Ensconsin, a Par-1 regulated microtubule associated protein, regulates kinesin dependent transport

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Ensconsin, a Par-1 regulated microtubule associated protein, regulates kinesin dependent transport

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僅此獻給

不辭辛苦的母親和在天之靈的父親

Summary

Maternal proteins and mRNA contribute to both oocyte and embryo development. I performed a genetic screen to identify genes with maternal function in *Drosophila*. From this screen, I isolated *PB4170*, which affects *CG14998* (*ensconsin*). The human homologue of this protein, E-MAP-115/Ensconsin, is known to be a microtubule binding protein that interacts dynamically with microtubules. However, its molecular function is poorly understood. From *ensconsin* mutant analysis in *Drosophila*, I have found that this gene is specifically required for microtubule-dependent polarity in the oocyte. My results suggest that Ensconsin does not directly affect the stability or orientation of microtubules, but acts through Kinesin, a plus end directed motor that moves cargos along microtubules.

I could also demonstrate that Ensconsin is a target of the Par-1 kinase, which has been shown to be required for the establishment of oocyte polarity in *Drosophila*. In mammals, the Par-1 homolog MARK destabilises microtubules through phosphorylation of microtubule associated proteins (MAPs).In *Drosophila,* Par-1 directly affects microtubule stability via unkown MAPs, as the MAPs identified so far do not show any polarity defects in the oocyte. As *ensconsin* mutants do show an oocyte polarity phenotype, this strongly suggests that in *Drosophila* Par-1 kinase controls oocyte polarity also through the regulation of Kinesin-based transport by phosphorylation of Ensconsin.

Zusammenfassung

Maternale Transkripte und Proteine steuern zur Entwicklung der Eizelle und des Embryos bei. Um Gene mit einer maternalen Funktion in Drosophila zu identifizieren führte ich einen genetischen Screen durch. Dabei isolierte ich PB4170, eine Mutante für das Gen CG14998 (ensconsin). Es ist bekannt, dass das menschliche Homolog dieses Gens, E-MAP-115/Ensconsin, Mikrotubuli binden und dynamisch mit ihnen interagieren kann. Die genaue Funktion von Esconsin ist jedoch nicht geklärt. Durch die Analyse der ensconsin Mutanten fand ich heraus, dass Ensconsin spezifisch für die Polarität der Oozyte, welche von den Mikrotubuli abhängig ist, benötigt wird. Meine Resultate deuten darauf hin, dass Ensconsin nicht direkt die Stabilität oder die Orientierung der Mikrotubuli beeinflusst, sondern spezifisch für die Funktion von Kinesin, einem Motorprotein welches sich den Mikrotubuli entlang bewegt, gebraucht wird.

Im weiteren konnte ich zeigen, dass Ensconsin durch die Par-1 Kinase, einem Enzym, welches für die Polarität der Oozyte und der Follikelzellen benötigt wird, phosphoryliert wird. In Säugetieren destabilisert das Par-1 homolog MARK Mikrotubuli durch Phosphorylierung von Mikrotubuli-Bindungsproteine (MAPs). Par-1 beeinflusst auch in Drosophila die Stabilität der Mikrotubuli, doch ist in den Mutanten der bisher bekannten MAPs die Polarität der Oozyte normal. Da in ensconsin Mutanten die Polarität der Oozyte beeinträchtigt ist, deuten meine Resultate darauf hin, dass die Par-1 Kinase diese Polarität durch die Regulation von Kinesin-basiertem Transport über die Phosphorylierung von Ensconsin kontrolliert.

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1 INTRODUCTION

1.1 Cell polarity

Multicellular organisms are composed of different types of tissues which are derived from a single fertilized egg. How does a single cell develop into a complex organism? Embryonic cells and stem cells use their polarity to generate different cell types by asymmetric cell division. Furthermore, differentiated cells also perform specific functions by using their polarity. For example, fibroblast cells form an actin-rich leading edge which aids in their migration. Neuronal cells form distinct axonal and dendritic compartments which are important for directional signaling. And epithelial cells have apical-basolateral domains necessary for maintaining tissue impermeability. (Gonzalez-Reyes, 2003; Siegrist and Doe, 2005; Spradling et al., 2001; Suzuki and Ohno, 2006)

What is cell polarity? Cell polarity has been studied in organisms ranging from bacteria, yeast, worms, and flies to mammals. Cell polarity is the asymmetric distribution of proteins that generates an asymmetry of cellular function. One way that asymmetric protein distribution is accomplished is by asymmetric mRNA localization. The factors involved in asymmetric localization of mRNA from yeast to *Drosophila* do not belong to one common family. Even though diverse molecular mechanisms can control mRNA localization, the most common method is the transport of mRNAs along cytoskeleton tracks. In this study, I will focus on the *Drosophila* oocyte, a relatively large cell with asymmetric mRNA localization (*oskar*, *gurken* and *bicoid*). It provides a good system for investigating cell polarity formation (Jan and Jan, 2001; Johnstone and Lasko, 2001; Nusslein-Volhard and Roth, 1989; Riechmann and Ephrussi, 2001; St Johnston, 2005).

1.2 *Drosophila* early oogenesis

Drosophila females have one pair of ovaries. Each ovary consists of 16-20 ovarioles, which can be subdivided into the anterior germarium and posterior vitellarium. The egg chamber is formed in the anterior germarium and matures in the posterior vitellarium (Figure 1). Thirteen different stages of egg chambers (stages 2-14) can be found in the vitellarium. The functional unit of oogenesis is the egg chamber which contains 15 nurse cells and an oocyte. The germ cells are surrounded by a monolayer of follicle cells (Figure 1). The oocyte and nurse cells are interconnected via cytoplasmic bridges called ring canals.

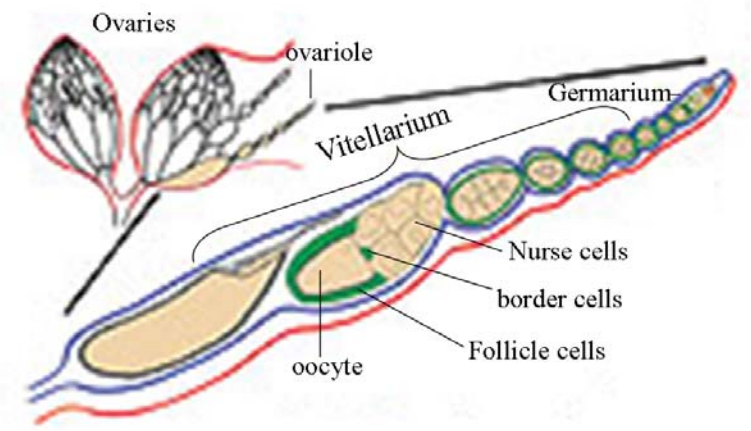

Figure 1. *Drosophila* ovary
The *Drosophila* ovary (top left) is surrounded by a thin peritoneal sheath (red). Each ovariole (middle) is surrounded by a layer of muscle (blue). Egg chambers arise in the germarium and mature in the vitellarium. Each egg chamber contains 15 nurse cells and one oocyte. The germ cells are surrounded by a monolayer of follicle cells (green). Adapted and edited from (Frydman et al., 2006)

1.2.1 The germarium development

The germarium is divided into four stages: stage 1, 2a, 2b and 3. The stage 1 area of the germarium contains two germ cells, associated with a stack of around 10 terminal filament cells. Terminal filament cells contribute to ovariole development and support stem cell activity (Figure 2) (King et al., 2001; Lin and Spradling, 1993; Sahut-Barnola et al., 1995). The germ line stem cell divides asymmetrically, forming a new stem cell and a cystoblast. The new stem cell remains attached to the neighboring somatic cap cells, at

the anterior (Gonzalez-Reyes, 2003; Spradling et al., 2001). The cystoblast undergoes four rounds of division with incomplete cytokinesis to produce 16 germ cells. During these divisions the fusome, a vesicle-rich organelle, becomes polarized, with one daughter cell getting more fusome material than the other during cell division. This asymmetric division is due to the anchoring of one pole of each spindle to the fusome at every division. This asymmetric division ensures that the cyst consists of two cells with four ring canals, two cells with three ring canals, four cells with two canals, and eight cells with one ring canals. When 16 germ cells are formed, the oogenesis enters region 2a of germarium (Lin et al., 1994).

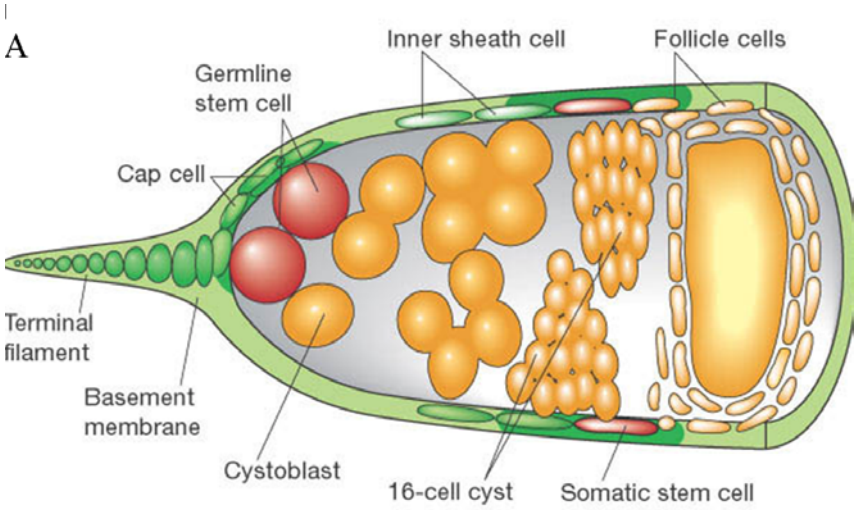

Figure 2. The structure of *Drosophila* germarium
The location of germline stem cells (red cells on left) and somatic stem cells (red cells on top and bottom). The terminal filament and cap cells (green) retain stem cell activity. The germ cells and dividing germ cells (yellow) move away from the anterior tip. At the end of germarium the somatic cells divide and give rise to the follicle cells which then migrate inwards, surrounding the germ cells. Adapted from (Spradling et al., 2001).

1.2.2 Oocyte specification

At stage 2a, the two cells with four ring canals are selected as pre-oocytes. At stage 2b, one of them becomes the oocyte, with the other cell becoming a nurse cell. The determination of the oocyte can be followed with several markers. (1) At stage 2a, the oocyte specific proteins Bicaudal (BicD), Orb, Barentsz (Btz), Cup, and Egalitarian (Egl) and mRNAs *oskar, BicD, and orb* become enriched in the two pre-oocytes. At the end of stage 2a, these proteins and mRNAs are concentrated in the oocyte (Suter et al., 1989) (Ephrussi et al., 1991; Keyes and Spradling, 1997; Lantz et al., 1994; van Eeden et al.,

2001; Wharton and Struhl, 1989). (2) Initially microtubules are distributed through the 16 germ cells. At the end of stage 2a, their minus ends gradually localize to the selected oocyte (Bolivar et al., 2001; Grieder et al., 2000). (3) By the end of stage 2a, after the last mitotic division, the centrosomes appear to become inactived and move into the oocyte (Cox and Spradling, 2003) (Grieder et al., 2000); (Bolivar et al., 2001). In region 2b, the germ cells change shape, forming a one cell-thick disc. At the same time, somatic follicle cells migrate inward and envelop the germ cells. As the germ cells reach stage 3, the mitochondria, centrosomes, Golgi vesicles, proteins and mRNAs accumulate at the anterior of the oocyte. The germ cells become rounded and form a sphere, with the oocyte always staying at the posterior pole. The germ cells leave the germarium and enter the vitellarium. The oocyte becomes further polarized, as proteins, mRNAs, centrosomes and a subset of the mitochondria move to the posterior cortex. The oocyte DNA becomes highly condensed to form a structure called the karyosome, whereas the nurse cells become polyploid (Dej and Spradling, 1999; Huynh et al., 2001).

1.2.3 Microtubules are involved in the oocyte specification

Microtubules are essential for the specification of the oocyte. Treatment of ovaries with the microtubule depolymerizing drug, colchicine, results in failure of oocyte specification, and instead 16 nurse cells are seen (Koch and Spitzer, 1983). Furthermore, the oocyte-specific proteins and mRNAs are not asymmetrically localized within germ cells (Theurkauf et al., 1993). Mutations in the microtubule minus-end directed motor, Dynein heavy chain (Dhc), or in its associated regulator, Lissencephaly (Lis1) affect the formation of the fusome. Germ cells mutant for these genes show fewer than 16 germ cells and sometimes lack oocytes (Bolivar et al., 2001; Liu et al., 1999; McGrail and Hays, 1997). This suggests that oocyte-specific proteins and mRNAs are transported using microtubule network.

1.2.4 Microtubules in early stages of the egg chamber

After the egg chamber leaves the germarium, it enters the vitellarium. The vitellarium is divided into two stages; the first 6 stages are called the previtellogenic stage and the later stages vitellogenesis. During the pre-vitellogenic stages, the germ cells grow at almost the

same rate. After the pre-vitellogenic stage, vitellogenesis begins and oocyte grows fast, causing it to increase in size with respect to the nurse cells. In the germarium and the pre-vitellogenic stages of the vitellarium, the microtubule minus-ends are enriched in the posterior part of the oocyte and microtubules extend their plus-ends through the ring canals into the nurse cells (Figure 3A). The oocyte nucleus is largely transcriptionally inactive throughout oogenesis, as it is arrested in meiotic prophase. At this time, the nurse cell nuclei start to endo-replicate, becoming highly polyploid (Spradling, 1993). Most of the RNA and proteins, including *grk*, *oskar* and *bicoid* mRNA, which are required for oogenesis and embryogenesis are synthesized in the nurse cells and transported into the oocyte. mRNA and proteins produced in the nurse cells are transported to oocyte on the microtubule network (Grieder et al., 2000; Pokrywka and Stephenson, 1995; Theurkauf et al., 1992).

1.3 Mid-oogenesis

1.3.1 Establishment of anterior-posterior axis of the oocyte

Establishment of the anterior-posterior (A-P) axis of the oocyte is mediated by *gurken*, which encodes a *Drosophila* TGF-α homolog, an EGF-receptor ligand. Its expression is associated with the oocyte nucleus (Nilson and Schupbach, 1999). In previtellogenic stage egg chambers, the microtubules minus ends are enriched in the posterior pole of the oocyte, and the oocyte nucleus also localize at posterior pole of the oocyte. mRNA and proteins which are produced in the nurse cells are transported along microtubules into the transcriptionally inactive oocyte (Figure 3A) (Spradling, 1993; Theurkauf et al., 1992). The first wave of Gurken signal is that Gurken produces from perinuclear region at posterior pole of oocyte and the signal is sent to the terminal follicle cells, specifying posterior follicle cells fate (stage 6) (Figure 3B) (Gonzalez-Reyes et al., 1995; Gonzalez-Reyes and St Johnston, 1998; Roth et al., 1995). Then an unknown signal is sent from the posterior follicle cells to the oocyte resulting in the repolarization of oocyte microtubule cytoskeleton. In response to this reverse signal, the microtubules minus-ends at the posterior of the oocyte disassemble and microtubules nucleate from the anterior and lateral cortex of the oocyte (Figure 3C) (Gonzalez-Reyes et al., 1995; Roth et al., 1995). This reorganization of the microtubule network is necessary for the oocyte nucleus to migrate to an anterior position (Figure 3C).

1.3.2 Patterning of the dorsal-ventral axis

The second wave of Grk signaling occurs from the anterior-dorsal corner of the oocyte and controls dorsal-ventral (D-V) patterning. First, Gurken and Dpp specify the dorsal follicle cell fate, which will form structures such as the dorsal appendages (Figure 3E, 4) (Peri and Roth, 2000). Furthermore, Gurken and PVF1 have been identified as guidance cues for border cells. Border cells, a group of 6-8 follicle cells, delaminate from the anterior follicle cells and migrate through the nurse cells to the oocyte. When they reach to the oocyte, they migrate toward to the dorsal part of the oocyte and then form a micropyle, a hole for sperm entry at the anterior end of the egg. Gurken and PVF1 signal through their tyrosine kinase receptors (RTKs), EGFR and PVR, respectively.

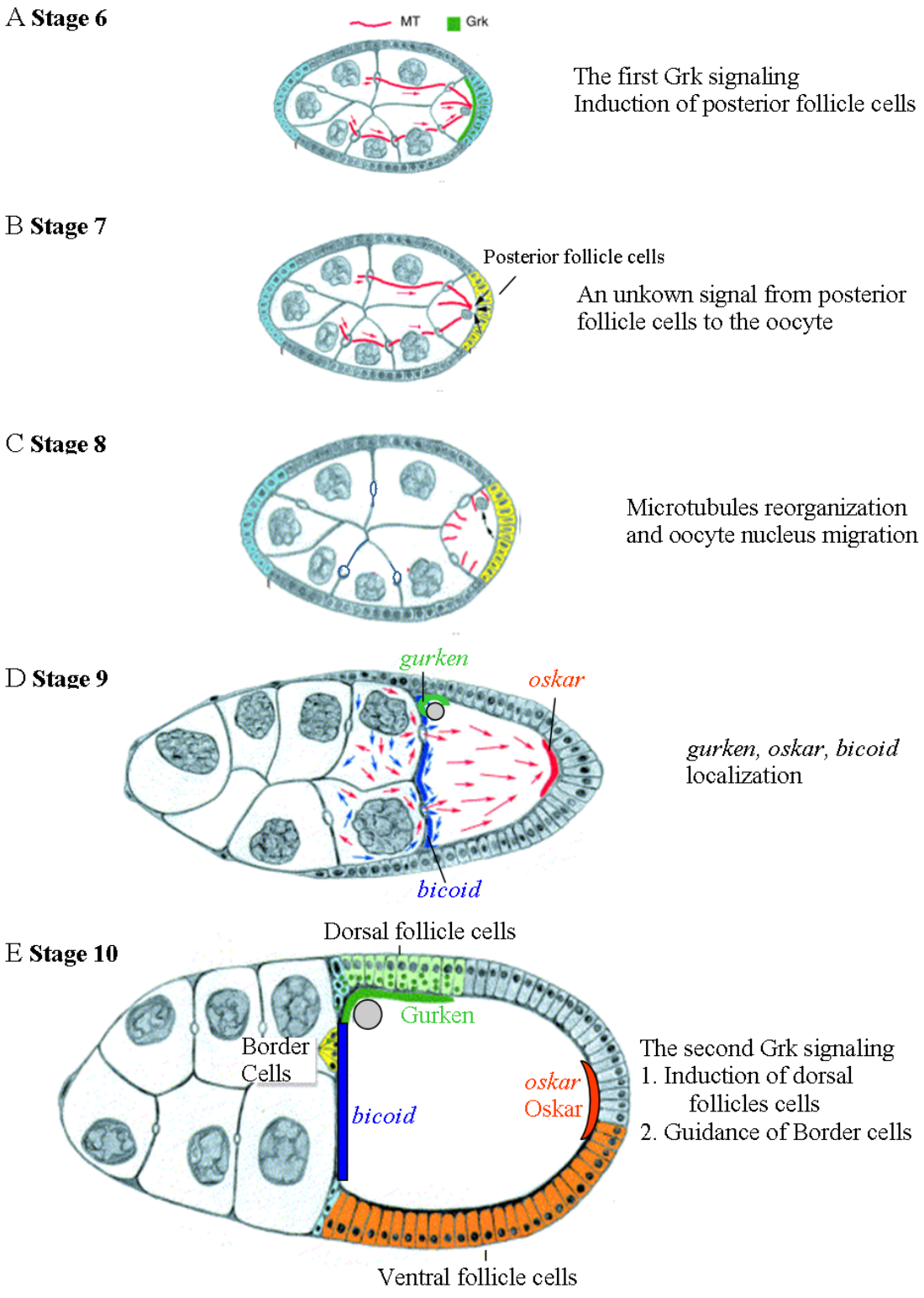

Figure 3. Patterning of the oocyte.
(A) At previtellogenic stage, mRNAs and proteins (red arrows) are produced in the nurse cells and transported along microtubules into the ooctyte. The oocyte nucleus localizes in the posterior part of the oocyte, and Gurken is expressed around oocyte nucleus. At stage 6, after receiving the Grk signal from the oocyte, the terminal follicle cells become posterior follicle cells fate. (B) After posterior follicle cells formation, posterior follicle cells send an unidentified signal back to the oocyte at stage 7. (C) The unidentified signal from posterior follicle cells causes the oocyte microtubules to reorganize. The microtubule minus-ends disassemble and microtubules

nucleate at the anterior and lateral cortex. Then the oocyte nucleus moves to the anterior corner of the oocyte to define the dorsal side of the egg chamber at stage 8. (D) *bicoid* and *oskar* mRNA which are produced in the nurse cells are transported to the oocyte and localized in the oocyte at stage 9. Microtubules extend from anterior to posterior in the oocyte. *bicoid* mRNA accumulates at the anterior cortex of the oocyte. *oskar* mRNA and Staufen protein are transported by Kinesin towards the posterior pole. (E) Patterning of the egg by the second Grk signal at stage 10 egg chamber. Grk protein, localized in the anterior-dorsal corner of the oocyte, specifies dorsal chorion structures and also guides the migration of border cells, which are required for the formation of a functional micropyle. Adapted from (Riechmann and Ephrussi, 2001).

Both RTKs have redundant activity in guiding the anterior-posterior migration of border cell toward the oocyte, while only the EGFR pathway is essential for dorsal migration (Figure 3E) (Duchek and Rorth, 2001; Duchek et al., 2001). Formation of the A-P and D-V axes of the oocyte depends on Gurken signaling. Mutations in *gurken* or genes involved in Gurken signaling disrupt oocyte polarity. In these cases, the oocyte nucleus fails to migrate from the posterior to the anterior-dorsal side and posterior terminal follicle cells adopt anterior cell fates (Gonzalez-Reyes et al., 1995; Roth et al., 1995).

1.3.3 Localization of the embryo polarity determinants

After the Gurken signaling sets up A-P and D-V axes of the oocyte, A-P and D-V axes determinants of the embryo start to localize in the oocyte during mid-oogenesis. In addition to the Gurken signaling specifies the dorsal cell fate of the embryo, the A-P axis of the embryo also requires for *bicoid* and *oskar* mRNA localization in mid-oogenesis (Figure 3D). *bicoid* mRNA localizes the anterior pole of the oocyte to determine the anterior cell fate of embryos, and *oskar* mRNA localizes to the posterior pole of the oocyte to define the posterior cell fates of the embryo (Figure 3E, 4) (Ephrussi et al., 1991; Kim-Ha et al., 1991; Neuman-Silberberg and Schupbach, 1993; Nusslein-Volhard et al., 1987; Nusslein-Volhard and Roth, 1989). Both mRNAs are produced in the nurse cells and transferred into the oocyte in mid-oogenesis. After transferring into the oocyte, both mRNAs are localized and anchored at the poles.

1.3.3.1 *bicoid* mRNA localization

The early stage of *bicoid* mRNA localization depends on Exuperantia (Exu), which is also localized to the anterior of the oocyte. Exu is required in nurse cells to recruit anterior-targeting factors to the *bicoid* mRNA. This anterior-targeting complex drives

bicoid mRNA to localize at anterior margin of the oocyte (Macdonald et al., 1991; Wang and Hazelrigg, 1994). *bicoid* mRNA is seen in a ring at stage 10a and then moves to cover the whole anterior cortex at stage 10b. From stage 10b onwards, maintaining *bicoid* mRNA in the anterior requires Swallow, which is also enriched at the anterior cortex. Swallow binds Dynein light chain which is a microtubule minus-end directed motor protein. This suggests that Dynein either anchors or transports *bicoid* mRNA to the microtubule minus-end (Berleth et al., 1988; Meng and Stephenson, 2002; Schnorrer et al., 2000; St Johnston et al., 1989). In addition to Swallow, *bicoid* mRNA localization also requires γ-tubulin complex components, γ-tubulin37C and Grip75 that nucleate microtubules. Thus, the γ-tubulin complex nucleates anterior microtubules, that anchor *bicoid* mRNA at right position at stage 10b-11 (Schnorrer et al., 2002). The final stage of this localization requires Staufen to maintain *bicoid* mRNA at anterior cortex after stage 11. This process is also microtubule dependent (Ferrandon et al., 1994; St Johnston et al., 1989).

1.3.3.2 *oskar* mRNA localization

oskar 3’ untranslated region (3’UTR) is required for the posterior localization of the mRNA in the oocyte (Kim-Ha et al., 1993). Hrp48 binds to the 5’and 3’UTRs of *oskar* mRNA. This represses *oskar* mRNA translation during its transport and regulates its localization (Huynh et al., 2004; Yano et al., 2004). Human homologues of Y14 and Magonashi are core components of the exon-exon junction complex (EJC). They bind to mRNAs 20-24 nucleotides upstream of exon-exon junctions in a splicing-dependent manner. *oskar* mRNA is mislocalized in the Y14/magonashi mutant egg chamber (Hachet and Ephrussi, 2004). eIF4AIII interacts with Y14 and magonashi and is also suggested to be involved in *oskar* mRNA localization (Palacios et al., 2004).

After *oskar* mRNA is exported from the nurse cell nuclei along with the Hrp48 and EJC complexes, eIF4AIII recruits Barentsz, a cytoplasmic protein, which also affects *oskar* mRNA localization (Palacios et al., 2004; van Eeden et al., 2001). Staufen associates with *oskar* mRNA and regulates its translation in the nurse cell cytoplasm (Ephrussi et al., 1991; Kim-Ha et al., 1991; St Johnston et al., 1991). During *oskar* mRNA transport, translational control is also essential for *oskar* mRNA localization. The translation of

oskar mRNA is repressed by Bruno and Hrp48. Bruno recruits Cup protein which binds to the translation initiation factor eIF4E inhibiting *oskar* mRNA translation (Chekulaeva et al., 2006; Kim-Ha et al., 1995; Wilhelm et al., 2003; Yano et al., 2004). This *oskar* RNP complex is moved to posterior part of the oocyte by plus-end motor protein on the microtubules (Cha et al., 2002). Only after *oskar* mRNA reaches to the posterior pole of oocyte, it starts to translate Oskar protein at stage 9. As microtubule traffic is important for the determinants localization, I will focus on one of the microtubule plus end directed motor, Kinesin.

1.3.4 Kinesin patterns the *Drosophila* oocyte

Kinesin, a microtubule plus-end directed motor protein, contains 2 Kinesin heavy chains (Khc) which can form a homodimer and 2 light chains (Klc) (Huang et al., 1994; Kozielski et al., 1997; Yang et al., 1989). The N-terminal region of Khc contains ATP and microtubule binding domains (Kull et al., 1996; Yang et al., 1990). Klc binding is mediated through the C-terminal region of Khc (Cyr et al., 1991; Gauger and Goldstein, 1993; Verhey et al., 1998). The C-terminal portion of Khc has two different functions. (1) The C-terminal tail (around 60 amino acids) may bind to cargos such as membrane-bound organelles or vesicles, as well as Klc (Bi et al., 1997; Skoufias et al., 1994). (2) The C-terminal tail domain also inhibits the ATPase and motor activities of the N-terminus rendering it inactive. Upon cargo binding, this inhibition is removed and Khc becomes active (Coy et al., 1999; Hackney et al., 1992). In midoogenesis, *oskar* mRNA localization to the posterior pole depends on active transport by motor protein along the microtubule network. Treatment of egg chambers with colchicine leads to a mislocalization of *oskar* mRNA to the oocyte cortex. In *khc* mutant egg chamber, *oskar* mRNA is mislocalized to the oocyte cortex like the phenotypes in the colchicine-treated egg chamber (Cha et al., 2002). Cha et al. have suggested that during stage 7, before the reorganizing of the microtubule network, Khc transports *oskar* mRNA away from cortex into the middle of the oocyte, then during stage 9-10, *oskar* mRNA is moved from the middle of the oocyte to the posterior pole by Khc (Cha et al., 2002). This is confused by the fact that there is no obvious *oskar* localization defect in *klc* mutants. In *khc* mutants, dorsal localization of *gurken* mRNA, proper anterior-dorsal localization of the oocyte nucleus and Dhc posterior localization are also disrupted, but *bicoid* mRNA localization

is not (Brendza et al., 2000; Duncan and Warrior, 2002). *Drosophila khc* mutants also show neuronal defects at the larvae stages (Saxton et al., 1991). Thus, Khc plays a role not only in oogenesis but also in neurongenesis. In neurons, cargos are transported at two different speeds along axons. Cytoskeleton elements such as neurofilaments, tubulins and actins, are transported slowly, whereas membrane bound organelles and synaptic membrane proteins are transported faster (Cyr et al., 1991; Hirokawa, 1996; Vallee and Bloom, 1991). *khc* mutations cause swellings in the axons that are filled with fast transported cargoes such as Synaptotagmin (syt), an synaptic vesicle membrane protein, normally concentrated in the terminal boutons (neurite varicosities). Impaired Khc function causes neuronal defects due to a general disruption of fast axon transport (Hurd and Saxton, 1996). For large cells, such as neurons or the oocyte, materials that need to be distributed over long distance are often large and diffuse slowly. Hence, rapid, active transport via Kinesin, along the microtubule network is necessary.

1.4 Late oogenesis

1.4.1 Ooplasmic streaming

After the polarity determinants of the embryo are localized, the nurse cells start to pump large amounts of their cytoplasm, which contribute the embryo development, into the oocyte. This occurs from stage 10b. At this stage, microtubules become highly dynamic, allowing fast and well ordered streaming. This streaming allows the equal distribution of nurse cell cytoplasm throughout the oocyte (Theurkauf et al., 1992). *oskar*, *bicoid*, and *gurken* mRNA are anchored to the oocyte cortex, preventing their delocalization during streaming. In weak *khc* mutant background, stratified egg chambers are observed, in which yolk granules accumulate in the posterior of the oocytes, leaving a clear zone in the anterior part. This stratified egg chamber is caused by a lack of ooplasmic streaming and remaining pumping force from the nurse cells. The plus-end directed motor (Khc) is essential for ooplasmic streaming. The fact that a weak allele of *khc*, in which streaming was slow or stopped, shows normal *oskar* mRNA localization, indicating that *oskar* localization does not require for ooplasmic streaming (Serbus et al., 2005). Injecting of Dhc antibodies blocking Dhc function or treatment of egg chambers with cytochalasin, which disrupts F-actin, leads to premature streaming, suggesting that Khc can drive the ooplamic streaming before stage 10B, but its function is blocked by Dhc and actin at earlier stages (Serbus et al., 2005; Theurkauf, 1994).

1.4.2 The anterior-posterior axis of the embryo

After fertilization, Bicoid protein is translated from its anteriorly localized mRNA, and diffuses towards the posterior of the embryo forming a gradient (Figure 4). The Bicoid gradient is required to regulate zygotic gap gene expression and pattern the anterior structures of embryo (Driever, 1993). During late stage oogenesis, Oskar recruits other posterior pole-plasm components, including Vasa protein and *nanos* mRNA, which encode the abdominal determinant of the embryo. Vasa, a DEAD-box RNA helicase, is essential for posterior patterning and germ line formation in the embryo. Nanos is also translated after fertilization and forms a gradient from posterior to anterior of the embryo.

Nanos also regulates zygotic gap gene expression and abdominal patterning (Bergsten and Gavis, 1999; Breitwieser et al., 1996; Ephrussi et al., 1991; Gavis and Lehmann, 1994).

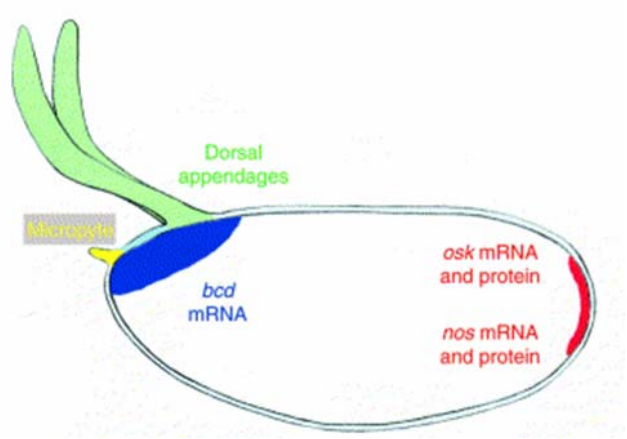

Figure 4. Localization of anterior and posterior determinants in the egg
A schematic of a late stage egg showing the dorsal appendages (green) and micropyle (yellow). Before fertilization, *bicoid* mRNA (blue) is anchored at the anterior pole and *oskar* and *nanos* mRNAs are at the posterior. Following fertilization, *bicoid* and *nanos* are translated. Bicoid protein diffuses from the anterior pole forming a gradient, concurrently the Nanos gradient forms from the posterior. The two gradients regulate zygotic gap gene expression and regulate early embryonic patterning. Adapted from (Riechmann and Ephrussi, 2001).

1.5 Par-1 function in oogenesis

1.5.1 Par-1 affects microtubule-dependent polarity in the oocyte

When the germ line cyst reaches region 2b, the oocyte specific proteins and mRNAs along with the centrosomes are transported into the presumptive oocyte, where they accumulate at the anterior (Cox and Spradling, 2003). Once the egg chamber reaches region 3, these proteins and mRNAs shift to the posterior of the oocyte (Huynh et al., 2001; Pare and Suter, 2000). Par-1 is involved in this transportation. Par-1 is the *Drosophila* homolog of *Caenorhabditis elegans* PAR-1: a serine/threonine kinase required for the polarization of the *C. elegans* zygote (Guo and Kemphues, 1995). *par-1* mutations disrupt the microtubule network in early oogenesis. In *par-1* mutants, the oocyte is specified and the centrosomes, oocyte specific mRNAs and proteins accumulate in oocyte in region 2b and 3. But the oocyte specific proteins and mRNAs can not be the transported to the posterior pole of the oocyte. Later the oocyte de-differentiates and becomes a nurse cell (Cox et al., 2001; Huynh et al., 2001). Par-1 also plays a role in axis specification during mid-oogenesis (Shulman et al., 2000; Tomancak et al., 2000). Mutations in Par-1 disrupt posterior localization of *osk* RNA and microtubule polarity at stage 9 (Benton et al., 2002; Shulman et al., 2000; Tomancak et al., 2000).

The proteins aPKC, Bazooka (Par-3) and Par-6 interact with each other and form a functional complex (aPKC complex). The aPKC complex is localized to the anterior of the oocyte and apical domains of follicle cells, corresponding to areas where microtubule minus-ends are enriched. Par-1 localization does not overlap with aPKC complex (Benton and St Johnston, 2003; Cox et al., 2001; Shulman et al., 2000; Vaccari and Ephrussi, 2002). Instead, Par-1 expresses in the posterior of the oocyte and basolateral regions of follicle cells (Shulman et al., 2000; Tomancak et al., 2000). The asymmetric localization of Par-1 and the aPKC complex is essential for cell polarity. PAR-1 can phosphorylate the conserved 14-3-3 (PAR-5) binding site in Bazooka, allowing the recruitment of 14-3-3. The phosphorylation of Bazooka destabilizes and delocalizes the aPKC complex, establishing the mutually exclusive localization of aPKC/Bazooka/Par-6 complex and Par-1 (Benton and St Johnston, 2003).

1.5.2 Par-1/MARK regulate microtubules through MAPs

Par-1 is a homologue of the mammalian MAP/MT affinity regulating kinase (MARK) family. The MARK family proteins are involved in establishing polarity in many different cells (Tassan and Le Goff, 2004). MARK proteins are known to phosphorylate microtubule associated proteins (MAP), such as Tau, MAP2 and MAP4 (Drewes et al., 1995; Illenberger et al., 1996). These MAPs were originally identified to be copurified with tubulin in microtubule binding assays. These MAPs contain three to four conserved microtubule binding domains. They are known to bind and stabilize microtubules. Most of microtubule associated proteins are found in neuronal cells. MAP2A and MAP2B are enriched in the dendrites, Tau is abundant in axons, whereas MAP4, originally isolated from HeLa cells, is abundant in a variety of cell and tissue types (review in (Mandelkow and Mandelkow, 1995).

Overexpression of Tau *in vivo* or addition of Tau in excess to microtubules *in vitro*, leads to its accumulation on the microtubules and interference with the movement of motor proteins (Ackmann et al., 2000; Stamer et al., 2002). At normal conditions, Tau and other MAPs clearly help motor transportation by creating space around microtubules (Chen et al., 1992). Phosphorylation of MAPs by MARK reduces their affinity for microtubules and consequently destabilizes microtubules (Drewes et al., 1997; Mandelkow et al., 2004). This suggests that Par-1 may act as a direct mediator of microtubule organization in the oocyte. However, mutations in *Drosophila* Tau or 205KMAP (MAP4 homologue) do not disrupt oocyte polarity, suggesting that Tau and MAP4 may not be essential Par-1 targets in the oocyte (Doerflinger et al., 2003; Pereira et al., 1992). *Drosophila* does not contain MAP2 homologues (Doerflinger et al., 2003). So to date the essential targets of Par-1 in *Drosophila* oocyte remain unknown.

1.5.3 E-MAP-115 (Ensconsin), another MAP

E-MAP-115 was originally isolated from HeLa cells (Bulinski and Borisy, 1979; Weatherbee et al., 1980). It contains two highly charged regions in the N- and C-terminal regions. A novel microtubule binding domain is also found in the highly charged region N-terminal region (Masson and Kreis, 1993; Masson and Kreis, 1995). Several studies

have shown that E-MAP-115 stabilizes and organize microtubules. First, cells expressing high level of E-MAP-115 can stabilize microtubules against nocodazole treatment (which can depolymerize microtubules). Second, association of E-MAP-115 with microtubules is reduced when microtubules become dynamic during mitosis. During interphase, microtubules are involved in the transportation of vesicles, whereas during mitosis, microtubules are used to ensure accurate chromosomes segregation. The transition between these two stages requires the rapid rearrangement of the microtubule network and is accompanied by changes in the dynamic properties of microtubules. The decreased association of E-MAP-115 with microtubules is correlated with serine/threonine phosphorylation of E-MAP-115. This suggest that phosphorylation of E-MAP-115 regulate its microtubule binding affinity. However, it is still not known which kinases regulate E-MAP-115 (Masson and Kreis, 1993; Masson and Kreis, 1995).

Faire et al. have shown that GFP labeled E-MAP115 is present along all microtubules during mitosis. This suggests that E-MAP-115 may function to modulate the stability or dynamics of microtubules (Faire et al., 1999). If E-MAP-115 is a microtubule stabilizing protein, overexpression of it would be expected to alter microtubule stability. Unfortunately, overexpressing E-MAP-115 4-10 times above endogenous levels does not result in more bundles of stabilized microtubules. This suggested that E-MAP-115 may modulate other microtubule functions or interactions with other cytoskeletal elements (Faire et al., 1999).

E-MAP-115 knock out mice have been generated and analyzed. Homozygous mice are viable but male sterile, and show microtubule disruption during spermatogenesis (Komada et al., 2000). However it is worth noting that mouse genome contains a similar protein, RPRC1, which may have redundant function with E-MAP-115 in the mouse genome.

1.6 The aim of this thesis

Border cells delaminate from the anterior follicle cells and migrate through nurse cells toward the oocyte. The original aim of my thesis was to understand the germ line function in border cell migration. I performed a genetic screen in *Drosophila*. Unfortunately, I did not find any good candidate to work on. I also identified genes required in germ line for oocyte or embryonic development in this screen. In this thesis, I will present the result of the screen and then focus on the gene, *CG14998 (ensconsin)* which is disrupted by one PiggyBac insertion, *PB4170*.

Oocyte and embryo polarization occur by the specific localization of axis determinants. Par-1 affects oocyte polarity by regulating microtubules through unkown Microtubule associated proteins. Microtubules and motor proteins are also important for the transportation and localization of axis determinants during *Drosophila* oogenesis. I show that *Drosophila* Ensconsin is a microtubule associated protein and its N-terminal domain binds microtubules, similar to its human homologue (E-MAP-115). In *ensconsin* mutant egg chambers, axis determinants are mislocalized in a manner similar (but weaker) to *Khc* mutants. Furthermore, *ensconsin* mutants disrupt the accumulation of Khc in the posterior part of the oocyte at stage 9. My experiments also show that Ensconsin is a direct target of Par-1. Par-1 phosphorylates Ensconsin and thereby regulates its localization. This is crucial for the correct localization of axis determinants. Here, I describe a novel Par-1 target, Ensconsin, which is a microtubule associated protein, which controls Khc-dependent motor transport.

2 MATERIALS AND METHODS

2.1 Fly genetics

2.1.1 Fly husbandry

Flies were grown on standard corn meal molasses agar. All crosses were carried out at 25ºC. Fly stocks were stored at 18 ºC and flipped once a month.

Fly food recipe

12 g agar, 18 g dry yeast, 10 g soy flour, 22 g turnip syrup, 80 g malt extract, 80 g corn powder, 6.25 ml propionic acid, and 2.4 g methyl 4-hydroxybenzoate (Nipagin) are mixed with one liter water.

2.1.2 Fly strains

Table 1. Fly strains used for experiments

Genotype	Source	Description
PB;;FRT40FRT42;;FRT80FRT82	(Mathieu et al., 2007)	PB jump starter
If, transposase/CyO	(Horn and Wimmer, 2000)	Transposase for PB
eyFLP;;FRT40/CyO;;FRT80/TM3ser	(Thompson et al., 2005)	For mapping
eyFLP;;FRT42/CyO;;FRT82/TM3ser	(Thompson et al., 2005)	For mapping
FRT40ovoD1/CyO	Bloomington #2121	For germ line clone analysis
FRT42ovoD1/CyO		For germ line clone analysis
FRT80ovoD1/TM3ser		For germ line clone analysis
FRT82ovoD1/TM3ser	Bloomington #2149	For germ line clone analysis
FRTG13Par-1^{9A}	(Tomancak et al., 2000)	For testing Ens localization
FRTG13Par-1^{W3}	(Shulman et al., 2000)	For testing Ens localization
kinesinLacZ	(Clark et al., 1994)	Plus end marker
tub-tauGFP	(Micklem et al., 1997)	For marking microtubules
FRTG13ubiGFP	Bloomington #5826	For creating mutant clone
FRT80ubiGFP	Bloomington #5630	For creating mutant clone
PB4170		*ensconsin mutant* allele
P{RS3}CB-5457-3	Szeged stock center	*ensconsin mutant* allele
Df(3L)ED4341	Szeged stock center	*Deficiency* for *ensconsin*
FRT80ens$^{\Delta N}$		*ensconsin* mutant allele from *P{RS3}CB-5457-3* imprecise excision
FRT80ens$^{\Delta C}$		*ensconsin* mutant allele from end-out experiment
UASP-ens	Pernille Rørth	
UASP-ens-mutant Δ3'UTR	Pernille Rørth	
Tub-ens	Pernille Rørth	
Tub-ensΔ3'UTR	Pernille Rørth	
Tub-ensmutantΔ3'UTR	Pernille Rørth	
elavGAL4	(Campos et al., 1987)	Pan-neuron driver

Continued Table 1.

P{70FLP}11,P{70I-SceI}2B noc^{Sco}/CyO	Bloomington #6934	For end-out experiment
Maternal:: GAL4 VP16	(Coutelis and Ephrussi, 2007)	Germ line specific driver

2.1.3 Ectopic expression using the GAL4/UAS system

In *Drosophila*, genes of interest can be expressed in a temporally and spatially regulated manner by using the GAL4/UAS system (Brand and Perrimon, 1993). This system uses the yeast transcription activator, GAL4, and its target sequence, upstream activation sequence (UAS). When GAL4 binds its UAS, it activates the transcription of the gene downstream of UAS. GAL4 can be expressed in many different patterns under the control of various *Drosophila* promoter sequences and activates expression of target gene or reporters placed downstream of a UAS. In order to express a target gene in to the pattern of a specific promoter, transgenic flies carrying the GAL4 driver under the control of a specific promoter are crossed to transgenic flies carrying UAS followed by the target gene.

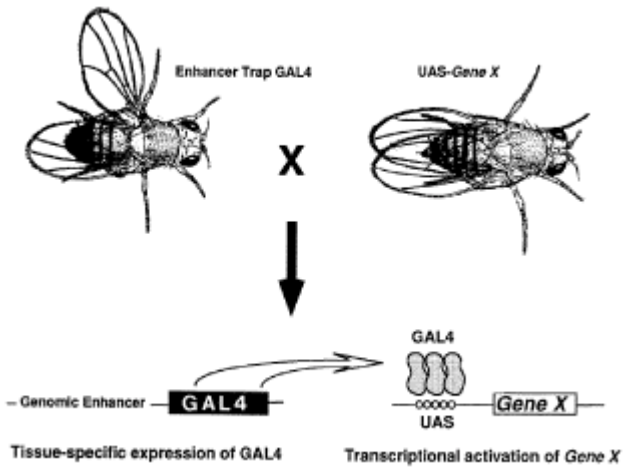

Figure 5. GAL4-UAS system
A fly line expressing GAL4 (a transcriptional activator protein) is crossed to a line carrying the UAS element upstream of gene X. In the progeny, GAL4, expressed in the tissue of interest, binds to the UAS element and activates transcription of the downstream gene X, specifically in that tissue. Adapted from (Brand and Perrimon, 1993).

2.1.4 Generation of mosaic clones using the FLP/FRT system

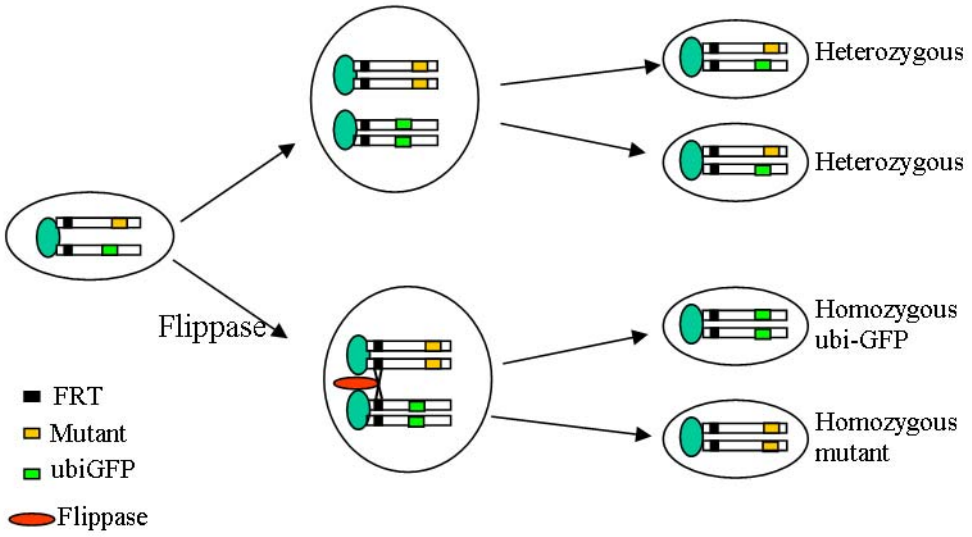

Figure 6. FLP-FRT system
FLP-recombinase can induce site-specific exchange at position of the FRT (FLP-recombinase target sequence) sequence during mitosis. Yellow is the mutant locus, and green the ubiGFP marker. In the absence of FLP, the genotype of daughter cells is like that of the mother cell (*FRTmutant/FRTubiGFP*). When FLP is added, homozygous mutant ovaries without ubiGFP can be obtained and also homozygous wild type.

In order to obtain a group of clonal cells that are homozygous mutant for a given allele, the FLP/FRT (flippase recombinase/ flippase recognition target) system was introduced into fly genetics (Golic, 1991). The mutant allele is recombined onto a suitable FRT chromosome (Xu and Rubin, 1993) and crossed to flies carrying a heat-shock induced flippase (*hs-FLP*) and marker (usually Ubi-GFP) on the matching FRT chromosome. Ubi-GFP expresses ubiquitous expression of green fluorescent protein under the ubiquitin promoter. To induce mitotic clones in the germ line or ovarian follicular cells, young larvae at 48-72 hours after eggs lying (AEL) are heat-shocked at 37°C for 1 hour. Following heat shock, the FLP induces recombination of homologous chromosomes at the FRT sites. The recombination event results in two daughter cells: one homozygous for the GFP transgene carrying chromosome; the other homozygous for the mutant allele. Subsequently, the two daughter cells will give rise to two clonal groups of cells: a wild type clone with a higher level of GFP and a mutant clone lacking GFP expression. So, homozygous mutant cells are marked by the absence of GFP in a background of either homozygous wild type, GFP-expressing cells (the twin clone) or heterozygous,

GFP-expressing (no recombination event) cells.

2.1.5 Generation of Germ line clones using the FLP-DFS system

This system is similar to FLP-FRT system. However, instead of ubi-GFP, an FRT chromosome carrying the dominant female sterile (DFS) transgene, *ovoD1* is used. In *ovoD1* ovaries, oogenesis stops at early stages, but does not affect adult viability. The mutant allele is recombined onto a suitable FRT chromosome (Xu and Rubin, 1993) and crossed to flies carrying heat-shock induced flippase (*hs-FLP*) and *ovoD1* on the same FRT chromosome. To induce mitotic clones in the germ line cells, the resulting larvae are heat-shocked at 48-72 hours AEL at 37°C for 1 hour. Following the heat shock, the FLP induces recombination at the FRT sites. This recombination event may result in two daughter cells: one homozygous for the *ovoD1* transgene chromosome; the other one homozygous for the mutant allele. Subsequently, the two daughter cells will give rise to two groups of clonal germ cells: The *ovoD1* clone will produce small arrested ovaries;

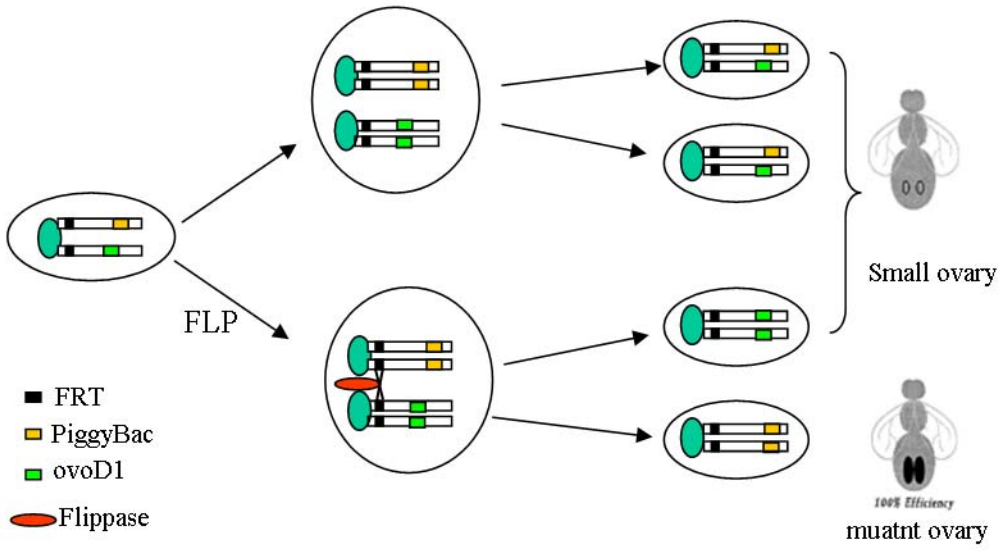

Figure 7. FLP-DFS system
The FLP-recombinase induces site-specific exchange at the FRT (FLP-recombinase target sequence) sequence during mitosis. Yellow is PiggyBac insertion, and green is *ovoD1*. *ovoD1* is a dominant female sterile transgene. Without FLP, the genotype of daughter cells is like mother cell (*FRTPiggyBac/FRTovoD1*). Ovaries of this genotype are small. With *FLP*, homozygous mutant ovaries can be obtained.

and the mutant clone, ovaries are different sizes depending on the mutant used (Chou and Perrimon, 1996).

2.2 Mutant analysis in the Screen

2.2.1 X-gal staining in the *Drosophila* egg chamber

Ovaries were dissected in cold PBS, fixed in 0.5% glutaraldehyde in PBS for 10 min, washed in PBS-0.1%Triton (PBST), and stained (in 10 mM NaH_2PO_4/Na_2HPO_4 (pH7.2), 150 mM NaCl, 1 mM $MgCl_2$, 3.1 mM $K_4(FeII(CN)_6)$, 3.1 mM $K_3(FeIII(CN)_6)$, 0.3% Triton X-100, 0.2% X-Gal) at room temperature in 1 hour. Ovaries were then washed in PBST and mounted in 50% glycerol.

2.2.2 Cuticle preparation

Embryos were collected and dechorinated in 50% bleach in 1-3 minutes. Embryos were washed thoroughly to remove the bleach and transferred to a 2 ml eppendorf tube containing a Methanol/ Heptane (1:1) solution and shaken vigorously for 1 minute to remove the vitalline membrane. This causes the devitallinzed embryos to sink to the bottom. All liquid and any embryos at the interface were removed. Embryos were mounted in the Hoyer's mount and incubated in 65^{o}C overnight.

Hoyer’s mount: 30 g of gum Arabic was added to 50 ml distilled water, and stirred overnight. While stirring, 200 g chloral hydrate was added in small quantities. Then 20 g glycerol was added. The solution was centrifuged at least 3 hours at 12000 g to clear. Lactic acid (1:1) was added in to increase contrast and decrease clearing time.

2.2.3 Inverse PCR

Inverse PCR (IPCR) is a method which allows the rapid amplification of DNA sequences flanking a region of known sequence (Ochman et al., 1988). The method uses PCR, but with primers oriented in the opposite direction to usual. The template for the reverse primers is a restriction fragment that has been self-ligated to form a circle. IPCR is routinely used to amplify and identify the sequences flanking transposable elements.

Fly genomic DNA was prepared using the DNAeasy kit (Quiagen). Then 15 µl of gDNA

was used for the enzyme digestion in total volume of 20 µl for 2 hours at 37°C. The enzymes used for inverse PCR are 4-cutters restriction enzymes with recognition sites present once in the 5' and 3' end of the PiggyBac element, such as Sau3AI or HinP1. After digestion, the mixture was incubated at 65°C for 20 minutes to heat-inactivate the restriction enzyme, and then transferred to ice immediately. The ligation mixture was set up in a total volume of 200 µl, (to promote self-ligation), and included T4 DNA ligase, ligase buffer, and the original 20 µl digest mixture. The reaction was then incubated at 16°C overnight. The next day, DNA was precipitated by adding 20 µl of 3M sodium acetate and two volumes of absolute ethanol. The mixture was left at -80°C for 30 minutes and centrifuged it at 4°C for 30 minutes. The DNA pellet was washed twice with 70% ethanol and resuspended it in 20 µl of water. The flanking region of the PiggyBac insertion was amplified with PiggyBac element specific primers, designed to amplify out from the transposon vector sequence. The primers PLF (5′-CTT GAC CTT GCC ACA GAG GAC TAT TAG AGG-3′) and PLR (5′-CAG TGA CAC TTA CCG CAT TGA CAA GCA CGC-3′) were used to amplify the genomic fragment from the 5' end of PiggyBac and the primers PRF (5′-CCT CGA TAT ACA GAC CGA TAA AAC ACA TGC-3′) and PRR (5′-AGT CAG TCA GAA ACA ACT TTG GCA CAT ATC-3′) were used for the 3' end. The annealing temperature of PCR reaction was set at 60°C. The amplified products were then sequenced and compared by BLAST search against the *Drosophila* genome to identify the insertion site.

2.3 *ensconsin* mutant analysis

The following *ensconsin* constructs were used: Full length *ens-wt* cDNA, *ens* without the 3'UTR, an *ens* mutant which removed all putative 14-3-3 binding sites, Ens-X which lacks amino acids 307-676 and Ens-C which lacks amino acids 1-370 (generated by Pernille Rørth).

ens$^{\Delta N}$ was obtained from imprecise excision of *P{RS3}CB-5457-3* and checked by single fly PCR (using the primers CB5' CAC TAC AGA GCT GGC CAC ACT G and PI3' GCA AGA CGA CAA AGG AAC AAC TGC AAG).

ens$^{\Delta C}$ was generated using the ends-out homologous recombination system. I designed primers to amplify genomic regions +4146-+7356 (primers A5' and A3' were used) and +9578-+12904 (primers B5' and B3' were used) relative to the transcription start site from genomic DNA. Primer sequences in red are restriction enzyme sites used for further cloning. The two flanking sequence were cloned into the PW25 vector and used to generate transgenic (donor) flies. The donor flies were then crossed with flies carrying the recombination transgenes (*P{70FLP}11 P{70I-SceI}2B noc*^{Sco}*/CyO*) which expresses the FLP site-specific recombinase and I-*Sce*I endonuclease. I used a heat-shock to induce expression of these enzymes. The FLP and *I-SceI* then linearize the donor molecule, allowing it to undergo homologous recombination with the target locus. These targeting events occur in the germ line. Using this method, I replaced the Ensconsin C-terminal region with the *mini-white* cassette (Figure 9) (Gong and Golic, 2004).

A5'GCGGCC GCT CGA TCT TCG ATT TGA ATC ACC CGC (sequences in red are Not1 cutting site).
A3'GGTACC TCA ATC TGC TGC AGG ACG TAG TCG C (sequences in red are Kpn1 cutting site).
B5' GGCGCGCC ATT ATT GCA GGG GGG AAA CAC AGG G (sequences in red are Asc1 cutting site).
B3' CGTACG CCC ACT TCC AAA CAA ACA GCC AAC G (sequences in red are BsiW cutting site)

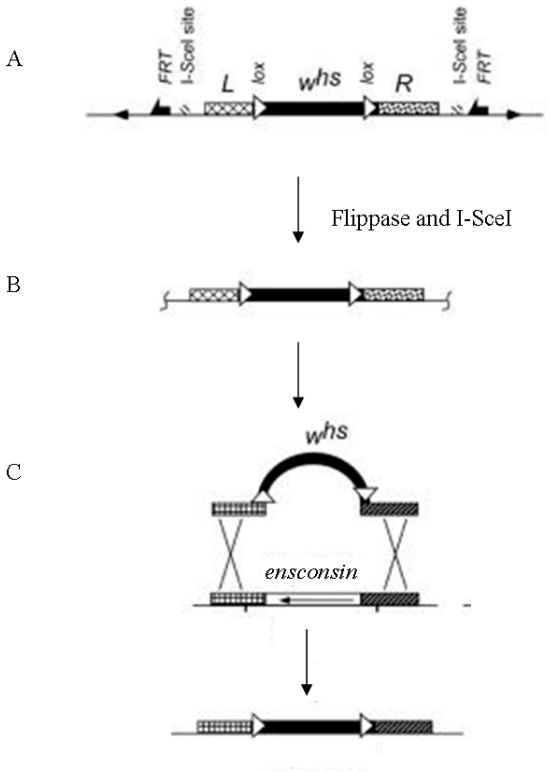

Figure 8. Ends-out system
The targeting scheme. (A) Regions flanking the locus to be deleted, indicated as L and R, were cloned into the *P*-element vector, pW25, with the indicated features. *FLP and I-SceI* generate the extra chromosomal linear donor, as shown (B). (C) Recombination of the *ensconsin* and *ensconsin* donor should generate the indicated deletions. Adapted from (Gong and Golic, 2004).

2.3.1 RNA isolation

5-10 adult ovaries were dissected and put into 1.5 ml eppendorf tube on ice. They where then dissociated by pipetting up and down with 200 µl of TRIzol (Invitrogen) until no large pieces could be seen. 800 µl of TRIzol was added and the lysate was kept at room temperature for 5 minutes. 200 µl of Chloroform was added and samples were vortexed for 15 seconds. The samples were incubated at room temperature for 15 minutes before a centrifugation at 14000 rpm at 4°C for 15 minutes. The aqueous phase (top or non-pink layer) was transferred into a new tube and 500 µl of Isopropanol was added to precipitate the RNA. Samples were incubated at room temperature for 10 minutes, and then centrifuged for 15 minutes at 4°C. The pellet was washed with 75 % ethanol (in RNase-free water) and dried at room temperature for 15 minutes. The pellet was the resuspended in 50 µl of RNase-free water. Samples were heated to 60°C in order to dissolve the RNA if necessary. Samples were stored at -80°C.

2.3.2 First-strand cDNA synthesis

First-strand cDNA for PCR reaction was synthesized by reverse transcriptase using the SuperScript™ first-strand synthesis system (Cat. 11904-018, Invitrogen). 1 μg of RNA was mixed with oligo(dT) and dNTPs in a final volume of 10 μl and heated to 65°C to denature secondary structures of RNA, and then placed them on ice for 1 minute. The reaction mixture composed of reaction buffer, $MgCl_2$, DTT, RNase inhibitor, and SuperScript™ II RT was prepared, and 10 μl of the mixture was added to each RNA/primer mix. Samples were incubated at 42°C for 50 minutes. The reactions were then terminated by heating at 70°C for 15 minutes, and transferred to ice for the next procedure or store them at -20°C. RT5', RT3' or CB5', 2RT3' were used to check different splicing forms and transcript in PB4170.

RT5' GCCGACAGTGGTGCTAAGAAGCG
Rt3'GCGATACTGGCGGGCCTCG
CB5' CACTACAGAGCTGGCCACACTG
2RT3' TACTCGCGGCGCTCCTTCTCG

2.3.3 Microtubule binding assay

1.5 ml 0-2 hours embryos or 100μl ovaries were collected. Embryos or ovaries were homogenized with the same volume of BRB-80 buffer (80 mM PIPEs pH6.6, 1 mM $MgCl_2$, 1 mM EGTA) with protease inhibitor (Roche) on ice, and supplemented with 0.1 % NP40, 1 mM DTT and 1 mM GTP. The solution was centrifuged at 100000g in TLA100.4 or TLA100 rotors for 20 minutes 4^{o}C. The supernatant was split into two tubes. The supernatant was heated to 25 ^{o}C in water bath. To one sample taxol was added to a final concentration of 20μM, and an equal volume of DMSO was added to the control. All reactions were kept at 25 ^{o}C 30 minutes to allow microtubules to polymerize. The solution was then layered onto the 1V cushion of 40 % glycerol in BRB80 buffer. All reactions were spun 100000g in TLA 100.4 or TLA 100 10 minutes 25 ^{o}C. 75 μl of the 1V supernatant and 25 μl 4X Laemmli Buffer were added. The pellet was dissolved in 1V BRB80 buffer. After dissolving, 75 μL solution was taken to mix with 25 μl 4X Laemmli

Buffer. Both samples were analyzed by western blot, probed with anti-tubulin and anti-Ensconsin.

2.3.4 Western blotting

For whole larvae or adults extracts, total protein was obtained by crushing 10 pairs of ovaries in 200 µl 1x Laemmli Buffer with a plastic pestle. Samples were boiled at 95°C for 5 minutes and 10 µl from each was loaded on an SDS-polyacrylamide gel. Proteins were separated by elecrophoresis with a constant current of 20 mA per mini-gel. Proteins were transferred to nitrocellulose membranes (10401196, Schleicher & Schuell) using a constant current at 45 mA per mini-gel for 1 hour. The membrane was rinsed with water and incubated in Ponceau S solution for several minutes. The membrane was then washed with water several times and checked the total protein levels. The membrane was blocked with 5 % milk/ PBT (PBS with 0.1% Tween20) for 1 hour. Next, it was incubated with primary antibody diluted in 5 % milk/ PBT at 4°C overnight. Primary antibodies used in this study are mouse anti-α-Tubulin DM1A (1:5000) and rat anti-Ensconsin (1:10000). The membrane was rinsed once and washed for 15 minutes 3 times with PBT. The membrane was incubated with HRP-conjugated secondary antibody (Jackson ImmunoResearch) diluted in 5 % milk/ PBT at room temperature for 1 hour. The membrane was rinsed once and washed for 3 times 15 minutes with PBT. The membrane was rinsed with enhanced chemiluminescence reagent by mixing equal volumes of the Enhanced Luminol Reagent and the Oxidizing Reagent (NEL105, PerkinElmer). The membrane was exposed to Kodak X-OMAT MR Film for 30 seconds to 15 minutes.

2.3.5 Single Fly PCR

One fly was squashed in 50 µl SB buffer (10 µM Tris-Cl, 1 µM EDTA, and 25 µM NaCl) with 1 µl ProteinaseK (10 mg/ml). The solution was incubated for 1 hour at 37^{o}C. The solution was then incubated at 95^{o}C for 2 minutes to inactive the proteinase K. 1 µl solution was used per PCR reaction.

PCR reaction:

The PCR reaction was performed in a final volume of 20 µl and contained 1 µl of extracted genomic DNA as amplification template, 1 U Taq DNA polymerase (Roche), 1x PCR Buffer containing $MgCl_2$ (Roche), 0.2 mM dNTPs (PCR grade, Roche), 1 µM of forward and reverse primers, and sterile distilled water. The PCR reaction was running on Thermal Cycler (PTC-200, MJ research) according to the following program: (1) 94°C for 3 minutes, (2) 94°C for 30 seconds, (3) 60°C for 30 seconds (depend on the Tm values of primers), (4) 72°C 30 seconds to 1 minute (depend on the length of the product), (5) repeat step (2)-(4) for 34 times, (6) 72°C 10 min, (7) end at 4°C. The amplified products were separated by electrophoresis on 1 % agarose gel and visualized by Ethidium Bromide staining and UV light.

2.3.6 Purification of Ensconsin protein

I designed primers to amplify fragments from LD09646, *enswt* and *ens* mutant without 14-3-3 binding sites plasmids which are constructed by Pernille Rørth. Then the fragments were cloned into PETM11vector (N-his tag labeling) to express the truncated protein. Plasmids were transformed into BL21 competent cells. The colony was cultured into in 5 ml LB overnight. 500 µl bacterial culture was mixed with 500 µl 30% glycerol, flash frozen and kept as a stock. 200 µl bacterial culture was used to inoculate 2 l of LB. The culture was incubated for about 2 hours at 30^{o}C until the OD reached 0.4-0.6 IPTG was then added to a final concentration 1 mM to induce protein expression. The culture was then grown at 30^{o}C for another 4 hours. The bacteria were pelleted and resuspended in 40 ml Lysis buffer. The lysate flash frozen in liquid nitrogen and kept in -80^{o}C. When required, the lysate was then thawed on ice. Proteinase inhibitor cocktail (Roche) and DNAase powder were added to digest DNA. The lysate was passed through a French press to lyse the cells. The sample was then sonicated 2 times 30sec to further disrupting the cells. The lysate was spun at 4000 rpm at 4°C. The supernatant was mixed with nickel beads for 2 hours at 4^{o}C on a shaker. Before mixing, water and Lysis buffer washes were used twice to remove methanol from 500 µl of beads. The beads were spun at 600 rpm for 5 min and the supernatant was removed. Beads were washed 3 times in wash to remove non-specific binding proteins. The protein was then eluted in 3 washes of 500 µl elution buffer.

Lysis buffer (500 mM NaH_2PO_4, 500 mM NaCl, 5 mM β-Mercaptoethanol, 10% glycerol, 1 mM $MgCl_2$, pH7.4 at room temperature)

Wash buffer (50 mM NaH_2PO_4, 1 M NaCl, 5 mM β-Mercaptoethanol, 10% glycerol, 1 mM $MgCl_2$, 40 mM Imidazole, pH7.4 at room temperature)

Elution buffer (500 mM NaH_2PO_4, 500 mM NaCl, 5 mM β-Mercaptoethanol, 10% glycerol, 1 mM $MgCl_2$, 500 mM Imidazole, pH7.4 at room temperature)

For antigen A, primersA5' andA3' were used to do a PCR. For antigen B, primer B5'-B3' were used. For Ens-N and Ens-N mutant, C5' and A3' were used. For C-terminal protein, D5'and D3' were used.

A5' CCATGGctTCCGCACAAACGACACCCAAACG (sequences in red is Nco1 cutting site)
A3' GAATTCCAGTTCCCGCACCTCTGAGTTC(sequences in red is EcoR1 cutting site)
B5' CCATGGctGCAGCTCCTGCCGCAACGG (sequences in red is Nco1 cutting site)
B3' GAATTCCAGCAGCGATATATCTTTATTTTCGTGACTATC (sequences in red is EcoR1 cutting site)
C5' CCATGGctATGGCGAGTCTTGGGGGCCAAC (sequences in red is Nco1 cutting site)
D5' CCATGGctTCCACCATCACAAAACCAGCTCCTG (sequences in red is Nco1 cutting site)
D3' GGATCCTCACAGCAGCGATATATCTTTATTTTCGTGAC (sequences in red is BamH1 cutting site)

2.3.7 In vitro kinase assay

The *in vitro* kinase assay was performed as described (Benton et al., 2002). Bacterially expressed Ensconsin fragments were mixed with the kinase source and 50 μM of γ-^{32}P label ATP or BnATP (synthesized by Piyi Papadaki) in kinase buffer (20 mM HEPES pH7.5, 150 mM NaCl, 5 mM $MgCl_2$) for 1 hour at 30^{o}C. The kinase source used was

bacterially expressed MBP-fused Par-1 kinase domain (amino acid 245-521, done by Piyi Papadaki) or GFP-fused full length GFP-Par-1^{M329G} (done by Piyi Papadaki) immuno-precipated from ovarian lysate using an anti-GFP monoclonal antibody (Molecular probes). Samples were run on a 10% protein gel, and then stained in Coomassiae solution (45% Methanol, 10% Acetic acid and 0.1% Coomassiae Brilliant Blue R-250) for 30 minutes. The gel was transferred to the destaining solution (50% H_2O, 40% Methanol and 10% Acetic acid) for 1 hour to remove the background signal. The gel was then dried and exposed to Kodak X-OMAT MR Film for 1-3 days.

2.3.8 The *ensconsin* Probe preparation

The *ensconsin* cDNA clone LD09626 was linearized using BamH1. 2 µl of the linearized plasmid was incubated with 2 µl DEPC- water, 2 µl 10X buffer for T7 polymerase, 2 µl each of ATP, CTP, GTP, 1.5 µl UTP, 4 µl DIG-11-UTP (10nmol/ul), 2 µl T7 polymerase 6 hours at 37°C. Then 1 µl DNase was added in the solution for 15 minutes at 37°C to digest the template DNA. The probe was purified using a Qiagen RNeasy Kit.

2.3.9 In situ hybridization of egg chambers

Fly ovaries were dissected in the cold PBS and fixed in 4% Paraformaldehyde for 30 minutes. Ovaries were washed for 3 times 10 minutes in PBT (PBS with 0.1% Tween-20). Ovaries were washed with 1:1 PBST/Hybridization buffer for 5 minutes. Ovaries were then washed in Hybridization buffer for 5 minutes. Ovaries were prehybridized in the 1ml Hybridization buffer containing 20 µl Salmon sperm DNA (5mg/ml) for at least 1.5 hours in a 55°C heat block. Ovaries were then hybridized in 50µl hybridization buffer containing the probe (1:1000), 10 µl ssDNA at 65°C water bath overnight. Probes used in this study were *oskar*, *gurken*, *bicoid* (gift from Anna Cyrklaff) and *ensconsin*. Ovaries were washed in hybridization buffer 30 minutes at 65°C, then in 1:1 hybridization buffer/PBST for 30 minutes at 65°C. Ovaries were washed for 4 times 20 minutes in PBST at 65°C, then in PBST for 20 minutes at room temperature. Ovaries were incubated with anti-DIG-Hrp (Roche) antibody for 2 hours in PBST. Ovaries were washed for 5x30 min in PBST. Ovaries were incubated in staining solution (0.5 mg/ml DAB and 0.003%

H_2O_2), washed in the PBST, and then mounted in 70% glycerol.

Hybridization buffer (50% Formamide, 5X SSC, 0.1% Tween-20)

2.3.10 In situ hybridization of embryos

Flies were allowed to lay eggs on apple juice plates. The eggs were then washed with into a tube sealed at one end with mesh. Embryos were dechorionated in a 50% bleach solution for 1-2 minutes. Embryos were washed thoroughly to remove bleach and transferred into a 2 ml eppendorf tube. They were then fixed by shaking in 4 % Formaldehyde/ PBS/ Heptane for 25 minutes. As the fixed embryos rest at the interface, the lower, aqueous phase was removed and 500 µl methanol added. The tube was vigorously shaken for 1 minute to remove the vitalline membrane. The devitallinized embryos will then sink to the bottom. All liquid and any embryos at interface were then removed. Embryos were washed twice in methanol and stored at -20°C. Embryos were then rehydrated in PBT (PBS with 0.1% Tween-20) and fixed again in 4% paraformadehyde for 30 minutes. They were then washed for 3 times 20 minutes in PBT (PBS with 0.1% Tween-20). Embryos were washed in a 1:1 mix of PBT/hybridization buffer for 5 minutes, then in hybridization buffer for 5 minutes. Embryos were prehybridized in 1 ml hybridization buffer containing 20 µl Salmon sperm DNA (5mg/ml) for at least 1.5 hours in 55^{o}C heat block. Embryos were hybridized in 50 µl hybridization buffer with probe (1:1000), 10 µl ssDNA at 65^{o}C water bath O/N. The probes, used in this study are *ftz*, and *eve* (gifts from Natasha Bushati). Embryos were washed in hybridization buffer for 30 minutes at 65^{o}C. They were then washed in 1:1 hybridization buffer/PBST for 30 minutes at 65^{o}C. Following this, embryos were washed for 4 times 20 minutes in PBST at 65^{o}C then once in PBST at room temperature. Embryos were incubated with anti-DIG-HRP antibody (Roche) in PBST for 2 hours. Embryos were washed for 5 times 30 minutes in PBST. Embryos were stained in 0.5 mg/ml DAB and 0.003% H_2O_2 for 10-30 minutes in the dark, and then washed in the PBST and mounted in 70% Glycerol.

Hybridization buffer (50% Formamide, 5X SSC, 0.1% Tween-20)

2.3.11 Immunofluorescence staining of larvae axons

Wandering third instar larvae were dissected in cold PBS as follows: The larva was cut in half and the head inverted by pushing the mouth hook with forceps. The fat body and salivary gland were removed from the head to prevent the endo-replicating tissues depleting the antibodies. Dissected tissues were transferred to a 2 ml eppendorf tube and fix samples with 4% paraformaldehyde in PBS for 20 minutes on the shocker. The tissues were washed in PBST (PBS with 0.1% Triton-X100) for 30 minutes. The tissues were then blocked in PBST with 5% NGS for 1 hour before being incubated with primary antibody overnight. Primary antibody was used in this study: rabbit anti-Syt (1:100, the gift from Hugo Bellen). The tissues were washed in several changes of PBST with 0.2% BSA for 2 hours, blocked in PBST with 5% NGS (1 hour), then incubate with Cy5, Rodamine and HRP conjugated secondary antibodies in PBST with 5% NGS for 2 hours. The tissues were washed repeatedly in PBST and mounted in 80% Glycerol with 0.4% n-Propyl gallate (P313 Sigma) in PBS. Axons were dissected out of the tissue prior to confocal imaging. Analyses were performed using a Leica TCS SP confocal microscope and images were editing using Photoshop CS (Adobe, San Jose, CA)

2.3.12 Immunofluorescence staining of embryos

Embryos were collected and fixed as with in situ analysis. Devitallinized, methanol washed embryos were washed in PBST (PBS with 0.1% Triton-X100) for 30 minutes, then blocked in PBST with 5% NGS for 1 hour. Embryos were incubated with primary antibody overnight. Primary antibodies were used in this study: mouse anti-En (1:10 DSHB), rabbit anti-Ftz (1:350), genie pig anti-Eve (1:100), rabbit anti-Knirpe (1:10), and rat anti-Kruppel (1:100) (a gift from Natasha Bushati). Embryos were washed several times in PBST with 0.2% BSA for 2 hours. Embryos were blocked in PBST with 5% NGS 1 hour. Embryos were incubated with FITC, Cy5, Rhodamine conjugated secondary antibodies (1:100) and Rhodamine conjugated Phallodin (1:500; Molecular probes) in PBST with 5% NGS for 2 hours. Embryos were finally washed in PBST, and mounted in 80% Glycerol with 0.4% *n*-Propyl gallate (P313 Sigma) in PBS. Analyses were performed using a Leica TCS SP confocal microscope and images were editing using Photoshop CS (Adobe, San Jose, CA).

2.3.13 Immunofluorescence staining of egg chambers

Ovaries were dissected from females in the cold PBS and fixed in 4% paraformaldehyde for 20 minutes. They were then washed in PBST (PBS with 0.1% Triton) for 10 minutes. Ovaries were permeablized in PBST (PBS with 1% Triton) for 1 hour then blocked in PBST with 5% NGS for 1 hour. Ovaries were incubated overnight in primary antibody. Primary antibodies were used in this study: Rat anti-Staufen (1:2000), rabbit anti-Oskar (1:2000) (gifts from Anne Ephrussi), rabbit anti-aPKC (1:100, Santa Cruz), and mouse anti-α-tubulin (1:100, Sigma clone DM1A), mouse anti-Gurken (1:100, DHSB), mouse anti-α-spectrin (1:100, DHSB). Ovaries were washed several times in the PBST with 0.2% BSA for 2 hours. Ovaries were then blocked in PBST with 5% NGS for 1 hour before incubation with FITC, Cy5, or Rhodamine conjugated secondary antibodies (1:100) and Rhodamine conjugated Phallodin (1:500; Molecular probes) in PBST with 5% NGS for 2 hours. Ovaries were washed repeatedly in PBST, and mounted in 80 % Glycerol and 0.4 % *n*-Propyl gallate (P313 Sigma) in PBS. Oskar and Staufen stained samples were dehydrated in methanol and mounted in clearing solution (1:2 Benzyol alcohol: Benzylbenzoate). Analyses were performed using a Leica TCS SP confocal microscope and images were editing using Photoshop CS (Adobe, San Jose, CA).

2.3.14 Dhc immunostaining of egg chambers

Ovaries were dissected in cold PBS and fixed in 4% PFA for 15 minutes. Ovaries were then washed in PBS with 0.1% Tween-20 (PBT) for 10 minutes, then dehydrated in methanol and keep in 100% methanol at -20^{o}C O/N. Ovaries were allowed to re-hydrate in PBT (PBS with 0.1% Tween) for 4-8 hours at 4^{o}C. Ovaries were blocked (PBT with 5% NGS) for 30 minutes. Ovaries were incubated overnight with mouse P1H4 anti-Dhc (McGrail and Hays, 1997). After staining ovaries were washed several changes of PBT with 0.2% BSA for 2 hours. Ovaries were blocked again (PBT with 5% NGS) then incubated in PBT with 5% NGS with secondary antibodies at 4^{o}C overnight. Ovaries were washing in PBT 3 times then dehydrated in the methanol for 5 minutes and mounted in cleaning solution (Benzyol alcohol: Benzylbenzoate = 1:2).

2.3.15 Khc immunostaining of *Drosophila* egg chambers

Ovaries were dissected in the cold PBS, fixed in 4% paraformadehyde in PBS for 30 minutes and washed 3 times 10 min in PBST (PBS with 0.1% Tween-20). Ovaries were washed in 1:1 PBST/hybridization buffer for 5 minutes, then in hybridization buffer for 5 minutes. Ovaries were prehybridized in 1 ml hybridization buffer for at least 1.5 hours at 55^{o}C. Ovaries were hybridized 50 µl hybridization buffer at 65^{o}C overnight. Ovaries were washed in hybridization buffer for 30 minutes at 65^{o}C. They were then washed in 1:1 hybridization buffer/PBST 30 minutes at 65^{o}C. Ovaries were washed for 4 times 20 minutes in PBT at 65^{o}C, then in PBT for 20 minutes at room temperature. Ovaries were incubated with rabbit anti-Khc (1:100, Cytoskeleton) for 2 hours in the PBT. Ovaries were washed for 3 times 30 minutes in PBT, then incubated in PBT with 5% NGS with secondary antibodies at 4^{o}C overnight. Ovaries were washed in PBT 3 times. Ovaries were dehydrated in methanol for 5 minutes and mounted in cleaning solution (Benzyol alcohol: Benzylbenzoate = 1:2).

Hybridization buffer (50% Formamide, 5X SSC, 0.1% Tween-20)

2.3.16 Live image of tau-GFP in the ovoD1 germ line clone

hsFLP/ +;; Tub-TauGFP/ +; FRT80 ens$^{\Delta C}$/FRT80 ovoD1 females were dissected and mounted on a glass slide in halocarbon oil (Halocarbon products Crop, USA). The samples were immediate imaged using a confocal microscope (Leica NT, Germany).

2.3.17 Live image for ooplasmic streaming in the egg chamber

Wild type and *PB4170* mutant ovaries were dissected in halocarbon oil (Halocarbon products Crop.USA) and transferred to glass bottomed chamber (MatTek Corp). The samples were immediately imaged using a by Perkin Elmer UltraView RS spinning disc confocal. Images were contrast enhanced and maximum projections of 30 sections per time point were made using ImageJ (NIH).

2.3.18 Climbing assay

A single 3 days old male fly was put into an empty vial. After a 30 minute recovery, the fly was tapped to the bottom of the vial and thenthe time it took to climb up 5cm was recording. If flies did not reach 5 cm in 2 minutes, counting was stopped.

2.4 Websites

BDGP http://www.fruitfly.org/

BLAST http://www.ncbi.nlm.nih.gov/BLAST/

ClustalW http://www.ebi.ac.uk/clustalw/index.html

DHSB http://www.uiowa.edu/~dshbwww/

Flybase http://flybase.bio.indiana.edu/

NEB cutter http://tools.neb.com/NEBcutter2/index.php

PubMed http://www.ncbi.nlm.nih.gov/entrez/query.fcgi

SMART http://smart.embl-heidelberg.de/

3 RESULTS

3.1 Screen for genes affecting border cell migration, oogenesis and embryogenesis

Most of the mRNA and proteins required for oogenesis and embryogenesis are synthesized in the nurse cells and transported into the oocyte. To investigate the functions of these mRNA and proteins, I performed a screen with Juliette Mathieu, a former postdoctor fellow in the lab, looking for genes required in border cells migration. I focused on genes required in the germ line that affect border cell migration as well as oocyte and embryo development.

3.1.1 Screening method

3.1.1.1 Generation of PiggyBac mutants in FRT background

Mutations in fly can be induced by transposon insertion or EMS (ethyl methanesulfonate). EMS can induce unbiased mutations but mapping those mutations is very time consuming. For transposon mutagenesis, the interrupted genes can easily be cloned by inverse PCR. However, different transposons display different preferential sites biasing the screen at certain hot spot (Liao et al., 2000). The P element is the most commonly used transposon in *Drosophila* screens. In order to perform the FLP-DFS system (see material and method, Figure 7) to create homozygous mutant germ line cells, we wanted to generate transposon insertions in FRT background (Chou and Perrimon, 1996). Unfortunately, P-elements can not be used in FRT background because the FRT sequences are inserted into the genome using by P elements. Therefore, another transponson, the PiggyBac which is not disrupted FRT site, was used as a mutagen in our screen. The original PiggyBac line used was on X chromosome so new PiggyBacs insertions in the autosomes could be selected, and readily segregated from the original one. Later we performed a mapping cross to localize the insertions to a chromosome arm.

3.1.1.2 Mapping

We mapped the chromosome arm on which the PiggyBacs were inserted using the eyFLP-FRT system (Figure 6). eyFLP-FRT drives expression of the FLP-recombinase

with the eyeless promoter (in the eye) and homozygous mutant clones (mosaic eyes) were created only in eye (the other tissues are still normal). Depending on which flies displayed mosaic eyes, we could distinguish the chromosome PiggyBac was inserted in. For example, if the PiggyBac was inserted in FRT42 chromosome, the only mosaic eyed flies were those lacking CyO in the X2 cross in (Figure 9).

3.1.1.3 Germ line clone analysis

After identification of the chromosome the PiggyBac was inserted in, we made stocks for each mutant line. I then collected mutant virgins to make germ line clones (the FLP-DFS system, Figure 7) and further analyzed the mutant phenotype. I used a *slbo: LacZ* marker which labels the border cell cluster to test for migration delays. To test for embryogenesis defects, I selected the crosses which produced dead embryos and made cuticle preparations to check for cuticle defects. If the eggs were unfertilized, I analysed ovaries stained with phalloidin and DAPI (labelling F-actin and DNA) to check for any obvious oogenesis defects.

3.1.1.4 Cloning the genes affected by the PiggyBac insertions

For mutants with interesting phenotypes, I used inverse PCR (IPCR) to isolate the flanking sequence of the insertion. I then blasted the sequences against the *Drosophila* genome to identify the insertion site.

3.1.1.5 Creation of new ovoD1 lines

In order to generate germ line clones, *FRT40ovoD1*, *FRT42DovoD1*, *FRT80ovoD1*, and *FRT82ovoD1* were required to create germ line mutant clones (Chou and Perrimon, 1996). But *FRT42ovoD1* and *FRT80ovoD1* were not available. It is not possible to create these lines using recombination in females because *ovoD1* is a dominant female sterile mutation. I chose X-ray to induce homologous recombination of FRT sites with *ovoD1*.

First, *FRTG13(42B)ovoD1* (w^+) and *FRT2A(79A)ovoD1* (w^+) males were crossed with *eyeflp;;FRT42D* (w^-) virgins, and *eyeflp;;FRT80* (w^-) virgins separately. When progenies

reached the second or third instars larvae, they were irradiated with an X-ray dose of 1000 rad (4 mA, 100 kV, 3'18", Philips MG102) to induce homologous recombination, including in the germ line. Using the eyFLP-FRT system, I confirmed the presence of the FRT site and *ovoD1* in the genome. I successfully generated 2 lines of *FRT80ovoD1* and 1 line of *FRT42DovoD1*.

Figure 9. The scheme of crosses

3.2 Screen result

We produced over 5000 individual PiggyBac insertions. From them, I checked about 3000 lines for defects in border cell migration, oogenesis or embryogenesis by making germ line clones. Unfortunately, I only found two mutants which were required in the germ line for border cell migration. The first mutant is an allele of *cornichon*, which is required for early Gurken signaling. Mutant egg chambers show a second, posterior set of border cells, as the posterior follicle cells are not specified and assume an anterior follicle cell fate (Roth et al., 1995). The second mutant is an allele of *cup*, which is a translation repressor of Gurken, and Oskar (Chekulaeva et al., 2006; Wilhelm et al., 2003). Mutant *cup* egg chambers show border cell migration defect and oocyte growth defect. Border cell migration defect may be a secondary effect, due to abnormal oocyte growth. Below, I will focus on other classes of mutants that I found in the screen. Mutants displaying embryonic phenotype or oogenesis defects are listed in Table 2. I divided all mutant lines into several groups using the well-established criteria: No maternal effect, Germ cell lethal, abnormal oogenesis and embryonic defects (Perrimon et al., 1989; Perrimon et al., 1996).

3.2.1 No maternal effect

All PiggyBac mutants in this class can lay eggs which are fertilized and hatched. This group corresponds to genes that are either not expressed in germ line or not required for oogenesis and embryogenesis. About 90% PB mutants belong to this group.

3.2.2 Germ cell lethal

In this class, mutant females have ovaries similar to *ovoD1*, in that oogenesis stops at very early stage. I identified 300 mutant lines in this category. This group corresponds to genes that may require for germ cell viability or early oogenesis (Perrimon et al., 1989). Some of the mtants belonging in this class may be present due to technical reasons, such as mapping problems. If the mutation was mapped to the wrong chromosome, failure of recombination will lead to germ cell lethal phenotype (as no clones will be made).

3.2.3 Abnormal oogenesis

Mutations in this class show ovaries that can develop further than *ovoD1* but with some defects in the oogenesis. I observed several different kinds of oogenesis defects in these mutants. I divided these mutants into several classes depending on phenotypes.

3.2.3.1 Nurse cell membrane defects

In mutants of this class some of nurse cell membranes are absent based on phalloidin staining. One such mutant: *PB1173*, an allele of *sds22*, mutant eggs can be fertilized and hatch. Most of *PB1173* egg chambers show some nurse cell nuclei that are close each other and lack any phalloidin-labelled cell membranes between them (Figure 10B). In yeast, *sds22* regulates *protein phosphatase 1* (Ceulemans et al., 2002). In *Drosophila*, *protein phosphatase 1* regulates nonmuscle myosin to control actin cytoskeleton (Vereshchagina et al., 2004). I proposed that *sds22* may affect nurse cell membrane formation through *protein phosphatase 1*.

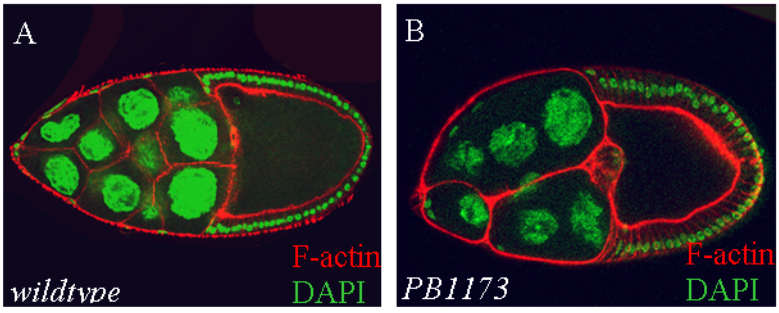

Figure 10. Nurse cell membrane defects
Green is DAPI staining and Red is phalloidin staining in (A) wild type egg chamber and (B) *PB1173* mutant egg chamber.

3.2.3.2 Gurken signaling defects

The dorsal-ventral axis of eggs and dorsal appendages are determined on the second wave of Gurken signaling. I isolated five mutant alleles of *squid*, a heterogeneous nuclear RNA-binding protein, which controls dorsoventral (DV) axis formation during

Drosophila oogenesis by localizing *gurken (grk)* mRNA (Kelley, 1993; Norvell et al., 1999). In late wild type egg chamber, Gurken is normally localized in the anterior-dorsal part of egg chamber to control formation of two dorsal appendages (Figure 11A, B, B'). In *PB4180* mutan, there are more dorsal appendages due to Gurken localizing not only to the anterior-dorsal site but also at ventral site of mutant egg chambers (Figure 11C, D, D').

Another mutant, *PB4329*, affects *cornichon*, which is required for the first Gurken signaling which is establishes the anterior-posterior polarization of the oocyte in mid-oogenesis and later in dorsal-ventral patterning (Roth et al., 1995). In wild type egg chambers, dorsal appendages and one group of border cells are present at the anterior part of egg (Figure 11E). In *cornichon* mutant egg chamber, posterior terminal follicle cells are transformed to anterior follicle cells, forming another set of border cells at the posterior part. Dorsal appendages are absent in this mutant (Figure 11F).

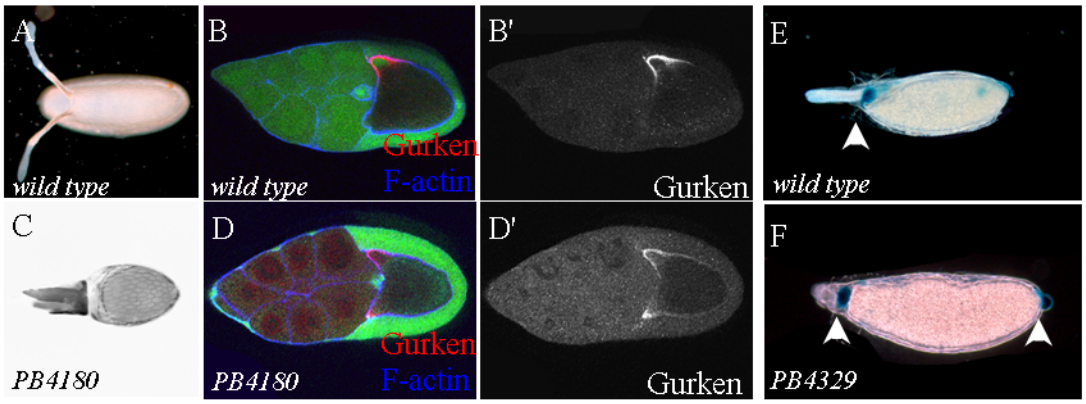

Figure 11. Gurken signaling defects
Dorsal appendages in wild type eggs (A) or *PB4180* eggs (C). Antibody staining detecting Gurken in wild type (B) or *PB4180* mutant germ line egg chamber (The mutant clone is marked by lack of GFP, shown in green) (D). Gurken antibody staining (red). (B', D') single channel of Gurken antibody staining (E, F) arrows indicate border cells, marked by X-gal. One cluster is present in wild type but two in *PB4329* germ line clone egg chamber.

3.2.3.3 Dumpless eggs

The dumpless phenotype is seen when nurse cells fail to transport protein and mRNA into the oocyte. The oocyte is smaller than in a normal egg chamber, but follicle cells appear normal. Later, nurse cells may fail to degenerate. One such mutant, *PB4057*, in the *Bullwinkle* locus, shows such a phenotype (Figure 12B). This mutant affects not only dumping in oogenesis but also anterior-posterior axis formation in embryogenesis

(Rittenhouse and Berg, 1995).

I found two mutants of *cup*, a translation repressor, in which egg chambers have smaller oocyte (Figure 12 D) and border cells are unable to attach to the oocyte at the right time. As the Cup protein normally represses the translation of axis determinants such as Oskar, and Gurken, loss of cup results in reduced oocyte size. Border cell migration defects may be a secondary effect due to the oocyte growth defects.

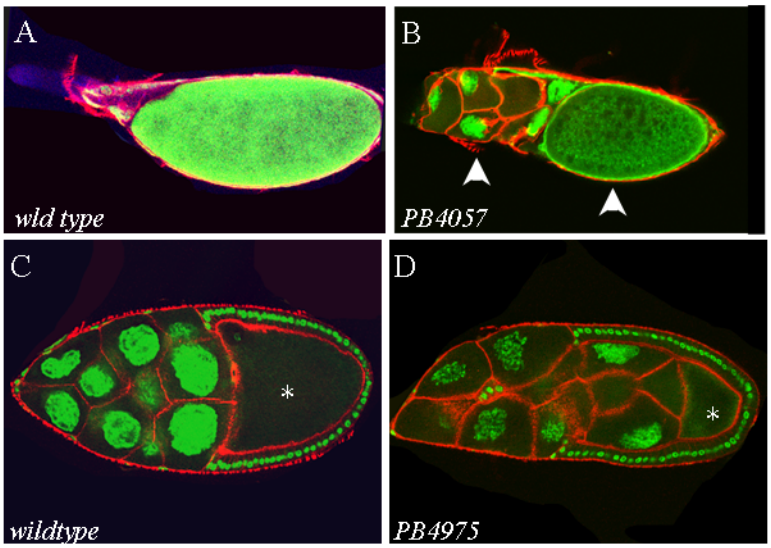

Figure 12. Dumpless phenotype
(A) Late stage wild type egg chamber with normal dorsal appendages. (B) Late stage *PB4057* mutant egg chamber. Arrows indicate smaller oocyte and non-degenerated nurse cells. Stage 10 egg chamber have a large oocyte in wild type (C) or smaller oocyte in PB4975 mutant egg chamber (D). Green is DAPI staining, and red is phalloidin staining.

3.2.3.4 Abnormal nurse cells

An abnormal number of nurse cells may due to egg chamber fusion or cell cycle defects. Wild type egg chambers are composed of 15 nurse cells and one oocyte (Figure 13A). *PB2955* affects *neuralized*, which encoded an E3 ubiquitin ligase. *PB2955* egg chambers showed more than 15 nurse cells and one oocyte (Figure 13B).

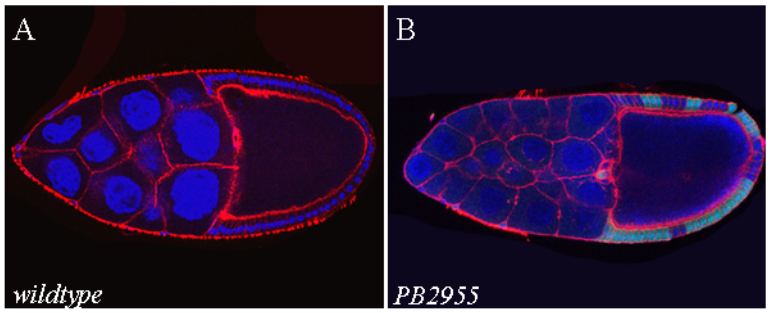

Figure 13. Abnormal nurse cells
Stage 10 egg chamber with 15 nurse cell nuclei (A) or more nurse nuclei in PB2955 germ line clone (B). DAPI staining is in blue and phalloidin staining is in red. Mutant clone is marked by lack of GFP (green).

3.2.3.5 Others

This class contains females with germline clones that can lay unfertilized eggs. There are no obvious oogenesis defects by phalloidin staining. To analyze this class further, I examined the localization of the axis determinants in some mutant ovaries. In wild type stage 9-10 egg chamber, Oskar localizes to the posterior pole of the oocyte (Figure 14A). About 10% of *PB3975* egg chambers showed Oskar mislocalization (Figure 14B). *PB3975* affects *cropped* which is predicted to be a transcription factor. I also observed Oskar anchoring defects in >10% of *PB4949* egg chambers (Figure 14C). PB4949 affects *pipsqueak*, a predicted transcription factor.

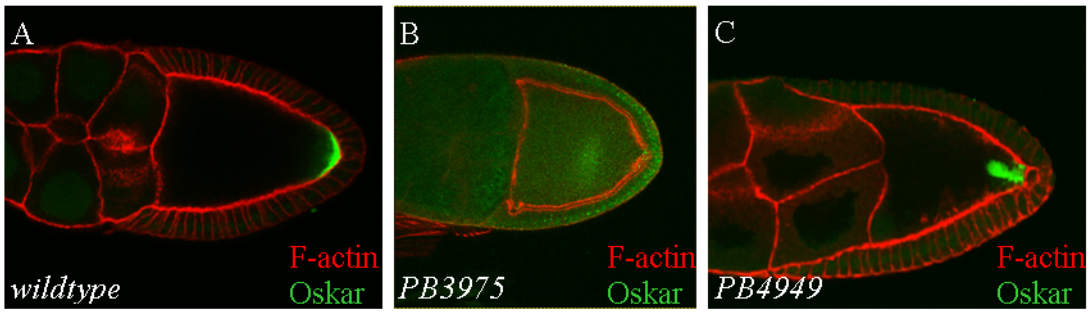

Figure 14. Oskar localization defects
(A) Wild type egg chamber. (B) *PB3975* germ line mutant egg chamber. (C) *PB4949* mutant egg chamber. Green is Oskar antibody staining, and red is phalloidin staining.

3.2.4 Mutations affecting embryo development

RNA and proteins produced by the nurse cells also contribute to early embryonic development. In this screen, I also identified genes required in the germ line that affect embryogenesis by checking the cuticle phenotype of embryos. I divided these mutations into 3 different classes.

3.2.4.1 Posterior group

A general feature in this class is that embryos lack abdominal segments and only contain the head and tail structure (Figure 15). Posterior group genes are involved in determination of germ cell fate and posterior cell fate in the embryos. *PB456* affects *hrp*

48, an RNA binding protein, which is already known to affect *oskar* RNA localization (Figure 15D) (Huynh et al., 2004; Yano et al., 2004). Four mutants, *PB122*, *680*, *1094*, *3747*, affect *vasa*, which is essential for the assembly of pole plasm, a special type of cytoplasm found in the posterior portion of the egg and early embryo (Figure 15E) (Schupbach and Wieschaus, 1986).

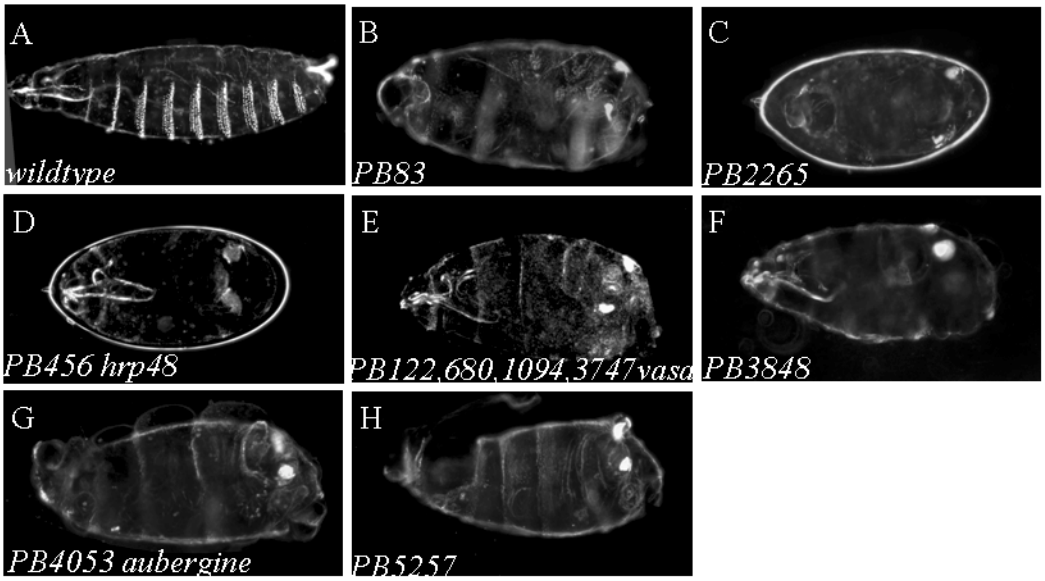

Figure 15. Posterior group phenotype
Anterior is left, and posterior is right in all panels. (A) wild type embryo cuticle. (B) *PB83*. (C) *PB2265*. (D) *PB456*. (E) *PB122, PB680, PB1094, PB3747*. (F) *PB3848*. (G) *PB4053*. (H) *PB5257*.

3.2.4.2 Segmentation defects

A general feature in this class is that the number of denticle belts is reduced or denticle belts are fused or mispatterned (Figure 16). Embryos from *PB5090*, affecting *eIF4E*, a translation initiation factor, show a pair-rule gene mutant like phenotype (Figure 16H). The *eIF4E* mutant enhances the phenotype of mutants in *ftz*, a pair-rule gene. But Kankel et al. did not see a pair-rule gene mutant like phenotype in their *eIF4E* mutant (Kankel et al., 2004). This may be due to the different alleles of *eIF4E* used.

One mutant in this category, *PB2691*, affects *CG17090* which encodes HIPK 2 kinase. *PB2691* are homozygous viable but embryos from homozygous females show denticle belt fusions (Figure 17B). Another transposon inserted in this region, *P{GT1}CG17090*^{BG00855} , is homozygous lethal. *P{GT1}CG17090*^{BG00855} and *PB2691* fail

to complement with each other or a *deficiency* lacking this region (Figure 17C). This suggested that both *PB2691* and *P{GT1}CG17090*^{BG00855} affect *CG17090*.

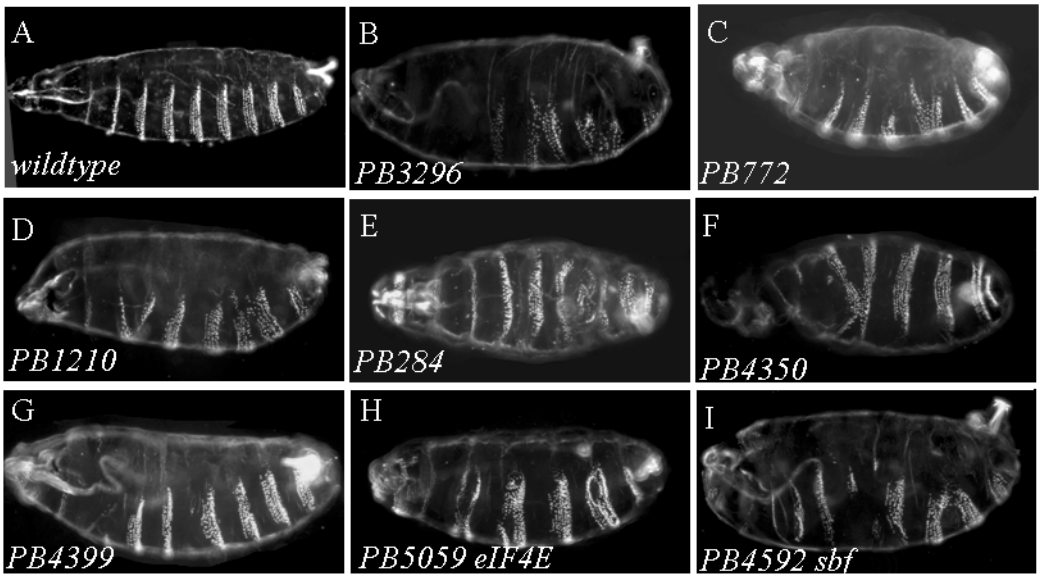

Figure 16. Segmentation defect
Anterior is left, and posterior is right in all panels. (A) wild type cuticle. (B) *PB3296*. (C) *PB772*. (D) *PB1210*. (E) *PB284*. (F) *PB4350*. (G) *PB4399*. (H) *PB5090*. (I) *PB4592*

In *Drosophila* embryogenesis, the A/P axis of the fly is structured by both the gradient of Bicoid and the posterior system that gives rise to Nanos localization (Nusslein-Volhard and Roth, 1989). Both Bicoid and Nanos gradients determine the location of gap gene transcription, such as *krupple*, *knirps*, and *giant*. Gap genes control the striped pattern of pair rule genes like *even-skipped (eve)*, and *fushi tarazu (ftz)*. These striped pattern leads to the segmental expression of segment polarity genes such as *wingless*, *hedgehog*, and *engrailed (en)* (Figure 17O). To test whether *PB2691* is directly involved in segmentation, I stained mutant embryos for En. Wild type stage 10-11 embryos express En in 14 stripes (Figure 17D). Some specific En stripes are missing or discontinuous in the *PB2691/Df* mutant embryos (Figure 17E). It suggested that HIPK2 is required in segmentation. As segment polarity genes are induced by Pair-rule genes, the loss of some En stripes may be due to a pair-rule gene defect. I examined expression of the pair-rule proteins, Eve and Ftz in *PB2691/Df* flies. Wild type embryos show 14 evenly spaced stripes, 7 Even-skipped stripes alternating with 7 Fushi tarazu stripes (Figure 17F). In *PB2691/Df* embryos, some specific pair-rule stripes are wider or narrower in mutant background and the distance between them is not always equal (Figure 17G). It is known that Pair-rule

gene regulation can be auto-regulatory (Schier and Gehring, 1992). To determine whether *PB2691* affected *gap* gene or *pair-rule* autoregulation, I checked the expression of the Pair-rule genes *PB2691/Df* embryo by in situ. Both *eve* and *ftz* in situ patterns are similar to the protein expression pattern in *PB2691/Df* background (Figure 17 I, K). This suggests that *PB2691* affects *gap* gene or the regulation of *pair-rule* genes by Gap protein, but not *pair–rule* gene autoregulation. To further test this, I check *gap* gene expression in the mutant background. Preliminary data suggests, that *gap* gene expression is not affected (Figure 17L, M). This suggested that *PB2691* does not affected *gap* gene expression pattern but may affect the gap gene regulatory network. Zhang et al. found that HIPK2 negatively regulates CtBP (Zhang et al., 2003). And CtBP, a transcriptional co-repressor, is involved in Gap protein repression in *Drosophila* (Nibu and Levine, 2001; Nibu et al., 1998; Strunk et al., 2001). These data suggest that the role of HIPK2 may warrant further study.

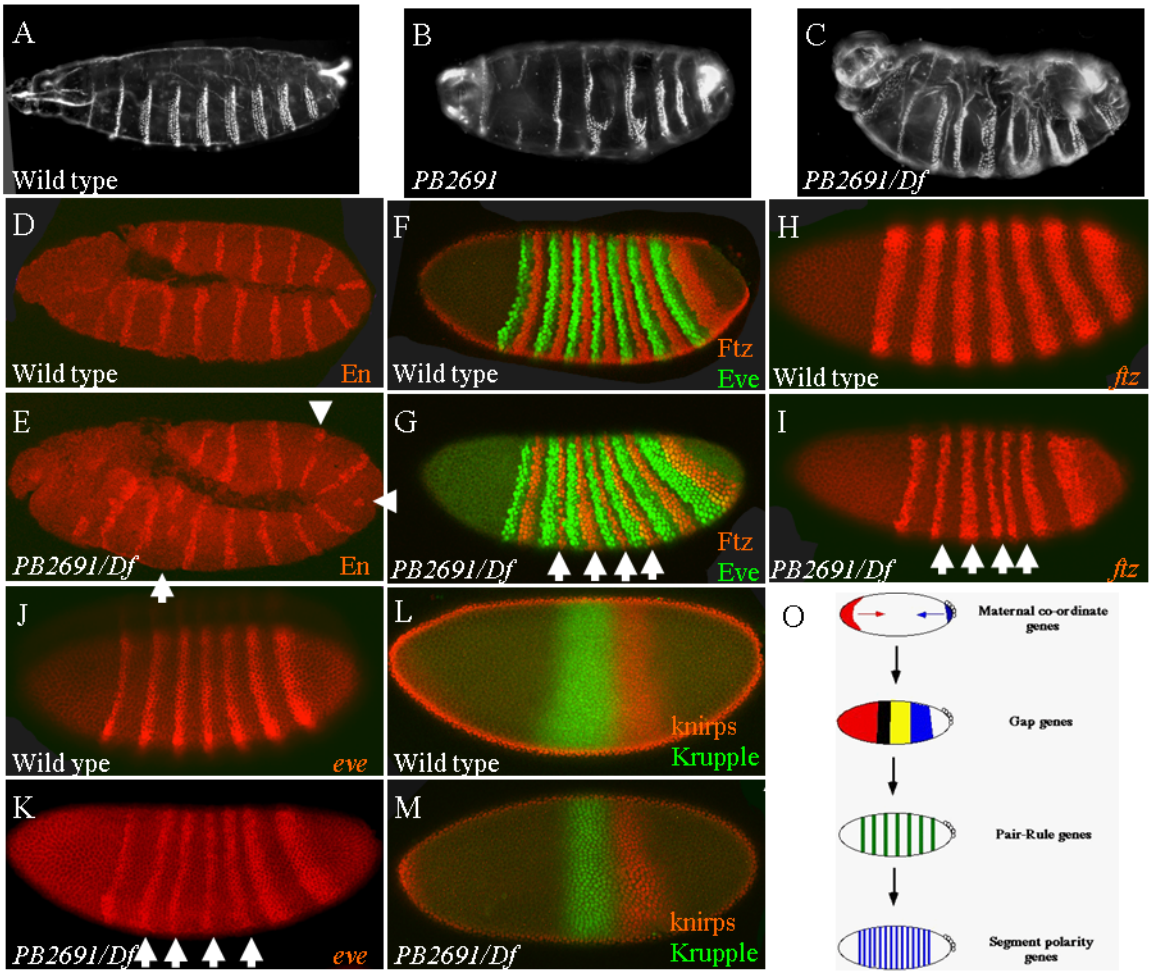

Figure 17. *PB2691* mutant phenotype
(A) wild type cuticle with head structure, tail structure and eight denticle belts. (B,C) *PB2691* (B) or *PB2691/Df* (C) cuticle still form head and tail structures but show disordered segmentation. Antibody staining detecting En (red) in wild type (D) or *PB2691/Df* (E). Arrows indicated these engrailed stripes that are missing or discontinuous. Antibody staining against Eve (green) and Ftz (red) in wild type (F) or *PB2691/Df* (G) . (G) Arrows indicated where the Ftz or Eve stripes are wider or narrower. Note that more cells are co-stained with Ftz and Eve. In situ hybridization detecting *ftz* in wild type (H) or *PB2691/Df* (I). Arrows indicated that *ftz* stripes are narrower and the distance of two stripes is not in order. In situ hybridization detecting *eve* in wild type (J) or *PB2691/Df* (K). Arrows indicated where *eve* stripes are narrower and the distance of two stripes is altered. Antibody detecting krupple (green) and knirps (red) in wild type (L) or *PB2691/Df* (M). (O) Cartoon of embryo segmentation. Adapted from Dynamic development.

3.2.5 Others

This class contains mutants whose phenotype does not belong to any of the above classes. *PB3292* affected the transcription factor *dorsal*, which activates and represses zygotic genes responsible for dorsal-ventral axis patterning in early stages of development. In the dorsal mutation, ventral cell fates are not formed, and embryos adopt a dorsal cell fate. *PB3292* mutant embryos show the dorsalization phenotype (Figure 18B). Embryos from PB4170 or PB4496 die at early stages and do not form cuticle (Figure 18E, F).

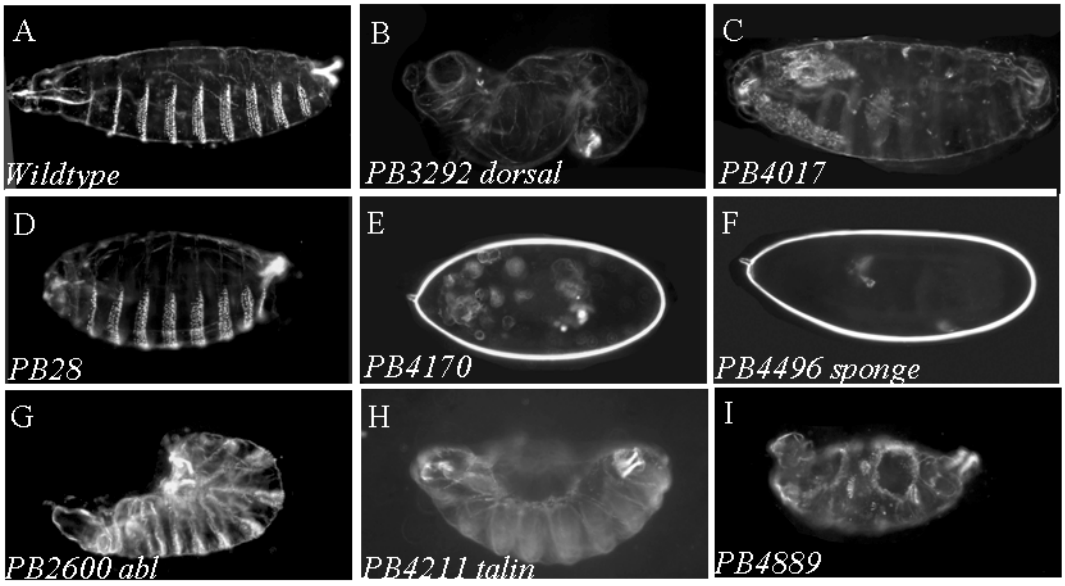

Figure 18. Others
Anterior is left, and posterior is right in all panels. (A) wild type cuticle. (B) *PB3292*, dorsalized cuticle. (C) *PB4017* (D) *PB28*, smaller embryo. (E) *PB4170*. (F) *PB4496*. (E,F) early embryo defects. (G) *PB2600*, germband retraction defect. (H) *PB4211*, dorsal opened embryo. (I) *PB4889*, opened embryos.

3.3 Ensconsin

3.3.1 PB4170 affects Ensconsin

From the screen, I retrieved one PiggyBac line, *PB4170*, which is homozygous viable but embryos laid by homozygous females arrest at early embryo stages (Figure 19B). By inverse PCR, I found that *PB4170* is inserted in the second intron of *CG14998* (*ensconsin*) (+1476 relative to transcription start site). I obtained another P element line, *P{RS3}CB-5457-3* which is inserted in the first exon of *CG14998* (at +30 relative to transcription start site) from the Bloomington stock center (Figure 19E). The embryonic phenotype of *P{RS3}CB-5457-3(PCB5457)* is similar to that of *PB4170*. To confirm that the phenotype is caused by the transposon insertions, I did a complementation test between *PB4170* and *PCB-5457* or between *PB4170* and a *deficiency*(*Df*) removing *ensconsin* and other neighboring genes. Embryos from both trans-heterozygous females gave embryos which died at early stages, like *PB4170* (Figures 19C, D). This suggests that both the PiggyBac and P element alleles affected *enconsin*, which is required in germ line for embryogenesis.

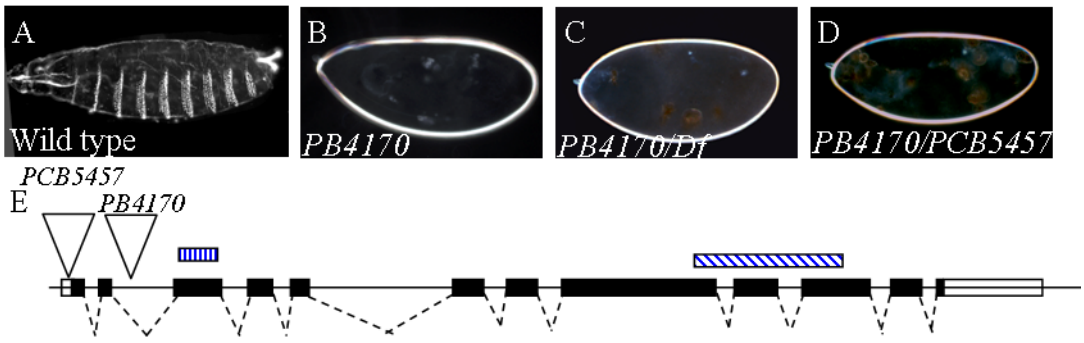

Figure 19. *PB4170* embryo phenotypes and genomic structure
(A, B, C, D) cuticle preparation. (A) Wild-type embryo. (B) Embryos from *PB4170* females. (C) Embryos from *PB4170/Df* females. (D) Embryos from *PB4170/PCB5457* females. (E) Schematic of isoform B of ensconsin and two arrows indicated the *PB4170* insertion and *PCB5457* insertion. The black bar marks the coding region; the white bar shows untranslated regions. The two blue bars mark regions conserved with human E-MAP115.

3.3.2 N-terminal region of Ensconsin can bind to microtubules

The *ensconsin* transcript is predicted to form five different splice forms (A, B, C, D, and

E) which differ in the 5' region and share most of 3' region. I found by RT-PCR that isoform B is the most highly expressed in wild type ovaries. In *PB4170* mutant ovaries, two more splicing forms are seen within the PiggyBac sequences, confirming that the PiggyBac insertion affects *ensconsin* (*ens*).

Ensconsin is predicted to be a microtubule associated protein with two conserved, highly charged regions, EHR1 and EHR2. There are two mouse homologues, E-MAP-115 and RPRC1 (arginine proline rich coiled-coil protein 1). Vertebrate E-MAP-115 has already been analyzed. When compared to the mouse homologue, E-MAP-115 shows 31% identity and 60% similarity in the EHR1, and 43% identity and 62% similarity in the EHR2 domain (Figure 20A). Masson and Kreis identified human E-MAP115 as a microtubule binding protein in cell culture (Masson and Kreis, 1993). To test whether *Drosophila* Ensconsin is also a microtubule binding protein in *Drosophila*, I performed a microtubule binding assay with Taxol (Karpova et al., 2006). Taxol, a yew tree extract,

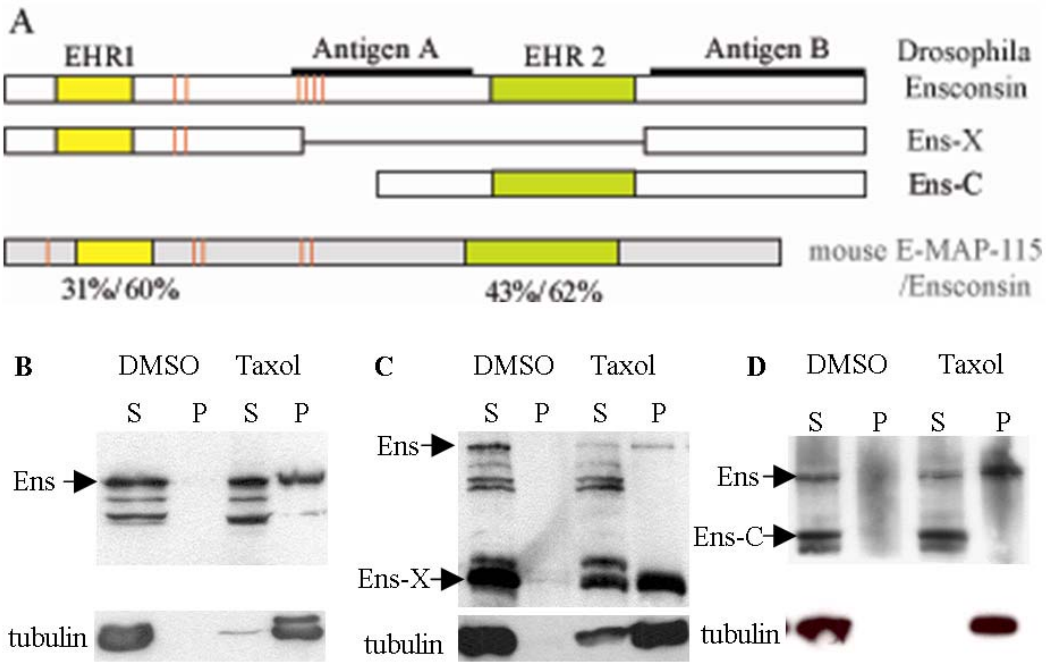

Figure 20. N-terminal of Ensconsin can bind microtubules
(A) Schematic of Ensconsin protein. EHR1 (Ensconsin homologue region 1) is shown as the yellow bar, and EHR2 (Ensconsin homologue region 2) is represent by the green bar. White bar represents *Drosophila* protein and gray bar is mouse E-MAP-115. Black bars mark the A and B antigens region. Identity and similarity between *Drosophila* and mouse E-MAP-115 are shown below the gray bar. (B, C, D) Microtubule binding assay showing supernatant (S) or pellet (P) after incubation of cleared exact with either Taxol or DMSO control. (B) 0-2 hours wild type embryo extracts (C, D) ovaries from *tub-Ens-x* or *tub-Ens-C* transgenic females. The blot was probes with anti-Ensconsin B and anti-tubulin.

binds and stabilizes microtubules. When microtubules are polymerized and stabilized with taxol, they can be spun down as a pellet. If a protein is microtubule associated, most of the protein will pellet with the microtubules. Such microtubule binding assay is frequently used to identify microtubule associated protein (MAPs). For microtubule binding assay, I produced two antigen fragments of E-MAP-115: antigen A (284-499 amino acids) and antigen B (767-851 amino acids) (Figure 20A). I used these to immunize rats and generate two polyclonal antibodies. When this assay is performed using taxol treated 0-2 hours wild type embryo extracts, most microtubules are sedimented, demonstrating that Taxol functions in this experiment. In this experiment I found that Ensconsin co-sediments with microtubules. This suggested that full length Ensconsin can bind microtubules in embryonic extract (Figure 20B).

The EHR1 domain of the human protein (77-228) is essential for microtubule binding (Masson and Kreis, 1993). To examine which regions of *Drosophila* Ensconsin are important for microtubule binding, two constructs, lacking either the EHR2 (Ens-X) or EHR1 domains of Ensconsin (Ens-C) were created by Pernille Rørth. From these, I generated transgenic flies which express the transgenes under control of tubulin promoter. I found that Ens-X could co-sediment with taxol treated microtubules (Figure 20C), but Ens-C could not. (Figure 20D). It has been suggested that the N-terminal region of *Drosophila* Ensconsin is required for microtubule binding as human homologue.

3.3.3 20% of PB4170 egg chambers showed Oskar and Staufen mislocalization

Microtubules are important for the transportation of axis-determinants during oogenesis. As Ensconsin is a microtubule binding protein, it seemed logical to test whether it played a role in these events. I examined the localization of the axis determinants Oskar and Gurken, in *PB4170* mutant ovaries. In wild type, from stage 9, Oskar protein is translated at, and localized to the posterior pole of the oocyte (Figure 21A'). Around 20% of *PB4170* egg chambers show Oskar accumulation in the middle of the oocyte (Figure 21 D'). Oskar protein localization depends on its mRNA localization. I therefore analyzed *oskar* mRNA localization by in situ hybridization and Staufen antibody staining. Staufen, a RNA binding protein, binds *oskar* RNA in the oocyte. Normally, in stage 9 egg chambers, *oskar*

mRNA is localized to the posterior pole of the oocyte in a pattern similar to Oskar protein (Figure 21A) and Staufen is also found at the posterior pole (Figure 21B). *oskar* mRNA and Staufen also are mislocalized in 20% of *PB 4170* egg chambers (Figure 21D,E). I also checked Gurken antibody staining. Gurken is usually localized to the anterior-dorsal site of the oocyte and the protein extends along the oocyte cortex to posterior pole (Figure 21C). In *PB4170*, Gurken is restricted to the anterior-dorsal side and extends less towards the posterior pole, and some punctate stainings are also found in the middle of the oocyte (Figure 21F). Interestingly, the localization of *bicoid* is unaffected (data not shown). This shows that the localizations of *oskar* mRNA, Oskar and Gurken proteins are affected, but localization of *bicoid* is not affected in *PB4170* mutants.

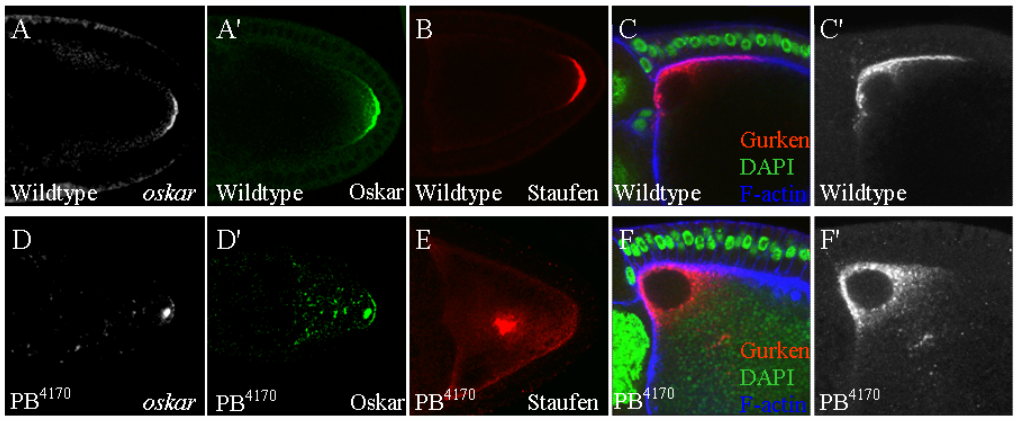

Figure 21. Oskar, Staufen and Gurken are mislocalized in PB4170
(A, B, C) wild type egg chambers. (D, E, F) PB4170 homozygous mutant egg chambers. (A, D) show *oskar* mRNA in situs. (A', D') show Oskar antibody staining. (B, E) show Staufen antibody staining. (C, E) red is Gurken antibody staining, green is DAPI and blue are phalloidin. (C', E') only Gurken antibody staining is shown.

3.3.4 Generating new alleles of *ensconsin*

Western blots and immunostaining of PB4170 ovaries show that the mutant is not a protein null allele. To fully investigate the function of Ensconsin, I created two new mutant alleles, $ens^{\Delta C}$ and $ens^{\Delta N}$. $ens^{\Delta N}$ was created by imprecise excision of *PCB-5457*. $ens^{\Delta N}$ (removing from +31-+1475 of the gene) homozygous flies are viable but embryos laid by homozygous females die at early stages, much like *PB4170* (Figure 22A). $ens^{\Delta C}$ was created by end-out homologous recombination (Gong and Golic, 2004). In $ens^{\Delta C}$, two third of Ens coding region, including EHR2 are deleted and replaced by the *mini-white* cassette (from +7077 to +9632, Figure3-13A). $ens^{\Delta C}$ homozygous flies are

viable but sick and most display wing eversion defects (Figure 22C). Embryos laid from homozygous females die at early stages. *ens* $^{\Delta C}$ mutant phenotypes can be completely rescued by *tub-ens*. It suggests that the phenotypes of *ens* $^{\Delta N}$ and *ens* $^{\Delta C}$ are caused by the deletion of *ens*. Western-blot analysis of mutant ovary extracts indicated that the mutant had decreased levels of Ensconsin protein (*wild type>PB4170>ens*$^{\Delta N}$*>ens*$^{\Delta C}$) (Figure 22B). *ens* $^{\Delta C}$ protein levels are hard to measure as the antigen region is removed in this mutant. In *ens*$^{\Delta N}$ mutant ovaries, the Ensconsin protein still present may due to alternate splice forms. I decided to perform most subsequent experiments with *ens*$^{\Delta C}$ flies.

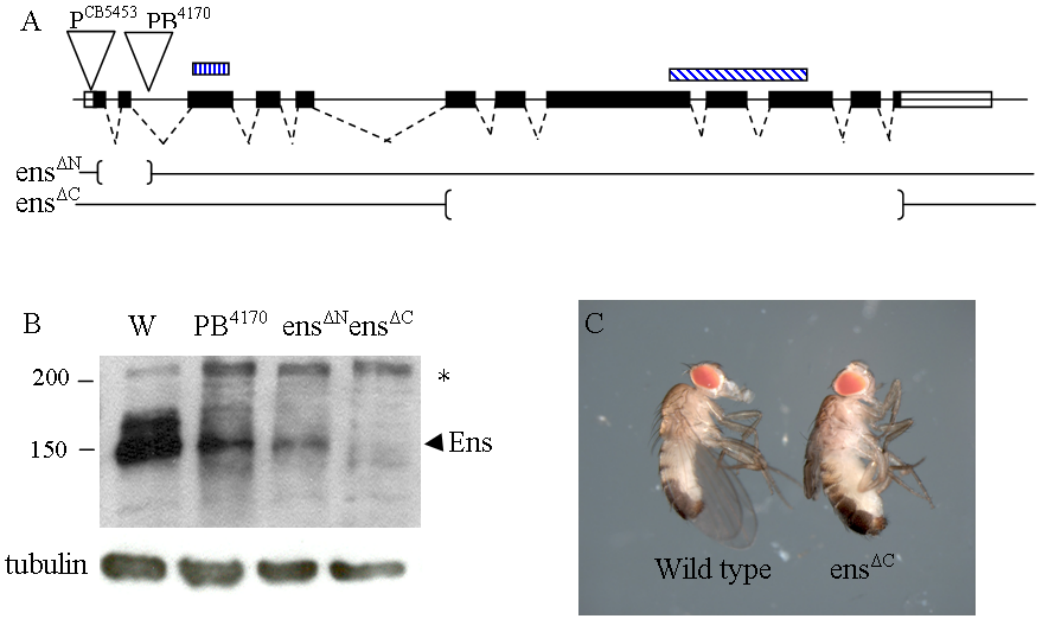

Figure 22. New mutant alleles of *ensconsin*
(A) Schematic of *ensconsin* gene structure. (B) Western blot of different mutants. Ensconsin is about a ~150kD protein. In *PB4170*, and *ens* $^{\Delta N}$, protein level is reduced. Note that the epitope is absent in *ens* $^{\Delta C}$. Tubulin is shown as a loading control. A Star indicates a background band. (C) Left is wild type adult, and right is *ens* $^{\Delta C}$ adult. Both are 1 day old. In *ens* $^{\Delta C}$, the wing can not properly expand.

3.3.5 Oskar, Staufen and Gurken localization are affected but *bicoid* is not in *ens*$^{\Delta C}$ mutant

My experiments with PB4170 suggest that Ensconsin is required for Oskar and Gurken localization. I decided to re-examined Oskar, Gurken, and *bicoid* in *ens*$^{\Delta C}$ mutants. *ens*$^{\Delta C}$ mutant stage 9 egg chambers show less accumulation of Staufen and Oskar protein at the

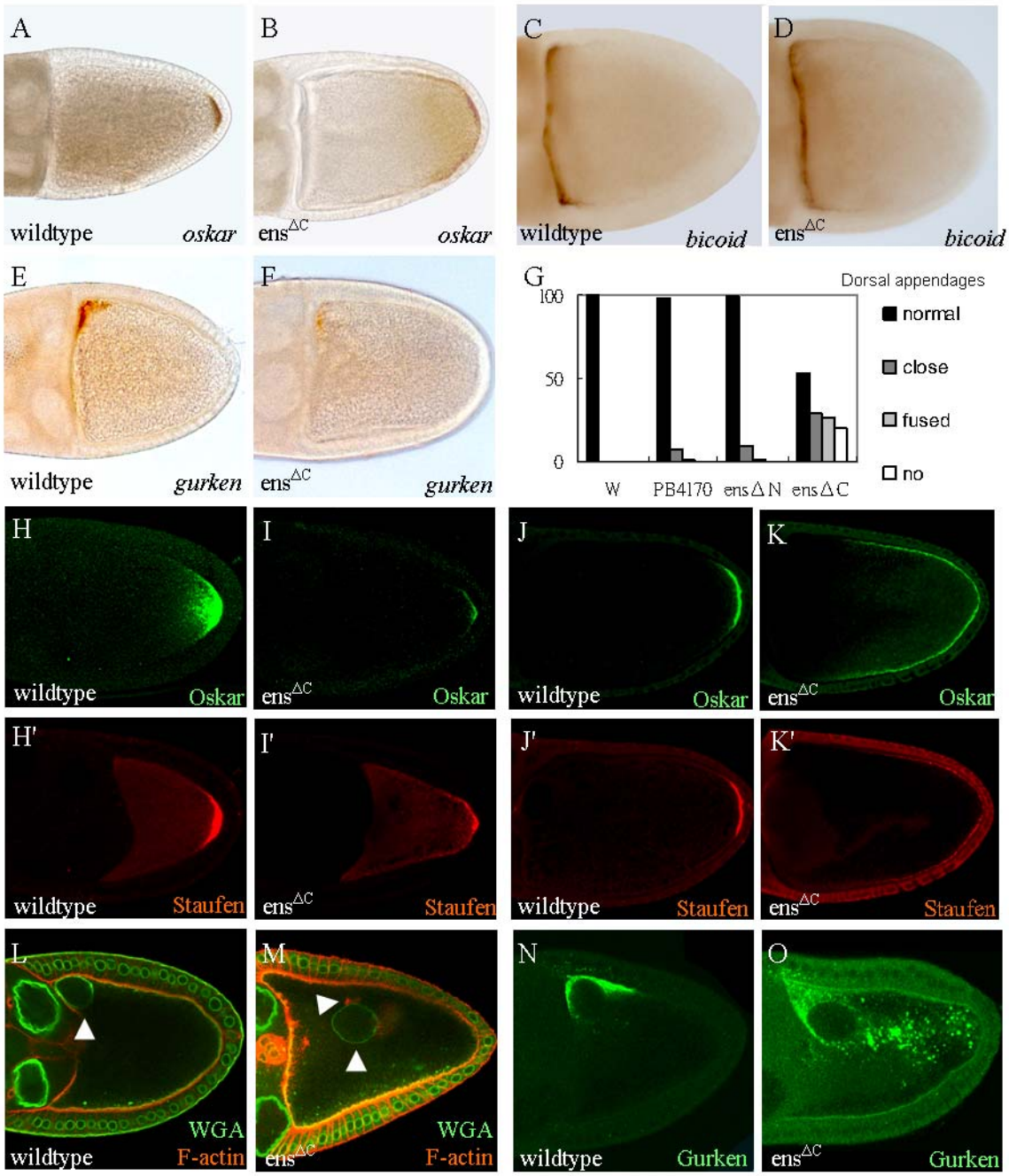

Figure 23. *ens*$^{\Delta C}$ mutant phenotype
(A-F) in situ hybridization of *oskar* in wild type (A) or in *ens*$^{\Delta C}$ (B), *bicoid* in wild type (C) or in *ens*$^{\Delta C}$ (D), and *gurken* in wild type (E) or in *ens*$^{\Delta C}$ (F). (G) Quantification of dorsal appendages in w, *PB4170*, *ens*$^{\Delta N}$, and *ens*$^{\Delta C}$. (H-K) antibody staining at stage 9 in wild type (H) or in *ens*$^{\Delta C}$ (I), at stage 10B or 11 egg chamber in wild type (J) or in *ens*$^{\Delta C}$ (K).Oskar staining is in green and Staufen in red. (L, M) nuclei marked by WGA staining and F-actin marked by phalloidin in the wild type (L) or in *ens*$^{\Delta C}$ (M) late stage 9 egg chambers. Arrows indicate the oocyte nucleus and abnormal accumulation of F-actin. (N, O) antibody staining detecting Gurken (green) in wild type (N) or in *ens*$^{\Delta C}$ (O). The oocyte nucleus can be seen as the round, unstained area, slightly mislocalized in (O)

posterior pole. Staufen is also seen around the oocyte cortex (Figure 23 I, I'). In *ens*$^{\Delta C}$ stage 10 egg chambers, both Staufen and Oskar showed a diffuse staining along a large section of the anterior cortex (Figure 23K, K'). Oskar protein is still translated and remains attached to the oocyte membrane. This suggests that Ensconsin is not involved in Oskar translation or anchoring. Normally, *oskar* mRNA is accumulated at the posterior pole of the oocyte at stage 10 (Figure 23A). In mutants, *oskar* mRNA, like Oskar protein is spread along the anterior cortex (Figure 23B). This confirms that Ensconsin is required for *oskar* localization.

gurken mRNA and protein were localized in the anterior-dorsal corner of egg chambers in wild type egg chamber (Figure 23N and E). In *ens*$^{\Delta C}$ mutant egg chamber, *gurken* mRNA levels appear to be reduced but still correctly localized (Figure 23F). Gurken protein was observed not only to be loosely cortical localization at anterior-dorsal site but also in the middle or posterior parts of the oocyte (Figure 23O). Gurken signaling is required to reorganize the microtubule network. This allows the oocyte nucleus to migrate from the posterior pole to the anterior-dorsal side of the oocyte at mid-oogenesis. Wheat germ agglutinin (WGA) is a carbohydrate-binding protein that selectively recognizes sialic acid and N-acetylglucosaminyl sugar residues which are predominantly found on the plasma membrane. I used WGA-conjugated with FITC to label the oocyte nucleus membrane. In wild type egg chambers, all oocyte nuclei localize to the anterior-dorsal part of the oocyte (Figure 23L). Around 10% of *ens*$^{\Delta C}$ mutant egg chamber show a strong mislocalization of the oocyte nucleus and the others show weaker mislocalization (Figure 23O). Abnormal F-actin accumulations are also found within the oocyte in *ens*$^{\Delta C}$ mutant egg chambers (Figure 24M).

Around 50% of *ens*$^{\Delta C}$ eggs showed dorsal appendage fusions which corresponds to the mis-localization of Gurken but few fusions are seen in *ens*$^{\Delta N}$ or *PB4170* egg chamber (Figure 23G). *bicioid* mRNA remains localized to the anterior of the oocyte in *ens*$^{\Delta c}$ mutant egg chamber (Figure 23D). This confirms that Ensconsin is required for Oskar and Gurken localization, but not *bicoid*.

3.3.6 *khc* interacts genetically with *ensconsin*

The Oskar and Gurken mislocalization phenotypes of $ens^{\Delta C}$ are similar to *kinesin heavy chain (khc)* mutant phenotypes but weaker. Kinesin heavy chain is a microtubule motor protein which moves its cargo toward the plus ends of microtubules. To test whether *ensconsin* interacts with *khc*, I anaylzed a genetic interaction between $ens^{\Delta C}$ and khc^{27} (null allele). I observed a strong interaction between *khc* and *ensconsin*, as flies lacking one copy of *khc* and homozygous for $ens^{\Delta C}$ are lethal. 10% of $ens^{\Delta C}$ egg chambers show oocyte nucleus mislocalization phenotype, adding one copy of khc^{27} mutant in $ens^{\Delta C}$ germ line clone background increases the oocyte nucleus mislocalization rate to 32%. This suggests that *khc* interacts with *ensconsin* genetically.

3.3.7 Ensconsin affects posterior localization of Khc

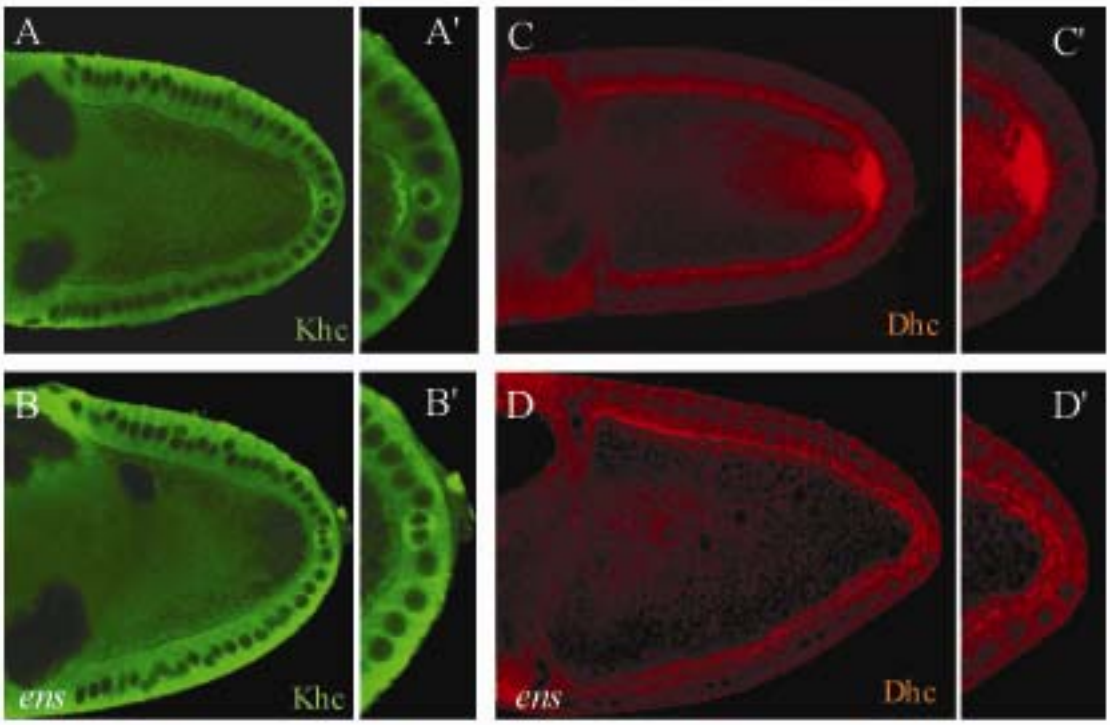

Figure 24. Ensconsin affects Dhc localization through Khc mislocalization
Antibody staining of Khc (green) at stage 9 in wild type (A) or in $ens^{\Delta C}$ (B). Antibody staining of Dhc (red) at stage 10 in wild type (C) or in $ens^{\Delta C}$ (D). Enlargements of posterior part of the egg chamber (A', B', C', D').

As I found that *khc* interacts genetically with *ensconsin*, I wanted to test if Ensconsin affects the localization of Khc. To examine this, I did Khc antibody staining in the $ens^{\Delta C}$

mutant background. In wild type stage 9 egg chambers, Khc is enriched the oocyte, and strongly accumulated in the posterior pole of the oocyte (Figure 24A). However in *ens*$^{\Delta C}$ mutants, Khc is still enriched in the oocyte, but no longer accumulated at the posterior pole (Figure 24B). Dynein heavy chain (Dhc), a minus-end directed motor protein, localizes to the posterior pole of the oocyte at stage 9-10 egg chamber. This localization requires Dhc to be transported in the oocyte by Khc (Figure 24C) (Januschke et al., 2002). Dhc is also mislocalized in stage 9-10 *ens*$^{\Delta C}$ mutant egg chambers (Figure 24D). Interestingly, Dhc is involved in early oocyte specification, but I did not find similar defect in the *ens*$^{\Delta C}$ mutants (McGrail and Hays, 1997). This suggests that Ensconsin affects Dhc indirectly, through the localization of Khc to the oocyte.

3.3.8 Microtubule polarity is not affected in *ens*$^{\Delta C}$ mutant

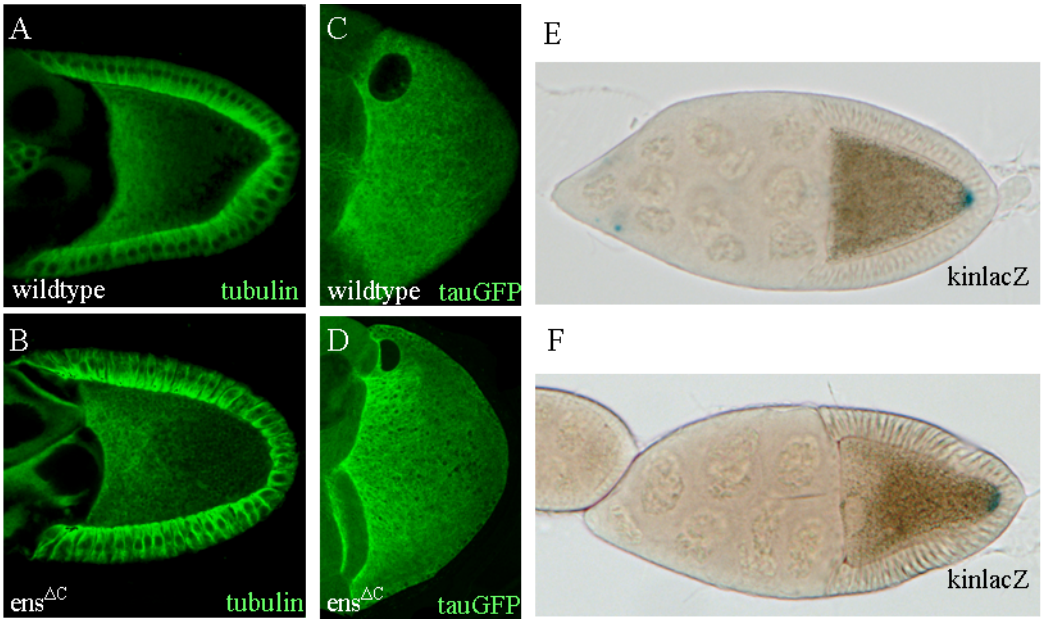

Figure 25. Microtubule formation and microtubule polarity in *ens*$^{\Delta C}$ mutant
Antibody staining of tubulin (green) at stage 9 in wild type (A) or in *ens*$^{\Delta C}$ (B). Images of live, early stage 9 samples expressing tauGFP in wild type (C) or in *ens*$^{\Delta C}$ (D). X-gal staining of Kinesin-lacZ at late stage 9 in wild type (E) or in *ens*$^{\Delta C}$ (F).

The effect of Ensconsin on Khc and Dhc localization may be caused in several ways: (1) microtubule formation defects, (2) microtubule polarity defects or (3) Impaired Khc movement. To test whether Ensconsin affects microtubule formation and microtubule polarity, I looked at microtubule staining and checked microtubule polarity markers in *ens*$^{\Delta C}$ egg chambers. In wild type egg chambers, microtubules are enriched in the anterior

part of the oocyte (Figure 25A). In *ens*$^{\Delta C}$ mutants, microtubules are also enriched in the anterior region (Figure 25B). In wild-type oocytes, microtubules are organized in an anterior to posterior gradient at stage 7–9 that can be visualized by using a Tau: GFP fusion protein (Figure 25C) (Micklem et al., 1997). Tau: GFP expression pattern is unaffected in *ens*$^{\Delta C}$ mutant oocyte (Figure 25D). Furthermore, microtubules are essential for early oocyte specification, as no oocyte is seen in egg chambers by treating with microtubule disrupting drugs (Theurkauf et al., 1993). The fact that *ens*$^{\Delta C}$ mutants still can specify an oocyte implies that Ensconsin is not simply required to assemble or maintain microtubules. To check microtubule polarity, I used Kinesin-LacZ, LacZ fused to Khc, lacking the C-terminal regulatory domain (Clark et al., 1994). This fusion protein is enriched at the posterior end of the oocytes and marks microtubule plus-end in wild type stage 9 egg chamber (Figure 25E). Kin-LacZ was unaffected in *ens*$^{\Delta C}$ mutants (Figure 25 F). This suggests that Ensconsin does not affect microtubule polarity.

3.3.9 Ooplasmic streaming is affected in *ensconsin* mutants

In late stage 10 egg chamber, the nurse cells start to pump RNA and proteins into the oocyte by a process referred to as cytoplasmic dumping. At the same time, the oocyte begins ooplasmic streaming. This process utilizes microtubules and motor protein to help distribute the content from the nurse cell within the oocyte (Theurkauf et al., 1992). Without ooplasmic streaming, nurse cells are still able to pump RNA and proteins into oocyte, but the lack of mixing causes a stratified ooplasm (Serbus et al., 2005). This stratification is due to an accumulation of yolk granules near the posterior of the oocyte and leaving a clear zone at anterior zone. This streaming can be visualised by imaging and tracking the large, yolk-filled endosomes in live egg chambers (Figure 26A). The speed and onset of streaming are sensitive to levels of Khc. Several *khc* point mutants display reduced or completely halted ooplasmic streaming. *khc* mutant egg chambers also show stratified ooplasm. As Ensconsin affects Khc localization, I decided to examine ooplasmic steaming in *ensconsin* egg chambers. In the wild type egg chambers, yolk granules are equally distributed (Figure 26C). All *ensconsin* mutant egg chambers show stratified ooplasm (Figure 26D). This suggests that ooplasmic streaming is blocked. To confirm this, I recorded of ooplasmic streaming. 7/8 wild type egg chambers show normal streaming (Figure 26A) but 16/16 *PB4170* egg chambers showed streaming defects

(Figure 26B). In *khc*^{27}/+; *ens*$^{\Delta C}$/+, there are around 10% stratified egg chambers. It suggested that Ensconsin affects Khc-dependent transport.

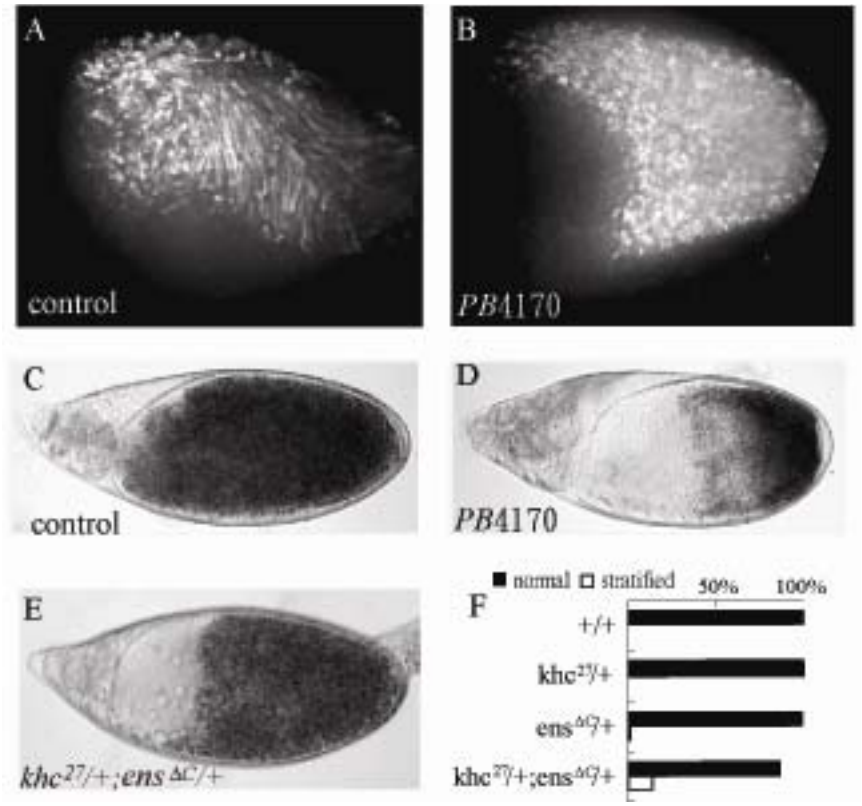

Figure 26. Ooplasmic streaming is abolished in *ens* mutants
(A,B) Yolk granules movement visualized by overlay multiple frames from movies from stage 11 (A) wild type or (B) *PB4170* mutants. The moving particles are seen as streaming in (A), but not in all *ensconsin* mutants (B). (C,D,E)Nomarski images of late stage egg chamber from control (C), *PB4170* (D), and transheterozygous *khc*^{27}/+;*ens*$^{\Delta C}$/+ (E) females. The dark material is yolk granules. (F) Quantification of stratified eggs in different mutant.

3.3.10 Ensconsin is required in neurons

Khc-dependent transportation is not only required to localize axis determinants in the oocyte, but is also for the delivery of synaptic components to the tips of axons. To examine whether Ensconsin is also important in the neuron, I checked for potential neuronal defects in *ens*$^{\Delta C}$ mutant. Most wild type flies climb to top of a vial in several seconds. *ens*$^{\Delta C}$ mutant showed wing eversion defects and coordination problems preventing them from climbing (Figure 27A). *ens*$^{\Delta N}$ mutants show normal wing eversion, but also show climbing defects (Figure 27A). Both the wing eversion defects and climbing defects can be rescue by expressing *UAS-ens* with *elavGal4*, a pan neuron driver. Unsurprisingly, neuronal expression can not rescue the observed oogenesis defects

(Figure 27A).

At late larval stages Ensconsin protein is also enriched in the axons connecting the optic lobe to the eye disc (Figure 27B). This suggests that Ensconsin is required for neuronal function. Synaptotagmin (Syt) is a major component of synaptic vesicles (DiAntonio et al., 1993). In Khc mutants, Syt accumulates in the axons (Hurd and Saxton, 1996). Ensconsin affecting Khc transportation in the oocyte and enrichment of Ensconsin in the axon suggest that the neuron function of Ensconsin may also be to promote Khc transportation. To look more directly at kinesin-dependent transport in neurons, I did Syt antibody staining in *ens*$^{\Delta C}$ mutant larvaes. In wild type larvae, syt is efficiently transported to synapses and no staining is seen in the axons (Figure 27C). In *ens*$^{\Delta C}$, I see accumulation of Syt along the axon indicating inefficient transport to the tip (Figure 27B). This also suggests that Ensconsin is required for efficient kinesin-dependent transport in multiple tissues.

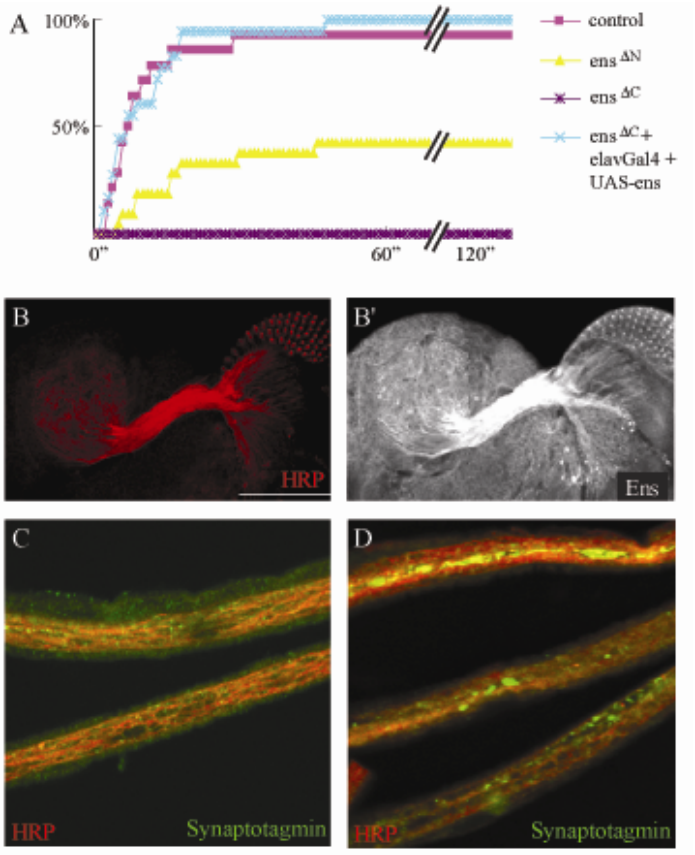

Figure 27. Neuronal functions of Ensconsin
(A) Climbing assay; A single male was put into the empty vial, tapped to the bottom and their climb to the top was timed. The graph shows the cumulative success rate of different genotypes. (B) HRP staining, which marks axons in red and Ensconsin antibody staining (white in B') of axons in the optic stalk (C,D)HRP (red) and Syt staining (green) in control (C) or *ens*$^{\Delta C}$ mutant (D). Syt accumulation in the axon shows impaired transport (D).

3.3.11 Ensconsin is localized to the anterior of the oocyte

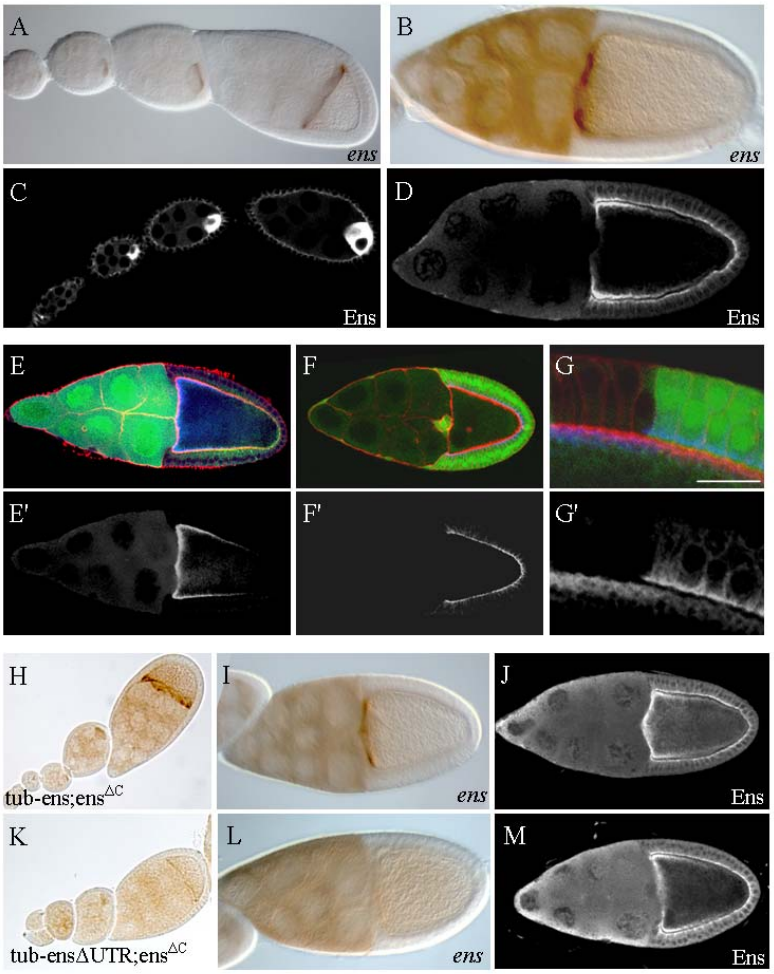

Figure 28. Drosophila Ensconsin is tightly localized during oogenesis
Anterior is left in each panel. (A,B) In situ hybridization of endogenous *ensconsin* in early (A) and stage 10 (B) egg chambers. (C,D) Antibody staining of endogenous Ensconsin protein early (C) and at stage 10 (B). (E-G) Staining of Ensconsin when all follicle cells (E), the germ line (F), or some follicle cells (G) are mutant (*ens*$^{\Delta C}$). Mutant cells are marked by lack of GFP (green); phalloidin (red). (E'-G') Show only the anti-Ensconsin channel. In situ hybridization detecting transgenically expressed *tub::ens* (H-I) or *tub::ensΔ3'UTR* (K,L) in *ens*$^{\Delta C}$. The diffuse and delocalized signal in K and L is significantly above background. Staining of transgenically expressed tub::ens (J) or *tub::ensΔ3'UTR* in *ens*$^{\Delta C}$(M). *ens*$^{\Delta C}$ alone shows no signal by in situ or antibody staining.

Correct localization is important in the regulation of motor protein transport. From in situ data, the *ensconsin* transcript is enriched in the oocyte from early stages (Figure 28A) and localizes to the anterior of the oocyte from stage 9 (Figure 28B). Ensconsin protein is also enriched in the oocyte from early stages (Figure 28C) and begins to form a gradient along the cortex from anterior to posterior at stage 9 (Figure 28E, D). Ensconsin protein is also

localized apically in follicle cells (Figure 28D). Apical localization of Ensconsin has been reported in the mouse intestinal epithelium cells, indicating that this is a conserved feature of this protein (Fabre-Jonca et al., 1998). This staining pattern is seen with both antibodies I generated and no signal can be detected in $ens^{\Delta C}$ mutant cells (which lack the epitope). (Figure 28E-G) The polarized localization of Ensconsin in the soma and germ line is similar to the distribution of microtubule minus ends in these tissues.

To determine the mechanism of Ensconsin localization, I first focused on the mRNA. In the cases of *oskar* and *bicoid*, the 3'UTR is an important determinant of RNA localization (Kim-Ha et al., 1993; Macdonald and Struhl, 1988)**.** I reasoned that as *ensconsin* has a 1.28Kb 3'UTR, this may play a role its localization. Pernille Rørth created *tub:ens* constructs with and without the *ensconsin* 3'UTR, from these I generated transgenic flies. When wild type *ensconsin* is expressed in $ens^{\Delta C}$ mutants, *ensconsin* mRNA and protein are correctly localized (figure 28H-J). Expression of *ensconsin* without its 3'UTR in $ens^{\Delta C}$ mutant results in mislocalization of the transcript (Figure 28K, L), but normal protein localization (Figure 28M). This suggests that 3'UTR is required for RNA localization, but that this is not essential for protein localization. It also suggests that there is another mechanism regulating Ensconsin protein localization. The polar localization of Ensconsin, in the follicle cells and the oocyte suggests that Par proteins may regulate its localization.

3.3.12 Ensconsin localization is affected by Par-1

Par-1 is localized to the posterior pole of the oocyte and the basal-lateral domain of follicle cells, and its localization does not overlap with Ensconsin. To test whether Par-1 regulates Ensconsin localization, I used a Par-1 hypomorphic combination ($par\text{-}1^{W3}$ / $par\text{-}1^{9A}$). This situation allows the need for Par-1 function in oocyte cell fate maintenance in early oogenesis, allowing me to analyze its potential later role in Ensconsin localization. In this genotype, most egg chambers are phenotypically normal (Figure 29A), but some showing mislocalization of posterior markers such as Staufen. In *Par-1* mutants, Ensconsin localization is clearly affected and it is no longer excluded from the posterior pole of the oocyte (Figure 29B). In $par\text{-}1^{W3}$ mutant follicle cells (which lack any par-1 function), Ensconsin is no longer apically localized, but is evenly distributed throughout

the cell (Figure 29C, D). This suggests that Par-1 functions, either directly or indirectly, to restrict Ensconsin localization.

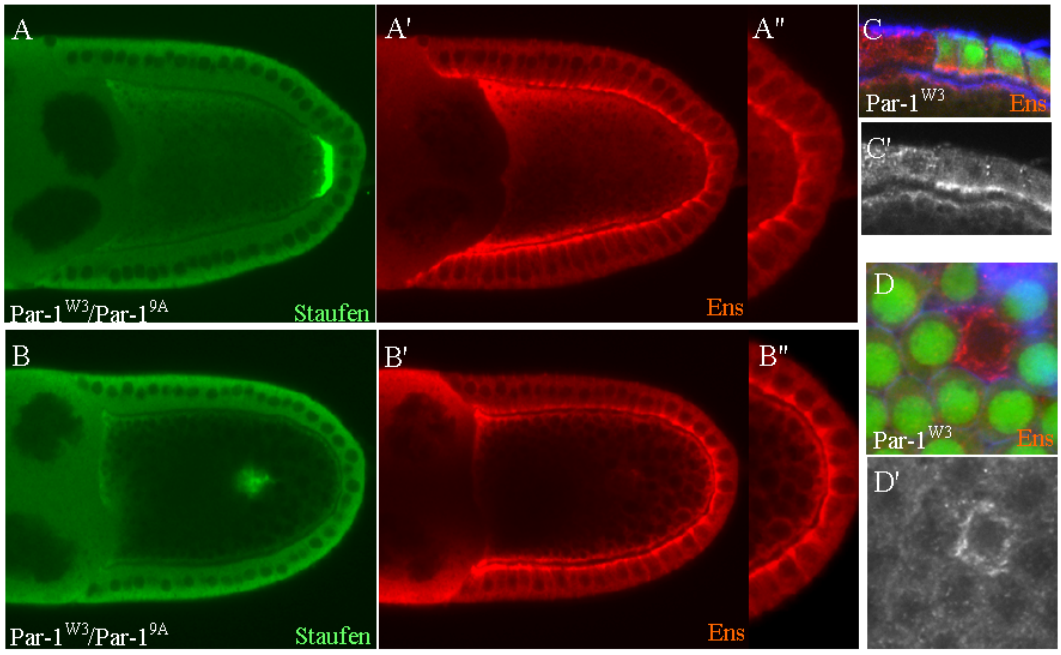

Figure 29. Ensconsin localization is restricted by Par-1
(A,B) Staufen (green) and Ensconsin (red) protein localization in Par-1 hypomorphic mutant egg chambers (*Par-1^{9A}/Par-1^{W3}*) at late stage 9. Only the posterior part of egg chamber (including the oocyte) is shown. In some egg chambers, both staufen and Ensconsin localization is affected at the same time. (A'',B'') An enlarged posterior image of the Ensconsin staining. (C,D) Ensconsin protein (red) in *Par-1^{W3}* mutant follicle cells. Mutant clones are labeled by the lack of GFP (green); phalloidin marks F-actin (blue). (C',D') Single channel image of Ensconsin staining. In (C), basal is up. (D) A tangential section through the middle of the cells showing Ensconsin delocalized from the apical domain in Par-1 mutant follicle cells.

3.3.13 Ensconsin is a direct target of Par-1

Par-1 may regulate Ensconsin localization directly or indirectly, for example, via microtubule polarity. Human E-MAP-115 (Ensconsin) is a highly phosphoylated protein, it is unclear which kinases it is a substrate for. Par-1 activity, together with the phosphorylation induced binding of 14-3-3 (Par-5) to its target proteins, such as Bazooka (Par-3) is required to restrict proteins to the apical domain of follicle cells. There is also evidence that a similar mechanism acts downstream of Par-1 in the germ line. We identified 6 conserved putative 14-3-3 binding sites in Ensconsin. To test whether Ensconsin is a Par-1 direct target, I performed an in vitro kinase assay with Piyi Papadaki,

a post-doctoral fellow in Anne Ephrussi's lab. Several point-mutant constructs of Ensconsin were generated by Pernille Rørth. I expressed these, and wild type Ensconsin in bacteria and purified them. Using Par-1 kinase domain purified by Piyi Papadaki, we first performed an in vitro kinase assay with purified Ensconsin and the kinase domain of Par-1. We found that an N-terminal truncation of Ensconsin can be strongly phosphorylated and a C-terminal fragment of Ensconsin was also somewhat phosphorylated. The N-terminal fragment with mutated 14-3-3 binding sites is more weakly phosphorylated (Figure 30B).

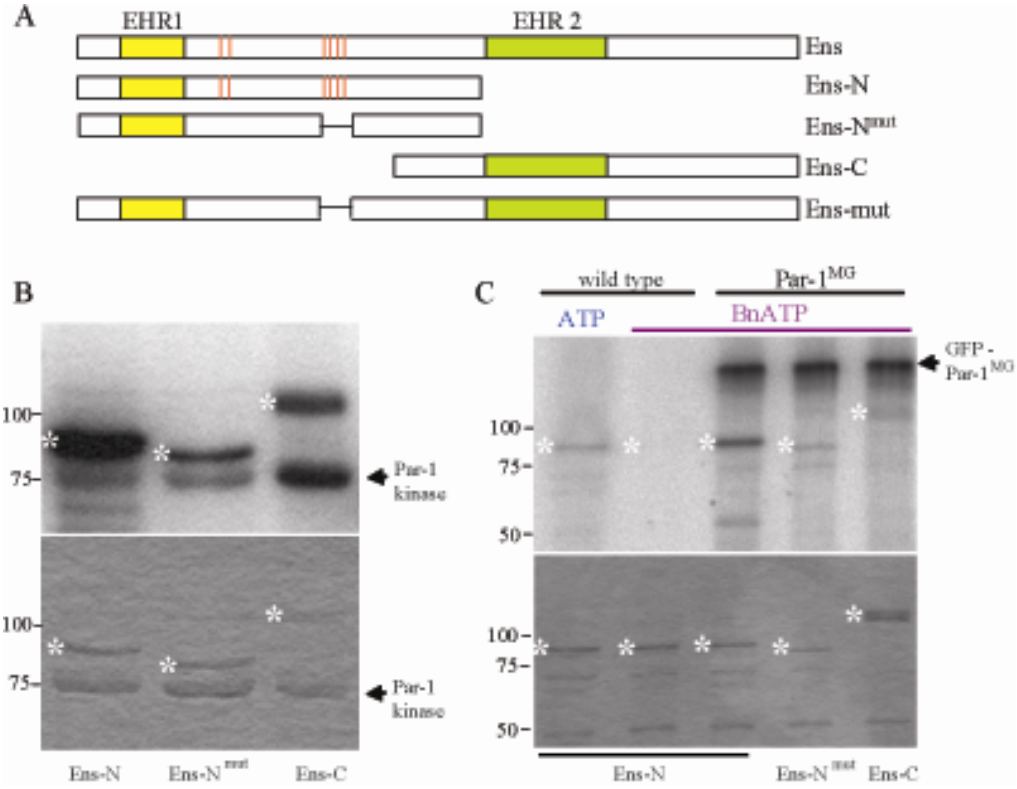

Figure 30. Ensconsin is a direct target of Par-1
(A) Schematic of Ensconsin constructs used. Red bars mark the predicted 14-3-3 binding sites. (B,C) Star indicated Ensconsin fragments. Top panel is the autoradiogram and bottom is the Coomassie staining of the same gel. Molecular weight markers are indicated on the left. Arrow indicates Par-1. (B) In vitro kinase assay using recombinant Par-1 kinase domain and recombinant Ensconsin fragments. (C) In vitro kinase assay using GFP-Par^{M329G} fusion protein. With normal ATP, phosphorylation of Ens-N is seen in the absence of the GFP-PAR-1 transgene.

To confirm the in vitro kinase data, we performed the in vitro kinase assay again, using GFP-Par-1 immunoprecipitated from ovaries (Benton et al., 2002). Unfortunately, the N-terminal Ensconsin fragment is phosphorylated in the control experiment, which lacks the GFP transgene. This suggests that an endogenous kinase is non-specifically copurified

and can phosphorylate Ensconsin. Piyi Papadaki had created a *GFP-Par-1*^{M329G} mutant which retains Par-1 kinase activity but only uses the ATP analogue, BenzoATP, as a substrate.This approach has been used successfully to identify targets of other kinases (Shah et al., 1997). We hoped that using *GFP-Par-1*^{M329G} would reduce the background in our assay. *GFP-Par-1*^{M329G} was immunoprecipated from ovaries of transgenic flies and mixed with bacterially purified Ensconsin fragments in the present of radio-labeled, benzyl-ATP. In the control, lacking the GFP construct, no phosphorylation of the Ensconsin N-terminal fragment was seen. As with the in vitro kinase assay, the N-terminal fragment can be phosphorylated, but the C-terminal fragment and mutated N-terminal fragments are weakly phosphorylated (Figure 30C). This suggests that Ensconsin is a Par-1 direct target and that its 14-3-3 binding sites mediate much of this phosphorylation.

3.3.14 Follicle cell polarity is not affected in *ens*$^{\Delta C}$ mutant

Par-1 affects follicle cell polarity (Doerflinger et al., 2003; Vaccari and Ephrussi, 2002). Since Ensconsin is a direct target of par-1, Ensconsin may also affect follicle cell polarity. To examine this, I made *ens*$^{\Delta C}$ mutant clones and checked the distribution of the polarity markers aPKC (an apical marker) and α-Spectrin (a lateral marker). In large *ens*$^{\Delta C}$ clones, both markers were unchanged (Figure 31A, B). This suggests that Ensconsin does not affect follicle cell polarity.

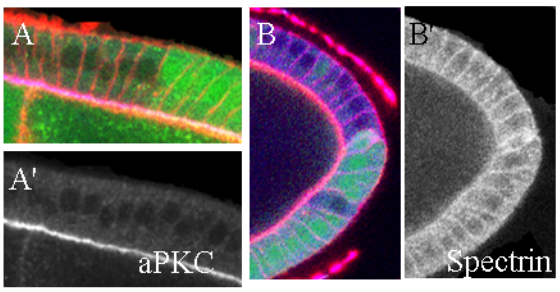

Figure 31. Ensconsin does not affect follicle cells polarity.
(A) Antibody staining of aPKC (blue) in *ens*$^{\Delta C}$. (B) Antibody staining of α-Spectrin (blue) in *ens*$^{\Delta C}$.(A',B') single channel of aPKC (A') or spectrin(B'). (A,B) Mutant cells are labelled by the lack of GFP; phalloidin staining is in red.

3.3.15 The Par-1 phosphorylation/14-3-3 binding sites in Ensconsin are essential for its localization

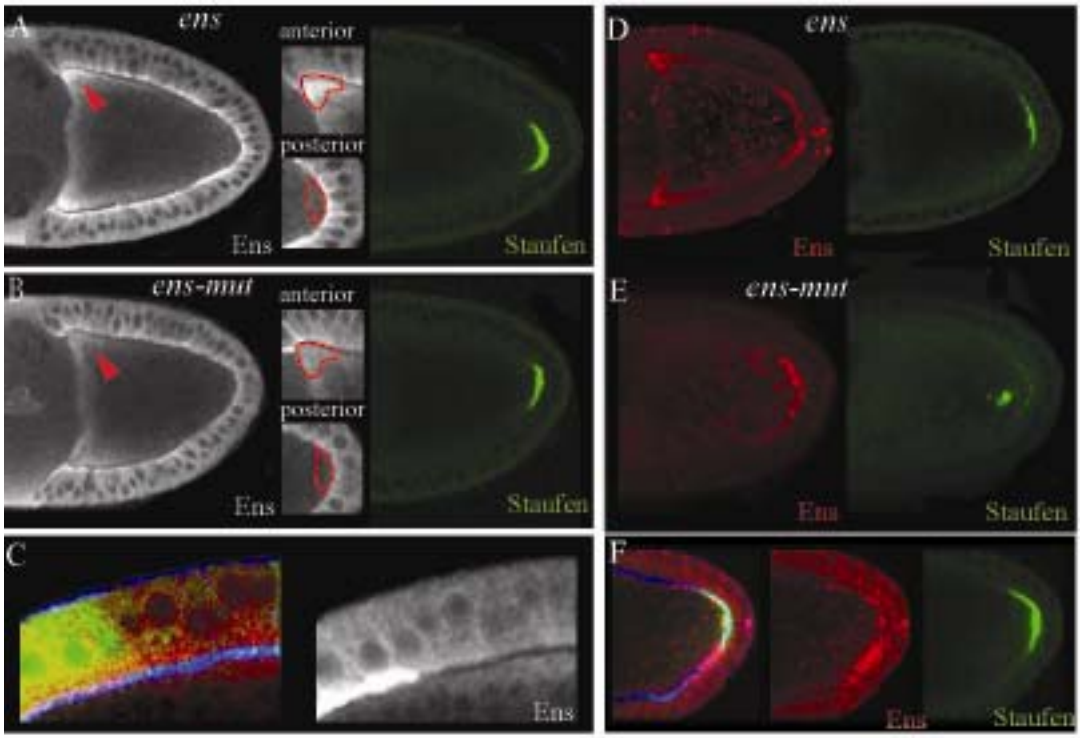

Figure 32. Ensconsin is regulated by Par-1 and controls proper posterior marker localization
(A,B,C) Localization of wild type or mutant tub::Ens, in $ens^{\Delta C}$ mutants. In (A,B) arrows indicate the anterior-dorsal accumulation of Ensconsin, which is reduced in Ens-mutant. Inserts show the areas used quantification, and green is Staufen staining. (C) Follicle cells expressing only the transgene (marked by lack of GFP, green) the adjacent cells are express both endogenous and transgenic *ensconsin*. (D,E,F). Localization of wild type (D) or Ens-mutant, expressed in the germ line using maternal-Gal4-VP16 driver. (F) The posterior region of the oocyte is shown at high magnification. (D,E,F) Late stage 9 egg chamber with minimal endogenous Ensconsin ($ens^{\Delta C}$/ $ens^{\Delta N}$) to visual the transgenic protein. Phalloidin is shown in blue

To investigate the significance of Par-1 phosphorylation *in vivo*, Pernille Rørth generated constructs containing the tubulin protmoter driving either full length *ensconsin* (Ens-wt) or *ensconsin* with mutations in the 6 14-3-3 binding sites and without the 3'UTR (Ens-mutant) to remove any contribution of mRNA localization. I generated transgenic flies of these constructs. Both constructs rescue the $ens^{\Delta C}$ mutant phenotypes, including the wing phenotype and staufen mislocalization (Figure 32A,B). But when *tub::ens-mutant* is expressed in the $ens^{\Delta C}$ background, the protein is less enriched in the anterior of the oocyte and is less apical in follicle cells (Figure 32C). To quantify the degree of mislocalization seen in the oocyte, I compared the intensity of staining in the

same area of the anterior and posterior of the oocyte. Confirming my earlier observations, I find 3 times more mutant protein at anterior than the posterior, where as for wild type the difference is about 5-fold (Figure 32A, B). The remaining Ens-mutant signal at the anterior could be attributed to other, unidentified par-1 phosphorylated sites.

As the *tub::ens-mut* was only partially delocalized in the oocyte, I tried expressing high levels of the Ensconsin transgenes (using maternal tubulin-Gal4) in $ens^{\Delta C}$ / $ens^{\Delta N}$ flies. Under this condition, Ensconsin-wt is found not only in the anterior region of the oocyte but also at the posterior pole and in punctate staining throughout the oocyte (Figure 32D). Under these conditions, Ens-mut is localized more to the posterior, with some egg chambers lacking any protein at the anterior. In 50% of stage 9 mutant expressing egg chambers, Ens-mut is strong mislocalized and Staufen is also mislocalized (Figure 32E). In other Ens-mut egg chambers, Staufen still can localize to posterior pole of the oocyte but no longer overlap with Ens-mut (Figure 32F), although it still appears mislocalized. This suggests localization of Ensconsin to the anterior of the oocyte is controlled by a saturable process, which may require Par-1 phosphorylation. Mislocalization of Ensconsin leads to Staufen mislocalization, suggesting that the distribution of posterior markers is controlled by Par-1, which acts via Ensconsin.

4 DISCUSSION

4.1 The genetic screen using PiggyBac Transposons

In this study, I performed a genetic screen for genes required, in the germ line, for oogenesis and embryogenesis. From the screen, I isolated the mutant *PB4170*, which affects *ensconsin*. I showed that the N-terminal region of Ensconsin can bind to microtubules, but does not regulate microtubule formation or polarity. The *ensconsin* transcript localization in the oocyte depends on its 3'UTR and Ensconsin protein localization is regulated by Par-1 phosphorylation. In flies lacking Ensconsin, Oskar and Gurken are mislocalized and this effect is due to the defects in Khc-dependent transport. I propose that Ensconsin is a Par-1 regulated microtubule associated protein, required for Kinesin-dependent transport.

4.1.1 The PiggyBac screen is effective but laborious

For this screen, we created around 5000 lines but I only managed to test 3000 with the GLC system over the course of a year. There are several reasons for this. First, the eye color of some PiggyBac lines was very weak, making mistakes in the mapping easy. Second, any lines where the insertion lay between two FRT sites or on the fourth chromosome could not be used for screening. Third, some mistakes cannot be avoided during the mapping crosses. Although this screen was laborious; we found that the phenotypes of some mutations showed identical phenotypes to those of known genes. For example, I isolated *dorsal*, *vasa*, *hrp48*, and *aubergine* in the screen. This suggested, in principle that our screen was working. It should be noted that since I have tested 3000 mutant lines and fly genome contain more than 10000 genes, this screen was not saturating.

4.1.2 The PiggyBac and P element have similar preferential sites

It has been reported that PiggyBac transposons do not share hot spots or preferential integration sites with P elements (Hacker et al., 2003; Thibault et al., 2004). In our screen, I found several PiggyBac "hot spots" , for examples, *squid* (hit six times), *vasa* and

cropped (hit four times), and cup (hit three times). Most of these mutants that we found interesting also have one or more P element insertions in them. This may reflect that the near saturating level of P element screens but may also indicate that the insertion sites of P element and PiggyBac element are perhaps not so different.

4.1.3 Germline clone analysis

From this screen, I found mutants required in germ line for oogenesis, embryogenesis and border cell migration. The FLP-DFS system works well to analyze germ line function because only egg chambers with mutant germlines can develop. For border cell migration, very few genes are known to be involved in the germ lines that affect this process. In this screen, only two mutants, *cup* and *cornichon*, were identified. This may be due to the limited number of genes that are required in germline for border cell migration or because relatively few mutants were tested in my screen. It is also possible that many of PiggyBac mutants were simply not strong enough to cause border cell migration defects.

For oogenesis and embryogenesis, this screen was done by checking for unfertilized eggs or dead embryos, which does not require dissecting flies, and therefore makes the screen less labour intensive. Some mutants with high penetrance phenotypes affecting novel genes like *PB2691,PB4350* are worth further analysis. From the ~3000 mutants, I still managed to find mutants affecting novel genes. This suggests that it may be worth to do a systemic or saturating screen to study the germ line function in *Drosophila*.

4.2 Ensconsin: a microtubule associated protein

4.2.1 Ensconsin affects microtubules in a subtle way

Most of the reported microtubule associated proteins bind microtubules and affect their stabilization. Tau knock out mice are viable, but show microtubule defects only in some small calibre axons (Harada et al., 1994) and removal of MAP4 in fibroblast cells does not cause microtubule defects (Wang et al., 1996). These may be due to a level of redundancy in MAP functions. In E-MAP-115/Ensconsin knock out mice, microtubule bundles appear to be thinner and less developed in spermatogenesis, but there are no obvious defects in microtubule staining in $ens^{\Delta C}$ mutant egg chamber (Figure 25B,D). It is still possible that Ensconsin affects microtubules in *Drosophila* egg chambers, but only in subtle way and these defects can not be detectable by immunostaining or live imaging. There may also be other, partially redundant MAPs in the oocyte, which can compensate for some of the $ens^{\Delta C}$ mutant defects.

4.2.2 Ensconsin affects Khc-dependent transport

In this study, I found that Ensconsin affects Khc-dependent transport. If there was a strict dependence of Khc on Ensconsin, then *ensconsin* mutant phenotype should as strong as *khc*. However, $ens^{\Delta C}$ phenotypes are similar to *khc* mutant phenotypes, but weaker. It is possible that the *ensconsin* mutants that I found are not null alleles, other redundant proteins exist in the genome or Ensconsin increases the activity or funtion of Khc but is not completely essential for its funtion.

Neither Ensconsin nor Khc are required for the polarity or morphology of follicle cells, but affect *oskar* mRNA localization in the oocyte and vesicle traffic in neurons. It is possible that because of the smaller size of follicle cells asymmetric protein localization can be achieved by diffusion rather than requiring active transport.

4.2.3 Ensconsin is regulated by Par-1 phosphorylation

Ensconsin localization in the oocyte is controlled at multiple levels: First, mRNA is

localized to the anterior part of the oocyte, which is dependent on the 3'UTR. However, this does not appear to be required for Ensconsin protein localization and function. Second, Ensconsin is enriched at the oocyte cortex. Third, Ensconsin is kept away from the posterior pole of the oocyte by Par-1-dependent phosphorylation. The axis determinants, *bicoid*, *gurken* and *oskar* are transported by microtubule motor proteins along with microtubule network in the oocyte. Par-1 regulates *bicoid* mRNA localization by phosphorylating Exuperantia and also *oskar* mRNA localization. Furthermore, Par-1 also regulates microtubule-dependent polarity through unknown MAP targets (Doerflinger et al., 2003; Riechmann and Ephrussi, 2004; Riechmann et al., 2002; Shulman et al., 2000). Here I have found that Ensconsin is a direct target of Par-1 and affects oocyte polarity through Kinesin. The phosphorylation of Par-1 sites in Ensconsin helps to control its proper localization. It is worth noting that mutations in the Par-1 phosphorylation sites do not severly affect the function of Ensconsin, as *tub::ens mutant* can completely rescue $ens^{\Delta C}$ mutant phenotype. It could be that there are still some Par-1 phosphorylation sites in the Ens-mutant, a hypothesis which is supported by the in vitro kinase assay. These sites may be enough to rescue some Ensconsin localization, or rescue some its function. I also found that Ensconsin was delocalized from the apical membrane of follicle cells and mislocalized to posterior pole of the oocyte in Par-1 mutant (Figure 29B, C, D). This suggests that Par-1 prevents Ensconsin localization to the posterior part of the oocyte.

4.2.4 Par-1 regulates motor protein transport through MAPs

Cell polarity is dependent on microtubule polarity and motor protein function. MARK/Par-1 regulates microtubule stabilization and polarity through phosphorylation of MAPs, like Tau, MAP2 and MAP4. However, mutations in *Drosophila* Tau do not disrupt oocyte polarity, implying that Tau may not be an essential target of Par-1 in the oocyte (Doerflinger et al., 2003). This suggests that either there are other redundant or unknown genes, like *futsch* (MAP1 homologue), or Par-1 can regulate cell polarity through different mechanisms. I propose that Par-1 can control transportation of the plus-end directed motor protein Kinesin through phosphorylation of Ensconsin.

4.2.5 How does Ensconsin affect Khc-dependent transport?

How does Ensconsin affect Khc transport? Ensconsin does not appear to directly affect the microtubule network. The microtubule binding assay shows that the N-terminal region of Ensconsin can bind to microtubule, but the C-terminal region can not (Figure 20C, D). Also *tub-ens-X*, which lacks the EHR2 conserved domain, can not rescue the $ens^{\Delta C}$ mutant. This suggests that, although the EHR2 region doesn't bind microtubules, is still important for the Ensconsin function. Kinesin-1 transports vesicles or other cargo in a strictly regulated manner. The C-terminal tail inhibits the motor domain until cargos binds, causing a change in conformation allowing it to become active (Coy et al., 1999). Kinesin-LacZ, which contains the motor domain but lacks C-terminal tail (Clark et al., 1994), still localizes normally in the oocyte of $ens^{\Delta C}$ mutants, whereas full length Khc protein does not(Figure 24B,25F). This suggests that the C-terminal domain of Khc may be regulated by Ensconsin or that Ensconsin somehow regulates cargo loading onto Khc. It is possible that the C-terminal domain of Ensconsin transiently or indirectly binds to C-terminal domain of Khc and affects this autoinhibition or Khc binding of microtubules. However, I have repeatedly searched for any such interaction between Khc and Ensconsin and have been unable to find one. This does not, however, completely exclude on the interaction between two.

Another possibility is that Ensconsin plays a role in removing obstacles or helping Kinesin to bypass obstacles that can block its movement. Tau is involved in neuron-degenerative disease in human. Aggreated hyperphosphorylated form of Tau causes neurofibrillary tangles which are the symptom of Alzheimer's disease and Parkinson disease. Too much Tau proteins accumulate on microtubule surfaces and interfere with the movement of motor proteins, like Kinesin, along microtubules, therefore affecting cargo transport (Ackmann et al., 2000; Stamer et al., 2002; Vershinin et al., 2007). The phosphorylation of Tau by Par-1/MARK causes Tau to be released from microtubules (Mandelkow et al., 2004). This suggests that Par-1 could regulate Kinesin movement via Tau. I have found that Ensconsin, a Par-1 target, also regulate Kinesin movement. It is worth noting that Ensconsin and Tau display antagonistic function in Khc transportation. Both Tau and Enconsin are expressed in the axon connecting the eye disc and optic lobe in *Drosophila* (Figure 27B') (Heidary and Fortini, 2001). Preliminary data

shows that expressing TauGFP in *ens*$^{\Delta N}$ mutant can enhance the *ens*$^{\Delta N}$ phenotype (data not shown). Overexpressing the *wild type or disease mutation of htau* causes the same symptom of the human disorder in the *Drosophila,* such as Tau accumulation, progressive neurondegenation, and early death (Ferber, 2001). If Ensconsin and Tau functions are antagonistic, it may be worth to test if overpressing *h-ensconsin* or *drosophila ensconsin* can suppress the *htau* overexpression phenotype in *Drosophila*. By using *Drosophila*, we may find new ways to approach the neurodegerenating diseases caused by Tau.

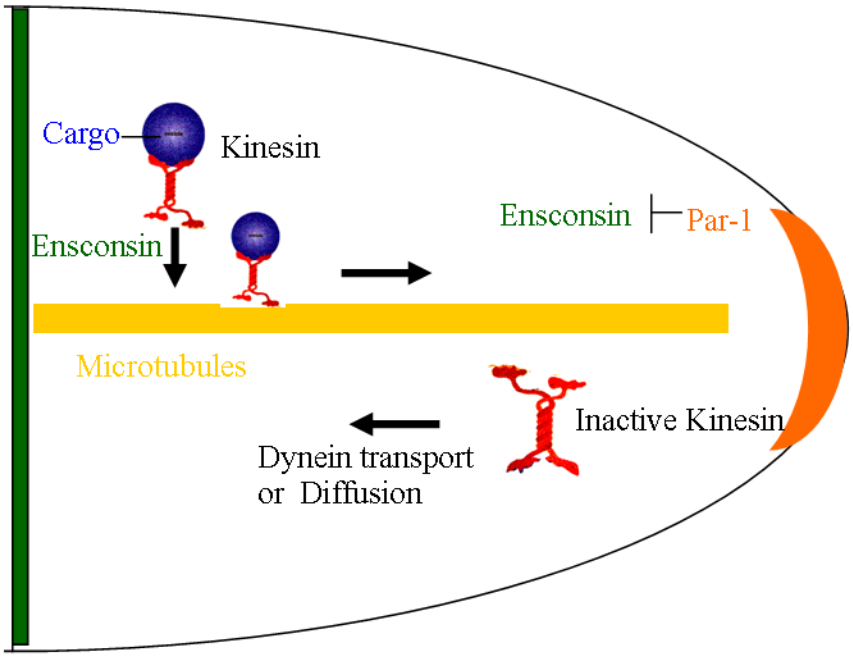

Figure 32. The model for Ensconsin function.
Anterior is left, and posterior is right in the oocyte in the stage 9 or 10 egg chamber. Kinesin carries cargo (blue) from anterior to the posterior pole of the oocyte along the microtubules (yellow). After Kinesin reaches to the posterior of oocyte, it may become inactive and diffuse or be transported by Dynein back to the anterior pole of the oocyte. Ensconsin (Green), accumulated in the anterior part of oocyte, helps Kinesin loading/ processivity. Par-1 prevents Ensconsin expression at the posterior pole of the oocyte to ensure the one direction Kinesin tranportation.

Under normal condition, microtubule minus-ends are abundant in the anterior pole of the oocyte and plus-ends are enriched in the posterior pole. Kinesin motor proteins transport cargos from minus- to plus-ends (anterior to posterior) in the oocyte. When Kinesin reaches to the posterior pole of the oocyte, Kinesin may become inactive. Then Kinesin may diffuse or be transported by other motor proteins to the anterior pole of the oocyte to continue its function (Duncan and Warrior, 2002). Ensconsin, a factor that has a positive

role on motor protein loading/or processivity, is accumulated in the anterior part of oocyte. Mislocalization of Ensconsin in the posterior pole of the oocyte may stimulate Kinesin to become active in the posterior pole of the oocyte. This may prevent the normal interaction and recycling of Kinesin, leading to an accumulation of Kinesin at the posterior or Kinesin-dependent transport may be stimulated on few microtubules which have their plus end oriented away from the posterior pole of the oocyte. Kinesin-LacZ may still localize normally in the *ensconsin* mutant due to its lack of cargo binding and regulatory domains. A function of Par-1 may be to ensure that Ensconsin is only present at the anterior, so allowing Kinesin to cycle normally or transport in one direction. The precise role of Ensconsin in Kinesin regulation remains to be elucidated and may make a promising avenue of investigation.

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6 APPENDIX

6.1 Screen result

Table 2. Summary of PiggyBac insertions

	FRT chromosome arm	Putatively affected genes	Molecular nature	Phenotype
Group 1. No maternal defects				
4278	82			rough eye
3297	80			wing can not expand
2344	42	*CG12765*	F-box protein	wing bigger and leg shorter and bigger
312	40	*rab6*		bristle miss
Group 3. Abnormal oogenesis				
55		*squid*		more dorsal appendages
1621	82	*squid*		more dorsal appendages
2178	82	*squid*		more dorsal appendages
3508	82	*squid*		more dorsal appendages
4180	82	*squid*		more dorsal appendages
5133	82	*squid*		more dorsal appendages
441		*mod*		
321	40	*lgl*		Few later stage egg chambers.
553	42	*CG12340*		cuticle defects in adult, Oskar protein mis-localization (<10%)
701	82	*CG5169*	Kinase	some nurse cell membranes gone, dumpless egg
1173	82	*sds22*	ppp 1, regulatory subunit 7	some nurse cell membrane gone, 10% border cell migration defects
2317	40	*pka*		some nurse cell membranes gone
2766	80	*CG11811*	Guanylate kinase	
308	40	*cropped*	transcription factor	collapsed eggs
2791	40	*cropped*	transcription factor	collapsed eggs
2877	40	*cropped*	transcription factor	Expression of Oskar protein is weaker.
3295	40	*cropped*	transcription factor	Oskar protein mislocalization, 10% border cells split
3594	42			Oocytes are smaller, Oskar protein mislocalization
3753	40	*fray*		some nurse cell membranes are undetectable
4044	82	*CG31232*	Cyclin-like domain	all germ cells degenerated
4256	40	*CG10664*	cytochrome c oxidase	Border cells migration defects in somatic clone.

Continued Table 2

	FRT chromosome arm	Putatively affected genes	Molecular nature	Phenotype
4329	82	*cornichon*		no dorsal appendage
4371	80			
4497	80	*Bab2*	Transcription factor	
4670	82			
4741	82			
4882	80	*CG2034*		
4949	42	*pipsqueak*	BTB/POZ, Helix-turn-helix, Protein kinase-like	Oskar unanchoring defect
4744	40	*cup*		Border cell migration defects.
4975	40	*cup*		Border cell migration defects
5016	40	*cup*		All germ cells die
5493	82	*fer1hch*	iron binding protein	Border cell migration defects in somatic clone
Group 4. Posterior group				
83	82			posterior phenotype in embryo
122	40	*vasa*		posterior phenotype in embryo
456		*hrp48*		posterior phenotype in embryo
680	40	*vasa*		posterior phenotype in embryo
1094	40	*vasa*		posterior phenotype in embryo
3269	82			posterior phenotype in embryo
3747	40			posterior phenotype in embryo
3848	82			posterior phenotype in embryo
4053	40	*aubergine*		posterior phenotype in embryo
4131				posterior phenotype in embryo
4825	82			posterior phenotype in embryo
5144	82			posterior phenotype in embryo
5175	80			posterior phenotype in embryo
5257	40			posterior phenotype in embryo
Group 5. Segmentation defects				
284	80			denticle belts fusion
772	80	*CG6854*	bess motif	denticle belts fusion
1210	82	*E2F*		open or denticle belts fusion
1280	80			denticle belts fusion
1338	82			denticle belts fusion
2265	82			denticle belts fusion
2670	80	*CG7339*	RNA polymerase Rpb7	
2691	80	*CG17090*	Kinase(hipk2)	denticle belts fusion or missed

Continued Table 2.

	FRT chromosome arm	Putatively affected genes	Molecular nature	Phenotype
2700	82	*fray*		denticle belts fusion
2860	82	*r1*		denticle belts fusion
2970	80	*CG7177*	Kinase (wnk)	posterior like or denticle belts fusion
3218	82	*CG31048*	GTPase	naked embryo or early embryo defects
3286	82			
4350	82	*CG33105*	emp24/gp25L/p24, GOLD	longer denticle and some denticle belts missed
4369	82			
4399	82	apc or CG31048		denticle belts missing
4542	80			
4592	82	*sbf*		some denticle belts missing
4824	82			
4889	82	*CG7023*	Ubiquitin hydrolase	open Embryo
5059	80	*eIF4E*		pair-rule like mutant phenotype
911	40	*kismet*		denticle belts fusion
5201	40	*kismet*		denticle belts fusion
Group 6. Others				
28	40	*CG6746*	Protein tyrosine phosphatase-like protein	smaller embryo
173	82			
2600	80	*abl*		germband retraction defects
3292	40	*dorsal*		dorsalized embryo
3311	42			
3900	80	*tsp66E*		embryo defects
4017	82			
4170	80	*CG14998*	Ensconsin	early embryo defects
4211	80	*talin*		dorsal open phenotype
4496	82	*CG31048*	Sponge	early embryo defects
4589	80			germband retraction defects
4735	40			

6.2 Publications

A Sensitized PiggyBac Based Screen for Regulators of Border Cell Migration in Drosophila. Mathieu, J**,** Sung, H.H.**,** Pugieux, C**,** Soetaert, J**,** Rørth, P**.** Genetics. 2007 May 4

Tumor suppressor properties of the ESCRT-II complex component Vps25 in Drosophila. Thompson, B.J., Mathieu, J., Sung, H.H., Loeser, E., Rørth, P. & Cohen, S.M. Dev Cell 2005 Nov;9(5):711-20.

Regulators of endocytosis maintain localized receptor tyrosine kinase signaling in guided migration. Jekely, G., Sung, H.H., Luque, C.M. & Rørth, P.
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