Characterization of the p21-activated kinase Pak during Drosophila oogenesis

by

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Abstract

Understanding the mechanisms involved in tissue reorganization and organ formation are fundamental questions that are of particular interest to those studying epithelial morphogenesis. Model organisms such as Drosophila melanogaster have been instrumental in identifying key components required for cellular processes that are critical for tissue morphogenesis as a whole. These include cell fate specification, cell shape change, cell growth and cell migration. The Rho subfamily of small GTPases Rho, Rac and Cdc42, are master regulators of actin cytoskeletal dynamics and the well-characterized Rac/Cdc42 effector kinase Pak has been implicated in morphogenesis of epithelia both in mammalian cell culture as well as Drosophila development.

Drosophila oogenesis is a highly favourable system for studying epithelial morphogenesis and differentiation of epithelia from a stem cell. Newly formed germline cysts become encapsulated by follicle cells that arise from follicle stem cells (FSCs) to form egg chambers. This study on Pak function during oogenesis, combined with the work of others, has opened the door to our understanding of how the FSC and its niche produce a simple yet very organized epithelium. Pak appears to be required during early stages of oogenesis at or around the time point at which FSCs and/or their niches are specified. Loss of Pak during early stages of oogenesis leads to a novel side-by-side egg chamber phenotype with pak mutant germaria having duplicated FSC niches, implicating Pak in FSC niche formation. This work has led to a model for stem cell niche formation that may be broadly applicable. Later in oogenesis Pak is required for the polarized organization of the basal F-actin in follicle cells, which drives egg chamber elongation. Further investigation of Pak’s role has demonstrated that it acts antagonistically to the Rho1-activated actomyosin contractility pathway in regulation of this F-actin. The basal F-actin of the follicle cells is similarly organized as the stress fibres of mammalian cells and insights gained from this work are likely to be relevant to understanding regulation of the mammalian cytoskeleton. Overall this work has revealed new roles for Pak in epithelial development.

Keywords: Drosophila; oogenesis; Pak; epithelial morphogenesis; follicle stem cell; actin cytoskeleton
Dedication

To Mom, Baba, Perry, Anna and Ryan.
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# Table of Contents

Approval.................................................................................................................................................. ii  
Abstract.................................................................................................................................................. iv  
Dedication.................................................................................................................................................. v  
Acknowledgements.................................................................................................................................... vi  
Table of Contents...................................................................................................................................... vii  
List of Tables............................................................................................................................................ x  
List of Figures.......................................................................................................................................... xi  

## 1. Introduction

1.1. Epithelial Morphogenesis.................................................................................................................. 1  
1.2. *Drosophila* oogenesis........................................................................................................................ 5  
1.3. Stem cells and their niches.................................................................................................................. 11  
1.4. Regulation and maintenance of the *Drosophila* germline stem cells........................................... 12  
1.5. The follicle stem cell and its niche..................................................................................................... 16  
1.6. Morphogenesis of the follicular epithelium....................................................................................... 21  
1.7. Basal F-actin of the follicular epithelium........................................................................................... 24  
1.8. *Drosophila* egg chamber rotation as a mechanism for egg chamber elongation.......................... 29  
1.9. Rho family of small GTPases.............................................................................................................. 32  
1.10. p21-activated kinases....................................................................................................................... 33  
1.11. Pak’s role in *Drosophila* oogenesis............................................................................................... 35  
1.12. Goal of this thesis............................................................................................................................. 37  

## 2. Materials & Methods

2.1. Fly stocks........................................................................................................................................... 38  
2.2. Generation of *Drosophila* somatic cell clones................................................................................. 39  
2.3. Fixation and staining of *Drosophila* ovaries.................................................................................... 40  
2.4. TUNEL method to detect apoptosis................................................................................................... 42  
2.5. Acridine Orange staining to detect apoptosis.................................................................................... 43  
2.6. Larval and pupal ovary dissection, fixation and staining................................................................... 44  
2.7. Measuring egg chamber length......................................................................................................... 45  
2.8. Rho-GTP activity assay...................................................................................................................... 45  
2.9. cDNAs................................................................................................................................................ 48  
2.10. Generation of the RhoGEF2-DH/PH domain – GST fusion protein and co-immunoprecipitation experiment........................................................................................................................................ 49  
2.11. Live imaging of egg chamber rotation............................................................................................. 51  
2.12. RNA and Fluorescence *in situ* hybridization.................................................................................. 54  

## 3. Results I: Analysis of a unique side-by-side egg chamber phenotype in *pak* mutant ovaries

3.1. *pak* mutant ovarioles exhibit a novel side-by-side egg chamber phenotype ................................ 58  
3.2. Somatic tissue specific loss of *pak* generates the side-by-side paired egg chamber phenotype ........ 64  
3.3. Polar cells and stalk cells are affected in *pak* mutants................................................................... 74  
3.4. Possible effect of Pak on DE-Cadherin distribution in the germarium......................................... 77
3.5. Pak functions during ovarian development ........................................... 83
3.6. The FSC niche in pak mutant gerariuma is mispositioned anteriorly .......... 84
3.7. The number of FSC niches is increased in pak mutant germaria .......... 90
3.8. Interactions between Pak and Hedgehog signaling ............................... 96

4. Discussion I ..................................................................................................................... 99
4.1. pak mutants are able to encapsulate side-by-side cysts due to the presence of extra FSC niches ........................................ ...................................................... 99
4.2. Pak function in the pupal ovary contributes to the establishment of the FSC niche ................................................ .............................................................. 103
4.3. A model for formation of the FSC niche ..................................................... 105
4.4. An interaction between DE-Cadherin and Pak may play a minor role in the pak mutant paired egg chamber phenotype ......................................................... 110
4.5. Reducing Hedgehog signaling in pak mutants affects follicle cell proliferation and generates compound egg chambers ......................... 112
4.6. Pak’s role in escort cell regulation may involve interactions with Rho1 signaling ................................................................................................................. 113
4.7. General conclusions ......................................................................................... 115

5. Results II: The role of Pak in actomyosin contractility during egg chamber elongation ...................................................................................................... 116
5.1. Deficiency screen to identify second site modifiers of pak mutant oogenesis defects ............................................................................................................... 116
5.2. Heterozygosity for components of the Rho1 signaling pathway controlling actomyosin contractility supresses the pak rounded egg chamber phenotype ......................................................................................................... 124
5.3. Evidence that RhoGEF2 is the major activator of Rho1 in regulation of the basal F-actin in the follicular epithelium ........................................................................... 126
5.4. Pak does not appear to regulate the levels of activated Rho1 during oogenesis but is required for RhoGEF2 localization .................................................. 130
5.5. Pak regulates the phosphorylation of the non-muscle myosin regulatory light chain in follicle cells ....................................................................................... 139
5.6. The other Group I Pak member Pak3, acts antagonistically to Pak during egg chamber elongation ................................................................. 142
5.7. Dpp pathway and other signaling components identified as pak interactors in the second chromosome deficiency screen .............................................. 143
5.8. pak mutant egg chambers undergo rotation but do not elongate ............... 146

6. Discussion II .............................................................................................................. 150
6.1. The follicular epithelium as a system for studying stress fibers and actomyosin contractility ........................................................................................................ 150
6.2. Confirmation of RhoGEF2 as the major activator of Rho1 in epithelia ........ 154
6.3. Pak3 functions antagonistically to Pak during egg chamber elongation ...... 155
6.4. Candidate regulators of the Rho pathway identified in the screen ............. 156
6.5. Egg chamber elongation requires both polarized ECM as well as basal F-actin ......................................................................................................................... 157
7. Appendix..................................................................................161
  7.1. Third chromosome deficiency screen .................................................................161
  7.2. Pak is required for sepatate junction formation ....................................................165
  7.3. dPix RNA localization does not correlate with its known association with Pak.........................................................................................................................172

8. Final conclusions..................................................................................176

References ..........................................................................................178
List of Tables

Table 3.1  Comparison of pak mutant and wild-type germaria........................................67

Table 5.1  Second chromosome deficiencies that suppress the pak rounded egg chamber phenotype ........................................................................................................120

Table 5.2  Quantification of stage 9 egg chamber length........................................................123

Table 5.3  Quantitative production of mature eggs..............................................................125

Table 5.4  Summary of chromosomal deficiencies and alleles used to test for effect of reductions in RhoGEFs on the pak rounded egg chamber phenotype .................................................................129

Table 7.1  Third chromosome deficiencies tested as modifiers of the pak rounded egg chamber phenotype .........................................................................................162
List of Figures

Figure 1.1 Mechanisms of epithelial morphogenesis ................................................................. 2
Figure 1.2 Overview of Drosophila oogenesis ............................................................................... 6
Figure 1.3 Structure and cell types within the germarium ........................................................... 9
Figure 1.4 Germline stem cell maintenance ..................................................................................14
Figure 1.5 Organization and regulation of the follicle stem cell niche ........................................ 17
Figure 1.6 Movement of follicle cells during mid-oogenesis ...................................................... 22
Figure 1.7 Basal F-actin of the follicular epithelium ................................................................. 26
Figure 1.8 Egg chamber rotation as a mechanism for egg chamber elongation ......................... 30
Figure 3.1 pak mutant ovarioles have side-by-side egg chamber that are present in the germarium ................................................................. 59
Figure 3.2 pak mutant ovarioles generate fully encapsulated egg chambers ......................... 62
Figure 3.3 GSC differentiation is not affected in pak mutant germaria ........................................ 65
Figure 3.4 Loss of Pak in the adult or FSCs does not appear to cause the paired egg chamber phenotype ......................................................................................... 69
Figure 3.5 Expression of a Pak-RNAi transgene with somatic cell drivers produces paired egg chambers ............................................................................................. 72
Figure 3.6 Polar cells are specified in pak mutant egg chambers but are abnormal .........................75
Figure 3.7 Loss of Pak affects stalk cells ......................................................................................78
Figure 3.8 Disruptions in DE-Cadherin are present in pak mutants ...........................................81
Figure 3.9 Larval and pupal ovary development in wild-type and pak mutant females ..................85
Figure 3.10 FSC niches are displaced towards the anterior end of germaria in pak mutants .............88
Figure 3.11 Evidence that pak mutant germaria have additional, internally located FSC niches ........................................................................................................... 91
Figure 3.12 Triple encapsulation of germline cysts generates triple-fused egg chambers ................94
Figure 3.13 Interactions between Pak and Hh signalling during oogenesis affect cyst encapsulation .................................................................97

Figure 4.1 Model of FSC niche formation.................................................................106

Figure 5.1 Mating scheme for second chromosome deficiency screen ..............117

Figure 5.2 Suppressors of the pak rounded egg chamber phenotype ..............121

Figure 5.3 Comparison of the basal F-actin of middle stage egg chambers .........127

Figure 5.4 Alignment of the DH/PH domains of Drosophila RhoGEF2 with the corresponding sequences of the DH/PH domains of mammalian p115-RhoGEF ............................................................... 131

Figure 5.5 Pak does not appear to regulate the levels of activated Rho1 .............134

Figure 5.6 RhoGEF2 is basally localized in the follicular epithelium and its localization is regulated by Pak .................................................................137

Figure 5.7 Pak regulates phosphorylation of MLC during development of the follicular epithelium .................................................................140

Figure 5.8 Pak3 acts antagonistically to Pak and its expression is regulated by actomyosin contractility...............................................................144

Figure 5.9 pak mutant egg chambers are able to rotate and lay down polarized ECM components .................................................................148

Figure 6.1 A model for Pak’s role in Rho1 mediated actomyosin contractility during egg chamber elongation .........................................................151

Figure 6.2 Cortical actin of follicle cells extends small actin-based protrusions on one side of the cell .................................................................159

Figure 7.1 Third chromosome deficiencies that suppress the pak rounded egg chamber phenotype and/or enhance the pak multilayering phenotype ......163

Figure 7.2 Components of the Scribble complex can be ectopically recruited to the membrane when pak is mis-expressed ..................................167

Figure 7.3 Interdependence between Scribble complex or septate junction proteins and integrins for localization in follicular epithelium ...............170

Figure 7.4 dpix is expressed in the dorsal epidermis but not in the DME cells during dorsal closure ............................................................................174
1. Introduction

1.1. Epithelial Morphogenesis

The first epithelia to develop in the common fruit fly, *Drosophila melanogaster*, referred to as primary epithelia, form in the developing embryo during cellularization of the blastoderm and typically share standard epithelial characteristics such as formation of apical belt-like adherens junctions, appearance of an apical extra cellular matrix (ECM) and an absence of a basal ECM (BROWN 2000; TEPASS 1997; TEPASS and HARTENSTEIN 1994a; TEPASS et al. 2001). Mesenchymal cells derived from primary epithelia can undergo a process known as epithelial-to-mesenchymal transition (EMT), which is conserved among invertebrates and vertebrates, and which involves conversion of an epithelial cell into a migratory mesenchymal cell (REVENU and GILMOUR 2009; TEPASS and HARTENSTEIN 1994b). Mesenchymal cells can undergo the process of mesenchymal-to-epithelial transition (MET) to form a structured epithelium known as a secondary epithelium (TEPASS and HARTENSTEIN 1994b). Secondary epithelia develop concurrently with or after formation of the basement membrane and receive cues from the basal ECM during their development (TEPASS 1997). During *Drosophila* development, a number of secondary epithelia undergo dramatic changes to give rise to nascent tissues (TEPASS 1997). Of particular interest is the polarized follicular epithelium (FE) of the *Drosophila* ovary that surrounds each maturing oocyte.

Epithelial morphogenesis is a fundamental process that is integral both during development of an organism where nascent tissues are formed and later during adulthood where tissue maintenance and homeostasis are important for function. The mechanisms that govern changes within individual cells (Figure 1.1 A), that later give rise to dramatic changes in tissues
Figure 1.1  Mechanisms of epithelial morphogenesis

(A) Stereotypical apical-basal polarity of an epithelium, here a pair of follicle cells in the Drosophila ovary. The germline cysts are interior on the apical side. DE-Cadherin-rich adherens junctions are apically localized while just basal to them on the lateral surface lie the septate junctions, which are functionally analogous to the tight junctions of vertebrate cells. Note that in vertebrate cells the tight junction is localized apical to the adherens junction. Bundles of basally localized F-actin bundles are found in the follicular epithelium. The ECM is on the outer, basal-most surface of the follicle cells. (B) Two examples of cell shape change that result in tissue morphogenesis of the follicular epithelium where one domain expands at the expense of an opposite domain are flattening and columnarization. The cuboidal epithelium over the nurse cells flattens as it transitions to a squamous epithelium (right). Cuboidal follicle cells that surround the oocyte transition to a columnar epithelium (left). (C) Cell intercalation cause dramatic changes in the organization of cells within a given tissue in a process also known as convergent extension. Pre-stalk cells intercalate to form a single row of disc-shaped cells. (D) During cell migration epithelial cells extend lamellipodia and filopodia at their leading edge. During border cell migration, cell-cell adhesions are modified to allow the cluster of cells to delaminate and migrate together as a single unit.
as a whole, are orchestrated in a manner that is conserved among vertebrates and invertebrates (KNUST and BOSSINGER 2002; SCHOCK and PERRIMON 2002; TEPASS et al. 2001). Much of what is understood about the mechanisms that regulate cell shape change and cell migration has come from studies performed in cell culture and although the results yielded are extremely informative, the application of these findings with regard to living tissues has been challenging. Genetic manipulation studies using Drosophila have been particularly beneficial in identifying the signaling mechanisms that function during epithelial morphogenesis. The results in this thesis focus on the FE of the Drosophila ovary that surrounds each developing germline cyst, in which several mechanisms of epithelial morphogenesis are employed (generation and maturation of the FE will be described in detail in section 1.2).

A driving force of epithelial morphogenesis is cell shape change of individual cells that collectively give rise to changes in tissue morphology (SCHOCK and PERRIMON 2002). These mechanisms include flattening, columnarization, shortening and elongation whereby one membrane or domain elongates or expands while the opposite domain concomitantly shortens or flattens (Figure 1.1 B, C) (KNUST and BOSSINGER 2002). An example of cell shape change in the FE is the transition from a cuboidal to a columnar epithelium in follicle cells that surround the oocyte and a concurrent transition of cuboidal follicle cells that surround the nurse cells to a squamous epithelium (HORNE-BADOVINAC and BILDER 2005). For these processes individual cells within the tissue undergo dynamic cytoskeletal rearrangements in order to change shape but there is no effect on cell position (SCHOCK and PERRIMON 2002). Another mechanism of epithelial morphogenesis involves changes in the cytoskeleton but also relies on breaking and remaking cell-cell adhesion complexes, including Cadherin-mediated adhesion, to allow for cells to move past one another and reorganize themselves into a new epithelial arrangement (Figure 1.1 C) (KNUST and BOSSINGER 2002; REVENU and GILMOUR 2009; SCHOCK and PERRIMON 2002; TEPASS 1997). Examples of this include intercalation, ingression, egression and epithelial sheet fusion. Many of these mechanisms result in a process known as convergent extension whereby polarized cells break their adhesions and intercalate along a given axis causing elongation along
the perpendicular axis (REVENU and GILMOUR 2009; SCHOCK and PERRIMON 2002; TEPASS 1997). During interfollicular stalk formation, pre-stalk cells are arranged in a cluster of 6-8 cells that intercalate to form a single row of disc-shaped cells (HORNE-BADOVINAC and BILDER 2005). The last mechanism of epithelial morphogenesis that will be discussed is cell migration where in addition to changes in the cytoskeleton, changes in polarity and adhesive properties are also required to allow a cell or group of cells to migrate (BROWN 2000; KNUST and BOSSINGER 2002; SCHOCK and PERRIMON 2002; TEPASS and HARTENSTEIN 1994b; TEPASS et al. 2001). The leading edge cells acquire characteristics of mesenchymal cells and extend actin-based protrusions required for cell migration (FULGA and RORTH 2002; MURPHY and MONTELL 1996). In the FE a group of cells called border cells at the anterior pole detaches from the FE and migrate posteriorly through the nurse cells to the oocyte (Figure 1.1 D) (FULGA and RORTH 2002; HORNE-BADOVINAC and BILDER 2005; MONTELL 2003; MURPHY and MONTELL 1996). Border cell migration has been studied extensively as an in vivo model for cell migration (FULGA and RORTH 2002; MONTELL 2003; MURPHY and MONTELL 1996; TANENTZAPF et al. 2000).

1.2. *Drosophila* oogenesis

*Drosophila* oogenesis has been widely used as a model to study epithelial morphogenesis as it involves interactions between cell types of different origins and the epithelium that surrounds the germline cells displays characteristic apical-basal polarity. *Drosophila* oogenesis occurs in the ovary which is comprised of 16-20 identical tube-like structures called ovarioles, each containing an assembly line of egg chambers with the youngest egg chambers at the anterior end and the oldest egg chambers at the posterior end (Figure 1.2 A, B) (KING 1970). Egg chambers, surrounded by a monolayer of cuboidal follicular epithelial cells (Figure 1.2 C), move towards the posterior end as they develop through 14 well-characterized stages until they become mature eggs that enter the oviduct, are fertilized and then laid by the
Figure 1.2  Overview of Drosophila oogenesis

Anterior is to the left. A) Schematic representation of a Drosophila ovariole. At the anterior-most end is the germarium, which houses the germline stem cell (GSC) and follicle stem cell (FSC) that give rise to all the cells of the developing egg chambers. As egg chambers bud off from the germarium the germline cells grow and the surrounding follicle cells divide and grow to accommodate this expansion through 14 well-characterized stages. Egg chambers of different stages are separated by a short row of stalk cells. B & C) Egg chambers are stained with phalloidin. B) Ovariole from a wild-type ovary. C) Stage 7 wild-type egg chamber showing the organized monolayer of follicle cells and slight elongation of the once spherical egg chamber. Scale bars: 50µm.
female (King 1970; Spradling 1993).

Egg chambers first arise from germline stem cells (GSCs) located at the anterior-most end of the ovariole in a structure called the germarium (Figure 1.2A & 1.3 A, B). Two to three GSCs located at the anterior tip of the germarium in region 1 divide asymmetrically to give rise to one GSC which remains in its niche and one daughter cystoblast that is immediately encased by squamous escort cells, which help move newly formed cysts posteriorly through the germarium by passing them from one escort cell to the next (Morris and Spradling 2011). Escort cells were originally hypothesized to arise from 5-6 anteriorly located escort stem cells that divide and move along with cysts as they travel posteriorly through the germarium, then undergo apoptosis at the follicle stem cell (FSC) niche where they are replaced by follicle cells (Morris and Spradling 2011). More recently lineage-tracing analysis has not been able to generate patterns consistent with that of a stem cell lineage (Kirilly et al. 2011) and in vivo escort cell tracking experiments show that they remain in a fixed position, actively pass cysts posteriorly and either get recycled or undergo apoptosis once the germline cysts have completed their posterior movement though region 1 and 2a (Morris and Spradling 2011). During this posterior movement through region 2a, daughter cystoblasts undergo 4 rounds of incomplete mitosis to form 16-cell cysts with a structure called the fusome connecting each of the 16 cells (Chen and McKearin 2003a; Spradling 1993). Once the cyst is passed to the last and posterior-most set of escort cells it flattens to form a lens-shaped disc that spans the whole width of the germarium at the region 2a/2b boundary located approximately halfway down the length of the germarium. Here, flattened cysts encounter two FSCs on opposite sides of the germarium, which encapsulate one cyst at a time in a monolayer of follicle cells (Kirilly and Xie 2007; Nystul and Spradling 2007). Once cysts reach the FSCs the germline cells continue to move posteriorly but stop dividing. Instead, the nurse cells enter into an endoreplicative and growth phase while the oocyte enters meiosis and remains in Prophase I (King and Vanoucek 1960). Each of the FSCs divide asymmetrically to each produce one daughter cell that collectively gives rise to approximately 80 cuboidal follicle
Anterior is to the left. A) Schematic of the organization of the *Drosophila* gerarium. B) Wild-type gerarium stained with phalloidin. The gerarium can be divided into four regions. At the anterior-most end is region 1 where the GSCs (green) reside along with their niche cells, the terminal filaments (white) and cap cells (yellow). GSCs divide asymmetrically to produce a GSC and a daughter cell that differentiates as a cystoblast (light green), which is immediately surrounded by escort cells (blue). In region 2a the cystoblast undergoes four rounds of incomplete mitosis to produce a 16-cell cyst that is interconnected by a structure called the fusome (black, branch-like structure). At the region 2a/2b border the cyst flattens and is passed from the last escort cell to the FSCs (dark pink) located at the 2a/2b border (asterix in ‘B’ denotes likely position of the FSC). In region 2b FSCs divide to produce an FSC and a daughter cell that either migrates posteriorly (light pink) or cross-migrates (light pink, starred) across the width of the gerarium to encapsulate the cyst. Daughter follicle cells continue dividing in region 3 where the now stage 1 egg chamber buds off from the gerarium.
cells that will encapsulate the developing germline cyst by the time it leaves the germarium as a stage 1 egg chamber (HORNE-BADOVINAC and BILDER 2005). As oogenesis continues the germline cells within the egg chamber increase in volume and the surrounding FE accommodates this expansive growth by continuously dividing until stage 6 upon which mitosis stops leaving the germline cells encased by approximately 650 follicle cells. Egg chambers begin to elongate at stage 6 along the anterior-posterior axis through actomyosin contractility of the basal F-actin in the follicle cells and ceases at stage 10a (GUTZEIT 1991). Dramatic changes in position and cell shape of follicle cells begin during stage 9 and continue through to stage 14 to accomplish critical developmental events necessary for proper egg formation (SPRADLING 1993). A more detailed look at the morphogenetic changes occurring in the FE will be discussed in section 1.6.

1.3. Stem cells and their niches

The field of stem cell research has grown exponentially in the past decade largely due to interest in their clinical applications with regards to human disease. Stem cells can be classified into two main categories; embryonic stem cells, whereby stem cells are pluripotent and have the ability to differentiate into any tissue type, and adult stem cells, which reside in differentiated tissues but are undifferentiated and have the ability to give rise to any of the specialized cells within a given tissue (EVANS 2011; LOSICK et al. 2011). Adult stems cells in humans have been found in a large number of tissues and organs including skeletal muscle, heart, gut and the ovarian epithelium. Stem cells found in adult tissues are required to maintain tissue homeostasis and to respond to injury, however the mechanisms that govern stem cell maintenance vary dramatically among tissue types and across species. The ability to divide asymmetrically to produce one self-renewing daughter stem cell and one differentiating daughter cell is a common theme that is carried across all species in all stem cells. As important as the stem cell itself are the cells that make up the niche, a microenvironment in a restricted location that supports the
self-renewing division of stem cells, thereby preventing them from differentiation (LIN 2002; SCHOFIELD 1978). The *Drosophila* gonad, with its relatively simple and well-characterized structure, has provided insight into the structure of the niche and the signalling mechanisms that govern stem cell anchoring, self-renewal and maintenance (LIN 2002).

There are two general categories of stem cell niches, stable and flexible. Stable otherwise known as classic niches, are made up of a small number of stromal cells within a given tissue that can exist in the presence or absence of stem cells and can indefinitely control stem cell self-renewal and differentiation (KAI and SPRADLING 2003; KAI and SPRADLING 2004; SPRADLING et al. 2001; WILSON and TRUMPP 2006; XIE and SPRADLING 2000). Stable niches are commonly fixed in a specific location within the tissue (LIN 2002; PEARSON et al. 2009) and an example of this is the *Drosophila* germline stem cell niche. Flexible niches make up the second category of stem cell niches and generally have an architecture and position that is less clearly defined with no obvious differentiated support cells within the tissue in which they reside (SPRADLING et al. 2001). In these niches, stem cells may have the ability to attach themselves to the basal lamina as observed with the *Drosophila* intestinal stem cell (OHLSTEIN and SPRADLING 2006). Since these stem cells do not have a positionally-defined microenvironment it is possible for them to change location in response to signals, thereby allowing them to function where and when they are needed (SPRADLING et al. 2001). Overall, studies in *Drosophila* have made important contributions to our understanding of stem cell niche regulation.

1.4. Regulation and maintenance of the *Drosophila* germline stem cells

The *Drosophila* ovarian GSC was one of the first stem cell niches identified and has been an excellent model to examine how adult stem cells are regulated *in vivo* (XIE and SPRADLING 1998; XIE and SPRADLING 2000). The simple structure of the germarium, in addition to the
availability of genetic tools, has allowed for easy identification of the GSCs and their supporting
niche cells, making the Drosophila GSC niche a desirable system to understand how stem cells
are regulated in higher organisms. The Drosophila germarium contains 2-3 GSCs and its niche is
comprised of three somatically derived cell populations, terminal filament (TF) cells, cap cells and
escort cells (Xie and Spradling 2000) (Figure 1.4).

GSCs are physically maintained in their anterior-most position in the germarium via DE-
Cadherin mediated adhesion to the cap cells. Anchoring of the GSCs to the cap cells is essential
for their maintenance and function (Song et al. 2002). GSC divisions occur along the anterior-
posterior axis whereby one daughter cell lies more anterior than the other. The anterior daughter
remains in the niche anchored by DE-Cadherin, while the other more-posterior daughter divides
away from the cap cells and differentiates into a cystoblast (Song et al. 2002). The Bone
Morphogenic Protein (BMP) pathway has been shown to play an important role in controlling
GSC function (Song et al. 2004; Xie and Spradling 1998). Two BMP-family ligands, encoded by
the genes decapentaplegic (Dpp) and glass bottom boat (Gbb), are expressed in the cap cells
and form a steep gradient of BMP at the anterior-most end of the germarium (Kiger et al. 2001;
Song et al. 2004; Song et al. 2002; Xie and Spradling 1998). Dpp/Gbb secreted by the cap
cells, act specifically on the GSCs through the canonical BMP signalling pathway to repress
transcription of the pro-differentiation gene bag of marbles (bam), thereby preventing their
differentiation and maintaining them in the niche (Chen and McKearin 2003b; Song et al. 2004).
The daughter cell that is not anchored to the cap cells is only one cell diameter away from the
niche but is distant enough from the GSC to not receive a BMP signal. This allows for
transcription of bam and differentiation of the daughter cystoblast, which will divide four times to
produce a 16-cell cyst, in which cells are interconnected by the fusome (Chen and McKearin
2003a). Although BMP signalling has been shown to work at long distances in other
developmental contexts, its activity in the germarium is dramatically restricted to regulate GSC
maintenance (Harris and Ashe 2011).
Figure 1.4  Germline stem cell maintenance

A) Schematic of GSC regulation. GSCs (dark green) are anchored to cap cells in their niche by DE-Cadherin mediated adhesion (purple). Cap cells (yellow) express Hh (teal), Dpp (red) and Gbb (red) that bind their appropriate receptors on the GSC to regulate self-renewal. Activation of the BMP pathway functions at a short distance to repress transcription of bam, a differentiating factor. This inhibits the GSC from differentiating. The daughter cystoblast (light green) that divides away from the niche differentiates as it is out of range to receive the Dpp signal. bam is no longer repressed, allowing for differentiation to occur in the cystoblast. B) Wild-type germarium stained with anti-Hts (red) and anti-Bam (green) antibodies. Dashed lines represent the anterior tip of the germarium where GSCs are located. The expression of Bam is restricted to the early cystoblast and is notably absent from the GSCs at the anterior-most end. Bam protein is not detectable with the antibody in all cystoblasts. Spec: spetrosome. Fus: fusome.
In addition to BMP signalling, expression of $fs(1)Yb$, $piwi$ and $hedgehog$ in the niche cells has been shown to be important for GSC self-renewal (Cox et al. 1998; Cox et al. 2000; King and Lin 1999; King et al. 2001). Jak/Stat signalling and Delta-Notch signalling have been shown to be important for maintenance of the niche (Lopez-Onieva et al. 2008; Song et al. 2007; Wang et al. 2008; Ward et al. 2006) and more recently Ecdysone signalling was shown to regulate GSC differentiation and stem cell niche size (Konig et al. 2011).

1.5. The follicle stem cell and its niche

Compared to the GSC, the follicle stem cell (FSC) is less well understood. The existence of the FSC has been known for many years (Margolis and Spradling 1995) but the absence of specific markers and problems with establishing a stereotypic morphology and location have impeded progress in characterizing the FSC niche. However, recent work performed by several groups has provided insight into the function of the FSC, thereby establishing the FSC niche as an excellent model for studying epithelial stem cell niches. As with other stem cells, the FSCs are characterized by their ability to divide asymmetrically, yielding another FSC for stem cell self-renewal and a differentiating daughter cell that will give rise to all of the somatic cells that make up a functional ovariole downstream of the FSCs. Using lineage tracing analysis, exactly 2 FSCs were identified and reside in fixed niches that are found on opposite sides of the germarium (Nystul and Spradling 2007) (Figure 1.5 A). The FSC niche is likely comprised of the escort cell that is immediately adjacent to the FSC, the flattened germline cyst, its own daughter follicle cell and the daughter cells of the opposite FSC (Nystul and Spradling 2007). Given that it is known that the escort cells maintain a fixed position, the posterior-most escort cells residing at the region 2a/2b border likely aid in defining the FSC niche (Morris and Spradling 2011). What makes the
Figure 1.5  Organization and regulation of the follicle stem cell niche

A) GSCs and cystoblasts are not depicted. Blue region represents the escort cell population in region 1 and 2a. Cap cells (yellow) secrete Hh (teal) and Wg (dark blue), which bind to their appropriate receptors on the FSC (dark pink). The germarium contains 2 FSCs that reside on opposite sides of the germarium at the 2a/2b border. One FSC daughter (pink) migrates posteriorly while the other FSC daughter (pink, starred) cross-migrates across the width of the germarium to the opposite FSC. B) FSCs are anchored to niche cells, the escort cells (blue) by DE-Cadherin mediated adhesion (purple). Hh and Wg signaling are active in the FSC. Laminin A (grey) secreted by the FSC activates integrin $\alpha_{PS1}\beta_{PS}$ (yellow, orange) to anchor the FSC to the ECM and to promote FSC proliferation. C) Wild-type germarium stained with anti-Laminin A antibody (red) and FITC-phalloidin (green). While Laminin A is expressed basally around the entire germarium, it is more highly expressed in the follicle cells beginning at the region 2a/2b border.
FSC niche unique from other niches described in section 1.3, is that two of the components of the niche are transient. The germline cyst and the daughter cells eventually move away from the niche, suggesting that this niche holds properties uniquely different from those of stable niches.

Much like the GSCs, the FSCs are anchored to one of their niche cells, here the posterior-most escort cells, by DE-Cadherin mediated adhesion (SONG and XIE 2002). Although not well characterized, the signalling mechanisms that have been found to regulate the FSC do not involve cells that are directly neighbouring the FSC as observed with the GSC, but instead involve cells located relatively far away. The cap cells and TF cells secrete Hedgehog (Hh) and Wingless (Wg), which have been shown to be important for FSC maintenance and follicle cell proliferation (FORBES et al. 1996a; KAI and SPRADLING 2003; KING et al. 2001; SONG and XIE 2003; ZHANG and KALDERON 2001). This suggests that the FSC niche may be comprised of more than just the cells immediately adjacent to the FSC and might exist as a microenvironment within the germarium (PEARSON et al. 2009). The function of the two FSCs is to give rise to daughter cells that migrate in two distinct patterns and then divide further to fully encapsulate germline cysts. The daughter cell of one FSC migrates in a posterior fashion while the daughter from the opposite FSC cross-migrates across the width of the germarium until it reaches the other FSC (NYSTUL and SPRADLING 2007), a process that is in part governed by -Delta-Notch signalling (NYSTUL and SPRADLING 2010). Interestingly the cross-migrating daughter is capable of displacing the opposite FSC, thereby replacing and “evicting” the old FSC from its niche, a process that again requires Notch signalling (NYSTUL and SPRADLING 2007; NYSTUL and SPRADLING 2010). FSC replacement is rare but demonstrates that a cross-migrating daughter cell may not yet be completely differentiated into a follicle cell and thereby may still retain some 'stemness', having the ability to either differentiate into a daughter follicle cell or remain as a stem cell and replace the opposite FSC. The posteriorly migrating daughter gives rise to the follicle cells that lie on the posterior side of the newly encapsulated cysts while the cross-migrating daughter gives rise to the anterior face of follicle cells (NYSTUL and SPRADLING 2007).
As mentioned previously, there are no known markers for the FSCs and so their position has largely been inferred through lineage tracing analysis and the expression patterns of various proteins within the germarium (Kirilly et al. 2011; Margolis and Spradling 1995; Nystul and Spradling 2007). More recently the FSCs have been shown to contribute to the formation of their own niche through secretion of the ECM protein Laminin A, which incorporates into the basal lamina and activates αPS1βPS integrin on the surface of the FSC (Figure 1.5B). This interaction anchors the FSC to the underlying actin cytoskeleton at its well-characterized position at the 2a/2b border and also controls FSC proliferation (O’Reilly et al. 2008). Germaria stained with an antibody against Laminin A shows that Laminin is basally present at low levels around the anterior end at region 1 and 2a, whereas high levels of Laminin are seen beginning at the region 2a/2b border (Figure 1.5C), suggesting that it is a critical component of the FSC niche (O’Reilly et al. 2008). Together the current published results demonstrate that the FSC niche is an example of a dynamic somatic stem cell niche in which the stem cell microenvironment demonstrates characteristics of both classic and flexible stem cell niches.

An unresolved question is how the FSC niches become positioned at the region 2a/2b border. Several studies have demonstrated that in agametic ovaries lacking GSCs and escort cells, the FSCs become mislocalized to the anterior-tip of the germarium where they function normally to produce daughter follicle cells and generate an ovary that is solely comprised of somatic cells (Besse et al. 2005; Kai and Spradling 2003; Kirilly et al. 2005; Margolis and Spradling 1995; Song et al. 2004). This suggests that germ cells and/or escort cells contribute to the determination of niche position but are not necessary for FSC proliferation. Given that the GSC generates the ‘target’ for the FSC to cover with follicle cells, it makes sense that the two niches may be functionally interconnected.

Specification of the FSC and establishment of the niche has yet to be resolved. Beginning in the ovary of the third instar larvae, GSCs are present and are in contact with newly established terminal filaments that are easily visualized as the ovary begins to develop (Godt and Laski 1995; Spradling 1993). Germline cells in the pupal ovary are observed to be
intermingled with somatic cells, and undergo four rounds of incomplete mitosis, just as they do in
the adult gerarium, to form the very first egg chambers for a given ovariole. Just basal to the
first egg chamber that develops lies a population of somatic cells in the larval ovary that
intercalate to form a distinct row of cells known as the basal stalk, a transient structure that exists
at the posterior-most end of each developing ovariole (GODT and LASKI 1995). The first and
posterior-most egg chamber forms continuously with the basal stalk (BESSE et al. 2005)
suggesting that the FSC and its niche are specified at some time during pupal ovary development
either before formation of the basal stalk or after its formation but just prior to posterior-
progression of the first germline cyst. This ‘black box’ during gonadogenesis has left a noticeable
gap in the current understanding of how oogenesis works. More specifically, nothing is yet known
about how the FSC and its niche cells are specified during larval/pupal development. A more
detailed description of ovary development is presented in section 3.5.

1.6. Morphogenesis of the follicular epithelium

The follicular epithelium of developing egg chambers has been an especially informative
model for studying epithelial morphogenesis. Continued work on the FE during oogenesis will
ultimately lead to a complete understanding of the “life of an epithelium” from its derivation from a
FSC through to its morphogenesis and on to its final roles in the construction of eggshell
components (HORNE-BADOVINAC and BILDER 2005). During development of the FE, follicle cells
continuously divide until stage 6 to encapsulate each germline cyst in a monolayer of epithelial
cells. Hereafter, the egg chambers begin to elongate along their anterior-posterior axis in a
mechanism that will be further described in detail in the following section. During stage 9 the
follicle cells undergo dramatic changes in cell shape and morphogenetic movement, with different
populations of follicle cells undergoing different reorganization events (Figure 1.6). Main body
follicle cells undergo a cuboidal to columnar transition in a wave of morphogenesis that begins
Figure 1.6 Movement of follicle cells during mid-oogenesis

(A) Schematic of a late stage 9 egg chamber depicting the posterior migration of main body follicle cells and their transition from a cuboidal to columnar epithelium over the oocyte, and from a cuboidal to a squamous epithelium over the nurse cells. Border cells have also begun their posterior migration between the nurse cells towards the oocyte. Migration of follicle cells and border cells continues through stage 10A. (B) Schematic of a stage 10b egg chamber in which the main body follicle cells and border cells have completed their posterior migrations. Columnar cells at the anterior edge of the oocyte also have begun to migrate inwards along the anterior edge of the oocyte. (C) Wild-type stage 10a egg chamber stained with anti-Scribble antibody marking the lateral membranes of the follicle cells and border cells. Arrow points to the newly formed squamous epithelium. Arrowhead points to border cell/ polar cell cluster.
with the cells at the posterior-most end and moves anteriorly, thereby causing the cells to migrate posteriorly towards the oocyte. By the end of this process only 50 follicle cells remain around the nurse cells and have been stretched to form a squamous epithelium (Figure 1.6A) while all the remaining columnar follicle cells cover the growing oocyte completely (Figure 1.6B) (SPRADLING 1993). At the same time as this posterior migration of the FE a subset of anterior follicle cells, called border cells delaminates from the FE and migrate between the nurse cells towards the oocyte, carrying a non-migratory anterior pair of polar cells within them (Figure 1.6 A, C) (MONTELL et al. 1992). At stage 10B, the columnar epithelium at the junction where the nurse cells meet the oocyte migrates centripetally along the anterior edge of the oocyte to accommodate the increasing volume of the oocyte (SCHOTMAN et al. 2008). In an effort to further allow for this growth, the columnar epithelial cells stretch and flatten out while transiently exposing a portion of their basolateral membranes to the ECM (SCHOTMAN et al. 2008). When the columnar follicle cells near completion of their posterior migration they begin to secrete components required for eggshell development and formation of specialized structures including the two dorsal appendages, the operculum and the micropyle, that are critical for proper egg and/or embryo development (DOBENS and RAFFERY 2000; WARING 2000) (reviewed in (HORNE-BADOVINAC and BILDER 2005)).

1.7. Basal F-actin of the follicular epithelium

In middle-stage egg chambers, after the follicle cells have ceased dividing, the F-actin network polarizes along the basal side of the follicle cells where it forms bundles of filaments aligned perpendicular to the anterior-posterior (A-P) axis of the egg chamber (GUTZEIT 1990; GUTZEIT 1991; GUTZEIT and HAAS-ASSENBAUM 1991). The role of the F-actin bundles during egg chamber elongation in middle-staged egg chambers has been characterized through analysis of mutants in which these bundles are disrupted, and has led to a model in which the polarized actin
bundles act as a ‘molecular corset’ to promote elongation of the egg chamber along the A-P axis through actomyosin contractility along the basal surface of the FE (Figure 1.7) (DENG et al. 2003; MIROUSE et al. 2009; VIKTORINOVA et al. 2009). This pattern of polarized actin is first established during stages 5-6 at the anterior- and posterior-most ends when egg chambers are virtually completely spherical, then spreads circumferentially around the egg chamber and lasts through stage 14 (FRYDMAN and SPRADLING 2001; GUTZEIT 1990). Evidence provided by several groups shows that regulation of the basal F-actin within each follicle cell occurs non-cell-autonomously whereby clones of mutant follicle cells with disrupted actin bundles can affect the basal F-actin in neighbouring wild-type cells and vice versa (BATEMAN et al. 2001; CONDER et al. 2007).

It has been noted that the basal F-actin bundles in the FE are similar to the stress fibers of mammalian cultured cells, and the FE provides an attractive system for the genetic analysis of the signaling events regulating the formation of parallel actin bundles (BATEMAN et al. 2001; BAUM and PERRIMON 2001). Stress fibers consist of 10-30 bundled actin filaments anchored at integrin-based focal adhesions (reviewed in (PELLEGRIN and MELLOR 2007)). Stress fiber formation is regulated by the Rho family small GTPase RhoA through the Rock-Rok-Rho kinase family of serine/threonine kinases (hereafter referred to as Rok) and the Diaphanous-related formin, mDia1 (LEUNG et al. 1996; WATANABE et al. 1999; WATANABE et al. 1997). Rok promotes stress fiber formation and actomyosin contractility by directly phosphorylating myosin light chain (MLC) and phosphorylating the regulatory myosin binding subunit of MLC phosphatase, thereby inhibiting the phosphatase activity (AMANO et al. 1996; KAWANO et al. 1999; KIMURA et al. 1996; TOTSUKAWA et al. 2000). Rok also phosphorylates LIM kinase, which in turn phosphorylates cofilin, inactivating its actin-depolymerizing function (OHASHI et al. 2000; SUMI et al. 2001). This signaling network regulating actomyosin contractility is conserved in Drosophila, where it has a number of roles in development (reviewed in (SETTLEMAN 2001)).

In addition to Rho-activated formation of stress fibers, regulation by integrin-mediated adhesion has also been observed (BARRY et al. 1997) (reviewed in (DEFILIPPI et al. 1999)).
The basal F-actin of the follicular epithelium acts like a ‘molecular corset’ around the circumference of the developing egg chamber. Left panels are schematics of the phalloidin stained wild-type egg chambers on the right. Red represents the basal F-actin. A) Stage 4 egg chamber has not yet started to elongate and therefore is spherical. B) Basal view of the outer surface of a stage 8 egg chamber shows the parallel organization of the basal F-actin filaments perpendicular to the A-P axis. C) Actomyosin contractility along these bundles causes elongation of the maturing egg chamber.
Integrins are transmembrane proteins that exist as heterodimers of α and β subunits (reviewed in (BROWN et al. 2000)) in which the extracellular domain interacts with the extracellular matrix and the intracellular domain interacts with the actin cytoskeleton through focal adhesion complexes (BROWN et al. 2000; PETIT and THIERY 2000). As single-pass transmembrane proteins, integrins function to relay signals bidirectionally. During inside-out signalling cytoplasmic proteins interact with the intracellular domains of integrin and causes the extracellular domains to have a higher affinity for ECM molecules such as laminins and collagens (BROWN et al. 2000; PETIT and THIERY 2000). During outside-in signalling interaction of the ECM with the extracellular domains causes the intracellular domains of integrin to interact with cytoplasmic proteins such as those that link integrins with the cytoskeleton (reviewed in (BROWN et al. 2000)).

Given the relationship between integrins and the cytoskeleton a lot of research has been focused on elucidating the mechanisms by which the basal F-actin bundles of the FE are organized in relation to the ECM. Seven Drosophila integrin subunits have been identified, five α-subunits, αPS1-5, and two β subunits, βPS1 and βν (BROWN 1994)(reviewed in (BROWN et al. 2000)). Work on the integrin subunits show that while αPS1, αPS2 and βPS integrins are related to their respective vertebrate α and β integrin subunits, αPS3-5 are equally unrelated to all integrin α subunits in vertebrates (BROWN et al. 2000). The αPS3-5 subunits have been shown to participate during late oogenesis in the determination of final egg length (DINKINS et al. 2008). The βν subunit has recently been shown to mediate phagocytosis of apoptotic cells in embryos (NAGAOSA et al. 2011). The best characterized subunits are the two α-Integrin subunits, αPS1, encoded by the gene multiple edematous wings (mew) and αPS2, encoded by the gene inflated (if), and one β-integrin subunit, βPS, encoded by the gene myospheroid (mys) (WEHRLI and TOMLINSON 1995). The two possible combinations of α and β subunits, either PS1 (αPS1 βPS) or PS2 (αPS2βPS) are differentially expressed throughout various tissues (BROWN et al. 2000). Both the PS1 and PS2 heterodimers localize to the basal surface of the FE beginning in the germarium (O’REILLY et al. 2008) and are localized to the ends of the basal F-actin filaments (BATEMAN et al. 2001; CONDER et al. 2007; FRYDMAN and SPRADLING 2001). In addition to integrin βPS, which is
required for egg chamber elongation, (BATEMAN et al. 2001; DUFFY et al. 1998) mutations in several other genes that disrupt the association of the ECM with the actin cytoskeleton cause defects in egg chamber elongation. These include the receptor-like tyrosine kinase Dlar (BATEMAN et al. 2001; FRYDMAN and SPRADLING 2001), the ECM component Dystroglycan (DENG et al. 2003) and the Rac/Cdc42 effector kinase, Pak (CONDER et al. 2007). Of particular interest is how these components work together to establish and maintain the polarized organization of the F-actin.

1.8. *Drosophila* egg chamber rotation as a mechanism for egg chamber elongation

Much of what is known about morphogenesis of the FE and the egg chamber as a whole has come from studies involving fixed tissue samples. Recent work on live tissues from David Bilder’s lab has provided insight on how the morphogenetic changes occurring within the follicle cells can function collectively to drive egg chamber elongation (HAIGO and BILDER 2011). In recent years live imaging of *Drosophila* egg chambers has advanced the study of cell migration in an *in vivo* context, but has been primarily focused on the migration of the border cells across the egg chamber from the anterior FE, between the nurse cells to the anterior end of the oocyte (PRASAD et al. 2007; TEKOTTE et al. 2007). The application of these live imaging techniques to various stages of oogenesis has led to the discovery of a new and extraordinary morphogenetic movement: developing egg chambers rotate around their lengthening A-P axis and lay down polarized ECM as they do so (Figure 1.8) (HAIGO and BILDER 2011). Mutants for the integrin βPS subunit or the ECM component collagen IV fail to rotate and elongate to generate spherical eggs (BATEMAN et al. 2001; HAIGO and BILDER 2011). These results suggest that secretion of and interaction with ECM components is required for egg chamber rotation and egg chamber elongation. Interestingly Haigo and Bilder suggest that the basal F-actin might provide only a minor contribution to this process and it is the polarized ECM, created by rotation, that functions
Rotation begins in stage 5 egg chambers. Within a given ovariole, the direction of egg chamber rotation about the anterior-posterior axis is independent from one egg chamber to the next. As egg chambers rotate circumferentially around their lengthening axis, polarized ECM components are laid down in the same direction. Formation of a polarized ECM around the egg chamber is required for elongation of the egg chamber.
Rotation

Formation of polarized ECM

Elongation
as the “molecular corset”, in contrast to actomyosin contractility. The revelation that the FE circumferentially migrates across the ECM and causes egg chambers to spin around their A-P axis has increased interest in this field and reveals tissue revolution as a new mechanism for epithelial morphogenesis. These recent findings urge groups to revisit the mechanisms that govern egg chamber elongation and ask old questions but with a new ‘spin’.

1.9. Rho family of small GTPases

The master regulators of actin cytoskeletal rearrangements are the Rho subfamily of small GTPases, which belong to the Ras-related superfamily of small GTPases. Rho, Rac and Cdc42 are members of the Rho subfamily that are highly conserved throughout eukaryotes, and are well-characterized members and shown to be regulated in a similar manner. These proteins are of low molecular weight and have similar the amino acid sequences in their effector domain ranging between 35-41 amino acids (KAIBUCHI et al. 1999). Rho GTPases function as molecular switches that cycle between an inactive GDP-bound state and an active GTP-bound state (HALL and NOBES 2000). The exchange of GDP for GTP is facilitated by guanine nucleotide exchange factors (GEFs). GTPase activating proteins (GAPs) stimulate the intrinsic GTPase activity of RhoGTPases to hydrolyze GTP to GDP. It remains unclear how GEFs and GAPs are universally regulated but it is likely related to how Rho family GTPases are spatially and temporally activated within a given cell or tissue (HALL 2005). Some RhoGEFs can activate multiple GTPases while others specifically activate a single target (KAIBUCHI et al. 1999) and similarly some GAPs can show activity towards multiple Rho family GTPases while others are more specific. Additional regulators at the level of the GDP/GTP exchange reaction are the GDP dissociation inhibitors (GDI), which inhibit the dissociation of GDP from RhoGTPases (HALL and NOBES 2000).

The field of cytoskeletal regulation has been of great interest to many avenues of research and the signaling pathways linking Rho, Rac and Cdc42 to the regulation of the actin
cytoskeleton are well established. Activated Rho promotes formation of stress fibers, bundles of
10-30 actin filaments that stretch across a cell, as mentioned previously. Activation of Rac
promotes the formation of lamellipodia and membrane ruffling, while activated Cdc42 induces the
formation of filopodia and both GTPases in this way contribute to cell migration (NOBES and HALL
1994; RIDLEY and HALL 1992; RIDLEY et al. 1992). By and large, the function of the Rho GTPases
and the processes in which they participate in such as cell shape change, cell division, cell
migration and adhesion, membrane trafficking, embryonic development, tissue regeneration and
epithelial morphogenesis are conserved among eukaryotes (HALL 1998). Put simply, the main
role of Rho, Rac and Cdc42 is to control the dynamic rearrangements of the actin cytoskeleton by
means of assembly and disassembly of actin filaments (HALL 1998; HALL and LALLI 2010; NOBES
and HALL 1995a).

1.10. p21-activated kinases

An important and well-characterized effector of Rac and Cdc42 is the p21-activated
kinase (Pak) (MANSER et al. 1994). Paks are conserved serine/threonine kinases that serve as
important regulators of cell cycle progression, apoptosis, MAPK cascades, cytoskeletal dynamics
and cell motility (BOKOCH 2003). Mammalian Paks are divided into two subfamilies based on their
structural organization and mode of regulation. The group I Paks consist of Paks 1-3 that have an
N-terminal regulatory region and a highly conserved C-terminal kinase domain, and the group II
Paks consist of Paks 4-6, which have significant structural differences and appear to also have
completely different substrates than group I Paks (ARIAS-ROMERO and CHERNOFF 2008; BOKOCH
2003). Regulation of the group I Paks largely relies on the Cdc42/Rac interacting binding (CRIB)
domain, which overlaps with the autoinhibitory domain (AID). When Pak is inactive it exists as a
homodimer in a ‘head-to-tail’ conformation with two Pak molecules bound to each other in
opposite orientations. The kinase domain of one protein interacts with the AID of the other
thereby suppressing each other’s kinase activity (Bokoch 2003). When Rac or Cdc42 are activated, they interact with the CRIB domains in the Pak homodimer causing the two Pak proteins in the homodimer to dissociate from one another, undergo autophosphorylation and further phosphorylate downstream substrates (reviewed in (Bokoch 2003)).

Much like Rac and Cdc42, Pak has also been implicated as a critical regulator of cytoskeletal dynamics (Manser et al. 1997; Manser et al. 1994; Sells et al. 1997). Pak was initially identified as a modulator of the actin cytoskeleton in mammalian cell culture studies whereby over-expression of Pak caused formation of filopodia, lamellipodia, membrane ruffling and an increase in focal adhesion turnover (Manser et al. 1997; Sells et al. 1997). Interestingly, the effects that Pak has on the actin cytoskeleton have been shown to be both dependent and independent of its kinase function (Arias Romero and Chernoff 2008; Bokoch 2003; Daniels et al. 1999). Several of the best-characterized substrates of mammalian Pak1 are key components of actin cytoskeletal regulation (reviewed in (Arias Romero and Chernoff 2008; Bokoch 2003)). Pak’s specificity for these substrates is relatively similar across species (Brzeska et al. 1999), suggesting that these interactions previously identified in mammalian cell culture likely also occur in Drosophila. The most-well characterized substrates for Pak include myosin light chain kinase (MLCK) (Sanders et al. 1999), myosin light chain (MLC) and Lim domain kinase (LIMK) (Yang et al. 1998). Phosphorylation of MLCK by Pak1 decreases MLC phosphorylation and leads to decreased actomyosin contractility (Sanders et al. 1999), whereas phosphorylation of LIMK and MLC leads to increased actomyosin contractility (Edwards et al. 1999), demonstrating that in mammalian cell culture studies, Pak1 can both positively and negatively regulate actomyosin contractility.

Much of the current literature focuses on the regulation and activity of the group I Paks and the same holds true for the Drosophila Paks, which are also divided into two subfamilies. The group I Paks in Drosophila are encoded by the genes pak (Harden et al. 1996) and pak3 (Pirone et al. 2001) and have been implicated in dorsal closure (Bahri et al. 2010; Conder et al. 2004; Harden et al. 1999), photoreceptor axon guidance (Hing et al. 1999), salivary gland formation
(PIRRAGLIA et al. 2010), neuromuscular junction development (ALBIN and DAVIS 2004; OZDOWSKI et al. 2011; PARNAS et al. 2001) and epithelial morphogenesis of the follicular epithelium (CONDER et al. 2007). The single group II Pak, encoded by the gene *mushroom bodies tiny* (SCHNEEBERGER and RAABE 2003) that has been implicated in the regulation of adherens junctions and the actin cytoskeleton during photoreceptor epithelium morphogenesis (MENZEL et al. 2007). Work done by our lab has further characterized a role for the group I Paks during dorsal closure of the *Drosophila* embryo (BAHRI et al. 2010; CONDER et al. 2004). The group I Paks are required for cell shape change, cell-cell junction establishment and actin cytoskeletal integrity at the leading edge of the migrating epidermis during dorsal closure (CONDER et al. 2004; HARDEN et al. 1996; HARDEN et al. 1999). Rac, Cdc42 and Pak regulate actin-based filopodia and lamellipodia at the leading edge of the dorsal-most epidermal cells required to seal the dorsal hole of the embryo (GENOVA et al. 2000; HAKEDA-SUZUKI et al. 2002; HARDEN et al. 1996; HARDEN et al. 1999; JACINTO et al. 2000). The group I Paks are also involved in the formation of septate junctions between the newly sealed epidermal flanks at the end of dorsal closure, which points to a role for Pak in apical-basal polarity specification in secondary epithelia (BAHRI et al. 2010), and this is borne out by studies in the follicular epithelium (appendix 7.2) (CONDER et al. 2007).

1.11. Pak’s role in *Drosophila* oogenesis

Work performed by a previous graduate student in the lab, Ryan Conder, showed that Pak functions during the development of the FE (CONDER et al. 2007). In females transheterozygous for hypomorphic *pak* alleles, the organization and density of the basal F-actin is disrupted and complete loss of the basal F-actin is observed in *pak* mutant follicle cell clones (CONDER et al. 2007). *pak* transheterozygous mutants have a striking rounded egg chamber phenotype in which egg chambers remain spherical and never elongate along the anterior-posterior axis, which is presumably due to the severe disruptions in the basal F-actin (CONDER et
The basal F-actin of follicle cells show end-to-end alignment with neighbouring cells and previous work has shown that Pak functions non-cell-autonomously to regulate the basal F-actin bundles (CONDÉR et al. 2007). Pak is also required for apical-basal polarity of the FE, likely as a component of the Scribble complex (BAHRI et al. 2010), which is an important component in the determination of apical-basal polarity. Multilayering of the FE is seen in pak mutants, a phenotype commonly seen in mutants that disrupt epithelial apical-basal polarity (CONDÉR et al. 2007). Finally, a former Harden lab graduate student, Judy Yu, described a striking pak mutant phenotype during oogenesis of side-by-side encapsulation of egg chambers (thesis of H. Yu, 2003). This phenotype has never before been described in the literature.
1.12. Goal of this thesis

The goal of this thesis was to further characterize Pak’s roles during the life of the follicular epithelium, from specification and division of the follicle stem cell, to maturation of the follicle cells during egg chamber elongation. Given that the side-by-side pak mutant egg chamber phenotype was unique and not previously analysed in detail by former members of the lab, I set out to characterize how and when Pak might be functioning during oogenesis, and why loss of pak is able to generate this phenotype. Previous work from this lab has demonstrated that Pak may participate in egg chamber elongation through its regulation of the basal F-actin in follicle cells. pak mutant egg chambers do not elongate along the anterior-posterior axis, and to gain insight into the mechanism by which Pak regulates elongation I screened for second site modifiers that suppressed the pak rounded egg chamber phenotype.
2. Materials & Methods

2.1. Fly stocks

pak6/TM3,Sb, pak11/TM3, Ser and UAS-pakmyr flies were gifts from H. Hing. pak14, pakRNAI FRT82B/TM6B and pak22 FRT82B/TM6B flies were from B. Dickson. pak14, pak3x76a/TM6B was generated in our lab by R. Conder. pakRNAI flies were from the Vienna Drosophila RNAi Center. Trafficjam Gal4 flies were from G. Tanentzapf and D. Godt. PZ1444 enhancer trap line was from A. Spradling (MARGOLIS and SPRADLING 1995). rho118/Cyo and rho12107rev5/Cyo flies were from S. Parkhurst. UAS-PKNG58Ae::GFP/TM3 flies were from A. Jacinto (SIMOES et al. 2006). moePL54/Cyo flies were from F. Payre (POLESELO et al. 2002). scrib4 FRT82B/TM6B flies were from D. Bilder. cora108713 FRT42D/Cyo flies were from V. Auld. mysXG43 FRT19A/FM7a flies were from the late D. Brower. 13c06-Gal4 flies were from Janelia Farm via T. Nystul (PFEIFFER et al. 2008). X15-33 and X15-29 flies were from N. Perrimon (HARRISON and PERRIMON 1993). Viking-GFP were from J. Fessler (MORIN et al. 2001). All other stocks used were obtained from the Drosophila Stock Centre in Bloomington, Indiana. These were c587-Gal4 (KAI and SPRADLING 2004), bab1-Gal4 (BOLIVAR et al. 2006), shg103401/Cyo (TEPASS et al. 1996), hh2/TM3 (PERRIMON and MAHOWALD 1987), rho1ev220/Cyo, rhoGEF204291/Cyo, rok2/FM7a, dia1/Cyo, flywG0172/FM7a, dpp2/Cyo, tkv7/Cyo, prv1/Cyo, prd-Gal4, UAS-zip ubi-GFP, FRT82B/TM3 Ser, ubi-GFP, FRT19A/FM7a, ubi-GFP, FRT42D/Cyo, and UAS-lacZ, arm-GFP second chromosome deficiency stocks from the Bloomington second chromosome deficiency kits, DK2L & DK2R, third chromosome deficiency stocks from the Bloomington third chromosome deficiency kits, DK3L & DK3R. w1118 was used as a wild-type control. Unless otherwise stated, all flies were raised at 25°C.
2.2. Generation of *Drosophila* somatic cell clones

The ability to generate genetic mosaics is extremely useful for studying genes that are essential for early developmental events, which are generally lethal when the whole organism is made homozygous mutant for a given gene. Mosaics or clones have been shown to be an important tool for studying how a given gene is required in a tissue-specific manner. In *Drosophila* clones are created through the site-specific recombination activity of the Flip Recombinase (FLP), which recognizes Flip Recombinase Target (FRT) sites and induces mitotic recombination between them (Xu and Harrison 1994). FLP-mediated recombination allows for the creation of cells that are homozygous for the allele in an otherwise heterozygous background. Upon further rounds of cell division, these cells result in patches or “clones” of cells mutant for the gene of interest.

Alleles of the gene of interest are recombined onto chromosomes containing FRT sequences that are proximal to the centromere. FLP is placed under the control of a heat shock promoter, hsp70, and can therefore be induced at 37°C. The developmental stage, duration and repetition of when heat shocks are applied depend completely on the experiment or question at hand. This allows for targeted induction of clones both spatially in a specific tissue and temporally at a specific developmental stage.

The ubi-GFP transgenic reporter is also utilized for the creation of genetic clones by allowing visualization of cells not homozygous mutant for the gene of interest. To generate somatic cell clones lacking *pak*, *hsFLP; FRT82B, ubi-GFP/TM3, Sb* females were crossed to *pak*^{22} *FRT82B/TM6B* males. The progeny was heat shocked for 2 hours at 37°C to induce genetic recombination in somatic cells undergoing mitosis. Generally this was done for three consecutive days 3-4 days after egg laying to ensure 2^{nd} and 3^{rd} larval instars were exposed to the heat shock during ovary development. Female progeny of the genotype *hsFLP; pak*^{22} *FRT82B/FRT82B, ubi-GFP* were aged on yeasted media for a couple days to allow for development of healthy ovaries. In addition to *pak* mutant FCCs I also created FCCs for other mutants. The procedure detailed
above was also used to generate clones in the context of these mutants. The flies that were
dissected following heat shock treatment as described above are as follows: $\text{hsFLP; cora}^{408713}$
$\text{FRT42D/FRT42D, ubi-GFP, hsFLP; scrib}^{4}$ $\text{FRT82B/FRT82B, ubi-GFP, and mys}^{XG43}$
$\text{FRT19A/FRT19A, ubi-GFP; hsFLP/+}$. The results of these clones are described in the Appendix.

### 2.3. Fixation and staining of *Drosophila* ovaries

**Preparation of solutions:**

**EBR:** (130 mM NaCl, 4.7mM KCl, 1.9 mM CaCl$_2$, 10 mM HEPES pH 6.9)

**Buffer B:** 100mM potassium phosphate pH 6.8, 450 mM KCl, 150 mM NaCl, 20mM MgCl$_2$

**Devitellinizing buffer:** 1 volume buffer B, 1 volume 36% formaldehyde, 4 volumes water

**Phosphate Buffered Saline (PBS):** 8g NaCl, 0.2g KCl, 1.44g Na$_2$PO$_4$ and 0.24g KH$_2$PO$_4$ were added to 800ml H$_2$O. The solution was adjusted to pH 7.4 then brought to 1L and sterilized by autoclave.

**PBT:** 1X PBS, 0.3% Triton-X

Ovaries fixation and staining was performed as described by (Verheyen and Cooley 1994). Ovaries were dissected in cold EBR using fine-point forceps and transferred to a microcentrifuge tube. EBR was removed and 100 µl devitellinizing buffer and 600 µl heptane were added. The sample was vigorously agitated to be sure that the buffer was saturated with heptane and then rotated for 10 minutes at room temperature. The solution was removed and ovaries were washed 3X 10 minutes in 1X PBS. To visualize F-actin the egg chambers were incubated with 1:1000 FITC- or TRITC-conjugated phalloidin (Sigma) for 30 minutes with rotation.

When ovaries were stained for a specific protein, the samples were blocked for 10 minutes in PBT + 0.5% BSA then were incubated with primary antibodies overnight at 4°C with rotation. The primary antibodies used were as follows: mouse anti-PY$_{100}$ (1:500) (Cell Signaling
rabbit anti-GFP (1:500) (Sigma), rabbit anti-phospho-MLC (Ser19, corresponding to Ser21 in Drosophila) (1:20) (Cell Signaling), rabbit anti-beta galactosidase (1:200) (AbCam), anti-phospho-histone H3 (1:500) (Upstate Biotech), rabbit anti-Pak (1:1000), mouse anti-Pak3 (1:1000), guinea pig anti-Traffic Jam (1:5000) provided by D. Godt, rabbit anti-RhoGEF2 (1:100) provided by S. Rogers, guinea pig anti-Scribble (1:500) provided by D. Bilder, mouse anti-Pak3 (1:1000), rat anti-Bam (1:50, Developmental Studies Hybridoma Bank (DSHB), mouse anti-Hts (1:5, DSHB), mouse anti-FasIII (1:100, DSHB), rat anti-Vasa (1:100, DSHB), rat anti- DE-Cadherin (1:100, DSHB), mouse anti-BicD (1:10, DSHB), mouse anti αPS1 (1:500, DSHB). Samples were then washed 3X10 minutes in PBT + 0.5% BSA. Fluorescent detection of antibody staining was conducted using the appropriate secondary antibodies conjugated to FITC (Vector Laboratories), TRITC (Vector Laboratories), or Cy5 (Invitogen) at a dilution of 1:200. Samples were incubated in the dark for 2 hours at room temperature then washed 3X10 minutes in PBT. Following removal of the last wash, Vectashield mountant (Vector Laboratories) was added. In some experiments Vectashield mountant containing DAPI (Vector Laboratories) was added to visualize nuclei. Images were acquired using Zeiss LSM 410 or Nikon A1R laser-scanning confocal microscopes or a Zeiss ApoTome structured illumination microscope and processed using Adobe Photoshop. For samples stained with mouse anti- αPS1, a different method for fixation and staining had to be used as this monoclonal antibody is formaldehyde-sensitive (BROWER et al. 1984; DINKINS et al. 2008). To observe the staining pattern of the anti- αPS1 antibody, and following advice of D. Brower (via personal communications) I stained unfixed, unpermeabilized ovaries. To preserve the sample as best as possible without fixing, ovaries were dissected in cell culture medium, then incubated with the antibody diluted in cell culture medium for 30-45 minutes at 37°C (BROWER et al. 1984). Samples were rinsed in PBS and then fixed as described above, washed 3X 10 minutes in PBT, then incubated with the appropriate secondary antibody as described above.
2.4. TUNEL method to detect apoptosis

To observe escort cell turnover (i.e. escort cell death) I followed methods used by other groups (Kai and Spradling 2003; Kirilly et al. 2011) and used the ApopTag Direct In Situ Apoptosis Detection Kit from Millipore (formerly Chemicon; Product number: S7160), which detects apoptosis by the direct TUNEL (Terminal dUTP Nick End-Labelling) method. This method is used to assay the endonuclease cleavage products by enzymatically end-labelling the DNA strand breaks. DNA fragmentation along with cell shrinkage, membrane blebbing, nuclear condensation and formation of apoptotic bodies are many characteristics of apoptotic cells (reviewed in (Elmore 2007)). Using this kit, the DNA fragments are tagged with a directly-labeled fluorescein nucleotide.

Kit Components: Equilibrium Buffer, Reaction Buffer, TdT Enzyme, Stop/Wash Buffer.

Experimental Preparation:

Working strength TdT Enzyme. TdT Enzyme provided is in a stabilization buffer to preserve its activity and was diluted with Reaction Buffer prior to use in a ratio of 70% Reaction Buffer to 30% TdT Enzyme. 77 µl Reaction Buffer and 33 µl TdT Enzyme (110 µl TOTAL) were mixed well by vortexing and stored on ice for no more than 6 hours.

Working Strength Stop/Wash Buffer. 20 µl Stop/Wash Buffer was mixed in 680 µl dH₂O.

Protocol: (modified from product manual to reflect goals of this thesis):

1.) Ovaries were dissected in cold EBR and placed in 500 µl 1% Paraformaldehyde in PBS for 15 minutes on ice.

2.) Fixative was removed and samples washed in 2 changes of 1X PBS for 5 minutes each time on ice.
3.) Ovaries were resuspended in 500 µl 70% Ethanol and kept at -20°C for at least 1-2 hours. During this time working strength TdT Enzyme, Stop/Wash Buffer, and 0.1% Triton X-100 in PBS were prepared.

4.) Samples were washed in 2 changes of 1X PBS for 5 minutes each wash.

5.) 50 µl Equilibrium Buffer was applied directly onto sample and incubated for at least 10 seconds at room temperature.

6.) Equilibrium Buffer was removed and 50 µl working strength TdT Enzyme added and incubated at 37°C for 30 minutes in the dark, with the tube being inverted several times halfway through the incubation.

7.) TdT Enzyme was removed, 1ml Stop/Wash Buffer added, and sample incubated on a Nutator for 10 minutes at room temperature. Wash was then repeated.

8.) Samples were mounted onto slides and viewed with a microscope using standard fluorescein excitation and emission filters.

2.5. Acridine Orange staining to detect apoptosis

Another method of looking at apoptosis of escort cells is to stain ovaries with the vital dye Acridine Orange (AO). Dying cells lose their permeability barrier, which allows the AO dye to enter the cell and intercalate between the base pairs of dsDNA, thereby causing it to fluoresce green in live, unfixed tissues (ELMORE 2007). Protocol was adapted from (ABRAMS et al. 1993). This offers a very quick method of looking at escort cell death, which is useful as this event is rare (KAI and SPRADLING 2003; KIRILLY et al. 2011).

Ovaries were dissected in cold EBR and egg chambers were placed in an equal volume of acridine orange solution (10 µg/ml, Sigma) and heptane for 3 minutes in the dark with agitation. Samples were then immediately placed onto glass slides and the excess dye was aspirated away. Egg chambers were covered with halocarbon oil 27 (Sigma) and a coverslip added and
imaged immediately. Elapsed time from dissection of the ovaries to the end of viewing was restricted to 20 minutes.

2.6. Larval and pupal ovary dissection, fixation and staining

**Preparation of 20% Paraformaldehyde fixation solution:** 10g paraformaldehyde was added to a 50ml Falcon tube. 35ml of dH₂O and 0.5ml of 1M NaOH was added to the tube. This was then heated to 65°C until the paraformaldehyde dissolved. 10ml of 5X PBS was then added.

Female 3rd instar larvae were dissected in 1X PBS and had ovaries removed that were still intact with the larval fat body. Fat bodies and ovaries were then transferred to a basket and fixed in 5% paraformaldehyde in PBS (GODT and LASKI 1995). To obtain pupal ovaries, 3rd instar larvae were sexed and females were isolated in a vial with food for a few days. Dissection of pupal ovaries depended on the developmental time point I was interested in. The protocol for pupal dissections was kindly given to me by Dorothea Godt via personal communication. A mouth pipette was made from a capillary tube to aid in dissection of pupal ovaries, using a needle puller (Sutter Instruments). The tip of the pulled pipette was broken off while viewing under a microscope such that the tube had a small opening. The blunt end of the capillary was attached to a plastic tube and a mouth-piece added to the other end of the plastic tube. Pupae were carefully dissected in 1X PBS using fine-tipped forceps. Outer pupal cases were carefully removed as well as heads and part of the thorax. The remaining abdomens were carefully washed using the mouth pipette by sucking up a small amount of PBS and expelling it into the abdomen to flush out the fat droplets, leaving behind the pupal ovaries. This tissue was kept submerged in buffer during the entire procedure. Abdomens were then transferred to a basket and fixed in 5% paraformaldehyde in PBS. Both larval and pupal ovaries were then washed and stained according to the procedure detailed in section 2.4.
2.7. Measuring egg chamber length

Egg chambers were dissected, fixed and stained with phalloidin as described above and measurements were acquired with Improvision OpenLab Version 5.5.0 software using a QImaging Retiga EXi camera mounted to a Zeiss Axiplan 2 microscope.

2.8. Rho-GTP activity assay

I used a pulldown assay to quantitate active GTP-Rho1 levels using the Rho binding domain (RBD) of rhotekin or mDia (KIMURA et al. 2000). pGEX-mDia-RBD and pGEX-rhotekin-RBD plasmids were gifts from S. Narumiya in the form of plasmid DNA (pGEX 4T-1).

Preparation of solutions:

**GST-lysis buffer**: 20mM Tris HCl pH 7.4, 300mM NaCl, 1mM EDTA, 1 complete protease tablet

**IP Buffer I**: 47.5ml Tris HCl pH 8.0, 2.5ml 10% Triton X-100, 1 complete protease inhibitor tablet per 50 ml (Roche)

**IP Buffer II**: 50% volume:volume, 1M NaCl and IP Buffer

**SDS-PAGE sample buffer (5X)**: 5ml 0.5 M Tris, pH 6.8, 8ml 50% Glycerol, 8ml 10% SDS, 16ml dH2O, 2ml 2-βmercaptoethanol, bromophenol blue

**SDS-PAGE Gels**: Volumes of each varied depending on the number and thickness of gels required.

- 30% acrylamide
- 10% SDS
- 10% APS (made fresh each time)

TEMED
1.5 M Tris, pH 8.8 (resolving gel)

1.0 M Tris, pH 6.8 (stacking gel)

5X SDS Running Buffer: 15g TrisBase, 72g Glycine, 5g SDS, topped to 1L with ddH2O

10X Transfer Buffer: 30g TrisBase, 144g Glycine, topped to 1L with ddH2O

Coomassie Blue Stain: 100ml MeOH, 20ml Acetic acid, 0.1g Brilliant Blue R-250, 80ml ddH2O

Destain Buffer: 2.5L MeOH, 500ml Acetic acid, 2L ddH2O

10X Tris Buffered Saline (TBS): 60.5g TrisBase, 87.65g NaCl, 800ml ddH2O, adjusted to pH 7.5 with approximately 9.5ml 1M HCl. Volume adjusted to 1L

TBS-Tween (TBST): 99ml 1X TBS + 1ml Tween-20

1% Blocking solution: 10ml Western blocking reagent (Roche) in 90ml 1X TBS

0.5% Blocking solution: 50ml 1% blocking solution diluted with 50ml TBS

Transformation of BL21 bacterial competent cells: Competent cells were kept on ice at all times. 100 µl aliquots of competent cells were added to round-bottom falcon tubes. 1.7 µl of β-mercaptoethanol (made from a fresh 1:10 dilution of a 14.2M stock solution) was added to each aliquot and gently swirled. Reactions were incubated on ice for 10 minutes, swirling gently every 2 minutes. 1 µl of plasmid DNA was added to each aliquot and reactions were then incubated on ice for 30 minutes. Each transformation reaction was heat pulsed in a 42°C water bath for 45 seconds, them immediately placed on ice for 2 minutes. 900 µl of preheated (42°C) SOC medium was added to each transformation reaction, which were then incubated at 37°C for 1 hour with shaking. Using a sterile spreader, transformed cells were spread onto LB-Ampicillin agar plates and incubated overnight at 37°C to allow bacterial colonies to grow.
**Induction of protein expression:** Single colonies were picked and isolated in 50ml LB-Ampicillin. Bacterial cultures were grown up overnight at 37°C then added to 150ml LB-Ampicillin until cell density reached an $A_{600}$ of 0.5-0.6. Expression of the fusion protein was induced by adding 1.0mM isopropyl-β-D-thiogalactoside (IPTG) at 29°C for 3-4 hours. Cultures were spun down at 9000rpm at 4°C for 20 minutes. The supernatant was removed and pellets were stored at -80°C.

**Purification of GST-fusion protein:** Pellets were resuspended thoroughly in 500 µl ice-cold GST-lysis buffer and kept on ice for 10 minutes, then aliquoted into microfuge tubes and lysed using 3 freeze/thaw cycles (liquid nitrogen/37°C water bath). To remove trans-membrane proteins 100 µl of 10% Triton-X was added. The lysed cells were then incubated with rotation at 4°C for 30 minutes, then spun down at 14000rpm for 30 minutes at 4°C. At the same time Glutathione Sepharose 4B beads (Amersham) were washed several times with ice-cold PBS, and supernatant removed each time. Both GST-mDia-RBD and GST-Rhotekin-RBD samples were incubated with beads at 4°C overnight, washed 3X with ice-cold lysis buffer, resuspended in SDS-PAGE sample buffer, boiled for 5 minutes and then run out on an SDS-PAGE gel according to standard procedures (SAMBROOK et al. 1989). Once complete, gels were stained with coomassie blue to visualize the purified fusion protein. Coomassie blue solution was added to gels and left shaking at room temperature for 2h. Coomassie blue was then removed and gel washed several times (often overnight) with destain buffer. This was done to confirm that the purified protein was of the correct size (approximately 35 kDa) before carrying on with the assay.

**Assay and western blot:** Ovaries were dissected out from wild-type and pak mutant flies and flash frozen using liquid nitrogen. 50 µl of ovarian tissues was collected for each sample and homogenized in 500 µl of IP Buffer I. The samples were then centrifuged for 10 minutes at 4°C. The supernatant was removed from the debris and 5% of this was kept for the lysate lane on the gel. The rest of the lysate was incubated overnight at 4°C with purified GST-mDia-RBD or GST-rhotekin-RBD bound to Glutathione Sepharose 4B beads (GE Healthcare) as detailed above. The beads were then centrifuged briefly, supernatants removed and beads washed 3 times with IP
Buffer II. These beads were then re-suspended in SDS-PAGE sample buffer, boiled for 10 minutes, run out on an SDS-PAGE gel together with the lysate sample, and subjected to western blot analysis as per standard procedures (SAMBRook et al. 1989). Gels were placed in Transfer Buffer and proteins were transferred to a nitrocellulose membrane (Bio-Rad) using the Semi-Dry Electrophoretic Transfer Cell (Bio-Rad). Membranes were then washed 3X10 minutes in 1X TBS then blocked for 1h at room temperature in 1% blocking solution. Primary antibodies were diluted in 0.5% blocking solution. Anti-Rho1 antibodies (1:50, DSHB) were used to determine the total amount of Rho and anti-GST antibodies (1:10,000, Cell Signaling Technologies) to determine the amount of GTP-bound Rho in the lysates. Antibodies were removed and membranes were washed 2x10 minutes in TBST at room temperature, then 2X10 minutes in 0.5% blocking solution at room temperature. An HRP-labelled secondary antibody (1:2000; Vector Laboratories) was diluted in 0.5% blocking solution and incubated with membranes for 30 minutes at room temperature. Membranes were washed 4X 15 minutes in TBST. Western blot signals were detected by the BM Chemoluminescence Western Blotting Substrate (POD) (Roche) as per manufacturer’s instructions. Last TBST wash was removed and detection buffer (1ml substrate A + 10 µl substrate B) was added and re-pipetted over the membrane repeatedly for about 1 minute. Detection buffer was removed and membrane was placed in plastic wrap in a film case and exposed on either Clonex Bioflex or Kodak Biomax film.

The assay was repeated several times for each RBD, and results analyzed by performing densitometry using Adobe Photoshop CS4 as described (http://www.lukemiller.org/journal/2007/08/quantifying-western-blots-without.html). GTP-Rho1 levels were normalized against levels of GST-RBD and then compared to total Rho1 levels.

### 2.9. cDNAs

All cDNAs were produced by the Berkeley Drosophila Genome Project. RE32772 (Pix) and SD04476 (RhoGEF2) were obtained from the Canadian Drosophila Microarray Centre. cDNA
constructs were transformed into DH5α bacterial competent cells (Invitrogen) for amplification and general maintenance.

2.10. Generation of the RhoGEF2-DH/PH domain – GST fusion protein and co-immunoprecipitation experiment

The initial idea for constructing the RhoGEF-DH/PH domain–GST fusion protein came from a desire to clone full-length RhoGEF2-GFP into pUAST with the hopes of making a RhoGEF2-GFP transgenic fly that could be used for biochemical assays using an anti-GFP antibody, since the RhoGEF2 antibody is in short supply. Several reagents kindly sent to me for this experiment included DRhoGEF2-RE in pUAST from Ron Vale, and DRhoGEF2-GFP in pMT/V5-hisA from Steve Rogers. Through personal communication with S. Rogers I came up with a cloning strategy that would allow me with the help of lab member Michael Chou, to generate the desired construct by piecing together various fragments, but this proved to be more difficult and time consuming than I anticipated. Full length RhoGEF2 is 6.62kb, the GFP tag is 716 bases and the pUAST plasmid is 9.05kb. Although there are various bacterial competent cell lines that accommodate plasmids with large inserts, I decided to try a different approach at looking the relationship between Pak and RhoGEF2 via biochemical approaches.

All reagents, buffers and solution are as described in Section 2.2.

Construction of the pGEX 4T-1–RhoGEF2 DH/PH plasmid: The DH/PH domain of RhoGEF2 was PCR amplified using the following primers:

RhoGEF2 DH/PH-Forward: TGCGGATCCCTGGAGAGCGAGGATGAAGAC

RhoGEF2 DH/PH-Reverse: TGCGAATTCTGCGTTCTTTGAACGATTCTT

The resulting PCR fragment was purified with the QIAquick PCR Purification kit (Qiagen), digested with restriction endonucleases BamHI and EcoRI, run out on an agarose gel, and gel purified using the QIAquick Gel Extraction Kit (Qiagen). At the same time the plasmid, pGEX 4T-1
was also digested with the same restriction endonucleases to linearize the vector DNA, run out on an agarose gel and gel purified using the same kit. The RhoGEF2-DH/PH insert and linearized pGEX 4T-1 were added to the ligation reaction with T4 DNA ligase (Fermentas) at a ratio of 3:1 insert to vector and incubated overnight at 4°C. The newly formed plasmids were then transformed as described above in section 2.8 into BL21 bacterial competent cells (New England BioLabs), a strain that is defective in OmpT and Lon protease production that allows for high level of expression of GST fusion proteins. Cultures were spread out onto LB-Ampicillin agar plates and grown overnight at 37°C. Single bacterial colonies were isolated, grown up in 3ml of LB-Ampicillin overnight at 37°C, and purified using the GeneJET Plasmid Miniprep Kit (Fermentas). Diagnostic double digests were performed to confirm successful ligation of RhoGEF2-DH/PH into pGEX 4T-1.

**Induction of protein expression & purification of GST-RhoGEF2-DH/PH fusion protein:** Bacterial cultures of pGEX 4T-1–RhoGEF2-DH/PH were grown, expression of the fusion protein was induced, and purification of the GST-RhoGEF2-DH/PH fusion protein was carried out as described in Section 2.8. Gels were stained with coomassie blue to visualize total proteins and the purified fusion protein (approximately 67kDa).

**Co-immunoprecipitation and western blot:** Protocol was followed as described in Section 2.8. Purified fusion protein and bead samples were incubated with whole fly lysates made from 30 wild-type flies homogenized in 500 µl IP Buffer I overnight at 4°C. Samples were then washed 3X with ice-cold IP Buffer I. The beads were then resuspended in SDS-PAGE sample buffer, boiled for 5 minutes and run out on an SDS-PAGE gel. Western blot analysis was performed using anti-GST antibodies (1:10,000, Cell Signaling Technologies) to ensure successful pull down of the GST fusion protein and anti-Pak antibody (1:1000) to identify potential interaction between Pak and the DH/PH domains of RhoGEF2. Western blot signals were detected by the BM Chemoluminescence Western Blotting Substrate (POD) (Roche) and exposed on either Clonex Bioflex or Kodak Biomax film.
2.11. Live imaging of egg chamber rotation

This protocol has been adapted from (PRASAD et al. 2007) and (HAIGO and BILDER 2011) but does not deviate significantly. Protocol must be followed as stated. All live imaging was done using a Quorum spinning disc confocal microscope.

Materials:

Schneider’s Drosophila media (Invitrogen)

Streptomycin/penicillin (Invitrogen)

Insulin, 10mg/ml (Sigma)

Halocarbon oil 27 (Sigma)

Depression slide or any welled-dish for dissecting in

Greiner Lumox culture dish hydrophilic (50mm) (Sigma)

Fetal Bovine Serum (FBS)

Reagent Setup:

**Preparation of Schneider’s medium cocktail:** Schneider’s medium was supplemented with 15%vol/vol FBS and 0.6X penicillin/streptomycin final pH to adjusted to 6.95-7.00. This cocktail was prepared in small batches of approximately 10ml and stored at 4°C. Just before each use, pH of the cocktail at room temperature was checked and if it exceeded 7.1, was re-prepared. Just before use, the cocktail was supplemented with a small aliquot of insulin to a final concentration of 0.2mg/ml.

**Streptomycin/penicillin:** 10,000U/ml of penicillin G-sodium, 10,000U/ml streptomycin sulphate in 0.85% Saline.

**Yeast Paste:** Baker’s yeast with water to make a thick paste, which is important to provide flies with to promote growth of ovarian tissue.
Acidified water: Add 1 µl of concentrated HCl to 1ml of water.

Dissection and mounting of egg chambers:

1.) One well of a depression slide was filled with Schneider’s medium cocktail and placed under a dissection microscope.

2.) A clean Lumox 50mm-hydrophilic dish was kept ready for dissected egg chambers.

3.) Ovaries were gently dissected out of females as described (VERHEYEN and COOLEY 1994). Ovaries were handled gently from the posterior end to avoid damaging mid-staged egg chambers, which might cause rotation to fail.

4.) The posterior part of the ovary where the oldest egg chambers are located was held with one pair of forceps, while the anterior tip of the ovary that contains the germarium and very early-stage egg chambers was held with another pair of forceps.

5.) Very slowly, the anterior end of the ovary was pulled, popping the ovariole out of the muscle sheath. This step was repeated a few times for each ovary to obtain a number of ovarioles from each. Care was taken to ensure that the forceps did not touch the dissected egg chambers, as even slight damage, although not obvious under the microscope, could still affect egg chamber rotation.

6.) If some late-stage egg chambers (stage 11 or older) were still attached to the mid-stage egg chambers, these were removed without touching the egg chambers of interest. Late-stage egg chambers were not included on the Lumox dish for two reasons. First, older egg chambers are large and exhaust the medium quickly, which can limit normal development of mid-stage egg
chambers. Second, the volume of late stage egg chambers is considerably larger than mid-stage egg chambers and so they inhibit immobilization and imaging of egg chamber rotation as the larger egg chambers allow for drift in the Z-plane to occur.

7.) For egg chamber mounting, a Lumox dish was prepared by smearing two thin lines of vacuum grease (Dow Corning) on the Lumox membrane at the position of the outer edges of a 18 X 40 mm² #1 coverslip (Fisher) using a cotton-swab (i.e. along the short edges of the coverslip; position was marked gently with a pen). This was done to cushion the egg chambers and prevent them from being crushed by the cover slip once mounted onto the Lumox membrane, and to help prevent the coverslip from moving during imaging. Only a very thin layer of vacuum grease was needed, as adding excess grease would not allow for immobilization of mid-stage egg chambers. Care was taken not to damage the membrane of the Lumox dish as it provides a gas-permeable base for O₂/CO₂ exchange. Any damage to the membrane will render the dish useless.

8.) The egg chambers were transferred with a pipette, with some Schneider’s cocktail media (30 µl), to the center of the dish, between the two strips of vacuum grease and covered with a 18 X 40 mm² cover slip.

9.) Excess Schneider's cocktail was removed from the sides of the coverslip until mid-stage egg chambers were seen to be immobile when the Lumox dish was tapped. Care was taken not to remove too much medium as the egg chambers may get crushed or fail to develop if there is insufficient medium. The sides of the coverslip were surrounded with a very thin layer of halocarbon oil 27 to minimize evaporation of the medium.
Time-lapse microscopy of living egg chambers

10.) Lumox dish was placed under the microscope and egg chamber(s) of the desired stage identified. Egg chambers that exhibited discontinuities in the outer follicle cells or any other defects were avoided, as were egg chambers that were near the germarium as it has an inherent pulsating movement that will cause any egg chamber which is next to it to move during imaging.

11.) Time-lapse image capture was done using a 25X, 40X or 63X oil immersion lens, with images acquired at 2.5 minute intervals with multiple z-step xy planes over a total scan time of < 1min per time point.

12.) Samples were sometimes refocused early during imaging between the time-lapse images as the compression of the lens onto the coverslip can sometimes cause initial drifting and/or shift.

After the experiment, the coverslip was slowly removed from the Lumox dish, the vacuum grease wiped away with a Kim Wipe, and the halocarbon oil and Schneider’s cocktail medium washed away with ethyl alcohol.

2.12. RNA and Fluorescence in situ hybridization

Embryo Fixation: Embryos were fixed as described in (ASHBURNER 1989). Embryos were allowed to develop as indicated and dechorionated using 50% bleach – 0.01% Triton-X mixture for 3 minutes and rinsed with 0.01% Triton-X. Removal of the protective chorion is required to allow the diffusion of molecular probes into the embryo. Embryos were fixed in a solution containing 1ml 20% paraformaldehyde, 4ml 1X PBS, and 5ml heptane. Vigorous shaking was performed for 25 minutes and the bottom aqueous layer was removed. 5ml methanol was added to the tube and shaken vigorously for 1 minute and the embryos were allowed to settle. Embryos were removed and washed 3X with methanol and stored in methanol at -80°C.
Preparation of solutions:

**Hybridization buffer:** 50% deionized formamide, 4X SSC (SAMBROOK et al. 1989), 1X Denhardts as per (SAMBROOK et al. 1989), 0.1% Tween-20, 5% dextran sulphate, 250 µg/ml salmon sperm DNA, 50 µg/ml heparin. Stored at -20°C (boiled for 10 minutes then put on ice just prior to use).

**Wash Buffer:** 100mM NaCl, 50mM MgCl₂, 100mM Tris pH 9.5, 0.1% Tween-20 (ASHBURNER 1989).

**PBSTw:** PBS with 0.1% Tween-20

RNA **in situ** and fluorescence **in situ** were performed as described in (SEM et al. 2002) and (LECUYER et al. 2007), respectively.

**Generation of RNA probe:** The Pix RNA probe was generated by **in vitro** transcription of the antisense strand of the Pix cDNA clone using the Digoxigenin-labelled (DIG) RNA labelling kit (Roche) according to the manufacturer’s instructions. Plasmid DNA was linearized with AccI and the DIG label was incorporated using the T3 RNA polymerase (anti-sense). Unincorporated nucleotides were then removed using MicroSpin S-200HR columns, spun for 2 minutes at 3000 rpm. 1 µl of the purified probe was run on a 1% DNA agarose gel to quantitate the concentration of RNA. The intensity of the RNA band was compared to the intensity of the 1.6kb band from a 1 kb DNA ladder (Invitrogen, 0.5 µg loaded). As a general rule, the amount of probe added to each hybridization reaction was two times the intensity of the 1.6 kb DNA marker.

**Colourimetric in situ hybridization:** Fixed embryos stored in methanol were serially rehydrated for two minutes in 3:1, 1:1 and 1:3 methanol – 4% paraformaldehyde mixtures, then fixed for 10 minutes in 4% paraformaldehyde and then rinsed 3X with PBSTw. Embryos were
then prehybridized with 500 µl warm hybridization buffer for 1 hour at 52°C. The probe was then added to the embryos, mixed gently and incubated overnight at 52°C without agitation. Following incubation the probe was removed and stored at -20°C for reuse (before reuse, probe was heated at 65°C for 15 minutes to denature the RNA). Embryos were washed at least four times throughout the day with wash buffer kept warm at 52°C, allowing the last wash to proceed overnight. Following this the wash buffer was removed and the embryos were rinsed briefly 3X with PBSTw and rinsed for 30 minutes in PBSTw with rotation. PBSTw was removed and anti-DIG-alkaline phosphatase (AP) (Roche) was diluted 1000X in 1ml PBSTw + 3% BSA and added. Embryos were incubated for 1 hour at room temperature with rotation. The antibody staining mixture was removed and discarded, the embryos were washed 3X 10 minutes in PBSTs and then washed again 3X 5 minutes in AP wash buffer (Ashburner’s solution + Tween-20 to make 0.1% PBTween). Embryos were kept in the last wash and 4.5 µl of NBT (4-nitro blue tetrazolium chloride at 100 µg/µl (Roche, 92451026)) and 3.5 µl BCIP (5-bromo-4-chloro-3-indolyl-phosphate at 50 µg/µl (Roche, 1383221)) were added. Embryos were incubated with rotation and monitored until the colour emerged. Colour development was further monitored under a dissecting microscope and stopped with removal of the AP buffer and washed several times with PBTween, when the desired resolution and pattern was obtained. Embryos were rotated in 70% glycerol for 1 hour at room temperature then stored at 4°C or mounted for visualization. Embryos were observed using differential interference contrast (DIC) microscopy on a Zeiss Axioplan microscope.

**Fluorescence in situ hybridization:** Embryos stored in methanol were rinsed 2X in PBTween and post-fixed for 20 minutes in 4% paraformaldehyde (made fresh in PBTween) with rotation. Embryos were then washed 3X for 2 minutes in PBTween. 500 µl of 3 µg/ml proteinase K (Sigma) was added and incubated at room temperature for 2 minutes while pipetting up and down 2-3 times during this incubation. Tubes of embryos were then transferred on ice and incubated for 1 hour. The proteinase K digestion was removed and blocked by washing 2X for 2
minutes with 2mg/ml glycine in PBTween. Embryos were then rinsed 2X PBTween to remove the glycine and post-fixed again for 20 minutes in 4% paraformaldehyde in PBTween. Embryos were washed 5X for 5 minutes in PBTween and rinsed in a 1:1 mixture of PBTween and hybridization buffer. 1 ml of hybridization buffer was boiled for 5 minutes then immediately placed on ice for 5 minutes. Embryos were then transferred to a new tube, hybridization buffer was removed and the boiled-then-cooled hybridization buffer added and then incubated for a minimum of 2 hours in a 56°C water bath or hybridization oven. RNA in situ probe was diluted in hybridization buffer, heated to 80°C for 3 minutes then cooled on ice for 5 minutes. Pre-hybridization buffer was removed from the embryos and 100 µl of the hybridization + probe solution was added. Embryos were hybridized for 12-16 hours at 56°C without rotation. With all solution warmed to 56°C, the probe was removed and stored for reuse and embryos were rinsed 2X for 30 minutes with pre-warmed hybridization buffer. Embryos were then washed for 15 minutes each with 500 µl 3:1, 1:1 and 1:3 mixtures of hybridization buffer – PBTween and then further washed 4X 5 minutes with 500 µl pre-warmed PBTween. Embryos were allowed to cool to room temperature before the last PBTween wash was removed. Embryos were then blocked in PBTween + 3% BSA for 10 minutes with rotation then incubated with anti-DIG-POD (Roche) at a dilution of 1:400 and other desired primary antibodies in PBTween + 3% BSA at room temperature for 2 hours with rotation. Embryos were then washed 5X for 2 hours in PBTween + 3% BSA and were incubated with the appropriate secondary antibodies at a 1:200 dilution for 2 hours at room temperature. Embryos were then washed overnight and the following day 5X for 2 hours in PBTween + 3% BSA followed by 2 washes in PBTween for 10 minutes each and 3 washes in PBS for 5 minutes each. The tyramide substrate was diluted in amplification buffer (Invitrogen) at a concentration of 1:100. The last PBS wash was removed and embryos were incubated with the tyramide solution for 2 hours in the dark with rotation. Embryos were then washed in PBS 5X for 2 hours then resuspended in Vectashield mountant (Vector Labs) and allowed to settle for 1-3 hour or overnight at 4°C before mounting.
3. Results I: Analysis of a unique side-by-side egg chamber phenotype in *pak* mutant ovaries

3.1. *pak* mutant ovarioles exhibit a novel side-by-side egg chamber phenotype

As mentioned in the introduction, females transheterozygous for the *pak*\(^6\) and *pak*\(^{11}\) alleles, which both encode a truncated Pak protein with no kinase domain (Hing et al. 1999) (Figure 3.1A), also have a striking side-by-side egg chamber phenotype which was first observed by former lab member Judy Yu (results in Yu thesis, 2003). Ovaries of *pak* mutants with a characteristic droopy wing phenotype (Hing et al. 1999) were comprised of ovarioles that contain side-by-side paired egg chambers as well as ovarioles with single egg chambers. Using her work as a starting point I further characterized this paired egg chamber phenotype and found that it is present in approximately 40% of ovarioles generated in the ovary. The paired egg chamber phenotype is initially evident in the germarium, where germline cysts sit side-by-side adjacent to each other as opposed to one behind the other as in wild-type (Figure 3.1 B-E). This side-by-side positioning of cysts continues and is present throughout the ovariole (Figure 3.1 F, G). What is most unique about this phenotype is that the paired egg chambers each have a wild-type number of germ cells and their own complete follicular epithelium, such that at the region of fusion there is a bilayered epithelium (Figure 3.2 D, E). To my knowledge this striking “siamese twin” ovariole phenotype has not been previously reported. Several mutants exist in the literature that have shown a germarium phenotype similar to that of *pak* whereby two 16-cell cysts are positioned side by side in the germarium at the boundary between region 2a and 2b
**Figure 3.1** *pak* mutant ovarioles have side-by-side egg chamber that are present in the germarium

Anterior is to the left in all panels. (A) Schematic of Pak domain organization. Pak has five canonical SH3 binding motifs (yellow), one non-canonical proline rich region shown to bind the GEF, Pix, in mammals (purple), an N-terminal Cdc42/Rac (CRIB) binding domain (red), an auto-inhibitory domain (blue) and a C-terminal catalytic/kinase domain (green). Together the CRIB and AI domains form the major regulatory component of Pak. (B) Schematic of a wild-type germarium. In regions 1 and 2a germline cysts (light green) descended from germline stem cells (dark green) move through escort cells (blue) to the region 2a/2b border where they encounter a pair of follicle stem cells (FSCs, magenta). The FSCs package one cyst at a time into a follicular epithelium (white cells) in region 2b to form egg chambers that bud off in region 3. (C) Schematic of *pak* mutant germarium. (D-G) images are stained with FITC-phalloidin to outline cells. (D) Wild-type germarium showing single cyst in region 3 (arrow). (E) *pak⁶/pak¹¹* germarium showing two cysts in region 3 (arrows). (F) Portion of a wild-type ovariole showing a single row of three egg chambers connected by stalk cells. (G) Portion of *pak⁶/pak¹¹* ovariole showing three pairs of side-by-side egg chambers connected by stalk cells. Scale bars, 25 µm (A, B), 50 µm (C, D).
(Frydman and Spradling 2001; McCaffrey et al. 2006; Muzzopappa and Wappner 2005; O'Reilly et al. 2006; Song and Xie 2003). In addition to these, other mutants have also been shown to result in compound follicles (encapsulation of two germline cysts into a single monolayer of follicle cells), or generate end-to-end fusions (Keller Larkin et al. 1999; Lopez-Schier and St Johnston 2001)(Forbes et al. 1996a; Ruohola et al. 1991)(McGregor et al. 2002; Zhang and Kalderon 2001). However none have been shown to generate two fully-encapsulated, side-by-side egg chambers. As several of these mutants have two cysts at the region 2a/2b border but do not generate the pak paired egg chamber phenotype, the existence of two or more cysts at the position of the FSC niche, or more specifically at the site of cyst encapsulation, is not sufficient by itself to generate side-by-side egg chambers. Interestingly, mutant ovarioles containing paired egg chambers did not produce paired egg chambers exclusively, as I observed that pak mutant ovarioles occasionally produced unpaired egg chambers in a chain of paired egg chambers (Figure 3.2 A-C). When the unpaired egg chamber was not the most mature egg chamber in the ovariole, it was tethered to the side of an aberrant “stalk” of follicle cells (Figure 3.2 A). When the unpaired egg chamber was the most mature egg chamber in the ovariole this abnormal stalk was often not present and the stalk formed at the anterior pole of the most mature egg chamber (Figure 3.2 B). Egg chambers without the aberrant stalk were always centered along the long axis of the ovariole, whereas younger paired and unpaired egg chambers in the same ovariole were positioned to one side or the other of the long axis along the aberrant stalk (Figure 3.2 B, C).

Does the FSC produce a fully functional follicular epithelium in the paired egg chambers of pak mutant females? Normally this cannot be addressed due to the degradation of all pak chambers at around stage 10 (Conder et al. 2007). However, in my work that is presented in sections 5 and 6 I show that heterozygosity for any component of the Rho-activated actomyosin contractility pathway suppresses this degradation (Vlachos and Harden 2011). Heterozygosity for an allele of rho1 enabled a small number of pak mutant paired egg chambers to develop into fused mature eggs, complete with dorsal appendages (Figure 3.2 F). The production of mature
Figure 3.2  

*pak* mutant ovarioles generate fully encapsulated egg chambers

Samples are stained with FITC-phalloidin to outline cells. (A) Portion of *pak*\(^5/pak^{11}\) ovariole showing paired egg chambers followed by three younger, unpaired egg chambers positioned on alternating sides of an aberrant stalk. (B) *pak*\(^6/pak^{11}\) ovariole showing unpaired egg chamber followed by two pairs of younger, paired egg chambers. There is an unpaired cyst in region 2b of the germarium (arrow). Asterisk marks mispositioned oocyte in the unpaired egg chamber. The oocyte is frequently mispositioned in *pak* mutant ovarioles bearing paired egg chambers. (C) *pak*\(^6/pak^{11}\) ovariole showing unpaired egg chamber followed by two younger, unpaired egg chambers on one side of an aberrant stalk. Dashed circles indicate positions of “missing” partner egg chambers if these unpaired egg chambers were generated as paired egg chambers. There is an unpaired cyst in region 2b of the germarium (arrow). (D) Wild-type stage 7 egg chamber highlighting the monolayer FE. (E) Stage 7 *pak*\(^6/pak^{11}\) paired egg chamber demonstrating that each cyst within the pair has its own complement of follicle cells. (F) Side-by-side fused eggs, each with a pair of dorsal appendages, from *rho*\(^{ko2107rev5}; pak*\(^6/pak^{11}\) female. Scale bars, 50 \(\mu\)m (A) (B, C) (D, E).
eggs suggests that the cuboidal epithelial follicle cells of fused egg chambers are fully functional when normal actomyosin contractility is restored. Thus, if later pak mutant phenotypes are suppressed, the FSC niches in pak mutant ovaries are capable of supplying a normal follicular epithelium to paired egg chambers.

3.2. Somatic tissue specific loss of pak generates the side-by-side paired egg chamber phenotype

There is a modest but statistically significant increase in the number of germline cysts in pak mutant germaria compared to that of wild-type germaria (8.13 and 6.88 cysts, respectively. Table 3.1). An idea I considered early on was that dual encapsulation of side-by-side cysts at the region 2a/2b border may be due to an increase in the number of differentiated germline cysts. Here, an overproduction of germline cysts might result in cysts having to sit side-by-side instead of single file to accommodate the increased number of cysts. I looked at the differentiating factor Bam, the expression pattern of which has been used as a hallmark for GSC niche asymmetry as it is only expressed in the differentiating cystoblasts (CHEN and MCKEARIN 2003a). I questioned if extra germline cysts could be due to changes in the location and timing of Bam repression. As discussed in section 1.4, bam is a pro-differentiation gene whose expression is repressed by Dpp signalling (CHEN and MCKEARIN 2003a). If Bam repression extended slightly more posteriorly, i.e. if the Dpp gradient extended past the GSC, more daughter cystoblasts might adopt a GSC-like fate and thus produce more germline cysts. Bam expression in wild-type germaria begins in early cystoblasts with no expression in the GSCs and high expression in the differentiating cystoblasts (Figure 3.3 A). I did not observe any abnormalities in Bam expression in pak mutant germaria (Figure 3.3 B) showing that cystoblast differentiation was not affected in pak mutants.

A previous graduate student Ryan Conder showed that pak germline clones failed to ever generate the paired egg chamber phenotype (personal communication). This work suggested that
Figure 3.3  GSC differentiation is not affected in *pak* mutant germaria

Germaria are stained with Hts (red) to show cell outlines and the Adducin-rich spectrosomes and fusomes of the GSCs and germline cysts, respectively, and the differentiating factor Bam (green), which is only expressed in cystoblasts. Both wild-type (A) and *pak* mutant (B) germaria show restricted Bam expression pattern. Scale bar 50 µm.
### Table 3.1 Comparison of pak mutant and wild-type germaria

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Each measurement/count was collected from separately from different ovarioles. *Germarium width was measured across the germarium from the position of 1 FSC to the opposite FSC. 
**Region 2a/2b boundary was measured from the anterior-most tip of the germarium lengthwise through the centre to the approximate location of the FSC. Escort cell numbers were counted using the enhancer trap PZ1444. P-values were calculated using the student’s T-test.
the \textit{pak} paired egg chamber phenotype is due to loss-of-function in somatic tissue. To gain a better understanding of how Pak might be functioning in somatic cells, I generated \textit{pak}^{22} somatic cell clones to examine the role of Pak in all somatic cells of the germarium; this includes follicle cells, follicle stem cells, escort cells and cap cells. During my semester in the Nystul Lab at UCSF I became interested in determining whether Pak was functioning in the ovary during adulthood or if it was functioning during development of the ovary. To separate these stages, I induced somatic cell clones either in the adult, when the ovary has fully developed, and during larval stages, when the ovary is developing. When clones are induced in adults only new follicle cells produced by the FSCs and the FSCs themselves are made mutant for \textit{pak} as most other somatic cells present in the ovary divide during ovary development, and the FSCs and daughter follicle cells are largely the only somatic cells undergoing mitosis in the adult ovary. Germaria with \textit{pak} mutant FSCs would produce egg chambers that are surrounded only by \textit{pak} mutant follicle cells. If the paired egg chamber phenotype was due to loss of \textit{pak} in FSCs and all of their progeny, clones induced in adults would be sufficient to generate paired egg chambers. If, on the other hand, the paired egg chamber phenotype was due to a loss of \textit{pak} during ovary development, clones induced during second and third larval instars might yield paired egg chambers.

I dissected ovaries from females at 7, 14 and 21 days after clone induction to allow clones induced in one or both of the FSCs to encapsulate cysts in region 2b and for these cysts to move out of the germarium. When I examined ovaries from flies that had clones induced in adults, I observed germaria with one or both FSCs mutant for \textit{pak} but never saw evidence of paired egg chambers (Figure 3.4 A). Other phenotypes associated with loss of Pak function were present such as multi-layering due to defects in apical-basal polarity. When clones were induced during the 3\textsuperscript{rd} larval instar, I frequently observed paired egg chambers where at least one FSC was wild-type for \textit{pak}, indicating that loss of Pak in all FSCs is not required to generate the phenotype (Figure 3.4 B, C). Importantly, I noticed that when paired egg chambers were present, the escort cells, a somatic cell population in the germarium, were consistently \textit{pak} mutant in the
Figure 3.4  Loss of Pak in the adult or FSCs does not appear to cause the paired egg chamber phenotype

Anterior is to the left. (A-B) Samples were stained with DAPI (blue) to mark all nuclei, anti-TJ (light blue) to mark somatic nuclei, anti-FasIII (red) to mark follicle cells, and anti-GFP to mark wild-type cells (A) pak22 somatic cell clones induced in adults do not produce paired egg chambers. (B) pak22 clones induced in 3rd instar larvae. One of the cysts is encapsulated by wild-type follicle cells (circled with a dashed line). (C) pak22 clones induced at 3rd instar stained with phalloidin (red) and GFP (green) shows two sets of side-by-side egg chambers with a mix of wild-type and pak mutant follicle cells. Dashed line in (B) and the two paired egg chambers in (C) indicate that not all FSCs need be pak mutant to generate the side-by-side egg chamber phenotype. Scale bars, 50µm (A, B) (C).
affected ovarioles (Figure 3.4 C) suggesting that the loss of pak in these if not all somatic cell populations of the germarium was contributing to the side-by-side egg chamber phenotype.

To confirm which cell type Pak is acting in to cause the paired egg chamber phenotype I used various somatic cell specific Gal4 lines, c587-Gal4, expressed in the escort cells (Kai and Spradling 2004), traffic tam-Gal4 (tj-Gal4), expressed in all somatic cells that contact the germline cells throughout the entire ovariole (Hayashi et al. 2002; Li et al. 2003; Tanentzapf et al. 2007), bab1-Gal4, expressed in all somatic cells of the germarium during ovary development (Bolivar et al. 2006) and 13c06-Gal4, specifically expressed in the posterior-most pair of escort cells that contact the FSC, the FSC and the immediate FSC daughters of adults (Pfeiffer et al. 2008) to express a pak RNAi transgene. I used anti-Hu-li Tai Shao (Hts) antibody as a general morphology marker but it is additionally informative as it not only provides cell outlines of follicle cells but is also expressed in the spectrosome of GSCs and the branched fusome in daughter germline cystoblasts (Lin and Spradling 1995). I was able to reproduce the side-by-side egg chamber phenotype with c587-Gal4, tj-Gal4 and bab1-Gal4 but not with 13c06-Gal4 (Figure 3.5 A-D). All of these drivers are expressed either in all or a subset of the somatic cell populations of the adult germarium but only c587-Gal4, tj-Gal4 and bab1-Gal4 are expressed throughout ovary development beginning in the larval ovary (Bolivar et al. 2006; Hayashi et al. 2002; Kai and Spradling 2004). From my RNAi data, together with my clonal analysis results, I conclude that loss of pak in somatic cells during ovarian development leads to side-by-side encapsulation of cysts. Consistent with these results Pak is expressed in the ovary from the larval stages through to adulthood (Figure 3.5 E-G).
Figure 3.5 Expression of a Pak-RNAi transgene with somatic cell drivers produces paired egg chambers

(A-D) Samples were stained with anti-Hts antibody. Expression of a \( \text{pak}^{\text{RNAi}} \) transgene with the somatic cell drivers \( c587\text{-Gal4} \) (A), \( tj\text{-Gal4} \) (B) or \( bab1\text{-Gal4} \) (C) results in side-by-side egg chambers. All of these Gal4 lines are expressed in the larval ovary through to the adult tissue. (D) expression of a \( \text{pak}^{\text{RNAi}} \) transgene with \( 13c06\text{-Gal4} \), which is only expressed in the adult germarium, does not produce paired egg chambers. (E) Wild-type larval, (F) late pupal (40h after puparium formation-APF), and (G) adult ovaries stained with anti-Pak antibody, revealing ubiquitous expression of Pak in the developing ovary and germarium. Scale bars, 50µm (A-D) (E) (F, G).
3.3. Polar cells and stalk cells are affected in pak mutants

To gain a better understanding of how Pak might be functioning in the FE I wanted to determine if various populations of somatic cells were being specified in maturing egg chambers. One of the first cell types to be specified during oogenesis are the polar cells in the germarium (Besse et al. 2005; Margolis and Spradling 1995), which are specified by Delta/Notch signalling (Grammont and Irvine 2001; Lopez-Schier and St Johnston 2001; Vachias et al. 2010). Wild-type egg chambers have two pairs of polar cells, one at the anterior end and another at the posterior end, each having a characteristic rounded cell shape (reviewed in (Wu et al. 2008))(Ruohola et al. 1991). Although recent work has shown that although there are no early markers for polar cells in the germarium, Notch signalling has been shown to induce anterior and posterior polar cell fates during the initial events of cyst encapsulation when only 16 follicle cells are surrounding the germline cyst (Nystul and Spradling 2010). Specification of polar cells is important for proper egg chamber formation, as they are critical for anterior-posterior axis formation (Grammont and Irvine 2001; Larkin et al. 1996; Ruohola et al. 1991). The homophilic adhesion molecule Fasciclin III (FasIII) has widely been used as a marker for polar cells in stage 3 to stage 10b egg chambers (Figure 3.7 A) (Ruohola et al. 1991; Snow et al. 1989). I stained pak mutant ovaries with anti-FasIII antibody to look at polar cells and found a variety of phenotypes in paired egg chambers ranging from expression of FasIII in cells that have a stalk-like morphology and are not within the monolayer of follicle cells, to reduced polar cell numbers, to ectopic polar cells at varying positions around the egg chamber (Figure 3.7 B-E). I also observed some of the same defects in non-paired pak mutant ovarioles suggesting that this phenotype is not always connected to the paired egg chamber phenotype and as such have not pursued this phenotype any further.

Another somatic cell-type I was interested in looking at was the stalk cell population. Stalk cells are initially observed as a thick grouping of 6-8 wedge-shaped cells separating one egg chamber from the more anterior egg chamber one as budding occurs from the germarium.
Figure 3.6  Polar cells are specified in pak mutant egg chambers but are abnormal

Samples are stained with phalloidin (red) to see cell outlines and FasIII (green) to look at polar cells. FasIII is expressed in all follicle cells starting in the gerarium but then is restricted to the polar cells in early stage egg chambers. (A) Wild-type ovariole shows the proper localization of polar cells at the anterior and posterior ends of each egg chamber. (B) \( pak^6/pak^{11} \) mutant ovariole shows polar cells at somewhat random locations. (C-E) \( pak/pak^{11} \) paired egg chambers showing polar cells with abnormal morphology, positioning and numbers. Scale bars, 50\( \mu \)m.
(reviewed in (HORNE-BADOVINAC and BILDER 2005)). Following specification of the anterior polar cell pair, JAK/STAT and Notch signaling from the polar cells have been shown to induce stalk cell fates (ASSA-KUNIK et al. 2007; LOPEZ-SCHIER and ST JOHNSTON 2001; McGRegor et al. 2002; TORReS et al. 2003). It was previously thought that polar cells and stalk cells arise from lineage-restricted polar/stalk cell precursors (TоворогеR et al. 1999) but recent work has demonstrated that polar and stalk cells arise from lineage-unrestricted progenitors that respond to local signals (NyстуL and SPRADLING 2010). I used an anti-integrin-αPS1 antibody as a general morphology marker as integrin αPS1 has been shown to be elevated in stalk cells as well as localized to follicle cells membranes (BaTEMаN et al. 2001; Fernаndеz-MиnаН et al. 2007; О’реilly et al. 2008). In wild-type ovarioles the wedge-shaped cells mentioned above, subsequently intercalate during budding resulting in a narrow row of disc-shaped cells (Figure 3.7A). As with the polar cells, pak mutant ovarioles have a variety of stalk cell defects. In some cases there was a double stalk in between two sets of paired egg chambers (Figure 3.7B). Ectopic stalk cells were often present and could be seen running through paired egg chambers along the fusion boundary (Figure 3.7C). When pak mutant ovarioles contain both paired and unpaired egg chambers, an unpaired egg chamber was tethered to the side of an aberrant stalk (Figure 3.7D) as mentioned in section 3.1. In pak mutant ovarioles that do not contain paired egg chambers, stalk cells appeared normal (Figure 3.7E).

3.4. Possible effect of Pak on DE-Cadherin distribution in the germarium

As part of my strategy for understanding how Pak might function during oogenesis, I looked for similarities between pak phenotypes and those of other mutants described in the literature. Two obvious defects in pak mutants are mispositioned oocytes in egg chambers and side-by-side cysts at the region 2a/2b border within the germarium. The position of the
Figure 3.7    Loss of Pak affects stalk cells

(A-E) All ovarioles are stained with anti-αPS1 antibody to look at stalk cells. (A) Wild-type stalk cells have a uniform disc-shaped morphology. (B-E) pak mutant stalk cells showing various defects. (B) Duplicated stalk between two sets of paired pak mutant egg chambers. (C) pak mutant stalk are often thicker with an abnormal morphology and often appear to run in between the two egg chambers in a pair, independent of the FE of each egg chamber. (D) pak mutant ovarioles that produce paired and unpaired egg chambers have aberrant stalks that run along side egg chambers. (E) pak mutant ovarioles that do not produce paired egg chambers appear to have normal stalk cells. Scale bars: 50µm (A, B) (C) (D), 100µm (E).
mislocalized oocytes in the \textit{pak} paired egg chambers was always somewhere along the bilayered epithelium in between the two cysts, sometimes positioned directly adjacent to each other (Figure 3.8A) but were not always positioned in this manner (Figure 3.8C). The homophilic adhesion molecule DE-Cadherin has been implicated in oocyte positioning where it is required in both the follicle cells and germ cells (GODT and TEPASS 1998; GONZALEZ-REYES and ST JOHNSTON 1998). In wild-type germaria, oocytes are positioned and anchored by high levels of DE-Cadherin expressed in the posterior-most follicle cells (Figure 3.8B). When these follicle cells are clonally made mutant for \textit{shotgun (shg)} , the gene encoding DE-Cadherin, oocytes are mispositioned towards other follicle cells that are not mutant for \textit{shg} (GODT and TEPASS 1998), demonstrating a requirement for DE-Cadherin in posterior follicle cells during oocyte positioning. Previous work from our lab has shown that DE-Cadherin distribution is aberrant in \textit{pak} mutant follicle cells (CONDER \textit{et al.} 2007) suggesting that the oocyte positioning defects observed in paired egg chambers might be due to aberrations in DE-Cadherin. The expression of DE-Cadherin in \textit{pak} mutant germaria appeared to be slightly disrupted as it was more diffuse than what was observed in wild-type, and some follicle cells appeared to inappropriately express higher levels of DE-Cadherin (Figure 3.8D). Using the anti-Bicaudal D (BicD) antibody, which provides a marker for the oocyte (SUTER and STEWARD 1991), I looked at DE-Cadherin expression in relation to oocyte position. Oocytes and posterior follicle cells in wild-type germaria establish strong DE-Cadherin mediated adhesions as both cell types were enriched in DE-Cadherin (Figure 3.8 E-E’’). However in \textit{pak} mutant germaria, follicle cells with increased DE-Cadherin expression and the position of nearby oocytes did not appear to correlate well as cells that had higher levels of DE-Cadherin were not always directly adjacent to BicD-positive oocytes (Figure 3.8 F-F’’).

As mentioned in section 3.1, \textit{pak} mutant germaria contain side-by-side cysts just prior to encapsulation and interestingly, germline clones of \textit{shg} show a similar side-by-side defect in the germarium (GONZALEZ-REYES and ST JOHNSTON 1998). Previous studies have shown that DE-Cadherin mediated adhesion is additionally required in the germline cyst to promote cell sorting as the oocyte “out-competes” the remaining fifteen germ cells for the posterior-most position, and
Figure 3.8  Disruptions in DE-Cadherin are present in pak mutants

pak\textsuperscript{6}/pak\textsuperscript{11} paired egg chamber stained with phalloidin (A) and anti-phospho-tyrosine (C) to look at cell outlines and mis-localization of oocytes. (A) Oocytes of this pak paired egg chamber are adjacent to each other at the posterior end of the bilayered FE (yellow lines). (C) Paired egg chamber with non-adjacent oocytes (yellow lines). (B, D) Germaria stained with anti-DE-Cadherin antibody. (B) Wild-type germarium showing DE-Cadherin expression in all follicle cells at cell-cell boundaries and in between germ cells. Expression is elevated in the posterior follicle cells (arrowhead) and oocyte to position and anchor the oocyte at the posterior pole. (D) DE-Cadherin expression in a pak mutant germarium is more diffuse in both somatic and germ cells. (E, F) Germaria stained with anti-BicD antibody show the position of the oocytes (arrows) in wild-type (E) and pak mutant germaria (F). Anti-DE-Cadherin antibody in wild-type (E\textprime) and pak mutant (F\textprime) germaria reveals elevated expression in posterior follicle cells (arrowheads). Merged image of wild-type germarium (E\textquoteright\textquoteright) shows that DE-Cadherin expression is increased between the oocyte and the posterior follicle cells (arrow and arrowhead are pointing at the same cell). Merged image of pak mutant germarium (F\textquoteright\textquoteright) shows follicle cells with higher levels of DE-Cadherin are not in direct contact with the oocyte (arrow and arrowhead are pointing to different cells). (G, H) Germaria stained with phalloidin (red) to outline cells and BicD (green) to show the position of the oocyte. (G) Wild-type germline cysts flatten to form a disc (dashed line) that spans the width of the gerarium at the region 2a/2b border. (H) pak mutant germaria also have germline cysts that flatten (dashed line). (I) pak mutant gerarium stained with Hts shows a 16-cell cysts at the region 2a/2b border that has not flattened (yellow dashed line). Scale bars: 50 µm (A, C, G, H, I); 25 µm (B, D) (E-E\textquoteright\textquoteright, F-F\textquoteright\textquoteright).
maximizes its adhesions with the posterior follicle cells (GODT and TEPASS 1998; GONZALEZ-REYES and ST JOHNSTON 1998). In wild-type ovarioles germline cysts form flattened discs that span the entire width of the germarium as they reach the FSCs at the 2a/2b border (Figure 3.8G). In the germaria of shg germline clones, cysts fail to flatten and remain spherical, ending up side-by-side (GONZALEZ-REYES and ST JOHNSTON 1998). This led me to question if the presence of side-by-side cysts in pak mutant germaria and in turn, paired egg chambers, was at least in part due to an inability of cysts to flatten. Cysts in pak mutant germaria were occasionally unflattened at the region 2a/2b border (Figure 3.8I) but largely flattened normally (Figure 3.8H) and I conclude that a failure to flatten does not make a significant contribution to the side-by-side egg chamber phenotype.

### 3.5. Pak functions during ovarian development

Given my results described in sections 3.1–3.4, I became interested in ovary development and started looking at ovaries of third instar larvae where discrete cell populations first become apparent during gonadogenesis (GODT and LASKI 1995; KING 1970). In wild-type larval ovaries the germ cell population, along with a small population of intermingled somatic cells, is centrally located between apical and basal somatic cell populations (BESSE et al. 2005; GILBOA and LEHMANN 2006; GODT and LASKI 1995). The ovarioles observed in the adult ovary begin to form during the third larval instar as stacks of 8-9 disc-shaped cells that make up the terminal filaments (TFs) (Figure 3.9A) (BESSE et al. 2005; GODT and LASKI 1995). During early pupal formation, a sub-population of apical somatic cells migrates basally in between each of the TFs and the central population of somatic and germline cells to give rise to individual ovarioles (Figure 3.9 B) (BESSE et al. 2005; GODT and LASKI 1995; KING 1970). At 30 hours after puparium formation (APF) clusters of somatic cells once part of the basal population of somatic cells in the larval ovary intercalate to form a distinct row of single cells known as the basal stalk, a transient
structure that exists at the posterior-most end of each developing ovariole and which is immediately followed by the first egg chamber produced in that ovariole (Figure 3.9 C) (BESSE et al. 2005; GODT and LASKI 1995; KING 1970). I examined pak mutant larval and mid-pupal ovaries (20-30h APF) for gross morphological defects but was not able to see anything significant in larval and early pupal ovaries (Figure 3.9 D-E, G-H). The basal stalk of pak mutant pupal ovaries at 30h APF appear to be wider and slightly longer than the basal stalk of wild-type ovaries at the same developmental timepoint (Figure 3.9 F, I). I observe some instances where germline cells were posterior to the basal stalk (Figure 3.9 H, I) but work done by others has shown that this occasionally occurs in wild-type ovaries (BESSE et al. 2005). During 30-45h APF pak mutant ovaries were clearly abnormal when compared with wild-type. In pak mutant ovarioles the basal stalk was frequently duplicated within an ovariole (Figure 3.9 J, K).

### 3.6. The FSC niche in pak mutant gerarium is mispositioned anteriorly

To extend my analysis of Pak’s effects on somatic cell specification I examined the somatic cells in the adult gerarium using the enhancer trap PZ1444, which positively marks the caps cells and escort cells (DECOTTO and SPRADLING 2005; KAI and SPRADLING 2003; MARGOLIS and SPRADLING 1995; XIE and SPRADLING 2000). Using this reporter I found that wild-type gerarium had an average of 29 escort cells whereas pak mutant gerarium had an average of 15 escort cells (Figure 3.10 A, B & Table 3.1, page 67). Interestingly, wild-type and pak mutant gerarium had similar numbers of germline cysts (Table 3.1), indicating that the ratio of escort cell number to number of germline cysts had been halved in pak mutant gerarium. Accompanying the reduction in escort cell number in pak mutant gerarium was a significant shortening of region 1/2a of the gerarium along the anterior-posterior axis (Table 3.1). To accommodate the number of germline cysts at the anterior tip of the gerarium pak mutant gerarium were significantly wider
Figure 3.9 Larval and pupal ovary development in wild-type and pak mutant females

Panels (A-I) are oriented with the apical (anterior) end at the top, whereas panels (J-K) are oriented with the anterior to the left. (D-K) Samples were stained with DAPI (blue) to mark nuclei, anti-Vasa (green) to mark germ cells, and anti-FasIII (magenta) to mark basal stalk cells. (A-C) Schematic of ovary development showing germline cells (green), terminal filaments (white), escort cells and/or escort cell precursors (blue), basal stalk cells (light pink), follicle cells (dark pink) and apical population of somatic cells that contribute to individualization of ovarioles (grey). (D-F) Wild-type and (G-I) pak\textsuperscript{6}/pak\textsuperscript{11} 3\textsuperscript{rd}-instar larval (A, D, G), early (20h APF) (B, E, H) and mid-pupal (30h APF) (C, F, I) ovaries. Early- and mid-pupal ovaries show Vasa-positive germ cells clustering together in small bundles as FasIII-positive cells begin to organize basally to form the basal stalks. At these stages pak\textsuperscript{6}/pak\textsuperscript{11} ovaries (G, H) are largely indistinguishable from wild-type (D, E). pak mutant pupal ovaries at 30h APF (I) appear to have more FasIII positive cells compared to the early basal stalk of wild-type ovaries at the same developmental time point (F). (J, K) Developing germaria in (J) wild-type and (K) pak\textsuperscript{6}/pak\textsuperscript{11} late-pupal ovaries (40h APF) showing FasIII-positive basal stalks. The wild-type ovary shows a single cyst in contact with a single basal stalk, whereas the pak\textsuperscript{6}/pak\textsuperscript{11} ovary shows two cysts in contact with two basal stalks. Scale bars, 50 \textmu m (D-I)(J, K).
than wild-type germaria (Table 3.1). This shortening of region 1/2a and widening of the
germarium allowed for germline cysts to sit side-by-side as they approached the FSC niche and
therefore promoted dual encapsulation of two cysts at the same time. The reduction in escort cell
number and the shortening of region 1/2a also repositioned the FSC niche towards the anterior
end (Table 3.1). Others in the field have used the anterior-most extent of FasIII expression as an
indirect marker for FSC position at the region 2a/2b boundary (DECOTTO and SPRADLING 2005;
KAI and SPRADLING 2003; MARGOLIS and SPRADLING 1995; ZHANG and KALDERON 2001). Using the
same method, I measured the distance from the anterior-most tip of the germarium along the
midline to the beginning of FasIII expression to determine the approximate location of the FSC.

To further demonstrate that the FSC niche has been mispositioned closer to the anterior
tip of the germarium I looked at the position of escort cell turnover as well as Laminin A
expression in the germarium. Previous work has shown that escort cells turnover albeit
infrequently, by undergoing apoptosis at the region 2a/2b border (DECOTTO and SPRADLING 2005;
KIRILLY et al. 2011). This posterior presence of escort cell death has been used as another
method to infer the position of the FSC niche (DECOTTO and SPRADLING 2005). Using a TUNEL
labelling system (ApopTag) in addition to in vivo staining with Acridine Orange, I examined wild-
type and pak mutant germaria for apoptotic cells. I was able to occasionally detect apoptotic cells
at the region 2a/2b border and measured the distance of these cells from the anterior tip of the
germarium to give an approximate measure of FSC niche position in wild-type and pak mutant
germaria (Figure 3.10 C, D). The average distance of apoptotic cells measured from the anterior-
most tip of the germarium was 36.43 µm and 19.09 µm for wild-type and pak mutant germaria,
respectively. Given that Laminin A is strongly localized to the basal surface of the germarium
beginning at the region 2a/2b boundary, it therefore provides an additional marking for estimating
the position of the FSC niche (O’REILLY et al. 2008). I looked at Laminin A distribution in wild-type
(Figure 3.10 E) and pak mutant ovaries and found that it often extended almost to the anterior
end of pak mutant germaria as well as showed ectopic internal accumulation (Figure 3.10 F).
Figure 3.10  FSC niches are displaced towards the anterior end of germaria in pak mutants

(A, B) Wild-type (A) and pak6/pak11 (B) germaria carrying the enhancer trap PZ1444, stained with anti-β-galactosidase (yellow) to mark cap cells and escort cells and anti-FasIII (magenta) to mark follicle cells and thus reveal region 2b. Compared to wild-type, the pak6/pak11 germarium is wider with a shorter region 1/2a and fewer escort cells. (C, D) Wild-type (C) and pak mutant (D) germaria stained with Acridine Orange to look at escort cell death as an indirect marker for FSC position. Region of death in pak mutant germaria is closer to the anterior-tip than in wild-type germaria. (E, F) Wild-type (E) and pak mutant (F) germaria stained with anti-Laminin A show increased Laminin A expression extending posteriorly from the approximate location of the FSCs (arrows). pak mutant germaria ectopically express Laminin A interiorly in the germarium (arrows). Scale bars, 50 μm (A, B, E, F)(C, D).
3.7. The number of FSC niches is increased in *pak* mutant germaria

With anteriorly displaced FSC niches, how are *pak* mutant germaria able to encapsulate two cysts approximately at the same time? How are each of the cysts in the *pak* mutant paired egg chambers surrounded by their own complement of follicle cells? My data suggests that FSC cell proliferation is not affected in *pak* mutants (section 3.8) and *pak* mutants likely have at least one extra FSC that enables dual packaging of germline cysts at the region 2a/2b border. Other mutants producing side-by-side cysts in the germarium do not show paired encapsulation of cysts, (Frydman and Spradling 2001; Gonzalez-Reyes and St Johnston 1998; Hartman et al. 2010; McCaffrey et al. 2006; Muzzopappa and Wappner 2005; O'Reilly et al. 2006; Song and Xie 2003), presumably because they have a wild-type number of FSC niches (i.e. two) that are incapable of supporting such side-by-side egg chamber formation. I predicted that extra FSC niches should be located internally in the germarium between paired cysts at the region 2a/2b boundary. I stained *pak* mutant ovarioles with anti-TJ to visualize all somatic cells in the germarium and anti-FasIII to detect follicle cells. *pak* mutant germaria were optically sectioned and the region 2a/2b boundary examined. I compared *pak* mutant germaria that did not yield paired egg chambers with *pak* mutant germaria that did produce paired egg chambers to normalize for other effects of loss of *pak*. Germaria from *pak* mutant ovarioles lacking side-by-side egg chamber fusions had no internally localized somatic cells at the 2a/2b boundary (Figure 3.11A), whereas germaria from *pak* mutant ovarioles with side-by-side egg chambers had a cluster of cells between paired cysts at the 2a/2b boundary (Figure 3.11B). I believe that this cluster of cells is comprised of one or more internal FSC niches and one or more daughter follicle cells.
Figure 3.11  Evidence that pak mutant germaria have additional, internally located FSC niches

Panels depict progressively deeper Z-sections of germaria stained with anti-TJ (yellow) to mark all somatic cells and anti-FasIII (magenta) to mark the follicle cells. Vertical lines mark region 2a/2b borders. (A) Control germarium containing pak mutant clones but which has not generated any paired egg chambers in the ovariole. There are no clusters of internally localized somatic cells at the region 2a/2b border. (B) Germarium containing pak mutant clones that has given rise to side-by-side egg chambers has internally localized clusters of somatic cells at the region 2a/2b border (arrows in B\textsubscript{III} and B\textsubscript{IV}). Region 2a/2b border in each panel is marked by vertical white lines. Scale Bar, 50 µm.
In further support of extra FSC niches, I occasionally observed triple-fused egg chambers in \( \textit{pak} \) mutant ovarioles in which three cysts were encapsulated instead of the two usually seen (Figure 3.12A). The frequency of triple-encapsulations was extremely rare. In a screen for putative modifiers of \( \textit{pak} \) oogenesis defects, I found that heterozygosity for an allele of \( \textit{moesin} \) resulted in triple encapsulation in \( \textit{pak} \) mutants more frequently than observed in \( \textit{pak} \) mutants alone (Figure 3.12 B, C), thereby supporting my previous results that \( \textit{pak} \) mutant germaria may contain ectopic FSC niches.

A common method for marking stem cells has been to positively mark the progeny of a stem cell, thereby allowing cell lineages to be traced (HARRISON and PERRIMON 1993). In the \textit{Drosophila} ovary this technique has been used for both the GSC and the FSC populations (DECOTTO and SPRADLING 2005; KIRILLY \textit{et al.} 2011; MARGOLIS and SPRADLING 1995; NYSTUL and SPRADLING 2007; ZHANG and KALDERON 2001). Using this technique, FLP-induced FRT mitotic recombination leads to the reconstitution of \( \textit{tub-lacZ} \) in mitotic cells and, \( \textit{tub-lacZ} \) expression subsequently positively and permanently marks mitotically active cells and their progeny (HARRISON and PERRIMON 1993). I attempted to set up this system in the \( \textit{pak} \) mutant background, but despite repeated attempts could not create the required stocks. I think that recombining the stocks used for making these positively marked clones to a \( \textit{pak} \) RNAi transgene and a Gal4 driver capable of generating the paired egg chamber phenotype will be beneficial in detecting ectopic FSCs in \( \textit{pak} \) mutant germaria.
Figure 3.12  Triple encapsulation of germline cysts generates triple-fused egg chambers

Egg chambers were stained with phalloidin to mark cell outlines. Triple encapsulated germline cysts in pak mutants (A) appear “fused” at a common point. (B, C). moe^{PL54/A; pak^6/pak^{11}} triplet egg chambers frequently contained germline cysts that appeared stacked. Triple fused egg chambers supports the presence of ectopic FSC niches in pak mutant germaria. Scale bar 50 µm.
3.8. Interactions between Pak and Hedgehog signaling

Looking at each pak mutant paired egg chamber as a single unit and comparing this to a wild-type egg chamber it became obvious that there were twice as many follicle cells in the paired egg chamber as non-paired egg chambers, albeit the follicle cell:germ cell ratio was not altered. Hedgehog (Hh) produced in the terminal filaments has been shown to directly stimulate proliferation of somatic cells in the germarium, and furthermore, has been shown to cause supernumerary stalk cells when Hh signalling is ectopically expressed (Zhang and Kalderon 2001). Conversely, reduced Hh signalling leads to the production of compound egg chambers in which two germline cysts are packaged into a single monolayer of follicle cells because not enough follicle cells are produced to encapsulate both cysts individually (Hartman et al. 2010; McCaffrey et al. 2006; Muzzopappa and Wappner 2005). Since pak mutants seems to have twice as many follicle cells leaving the germarium at a single time I wanted to see if Hh signalling was affected in the pak mutants. To see if pak mutants had an increase in mitotically active cells I stained ovaries with anti-phospho-histone H3 antibody to mark cells undergoing mitosis.

Compared to wild-type, I did not observe a noticeable difference in the expression pattern of anti-phospho-histone H3 in pak mutant germaria. To see if there was a genetic interaction between hh and pak, I recombined an allele of hh with the pak6 allele, then crossed these recombinant lines to flies carrying the pak11 allele. Interestingly, this appeared to suppress the pak rounded egg chamber phenotype (Figure 3.13 A-C), but more strikingly, the paired hh2, pak6/pak11 egg chambers appeared to resemble compound egg chambers that result due to insufficient follicle cell proliferation (Figure 3.13 B, C). These paired egg chambers also appeared to have a small portion of the epithelial bilayer found in pak paired egg chambers but do not completely surround the inner surface of the two germline cysts (Figure 3.13 B, C arrows). hh2, pak6/pak11 mutant ovarioles also generate aberrant stalks as previously observed in pak mutant ovarioles (Figure 3.2A-C).
Figure 3.13 Interactions between Pak and Hh signalling during oogenesis affect cyst encapsulation

$hh^2$, $pak^6/pak^{11}$ ovarioles were stained with phalloidin to mark cell outlines. (A) Production of a stage 12 egg chamber suggests that reduction of $hh$ suppresses the $pak$ rounded egg chamber phenotype. Stage 10A/B egg chamber (B) and younger egg chambers within an ovariole (C) do not appear to have cysts that are individually surrounded by a monolayer of follicle cells. Squamous epithelial cells appear in between a few germ cells (arrow in B) and the bilayered epithelium characteristic of $pak$ paired egg chambers is present in $hh$, $pak$ mutant paired egg chambers but only partway between the two germline cysts (arrow in C). Generation of an aberrant stalk (bracket in C) suggests that ectopic FSCs in these germaria may be present. Scale bar: 50 µm.
4. Discussion I

4.1. *pak* mutants are able to encapsulate side-by-side cysts due to the presence of extra FSC niches

In the preceding chapter, I have described a novel phenotype that is truly distinct from other oogenesis mutants published in the literature to date. Our lab had previously described roles for Pak during later stages of oogenesis (Conder *et al.* 2007) but an early function of Pak during ovary morphogenesis had not been investigated. This correlates with the existing literature in the field, which has largely focused on the development of the follicular epithelium throughout oogenesis in the adult fly. This includes changes in cell morphology, movement/migration of follicle cells and fate specification of different subsets of somatic cells. I have always been intrigued with how *pak* mutants are able to produce paired egg chambers with two complete monolayered follicular epithelia that do not invade the encapsulated germline cysts and are able to correctly specify different cell types within the follicular epithelium. Even more striking than this is the ability of *pak* mutants to form side-by-side fused eggs when other *pak* oogenesis defects are suppressed by removal of a single copy of the small GTPase, *rho1* (Pak–Rho1 interactions will be described in detail in sections 5 and 6). The presence of fused eggs demonstrates that the follicle cells surrounding cysts in *pak* paired egg chambers are fully functional when actomyosin contractility defects are suppressed. These fused eggs exhibit three key features required for proper egg maturation: one, proliferation, growth and endoreplication; two, establishment and maintenance of apical-basal polarity; and three, formation of polarized actin filaments (reviewed in (Horne-Badovinac and Bilder 2005)). Through my analysis of the *pak* paired egg chamber phenotype I have demonstrated that a FSC and its niche are robust in that they can function in a variety of environments and still manage to generate a fully functional follicular epithelium. My
work demonstrates through loss-of-function, clonal analysis, and RNAi studies that the adult side-by-side paired egg chamber phenotype is the ultimate result of the loss of Pak function in the somatic cells of the pupal ovary. The observation that unpaired egg chambers can be followed by paired egg chambers suggests that during the development of pak mutant ovarioles that there is a time point at which the structure of the germarium changes and follicle cell production increases enabling a transition to paired encapsulation of cysts. The presence of the extra somatic cells in the form of the aberrant stalk shows that there are excess follicle cells even in the absence of a paired egg chamber.

Two features of pak mutant germaria that appear to facilitate dual packaging of cysts are an increase in width and shortening of region 1/2a, which may at least in part be due to a reduction in escort cell number. Escort cells play an active role in transporting cysts through the germarium from the GSC to the FSC while maintaining a fixed position (Decotto and Spradling 2005; Kirilly et al. 2011; Liu et al. 2010; Morris and Spradling 2011). The mechanisms that regulate germarium size are not yet known, but I hypothesize that the escort cells likely play a large role. Removal of the outer epithelial sheath that surrounds each ovariole does not affect the size, shape or function of the germarium (Morris and Spradling 2011), suggesting that the predominant somatic cells of the germarium, i.e. the escort cells, likely contribute to its morphology. Since the escort cells do not move they are able to make stable connections with the ECM (O’Reilly et al. 2008) and are thereby able to provide structural support to region 1 and 2a of the germarium. The regulation of cell-ECM interactions is critical for changes in cell shape that drive epithelial morphogenesis and in turn, formation of organs and tissues such as the Drosophila wing (Dominguez-Gimenez et al. 2007), suggesting that interactions between escort cells and the ECM could function in regulating germarium morphology. Apical cells in the pupal ovary (Figure 3.10B, C) migrate basally/posteriorly to separate and eventually form the epithelial muscle sheath that separates each ovariole (King 1970). This movement may help to guide the placement of newly specified escort cells or the escort cell precursors posteriorly such that the escort cells end up distributed throughout the outer surface of region 1 and 2a as observed in the
adult germarium. The arrangement of escort cells can be thought of as the ‘walls of a long hallway’ that accommodates approximately seven germline cysts in wild-type. The reduction in escort cell number in pak mutant germaria does not allow for formation of a long hallway but rather results in the formation of a shorter, wider germarium. This ‘short, wide hallway’ has to accommodate approximately eight germline cysts in pak mutant germaria. Since the posterior-most escort cells immediately adjacent to the FSC help define the FSC niche (MORRIS and SPRADLING 2011), the end result of having fewer escort cells is the displacement of the FSC towards the anterior end. Since germline cysts have more room to sit side-by-side in the “short, fat” anterior region of pak mutant germaria, they can push cysts through two-by-two and encapsulate them two-by-two.

Paired pak mutant egg chambers cannot solely be attributed to altered shape of the germarium. As mentioned in section 3.8, several other mutants have been described with side-by-side cysts in the germarium but never exhibit side-by-side encapsulation of cysts (FRYDMAN and SPRADLING 2001; GONZALEZ-REYES and ST JOHNSTON 1998; HARTMAN et al. 2010; McCAFFREY et al. 2006; MUZZOPAPPA and WAPPNER 2005; O’REILLY et al. 2006; SONG and XIE 2003). This suggests that the presence of two cysts at the position of the FSC niche is not sufficient by itself to generate side-by-side egg chambers as observed in pak mutants. Compound egg chambers observed in some of the mutants listed above are never present in pak mutant ovarioles because there are sufficient numbers of follicle cells to surround each of the two cysts. Previous studies have shown that a coordinated relationship exists between GSC division and cyst encapsulation to maintain precise packaging of one germline cyst at a time (DRUMMOND-BARBOSA and SPRADLING 2001; KING and LIN 1999; KING et al. 2001; ZHANG and KALDERON 2001). Mutants where this parity is disrupted display different phenotypes that are never seen in pak mutants, including compound egg chambers and apoptosis of germline cysts (DRUMMOND-BARBOSA and SPRADLING 2001) (SONG and XIE 2003) suggesting that the germline cyst-to-follicle cell ratio is largely unaffected in pak mutants. The inability of other mutants with side-by-side cysts at the region 2a/2b border to produce paired egg chambers is presumably because they
have a wild-type number of FSC niches (i.e. two) that are incapable of supporting such side-by-side egg chamber formation. My data provides evidence that the \textit{pak} mutant paired egg chambers are due to extra FSC niches within the germarium.

Given that there are no known markers specific for the FSCs, their position cannot be detected directly. Using an antibody against TJ to mark all somatic cells that contact the germline and an anti-FasIII antibody to mark follicle cells, the location of the FSC has been inferred. The FSC weakly expresses FasIII and the follicle cell daughters express higher levels of FasIII. In this way, cross-migrating daughters cells have been detected crossing the germarium as they move from one FSC niche to the other. During their migration, the cross-migrating daughter cells thereby occupy a position in the center of the germarium at the region 2a/2b boundary (\textsc{Nystul and Spradling} 2007). Other than these single cells, clusters of cells are not seen between the FSC niches in wild-type germaria or \textit{pak} mutant germaria in which side-by-side cyst packaging is not exhibited. In contrast, \textit{pak} mutant germaria yielding paired egg chambers have an internally localized cluster of TJ-positive somatic cells that I speculate are not cross-migrating daughter cells, but rather are composed of one or more FSC niches. The presence of at least one ectopic, internally localized FSC niche and at least one ectopic FSC makes it possible for \textit{pak} mutants to fully encapsulate two germline cysts at a time in their own monolayer of follicle cells.

The FSC niche has been identified as a dynamic somatic stem cell niche that demonstrates characteristics found in both classic and flexible stem cell niches (\textsc{O’Reilly et al.} 2008). Previous studies have shown that germaria lacking germ cells due to GSC differentiation and loss also exhibit loss of the escort cell population (\textsc{Besse et al.} 2005; \textsc{Kai and Spradling} 2003; \textsc{Kirilly et al.} 2005; \textsc{Margolis and Spradling} 1995). As a result these agametic germaria fill up with follicle cells from the anterior end, demonstrating that the FSCs are able to function and divide in the absence of germline cells and are thereby able to establish their own niches even when mispositioned. This dynamic characteristic can be applied to the extra internal FSC niches in \textit{pak} mutant germaria, which are able to function normally to produce follicle cells that then surround two adjacent 16-cell cysts. My analysis of \textit{pak} mutant ovarioles demonstrates that
the position and number of FSC niches can vary in the germarium and still allow cysts that pass through the region 2a/2b border to get encapsulated in their own follicular epithelium.

4.2. Pak function in the pupal ovary contributes to the establishment of the FSC niche

My characterization of the pak paired egg chamber phenotype began in adult ovarioles but was subsequently focused on the developing pupal ovaries. My results with expression of a pak RNAi transgene in addition to clonal analysis of pak mosaic ovarioles indicated that the "short, fat" germarium observed in pak mutant adults is due to defects occurring prior to formation of the adult ovary.

The general morphological changes necessary to shape the ovary throughout development from the 3\textsuperscript{rd} larval instar, through the puparium to the adult fly were determined over 40 years ago (King 1970), but since then only a handful of papers have looked at the specification and reorganization of various cell populations during ovarian morphogenesis (Besse \textit{et al.} 2005; Gilboa and Lehmann 2006; Godt and Laski 1995; Li \textit{et al.} 2003). Compared to the germ cell population, very little is known about the development of the somatic cell populations from the onset of female gonadogenesis to the appearance of the very first egg chamber. Are there one or several somatic progenitor cells? When and where are the FSCs specified? By and large the developmental time points between larval gonadogenesis and adult oogenesis have been a black box and as such, these questions have yet to be answered. I believe that the lack of specific markers for somatic progenitor cells in addition to the level of difficulty associated with pupal ovary dissections has limited the amount of literature in the field regarding this stage of ovary development. Insight into ovary development has come from looking at the primordial germline cell (PGC) population of third larval instar ovaries. The PGCs are surrounded by a population of TJ-positive somatic cells termed intermingled cells (ICs) (Gilboa and Lehmann 2006; Li \textit{et al.} 2003) that have been suggested to give rise to the follicle cell precursors, i.e. the
FSC (GODT and LASKI 1995). The enhancer trap MA33 is also expressed in ICs and interestingly is later expressed in the squamous follicle cell population that covers the nurse cells in stage 10 egg chambers (GILBOA and LEHMANN 2006). This unique expression pattern supports the idea that the follicle cell population might arise from the ICs or from a common early progenitor.

Although the identity of the ICs is not yet known and their function not fully understood, parallels can be drawn between the ICs and the escort cells of the adult germarium. Both these populations are immediately adjacent to and reside in between germline cells or in the case of escort cells, germline cysts, suggesting that the ICs could possibly function as escort cell precursors during gonadogenesis.

A recent study on escort cell morphology has provided further insight into their function. Escort cells surrounding newly formed GSC daughters have short cellular processes whereas the more-posterior escort cells that surround 16-cell germline cysts have longer cellular extensions (KIRILLY et al. 2011). In germaria where daughter cystoblasts fail to differentiate and maintain a GSC-like appearance due to loss of the differentiating factor Bam (CHEN and MCKEARIN 2003a), or over-expression of dpp (SONG et al. 2004), escort cell cellular extensions remain short (KIRILLY et al. 2011). These authors show that germline cysts regulate the cellular extensions of their surrounding escort cells and conversely that escort cells assist in regulating germ cell differentiation. I postulate that the germ cells may function in the same manner at earlier time points during ovary morphogenesis to regulate escort cells when they are participating in establishment of the FSC niche in the pupal ovary.

My results implicate Pak in the development of the basal stalk in the pupal ovary. The generation of duplicated basal stalks in pak mutants strongly suggests that the defects that promote formation of extra FSC niches originate in the pupal ovary. The basal migration of apical cells that separate the germ cell and basal stalk cell populations into individual ovarioles aids in defining the morphology of the basal stalk, however the basal stalk cell population has previously been assumed to originate from a somatic cell population distinct from that supplying the FSCs (GODT and LASKI 1995; KING 1970). Transition of the basal stalk from a row of cells that is three
105
cell diameters wide to a long row of cells a single cell wide occurs during the second day after puparium formation and occurs via cell intercalation (Godt and Laski 1995). Duplication of the basal stalk in pak mutants could arise from defects in cell reorganization, sorting and intercalation. In some instances pak mutant basal stalks do not appear as two separate stalks but instead appear as a thickened single stalk. Failure to complete cell intercalation may be the cause of both duplicated and thick stalks, but when duplicated basal stalks are present, the giant stalk may have split or sorted into two distinct rows, thereby generating two separate stalks. It will be of interest to look at basal stalk length of wild-type and pak mutant pupal ovaries. If basal stalk duplication arises because intercalation fails, then pak mutant basal stalks should be shorter than wild-type. Alternatively, if pak mutant stalks are not shorter than wild-type basal stalks then I would postulate that Pak might be involved in proliferation of the basal stalk cell population. If the basal stalk cell population is increased in pak mutants, more cells may be segregated into individual ovarioles by the apical cells. Previous studies have demonstrated a role for Pak in cell division and this will be discussed in detail in section 4.6. Another possibility is that an alteration in somatic cell fate in pak mutant pupal ovaries leads to basal stalk duplication. Basal stalk cells and escort cells might be derived from the same somatic cell population in the pupal ovary and a cell fate specification defect in pak mutant ovaries could lead to an increase in basal stalk cells at the expense of escort cells.

4.3. A model for formation of the FSC niche

I have provided evidence that extra FSC niches are present in pak mutant germaria that allow for packaging of two cysts at a time. I postulate that the duplicated basal stalk in pak mutant pupal ovaries is likely linked to the creation of extra niches. Taking my data into consideration with previous studies, I have developed a model for formation of the FSC niche (Figure 4.1). The FSC must be ready to encapsulate the first germline cyst that matures in the pupal ovary, that is,
Figure 4.1  Model of FSC niche formation

Anterior is to the left in all panels. (A) Development of wild-type ovariole. Mature germline cyst at tip of the basal stalk in the pupal ovary guides establishment of two FSC niches containing FSCs (magenta). Cysts are encapsulated one at a time in the subsequent ovariole. (B) Development of pak mutant ovariole. pak mutants form duplicate basal stalks, enabling the anchoring of four FSCs in at least three niches (the two internal FSCs might share a niche). In the case shown in the left panel of (B) the first cyst has arrived at the lower basal stalk before the second cyst arrives at the upper basal stalk. At this point in time there is a single pair of FSC niches, leading to normal encapsulation of the first cyst as a single egg chamber. Second and third cysts subsequently arrive at the upper and lower basal stalks, respectively. There are now four FSCs in niches, enabling side-by-side encapsulation of second and third (and subsequent) cysts. The panels in (B) depict an ovariole like those in Figure 3.2B and Figure 3.5B. (C) One-by-one encapsulation of cysts by single pair of FSC niches in wild-type gerarium. (D) Once four FSCs are anchored in niches in the pak mutant gerarium, germline cysts can be encapsulated by the upper pair of FSCs, the lower pair of FSCs, or both. In the example shown here the first two cysts were encapsulated simultaneously using both pairs of FSCs, leading to side-by-side egg chambers. The third and fourth cysts were encapsulated by one pair of FSCs (upper pair for third, lower pair for fourth) when there was no cyst passing through the other pair of FSCs and hence they appear as single egg chambers on opposite sides of the ovariole. The panels in (D) depict an ovariole like that in Figure 3.2A.
all the components of the FSC niche must be correctly specified and positioned either just prior to
or just as the oldest, posterior-most 16-cell cyst reaches the FSC. One way to ensure that the
timing of this occurs properly is to have cyst maturation initiate development of the niche. The
posterior-most escort cell forms a component of the FSC niche and escort cell long cellular
extensions can only develop in the presence of differentiated germ cells (KIRILLY et al. 2011;
NYSTUL and SPRADLING 2007; SONG and XIE 2002). These studies focused on the adult
germarium but the same is likely to occur in the pupal ovary when escort cell maturation is first
established. The basal stalk at the posterior end of each pupal ovariole may act as a physical
barrier and I propose that contact of the first mature germline cyst with the basal stalk is required
for formation of a pair of FSC niches, one on opposite sides of the cyst. In other words, the
posterior-most extent of cyst positioning, and hence location of the FSC niches, is determined by
the basal stalk, which immobilizes the first germline cyst (Figure 4.1A). Cyst immobilization may
favour the formation of cadherin-based adhesion between the FSC and neighbouring escort cells
(SONG and XIE 2002). Unpublished results from our collaborators suggest that FSCs and adjacent
escort cells may arise from a common pupal progenitor (personal communication, T. Nystul). One
possibility during FSC niche formation is that the escort cell and FSC or their common progenitor,
move together with the first germline cyst. Here, the maturation, arrival and immobilization of the
first cyst enables the neighbouring escort cells to extend long processes, thereby making them
competent to form a niche for the nearby FSC. If the FSC and escort cell are from a common
progenitor, arrival of the cyst could cause the progenitor to divide, with the more-anterior cell
forming an escort cell and the more-posterior cell in contact with the basal stalk becoming the
FSC. Arrival and immobilization of the germline cyst, escort cell and FSC at the basal stalk allows
for integrin-mediated adhesion of the FSC (O’REILLY et al. 2008), thereby maintaining the FSC
and establishing the niche at this fixed location. Anchoring via integrin/ Laminin A adhesion also
promotes FSC proliferation (O’REILLY et al. 2008), which allows the first germline cyst to become
encapsulated by daughter follicle cells and move posteriorly through the germarium as seen in
the adult ovary (Figure 4.1C). In pak mutant ovarioles, duplication of the basal stalk forms a
second "cyst docking station" allowing a second cyst to guide establishment of a second pair of FSCs. If the first germline cyst arrives alone at one of the basal stalks, there will be a period of time when the ovariole has only one pair of niches and there will be normal encapsulation of single egg chambers (Figure 3.2 B, C) (Figure 4.1B). The arrival of a mature cyst at the second basal stalk leads to creation of new FSC niches allowing side-by-side encapsulation of egg chambers. After this increase in the number of niches there will be occasions where only one pair of niches is in use at a time, leading to unpaired cysts (as observed in Figure 3.2 A-C) (Figure 4.1D). These unpaired cysts are found on one side or the other of the chain of egg chambers in the ovariole, depending on which pair of FSC niches was used. Interestingly the follicle cells generated from the unused niche always develop into aberrant stalks that accompany the unpaired egg chamber. The cells in these stalks appear to have adopted a stalk cell fate as they express high levels of integrin PS1. This specification of stalk cell fate likely arises from lack of contact with the germline but may also be linked to the polar cell defects observed in pak mutants. Work by others has shown that expression of the JAK/STAT ligand Unpaired in polar cells leads to stalk cell differentiation in neighbouring cells (BAKSA et al. 2002; GHIGLIONE et al. 2002; LOPEZ-SCHIER and ST JOHNSTON 2001; McGREGOR et al. 2002; NYSTUL and SPRADLING 2010) and ectopic expression of Unpaired has been shown to induce ectopic stalk cells (ASSA-KUNIK et al. 2007). If pak mutant polar cells are adjacent to the extra follicle cells that arose from the unused niche, these extra follicle cells may receive excess levels of JAK/STAT activation and in turn, acquire a stalk cell fate.

The FSC niche is faced with a unique challenge in that it must generate an epithelium to package a "moving target", the germline cyst. Having the germline cyst deliver the FSC to the niche ensures that the niche always forms in a position in line with the flow of cysts from the anterior of the germarium. My results and these previous studies indicate that the FSC can form its own niche wherever it is deposited and function normally to produce follicle cells when mispositioned (BESSE et al. 2005; KAI and SPRADLING 2003; KIRILLY et al. 2005; MARGOLIS and SPRADLING 1995). My data suggest that pak mutant germaria with paired follicles have at least
three FSC niches (two lateral niches and one internal niche), and possibly four separate niches, one for each FSC. Although I am unable to show the exact number of niches and/or FSCs, I propose that there are likely four FSCs due to the parity observed in pak paired egg chambers. It is possible that the internal pair of ectopic FSCs are able to share a single niche and the reduced number of escort cells in pak mutant germaria might support this idea. Intriguingly, Laminin A is inappropriately expressed in the centre of pak mutant germaria. This ectopic Laminin A expression could reveal the creation of an ectopic basement membrane between the ectopic FSCs and in turn, their daughter follicle cells.

The remarkable plasticity of the FSC niche enables encapsulation of two cysts at a time in the pak mutant ovary. Encapsulation of even three cysts at a time is possible as heterozygosity for an allele of moesin resulted in triple encapsulation in pak mutants. Triple encapsulation in pak mutants alone has been observed however it is extremely rare. Together these results suggests that it is possible to create even more extra FSC niches in the pak mutant ovary and that there are at least six FSC precursors present in the developing germarium.

4.4. An interaction between DE-Cadherin and Pak may play a minor role in the pak mutant paired egg chamber phenotype

Mutant phenotypes associated with the loss of DE-Cadherin in both the germline and soma resemble some of the defects observed in pak mutant paired egg chambers (GODT and TEPASS 1998; GONZALEZ-REYES and ST JOHNSTON 1998), suggesting that pak paired egg chambers may be due in part to disruptions in DE-Cadherin. Former lab member Ryan Conder previously demonstrated a relationship between Pak and DE-Cadherin where he characterized the role of Pak in apical basal polarity in the follicular epithelium. pak mutant egg chambers that exhibit multi-layered follicle cells due to a loss of epithelial integrity, showed disruptions in the
polarized expression of DE-Cadherin in addition to other markers of apical-basal polarity such as Discs Large (Dlg) (CONDER et al. 2007). These proteins were no longer restricted to their respective membranes but were instead inappropriately expressed on all membranes. My preliminary evidence that DE-Cadherin expression in pak mutant germaria is more diffuse than in wild-type germaria corroborates with these previous observations of R. Conder and suggests that Pak’s effect on DE-Cadherin at early stages of oogenesis could contribute to pak mutant defects. pak mutant germaria contained cells with enriched DE-Cadherin expression that, unlike wild-type, did not correlate with the position of the oocyte. These aberrations in DE-Cadherin distribution likely contribute to the mislocalized oocytes observed in pak mutants. Because oocyte positioning also requires germline expression of DE-Cadherin to change the morphology of germline cysts from a round cluster to a flattened disc (GONZALEZ-REYES and ST JOHNSTON 1998), I was also interested in cyst morphology of pak mutants. Flattened cysts were often observed in pak mutants suggesting that Pak might not affect DE-Cadherin in germ cells. Consistent with this, pak germline clones do not result in side-by-side cysts in the gerarium. Taking all these data together, I believe that the disruptions in DE-Cadherin observed in pak mutant follicle cells might contribute to oocyte mislocalization but do not contribute to the formation of paired egg chambers. As these observations are preliminary, I believe that this is an avenue that should be pursued further.

The small GTPase Rac1 has been shown to be an important regulator of Cadherin in mammalian cell culture studies as well as in Drosophila (LEE and THOMAS 2011; LOZANO et al. 2008; XIAO et al. 2007). The nature of Cadherin regulation by Rac, either negative or positive, appears to be tissue and context dependent (XIAO et al. 2007). As the best-characterized effector of Rac and Cdc42, Pak has also been implicated in Cadherin regulation. In human keratinocytes, discontinuous and ectopic Cadherin at cell-cell contacts caused by expression of a constitutively active form of Rac is rescued by expression of either a dominant-negative form of Pak1 or depletion of endogenous Pak1 by RNAi, suggesting that Pak might regulate Cadherin stability following Rac1 activation (LOZANO et al. 2008). In Drosophila, the sole member of the Group II
Paks, Mushroom Bodies Tiny (Mbt) has been shown to maintain adherens junctions by stabilizing and destabilizing DE-Cadherin during eye development (MENZEL et al. 2008; MENZEL et al. 2007). Moreover, Pak has been demonstrated to regulate DE-Cadherin during morphogenesis of the embryonic salivary gland lumen (PIRAGLIA et al. 2010). The apical domains of pak mutant salivary gland cells are expanded due to loss of DE-Cadherin from the basolateral membranes and a concomitant increase at the adherens junctions (PIRAGLIA et al. 2010). This study showed that Pak differentially regulates the localization DE-Cadherin to restrict apical domain size, suggesting that Pak may similarly regulate DE-Cadherin in the gerarium during oogenesis. A common theme from these studies is that Pak functions to regulate the localization or stability of DE-Cadherin. Perhaps in pak mutant germaria, DE-Cadherin enrichment is lost or aberrantly expressed in the posterior-most follicle cells, which might inhibit DE-Cadherin expression in the oocyte, thereby causing it to be misguided and/or not find the posterior follicle cells at all. Interestingly, oocytes in pak mutant egg chambers always contact the bilayered epithelium between the two cysts but are not always pointing towards each other, suggesting that the disruptions in DE-Cadherin expression are not overly severe as oocytes were never observed in any other position.

4.5. Reducing Hedgehog signaling in pak mutants affects follicle cell proliferation and generates compound egg chambers

The aberrant stalk observed in pak mutant ovarioles where only one pair of niches is in use (Figure 3.2 A-C) (Figure 4.1D) is reminiscent of the giant stalks seen with increased Hedgehog signaling in the ovariole (FORBES et al. 1996a; FORBES et al. 1996b; HARTMAN et al. 2010; TWOROGER et al. 1999; ZHANG and KALDERON 2000; ZHANG and KALDERON 2001). In the case of increased Hh signaling, extra follicle cells in the giant stalks are believed to be derived from excessive proliferation of follicle cells. In the case of pak mutants, extra follicle cells are a
result of extra FSCs. The $hh^2$, $pak^6/pak^{11}$ paired egg chambers appear different from $pak^6/pak^{11}$ paired egg chambers. As previously described, each germline cyst in $pak$ mutant paired egg chambers is encapsulated in its own monolayer epithelium resulting in a bilayer of follicle cells in between the two cysts. However in $hh^2$, $pak^6/pak^{11}$ ovarioles, the follicular epithelium appears to only surround the outer surface of the two germline cysts and does not individually encapsulate each cyst, thereby forming a compound egg chamber. I postulate that the extra FSCs and FSC niches are still present in these double mutants but the reduction of Hh signaling by half affects proliferation of the follicle cells such that there are no longer enough to separately encapsulate each cyst. Thus the $pak$ mutant phenotype becomes similar to other mutants in which there are side-by-side cysts that are not individually encapsulated when Hh signalling is reduced (HARTMAN et al. 2010; MUZZOPAPPA and WAPPNER 2005).

4.6. Pak’s role in escort cell regulation may involve interactions with Rho1 signaling

Insights into escort cell morphology and function have been greatly expanded in the past year due to the findings of two studies (KIRILLY et al. 2011; MORRIS and SPRADLING 2011), some of which have been discussed in sections 4.1–4.3. Escort cell extension length changes depending on the maturation of the underlying germ cells, and so as key regulators of cellular protrusion mechanisms, the Rho family small GTPases were candidates for regulators of escort cell extension. Expression of a dominant-negative form of Rho1 ($Rho1^{DN}$) in escort cells causes a reduction in escort cell number and a reduction in cellular extension length (KIRILLY et al. 2011), thereby implicating Rho1 in the regulation of escort cell processes and suggesting that actin regulation is important for escort cell function (KIRILLY et al. 2011). Furthermore, expression of $Rho1^{DN}$ results in an increased number of undifferentiated germline daughters and increased BMP signalling activity. Results from these authors and from other studies collectively
demonstrate that Rho1 is required in escort cells to control cystoblast differentiation by restricting 
BMP signalling activity to the GSC niche (Kirilly et al. 2011; Liu et al. 2010). This by extension 
implicates actin regulation as a mechanism of regulation of germline cyst maturation.

These previous findings are of particular interest to me as I have found an antagonistic 
relationship between Pak and Rho1 during egg chamber elongation (sections 5 & 6). The work 
presented in the latter half of this thesis demonstrates that Rho1 signaling is increased in the 
absence of Pak. Applying my findings of an antagonistic relationship between Rho1 and Pak 
signalling to the escort cell population in the germarium leads to the hypothesis that Pak functions 
in escort cell maintenance in the adult ovary. When a pak RNAi transgene is expressed in a 
subset of escort cells specifically in the adult ovary using the puc-Gal4 driver, escort cell numbers 
are dramatically increased, suggesting that Pak may indeed be required in the escort cells of the 
adult germarium to restrict their proliferation. In contrast, when Pak is lost earlier in development, 
escort cell numbers decrease, which may be due to a specification defect. Escort cell 
replacement in the adult germarium has been shown to occur in the event of escort cell death or 
injury, two events that are quite rare (Kirilly et al. 2011; Morris and Spradling 2011). 
Intriguingly, I have preliminary evidence showing that when I induced pak somatic cell clones at 
the 3rd larval instar and looked at pak mosaic germaria at 7 and 14 days after clone induction 
(ACI), I observed a significant increase in the number pak mutant escort cells between the two 
time-points (7 days ACI: 18.05, 14 days ACI: 31.54).

My findings indicate that when Pak is absent from escort cells in the adult, escort cells 
proliferate at a greater rate than observed in wild-type to yield an increase in the total escort cell 
number. This raises the possibility that Pak might regulate mitosis of escort cells. Several lines of 
evidence have implicated the Group I Paks as possible regulators of microtubule and actin 
cytoskeletal dynamics during cell division (Banerjee et al. 2002; Bokoch 2003; Cau et al. 2000; 
Daub et al. 2001; Li et al. 2002; Thiel et al. 2002; Zhao et al. 2005). Phosphorylation of 
mammalian Pak1 on Thr212 in mitotically active cells is mediated by the checkpoint protein 
cyclinB1/Cdc2 and is required to target Pak1 to the microtubule organizing centre (MTOC)
(BANERJEE et al. 2002; THIEL et al. 2002). Pak1 has also been shown to localize to the spindle mid body at the onset of mitosis and at the contractile ring during cytokinesis (Li et al. 2002). More recently the Pak-related protein Pak1p in the fission yeast Schizosaccharomyces pombe, has been shown to localize to the actomyosin contractile ring during mitosis and cytokinesis where it functions to phosphorylate the myosin II regulatory light chain protein, Rlc1p (Loo and BALASUBRAMANIAN 2008). Loss of pak1p leads to premature constriction of the contractile ring due and in turn, accelerated cytokinesis. These previous findings demonstrate a role for Pak during various stages of mitosis and suggest that a similar role for Pak could be present during escort cell maintenance in the adult gerarium. It will be of interest to see if escort cells in puc>pkRNAi adults proliferate at a higher rate than wild-type and to see if Pak associates with any component of the spindle apparatus or the contractile ring during mitosis in escort cells.

4.7. General conclusions

My findings demonstrate a role for Pak in the establishment of the FSC niche and in the regulation of escort cells. Ultimately Pak may function to regulate escort cells in a temporally distinct manner. In the pupal ovary during gonadogenesis, Pak either in the basal stalk population or the escort cell/FSC precursors, functions to establish the FSC niche as the first germline cyst arrives at the basal stalk, which may be mediated by immobilization of the cyst by the basal stalk. Pak’s role here is to correctly position the FSC niche and establish the general morphology of the gerarium such that the FSC niche is position half-way down the long axis of the gerarium, thus separating region 1/2a from 2b and allowing proper cyst encapsulation. In the adult ovary, Pak may be required in the escort cells to control their proliferation.
5. **Results II: The role of Pak in actomyosin contractility during egg chamber elongation**

Portions of this chapter were published in Stephanie Vlachos and Nicolas Harden, "Genetic evidence for antagonism between Pak protein kinase and Rho1 small GTPase signaling in regulation of the actin cytoskeleton during Drosophila oogenesis" Genetics 187: 501-512, 2011.

5.1. **Deficiency screen to identify second site modifiers of pak mutant oogenesis defects**

Characterization of pak mutant egg chambers previously done in our lab by Ryan Conder determined that Pak is required for the assembly of parallel basal F-actin bundles in the FE. Given that pak mutant egg chambers remain spherical (CONDER et al. 2007) I was interested in further characterizing the role of Pak during egg chamber elongation to see if I could identify modifiers of the rounded egg chamber phenotype by performing a genetic deficiency screen of the second chromosome. pak transheterozygous mutants are of poor health and the oogenesis defects are so severe that identifying modifiers that would make the phenotype worse was extremely difficult and so I only looked for modifiers that suppressed the rounded egg chamber phenotype. For this screen I used flies, which I refer to as pak mutants, trans-heterozygous for the pak\(^6\) and pak\(^{11}\) alleles, which both encode a truncated Pak protein with no kinase domain (HING et al. 1999). I tested 104 deficiencies spanning the second chromosome by comparing egg chambers from females of the genotype Df(2)/+; pak\(^6\)/pak\(^{11}\) (where Df(2) denotes any given deficiency on the second chromosome) to egg chambers from pak\(^6\)/pak\(^{11}\) females, with a focus on looking for elongation in the egg chambers of Df(2)-bearing flies (Figure 5.1). As a control, in tandem I made pak mutant flies heterozygous for the second chromosome balancers from each
Figure 5.1 Mating scheme for second chromosome deficiency screen

In the screen $Df(2)/+; pak^6/pak^{11}$ flies were generated by crossing $pak^6$ females to males carrying the second chromosome deficiency. The male progeny of this cross were then crossed to females carrying the second $pak^{11}$ allele.
deficiency strain and found no effects on the egg chamber elongation defect. I originally intended to use pak allele stocks double balanced for the second and third chromosomes in these experiments so that I could follow all chromosomes, but this was not possible due to the poor health of the stocks. In any case, I could unambiguously identify pak mutant females in my crosses by their characteristic crumpled, droopy wings and uncoordinated behavior (HING et al. 1999) (none of the deficiencies I tested suppressed these phenotypes). Half of the pak mutant females would be heterozygous for a second chromosome deficiency and might show suppression of oogenesis defects. I aged pak females on yeasted media for several days to allow for sufficient ovary development and then dissected out their ovaries. In my dissections I looked for pak females with ovaries larger than typical, as these individuals likely contained suppressing deficiencies, and assessed their ovarioles using phalloidin staining. All putative suppressors were rechecked by repeating the cross with the deficiency stock.

From my screen of the second chromosome I identified 8 deficiencies of the 104 that were able to partially suppress the pak elongation defect when made heterozygous in the pak mutant background (Table 5.1). Given that previous studies in mammalian cell culture have indicated that Pak can have an antagonistic affect on RhoA signaling, and the central role of RhoA signaling in stress fiber formation, I focused my attention on one deficiency, Df(2R)Jp8, which removes cytological region 52F5–53A1 and that fails to complement alleles of the rho1 locus, which encodes the Drosophila orthologue of RhoA (HALSELL et al. 2000; ROSENFELDT et al. 2006; SANDERS et al. 1999). pak mutant flies heterozygous for Df(2R)Jp8 were healthier than flies that were solely mutant for pak as they survived for a longer period of time, living for 5–6 days compared to the 2- or 3-day life span typical of pak mutant flies. The ovaries of Df(2R)Jp8/+; pak6/pak11 females were notably larger than pak6/pak11 mutant ovaries and contained elongated egg chambers and egg chambers that were older than stage 10, including mature eggs (Figure 5.2, compare C with A and B). I quantified the suppression by comparing the lengths of stage 9 egg chambers from the genotype and found that Df(2R)Jp8/+; pak6/pak11 individuals had significantly longer egg chambers than pak6/pak11 females (Table 5.2).
Table 5.1  Second chromosome deficiencies that suppress the *pak* rounded egg chamber phenotype

<table>
<thead>
<tr>
<th>Deficiency Name</th>
<th>Region removed by deficiency</th>
<th>Suppresing gene(s) in deficiency</th>
</tr>
</thead>
<tbody>
<tr>
<td>Df(2L)dpp^{314}</td>
<td>22E4-F2; 22F3-23A1</td>
<td><em>dpp</em></td>
</tr>
<tr>
<td>Df(2L)BSC28</td>
<td>23C5-D1; 23E2</td>
<td><em>mad</em></td>
</tr>
<tr>
<td>Df(2L)BSC111</td>
<td>28F5; 39B1</td>
<td><em>pvr</em></td>
</tr>
<tr>
<td>Df(2L)BSC32</td>
<td>32A1-2; 32C5-D1</td>
<td>– *</td>
</tr>
<tr>
<td>Df(2R)Np5</td>
<td>44F10; 45D9-E1</td>
<td><em>wun</em></td>
</tr>
<tr>
<td>Df(2R)BSC29</td>
<td>45D3-4; 45F2-6</td>
<td><em>wun; wun2</em></td>
</tr>
<tr>
<td>Df(2R)Jp8</td>
<td>52F5-9; 52F10-53A1</td>
<td><em>rho1</em></td>
</tr>
<tr>
<td>Df(2L)14H10W-35</td>
<td>54E5-7; 55B5-7</td>
<td>– *</td>
</tr>
</tbody>
</table>

–, candidate genes have not yet been identified.
Figure 5.2  Suppressors of the pak rounded egg chamber phenotype

Suppressors of pak rounded egg chamber phenotype. Panels show stage 8 or older egg chambers or eggs stained with phalloidin. Genotypes are shown on panels. (A) Wild-type egg chamber elongated along the A-P axis. (B) Spherical pak mutant egg chamber. (C-G) Heterozygosity for various components of the Rho1 activated actomyosin contractility signaling pathway suppresses the pak rounded egg chamber phenotype. (H) Heterozygosity for an allele of the MLC phosphatase flw suppresses pak elongation defect, allowing development of a mature egg. (I-L) Heterozygosity for candidate genes identified in the screen suppresses the pak rounded egg chamber phenotype. Note that panels are not all to same scale. Scale bars: 100 µm in (A, D, E, I, J); 50 µm in (B, C, F, L); 150 µm (G, H, K).
Table 5.2    Quantification of stage 9 egg chamber length

<table>
<thead>
<tr>
<th>Genotype</th>
<th>EC length (μm)</th>
<th>SD</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>pak^6/pak^11</td>
<td>150.758</td>
<td>19.8</td>
<td>20</td>
</tr>
<tr>
<td>w^118</td>
<td>218.527</td>
<td>33.9</td>
<td>21</td>
</tr>
<tr>
<td>rhoGEF2^{D4291}/+; pak^6/pak^11</td>
<td>209.975</td>
<td>28.6</td>
<td>25</td>
</tr>
<tr>
<td>Df(2R)Jp8/+; pak^6/pak^11</td>
<td>207.072</td>
<td>30</td>
<td>19</td>
</tr>
<tr>
<td>rho1^{1B}/+; pak^6/pak^11</td>
<td>204.717</td>
<td>19.8</td>
<td>21</td>
</tr>
<tr>
<td>sqh^2/X;pak^6/pak^11</td>
<td>187.292</td>
<td>20.1</td>
<td>20</td>
</tr>
<tr>
<td>dia^1/+;pak^6/pak^11</td>
<td>175.085</td>
<td>15.3</td>
<td>20</td>
</tr>
</tbody>
</table>

Egg chambers were measured from the anterior-most end to the apex of the oocyte at the posterior end. In comparison to pak^6/pak^11, all other genotypes are significantly different with P-values, ≤ 0.005. All heterozygous mutations in pak^6/pak^11 individuals are strong alleles with the exception of dia^1, which is a hypomorph.
5.2. Heterozygosity for components of the Rho1 signaling pathway controlling actomyosin contractility supresses the pak rounded egg chamber phenotype

Two hypomorphic alleles, \( \text{rho1}^{\text{rev}220} \) and \( \text{rho1}^{\text{ho2107rev}5} \), and a null allele, \( \text{rho1}^{1B} \), were tested using the same genetic crossing scheme described for the deficiency screen (Figure 5.1) (MAGIE et al. 1999; MAGIE and PARKHURST 2005; SANNY et al. 2006). All three alleles suppressed the pak mutant phenotype to a similar extent as \( \text{Df(2R)Jp8} \), indicating that a loss of Rho1 in this deficiency allows it to suppress and that Pak is a negative regulator of the Rho1 signaling pathway during oogenesis (Figure 5.2D, Table 5.2 and 5.3).

As discussed in section 1.7, RhoA signals to activate actomyosin contractility and stress fiber formation (Figure 6.1, page 151), and so I checked to see if reduction of various components of this signaling cascade would similarly suppress pak mutant oogenesis defects. I obtained alleles of components acting at different points in the signaling network, extending from activation of Rho1 through to myosin at the end of the cascade. The guanine nucleotide exchange factor RhoGEF2 has been shown to participate in actin regulation in other tissues, likely as an activator specifically of Rho1 (BARRETT et al. 1997; CAO et al. 2008; DAWES-HOANG et al. 2005; FOX and PEIFER 2007; GROSSHANS et al. 2005; HACKER and PERRIMON 1998; HALSELL et al. 2000; KOLSCH et al. 2007; MULINARI et al. 2008; NIKOLAIDOU and BARRETT 2004; PADASH BARMCHI et al. 2005; ROGERS et al. 2004; SIMOES et al. 2006). An allele of RhoGEF2 was an effective suppressor, as were alleles of rok, \( \text{LIM kinase 1 (LIMK1)} \), \( \text{diaphanous (dia)} \), \( \text{spaghetti squash (sqh)} \) encoding the regulatory light chain of nonmuscle myosin, and \( \text{zipper} \), encoding nonmuscle myosin heavy chain (ANG et al. 2006; BARRETT et al. 1997; CASTRILLON and WASSERMAN 1994; EDWARDS and KIEHART 1996; WINTER et al. 2001; YOUNG et al. 1993) (Figure 5.2 E-G, Tables 5.2 and 5.3). Suppression of the pak mutant elongation phenotype by removal of any single component of the Rho1-activated actomyosin contractility pathway suggests that Pak regulates this pathway during egg chamber elongation.
Table 5.3  Quantitative production of mature eggs

<table>
<thead>
<tr>
<th>Genotype</th>
<th>no. ovarioles</th>
<th>Total no. eggs</th>
<th>Ovarioles with eggs (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>$w^{118}$</td>
<td>n/c</td>
<td>n/c</td>
<td>100</td>
</tr>
<tr>
<td>pak$^{5}$/pak$^{11}$</td>
<td>55</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>rho$^{110}$/+; pak$^{5}$/pak$^{11}$</td>
<td>215</td>
<td>22</td>
<td>10.23</td>
</tr>
<tr>
<td>rok$^{2}$/X; pak$^{5}$/pak$^{11}$</td>
<td>47</td>
<td>4</td>
<td>8.55</td>
</tr>
<tr>
<td>limk$^{2}$/X; pak$^{5}$/pak$^{11}$</td>
<td>94</td>
<td>4</td>
<td>4.25</td>
</tr>
<tr>
<td>rhoGEF2$^{p329}$/+; pak$^{5}$/pak$^{11}$</td>
<td>430</td>
<td>105</td>
<td>24.42</td>
</tr>
<tr>
<td>dia$^{1}$/+; pak$^{5}$/pak$^{11}$</td>
<td>86</td>
<td>2</td>
<td>2.32</td>
</tr>
<tr>
<td>filw$^{G0172}$/X; pak$^{5}$/pak$^{11}$</td>
<td>139</td>
<td>6</td>
<td>4.32</td>
</tr>
<tr>
<td>Df(3L)Exel6102, pak$^{5}$/pak$^{11}$</td>
<td>100</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

n/c, not counted as each ovariole in the wild-type sample always contained at least one egg. Df(3L)Exel6102 removes the RhoGEF64C locus. All heterozygous mutations in pak$^{5}$/pak$^{11}$ individuals are strong alleles with the exception of dia$^{1}$, which is a hypomorph.
5.3. Evidence that RhoGEF2 is the major activator of Rho1 in regulation of the basal F-actin in the follicular epithelium

Heterozygosity for RhoGEF2 was as effective as heterozygosity for alleles of Rho1 signaling components in suppressing pak mutant oogenesis defects, and I checked to see if heterozygosity for RhoGEF2 was suppressing the basal actin defects in the pak mutant egg chambers. I observed a clear suppression of the disorganized basal F-actin of the pak mutants. As in wildtype, the basal F-actin of RhoGEF2^{04291}/+; pak^{6}/pak^{11} egg chambers was arranged in parallel bundles lying perpendicular to the A-P axis, which was significantly different from the basal F-actin of the pak mutants (Figure 5.3). Reduction of other components of the Rho1 pathway similarly rescued the F-actin disruption in pak mutant females (data not shown).

Additionally, heterozygosity for RhoGEF2 was notably more effective than other Rho pathway components at extending the life span of pak mutant females, with flies surviving $\geq 2$ weeks. In addition to RhoGEF2, two other GEFs in Drosophila have been demonstrated to be involved in activating Rho1 signaling to the actin cytoskeleton. Pebble activates Rho1 during cytokinesis and RhoGEF64C is a Rho1 activator participating in formation of the spiracular chamber in axon attraction (BASHAW et al. 2001; PROKOPENKO et al. 1999; SIMOES et al. 2006; SOMERS and SAINT 2003). Furthermore, RhoGEF64C can promote stress fiber formation in mammalian fibroblasts in a RhoA-dependent manner (BASHAW et al. 2001). Reducing the levels of either of these GEFs using deficiencies or loss-of-function mutations had no effect on pak oogenesis defects, as all resulting egg chambers were indistinguishable from pak mutant egg chambers (Table 5.3). I also tested deficiencies and/or alleles disrupting 20 other predicted RhoGEFs (Table 5.4) for the ability to suppress the pak mutant egg chamber phenotype and found that none were effective.

Similarly, a recent study tested predicted RhoGEFs as Rho1 regulators in driving epithelial morphogenesis during imaginal disc morphogenesis and concluded that RhoGEF2 is a key regulator (PATCH et al. 2009).
Figure 5.3  Comparison of the basal F-actin of middle stage egg chambers

All panels are stained with phalloidin. (A) Basal F-actin of wild-type egg chamber is organized in parallel bundles that are oriented perpendicular to the A-P axis. (B) Basal F-actin of a pak⁵/pak¹¹ egg chamber displaying disorganized F-actin bundles that have no specific orientation with respect to the A-P axis. (C) Basal F-actin of rhoGEF2⁰⁴²⁹¹/+; pak⁶/pak¹¹ egg chamber showing that a suppressor of the pak rounded egg chamber phenotype suppresses the basal F-actin disorganization. Scale bar: 25 μm.
Table 5.4 Summary of chromosomal deficiencies and alleles used to test for effect of reductions in RhoGEFs on the \textit{pak} rounded egg chamber phenotype

<table>
<thead>
<tr>
<th>Gene</th>
<th>Chromosome</th>
<th>Cytology</th>
</tr>
</thead>
<tbody>
<tr>
<td>RhoGAP1A</td>
<td>X</td>
<td>1A1</td>
</tr>
<tr>
<td>CG14045</td>
<td>X</td>
<td>3A2</td>
</tr>
<tr>
<td>CG8557</td>
<td>X</td>
<td>16 A5-B1</td>
</tr>
<tr>
<td>Vav</td>
<td>X</td>
<td>18 B6-7</td>
</tr>
<tr>
<td>Sos</td>
<td>2L</td>
<td>34 D1</td>
</tr>
<tr>
<td>CG10188</td>
<td>2L</td>
<td>37 E4-5</td>
</tr>
<tr>
<td>Pix</td>
<td>2L</td>
<td>38 C5</td>
</tr>
<tr>
<td>CG30440</td>
<td>2R</td>
<td>41 F2</td>
</tr>
<tr>
<td>RhoGEF2</td>
<td>2R</td>
<td>53 E4-F1</td>
</tr>
<tr>
<td>CG15611</td>
<td>2R</td>
<td>53 F10-11</td>
</tr>
<tr>
<td>CG30456</td>
<td>2R</td>
<td>53 F8-9</td>
</tr>
<tr>
<td>CG30115</td>
<td>2R</td>
<td>55 D3-4</td>
</tr>
<tr>
<td>RhoGEF3</td>
<td>3L</td>
<td>61 B3-C1</td>
</tr>
<tr>
<td>Trio</td>
<td>3L</td>
<td>61 E1-2</td>
</tr>
<tr>
<td>GEF64C</td>
<td>3L</td>
<td>64 B13-17</td>
</tr>
<tr>
<td>Sif</td>
<td>3L</td>
<td>64 E1</td>
</tr>
<tr>
<td>RhoGEF4</td>
<td>3L</td>
<td>65 F4</td>
</tr>
<tr>
<td>Pebble</td>
<td>3L</td>
<td>66 A18-19</td>
</tr>
<tr>
<td>CG33275</td>
<td>3L</td>
<td>66 A6-8</td>
</tr>
<tr>
<td>CG3798</td>
<td>3L</td>
<td>73 E1-2</td>
</tr>
<tr>
<td>CG7323</td>
<td>3L</td>
<td>76 F2-3</td>
</tr>
<tr>
<td>Cdep</td>
<td>3R</td>
<td>82 E2-3</td>
</tr>
<tr>
<td>CG7397</td>
<td>3R</td>
<td>90 C3-4</td>
</tr>
</tbody>
</table>

Listed are the 20 known and/or predicted RhoGEFs in \textit{Drosophila} and their cytological position.
5.4. Pak does not appear to regulate the levels of activated Rho1 during oogenesis but is required for RhoGEF2 localization

A common and characteristic feature for GEFs in general is the presence of a Dbl homology (DH) domain adjacent to a pleckstrin homology (PH) domain, which are both required for proper GEF function (reviewed in (SCHOCK and PERRIMON 2002)). It remains unclear how GEFs are universally regulated but it is likely related to how RhoGTPases are spatially and temporally activated within a given cell or tissue (HALL 2005). Some RhoGEFs can activate multiple GTPases while others specifically activate a single target (KAIBUCHI et al. 1999). One of the better-characterized GEFs in mammals is the Rho specific p115-RhoGEF, a member of the RGS-family of GEFs, which has been shown to act downstream from the α subunit of the heterotrimeric G proteins of the G12/13 family (KOZASA 2001; KOZASA et al. 1998; OFFERMANNS and SCHULTZ 1994; ROSENFELDT et al. 2006; SEASHOLTZ et al. 2004). Previous studies have also placed RhoGEF2 genetically downstream of a G-protein α subunit of the G12/13 family, encoded by the gene concertina, and its upstream ligand encoded by the gene folded gastrulation (fog) (BARRETT et al. 1997; COSTA et al. 1994; DAWES-HOANG et al. 2005; PARKS and WIESCHAUS 1991). Additionally, p115-RhoGEF, but not other GEFs included in the RGS-containing family of GEFs, was co-immunoprecipitated with mammalian Pak1 (ROSENFELDT et al. 2006). Because of these previous findings, I decided to assess whether Pak is negatively regulating Rho1 signaling by direct interaction with RhoGEF2.

Mammalian Pak1 is able to interact with the DH-PH domain of the RGS-containing p115-RhoGEF, leading to a disruption in G protein coupled receptor-dependent RhoA signaling, thereby implicating Pak1 in the negative regulation of RhoA signaling events (ROSENFELDT et al. 2006). I wondered if Pak similarly physically interacts with RhoGEF2 and thus, I initially attempted to create a transgenic fly containing UAS-RhoGEF2-GFP in preparation for co-immunoprecipitation reactions between RhoGEF2 and Pak. Despite concerted efforts by my lab mate Michael Chou and myself, cloning RhoGEF2 along with GFP into the pUAST vector proved
Figure 5.4 Alignment of the DH/PH domains of *Drosophila* RhoGEF2 with the corresponding sequences of the DH/PH domains of mammalian p115-RhoGEF

Alignment of the DH/PH domain of *Drosophila* RhoGEF2 (accession no. NP_477317.1) with sequences of the DH/PH domain of mammalian p115-RhoGEF (accession no. Q92888.2). These domains share 37% identity and 59% similar non-identical residues (noted by the ‘+’ above the residues).
to be impossible in our hands, probably due to the size of RhoGEF2 (detailed in section 2.10).

From personal correspondence with other groups previously working with RhoGEF2, I decided to forego creating the RhoGEF2 transgenic line and instead opted to focus on the DH-PH domains of RhoGEF2, which were shown to be important for the interaction between mammalian Pak1 and p115-RhoGEF (ROSENFELDT et al. 2006). A sequence alignment of the DH-PH domains of Drosophila RhoGEF2 with that of mammalian p115-RhoGEF showed that these domains are conserved (Figure 5.4) and suggested that the interaction observed between mammalian Pak1 and the DH-PH domains of p115RhoGEF might also be occurring in Drosophila. I cloned the DH-PH domains of RhoGEF2 into a pGEX vector containing Glutathione S-transferase (GST) to create a GST-fusion protein containing the DH-PH domain of RhoGEF2 (detailed in section 2.10), in an attempt to pull down Pak along with RhoGEF2 but this was not successful.

Although I did not find a direct interaction between Pak and RhoGEF2, I was still interested in determining if Pak was regulating the activity of RhoGEF2 during oogenesis, and consequently the activation of Rho1. I assessed the levels of active, GTP-bound Rho1 in ovarian tissue lysates collected from wild-type flies and pak mutant flies using a pulldown assay (detailed in section 2.8) (KIMURA et al. 2000). GST fusions of the RhoA binding domain (RBD) of mDia or Rhotekin were used to pull down GTP-Rho1 from the lysates, and levels of GTP-Rho1 were compared to the total levels of Rho1 (Figure 5.5A; since GST-mDia and Rhotekin produced the same results I only show the assays using GST-Rhotekin). If Pak were negatively regulating Rho1 activation, the level of GTP-Rho1 in the pak mutant lysates would be greater than that observed in wild-type lysates, however I did not detect a significant increase in GTP-Rho1 levels in pak mutant lysates.

If Pak were regulating Rho1 activation only regionally in the ovary, this might not be detectable using the pulldown assay since a greater volume of the total ovary was comprised of germline tissue, where I do not think that Pak is functioning. Using a transgenic GFP-based reporter, PKNG58AeGFP, that binds to GTP-Rho1 and results in an intense GFP localization wherever GTP-Rho1 accumulates (SIMOES et al. 2006) I visualized Rho1 activation in situ in the ovary. I expressed the reporter in the follicular epithelium of wild-type and pak mutant embryos using
Figure 5.5  Pak does not appear to regulate the levels of activated Rho1

(A) Rho activity assay. Using a fusion protein comprised of the Rho binding domain (RBD) of the Rho1 binding protein Rhotekin fused to GST in a GST-pulldown assay allowed for the detection of GTP-bound Rho1 in wild-type and pak mutant ovarian tissue lysates. Panel shows representative SDS-PAGE gel Western blotted with anti-Rho1 antibody. On left side of panel A are lanes containing equal volumes of ovarian tissue lysates from wild-type and pak mutant flies. For the lanes on the right side of the panel, equal volumes of ovarian tissue lysates were passed through columns of GST-Rhotekin-RBD Sepharose beads and precipitated beads run on gel. Incubation of these same lanes with anti-GST antibodies revealed amounts of GST-Rhotekin-RBD in each lane. Intensity of Rho1 pulldown bands was normalized against intensity of GST bands and compared to total Rho1 input. (B-E) GFP-based in vivo reporter to detect subcellular changes in activated Rho1 levels. The follicle cell-specific driver tj-Gal4 was used to express UAS-PKNG58AeGFP in a wild-type background (B, C) or a pak mutant background (D, E). Anti-Rho1 antibody shows the level and distribution of total Rho1 whereas anti-GFP antibody shows the level and distribution of activated Rho1. Scale bar: 50 µm.
<table>
<thead>
<tr>
<th></th>
<th>Lysates</th>
<th>GST-RBD</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>WT</td>
<td>dpak</td>
</tr>
</tbody>
</table>

**A**

- anti-Rho
- anti-GST

**B**

- Rho1
- tj>PKNG

**C**

- GFP

**D**

- tj>PKNG; pak5/pak11
198Y-GAL4, 185Y-GAL4 or traffic jam (tj)-GAL4 drivers (Hayashi et al. 2002; Li et al. 2003; Manseau et al. 1997; Tanentzapf et al. 2007) and detected GTP-Rho1 with anti-GFP antibody and total Rho1 levels with anti-Rho1 antibody. As Pak becomes localized basally when the basal F-actin begins to polarize, it could be negatively regulating Rho1 activation only at this end of the follicle cells. To see if this was the case I looked for an increase in GTP-Rho1 at the basal end of follicle cells in pak mutant egg chambers but saw no obvious difference compared to wild-type (Figure 5.5 B-E) (Conder et al. 2007).

To explore further the relationship between Pak and RhoGEF2 I looked at RhoGEF2 distribution in wild-type and pak mutant follicle cells. I used an available FRT-recombined allele, pak^{14} (Newsome et al. 2000), which encodes a protein two amino acids shorter than that encoded by pak^{6}, to make follicle cell clones (FCC) lacking pak (marked by the absence of GFP), and assessed the distribution of RhoGEF2 using an anti-RhoGEF2 antibody. In wild-type egg chambers RhoGEF2 was enriched throughout oogenesis to the basal end of follicle cells, including at the basolateral domain between follicle cells in stage 10B egg chambers, which parts as follicle cells flatten to accommodate the growth of the oocyte (Figure 5.6 A´-D´) (Schotman et al. 2008). During early oogenesis loss of Pak had little or no effect on RhoGEF2 (Figure 5.6 A´´), whereas pak^{14} FCCs in stage 10A or older egg chambers showed delocalization of RhoGEF2 such that it was no longer basally restricted or highly enriched at the basolateral junction as seen in the neighbouring wild-type cells, but rather was distributed throughout the cell (Figure 5.6 D´´-D´´).
Figure 5.6  RhoGEF2 is basally localized in the follicular epithelium and its localization is regulated by Pak

(A-D) anti-GFP. (A´-D´) anti-RhoGEF2. (A´´-D´´) merge. FCC are distinguished by a lack of GFP staining. Arrowheads mark some clone boundaries. (A-A´) pak^{14} FCC in a stage 5 egg chamber showing that the basal localization of RhoGEF2 is slightly reduced. (B-B´) pak^{14} FCC in columnar cells of a stage 10A egg chamber showing that the localization of RhoGEF2 is no longer restricted to the basal end of follicle cells in the absence of Pak. Yellow arrows mark basal punctate localization of RhoGEF2 in wild-type cells. (C-C´) pak^{14} FCC in a stage 10B egg chamber showing ectopic RhoGEF2 distribution throughout mutant cells. (D-D´) pak^{14} FCC imaged at the basal surface of a stage 10B egg chamber showing that RhoGEF2 accumulation at the points of basal membrane separation is lost in the absence of Pak. White arrows mark obvious sites of basal membrane separation in wild-type tissue. Scale bar: 50 μm.
5.5. Pak regulates the phosphorylation of the non-muscle myosin regulatory light chain in follicle cells

A major output of RhoA signaling is the phosphorylation of the nonmuscle myosin regulatory light chain (MLC). Mammalian Pak1 phosphorylates and inhibits the activity of myosin light chain kinase, MLCK, leading to a reduction in phosphorylation of MLC (SANDERS et al. 1999). To determine if loss of Pak affected MLC phosphorylation I created pak14 FCC and assessed the levels of phospho-MLC (pMLC). In wild-type egg chambers pMLC is largely restricted to the apical end of follicle cells from stages 3 to 6 but can also be seen at the lateral membrane. During and following stage 7 pMLC also accumulates at the basal end of follicle cells around the time that the parallel actin bundles emerge, and can be detected with an antibody against human pMLC (WANG and RIECHMANN 2007). Staining with this anti-pMLC antibody revealed that pak14 mutant cells in early egg chambers had a loss of pMLC with respect to the neighbouring wild-type cells (5.7 A-A`). pak14 FCCs in older, elongating egg chambers showed an ectopic distribution of pMLC throughout the cytoplasm of the follicle cells compared to their wild-type neighbours (Figure 5.7 B-B`). However, in follicle cells that had begun their cuboidal to columnar transition and were positioned over the oocyte, pak14 FCCs did not show ectopic pMLC (Figure 5.7 C-C`).

To determine if the ectopic pMLC in pak mutant follicle cells was due to a failure to negatively regulate myosin light chain kinase (MLCK), I tested to see if reducing MLCK function in pak mutant flies would suppress the egg chamber elongation defect. Three Drosophila loci have been identified encoding members of the Titin/MLCK family, Stretchin-MLCK, bent, and CG1776, although the products of these genes have yet to be tested with regard to effects on MLC (CHAMPAGNE et al. 2000). I saw no suppression of oogenesis defects in pak mutants made heterozygous for alleles of these genes. In addition to this I also tried the converse experiment by over-expressing a constitutively active MLCK transgene using the same drivers mentioned in section 5.4 but was unable to see any phenotypic effects. The loss of pMLC in pak mutant FCCs
Figure 5.7 Pak regulates phosphorylation of MLC during development of the follicular epithelium

(A-C) anti-GFP. (A’-C’) anti-pMLC. (A’’-C’’) merge. FCC are distinguished by a lack of GFP staining. Arrowheads mark some clone boundaries. (A-A’) pak^{14} FCC in a stage 5 egg chamber showing a loss of pMLC staining from the apical and lateral membranes. (B-B’) pak^{14} FCCs in main-body follicle cells of stage 9 egg chamber showing ectopic pMLC in the absence of Pak. (C-C’) Stage 9 egg chamber in which pak^{14} mutant clone contains both main-body follicle cells and follicle cells over the oocyte. Arrow denotes the junction between these two cell types. In mutant follicle cells that are over the oocyte, pMLC levels are slightly reduced at the apical membrane but are otherwise unaffected. However, in the mutant main-body follicle cells there is ectopic pMLC. Scale bar: 25 µm.
in early egg chambers indicates that during oogenesis Pak positively contributes to phosphorylation of MLC prior to its role as a negative regulator. A role for Pak in driving MLC phosphorylation is further supported by a genetic interaction between pak and an allele of flapwing (flw), encoding the MLC phosphatase PP1b (VERESHCHAGINA et al. 2004). Flw suppresses basolateral MLC phosphorylation in the follicular epithelium and I therefore tested for a genetic interaction with pak (VERESHCHAGINA et al. 2004; WANG and RIECHMANN 2007). Heterozygosity for flw suppressed the egg chamber elongation defect of pak mutant flies and allowed the development of mature eggs (Figure 5.1H, Table 5.3). These results demonstrate that Pak both promotes and restricts myosin light chain phosphorylation in a temporally and spatially distinct manner throughout oogenesis.

5.6. The other Group I Pak member Pak3, acts antagonistically to Pak during egg chamber elongation

I became interested in the relationship between the two Group I Pak members because work done by Simon Wang in our lab demonstrated that Pak and Pak3 act cooperatively during embryogenesis (BAHRI et al. 2010). Preliminary results made by Ryan Conder showed that Pak3 may have a function during oogenesis as it is expressed in egg chambers in a ubiquitous pattern in both germline and follicle cells during early stages of oogenesis, but then becomes more restricted to the apical and basolateral membranes beginning at stage 6 (results in thesis of R. Conder, 2007). Together with results from the embryo I became curious as to what relationship Pak and Pak3 had with each other throughout oogenesis. I looked at ovaries of flies that were heterozygous for pak3 in a pak11/pak14 background. Here I used the pak14 allele with the pak11 allele as my pak mutant background because the pak3ex76a allele was recombined with the pak14 allele. R.C. has previously shown that like the pak6 allele, pak14 encodes a truncated protein (NEWSOME et al. 2000) that when crossed with pak6 also produced semi-viable adults with droopy
wings (results in thesis of R. Conder, 2007). Interestingly I observed that flies of the genotype $pak^{14}pak^{3_{ex76a}}/pak^{11}$ produced elongated egg chambers similar to those seen when $pak$ mutant flies were made heterozygous for mutations in Rho1 signaling components (Figure 5.8A). Actomyosin contractility can regulate gene expression through the MAL/SRF transcription factor complex, which has been shown to function in the follicle cells (MIRALLES et al. 2003; POSERN and TREISMAN 2006; SOMOGYI and RORTH 2004), and I considered the possibility that Pak3 expression might be controlled by contractility downstream of Rho1. I manipulated actomyosin contractility signalling by manipulating zipper ($zip$), the gene that encodes non-muscle myosin heavy chain. $zip$ follicle cell clones stained with anti-Pak3 antibodies showed that in the absence of $zip$, Pak3 expression was also lost (figure 5.8C-C’) and conversely, when $zip$ was over-expressed in embryos using the Gal4 driver paired-Gal4 Pak3 levels appeared to increase (Figure 5.8B).

5.7. Dpp pathway and other signaling components identified as $pak$ interactors in the second chromosome deficiency screen

In addition to identifying the Rho1 pathway members as Pak interactors, I have evidence that other signaling proteins interact with Pak in regulating egg chamber elongation. Given the recent demonstration of a link between Decapentaplegic (Dpp) signaling and the Rho1 pathway, I was interested that two of the deficiencies identified as suppressors in my screen deleted genes encoding components of the Dpp pathway (WIDMANN and DAHMANN 2009). $Df(2L)dpp^{914}$ removes $dpp$, and $Df(2L)BSC28$ removes mothers against Dpp (mad), a R-Smad mediating Dpp signaling to the nucleus (PARKS et al. 2004; SEGAL and GELBART 1985)(Table 5.4). To determine if $pak$ had an antagonistic relationship with the Dpp pathway, I made $pak$ mutants heterozygous for alleles of $dpp$, $mad$ and thickveins ($tkv$), encoding a type I Dpp receptor, and found that removing a single copy of any of these Dpp signaling components was sufficient to suppress the $pak$ mutant elongation defect (Figure 5.1I, J).
Figure 5.8 Pak3 acts antagonistically to Pak and its expression is regulated by actomyosin contractility

(A) Stage 10 pak^{14}, pak^{ex76a}/pak^{11} egg chamber stained with phalloidin to look at cell outlines, showing that heterozygosity for pak3 suppresses the pak rounded egg chamber phenotype. (B) paired-Gal4>UAS-zipper embryo stained with anti-Pak3 antibody. Over-expressing zip, the gene encode non-muscle myosin heavy chain, in paired stripes caused an increase in Pak3 expression. (C-C´´) Stage 8 zip FCC stained with GFP (C, green in C´´) and Pak3 (C´, red in C´´). In the absence of zip, Pak3 expression is also lost. scale bars: (A)(C-C´´) 100um, (B) 50um.
I identified a pair of overlapping deficiencies, \textit{Df(2R)Np5} and \textit{Df(2R)BSC29}, in the screen that remove \textit{wunen (wun)}, which encodes a phosphatidic acid phosphatase involved in germ cell migration that also interacts with Rho1 signaling (PARKS \textit{et al.} 2004; ZHANG \textit{et al.} 1996; ZHANG \textit{et al.} 1997)(Table 1.4, Figure 5.1K and data not shown). \textit{Df(2R)BSC29} also removes the related gene \textit{wun2}, which works together with \textit{wun} in regulating germ cell migration (STARZ-GAIANO \textit{et al.} 2001), and I found that heterozygosity for alleles of either \textit{wun} or \textit{wun2} suppressed \textit{pak} elongation defects (data not shown).

\textit{PDGF- and VEGF-receptor related (Pvr)} is disrupted in \textit{Df(2L)BSC111} and encodes a receptor tyrosine kinase guiding migration of the border cells (DUCHEK \textit{et al.} 2001) (Table 1). Given that \textit{Pvr} regulates the actin cytoskeleton in a Rac-dependent manner during border cell migration (DUCHEK \textit{et al.} 2001), it was a suitable candidate for a Pak-interacting protein and I determined that heterozygosity for a \textit{Pvr} allele suppressed \textit{pak} elongation defects (Figure 5.1L).

### 5.8. \textbf{pak mutant egg chambers undergo rotation but do not elongate}

A recent study revealed that one of the mechanisms driving egg chamber elongation is circumferential rotation of the egg chambers around their anterior-posterior axes (Section 1.7) (HAIGO and BILDER 2011). This study concentrated on the role of the ECM in egg chamber elongation and built on work done by other groups showing that mutants with disruptions in the ECM produced egg chambers that do not elongate (BATEMAN \textit{et al.} 2001; HAIGO and BILDER 2011). Results from imaging of live tissues indicate that egg chamber rotation begins at stage 5 and that polarized ECM is laid down as a result. The polarized ECM then acts as the "molecular corset" driving egg chamber elongation. All of my work in this chapter regarding egg chamber elongation has focused on the role that the basal F-actin plays as the "molecular corset" (GUTZEIT 1991). Unpublished results made by Ryan Conder showed that the polarized organization of the...
ECM component Laminin A, was not affected in pak mutants (results in R. Conder thesis, 2007). With the help of my student Lindsay Wainwright, I further examined the ECM in pak mutants using a stock with a GFP protein trap fused to the Collagen IV protein (viking-GFP) and observed that the circumferentially oriented fibrils of Collagen IV were unaffected in pak mutant egg chambers (Figure 5.9A). If the ECM is the molecular corset, why do pak mutant egg chambers fail to elongate? This was extremely intriguing to me and so Lindsay and I set out to determine if rotation occurred in pak mutant egg chambers. To analyse egg chamber rotation we utilized an Armadillo-GFP protein fusion to view follicle cell outlines. Similar to arm-GFP egg chambers, we observed arm-GFP/+; pak\(^{24}/pak^{11}\) rounded egg chambers rotating around their anterior-posterior axes (Figure 5.9 B, C). Our data show that egg chamber rotation and the ECM are not affected in pak mutants, and yet there is a failure to elongate.
Rotation occurs within a static ECM of circumferentially polarized Collagen IV fibrils. (A) Collagen IV-GFP visualized in a vkg-GFP/+; pak^{6}/pak^{11} stage 8 egg chamber to show collagen fibrils during egg chamber rotation. (B, C) Still pictures of egg chamber rotation in live tissues. Follicle cell tracking (red, green, purple, yellow dots) during egg chamber rotation of stage 8 arm-GFP (A) and arm-GFP/+; pak^{6}/pak^{11} mutant (B) egg chambers. Time is depicted in minutes.
6. Discussion II

6.1. The follicular epithelium as a system for studying stress fibers and actomyosin contractility

My data in section 5 establishes the basal F-actin of the follicular epithelium as an attractive system for the genetic analysis of the signaling pathways regulating the formation of stress fiber-like structures. The actin bundles in the follicle cells appear to be similar to the ventral stress fibers of non-motile cultured cells, for which one model of stress fiber formation is that it is driven by bundling of actin filaments by actomyosin contractility (CHRZANOWSKA-WODNICKA and BURRIDGE 1996; PELLEGRIN and MELLOR 2007). Consistent with this model, my results indicate that the major cause of basal F-actin disruption in pak mutant cells is misregulated actomyosin contractility that can be suppressed by reduction of the Rho1 pathway. I have found that Pak regulates pMLC distribution during oogenesis, at first being required for pMLC and later restricting where it is present. Such conflicting roles for Pak have been reported in isolation in mammalian cell culture studies, but my results are the first to show that they can be temporally separated during development of an epithelial cell (see model in Figure 6.1). Paks from diverse species can function as MLCKs (BISSON et al. 2003; CHEW et al. 1998; LOO and BALASUBRAMANIAN 2008; RAMOS et al. 1997; SZCZEPANOWSKA et al. 2006; ZENG et al. 2000; ZHANG et al. 2005), and such an activity for Pak is indicated in early stage egg chambers, where Pak’s MLCK function is opposed by the Flw MLC phosphatase. Later in oogenesis, around the time of egg chamber elongation, Pak restricts the distribution of MLC phosphorylation, and comes into conflict with the Rho1/Rok pathway. There are a number of ways that Pak could impinge on the Rho1 pathway, with one being at the level of RhoGEF2 at the top of the pathway (Figure 6.1). Pak is required for
Pak functions to both promote and restrict MLC phosphorylation. (A) During early oogenesis Pak acts as a positive contributor to MLC phosphorylation, probably functioning as an MLCK. This function is inhibited by the MLC phosphatase Flw. (B) During later stages of oogenesis, Pak restricts MLC phosphorylation, counteracting the Rho1 actomyosin contractility pathway. Consistent with the literature on mammalian Pak, this could be occurring through negative regulation of an MLCK (1). Another possibility is that Pak negatively regulates Rok (2). Pak likely also controls the distribution of MLC phosphorylation by regulation of RhoGEF2 localization (3).
(A) Early

flapwing → MLC-©

(B) Late

RhoGEF2 → Rho1 → Rok → LIMK-© → Diaphanous

MLCK → MLC-© → Coflin-©

Stabilization of F-actin → Actin polymerization → Actomyosin contractility
the basal localization of RhoGEF2, and the mislocalized RhoGEF2 seen in pak mutant clones could at least in part be responsible for the ectopic pMLC seen in older egg chambers. A protein similar to RhoGEF2 in mammals, P115-RhoGEF, appears to be negatively regulated by Pak binding to its DH-PH domain but I have been unable to find a similar physical interaction between Pak and the RhoGEF2 DH-PH, nor have I detected an effect of Pak on Rho1-GTP levels, although it is possible that there could be an effect not detectable by my assays (ROSENFELDT et al. 2006). Mammalian Pak1 has been shown to phosphorylate p115-RhoGEF (ROSENFELDT et al. 2006) suggesting that more work should be concentrated on looking for a physical interaction between RhoGEF2 and Pak.

A recent study has shown that the PDZ domain of RhoGEF2 is required for its localization at the furrow canal during cellularization (WENZL et al. 2010). Furthermore, the novel protein Slam, which complexes with the RhoGEF2 PDZ domain, is required for RhoGEF2 localization during cellularization, and it will be of interest to determine if Pak regulation of RhoGEF2 localization in the follicular epithelium involves the PDZ domain and/or Slam (WENZL et al. 2010). Another possibility is that Pak regulates RhoGEF2 through a trimeric G protein interaction. RhoGEF2 is a member of the RGS containing family of GEFs that interact with the activated $G_\alpha$ subunits of trimeric G proteins through their RGS domain (reviewed in (STERNWEIS et al. 2007)) and members of the Pak family bind the $G_{\beta\gamma}$ subunit complex through a motif conserved in Drosophila Pak (LEBERER et al. 2000; LEEUW et al. 1998).

Another route by which Pak could be restricting pMLC distribution is through regulation of a MLCK co-operating with Rok. Work on mammalian Pak has demonstrated that Pak can negatively regulate the activity of MLCK, thus reducing the level of MLC phosphorylation and I considered three potential MLCKs as candidate Pak targets (SANDERS et al. 1999). Alleles of these genes did not suppress pak oogenesis defects, suggesting either that they are not regulated by pak during oogenesis, or that more than one is being regulated by Pak. Another possibility is that Pak is directly regulating Rok in some manner to restrict the output of this
pathway. Finally, I have not eliminated the possibility that Pak could be regulating pMLC levels simply by controlling the overall amount of MLC, but this seems unlikely given the considerable evidence that vertebrate Pak regulates MLC phosphorylation. Interestingly, in the columnar epithelial cells over the oocyte in late egg chambers, Pak does not regulate MLC phosphorylation and this may be to allow the extensive actomyosin contractility likely to be required to shape these cells.

6.2. Confirmation of RhoGEF2 as the major activator of Rho1 in epithelia

My finding that RhoGEF2 is a basally localized regulator of actomyosin contractility in the follicular epithelium is consistent with numerous previous studies indicating that RhoGEF2 is the major activator of Rho1 during epithelial morphogenesis (BARRETT et al. 1997; BAYER et al. 2003; DAWES-HOANG et al. 2005; FOX and PEIFER 2007; HACKER and PERRIMON 1998; HALSELL et al. 2000; KOLSCH et al. 2007; MULINARI et al. 2008; NIKOLAIDOU and BARRETT 2004; PADASH BARMCHI et al. 2005; SIMOES et al. 2006). As mentioned in section 5.3, two other RhoGEFs known to regulate actin, Pebble and RhoGEF64C, did not impact the pak mutant egg chamber phenotype. Likewise, deficiencies and/or alleles disrupting 20 other predicted RhoGEFs were not effective at suppressing the pak rounded egg chamber phenotype. Similarly, a recent study tested predicted RhoGEFs as Rho1 regulators in driving epithelial morphogenesis during imaginal disc morphogenesis and concluded that RhoGEF2 is a key regulator (PATCH et al. 2009). Many of the RhoGEFs have not been characterized functionally, although some have been shown to be GEFs for GTPases other than Rho1 and to function in non-epithelial cells such as neurons. I conclude that RhoGEF2 is the major or only GEF regulating Rho1 activation in control of the basal F-actin in the FE.
RhoGEF2 is enriched at the basal end of the follicle cells throughout oogenesis including the points of basal membrane separation between follicle cells that occurs during follicle cell flattening in late stage egg chambers (SCHOTMAN et al. 2008). Recently, it has been shown that the Rho1 actomyosin contractility pathway is required for this separation between follicle cells at the basal membrane and presumably this signaling is activated by RhoGEF2 (SCHOTMAN et al. 2009).

*RhoGEF2* alleles are much more effective than alleles of other Rho1 pathway components at extending the lifespan of *pak* mutant females, implying that RhoGEF2 may have roles independent of the Rho1 actomyosin contractility pathway that could be regulated by Pak. There is evidence that RhoGEFs have functions distinct from small GTPase activation, for example Pebble has a Rho1-independent role in mesoderm migration (ROSSMAN et al. 2005; SCHUMACHER et al. 2004).

Previous work has identified the Gα receptor encoded by the gene *concertina (cta)* and its ligand encoded by the gene *folded in gastrulation (fog)*, as upstream components or RhoGEF2 in Rho1-activated signalling during gastrulation (BARRETT et al. 1997; COSTA et al. 1994; PARKS and WIESCHAUS 1991). I have some preliminary evidence suggesting that heterozygosity for an allele of *fog* in the *pak6/pak11* mutant background suppresses the rounded egg chamber phenotype. This needs to be pursued further but it will also be important to identify the G-protein β and γ subunits as these have not yet been identified in *Drosophila*.

### 6.3. Pak3 functions antagonistically to Pak during egg chamber elongation

While I expected *pak* mutant flies with a single copy of *pak3* removed to show enhanced *pak* oogenesis phenotypes, I was surprised to observe suppression of the *pak* rounded egg chamber phenotype instead. When *zipper*, the gene encoding non-muscle myosin is over-
expressed in the embryo, Pak3 expression is elevated, and conversely, in zipper follicle cell clones, Pak3 expression is lost, suggesting that Pak3 levels are regulated in response to actomyosin contractility. One possible mechanism by which Rho1-actomyosin contractility could be regulating Pak3 is through the transcription factor complex consisting of MAL-D and serum response factor (SRF). Nuclear accumulation of MAL-D during border cell migration has been shown to be regulated by perceived tension (SOMOGYI and RORTH 2004). A model for this regulation suggests that monomeric G-actin is bound to and inhibits MAL-D. In response to Rho1-activated signaling, F-actin stress fibers form and dissociate from MAL-D. The free MAL-D enters into the nucleus and binds and activates SRF to promote transcription of target genes, such as Pak3 (MIRALLES et al. 2003; POSERN and TREISMAN 2006; SOMOGYI and RORTH 2004). My preliminary data suggest that Pak3 acts antagonistically to Pak via a feedback loop that functions through the Rho-activated actomyosin contractility pathway. Work on this project is further being pursued by Michael Chou.

6.4. Candidate regulators of the Rho pathway identified in the screen

In addition to the Rho pathway, I uncovered an antagonistic relationship between Pak and the Dpp pathway in the regulation of egg chamber elongation. A recent study in the Drosophila wing disc has demonstrated that Dpp signaling regulates the subcellular distribution of Rho1 activity and MLC phosphorylation in epithelial cells (WIDMANN and DAHMANN 2009). If this link between pathways also occurs in the follicular epithelium, it may be that loss of Dpp is suppressing the pak mutant phenotype through disruption of Rho1 signaling. Another possibility is that Dpp regulation of the actin filament crosslinking protein a-actinin in the follicular epithelium is relevant (WAHLSTROM et al. 2006).
The ability of \textit{wun} and \textit{wun2} alleles to suppress the \textit{pak} egg chamber elongation defect might also be due to down regulation of the Rho1 pathway, as \textit{wun} was picked up in an over-expression screen for suppressors of impaired Rho1 signaling (GREGORY \textit{et al.} 2007). Wun and Wun2 belong to a family of lipid phosphate phosphatases that regulate the levels of lipids involved in signaling including lysophosphatidic acid, which is an important activator of the RhoA pathway (MOOLENAAR \textit{et al.} 2004; PYNE \textit{et al.} 2004).

\textbf{6.5. Egg chamber elongation requires both polarized ECM as well as basal F-actin}

Living imaging of elongating egg chambers has provided a new mechanism for epithelial morphogenesis (section 1.7). Recent findings from the Bilder Lab indicate that the polarization of the ECM in response to tissue rotation promotes directed tissue elongation (HAIGO and BILDER 2011). My data in section 5.8 show that \textit{pak}, mutant egg chambers are able to rotate and thus, are able to generate polarized ECM components. However, this polarized ECM is not sufficient to generate elongated egg chambers. Given that the ECM and the underlying basal F-actin of follicle cells are polarized in the same circumferential orientation around the egg chamber, polarization of one (i.e. the ECM) likely influences the polarization of the other, the F-actin. In support of this idea, egg chamber rotation has been shown to start at stage 5 and the polarization of the basal F-actin occurs at stage 6. What are the mechanisms that govern this? Since \textit{pak} mutant egg chambers are able to rotate but cannot elongate, I postulate that Pak is required to relay signals from the ECM to the underlying actin cytoskeleton to direct basal F-actin polarization and ultimately regulate elongation. Work done in our lab collaboratively by myself and Simon Wang showed that the localization of Pak both in the follicular epithelium and in the epidermis of the embryo is integrin-dependent (my contributions are found in the appendix, section 7.2) (BAHRI \textit{et al.} 2010). Previous work demonstrates that stress fibers are anchored at integrin-based focal adhesion complexes, which link the ECM to the actin cytoskeleton (BURRIDGE and CHRZANOWSKA-
Moreover, Pak and mammalian Pak have both been shown to localize at focal adhesion complexes (BOKOCH 2003; HARDEN et al. 1996; MANSER et al. 1997; SANDER and COLLARD 1999; SELLS et al. 2000; ZHAO et al. 2000). Follicle cells have been shown to secrete ECM components such as Laminin A (O'REILLY et al. 2008), which activates integrin receptors which may in turn recruit Pak to focal adhesion complexes. The ability of pak rounded egg chambers to rotate also suggests that egg chamber rotation is not initiated by the basal F-actin, since the basal F-actin in pak mutants is disorganized and reduced (Figure 1.9C & 5.3B) (CONDER et al. 2007), and egg chamber rotation begins prior to polarization of the basal F-actin. Rather, rotation may be driven by accumulations of polarized cortical, actin-based extensions of the follicle cells. In images of fixed wild-type ovaries it appears that the cortical actin of follicle cells is polarized, with small filopodia-like extensions largely pointing in one direction (Figure 6.2). The follicular epithelium can be thought of as a migratory epithelial sheet without one specific “leading edge”. It may be that all the follicle cells participate in epithelial sheet migration by extending short filopodia to propel egg chamber rotation by crawling over the ECM. Live imaging of follicle cells expressing Actin-GFP will enable visualization of the dynamic behaviour of these filopodia and will show if they point in the direction of rotation. I predict that since pak mutant egg chambers are able to rotate, the cortical actin will not be affected and so, small actin-based protrusions will be maintained.
Figure 6.2  Cortical actin of follicle cells extends small actin-based protrusions on one side of the cell

Stage 9 egg chamber stained with phalloidin to look at cortical and basal actin. Actin based protrusions of follicle cells (arrowheads) largely appear to be pointing in the same direction (arrow). In this case, most filopodia are pointing up, which may indicate the direction of rotation for this particular egg chamber.
7. Appendix

7.1. Third chromosome deficiency screen

In an effort to ultimately do a genome wide deficiency screen looking for modifiers for the pak rounded egg chamber phenotype I utilized the 3rd chromosome deficiency kit available from the Bloomington Stock Center. Michael Chou, Julienne Jagdeo and Chloe Chen carried out the recombinations of various deficiencies to the pak\(^6\) allele. The third chromosome deficiencies that have been tested are listed in Table 7.1. Two of these deficiencies *Df(3L)R-G7* and *Df(3R)DG2* appeared to suppress the pak rounded egg chamber phenotype (Figure 7.1 A, B). Interestingly, *Df(3R)DG2* in addition to two other deficiencies, *Df(3)Exel-6197* and *Df(3R)3450*, appeared to enhance follicle cell multilayering (Figure 7.1C, D), another pak phenotype attributed to Pak’s role in epithelial integrity (BAHRI et al. 2010; CONDER et al. 2007). Candidate genes from each of these deficiencies have not yet been identified or tested as I have not pursued this screen any further.
Table 7.1  Third chromosome deficiencies tested as modifiers of the *pak* rounded egg chamber phenotype

<table>
<thead>
<tr>
<th>Deficiencies Tested</th>
<th>Breakpoints</th>
<th>Modifying effect</th>
</tr>
</thead>
<tbody>
<tr>
<td>Df(3L)emc-E12</td>
<td>61A-61D</td>
<td>No effect (NE)</td>
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<tr>
<td>Df(3L)Ar14-8</td>
<td>61C4-62A8</td>
<td>NE</td>
</tr>
<tr>
<td>Df(3L)R-G7</td>
<td>62B9-62E7</td>
<td>Suppressor</td>
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<td>63F6-64C9</td>
<td>NE</td>
</tr>
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<td>NE</td>
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<td>NE</td>
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</tr>
<tr>
<td>Df(3L)hi79c</td>
<td>77B7-77F5</td>
<td>NE</td>
</tr>
<tr>
<td>Df(3R)sbd105</td>
<td>89A1-89B10</td>
<td>NE</td>
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<tr>
<td>Df(3R)P115</td>
<td>89B7-89E7</td>
<td>NE</td>
</tr>
<tr>
<td>Df(3R)sbd104</td>
<td>89B6-89B18</td>
<td>NE</td>
</tr>
<tr>
<td>Df(3R)DG2</td>
<td>89E-91B2</td>
<td>Supp &amp; Enhancer</td>
</tr>
<tr>
<td>Df(3R)Ex6197</td>
<td>95D8-95E1</td>
<td>Enhancer</td>
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<tr>
<td>Df(3R)orb87-5</td>
<td>95F7-96A20</td>
<td>NE</td>
</tr>
<tr>
<td>Df(3R)Esp13</td>
<td>96F1-97B1</td>
<td>NE</td>
</tr>
<tr>
<td>Df(3R)T1-P</td>
<td>97A-98A2</td>
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<tr>
<td>Df(3R)3450</td>
<td>98E3-99A6</td>
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</tr>
<tr>
<td>Df(3R)Dr-rvl</td>
<td>99A1-99B11</td>
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Listed are the deficiencies tested and their respective estimated cytological breakpoints. Two deficiencies appeared to suppress the *pak* rounded egg chamber phenotype and two deficiencies appeared to enhance the *pak* multilayering phenotype. One deficiency appeared to both suppress and enhance these *pak* mutant phenotypes. Candidate genes have not been identified.
Figure 7.1 Third chromosome deficiencies that suppress the pak rounded egg chamber phenotype and/or enhance the pak multilayering phenotype

Ovaries stained with phalloidin to look at cell outlines. Production of a stage 12 egg chamber in Df(3L)R-G7, pak6/pak11 flies (A) and an elongated stage 10b egg chamber in Df(3R)DG2, pak6/pak11 (B) suggests that these two deficiencies may contain a suppressor of the pak rounded egg chamber phenotype. Df(3R)DG2, pak6/pak11 (C) and Df(3R)Exel6197, pak6/pak11 (D) egg chambers exhibit multilayering that appears to be slightly more severe than that observed in pak mutants alone, suggesting that these deficiencies may contain an enhancer of pak loss of epithelial integrity phenotype. Scale bars: 150µm (A, B) and 50µm (C, D).
7.2. Pak is required for separate junction formation


The follicular epithelium provides an excellent model for looking at the apical-basal polarity. Previous work performed by Ryan Conder demonstrated an early role of Pak in the specification of apical-basal polarity during oogenesis (Conder et al. 2007). pak follicle cell clones exhibit bilayering/multilayering that resemble the multilayering observed in follicle cell clones mutant for components of the Scribble complex, encoded by scribble (scrib), lethal giant larvae (lgl) and discs large (dlg) (Goode and Perrimon 1997) (Bilder et al. 2000; Bilder and Perrimon 2000; Goode et al. 2005), suggesting that Pak may participate with the Scribble complex during the formation and maintenance of apical-basal polarity (Conder et al. 2007). Recent studies in mammalian cell culture studies show that Scrib can recruit Pak to the leading edge of migrating cells (Nola et al. 2008; Osmani et al. 2006) suggesting that Pak and Scrib may form a complex together during epithelial morphogenesis. In our lab, Simon Wang has been focused on the requirement of Pak in the restoration of cell-cell junctions at the leading edge (LE) of the dorsal-most epidermal cells (DME) during dorsal closure of the Drosophila embryo. Dorsal closure has been a useful tool for the study of developmental epithelial fusions, wound healing and cell migration. The DME cells are of particular interest because they acquire specific characteristics at the LE that allow for migration of the epidermal flanks toward each other, while the remaining sides of the DME cells show no obvious changes (Arquier et al. 2001; Harden 2002; Kaltschmidt et al. 2002; Woolner et al. 2005; Zeitler et al. 2004). Previous findings show that Pak and its upstream activators Rac and Cdc42 participate in dorsal closure, as well as all the components of the Scribble complex (Bilder et al. 2000; Harden et al. 1995; Harden et
al. 1999; Jacinto et al. 2000; Manfruelli et al. 1996; Woods et al. 1996) (Arguier et al. 2001; Woolner et al. 2005; Zeitler et al. 2004). Work done by R. Conder showed that Scrib and Pak could be coimmunoprecipitated together (results in thesis of R. Conder, 2007) and furthermore, S. Wang has shown that Pak localizes to integrin complexes on the lateral membrane of DME cells where it recruits the Scribble complex (Bahri et al. 2010). To complement these results, I focused on the follicular epithelium to see if Pak could function similarly in a different tissue. The basal localization of Pak (Figure 7.2A) has been shown to be disrupted in scrib and lgl clones (results in thesis of R. Conder, 2007). To determine if Pak could regulate membrane localization of the Scrib complex, I over-expressed a version of Pak constitutively localized to the membrane by myristoylation (Pakmyr) (Hing et al. 1999), in the FE using traffic jam (TJ)-Gal4, and looked at the distribution of Scrib and Dlg. Normally the Scrib complex proteins are restricted to the lateral membranes (Figure 7.2 B-D), but over-expression of Pakmyr caused Scrib, Dlg and Lgl to additionally accumulate along the apical membrane (Figure 7.2 E-E’, F-F’). Previous findings show that the lateral membrane localization of Dlg was disrupted in pak follicle cell clones in the FE (Conder et al. 2007) and this was also the case for Scrib and Lgl (Figure 7.2 G-G’, H-H’). These results complement the work of S. Wang and R. Conder and together indicate that Pak is a component of the Scrib complex in epithelia and shows a mutual dependence with Scrib, Dlg and Lgl for membrane localization. Studies in mammalian cell culture suggests that Pak is a component of integrin-based focal adhesion complexes (reviewed in (Bokoch 2003)) and previous findings from our lab suggests that this mechanism of membrane localization appears to be conserved in Drosophila as Pak membrane localization in the FE is integrin-dependent (Conder et al. 2007) (Figure 7.3 A-A’). Lateral Pak recruitment would require lateral integrins and βPS- and αPS-integrins are found at the lateral and apical membranes of follicle cells in addition to their expected basal localization (Dinkins et al. 2008; Fernandez-Minan et al. 2007; Goode et al. 1996) (Figure. 7.3B). Results from S. Wang’s work in the embryo suggest that in situations where Pak participates in Scrib complex and/or septate junction recruitment, integrins should similarly
Figure 7.2  Components of the Scribble complex can be ectopically recruited to the membrane when pak is mis-expressed

(A-D) Wild-type egg chamber showing Pak distribution (A), localization of Dlg (B), Scrib (C) and Lgl (D) to lateral membranes of follicle cells. (E-E´, F-F´) pak<sup>myr</sup>-expressing egg chambers showing heavy accumulation of Pak<sup>myr</sup> at follicle cell membranes (E,F) and ectopic Dlg (E´) and Scrib (F´) accumulation at the apical membrane (arrowheads). (G-G´) pak mutant follicle cell clones stained with GFP (G) showing that Scrib (G´) localization is dependent on Pak.
be required. I looked at Scrib complex recruitment in clones of mys mutant follicle cells and found that similar to pak follicle cell clones, lateral localization of Dlg was disrupted (Figure 7.3 C-C’). This result is in contrast to previous results in which follicle clones of the same mys allele showed retention of lateral Dlg (FERNANDEZ-MINAN et al. 2008; FERNANDEZ-MINAN et al. 2007). The reason why my results differ from these previous findings might be the different approach I used to create mys clones. Whereas the earlier studies used e22c-Gal4 to drive UAS-FLP, I used heatshock and hsFLP. mys embryos fail to accumulate the septate junction protein Fasciclin III to the dorsal midline upon completion of dorsal closure (BAHRI et al. 2010). To further investigate this relationship between Pak, SJs and integrins in the FE I created FCCs mutant for coracle (another component of the septate junction) and scrib and looked at the effects on β-Integrin expression. Ectopic integrin accumulates in coracle mutant stalk cells and scrib follicle cell clones (Figure 7.3 D-D’, E-E’). Interestingly, when scrib follicle cell clones occurred in contact with scrib germline clones, integrin staining was lost in the follicle cells, indicating a cell-non-autonomous effect of loss of Scribble in the germline. This may be due to disruptions of germline-to-somatic cell signaling (Figure 7.3 F-F’).

The FE is an example of an epithelium derived from a mesenchymal-like intermediate and collectively our data suggest that Pak might only be required for the recruitment of the Scrib complex in this type of epithelium also known as a secondary epithelium. Localization of Pak at the lateral membranes is integrin dependent and previous studies have demonstrated a requirement for integrins in the maintenance of apical-basal polarity (CONDER et al. 2007; DEVENPORT and BROWN 2004; FERNANDEZ-MINAN et al. 2008; FERNANDEZ-MINAN et al. 2007). During development of the follicular epithelium, integrin activation is first observed in FSCs and has been demonstrated to be required for FSC proliferation (O’REILLY et al. 2008), which may show a role for integrins during the initial stages of follicle cell polarization.
Figure 7.3  Interdependence between Scrib complex or septate junction proteins and integrins for localization in follicular epithelium

(A, C, D, E, F) FCC are distinguished by a lack of GFP staining. Arrowheads mark some clone boundaries. (A, A’) Clone of mys mutant cells showing disruption of Pak localization in follicular epithelium in the absence of βPS-integrin. (B) Egg chamber showing βPS-integrin at the lateral and apical membranes in addition to basal membrane. (C, C’) Membrane localization of Dlg is disrupted in mys mutant cells. (D, D’) cora clones in stalk cells show elevated levels of βPS-integrin at the membrane. (E, E’) Egg chamber showing elevated integrin staining at the apical membrane in a scrib mutant clone. (F, F) In egg chambers where a scrib follicle cell clone overlies a germline scrib clone (indicated by lack of GFP staining in the germline), integrin staining is lost in the follicle cell clone. Arrowheads indicate clone borders. Scale bar: 10 mm.
7.3. dPix RNA localization does not correlate with its known association with Pak

Group I Paks have been shown to interact directly with a Rac/Cdc42 guanine exchange factor βPix (Pak interacting exchange factor) at focal adhesion complexes (FENG et al. 2002; HOEFEN and BERK 2006; LOO et al. 2004; MANSER et al. 1997; ZHAO et al. 2005; ZHAO et al. 2000). βPix also interacts with (GPCR)-kinase-interacting protein 1 (Git1) (MANSER et al. 1998) and this βPix/Git1 complex has been shown to be essential in the activation of Pak at focal complexes (LOO et al. 2004). Recent mammalian cell culture studies have shown that Scrib interacts with βPix directly at the leading edge during cell migration (OSMANI et al. 2006), and this interaction is required to recruit Pak to the leading edge (NOLA et al. 2008; OSMANI et al. 2006).

Preliminary work done by Ryan Conder has shown that pak, dpix and git1 mutants all share similar oogenesis defects exhibiting gaps and multilayering within the follicular epithelium, and all three proteins co-localize to the basolateral membranes of the FE, suggesting that βPix and Git1 may be interacting with Pak to regulate apical-basal polarity (results in thesis of R. Conder, 2007). Anti-dPix antibodies obtained from out collaborator Sami Bahri, did not work well in the embryo (unpublished results, R.C). Therefore, to look at the role that dPix might have during dorsal closure of the embryo I preformed an RNA \textit{in situ} hybridization using a pix RNA probe on wild-type embryos (described in section 2.12). The colorimetric reaction did not provide a high enough resolution for me to determine which cells pix RNA was transcribed in (Figure 7.4A). I went on to perform fluorescence \textit{in situ} hybridization using the same probe. Staining these wild-type embryos with an antibody again phosphorylated-tyrosine to mark cell outlines, I was able to detect localization of pix transcripts at a more detailed level. Given the known roles of βPix at focal adhesion complexes and its ability to interact with Pak, Scrib, Git1 as detailed above, I was expecting the dpix probe to be expressed at the leading edge or in the DME cells during dorsal closure. Surprisingly, dpix RNA appears to be expressed two-cell rows behind the DME cells in a unique pattern down the center of each segment (Figure 7.4 B-B″, C-C″). This is intriguing and
suggests that further work should be done to determine how dPix might be functioning during dorsal closure.
Figure 7.4  *dpix* is expressed in the dorsal epidermis but not in the DME cells during dorsal closure

RNA *in situ* hybridization of *dpix* on wild-type embryos. (A) Colorimetric reaction shows a distinct expression pattern. (B-B', C-C') Fluorescence *in situ* hybridization of *dpix* (red) co-stained with anti-PY antibodies (green) to show cell outlines. (C-C’) provides a higher magnification of an embryo during dorsal closure. *dpix* transcript is not found in the DME cells but instead two-cell rows back behind the leading edge. Scale bars: 100µm (A), 50µm (B-B')(C-C’).
8. Final conclusions

The follicular epithelium has been a useful system for studying key signalling and structural components required for establishment and maintenance of epithelial integrity. My work and that of my former lab mates has demonstrated roles for Pak several times during the life of the follicular epithelium, from the specification and establishment of the follicle stem cell niche in the pupal ovary to regulation of the basal F-actin cytoskeleton during egg chamber elongation. The follicular epithelium is similar to other epithelia in which cells arise from a stem cell and eventually slough off or undergo apoptosis. Beginning at the division from the FSC, daughter follicle cells are responsible for encapsulating the germline cyst in a monolayer of cells and manage to do so in approximately 40 hours. Proliferation of the follicular epithelium occurs over the next 30 hours and egg chamber elongation proceeds over the following 18-20 hours whereby follicle cells stop proliferating and enter into endoreplicative cycles. Over the next 13-15 hours, follicle cells migrate, undergo dramatic changes in cell shape and secrete components required for eggshell formation (HORNE-BADOVINAC and BILDER 2005; SPRADLING 1993). Once the egg is laid, the larva hatches from the eggshell at the end of embryogenesis, thus completing the life of the follicular epithelium.

My work has shown that Pak function varies throughout gonadogenesis and oogenesis. Although I have not been able to define how Pak is acting in the pupal ovary during establishment of the FSC niche, I have demonstrated that Pak is required early in the formation of the adult germarium (Section 3). Together with previous work done in this lab and the work of lab mate Simon Wang, I have shown that Pak participates in establishing epithelial integrity of the follicular epithelium (Appendix). Pak’s ability to recruit the Scribble complex and mediate mesenchymal-to-epithelial-like transitions suggests a possible mechanism for establishment of the FSC niche. Escort cell/FSC precursors may exhibit mesenchymal qualities and could undergo a
mesenchymal-to-epithelial-like transition as they become immobilized and specified. I have shown that Pak may have temporally distinct roles in the escort cell/FSC precursors in the pupal ovary and the escort cells of the adult germarium. Escort cells appear to have mesenchymal-like properties with highly dynamic protrusions but ultimately assume a fixed position. Other examples in *Drosophila* of cells with partial mesenchymal-like features include the dorsal-most epidermal cells found in embryos undergoing dorsal closure and the border cell cluster of cells that detach from the follicular epithelium in stage 10A egg chambers (reviewed in (REVENU and GILMOUR 2009)). These two types of cells exhibit reduced epithelial integrity at one side of the cell while maintaining cell adhesions and apical-basal polarity at the other sides. Pak’s function in cell migration and the establishment of apical-basal polarity suggests that it may have a role in escort cells/FSC precursors by locally regulating mesenchymal transitions within the cell.

During egg chamber elongation I have shown that Pak acts antagonistically to the Rho1-activated actomyosin contractility pathway in regulating organization of the basal F-actin in the follicle cells (Section 5). What could be upstream of Pak in this function? Given that Pak localization has been shown to be integrin-dependent, Pak may be recruited to the basal domain of follicle cells during egg chamber rotation and may act as the link between the ECM and the underlying actin cytoskeleton to participate in egg chamber elongation.

My work has focused on the where and when of Pak’s participation in development of the follicular epithelium. Hopefully, future studies in the lab will unveil mechanisms of action, for example, targets for Pak phosphorylation.
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