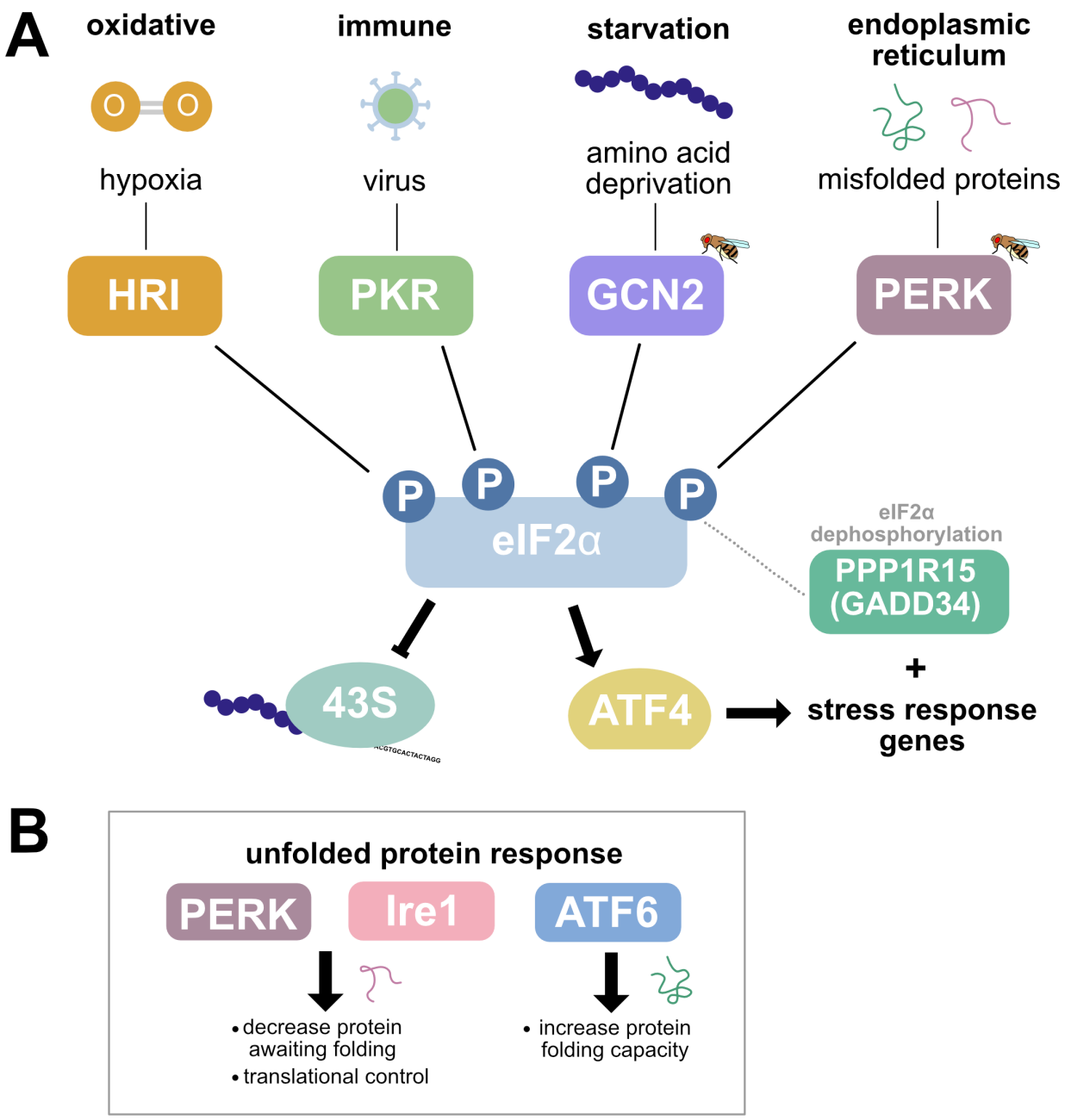


Figure 1.6 The integrated stress response mediates cellular recovery and homeostasis.

A) The integrated stress response (ISR) is a common downstream response to a wide variety of physiological stressors, including oxidative stress, immune threats, starvation, and endoplasmic reticulum (ER) stress. Four specific stress-sensing kinases (HRI, PKR, GCN2, and PERK) phosphorylate eIF2α in response to stress, thereby reducing global translation. In *Drosophila,*

only homologs for GCN2 and PERK have been identified. Paradoxically, *activating transcription factor 4* (ATF4) is activated during a global decrease in translation to up-regulate genes that will aid the cell in its return to homeostasis. Importantly, a target of ATF4, PPP1R15 (also known as GADD34), acts in a negative feedback loop to dephosphorylate eIF2α and return the cells to normal levels of translation. **B)** The kinases PERK and Ire1 and the transcription factor ATF6 comprise the three branches of the unfolded protein response (UPR), a specific response to an accumulation of unfolded protein in the ER. Downstream targets of PERK and Ire1 function to decrease global translation and the amount of protein awaiting proper folding in the ER. ATF6 upregulates genes to increase the protein folding capacity of the ER, and therefore ease the protein folding burden.

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Chapter 2 - Transcriptome analysis reveals temporally regulated genetic networks during *Drosophila* border cell collective migration

Emily Burghardt[1], Jessica Rakijas[1], Antariksh Tyagi[1], Pralay Majumder[2], Bradley J.S.C. Olson[1,*], and Jocelyn A. McDonald[1,*]

[1]Division of Biology, Kansas State University, Manhattan, KS 66506, USA

[2]Department of Life Sciences, Presidency University, Kolkata, 700073, West Bengal, India

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Author contributions

Conceptualization – E.B., B.J.S.C.O., and J.A.M.; Data curation – J.R., A.T., and B.J.S.C.O.; Formal analysis – E.B., J.R., and B.J.S.C.O.; Funding acquisition – E.B., B.J.S.C.O. and J.A.M.; Investigation – E.B., J.R., A.T., P.M., and B.J.S.C.O.; Validation – E.B., J.R., and B.J.S.C.O.; Writing original draft – E.B., J.R., B.J.S.C.O., and J.A.M.; Writing – review & editing – E.B., J.R., A.T., P.M., B.J.S.C.O., and J.A.M.

2.1 Abstract

Background

Collective cell migration underlies many essential processes, including sculpting organs during embryogenesis, wound healing in the adult, and metastasis of cancer cells. At mid-oogenesis, *Drosophila* border cells undergo collective migration. Border cells round up into a small group at the pre-migration stage, detach from the epithelium and undergo a dynamic and highly regulated migration at the mid-migration stage, and stop at the oocyte, their final destination, at the post-migration stage. While specific genes that promote cell signaling, polarization of the cluster, formation of protrusions, and cell-cell adhesion are known to regulate border cell migration, there may be additional genes that promote these distinct active phases of border cell migration. Therefore, we sought to identify genes whose expression patterns changed during border cell migration.

Results

We performed RNA-sequencing on border cells isolated at pre-, mid-, and post-migration stages. We report that 1,729 transcripts, in nine co-expression gene clusters, are temporally and differentially expressed across the three migration stages. Gene ontology analyses and constructed protein-protein interaction networks identified genes expected to function in collective migration, such as regulators of the cytoskeleton, adhesion, and tissue morphogenesis, but also uncovered a notable enrichment of genes involved in immune signaling, ribosome biogenesis, and stress responses. Finally, we validated the *in vivo* expression and function of a subset of identified genes in border cells.

Conclusions

Overall, our results identified differentially and temporally expressed genetic networks that may facilitate the efficient development and migration of border cells. The genes identified here represent a wealth of new candidates to investigate the molecular nature of dynamic collective cell migrations in developing tissues.

2.2 Introduction

Cell migration shapes tissues throughout the life of an organism, from embryonic development to wound healing in the adult, including in various disease states such as in cancer. Cells can migrate individually, like in immune surveillance, or collectively in larger interconnected and coordinated groups of cells (Friedl and Gilmour, 2009; Mayor and Etienne-Manville, 2016; Scarpa and Mayor, 2016). A wide variety of collective cell migrations occur *in vivo*, including *Drosophila* dorsal closure, vertebrate blood vessel formation and remodeling, vertebrate neural crest migration, wound healing, and tumor metastasis. Despite the diversity of cell types and organisms, the cellular and molecular mechanisms that govern collective cell migration are remarkably conserved. In response to external signals, cell polarization and cytoskeletal rearrangements define the collective's leader cells, which form F-actin-rich protrusions to provide traction but also sense the extracellular environment (Mayor and Etienne-Manville, 2016; Roberto and Emery, 2022). Leader and follower cells are linked through adhesion proteins and the actomyosin cytoskeleton, which together promote contraction and movement of the entire group. Because cells move inside developing tissues, organs, and organisms, our understanding of the molecular mechanisms that coordinate various collective cell migration behaviors is still incomplete.

One of the best studied genetic models of collective cell migration is the *Drosophila* border cells, which move as a small group during development of the ovary. The ovary is composed of multiple strings of progressively developing egg chambers bundled together (Montell et al., 2012). Each egg chamber consists of an oocyte and 15 supportive germline nurse cells in the center, surrounded by a monolayer of somatic epithelial follicle cells (Saadin and Starz-Gaiano, 2016). During mid-oogenesis, four to six follicle cells at the very anterior of the

egg chambers are specified to become border cells by the polar cells, a pair of non-motile follicle cells (Figure 2.1A-D; Montell et al., 2012; Silver and Montell, 2001). Border cells then surround the polar cells to form a migratory cluster (Figure 2.1A, D). After assembly, the border cell cluster delaminates from the follicular epithelium and migrates between the large nurse cells (Figure 2.1B). Border cell migration is highly dynamic and active. Throughout their migration, border cells continuously extend and retract protrusions. There is also no fixed leader cell. Instead, individual border cells exchange places and move within the cluster (Prasad and Montell, 2007). Border cells stop migrating when they reach the oocyte, where they become epithelial again in a process termed "neolamination" (Figure 2.1C; Miao et al., 2020). The entire process from specification to reaching the oocyte takes ~6 hours (He et al., 2011; Prasad and Montell, 2007). After delamination, it can take border cells 3-to-4 hours to actively migrate the entire ~150-200 μm distance (Prasad and Montell, 2007). Border cells are then joined by the migrating centripetal cells to completely enclose the anterior side of the oocyte (Parsons et al., 2023). Eventually, border cells, polar cells, and a subset of centripetal cells form a structure in the eggshell called the micropyle, which serves as an entry point for sperm to fertilize the oocyte (Horne-Badovinac, 2020; Montell et al., 1992; Spradling, 1993).

The formation and migration of border cells requires multiple signals that coordinate dynamic cellular behaviors. First, border cells are specified and recruited by Janus kinase/signal transducer and activator of transcription (JAK/STAT) signaling (Beccari et al., 2002; Ghiglione et al., 2002; Silver and Montell, 2001; Xi et al., 2003). The anterior polar cells secrete a cytokine-like ligand, Unpaired (Upd1), which activates JAK/STAT in a gradient within the adjacent follicle cells. Cells with highest levels of JAK/STAT activity turn on a critical downstream transcription factor, the C/EBP ortholog *Slow border cells* (Slbo). Slbo activates a

number of genes which together induce cells to become migratory border cells (Borghese et al., 2006; Montell et al., 1992; Silver and Montell, 2001; Wang et al., 2006). A key target of Slbo is E-cadherin, which promotes the ability of border cells to migrate upon and between the nurse cells (Niewiadomska et al., 1999). E-cadherin also keeps border cells adhered to each other and to the polar cells, particularly as the cluster navigates the dense and crowded tissue environment (Cai et al., 2014; Chen et al., 2020; Dai et al., 2020; Raza et al., 2019). Continuous JAK/STAT activation is required throughout migration to keep border cells motile, although how it does so is still unclear (Silver et al., 2005). Second, a pulse of ecdysone at stage 9 of oogenesis activates the Ecdysone receptor (EcR) and the transcriptional co-activator *Taiman* (Tai) so that border cells begin their migration at the right time (Bai et al., 2000; Domanitskaya et al., 2014; Jang et al., 2009; Manning et al., 2017). Ecdysone helps localize E-cadherin within the cluster but also stimulates the expression of multiple genes that promote migration (Bai et al., 2000; Manning et al., 2017). Third, once specified, border cells are guided to the oocyte by secreted chemoattractant ligands from the oocyte that activate two receptor tyrosine kinases on border cells, *PDGF- and VEGF-receptor related* (PVR) and *Epidermal Growth Factor Receptor* (EGFR) (Duchek and Rørth, 2001; Duchek et al., 2001; McDonald et al., 2003; McDonald et al., 2006). PVR and EGFR together stimulate the formation of dynamic F-actin-rich protrusions at the front of the cluster via the small GTPase Rac (Duchek et al., 2001; Prasad and Montell, 2007; Ramel et al., 2013; Wang et al., 2010). Extension and retraction of these protrusions help the cluster navigate between the nurse cells and move posteriorly towards the oocyte (Bianco et al., 2007; Dai et al., 2020; Prasad and Montell, 2007). Additional signaling pathways actively contribute to keeping border cells organized and motile. For example, Jun-kinase (JNK) helps border cells stay cohesive through regulation of the polarity protein Par-3 (*Bazooka*; Baz),

whereas Hippo/Warts polarizes F-actin to the periphery of the cluster through the actin-regulatory proteins *Enabled* (Ena) and *Capping protein* (Lin et al., 2014; Llense and Martín-Blanco, 2008; Lucas et al., 2013).

Many of these signals and their downstream targets are conserved, making border cells a powerful model to identify additional genes that promote distinct aspects of collective cell migration. Previous microarray analyses of isolated border cells, along with multiple genetic screens, have uncovered genes expressed in and required for border cell formation and collective movement (Borghese et al., 2006; Manning et al., 2017; Montell et al., 2012; Saadin and Starz-Gaiano, 2016; Wang et al., 2006). These expression screens pooled isolated border cells at all stages of the migration process, and thus were unable to determine if specific genes, or networks of genes, were dynamically expressed. Moreover, newer technologies have improved both the specificity and dynamic range of identifying transcripts. Therefore, we sought to comprehensively identify molecular determinants of dynamic border cell migration. In this study, we sequenced RNA isolated from border cells at three specific stages of migration: pre-migration, just prior to delamination; mid-migration, during movement of border cells between the nurse cells; and post-migration, when border cells reached the oocyte. We report here that 1,729 transcripts from 1,394 unique genes are temporally and differentially expressed at the three migration stages. These genes fall into nine clusters of co-expressed genes. Further gene ontology analyses and constructed protein-protein (PPI) interaction networks identify multiple categories of differentially expressed genes. In addition to genes expected to function in collective cell migration, such as those that regulate the cytoskeleton, cell adhesion, and tissue morphogenesis, we unexpectedly found an enrichment of genes involved in immune signaling, stress response pathways, and ribosome biogenesis. Finally, we characterized and confirmed the

in vivo expression and functions of a subset of the identified genes in migrating border cells, thus validating this approach. Together, our results highlight new networks of genes that are differentially expressed during border cell migration and that represent potential regulators of dynamic collective cell behaviors.

2.3 Results

2.3.1 Transcriptional profile of temporally expressed genes at three border cell migration stages

Previous studies identified genes enriched in border cells using microarray analyses (Borghese et al., 2006; Wang et al., 2006). However, whether some genes are differentially expressed at different stages of migration was unknown. Moreover, a subsequent analysis found that only 10% of genes between these two border cell microarray experiments overlapped (Bae et al., 2017). Thus, our current picture of the dynamics of transcripts expressed in migrating border cells is incomplete. Therefore, we sought to identify temporally-expressed genes at three distinct stages of border cell migration: pre-migration, when border cells have rounded up into a cluster but have not yet left the follicle cell epithelium (Figure 2.1A); mid-migration, when border cells have delaminated and migrated anywhere along the migration pathway (Figure 2.1B); and post-migration, when border cells have finished their migration at the oocyte boundary (Figure 2.1C). We performed bulk RNA sequencing of border cells isolated at these three distinct stages using a previously validated method of magnetic-bead cell sorting (*see* Methods; Wang et al., 2006). The *slbo* enhancer was used to directly drive mCD8:GFP in border cells (Figure 2.1A-D). A few additional follicle cells, including the centripetal cells at stage 10 (Figure 2.1C) and central polar cells (Figure 2.1D), also express *slbo*-mCD8:GFP. Egg chambers at the relevant stage were manually sorted using the GFP fluorescence, dissected, and pooled, followed by isolation and

cell sorting of individual mCD8:GFP-expressing cells (Figure 2.1E). Three biological replicates were performed for each stage of migration, except the pre-migration stage in which one replicate failed due to low levels of RNA in the sample. RNA sequencing of these sorted border cell populations was then performed (Figure 2.1E).

After mapping the reads to the genome, expression for 25,645 of 30,504 *Drosophila* transcripts was detected in the genome (FlyBase version FB2021_02 Dmel Release 6.39; Supplemental Table 2.1; Gramates et al., 2022). A total of 1,729 transcripts from 1,394 unique genes at the three different migration stages were determined to be differentially expressed with statistical significance (EBSeq-HMM FDR<0.05; Figure 2.1F; Supplemental Tables 2.2 and 2.3). As a positive control, expression of *slbo* (FBtr0339359), a gene known to regulate border cell identity and migration, was found to increase its expression during border cell migration (Figure 2.1F, Supplemental Table 2.3). As expected, GFP expression was also observed from *slbo*-mCD8:GFP samples and matched the levels of *slbo* transcript due to being driven by the *slbo*-enhancer, indicating consistency across the biological replicates (Supplemental Table 2.1). A significant number of genes known to be expressed in border cells and required for their migration had differing levels of expression from pre- to post-migration (Figure 2.1F). Border cell-related genes that changed expression levels during migration included *drongo*, *Pvr*, *Rab11, Rac2, Raskol, Septin1*, and *spaghetti squash* (*sqh*) (Gabbert et al., 2023; Montell et al., 2012; Saadin and Starz-Gaiano, 2016; Raza et al., 2019; Zeledon et al., 2019). Additional genes such as *jing*, *Notch* (*N*), and *Stat92E*, known to be primarily expressed or activated in border cells at these stages, were also differentially expressed (Liu and Montell, 2001; Schober et al., 2005; Silver et al., 2005; Wang et al., 2007). Interestingly, distinct gene isoforms were also observed (e.g., *Cdc42*, *par-1*, *Pvr*, *raskol*, *STAT92E*, and *sqh*; Figure 2.1F, Supplemental Table 2.1).

However, the single end-read based approach we used limits the quantification of transcript isoforms, so we did not further focus on isoform-specific transcription in this study (Li and Dewey, 2011). Our data also support the idea that while other follicle cells, including polar cells, were potentially isolated, relevant genes for border cell migration were enriched due to consistently higher levels of *slbo*-mCD8:GFP in border cells, and thus enrichment of border cells in the isolated cell populations (Figure 2.1A-D; *see* Methods).

We next asked if these temporally expressed genes overlapped with other genes known to be expressed in border cells or required for their migration. We compiled a comprehensive list of genes required for border cell migration from the published literature (Supplemental Table 2.4). Using the entire sequencing dataset, we then analyzed if any of these genes were differentially expressed. We identified 496 border cell migration transcripts (representing 136 unique genes) that exhibited significant differential expression from pre- to post-migration stages (Figure 2.2A; Supplemental Table 2.4). We next compared our sequencing data to two previous microarray gene expression studies, which identified genes that were up- or down-regulated in border cells relative to other follicle cells and compared to *slbo* mutant border cells (Borghese et al., 2006; Wang et al., 2006). Both microarray studies assessed the expression of genes in border cells that were isolated from whole ovaries but did not perform temporal staging of egg chambers. Thus, it was unknown if any of these genes were temporally expressed. Our analyses found that 1,145 transcripts from the Borghese et al. (Borghese et al., 2006) study, representing 85.8% of total genes within the microarray, exhibited distinct patterns of expression during border cell migration (Figure 2.2B; Supplemental Table 2.4). Similarly, we found 853 transcripts, representing 71.7% of identified genes from the Wang et al. (Wang et al., 2006) microarray, that were differentially expressed (Figure 2.2C; Supplemental Table 2.4). Due to technical

differences, neither of the microarray studies isolated polar cells along with border cells, in contrast with our data that included polar cells (Figure 2.1D). Nonetheless, many differentially expressed transcripts from our analysis overlap with Borghese et al. (Borghese et al., 2006) and Wang et al. (Wang et al., 2006). This may be because polar cells form early in oogenesis (Horne-Badovinac, 2020). One possibility may be that polar cells do not undergo major transcriptional changes from pre- to post-border cell migration, though this remains to be tested.

Next, we asked whether genes that might be expected to function in border cell collective migration or development were temporally expressed from pre- to post-migration stages. Collectively migrating cells, including border cells, require the actin cytoskeleton and adhesion for movement and to organize the collective (Montell et al., 2012). Indeed, 154 actin cytoskeleton transcripts and 147 adhesion transcripts were differentially expressed (Figure 2.2D, E; Supplemental Table 2.4). These included actin binding genes *Gelsolin (Gel)*, *Zasp52,* and *Vinculin (Vinc),* and adhesion-related genes *rhea, p120 catenin (p120ctn),* and *Fasciclin 1 (Fas1).* We also found 191 transcripts associated with epithelial-mesenchymal transitions (EMT) that exhibited differential expression during migration (Figure 2.2F; Supplemental Table 2.4). Even though border cells retain many epithelial features, including high levels of apical-basal cell polarity proteins and E-cadherin, and thus do not undergo true EMT (Montell et al., 2012), genes such as *Goosecoid (GSC), snail (sna),* and *twist (twi)* were differentially expressed in border cells (Nieto et al., 2016; Yang et al., 2020). Transcripts of genes associated with oogenesis and transcription factors were also differentially expressed in migrating border cells (Figure 2.2G, H; Supplemental Table 2.4).

2.3.2 Identification of distinct patterns and classes of co-expressed genes during border cell migration

To better understand the molecular control of border cell migration, we next used *clust* to determine which of the significantly differentially expressed genes were co-expressed from pre- to post-border cell migration. *Clust* analysis automatically extracts optimal gene co-expression patterns in RNA-seq data that have a high correlation with similar biological activity (Abu-Jamous and Kelly, 2018). Here, our *clust* analysis resulted in nine distinct clusters of temporally co-expressed genes (Figures 2.3-2.4, Appendix Figure A.1). Of the transcripts with significant differential expression, 587 were grouped into three distinct co-expressed clusters with various patterns of increased expression across the migration stages (C0, C1, and C8; Figure 2.3). These clusters included regulators of border cell migration such as *spaghetti squash* (*sqh*; C0), *big bang (bbg*, C1*)*, and *slbo* (C8; Supplemental Table 2.5). Another 556 transcripts were grouped into co-expressed clusters with various patterns of decreased expression across the migration stages (C4, C5, and C6; Figure 2.4). Several genes known to be expressed or required in migrating border cells were found in these clusters, including *singed* (*sn*; C4)*, par-6* (C4)*, STAT92E* (C5)*,* and rolling pebbles (*rols*; C5; Supplemental Table 2.5). Finally, the remaining 119 transcripts sorted into three co-expressed clusters with variable expression patterns that specifically increased or decreased their expression only at mid-migration stages (C2, C3, and C7; Appendix Figure A.1). Known border cell migration regulators *Patj* (C2) and *Rac2* (C7) are found in these clusters, along with F-actin regulators (*HSPC300* in C3, *Arpc2* in C7) and several RhoGEFs (*RhoGEF2* and *RhoGEF3* in C3). These findings suggest that, despite their unique expression patterns and fewer transcript numbers, the three variable gene expression clusters are still biologically relevant (Supplemental Table 2.5).

To obtain further insight into potential biological functions of the significantly differentially expressed clusters of genes, we performed a Metascape enrichment analysis (Zhou et al., 2019). This analysis identified top significantly enriched gene annotation terms, including those from gene ontology (GO), KEGG, Reactome, and other databases, for each clustered group of genes (Figures 2.3-2.4, Appendix Figure A.1; Supplemental Table 2.6). Clusters with increased expression from pre- to post-migration were notably enriched for genes that had four broadly shared functions (C0, C1, and C8; Figure 2.3), specifically cell-cell junction regulation (C0: septate junction assembly; C1: cell-cell junction assembly), cytoskeletal organization (C0: actin cytoskeleton organization; C1: supramolecular fiber organization and actomyosin structure organization; C8: cytoskeleton organization), morphogenesis (C0: cellular component morphogenesis and morphogenesis of an epithelium; C1: regulation of cellular component organization and epithelial cell differentiation; C8: animal organ morphogenesis, cellular component morphogenesis, and regulation cellular component organization), and developmental processes (C0: oogenesis; C1: pupariation; C8: regulation of developmental processes). Clusters with decreased expression from pre- to post-migration were enriched for genes that shared a variety of functions ranging from development and signaling to basic cellular metabolic processes, such as ribosome biogenesis, rRNA processing, and ribonucleoprotein complex biogenesis (C4, C5, and C6; Figure 2.4). The clusters with variable expression patterns at mid-migration were enriched for genes expected to function in cell migration (C2, C3, and C7; Appendix Figure A.1), such as regulation of morphogenesis (C2: morphogenesis of embryonic epithelium; C3: morphogenesis of an epithelium), regulation of cell projections (C3: plasma membrane bounded cell projection organization; C7: cell projection organization), and cytoskeletal organization (C2: supramolecular fiber organization; C3: Rac1 GTPase cycle).

Together, these analyses highlight the complexity of border cell migration, with both expected (e.g. cytoskeletal genes, morphogenesis) and unexpected (e.g. metabolic processes) groups of differentially co-expressed genes.

2.3.4 Interaction networks reveal enrichment of migration-related, immune signaling, and ribosome biogenesis genes

The above-described *clust* analysis positioned the significantly co-expressed genes into clusters with predicted or known annotated functions but did not capture relationships such as protein-protein interactions (PPI) among the gene products. Specifically, we wanted to understand how the proteins encoded by the differentially expressed genes might interact during border cell migration. Therefore, we examined potential PPI networks present in each of the *clust* co-expression clusters during border cell migration. We specifically focused on those co-expressed clusters with at least 150 significantly differentially expressed genes, namely clusters C0, C1, C4, C5, C6, and C8 (Figures 2.3-2.4). To identify PPI networks, we used Cytoscape to integrate Metascape annotations, which includes FlyBase PPIs and STRING-based PPIs (Figure 2.5; Appendix Figure A.2; *see* Methods; Shannon et al., 2003; Szklarczyk et al., 2022). The PPI networks were further refined by manual curation using functional data from FlyBase to enhance the predictions of biological functions (Supplemental Table 2.7). Demonstrating the utility of this approach, we found that at least 40% of the genes in each of these co-expressed clusters contained known physical protein interactions.

From this analysis, we identified major interaction networks within each of these significantly differentially expressed clusters (Figure 2.5; Appendix Figure A.2; Supplemental Table 2.7). We first analyzed networks of protein-protein interactions found in three clusters with patterns of increasing expression, C0, C1, and C8. Two of these co-expression clusters, C0

and C8, had modules of protein interactions with functions known or predicted to be important for collective cell migration (Figure 2.5A-B). These migration-related activities included regulation of the cytoskeleton, cell adhesion, small GTPase activity, and cell polarity. Cluster C0 had additional functional interaction modules with proteins required for septate junction formation and proteins involved in ecdysone signaling response, both of which are required for border cell migration (Figure 2.5A; Alhadyian et al., 2021; Bai et al., 2000; Jang et al., 2009; Wang et al., 2020). Cluster C8 was further enriched with modules of proteins with annotated broad functions in development, including cell signaling, serine-threonine kinase activities, and regulation of gene expression (Figure 2.5B). Notably, both C0 and C8 also included proteins with annotated functions in stress signaling (Figure 2,5A-B). The third co-expression cluster with a pattern of increasing expression, C1, had fewer protein interactions (Appendix Figure A.2A). However, the C1 nodes still formed functional modules with predicted or known migration-related activities, along with cellular transport and biosynthesis/metabolism. Next, we analyzed networks of protein interactions in the three co-expression clusters with various patterns of decreasing expression, C4, C5, and C6. Two of these co-expression clusters, C4 and C5, had modules of protein interactions with migration-related functions (Appendix Figure A.2B and A.2C). Both C4 and C5 also had modules of proteins with functions that regulate gene expression and cell signaling. Cluster C6 was enriched for functional modules of proteins that regulate gene expression and proteins involved in biosynthesis and metabolism (Figure 2.5C). Overall, the assembled protein interaction networks reveal functions predicted or known to be important in border cell migration, but also new and unexpected functions.

The network analysis identified two major molecular pathways not typically associated with collective cell migration, immune response regulation in cluster C4 and ribosome-related

functions in clusters C5 and C6 (Figure 2.5C; Appendix Figure A.2B, C; Supplemental Table 2.7). To enhance this analysis, we investigated if additional genes involved in immune response or ribosome-related activities were enriched throughout the co-expression networks. We used Metascape and GO enrichment categories (Supplemental Table 2.6) to curate genes with annotated "immune" or "ribosome" functions from each of the differentially co-expressed clusters (Supplemental Tables 2.8 and 2.9). We used additional pathway and function information from FlyBase and the primary literature to assemble these genes into graphical maps of immune signaling (Figure 2.6A; Appendix Figure A.3) and ribosome functions (Figure 2.6B).

Multiple immune pathway genes were identified. These genes were mainly found in co-expression clusters C4, C0, and C8, which had patterns of increasing or decreasing expression (Figure 2.6A; Appendix Figure A.3; Supplemental Table 2.8). The largest number of genes were classified as having general immune response functions. These 21 genes included those with specific functions in fungal, antiviral, or antibacterial responses, and those with less specific immune roles such as phagocytosis, encapsulation, or hemocyte proliferation (Figure 2.6A; Supplemental Table 2.8). Importantly, we identified core components and genetic regulators of both the *Drosophila* Toll and Immune Deficiency (Imd) innate immune signaling pathways (Figure 2.6A; Appendix Figure A.3A-B). Six genes involved in the Toll pathway and twelve genes involved in the Imd pathway were differentially expressed in border cells. Some critical genes in the canonical Toll cascade such as Toll itself, Cactus, and Dorsal, which also promote embryonic development, were not differentially expressed in border cells (Belvin and Anderson, 1996; Lindsay and Wasserman, 2014). The immune-specific transcription factor Dorsal-related immunity factor (Dif) was also not differentially expressed. However, other key genes such as the adaptor protein *Myd88* and the ligand *spatzle* (*spz*) in the Toll pathway and the adaptor

protein *Imd* and the downstream transcription factor *Relish* (*Rel*) in the Imd pathway were differentially expressed in border cells (Appendix Figure A.3A, B). Jun-kinase (JNK) signaling contributes to the immune response by promoting differentiation of immune cells and sensing stresses such as infections, triggering production of antimicrobial peptides, and promoting wound healing (Yu et al., 2022). JNK can also have other functions, including regulating polarity and cluster cohesion in border cells (Llense and Martín-Blanco, 2008; Melani et al., 2008). Nonetheless, five regulators of the JNK signaling pathway were significantly differentially expressed in border cells (Figure 2.6A; Appendix Figure A.3C). Thus, some, though not all key members of the innate immune pathways, along with known regulators, are differentially expressed during border cell migration.

A substantial number of genes with ribosome functions were identified (87 in total). Ribosomal-related genes were primarily found in co-expression clusters C5 and C6, with decreasing patterns of expression during border cell migration, with only four genes exhibiting increased expression (Figure 2.5C; Appendix Figure A.2C; Supplemental Table 2.9). Ribosome biogenesis primarily takes place in the nucleolus and requires hundreds of proteins to assemble the ribosomal subunits (Ni and Buszczak, 2022). The greatest number of genes found in border cells function in ribosome biogenesis within the nucleolus (Figure 2.6B; Supplemental Table 2.9). These genes (34 in total) encode proteins annotated to regulate transcription of ribosomal RNA (rRNA), modify rRNA, or form the immature large and small ribosomal subunits. Another four genes encode proteins that export ribosomes from the nucleus to the cytoplasm. Genes with annotated functions in the maturation of the ribosomal subunits after export to the cytoplasm (six genes) or mitochondrial ribosome function (eight genes) were also differentially expressed. Additional differentially expressed genes include those that encode the large and small ribosomal

subunits, function in the mature ribosome to regulate cytoplasmic translation/modification, and methylate/bind RNA, as well as other various functions (Figure 2.6B; Supplemental Table 2.9). These data together indicate that, within border cells, ribosome biogenesis genes are differentially expressed and broadly decrease from pre- to post-border cell migration.

2.3.5 Genes identified by transcriptomics are expressed in migrating border cells *in vivo*

The transcriptomic analyses described above revealed large sets of significantly differentially expressed genes. We wanted to further validate the expression of a subset of these genes in migrating border cells *in vivo*. To do this, we took advantage of an open repository of ovarian RNA fluorescence *in situ* hybridization images, the Dresden Ovary Table (DOT) (Jambor et al., 2015). Of the 1,262 significantly differentially expressed genes (Figures 2.3-4, Appendix Figure A.1), 17% had available DOT FISH images at oogenesis stages 8-10 when border cells form and migrate (Supplemental Table 2.10). To validate this approach, we selected DOT images for two genes known to be expressed in border cells (Borghese et al., 2006; Montell et al., 1992; Wang et al., 2006), *slbo* (C8*)* and *sn* (C4), that were also found to be significantly differentially expressed in the *clust* datasets. As expected, both *slbo* and *sn* transcripts were enriched in border cells at pre-, mid-, and post-migratory stages (Figure 2.7A-B, M; Supplemental Table 2.10). DOT data for two additional genes with known functions in border cells, *rols* and tramtrack (ttk), also revealed strong expression in border cells at these stages (Supplemental Table 2.10) (Borghese et al., 2006; Wang et al., 2006). Fourteen additional genes from various enriched clusters showed strong evidence of border cell expression in the DOT stages 8 to 10 data (Figure 2.7M; Supplemental Table 2.10), including *CG11147* (Figure 2.7C), *couch potato* (*cpo*; Figure 2.7D*), Neprilysin 2* (*Nep2*; Figure 2.7E*),* and *Cadherin 74A* (*Cad74A*;

Figure 2.7F). A further six genes had visible expression in border cells during at least one stage of migration in the DOT data: *B4* (Figure 2.7G), *fng* (Figure 2.7H), *CG11007* (Figure 2.7I), *Lipase 4* (*Lip4*; Figure 2.7J*), CG17124* (Figure 2.7K), and *varicose* (*vari*; Figure 2.7L). It is possible that these genes are expressed at other stages of border cell migration but clear images for these genes in the DOT were limited. Moreover, the dynamic range of standard fluorescent RNA *in situ* hybridizations, especially those done in high-throughput screens such as by Jambor et al. (Jambor et al., 2015) in the DOT, does not allow quantitative assessment of differences in transcript levels from pre- to post-migration stages. Nonetheless, these data provide visual confirmation that many genes identified in our RNA sequencing datasets are expressed in migrating border cells *in vivo*.

2.3.6 Functional validation of temporally expressed genes in border cell migration

Finally, we wanted to functionally validate that the temporally expressed genes in border cells were also required for their migration. We identified a subset of genes to be tested from the entire set of significantly differentially expressed clustered genes. Specifically, we tested genes with various GO terms that ranged from transcription, cell differentiation, adhesion, actin binding, and other various molecular functions, that were found across the different gene expression clusters (Figures 2.3-4; Supplemental Table 2.11). A total of 42 genes were knocked down by RNAi in border cells. We drove RNAi expression using the *c306*-GAL4 driver, which is expressed in border cells as well as in some anterior and posterior follicle cells (Figure 2.8A-C). Where possible, we tested at least two RNAi lines per gene. Migration was scored at stage 10 of oogenesis, by which time border cells have normally completed their migration at the oocyte anterior boundary (Figure 2.8C-F). Border cells that did not reach the last quarter of the migration route were considered to have a migration defect (0-75% of the distance away from

the anterior tip of the egg chamber; Figure 2.8D, E). As positive controls for this screen, we performed RNAi knockdown of two genes known to be required for border cell migration, *bazooka (baz)* and *Rap1*, both of which impaired migration in ~25% of egg chambers (Figure 2.8D, E, G; Chang et al., 2018; Pinheiro and Montell, 2004; Sawant et al., 2018). RNAi against *mCherry*, which encodes a fluorescent protein not normally found in *Drosophila*, was used as a negative control; *mCherry* RNAi did not significantly disrupt border cell migration (5% migration defects; Figure 2.8E, F). RNAi knockdown of 21 total genes (22 RNAi lines) resulted in border cell migration defects in >10% of egg chambers (Supplemental Table 2.11). Knockdown of eight of these genes resulted in stronger migration defects, with a range of 14-to-45% of border cells failing to complete their migration by stage 10 (Figure 2.8E, H-K). The genes identified here have a variety of predicted or known functions, including transcriptional regulation (*held out wings, how*; *Ches-1-like*; Figure 2.8E), small GTPase activity (*Arf51F*, also known as *Arf6*; Figure 2.8E, H), cell polarity (*serrano*, *sano*; Figure 2.8E, I), cell adhesion (*Basigin*, *Bsg*; Figure 2.8E, J; *mspo*, Figure 2.8E), cell membrane organization (*Cip4*; Figure 2.8E, K), and other various functions (e.g., cell signaling, F-actin regulation, etc.; Supplemental Table 2.11). Knockdown of the transcription factor *Ches-1-like* had the strongest migration defects (Figure 2.8E; Supplemental Table 2.11). This RNAi line also has a predicted off-target match to *taiman* (*tai*). Tai is an Ecdysone Receptor coactivator that is required for border cell migration (Bai et al., 2000; Jang et al., 2009; Manning et al., 2017). Thus, the migration defects seen by *Ches-1-like* RNAi could be due to knockdown of *tai* or reflect co-knockdown of both genes. However, a previous RNAi screen used an independent RNAi line to *Ches-1-like* with no predicted off-target matches and found that *Ches-1-like* knockdown completely prevented border cell migration (Luo et al., 2015), suggesting that *Ches-1-like* is required. The gene *karst*, which

encodes βH-spectrin, was previously shown to help organize the border cell cluster (Zarnescu and Thomas, 1999). Here we observed mild migration defects when *karst* was knocked down using either of two RNAi lines (Figure 2.8E; Supplemental Table 2.11). Knockdown of most genes resulted in mild migration defects, which could reflect incomplete knockdown due to RNAi efficiency or redundancy with other genes. Nevertheless, these data together demonstrate that at least a subset of the temporally expressed genes identified through RNA sequencing are required for normal border cell migration. Other genes identified here likely also function in border cells but will require further work to determine their exact contributions.

2.4 Discussion

Border cell migration is a tightly regulated process in which multiple signaling pathways converge to specify cell fate and control dynamic cellular behaviors. This is reflected in our results from sequencing of transcripts from temporally staged populations of border cells. Here, we identified nine clusters of significantly differentially expressed transcripts with shared patterns of co-expression from pre- to post-migration. The Metascape and network analyses further revealed distinct molecular pathways and PPI networks that occur during various stages of the migration process. Our study found differential expression of genes involved in many cellular processes known or predicted to be important for collective migration. This included genes that function in the regulation of cell adhesions, cell and tissue morphogenesis, organization of the cytoskeleton, and cell polarity. In addition to these expected migration-related genes, genes that function in immune signaling, ribosome biogenesis, and stress response were also found to be differentially expressed in border cells. The relevance of a subset of differentially expressed genes was confirmed through RNA *in situ* patterns and by functional RNAi in border cells. Importantly, many of the differentially-expressed genes identified here

overlapped with two previous microarray studies (Borghese et al., 2006; Wang et al., 2006). However, these previous microarray studies pooled all migration stages together and focused only on genes upregulated in border cells versus non-migratory follicle cells. Here, in contrast, we report time-specific changes in the expression of genes in border cells. Thus, results from our study can help further identify the targets of temporally regulated molecular pathways required for border cell migration, such as critical signaling pathways and their downstream transcription factors in border cells. The genes identified in our study represent a rich resource of gene expression networks that may function during different stages of border cell migration and thus could inform our understanding of other migrating cell collectives during development and in disease.

Among the top categories of differentially expressed genes in border cells were adhesion- and morphogenesis-related genes. A variety of adhesion proteins help collectively migrating cells stay together, maintain their cell shapes, communicate among cells, and move upon other cells and extracellular matrices (Fierro Morales et al., 2022; Friedl and Mayor, 2017; Messer and McDonald, 2023). Similarly, border cells require complex regulation of cell-cell adhesions for their collective migration to maintain cluster integrity and shape as well as to migrate on and between nurse cells (Cai et al., 2014; Niewiadomska et al., 1999; Saadin and Starz-Gaiano, 2016; Raza et al., 2019). Recent genetic screens and targeted approaches have uncovered various adhesion genes, including septate junction genes, that promote border cell collective cohesion and migration (Alhadyian et al., 2021; Kotian et al., 2022; Raza et al., 2019). Consistent with this, we observed a broad pattern of dynamic adhesion-related gene expression from pre- to post-migration stages. Septate junction assembly, cell-cell junction assembly, and cellular and epithelial morphogenesis genes were specifically enriched in co-expressed clusters whose

expression increased from pre- to post-migration stages. We further confirmed the expression and/or functions of several cell junction and morphogenesis genes, including *Cad74A*, *B4*, *fng*, *Arf51f*, *sano*, and *Cip4*. The adherens junction protein E-cadherin plays a major role in border cell cohesion and migration upon nurse cells (Cai et al., 2014; Chen et al., 2020; Niewiadomska et al., 1999) but surprisingly was not differentially expressed. However, we observed differential expression of *Stat92E* and *slbo*, which upregulate expression of *E-cadherin* just prior to border cell migration (Niewiadomska et al., 1999; Silver and Montell, 2001). Moreover, other regulators of E-cadherin protein localization and/or levels in border cells, such as *raskol*, *Hrb98DE*, and *par-1* were also differentially expressed (Supplemental Table 2.6; McDonald et al., 2008; Raza et al., 2019). The identified differentially expressed cell adhesion, cell junction and morphogenesis genes are thus candidates to regulate critical features of border cell adhesion, cluster integrity, and migration.

We also identified differentially expressed "migration-related" genes within most of the co-expressed clusters and predicted PPI networks. These migration-related genes included those that encode regulators of the actin cytoskeleton, actomyosin structure, and regulators of Rho and Rac small GTPases. Border cells, like all migrating collectives, require finely-tuned Rac-dependent F-actin to extend and retract protrusions specifically at the cluster front for efficient movement (Mayor and Etienne-Manville, 2016; Roberto and Emery, 2022). Non-muscle myosin II (myosin) contracts the rear to facilitate delamination from the epithelium (Majumder et al., 2012; Zeledon et al., 2019). Actomyosin contraction via RhoA activation also helps border cells maintain an optimal cluster morphology as the group moves through the tight spaces between nurse cells (Aranjuez et al., 2016; Gabbert et al., 2023). Regulators of actin and actomyosin were among the top annotated terms in the co-expressed clusters with increasing expression from pre-

to post-migration (e.g., clusters C0 and C1), consistent with their central roles in active cell migration. The differential expression of F-actin and myosin regulatory genes could help border cells quickly adapt to changing conditions in the immediate environment as border cells delaminate and move through the tissue. Supporting this idea, border cells actively respond to physical and chemical cues in the egg chamber by altering protrusion numbers, the shape of the collective itself, and the speed of their migration (Aranjuez et al., 2016; Dai et al., 2020; Gabbert et al., 2023; Lamb et al., 2021; Molina López et al., 2023; Prasad and Montell, 2007; Wang et al., 2010).

Genes important for cell polarity, along with genes associated with EMT, were also differentially expressed. Border cells do not undergo the stereotypical EMTs found in other migratory cell types during development or in cancer. Instead, border cells begin as epithelial cells with notable apical-basal polarity that is retained upon formation of the cluster and subsequent migration (Campanale et al., 2022; McDonald et al., 2008; Pinheiro and Montell, 2004). Border cells also upregulate E-cadherin, which contrasts with cells that undergo complete EMT and downregulate E-cadherin (Nieto et al., 2016). Therefore, it was surprising that genes associated with EMT, including EMT-associated transcription factors *sna* and *twi*, were differentially expressed in border cells. Not all EMT genes play exclusive roles in EMT, however, and could have additional roles in cell migration. For example, two of the differentially expressed EMT genes, *crb* and *Notch*, also promote the polarity and cohesion of border cells (Wang et al., 2007; Wang et al., 2018). Moreover, EMT itself is quite complex (Nieto, 2020; Yang et al., 2020). Many cells that undergo EMT during development and in cancer exhibit "plasticity" during their migration, and can transition back and forth between different epithelial and mesenchymal states (Nieto et al., 2016; Yang et al., 2020). The high levels of apical-basal

proteins in border cells promote various aspects of their migration, from delamination from the epithelium, extension of protrusions, follower cell behaviors, and maintenance of junctions between migrating border cells (Campanale et al., 2022; Llense and Martín-Blanco, 2008; McDonald et al., 2008; Pinheiro and Montell, 2004; Wang et al., 2018). Supporting this role, we found that apical complex genes (e.g., *par-6*) and basal complex genes (e.g., *par-1*, *l(2)gl*, *dlg1*, and *scrib*) were differentially co-expressed and found in multiple PPI networks. Thus, while border cells retain epithelial polarity and require polarity genes for their migration, they may share features in common with other cells that undergo EMT.

Ribosome biogenesis and function genes were among the most highly represented genes whose expression was high early in border cells but decreased during the course of migration. The differentially expressed ribosomal genes included those that function in the transcription, processing, and modification of rRNAs and in the production and nuclear export of the small and large ribosomal subunits. Ribosome biogenesis is tightly linked to translation efficiency, which in turn regulates the levels of proteins. This suggests that border cells need high levels of proteins early in the migration process. In development, homeostatic levels of ribosomal biogenesis proteins and subsequent protein translation are critical, particularly for the specification of stem cells and germ cells and during times of tissue growth (Breznak et al., 2022; Ni and Buszczak, 2023; Yelick and Trainor, 2015). *Drosophila* germline stem cells require an increase in ribosome assembly, including higher expression of ribosome biogenesis and RNA processing genes, for proper growth and division (Breznak et al., 2022; Sanchez et al., 2016). Conversely, altered ribosome biogenesis leads to defects such as skeletal and craniofacial abnormalities and to diseases termed “ribosomopathies” (Armistead and Triggs-Raine, 2014;

Breznak et al., 2022; Ni and Buszczak, 2023). Such defects are due to some mRNAs being more sensitive than others to the overall availability of ribosomes (Ni and Buszczak, 2023).

Why might border cells have higher levels of ribosomal biogenesis genes early in the migration process? Recent work indicates that increased levels of rRNA and ribosomes are important for migrating and invading cells. For instance, differential levels of ribosome biogenesis genes occur during the early phases of anchor cell invasion in *C. elegans* vulval development (Costa et al., 2023). In this case, a burst in ribosome production coincides with an increase in the levels of pro-invasive proteins, which are needed for the anchor cell to breach the basement membrane (Costa et al., 2023; Kenny-Ganzert and Sherwood, 2024). Similarly, in neural crest cells and breast cancer cells, rRNA and ribosome biogenesis is high and required to initiate the EMT program (Prakash et al., 2019). In some migrating cells, cellular protrusions such as lamellipodia have an enrichment of localized mRNAs, eukaryotic initiation factors, and ribosomes required for movement (Dermit et al., 2020; Liao et al., 2015; Mardakheh et al., 2015; Willett et al., 2011). Border cells similarly produce dynamic lead cell protrusions that detect guidance cues and help the cluster move between the nurse cells (Cai et al., 2014; Dai et al., 2020; Poukkula et al., 2011; Wang et al., 2010). However, there is no evidence yet for localized enrichment of mRNAs or ribosomes in border cell protrusions. Nevertheless, an increase in ribosome biogenesis could support high levels of protein translation in border cells that leads to more efficient protrusion formation, delamination, motility, and/or collective invasion into the tissue. Interestingly, we found that the expression levels of ribosomal biogenesis genes significantly decreased by the end of border cell migration. Building the ribosome is energy intensive (Baßler and Hurt, 2019; Ni and Buszczak, 2022; Thomson et al., 2013). Thus, when border cells finish their migration, they may need to conserve energy and/or decrease their protein production. One caveat is that it is yet

unknown whether or if other cells in the ovary at this stage of development also have changes in ribosomal biogenesis genes. As such, it is possible that there is a global change in the expression of ribosome biogenesis genes at these stages of oogenesis. Further work will be needed to formally test how differences in the levels of ribosomal biogenesis genes specifically contribute to border cell migration and/or if it more generally promotes active development during these stages of oogenesis.

The transcriptome and PPI network analyses further revealed enrichment of genes whose protein products are annotated to function in immune signaling, in biosynthesis and metabolism, and stress response. Immune and biosynthesis/metabolic genes were found throughout all the major co-expressed clusters and networks. Thus, while differentially expressed, these genes were not associated with one specific stage of migration. Border cells express some, though not all, key components and regulators of the Toll and Imd signaling pathways, including genes that function in defense against viral, bacterial, fungal, or parasitic threats (Lindsay and Wasserman, 2014). For example, Spatzle, Myd88, Imd and Relish were significantly differentially expressed during border cell migration, but Toll, Cactus, and Dorsal were not. The significance of these immune signaling genes is unknown since border cells are migratory epithelial cells and as such are not expected to have immune signaling functions. While it is formally possible that the observed expression of immune genes could have been induced due to technical issues with sample isolation, the differential expression of some immune pathway components but not others suggests a more specific role. Supporting this idea, a previous RNAi screen found a requirement for multiple members of the Toll signaling pathway including Dorsal and Dif in border cell migration (Luo et al., 2015). The biosynthesis/metabolic genes found to be differentially expressed in border cells have a wide variety of cellular functions. These functions include lipid

metabolism and synthesis (e.g. Agpat3, SNF4Agamma), membrane homeostasis (e.g. CDase), and metabolic processes in mitochondria (e.g. Idh, Idh3g, SdhA). Stress response genes were found in networks within C0 and C8, two clusters that had increased expression from pre- to post-migration. Differentially expressed stress genes include those whose protein products regulate oxidative stress (e.g., Cnc; Whd) and the unfolded protein response (e.g., Atf6, Hsc70-3). Recent work has demonstrated roles for metabolic and mitochondrial genes in immune cell (hemocyte) invasion during *Drosophila* embryogenesis (Emtenani et al., 2022). While intriguing, it remains to be determined whether the immune, biosynthesis/metabolism, and stress response genes identified here have specific roles in border cell development or migration, or if they mainly provide basal homeostatic functions.

Conclusions

Collective cell migration is a highly dynamic process that requires intricate coordination of multiple developmental processes ranging from differential adhesion that keeps cells together, to a dynamic actin cytoskeleton that directs cell movements and migratory protrusions. Because of this complexity, it has been a challenge to understand the dynamic changes that occur in the molecular architecture of cell collectives as they migrate. Our transcriptome analyses, and functional validations, in border cells identified known biological processes in collective cell migration such as adhesion and polarity, but also implicated a novel role for the ribosome in border cell migration. Given the striking similarities between border cells and other cell collectives including cancer cells (Friedl et al., 2012; Scarpa and Mayor 2016; Stuelten et al., 2018), the genes identified here represent a wealth of new candidates to investigate the molecular nature of collective cell migration.

2.5 Methods

Drosophila genetics

All fly stocks and crosses were maintained at 25˚C, unless otherwise indicated. A *slbo*-mCD8:GFP/CyO stock (gift of X. Wang, CNRS/University of Toulouse), which drives Mmus\Cd8a (FBgn0026406) tagged with eGFP under control of the *slbo* enhancer, was outcrossed to w^{1118} and used to obtain sorted border cell populations. For RNAi screening, stocks were obtained from the Bloomington Drosophila Stock Center (BDSC), the Vienna Drosophila Resource Center (VDRC), and NIG-Fly. Where possible, two independent non-overlapping RNAi lines were chosen per gene. Females from a *c306*-Gal4; tsGAL80/CyO stock were crossed to UAS-RNAi males to drive expression of the knockdown construct in border cells. RNAi against *mCherry* (BDSC 35785) was used as a negative control, and *bazooka* (*Baz*; VDRC 2914) and *Rap1* (BDSC 57851) were used as positive controls. Female progeny from these crosses were selected and fattened on wet yeast paste overnight at 29˚C to allow maximal GAL4/UAS expression and inactivation of tsGAL80. All RNAi lines are listed in Supplemental Table 2.11.

Isolation of staged border cell RNA, library preparation, and Illumina sequencing

To isolate staged egg chambers for cell sorting, whole ovaries were first dissected from fattened 3- to 5-day old *slbo*-mCD8:GFP/+ females followed by dissection into ovarioles in room temperature “live imaging media” (Schneider’s media, pH 6.95 [Thermo Fisher Scientific],15% fetal bovine serum [Seradigm FBS; FBS (VWR), 200 µg/mL insulin (bovine pancreatic, Sigma-Millipore, cat. no. I5500)]; Chen et al., 2023). Stages 9 and 10 egg chambers (Spradling, 1993) were manually selected in a two-well concave slide by observing GFP in the border cells (*slbo*-mCD8:GFP) on a fluorescence stereomicroscope. Egg chambers were

considered to be at the “pre-migration” stage if border cells had rounded up as a cluster within the anterior follicular epithelium; these border cell clusters often had a visible protrusion at the time of selection as viewed by GFP-expression under the microscope. Egg chambers with border cells that detached from the epithelium and had moved into the egg chamber anywhere along the path of migration but had not yet reached the oocyte were considered “mid-migration.” Egg chambers with border cells that had reached the oocyte were considered “post-migration.” The stalk between egg chambers was cut with a needle and 40-60 GFP-positive egg chambers at each stage (pre-, mid-, and post-migration) were individually pooled and transferred to separate microcentrifuge tubes. Egg chambers were washed twice with cell dissociation buffer (Sigma-Millipore, cat. no. C5914-100ML), then treated with 10 mg/mL elastase (Sigma-Millipore SIGMA, cat. no. E0258-5MG; lot number SLBL4608V) in cell dissociation buffer for 30 min at 25ºC with occasional agitation, followed by pipetting the mixture. Full cell dissociation was confirmed by visual inspection on a fluorescence stereomicroscope. The elastase reaction was stopped by addition of Schneider’s media. Cells were collected by centrifugation at 1000 rpm for 2 minutes and resuspended in PBS, 0.5 mg/mL Bovine Serum Albumin (BSA). Cells were washed twice in PBS supplemented with 0.5 mg/mL PBS prior to selection for GFP-positive cells.

Dissociated cells were incubated with anti-mCD8 antibody (Thermo Fisher Scientific, catalog number MA1-145) and 5 µl of protein-G Dynabeads Magnetic Beads (Thermo Fisher Scientific, cat. no. 10003D) for 30 minutes at room temperature in the dark. Cells bound to the beads were isolated using a magnetic rack (Thermo Fisher Scientific, cat. no. 12321D) for 5 min at 25ºC, followed by two washes with PBS supplemented with 0.5 mg/mL BSA. Magnetic isolation yielded ~50-120 GFP-positive cells. The yield was assessed by fluorescence

microscopy to ensure that all cells were GFP-positive. Captured cells were then suspended in 100 µl of Trizol™ (Thermo Fisher Scientific, cat. no. 15596026) and frozen at -20˚C. RNA was isolated using the manufacturer's instructions. The resultant purified RNA was stored in 75% ethanol at -80ºC prior to library preparation. Each cell isolation yielded ~50-100 ng of total high-quality RNA, as assessed by TapeStation analysis (Agilent Technologies, RIN^e >8.0). RNA-sequencing libraries were prepared with the Illumina TruSeq® version 2 stranded library preparation kit with 12 multiplexing barcodes. Prior to combination and loading, libraries were quantified by qPCR using a library quantification kit (NEBNext® Library Quantification Kit, E7630, New England Biolabs). Illumina sequencing was performed on a HiSeq2500 instrument for 100 single end read cycles (Genome Sequencing Core, University of Kansas).

Read mapping, quantification, and normalization

Read mapping and quantification were performed using RSEM version 1.3.3 (Li and Dewey, 2011) into a Snakemake version 5.5.4 (Mölder et al., 2021) pipeline (https://bitbucket.org/olsonlab/border_cell_migration). Illumina reads were trimmed for quality and adapter presence with Sickle version 1.33 Joshi2011-yt and Scythe version 0.994 Buffalo2011-za. Reads with quality less than PHRED 30 or less than 4 base pairs in length were discarded prior to mapping. Reads were checked for quality with FastQC version 0.11.6 Andrews2019-hi before and after trimming. Reads were mapped to the *Drosophila melanogaster* FlyBase predicted transcriptome (FB2021_02 Dmel Release 6.39), including splice variants, using bowtie RSEM included in the RSEM distribution Langmead2012-uk (for information on aligned reads, refer to Supplemental Table 2.2). For all sequences, the transcripts per million (TPM) were used for absolute counts. Subsequent analysis used quantile normalization data that

was log2 transformation and z-score normalization of expression in Python 3.11 and Pandas 1.5.3. Statistically significant differential expression using EBSeq-HMM was included in the RSEM distribution Leng2015-yz. EBSeq-HMM normalized differential expression was performed by quantile normalization. The significance for cutoff for differential expression was a False Discovery Rate (FDR) of <0.05. A CDS sequence for eGFP (Genbank: AAB02572.1) was included during transcript mapping to ensure that the RNA from sorted cells were indeed GFP-positive (Supplemental Table 2.1). For downstream analyses, alternate transcripts in the *D. melanogaster* genome were aggregated prior to gene level analysis using a custom script using Pandas 1.5.3 to aggregate data (https://bitbucket.org/olsonlab/border_cell_migration).

Gene expression analyses

Co-expression clustering: Genome-wide co-expression clustering was performed using quantile normalized TPM counts per transcript, followed by log2 transformation and z-score transformation. Heatmaps were prepared in Python 3.11, Pandas 1.5.3, and Seaborn 0.12.2 clustermap function that implements the SciPy 1.10.0. linkage algorithm using Euclidean distance (https://bitbucket.org/olsonlab/border_cell_migration). Co-expression clustering was performed using the *clust* version 1.12.0 (Abu-Jamous and Kelly, 2018) on TPM counts for each transcript corresponding to genes identified as having statistically significant differential expression during the stages of border cell migration. *Clust* automatically performs quality control and normalization of TPM counts per gene and clusters expression patterns represent biological expectations (Abu-Jamous and Kelly, 2018).

Gene annotation enrichment and protein network analysis: Metascape was used for annotation enrichment analysis (Zhou et al., 2019) of genes identified in each of the *clust* co-expression clusters. Default analysis parameters were used and functional annotation results were further analyzed with customized visualizations in Python (available at https://bitbucket.org/olsonlab/border_cell_migration). Protein interaction networks derived from Metascape analysis of *clust* co-expression clusters were augmented with additional protein interactions and annotations from STRING (Szklarczyk et al., 2022), with a confidence cutoff score of 0.4 implemented as a plug-in in CytoScape version 3.9.1 (Shannon et al., 2003). Protein/gene nodes in networks without interactions, or those with a single interaction, were removed from further analysis. Metascape functional annotations augmented with STRING annotations were manually curated into a consistent set of functional terms reflective of the gene function based on gene summary, gene group and protein family, pathway, additional gene ontology (GO), and phenotype data from Flybase 2023_01 and Dmel Release 6.50 ("Annotations" column, Supplemental Table 2.7). For clarity, one annotation "keyword" was chosen for each protein in the interaction network ("Annotation Keyword" column, Supplemental Table 2.7). Protein/gene nodes were then colored based on annotation keyword to highlight regions of the network with similar functions as indicated. Additional manual curation was performed on immune and ribosome biogenesis related genes.

Immune and ribosome biogenesis pathway analysis: Differentially expressed genes were chosen based on Metascape enrichment for immune and ribosome related GO terms (including "innate immune response," "immune effector process," "ribonucleoprotein complex biogenesis," "ribosomal small/large subunit complex biogenesis," and "rRNA processing" (Supplemental

Tables 2.8-9; Metascape). To confirm biological functions for these chosen genes, we used FlyBase data and primary literature to annotate each gene ("Annotation Notes" and "References" columns; Supplemental Tables 2.8-9). Based on this annotation, each gene was then either assigned a category or excluded from analysis ("Category" column; Supplemental Tables 2.8-9). Differentially expressed genes enriched for immune GO terms were then placed into either the Toll, Imd, JNK, or other signalings categories, or into a more general "immune response" category (Figure 2.5). For a more detailed view of each pathway and differential expression patterns for each gene, we combined expression and pathway information in Appendix Figure A.3.

Heatmaps of gene groups of interest: FlyBase gene numbers (FBgns) associated with GO IDs for oogenesis (GO:0048477), actin cytoskeleton (GO:0015629), and adhesion (GO:0007155, GO:0022610) were downloaded from FlyBase (FlyBase). FBgn numbers for transcription factors were taken from the "PL FlyTF_trusted_TFs" list on FlyMine (Lyne et al., 2007). Genes for epithelial-to-mesenchymal transition (EMT; GO:0001837) were obtained using the high rank orthologs from mouse EMT genes as determined by the DRSC Integrative Ortholog Prediction Tool (DIOPT) (Hu et al., 2011) in addition to FBgns associated with the indicated GO IDs downloaded from FlyBase. "Border cell migration genes" were manually curated using primary literature to include genes likely to function in border cell migration (genes and references available in Supplemental Table 4). FBgns from Wang et al. (Wang et al., 2006) were taken from their Supplemental Table 1 ("Genes Enriched in Migratory Cells"). FBgns from Borghese et al. (Borghese et al., 2006) were taken from their supplemental tables S1 and S4 (S1: "Genes significantly up-regulated; $P<0.05$, in wild type border cells [BCs] compared to follicle cells

[WT BCs > FCs]", S4: "Genes significantly [P<0.05] down-regulated in wild type border cells compared to follicle cells [WT BCs < FCs]"). FBgns were translated to FlyBase transcript numbers (FBtr) using FlyBase and expression of all significantly differentially expressed transcripts (EBseq-HMM FDR<0.05) was assessed.

Dresden Ovary Table RNA situ hybridization database analyses: Fluorescence *in situ* hybridization images for genes present in the dataset were accessed from the Dresden Ovary Table (DOT) website (Pavel Tomancak; Jambor et al., 2015). The "gene search" and "table" functions were used individually to search for expression data for the 1,262 significantly differentially expressed genes identified in our analyses. Although the DOT provides developmentally staged images with annotated cell types, it was necessary to empirically validate these results. Therefore, images were manually assessed for each gene that had data for oogenesis stages 8-10 and the relevant cell types visible, including border cells, polar cells, centripetal cells, and/or posterior terminal cells (Supplemental Table 2.10). Expression data was manually curated for significantly differentially expressed genes except where images were not available for the given gene. In this case, the DOT annotation was used, which is denoted by use of italicized text in Supplemental Table 2.10. A homogenous RNA signal throughout the egg chamber was denoted as "ubiquitous signal." Representative images were chosen for a subset of genes with strong RNA signal in border cells at stages 9 and/or 10 (Figure 2.7).

Immunostaining and Microscopy

To analyze border cell migration, whole ovaries were dissected in Schneider's *Drosophila* medium (Thermo Fisher Scientific) and fixed in 4% methanol-free formaldehyde

(Polysciences) and 0.1 M potassium phosphate buffer (pH 7.4) for 10 minutes. To analyze migration, egg chambers were stained for E-cadherin (E-cad; 1:10 dilution, rat monoclonal DCAD2; Developmental Studies Hybridoma Bank [DSHB]), Singed (Sn; 1:25 dilution, mouse monoclonal Sn7C; DSHB), and DAPI to label nuclei (2.5 µg/mL; Millipore Sigma, cat. no. D954). Isotype-specific anti-mouse or anti-rat secondary antibodies conjugated to AlexaFluor–488 or –568 (Thermo Fisher Scientific) were used at a concentration of 1:400. All slides were mounted using Fluorsave Reagent mounting media (Millipore Sigma, cat. no. 345789). Migration defects were analyzed on an upright Zeiss AxioImager Z1. Primary antibodies against GFP, either mouse monoclonal 12E6 (DSHB, 1:200 dilution; Figure 2.1A-C) or chicken polyclonal ab13970 (Abcam, 1:500 dilution; Figure 2.1D) were used to improve visualization of the *slbo*-mCD8:GFP pattern in fixed egg chambers (Figure 2.1A-D). To visualize the *c306*-Gal4 expression pattern (Figure 2.7A-C), *c306*-Gal4; tsGAL80>UAS-LacZ egg chambers were stained with a primary antibody against beta-galactosidase (1:10 dilution, mouse monoclonal 40-1a; DSHB). Other antibodies used were Eyes Absent (Eya; 1:50 dilution, mouse monoclonal eya10H6; DSHB) and Fasciclin III (Fas3; 1:5 dilution, mouse monoclonal 7G10; DSHB). All images were acquired on a Zeiss LSM 880 confocal microscope at the Kansas State University (KSU) College of Veterinary Medicine (CVM) Confocal Core using a 20x (Figure 2.1A-C, Figure 2.8) or 40x oil (Figure 2.1D) numerical aperture (NA) objective. All images were processed in ImageJ (FIJI).

Statistical Methods, Graphs, and Figure Assembly

Bioinformatic analyses were performed in Python version 3.11, Pandas version 1.5.2, Numpy version 1.24.1, SciPy version 1.10.0, and Seaborn version 0.12.2 overlaid on Matplotlib

version 3.6.3. Data tables were analyzed in Python and Pandas. Tables were exported to Microsoft Excel format and functions using OpenPyxl version 3.0.10. Additional graphs were assembled and statistical analyses were performed in GraphPad (version 7.04). Figures were assembled using Affinity Photo, Affinity Designer, and Adobe Illustrator. Three trials per RNAi line were used for the RNAi screen. The cutoff value for migration defects was determined by the background mean migration defect, calculated using an average of the migration defect observed in the negative control (*c306*-Gal4; tsgal80/+; UAS-mCherry RNAi/+). To assess the significance of the migration defects, the empirical rule for a normal distribution was used, where the migration defect cutoff is equal to the mean plus three times the standard deviation.

Availability of Data and Materials

Data and code used to generate figures are available at https://bitbucket.org/olsonlab/border_cell_migration. RNA-sequencing data is available at NCBI Sequence Read Archive (SRA) BioProject ID (PRJNA1021332; http://www.ncbi.nlm.nih.gov/bioproject/1021332). Major results are available in the supplemental tables. All other data and materials will be made available on request.

2.6 Figures

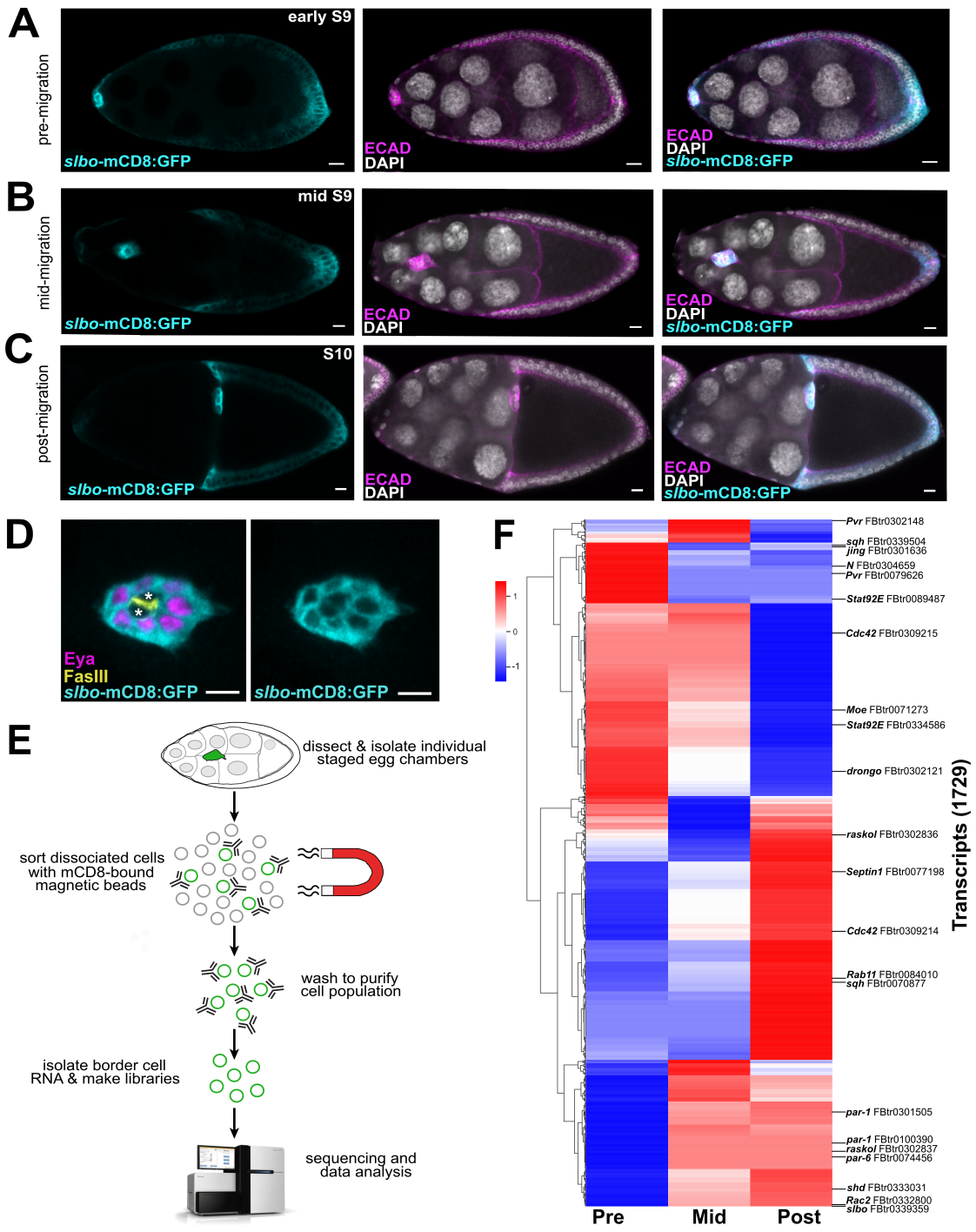


Figure 2.1 RNA sequencing of isolated populations of border cells reveals temporal change in gene expression.

(A-C) Representative egg chambers showing the patterns of *slbo*-mCD8:GFP expression in the border cell cluster prior to **(A)**, during **(B)**, and after migration **(C)**. **(D)** Representative migrating border cell cluster showing the pattern of *slbo*-mCD8:GFP expression in individual border cells (expressing Eyes absent, Eya) and polar cells (FasIII-positive, marked with asterisks). **(E)** Schematic of how the mCD8:GFP-positive border cells were sorted and selected using mCD8-bound magnetic beads. RNA from isolated border cells was then prepared for RNA sequencing and downstream analyses. **(F)** Heatmap of all sequencing results, identifying significant differential expression for 1,729 transcripts from isolated border cells during their migration (EBSeq-HMM FDR<0.05). Several border cell migration-related genes, along with their transcript number ("FBtr"), are highlighted. Scale bars represent 10 μm **(A-C)** or 5 μm **(D)**. All significantly differentially expressed transcripts are shown in Supplemental Table 2.3. HiSeq 2500 graphic courtesy of Illumina, Inc.

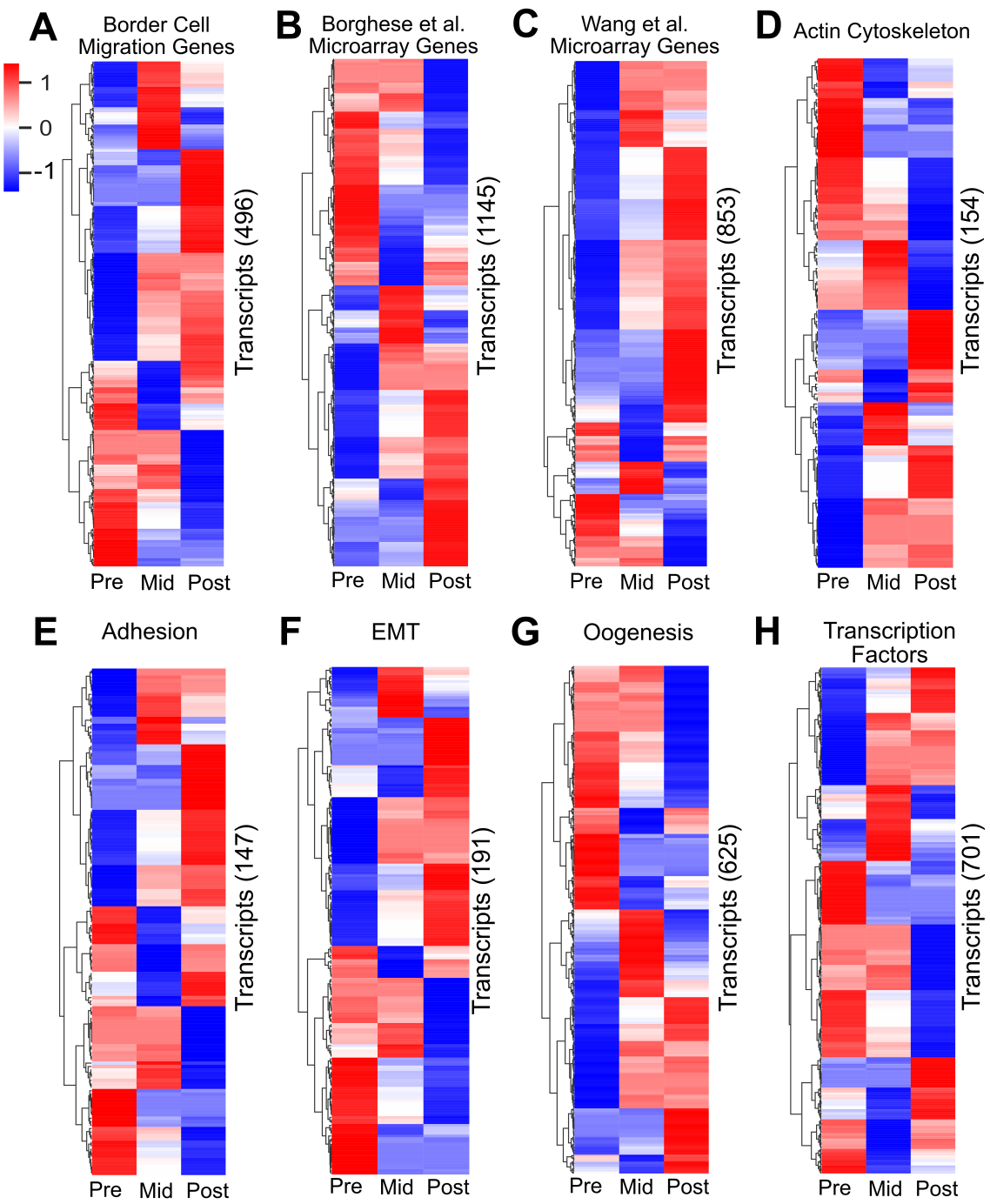


Figure 2.2 Migration and development-related genes are temporally expressed during border cell migration.

(A-H) Heatmaps of significantly differentially expressed transcripts (EBSeq-HMM FDR<0.05), focused on subsets of migration-related **(A-F)** or developmentally related **(G, H)** gene categories. The primary literature or other databases were used to identify genes and GO terms (*see* Methods

for details). Heatmaps represent differential expression in border cells at pre-, mid-, or post-migration. A *z*-score of 1 (shown in red) signifies up-regulation; a *z*-score of -1 (shown in blue) signifies down-regulation. **(A-C)** Differentially expressed transcripts known to be required or expressed in border cells during their migration (A, 496 transcripts, representing 136 unique genes), or shown to be significantly upregulated in border cells versus non-migratory follicle cells from two microarray studies, (Borghese et al., 2006) (**B**, 1145 transcripts representing 475 unique genes) and (Wang et al., 2006) (**C**, 853 transcripts, 297 unique genes). **(D-H)** Transcripts for migration-related **(D-F)** or developmentally-related **(G, H)** genes, including the actin cytoskeleton (**D**, 154 transcripts, 38 unique genes), adhesion (**E**, 147 transcripts, 46 unique genes), epithelial-to-mesenchymal transition (EMT; **F**, 191 transcripts, 66 unique genes), oogenesis (**G**, 625 transcripts, 184 unique genes), or transcription factors (**H**, 701 transcripts, 263 unique genes). All data are shown in Supplemental Table 2.4.

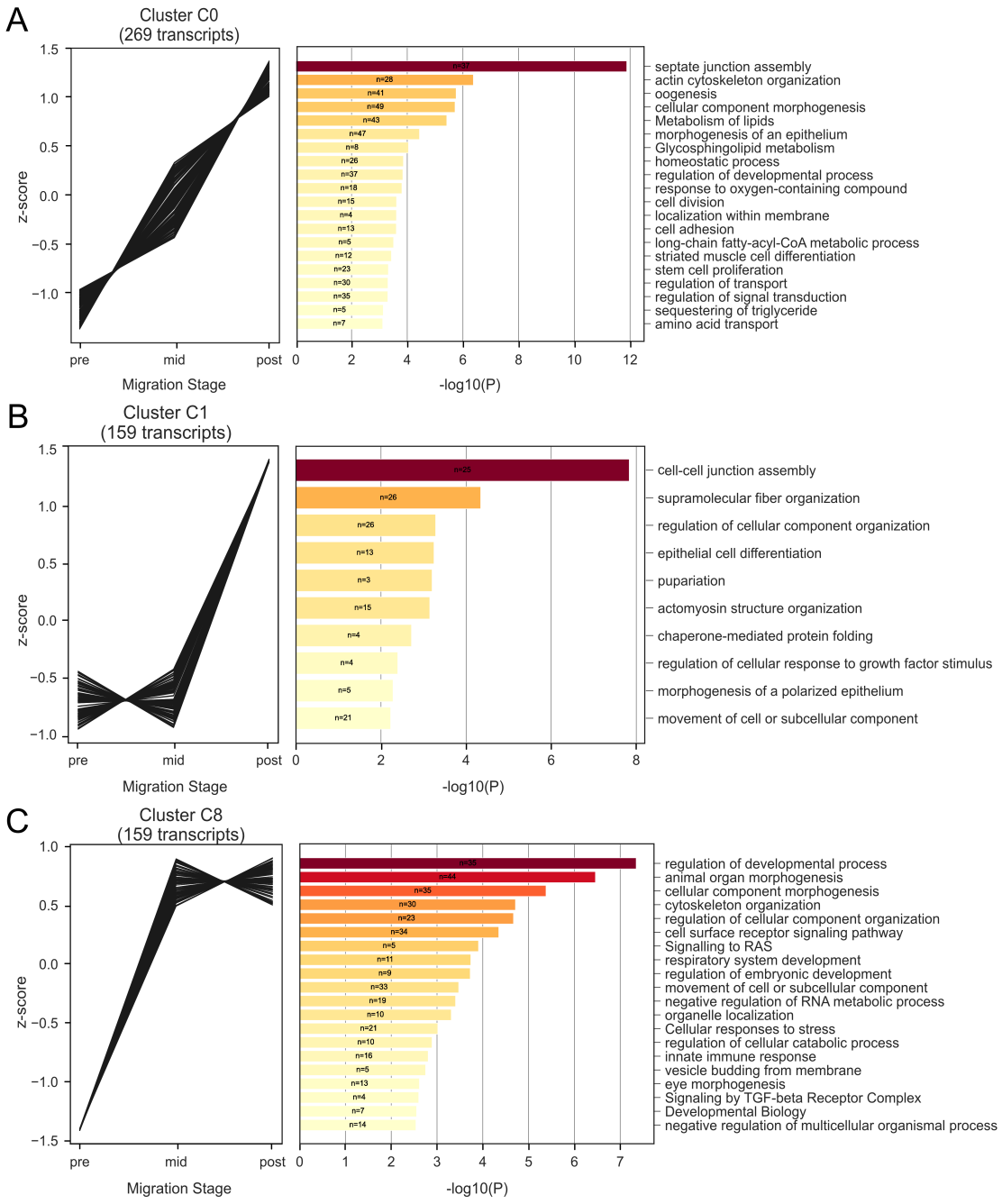


Figure 2.3 Differentially co-expressed transcripts are up-regulated during border cell migration and enriched for shared biological functions.

(**A-C**, left graphs) Significantly differentially expressed transcripts sorted by *clust* into shared co-expression patterns in pre-, mid-, and post-migration stages. (**A-C**, right graphs) Metascape pathway and process enrichment analysis results for each co-expression cluster, showing the

most significantly enriched terms. *N,* number of genes enriched for a given annotation term; bars show significance of annotation terms, sorted by *p* values (-log10P; darker color, more significant values). Note that genes can be found in multiple Metascape annotation categories. Clusters C0 **(A)**, C1 **(B)**, and C8 **(C)** show patterns of increased expression during border cell migration. All data are shown in Supplemental Tables 2.5 and 2.6.

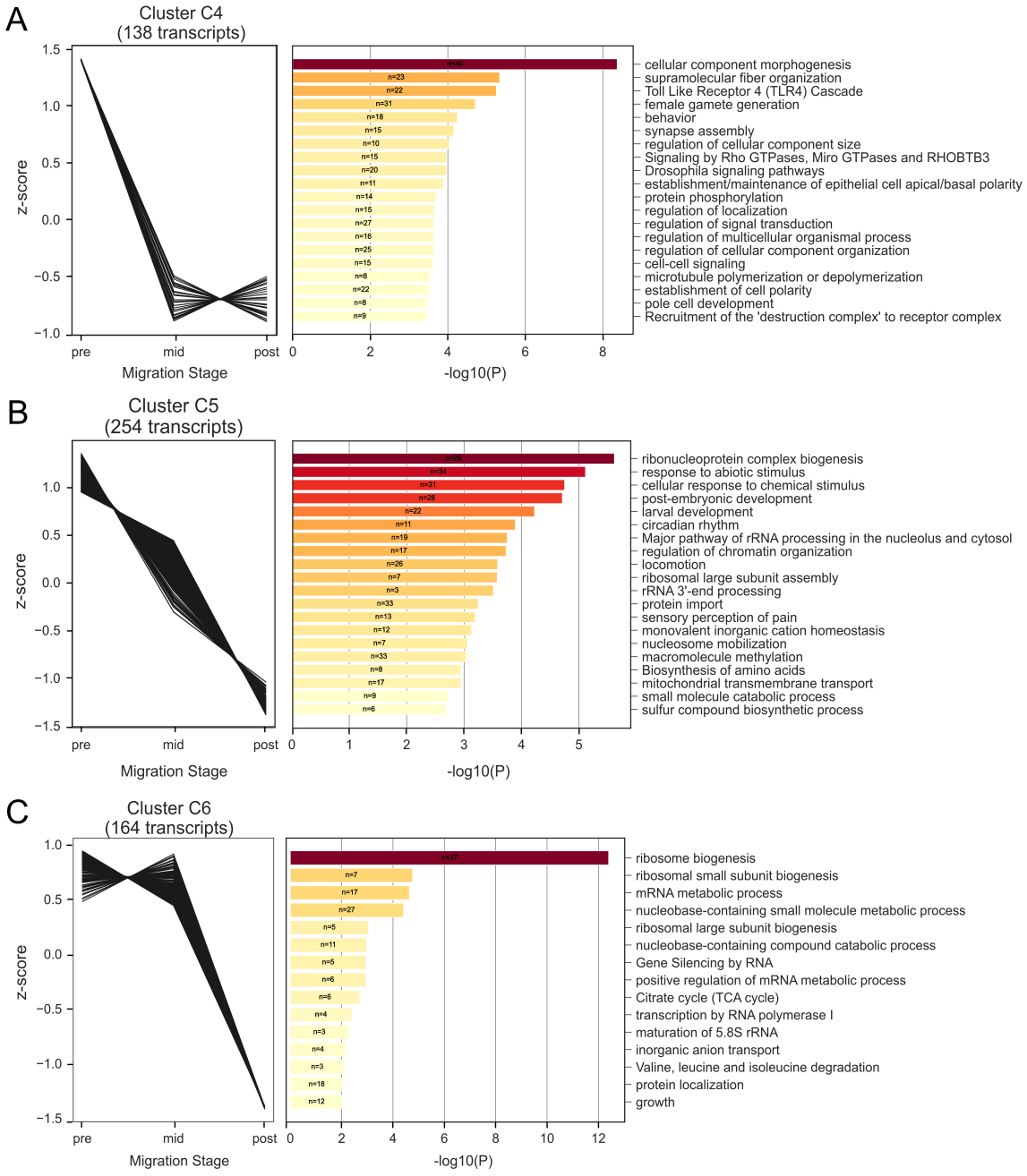


Figure 2.4 Differentially co-expressed transcripts are down-regulated during border cell migration and enriched for shared biological function.

(**A-C**, left graphs) Significantly differentially expressed transcripts sorted by *clust* into shared co-expression patterns in pre-, mid-, and post-migration stages. (**A-C**, right graphs) Metascape pathway and process enrichment analysis results for each co-expression cluster, showing the mostly significantly enriched terms. *N*, number of genes enriched for a given annotation term;

bars show significance of annotation terms, sorted by *p* values (-log10P; darker color, more significant values). Note that genes can be found in multiple Metascape annotation categories. Cluster C4 **(A)**, C5 **(B)**, and C6 **(C)** show patterns of decreased expression during border cell migration. All data are shown in Supplemental Tables 2.5 and 2.6.

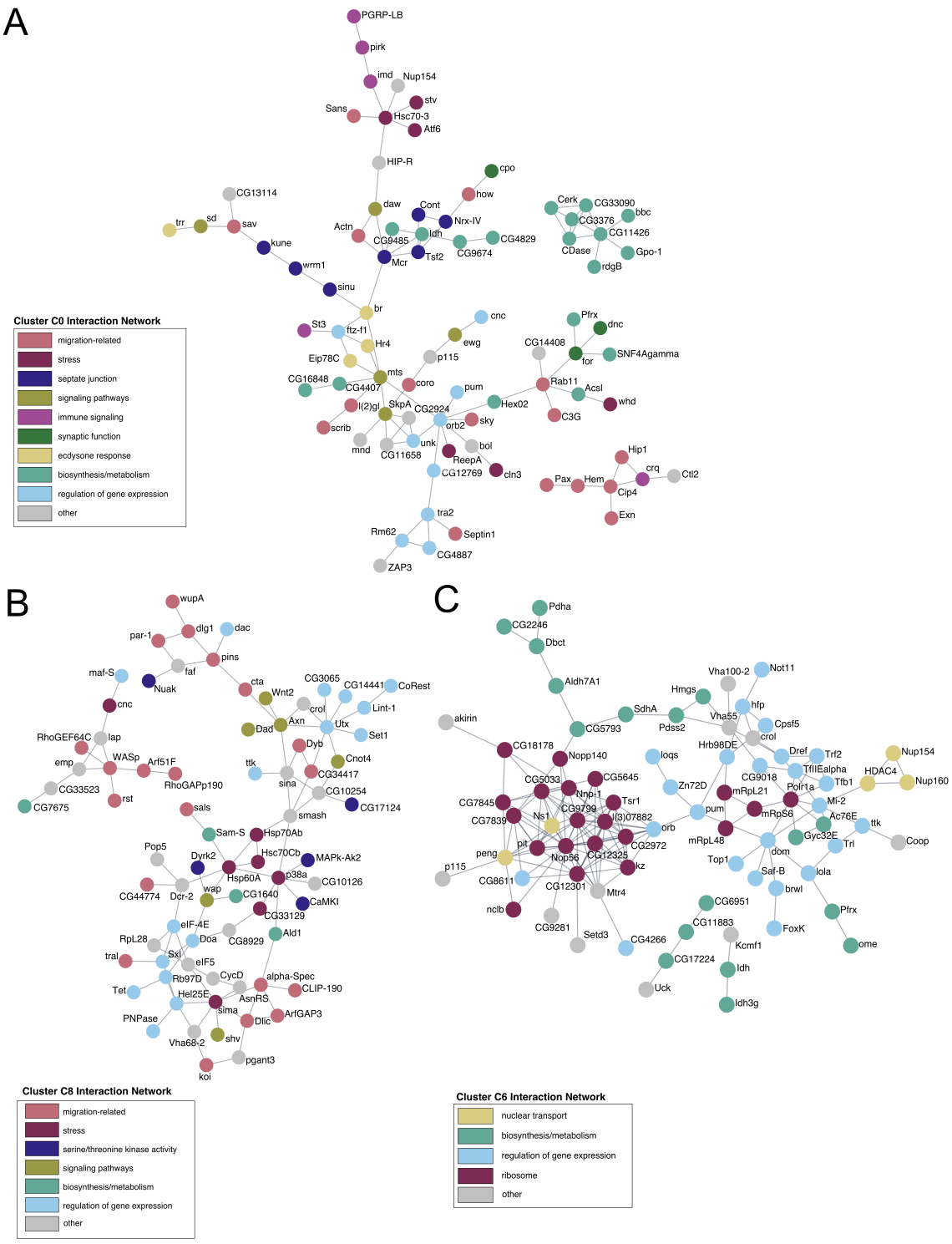
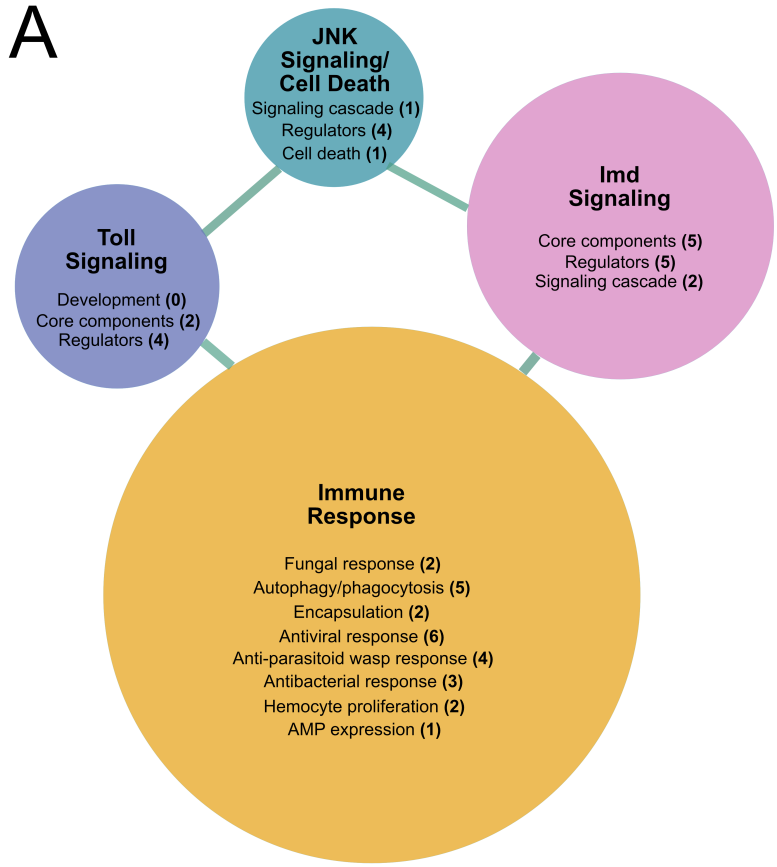


Figure 2.5 Differentially co-expressed genes form protein-protein interaction networks.

Physical protein interaction (PPI) network analysis of gene products from selected co-expression clusters. Functional annotation keywords were used to assign color to proteins in the networks. **(A)** Co-expression cluster C0, encompassing genes that increase expression during migration, contains PPI nodes with migration-related functions (pink), including functions known to regulate border cell migration such as septate junction regulation (dark blue) and ecdysone response function (mustard). Individual networks consist of proteins with biosynthesis/metabolism (cyan, upper right) and migration-related (pink, lower right) functions. **(B)** Co-expression cluster C8, encompassing genes that increase expression, forms one major protein interaction network with migration-related (pink), multiple signaling pathways (light green), and gene expression regulation (light blue) functions. One individual network consists of proteins with migration-related and additional categories (upper left). **(C)** Co-expression cluster C6, encompassing genes that decrease during migration, forms large nodes for ribosome function (magenta), biosynthesis/metabolism (cyan), and regulation of gene expression (light blue). Individual networks consist of proteins with biosynthesis/metabolism (cyan, lower center). For each PPI network, the "other" category (gray) either denotes genes that do not have available FlyBase annotations/data or for which three or fewer genes were annotated. All data, including annotations and keywords, are shown in Supplemental Table 2.7.


A
JNK Signaling/ Cell Death
Signaling cascade (1)
Regulators (4)
Cell death (1)
Imd Signaling
Core components (5)
Regulators (5)
Signaling cascade (2)
Toll Signaling
Development (0)
Core components (2)
Regulators (4)
Immune Response
Fungal response (2)
Autophagy/phagocytosis (5)
Encapsulation (2)
Antiviral response (6)
Anti-parasitoid wasp response (4)
Antibacterial response (3)
Hemocyte proliferation (2)
AMP expression (1)

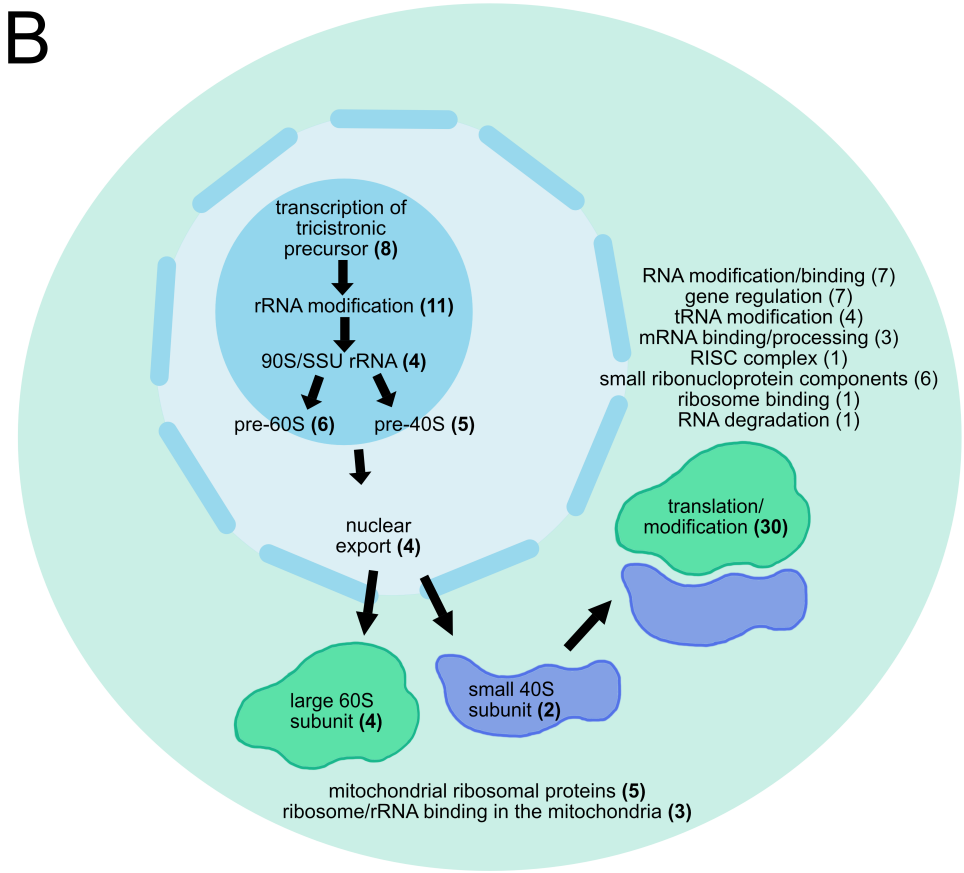
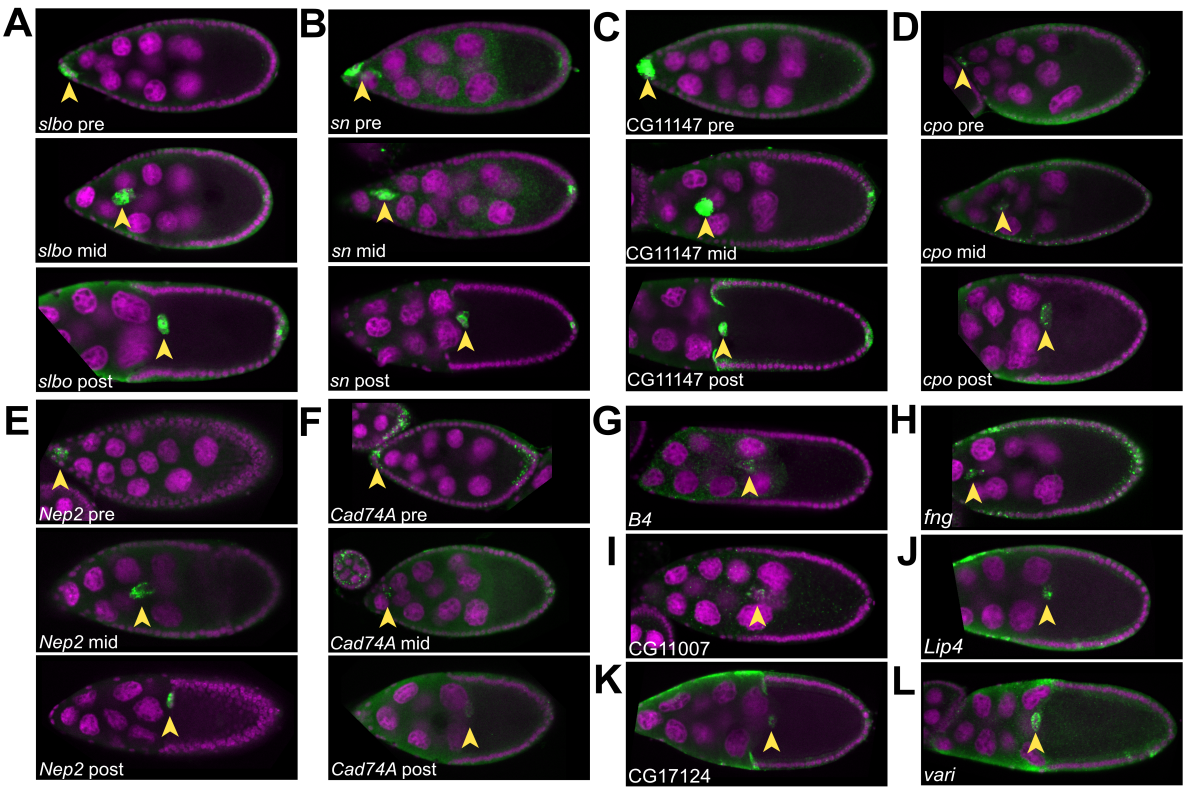

B
transcription of tricistronic precursor (8)
rRNA modification (11)
90S/SSU rRNA (4)
pre-60S (6)
pre-40S (5)
nuclear export (4)
RNA modification/binding (7)
gene regulation (7)
tRNA modification (4)
mRNA binding/processing (3)
RISC complex (1)
small ribonucloprotein components (6)
ribosome binding (1)
RNA degradation (1)
translation/ modification (30)
large 60S subunit (4)
small 40S subunit (2)
mitochondrial ribosomal proteins (5)
ribosome/rRNA binding in the mitochondria (3)

Figure 2.6 Networks of immune signaling and ribosome biogenesis genes differentially expressed during border cell migration.

Graphical representation of the networks of differentially co-expressed genes in border cells annotated with immune **(A)** or ribosome-related **(B)** functions. **(A)** Co-expressed genes enriched for immune functions were sorted into Toll Signaling, JNK Signaling/Cell Death, Imd Signaling, or Immune Response categories. Genes involved in defense against fungus, virus, bacteria, or parasitoid wasp, as well as genes implicated more broadly in immune cell or cell death functions, but not linked to a signaling pathway, comprise the "immune response" category. **(B)** Co-expressed genes enriched for ribosome function were sorted into ribosome biogenesis in the nucleolus, nuclear export, large and small ribosomal subunit, translation/modification and other categories as indicated. For simplicity, the total number of genes enriched for small ribonuclear protein component functions is shown in the cytoplasm; a subset of these genes have predicted or demonstrated nuclear localization. All data are shown in Supplemental Table 2.8 (**A**, immune functions) and Supplemental Table 2.9 (**B**, ribosome functions).



M

Gene Symbol	Gene Name	Expression Data	SigDE Cluster
slbo	slow border cells	BC, PC, FC surrounding oocyte, CC	C8
sn	singed	BC	C4
CG11147	-	BC	C0, C1
cpo	couch potato	BC, follicle cells	C0, C1, C4
Nep2	Neprilysin 2	BC	C1
Cad74A	Cadherin 74A	ubiquitous signal/ BC	C0
B4	B4	BC	C8
fng	fringe	BC/main body FC	C0
CG11007	-	puncta BC expression	C0
Lip4	Lipase 4	BC/ squamous FC	C0, C8
CG17124	-	ubiquitous signal/BC	C1, C8
vari	varicose	BC, follicle cells	C5

Figure 2.7 Expression patterns of genes in migrating border cells *in vivo*.
Fluorescence RNA in situ hybridization patterns of differentially expressed genes in migrating border cells from the transcriptomics analysis in migrating border cells using data and images from the Dresden Ovary Table (DOT). Representative images of stage 9 and/or stage 10 egg chambers were chosen for given genes of interest. RNA signal is in green and DAPI labels the nuclei in magenta. Anterior is to the left in all images. **(A-F)** RNA signal (green) is shown in pre-, mid-, and post- migratory border cell clusters. **(G-L)** RNA signal (green) is shown at one stage

of migration. **(A, B)** Expression of *slbo* (A) and *sn* (B), two genes known to regulate border cell migration. **(C-L)** Expression of multiple genes identified from the transcriptome analyses with previously uncharacterized roles in border cell migration. **(M)** Table of genes found in the DOT, along with their annotated expression patterns in the ovary, based on all available images, and location within one of the significantly differentially co-expressed gene clusters (SigDE Cluster). Arrowheads mark the position of border cells. All data are shown in Supplemental Table 2.10.

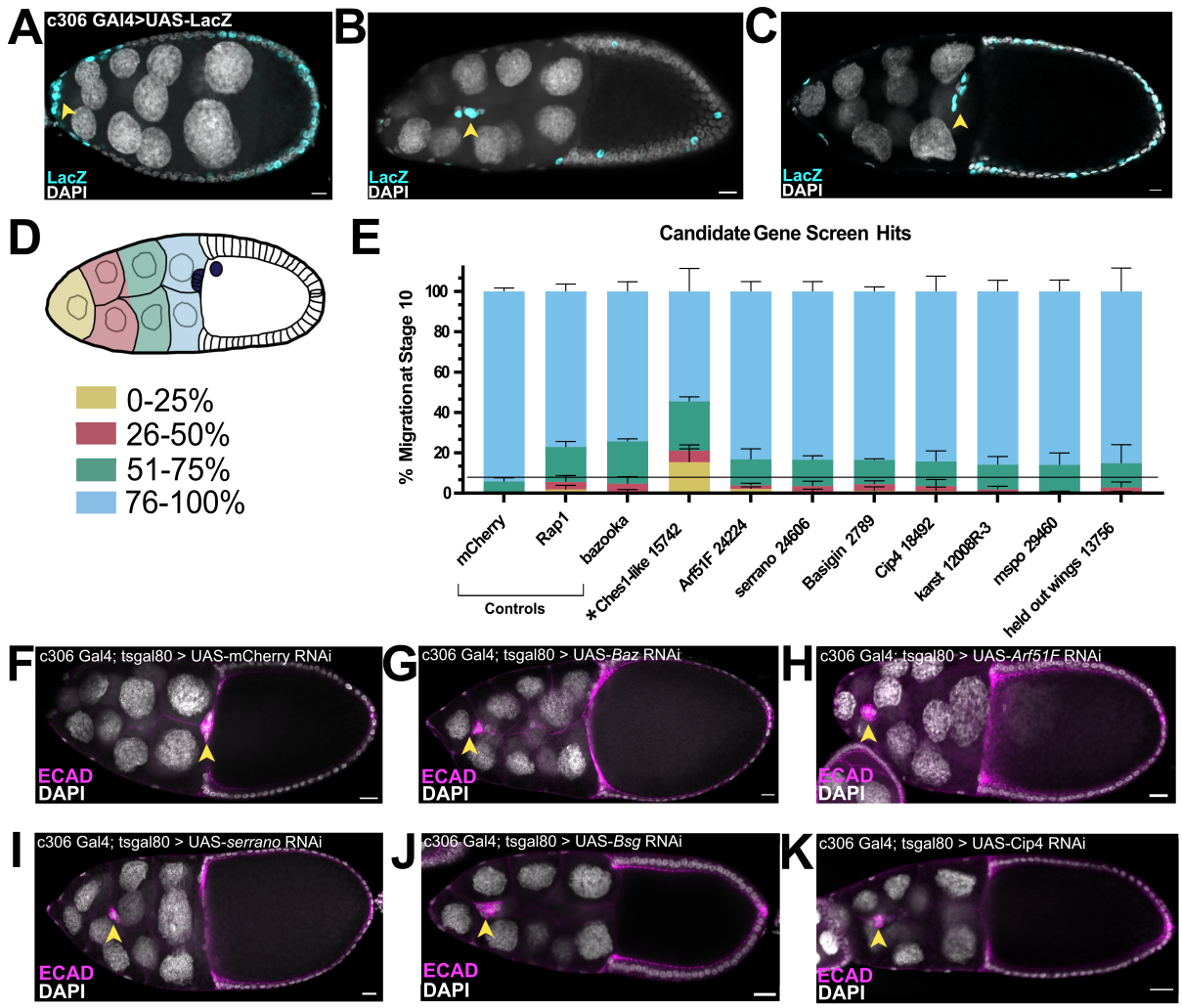


Figure 2.8 Functional assessment of temporally expressed genes in border cell migration. Validation of differentially expressed genes in border cell migration using RNAi. **(A-C)** Pattern of *c306*-GAL4, driving UAS-LacZ (cyan), in stages 9-10 pre-, mid-, and post-migratory border cells and a few additional follicle cells. DAPI (gray) labels all nuclei in the egg chambers. **(D-K)** Knockdown of candidate genes driven by *c306*-Gal4; genotypes are *c306*-Gal4; tsGAL80 > UAS-RNAi. **(D)** Migration of border cells was scored based on the percentage of border cells that completed their migration (last quartile of the migration distance; 76-100%, blue) by stage 10 of oogenesis, by which time migration should be complete, or if the border cells stalled along the migration pathway, shown as the quartile distance migrated away from the anterior tip of the

egg chamber (0-25%, yellow; 25-50%, pink; 51-75%, green) as shown in the schematic. **(E)** Quantification of migration defects for genes from the transcriptome analyses, along with the negative control (*mCherry* RNAi) and positive controls *bazooka* (*Baz*) and *Rap1* RNAi, two genes known to regulate border cell migration. Shown are results for knockdown of 8 genes that resulted in significant migration defects at stage 10 (line); *Ches-1-like* (asterisk) RNAi had a strong migration defect but may partly be due to off-target effects. **(F-K)** Images of border cell migration at stage 10 for control **(F, G)** and experimental RNAi **(H-K)** egg chambers. Border cells (arrowheads) are labeled with E-cadherin (magenta); DAPI (gray) labels the nuclei of all cells. **(F)** Complete migration in a stage 10 *mCherry* RNAi control egg chamber. **(G)** RNAi knockdown of a positive control, *Baz,* to show representative migration defects. **(H-K)** Migration defects in *Arf51F* **(H)**, *serrano* (*sano*; **I**), *Bsg* **(J)**, and *Cip4* **(K)** RNAi egg chambers. Anterior is to the left in all images. Arrowheads mark the position of border cells. All scale bars represent 10 μm. RNAi knockdown for each gene/line was performed in triplicate, with an average of 111 egg chambers per replicate. Exact *N* values, raw data and reagents used are available in Supplemental Table 2.11.

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Chapter 3 - Balanced levels of Activating transcription factor 4 promote border cell collective migration

Emily Burghardt and Jocelyn A. McDonald

Division of Biology, Kansas State University, Manhattan, KS 66506

3.1 Abstract

Collective cell migration, whether in development or disease, presents cells with new environments and new challenges. *Drosophila* border cells, which migrate collectively in the developing egg chamber, must remain connected to one other, undergo dynamic cell movements and adhesion remodeling, and move rapidly forward into a new environment. Despite border cell migration occurring during normal development of the ovary, we find that multiple genes in the integrated stress response (ISR) pathway are differentially expressed in border cells during their migration. *Activating transcription factor 4* (ATF4), a downstream effector of the ISR, is among the most highly expressed genes in border cells. Both ATF4 overexpression and RNAi knockdown result in migration defects, including a significant decrease in the number of border cells within the cluster and altered cluster shape. Finally, we demonstrate that ATF4 overexpression prevents border cell motility, restricting border cells with elevated ATF4 to the rear of the cluster during migration. Together, our data implicate a balance in the levels of ATF4 expression required to promote normal border cell collective migration *in vivo*.

3.2 Introduction

Collective cell migration, in which interconnected cells move as a group, instead of individually, drives the formation of structures during development and maintains balance in organisms. During organogenesis, cell collectives transform simple epithelial sheets into complex structures and deliver progenitor cells to their required locations (Affolter and Caussinus, 2008; Mayor and Theveneau, 2013). In adult organisms, collectives maintain homeostasis. Collective migrations of epithelial sheets contribute to wound healing (Friedl and Gilmour, 2009). In the immune system, migrating lymphocytes can organize into collective swarms and clusters as they move through varied tissue environments to respond to infection and threats (Rey-Barroso et al., 2018). In addition, the molecular machinery of collective migration is co-opted by invasive cancer cells to metastasize and spread these cells into new environments (Nieto, 2020). Balance within each collective additionally contributes to migration. Feedback from the surrounding environment, including external signals, spatial cues, and mechanosensory information, drive cell behavior changes to respond and adapt accordingly (Charras and Sahai, 2014; Ventura and Sedzinski, 2022). Up-regulation of regulatory programs, including metabolic process and stress response genes, may allow migratory cells in both disease and developmental contexts to cope with physiological stressors (DeWane et al., 2021). Collective migrations underlie transitions throughout the life of an organism and often represent critical drivers of a return to, or deviation from, homeostasis. It is therefore critical to identify genes and regulatory programs that control collective behavior and adaptation during migration. Due to their relative simplicity and accessibility to genetic and cellular manipulations, model organisms such as *Drosophila* are widely used to characterize the complex networks of gene expression, cell signaling, and environmental cues that drive collective behavior *in vivo.*

Border cells, which migrate during *Drosophila* oogenesis, are a genetically tractable model with which to investigate the role of cellular adaptation in an *in vivo* collective migration. In stage 9 of oogenesis, the border cell cluster delaminates from the underlying epithelium and migrates on and between the large nurse cells that fill the egg chamber (Figure 3.1A). Migration is completed when the outer, migratory border cells and the two nonmotile polar cells, carried in the cluster center, reach the anterior boundary of the developing oocyte (Figure 3.1A). Both cell types are required for formation of the micropyle, a sperm entry port in the mature egg, and therefore fertility of the adult fly (Horne-Badovinac, 2020).

As in other collectives, border cells face numerous challenges during migration, both in coordination of group behavior and interaction with the microenvironment. Establishment and maintenance of group behavior among the border cells must be continually directed by cell signaling throughout migration. Border cells are specified from the anterior follicle epithelium by the polar cell organizers during stage 8 of oogenesis. The polar cells secrete *Unpaired* (Upd), a JAK/STAT ligand. The four to six surrounding follicle cells with the highest levels of STAT activity, triggered by Upd, become border cells (Beccari et al., 2002; Ghiglione et al., 2002; Silver and Montell, 2001; Xi et al., 2003). Expression of the border cell migration regulator *slow border cells* (Slbo) is then activated downstream of JAK/STAT signaling in the newly organized border cell cluster (Montell et al., 1992; Silver and Montell, 2001). Both Slbo and ecdysone, a steroid hormone which precisely times the initiation of border cell migration, stimulate the activation of additional genes required for migration (Borghese et al., 2006; Manning et al., 2017; Wang et al., 2006). Throughout migration, proper expression of Slbo and other key transcription factors must be carefully regulated in border cells: neither an increase nor decrease of Slbo is tolerated by border cells for timely migration to occur (Rørth et al., 2000).

Downstream signaling, including targets of Slbo and ecdysone, additional pathways, and cell-cell interactions then direct and coordinate protrusion formation, adhesion remodeling, and other behavior at the collective level.

For the border cell collective to delaminate and begin its forward movement, leader cells extend actin-rich protrusions. Chemoattractant ligands PVR and EGFR, secreted by the oocyte, initiate this protrusion formation and establish direction of migration. Binding of PVR and EGFR to receptor tyrosine kinases activates the GTPase Rac in one to two border cells that will lead the cluster (Duchek et al., 2001; Ramel et al., 2013; Wang et al., 2010). Rac activity induces protrusion formation in the lead border cells (Murphy and Montell, 1996; Wang et al., 2010). In this way, PVR and EGFR polarize the cluster through migration direction and Rac-dependent protrusion formation at the cluster front. In other developmental collective migrations, external signals induce gene expression and migratory behaviors specific to a subset of leader cells. Each outer, migratory border cell in the cluster is capable of leading the collective (Bianco et al., 2007). Focal activation of Rac in other border cells induces protrusion formation and movement in a new direction (Wang et al., 2010). In addition, border cells can change position throughout migration (Bianco et al., 2007; Prasad and Montell, 2007). The mechanism of their exchange within the cluster is poorly understood.

Collective migration depends on, and is challenged by, interactions with the surrounding environment. The dynamic formation and remodeling of adhesions between migratory cells and their substrate is necessary for the generation of traction to move the collective forward (Macabenta and Stathopoulos, 2019). In the border cell cluster, maintenance and remodeling of E-cadherin-based cell adhesions is required not only among the cells of the cluster, but between the border cells and the nurse cells for proper migration (Niewiadomska et al., 1999). Expression

of E-cadherin, the major *Drosophila* epithelial cadherin, is required in both cell types for migration (Cai et al., 2014; Niewiadomska et al., 1999). Both protrusions and cell-cell contacts with the substrate transmit mechanosensory information throughout the collective to promote group behavior. E-cadherin-based cell adhesions between lead border cells and nurse cells create a positive feedback loop to promote Rac protrusion formation (Cai et al., 2014). In addition, E-cadherin throughout the border cell cluster facilitates cell-cell communication of direction (Cai et al., 2014).

The migratory environment poses additional physical challenges to the collective. Continual remodeling of the actin cytoskeleton is energy intensive (DeWane et al., 2021). As the border cell cluster moves through the egg chamber, these actin cytoskeleton dynamics allow cell shape changes and adaptations to the surrounding environment required for migration. Physical properties of the substrate, including stiffness, can affect migration of the collective. An optimal level of nurse cell stiffness promotes border cell migration (Aranjuez et al., 2016; Lamb et al., 2021, 2020). Border cells pull on nurse cell membranes as they migrate past (Dai et al., 2020). In the border cell cluster, myosin II (MyoII) regulates cortical tension and cluster shape through its activity on F-actin (Aranjuez et al., 2016). Increased stiffness of the nurse cells via increased MyoII impairs border cell migration, resulting in a compressed, elongated cluster (Aranjuez et al., 2016). During migration, the actin bundling protein Fascin (*singed* in *Drosophila*) regulates myosin activation in border cells and nurse cells (Lamb et al., 2021). As the cluster migrates, Fascin limits MyoII activity to balance stiffness of both collective and substrate (Lamb et al., 2021, 2020). The migratory path can be obstructed by surrounding cells, including the substrate, or extracellular matrix (ECM). Large collectives in these confined microenvironments can encounter spaces smaller than a single cell (McGregor et al., 2016). Recent work has shown that

border cells preferentially migrate along a central path in the egg chamber (Dai et al., 2020). Here, spaces where three or more nurse cells contact one another create more permissible spaces for migration (Dai et al., 2020). In comparison, other interfaces between two nurse cells along "side paths" are tighter and represent more challenging spaces for the border cell cluster to move through (Dai et al., 2020). In border cells and other collectives, characterization of the molecular wiring and gene expression that allow continual adaption to these and other challenges to promote migration is critical for our understanding of development, homeostasis, and disease.

In addition to physical stressors, all cells must be prepared to respond and adapt to physiological stress. Cellular adaptation to physiological challenges of nutrient stress, infection, or misfolded proteins is mediated by the integrated stress response (ISR) (Ryoo and Vasudevan, 2017). The ISR is a common downstream pathway activated in cells upon detection of a diverse array of stressors. Four stress sensor kinases are widely studied in mammalian cells: GCN2 (amino acid starvation), PKR (viral infection), PERK (ER stress), or HRI (heme insufficiency) (Neill and Masson, 2023; see also Chapter 1, Figure 1.7). *Drosophila* has orthologs for only PERK and GCN2 (Malzer et al., 2013). During cellular stress, each kinase phosphorylates the alpha subunit of eukaryotic initiation factor 2 (eIF2α), thereby downregulating translation (Neill and Masson, 2023). Although global translation is temporarily decreased to allow conservation of amino acids and respite for the cellular protein folding system, a simultaneous up-regulation of a specific subset of genes promotes cellular recovery (Ryoo and Vasudevan, 2017). Under these conditions, genes with a uORF (upstream open reading frame) are preferentially translated, including the bZIP transcription factor ATF4 (Neill and Masson, 2023). ATF4 further activates expression of genes important for amino acid metabolism, protein biosynthesis, and oxidative or ER stress response (Harding et al., 2003; Tameire et al., 2019). Another critical target of ATF4,

GADD34 (PPP1R15 in *Drosophila*), acts in a negative feedback loop to dephosphorylate eIF2α, allowing a return to normal translation levels and therefore, cell homeostasis (Ameri and Harris, 2008).

Recent work from our lab demonstrated a role for components of the ISR, including PERK and PPP1R15, in the adaptation of border cells to the challenge of migration. In wild type border cells, PPP1R15 controls collective movement (Chen and McDonald, 2024). Misexpression of PPP1R15 in border cells resulted in cluster delamination, cohesion, and protrusion formation defects (Chen and McDonald, 2024). Furthermore, constitutive activation of cell stress via overexpression of PERK, ATF4, or phosphorylated eIF2α (phosphomimetic mutant) resulted in strong border cell migration defects (Chen and McDonald, 2024). Removal of upstream (knockdown of PERK) or downstream (knockdown of ATF4) stress signals in PPP1R15-diminished border cells decreased migration defects (Chen and McDonald, 2024). Together, these data indicate that PPP1R15 in border cells continually works against an active intrinsic stress signaling pathway (Chen and McDonald, 2024). The roles of other ISR components in collective cell migration, especially ATF4, are less well characterized.

Drosophila border cells are an *in vivo* model with which to understand how collectively migrating cells respond and adapt to physical or physiological stressors. Because border cells require the stress response genes PERK and PPP1R15 for their migration, we used the border cell model to investigate the role of the major effector of the integrated stress response (ISR), ATF4, in collective cell migration. Using sequencing data in border cells during their migration, we identified expression of ATF4 and other stress response pathway members, including endoplasmic reticulum (ER) stress pathway components, in border cells. With both ATF4 knockdown and overexpression constructs, we demonstrated that proper levels of ATF4 are

required for border cell migration. Additionally, ATF4 misexpression in border cells resulted in a reduced border cell number and reduced cluster aspect ratio. To understand the strong border cell migration defect caused by ATF4 overexpression, we used mosaic clone analyses. In these mosaic border cell clusters, ATF4-overexpressing border cells are improperly motile and restricted to the cluster back throughout migration. These results identify a balance in ATF4 expression required to promote border cell collective migration.

3.3 Results

3.3.1 Integrated stress response components are expressed in border cells during migration

Stress response genes PERK and PPP1R15 are required in border cells to promote their collective migration (Chen and McDonald, 2024). To search for other genes in the integrated stress response (ISR) pathway that may be required for migration, we used a previous RNA sequencing dataset of wild-type border cells during three stages of migration (Burghardt et al., 2023; see also Chapter 2). We identified differential expression of key components of the ISR in border cells across these three stages of migration (pre-, mid-, and end-migration; Figure 3.1B; see also Chapter 1, Figure 1.7). The kinases GCN2 and PERK (PEK in *Drosophila*), sensors of nutrient and endoplasmic reticulum (ER) stress, respectively, increase in expression during border cell migration (Figure 3.1B). Accumulation of unfolded or misfolded proteins triggers PERK activation and stress responses, including the unfolded protein response (UPR). Interestingly, Ire1 and ATF6, two additional sensors of the UPR, are also expressed in border cells. One isoform of ATF6 was significantly differentially expressed in border cells during their migration, increasing in expression over time (Figure 3.1B, denoted by asterisk [*]). In order for the cell to resume normal translation, a protein phosphatase dephosphorylates eukaryotic

initiation factor 2 α (eIF2α). In *Drosophila,* this is carried out by dPPP1R15, also known as GADD34 (Malzer et al., 2013). Both PPP1R15 and eIF2α are differentially expressed in border cells across migration timepoints (Figure 3.1B, denoted by asterisk [*]). For a complete list of significantly differentially expressed genes in border cells during migration, see Chapter 2 and Supplemental Table 2.3.

In addition to characterization of differential gene expression, our data identified the most highly expressed transcripts in border cells during migration. The transcription factor ATF4, known as *cryptocephal* (crc) in *Drosophila*, was among the 200 most highly expressed transcripts in border cells and represents one of the most highly expressed transcription factors in border cells (Supplemental Table 2.12). ATF4 mediates cell recovery from stress by activating expression of genes important for amino acid and protein biosynthesis, protein folding chaperones, and metabolic enzymes (Harding et al., 2003; Wortel et al., 2017). All four isoforms of ATF4 were expressed in border cells. Expression increases for three of the four isoforms over the course of migration (Figure 3.1B). Two other ISR-associated genes, the transcription factor Xrp1 and chaperone Hsc70-3, were also found in the top 200 transcripts expressed in border cells (Figure 3.1B, Supplemental Table 2.12). Xrp1 mediates cellular response to stress downstream of PERK, but independently of ATF4 (Brown et al., 2021). Hsc70-3, also known as BIP, is a protein folding chaperone and repressor of the unfolded protein response (UPR) (Walter and Ron, 2011). During border cell migration, an isoform of each of these genes significantly changed expression (Figure 3.1B, denoted by asterisk [*]; see also Chapter 2).

Although ATF4 mRNA is widely expressed in border cells, ATF4 protein is only elevated under stress conditions (Ryoo, 2015). In wild type border cells, very little ATF4 protein

is present (Chen and McDonald, 2024). Therefore, to further visualize the activity of ATF4 in the egg chamber, we made use of a *Thor*-LacZ construct (Kang et al., 2017). *Thor*, also known as eukaryotic translation initiation factor 4E binding protein (4E-BP), is a known ATF4 target that regulates translation through binding of eukaryotic translation initiation factor 4E (eIF4E) (Grmai et al., 2023; Kang et al., 2017). Both *Thor* isoforms are expressed in border cells during their migration (Figure 3.1B). *Thor*-LacZ signal is expressed in the border cell cluster, as well as additional follicle cells and nurse cells (Figure 3.1C-C'). *Thor*, or 4E-BP, is a known target of other pathways, including the Torso (Tor) and insulin signaling pathways (Chatterjee and Perrimon, 2021). *Thor*-LacZ reporter activity has been shown to be induced by the insulin signaling pathway transcription factor FoxO in other *Drosophila* systems during development, such as the larval thoracic muscles and the wing imaginal disc (Demontis and Perrimon, 2010; Galagovsky et al., 2014). However, recent experiments have demonstrated that the *Thor*-LacZ reporter used here is specifically induced in response to stress, even in a FoxO mutant background (Kang et al., 2017). Further work, including the use of additional reporters and genetic backgrounds, will be important to validate the use of this reporter to visualize ATF4 activity in the border cells. In total, we identified expression of 10 genes associated with the ISR and other cell stress pathways, including the highly expressed transcription factor ATF4 and its target *Thor*, in border cells during their migration.

3.3.2 Proper levels of ATF4 expression are required for border cell migration

Cells experiencing stress employ transcriptional and translational responses to return to homeostasis. Activation of ATF4 during the stress response regulates cellular recovery through downstream targets (Ameri and Harris, 2008). However, overexpression of ATF4 results in border cell migration defects (Chen and McDonald, 2024). To further investigate the

requirement for ATF4 in border cells, we altered the expression levels of ATF4 and scored migration. Wild type or negative control (mCherry RNAi) border cells migrate from the anterior end of the egg chamber at stage 9 of oogenesis, moving on and between the large nurse cells that surround them to reach the oocyte boundary by stage 10 (Figure 3.2A-B). Either knockdown or overexpression of ATF4 was driven in border cells using a *c306* GAL4; tsGAL80 driver (for genotypes used in this study, see Table 3.1). Border cell migration was then scored in stage 10 egg chambers as a percentage of the total migration distance (Figure 3.2B). Knockdown of ATF4 using two of three RNAi lines resulted in a significant migration defect compared to control (Figure 3.2C-E). Migration defects were also analyzed using heterozygotes of a hypomorphic ATF4 allele (Figure 3.2F-G; Hewes et al., 2000). In this background, border cell migration was also significantly impaired compared to control border cells (Figure 3.2E). Border cell migration defect upon overexpression of ATF4 was confirmed using either of two overexpression constructs (Figure 3.2H-J). In both knockdown and overexpression experiments, many border cell clusters migrated less than 50% of the total distance to the oocyte boundary (Figure 3.2C, D, F, H). In addition, when there was a migration defect, many of these border cell clusters never fully delaminated from the follicular epithelium, with some clusters even migrating along the side of the egg chamber (Figure 3.2D, F, H). Taken together, our data indicate that proper levels of ATF4 expression are essential for proper border cell migration.

3.3.3 Proper levels of ATF4 expression ensure overall cluster shape and border cell number

Either overexpression or RNAi knockdown of ATF4 in border cells resulted in clusters that appeared rounder than controls, with little to no visible protrusions (Figure 3.2). To quantify differences in the overall cluster shape during migration, we measured border cell cluster aspect

ratio. Briefly, the anterior-to-posterior axis of each border cell cluster was defined. A line measurement from cluster rear to front was used to represent cluster length. A line drawn through the midpoint of the length line was then used to represent cluster width (Figure 3.3A-B). E-cadherin staining was used to define cluster boundaries. Protrusions, whether at the front or sides of the cluster, were only included in measurements if they fell within either line. Control clusters expressing either mCherry RNAi or UAS-LacZ are slightly longer than they are wide, with an average aspect ratio of 1.83 or 1.93, respectively (Figure 3.3A-A', F-F'). When ATF4 expression was decreased by RNAi, clusters were shorter in length, resulting in a significantly decreased average cluster aspect ratio of 1.22 or 1.30 with knockdown of either ATF4 RNAi #1 or #2, respectively (Figure 3.3C-E). When ATF4 was overexpressed using either of two lines, the average cluster aspect ratio was also significantly decreased to 1.23 or 1.07, respectively (Figure 3.3F-I). Cluster shape upon ATF4 overexpression was more circular than controls, with an aspect ratio for many samples approaching 1.0. Overall, ATF4 misexpression (either knockdown or overexpression) in border cells resulted in an altered cluster shape; specifically, a significant decrease in cluster aspect ratio.

Border cell clusters appeared rounder upon ATF4 misexpression; however, they also seemed smaller. Apparent changes in size in border cell clusters misexpressing ATF4 could be due to a more compact cluster, since cluster aspect ratio was significantly decreased in either RNAi knockdown or ATF4 overexpression. However, a smaller cluster could also be due to a lower border cell number. To analyze this, we counted the number of border cells in each cluster using DAPI labeling of cell nuclei. Wild type border cell clusters are comprised of 4 to 6 border cells surrounding the two nonmotile polar cells, which are carried at the center of the cluster (Niewiadomska et al., 1999). Here, control clusters expressing either mCherry RNAi or UAS-

LacZ were comprised of an average 6.83 or 6.43 cells per cluster, respectively (Figure 3.4A, D, E, H). Knockdown of ATF4 in border cells by either of two RNAi lines resulted in a significant decrease in border cell number, with an average of 5.42 or 4.75 cells per cluster (Figure 3.4B-D). Interestingly, both RNAi knockdown and overexpression of ATF4 resulted in similar phenotypes, as in cluster aspect ratio. ATF4 overexpression using either of two constructs resulted in a significant decrease in border cell number to average of 4.83 or 4.35 cells per cluster (Figure 3.4F-H). Taken together, our data indicate that proper levels of ATF4 expression are required for multiple aspects of border cell migration, including correct border cell number and overall cluster shape.

3.3.4 ATF4-overexpressing border cells are restricted to the back of the cluster during migration

As described above, misexpression of ATF4 in the entire border cell cluster results in migration defects, including stalled migration, defects in cell number, and a decreased cluster aspect ratio (Figures 3.2-4). In particular, ATF4 overexpression results in strong migration defects; however, the mechanism by which ATF4 regulates these aspects of migration is unknown. To test the motility of ATF4-overexpressing cells within the cluster, we used the 'FLP-out' GAL4 system (Ito et al., 1997) to overexpress ATF4 in random groups of follicle cells. Border cells overexpressing ATF4 in the mosaic cluster were marked with the nuclear marker UAS-Red Stinger (see Methods; Table 3.1). In clusters that had a mix of wild-type and ATF4-overexpressing cells ('clone cells'), the ATF4-overexpressing border cells were frequently found at the back of the cluster. In contrast, ATF4-overexpressing cells were almost never found at the front of the cluster. To quantify this, we scored the position of clone cells (at the cluster front, middle, or back) of each border cell cluster that had at least one clone cell overexpressing

either the control (UAS-LacZ or UAS-GFP) or ATF4 (Figure 3.5A). Because cluster rotation and oocyte "docking" occurs just prior to the end of migration, only border cell clusters at pre- and early or mid-migration were scored for position (see Section 3.5, Methods; Miao et al., 2020).

Clonal cells overexpressing a control construct, UAS-LacZ, were generally positioned throughout the cluster, though slightly biased towards the cluster front both at pre/early and mid-migration stages (Figure 3.5B-B', F-G). Overexpression of a second control construct, UAS-GFP, resulted in a more even distribution of cell positions throughout the cluster (Figure 3.5C-C", F-G). However, clone cells overexpressing ATF4 were significantly biased to the cluster back throughout migration (Figure 3.5D-E, F-G). Considering each stage of migration, in pre-migratory clusters ATF4-overexpressing clone cells can be located at the cluster front, although most were positioned at the cluster back (Figure 3.5F). On average, 24.1% of ATF4-overexpressing clone cells were positioned at the cluster front in pre-migratory clusters, compared to 32.1% or 43.7% of UAS-GFP or UAS-LacZ cells, respectively (Figure 3.5F). However, by mid-migration, the majority (72.5%) of ATF4-overexpressing border cells were significantly restricted to the cluster back (compared to averages of 28.5% of UAS-GFP and 20.3% of UAS-LacZ-expressing cells at the cluster back; Figure 3.5G). Interestingly, in nearly 18% of analyzed clusters, one to two ATF4-overexpressing border cells found at the cluster back "lagged" behind the rest of the cluster (Figure 3.5E). In contrast, only 1.19% of LacZ-overexpressing control cells "lagged" behind the cluster. Together, our data suggest that ATF4-overexpressing border cells are improperly motile. During migration, ATF4-overexpressing cells either do not move to the lead position or generally sort to the cluster back. Proper levels of ATF4 expression are required for border cell migration, including in proper border cell number

and overall cluster shape. In addition, our cell position data indicate that proper ATF4 expression may be required for overall cell motility or position exchange within the cluster.

3.4 Discussion

Border cell migration is a dynamic process in which cell signaling, environmental cues, and mechanosensory information trigger downstream signaling and gene expression to promote collective migration. Components of the integrated stress response (ISR) are required for both cellular stress response and border cell migration (Chen et al., 2020; Chen and McDonald, 2024). Here, we identified expression of additional ISR genes in border cells during their migration. We sought to further characterize the role of ATF4, one of the most highly expressed genes in border cells, in their collective migration. Interestingly, either RNAi knockdown or overexpression of ATF4 in border cells resulted in migration defects. Either knockdown or overexpression of ATF4 in border cells also resulted in significant differences in border cell number and overall cluster shape. Finally, ATF4-overexpressing border cells in clonally mosaic clusters were restricted to the cluster back, especially by mid-migration. Taken together, our data indicate a requirement for a balance in the levels of ATF4 expression in a non-canonical role to promote multiple aspects of border cell collective migration.

We identified expression of several ISR genes in border cells during their migration, including core pathway components (eIF2α, ATF4, PPP1R15), specific stress sensor kinases (GCN2, PERK), and unfolded protein response (UPR)-specific genes (Ire1, Hsc70-3, ATF6). Elevated expression and a requirement for these and other ISR components has been characterized in cancer metastasis, in which migratory cancer cells are thought to co-opt the stress response to survive the challenges of rapid growth and protein folding (Wortel et al.,

2017). However, potential roles outside of stress signaling have been described for a subset of these genes in other cells and tissues. PERK, ATF4, and PPP1R15 are required during *Drosophila* development and have roles in larval molting, pupariation, and eclosion (Malzer et al., 2013; Ryoo, 2015). In *Drosophila* adult intestinal stem cells, PERK is active and required for proper proliferation (Ryoo, 2015). Elevated levels of GCN2 expression (both transcript and protein) and phosphorylated eIF2α are found in unfertilized mouse eggs, and possibly contribute to translation control during oocyte maturation (Alves et al., 2009). Upon wounding of keratinocytes, GCN2 is rapidly activated and required for the subsequent collective migration to close the wound, including proper protrusion formation at the leading edge (Miles et al., 2021).

Interestingly, differentially expressed genes with roles in the unfolded protein response (UPR) were expressed in border cells during their migration (see Chapter 2). Endoplasmic reticulum (ER) stress activates three stress sensors, PERK, Ire1, and ATF6, often referred to as three "branches" of the UPR (Walter and Ron, 2011). Transcriptional changes downstream of the transcription factor ATF6 increase protein folding capacity of the ER, while Ire1 and PERK function to decrease the amount of protein awaiting proper folding (Walter and Ron, 2011). Hsc70-3 (also known as BiP) and Xbp1 were among the top transcripts expressed in border cells during their migration. Hsc70-3 associates with the luminal domains of the three sensors, repressing their activation until release is triggered by ER stress (Limia et al., 2019). Downstream of Ire1 activation, cleavage of Xbp1 regulates ER-associated degradation components and promotes development of additional ER machinery for protein folding (Walter and Ron, 2011). In border cells, overexpression of UPR sensor PERK results in defects in delamination, protrusion formation, and a decrease in E-cadherin expression between border cells (Chen and McDonald, 2024). Induction of the UPR (via knockdown of SERCA to alter ER

calcium homeostasis) also results in migration defects (Chen and McDonald, 2024; Guo et al., 2019). Rapidly proliferative cancer cells, which can be highly susceptible to misfolded protein stress, are thought to utilize the ISR and UPR to adapt to these intrinsic challenges (Boyle et al., 2020; Holcik, 2015). Indeed, activation of the UPR, including activation of PERK and high Hsc70-3 and Ire1 expression, is associated with tumor development, aggressiveness, and metastasis (Limia et al., 2019). How stereotypic collective migrations, including border cell migration, may utilize these pathways during development remains poorly understood.

Activating transcription factor 4 (ATF4, also known as *cryptocephal* in *Drosophila*) is one of the most highly expressed transcription factors in border cells during their migration (Burghardt et al., 2023). During mammalian development, ATF4 expression is critical for osteoblast differentiation and may play a role in bone metastasis (Ameri and Harris, 2008). Additionally, PERK, ATF4, and eIF2α are linked to lipogenic signaling and lipid homeostasis during development (Anderson et al., 2021; Bobrovnikova-Marjon et al., 2008). Constitutive ISR activity has been reported in adipocytes and hepatocytes, possibly to maintain homeostasis in these highly metabolic fatty tissues (Grmai et al., 2023; Kang et al., 2017). In the *Drosophila* ovary, the fat body is important for vitellogenesis, the uptake of yolk and lipid into the oocyte between stages 8 and 14 of oogenesis (Mirth et al., 2019). Loss of ATF4 in the fat body caused egg chamber death, arrest of oogenesis, and a decrease in yolk granule accumulation (Grmai et al., 2023; Vasudevan et al., 2022). ATF4 may also regulate oogenesis, yolk lipoprotein synthesis, and ovulation through the promotion of downstream targets (Grmai et al., 2023). Expression of ATF4 and its target genes are also associated with disease, including reduced patient survival, angiogenesis, and metastasis in cancer (Boyle et al., 2020; Holcik, 2015; Wortel et al., 2017).

In border cells, either overexpression or knockdown of ATF4 resulted in strong migration defects. As a transcription factor with multiple downstream targets, ATF4 may interact with other genes and proteins required for border cell migration. ATF4 has been found to promote adult molting behavior through binding of an isoform of the ecdysone receptor (EcR) (Gauthier et al., 2012). Ecdysone shapes differentiation of multiple tissues during *Drosophila* development and is specifically required during oogenesis for proper egg chamber production in stages 9 and 10 (Montell et al., 1992). During stage 9, border cell migration is precisely timed by a pulse of ecdysone. In addition, EcR is expressed and required in border cells to regulate protrusion formation, apicobasal polarity, and distribution of E-cadherin throughout the cluster (Bhattacharya and Starz-Gaiano, 2024). The mammalian ATF4 can form homo- or heterodimers with transcription factors of the C/EBP family (Ameri and Harris, 2008; Neill and Masson, 2023). Slbo, or *slow border cells*, is a *Drosophila* C/EBP transcription factor critical for border cell migration (Montell et al., 1992). *Drosophila* ATF4 was demonstrated to heterodimerze with Slbo in a FRET assay (Reinke et al., 2013). During migration, Slbo is negatively regulated by *tribbles* (Trb) and targeted for degradation via the proteasome pathway (Rørth et al., 2000). *Tribbles 3*, the human ortholog of *Drosophila* Trb, is induced during ER stress via the ATF4-CHOP pathway and inhibits transcriptional activity of ATF4 (Neill and Masson, 2023; Ohoka et al., 2005). Although untested in border cell migration, interactions between Slbo, Trb, and ATF4 may represent a potential circuit active during migration.

Either overexpression or RNAi knockdown of ATF4 in border cells resulted in a significant decrease in border cell number. A loss of border cells in the cluster could be due to a recruitment defect. Border cells are specified from the follicle epithelium during stage 8 of oogenesis, when JAK/STAT signaling is activated in response to polar cell secretion of the

JAK/STAT ligand *Unpaired* (Beccari et al., 2002; Ghiglione et al., 2002; Silver and Montell, 2001). However, expression of the STAT activity reporter (STAT92E-GFP) in ATF4-overexpressing border cells resembles control samples (data not shown). This suggests that ATF4-overexpressing border cells are properly specified. It is possible that ATF4 affects polar cell function and therefore recruitment of the border cells to the cluster, though this remains to be tested. A defect in expression of other border cell migration regulators, including the small GTPase Rap1, also results in decreased cell number in the cluster. Rap1 inhibition resulted in cell death of polar cells and other follicle cells during stages 7-8 of oogenesis, with fewer cells incorporated into the border cell cluster (Messer and McDonald, 2023). In mammalian and yeast cells, the C/EBP transcription factor CHOP, downstream of ATF4, activates pro-apoptotic pathways (Marciniak et al., 2004; Zinszner et al., 1998). However, no CHOP homolog has been identified in *Drosophila* (Ryoo et al., 2007). Although loss of ATF4 expression in the *Drosophila* larval fat body caused death and oogenesis arrest, this death resembles overall egg chamber degeneration rather than death of individual cells (Vasudevan et al., 2022). Egg chamber health and viability are assessed at multiple checkpoints throughout oogenesis (Lebo and McCall, 2021). A final checkpoint occurs prior to the energy-intensive yolk production, which begins at stage 8 of oogenesis. Here, entire egg chambers that do not meet health and environmental standards are eliminated prior to progression into stages 8-9, when border cell migration takes place (Lebo and McCall, 2021). In unpublished observations, overexpression of PERK, which activates the stress response at high levels, can cause border cell death and results in visible pyknotic nuclei. DAPI was used in our study to label cell nuclei for border cell number analyses, but no pyknotic nuclei were observed in either ATF4 RNAi knockdown or overexpression conditions. Therefore, it is likely that ATF4 misexpression does not trigger

apoptosis in border cells, but rather affects border cell recruitment to the cluster through another mechanism. Although border cells may be specified properly in these conditions, they must properly adhere to one another to delaminate and begin their migration as a group. Currently, we suggest that a cohesion defect, in which border cells do not properly adhere to one another at delamination and initiation of their migration, contributes to this phenotype. Overexpression of PP1R15, in which eIF2α cannot be dephosphorylated and stress remains active, induces cohesion defects in border cells (Chen and McDonald, 2024). In these instances, border cell clusters are split into up to 3 parts during migration. Further, the ecdysone receptor (EcR) and downstream ecdysone signaling are required for proper distribution of E-cadherin throughout the border cell cluster (Bai et al., 2000; Bhattacharya and Starz-Gaiano, 2024). If the interaction of ATF4 with the ecdysone receptor (EcR) is functionally relevant in border cells, the distribution of E-cadherin could be altered and result in an early cluster cohesion defect. Further work will be needed to analyze cluster organization and cohesion versus apoptosis in ATF4-misexpressing border cells.

ATF4 RNAi knockdown or overexpression resulted in a decrease in border cell number and might therefore be expected to additionally cause a decrease in cluster size. However, ATF4-misexpressing border cells clusters were also rounder than controls. During border cell migration, overall cluster shape is carefully balanced to adapt to their environment (Aranjuez et al., 2016). In response to physical pressure from the nurse cells during migration, dynamic Myosin II activation at the cluster periphery adjusts and maintains normal border cell cluster shape, which is slightly longer than it is wide (Aranjuez et al., 2016; Gabbert et al., 2023). Myosin II activation is regulated by the small GTPase RhoA. Constitutive activation of RhoA, and therefore increased Myosin II activation, results in rounded individual border cells with

altered F-actin distribution (Aranjuez et al., 2016). Also regulated by RhoA are septins, filament-forming GTPases expressed in border cells (Gabbert et al., 2023). Overexpression of septins in the border cells results in migration defects and clusters which are rounder than controls (Gabbert et al., 2023). These rounded border cell clusters also form less protrusions that controls (Gabbert et al., 2023). Cytoskeletal dynamics, including control of cluster shape through RhoA, Myosin II, or septins, may be altered upon ATF4 misexpression, resulting in a rounder cluster and decreased cluster aspect ratio.

Dynamic shape regulation of both the overall collective and individual cells within are important for migration efficiency. The ability of the individual cells to deform, change shape, and adapt to the available migratory space is critical for *in vivo* collective migration (Friedl and Wolf, 2010). In our analysis of mosaic border cell clusters, we found that ATF4-overexpressing border cells are restricted to the cluster back throughout migration. In the one to two lead cells of the border cell clusters, binding of the chemoattractant ligands PVR and EGFR activates the small GTPase Rac and induces protrusion formation (Ramel et al., 2013; Wang et al., 2010). During border cell migration, protrusion formation is then mostly restricted to the lead cell (Mishra et al., 2019). However, recent work has shown that follower cells change shape, extend dynamic protrusions, and "crawl" over other cells during migration (Campanale et al., 2022). Expression of Rac is required in all cells of the cluster to drive this behavior. In mosaic border cell clusters, cells expressing a dominant negative version of Rac were also restricted to the cluster back (Campanale et al., 2022). In addition, Rac dominant negative clonal cells exhibited little to no changes in shape during migration (Campanale et al., 2022). In the rounder border cell clusters observed upon septin overexpression, cell membranes of the individual border cells were more rigid and less deformable (Gabbert et al., 2023). One possibility is that ATF4-

overexpressing border cells are less capable of shape changes, including deformability, required to move through the tissue and therefore move to the cluster back. The other border cells capable of these dynamic changes then occupy the lead position of the cluster. This is supported by our observation that some ATF4-overexpressing cells can occupy the lead position in pre- or early migration stages, but move to the cluster back by mid-migration. Another possibility is that protrusion formation is affected in ATF4-overexpressing cells. RNAi knockdown of PPP1R15 and overexpression of PERK in border cells affect protrusion coordination and formation, respectively (Chen and McDonald, 2024). If protrusion formation is affected in the ATF4-overexpressing clonal cells in the mosaic cluster, the cells might move to the cluster back in favor of cells able to form the dynamic protrusions required in the lead cell position. Live imaging of both mosaic and ATF4-misexpressing border cell clusters will be required to determine the effects of ATF4 on cell shape changes, both in individual border cells and the entire collective.

Adaptation to challenges encountered along the migration path is necessary for all cell collectives in development, disease, and homeostasis. Our data in *Drosophila* border cells identified expression of ISR genes and a new role for the stress response transcription factor ATF4 in a developmental collective cell migration. Co-option of the ISR pathway, including ATF4 expression, in cancer provides an advantage to cells challenged by limited resources and collective invasion into new environments. A balance in expression of the ISR downstream effector ATF4 may allow border cells to adapt to challenges encountered during their migration. Further work will be needed to characterize the molecular mechanism and downstream targets of ATF4 in border cells.

3.5 Methods

Drosophila genetics

All fly stocks and crosses were maintained at 25˚ C. RNAi lines were obtained from the Bloomington Drosophila Stock Center (BDSC) and Vienna Drosophila Resource Center (VDRC). ATF4-GFP, *Thor*-LacZ, and ATF4 hypomorphic allele stocks were obtained from the Bloomington Drosophila Stock Center (BDSC). ATF4 overexpression constructs were obtained from the Zurich ORFeome Project (identifiers: FlyORF F000106 and F004853). For all crosses, virgin females from a *c306*-Gal4; tsGAL80/CyO stock were crossed to UAS-RNAi or UAS-OE (overexpression) males to drive construct expression in border cells. RNAi against *mCherry* (BDSC 35785) or a UAS-LacZ overexpression construct (D. Montell lab) were used as negative controls. Female progeny from these crosses were selected and fattened on wet yeast paste overnight at 29˚ C to allow maximal Gal4/UAS expression and inactivation of the tsGAL80 construct.

Gene expression analyses

A heatmap (Figure 3.1B) was built in heatmapper.ca (http://heatmapper.ca/; Babicki et al., 2016) using Z-score data calculated from raw transcript per million (TPM) values from border cell transcriptome data (Burghardt et al., 2023). An average linkage clustering method and Euclidean distance measurement method were applied.

Immunostaining and Microscopy

Whole ovaries were fixed in 4% methanol-free formaldehyde (Polysciences) and 0.1 M potassium phosphate buffer (pH 7.4) for 10 minutes. To analyze border cell clusters, including

migration defects, cell number, and cluster aspect ratio, egg chambers were stained for E-cadherin (E-cad; 1:10 dilution, rat monoclonal DCAD2; Developmental Studies Hybridoma Bank [DSHB]) and DAPI to label nuclei (2.5 μg/mL; Millipore Sigma, cat. no. D954). An isotype-specific anti-rat secondary antibody conjugated to AlexaFluor–488 (Thermo Fisher Scientific) was used at a concentration of 1:400. DAPI was used at a concentration of 1:400 to label cell nuclei. All slides were mounted using Fluorsave Reagent mounting media (Millipore Sigma, cat. no. 345789). Migration defects were analyzed using an upright Zeiss AxioImager Z1. *Thor*-LacZ (BDSC line # 9558) signal was visualized in border cells using anti-β-galactosidase primary antibody (40-1A, Developmental Studies Hybridoma Bank [DSHB], 1:10 concentration) and anti-mouse IgG1 488 secondary antibody (Fisher Scientific, cat. # A21121) (Figure 3.1C-C'). Phalloidin 647 dye (Sigma-Aldrich cat. # 65906, 1:400 concentration) was used to label the border cell cluster (Figure 3.1C). Images were obtained as Z-stacks using an upright Zeiss AxioImager Z1 with Apotome2 and 20x objective (Figures 3.1C-C'; 3.2, 3.4, 3.5) or Zeiss LSM 880 confocal microscope at the Kansas State University (KSU) College of Veterinary Medicine (CVM) Confocal Core using a 20x (Figure 3.4) or numerical aperture (NA) objective.

Border Cell Number and Aspect Ratio Analyses

Z-stacks were obtained of the entire border cell cluster in ATF4 RNAi or ATF4 overexpression border cell clusters with paired mCherry RNAi or LacZ overexpression negative controls using an upright Zeiss AxioImager Z1 with ApoTome2 and 20x objective. For each condition, only clusters that were anywhere along the migratory path (fully delaminated from the follicle epithelium, but had not yet stopped at the oocyte) and not immediately above or below a nurse cell were scored to obtain a full view of the cluster and all nuclei. DAPI staining of nuclei

was used to score border cell number. For ATF4 RNAi border cell number, $n \geq 8$ border cell clusters. For ATF4 overexpression border cell number, $n \geq 12$ border cell clusters. Individual cell number values are plotted in Figure 3.3.

For cluster aspect ratio measurements, Z stacks were obtained of both ATF4 RNAi or ATF4 overexpression border cell clusters with paired mCherry RNAi or LacZ overexpression negative controls using an upright Zeiss AxioImager Z1 with ApoTome2 and 20x objective. For each condition, only clusters that were anywhere along the migratory path (fully delaminated and not yet stopped at the oocyte, including both stage 9 and stage 10 egg chambers) were used to score cluster aspect ratio. E-cadherin was used to label the border cell cluster and define cluster boundaries, including protrusions. In FIJI, Z stack images were rotated into the same orientation (anterior to posterior of the egg chamber, left to right). Slices from the bottom to the top of the cluster were projected together with average intensity. The back of the cluster was defined using the anterior/posterior axis of the egg chamber. A straight line was drawn from the back of the cluster to the front of the cluster (end of the E-cadherin staining boundary) using the "straight line" tool in FIJI. A measurement of the length of this line was used for "cluster length." For cluster width, one straight line was drawn through the middle of the "cluster length" line across the line midpoint (marked with a single white dot by the FIJI program). A measurement of the length of this line was used for "cluster width." Cellular protrusions were included in cluster length or width measurements only when they fell into either defined line drawn over the cluster (front or rear protrusions for cluster length, side protrusions for cluster width). Aspect ratio was then calculated for each cluster by dividing length measurement by width measurement. Individual cluster aspect ratio values are plotted in Figure 3.4. For ATF4 RNAi cluster aspect

ratio, $n \geq 10$ border cell clusters. For ATF4 overexpression aspect ratio, $n \geq 17$ border cell clusters.

Cluster Position Scoring Analysis

Virgin females from a *hsflp;* Ay-Gal4, UAS-Red Stinger/CyO stock were crossed to males from either an ATF4 overexpression construct (ATF4 overexpression #1, UAS-ATF4 F000106) or a UAS-LacZ or UAS-GFP nuclear localization signal (BDSC #4776) control. Female progeny were collected at 1-2 days old and subjected to heat shock for 1 hour in a 39˚ C water bath at 3-4 days old. After heat shock, flies were allowed to recover for 3 days total in a new vial at 25˚ C before fattening with wet yeast paste overnight at 29˚ C. E-cadherin and DAPI staining were used to label the border cell cluster and all border cell nuclei of the cluster. To score position in the experimental condition or a paired control, Z stacks were obtained of each border cell cluster using an upright Zeiss AxioImager Z1 with ApoTome2 and 20X objective. UAS-Red Stinger signal in each image was then used to identify and score position of each clone cell at front, middle, or back of the cluster (schematic of cluster position shown in Figure 3.5A). For data analysis, border cell clusters at pre- or early migration (not yet delaminated or migrated less than 25% of the total migration distance to the oocyte) and clusters at mid-migration (clusters in mid-migration until cluster rotation begins, roughly 26-90% of the total migration distance) were grouped into “pre/early” and “mid” migration groups, respectively. “Lagging” border cells were defined as any cells that appeared disconnected from the main cluster, specifically from other cells in the cluster back position. These cells were included in the “cluster back” position for data analysis. For pre- or early migration cluster position scoring, $n \geq 4$ replicates with $n \geq 11$ border cell clusters assessed per condition (experimental or control). For

mid-migration cluster position scoring, $n \geq 4$ replicates with $n \geq 20$ border cell clusters assessed per each condition.

Statistical Methods, Graphs, and Figure Assembly

All images were processed in FIJI. Graphics were created and figures were assembled in Affinity Designer. Graphs were created in GraphPad (version 7.04). Three trials were used per RNAi line or overexpression construct compared to either mCherry RNAi or LacZ negative controls. Unpaired two-tailed *t* tests were used to assign significance of migration defect, border cell number, cluster aspect ratio, or percentage of cells at each cluster position for each ATF4 RNAi or overexpression line compared to either mCherry RNAi or UAS-LacZ negative control. Standard deviation error bars were applied to each graph in GraphPad.

3.6 Figures

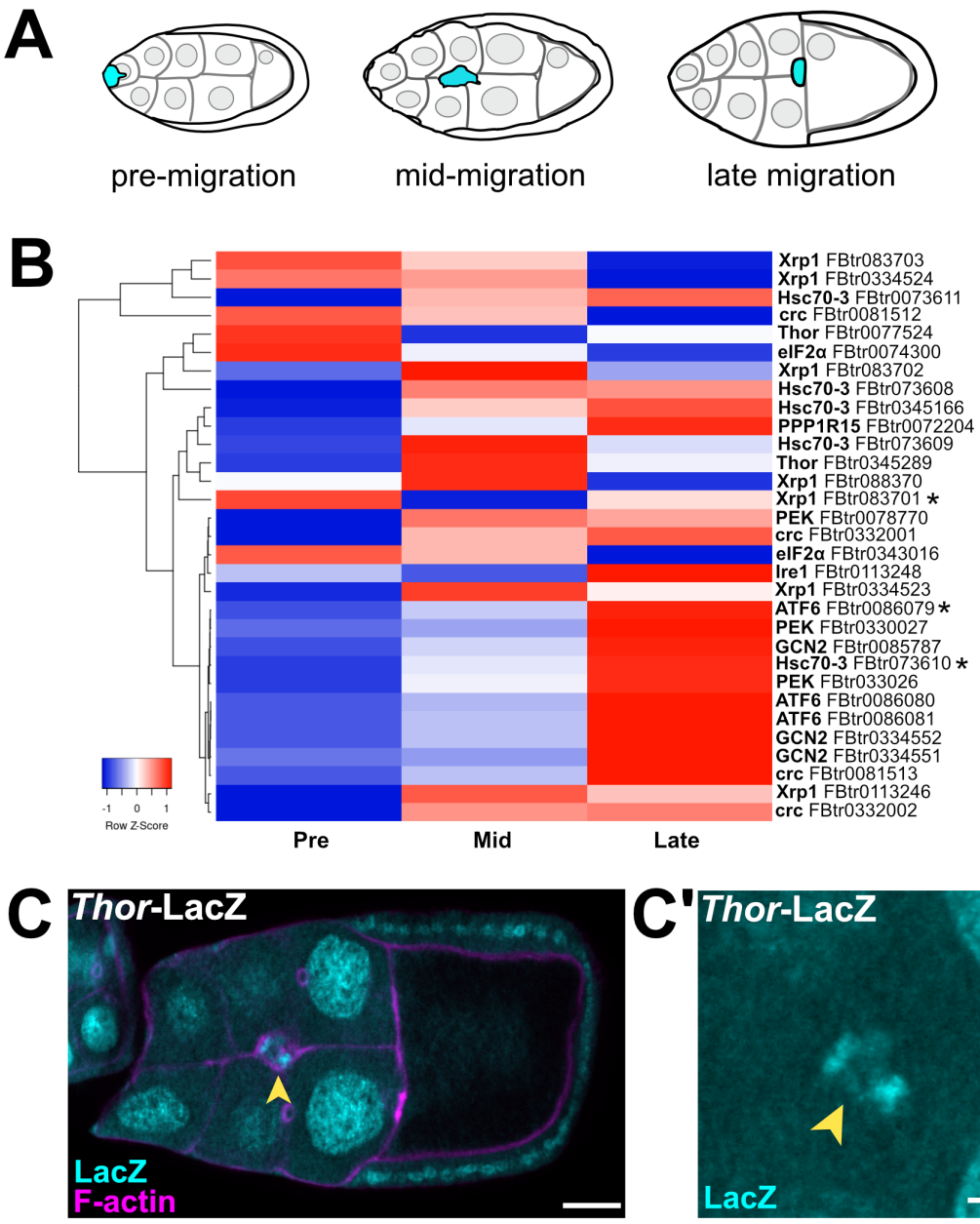


Figure 3.1 Integrated stress response genes are expressed in border cells during migration. **A)** Border cells migrate in the developing egg chamber. The border cells (cyan) round up into a cluster and begins forming protrusions at the pre-migration stage (left). At stage 9 of oogenesis, the border cell cluster delaminates from the underlying follicle epithelium and migrates through

the nurse cells that fill the egg chamber (gray) (mid-migration, middle). By stage 10 of oogenesis, the border cell cluster reaches the anterior oocyte boundary and stops migrating (late migration, right). **B)** Isolation and sequencing of border cells at pre-, mid-, and late migration identified differential gene expression across the three stages of migration Burghardt et al 2023. The heatmap shows differential expression and clustering of genes associated with the integrated stress response in border cells during their migration. Red denotes high expression; blue denotes low expression. Asterisks (*) denote significant differential expression determined by Clust in border cells during migration. Multiple isoforms for each gene are labeled using gene symbol and FlyBase transcript number (FBtr). **C-C')** *Thor*-LacZ is expressed throughout the egg chamber, including in the border cell cluster at mid-migration. F-actin (in magenta) highlights the border cell clusters and anti-β-galactosidase staining allows visualization of the LacZ signal. Scale bars represent 20 μm **(C)** and 5 μm **(C').**

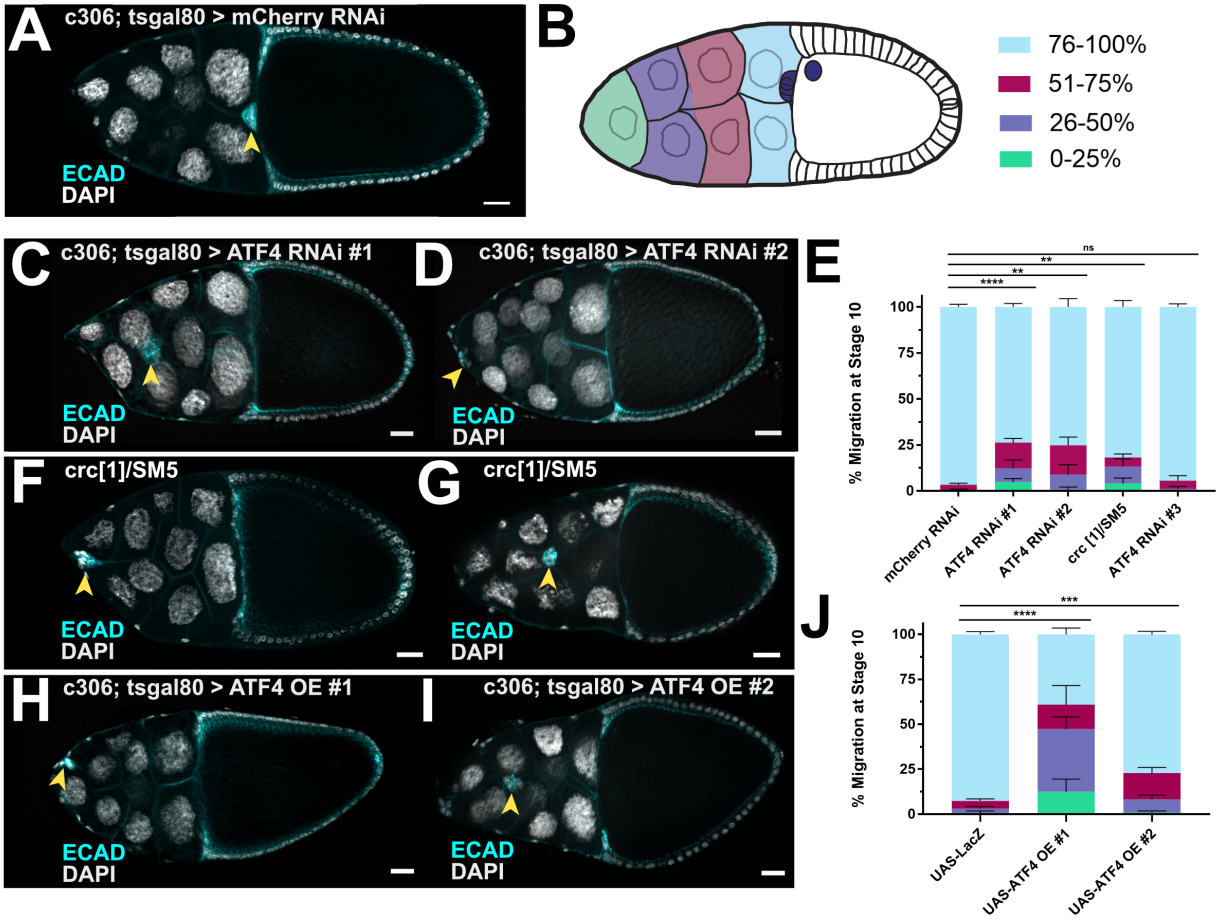


Figure 3.2 A balance in ATF4 expression is required for border cell migration.

A) RNAi Knockdown of an mCherry negative control in border cells using *c306* GAL4; tsGAL80 results in complete border cell migration by stage 10 of oogenesis. **B)** Schematic of an egg chamber at stage 10, by which time border cell migration should be complete. Migration is scored based on the percentage of border cells that completed their migration (last quartile of the migration distance; 76-100%, blue) by stage 10 of oogenesis, or if the border cells stalled along the migration pathway, shown by quartile distance migrated from the anterior tip of the egg chamber (0-25%, green; 26-50%, purple; 51-75%, magenta). **C-G)** Decreased expression of ATF4 in border cells, either knockdown of ATF4 in border cells (with two of three RNAi lines, driven by *c306* GAL4; tsGAL80) or in a hypomorphic (ATF4 allele, *crc* [1]/SM5) background) resulted in a significant migration defect compared to an UAS-mCherry RNAi negative control.

H-J) Overexpression of ATF4 in border cells (using two overexpression constructs, driven by *c306* GAL4; tsGAL80) results in a significant migration defect compared to a UAS-LacZ overexpression negative control. Each trial consists of three replicates, with $n > 30$ egg chambers per sample. $^{**}p < 0.01$; $^{***}p < 0.001$; $^{****}p < 0.0001$; *ns* represents a non-significant difference in migration defect. Unpaired two-tailed *t* test comparing complete migration between experimental and mCherry RNAi or UAS-LacZ overexpression negative control. DAPI labels cell nuclei and E-cadherin (ECAD) labels the border cell cluster. Arrowheads denote border cell cluster position. Scale bars represent 20 μm.

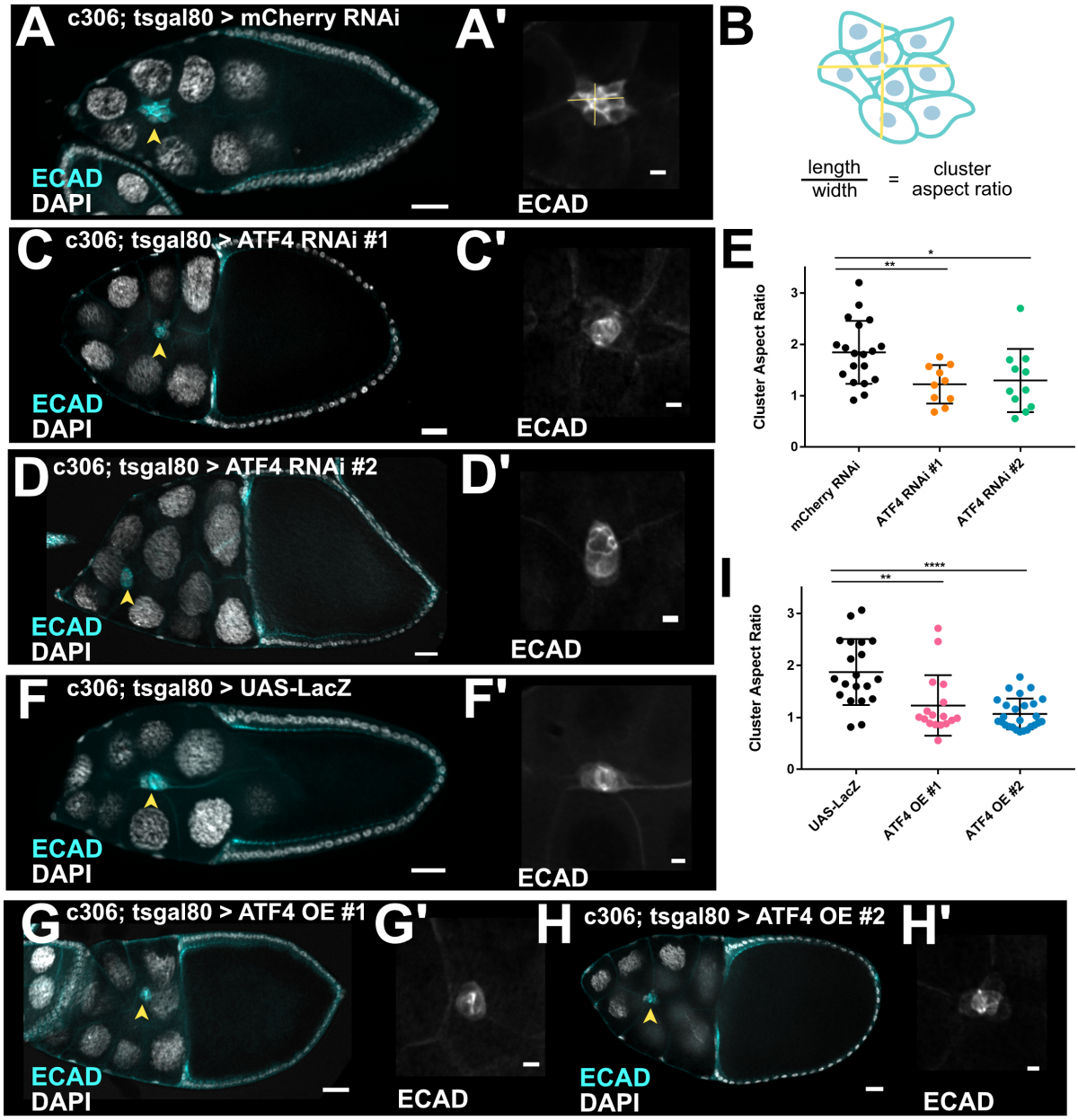


Figure 3.3 ATF4 knockdown or overexpression in border cells results in a decreased cluster aspect ratio.

A) Border cells at mid-migration expressing UAS-mCherry RNAi as a negative control form a slightly elongated cluster. **A')** Representative image of a UAS-mCherry RNAi-expressing border cluster. E-cadherin staining (shown in gray) defines the cluster boundaries. Yellow lines from the anterior-posterior axis and across the border cell cluster at the midpoint (denoted by a white dot)

represent length and width measurements taken in FIJI, respectively. **B)** Schematic of the border cell cluster shown in A-A' with sample length and width measurements and cluster aspect ratio formula. **C-E)** Expression of either ATF4 RNAi line #1 or line #2 results in a significantly decreased cluster aspect ratio. * $p < 0.05$; ** $p < 0.01$; **** $p < 0.0001$. Unpaired two tailed *t* test comparing cluster aspect ratio in ATF4 RNAi background to cluster aspect ratio in mCherry RNAi control background. **F-F')** Border cells at mid-migration expressing UAS-LacZ as a negative overexpression control form a slightly elongated cluster. **G-I)** Expression of either ATF4 overexpression line #1 or line #2 results in a significantly decreased cluster aspect ratio. * $p < 0.05$; ** $p < 0.01$; **** $p < 0.0001$. Unpaired two tailed *t* test comparing cluster aspect ratio in ATF4 overexpression background to cluster aspect ratio in LacZ overexpression background. Genotypes are *c306* GAL4; tsGAL80 > UAS-ATF4 RNAi or UAS-ATF4 overexpression line. Scale bars represent 20 μm (A, C, D, F, G) or 5 μm (A', C', D', F', G'). Border cells clusters are denoted by yellow arrowheads (A, C, D, F, G).

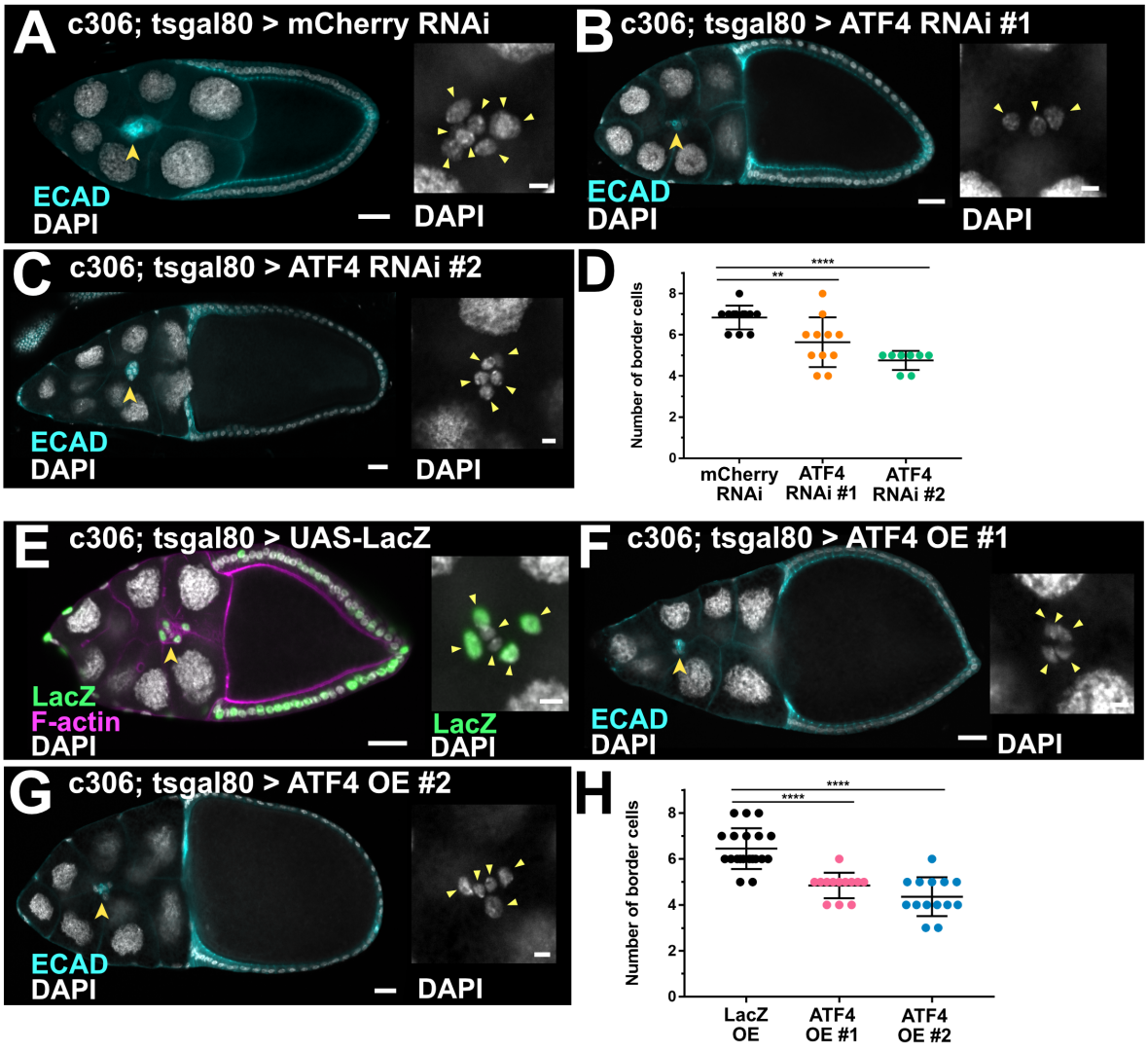


Figure 3.4 Proper levels of ATF4 expression are required for border cell number.

A) A border cell cluster expressing mCherry RNAi as a negative control is comprised of 8 total cells, denoted by arrowheads. **B-C)** Border cell migration is stalled and border cell number is decreased to 3 or 5 total cells when border cells express either ATF4 RNAi line #1 (B, VDRC line v2934) or line #2 (C, VDRC line v2935), respectively. **D)** RNAi knockdown of ATF4 in border cells results in a decrease in cell number per cluster (on average, 5.42 cells for ATF4 RNAi #1 or 4.75 cells for ATF4 RNAi #2). $^{**}p < 0.01$; $^{***}p < 0.001$; $^{****}p < 0.0001$. Unpaired two-tailed *t* test comparing cell number in ATF4-RNAi background to cell number in mCherry negative control background. **E)** A border cell cluster expressing UAS-LacZ as a negative

control is comprised of 6 total cells. *c306* GAL4; tsGAL80 > UAS- LacZ signal (in green) labels outer migratory border cells of th migration. **F-G)** Migration is stalled and border cell number is decreased to 5 total cells when ATF4 is overexpressed in border cells using either of two ATF4 overexpression constructs (F, ATF4 overexpression #1, ATF4 F000106; G, ATF4 overexpression #2, ATF4 F0004853). **H)** Overexpression of ATF4 in border cells using either of two lines results in a decrease in cell number per cluster (on average, 4.83 cells for ATF4 overexpression #1 or 4.83 cells for ATF4 overexpression #2). $^{**}p < 0.01$; $^{***}p < 0.001$; $^{****}p < 0.0001$. Unpaired two-tailed *t* test comparing cell number in ATF4 overexpression background to cell number in LacZ negative control background. Genotypes are *c306* GAL4; tsGAL80 > UAS-ATF4 RNAi or UAS-ATF4 overexpression line. Scale bars represent 20 μm (A, B, C, E, F, G) or 5 μm (A', B', C', E', F', G'). Individual border cells are denoted by yellow arrowheads.

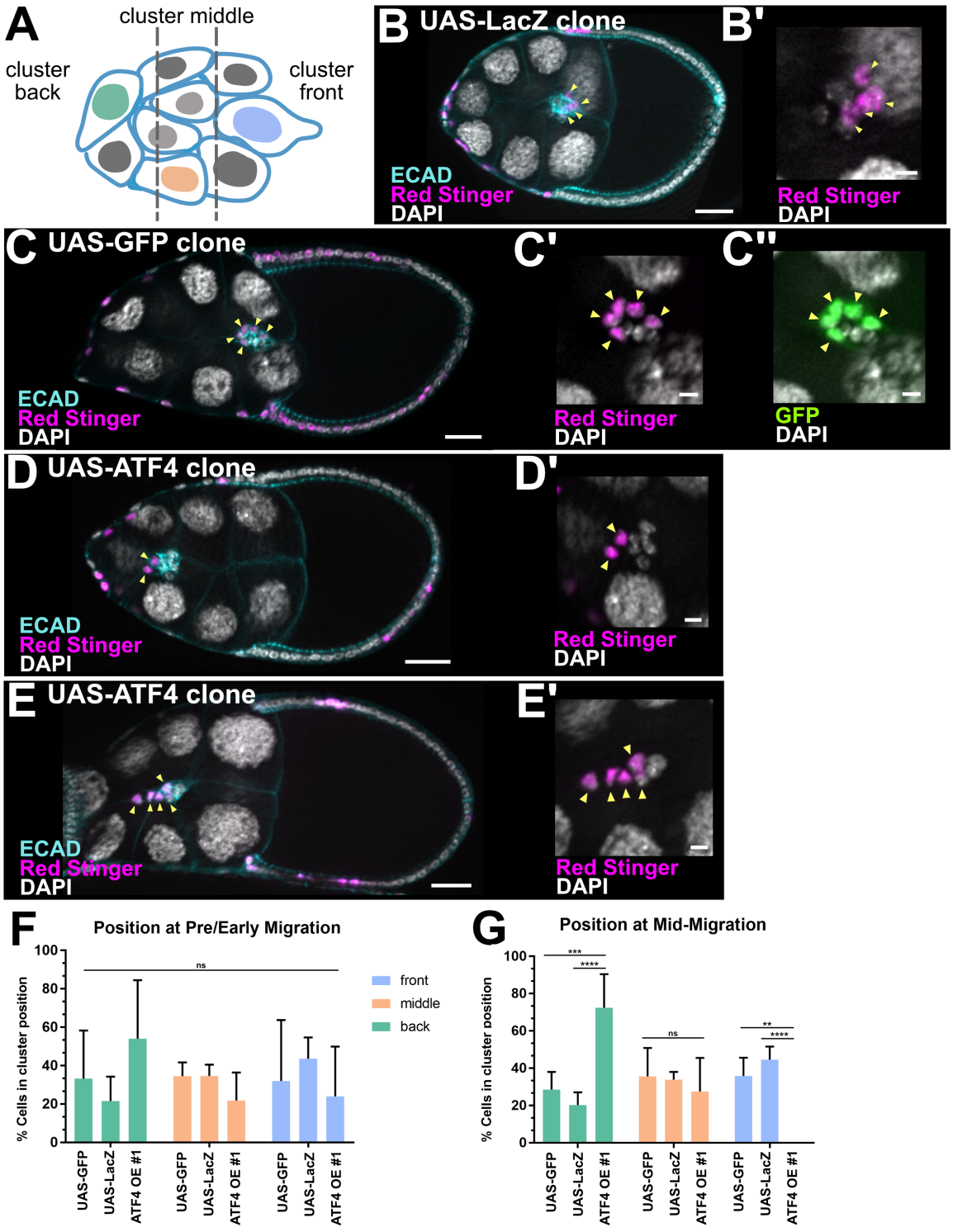


Figure 3.5 ATF4-overexpressing border cells are restricted to the cluster back during migration.

A) Schematic illustrates position of three example clone border cells at the front (light blue), middle (peach), or back (green) position within a cluster at mid-migration. Nuclei are marked to represent UAS-Red Stinger nuclear localization signal, used for scoring cell position. **B-B')** Representative image of clone cells overexpressing a UAS-LacZ negative control construct positioned at the cluster front. UAS-Red Stinger signal (shown in magenta) marks the clone cells within the cluster. **C)** Representative image of clone cells overexpressing a UAS-GFP negative control construct positioned throughout the cluster. **C-C")** UAS-Red Stinger (shown in magenta) or UAS-GFP (shown in green) signal marks the clone cells within the cluster. **D-D')** Representative images of UAS-ATF4 overexpressing clone cells restricted to the back of the border cell cluster during migration. **E-E')** Representative images of UAS-ATF4 overexpressing clone cells "lagging" behind the main cluster during migration. **F)** Percentage of clone cells at each position (front, middle, back) within the cluster at pre- or early migration. Data did not reach statistical significance with an unpaired, two tailed *t* test comparing percentage of ATF4-overexpressing border cells at each position to percentage at same position in either LacZ or ATF4 control. **G)** Percentage of clone cells at each position (front, middle, back) within the cluster at mid-migration. ATF4-overexpressing border cell position at the cluster front and back reached statistical significance compared to both UAS-LacZ and UAS-GFP controls. $^{**}p < 0.01$; $^{***}p < 0.001$; $^{****}p < 0.0001$. Unpaired two tailed *t* test comparing percentage of ATF4-overexpressing border cells at each position to percentage at same position in either LacZ or ATF4 control. E-cadherin (shown in cyan) labels the border cell cluster and DAPI (shown in gray) labels cell nuclei. Scale bars represent 20 μm (B, C, D, E) or 5 μm (B', C'-C", D', E'.) Individual clone border cells are denoted by yellow arrowheads.

Table 3.1 Genotypes used in this study.

Figure	Genotype
3.2A, 3.3A, 3.4A	c306 GAL4; tsGAL80/UAS-mCherry RNAi (BDSC #35785)
3.2C, 3.3C, 3.4B	c306 GAL4; tsgal80/+; UAS-ATF4 RNAi (VDRC #v2934)
3.2D, 3.3D, 3.4C	c306 GAL4; tsgal80/+; UAS-ATF4 RNAi (VDRC #v2935)
3.2J, 3.3E, 3.4E	c306 GAL4; tsgal80/+; UAS-LacZ (nls)/+ (D. Montell lab)
3.2H, 3.3F, 3.4F	c306 GAL4; tsgal80/+; UAS-ATF4/+ (FlyORF F000106)
3.2I, 3.3G, 3.4F	c306 GAL4; tsgal80/+; UAS-ATF4/+ (FlyORF F004853)
3.5B	hsflp; AyGal4-25b, UAS-Red Stinger-nls-6/+; UAS-LacZ (nls) (D. Montell lab)
3.5C	hsflp; AyGal4-25b, UAS-Red Stinger-nls-6/+; UAS-GFP.nls (BDSC #4776)
3.5D-E	hsflp; AyGal4-25b, UAS-Red Stinger-nls-6/+; UAS-ATF4 (FlyORF F000106)

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Chapter 4 - Discussion

Drosophila border cells undergo a dynamic *in vivo* collective migration and are a useful model with which to further our understanding of development and disease. This dissertation includes my findings on differential gene expression analysis in border cells, which allowed us to identify a wealth of new candidate genes dynamically expressed during their migration. Additional bioinformatic analyses to characterize function (gene ontology) and interactions (protein-protein interaction networks) of differentially expressed genes revealed transcriptional networks temporally regulated during migration. Among these, we identified a surprising temporal regulation of genes with roles in immune signaling and ribosome biogenesis. Expression and function analyses confirmed that a subset of differentially expressed genes are required in border cells for proper migration. In follow up work, using this sequencing data, we identified differential expression of integrated stress response (ISR) gene transcripts during border cell migration, including the highly expressed stress response effector *Activating transcription factor 4* (ATF4). We found that either knockdown or overexpression of ATF4 in border cells caused defects in migration, including both a reduced border cell number in the cluster and altered cluster aspect ratio. Together, our data demonstrate a balance in cellular requirements, both in expression of ATF4 and up- or down-regulation of migration-transcriptional programs, in border cells to promote collective migration.

4.1 Expression, interaction, and cooperative function: next steps for candidate nodes differentially expressed during border cell migration

The changes in cellular behavior, cell-cell communication, and collective movement required for border cell migration rely on a network of external signals, signaling pathways, and downstream responses. Bulk RNA sequencing of border cells allowed us to obtain a global view

of gene expression changes from cluster delamination to docking at the oocyte. Combining expression, protein interaction, and functional data, we identified small subsets of candidate genes that are expressed in the border cell cluster at the same time and in the same expression pattern throughout migration. Although the time, place, and demonstrated ability to physically interact are present, we have yet to determine which of these candidate gene groups represent functional nodes required for border cell migration. In this first section of the discussion chapter, I will highlight one such example of a potential functional node in our data.

In co-expression cluster C0, we identified a small node of genes with roles in ecdysone response and signaling. Throughout oogenesis, ecdysone controls developmental transitions. Although the steroid hormone ecdysone is present in high levels during stage 9 of oogenesis, the response to ecdysone is specifically high within the anterior follicle cells, including border cells (Jang et al., 2009; Montell et al., 2012). Ecdysone times the migration of the border cells, and ecdysone signaling further regulates the distribution of E-cadherin, polarity proteins, and activated myosin within the cluster (Bhattacharya and Starz-Gaiano, 2024). In border cells, the genes *ecdysone-induced protein response 78C* (Eip78C), *hormone receptor 4* (Hr4), and *broad* (Br) significantly increase in expression throughout migration and encode proteins that physically interact with one another. Hr4 is a nuclear receptor and downstream target of the ecdysone receptor (EcR) in border cells (Manning et al., 2017). Both Hr4 RNA and protein were identified in border cells at the time of specification, likely in response to the initiation of ecdysone signaling (Manning et al., 2017). In addition, knockdown of Hr4 in border cells resulted in mild migration defects, indicating a role in border cells (Manning et al., 2017). *Broad* (Br) is a well characterized downstream target of ecdysone signaling activated by *Notch* at stage 5 of oogenesis (Jia et al., 2014; Mugat et al., 2000; Rust et al., 2020) Both ecdysone and

JAK/STAT signaling act on Br to enact shape changes in the squamous follicle cells, which stretch to cover the growing anterior half of the egg chamber, from stages 8-10 (Jia et al., 2022). Knockdown of Br in border cells in this study resulted in a mild, but significant, migration defect.

Proper expression levels of the integrated stress response (ISR) effector ATF4 is required in border cells for their migration. ATF4 may regulate ecdysone in a non-canonical role, representing an additional potential link between the stress response and oogenesis. The ecdysone receptor (EcR) is directly bound by ATF4 to promote ecdysone-dependent expression of ecdysis-triggering hormone (ETF), essential for molting behavior (Gauthier et al., 2012). In the ovary, single cell RNA sequencing identified ATF4 as an ecdysone signaling transcription factor alongside EcR and Hr4 in main body follicle cells, which cover the developing egg chamber during oogenesis (Rust et al., 2020). Levels of ecdysone during developmental transitions, including metamorphosis from the larval stage to the adult, are regulated by a suite of genes, including *headcase* (Hdc). Loss of Hdc expression in *Drosophila* adult progenitor cells induces the stress response (Giannios and Casanova, 2021). Genes enriched for ecdysone response functions were found to significantly increase during border cell migration (Burghardt et al., 2023). Interestingly, as expression of these ecdysone response genes increase, expression of Hdc significantly decreases in border cells (Burghardt et al., 2023). The rise in expression of ecdysone-related genes could be important for the promotion of border cell migration, while a decrease in Hdc expression could be correlated with the expression of, and requirement for, stress response components we observe in border cells.

The relationship between expression, physical interaction, and known regulators of migration can provide a new use for our data: identification of candidate functional nodes for

testing in border cells. Knockdown of many of the genes tested in this study resulted in mild, yet significant, migration defects. In protein-protein interaction networks for each co-expression cluster, these genes form small nodes of interaction with other genes that could potentially function together in border cell migration. Co-expression cluster C0 contains a node of potential cytoskeleton regulators, including Cdc42-interacting protein 4 (Cip4) and Ephexin (Exn). RNAi knockdown of each gene resulted in mild, but significant, migration defects in border cells. Nodes in co-expression cluster C8 include Roughest (Rst), a gene required to maintain adhesion in the border cell cluster, and both Arf51F and WASp, potential cytoskeletal regulators in border cell migration identified in this study (Burghardt et al., 2023; Kotian et al., 2022). Here, these mild defects in multiple related genes could point to evidence of cooperative, functional nodes up-regulated together to promote border cell migration.

4.2 Ribosome biogenesis as a potential regulator of border cell migration

Our transcriptome analysis also uncovered a surprising temporal expression of many genes involved in ribosome biogenesis during border cell migration. A majority of these genes perform critical roles within the nucleolus, in both ribosomal RNA processing and ribosome subunit assembly. Ribosome biogenesis gene expression is high in border cells at the pre-migration stage, followed by a sharp and significant decrease in expression over the course of migration. Why might high levels of ribosome biogenesis be required in early border cell migration? Changes in ribosome biogenesis have been implicated in invasive cells to increase translation and provide the high levels of cytoskeletal, adhesion, and signaling proteins required for invasion (Sherwood et al., 2023). In tumor metastasis, ribosome biogenesis is commonly associated with the epithelial-to-mesenchymal transition (EMT) required for invasion (Prakash et al., 2019). One possibility is that border cells, like invasive cancer cells, utilize high levels of

ribosome biogenesis to support their invasion into the egg chamber. During early uterine and vulval development in *C. elegans*, a large single cell known as the anchor cell undergoes invasion into the basement membrane during early uterine and vulval development (Kenny-Ganzert and Sherwood, 2024). Before invasion, a "burst" of ribosome biogenesis results in an increase in ribosome levels in the anchor cell (Costa et al., 2023). This ensures ribosome quantity for the rapid translation of pro-invasive proteins. Despite this increase occurring nearly 4 hours prior to invasion, mRNAs encoding ribosomal proteins continue to be enriched in the anchor cell and are required for its invasion (Costa et al., 2023). In border cells, we identified 6 ribosome biogenesis genes for which knockdown resulted in a significant migration defect (Nop56, *peter pan, Tsr1 ribosome assembly factor, no child left behind, stubarista,* and Nopp140; see Appendix B). Each gene has a previously demonstrated role in ribosomal subunit assembly, ribosomal RNA (rRNA) processing and maturation, or ribonucleoprotein complex formation. Upon knockdown of a subset of these genes (Nop56, *Tsr1 ribosome assembly factor, stubarista*) we frequently observed border cell clusters that did not fully delaminate or begin their migration (Appendix B). High levels of ribosome biogenesis genes in early border cells, combined with the strong migration defects observed upon knockdown of a subset of these genes, could point to a role for ribosome biogenesis in the promotion of invasion. During migration, expression of these genes sharply and significantly decreases. Down-regulation of the ribosome biogenesis transcriptional program could represent a specific requirement during invasion of the border cell cluster that decreases by late migration, when border cells dock to the oocyte and return to their former epithelial state (Miao et al., 2020).

In collectively migrating cells, including border cells, dynamic cell behaviors are critical to interact with and respond to the surrounding environment. Migratory cells alter their speed,

focal adhesions with the substrate, and formation of protrusive structures in response to physical feedback from the surrounding tissue (Charras and Sahai, 2014). In some cell types, mRNAs are specifically localized within the cell and translated to form an increased and "on demand" local concentration of protein. To achieve this, mRNAs encoding ribosomal proteins, RNA binding proteins, translation initiation factors, and elongation factors have been demonstrated to localize to the protrusive front (Buoso et al., 2022; Dermit et al., 2020). In addition, ribosome tagging experiments have revealed sites of active translation in F-actin rich protrusions (Sherwood et al., 2023). Border cells extend actin-rich protrusions throughout their migration to sense their environment and to form focal adhesions with the nurse cells substrate (Montell et al., 2012). Continual remodeling of these adhesions between border and nurse cells is required for migration. During *in vitro* migration, mRNAs and ribosomal RNAs rapidly and specifically localize to sites of focal adhesion (Liao et al., 2015). Delocalization of some of these mRNAs has been demonstrated to impair migration (Liao et al., 2015). The high levels of ribosome biogenesis gene expression observed in early border cell migration may supply the translation machinery that will drive localized protein production specifically required for interactions with their environment. Further work will be needed to analyze ribosome abundance, localization, and active translation in the border cell cluster during migration.

4.3 ATF4 in border cells: perspectives on the stress response during migration

The downstream stress response effector *Activating transcription factor 4* (ATF4) is both highly expressed and required in border cells during their migration. ATF4 is activated in response to stress, including endoplasmic reticulum (ER) stress, in the cell, to activate transcription of cytoprotective and adaptive genes that promote cell recovery (Neill and Masson, 2023). To inhibit the stress response and restore homeostasis, *Protein phosphatase 1 regulatory*

subunit 15 (PPP1R15) dephosphorylates eIF2a to return the cell to normal levels of protein translation. The expression and requirement for a balanced level of PPP1R15, PERK, and ATF4 in border cell migration indicates a fine-tuned level of stress that promotes collective migration (Chen and McDonald, 2024). Data from our transcriptome analysis in border cells provides further insight into a potential ATF4 transcriptional network regulated during migration.

Our protein interaction network analyses identified small nodes of genes specific to the stress response, including the unfolded protein response (UPR), in border cells. Activation of Hsc70-3 (also known as BiP) and ATF6 in response to unfolded or misfolded proteins trigger transcriptional responses to promote cell recovery (Limia et al., 2019; Shen et al., 2002). Both genes form a physical interaction node with *starvin* (Stv). Expression of all three genes significantly increases during border cell migration (co-expression cluster C0). Stv is a specific chaperone of *heat shock protein 70* (Hsp70) genes, which can be mobilized during oxidative stress to promote protein folding (Donovan and Marr, 2016; Telonis-Scott et al., 2021). In co-expression cluster C8, Hsp70 chaperones *heat shock chaperone 70 Ab* (Hsc70Ab) and *Cb* (Hsc70Cb) form a second interaction node. Although not significantly differentially expressed, the UPR sensor Ire1 and its downstream effector Xpb1 were among the most highly expressed genes in border cells during migration. In mammalian cells, Xbp1 activation promotes ER biogenesis as an additional way to cope with the high protein folding load (Bommiasamy et al., 2009). If the unfolded protein response is active and required in border cells during their migration, the simultaneous up-regulation of protein folding chaperones and ER machinery may be used to balance any deleterious effects to the cell.

As a transcription factor, ATF4 upregulates expression of gene targets to promote recovery from stress (Neill and Masson, 2023). Characterized downstream targets of ATF4

include genes with roles in mitochondrial function, protein folding, amino acid synthesis and transport, and redox chemistry (Ameri and Harris, 2008; Harding et al., 2003; Poothong et al., 2021; Tameire et al., 2019). In border cells, amino acid and mitochondrial membrane transport, chaperone-mediated protein folding, and amino acid biosynthesis were among the top enriched functions for co-expression clusters C0, C1, and C5 (see Chapter 2). Another major target of ATF4 is glutathione biosynthesis (Harding et al., 2003). High levels of glutathione are required in the ER lumen to maintain homeostasis during the oxidative protein folding process (Chakravarthi et al., 2006). A total of four glutathione synthetases and transferases were significantly differentially expressed in border cells, albeit in varying co-expression patterns (both up- and down-regulated during migration). In addition, 3 members of the *Glutathione S tranferase D* (GstD) family were among the top 200 genes expressed in border cells. In the *Drosophila* eye imaginal disc, the GstD family was strongly induced upon activation of the UPR (Brown et al., 2021). As ATF4 expression is required in border cell migration, it is likely that some ATF4 targets also have a functional role in their migration. ATF4 targets may directly include or intersect with members of the ecdysone signaling pathway. Additionally, interactions between ATF4 with the C/EBP transcription factor *slow border cells* (Slbo) or its regulatory kinase, *tribbles* (Trb), may represent a regulatory loop in border cells during migration. RNA sequencing of border cells upon ATF4 knockdown will be critical to differentiate between general biosynthetic processes and specific ATF4 targets used to balance ER stress and promote border cell migration.

In border cells, alterations in ATF4 expression resulted in migration defects, including significant decreases in the number of cells in the border cell cluster and overall cluster shape. Why might this precise balance of ATF4 expression be required in border cells during

migration? What advantage does “mild” activation of the stress response offer cells? Activation of the ISR in the cell accomplishes two aims: the first, to pause translation and protein synthesis; and second, to allow expression of specific genes and promote cellular recovery. Strong activation, in either levels or duration of response, can induce pro-apoptotic pathways in the cell (Ryoo and Vasudevan, 2017). In medulloblastoma cells, strong activation of the kinase PERK suppresses, while moderate activation promotes, cell migration and invasion (Jamison et al., 2015). Data from this and other studies implicate a biphasic effect of the stress response, in which high expression triggers cell death, but moderate activation of stress or stress response gene expression promotes migration and invasion (Chen et al., 2021; Jamison et al., 2015; Xu et al., 2020).

Activation of the ER stress response pathway, including induction of ATF4, offers a selective growth advantage to cancer cells challenged by limited oxygen, glucose, and amino acids (Nagelkerke et al., 2013; Wortel et al., 2017). ATF4 transcriptional responses are also induced in cancer cells to promote growth during oxidative stress (Pathria et al., 2018; Sorge et al., 2020). Rapid protein synthesis and folding are also a source of intrinsic stress (Tu and Weissman, 2004). Amidst the rapid protein synthesis prior to invasion in the *C. elegans* anchor cell, Xbp1, a downstream effector of the UPR and a marker for ER stress, is highly expressed and localized to the invasive front (Costa et al., 2023). Border cells, in which UPR signaling components are also highly expressed, may have similar protein synthesis needs during invasion and initiation of migration. Activation of the ER stress response and ATF4 to increase amino acid biosynthesis, transport, and protein folding could combat oxidative stress and promote collective migration.

During migration, border cells undergo dynamic changes to the actin cytoskeleton to allow for movements through and around the large nurse cells that fill the egg chamber. The transcription factor MRTF regulates levels of actin to allow response of the border cell cluster to the environment during migration (Somogyi and Rørth, 2004). Chromatin immunoprecipitation (CHiP) sequencing data revealed binding of MRTF to heat shock proteins in addition to its major target, Actin5C (Salvany et al., 2014). Other bound genes were enriched for protein folding functions via gene ontology terms (Salvany et al., 2014). Some heatshock proteins, including a subset of those bound by MRTF, have been shown to interact with the actin cytoskeleton under non-stress conditions (Morrow and Tanguay, 2015; Mounier and Arrigo, 2002). Upon ATF4 knockdown or overexpression in border cells, cluster aspect ratio was significantly decreased. Clusters appeared smaller, more rounded, and with less visible protrusions. It is possible that downstream targets of ATF4 required for border cell migration include cytoskeletal proteins or interact with cytoskeletal components in a non-canonical role.

Border cell migration is a dynamic feat of collective behavior, in which invasion and migration, feedback from the environment, and maintenance of epithelial integrity are precisely maintained and communicated through the input and intersection of multiple signaling pathways. Our transcriptome analysis revealed networks of temporal gene expression just as dynamic as migration itself. The combination of our expression, interaction, and functional data reveals a wealth of candidates in the right time and place to regulate border cell migration. Up- and down-regulation of some of these transcriptional networks, like ribosome biogenesis, may be critical to balance early cellular requirements like invasion. Finally, the expression and requirement of ATF4 and other stress response genes in border cells suggests an active stress response during migration. During migration, ATF4 may activate expression of its canonical targets to regulate

changes in amino acid and glutathione metabolism in border cells experiencing ER stress. ATF4 could also regulate, or be regulated by, additional targets required for border cell migration. If ATF4 is required to balance physiological and physical stressors during migration, this could reflect the need for precise levels of ATF4 in regulating the proper border cell number and cluster shape, which are required for a successful collective migration. Taken together, our data provide evidence of dynamic changes in cells that drive forward movement, balanced with recovery from the consequences of those changes.

4.4 References

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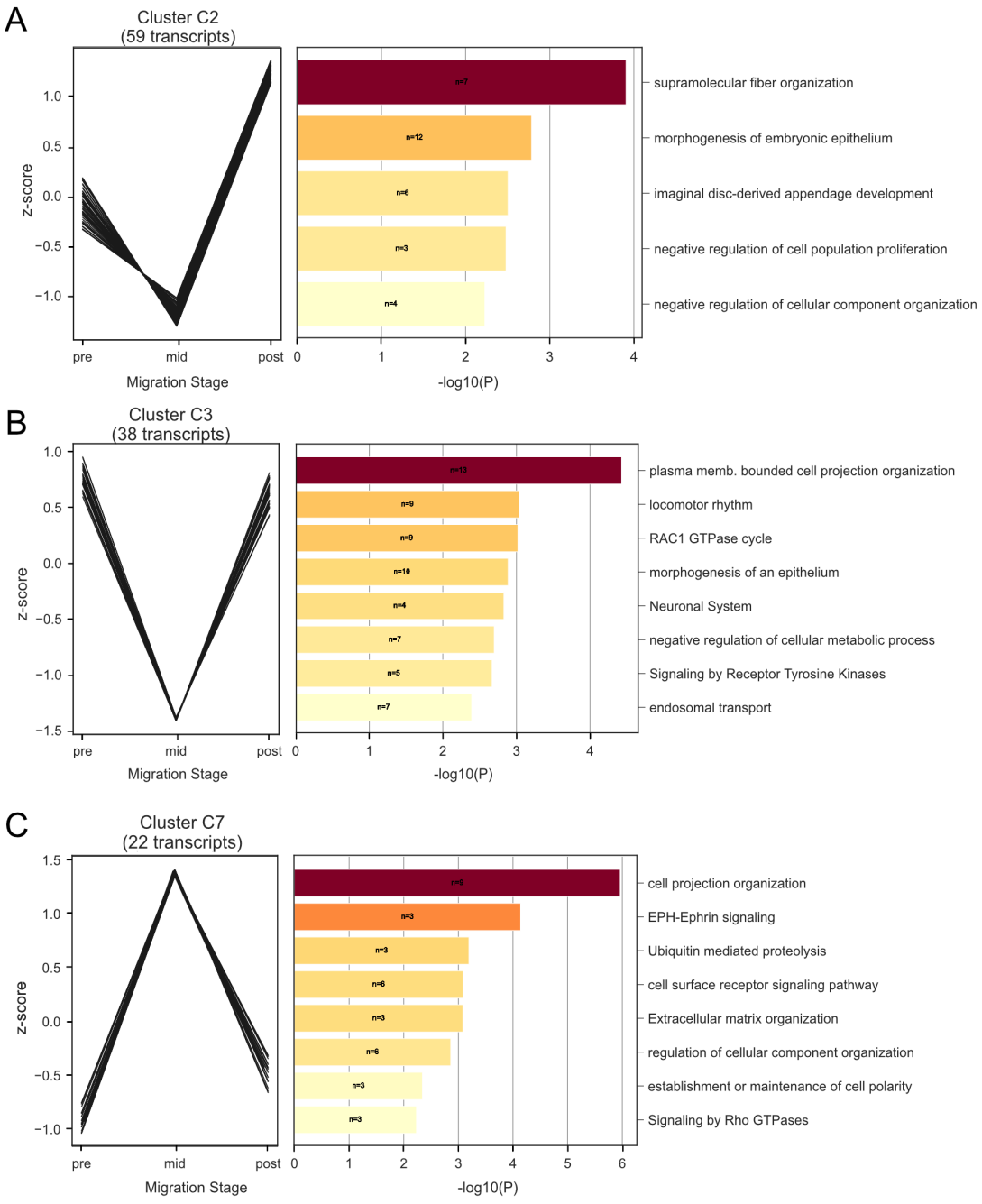
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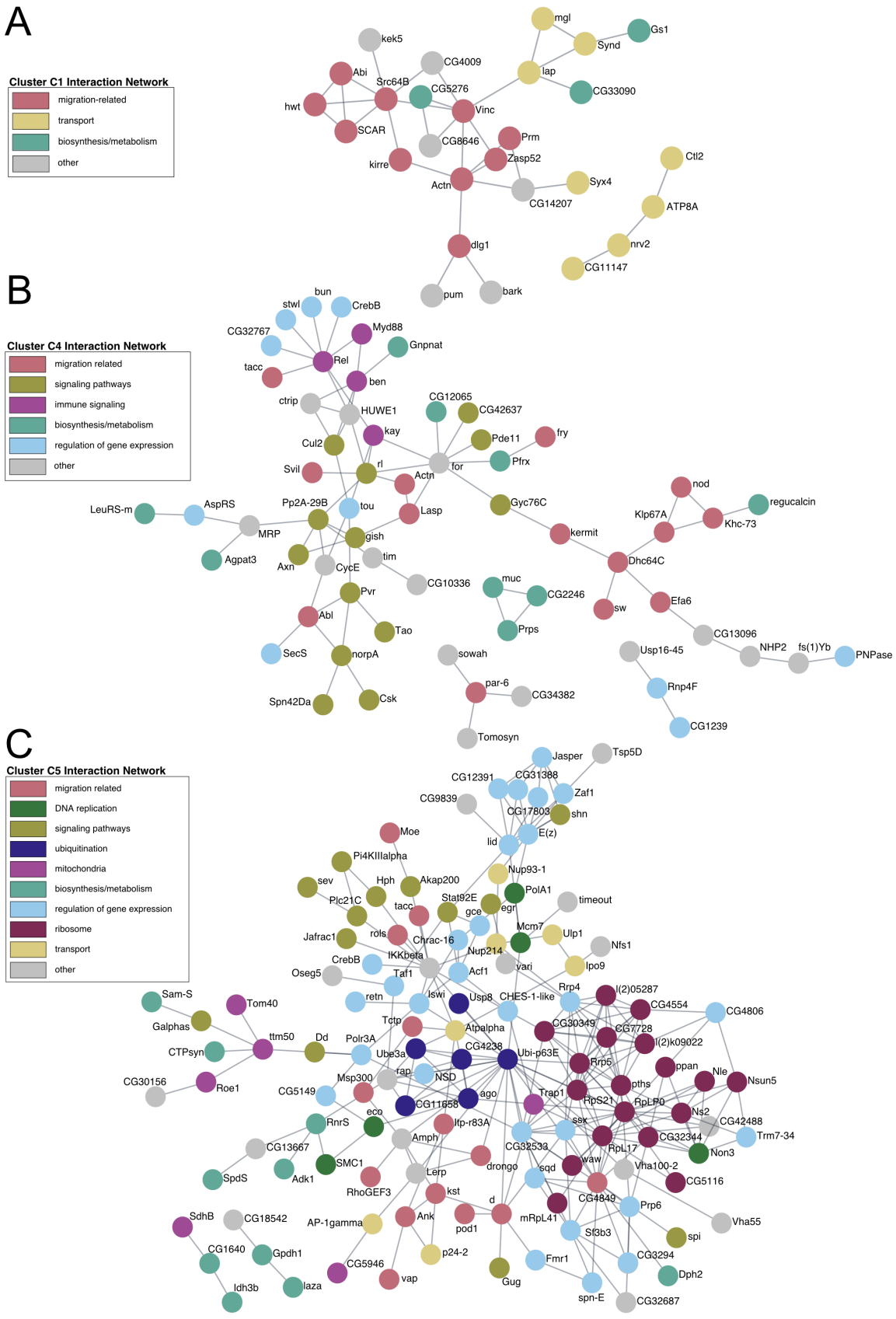
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Appendix A – Chapter 2 Supplemental Materials



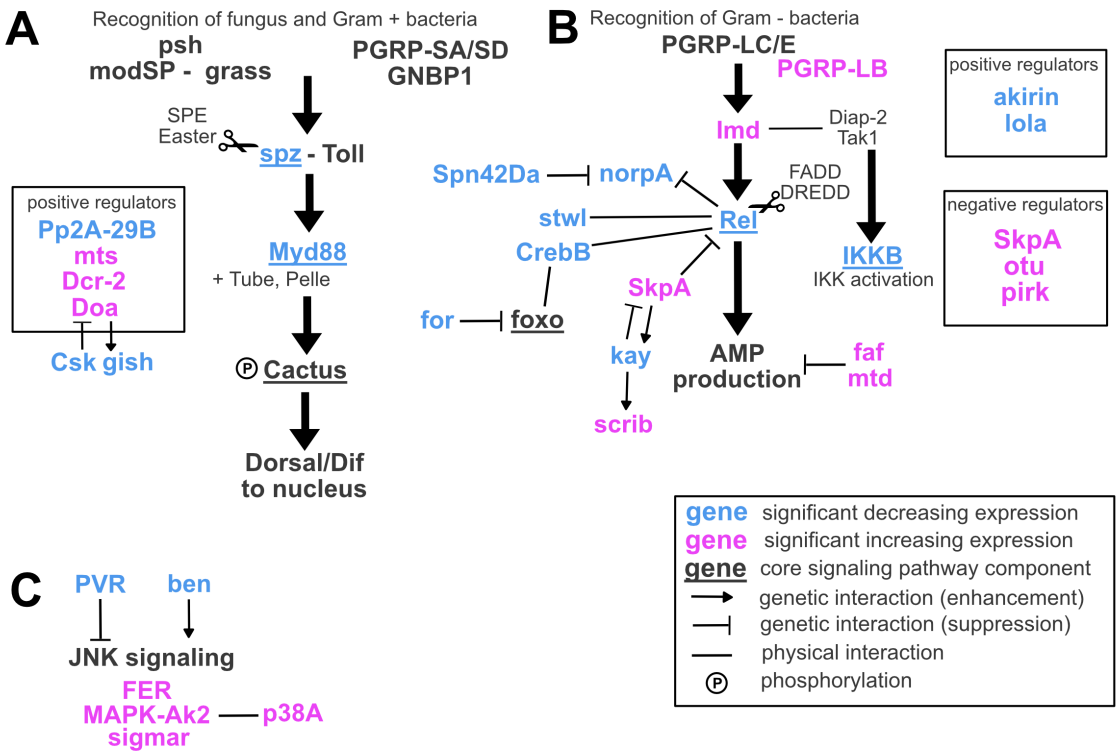
Appendix Figure A.1 Supplemental Figure 2.1. Differentially co-expressed transcripts are up- and down-regulated in border cells at mid-migration and enriched for shared biological function.

(**A-C**, left graphs) Significantly differentially expressed transcripts sorted by *clust* into shared co-expression patterns in pre-, mid-, and post-migration stages. (**A-C**, right graphs) Metascape pathway and process enrichment analysis results for each co-expression cluster, showing the mostly significantly enriched terms. N, number of genes enriched for a given annotation term; bars show significance of annotation terms, sorted by p values (-log10P; darker color, more significant values). Note that genes can be found in multiple Metascape annotation categories. Cluster C2 **(A)**, C3 **(B)**, and C7 **(C)** share patterns of stage-specific increased or decreased expression at mid-migration stages. All data are shown in Supplemental Tables 2.5 and 2.6.


A
Cluster C1 Interaction Network
migration-related
transport
biosynthesis/metabolism
other
mgl
Gs1
kek5
Synd
CG4009
Abi
Src64B
Iap
CG5276
CG33090
hwt
Vinc
SCAR
CG8646
Prm
kirre
Zasp52
Ctl2
Actn
Syx4
CG14207
ATP8A
nrv2
dlg1
CG11147
pum
bark
B
Cluster C4 Interaction Network
migration related
signaling pathways
immune signaling
biosynthesis/metabolism
regulation of gene expression
other
bun
stwl
CrebB
CG32767
Myd88
Gnpnat
tacc
Rel
ben
ctrip
CG12065
HUWE1
CG42637
kay
Pde11
fry
Cul2
Pfrx
for
Svil
rl
Actn
nod
regucalcin
LeuRS-m
AspRS
Pp2A-29B
tou
Lasp
Gyc76C
Klp67A
Khc-73
MRP
kermit
gish
Agpat3
Axn
tim
Dhc64C
CycE
CG10336
muc
CG2246
Pvr
sw
Efa6
Abl
Tao
Prps
CG13096
SecS
norpA
sowah
Usp16-45
NHP2
fs(1)Yb
PNPase
par-6
CG34382
Rnp4F
Spn42Da
Csk
CG1239
Tomosyn
C
Cluster C5 Interaction Network
migration related
DNA replication
signaling pathways
ubiquitination
mitochondria
biosynthesis/metabolism
regulation of gene expression
ribosome
transport
other
Jasper
Tsp5D
CG12391
CG31388
Zaf1
CG9839
CG17803
shn
E(z)
Moe
lid
Pi4KIIIalpha
Nup93-1
sev
Hph
Akap200
PolA1
Plc21C
tacc
Stat92E
egr
timeout
gce
Mcm7
Jafrac1
rols
Chrac-16
Ulp1
Nfs1
Nup214
IKKbeta
vari
Ipo9
CrebB
Oseg5
Taf1
Acf1
Sam-S
retn
Iswi
Usp8
CHES-1-like
Rrp4
l(2)05287
CG4554
CG4806
Tom40
Galphas
Tctp
Atpalpha
CG30349
CG7728
ttm50
Dd
Polr3A
CG4238
Ubi-p63E
l(2)k09022
CTPsyn
Ube3a
Rrp5
ppan
Nle
Nsun5
CG30156
Roe1
rap
NSD
Msp300
pths
CG5149
ago
Trap1
RpS21
RpLP0
CG11658
Ns2
eco
ssr
CG42488
RnrS
Itp-r83A
Amph
CG32533
RpL17
CG32344
Trm7-34
CG13667
Non3
SMC1
drongo
sqd
waw
Vha100-2
SpdS
Adk1
Lerp
CG5116
RhoGEF3
kst
CG4849
Prp6
d
Ank
mRpL41
Vha55
SdhB
CG18542
AP-1gamma
pod1
Sf3b3
spi
CG1640
Gpdh1
p24-2
Fmr1
CG3294
CG5946
Dph2
vap
Gug
laza
spn-E
Idh3b
CG32687

Appendix Figure A.2 Supplemental Figure 2.2. Protein-protein interaction networks for co-expression clusters C1, C4, and C5.

(A-C) Physical PPI network analysis of genes in selected co-expression clusters C1 **(A)**, C4 **(B)** and C5 **(C)**. Individual small networks are found in each cluster in addition to the larger networks. **(A)** Network of C1 co-expressed genes with expression that increased during border cell migration. **(B, C)** Networks of C4 **(B)** and C5 **(C)** co-expressed genes with expression patterns that decrease during border cell migration. Functional annotation keywords (Supplemental Table 2.7) were used to assign color to proteins in the networks. **(A)** Co-expression cluster C1 forms a network with migration-related functions (pink). An individual node (top right) and network (lower right) have transport-related functions (mustard). **(B)** The co-expression cluster C4 network forms nodes with immune signaling (purple) and regulation of gene expression (light blue, upper left), biosynthesis/metabolism (cyan, center), and signaling pathways (green, center). **(C)** Co-expression cluster C5 forms a large node for ribosome function (magenta, right), nodes for regulation of gene expression (light blue, top, center, and lower right), and a node for signaling pathways (green, upper left).



Appendix Figure A.3 Supplemental Figure 2.3. Toll, Imd, and JNK signaling pathway genes differentially expressed during border cell migration.

Diagrams of key components and regulators of the Toll **(A)**, Imd **(B)**, and JNK **(C)** signaling pathways, some of which are differentially expressed during border cell migration. Genes that are differentially expressed in border cells are in bold text, while those that increase in expression are shown in magenta and those that decrease in expression are shown in blue. Genes denoted as core signaling pathway components on Flybase are underlined.

Supplemental Table 2.1. Raw transcript quantification data in border cells during their migration.

Supplemental Table 2.2. Alignment to *Drosophila melanogaster* reference genome.

Supplemental Table 2.3. Significantly differentially expressed genes during border cell migration based on quantile normalized genome-wide gene expression with hierarchical clustering.

Supplemental Table 2.4. Supplemental Table 2.4 contains 8 tabs, each detailing genes identified in individual gene classes of interest that were differentially expressed during border cell migration, based on quantile normalized genome-wide expression with hierarchical clustering. Classes of interest include border cell migration genes (tab 1), genes identified in Borghese et al. or Wang et al. microarrays (tabs 2-3), genes enriched for adhesion, epithelial-to-mesenchymal transition, or oogenesis functions (tabs 4-7, respectively), and transcription factors (tab 8).

Supplemental Table 2.5. Significantly differentially expressed genes during border cell migration based on *clust* expression patterns. Data for co-expression clusters C0-C8 are displayed in tab 1.

Supplemental Table 2.6. Supplemental Table 2.6 contains 9 tabs, each with Metascape annotation for genes significantly differentially expressed during border cell migration in an

individual co-expression cluster. Data for co-expression clusters C0-C8 are displayed in tabs 1-9, respectively.

Supplemental Table 2.7. Supplemental Table 2.7 contains 6 tabs, each with STRING and functional annotation for Cytoscape protein interaction networks for analyzed co-expression clusters. Data for clusters C0, C1, C4, C5, C6, and C8 can be found in tabs 1-6, respectively.

Supplemental Table 2.8. Significantly differentially expressed genes enriched for immune function, with annotations.

Supplemental Table 2.9. Significantly differentially expressed genes enriched for ribosomal function, with annotations, including GO terms.

Supplemental Table 2.10. Supplemental Table 2.10 contains 10 tabs detailing the significantly differentially expressed genes identified in the Dresden Ovary Table (DOT). A summary of the available DOT data, including annotations for stage and cell type-specific expression, are provided for co-expression clusters C0-C8 (tabs 1-9, respectively). Tab 10 contains a summary of the available DOT data for genes with identified border cell-specific expression.

Supplemental Table 2.11. Supplemental Table 2.11 contains 4 tabs detailing the genes that were chosen for the RNAi screen testing function of selected differentially expressed genes in border cells (tab 1). Tab 2 details the RNAi reagents used. The raw migration defect data are shown in tab 3, and the summary of significant migration defect data is in tab 4.

Supplemental Table 2.12. Supplemental Table 2.12 contains three tabs listing the top 200 transcripts expressed in border cells at pre-, mid-, and late migration stages.

Appendix B – A functional role for ribosome biogenesis genes in border cell collective migration

Aim

A suite of signaling pathways regulate collective cell migrations throughout the life of an organism, including in development and disease. Recent transcriptomics studies have shed light on previously unappreciated gene expression programs in migrating cells (Morrison et al. 2017; McLennan et al. 2015; Costa et al. 2023). During *Drosophila* oogenesis, a group of 6-10 cells known as border cells undergo a collective migration through the developing egg chamber. To characterize gene expression changes in border cells during their migration, we isolated and sequenced border cells at three stages of migration (Burghardt et al. 2023). Surprisingly, our transcriptome analysis identified a transcriptional network of differentially expressed ribosome biogenesis genes downregulated in border cells during their migration. To test the function of ribosome biogenesis genes in migration, we used 15 RNAi lines to knock down function of 9 genes significantly differentially expressed in border cells.

Materials and Methods

All fly stocks and crosses were maintained at 25°C. Genes were chosen to test from the list of significantly differentially expressed ribosome biogenesis genes in border cells (Supplemental Table 2.9). Because many ribosome biogenesis genes have been identified based solely on sequence similarity to yeast or mammalian homologs, gene choice was further based on previous publication in ribosome biogenesis. For RNAi screening, stocks were obtained from the Bloomington Drosophila Stock Center (BDSC) and the Vienna Drosophila Resource Center (VDRC). Where possible, two independent, non-overlapping RNAi lines were chosen per gene. All RNAi lines tested are listed in Table A.1. Virgin females from a *c306* GAL4; tsgal80/Cyo stock were crossed to UAS-RNAi males to drive expression of the knockdown construct in

border cells. RNAi against *mCherry* (BDSC #35785) was used as a negative control, and *bazooka* (*Baz*; VDRC #v2914) was used as a positive control. Female progeny from these crosses were selected and fattened on wet yeast past overnight at 29˚C to allow maximal GAL4/UAS expression and inactivation of tsGAL80.

To analyze border cell migration, whole ovaries were dissected in Schneider's *Drosophila* medium (Thermo Fisher Scientific) and fixed in 4% methanol-free formaldehyde (Polysciences) and 0.1 M potassium phosphate buffer (pH 7.4) for 10 minutes. Egg chambers were stained for E-cadherin (E-cad; 1:10 dilution, rat monoclonal DCAD2; Developmental Studies Hybridoma Bank [DSHB]), Singed (Sn; 1:25 dilution, mouse monoclonal Sn7C; DSHB), and DAPI to label nuclei (2.5 μg/mL; Millipore Sigma, cat. no. D954). Isotype-specific anti-mouse or anti-rat secondary antibodies conjugated to AlexaFluor–488 or –568 (Thermo Fisher Scientific) were used at a concentration of 1:400. All slides were mounted using Fluorsave Reagent mounting media (Millipore Sigma, cat. no. 345789). Migration defects were analyzed and images were obtained on an upright Zeiss AxioImager Z1 with ApoTome2 using a 20X objective. All images were processed in FIJI.

Three trials per line were used. The cutoff value for migration defects was determined by the background mean migration defect, calculated using an average of the migration defect observed in the negative control (*c306*-Gal4; tsgal80/+; UAS-mCherry RNAi/+). To assess significance of migration defects, the empirical rule for a normal distribution was used, where the threshold for migration defect significance is equal to the mean plus three times the standard deviation. Graph

was assembled using Graphpad (version 7.04). Graphing schematic and figure were assembled using Affinity Designer.

Results and Conclusions

A total of 52 genes with predicted or functional roles in ribosome biogenesis (and an additional 30 genes in cytoplasmic ribosome translation or RNA modification categories) were significantly differentially expressed in border cells during their migration. Interestingly, expression of all but 3 of these genes began high at the pre-migration stage and sharply decreased during migration. To test the function of ribosome biogenesis in border cells during migration, we chose 9 genes from the list of significantly differentially expressed genes. Border cell migration upon RNAi knockdown was scored at stage 10 of oogenesis, by which time border cells should have reached the oocyte boundary and stopped their migration (Appendix Figure B.1A). Migration defect was scored in egg chamber as a percentage of the total distance migrated to the oocyte (0-25%, 26-50%, 51-75%, or 76-100%; Appendix Figure B.1B). RNAi knockdown of a negative control, mCherry RNAi, results in complete migration by stage 10 (Appendix Figure B.1C). An RNAi line against *Bazooka,* a gene known to be required for border cell migration, was used as a positive control for migration defect (Appendix Figure B.1D). Genes, RNAi lines used, replicates, and average migration defects are listed in Appendix Table B.1.

Knockdown of *Nop56* function in border cells using either of two RNAi lines resulted in a strong migration defect (Appendix Figure B.1B, E-G). *Drosophila* Nop56 is orthologous to the human gene Nop56, both of which are required for ribosome biogenesis (Gautier et al. 1997). A member of the box C/D snoRNP family, Nop56 and other genes form a small ribonucleoprotein

complex to catalyze site-specific rRNA methylation and assemble ribosomes (FlyBase). Interestingly, knockdown of Nop56 sometimes resulted in a cluster splitting defect, in which border cells split off the main cluster while migrating forward (Appendix Figure B.1G). The RNAi line in which this defect was observed (VDRC # v103783) has a potential off-target effect to *Hormone receptor 4* (Hr4). Hr4 is significantly differentially expressed in border cells during migration and loss results in a slight migration defect (Manning et al. 2017; Burghardt et al. 2023). However, here we demonstrate a strong migration defect upon Nop56 knockdown, matched across two non-overlapping RNAi lines. Further work, including live imaging, will be needed to assess the cluster splitting defect caused by Nop56 knockdown in border cells. Knockdown of a second gene in ribonucleoprotein assembly, Nopp140, resulted in a significant defect with one of two RNAi lines (Appendix Figure B.1B). Loss of Nopp140 causes abnormal ribonucleoprotein granule formation in *Drosophila* larval cells, indicating a normal role in small ribonucleoprotein particle assembly (He et al. 2015). An additional 25 genes significantly differentially expressed in border cells were enriched for gene ontology terms related to ribonucleoprotein complex biogenesis or assembly; their function in border cell migration remains to be tested (for a complete list, see Supplemental Table A.9).

Three genes tested in border cell migration have functional roles in ribosomal subunit assembly (Ameismeier et al. 2018; Marygold et al. 2007; Zielke et al. 2022). Knockdown of *Tsr1 ribosome assembly factor, peter pan,* or *stubarista* each resulted in a significant migration defect with one RNAi line (Appendix Figure B.1 B, H, I, K). Interestingly, for each gene, attempted knockdown using a second RNAi line resulted in little to no progeny, or no progeny of the correct phenotype for selection (Appendix Table A.1). As multiple genetic crosses yielded the same result, we conclude that knockdown of these genes in border cells and the additional

follicle cells in which the driver, *c306* GAL4, is expressed, is likely lethal. Ribosome biogenesis is a critical process in all cells, especially during time periods of rapid growth and differentiation (Ni and Buszczak 2022). Lethality upon alteration of ribosome biogenesis or ribosome gene expression is likely to be a continuing issue in our analyses. The use of additional GAL4 drivers, multiple non-overlapping RNAi lines, and careful analysis of any cell death in border cells will be needed to address this issue. Finally, knockdown of one gene important for maturation of ribosomal RNA, *no child left behind*, also resulted in a significant migration defect (Appendix Figure B.1B, J; Liu et al. 2018).

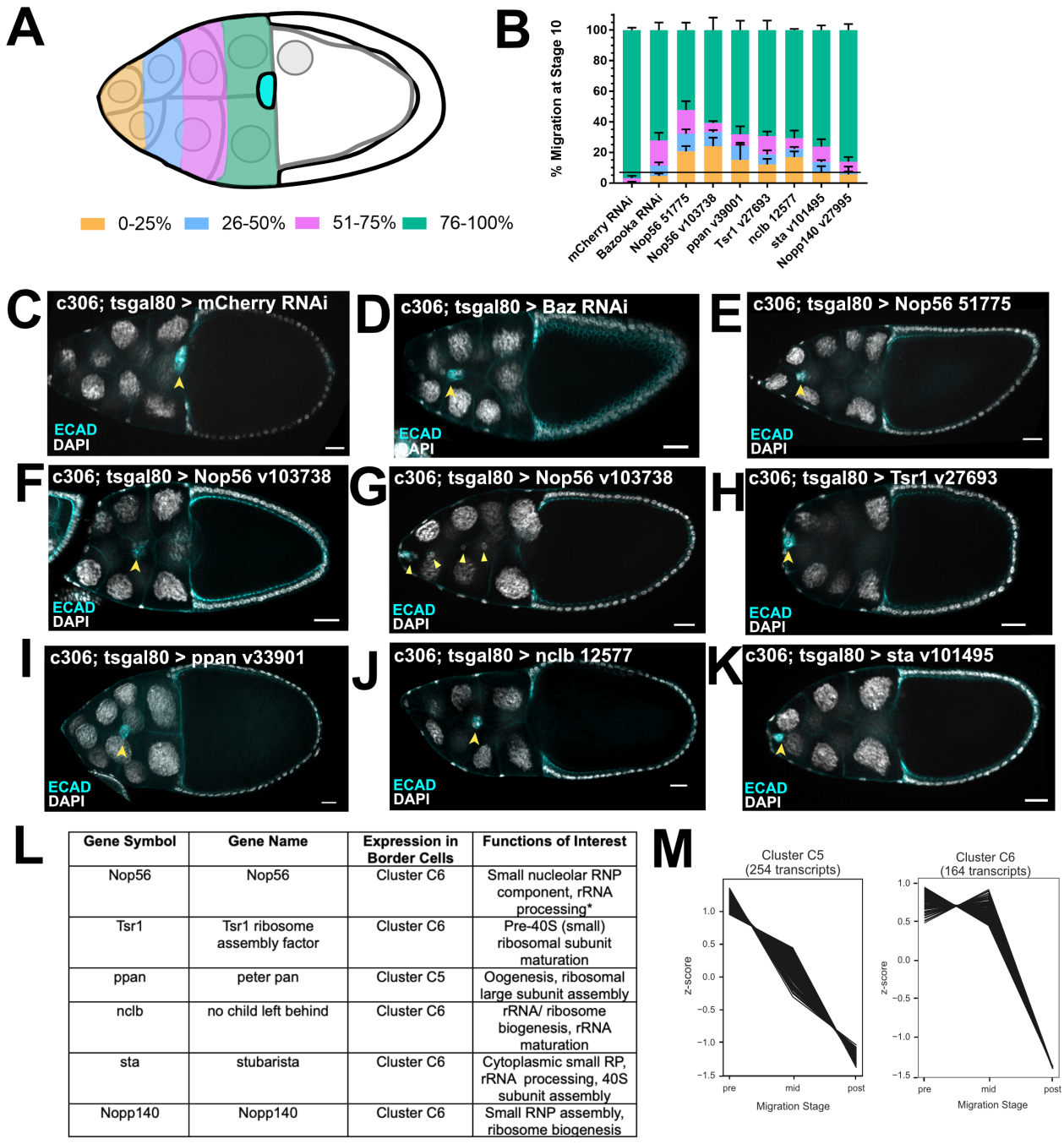
Our transcriptome analysis in border cells identified significant differential expression of genes with functional roles in ribosome biogenesis and assembly during migration (Appendix Figure B.1L). Strikingly, expression of nearly all ribosome biogenesis genes decreased sharply during migration in one of two co-expression clusters (Appendix Figure B.1M, also see Chapter 2). Here, we report a functional role for a subset of these genes during border cell migration. Knockdown of 6 genes (using 7 RNAi lines) resulted in a significant border cell migration defect. Recently, the ribosome has emerged as a potential regulator of invasive and migratory behavior in collectives. Our data represent a wealth of candidates, including 6 with demonstrated function, to further our understanding of ribosome biogenesis as a regulator of these behaviors in border cells.

Appendix Table B.1. RNAi knockdown of a subset of ribosome biogenesis genes results in border cell migration defects.

Gene Symbol/Name	RNAi line	Replicates	Phenotype
mCherry	35785	1.65%	3.18% average migration defect
		3.30%	
		4.60%	
Baz; *bazooka*	v2914	25.93%	27.77% average migration defect
		33.33%	
		24.07%	
Nop56	51775	49.33%	47.68% average migration defect
		51.47%	
		42.22%	
	103738	34.62%	45.40% average migration defect
		48.57%	
		34.33%	
ppan; *peter pan*	v39001	25.93%	31.95% average migration defect
		38.16%	
		31.76%	
	80392	3 replicates	potential lethal
Tsr1; *Tsr1 ribosome assembly factor*	v27693	35.90%	30.67% average migration defect
		32.26%	
		23.85%	
	62305	3 replicates	potential lethal
nclb; *no child left behind*	12577	28.38%	29.30% average migration defect
		29.73%	
		29.79%	
sta; *stubarista*	v101495	24.49%	28.83% average migration defect
		24	
		26.56%	
	77365	3 replicates	potential lethal
Nopp140	v27995	12.50%	14.01% average migration defect
		18.42%	
		11.11%	
	33694	2.20%	5.16% average migration defect
		6.14%	
		7.14%	
RpLP0; *Ribosomal protein LP0*	31370	3.60%	5.65% average migration defect
		7.69%	

		6.25%	
RpL17 ; *Ribosomal protein L17*	54048	2.73%	2.66% average migration defect
		2.59%	
CG5033	42930	2 replicates	potential lethal
	44118	2 replicates	potential lethal

Figures



L

Gene Symbol	Gene Name	Expression in Border Cells	Functions of Interest
Nop56	Nop56	Cluster C6	Small nucleolar RNP component, rRNA processing*
Tsr1	Tsr1 ribosome assembly factor	Cluster C6	Pre-40S (small) ribosomal subunit maturation
ppan	peter pan	Cluster C5	Oogenesis, ribosomal large subunit assembly
nclb	no child left behind	Cluster C6	rRNA/ ribosome biogenesis, rRNA maturation
sta	stubarista	Cluster C6	Cytoplasmic small RP, rRNA processing, 40S subunit assembly
Nopp140	Nopp140	Cluster C6	Small RNP assembly, ribosome biogenesis

Appendix Figure B.1 Differentially expressed ribosome biogenesis genes are required for border cell migration.

A) Schematic illustrates scoring of migration defect in border cells upon RNAi knockdown of ribosome biogenesis genes. Migration defect is scored as a percentage of the total migration

distance (0-25%, orange; 26-50%, blue; 51-75%, pink; 76-100%, green). **B)** Quantification of border cell migration defects upon knockdown of ribosome biogenesis genes compared to knockdown of mCherry RNAi (negative control, left). Migration defect upon mCherry RNAi knockdown is used to determine the threshold for migration defect significance, represented as a black line (6% migration defect; see Methods). Error bars represent standard deviation. **C)** RNAi knockdown of mCherry in border cells results in complete migration by stage 10 of oogenesis. **D)** RNAi knockdown of *Bazooka* (positive control) in border cells results in migration defects at stage 10 of oogenesis. **E-K)** RNAi knockdown of significantly differentially expressed genes with roles in ribosome biogenesis (Nop56, (**E-G)**; *Tsr1 ribosome assembly factor*, (**H)**; *peter pan*, (**I)**; *no child left behind*, (**J)**; or *stubarista*, (**K)**) results in migration defects. Cluster splitting was observed for RNAi knockdown of Nop56 with one line (shown in **G**). E-cadherin labels the border cell cluster (in cyan) and DAPI labels cell nuclei (in white). Yellow arrowheads denote the border cell cluster (**C-F**, **H-K**) or individual border cells (**G**). Scale bars represent 20 μm. **L)** Each gene has previously described functions in ribosome biogenesis. For a complete list of annotated functions, see Supplemental Table 2.9. **M)** Expression of each gene significantly decreases during border cell migration. For a complete list of significantly differentially expressed transcripts in co-expression clusters C5 and C6, see Supplemental Table 2.5.

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