

**dPak functions in cytoskeletal polarization  
and epithelial morphogenesis  
during *Drosophila* embryogenesis and oogenesis**

by

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**Abstract**

The Rho GTPases (Rho, Rac and Cdc42) play key roles in regulating cytoskeletal rearrangement, membrane trafficking, transcription activation and development. Many putative effector molecules for each member of the Rho family have been identified, of which the p21-activated kinases (Paks) are shared effectors for Rac/Cdc42. I was interested in the characterization of a *Drosophila* homologue of Pak (dPak) during embryonic dorsal closure (DC) and oogenesis.

During DC, a hole in the dorsal epidermis, occupied by an epithelium called amnioserosa, is sealed by dorsalward migration of the epidermal flanks driven by a contractile apparatus at the dorsal end of the advancing epidermis in the leading edge cells (LE). I examined phenotypes in DC of constitutively active versions of Drac1 (Drac1V12) and Dcdc42 (Dcdc42V12) induced in a *dpak*<sup>6</sup> mutant background. I expressed transgenes bearing an autoinhibitory domain (AID) of dPak, which blocks the endogenous kinase activity of Pak-I subfamily kinases, in the amnioserosa and the LE during DC. Pak-I subfamily kinase activity is required in the amnioserosa for morphogenesis of the epidermis but not for amnioserosa cell shape change. Inhibition of the Pak-I kinase activity in the LE cells

results in cell shape defects but does not eliminate c-Jun N-terminal kinase (JNK) pathway function, a kinase cascade involved in Rac/Cdc42 signaling.

Prominent defects were found in ovarioles transheterozygous for the *dpak<sup>6</sup>* and *dpak<sup>10</sup>* mutant alleles. Egg chambers were spherical in shape and degenerated by stage 9 with frequent fusions of egg chambers in a side-by-side configuration. Discontinuities in the follicular epithelium were frequent and a bilayer of follicle cells (FCs) piled up at the posterior end over the oocyte, losing their ability to change to columnar in shape. Mutant egg chambers were defective in both the assembly and organization of the basal F-actin. The results indicate that dPak participates in the organization of the follicular epithelium during oogenesis and is required for proper egg chamber elongation.

In conclusion, dPak has important functions in epithelial morphogenetic events during DC and oogenesis. Further work should help elucidate the pathways dPak participates in during the regulation of the actin cytoskeleton and cell shape change.

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## **Introduction**

## **The Rho subfamily of Ras-related small GTPases**

The Rho subfamily of Ras-related small GTPases (p21s) function in signal transduction cascades and participate in a wide range of cellular events including regulation of cytoskeleton, membrane trafficking, transcription activation, cell growth and development (reviewed in Settleman, 1999). Different p21s with multiple isoforms have been identified such as Rho (A, B, C, D, E), Rac (1, 2, 3), Cdc42 (Cdc42Hs, G25K), and TC10 etc. (reviewed in Van Aelst and D'Souza-Schorey, 1997). These closely related proteins are ubiquitously expressed and highly conserved through species from yeast to human beings. Like all members of the Ras superfamily, the Rho GTPases cycle between a GTP-bound activated state and a GDP-bound inactivated state regulated by two opposing families of molecules. Guanine nucleotide exchange factors (GEFs) promote the binding of GTP to the GTPases and the GTPase-activating proteins (GAPs) increase the intrinsic hydrolysis of bound GTP to GDP. In addition, p21s are regulated further by guanine nucleotide dissociation inhibitors (GDI), which can inhibit both the exchange of GTP and the hydrolysis of bound GTP, but the precise role of GDI is still poorly understood (reviewed in Ridley, 1999; Van Aelst and D'Souza-Schorey, 1997).

One of the major functions ascribed to p21s is their role in the organization of the actin cytoskeleton, which has been extensively studied using cell culture (reviewed in Van Aelst and D'Souza-Schorey, 1997). Constitutively active Rho can induce cultured cells to form actin stress fibers, regions where stress fibers are anchored to the plasma membrane and where the cell adheres most tightly to the substratum. Activated Rac can induce lamellipodia and membrane ruffles and Cdc42 can induce the formation of filopodia, more dynamic peripheral structures. Both Rac and Cdc42 have been shown to induce the assembly of multi-molecular focal adhesions/focal complexes at the plasma membrane of fibroblasts (Nobes and Hall, 1995b). A hierarchical cascade is found in the Rho subfamily, where activation of Cdc42 leads to Rac activation, and Rac activation leads to Rho activation (reviewed in Allen *et al.*, 1997; Nobes and Hall, 1995a; Ridley *et al.*, 1992). It has also been shown that Rac/Cdc42 are potent activators of the c-jun N-terminal kinase/stress-activated protein kinase (JNK/SAPK) and p38 mitogen-activated protein kinase (MAPK) cascades, which, in *Drosophila*, lead to the transcription of *decapentaplegic (dpp)*, a transforming-growth-factor-beta-like (TGF- $\beta$ ) ligand (reviewed in Boettner and Van Aelst, 1999; Harden, 2002).

Rho1 functions through Rho-specific downstream targets and often in pathways distinct from those mediated by Cdc42/Rac. However, studies also show that Rho pathways and Cdc42/Rac pathways are interwoven and coordinated in regulating cytoskeletal morphogenesis (Lu and Settleman, 1999; Settleman, 1999). As my project is focused on Pak (p21-activated kinase), a Cdc42/Rac effector, I will focus my introduction on signaling by Cdc42/Rac. At this point I will describe two morphogenetic processes involving Cdc42/Rac that I have studied with respect to *Drosophila* Pak (dPak) in this thesis.

### **Two actin-rich model systems in *Drosophila* for the study of cytoskeletal regulation and signal transduction in development: dorsal closure and oogenesis**

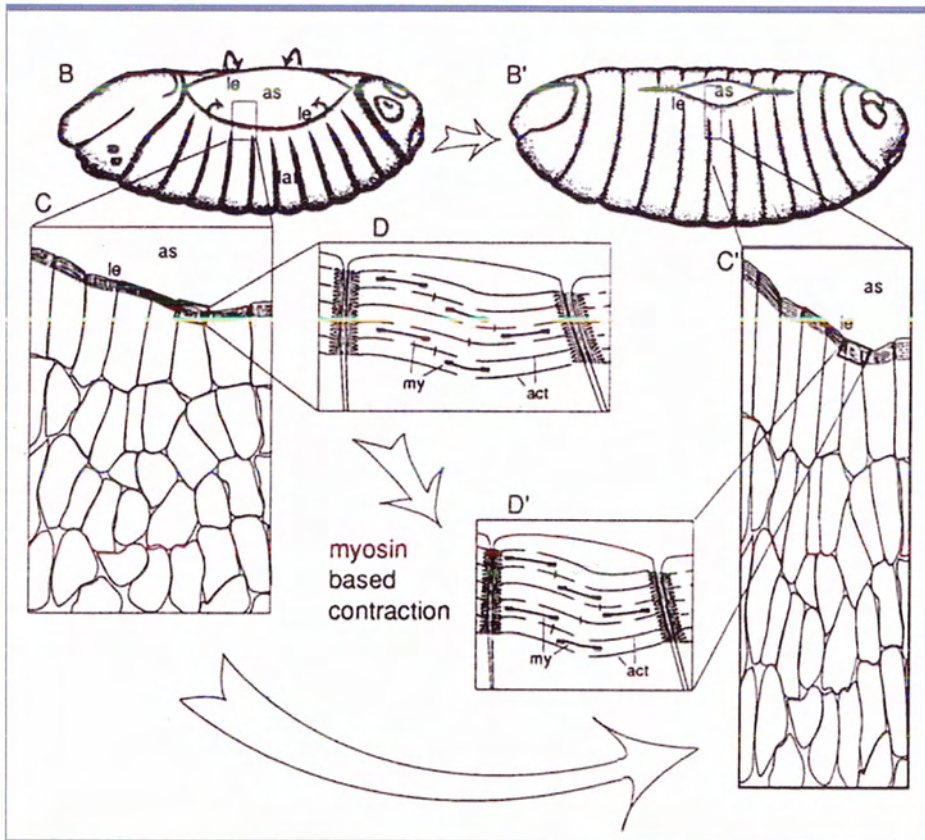
A morphogenetic process called dorsal closure (DC) occurs during *Drosophila* embryonic development (reviewed in Harden, 2002). Following germ band retraction, a hole is left in the epidermis of the developing embryo, which is covered by a layer of squamous cells called the amnioserosa. Beginning at stage 14, the two flanks of the lateral epidermis migrate dorsally, moving over the amnioserosa and completely suture the hole at the dorsal midline by stage 15. This dramatic morphological change is partly

mediated by cell elongation via an acto-myosin contractile apparatus consisting of filamentous actin (F-actin) and non-muscle myosin heavy chain (hereafter referred to as myosin) in the leading edge cells (LE), the single row of cells at the dorsal end of the epidermis (reviewed in Harden, 2002; Knust, 1997; Martin *et al.*, 1997; Noselli and Agnes, 1999). In *zipper* mutant embryos lacking zygotically-encoded myosin, the LE fails to advance properly (Young *et al.*, 1993). Phosphotyrosine and other components of adherens junctions form triangular nodes that may represent specialized adherens junctions organizing the contractile apparatus (reviewed in Harden, 2002) (Fig. 1).

Multiple forces appear to be required for the process of DC, but none of them alone can account for its completion. The contractile purse-string like apparatus at the LE facilitates the closure driving the constriction of the cells in an anterior-posterior (A-P) direction. Actin based filopodia and lamellipodia reaching out from the opposing sheets of the LE help cell matching when the two sides of the epidermis come together (Jacinto *et al.*, 2000). The more lateral epidermis produces forces that oppose DC, while the autonomous contractility of amnioserosa contributes to DC (Kiehart *et al.*, 2000). Following germ band retraction but prior to the onset of DC, cells in the amnioserosa are elongated perpendicular to the A-P axis of the embryos and there are no major accumulations of



Fig. 1 Kiehart model of dorsal closure (DC) (taken from Young *et al.*, 1993). as: the amnioserosa. le: the leading edge cells. act: F-actin. my: non-muscle myosin. (B, B') Dorsal view of the beginning of DC (B) to near completion (B'). (C, C') Expanded representation of the acto-myosin contractile apparatus deposited at the dorsal end of the LE cells driving cell elongation from beginning of DC (C) to near completion (C'). (D, D') The constriction of the acto-myosin apparatus consisting of F-actin and non-muscle myosin.



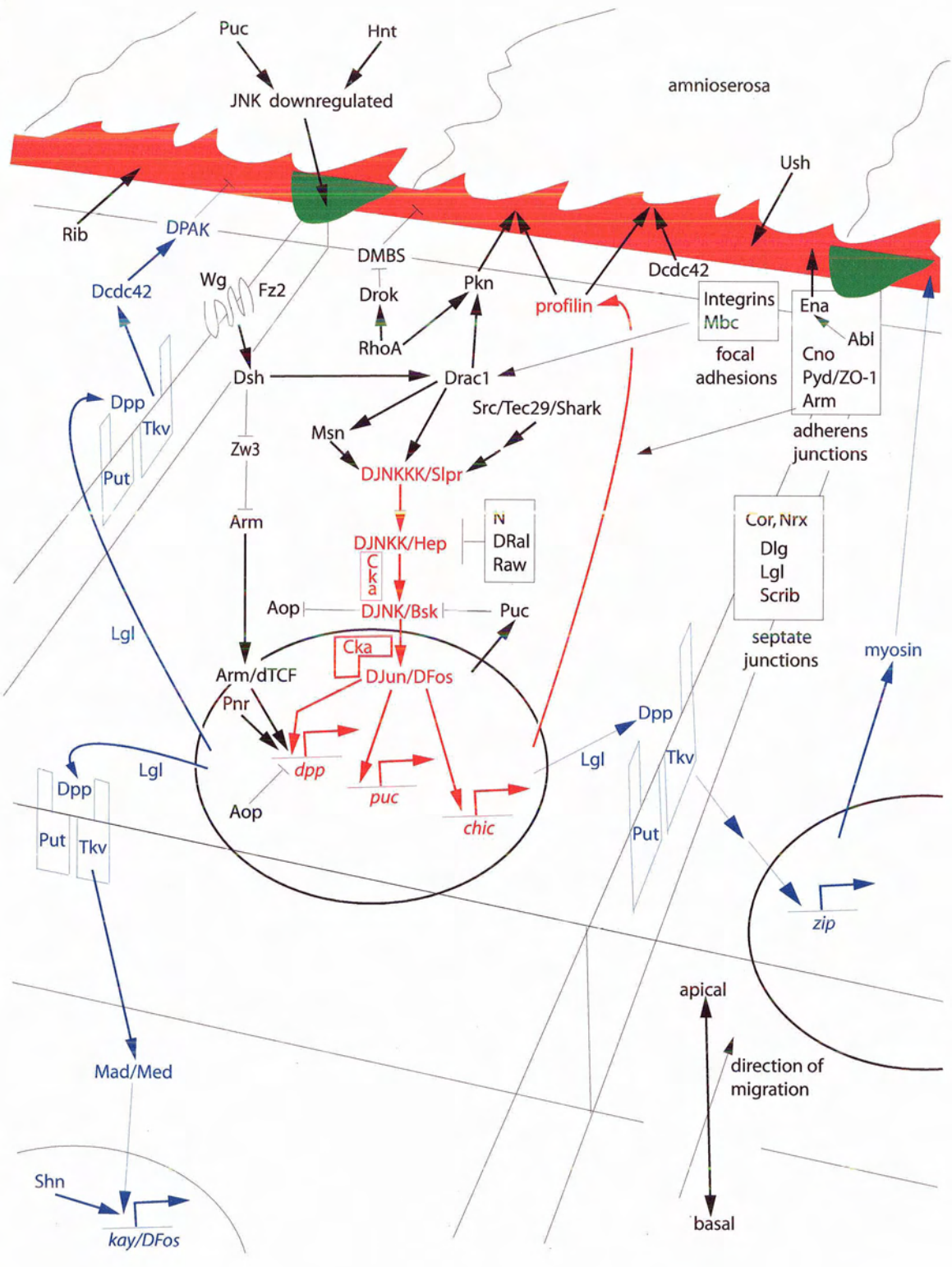
myosin and F-actin. Occurring simultaneously with the alteration of epidermal morphology, the amnioserosa changes from an elliptically shaped squamous epithelium to a narrow, tubular structure. As DC proceeds, cells of the amnioserosa constrict at their apical ends with heavy accumulation of myosin, F-actin and phosphotyrosine. This cell constriction, driven by the genes expressed in this tissue, contributes to the change in the morphology of the tissue (Harden *et al.*, 2002; Kiehart *et al.*, 2000).

Among the five families of MAPK cascades including extracellular regulated kinase 1 and 2 (ERK1/2), JNK, p38, ERK3/4, and ERK5, the JNK cascade is a major regulator of DC (reviewed in (Harden, 2002)). *Drosophila* JNKKK (DJNKKK) encoded by *slipper* (*slpr*) activates *Drosophila* JNKK (DJNKK) encoded by *hemipterous* (*hep*), which activates *Drosophila* JNK (DJNK) encoded by *basket* (*bsk*). DJNK further activates DJun/DFos leading to the transcriptional regulation of *dpp*, *puckered* (*puc*), *chickadee* (*chic*), and probably many other genes (reviewed in Harden, 2002). Dpp, encoded by *dpp*, binds to TGF- $\beta$  receptors including Thickveins (Tkv) and Punt (Put) on the LE cells activating a TGF- $\beta$  pathway that is also required for DC (Ricos *et al.*, 1999). Puc encoded by *puc* is a dual specificity MAPK phosphatase that negatively regulates the JNK pathway (reviewed in Harden, 2002). *Chic* encodes *Drosophila* profilin, an actin binding

protein involved in F-actin polymerization (Cooley *et al.*, 1992). DC defects result from lack of any of the JNK pathway molecules. Multiple signal transduction pathways participate during DC, for example nonreceptor tyrosine kinases function as upstream activators of JNK cascade and the Wingless pathway is also involved (reviewed in Harden, 2002) (Fig. 2).

*Drosophila* oogenesis provides another excellent system for studying morphogenetic changes such as cell migration, membrane fusion and cytoskeletal remodeling (reviewed in Spradling, 1993; Verheyen and Cooley, 1994). An ovary contains 15-17 ovarioles, each containing 6 to 7 sequentially more mature egg chambers starting from the very anterior end, which is named the germarium. Each egg chamber has 16 germ-line cells, one of which develops into the oocyte, whereas the rest stay as nurse cells. An epithelial monolayer of somatic follicle cells (FCs) surrounds the germ-line clusters, whose cells are cuboidal in shape by stage 9 (Frydman and Spradling, 2001) (Fig. 3). All these three disparate cell types display a distinct array of actin-rich structures. Ring canals, actin-rich cytoplasmic bridges, connect the 16 germ line cells throughout oogenesis, and are derived from incomplete cytokinesis when the single germline precursor cell experiences four rounds of cell division. F-actin is concentrated at the cortices of the nurse cells and

Fig. 2 Schematic diagram summarizing signaling occurring during DC (taken from Harden, 2002).

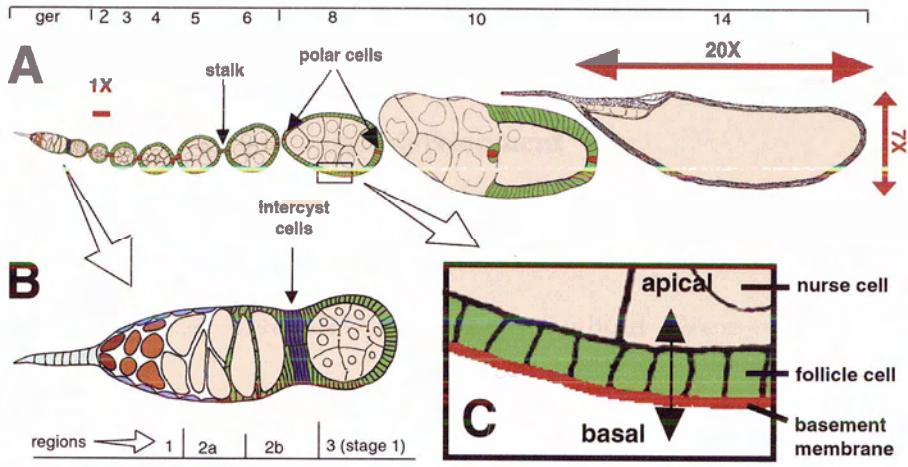


oocyte. In somatic cells, F-actin bundles accumulate throughout the basal surface in the columnar FCs. F-actin also localizes at the adherens junctions between the FCs and the follicle-germ cell interface (reviewed in Spradling, 1993; Verheyen and Cooley, 1994).

During oogenesis several morphogenetic events occur accompanied by dramatic changes in the actin cytoskeleton, providing a simple model system to analyze signaling pathways regulating cell motility (reviewed in Spradling, 1993; Verheyen and Cooley, 1994). One dramatic morphogenetic change is the rapid transport of nurse cell content, including proteins and mRNAs into the oocyte through the ring canals in late oogenesis (stage 11). The dumping process, resulting from the myosin-based contraction of subcortical actin, is preceded by the polymerization of a meshwork of actin filaments that creates a cage surrounding the nurse cell nuclei. The actin filamentous cages are assumed to anchor the nuclei when the nurse cell contents are passing into the oocyte. The somatic FCs undergo dynamic rearrangement and cell shape changes throughout oogenesis. The majority of the uniformly cuboidally shaped FCs rearrange into a columnar epithelium surrounding the oocyte in mid-oogenesis (stage 9), whereas the few (approximately 30-50) remaining nurse cell-associated FCs become squamous in shape. Meanwhile, 6-10 FCs, known as border cells, migrate from the anterior of the egg chamber through the nurse cell cluster

Fig. 3 Structure and development of *Drosophila* ovarian follicles (taken from Frydman and Spradling, 2001). (A) A schematic diagram of an ovariole consisting of progressively more mature egg chambers. (B) Germ line stem cell division in germarium. (C) Enlargement of follicle cells overlying the germ line.





to the anterior end of the oocyte to generate the micropyle pore for sperm entry. This protrusion or extension process of the border cells is accomplished with F-actin and myosin. Beginning in stage 10B, the centripetally migrating FCs move inward between the nurse cells and oocyte to eventually cover the oocyte and to create anterior eggshell structures such as the operculum (reviewed in Spradling, 1993; Verheyen and Cooley, 1994).

### **Drac1 and Dcdc42 in *Drosophila* development**

Rho-GTPases have multiple-functions throughout *Drosophila* development. The *Drosophila* Rac proteins participate in myoblast fusion (Luo *et al.*, 1994), synapse assembly (Allen *et al.*, 2000), axonal outgrowth (Kaufmann *et al.*, 1998) and photoreceptor morphogenesis (Chang and Ready, 2000; Hakeda-Suzuki *et al.*, 2002; Ng *et al.*, 2002). In the wing disc epithelium, Drac1 is involved in actin recruitment at adheren junctions and restricting the sites at which hairs grow (Eaton *et al.*, 1995; Eaton *et al.*, 1996). Drac1 is widely implicated in tissues undergoing dramatic morphogenetic change including germ band retraction, DC (Harden *et al.*, 1999; Ricos *et al.*, 1999), amnioserosa contraction (Harden *et al.*, 2002) and oogenesis (Murphy and Montell,

1996). The three *Drosophila* Rac genes including *rac1*, *rac2*, *mtl* have largely overlapping functions. However, the study of loss-of-function mutations in these genes indicates that different degrees of Rac signaling are required for different processes. This may reflect variation in upstream regulators and/or downstream effectors (Hakeda-Suzuki *et al.*, 2002; Ng *et al.*, 2002). For example, the GEF factor, Trio, is essential for Rac function in axon growth and guidance, but not for epithelial morphogenesis and myoblast fusion, and the Rac binding effector Pak is not required for axon growth but axon guidance and branching (Hakeda-Suzuki *et al.*, 2002; Ng *et al.*, 2002).

Similarly, Dcdc42, a *Drosophila* homologue of Cdc42, participates in the regulation of myoblast fusion (Luo *et al.*, 1994), neurite outgrowth (Allen *et al.*, 2000), apico-basal epithelial elongation in wing disc (Eaton *et al.*, 1995; Eaton *et al.*, 1996), cell fate determination of wing veins as a negative regulator of Notch signaling (Baron *et al.*, 2000), peripodial membrane edge differentiation in imaginal discs (Agnes *et al.*, 1999), DC (Harden *et al.*, 1999; Ricos *et al.*, 1999) and oogenesis (Genova *et al.*, 2000; Murphy and Montell, 1996). It is clear that these Rho-GTPases have distinct tissue-specific functions as well as overlapping roles. I will focus on describing the roles of Drac1 and Dcdc42 in DC and oogenesis.

The involvement of *Drosophila* homologs of Rac in DC was first described (Harden *et al.*, 1995) using transgenic flies bearing a dominant negative version of Drac1, Drac1N17. The expression of *Hs-Drac1N17* induced by heat shock promoter results in a high frequency of embryos with failures in DC accompanied by disruption of actin and myosin along the LE (Harden *et al.*, 1995). Consistent with this, using Gal4 driven by the *patched* (*ptc*) ectoderm promoter to express *UAS-Drac1N17* causes DC defects with dorsal holes ranging in size from a small 'scab' to a wide hole with missing cytoskeletal component in *ptc* stripes along the LE (Harden *et al.*, 1996). A constitutively active version of Drac1 (Drac1V12), expressed with *ptc-Gal4* or *Hs-Gal4<sup>M-4</sup>*, has a drastic effect on the embryonic cuticle, almost completely blocking its development (Harden *et al.*, 1999). Co-expression of p21 transgenes shows that Drac1V12 overrides the Drac1N17 (Harden *et al.*, 1999). The study of loss-of-function mutations in the *Drosophila* Rac genes indicates that all three Rac proteins contribute to DC, however Drac1 is a major player (Hakeda-Suzuki *et al.*, 2002; Ng *et al.*, 2002). Recent studies show that Drac1 also participates in the active morphogenesis of the amnioserosa during DC (Harden *et al.*, 2002). Amnioserosa-specific expression of Drac1N17 inhibits the contraction of this tissue and impedes the movement of the LE. Expression of Drac1V12 in the amnioserosa

causes excessive contraction of the tissue with excessive accumulation of F-actin, myosin and phosphotyrosine in the amnioserosa cells (Harden *et al.*, 2002).

A high frequency of DC defects were also seen with the expression of the dominant negative version of Dcdc42 (Dcdc42N17) in embryos using *ptc-Gal4*, but the dorsal phenotype is more uniform with most showing a large hole towards the posterior of the embryos (Harden *et al.*, 1999). Using heat shock promoter, many *Hs-Gal4<sup>M-4</sup>;UAS-Dcdc42N17* embryos survive to larval stage, and phenotypes are generally limited to a mild puckering of the cuticle, with very few dorsal holes occurring. Expression of *UAS-Dcdc42N17* by a weaker heat shock driver, *Hs-Gal4<sup>2077</sup>*, surprisingly produces a greater number of dorsal holes than *Hs-Gal4<sup>M-4</sup>* and some embryos exhibit dorsal ends of segments being pulled together at the LE into bunches. The expression of Dcdc42V12 by the above drivers causes a high frequency of puckers in the dorsal surface (Harden *et al.*, 1999). Germ band retraction and DC defects are also seen in embryos produced by Dcdc42 mutant females bearing heteroallelic combinations of weak and strong *dcdc42* alleles of *dcdc42<sup>3</sup>/dcdc42<sup>6</sup>* and *dcdc42<sup>4</sup>/dcdc42<sup>6</sup>* with 70% embryonic lethality (Genova *et al.*, 2000). Partial but not complete loss of the LE cytoskeletal components in Dcdc42N17 embryos suggests Dcdc42 as a contributor to the LE cytoskeleton but to a lesser degree

than Drac1 (Harden *et al.*, 1999). Dcdc42V12 expression can cause some rescue of Drac1N17 induced effects, suggesting that in the absence of Drac1 signaling Dcdc42V12 is capable of causing the accumulation of cytoskeletal components. The complicated phenotypes of Dcdc42 transgenes suggest that Dcdc42 contributes to both establishment and down regulation of the LE cytoskeleton during DC. In support of the down regulatory role, about 10% of the embryos expressing Dcdc42V12 show losses of LE F-actin and myosin (Harden *et al.*, 1999). Furthermore, Drac1N17 phenotypes are worsened by coexpression of Dcdc42N17 before the commencement of DC, 4-8 hours after egg laying (AEL), but are partially rescued by co-expression of Dcdc42N17 in the later stages of DC (8 to 12 hours AEL) (Harden *et al.*, 1999).

Extensive studies on Dcdc42, Drac1, Drho1 and Dras functions during DC show that the Cdc42→Rac1→RhoA hierarchy in cultured mammalian cells (reviewed in Allen *et al.*, 1997; Nobes and Hall, 1995a; Nobes and Hall, 1995b; Ridley and Hall, 1992) is not apparent during DC, rather they may act largely in parallel (Harden *et al.*, 1999). Drac1 is likely a major regulator, while Dcdc42 may have a lesser role of the establishment and/or maintenance of the LE cytoskeleton with different signaling pathways involved (Harden *et al.*, 1999).

Mutations in JNK signaling components cause a common phenotype with complete loss of the actin, myosin, phosphotyrosine and dPak along the LE and with no elongation of the lateral epidermis, which is consistent with the loss of Rac phenotypes. However, mutations in TGF- $\beta$  signaling maintain relatively normal accumulation of cytoskeletal components with normal initiation of epidermal elongation in early DC, and in late DC cause an epidermal bunching phenotype similar to *Dcdc42N17* (Ricos *et al.*, 1999). There is evidence that *Cdc42* can function in the TGF- $\beta$  cascades during DC, as *Dcdc42V12* can partially rescue the DC defects of *tkv* mutants (Ricos *et al.*, 1999). Thus, related but distinct functions for the *Drac1/JNK* and *Dcdc42/TGF- $\beta$*  cascades are proposed in the multiple signal transduction networks regulating cytoskeleton during DC (Ricos *et al.*, 1999) (reviewed in Harden, 2002). A possible integrin mediated signaling through Myoblast city encoded by *myoblast city (mbc)* leads to *Drac1* activation, followed by the activation of JNK pathway, which is required for the identity of the LE cells contributing to the major driving force of DC. The TGF- $\beta$  ligand *dpp*, a JNK pathway product, activates signaling in epidermal cells via TGF- $\beta$  receptor complexes containing type I and type II receptors (encoded by *tkv* and *put*) leading to *Dcdc42*-mediated TGF- $\beta$  signaling, which may be required for the down-regulation of the cytoskeleton in the

segment border cells contributing to the proper movement of the epidermis toward the midline, possibly through dPak (Ricos *et al.*, 1999; reviewed in Harden, 2002).

Germline defects including nurse cell collapse and fusion accompanied by subcortical actin breakdown and free ring canals are found in egg chambers expressing heat shock induced Dcdc42N17. Dcdc42V12 causes reduced and discontinuous staining of irregular F-actin in cortices of nurse cells and aggregation of ring canals, which are not anchored to the membrane (Murphy and Montell, 1996). *dcdc42*<sup>-</sup> somatic FC clones have highly penetrant phenotypes with loss of columnar cell shape, formation of multiple layers of cells at the posterior end of the egg chamber, and apparent fusion of adjacent egg chambers. *dcdc42*<sup>-</sup> germ-line clones fail to produce a normal oocyte with delayed and incomplete dumping process and decreased actin filaments in the nurse cells (Genova *et al.*, 2000). Expression of Drac1V12 and Dcdc42N17 in the posterior pole of FCs results in loss of monolayer organization of the FCs (Dobens *et al.*, 2001). Further, stalk cells are greater in number in *dcdc42*<sup>-</sup> germ-line clones than wild type, which suggests a function of Dcdc42 for the differentiation of the stalk cells resulting from the interactions between the germ line and the FCs (Genova *et al.*, 2000).



Border cell migration defects are found when *Drac1N17* is expressed with follicle drivers *Gal4-198Y* (expressed in all follicle cells after stage 9) and *Gal4-306* (expressed in border cells and posterior follicle cells during the migration), but not with expression of *Dcdc42N17* (Murphy and Montell, 1996). Studies also show that at late stage of oogenesis, FCs show regional differences in responsiveness to small GTPases, which may be mediated by the activity of *puc* (Dobens *et al.*, 2001). Increased or decreased Puc activity results in incomplete nurse cell dumping and aberrant dorsal appendages.

### **Paks are among the downstream effectors for Cdc42/Rac**

To identify the downstream effector proteins of Rho-GTPases is an important step in revealing the molecular basis underlying the functions of the small p21s in diverse biological processes. A large number of Rac/Cdc42 target proteins have been identified primarily using affinity chromatography and the yeast two-hybrid system (reviewed in Van Aelst and D'Souza-Schorey, 1997). For example, Cdc42 effectors include Wiskott-Aldrich syndrome protein (WASP), Cdc42 interacted protein (CIP4) and activated Cdc42-associated tyrosine kinase (ACK), whereas partner of Rac (POR1) is relatively selective for Rac. Shared Cdc42/Rac effectors include Pak, IQGAP, mixed-lineage kinase

(MLK3), MEKK1 and PI3K (reviewed in Boettner and Van Aelst, 1999). Mutational studies indicate that distinct regions in Rac/Cdc42 have been used by effector proteins to discriminate between them and make essential contacts (Bishop and Hall, 2000).

The Paks were discovered in [ $\gamma^{32}$ p]GTP-Cdc42 and [ $\gamma^{32}$ p]GTP-Rac1 overlays of SDS-polyacrylamide gel fractionated mammalian brain proteins. The serine/threonine protein kinase activity of Pak can be stimulated by binding to GTP-bound Cdc42 and Rac, but not other GTPases (Manser *et al.*, 1994). Paks show significant sequence homology to the Ste20 protein kinase, which participates in the pheromone/mating-responsive MAP kinase cascade in budding yeast (Leberer *et al.*, 1997). Multiple Paks have been found in mammals. Based on their conserved structure, Pak1, Pak2, and Pak3 are classed together as the Pak-I/Group I Paks (Manser *et al.*, 1995), distinguished from the newly defined Pak-II/Group II Paks including Pak4, Pak5, and Pak6 (reviewed in Dan *et al.*, 2001a; Jaffer and Chernoff, 2002). Pak4 was identified from a PCR screen with degenerate primers based on the Pak2 kinase domain and is implicated in cytoskeletal morphogenesis in Golgi responding to Cdc42Hs (Abo *et al.*, 1998). Pak6 was identified as an androgen receptor (AR) interacting protein in a yeast two-hybrid screen (Yang *et al.*, 2001). Pak5 was predicted from the sequence databases but has not yet been

described in the literature (reviewed in Jaffer and Chernoff, 2002). Paks are highly conserved from yeast to human, for example dPak has an amino acid sequence highly similar to Pak1 in mammals and *C. elegans* (Harden *et al.*, 1996).

### **Structural features and regulation of Paks**

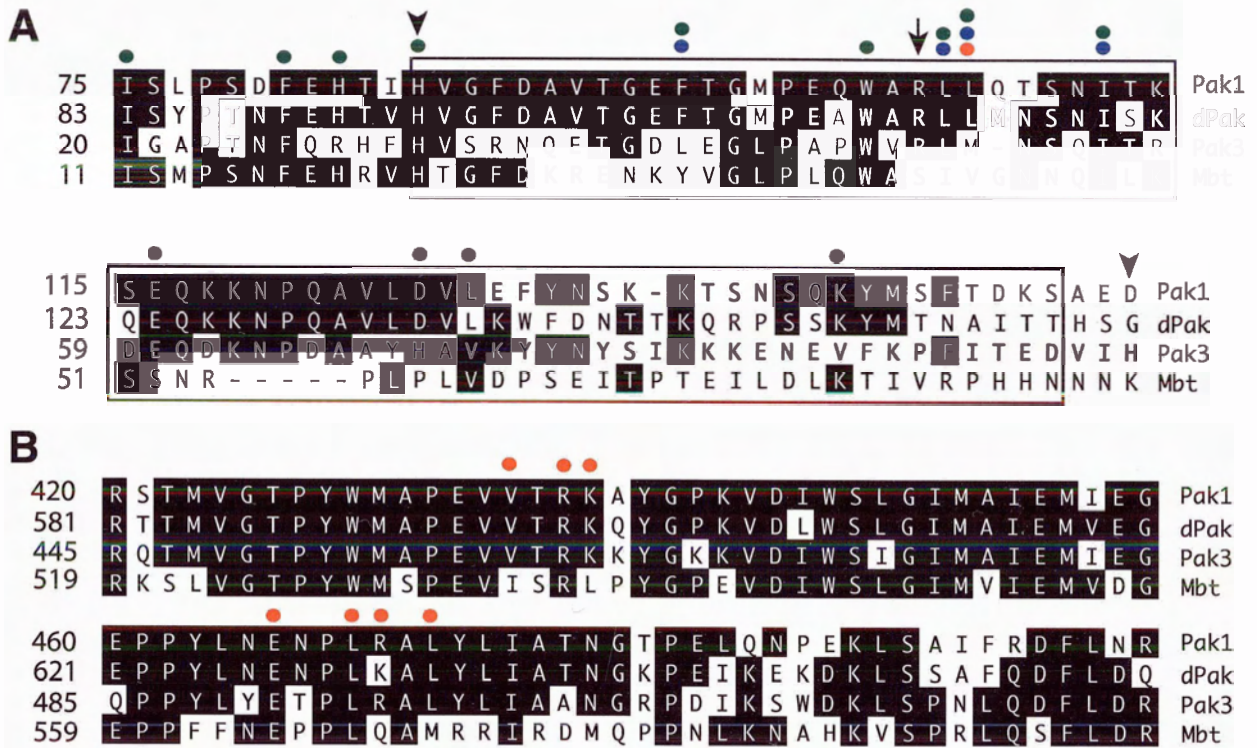
The Group I Paks share a number of defining structural characteristics including an N-terminal Cdc42/Rac interaction and binding motif (CRIB) and a C-terminal serine/threonine kinase domain (reviewed in Manser and Lim, 1999). The CRIB domain is a motif, conserved among many Rac/Cdc42 effectors, which is used for Cdc42/Rac binding (Burbelo *et al.*, 1995). A conserved proline-rich sequence at the extreme N-terminus has been shown to mediate binding of Pak to the second Src-homology 3 (SH3-2) domain of the adaptor protein Nck (Lu *et al.*, 1997). Several other proline-rich regions exist in the N-terminal regulatory domain and may possibly bind to other SH3-containing proteins, for example Pak-interacting exchange factor (Pix) (Manser *et al.*, 1998). A region contained in the C-terminus can mediate direct interactions between yeast Pak and the  $\beta\gamma$  subunits of heterotrimeric G-proteins involved in signaling from mating factor receptors (reviewed in Daniels and Bokoch, 1999). Based on studies of mammalian Pak1,

an autoinhibitory domain (AID) overlapping with the C-terminal end of the CRIB domain acts to inhibit Pak kinase activity in the absence of binding by activated GTP-bound Cdc42 or Rac (Zhao *et al.*, 1998; Frost *et al.*, 1998; Lei *et al.*, 2000; Tu and Wigler, 1999; Zenke *et al.*, 1999). Binding of GTP-Cdc42/Rac to CRIB initiates a conformational change and release of AID allowing for autophosphorylation and kinase activity. The Group II Paks also possess the CRIBs and C-terminal kinase domains, but these domains are very diverged from Group I Paks and they do not have an identifiable AID. These differences imply that Group II Paks are regulated by different mechanisms from Group I Paks and involved in distinct cellular processes (reviewed in Dan *et al.*, 2001b; Jaffer and Chernoff, 2002). Two Pak-I subfamily members are known in *Drosophila*, dPak (Harden *et al.*, 1996; Harden *et al.*, 1999) and Pak3 (Dan *et al.*, 2001b; Morrison *et al.*, 2000; Pirone *et al.*, 2001), and a single Pak-II family member, Mushroom bodies tiny (Mbt), has been identified (Melzig *et al.*, 1998).

The AID is present in a peptide containing residues 83-149 of Pak1, which can inhibit Pak function *in trans* without binding of GTP-Cdc42/Rac (Frost *et al.*, 1998; Zenke *et al.*, 1999; Zhao *et al.*, 1998). Mutations in particular residues of the AID of Pak1 result in constitutively active kinases (Brown *et al.*, 1996; Tu and Wigler, 1999; Zhao *et al.*,

1998). Crystal structure analysis of Pak1 in the auto-inhibited conformation has revealed additional residues that are important for the interaction between the AID and the kinase domain (Lei *et al.*, 2000). An alignment of the three *Drosophila* Pak kinases with Pak1 reveals that, of eight residues critical for the function of the AID, all are conserved in dPak (Harden *et al.*, 1996), three are conserved in Pak3 (Dan *et al.*, 2001b; Morrison *et al.*, 2000; Pirone *et al.*, 2001), and two are conserved in Mbt, Pak-II subfamily kinase in *Drosophila* (Melzig *et al.*, 1998) (Fig. 4A). In order to predict if the kinase activity of the *Drosophila* Paks would be blocked by the autoinhibitory motif, we examined their kinase domains for conservation of residues known to interact with this domain (Lei *et al.*, 2000). Of seven residues in Pak1 shown to interact with the AID, six are conserved in dPak, all are conserved in Pak3 and three are conserved in Mbt (Fig. 4B). dPak, with excellent conservation of both the AID and target sequences in the kinase domain, is predicted to use autoinhibition as a regulatory mechanism. Although the AID is less well conserved in Pak3, the conservation of key target amino acids in the Pak3 kinase domain suggests that expression of a Pak-AID would interfere with Pak3 kinase activity. Mbt shows poor conservation of both the AID and target sequences in the kinase domain and seems unlikely to be regulated by the same mechanism. Current data indicate that the Pak-II subfamily, to which Mbt belongs (Dan *et al.*, 2001a), is regulated in a distinct

Fig. 4 Alignments of human Pak1 sequences with corresponding sequences in the *Drosophila* Pak kinases, dPak, Pak3 and Mbt. (A) An alignment of the N-terminal CRIB-AID domains. Residues in Pak1 important for binding with Rac/Cdc42 are marked with green dots. The AID of Pak1 and corresponding sequences in the *Drosophila* Paks are boxed. Arrowheads delimit the sequences from dPak encoded by *UAS-dPak-AID* transgenes. Mutation in the residues in the Pak1 AID marked by red dot leads to its kinase activation. Residues in the Pak1 AID marked by blue dots interact with the kinase domain of Pak1. The codon for the arginine residue in dPak labeled by arrow is mutated to a stop codon in the *dpak<sup>6</sup>* allele, which produces a truncated protein with no functional AID or kinase domain. (B) Alignment of a portion of the kinase domains, showing residues in Pak1 that bind to the AID (red dots).



manner from the Pak-I subfamily in that binding of GTP-Cdc42 or GTP-Rac to these proteins does not increase kinase activity (reviewed in Dan *et al.*, 2001a; Jaffer and Chernoff, 2002).

The key initiation event for Pak activation is autophosphorylation. Upon Pak1 activation by GTP-Cdc42/Rac, six serines in the regulatory N-terminal region and a single threonine residue in the catalytic domain are phosphorylated (Manser *et al.*, 1997). A serine phosphorylation site, which lies in the kinase inhibitory domain, significantly contributes to activation (Chong *et al.*, 2001). However, amino acid substitutions that affect p21 binding does not prevent Pak recruitment to focal adhesions/focal complexes, which can be independent of direct association with Cdc42/Rac (Zhao *et al.*, 1998). Sphingosine and other lipids can cause a dose-dependent activation of Pak1 without binding to Cdc42/Rac (Bokoch *et al.*, 1998). Pak2 can also be activated by the proteolytic removal of the regulatory N terminus by caspases during apoptosis (Walter *et al.*, 1998). Pak function mediated by Cdc42/Rac-independent and kinase-independent pathways is apparent as a kinase-defective Pak1 can still promote lamellipodia formation and membrane ruffling (Frost *et al.*, 1998; Obermeier *et al.*, 1998), suggesting Pak family kinases can function through important interactions outside the catalytic domain. Paks are known as



downstream effectors for Rac/Cdc42. However, Pak may also act upstream of Rac mediating lamellipodia formation through interactions with Pix (Obermeier *et al.*, 1998).

The identification of Pak downstream targets or Pak-binding partners will help elaborate Pak signaling pathways. Two Pak1-binding proteins containing Src-homolog 3 (SH3), Dbl-homology (DH) and pleckstrin-homology (PH) domains, are named Pak-interacting exchange factor  $\alpha$ -Pix (Cool-2) and  $\beta$ -Pix (p85Cool-1) (Manser *et al.*, 1998; Parnas *et al.*, 2001) (reviewed in Bagrodia and Cerione, 1999). It has been shown that Cool/Pix recruits Pak from the cytoplasm to the Cdc42/Rac stimulated peripheral adhesions/complexes and it is likely that Cool/Pix proteins are indeed guanine nucleotide-exchange factors (GEFs) (reviewed in Bagrodia and Cerione, 1999). In *Drosophila*, dPak interacts with the SH3 domain in the GEF dPix through its proline rich sequence to function in postsynaptic structure and at the glutamatergic neuromuscular junction (Parnas *et al.*, 2001). Newly identified proteins containing an N-terminal zinc-finger motif, named Cool-associated tyrosine-phosphorylated protein 1 (Cat-1) and Cat-2, bind to Cool/Pix complexes to promote the stimulation of Pak activity (reviewed in Bagrodia and Cerione, 1999). Cat proteins might serve as a possible point of convergence for other signaling pathways (reviewed in Bagrodia and Cerione, 1999). Another N-terminal proline-rich sequence

(PXXP) in Paks binds to the SH3 domain in the adaptor protein of Nck (Bokoch *et al.*, 1996; Howe, 2001). Dock, the *Drosophila* adaptor protein homolog of mammalian Nck, possesses 1 SH2 domain, which binds to a number of receptor tyrosine kinases (RTK), and 3 SH3 domains interacting with dPak, regulating growth cone motility (Hing *et al.*, 1999).

### **Characterization of dPak in *Drosophila* development**

Loss-of-function mutations in *dpak* (Hing *et al.*, 1999) and *mbt* (Melzig *et al.*, 1998) have been isolated and indicate roles for Pak kinases in neuronal development. dPak participates in axon guidance (Hing *et al.*, 1999; Hu *et al.*, 2001; Newsome *et al.*, 2000; Schmucker *et al.*, 2000) and the regulation of postsynaptic protein localization and structure (Parnas *et al.*, 2001). *mbt* mutants have defects in the mushroom body of the adult brain (Melzig *et al.*, 1998) whereas mutations in *Drosophila pak3* have not been described.

Cloning of dPak cDNA was accomplished by screening a *Drosophila* cDNA library with human Pak1 cDNA and dPak transcripts can be detected throughout embryonic

development (Harden *et al.*, 1996). An elevation of *dpak* mRNA in the LE starts at stage 11, being particularly pronounced at stage 14 when DC initiates. Antibody staining shows that dPak colocalizes with F-actin, myosin, integrin, and phosphotyrosine, components of the actin cytoskeleton and focal adhesions/focal complexes (Harden *et al.*, 1996). Mammalian Paks colocalize with polymerized actin at the edge of lamellipodia (Dharmawardhane *et al.*, 1997) and Paks are recruited to adhesions/complexes and membrane ruffles in response to growth factor or activated Cdc42 (Manser *et al.*, 1997), suggesting that Paks have a primary role at adhesions/complexes and cell-cell junctions. In the early stages of DC, cells flanking segment borders show elevated dPak; as DC proceeds high levels of dPak appear all along the LE. Heat shock induction of *Drac1N17* transgene causes loss of dPak in the LE accompanied by a loss of cell elongation in the epidermis and a failure of DC, suggesting a function for dPak in Rac signaling during DC (Harden *et al.*, 1996).

It is difficult to evaluate the dPak levels at the LE in heat shock induced *Dcdc42N17* embryos due to the elevation of dPak in the amnioserosa (Harden *et al.*, 1999). *UAS-Dcdc42V12;Hs-Gal4<sup>M-4</sup>* embryos exhibit varied effects on LE components. 50% of these embryos show increased dPak staining extending along the LE beyond the segment

borders early in DC, at a time when dPak is normally restricted to segment borders in wild type embryos. In some embryos, patches of LE can be found that are deficient in both dPak and F-actin. 10% of the embryos lack dPak all along the LE, accompanied with severe reduction of F-actin. Thus, expression of Dcdc42V12 can both increase dPak level and the cytoskeleton and cause loss of dPak and the cytoskeleton at the LE during DC, supporting the idea that Dcdc42 has a conflicting roles in DC (Harden *et al.*, 1999).

Dcdc42 may have two opposing effects on cytoskeleton in the LE: to establish the cytoskeleton at early stages in DC and to break it down in late stages possibly through dPak (Harden *et al.*, 1999). There are transient gaps in actin, myosin and phosphotyrosine in the segment border cells of the LE midway through DC. These cells display a high level of dPak at an earlier stage (Harden *et al.*, 1996). Early in closure dPak may function in the JNK pathway to establish the LE cytoskeleton, while later it may contribute to F-actin disassembly, a Pak function that has been described in cultured cells (Manser *et al.*, 1997). In cultured cells, Paks can induce two types of morphological changes: disassembly of focal adhesions/complexes and promotion of lamellipodia formation and membrane ruffling (Manser *et al.*, 1997). Microinjection of plasmids encoding constitutively active forms of Pak1 in fibroblast cells leads to dramatic loss of focal

adhesions and actin stress fibers with concomitant cell contraction (Manser *et al.*, 1997), suggesting Pak1 promotes turnover of adhesions/complexes. It is apparent that the roles of Paks in controlling the cytoskeleton and cell shape/motility are still not well defined.

### **Experimental approaches**

The aim of this thesis was to further characterize dPak function in *Drosophila* development using transgenes and loss-of-function mutations. Although dPak loss-of-function mutant embryos successfully complete embryogenesis, dPak expression patterns in embryo suggest roles in embryonic development (Harden *et al.*, 1996). Maternally deposited dPak and/or other Pak family kinases in *Drosophila*, Mbt (Melzig *et al.*, 1998) and Pak3 (Dan *et al.*, 2001a; Morrison *et al.*, 2000) may substitute for the embryonic functions of zygotic dPak. Previous results on dPak expression in LE epidermal cells flanking segment borders indicate that dPak could function in DC (Harden *et al.*, 1996). Additionally, a response of dPak transcript levels in the amnioserosa to changes in small GTPase signaling has been previously described (Harden *et al.*, 1996; Sem *et al.*, 2002), suggesting a signaling function for dPak in this tissue. Genes expressed in the amnioserosa have been implicated in DC through roles in amnioserosa morphogenesis

and signaling to the epidermis (Frank and Rushlow, 1996; Harden, 2002; Harden *et al.*, 2002; Reed *et al.*, 2001; Stronach and Perrimon, 2001).

A number of approaches were used to further study dPak function in DC. First, I examined phenotypes of constitutively active versions of Drac1 and Dcdc42 in a *dpak*<sup>Δ</sup> mutant background. To further characterize dPak involvement in Dcdc42/Drac1 signaling, I set out to make transgenic flies bearing constitutively active versions of these p21s, which were no longer capable of interacting with dPak. The Y40C effector site substitution in constitutively active Rac/Cdc42 renders these GTPases incapable of binding Pak or activating the JNK cascade (Lamarche *et al.*, 1996; Owen *et al.*, 2000).

During DC, there are high levels of dPak in the LE cells (Harden *et al.*, 1996). To study the effects of impairing Pak-I subfamily kinase activity in the LE cells during DC, I expressed *UAS-dPak-AID transgenes* using the *LE-Gal4* driver, which targets transgene expression specifically in the LE cells (Glise and Noselli, 1997). I also expressed *dPak-AID transgenes* in amnioserosa by using amnioserosa-specific drivers to examine the function of dPak in this tissue.

I hoped to test the embryonic effects of eliminating maternal dPak by creating germline clones of the *dpak<sup>δ</sup>* mutation. These germline clones would be created using the FLP/FRT recombination system coupled with the dominant female sterile (DFS) mutation *ovo<sup>D1</sup>* (Chou and Perrimon, 1992). The theory is to induce mitotic recombination between the *dpak<sup>δ</sup>* mutation on an FRT chromosome and a *FRT/ovo<sup>D1</sup>* chromosome using *Hs-FLP*. Only germline tissue that is homozygous for *dpak<sup>δ</sup>* would be able to yield progeny. The recombination between *dpak<sup>δ</sup>* and *FRT* is not easy to achieve due to their close proximity. After screening hundreds of chromosomes for recombinants, I abandoned this approach when I discovered that *dpak<sup>δ</sup>/dpak<sup>10</sup>* flies were sterile. However, this finding led to a detailed investigation of the participation of dPak in oogenesis.

## **Materials and Methods**



## 1. Fly stocks and transgene expression

Standard *Drosophila* procedures were followed. Unless otherwise stated, all flies were raised and crossed at 25°C. Fly strains bearing various small GTPase transgenes in pUAST have been described previously (Harden *et al.*, 1999). Females from Gal4 lines were crossed to males from the pUAST transgenic lines and the progeny examined as embryos or adults (Brand and Perrimon, 1993). Most of the strains used were obtained from Bloomington Stock Center unless otherwise stated. The list of fly strains used:

Small GTPase transgenes:

*UAS-Drac1V12 /UAS-Drac1V12* was obtained from L. Luo (Luo *et al.*, 1994)

*UAS-Dcdc42V12/ UAS-Dcdc42V12*

*UAS-Drac1N17/ UAS-Drac1N17*

*dpak* mutations:

*dpak* deficiency line *Df(3R)Win11/TM3*

*dpak<sup>1</sup>/TM3* G586S dominant negative? Kinase dead?

*dpak<sup>3</sup>/TM3* G569D loss-of-function, kinase dead

*dpak*<sup>6</sup>/*TM3* R113 Stop loss-of-function, truncated

*dpak*<sup>10</sup>/*TM3* ORF normal, null?

*UAS-dPak-AID* transgenes were made by N. Harden and M. G. Ricos previously:

LineA: *dPak-AID*, *FLAG/cyo* 2<sup>nd</sup> chromosome viable

LineB: *dPak-AID*, *FLAG/cyo* 2<sup>nd</sup> chromosome lethal

LineC: *dPak-AID*, *FLAG/Tm3Ser* 3<sup>rd</sup> chromosome viable

*UAS-ΔC1/2-FLAG* flies were provided by V. Budnik

Gal4 drivers:

*Gal4*<sup>c381</sup>, *ptc-Gal4*, *Da-Gal4*, *Gal4-69B*, *Gal4-198*

*LE-Gal4/LE-Gal4* flies were provided by S. Noselli

*Hs-Gal4*<sup>M-4</sup>/*Hs-Gal4*<sup>M-4</sup> flies were provided by J. Roote

*en-Gal4/en-Gal4* flies were provided by M. Nakamura

Miscellaneous loss-of-function mutations:

*Mys*<sup>1</sup>/*Fm4*

*ij*<sup>k27e</sup>/*Fm7c*

*Df(2L)E55/cyo*

*Df(2L)TW84/cyo*

Justina S. P. Sanny and I created the following recombinant chromosomes:

*UAS-Drac1V12, dpak<sup>6</sup>/TM3*

*UAS-Dcdc42V12, dpak<sup>6</sup>/TM3*

*69B-Gal4, dpak<sup>6</sup>/TM3<sup>6</sup>*

*Hs-Gal4<sup>M-4</sup>, dpak<sup>6</sup>/TM3*

*dPak-AID, dpak<sup>6</sup>/TM3*

## **2. Standard Molecular Techniques**

The techniques used in this study were as described in Sambrook et al. (Sambrook *et al.*, 1989).

### **2.1. Agarose gel electrophoresis**

1% agarose gel:

1g agarose

100ml 1% TAE

50x TAE buffer:

242g Tris base

57.1ml glacial acetic acid

100ml 0.5M EDTA, pH 8.0

Volume brought to 1L with dH<sub>2</sub>O

Ethidium Bromide (10mg/ml):

1g EtBr

100ml dH<sub>2</sub>O

Agarose was dissolved by heating in buffer to 70°C. Ethidium bromide was added to a final concentration of 0.5µg/ml. DNA samples were run with loading dye.

## 2.2 Plasmid DNA Purification

## I. Small scale preparation of plasmid DNA by alkaline lysis method

### *Solution I:*

50mM glucose

25mM Tris•Cl (pH 8.0)

10mM EDTA (pH8.0)

### *Solution II:*

0.2N NaOH

1% SDS

### *Solution III:*

5M potassium acetate 60ml

glacial acetic acid 11.5ml

H<sub>2</sub>O 28.5ml

A single bacterial colony was picked up from a LB agar plate and then inoculated in liquid LB medium with 50 $\mu$ g/ml ampicillin for at least 8 hours at 37°C with vigorous

shaking before harvesting. 1.5ml of this culture was centrifuged at 12,000g for 30 seconds at 4°C. The bacterial pellet was dried by aspiration and was resuspended in 100µl of ice-cold Solution I by vigorous vortexing. 200µl of freshly made Solution II and 150µl of ice-cold solution III were added. Samples were centrifuged at 12,000g for 5 minutes at 4°C and the supernatant was transferred to a fresh tube. An equal volume of phenol:chloroform was added and mixed by vortexing. Samples were centrifuged at 12,000g for 2 minutes at 4°C and the supernatant was transferred to a fresh tube. The DNA was precipitated with 2 volumes of ethanol at room temperature and mixed by vortexing. The mixture was allowed to stand for 2 minutes at room temperature, centrifuged at 12,000g for 5 minutes at 4°C and the supernatant was removed by aspiration. The pellet was rinsed with 1 ml of 70% ethanol at 4°C. Samples were vortexed briefly and then centrifuged for 5 minutes. The supernatant was removed and the pellet was dried in a vacuum desiccator. The DNA was redissolved in 50µl TE (pH 8.0) containing RNAase (20µg/ml) (Sambrook *et al.*, 1989).

## II. Small scale preparation of plasmid DNA by boiling method

*STET:*

0.1M NaCl

10mM Tris•Cl (pH 8.0)

1mM EDTA (pH 8.0)

5% Triton X-100

The bacterial pellet was resuspended in 350 $\mu$ l of *STET* and 25 $\mu$ l of freshly prepared solution of lysozyme (10mg/ml in 10mM Tris•Cl [pH 8.0]) was added. The tube was placed in a boiling-water bath for 40 seconds. The bacterial lysate was centrifuged at 12,000g for 10 minutes at room temperature. The pellet of bacterial debris was removed with a sterile toothpick. 40 $\mu$ l of 2.5M sodium acetate (pH 5.2) and 420  $\mu$ l of isopropanol were added to the supernatant. Samples were mixed by vortexing and stored for 5 minutes at room temperature followed by a centrifugation at 12,000g for 2 minutes. The supernatant was removed by gentle aspiration. 1 ml of 70% ethanol was added and samples were centrifuged at 12,000g for 2 minutes at 4°C. All the supernatant was removed by aspiration. The nucleic acids were redissolved in 50 $\mu$ l of TE (pH 8.0) containing RNAase (20 $\mu$ g/ml) (Sambrook *et al.*, 1989).

III. Small and large scale preparation of plasmid DNA by QIA mini prep

Purified DNA template used for sequencing was generated using the QIAprep spin miniprep kit, according to the manufacturers' directions (Qiagen).

Pelleted bacterial cells were resuspended in 250 $\mu$ l of Buffer P1. 250 $\mu$ l of Buffer P2 were added and the tube was gently inverted 4-6 times. 350 $\mu$ l of Buffer N3 was added and the tube was inverted immediately but gently 4-6 times. Samples were centrifuged for 10 minutes. During centrifugation, a QIAprep spin column was placed in a 2-ml collection tube. The supernatant was applied to the QIAprep spin column by decanting. QIAprep column was centrifuged for 30-60 seconds and the flow-through was discarded. QIAprep column was washed by adding 0.5ml of Buffer PB and centrifuged 30-60 seconds. QIAprep column was washed by adding 0.75ml of Buffer PE and centrifuged 30-60 seconds. The sample was centrifuged for an additional 1 minute to remove residual wash buffer. QIAprep column was placed in a clean 1.5ml-microfuge tube. To elute DNA, 50 $\mu$ l of Buffer EB (10mM Tris•Cl, pH 8.5) or H<sub>2</sub>O was added to the center of each QIAprep column. Column was left to stand for 1 minute and centrifuged for 1 minute (Qiagen).

### 2.3 Restriction endonuclease digestion of DNA



Buffers from Gibco-BRL or New England Biolabs were selected to add to the restriction digestion reaction, which was normally incubated at 37°C for 2 hours.

#### 2.4 Ligation of DNA with vector

1 volume of vector, 3 volumes of insert (desired DNA), 4 $\mu$ l of reaction buffer, 1 $\mu$ l of DNA ligase, and ddH<sub>2</sub>O were added to make the total ligation reaction volume of 20 $\mu$ l.

The ligation reaction was incubated at 16°C overnight.

#### 2.5 Gel purification of DNA fragment

NaI solution:

0.75g Na<sub>2</sub>SO<sub>3</sub>

45g NaI

40ml dH<sub>2</sub>O

EZ-Glass Milk (Silica):

10g Silica (Sigma S5631)

100ml PBS

Mixed and removed the supernatant. Resuspend silica pellet in 6M guanidine hydrochloride at 100mg/ml.

Samples of DNA were loaded and electrophoresis was carried out at 4°C. The desired fragment was cut out and 3 volume of NaI solution (final concentration at 4M) was added. The gel was heated for 5 minutes at 45~55°C. 10 $\mu$ l of EZ-Glass Milk suspension was added to solution containing 5 $\mu$ g of DNA and incubated at room temperature for 5 minutes to allow binding of the DNA to the silica matrix with mixing every 1~2 minutes to ensure that EZ-Glass milk stayed suspended. Samples were spun 5 seconds to pellet the silica matrix with bound DNA. 10-50 volumes new wash was added to resuspend the pellet. Samples were spun 5 seconds and the supernatant was discarded. The wash was repeated two more times. Samples were spun for 5 seconds and the last bit of liquid was removed. The DNA from EZ-Glass Milk was eluted with 10 $\mu$ l of ddH<sub>2</sub>O. Samples were centrifuged for 30 seconds to make a solid pellet. The supernatant containing the eluted DNA was transferred to a new tube (Sambrook *et al.*, 1989).

## 2.6 Site-directed mutagenesis

Mutagenesis was performed using the QuikChange site-directed mutagenesis kit, according to the manufacturer's instructions (Stratagene). Primers, designed by Dr. Nick Harden, involved synthesizing two complimentary oligonucleotides containing the desired mutation, flanked by the unmodified nucleotide sequence.

Primers (bases in the brackets are the original cDNA sequence):

Rac1Y40C#1: CCGTGTTTCGACA(A)ACTG(A)CTCGGCCAACGTG

Rac1Y40C#2: CACGTTGGCCGAGC(T)AGTTGTCGAACACGG

Cdc42G12V#1: GTCGTCGGCGACGT(G)AGCCGTGGGTAAG

Cdc42G12V#2: CTTACCCACGGCTA(C)CGTCGCCGACGAC

Cdc42Y40C#1: CGGTGTTTCGACA(A)ACTG(A)TGCGGTCACTGTG

Cdc42Y40C#2: CACAGTGACCGCAC(T)AGTTGTCGAACACCG

The concentrations of the DNA in pBluscript were measured using an Ultraspec 1000E before the site-directed mutagenesis.

The control reactions:

5 $\mu$ l of 10x reaction buffer

2 $\mu$ l (10ng) of pWhitescript™ 4.5-kb control plasmid

1.25 $\mu$ l (125ng) of oligonucleotide control primer #1

1.25 $\mu$ l (125ng) of oligonucleotide control primer #2

1 $\mu$ l of dNTP mix

Double-distilled water to a final volume of 50 $\mu$ l

Then 1 $\mu$ l of *PfuTurbo*DNA polymerase (2.5U/ $\mu$ l) was added

Sample reactions:

5 $\mu$ l of 10x reaction buffer

50ng of dsDNA template

125ng of oligonucleotide primer #1

125ng of oligonucleotide primer #2

1 $\mu$ l of dNTP mix

Double-distilled water added to a final volume of 50 $\mu$ l

Then 1 $\mu$ l of *PfuTurbo*DNA polymerase (2.5U/ $\mu$ l) was added

Programming PCR machine:

Segment	Cycle	Temperature	Time
1	1	95°C	30 sec
2	16	95°C	30 sec
		55°C	30 sec
		68°C	2 min

Each reaction was cycled using the above cycling parameters and PCR machine was set to 4°C overnight following temperature cycling.

1 $\mu$ l of the *Dpn* I restriction enzyme (10U/ $\mu$ l) was added to each amplification reaction and was mixed by pipetting the solution up and down several times. The reaction mixtures were spun down for 1 minute and immediately incubated at 37°C for 1 hour to digest the parental supercoiled dsDNA.

Transforming into Epicurian Coli XL1-Blue supercompetent cells:

The E-Coli XL1-Blue supercompetent cells were gently thawed on ice. 50 $\mu$ l of the cells was aliquoted to a prechilled Falcon 2059 polypylene tube. 4 $\mu$ l of the *Dpn* I-treated DNA was transferred from each reaction to separate aliquots of the supercompetent cells. The

transformation reactions were swirled gently and incubated on ice for 30 minutes. Heat pulse was applied to the transformation reaction for 45 seconds at 42°C and then the reactions were placed on ice for 2 minutes. 0.5ml of NZY<sup>+</sup> broth preheated to 42°C was added to the transformation reactions and incubated at 37°C for 1 hour with shaking at 225-250 rpm. The entire volume of the sample transformation reaction was plated on LB-ampicillin agar plate and incubated at 37°C for >16 hours.

## 2.7 DNA sequencing

$\alpha$ -<sup>33</sup>P dideoxynucleotide (ddNTP) terminators

Preparation and running of the sequencing gel:

Acrylamide gel (1750ml):

100ml gel mix

46g urea

20ml 5x TBE

12.5ml 40% acrylamide

80 $\mu$ l TEMED

350 $\mu$ l 20% APS

20x glycerol tolerant buffer:

216g Tris base

72g Taurine

4g Na<sub>2</sub>EDTA•2H<sub>2</sub>O

Add H<sub>2</sub>O to 1000ml

The gel plates were washed with sparkleen and then with ddH<sub>2</sub>O. The gel appliances were washed with hand soap and ddH<sub>2</sub>O and then ethanol. One gel plate was polished with Rain-X till it was shining. Spacers were inserted in between the gel plates and the plates were held together with the locking device. The acrylamide gel was poured and left overnight. The next morning, the teeth was inserted on top of the gel and the plates were set up in the chamber. The sequencing buffer was poured into the bottom chamber and the upper buffer chamber. The gel was pre-run for 45 minutes to 1 hour at 45°C and 50V. Then the sequencing DNA samples were loaded and the gel was run until the short run (light blue) reached the end of the gel. After the run, the gel was transferred to a piece of

filter paper and put into dryer at 80°C for 1 hour. Then the gel was put on film for 3-5 days before the film was developed (Amersham Life Science).

## 2.8 Chemical mediated transformation

LB agar plate:

5g bactotryptone

2.5g bacto-yeast extract

5g NaCl

7.5g bacto-agar

500 ml dH<sub>2</sub>O

Liquid LB medium:

5g bactotryptone

2.5g bacto-yeast extract

5g NaCl

500 ml ddH<sub>2</sub>O



15-ml Falcon 2059 polypropylene tubes were prechilled and water bath was set to 42°C. 100µl SL1-Blue competent cells were thawed on ice. 1.7µl of diluted β-mercaptoethanol (1 in 10 dilution) was added, giving a final concentration of 25 mM. The cells were incubated on ice for 10 minutes, swirling gently every 2 minutes. 10µl of ligation reaction was added to cells and left on ice for 30 minutes. Then heat shock was applied at 42°C for 45 seconds. Samples were put on ice for 2 minutes and then 900µl of preheated LB (at 42°C) was added. Samples were incubated with shaking for 1 hour at 37°C at 225-250 rpm overnight with the lid loosely on. 400µl of transformation mixture was plated on LB+agar+ampicillin plate using a sterile spreader and incubated at 37°C overnight.

### **3. In situ hybridization with digoxigenin-labeled RNA probes**

Preparation of dPak probe:

dPak Ori2 cDNA cloned into pNB was obtained from Dr. Nick Harden. StuI was used to cut 1.1kb upstream of 5' end of dPak cDNA to linearize the DNA. 10µl of dPak cDNA, 5µl of StuI (Gibco), 10µl of reaction buffer 2 (Gibco) and 75µl of dH<sub>2</sub>O were added to incubate at 37°C for 2 hours. Phenol/chloroform were used to extract the StuI digestion reaction. Then 0.1 volume of 3.3m NaAC and 2 volume of 95% of ethanol were added

and samples were left at  $-80^{\circ}\text{C}$  for 1/2 hour. Samples were spun at maximum speed for 10 minutes and the supernatant was discarded. The pellet was washed using 70% ethanol and dried under vacuum.  $10\mu\text{l}$  of DEPC-treated  $\text{dH}_2\text{O}$  was added.  $2\mu\text{l}$  was used in the labeling reaction.  $2\mu\text{l}$  of NTP mix,  $2\mu\text{l}$  of transcription buffer,  $1\mu\text{l}$  of RNAase inhibitor,  $11\mu\text{l}$  of DEPC-treated  $\text{dH}_2\text{O}$ , and  $2\mu\text{l}$  of T7 RNA polymerase were added. Samples were incubated 2 hours at  $37^{\circ}\text{C}$  and then  $2\mu\text{l}$  of DNAase1 was added. Samples were incubated at  $37^{\circ}\text{C}$  for 15 minutes and the reaction was killed with  $1\mu\text{l}$  of 0.5M EDTA.  $7\mu\text{l}$  was used as dPak probe for hybridization to embryos.

*dpp* probe was obtained from Dr. Esther Verheyen's lab. mRNA *in situ* hybridizations were performed using DIG RNA labeling kit (SP6/T7) (Boehringer Mannheim).

1<sup>st</sup> wash buffer:

2.5ml formamide

0.5ml 20x SSC

$50\mu\text{l}$  10% Tween-20

20x SSC:

175.3g NaCl

88.2g Sodium citrate (Na<sub>3</sub> citrate)

800ml dH<sub>2</sub>O

Adjust volume to 1L

Ashburner wash buffer:

100mM NaCl

50mM MgCl<sub>2</sub>

100mM Tris, pH 9.5

0.1% Tween 20

Hybridization buffer:

50% deionized formamide

4x SSC

1x Denhardts

0.1% Tween 20

5% dextran sulfate

250µl/ml salmon sperm DNA

50µg/ml heparin (Sigma, H3393, 100000 units)

50x Denhardts:

5g polyvinylpyrrolidone

5g BSA (Pentax Fraction V)

H<sub>2</sub>O was added to total volume of 500ml

Digoxigenin-labeled RNA probes were generated by *in vitro* transcription of the antisense strand of the cDNA clone using the DIG RNA labeling kit (Roche molecular Biochemicals) (Van Vactor and Kopczynski, 1999). The methanol was removed from fixed embryos and the embryos were rehydrated with 500µl of 3:1 methanol: 4% Paraformaldehyde/PBS for 2 minutes and then 1:3 methanol: 4% Paraformaldehyde/PBS for 5 minutes. The embryos were then fixed in 4% paraformaldehyde/PBS for 10 minutes, followed by rinsing 3x with PBS + 0.1% Tween 20 (PBT). Then 500µl of hybridization mix and 10µl of preboiled ssDNA were added. The mix was boiled for 1 minute and left on ice. Embryos were put on a roller at 52°C for 1 hour. Then 7µl of dPak probe was added and samples were left at 52°C on the roller overnight. Samples were washed with

first wash buffer several times through the second day and then rotated at 52°C overnight. Then samples were rinsed several times in PBT, and rotated at 52°C for 1/2 hour. Samples were incubated in 1/2000 of anti-digoxigenin-alkaline phosphatase (AP, Roche Molecular Biochemicas) in 3% BSA in PBT for 1 hour and then washed 3 x 20 minutes in PBT at room temperature. Samples were washed 3 times, 5 minutes each, in Ashburner's wash. The last wash (1ml) was kept in the tube and 4.5µl of 4-Nitro blue tetrazolium chloride (NBT) and 3.5µl of X-phosphate were added. Samples were rotated on the roller for 1 to 2 hours, checking every 10 minutes. The reaction was stopped when samples stained to satisfaction. Samples were rinsed 3x in PBT and 70% glycerol was added and rotated for at least 1 hour. Embryos were observed using differential interference contrast (DIC) microscopy.

#### **4. Western analysis**

SDS sample buffer 1x:

Distilled water 4.4ml

0.5M Tris-HCl, pH 6.8: 1ml

Glycerol: 0.8ml

10% SDS: 1.6ml

DTT: 123mg

0.05% BPB: 0.2ml

B-Me: 0.4ml

5x Running Buffer 1L:

Tris Base: 15g

Glycine: 72g

SDS: 5g

5x Transfer Buffer 1L:

Tris: 5.8g

Glycine: 2.9g

10% SDS: 3.7ml

MeOH: 200ml

Water: 800ml

10% Separating gel 30ml:

Distilled water: 12.1ml

1.5M Tris-HCl, pH 8.8: 7.5ml

10% SDS 0.3ml

30% Acrylamide/Bis: 9.9ml

10% APS: 150 $\mu$ l

TEMED: 15 $\mu$ l

Stacking gel 3ml:

Distilled water: 1.83ml

1.5M Tris-HCl, pH 8.8: 750 $\mu$ l

10% SDS: 30 $\mu$ l

30% Acrylamide/Bis: 390 $\mu$ l

10% APS: 15 $\mu$ l

TEMED: 3 $\mu$ l

Tris buffered (TBS):

6.05g Tris base (50mM)

8.76g sodium chloride (150mM)

adjust volume to 1L

1% blocking solution:

10ml of blocking reagent

90ml of TBS

10 flies were ground in 100 $\mu$ l SDS sample buffer, then boiled for 10 minutes. 25 $\mu$ l of protein extract was loaded on the SDS gel and the running buffer was poured over the gel. The gel was run for 12 minutes at 300 volts. Then the stacking gel was removed. The gel was incubated in 1x new transfer buffer. Membrane and filter paper was cut to the gel size and wet in buffer. Gel sandwich was prepared. The blot was transferred at 15V for 20 minutes. The membrane was removed from the sandwich and stained for 1 min in 1x ponceau. Then blot was rinsed with water for several times. The membrane was labelled and covered with Saran wrap. The membrane was put back to the box, and 10ml 1x blocking solution was added for 1 hour. The blocking solution was decanted and 1/2000 diluted dPak antibody in 1% blocking solution in TBS was added. Membrane was incubated at room temperature for 1 hour. Membrane was washed 2x 10 minutes with 15ml TBS-T (0.1% Triton +TBS). The membrane was then washed 2x 10mins with 15ml



TBS + 0.5% block. 10ml secondary antibody (goat anti mouse) diluted 1/5000 in TBS + 0.5% block was added at room temperature for 1 hour. Secondary antibody was discarded. Membrane was washed 4x 15 minutes with TBS-T. During the second wash, 15µl blotting substrate + 5ml luminescence substrate solution were mixed. Detection was for 1 minute and then membrane was wrapped with saran wrap for storage. A sheet of film was placed onto the blot and the film cassette was exposed for 60 seconds (in dark). A second film was placed onto the blot and was exposed for 1 hour.

## **5. Fixation of embryos**

Apple juice medium:

1.5g agar in 50ml H<sub>2</sub>O

16.6ml apple juice

0.1g Tegosept and 1.67g sugar

20% paraformaldehyde in 1x PBS:

10g paraformaldehyde

10ml 5x PBS

35ml H<sub>2</sub>O

0.5MI 1M NaOH

10ml 5x PBS and top up H<sub>2</sub>O till 50ml.

1x PBS:

8g NaCl

0.2g KCl

1.44g Na<sub>2</sub>HPO<sub>4</sub>

0.24g KH<sub>2</sub>PO<sub>4</sub>

800ml dH<sub>2</sub>O

H<sub>2</sub>O was added to total volume of 1L

Embryos were washed off plates with 0.01 % PBT and dechorionated for 3 minutes in 50% household bleach in 0.01% PBT. Embryos were then washed in 0.01 % PBT for 5 min. Embryos were then transferred to a scintillation vial containing 4 ml PBS, 5 ml heptane and 1ml 20% paraformaldehyde. Embryos were agitated in this mix for 25 minutes. Following the agitation the lower, aqueous phase was removed and 5 ml of methanol was added. The tube was then shaken vigorously for 1 minute. The heptane

layer was then removed and the embryos were washed several times with methanol before storage at -20°C (Harden *et al.*, 1996; Harden *et al.*, 1999).

## **6. *Drosophila* embryonic cuticle preparations**

Preparation of Hoyer's medium:

30g of gum arabic was dissolved overnight in 50ml distilled water. 200g of chloral hydrate was added slowly with stirring, and then 20g glycerol was added. The mixture was centrifuged at 12,000g (10,000 rpm in SS34) for 3 hours and Hoyer's supernatant was transferred to bottles.

Cuticle preparations:

Embryos were washed off plates with 0.01 % PBT and dechorionated for 3 minutes in 50% household bleach in 0.01% PBT. Embryos were then washed in 0.01 % PBT for 5 min. The embryos were transferred to a drop of Hoyer's medium on a slide and the coverslip was placed on top. The embryos were baked at 60°C until clear (takes from one to several days). Embryos were examined with phase contrast optics (Harden *et al.*, 1996; Harden *et al.*, 1999).

## 7. Heat shock induction of transgenes

In order to obtain the phenotypes of the heat shock-induced transgenes, embryos were collected 4 hours after egg laying (AEL) and aged 8 hours at 25°C. Then the embryos were heat shocked for 1 hour, except for *UAS-Drac1V12* transgene, which was only heat shocked for 10 min. The embryos were allowed to recover at 18°C for 7 hours for phosphotyrosine staining, and for 36 hours for cuticle preparation (Harden *et al.*, 1996; Harden *et al.*, 1999).

## 8. Dissection and fixation of ovarioles

1 x EBR (Ringer's solution):

130 mM NaCl

4.7 mM KCl

1.9 mM CaCl<sub>2</sub>

10 mM Hepes, pH 6.9

Devitellinizing Buffer:

1 vol Buffer B

1 vol formaldehyde 36%

4 vol H<sub>2</sub>

*Buffer B:*

100 mM KH<sub>2</sub>PO<sub>4</sub>/K<sub>2</sub>HPO<sub>4</sub> (pH 6.8)

450 mM KCl

150 mM NaCl

20 mM MgCl<sub>2</sub>•6H<sub>2</sub>O

**PBT:**

1 x PBS

0.3 % Triton X-100

0.5% BSA (Fraction V form sigma)

The flies were fed in yeast-rich fresh apple juice food for 2-4 days before dissection. A small spot plate containing cold EBR was placed on a black background under dissecting microscope with light coming from the sides. The ovary tissue was gently pulled out of

the abdomen and the ovarioles were teased apart to remove the muscle-containing sheath surrounding the single ovariole. The ovarioles were transferred into an Eppendorf tube and EBR was removed. 100  $\mu$ l Devitellinizing buffer and 600 $\mu$ l heptane were added. Ovarioles were agitated vigorously to ensure that the buffer was saturated with heptane and then gently for 10 min at room temperature. Solution was removed and the ovarioles were rinsed with 1 x PBS and stored at 4°C (reviewed in Verheyen and Cooley, 1994).

### **9. SEM examination of *Drosophila* eyes**

Samples were sent to the lab of UBC for Scanning Electron Microscopy (SEM) analysis. Specimen used for SEM was fixed on a flat metal and gold coated by Nanotech SEMPRep II Sputter Coater. Then *Drosophila* eye pictures were taken using the scanning electron microscope Cambridge 250T (Sem *et al.*, 2002).

### **10. Wing fixation**

Wings were mounted in Aquamount (BDH) (Sem *et al.*, 2002).

## 11. Immunohistochemistry

### A. Staining fixed embryos with anti-phosphotyrosine antibody:

Methanol was removed from fixed embryos and the embryos were washed 3 x 20 minutes in PBT (PBS + 0.1% Triton). Embryos were blocked for 1 hour in PBT + 1% BSA. Embryos were incubated in PBT + 1% BSA containing the 1:500 diluted anti-phosphotyrosine mouse monoclonal IgG and put on roller overnight at 4°C. Antibody was removed and embryos were washed 3 x 20 minutes in PBT. Goat anti-mouse FITC-labeled secondary antibody (1:200) was added and incubated for 2 hours. Embryos were washed 3 x 20 minutes in PBT. 500  $\mu$ l of Vectashield (Vector laboratories) was added and embryos were left on roller at least for 1 hour. 80  $\mu$ l of Vectashield containing the embryos were mounted on a slide. The embryos were observed under LSM 410 confocal microscopy using 25x or 60x objective. Images were processed using Adobe Photoshop (Harden *et al.*, 1996; Harden *et al.*, 1999).

B. For dPak antibody staining on embryos, the dPak primary antibody was diluted 1:2000 in PBT + BSA, and FITC conjugated goat anti-rabbit secondary antibody was used. For dPak antibody staining on ovarioles, the dPak antibody was diluted 1:500 in PBT, and

biotin conjugated anti-rabbit secondary antibody was used followed by the staining with Texas Red Streptavidin.

#### C. DAPI staining for nuclei and FITC-Phalloidin staining of actin on ovarioles:

The fixed ovarioles were rinsed with PBS. 100  $\mu$ l of DAPI (1  $\mu$ g/ml) in PBS was added and incubated for 5 min. DAPI was removed and ovarioles were rinsed with EBR. 100  $\mu$ l FITC-phalloidin (Sigma) was added to stain in the dark for 20 minutes. Phalloidin was removed and rinsed with PBS. Ovarioles were incubated in Vectashield for at least one hour before observing. 80  $\mu$ l of ovaries were mounted on a slide and examined with the UV filter under a fluorescence microscope (reviewed in Verheyen and Cooley, 1994).

#### D. X-Gal staining:

X-Gal staining solution:

10mM NaHPO<sub>4</sub>

150mM NaCl

1mM MgCl<sub>2</sub>

0.3% Triton x-100

3mM K<sub>4</sub>[FeII(CN)<sub>6</sub>]



3mM  $K_3[FeII(CN)_6]$

Collected embryos were dechorinated in 50% bleach for 5 minutes and then the embryos were picked up with brush and dipped into 500ul heptane in 500ul of PBS, and agitated for 20 minutes. The embryos were put back into the mesh and dipped in 10ml of PBS with 100ul of Glutaldehyde (25% stock) added for 5 minutes. Embryos were rinsed 2x in PBT, followed by 2x15 minutes wash in PBT. 200ul X-Gal stock (8% X-Gal in DMSO) was added in 10ml of X-Gal staining solution and incubated at 37°C for 3 hours. The embryos were then washed in PBT and rotated in 70% glycerol for 1 hour before observation.

## Results

## **dPak function in embryogenesis**

### **Phenotypes generated by expression of *Dcdc42* and *Drac1* transgenes in a *dpak*<sup>6</sup> mutant background**

It has been shown that Paks have fundamental functions in cellular morphogenesis through studies from yeast to mammalian cells (reviewed in Manser and Lim, 1999). dPak accumulates along the LE, co-localizing with F-actin, myosin and phosphotyrosine, indicating that dPak could function in embryogenesis during DC (Harden *et al.*, 1996). However, embryos homozygous for *dpak* mutations survive until pharate adult stage. One possible explanation for this apparent paradox is that *dpak* is maternally expressed and loaded into the egg, thereby providing sufficient dPak function for embryogenesis. In order to see if zygotic dPak made any contribution to the effects of the p21s on embryonic, epithelial morphogenesis, I examined the phenotypes generated by expression of *Dcdc42* and *Drac1* transgenes in a *dpak*<sup>6</sup> mutant background.

The molecular lesions associated with the previously isolated *dpak* mutations have been characterized (Hing *et al.*, 1999). The *dpak*<sup>6</sup> mutant protein, the product of one of the

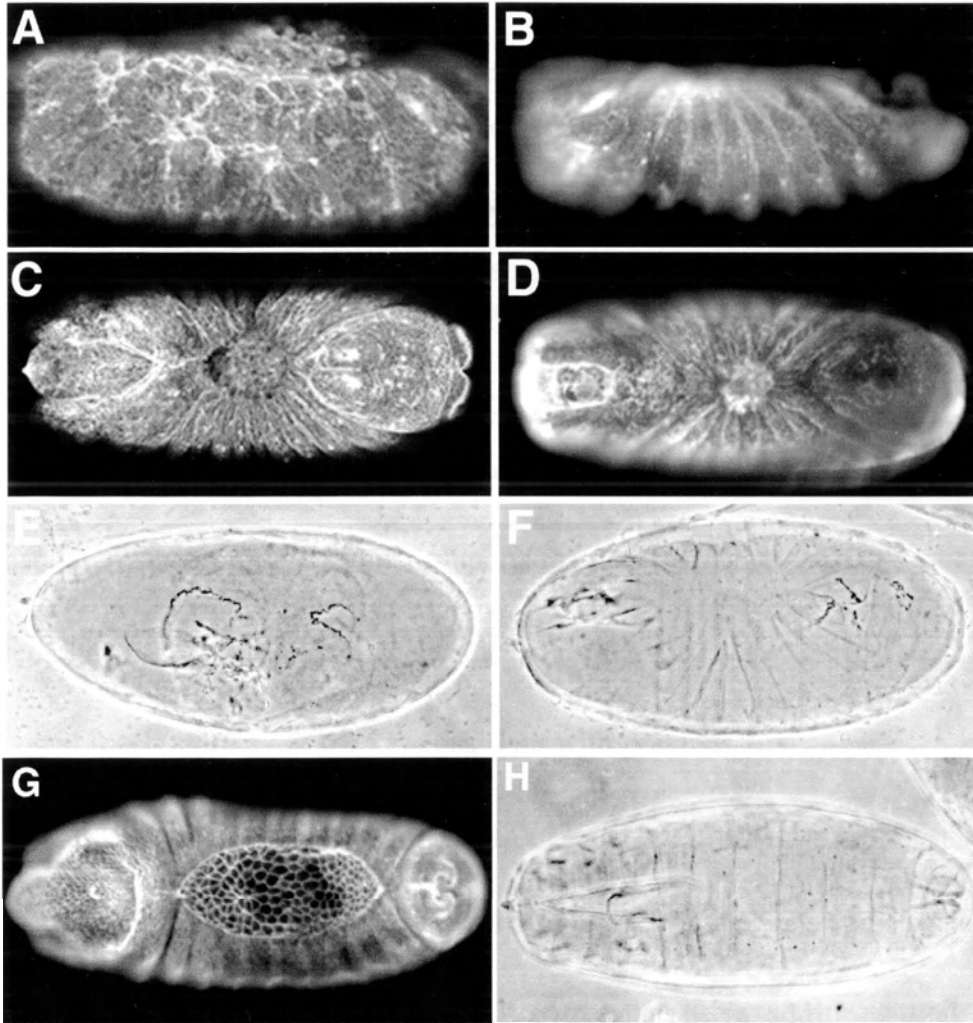
loss-of-function mutations, is truncated due to the presence of a stop codon in the CRIB motif, thus it cannot bind p21s and has no kinase activity (Hing *et al.*, 1999). I established strains in which constitutively active versions of the p21 transgenes and *dpak<sup>6</sup>* were recombined onto the same chromosome (the third). To test whether *dpak<sup>6</sup>* was successfully recombined with the p21 transgenes, I first selected strains displaying homozygous lethality for the reason that flies homozygous for *dpak* mutations are lethal. Second, I crossed the putative recombinant lines to another *dpak* mutation, *dpak<sup>10</sup>*, a null allele, and looked for the transheterozygous *dpak<sup>6</sup>/dpak<sup>10</sup>* adult escapers that were uncoordinated with crumpled wings (Hing *et al.*, 1999). The recombinant lines should have orange eye color due to p21 transgenes.

A heat shock-activated Gal4 driver, *Hs-Gal4<sup>M-4</sup>*, was recombined onto the *dpak<sup>6</sup>* chromosome. This was crossed to the following recombinant lines *Drac1V12, dpak<sup>6</sup>/TM3* and *Dcdc42V12, dpak<sup>6</sup>/TM3*. Expression of the transgenes was induced in embryonic progeny by 1-hour heat shock induction in the case of *Dcdc42V12, dpak<sup>6</sup>/TM3* or 10 minutes induction in the case of *Drac1V12, dpak<sup>6</sup>/TM3*. The reason for the shorter *Drac1V12, dpak<sup>6</sup>* induction is the potency of this transgene (Harden *et al.*, 1999). Embryos were examined with cuticle preparations and with anti-phosphotyrosine staining

to look at cell shape and the status of the cytoskeleton. Phosphotyrosine staining is along the plasma membrane, with very little staining visible in the cytoplasm, thus cell outlines are distinguishable. In addition to revealing cell shape, anti-phosphotyrosine staining provides information about the organization of the LE cytoskeleton. Nodes of phosphotyrosine staining at the LE mark specialized adherens junctions that appear to be required for the assembly of the LE contractile apparatus, and there is a good correlation between losses of these nodes and losses of the LE contractile apparatus (Harden *et al.*, 1996) (reviewed in Harden, 2002).

In the control cross of Drac1V12 with *Hs-Gal4<sup>M-4</sup>*, previously characterized Drac1-induced phenotypes were seen (Harden *et al.*, 1999). The amnioserosa contracted dramatically, which we believe reveals a role for Drac1 in amnioserosa morphogenesis (Harden *et al.*, 2002) (Fig. 5C). The LE bunched up and pinched together, causing pulling up of the head and tail ends of the embryo. There were patches of accumulation of proteins containing phosphotyrosine accompanied by distorted cell shape in the ventro-lateral epidermis (Fig. 5A), in what we believe are the histoblast nests that will form the adult abdomen. When Drac1V12 was induced in a *dpak<sup>6</sup>* mutant background, the amnioserosa also contracted and the LE bunched, but the ventro-lateral epidermis was no

Fig. 5 Phenotypes generated by expression of Drac1 transgene in a *dpak*<sup>6</sup> mutant background using the *Hs-Gal*<sup>M-4</sup> driver with heat shock for 10 minutes at 37°C. (A-D and G) is anti-phosphotyrosine staining on embryo showing cell shape change and status of cytoskeleton. (A) Lateral view of Drac1V12-expressing embryo, showing patches of accumulation of proteins containing phosphotyrosine accompanied by distorted cell shape in the ventro-lateral epidermis. (B) The ventro-lateral epidermis is no longer disrupted when inducing Drac1V12 in a *dpak*<sup>6</sup> mutant background. (C) Dorsal view of Drac1-expressing embryo, showing the dramatic contraction of the amnioserosa. (D) The amnioserosa is still contracted when inducing Drac1V12 in a *dpak*<sup>6</sup> mutant background. (E) The cuticle secretion is dramatically disrupted in Drac1V12-expressing embryo. (F) The cuticle secretion is much more extensive when inducing Drac1V12 in a *dpak*<sup>6</sup> mutant background. (G) Wild-type embryo showing amnioserosa. (H) Wild-type cuticle.

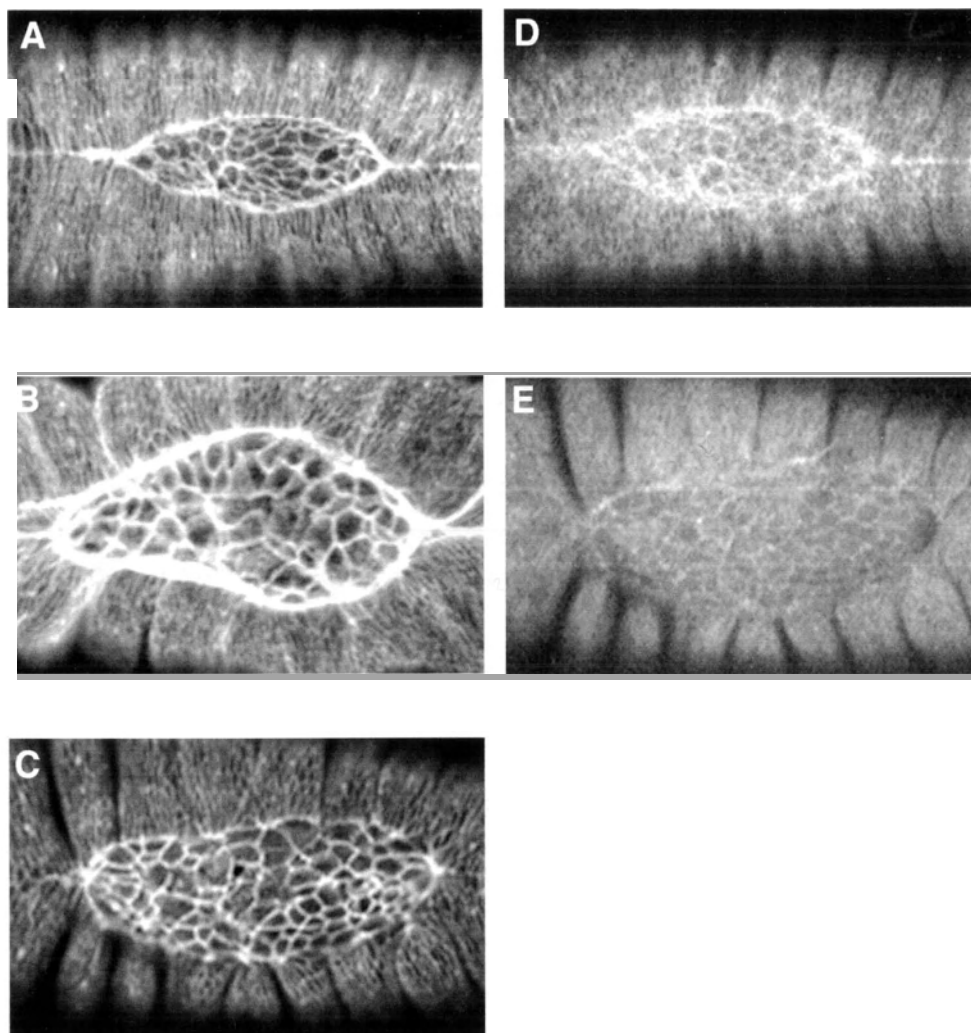


longer affected (Fig. 5B, D). This result suggests that dPak isn't involved in the contraction of the amnioserosa by Drac1 during DC, but may play a role in Drac1's effects on the histoblast nests. In cuticle preparations of Drac1V12-expressing embryos, I observed that the cuticle was dramatically disrupted and only small fragments of cuticle were secreted. But in the *dpak*<sup>δ</sup> mutant background, the cuticle secretion was much more extensive (Fig. 5E, F). Thus, Drac1V12 may require dPak to exert its effects on cuticle formation.

In control embryos of Dcdc42V12 induced by *Hs-Gal4*<sup>M-4</sup>, I found increased staining of phosphotyrosine at the LE, which supports the idea that Dcdc42 is a driving force in the assembly of the cytoskeleton at the LE (Fig. 6B). Dcdc42V12 expression also caused elevated levels of dPak at the LE consistent with previous studies (Harden *et al.*, 1999). In the *dpak*<sup>δ</sup> mutant background, Dcdc42V12 can no longer elevate phosphotyrosine at the LE, and the LE staining of dPak was also weak (Fig. 6C, E). Thus, Dcdc42 seems to act through dPak in setting up the LE cytoskeleton. In addition, dPak staining in *Dcdc42V12*, *dpak*<sup>δ</sup> mutant tended to be in the cytoplasm rather than enriched at the periphery of cells as seen normally (Fig. 6E). I speculate that the dPak antibody I used



Fig. 6 Phenotypes generated by expression of Dcdc42V12 transgene in a *dpak*<sup>6</sup> mutant background using the *Hs-Gal4*<sup>M-4</sup> driver by heat shock for 1 hour at 37°C. (A) Confocal fluorescent observation of anti-phosphotyrosine staining of a wild-type embryo. Phosphotyrosine staining is along the plasma membrane, thus outlining the cell shape and also reflecting the status of the LE contractile cytoskeletal apparatus. (B) The phosphotyrosine staining is increased along the LE in the Dcdc42V12-expressing embryo. (C) The phosphotyrosine staining is no longer elevated when inducing Dcdc42V12 in a *dpak*<sup>6</sup> mutant background. (D) Anti-dPak antibody staining on a wild-type embryo showing accumulation of dPak protein along the LE in the cell periphery. (E) Diffused dPak staining in a Dcdc42V12-expressing *dpak*<sup>6</sup> mutant embryo.



can detect the truncated protein in *dpak*<sup>δ</sup> mutant, which is no longer capable of localizing properly. I tried several times to test the capability of the dPak antibody I used to recognize the truncated form of dPak encoded from *dpak*<sup>δ</sup> on a Western blot, but had problems with high background (data not shown).

I was also interested in looking at the phenotypes of the dominant negative versions of Drac1 and Dcdc42 transgenes in the *dpak*<sup>δ</sup> mutant background. I was specifically interested in comparing the phenotypes induced by Drac1N17 transgenes in the *dpak*<sup>δ</sup> background at early (induced 4 to 8 hours AEL) or late (induced 8 to 12 hours AEL) stages of DC. Previous studies show that Dcdc42N17 worsens the Drac1N17 phenotype at early stages of DC, but suppresses the phenotype of Drac1N17 by elevating the cytoskeletal components at the LE at later stages of DC (Harden *et al.*, 1999). Two opposing effects for Dcdc42 in establishment versus breakdown of the LE cytoskeleton have been proposed and both may act through dPak (Harden *et al.*, 1999). I wondered if *dpak*<sup>δ</sup> mutation would have a rescuing effect on the effects of Drac1N17 expression in late DC.

However, when I was trying to recombine *UAS-Drac1N17* transgene with *dpak<sup>6</sup>* onto the same chromosome and testing the recombinant homozygous lethal lines by crossing with *dpak<sup>10</sup>*, I surprisingly found that the putative *dpak<sup>6</sup>/dpak<sup>10</sup>* transheterozygotes had straight wings and were fertile. At first I thought the leaky expression of *UAS-Drac1N17* rescued the *dpak<sup>6</sup>/dpak<sup>10</sup>* mutant phenotypes, but the theory seemed unconvincing. By outcrossing the recombinant lines to *dpak* deficiency line *Df(3R)Win11*, which removes the *dpak* gene, I determined that these chromosomes lacked the *dpak<sup>6</sup>* mutation. I assumed that there was a second site lethal mutation on the *dpak<sup>6</sup>* chromosome that had recombined with *Drac1N17* giving the homozygous lethal phenotype. Then I recombined the putative *dpak<sup>6</sup>, 2<sup>nd</sup>site* mutant chromosome with wild-type flies to separate the *dpak<sup>6</sup>* mutation and *2<sup>nd</sup>site* lethal gene. We successfully outcrossed the *dpak<sup>6</sup>* mutation from the *2<sup>nd</sup>site*, which was homozygous lethal. The *2<sup>nd</sup>site* mutant when crossed to *dpak<sup>10</sup>* showed normal wings and were fertile. I have not pursued the study of *Drac1N17* expression in the *dpak<sup>6</sup>* background due to a shift in my work to looking at oogenesis.

The fact that a *dpak<sup>6</sup>* mutant background could suppress certain aspects of the *Drac1V12* and *Dcdc42V12* phenotypes indicated that dPak was used by these small GTPases in generating their effects. I repeated the experiments with a different form of induction of

the transgenes. For this, I used the epidermal Gal4 driver *69B*, *69B-Gal4*, which drives transgene expression over many hours on epidermis in the embryo. I recombined *dpak<sup>6</sup>* onto this driver so that I could express small Drac1V12 and Dcdc42V12 with *69B-Gal4* in the *dpak<sup>6</sup>* mutant background. I expressed Drac1V12 and Dcdc42V12 under these conditions. Surprisingly, I found no significant effects in the *dpak<sup>6</sup>* mutant background on the phenotypes induced by these transgenes (data not shown). This may be due to the long period of time the *69B-Gal4* is turned on compared to our heat shock inductions.

### **Creating mutant versions of Drac1 and Dcdc42 that are constitutively active but cannot signal through dPak**

To further explore the participation of dPak in signaling by Drac1 and Dcdc42, I wished to make transgenic flies bearing constitutively active versions of these p21s, which were no longer capable of interacting with dPak. The Y40C effector site substitution in constitutively active Rac or Cdc42 renders these GTPases incapable of binding Pak or activating the JNK cascade (Lamarche *et al.*, 1996; Owen *et al.*, 2000). Dcdc42 cDNA was excised from the pGEX vector through an *EcoRI/BamHI* digestion and cloned into the *EcoRI* and *BamHI* sites of the vector pBluescript to generate *Dcdc42V12* mutation

through *in vitro* mutagenesis. The *Drac1V12* mutation was generated previously (Harden *et al.*, 1996). *Drac1V12Y40C* and *Dcdc42V12Y40C* were generated through *in vitro* mutagenesis. The presence of the mutation was confirmed by manual sequencing (data not shown). I digested the mutated genes out of the pBluscript using *BglII* and *KpnI* and tried to clone them into the vector pUASP, which can be expressed in all *Drosophila* tissues, including the germline, with the appropriate Gal4 drivers. I had problems in ligating the inserts into pUASP, and this was not further pursued due to the fact that these *Rac/Cdc42V12Y40C* flies were generated recently by other labs (Kim *et al.*, 2002; Ng *et al.*, 2002).

### **Pak-I subfamily kinase activity is required in the amnioserosa for morphogenesis of the epidermis but not for amnioserosa cell shape change**

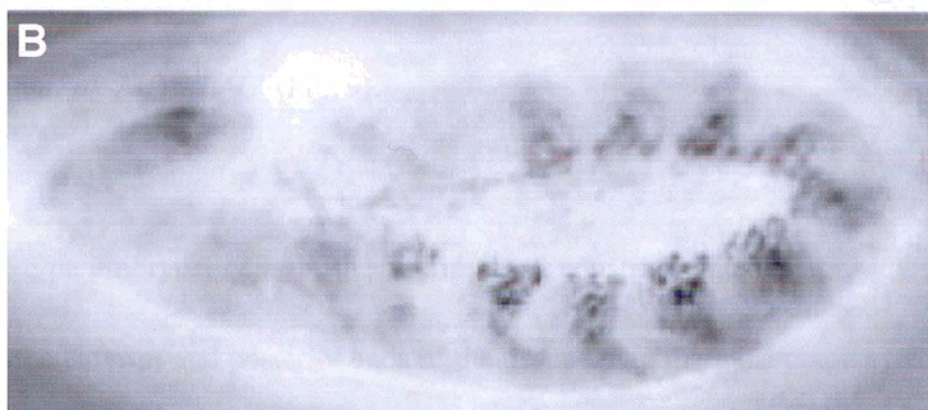
Previous studies describe a response of dPak transcript levels in the amnioserosa to changes in p21s signaling induced by expression of *Drac1V12* and *Dcdc42N17* (Harden *et al.*, 1999; Sem *et al.*, 2002), suggesting a function for dPak in this tissue. Genes expressed in the amnioserosa have been implicated in DC through roles in amnioserosa morphogenesis and signaling to the epidermis (Frank and Rushlow, 1996; Harden, 2002;

Reed *et al.*, 2001; Stronach and Perrimon, 2001). To explore Pak-I kinase family function in this tissue, I expressed AID of dPak that, based on studies of mammalian Pak1, should block the kinase activity of endogenous Pak-I family kinases bearing the appropriate target amino acids. In *Drosophila*, the kinases predicted to be inhibited by dPak-AID are dPak and Pak3 (Frost *et al.*, 1998; Lei *et al.*, 2000; Tu and Wigler, 1999; Zenke *et al.*, 1999; Zhao *et al.*, 1998). Sequences encoding dPak-AID were cloned into the vector pXJ-FLAG in order to add an initiator methionine and N-terminal FLAG tag, and then subcloned into pUAST to establish transgenic lines (made by M. Ricos and N. Harden). I tested the ability of the *dPak-AID* transgenes to drive dPak-AID protein expression by crossing to an *engrailed Gal4* driver and it revealed *en* stripes in embryos stained with anti-FLAG antibody (Fig. 7B).

I used two amnioserosa Gal4 driver drivers, *Gal4<sup>332.3</sup>* (Wodarz *et al.*, 1995) and *Gal4<sup>c381</sup>* (Manseau *et al.*, 1997), to express dPak-AID in this tissue during embryogenesis and examined transgene-expressing embryos by cuticle preparation. Expression of single *UAS-dPak-AID* transgene caused a low frequency of dorsal open embryos and failures to form cuticle. I examined embryos expressing two *UAS-dPak-AID* transgenes by crossing flies heterozygous for second and third chromosome dPak-AID insertions with the

Fig. 7 Anti-FLAG staining of wild-type embryo and embryo expressing dPak-AID with *engrailed Gal4* driver. An N-terminal FLAG tag was added in sequences encoding *dPak-AID* transgenes. (A) There are no *en* stripes in wild-type embryo. (B) *en* stripes are seen in dPak-AID-expressing embryo.





amnioserosa Gal4 drivers. This resulted in an increased frequency of DC defects in transgene expressing embryos (Fig. 8B, C). I examined the morphology of dPak-AID-expressing embryos by staining fixed embryos with anti-phosphotyrosine antibodies and viewing them by confocal microscopy. Embryos in which dPak-AID had been expressed exhibited serious defects in head involution and incomplete or “puckered” DC (Fig. 8D-F). To check that expression of FLAG itself was not having phenotypic effects, the various Gal4 drivers I used in this study were crossed to another FLAG fused transgene (*UAS-ΔC1/2-FLAG*, Thomas *et al.*, 2000) encoding a FLAG-tagged fragment of an unrelated protein, Discs-large. This induction did not produce the phenotypic effects I saw with *dPak-AID-FLAG* (Fig. 9).

In wild-type embryos, cells in the amnioserosa constrict apically during DC, with the cells at the anterior and posterior ends of the tissue being the most constricted (Harden *et al.*, 2002; Rugendorff *et al.*, 1994) (Fig. 8G). These cell shape changes underlie the morphogenesis of the amnioserosa from an elliptically shaped squamous epithelium to a narrow, tubular structure. In the dPak-AID-expressing embryos, apical constriction of amnioserosa cells occurred, and was more pronounced at the anterior and posterior ends than in wild type (arrowheads in Fig. 8D). This result suggests that dPak activity in the

Fig. 8 Effects of expressing *UAS-dPak-AID* in the amnioserosa. (A) Dorsal view of cuticle of wild-type embryo. (B) DC defects in dPak-AID expressing embryo using the *Gal4<sup>332.3</sup>* driver showing dorsal hole extending from middle of embryo to anterior end. (C) Identical DC defects seen in dPak-AID-expressing embryo using the *Gal4<sup>c381</sup>* driver. (D-H) Confocal fluorescent micrographs of dorsal views of embryos, assembled from Z-sections, stained with anti-phosphotyrosine antibodies to reveal morphology. (D, E) dPak-AID-expressing embryos at stage 14 (D) and stage 15 (E) with the *Gal4<sup>332.3</sup>* driver showed “bunched” closure of the epidermis and defects in head involution. The constriction of the anterior and posterior ends of the amnioserosa is maintained in embryo shown in (D) (arrowheads). (F) dPak-AID-expressing embryo at stage 15 with the *Gal4<sup>c381</sup>* driver showed head involution defect and failure of dorsal closure with hindgut rupturing amnioserosa. (G) Stage 14 wild-type embryo showing constriction of cells at ends of the amnioserosa (arrowheads). (H) Stage 15 wild-type embryo.

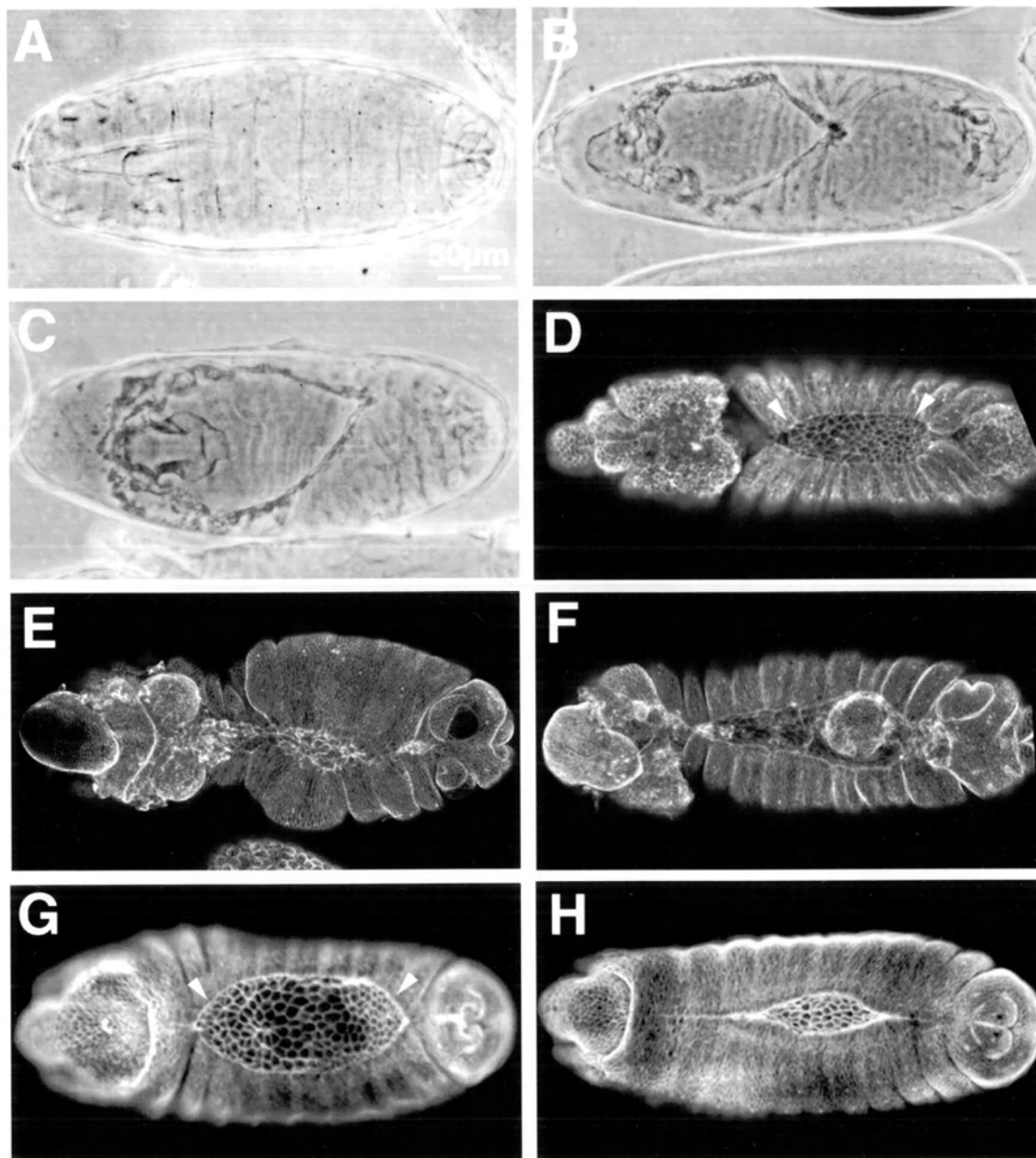
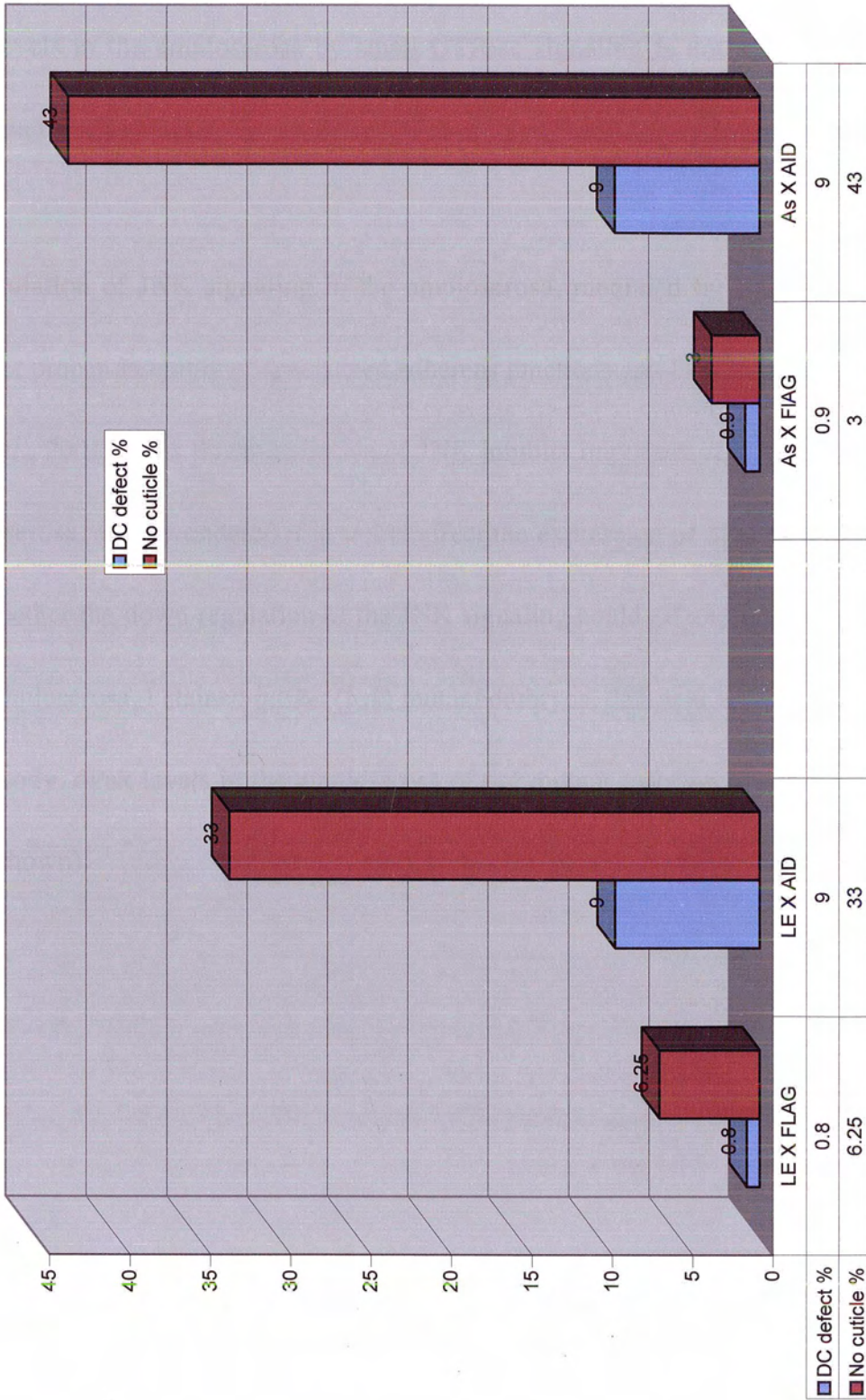


Fig. 9 Expression of *UAS-dPak-AID* with LE and amnioserosa specific drivers. Pink boxes represent the percentage of no cuticle phenotype. Blue boxes represent the percentage of DC defect. LE: *LE-Gal4* driver. As: amnioserosa driver *Gal4<sup>c381</sup>*. AID: transgenes *UAS-dPak-AID*. FLAG: *UAS-ΔC1/2-FLAG*.

Expression of UAS-dPak-AID with LE and amnioserosa drivers



amnioserosa is not required for the morphogenesis of this tissue, and that the regulation of dPak levels in the amnioserosa by small GTPase signaling is not a component of amnioserosa morphogenesis.

Down regulation of JNK signaling in the amnioserosa, mediated by Hnt and Puc, is required for proper assembly of specialized adherens junctions and F-actin in the LE cells (Reed *et al.*, 2001). This downregulation of JNK inhibits important signaling events in the amnioserosa, and I wondered if it would affect the expression of dPak in this tissue. To test whether the down regulation of the JNK signaling could affect the expression of dPak in amnioserosa, I stained *basket (bsk)* mutant embryos, deficient in JNK, with anti-dPak antibody. dPak levels in the amnioserosa of *bsk* mutant embryos appeared normal (data not shown).

**Inhibition of the kinase activity of Pak-I subfamily members in leading edge cells during dorsal closure results in cell shape defects but does not eliminate JNK pathway function**

During DC, there are high levels of dPak in the LE cells of the advancing epidermis (Harden *et al.*, 1996). The LE cells are characterized by signaling events and cytoskeletal changes that are essential for the migration and fusion of the epidermal flanks (reviewed in Goberdhan and Wilson, 1998; Harden, 2002; Jacinto *et al.*, 2001; Noselli and Agnes, 1999). A contractile apparatus of F-actin and non-muscle myosin, as well as F-actin-based lamellipodia and filopodia, accumulates at the dorsal end of each LE cell. The LE cells, and subsequently more ventrally located epidermal cells, constrict in the anterior-posterior direction, thereby elongating in the dorsal-ventral direction, and the epidermal flank “stretches” until it meets with the opposing flank, sealing the epidermis over the amnioserosa (Fig. 10C) (reviewed in Harden, 2002). A JNK cascade operating in LE cells is required for the LE expression of various genes participating in DC, including *dpp*, which encodes a member of the TGF- $\beta$  superfamily. The JNK cascade is also required for the integrity of the F-actin-based structures in the LE cells and correct cell shape change (reviewed in Harden, 2002).



To study the effects of impairing Pak-I subfamily kinase activity in the LE cells during DC, I expressed *UAS-dPak-AID* using the driver *LE-Gal4*, which targets transgene expression specifically in the LE cells (Glise and Noselli, 1997). I tested the expression pattern of this LE driver by crossing to an *UAS-lacZ* reporter and did X-Gal staining on the embryos, and this revealed that only the LE cells were stained blue (Fig. 11). Flies bearing one or two copies of *UAS-dPak-AID* were crossed with *LE-Gal4* and the progeny assessed as embryos by either cuticle preparation or antiphosphotyrosine staining. Cuticle preparation of *UAS-dPak-AID*-expressing embryos demonstrated DC defects in the form of small holes in the dorsal surface of the embryos (Fig. 10B). Staining for phosphotyrosine revealed irregularities in cell shape change in the epidermis. Some sections of the epidermis exhibited elongation of the epidermal cells in the dorsal-ventral direction comparable to wild-type embryos, while other areas lacked such elongation and had “splayed” cells at the LE (Fig. 10D). Elongated LE cells showed retention of phosphotyrosine nodes, but the splayed cells tended to lack nodes.

To see if inhibition of Pak-I subfamily kinase activity affected the function of the JNK cascade in the LE cells, I looked at LE *dpp* transcription in *UAS-dPak-AID*-expressing embryos by RNA *in situ* hybridization. These embryos showed *dpp* expression along the

Fig. 10 Effects of expressing *UAS-dPak-AID* in the LE cells. (A) Dorsal view of cuticle of wild-type embryo. (B) dPak-AID-expressing embryo with the *LE-Gal4* driver showed dorsal hole. (C, D) Confocal fluorescent micrographs, assembled from Z-sections, of the boundary between the amnioserosa (top) and epidermis midway through DC, stained with anti-phosphotyrosine antibody. (C) Wild-type embryo showing nodes of phosphotyrosine (arrowhead) at dorsal end of the LE cells which have elongated in the D-V direction. (D) dPak-AID-expressing embryo showing lack of cell elongation in the epidermis and loss of LE phosphotyrosine nodes. Arrow marks area of the LE where cells are exceptionally “splayed out”, having failed to constrict in the A-P direction. Arrowhead marks a cluster of LE cells that have maintained their phosphotyrosine nodes and have successfully elongated. (E, F) Lateral views of wild-type and dPak-AID-expressing embryos hybridized with a *dpp* riboprobe as a reporter for JNK cascade activation. Note that the JNK cascade is functional in the LE cells in both cases (arrowheads).

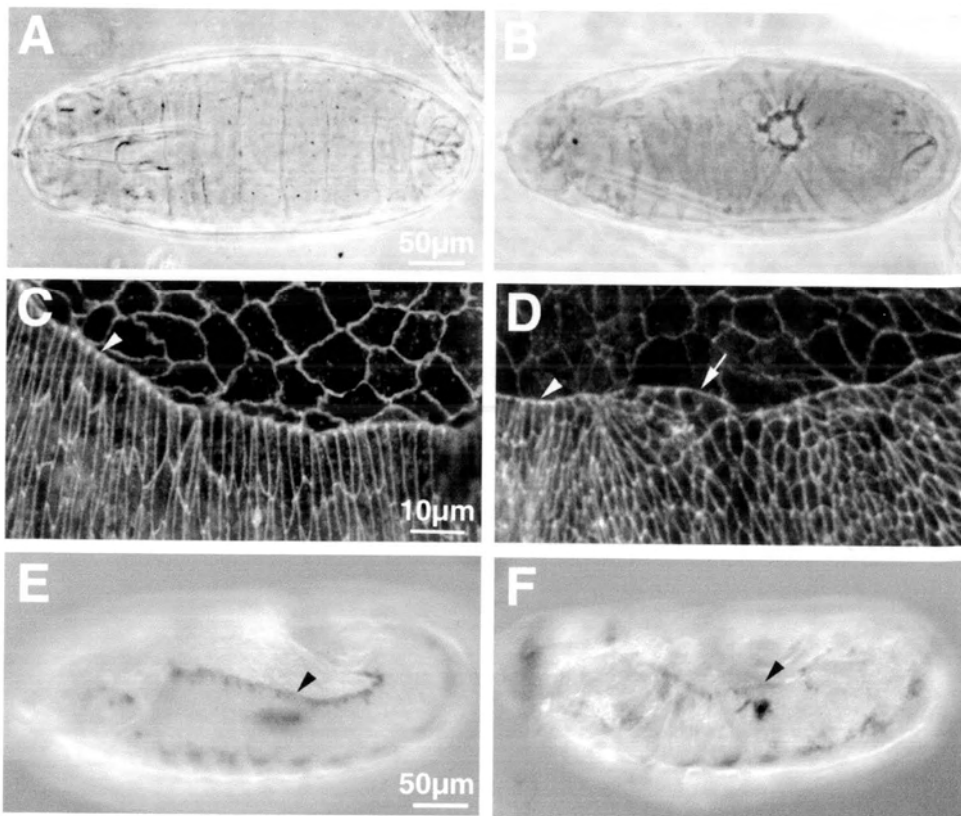
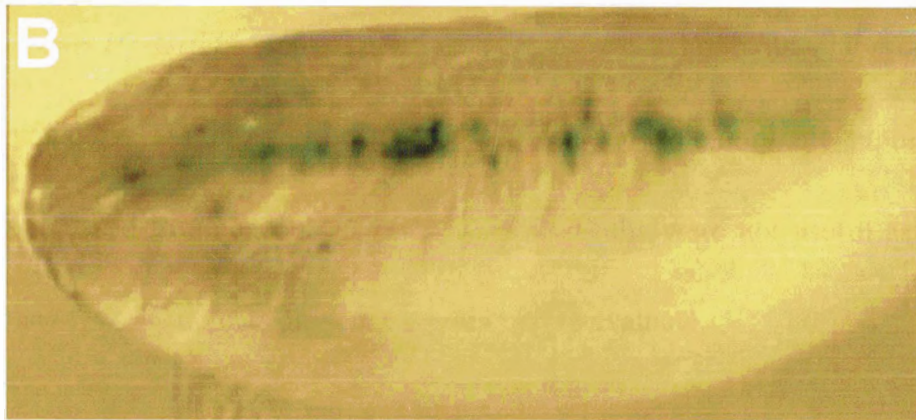
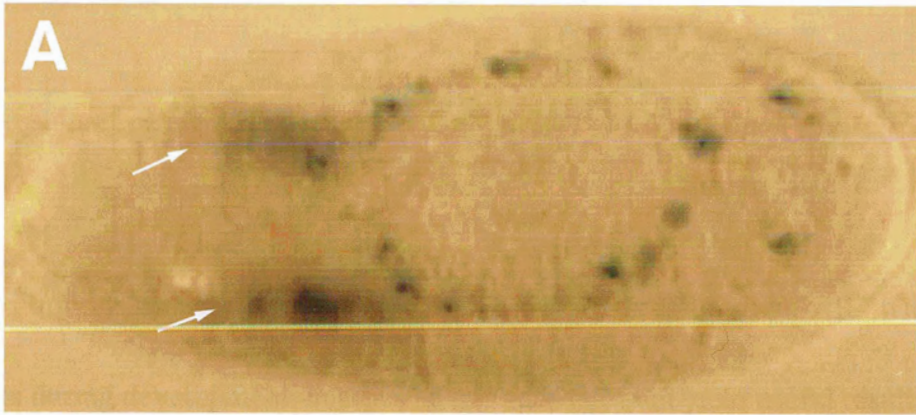


Fig. 11 X-Gal staining on embryo with the *LE-Gal4* driver crossed to an *UAS-lacZ* reporter. From the start of DC (A) to the end (B), the *LE-Gal4* driver is expressed in the LE cells, which are stained blue. There is also source of staining in the salivary gland (arrows).



LE. Although the expression appeared somewhat patchy, I could not determine if this represented a genuine reduction in *dpp* expression or was simply due to distortion of the embryo (Fig. 10E, F). I conclude that dPak is not a major regulator of JNK signaling in the LE during DC.

### **Expression of dPak-AID with other Gal4 drivers and in a *dpak*<sup>6</sup> mutant background**

In addition to the amnioserosa and LE drivers, I expressed dPak-AID with other Gal4 drivers during development, but these expressions did not yield useful results. The drivers tested were *Hs-Gal4*<sup>M-4</sup> (one hour heat shock on 4-8 hour old embryos), *ptc-Gal4* (embryonic expression in stripes), *da-Gal4* (wide-spread embryonic expression), *Gal4-69B* (expressed in embryonic epidermis). The results were not useful as the embryos either had few DC defects or were too disrupted to evaluate.

In order to test if the presence of the *dpak*<sup>6</sup> mutation can strengthen dPak-AID-induced phenotypes, two other dPak-AID inductions were done in a *dpak*<sup>6</sup> mutant background with *Gal4-69B* or *Hs-Gal4*<sup>M-4</sup> drivers. I recombined *dPak-AID* transgenic line C (on the third chromosome) with *dpak*<sup>6</sup> onto the same chromosome. I crossed *dPak-AID* line A

(on the second chromosome) and *dpak*<sup>6</sup>, so a quarter of the progeny should both express dPak-AID and be homozygous for *dpak*<sup>6</sup>. Both inductions under these conditions were indistinguishable from those done in a wild type dPak background.

**Expression of dPak-AID transgenes had no effect on phenotypes induced by Drac1V12 and Dcdc42V12, unlike the *dpak*<sup>6</sup> mutation**

To see if expression of dPak-AID had similar effects to homozygosity for *dpak*<sup>6</sup> on Drac1V12 and Dcdc42V12 induced phenotypes, I recombined *dPak-AID* transgenic line C with *Hs-Gal4*<sup>M-4</sup> to induce the expression of Drac1V12 and Dcdc42V12 by heat shock. dPak-AID did not suppress the effects of Drac1V12 and Dcdc42V12 unlike the effects I observed in the *dpak*<sup>6</sup> mutant background. I assume that one copy of dPak-AID activity is not sufficient to block all Pak kinase activity.

## **dPak function during *Drosophila* oogenesis**

### **dPak participates in organization of the follicular epithelium during oogenesis**

All homozygous loss-of-function *dpak* mutations die at pharate adult stage, including *dpak<sup>1</sup>*, *dpak<sup>3</sup>*, *dpak<sup>6</sup>*, and *dpak<sup>10</sup>* (the lesions and genetic characteristics of the alleles are shown in Materials and Methods) (Hing *et al.*, 1999). I tested various heteroallelic combinations between the above alleles trying to produce females that would produce embryos lacking the maternal dPak. All combinations were lethal at pharate stage except one, in which flies transheterozygous for *dpak<sup>6</sup>* and *dpak<sup>10</sup>* were viable but uncoordinated with crumpled wings (Hing *et al.*, 1999). I found both *dpak<sup>6</sup>/dpak<sup>10</sup>* males and females were sterile.

I visualized nuclei in ovarioles by DAPI staining and examined cell shape and evaluated the F-actin cytoskeleton by FITC-phalloidin staining (reviewed in Verheyen and Cooley, 1994). There were several prominent defects in *dpak<sup>6</sup>/dpak<sup>10</sup>* ovarioles. Egg chambers always degenerated after stage 8 or 9 and no mature eggs were ever produced. The emergence of the oocyte prior to degeneration could be distinguished by the increased F-

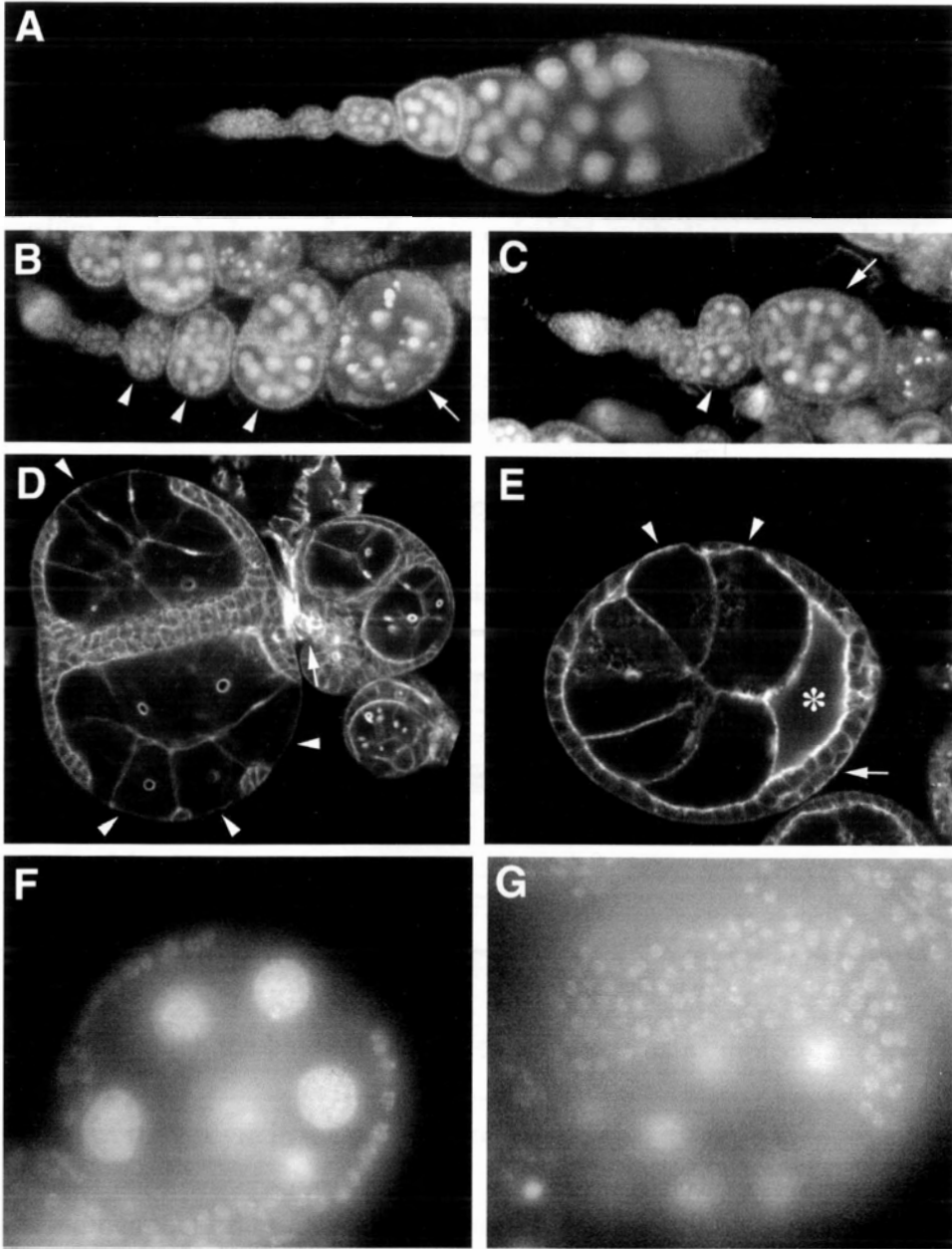


actin staining in this cell, similar to the wild-type oocyte (reviewed in Spradling, 1993; Verheyen and Cooley, 1994).

Another phenotype was the frequent fusion of egg chambers in an unusual side-by-side configuration (Fig. 12B, C). A bilayer of FCs usually divided the chambers in a fused pair, with each chamber containing the normal number of 16 nuclei. In later stage chambers, the bilayer of FCs dividing the fused chambers was occasionally missing, which may reflect degeneration (Fig. 12B, C). It is not clear how the fusion is formed, and whether it is related to cell polarity. Other cases of egg chamber fusion appear to be due to stalk cell loss (Genova *et al.*, 2000; McGregor *et al.*, 2002). The *dpak<sup>6</sup>/dpak<sup>10</sup>* egg chamber fusions do not appear to be due to stalk cell loss as the plane of fusion is perpendicular to that seen in these other cases and intact stalk cells can be seen (Fig. 12D). I have not characterized the *dpak<sup>6</sup>/dpak<sup>10</sup>* egg chamber fusion phenotype further, but have instead chosen to focus on analyzing defects in the morphogenesis of the follicular epithelium.

The F-actin rich follicular epithelium during oogenesis has become a model system for studying epithelial morphogenesis as the developmental events that occur in the ovary

Fig. 12 Egg chamber defects in *dpak<sup>6</sup>/dpak<sup>10</sup>* mutant ovaries. In this and subsequent oogenesis figures, the A-P axis is oriented left to right. (A) DAPI-stained wild-type ovariole, containing 6-7 egg chambers that are sequentially more mature as they move to the posterior end. (B, C) DAPI-stained *dpak<sup>6</sup>/dpak<sup>10</sup>* mutant ovarioles showing side-by-side fusions of egg chambers (arrowheads) and fused egg chambers without a dividing bilayer of FCs (arrows). (D) Stalk cells are intact between two pairs of fused *dpak<sup>6</sup>/dpak<sup>10</sup>* egg chambers near the plane of fusion (arrow), shown by FITC-phalloidin staining. Discontinuities in the follicular epithelium are seen (arrowheads). This phenotype is also seen in E, a single mutant egg chamber (arrowheads). (E) *dpak<sup>6</sup>/dpak<sup>10</sup>* mutant egg chamber estimated to be around stage 8/9 on the basis of size of oocyte (asterisk), which can be compared to a similarly aged wild-type egg chamber in Fig. 13C. Mutant egg chamber has failed to elongate and FCs at the posterior end have failed to change to columnar in shape and are in a bilayer (arrow). (F, G) DAPI-stained *dpak<sup>6</sup>/dpak<sup>10</sup>* mutant egg chambers showing that discontinuities in FCs are due to cell loss rather than flattening of epithelial cells. Cross-sectional (F) and basal (G) views of the follicular epithelium reveal regions with no FC nuclei.



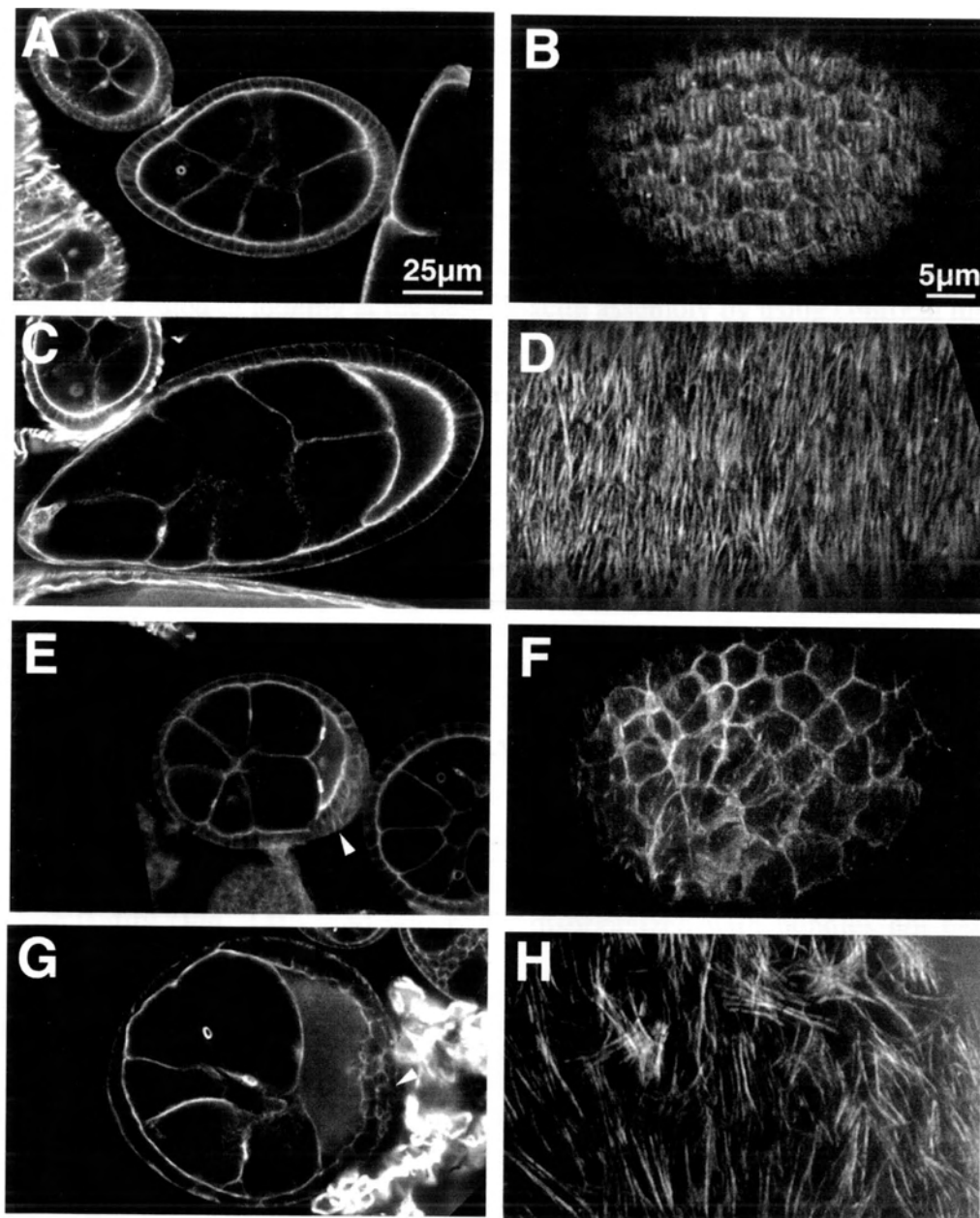
parallel those seen in other epithelia, for example the epithelium in DC during embryogenesis (reviewed in Dobens and Raftery, 2000; Spradling, 1993; Verheyen and Cooley, 1994). Dramatic morphogenetic changes occur throughout the ovariole during development, which are driven by the cytoskeletal system in the FCs. Starting from the germarium, a single layer of uniformly cuboidal epithelium covers the developing egg chamber. As egg chamber development progresses and the oocyte starts to emerge (stage 9), the FCs become columnar in shape over the oocyte at the posterior of the egg chamber, and the anterior epithelium becomes squamous in shape over the nurse cells (reviewed in Verheyen and Cooley, 1994). A notable feature of the FCs in mid-stage egg chambers is the polarized organization of basal F-actin bundles such that the filaments lie perpendicular to the A-P axis of the egg chamber (Gutzeit, 1990). The basal F-actin is disorganized in the FCs of *kugel* mutant egg chambers, and oocytes frequently remain rounded due to a failure to elongate along the A-P axis (Gutzeit *et al.*, 1991). This has led to a model in which the basal F-actin of the FCs acts as a “molecular corset” to promote elongation of the oocyte in the A-P axis (Gutzeit *et al.*, 1991). The model is supported by recent findings that other mutations disrupting the basal F-actin of the follicle cells also result in rounded oocytes (Bateman *et al.*, 2001; Frydman and Spradling, 2001; Gutzeit *et al.*, 1991). The *dpak<sup>6</sup>/dpak<sup>10</sup>* mutant egg chambers exhibited almost spherical shape,

indicating failure of elongation along the A-P axis and leading me to check the F-actin organization in the follicle cells.

### **dPak is required for the polarized accumulation of basal F-actin filaments in follicle cells and egg chamber elongation**

The rounded egg chambers in *dpak<sup>6</sup>/dpak<sup>10</sup>* mutant indicated the failure of elongation along the A-P axis (fig. 12E, Fig. 13E, G). A variety of defects were seen associated with the F-actin in the follicular epithelium covering these egg chambers. Both fused and unfused *dpak<sup>6</sup>/dpak<sup>10</sup>* mutant egg chambers exhibited frequent large openings in the follicular epithelium, seen in cross sectional and surface views (Fig. 12D-G). To avoid complications arising from the fused egg chamber phenotype, I restricted further analysis of FCs to unfused *dpak<sup>6</sup>/dpak<sup>10</sup>* egg chambers. Early in stage 9, most of the FCs in the wild-type egg chamber migrate to the posterior end and elongate in the apical-basal axis to form columnar epithelium covering the oocyte (Fig. 13C). But in *dpak<sup>6</sup>/dpak<sup>10</sup>* mutant egg chambers, the FCs failed to elongate to the columnar shape and became disorganized, losing the ability to maintain a monolayered epithelium and often forming bilayers in patches (Fig. 12E, 13E, G). In wild-type egg chambers, as FCs migrate to the posterior

Fig. 13 Both F-actin accumulation and organization in the FCs are disrupted in *dpak<sup>6</sup>/dpak<sup>10</sup>* mutant egg chamber revealed by FITC-phalloidin staining. Left panels show cross sectional views of egg chambers, while right panels show higher magnification views of the basal F-actin filaments in the FCs of these egg chambers. (A, B) Stage 7 wild-type egg chamber showing polarization of basal F-actin filaments perpendicular to the A-P axis of the chamber. (C, D) Stage 8/9 wild-type egg chamber showing dense, polarized accumulation of basal F-actin filaments. (E, F) Stage 8 *dpak<sup>6</sup>/dpak<sup>10</sup>* mutant egg chamber showing spherical shape and bilayer of FCs over oocyte (arrowhead). The basal F-actin filaments are decreased in quantity and disorganized. (G, H) Stage 9 *dpak<sup>6</sup>/dpak<sup>10</sup>* mutant egg chamber showing spherical shape and bilayer of FCs over oocyte (arrowhead). Basal F-actin filaments are less dense and disorganized.



end, the FCs remaining over the nurse cells flatten into squamous epithelium (Fig. 13C). In stage 9, *dpak<sup>δ</sup>/dpak<sup>10</sup>* mutant egg chambers, most of the FCs covering nurse cells remained cuboidal (Fig. 13G). The various defects in the follicular epithelium suggest that FCs are abnormally shaped and the distribution is disorganized.

I was interested in looking at the basal F-actin assembly by using confocal microscopy on the *dpak<sup>δ</sup>/dpak<sup>10</sup>* FCs, because of the rounded egg chamber morphology and the dramatic morphological effects on the FCs. *dpak<sup>δ</sup>/dpak<sup>10</sup>* mutant egg chambers were defective in both the assembly and organization of the basal F-actin. Starting at around stage 7, the basal actin filaments of the wild type follicular epithelium become organized into bundles that are oriented perpendicular to the A-P axis (Fig. 13B, D). In *dpak<sup>δ</sup>/dpak<sup>10</sup>* mutant egg later than stage 7, the basal F-actin in the FCs ranged from almost nonexistent to reduced in quantity (Fig. 13F), and was badly disorganized with bundles not being properly polarized (Fig. 13H).



**dPak is localized to the basal end of follicle cells during oogenesis and becomes enriched at the cell periphery in follicle cells forming the columnar epithelium over the oocyte**

I observed the distribution of dPak protein during oogenesis by staining ovarioles with anti-dPak antibody (Harden *et al.*, 1996), together with FITC-phalloidin to visualize the cytoskeleton. Throughout egg chamber development, dPak was found to be restricted to the basal end of FCs, as seen in cross sectional views (Fig. 14), and this was confirmed when the two images from the double staining of dPak and FITC-phalloidin were merged (Fig. 14). In the early stage of oogenesis before the oocyte developed, the dPak was diffusely distributed throughout the base of the FCs and it was only weakly localized to the cell periphery. At later stages of oogenesis, there was strong peripheral localization of dPak in the columnar FCs, and there was less dPak staining in the squamous FCs over the nurse cells. I could not see any dPak staining in nurse cells or the oocyte.

In the *dpak<sup>6</sup>/dpak<sup>10</sup>* mutant egg chambers, mutant dPak protein is basally localized in the follicular epithelium, as in wild type (Fig. 14), but does not show localization to the cell periphery (Fig. 15L). A similar failure of mutant dPak protein to localize to the cell

Fig. 14 dPak is localized to the basal end of FCs both on wild-type and *dpak<sup>6</sup>/dpak<sup>10</sup>* mutant egg chambers during oogenesis revealed by dPak antibody staining. Left panels show FITC-phalloidin stained egg chambers and right panels show anti-dPak staining on the same chambers, while the middle panels show merged images of both staining. (A-C) Early wild-type chambers. (D-F) Stage 8/9 wild-type chambers. (G-I) Stage 9 *dpak<sup>6</sup>/dpak<sup>10</sup>* mutant egg chamber showing that mutant dPak protein is still localized to the basal end of FCs.

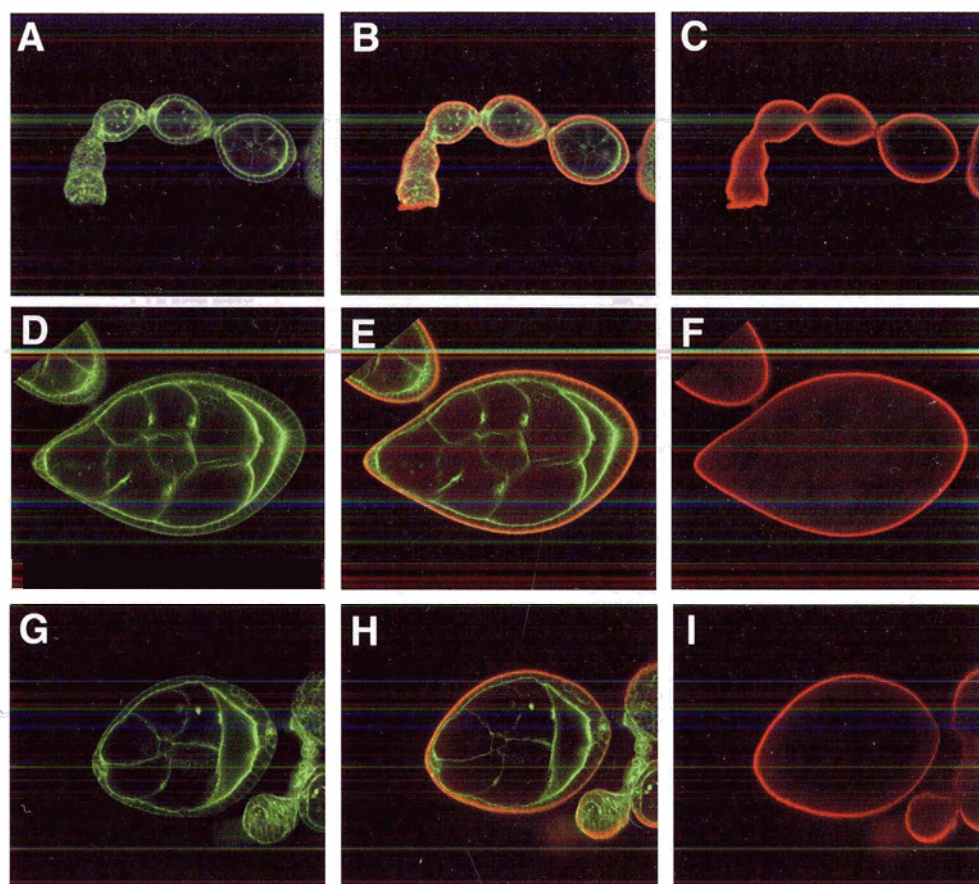
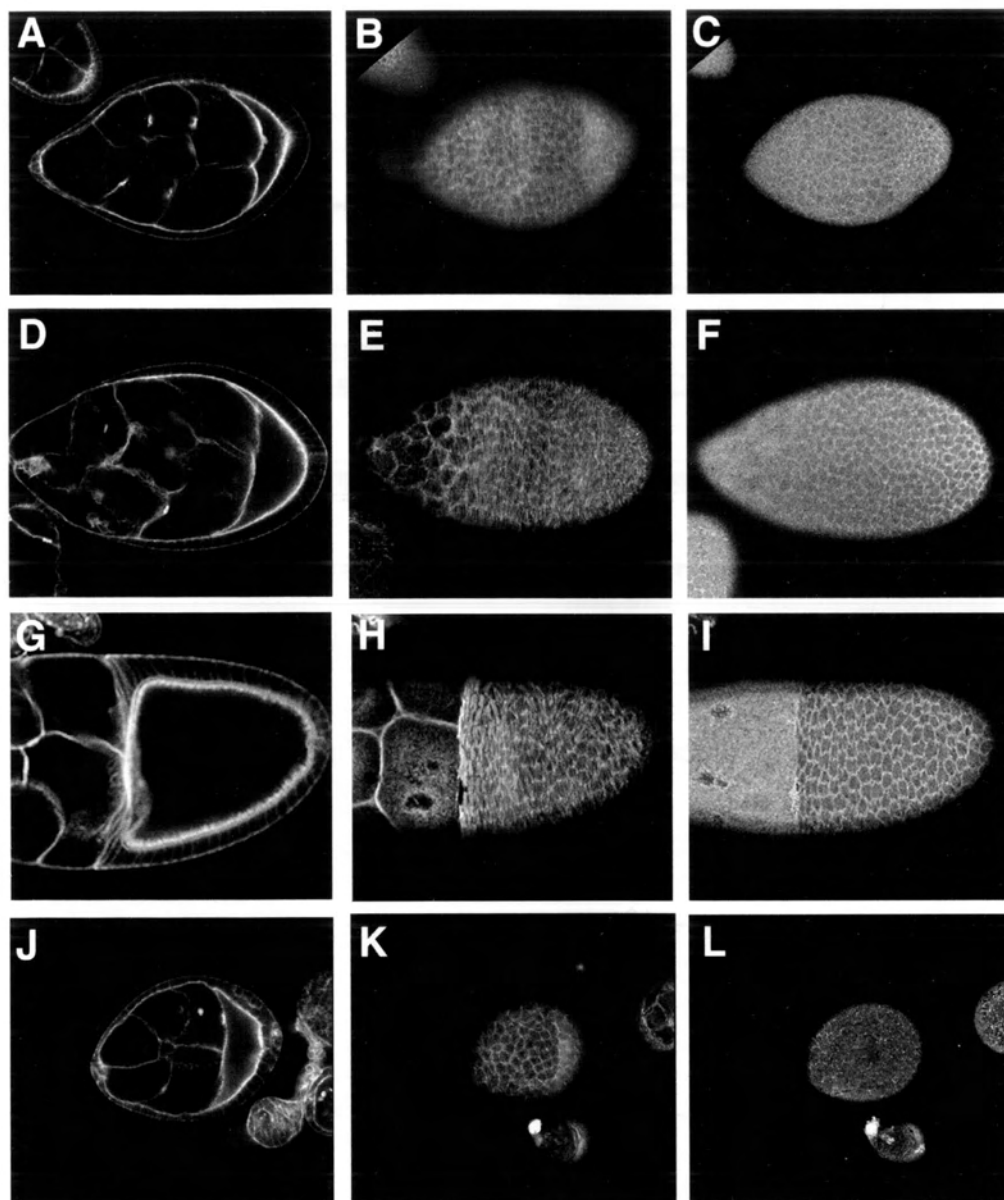


Fig. 15 dPak becomes strongly localized to the cell periphery in columnar FCs over the oocyte in wild-type but not in *dpak<sup>δ</sup>/dpak<sup>10</sup>* mutant egg chambers. Left panels are cross sectional views of FITC-phalloidin-stained egg chambers. Middle panel show basal F-actin of the same egg chambers, while right panels show basal views of the same egg chambers stained with anti-dPak antibodies. (A-C) Same wild-type chambers as in Fig. 14D-F. (D-F) Stage 9 wild-type egg chamber. (G-I) Stage 10 wild-type egg chamber. (J-L) Same *dpak<sup>δ</sup>/dpak<sup>10</sup>* mutant egg chamber as in Fig. 14G-I, showing that mutant dPak protein has lost the ability to localize peripherally (L).

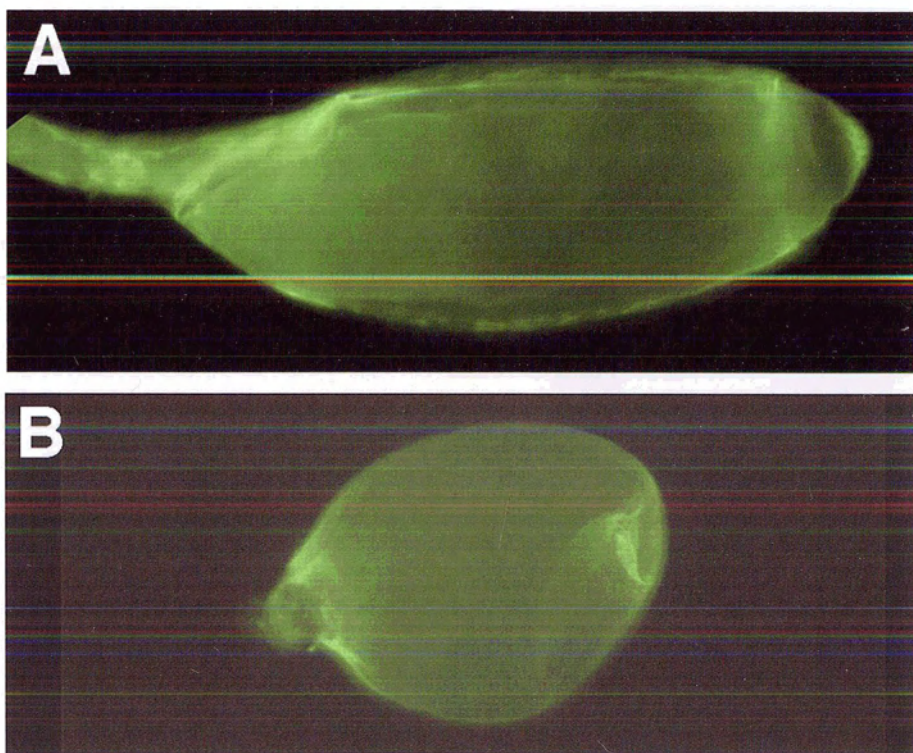


periphery was seen in the epidermis of *dpak<sup>6</sup>/dpak<sup>6</sup>* embryos (Fig. 6E). Overall the dPak staining is weaker in the *dpak<sup>6</sup>/dpak<sup>10</sup>* mutant egg chambers than in wild-type chambers.

**During egg chamber elongation *dpak* interacts genetically with *inflated*, which encodes an  $\alpha$ -integrin subunit**

Recent evidence indicates that signaling from the ECM, through heterodimers of  $\alpha$ - and  $\beta$ -integrin subunits and the receptor tyrosine phosphatase Dlar, participates in egg chamber elongation through the regulation of the basal actin cytoskeleton in the FE (Bateman *et al.*, 2001; Duffy *et al.*, 1998; Frydman and Spradling, 2001). I looked for genetic interaction between *dpak<sup>6</sup>* and the genes encoding  $\beta$ -integrin (*mysospheroid*, *mys*),  $\alpha$ -integrin (*inflated*, *if*) and Dlar by examining oogenesis in females transheterozygous for *dpak<sup>6</sup>* and either a loss-of-function *mys* allele, *mys<sup>1</sup>* (Bunch *et al.*, 1992) a null allele of *if*, *if<sup>k27e</sup>* (Bloor and Brown, 1998; Falk *et al.*, 1984) or one of two deficiencies disrupting the *Dlar* locus, *Df(2L)E55* and *Df(2L)TW84* (Krueger *et al.*, 1996). All of the various transheterozygous females were fertile and produced normally elongated eggs. However, about 16% of the eggs produced by *if<sup>k27e</sup>/+;dpak<sup>6</sup>/+* transheterozygotes were distinctly rounded (Fig. 16).

Fig. 16 Females transheterozygous for *dpak*<sup>6</sup> and *if*<sup>k27e</sup>, a gene encoding  $\alpha$ -integrin subunit, produce some rounded eggs. (A) Proper egg elongation shown in wild-type. (B) A rounded egg produced by transheterozygosity for *dpak*<sup>6</sup> and *if*<sup>k27e</sup>.





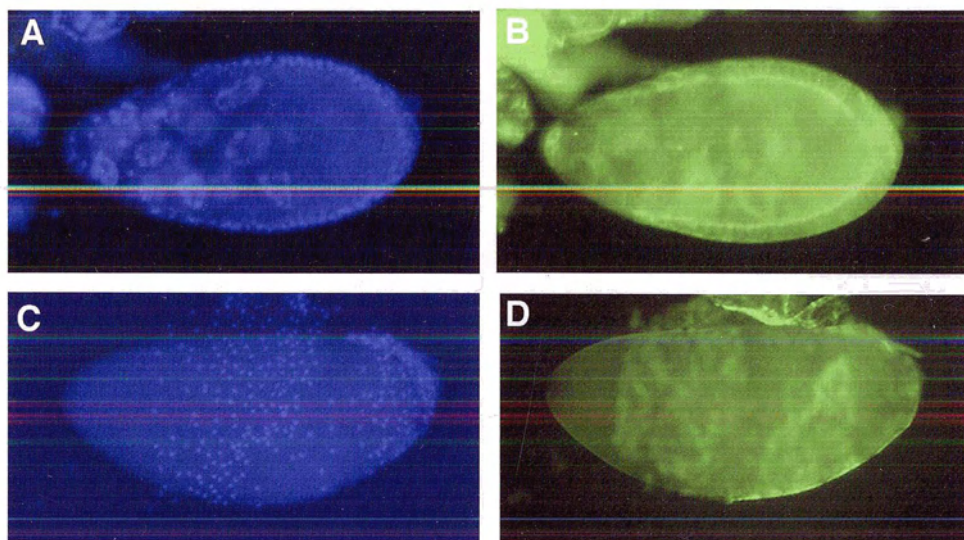
**Expressing *UAS-dpak* transgene in the *dpak<sup>6</sup>/dpak<sup>10</sup>* mutant egg chambers using *198Y-Gal4* resulted in normally elongated egg chamber**

That failure of *dpak<sup>6</sup>/dpak<sup>10</sup>* egg chambers to elongate was due to lack of dPak function was confirmed by expressing *UAS-dpak* in the FCs of these mutant chambers using the FC *Gal4 driver 198Y-Gal4* (Murphy and Montell, 1996). This expression of wild-type dPak rescued most of the oogenesis defects and resulted in normally elongated egg chambers, indicating that it is indeed a lack of dPak function in these cells that is causing the phenotypic effects (Fig. 17).

**No prominent defects were found in egg chambers expressing dPak-AID transgenes using follicle drivers**

Expression of dPak-AID transgenes resulted in epidermal phenotypes using *amnioserosa* and LE specific drivers during embryogenesis. I wished to see if expressing these transgenes in the FCs during oogenesis would yield any phenotypic effects. Transgenic flies bearing one or two copies of dPak-AID were expressed using two FC drivers (*198Y-Gal4*, *185Y-Gal4*) (Murphy and Montell, 1996) to explore Pak-I subfamily kinase

Fig. 17 Expressing *UAS-dpak* transgene in *dpak<sup>6</sup>/dpak<sup>10</sup>* mutant egg chambers using FC driver, *198-Gal4*, results in normally elongated egg chamber revealed by DAPI staining in (A, C) and FITC-phalloidin staining in (B, D). (A, B) Egg chamber was elongated with a monolayer of FCs, which have changed to columnar in shape at the posterior over the oocyte and to squamous in shape at the anterior over the nurse cells. (C, D) An almost mature egg has formed.



function during oogenesis. All the female progeny expressing dPak-AID were fertile, producing normally elongated eggs, and no prominent defects were found by examining DAPI/Phalloidin stained ovarioles (data not shown). The levels and/or timing of *dPak-AID* may not be sufficient to disrupt Pak-I function in FC morphogenesis.

### **Other defects found in *dpak<sup>6</sup>/dpak<sup>10</sup>* mutant flies**

*Dcdc42* mutants show ectopic wing veins and wing blisters where dorsal and ventral surfaces are not fully apposed; similar phenotypes are seen when expressing *Dcdc42N17* in the developing wing (Baron *et al.*, 2000; Eaton *et al.*, 1995; Genova *et al.*, 2000). Both cell shape and the actin cytoskeleton are highly dynamic during pupal wing morphogenesis. Impairment of DACK, a downstream effector kinase for *Dcdc42*, using a kinase dead transgene (KD-ACK) causes wing blisters and ectopic veins (Sem *et al.*, 2002). The crumpled wings of *dpak<sup>6</sup>/dpak<sup>10</sup>* mutant adults prompted me to examine them more closely (Hing *et al.*, 1999). I mounted the crumpled wings and found evidence of blisters and ectopic vein tissue (Fig. 18). There are parallels in the epithelial morphogenesis in the wing and embryonic morphogenesis, thus the wing might provide

Fig. 18 Wing defect in *dpak<sup>6</sup>/dpak<sup>10</sup>* mutant adult fly. (A) Wild-type wing. (B) *dpak<sup>6</sup>/dpak<sup>10</sup>* mutant wing with extra cross veins (arrow).

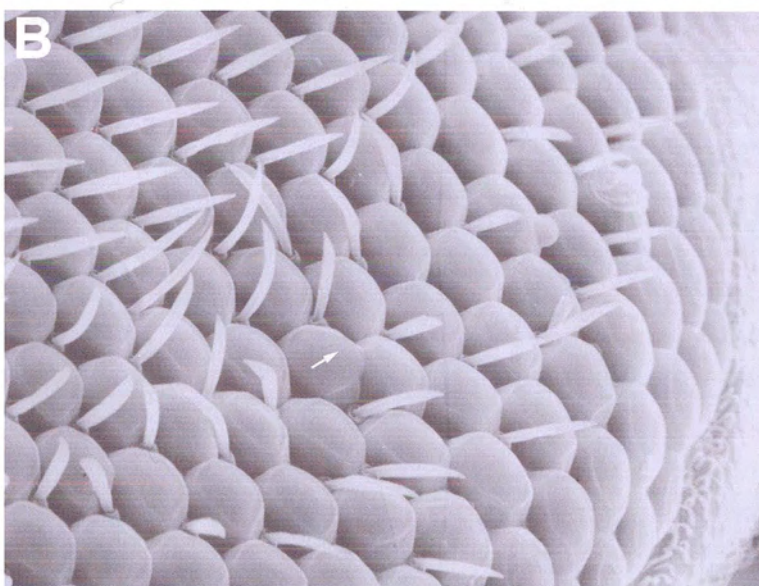
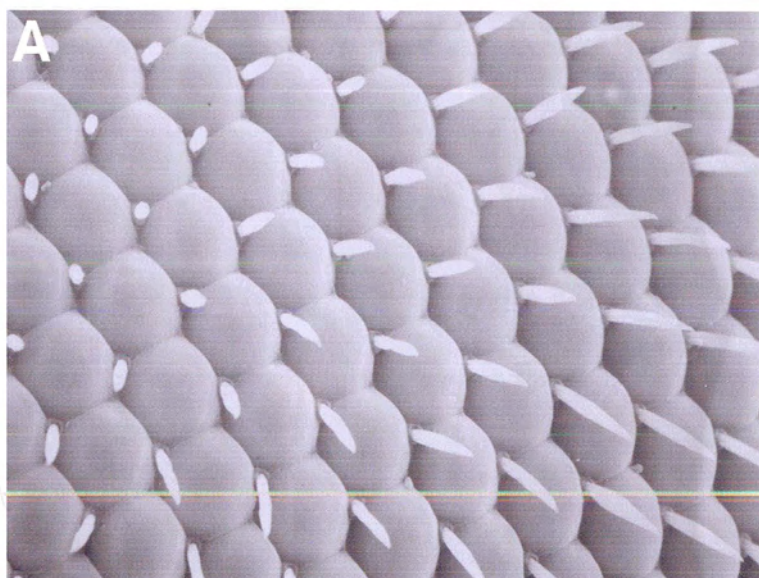


another useful system to analyze dPak involved signaling pathways and epithelial morphogenesis.

Expression of KD-ACK leads to a rough eye phenotype and missing bristles at the posterior end of the eye (Sem *et al.*, 2002). Scanning Electron Microscope (SEM) examination of *dcdc42* mutant eyes reveals minor flaws in the ommatidial array, primarily in the dorsal posterior quadrant, where occasional fusions of the ommatidia and frequent loss or duplication of bristles are found. All the photoreceptors are normal with histological examination (Genova *et al.*, 2000). I examined the adult eye of *dpak<sup>6</sup>/dpak<sup>10</sup>* mutant by SEM. There were a few fused ommatidia and missing bristles (Fig. 19).

Fig. 19 Scanning electronic micrographs (SEM) of wild-type eye (A) and *dpak<sup>6</sup>/dpak<sup>10</sup>* mutant eye (B). Minor defects were revealed in *dpak<sup>6</sup>/dpak<sup>10</sup>* mutants with occasional fusion of ommatidia (arrow) and missing bristles.





## **Discussion**

Rho GTPases have multiple essential functions in diverse cellular events such as membrane trafficking, transcriptional regulation, cell growth control and development. Particularly, Rho GTPases play key roles in the generation of distinct cytoskeletal structures in order to regulate tissue remodeling (reviewed in Van Aelst and D'Souza-Schorey, 1997). Controlled changes to the actin cytoskeleton are vital for almost all cellular processes including motility, adhesion, cell division, cell death and phagocytosis. These diverse processes can be induced by various extracellular signals that bind to their corresponding receptors to stimulate the cell changes. It is still elusive how Rho GTPases participate in such versatile signaling pathways and interact with a large number of proteins. One possibility is that distinct signaling pathways downstream of the extracellular ligands and receptors converge at the level of Rho GTPases to regulate the essential cytoskeletal rearrangement (reviewed in Daniels and Bokoch, 1999). The study of Rho GTPases in morphological signaling involves a search for the crucial effectors that Rho GTPases activate, among which Pak appears to be a pivotal molecule (reviewed in Daniels and Bokoch, 1999). Paks were initially discovered in rat brain as a protein binding to GTP-bound Rac/Cdc42 (Manser *et al.*, 1994), and mammalian Paks can induce Rac/Cdc42-mediated cytoskeletal structures such as lamellipodia and filopodia in Swiss 3T3 fibroblasts (Sells *et al.*, 1997). Paks are recruited to focal adhesions/focal

complexes in HeLa cells and co-localize with activated Rac/Cdc42 (Manser *et al.*, 1997). Thus, Paks have important functions in regulating the dynamic actin cytoskeleton downstream of Rac/Cdc42. In *Drosophila*, dPak accumulates with the actin-myosin apparatus along the LE, possibly mediating the establishment and/or the down regulation of the cytoskeleton in the LE with Drac/Dcdc42 (Harden *et al.*, 1996; Harden *et al.*, 1999).

Coordinated epithelial cell movements driven by the actin-myosin cytoskeletal system play important roles in a number of events, such as in wound healing or in determining the morphology of nascent tissues. In *Drosophila*, the migration of the lateral epidermis over amnioserosa to seal the dorsal hole bears similarities to the morphogenetic changes in oogenesis. Consistent with this scenario, we have found that dPak functions in both DC and oogenesis.

### **dPak function in embryogenesis**

#### **Pak-I subfamily kinase activity in the amnioserosa regulates epidermal morphogenesis during dorsal closure**

Previous studies have shown that p21s can regulate *dpak* transcript levels in the amnioserosa, indicating that dPak may function in this tissue (Harden *et al.*, 1999; Sem *et al.*, 2002). Expression of either *UAS-Drac1V12* or *UAS-Dcdc42N17* results in elevated dPak transcript levels in the amnioserosa, while transcription of another Dcdc42 effector, DACK, is upregulated in the amnioserosa by *UAS-Dcdc42V12* (Sem *et al.*, 2002). These results suggest that small GTPases can regulate the expression of their own downstream effectors in a tissue-specific manner. Genes expressed in the amnioserosa have been implicated in DC through roles in amnioserosa morphogenesis and signaling to the epidermis (Frank and Rushlow, 1996; Harden, 2002; Reed *et al.*, 2001; Stronach and Perrimon, 2001).

To explore Pak-I kinase family function in the amnioserosa, I expressed *dPak-AID* transgenes in this tissue with amnioserosa-specific drivers. This transgene should block

Pak-I subfamily kinase activity, i.e. it should impede dPak and Pak3 in *Drosophila* (Frost *et al.*, 1998; Lei *et al.*, 2000; Tu and Wigler, 1999; Zenke *et al.*, 1999; Zhao *et al.*, 1998). Expression of dPak-AID in the amnioserosa caused head involution and DC defects, suggesting that Pak-I subfamily kinase activity in this tissue is a component of signaling from the amnioserosa known to be required for epidermal morphogenesis. LE cell fate is dependent on communication between the amnioserosa and the dorsal epidermis. Down regulation of JNK signaling in the amnioserosa, mediated by Hnt and Puc, is required for proper assembly of specialized adherens junctions and F-actin in the LE cells (Reed *et al.*, 2001). Furthermore, a single row of LE cells is always specified at the interface between amnioserosa and dorsal epidermis (Stronach and Perrimon, 2001). To determine if Pak-I kinase activity may signal from the amnioserosa to induce epidermal morphogenesis will require further study. JNK downregulation in the amnioserosa does not induce dPak expression, as *dpak* transcript levels remain unaltered in *bsk* mutant embryos, which are deficient in JNK signaling.

Cell shape change in the amnioserosa in the dPak-AID-expressing embryos was similar to wild-type, with cells in the amnioserosa constricting apically at the anterior and posterior ends of the tissue during DC (Harden *et al.*, 2002; Rugendorff *et al.*, 1994). The

excessive contraction of the amnioserosa induced by Drac1V12 was still seen in a *dpak*<sup>o</sup> mutant background, suggesting that dPak is not involved in amnioserosa morphogenesis downstream of Drac1. Furthermore, in embryos co-expressing Drac1V12 and RhoAN19, dPak expression in the amnioserosa is not elevated above wild-type levels, but the amnioserosa shows excessive contraction similar to when Drac1V12 is expressed alone (N. Harden, unpublished observations). These various results indicate that Pak-I kinase activity in the amnioserosa is not required for the morphogenesis of this tissue.

### **dPak can function downstream of Drac1 during embryogenesis**

I showed that a *dpak* mutation suppressed certain aspects of the Drac1V12-induced embryonic phenotype, indicating that dPak was used by this p21 in generating effects during embryogenesis. In Drac1V12-expressing embryos, there were patches of accumulation of proteins containing phosphotyrosine accompanied by distorted cell shape in the ventro-lateral epidermis, in what we believe are the histoblast nests that will form the adult abdomen. However, when Drac1V12 was expressed in a *dpak*<sup>o</sup> mutant background this phenotype was no longer seen. This result suggests that dPak may play a role downstream of Drac1 in histoblast nest formation. That Drac1 may have a role in

development of the abdominal histoblast is supported by the finding that expression of Drac1<sup>N17</sup> during pupa development can result in adult flies with an undeveloped abdomen (N. Harden, unpublished observation). Another phenotypic effect of Drac1<sup>V12</sup> that can be suppressed by the *dpak*<sup>6</sup> mutation is the disruption of cuticle formation. Expression of Drac1<sup>V12</sup> severely disrupts cuticle secretion, possibly due to effects on the apical-basal polarity of the cuticle-secreting epidermis (Harden *et al.*, 1999). Cuticle secretion was much less disrupted when Drac1<sup>V12</sup> was expressed in the *dpak*<sup>6</sup> mutant background. Studies on Drac1 in wing development also indicate roles in establishing the apical-basal polarity of epithelia (Eaton *et al.*, 1995). My result raises the possibility that dPak is involved in regulation of epithelial polarity by Drac1.

**Pak-I subfamily kinase function is required for correct assembly of the cytoskeleton and cell shape change in the leading edge cells during dorsal closure**

Previous studies have shown that Drac1, and to a lesser extent, Dcdc42, are the driving forces setting up cytoskeletal components, including F-actin and non-muscle myosin, in the LE during DC (Harden *et al.*, 1996; Harden *et al.*, 1999; Hakeda-Suzuki *et al.*, 2002). During DC, there are high levels of dPak in the LE cells of the advancing epidermis,



especially at the segment border cells (Harden *et al.*, 1996). *Dcdc42V12* can cause increased accumulation of dPak and phosphotyrosine along the LE, but in the *dpak<sup>o</sup>* mutant background, dPak and phosphotyrosine are no longer elevated. In addition to revealing cell shape, anti-phosphotyrosine staining provides information about the organization of the LE cytoskeleton. Nodes of phosphotyrosine staining at the dorsal end of each LE cell mark specialized adherens junctions required for the assembly of the LE contractile apparatus (reviewed in Harden, 2002). Thus, *Dcdc42* may use dPak to set up cytoskeletal components along the LE.

Further evidence that Pak-I subfamily kinase function is involved in regulating the LE cytoskeleton comes from the fact that expression of dPak-AID transgenes in the LE cells causes DC defects. Phosphotyrosine staining of dPak-AID-expressing embryos reveals “splayed” LE cells lacking phosphotyrosine nodes and a corresponding loss of cell elongation in the D-V axis.

The JNK pathway, a MAPK cascade, operates in the LE cells and is required for the LE expression of various genes participating in DC, including *dpp*, which encodes a member of the TGF- $\beta$  superfamily (reviewed in Glise and Noselli, 1997; Harden, 2002; Knust,

1997; Noselli and Agnes, 1999) (Ricos *et al.*, 1999). The JNK cascade is also required for the integrity of the LE cytoskeleton. Considerable evidence from various systems indicates that the Ste20 group kinases, of which the Paks are a subgrouping, can operate as MAPKKKKs (Dan *et al.*, 2001b). In *Drosophila*, a role for dPak in activation of JNK cascade has not been addressed to date (reviewed in Harden, 2002). There is already a Ste20 group kinase known to function as a MAPKKKK in the JNK cascade during DC. *misshapen (msn)*, encoding a member of the germinal center kinase (GCK) family, a subgrouping of the Ste20 group kinases, is required upstream of JNKKK/*slipper (slpr)*, which in turn activates JNKK/Hep, which in turn activates JNK/Bsk (reviewed in Harden, 2002; Stronach and Perrimon, 2001). In embryos expressing dPak-AID in the LE cells, I still detected LE *dpp* expression. I conclude that dPak is not a major regulator of JNK signaling in the LE during DC. Similarly, *dpp* mRNA staining is normal in embryos deficient for Dcdc42, suggesting that Dcdc42, a likely dPak activator, is not essential for activation of the JNK pathway (Genova *et al.*, 2000). These results indicate that the regulation of the LE cytoskeleton by Dcdc42 and dPak is probably not channeled through the JNK pathway.

## **dPak function during oogenesis**

### **dPak function is required for F-actin filaments driving egg chamber elongation**

The follicular epithelium surrounding the germline cluster in *Drosophila* ovaries provides a genetically tractable system to study the cell biology of patterning and morphogenesis (reviewed in Dobens and Raftery, 2000; Spradling, 1993). A dense system of polarized F-actin filaments accumulates along the basal surface of FCs, perpendicular to the A-P axis of the oocyte (Bateman *et al.*, 2001; Frydman and Spradling, 2001; Gutzeit, 1990). This actin array is postulated to aid in oocyte elongation by acting as a “molecular corset” to restrict growth in the short axis (Gutzeit, 1991). In support of this model, a round egg chamber phenotype has been found in mutants in which disruption of the basal F-actin is obvious (Bateman *et al.*, 2001; Frydman and Spradling, 2001; Gutzeit *et al.*, 1991).

The basal F-actin in the FCs of *dpak<sup>6</sup>/dpak<sup>10</sup>* mutant egg chambers is reduced in quantity and disorganized. This is direct evidence that dPak functions in the organization of F-actin cytoskeleton during oogenesis and contributes to the “molecular corset”.

Preliminary genetic evidence indicates that dPak may function in signaling from the

ECM through integrin to regulate the F-actin cytoskeleton in FCs (del Pozo *et al.*, 1999; Howe, 2001; Takagi *et al.*, 1999). Consistent with the circumferential orientation of F-actin in the basal surface of FCs, laminin, a component of the ECM in the basement membrane, is also oriented perpendicular to A-P axis, and may contribute to the exerting forces for egg chamber elongation (Bateman *et al.*, 2001; Gutzeit, 1990). Both laminin and F-actin are disorganized in *kugel* mutant egg chambers (Gutzeit *et al.*, 1991) and disruption of the ECM through collagenase treatment affects egg chamber morphology (Gutzeit *et al.*, 1991). Furthermore, receptors for ECM components have been implicated in organization of the basal F-actin in the FCs. The  $\alpha_{PS1}$  integrin subunit, encoded by *multiple edematous wings (mew)*, functions together with the  $\beta_{PS}$  integrin subunit, encoded by *myospheroid (mys)*, as a receptor for the laminin  $\alpha$  chain, while the  $\alpha_{PS2}$  subunit encoded by *inflated (if)*, acts with  $\beta_{PS}$  as a receptor for ECM proteins bearing the amino acid motif RGD (reviewed in Brown *et al.*, 2000). *mew*, *mys*, and *if* mutant clones in FCs cause rounded oocytes and disruption of the organization of the basal F-actin cytoskeleton (Bateman *et al.*, 2001). Cell culture studies in a *Drosophila* neuronal cell line indicate that dPak colocalizes with integrin upon laminin stimulation (Takagi *et al.*, 1999). Cell adhesion and membrane targeted Rac1V12 can activate Pak, however, non-adherent Rac1V12-expressing cells fail to stimulate Pak (del Pozo *et al.*, 2000). Further,

the recruiting of Pak to the membrane by the adaptor protein Nck is also mediated by cell adhesion (Howe, 2001). Thus, integrins on the basal surface of FC may accept signals from components of the ECM, such as laminin, then send them to dPak through Rac.

To date, the requirement for the *Drosophila* Rac proteins in the FCs has only been tested using expression of Drac1 transgenes. No effect of Drac1N17 expression on oocyte elongation has been reported, although effects on border cell migration, dorsal appendage morphogenesis and FC morphology have been described (Dobens *et al.*, 2001; Murphy and Montell, 1996; Suzanne *et al.*, 2001). A definitive evaluation of Rac protein function in FC morphogenesis will require the creation of triple mutant FC clones bearing null alleles of the *Drosophila* Rac genes, *drac1*, *drac2* and *mtl* (Hakeda-Suzuki *et al.*, 2002; Ng *et al.*, 2002). Dcdc42 may not function as an activator of dPak in regulation of oocyte elongation as neither egg chambers expressing Dcdc42N17 in FCs nor those containing Dcdc42 mutant FC clones show elongation defects (Dobens *et al.*, 2001; Genova *et al.*, 2000; Murphy and Montell, 1996; Suzanne *et al.*, 2001).

A rounded egg chamber phenotype was seen in egg chambers deficient in the receptor tyrosine phosphatase (RPTP) Dlar, a phosphatase genetically interacting with  $\alpha$  and  $\beta$  integrin subunits (Bateman *et al.*, 2001; Frydman and Spradling, 2001; Fu and Galan,

1999). In flies transheterozygous for *dpak<sup>6</sup>* and *mys*, *Dlar*, or *if* alleles, a low frequency of rounded oocytes were found in *dpak<sup>6</sup>/+;if/+*, but not in the other combinations. These results suggest that dPak functions with integrin in regulation of the basal F-actin cytoskeleton. The lack of rounded eggs in *Dlar/dpak<sup>6</sup>* transheterozygotes does not preclude a role for dPak in Dlar signaling, as even in flies bearing two *Dlar* loss-of-function alleles, the frequency of rounded oocytes can be as little as 14-20% (Bateman *et al.*, 2001; Frydman and Spradling, 2001; Fu and Galan, 1999). In axonal guidance, Dlar genetically interacts with Trio, a GEF for Rac which itself genetically interacts with Rac and dPak (Bateman *et al.*, 2000; Hakeda-Suzuki *et al.*, 2002; Newsome *et al.*, 2000). Female sterile alleles of *trio* are available, thus implicating it in oogenesis (Awasaki *et al.*, 2000). Thus, a potential pathway linking ECM (laminin)→integrin→Dlar→Trio→Rac→dPak is proposed in regulating the F-actin in FCs during oogenesis.

Additional evidence for the involvement of dPak in integrin signaling is the blister defect I found in the adult wings of flies heterozygous for *dpak<sup>6</sup>/dpak<sup>10</sup>*. The elongated columnar cells of the wing pouch undergo profound changes in shape and are folded into an epithelial bilayer whose basal sides are apposed and adhere to each other via integrin-

dependent focal contacts (reviewed in Brown *et al.*, 2000). Loss-of-function mutations in genes encoding integrins  $\alpha_{PS1}$ ,  $\alpha_{PS2}$ , and  $\beta_{PS}$  also have wing blister phenotypes (reviewed in Brown *et al.*, 2000), suggesting a common signaling pathway involving integrin and dPak. Interestingly, *Dcdc42* mutants and flies deficient in the *Dcdc42* effector DACK also show wing blisters (Genova *et al.*, 2000; Sem *et al.*, 2002).

### **dPak function is required for the formation and/or maintenance of the follicular epithelium**

Gaps in the FCs were found in the *dpak<sup>6</sup>/dpak<sup>10</sup>* mutant egg chambers, suggesting dPak function is required for the formation and/or maintenance of the follicular epithelium. The gaps are likely caused by the failure of the FCs to integrate into intact epithelium to cover the germline. A bilayer of FCs accumulated at the posterior end of the FCs over the oocyte in *dpak<sup>6</sup>/dpak<sup>10</sup>* mutant egg chambers indicating a role for dPak in the organization of the follicular epithelium. The mutant egg chamber consistently degenerated by stage 9 and the FCs remained cuboidal, losing the ability to change to columnar at the posterior and to squamous at the anterior. It has been suggested that FCs elongate over the oocyte in order to accommodate the cells migrating to this region of the egg chamber (reviewed

in Spradling, 1993). One possibility was that the posteriorly migrating FCs piled up as a bilayer due to the loss of the cell change to columnar. This idea is supported by the observation that multilayering of the FCs caused by other mutations is limited to those FCs over the oocyte (Genova *et al.*, 2000; Goode *et al.*, 1996a; Lee *et al.*, 1997; Tanentzapf *et al.*, 2000).

Disruption of monolayer organization can also be found in egg chambers lacking Dcdc42 in the posterior pole FCs (Dobens and Raftery, 2000; Genova *et al.*, 2000). As the Cdc42 proteins are well established as activators of Paks (reviewed in Bagrodia and Cerione, 1999; Daniels and Bokoch, 1999; Manser and Lim, 1999), these data suggest that Dcdc42 acts through dPak during FC morphogenesis. Whether the multilayering of FCs results from over proliferation or loss of cell adhesion integrity is not clear. In *dc42* follicle clones, the multiple layers of FCs at the poles of the egg chamber are not caused by cell overproliferation, but rather loss of the ability to maintain a single layered epithelium (Genova *et al.*, 2000). However, a similar phenotype results from extra cell divisions in somatic clones lacking  $\alpha$ -spectrin (Lee *et al.*, 1997).  $\alpha$ -spectrin is generally localized at the apical-lateral surface of the FCs. It heterodimerizes with the 265kDa  $\beta$ -subunit at the lateral domain, and at the apical surface it heterodimerizes with the 430kDa  $\beta_{\text{Heavy}}$  ( $\beta_{\text{H}}$ )-



subunit. It is the  $\alpha$ -spectrin binding the apical  $\beta_H$ -subunit that controls the cell monolayer integrity in the posterior subgroup of FCs (Lee *et al.*, 1997). It will be interesting to see if there is a link between apically localized molecules, such as  $\alpha$ -spectrin and basally localized dPak in organizing epithelial polarity.

The follicular epithelium surrounding the *Drosophila* egg chamber is a unique polarization system with three surface domains: basal, apical and lateral (reviewed in Tanentzapf *et al.*, 2000). Transmembrane proteins that specifically localize to one of the three surface domains mediate cell polarity and the subsequent reorganization of the cytoskeleton (reviewed in Tanentzapf *et al.*, 2000). Lateral cadherin-catenin complex mediated adhesion is critical for the assembly of the lateral membrane domain (Peifer *et al.*, 1993). Mutations in a number of apically distributed proteins, including Crumbs (Crb), epidermal growth factor receptor (Egfr) and Notch have similar phenotypes with discontinuities and multilayering of the FCs (Tanentzapf *et al.*, 2000). *Crumbs*, encoding a transmembrane protein, also participates in the Drac1 signaling in the regulation of amnioserosa morphogenesis (Harden *et al.*, 2002). The accumulation of Crb and Discs Lost (Dlt), a cytoplasmic PDZ domain protein interacting with the cytoplasmic tail of Crb, requires the contact of FC with the germ line, since Crb and Dlt are missing from

FCs in agametic follicles (Tanentzapf *et al.*, 2000). Interestingly, similar phenotypes have been discovered in mutations in germ line-expressed secreted proteins Brainiac (Brn) and Egghead (Egh) (Goode *et al.*, 1996a; Goode *et al.*, 1996b; Goode *et al.*, 1992; Tanentzapf *et al.*, 2000). Interaction of the apical surface of the FCs with the germ line is critical for the formation and maintenance of the FCs (reviewed in Dobens and Raftery, 2000). Consistent with this, mutation in *gurken*, a germline transforming growth factor- $\alpha$ -like (TGF- $\alpha$ ) ligand for EGF receptor (Egfr) on FCs causes a similar phenotype (Goode *et al.*, 1996b). Thus the stability of the follicular epithelium is controlled by several molecular pathways that act in parallel or may overlap in function, including Egfr-TGF pathway, Notch pathway and a number of neurogenic loci, such as *brn* and *egh*.

The basal surface of the FC contacts the basement membrane, a layer consisting of extracellular matrix (ECM) components, such as laminin, surrounding the egg chamber underneath the muscle sheath (Bateman *et al.*, 2001; Gutzeit, 1990). Contact of FCs with the basement membrane contributes to the initial polarization of these cells which is further refined by contact with the germline at the apical surface (Tanentzapf *et al.*, 2000). dPak, which is localized to the basal end of follicle cells, may contribute to polarization through communication with the basement membrane.

### **dPak function may be required for the germ line stem cell divisions**

A side-by-side egg chamber fusion phenotype was seen in *dpak<sup>6</sup>/dpak<sup>10</sup>* mutants, a novel phenotype that has never been described before. In the germarium, germ cells divide in region 1 to form a 16-cell cyst, which flattens when moving posteriorly to region 2A. One germ cell is selected to become the oocyte, while the remaining 15 germ cells become nurse cells. In region 2B, FCs proliferate and migrate to contact the posterior side of the flattened cyst and further envelop it to form a stage 1 egg chamber (reviewed in Deng and Lin, 2001; Lin and Spradling, 1993). FCs that do not contact the germ cyst interleave to form stalk cells that connect to adjacent egg chambers at the pole cells (reviewed in Dobens and Raftery, 2000). The formation of the egg chamber involves three interdependent processes: 1) FC proliferation from stem cells, 2) FC migration and envelopment of a germ-line cyst, and 3) FC differentiation to create the stalk cells (reviewed in Deng and Lin, 2001; Lin and Spradling, 1993). Reduced FC proliferation results in fused egg chambers and gaps in the FC epithelium. Assembly of discrete egg chambers requires that FCs recognize individual germline cysts and migrate between them (reviewed in Dobens and Raftery, 2000; Spradling, 1993). Encapsulation of the germline cyst involves both the envelopment of the cyst and the separation of egg

chambers by differentiation of stalk cells, in which processes Hh and Notch signaling is required (reviewed in Dobens and Raftery, 2000; Spradling, 1993). An end-to-end fusion phenotype has been discovered in *dcdc42* mutant egg chambers, which reflects a lack of stalk cell differentiation (Genova *et al.*, 2000). Fused egg chambers generally arise from mutations that cause the early absence of stalk cell precursors (Forbes *et al.*, 1996; Ruohola *et al.*, 1991) or the later degeneration of the stalks (Edwards and Kiehart, 1996). But in *dpak<sup>6</sup>/dpak<sup>10</sup>* mutant egg chambers, an unusual side-by-side egg chamber fusion was found and the stalk cells linking adjacent egg chambers were intact, suggesting that a defect of stalk cells was not the cause.

Two to three germline stem cells (GSC) reside at the germarium and contact with a cluster of apical somatic cells called the terminal filament (TF) (reviewed in Deng and Lin, 2001; Lin and Spradling, 1993). A self-renewing germline stem cell division mechanism exists, by which asymmetric divisions produce a daughter GSC that remains in contact with TF and a cystoblast that is one cell away from the TF (reviewed in Deng and Lin, 2001; Lin and Spradling, 1993). GSC divisions are controlled by both extrinsic signals from the apical somatic cells and intrinsic factors that act within the germline stem cells. One possibility for the *dpak<sup>6</sup>/dpak<sup>10</sup>* mutant egg chamber fusion phenotype is

that two daughter cystoblasts derive from the primitive GSC instead of one GSC and one cystoblast and lose the ability to further differentiate, which could have been caused by the disorientation of the spectrum, a cytoplasmic structure controlling the asymmetrical differential segregation and GSC division (reviewed in Deng and Lin, 2001; Lin and Spradling, 1995). However, this situation seems unlikely because at least 6-7 egg chambers were found in a single ovariole. If the GSC divided to produce two cystoblasts, there could only be two egg chambers derived from the self-differentiation of each of the two to three GSCs in the germarium. I conclude that the GSC still remains active and in contact with the TF, and that the phenotype perhaps might be due to an extra round of division in the GSC or cystoblast.

Laser ablation studies show that GSC remains active without somatic cells, and ablation of the somatic cells in the TF results in increased rate of oogenesis, suggesting a negative regulation of the soma on the GSC division (Lin and Spradling, 1993). Although I couldn't determine whether dPak functions in the germ line, antibody staining showed that dPak localized in the somatic cells of the gemarium. The side-by-side fusion phenotype suggested an extra round of complete division happened in the *dpak<sup>6</sup>/dpak<sup>10</sup>* cystoblast resulting in two daughter cystoblasts which then went through the normal four

rounds of incomplete cell division to produce the 16 germ cell cluster (reviewed in Spradling, 1993; Spradling *et al.*, 1997; Verheyen and Cooley, 1994). Each chamber in a fused pair had exactly 16 nuclei and the oocyte determination was not affected. Another possible explanation would be that two GSCs start to differentiate at the same time in the germarium, giving two cystoblasts side-by-side, which were then encapsulated by the FCs. Both explanations suggest that lack of dPak in the soma could lead to the lack of the inhibitory signals flowing from soma to germ line dividing cells. However, there are no clear studies showing which molecules are the negative regulators. The laser ablation study only showed that the number of egg chambers increases by 40% without the control of TF, but did not mention a defect similar to our side-by-side fusion (Lin and Spradling, 1993). Quite a number of molecules exert functions either in the TF or in the GSC to regulate the asymmetric division (Deng and Lin, 1997; Lin and Spradling, 1993; Lin and Spradling, 1995; Lin *et al.*, 1994). For example, the genes *piwi*, *fs(1)Yb* (*Yb*), *hedghog* (*hh*), send signals from TF to GSC, but all seem to have a positive regulatory function in division. *Piwi* mutant ovarioles contain only two to three egg chambers. *Yb* not only promotes GSC differentiation, but also controls the proliferation of the somatic stem cells. *Dpp* also seems to be a positive regulator, since overexpression of *dpp* induces hyperproliferation of GSC without differentiation, and mutants in the *Dpp* receptors,

thick vein (*tkv*) and *punt*, reduce the GSC division rate (Deng and Lin, 1997; Lin and Spradling, 1993; Lin and Spradling, 1995; Lin *et al.*, 1994). Thus, how dPak loss causes overproliferation of cystoblasts and impairs the formation of the FC requires further study.

**The truncated dPak protein encoded by the *dpak<sup>6</sup>* allele failed to localize to the cell periphery in the egg chamber and embryos**

In the early stages of oogenesis, dPak was diffusely distributed through the base of the FCs and it was weakly localized to the cell periphery. At later stages of oogenesis, the peripheral distribution of dPak was stronger, especially in the columnar FCs over the oocyte, suggesting a dPak function resulting from translocation to the membrane. The *dpak<sup>6</sup>* allele is predicted to encode a truncated protein lacking the kinase domain due to a stop codon in the CRIB motif, whereas the *dpak<sup>10</sup>* allele produces no detectable protein product (Hing *et al.*, 1999 and Hing, personal communication). In *dpak<sup>6</sup>/dpak<sup>10</sup>* mutant egg chambers, dPak was still basally localized but no longer peripheral. Similarly, in the *dpak<sup>6</sup>* mutant embryos, the truncated dPak was no longer localized to the cell membrane, but diffuse in the cytosol.

At least two proline rich sequences (PXXP) exist in the N-terminal regulatory region of Paks, which contribute to its localization to the cell periphery. One PXXP is located upstream of the CRIB motif and binds to the SH3 domain in the adaptor protein Nck, which can link Pak to transmembrane receptors (Bokoch *et al.*, 1996; Howe, 2001). A *Drosophila* homolog of Nck, Dock, recruits dPak to the plasma membrane in photoreceptor axon guidance (Hing *et al.*, 1999; Newsome *et al.*, 2000). Another PXXP downstream of CRIB binds to the SH3 in Pix/Cool, a GEF that leads to Pak 1 localization to focal adhesions/focal complexes at the cell periphery (Manser *et al.*, 1998; Obermeier *et al.*, 1998). Flies mutant for a *Drosophila* homolog of Pix, dPix, exhibit disruption of dPak localization at the neuromuscular junction (Parnas *et al.*, 2001). Pak-Pix membrane targeting is mediated by binding of Pix to the focal adhesion protein Paxillin (Turner *et al.*, 1999). In *dpak<sup>6</sup>/dpak<sup>10</sup>* mutant, the first PXXP region still exists in the truncated protein encoded by the *dpak<sup>6</sup>* allele, but the second PXXP which binds to dPix is missing (Hing *et al.*, 1999). Thus, the lack of peripheral localization of the truncated dPak protein may be due to an inability to bind dPix.

Pak localization to the cell membrane has important functions, as an engineered membrane-tethered dPak has a dominant gain-of-function effect in R-cell axon guidance,



rescuing defects in *dPak* mutants (Hing *et al.*, 1999). Relocalization to the membrane places Paks in close proximity with membrane-associated activating factors, such as Rac/Cdc42. Membrane association of Rac is required for Pak activation and this membrane-localized Rac can restore Pak activation in non-adherent cells (del Pozo *et al.*, 2000). Membrane localization of Paks also places them in an appropriate spatial closeness to dynamic cytoskeletal structures, such as lamellipodia, filopodia and membrane ruffles. Pak mutant proteins unable to bind Nck are severely impaired in their ability to promote cytoskeletal organization (Sells *et al.*, 1997). The translocation of Paks to cell membrane also links Paks to the ECM through receptors such as the integrins, sending signals to and fro, helping establish cell polarity (Takagi *et al.*, 1999).

### **Pak-I kinase activity may not be required during oogenesis**

*dPak* consists of a number of functional domains, including N-terminal regulatory domain and C-terminal kinase domain. A model for Pak activation has been proposed in which, in the inactive state, an AID adjacent to the CRIB motif inhibits Pak kinase activity, while upon binding of GTP-bound Dcdc42/Drac1 to the CRIB domain, the C-terminal kinase domain is unmasked from AID and becomes active (Frost *et al.*, 1998;

Lei *et al.*, 2000; Tu and Wigler, 1999; Zenke *et al.*, 1999; Zhao *et al.*, 1998). However, cytoskeletal changes are partly independent of Pak1's kinase activity and the induction of neurite outgrowth is also independent of Pak's kinase activity, but relies on both N- and C- portions of the protein (Sells *et al.*, 1997; Daniels *et al.*, 1998). Thus, Pak function can be divided into kinase-dependent and kinase-independent mechanisms, with the latter indicating an adaptor function for Pak (reviewed in Daniels and Bokoch, 1999). Kinase-negative or Rac/Cdc42 binding-deficient Pak mutants still induce cell spreading and membrane ruffling in mammalian cells, presumably acting upstream of Rac (Obermeier *et al.*, 1998). Mutant versions of Rac and Cdc42 which are no longer capable of binding Pak can still induce cytoskeletal changes (Kim *et al.*, 2002; Lamarche *et al.*, 1996; Owen *et al.*, 2000). However, as described below, it is clear that at least some of Pak's cytoskeletal effects are due to its phosphorylation of proteins regulating the cytoskeleton. In my work, *dpak<sup>6</sup>/dpak<sup>10</sup>* mutant egg chambers displayed severe damage of the F-actin organization in the FCs, but I didn't find this abnormality in egg chambers in which kinase-inhibiting *dPak-AID* transgenes were expressed by FC drivers (Murphy and Montell, 1996). This result suggests that the kinase activity of dPak might not have a major function in the regulation of actin cytoskeleton in FCs. When I introduced a full length dPak transgene into the *dpak<sup>6</sup>/dpak<sup>10</sup>* mutant egg chamber by follicle drivers,

partial rescue of the mutation was found. I would like to repeat this experiment with a kinase-dead *dpak* transgene to see if any rescue can be achieved.

### **Parallels between dPak function at the leading edge cells during dorsal closure and in the follicle cells during oogenesis**

The morphogenetic changes during oogenesis and DC provide excellent model systems for studying cytoskeletal regulation, cell polarity establishment and cell signaling. During DC, the two lateral epidermal sheets driven by the actin-myosin apparatus migrate over the squamous amnioserosa to meet at the dorsal midline (Edwards and Kiehart, 1996; Young *et al.*, 1993) (reviewed in Harden, 2002). During oogenesis, FCs at the nurse cell-oocyte boundary migrate centripetally, to close the anterior end of the oocyte covered by the squamous nurse cell FCs (reviewed in Spradling, 1993; Verheyen and Cooley, 1994). Thus, the spreading of the lateral epithelia during DC to cover the amnioserosa and the spreading of anterior FCs over the nurse cells during oogenesis bear several similarities. Both involve actin-myosin driven changes in cell shape coordinated with a spreading of epithelia to enclose, respectively, the dorsal side of the embryo and the anterior of the oocyte. Both involve the JNK cascade and dPak (Harden, 2002; Suzanne *et al.*, 2001).

In this thesis I have described the participation of dPak in the morphogenesis of two epithelia: the LE of the epidermis during DC and the FCs during oogenesis. There are a number of parallels between these two processes. In both cases there is deposition of dPak protein at one end of the cell (basal in the FCs, dorsal in the LE) that co-localizes with a polarized accumulation of F-actin. In both cases this polarized F-actin is proposed to drive cell constriction, perpendicular to the A-P axis in the case of the FCs, and along the A-P axis in the case of LE. Loss of dPak function in the FCs is accompanied by disruption of the basal cytoskeleton and failure of cells to constrict properly perpendicular to the A-P axis. Impairment of the function of dPak (and possibly Pak3) through expression of *UAS-dPak-AID* in the LE cells is accompanied by disruption of the dorsally located LE cytoskeleton and failure of the LE cells to constrict properly in the A-P direction. It will be of interest in future to see what further parallels emerge between these two dPak-dependent processes, for example how many of the proteins known to have roles in the LE cells during DC (reviewed in Harden, 2002) will also participate in the morphogenesis of the FCs.

**A germ line clone would provide information of dPak function either in oogenesis or embryogenesis**

As mentioned previously, although *dpak* loss-of-function mutant embryos successfully complete embryogenesis, maternally deposited dPak and/or other Pak family kinases may substitute for embryonic functions of zygotic dPak. The creation of a dPak germ line clone could address this question by using the dominant female sterile (DFS) technique (Chou and Perrimon, 1992). Due to the close proximity between dPak and FRT on the chromosome, I wasn't successful in recombining the dPak alleles and FRT onto the same chromosome despite generating numerous lines.

However, it is not clear whether a dPak germ line clone would be sterile or would produce embryos. My dPak antibody staining of wild type egg chambers showed that there was no dPak accumulation in the germ line including the nurse cells and oocyte. Considering this, a germ line clone may not affect oogenesis and eggs may be produced. However, an unpublished study indicated that a *dpak* mutant germ-line clone was sterile and ring canal defects were found (S. Jackson, personal communication).

### **Routes for regulation of the cytoskeleton by Pak kinases**

Growing evidence indicates that Paks can regulate the actin cytoskeleton by routes more direct than signaling to the nucleus through MAPK cascades. Pak1 co-localizes with peripheral actin structures and focal adhesions/focal complexes in cultured mammalian cells (Dharmawardhane *et al.*, 1997; Manser *et al.*, 1997) and dPak accumulates in the LE cells during DC in *Drosophila* (Harden *et al.*, 1996; Harden *et al.*, 1999). In mammalian cells, two opposing effects on the actin cytoskeleton have been ascribed to Paks: the formation and the dissolution of the actin-based structures (Daniels *et al.*, 1998; Manser *et al.*, 1997; Sells *et al.*, 1997). Activated Pak1 can induce both filopodia and membrane ruffles in Swiss3T3 cells and cause neurite outgrowth in PC12 cells, similar to effects of constitutively active Cdc42 and Rac (Daniels *et al.*, 1998; Sells *et al.*, 1997). On the other hand, overexpression of constitutively active Pak1 in Hela cells results in the loss of stress fibers and a general retraction of the cell periphery, indicating that Pak can function in the dissolution of actin polymers (Manser *et al.*, 1997).

Two proteins have been described which participate in the positive regulation of the actin cytoskeleton by Pak. LIM-domain-containing kinase (LIM-kinase) has been identified as

a downstream effector for Pak1 in mediating actin cytoskeletal responses to Rac (Edwards *et al.*, 1999) (reviewed in Chernoff, 1999). Pak1 phosphorylates LIM-kinase at threonine residue 508 within its activation loop, and increases LIM-kinase-mediated phosphorylation of cofilin. Cofilin is a protein involved in actin depolymerization that can be inactivated by phosphorylation (Edwards *et al.*, 1999) (reviewed in Chernoff, 1999). Thus, Rac/Cdc42→Pak1→LIM-kinase→cofilin pathway causes the *decreased* depolymerization of actin and stabilizes actin structures (Fig. 20). Filamin (FLNa) crosslinks Pak1 to actin filaments, and is required for Pak-induced membrane ruffling (Vadlamudi *et al.*, 2002). The interaction between FLNa and Pak 1 is bidirectional in that not only does Pak1 phosphorylate FLNa, but FLNa promotes Pak1 kinase activity by binding to the Pak1 CRIB domain.

A protein that appears to contribute to the dissolution of F-actin stress fibres by Pak1 is myosin light chain kinase (MLCK) (Sanders *et al.*, 1999). Pak1 phosphorylates MLCK, decreasing its ability to phosphorylate MLC. Reduced phosphorylation of MLC causes reduced myosin contractility, which subsequently results in impairment of stress fiber formation.

Fig. 20 Schematic representation of regulation of actin by Pak1. Pak1 phosphorylates LIM-kinase and thus increases LIM-kinase-mediated phosphorylation of cofilin. The phosphorylation of cofilin inactivates its activity and inhibits its ability to depolymerize actin, leading to actin accumulation. Pak1 also downregulates MLCK by phosphorylation and thus decreases its ability to phosphorylate MLC, contributing to the loss of stress fibers. Another substrate for Pak1 is filamin, which directly binds to actin and crosslinks to crosslinking of filaments, and contributes to crosslinking of and which also in turn regulates Pak1 by binding to its CRIB.



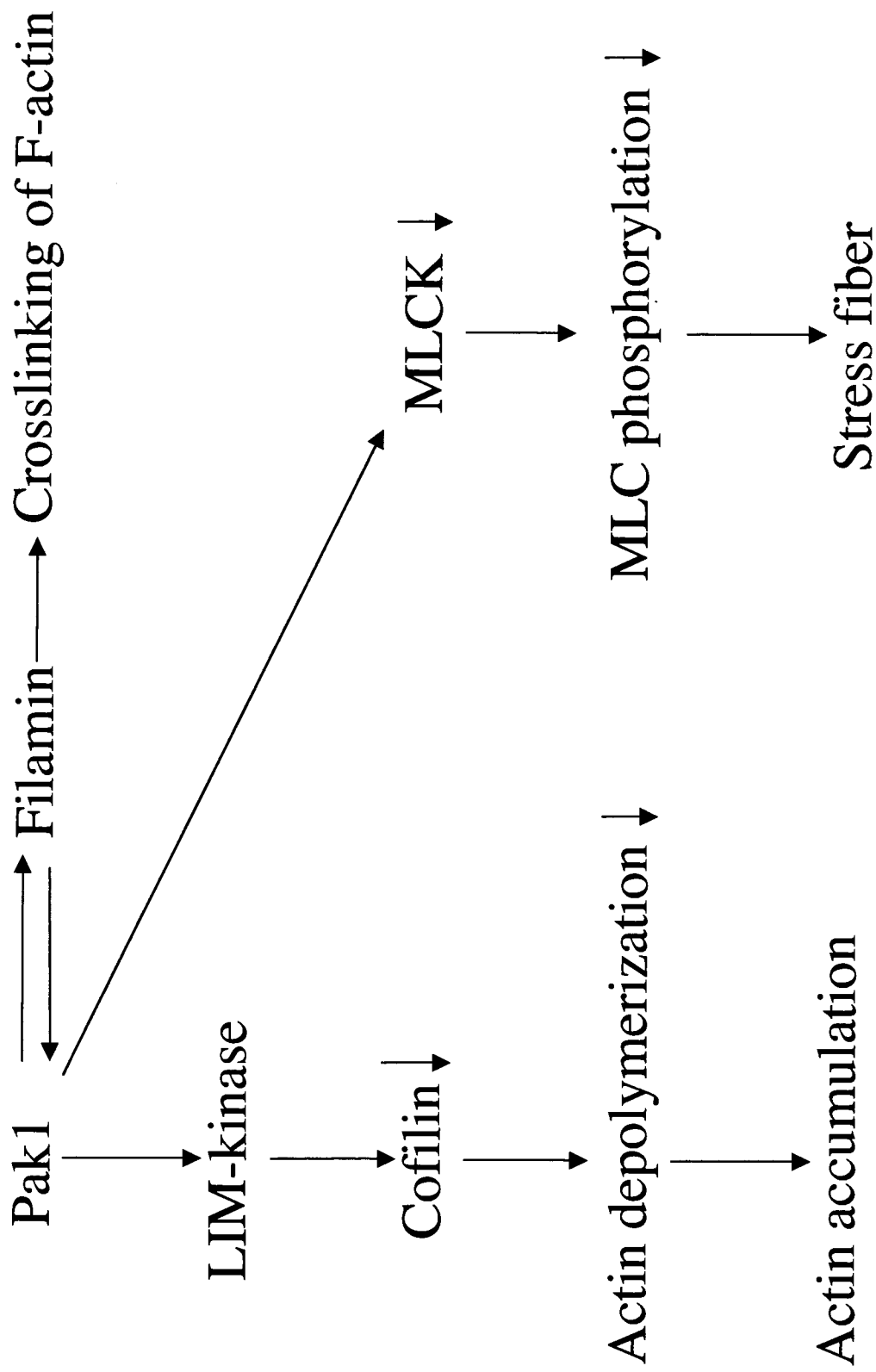


Fig. 20 Regulation of F-actin by Pak1

My experiments provided three pieces of evidence that Pak can contribute to the establishment of the cytoskeleton during *Drosophila* development. First, when Dcdc42V12 was expressed in a *dpak*<sup>6</sup> mutant background, the elevation of phosphotyrosine along the LE was no longer seen, suggesting dPak functions downstream of Dcdc42 in setting up the cytoskeleton. Second, in embryos expressing dPak-AID via a LE-specific driver, phosphotyrosine was missing in “splayed” LE cells that also failed to elongate in the dorsal-ventral direction. Third, in *dpak*<sup>6</sup>/*dpak*<sup>10</sup> mutant egg chambers, the basal F-actin in FCs ranged from almost nonexistent to reduced in quantity. It will be of interest to determine if dPak’s regulation of the actin cytoskeleton in these processes involves any of the effectors described in the mammalian system.

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