INFORMATION TO USERS

This manuscript has been reproduced from the microfilm master. UMI films the text directly from the original or copy submitted. Thus, some thesis and dissertation copies are in typewriter face, while others may be from any type of computer printer.

The quality of this reproduction is dependent upon the quality of the copy submitted. Broken or indistinct print, colored or poor quality illustrations and photographs, print bleedthrough, substandard margins, and improper alignment can adversely affect reproduction.

In the unlikely event that the author did not send UMI a complete manuscript and there are missing pages, these will be noted. Also, if unauthorized copyright material had to be removed, a note will indicate the deletion.

Oversize materials (e.g., maps, drawings, charts) are reproduced by sectioning the original, beginning at the upper left-hand corner and continuing from left to right in equal sections with small overlaps.

Photographs included in the original manuscript have been reproduced xerographically in this copy. Higher quality 6" x 9" black and white photographic prints are available for any photographs or illustrations appearing in this copy for an additional charge. Contact UMI directly to order.

ProQuest Information and Learning
300 North Zeeb Road, Ann Arbor, MI 48106-1346 USA
800-521-0600

THE DROSOPHILA GENES CAPPUCCINO AND SPIRE INTERACT WITH RHO FAMILY GTPASES TO REGULATE THE CYTOSKELETON DURING OOGENESIS

by

Brian Patrick James

A Dissertation Submitted to the Faculty of the

DEPARTMENT OF MOLECULAR AND CELLULAR BIOLOGY

In Partial Fulfillment of the Requirements

For the Degree of

DOCTOR OF PHILOSOPHY

In the Graduate College

THE UNIVERSITY OF ARIZONA

2001

UMI Number: 3026573

UMI Microform 3026573

Bell & Howell Information and Learning Company
300 North Zeeb Road
P.O. Box 1346
Ann Arbor, MI 48106-1346

THE UNIVERSITY OF ARIZONA ®
GRADUATE COLLEGE

As members of the Final Examination Committee, we certify that we have read the dissertation prepared by BRIAN PATRICK JAMES

entitled THE DROSOPHILA GENES CAPPUCCINO AND SPIRE INTERACT WITH RHO FAMILY GTPASES TO REGULATE THE CYTOSKELETON DURING OOGENESIS

and recommend that it be accepted as fulfilling the dissertation requirement for the Degree of DOCTOR OF PHILOSOPHY

LYNN MANSEAU	Date 10/4/01
MANI RAMASWAMI	Date 10-4-01
TED WEINERT	Date 10-4-01
CAROL DIECKMANN	Date 10/4/01
	Date

Final approval and acceptance of this dissertation is contingent upon the candidate's submission of the final copy of the dissertation to the Graduate College.

I hereby certify that I have read this dissertation prepared under my direction and recommend that it be accepted as fulfilling the dissertation requirement.

Dissertation Director — Date 10/4/01

STATEMENT BY AUTHOR

This dissertation has been submitted in partial fulfillment of requirements for an advanced degree at The University of Arizona and is deposited in the University Library to be made available to borrowers under rules of the Library.

Brief quotations from this dissertation are allowable without special permission, provided that accurate acknowledgment of source is made. Requests for permission for extended quotation from or reproduction of this manuscript in whole or in part may be granted by the head of the major department or the Dean of the Graduate College when in his or her judgment the proposed use of the material is in the interests of scholarship. In all other instances, however, permission must be obtained from the author.

SIGNED: 

ACKNOWLEDGEMENTS

I would like to thank my advisor, Dr. Lynn Manseau, for welcoming me into her lab and providing the open and supportive environment that allowed me to pursue many avenues during my time at the University of Arizona. I would also like to thank the past and present members of the Manseau lab, and the members of the Brower, Ramasawami, and Selleck labs for making life and science a rewarding experience at the University of Arizona.

I must also thank my wife, Dr. Sharon Hesterlee, for her patience and support over these last few months.

TABLE OF CONTENTS

TABLE OF CONTENTS -- *CONTINUED*

TABLE OF CONTENTS -- *CONTINUED*

TABLE OF CONTENTS -- *CONTINUED*

LIST OF FIGURES

LIST OF FIGURES -- *CONTINUED*

LIST OF TABLES

ABSTRACT

The genes cappuccino (*capu*) and *spire* (*spir*) are required for establishment of the anterior/posterior (A/P) and dorsal/ventral (D/V) axes during oogenesis in *Drosophila melanogaster*. In the oocytes of *capu* and *spir* mutant females, axis-defining determinants are either mislocalized or not localized at all. Mounting evidence suggests that this localization defect is due to misregulation of the microtubule and actin cytoskeletons, and by extension, suggests that the wild type function of *capu* and *spir* is to regulate the cytoskeleton during oogenesis.

In support of this hypothesis, previous data from two-hybrid experiments have suggested that SPIR binds to actin through its two WH2 domains. Here I show the interaction between SPIR and ACTIN is direct using *in vitro* binding assays.

Both genetic and yeast two-hybrid evidence suggested that CAPU and SPIR also interact with Rho family GTPases, which include RHOA, RHOL, CDC42 and RAC1. GST pull-down experiments, performed to test the directness of these interactions, revealed that CAPU and SPIR both bind strongly to RHOA and weakly to CDC42. SPIR also binds strongly to RHOL and weakly to RAC1.

I also present here the first evidence for *capu* function outside of oogenesis and for three splice variants of *capu*. Searches of the Drosophila genome database reveal that two splice variants of *capu* are expressed in the adult head, and *in situ* hybridization

results reveal that *capu* message is expressed in the developing larval brain. Additionally, phenotypes in the adult wing are described. One representative of each of two classes of EST from the database that appeared to define two novel classes of *capu* splice forms are sequenced and compared to the existing *capu* splice form.

Taken together, the data described here help demonstrate that *capu* and *spir* play a role in early axis determination in Drosophila, and in regulation of the cytoskeleton. It remains to be determined if *capu* and *spir* act to regulate the actin cytoskeleton which in turn regulates the microtubule cytoskeleton, or if these genes directly regulate the microtubule cytoskeleton.

CHAPTER 1. INTRODUCTION

1.1 OVERVIEW

Establishment of cell polarity is a fundamental step in the development of all multicellular organisms. In this respect, the cytoskeleton has been shown to play a key role in the development and maintenance of cellular polarity in diverse organisms. For example, during development of the Xenopus oocyte, Vg1 mRNA is moved towards the vegetal cortex in a microtubule dependent manner (Yisraeli, Sokol et al. 1990). In C. elegans, disruption of the actin cytoskeleton by cytochalasin-D in the one cell embryo interrupts the proper distribution of determinants and leads to embryos with defects in polarity (Hill and Strome 1990). And finally, localization of specific mRNAs to the cell periphery in fibroblasts has been shown to be dependent on intact actin microfilimants (Sundell and Singer 1991). While it is clear that the cytoskeleton is required for the establishment of polarity, it is less clear how the complex organization of the cytoskeleton is established or maintained during early stages of development when polarity is first determined.

The developing Drosophila oocyte provides an excellent model system in which to study the role of the cytoskeleton in the establishment of polarity. Both the microtubule and actin cytoskeletons are implicated in the establishment of the anterior -- posterior (AP) and dorsal -- ventral (DV) axes during oogenesis (see below). Many molecular markers

of oocyte polarity are available (Kim-Ha, Smith et al. 1991; St.Johnston, Beuchle et al. 1991), and the microtubule cytoskeleton of the oocyte undergoes dynamic, observable, reorganization during establishment of polarity (Theurkauf, Smiley et al. 1992).

Two Drosophila mutations, *cappuccino* (*capu*) and *spire* (*spir*), have been shown to disrupt establishment of polarity early in Drosophila oogenesis (Manseau and Schüpbach 1989). Embryos from females mutant for either *capu* or *spir* are defective in both their AP and DV axes. Evidence suggests that the phenotype of these mutations is likely due to a misregulation of either the actin or microtubule cytoskeletons (Theurkauf 1994; Emmons, Phan et al. 1995; Manseau, Calley et al. 1996; Wellington, Emmons et al. 1999) (and see figs. 1.1 &1.2). The microtubule cytoskeleton of oocytes from *capu* and *spir* mutant females is grossly misorganized, a phenotype that can be phenocopied by disrupting the actin cytoskeleton with cytochalasin D. This observation suggest that the wild type function of *capu* and *spir* is either to mediate the regulation of the microtubule cytoskeleton, possibly through the actin cytoskeleton, or to regulate the actin cytoskeleton which in turn regulates the microtubule cytoskeleton.

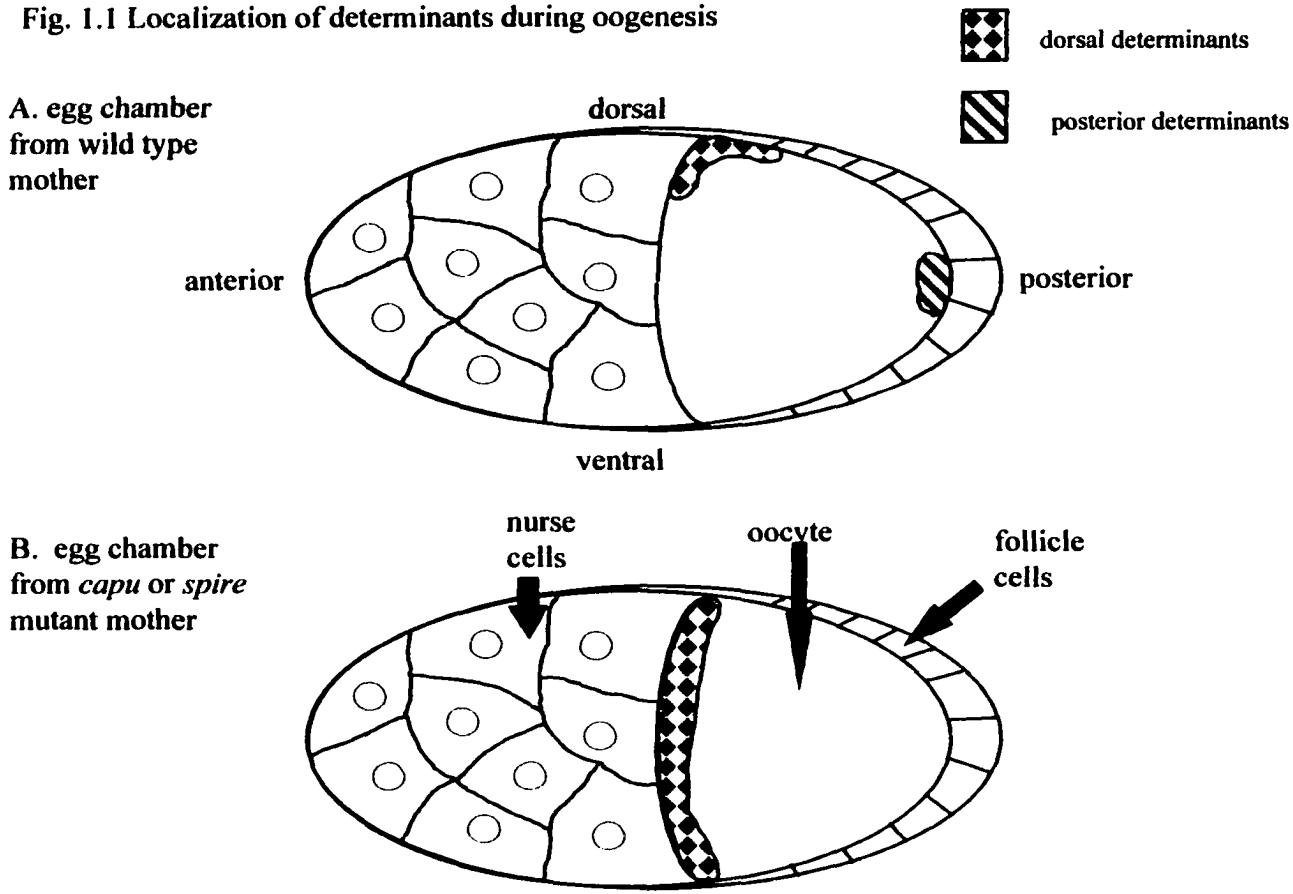
There are several lines of evidence supporting the idea that *capu* and *spir* act with the actin cytoskeleton to regulate development of polarity during oogenesis. Disruption of the actin cytoskeleton phenocopies several of the phenotypes seen in *capu* and *spir* mutants. *capu* binds profilin, an actin regulating protein, in the two-hybrid system (Manseau, Calley et al. 1996). Mutations in *chickadee* (*chic*) the gene that encodes

profilin, enhances *capu*'s DV phenotype, have microtubule phenotypes during oogenesis similar to *capu* and *spir* mutants and, like mutations in *capu* and *spir*, fail to localize STAUFEN and oskar mRNA to the posterior pole during oogenesis (Manseau, Calley et al. 1996). SPIR has been shown to interact with ACTIN itself (see chapter 3) (Wellington, Emmons et al. 1999). Additionally, *capu* is a member of the formin homology family whose members have been shown to be involved in actin-dependent processes, and to interact with Rho family members, known regulators of the actin cytoskeleton (see section 1.4).

There is also evidence that *capu* and *spir* are acting directly on the microtubule cytoskeleton. The most obvious is that the only cytoskeletal phenotype seen in *capu* and *spir* mutants is in the microtubule cytoskeleton(Theurkauf 1994; Emmons, Phan et al. 1995; Wellington, Emmons et al. 1999). There are two recent papers that link the mammalian formin family member, mDia, to the microtubule cytoskeleton. First, overexpression of a truncated form of mDia induces the elongation of HeLa cells and microtubules and actin filaments are reorganized parallel to the cellular elongation (Ishizaki, Morishima et al. 2001). Second, overexpression of a truncated mDia in 3T3 fibroblasts stimulates formation of stable microtubules, and it was shown that not only did mDia colocalize with these stable microtubules, but it also bound microtubules *in vitro* (Palazzo, Cook et al. 2001).

Fig. 1.1 Localization of determinants during oogenesis. (A) Location of posterior (diagonal lines) and dorsal anterior (checkered area) determinants in a wild-type oocyte during oogenesis. (B) Localization of determinants in an oocyte from a *capu* or *spir* mutant mother. Posterior determinants (absent) do not localize to the posterior pole. Determinants that are normally localized to the dorsal anterior (green) are localized along the anterior cortex of the oocyte.

Fig. 1.1 Localization of determinants during oogenesis



1.2 THE DROSOPHILA EGG CHAMBER, OOCYTE AND INITIAL POLARITY.

Oogenesis begins in Drosophila with the division of a germline stem cell to produce both a cystoblast, the progenitor of the germline contribution to the egg chamber, and a new stem cell. The cystoblast divides four times to produce a sixteen-cell germline cyst. Fifteen of these cells will become nurse cells, with large poly-ploid nuclei that provide most of the materials, mRNA and proteins required by the developing oocyte, while the remaining cell will become the oocyte. In each of the four divisions that produce the nurse cells and oocyte, cytokinesis is incomplete, leaving an opening between each daughter cell that is subsequently lined by actin rich structures, called ring canals. The number of resulting ring canals in each cell of the germline cyst depends on the round of division from which it was derived. The two cells from the first division both develop connections to four other cells, while the last eight cells to come from the last of the four divisions only become connected to one other cell (see figure 1.3). One of the two cells that maintains four connections develops into the oocyte, while the other fifteen become nurse cells. For review see (King 1970; Spradling 1993; Theurkauf 1997; Morris and Lehmann 1999; VanEeden and Johnston 1999; Dobens and Raftery 2000).

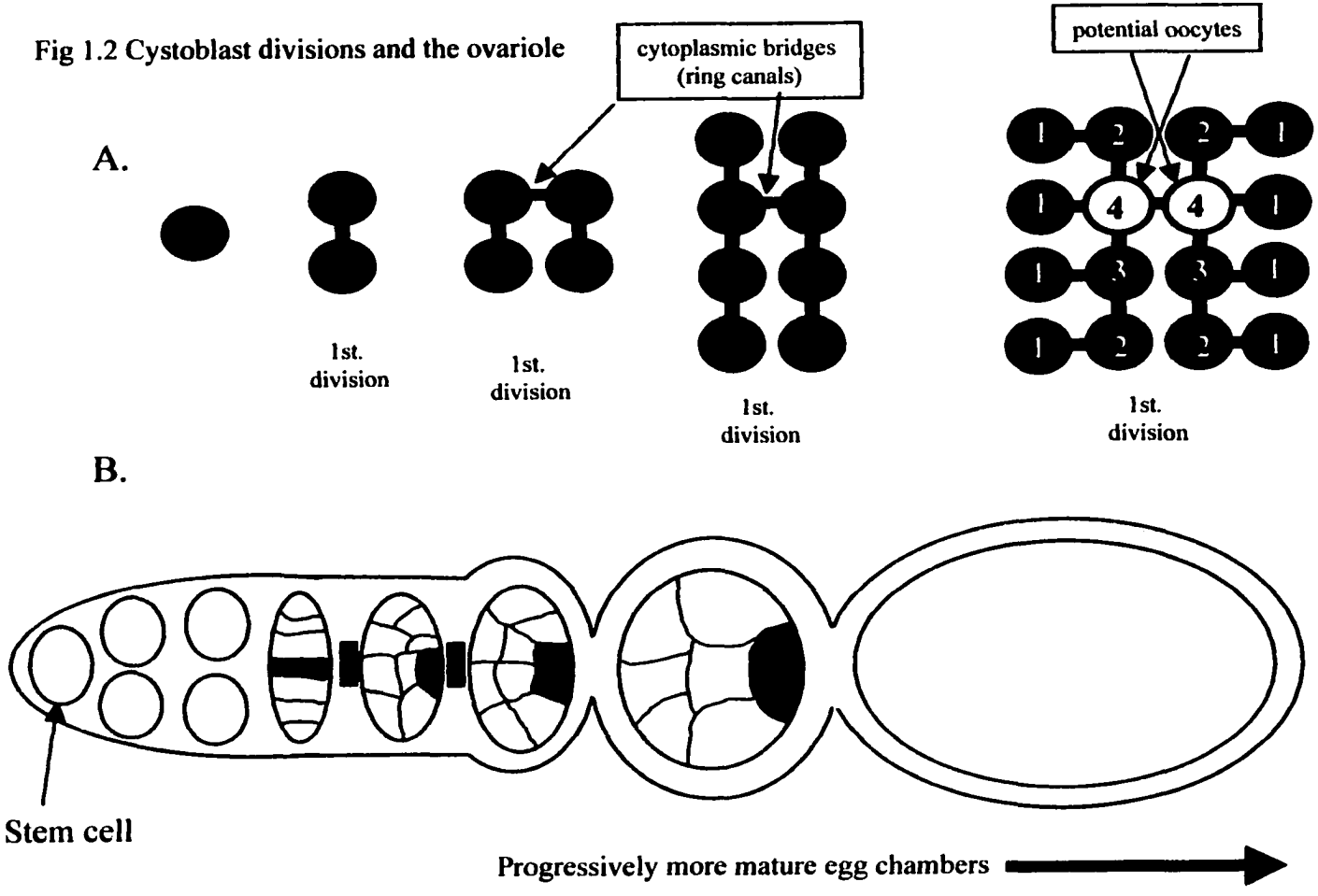
The fifteen nurse cells and the oocyte are surrounded early in oogenesis, including the stages when polarity is determined, by a layer of somatically derived epithelial cells called follicle cells (VanEeden and Johnston 1999). Signaling between the oocyte and the layer of surrounding follicles cells determines both the posterior position of the

oocyte in the egg chamber, and subsequently, the position of the posterior and dorsal anterior poles of the oocyte itself (see section 1.3).

The egg chamber develops in a structure called an ovariole, 10 to 15 of which make up the ovary. The ovariole can be likened to an "egg chamber assembly line", consisting of a chain of egg chambers with the stem cells and early stage egg chambers at the anterior end, and progressively more mature egg chambers towards the posterior end. As an egg chamber matures it is pushed along the ovariole by new younger egg chambers until it has developed enough to leave the ovary.

Fig. 1.2. Cystoblast divisions and the ovariole A). Cartoon showing the origin of the oocyte. As the cystoblast divides each daughter cell maintains a connection with the mother cell which is subsequently lined with actin to form ring canals. After four divisions two cells will have connections with four other cells. One of these two cells will become the oocyte. B) Diagram of the ovariole showing the position of the germline stem cell and the progression of developing egg chambers. As the cystoblasts divide to become the germline cysts, they are surrounded by follicle cells and, through a cadherin dependent process, (black rectangles) localize the oocyte (gray cell) to the posterior pole of the egg chamber.

Fig 1.2 Cystoblast divisions and the ovariole



1.3 DEVELOPMENT OF AXIAL POLARITY

Before the eventual position of the head, abdomen and any other structures are determined in Drosophila development, the DV and AP axes are defined. These initial axes of polarity in Drosophila are laid out early in oogenesis by a complement of mRNAs and proteins provided by the mother's genome to the developing oocyte. In a set of reciprocal signaling events, that go from the oocyte to the surrounding follicle layer then back to the oocyte, the AP axis is first established and then the DV axis is established. It is the structure of the ovary, made up of 10-15 ovarioles, chains of progressively mature egg chambers, that defines the first axis to develop, the AP axis.

The first step in specifying the posterior of the oocyte occurs as the sixteen-cell germline cyst matures and moves along the ovariole away from the stem cell, which is continually dividing, displacing the more mature germline cyst. The stem cell and the developing cystoblasts are all surrounded by a layer of epithelial somatic follicle cells which subsequently migrate around and between the individual sets of sixteen germline cells. The set of follicle cells that first migrate and separate a cyst from its neighboring, more mature cyst, send a signal that causes the oocyte to migrate to a posterior position within the cyst (King 1970). This movement of the oocyte to the posterior of the egg chamber provides what appears to be the first cue required for the development of asymmetry (Gonzalez-Reyes and St.Johnston 1998).

The first step in setting up the AP axis is the establishment of a posterior pole through signaling to surrounding follicle cells (Schüpbach 1987; Manseau and Schüpbach 1989). In the absence of a posterior signal these cells maintain characteristics similar to anterior follicle cells. For the DV axis, it is the dorsal follicle cells that adopt a dorsal fate after signaling from the oocyte (Wasserman 1998). In the absence of a dorsalizing signal default state is ventral.

It is this positioning of the oocyte between the posterior follicle cells and the nurse cells that sets into motion the process by which, not only the posterior pole of the oocyte is defined, but also, subsequently, the dorsal anterior of the oocyte by the product of the *gurken* gene. Soon after the oocyte is positioned at the posterior of the oocyte, *gurken* message is transcribed in the oocyte nucleus. The *gurken* message encodes a TGF alpha-like protein which signals to the surrounding follicle cells to adopt a posterior fate (Neuman-Silberberg and Schüpbach 1993). A subset of about 200 follicle cells at each end of the egg chamber is competent to receive the *gurken* signal and become posterior follicle cells. Those at the anterior end of the egg chamber are insulated from the *gurken* signal by the nurse cells, and do not change fate (Gonzalez-Reyes and St.Johnston 1998). It is not known what signal sets aside these sets of *gurken* competent follicle cells.

Upon adopting a posterior fate, the follicle cells send a signal back to the oocyte that repolarizes the microtubule cytoskeleton. Prior to the signal from the posterior follicle

cells, the microtubule cytoskeleton is nucleated at the posterior pole of the oocyte with the microtubule minus ends at the posterior pole, and plus ends directed towards the anterior of the oocyte. After the signal from the posterior follicle cells the microtubules are nucleated along the anterior cortex of the oocyte with the plus ends of the microtubules at the posterior and the minus ends at the anterior end of the oocyte (see Fig. 1.3) (Theurkauf, Smiley et al. 1992). The nature of the signal from the posterior follicle cells to the oocyte has not been identified, but in four mutants, *capu*, *spir*, *mago nashi* and *pka*, the posterior follicle cells adopt the proper fate but the signal back to the oocyte is not properly acted on (Gonzalez-Reyes, Elliott et al. 1995; Lane and Kalderon 1995; Newmark, Mohr et al. 1997).

Positioning of determinants along the AP axis of the oocyte later in development has been shown to be dependent on this follicle cell induced repolarization of the microtubule cytoskeleton. Drugs that disrupt the microtubule cytoskeleton prevent the proper localization of determinants (Pokrywka and Stephenson 1991), and dynein, a minus ended microtubule motor, has been shown to be involved in targeting determinants to the anterior pole of the oocyte (Schnorrer, Bohmann et al. 2000).

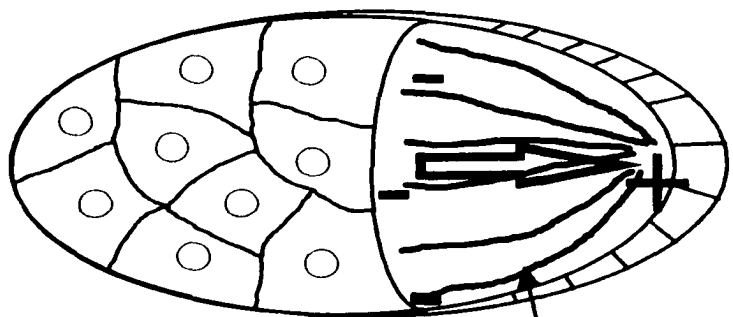
After the AP axis is established, the dorsal anterior of the oocyte is fixed by a second round of *gurken* signaling. *gurken* mRNA is translocated to the anterior cortex of the oocyte and the oocyte nucleus moves to what will become the dorsal anterior cortex. Only the *gurken* message immediately adjacent to the oocyte nucleus is translated into

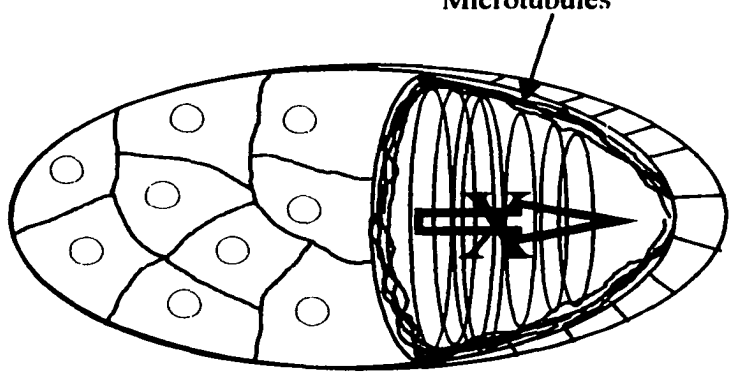
protein thus limiting this second round of *gurken* signaling to the dorsal anterior of the oocyte (Saunders and Cohen 1999). The follicle cells immediately adjacent to the region of the oocyte nucleus then adopt a dorsal cell fate, and in the absence of a *gurken* signal, these dorsal anterior follicle cells will take on a default ventral cell fate (Neuman-Silberberg and Schüpbach 1993). The different responses to the *gurken* signal suggest that there are two differently competent classes of follicle cells responding to each signal. Between the first, posterior *gurken*, and the second, dorsal *gurken* signal, not only does the egg chamber grow, but the proportion of the egg chamber taken up by the oocyte increases. Consequently, the follicle cells next to the oocyte nucleus that receive the later, dorsalizing *gurken* signal are a different subset of follicle cells than those near the oocyte nucleus when the first *gurken* signal was sent (see fig. 1.4) (VanEeden and Johnston 1999).

Fig. 1.3 Microtubule phenotype seen in *capu* and *spir* mutants. (A) Drawing of microtubules at stage 8 of a wild-type oocyte. Microtubules are polarized with their plus ends at the posterior pole of the oocyte. This organization is required for the transport of determinants to the posterior pole via plus ended microtubule motors (arrow). (B) Microtubule organization in an egg chamber from a *capu* or *spir* mutant mother. Microtubules are bundled around the cortex in mutant oocytes preventing the proper localization of posterior determinants.

Fig. 1.3 Microtubule phenotype seen in *capu* and *spir* mutants

A. egg chamber from wild type mother

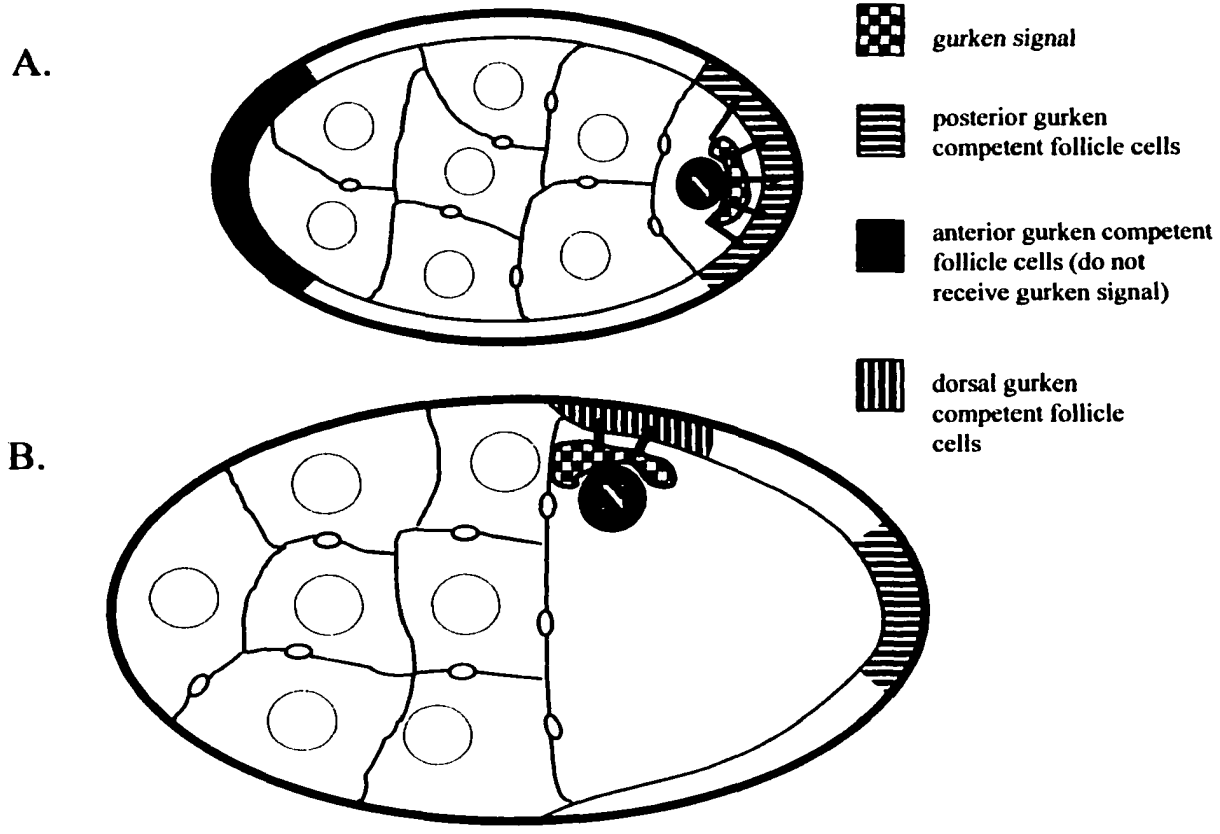




B. egg chamber from *capu* or *spire* mutant mother

Fig 1.4 Two separate rounds of *gurken* signaling define the A--P then the D--V axes. A.) *gurken* mRNA is localized and translated into GURKEN protein (checkered area) near the oocyte nucleus at stage 2 of oogenesis. About 200 cells at the anterior (stippled region) and at the posterior (horizontal lines) of the egg chamber, are competent to receive the *gurken* signal. Only those cells competent and at the posterior are exposed to this round of signaling and adopt the posterior cell fate. B). The posterior follicle cells signal back to the oocyte prompting a rearrangement of the microtubule cytoskeleton that causes the oocyte nucleus to migrate to what will become the Dorsal Anterior cortex of the oocyte. *gurken* mRNA is again localized and translated at the oocyte nucleus. This exposes a different class of follicle cells to a GURKEN signal than in the first round. These cells adopt a Dorsal Anterior cell fate (vertical lines).

Fig 1.4 Two separate rounds of gurken signaling define the A--P then the D--V axes



1.4 *CAPU* AND *SPIR* ARE REQUIRED FOR PROPER DEVELOPMENT OF BOTH THE ANTERIOR-POSTERIOR AND DORSAL-VENTRAL AXES DURING OOGENESIS.

1.4.1 *capu* and *spir* phenotype

capu and *spir* are maternal effect mutations that affect both the DV and AP axes of the developing egg and embryo (Manseau and Schüpbach 1989). Eggs and embryos from *capu* and *spir* mutant females are dorsalized, lack pole cells and abdominal segments. For the egg and embryo to develop normally, the products of the *capu* and *spir* genes are required during oogenesis. At the stages when *capu* and *spir* function, it is the mother's genome that provides all materials required for development. Other than those seen in the female germline, male and female adult mutants in *capu* and *spir* appear to have no significant phenotypes with one exception discussed in detail in chapter four.

capu and *spir* function is required in the germline. The developing oocyte is made up of both somatic cells (follicle cells), and germline tissues(the oocyte and nurse cells). Mosaic analysis has shown that being mutant in the germline for *capu* or *spir* is sufficient for the phenotypes seen in *capu* and *spir* mutants (Manseau and Schüpbach 1989).

The AP axis is not properly established in offspring of *capu* and *spir* mutant females. *capu* and *spir* are members of the posterior group genes, which produce embryos that have normal anterior structures but have fused or missing abdominal segments (Manseau and Schüpbach 1989). It is known that signaling from the oocyte to the posterior follicle cells is normal in *capu* and *spir* mutants as the posterior follicle cells in *capu* and *spir* egg chambers adopt the proper posterior fate (Gonzalez-Reyes, Elliott et al. 1995). Because the follicle cell induced reorganization of the microtubule cytoskeleton is defective, the problem in *capu* and *spir* mutants must be in the signal back from the follicle cells to the oocyte, or in the oocytes response to this signal (Theurkauf 1994; Emmons, Phan et al. 1995).

Segregation of the germline progenitor cells, the pole cells, is one aspect of proper posterior pole establishment that is defective in *capu* and *spir* mutants (Manseau and Schüpbach 1989). Pole cells segregate to the posterior pole of the embryo during normal development and go on to develop into the germline of the adult. The proper development of pole cells is very sensitive to *capu* or *spir* gene dose and provides a useful assay by which to analyze genetic interactions (see chapter 2). Very weak alleles of *spir* and *capu* will occasionally produce embryos that are normal in appearance and can develop into normal looking adults, but these adults are always completely sterile and lack gametes. This phenotype is called "grandchildless".

capu and *spir* mutant females lay eggs and embryos that are dorsalized (Manseau and Schüpbach 1989). *gurken* message is mislocalized in oocytes from *capu* or *spir* mutant females. In *capu* and *spir* mutants the *gurken* message ends up localized along the entire anterior cortex of the oocyte (Fig. 1.1) (Neuman-Silberberg and Schüpbach 1993). This mislocalization leads to aberrant signaling to the surrounding follicle cells, the majority of which take on a dorsal cell fate in strong alleles of *capu* and *spir*. The penetrance of the DV phenotype is variable in *capu* and *spir* mutants as only about 14% of eggs laid by the strongest *capu* alleles are normal, while about 84% of eggs laid by weak *capu* alleles are normal . The numbers for *spir* mutants are similar (Manseau and Schüpbach 1989).

1.4.2 *capu* and *spir* regulate the microtubule cytoskeleton during oogenesis.

The microtubule cytoskeleton of *capu* and *spir* mutant oocytes is visibly misorganized at stage 8 of oogenesis. In wild type egg chambers at stage 8 the microtubule cytoskeleton is polarized with the plus ends of the microtubules at the anterior end of the oocyte and the minus ends at the posterior of the oocyte, In *capu* and *spir* mutant egg chambers the microtubule cytoskeleton is not polarized, but rather is bundled around the entire cortex of the egg chamber (Theurkauf 1994; Wellington, Emmons et al. 1999). This bundled conformation of the microtubule cytoskeleton is similar to what is seen later in normal oogenesis, at stage 10. Also at stage 10, coincident with the nurse cells dumping their contents into the oocyte, there is a rapid, microtubule dependent cytoplasmic streaming

(Theurkauf 1994). The cytoplasm of stage 8 *capu* and *spir* oocytes exhibits a similar, but premature, rapid microtubule dependent cytoplasmic streaming (Theurkauf 1994; Emmons, Phan et al. 1995; Wellington, Emmons et al. 1999).

The rates of premature streaming at stage 8 were analyzed in order to see if they correlated with the variably penetrant DV phenotypes of *capu* mutants (unpublished results J. Calley). It was found that there was no correlation between steaming speed and phenotype with the strongest *capu* alleles streaming at about the same rate as the weakest alleles of *capu*. Thus it does not appear that the premature cytoplasmic streaming seen in *capu* and *spir* mutants is simply sweeping away determinants during oogenesis. However, the misorganization of the microtubule cytoskeleton clearly can account for the posterior phenotype seen in *capu* and *spir* mutants since localization of determinants to the posterior pole is dependent on the microtubule cytoskeleton.

1.4.3 The microtubule phenotype seen in *capu* and *spir* mutants can be phenocopied by disruption of the actin cytoskeleton.

Treatment of wild type egg chambers with the actin depolymerizing drug, Cytochalasin D, phenocopies the *capu* and *spir* mutant microtubule and premature cytoplasmic streaming phenotypes. Additionally, posterior determinants are mislocalized in Cytochalasin D treated egg chambers. DV determinants do not appear to be affected by Cytochalasin D treatments.

The disruption of the actin cytoskeleton causing microtubule phenotypes similar to those seen in *capu* and *spir* mutants suggest that the actin cytoskeleton is regulating the microtubule cytoskeleton during oogenesis. From this three simple models for the wild type function of *capu* and *spir* can be formed: 1) *capu* and *spir* act to regulate the actin cytoskeleton which in turn regulates the microtubule cytoskeleton; 2) *capu* and *spir* are regulated by the actin cytoskeleton and in turn regulate the microtubule cytoskeleton; 3) *capu* and *spir* are regulating both the actin and microtubule cytoskeletons in parallel.

1.5 *CAPU* IS A MEMBER OF THE FORMIN FAMILY

CAPU is a member of the formin homology family of proteins. Formin family members are found in a variety of organisms and were originally defined by the presence of two domains called the formin homology 1&2 (FH1, & FH2) domains (see fig. 1.5) (Castrillon and Wasserman 1994). The formin family is named after the vertebrate formins (Woychik, Maas et al. 1990), a family of alternately spliced genes found in mice and chickens. The formins have been subdivided into three families based on sequence, the formin, the diaphanous and the BNI1 subfamilies (Zeller, Haramis et al. 1999). *capu* is a member of the formin subfamily.

The FH1 domain of formins is thought to interact with profilin (Manseau, Calley et al. 1996; Imamura, Tanaka et al. 1997; Watanabe, Madaule et al. 1997), SH3 domain binding proteins (Uetz, Fumagalli et al. 1996; Bedford, Chan et al. 1997; Tominaga, Sahai et al. 2000) and with WW/P domains (Chan, Bedford et al. 1996; Bedford, Chan et al. 1997). The FH2 domain is a highly conserved region about 80 amino acids long with no, as yet, defined function.

In addition to the two domains initially identified, FH1 and FH2, formins have three other regions with lower sequence conservation, but that have functional conservation. These domains consist of, an amino terminal Rho binding domain, a carboxyl diaphanous autoregulatory domain (DAD) (Alberts 2001), and an FH3 domain

(Petersen, Nielsen et al. 1998). The latter is required in S. pombe for proper subcellular localization of the formin Fus1 (Petersen, Nielsen et al. 1998).

Many formins have been shown to interact with Rho family members through a Rho binding domain (RBD) at the amino terminus(Kohno, Tanaka et al. 1996; Evangelista, Blundell et al. 1997; Watanabe, Madaule et al. 1997; Westwick, Lambert et al. 1997; Kamei, Tanaka et al. 1998; Kikyo, Tanaka et al. 1999; Tominaga, Sahai et al. 2000; Ishizaki, Morishima et al. 2001; Kato, Watanabe et al. 2001; Ozaki-Kuroda, Yamamoto et al. 2001). Rhos are small GTP binding proteins that act as bimodal switches which are on in the GTP bound state and off in the GDP bound state. Additionally, Rhos are implicated in the regulation of the actin cytoskeleton(Hall 1992; Norman, Price et al. 1994; Eaton, Auvinen et al. 1995; Li, Zheng et al. 1995; Nobes and Hall 1995; Imamura, Tanaka et al. 1997; Tapon and Hall 1997; Tanaka and Takai 1998; Umikawa, Tanaka et al. 1998; Umikawa, Tanaka et al. 1998; Watanabe, Kato et al. 1999). Some of the interactions between formins and Rho family members have been demonstrated to be direct and GTP dependent through *in vitro* binding assays, including the S. cerevisiae formin, BNI1 which binds directly to CDC42 (Evangelista, Blundell et al. 1997), and the mammalian p140mDiaphanous, which has been shown to bind RhoA (Watanabe, Madaule et al. 1997). The mammalian formin family member, FRL, has been shown to bind Rac in a GTP independent manner (Yayoshi-Yamamoto, Taniuchi et al. 2000).

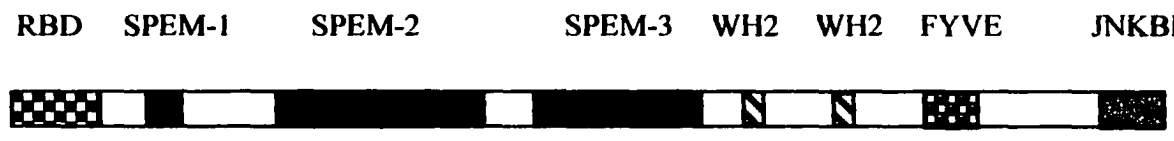
A fifth domain found in formin family members was recently described called the Diaphanous Autoregulatory Domain (DAD) (Alberts 2001). The DAD acts by binding the amino terminal RBD. In this configuration it is thought that the FH1 domain of the formin would be unable to interact with any downstream molecules, like profilin or SRC. GTP bound Rho is thought to bind the RBD displacing the DAD and unfolding the formin, thus allowing the interaction of the FH1 domain and downstream interactors. An alignment of the DAD published with the description of the DAD shows conserved amino acids among the members of the diaphanous subfamily of the formin family (Alberts 2001).

Figure 1.5 Diagram of Formin and SPIR domain structure A. Anatomy of a formin. Formins were initially defined by the presence of a proline rich FH1 domain and a highly conserved FH2 domain. Additional study has revealed a weakly conserved FH3 domain, a Rho binding domain (RBD) and the DAD domain in members of the diaphanous subfamily (see text). B. Diagram of SPIR showing the relative positions of the SPEM domains (black boxes), the Rho binding domain (RBD), the two WH2 domains, the FYVE zinc finger domain (FYVE), and the Jun N-terminal kinase binding domain (JNKBD).

Figure 1.5 Diagram of Formin and SPIR domain structure

A. Formin

B. SPIR



1.6 *spir* CONTAINS TWO WH2 ACTIN BINDING DOMAINS

Overall, *spir* is not similar to many described proteins, but does have some homology to a protein called posterior end mark-5 (pem-5) and several human EST sequences (Wellington, Emmons et al. 1999). Specifically, there are three regions of similarity between *spir* and pem-5, called spem (*spir* and pem) 1,2 and 3. Pem-5 is a posteriorly localized message in the acidian *Ciona seviagnyi* of unknown function. *spir* also contains two WH2 domains, which are named for regions first identified in n-wasp, a bovine protein which is related to the human Wiskott Aldrich Syndrome Protein (WASP). I have shown that SPIR binds actin monomers *in vitro* (See chapter 3) (Wellington, Emmons et al. 1999).

SPIR has also been shown to link c-Jun N-Terminal kinase (JNK) to the actin cytoskeleton in a heterologous system (Otto, Raabe et al. 2000). SPIR was identified in a two-hybrid screen for interactors with the Drosophila JNK homologue Basket (DJUNK). Expression of Myc-tagged SPIR in mouse fibroblasts led to the formation of filamentous actin clusters around the nucleus. SPIR protein was found to colocalize with the SPIR-induced actin clusters and was also found to be a phosphorylation target of JNK *in vitro* (Otto, Raabe et al. 2000).

1.7 RHO FAMILY GTPASES

Rho family GTPases, including Rho, Rac, RhoL, and CDC42, comprise a subfamily of the Ras superfamily of small GTPases. Members of the Rho family form a distinct subgroup based not only on sequence but also their involvement in a set of cellular functions including, regulation of the actin cytoskeleton (Hall 1998), cytokinesis (Drechsel, Hyman et al. 1997), transcriptional activation and endocytosis (Ridley, Paterson et al. 1992; Caron and Hall 1998; Patel, Hall et al. 2000).

Like all Ras GTPases, Rho GTPases act as bimodal switches to regulate cellular function. Rho GTPases are thought to be in the "inactive" state when bound to GDP and the "active" state when bound to GTP. There are two conserved factors that are primarily responsible for regulating the GTP state of Rho GTPases, guanine nucleotide exchange factors (GEFs) and GTPases activating proteins (GAPs). GEFs activate Rho GTPases by displacing the bound GDP and allowing it to be replaced by a GTP, which is present in the cytosol in much higher concentrations than GDP. GAPs activate the intrinsic GTPases activity of Rho GTPases converting GTP to GDP and thereby inactivating the Rho GTPases.

Two common tools used in the study of Rho GTPases are dominant negative (DN) and constitutively active (CA) forms of Rho. The DN and CA forms of Rho are similar to those found used in Ras GTPases. The mutation found in the CA forms of both Rho and

Ras disrupts the intrinsic GTPase activity of the protein thereby locking it in the GTP bound state (Barbacid 1987). The mechanism of the dominant negative nature of the DN forms of Rho and Ras is a little less clear. The mutations that are found in DN versions of Rho and Ras lead to preferential binding of GDP over GTP and this likely leads to a sequestration of upstream activating proteins, GEFs (Schweighoffer, Cai et al. 1993).

There are two simple ways that Rho GTPases are thought to activate downstream effectors: 1) Rho GTPases are associated with membrane and binding a Rho by an effector could alter local concentration of the effector bringing it into contact with its substrate; 2) Binding of the Rho GTPases to the downstream effector could displace an inhibitor of the effectors, or cause a conformational change in the effector that allows activation of the effector.

1.7.1 Rho function in Drosophila

There is currently limited information on Rho function during Drosophila oogenesis. This is most likely due to the lack of mutant alleles of most Rho GTPases in Drosophila until recently. Additionally, many Rho GTPases are essential for cell viability, thus preventing their use in mosaic analysis. One example of this is the Drosophila RhoA gene-germline clones of null alleles of RhoA cannot be produced as the clonal cells are not viable (Magie, Meyer et al. 1999). The requirement for RhoA was assessed in the

germline by combining a mutant allele of *rhoA* with a mutation in the gene *wimp*, which encodes an RNA polymerase II (Parkhurst and Ish-Horowicz 1991; Poortinga, Watanabe et al. 1998) subunit in hopes of reducing RhoA expression to a level that was viable but, would also uncover phenotypes (Magie, Meyer et al. 1999). Ovaries from these mutants had defects in their actin cytoskeleton at ring canals and at the oocyte cortex (Magie, Meyer et al. 1999).

Experiments using transgenically expressed CA and DV versions of rac1, cdc42 and rhoL during oogenesis did show a requirement for cdc42 and rhoL in the germline at stages when *capu* and *spir* normally function (Murphy and Montell 1996). F-actin staining was reduced and irregular in nurse cells of egg chambers from females expressing a CA version of the cdc42 gene under control of a heat shock promoter several hours after heat shock. Defects were also seen in cellularization of nurse cells, due to a break down of the membrane dividing these cells. Finally the ring canals, actin rich structures that line the openings between nurse cells and between nurse cells and the oocyte, in these egg chambers were released from the plasma membrane intact, occasionally forming concatamers. Expression of the DN version of cdc42 showed similar, but less severe, phenotypes to those seen when the CA version is expressed (Murphy and Montell 1996). Expression of the DN version of RhoL induced a nurse cell fusion phenotype similar to that seen when the cdc42 CA construct was expressed(Murphy and Montell 1996). No defects were seen in localization of determinants in these experiments (Murphy and Montell 1996). Another study looked at

germline clones of mutations in cdc42 and observed a reduction in cytoplasmic actin filaments in nurse cells (Genova, Jong et al. 2000). While *capu* mutants show no obvious actin phenotypes like those seen in the above experiments, *capu* mutants do have a weak nurse cell cellularization phenotype similar to that in the CA and DN expressing cdc42 and RhoL transgenic lines (Manseau, Calley et al. 1996).

CHAPTER 2. CAPU AND SPIR DIRECTLY INTERACT WITH RHO FAMILY GTPASES

2.1 BACKGROUND

2.1.1 Mutations in Rho GTPases interact dominantly with mutations in *capu* and *spir*.

A weak enhancement of a RhoA phenotype by the addition of one mutant allele of *capu* during oogenesis has been described (Magie, Meyer et al. 1999). According to the authors oocytes from females heterozygous for mutations in both *capu* and *rhoA* have actin phenotypes similar to those seen in certain *rhoA* mutant combinations. Specifically, the authors see a disorganization of actin at the oocyte cortex and in some ring canals in these *capu rhoA* combinations. It should be noted that these phenotypes are not seen in simple transheterozygous females (*capu* + / + *rhoA*). They are only seen in female offspring of *capu rhoA* / + + females that are themselves *capu rhoA* / + +. This was seen with a recombinant chromosome (mutant for both *capu* and *rhoA*) and required a maternal mutation to see the described phenotype (See fig 2.2) (Susan Parkhurst personal communication).

When investigating this phenotype we found that there was a much more obvious phenotype that more resembles a *capu* phenotype. The interactions seen between *capu* and *rhoA* required mutations in *capu* and *rhoA* to be on the same chromosome (*capu*

rhoA / + +). In an attempt to make the required recombinant chromosome one must first cross an allele of *capu* to an allele of *rhoA* and collect females from the first generation (the F1 *capu* + / + *rhoA*). These females are then crossed and their offspring (the F2) will be putative recombinants. We found that a strikingly large number of F2 individuals were sterile. On further investigation we found that these sterile F2 males and females were agametic (see Fig 2.2). Production of gametes requires proper localization of pole cells, the progenitors of the germline, early in embryogenesis. The embryos from these sterile adults were checked and they were lacking pole cells. This phenotype is seen in weak alleles of both *capu* and *spir*.

Mutations in *capu* strongly interact with mutations in *rhoA*; more than 80% of the eggs laid by females heterozygous for both *capu* and *rhoA* lack pole cells (see Fig. 1). *capu* also interacts with mutations in *cdc42* and a deficiency for *rac2* (rac2(Df)) but this interaction is much weaker than that seen between *capu* and *rhoA*. As a control, eggs from siblings of the cross that produced the *capu* + / + *rhoGTPase* females (*capu* + / + + and + *rhoGTPase* / + +) were checked for pole cells. All of the females of either single mutation laid eggs with normal pole cells.

Mutations in *spir* were tested for an interaction with *rhoA*, cdc42, and a deficiency for *rac1* (rac1(Df)) (see fig. 2.4). The interactions between *spir* and rho GTPases appears to be somewhat more indiscriminate than that between *capu* and rhoGTPases. None of the interactions seen with *spir* are as strong as that seen between *capu* and *rhoA*, while

spir's interactions with cdc42 and rac1 are much stronger than any seen with *capu*, other than *rhoA*. It is also interesting to note that an allele of *spir* that has a premature stop codon after *spir*'s RBD is a stronger interaction partner with various rho alleles than a deficiency for *spir*. This suggests that this allele of *spir* is slightly dominant negative for this interaction. Like the interaction with *capu*, all control females laid normal eggs.

Fig. 2.1 Pole cells are absent in embryos from *capu* or *spir*/+ rhoA/+ mothers

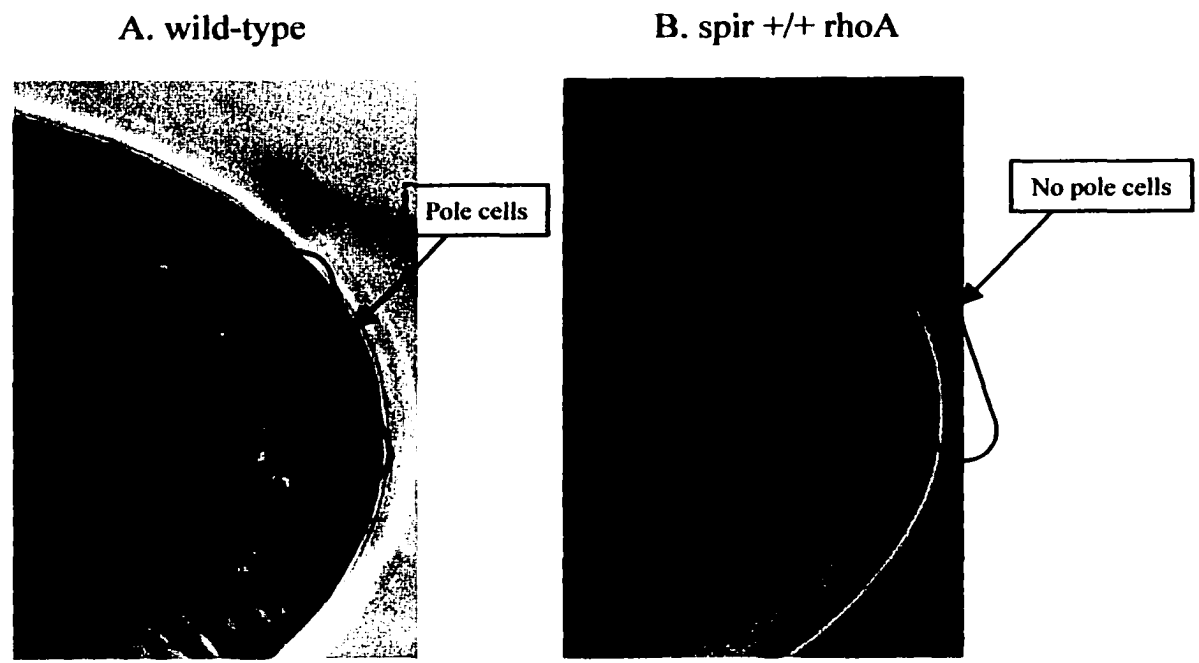


Figure 2.1. Double heterozygotes between *capu* or *spir* and certain Rho family GTPases lay embryos with no pole cells. A. A wild-type embryo during cellularization with pole cells at the posterior pole. B. An embryo from a $spir^{RP}$ +/+ $rhoA^{720}$ female during cellularization with no pole cells at the posterior pole.

Fig 2.2 Genetics of the observed interactions between *capu* and *rhoA*. Diagram of genetic crosses that yield phenotype seen reported in Magie et al. 1999 (box at F4) and in James et al. in prep (box at F1).

Fig 2.2 Genetic interactions between *capu* and *rhoA*

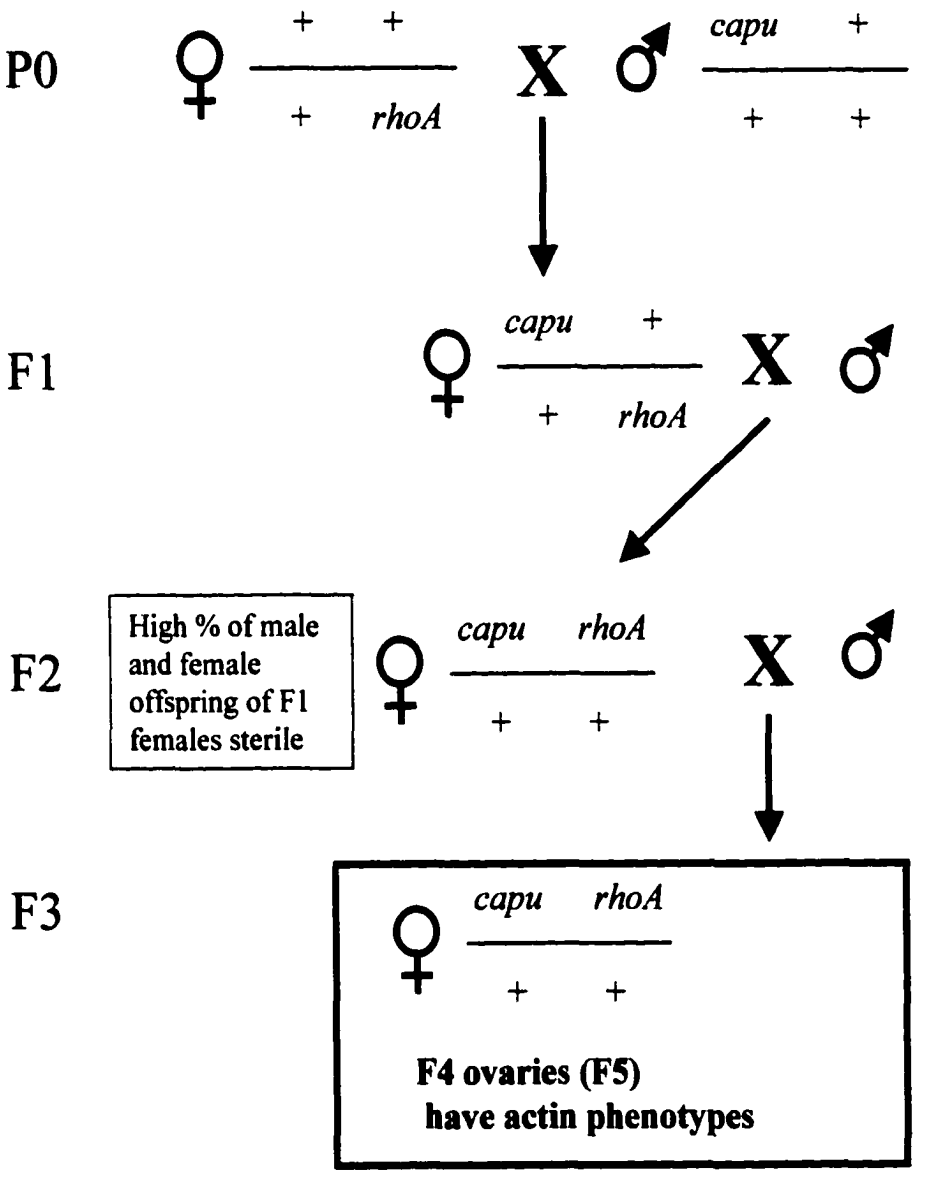


Fig. 2.3. capu* +/ + rho females lay embryos with no pole cells

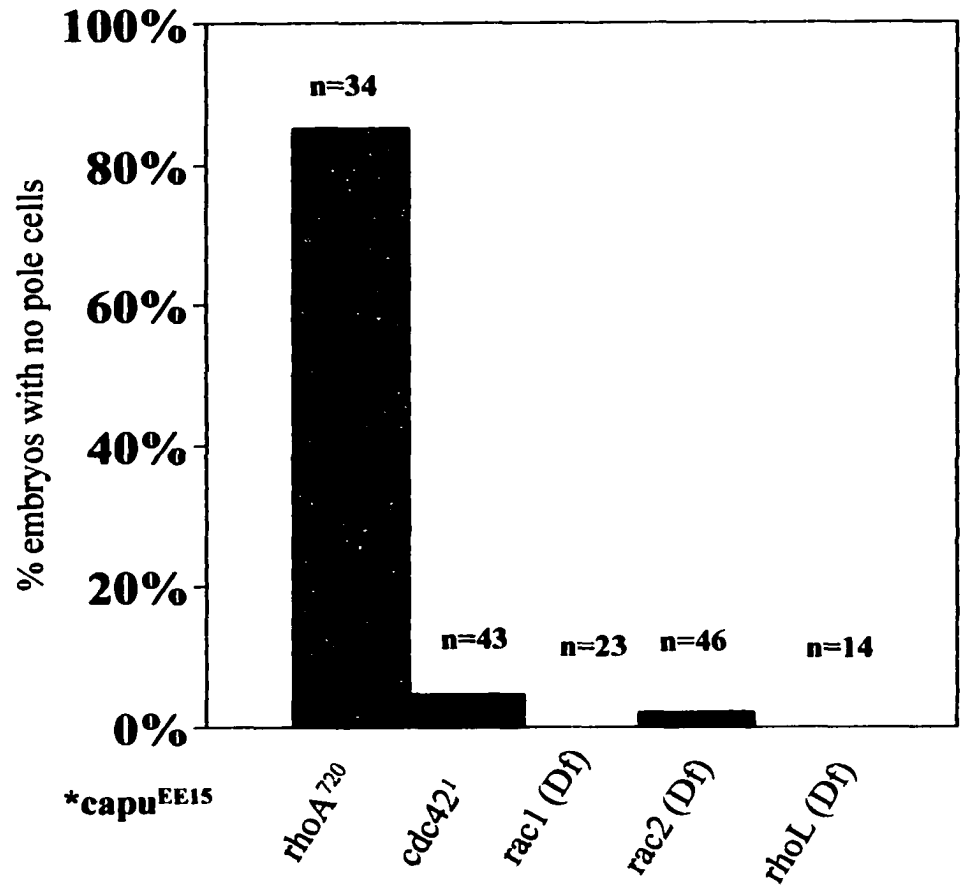
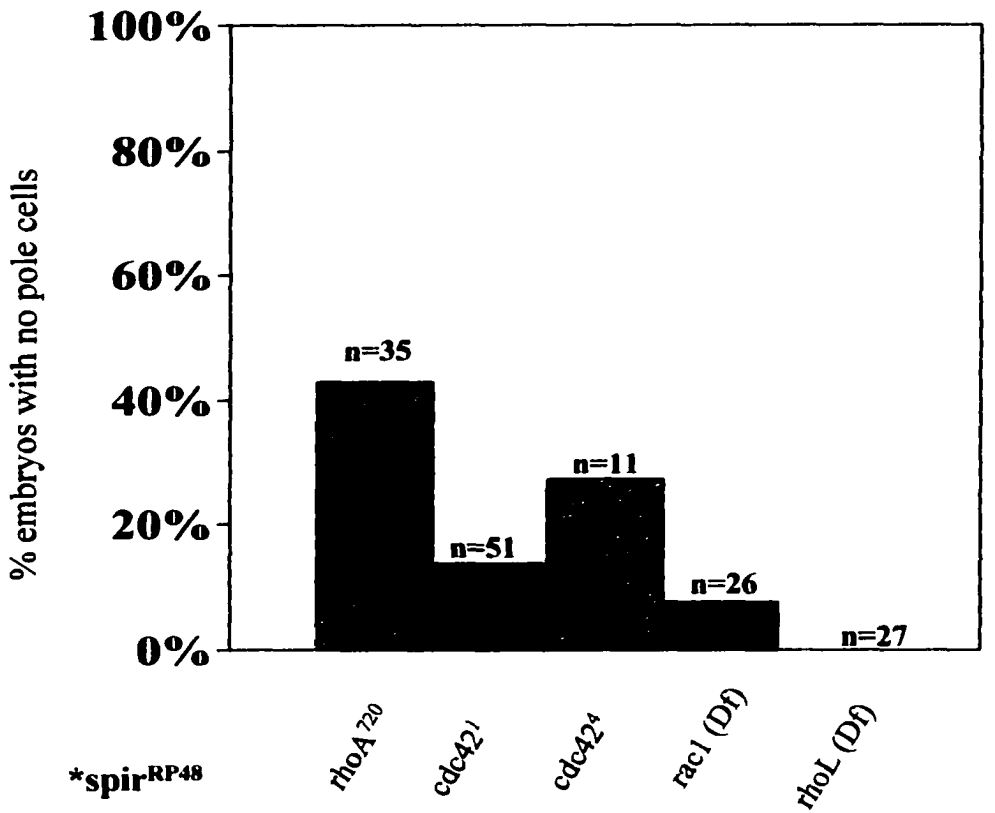


Fig. 2.4. spir* +/ + rho females lay embryos with no pole cells



2.1.2 Formin Rho physical interactions

Mounting evidence suggests that formin family members interact with Rho family GTPases. BNI1, a yeast formin, was originally identified as a Rho1 interactor in a two-hybrid screen in 1996 (Kohno, Tanaka et al. 1996). More direct evidence for an interaction between a formin and a Rho family member was provided in two papers from 1997. First, it was shown that the S. cerevisiae formin, BNI1, not only interacted with the Rho family GTPase cdc42 in the two-hybrid system, but that it also interacted directly and in a GTP dependent manner in *in vitro* binding assays. BNI1 and cdc42 were also shown to co-localize to the tips of yeast mating projections (Evangelista, Blundell et al. 1997). Watanabe et al. showed that the mammalian formin p140mDia binds RhoA in the two–hybrid system and in a GTP dependent manner *in vitro* (Watanabe, Madaule et al. 1997). And finally, p140mDia and RhoA also co-localize to membrane ruffles and p140mDia is recruited to the site of activated RhoA (Watanabe, Madaule et al. 1997).

2.1.3 CAPU and SPIR interact with Rho GTPases in the two-hybrid system

The two- hybrid system was used to determine if CAPU and SPIR interact with Rho GTPases and to determine if any interaction seen was GTP dependent. Mutant Rho constructs that are either locked in the GTP or GDP state were tested against both CAPU and SPIR. Unfortunately, the constitutively active (GTP bound) mutant

constructs of CDC42, RAC1 and RHOA were self activating in the two-hybrid system, requiring no interacting partner for growth on leucine (growth on leucine is indicative of a positive interaction). This confounding factor makes it difficult to draw a clear conclusion about CAPU or SPIR's ability to interact with these constructs. Additionally, both CAPU and SPIR were able to interact with the dominant negative Rho GTPase constructs of CDC42, RhoA and Rac1, suggesting that they are both capable of interacting with GDP bound "inactive" Rho GTPases.

CAPU was found to interact with several Drosophila Rho GTPases in the two-hybrid system(James, Grover et al. in prep.). CAPU interacted with the DN and wild-type versions of RhoA (Hariharan, Hu et al. 1995) and CDC42 (Eaton, Auvinen et al. 1995; Murphy and Montell 1996), with only the DN version of Rac1 (Luo, Liao et al. 1994; Eaton, Auvinen et al. 1995; Harden, Loh et al. 1995), and only very weakly with RhoL (Murphy and Montell 1996) (see Table 1). Deletion analysis was used to map the Rho Binding Domain (RBD) to the first 168 amino acids of CAPU (James, Grover et al. in prep.).

SPIR was also tested in the two-hybrid system and interactions were seen between both the wild-type and DN versions of RHOA, RAC1, and CDC42, but not at all with RHOL (CA, DN or wild-type) (see table 2) (Wellington, Emmons et al. 1999).

One common critique of the two-hybrid system is that a positive interaction does not necessarily indicate a direct protein—protein interaction. It is possible that an endogenous yeast protein is bridging the interaction. The *in vitro* binding assays outlined in this chapter are an attempt to determine if the interactions seen between CAPU and Rho GTPases in the two-hybrid system are direct and also to more clearly determine if these interactions are GTP dependent (James, Grover et al. in prep.).

TABLE 1. CAPU interacts with rho family GTPases in the two-hybrid system

	wild-type	dominant negative	constitutively active
rhoA	++	+++	**N/D***
rac1	-	+++	**N/D***
cdc42	+	+	**N/D***
rhoL	-	-	+

Table 1. Positive interactions in the two-hybrid system are indicated by growth on media lacking leucine (+, ++ or +++). Data is by John Calley and will be published in (James et al. In prep.).

* These constitutively active Rho constructs were self activating in the two hybrid system.

TABLE 2. SPIR interacts with rho family GTPases in the two-hybrid system

	wild-type	dominant negative	constitutively active
rhoA	++	++	**N/D***
rac1	++	++	**N/D***
cdc42	++	++	**N/D***
rhoL	-	-	-

Table 1. Positive interactions in the two-hybrid system are indicated by growth on media lacking leucine (+, ++ or +++). Data from Wellington et al. 1999.
* These constitutively active Rho constructs were self activating in the two hybrid system.

2.2 RESULTS

2.2.1 Transheterozygous *capu rhoA* and *spir rhoA* mutant females do not have an oocyte microtubule phenotype.

The microtubule phenotype seen in *capu* and *spir* mutants is thought to be responsible for posterior patterning phenotypes, and since lack of pole cells is indicative of this process being defective, I checked the oocytes of *capu rhoA* and *spir rhoA* mutant females for microtubule phenotypes similar to those seen in *capu* and *spir* mutants. Using alpha-tubulin antibodies I looked at oocytes from transheterozygous females and saw no difference from wild type (see fig. 2.5).

2.2.2 Preparations of Rho Fusion Proteins and *in vitro* translated CAPU and SPIR

The Drosophila Rho GTPases RAC1, CDC42, RHOA and RHOL were expressed as fusions with glutathione S-transferase (GST) in E. coli. RAC1-GST and CDC42-GST were relatively easy to express and purify with yields being similar to that of GST alone preparations (See fig. 2.4 for results from a typical expression). On the other hand, RHOA and RHOL proved to be insoluble under typical conditions for GST-fusion protein purifications. It was necessary to induce expression of both RHOA-GST and RHOL-GST at 30° as opposed to 37° and limit expression to less than 40 minutes (See methods for details). RHOA-GST and RHOL-GST preps typically yielded about 20% as

much pure fusion protein as a corresponding CDC42-GST or RAC1-GST prep. RHOA-GST and RHOL-GST preps were less stable than CDC42 and RAC1 and significant breakdown of RHOA-GST and RHOL-GST was seen within one week. Accordingly, all GST fusion proteins were used within two days of purification.

Both CAPU and SPIR have proven extremely difficult to express and purify as fusion proteins in E. coli. Steve Emmons, in the Manseau lab, attempted to purify CAPU as a GST fusion in 1994 and was not able to isolate any soluble recombinant protein. I attempted to purify a truncation of CAPU that contained only CAPU's Rho Binding Domain (first 186 amino acids of CAPU) as a GST fusion and was unable to purify any soluble CAPU-GST. SPIR also proved to be extremely difficult to purify from E. coli. Full length SPIR expressed at very low levels, and what was expressed also tended to degrade very rapidly. I was able to express and purify some fragments of SPIR, but none with SPIR's Rho binding Domain. It is for these reasons that I used 35-S methionine labeled *in vitro* translated SPIR and CAPU in these experiments.

2.2.3 CAPU physically interacts with RHOA

In order to determine if the binding seen in the two-hybrid system is direct, I performed glutathione S-transferase (GST) pull down assays with Drosophila rhoGTPases and *in vitro* translated CAPU. RHO GTPases fused to GST and bound to glutathione agarose beads were incubated with *in vitro* translated CAPU labeled with ^{35}S-Met. CAPU

binds strongly to RHOA and weakly to CDC42 (see Figure 2.7). No *in vitro* binding was seen between CAPU and RAC1 or RHOL. The *in vitro* interactions seen with CAPU were not GTP dependent. CAPU was able to bind to both the GTP (including the nonhydrolyzable form of GTP, GTP-γ-S) and GDP bound forms of RHOA and CDC42.

Fig 2.5 Oocytes from capu / rhoA mutant females have normal microtubules

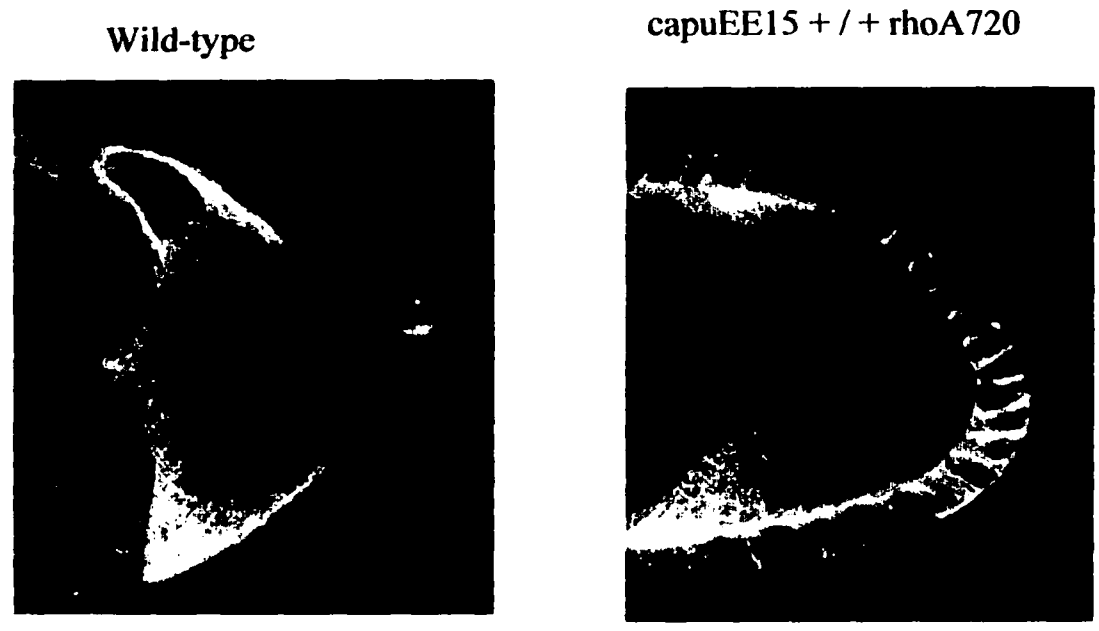


Fig 2.5 Confolcal image of oocytes from capu / rhoA mutant females stained with antibodies against tubulin. Microtubules in oocytes from capu / rhoA and spir / rhoA (data not shown) mutant females appear no different than wild type.

Fig. 2.6 Acrylamide gel of typical Rho-GST protein preps.

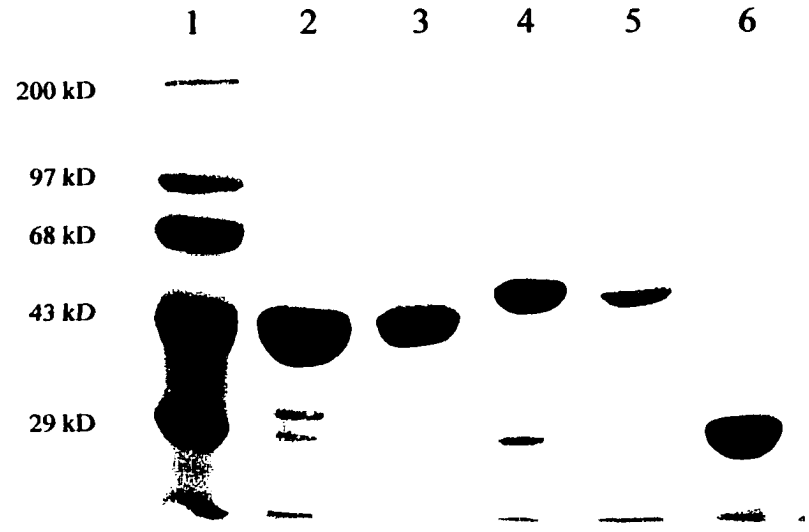


Fig. 2.6 Acrylamide gel of typical Rho-GST protein preps. Each lane represents about 0.5% of a 125 ml culture: Lane 1. molecular weight markers, Lane 2. GST-CDC42, Lane 3. GST-RAC1, Lane 4. GST-RHOA, Lane 5. GST-RHO and Lane 6. GST only.

Fig. 2.7 *In vitro* binding of CAPU to Rho GTPases.

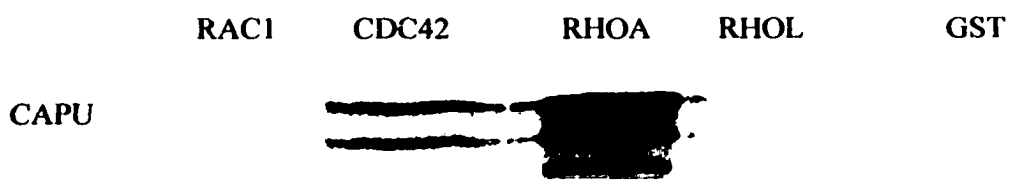


Figure 7. In vitro translated CAPU labeled with 35S-Met was incubated with purified Drosophila RAC1, CDC42, RHOA, and RHOL fused to GST and bound to glutathione beads. CAPU binds RHOA but also binds weakly to CDC42. We see no binding between CAPU and RHOL.

2.2.4 SPIR physically interacts with Rho family GTPases.

In order to determine if the binding seen between SPIR and RHO family GTPases in the two-hybrid system is direct I performed glutathione S-transferase (GST) pull down assays with Drosophila rhoGTPases and *in vitro* translated SPIR. RHO GTPases fused to GST and bound to glutathione agarose beads were incubated with *in vitro* translated SPIR labeled with ^{35}S-Met. SPIR binds strongly to RHOA and RHOL. SPIR also bound weakly to CDC42 and RAC1 (see Figure 2.8). The *in vitro* interactions seen with SPIR were not GTP dependent. Like CAPU, SPIR was able to bind to both the GTP (including the nonhydrolyzable form of GTP, GTP-γ-S) and GDP bound forms of RHOA, RHOL, RAC1 and CDC42.

Fig. 2.8 In vitro binding of SPIR to Rho GTPases.

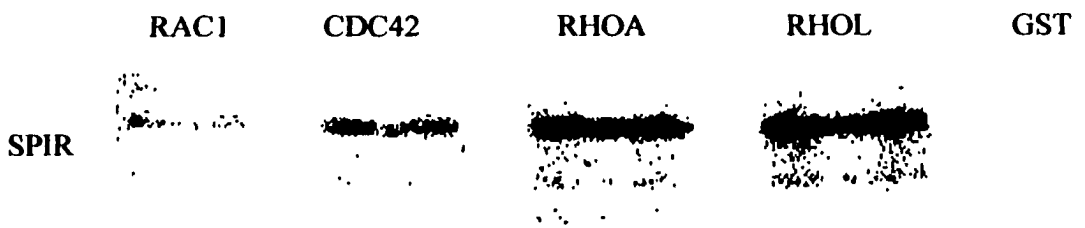


Figure 7. In vitro translated SPIR labeled with 35S-Met was incubated with purified Drosophila RAC1, CDC42, RHOA, and RHOL fused to GST and bound to glutathione beads. SPIRE binds RHOA and RHOL strongly but also binds weakly to CDC42 and very weakly to RAC1.

2.3 DISCUSSION

2.3.1 *capu* and rho family members interact during oogenesis

I show here that CAPU binds directly to both CDC42 and RHOA, and that this binding is GTP independent. For the most part, these results closely match previous data from two-hybrid and genetic assays about *capu*'s interaction with rhoGTPases. The exception is RAC1, the DN form of which shows a strong interaction with CAPU in the two-hybrid system, but no genetic or *in vitro* interaction with *capu.* The interactions between *capu* and *rhoA* appear solid in all assays, genetic (>80% eggs have no pole cells); *in vitro* (strong binding); and two-hybrid (strong interaction with both wild-type and DN forms of RHOA). The interaction between *capu* and cdc42 is weaker in all assays than that seen between *capu* and *rhoA.*

There are three lines of evidence that support an interaction between *capu* and rhoGTPases during oogenesis: 1) CAPU interacts with rhoGTPases in the two-hybrid system; 2) Mutations in *capu* dominantly enhance heterozygous mutations in *rhoA* and *cdc42* to produce a *capu* like phenotype; 3) *In vitro* translated CAPU binds CDC42 and RHOA in GST pull down experiments (James, Grover et al. in prep.). With this in mind, it is troubling that the physical interactions seen between CAPU and rhoGTPases

are not GTP dependent, as most rhoGTPase interactors interact in a GTP dependent manner.

Existing examples of formin / Rho interactions share a common theme. The evidence for the interaction is usually comprised of a physical interaction, two-hybrid or *in vitro* binding assay, co-localization and genetic evidence in the form of overexpression phenotypes (Kohno, Tanaka et al. 1996; Evangelista, Blundell et al. 1997; Watanabe, Madaule et al. 1997; Fujiwara, Tanaka et al. 1998; Watanabe, Kato et al. 1999; Bi, Chiavetta et al. 2000; Fujiwara, Mammoto et al. 2000; Tominaga, Sahai et al. 2000; Yayoshi-Yamamoto, Taniuchi et al. 2000). Mutations in *rhoA*, and to a lesser extent cdc42 and rac1, dominantly enhance *capu* alleles to produce a weak *capu* phenotype. *capu* is the only formin family member that has been shown to interact with a rhoGTPase in this way. Additionally, all of the demonstrated interactions between formins and Rho GTPases occur between members of the BNI1 and Dia subfamilies of the formin family. *capu* is the only member of the formin subfamily of formins that has a demonstrated interaction with rhoGTPases (Magie, Meyer et al. 1999; James, Grover et al. in prep.).

2.3.2 *spir* and rho family members interact during oogenesis

I show here that SPIR binds directly to RHOA, RHOL, CDC42 AND RAC1, and that this binding is GTP independent. SPIR's *in vitro* interactions between RHOA, CDC42

and RAC1 are supported by both two-hybrid and genetic data. SPIR's relationship with RHOL remains unclear as SPIR's strong *in vitro* interaction with RHOL contradicts both genetic and two hybrid data. The RHOL two-hybrid data lacks a positive control, as there is no protein identified in the literature as a RHOL interactor. A negative result in a genetic assay does not negate the possibility of an interaction. For example, there is strong evidence that *capu* and *spir* interact, they interact in the two-hybrid system (James, Grover et al. in prep.) and mutations in *capu* and *spir* enhance each others DV phenotype, but transheterozygous mutations in *capu* and *spir* do not have a grandchildless phenotype like that seen in *spir / rhoA* mutants.

2.3.3 CAPU and SPIR's interactions with rhoGTPases are not GTP dependent.

There are a few explanations why the interactions between CAPU and rhoGTPases appear to not be GTP dependent that are also relevant to the interactions seen between SPIR and rhoGTPases. First, it might be that the *in vivo* interaction between CAPU or SPIR and rhoGTPases is simply not GTP dependent. This is unusual but not unheard of. In S. cerevisiae protein, BEM4, binds to the GTP, GDP and nucleotide free forms of RHO1(Hirano, Tanaka et al. 1996). Another S. cerevisiae protein, BEM1, binds to the GDP bound form of the rhoGTPase BUD1(Park, Bi et al. 1997). And, a mammalian formin, FRL, has been shown to bind both the GTP and GDP bound forms of RAC (Yayoshi-Yamamoto, Taniuchi et al. 2000).

Second, the interaction may be GTP dependent *in vivo*, but I was unable to determine that *in vitro*. The *in vitro* translated CAPU and SPIR used was not purified and when added to the assay the components of the translation reactions they were made in were also added. This includes GTP which is a required component of translation. The maker of the translation system I used, Ambion inc., could not tell me the concentration of GTP in their kits, but based on other manufacturer product descriptions it is likely that the final concentration of GTP added with the translation mixture to the GST pull down assay was no more the 5% of the total GTP or GDP used in the assay. Additionally, at the point that the translated proteins are added to the GST pull down assay the concentration of magnesium (20mM) is a level that is reported to prevent the free exchange of GTP or GDP from rhoGTPases (Diekmann and Hall 1995; Miki 1995). In light of two-hybrid results that also demonstrate CAPU and SPIR's ability to interact with GDP bound forms of rhoGTPases it does seem clear that CAPU and SPIR are able to bind GDP bound rhoGTPases. The *in vivo* relevance of this is still not clear.

A third possibility is that the physical interactions seen between CAPU / SPIR and rhoGTPases *in vitro* do not occur *in vivo*. The genetic interactions seen between both *capu* and *spir* and rhoGTPases suggest that they interact directly *in vivo* or are at least in the same pathway. The dominant enhancement of the *capu / spir* phenotype by mutations in rhoGTPases is similar to synthetic lethality in yeast. A synthetic lethal mutant combination in yeast is a combination of mutants that individually are viable, but when combined have a lethal phenotype. In a review by Hartman et al. (Hartman,

Garvik et al. 2001) the authors compiled information about 173 synthetic lethal combinations where one of the members of the combination was involved in secretion. The authors noted that half of all synthetic lethal combinations involved genes acting at the same step in secretion. About a quarter of the interactions involved genes acting at different steps in secretion and one quarter of the interactions involved one gene not known to be involved in secretion.

2.3.4 *capu* and *spir*'s interactions with rhoGTPases suggest that they are regulating the actin cytoskeleton.

capu and *spir's* interaction with rhoGTPases is just one more piece of evidence that they work to regulate the actin cytoskeleton during oogenesis. First, disruption of the actin cytoskeleton with the actin depolymerizing drug cytochalasin D phenocopies several *capu* and *spir* phenotypes (Emmons, Phan et al. 1995). Second, *capu* is a member of the formin family whose members have been shown to be involved in several actin dependent processes (Evangelista, Blundell et al. 1997; Frazier and Field 1997; Helliwell, Schmidt et al. 1998; Palmieri and Haarer 1998; Kikyo, Tanaka et al. 1999; Afshar, Stuart et al. 2000; Goode, Drubin et al. 2000; Tanaka 2000; Ishizaki, Morishima et al. 2001). And finally, CAPU has been shown to interact with and bind PROFILIN, a regulator of the actin cytoskeleton (Manseau, Calley et al. 1996), and SPIR has been shown to bind ACTIN directly (see chapter 3) (Wellington, Emmons et al. 1999).

Unfortunately, there has been no observed changes in the actin cytoskeleton in oocytes of *capu* and *spir* mutants.

The possibility that *capu* is directly regulating the microtubule cytoskeleton cannot be dismissed. The microtubule cytoskeleton is clearly misregulated in *capu* mutants and no actin phenotypes are seen in *capu* mutants. Additionally, there is data that links at least some members of the formin family to the microtubule cytoskeleton. Activation of the formin mDia in 3T3 fibroblasts leads to the stable formation of microtubules and the colocalization of mDia with these microtubules (Palazzo, Cook et al. 2001). Also, mDia has been shown to interact with microtubules in vitro (Palazzo, Cook et al. 2001). The Rho family member, Rac1, has been shown to bind tubulin and also colocalize with microtubules in 3T3 fibroblasts (Best, Ahmed et al. 1996).

The relevance of *capu* and *spir*'s interaction with more than one rhoGTPase and the involvement of more than one rhoGTPase in posterior patterning is not clear. It could be that rhoA, cdc42 and rac1 all play semi-redundant roles during oogenesis. On the other hand there is precedent for rhoGTPases to function in a cascade where activation of one rhoGTPase will, in turn, activate a second rhoGTPase (Nobes and Hall 1995; Van Aelst and D'Souza-Schorey 1997).

CHAPTER 3. SPIR BINDS ACTIN MONOMERS *IN VITRO*

3.1 BACKGROUND

SPIR contains two domains with sequence similarity to WASP homology 2 domains (WH2) found in Wiskott-Aldrich syndrome protein (WASP) (Derry, Ochs et al. 1994), verprolin (Donnelly, Pocklington et al. 1993) and cofilin (Nishida, Maekawa et al. 1984). WH2 domains have previously been shown to bind actin monomers *in vitro* (Machesky and Insall 1998; Miki, Sasaki et al. 1998). Andrea Wellington showed that SPIR interacted with ACTIN in the two-hybrid system and used deletion constructs to narrow this interaction down to a construct that contained SPIR's two WH2 domains (Wellington, Emmons et al. 1999).

Though *spir* does not share significant similarity with WASP outside of its two WH2 domains, but like *spir*, the WASP related proteins have been linked to rho family GTPases. WASP has a cdc42 binding domain near its amino terminus (Mullins 2000) and the WASP related protein, WAVE, is thought to function downstream of the rho GTPase rac (Miki, Suetsugu et al. 1998).

WASP related proteins act downstream of cdc42 to initiate actin polymerization (Symons, Derry et al. 1996). It is thought that the binding of WASP by activated cdc42 disrupts an autoinhibitory interaction between the N-terminal and C-terminal ends of

WASP allowing WASP to interact with actin and profilin to induce actin polymerization. WASP also has an acidic domain that interacts with Arp2/3, a seven protein complex that nucleates actin polymerization (Welch 1999), to induce actin polymerization (Machesky, Mullins et al. 1999; Yarar, To et al. 1999).

3.2 RESULTS

3.2.1 SPIR binds ACTIN monomers *in vivo*.

In order to determine if the interaction seen between SPIR and ACTIN in the two-hybrid system is direct, I performed *in vitro* binding assays using *in vitro* translated SPIR and purified chicken G-ACTIN. Chicken ACTIN is 97% identical to Drosophila ACTIN and was readily available from Bruce Patterson (University of Arizona). I originally attempted to make purified SPIR protein expressed in E. coli, but found the resulting protein to be extremely difficult to express and very unstable. I was able to isolate only very small amounts of full length SPIR protein. I then attempted to make constructs of SPIR that contained only the regions that were shown to be necessary for the SPIR, ACTIN interaction seen in the two-hybrid system (SPIR amino acids 296-585), and found that these, too, did not express well in E. Coli. Thus, this method was abandoned and I used a reticulocyte lysate system to translate S-^{35} methionine labeled SPIR protein to use in binding assays with ACTIN.

In vitro translated S-^{35} methionine labeled SPIR protein was added to wells of microtiter plates coated with G-ACTIN from *chic*ken muscle (Fig. 3.1 provided by Bruce Patterson) or wells with no G-ACTIN. The wells of the microtiter plates were then washed to remove unbound protein. Remaining bound protein was removed by washing the plates with hot SDS-protein sample buffer and separated on an SDS-PAGE acrylamide gel.

A phosphoimager cassette was used to visualize any bound radioactive SPIR protein. Samples from microtiter wells coated with G-ACTIN and blocked with bovine albumin serum (BSA) contained bound SPIR protein and those from wells with no G-ACTIN (BSA only) had no bound SPIR protein (Fig 3.2)

Fig. 3.1 G-ACTIN used in in vitro binding assay with SPIR

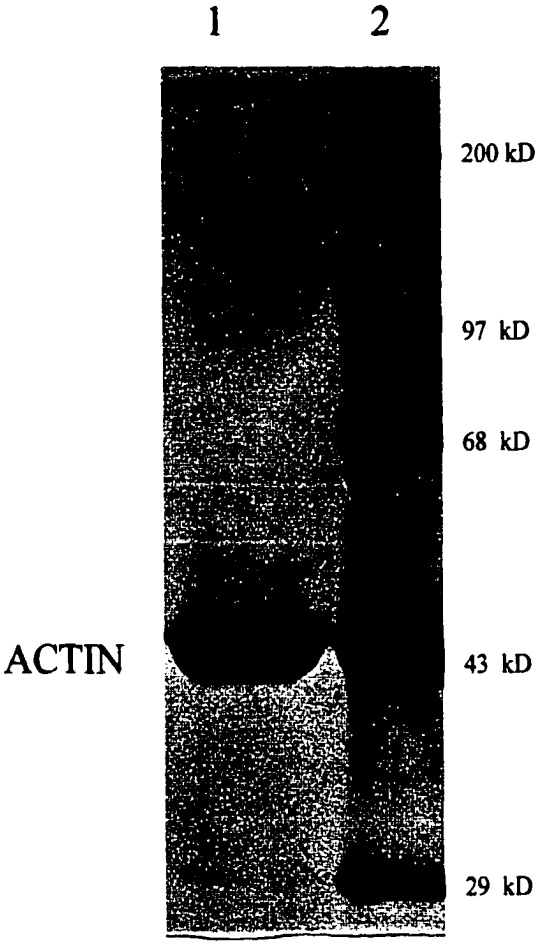


Fig. 3.1 G-ACTIN used in *in vitro* binding assay with SPIR. Coomassie-stained SDS-PAGE gel showing two micrograms of G-ACTIN preparation used in in vitro binding assay with SPIR (Lane 1). Lane 2 is molecular mass markers.

Fig. 3.2 Results of *in vitro* binding assay with SPIR and ACTIN

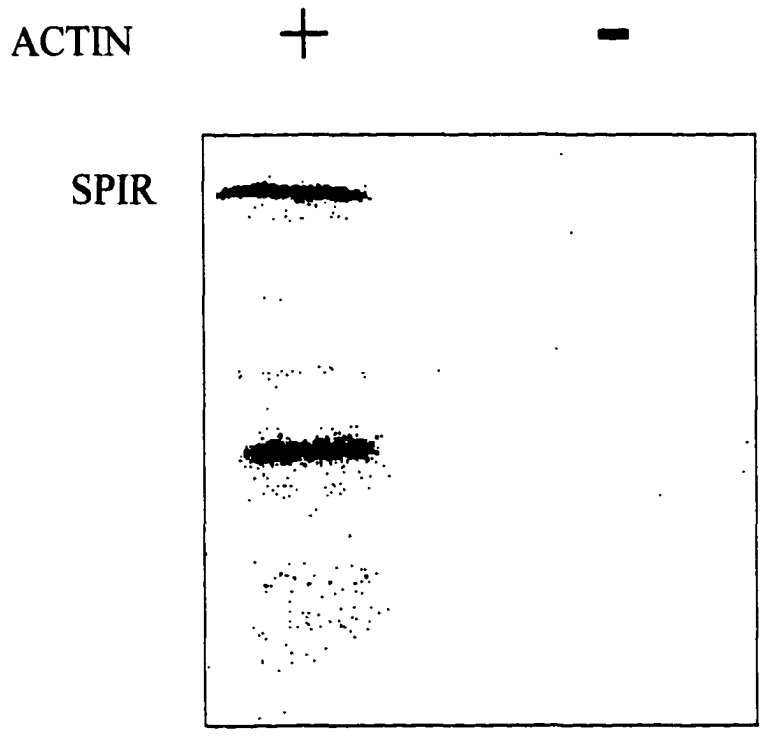


Fig. 3.2 Results of *in vitro* binding assay with SPIR and ACTIN. SDS-PAGE gel showing 35-S labeled SPIR protein from wells coated with (+) and without (-) G-ACTIN. Full length SPIR protein migrates at about 85 kD and is labeled in figure. The smaller molecular weight band is a SPIR breakdown product that binds G-ACTIN.

3.3 DISCUSSION

I show here that SPIR directly binds ACTIN monomers *in vitro*. There is additional evidence that *spir* regulates actin *in vivo*. First, disruption of the actin cytoskeleton during oogenesis phenocopies many phenotypes seen in *spir* mutants (Emmons, Phan et al. 1995). Second, *spir* interacts with rho family GTPases, which are known to regulate the actin cytoskeleton, both genetically and physically (James, Grover et al. in prep.). And third, we have recently found that *capu* and *spir*, in addition to having very similar phenotypes, interact genetically and physically with each other (James, Grover et al. in prep.), and CAPU has been shown to interact with the actin regulating protein PROFILIN (Manseau, Calley et al. 1996).

Two proteins, WASP and SCAR1, also have WH2 domains like SPIR and have additional domains that the have been shown to regulate the polymerization state of actin *in vitro*(Miki and Takenawa 1998; Machesky, Mullins et al. 1999). Specifically, the cofilin domain of WASP has been shown to act with cofilin's WH2 domain to sever actin filaments *in vitro* (Machesky, Mullins et al.1999; Miki, Sasaki, et al.1998). SPIR does not share homology with these proteins outside of its WH2 domains, but the function of these domains in other proteins does raise the possibility that SPIR itself may have an effect on the polymerization state of ACTIN filaments. Unfortunately, without purified SPIR protein, the biochemistry that would address this question is not possible.

Recently it has been shown that a WASP family member, WAVE, interacts with cAMP-dependent protein kinase (PKA) (Westphal, Soderling et al. 2000) and Andrea Wellington is checking in the two hybrid system to see if SPIR interacts with Drosophila PKA. Additionally, an epitope tagged version of *spir* has been transformed into flies by Andrea Wellington and she has found that SPIR protein is localized to the oocyte cytoplasm.

CHAPTER 4. THE REGULATION OF THE *capu* GENE

4.1 BACKGROUND

capu mRNA is expressed in several tissues in which there is little or no evidence of a requirement for *capu* function. A developmental northern probed with *capu* cDNA published by Emmons et al. 1995 shows that at least two splice forms of *capu* are expressed throughout development. The published *capu* cDNA sequence is roughly 4kb and corresponds with a band that is enriched in, but not limited to, oocytes. This will be referred to as the ovarian splice. A second slightly larger transcript, approximately 4.3kb, is also seen throughout development, but appears to be enriched in somatic tissues. This 4.3kb splice is referred to as the somatic *capu* splice. These observations beg the question of whether *capu*'s function is limited to oogenesis, as most of the observed phenotypes are.

Several possibilities exist that could explain why no *capu* phenotype has been seen outside of oogenesis: 1) *capu* function is required only during oogenesis and *capu* message expressed at other times either is not translated into protein, or the translated protein serves no function; 2) a second, similar gene, substitutes for *capu* function at other times in development; 3) alternate splice forms of *capu* function at other times in development; 4) many of the existing alleles of *capu* have come from screens where an oogenesis specific phenotype was selected for, eliminating any alleles that had severe

adult phenotypes and 5) no one has looked hard enough for a phenotype. It is likely that the true answer will contain some combination of these possibilities.

The bulk of the experiments in this chapter help address these possibilities by: 1) Identifying and sequencing the alternate splice forms of *capu* in order to determine if they may be compensating for *capu* function at other times during development; 2) screening for a p-element insertion in the *capu* gene in order to generate a protein null allele, and designing a "knock out" construct based on the method described by (Rong and Golic 2000); 3) describing the first non-oogenesis *capu* phenotype, one found in the adult wing; 4) identifying and sequencing a new formin gene that is expressed during oogenesis.

4.2 RESULTS

4.2.1 *capu* message is found early in oogenesis in the nurse cells and oocyte.

I examined *capu* mRNA expression during oogenesis using DIG labeled RNA probes made from the ovarian *capu* cDNA clone. Two DIG labeled RNA probes were made, one corresponding to the first 1Kb of *capu* and one corresponding to the last 0.9 Kb of *capu*. These probes both contain sequence that is common to all known splice variants of *capu* (Discussed in section 4.2.3). Both probes showed the same pattern in the oocyte. Generally it can be said that *capu* mRNA expression begins at stage two of the

germarium (Fig. 4.1A) and continues through stage 13 of oogenesis (Fig. 4.1). Specifically, *capu* mRNA is found abundantly in the nurse cells from stage 1-13 (Fig 4.1B), around the oocyte nucleus in stage 4-9 egg chambers (Fig. 4.1 C) and in the follicle cells in stages 4-11 (Fig. 4.1 A,B).

capu mRNA is expressed during the stages that *capu* is thought to affect patterning of the oocyte. The staining of the oocyte nucleus includes the earliest stage in which an effect of *capu* is seen on patterning, specifically, the stage at which *capu* mutants fail to localize STAFUN protein to the posterior of the oocyte at stage 8 (St.Johnston, Beuchle et al. 1991). *capu* function has been shown, by mosaic analysis to not be required in the follicle cells (Manseau and Schüpbach 1989), yet *capu* mRNA is expressed in this tissue (Fig. 4.1 A,B).

4.2.2 *capu* message is found in the larval and adult brain.

I looked at *capu* expression in the brains of 3rd instar larvae using the same DIG labeled RNA probes described in section 4.2.1. The staining seen with *capu* probe in the brain resembles the pattern seen when a synaptobrevin-GFP fusion is expressed in larval brains (Estes, Ho et al. 2000). This GFP-synaptobrevin fusion protein specifically labels the axons (Estes, Ho et al. 2000). This is an interesting pattern, as RNA is first expressed in the cell body, and this pattern would require active transport of *capu* mRNA to the axons. This observation is particularly interesting as active transport of

capu mRNA is also seen during oogenesis when *capu* mRNA is transported from the nurse cells to the oocyte.

The finding that two splice forms of *capu*, discussed in section 4.2.4, were found in libraries made from Drosophila adult heads, GH26143 and GH07742 (Harvey, Brokstein et al. 2001), is consistent with the idea that *capu* is expressed in the brain.

Fig 4.1 *capu* mRNA expression during oogenesis. Tissue in situ hybridization using DIG labeled mRNA probes corresponding to the *capu* mRNA. (A) Expression begins in the germarium region 2. (B) *capu* is expressed in the nurse cells and follicle cells from the germarium to stage 8 of oogenesis. (C) *capu* mRNA colocalizing with the oocyte nucleus at stage 9.

Fig 4.1 *capu* mRNA expression during oogenesis

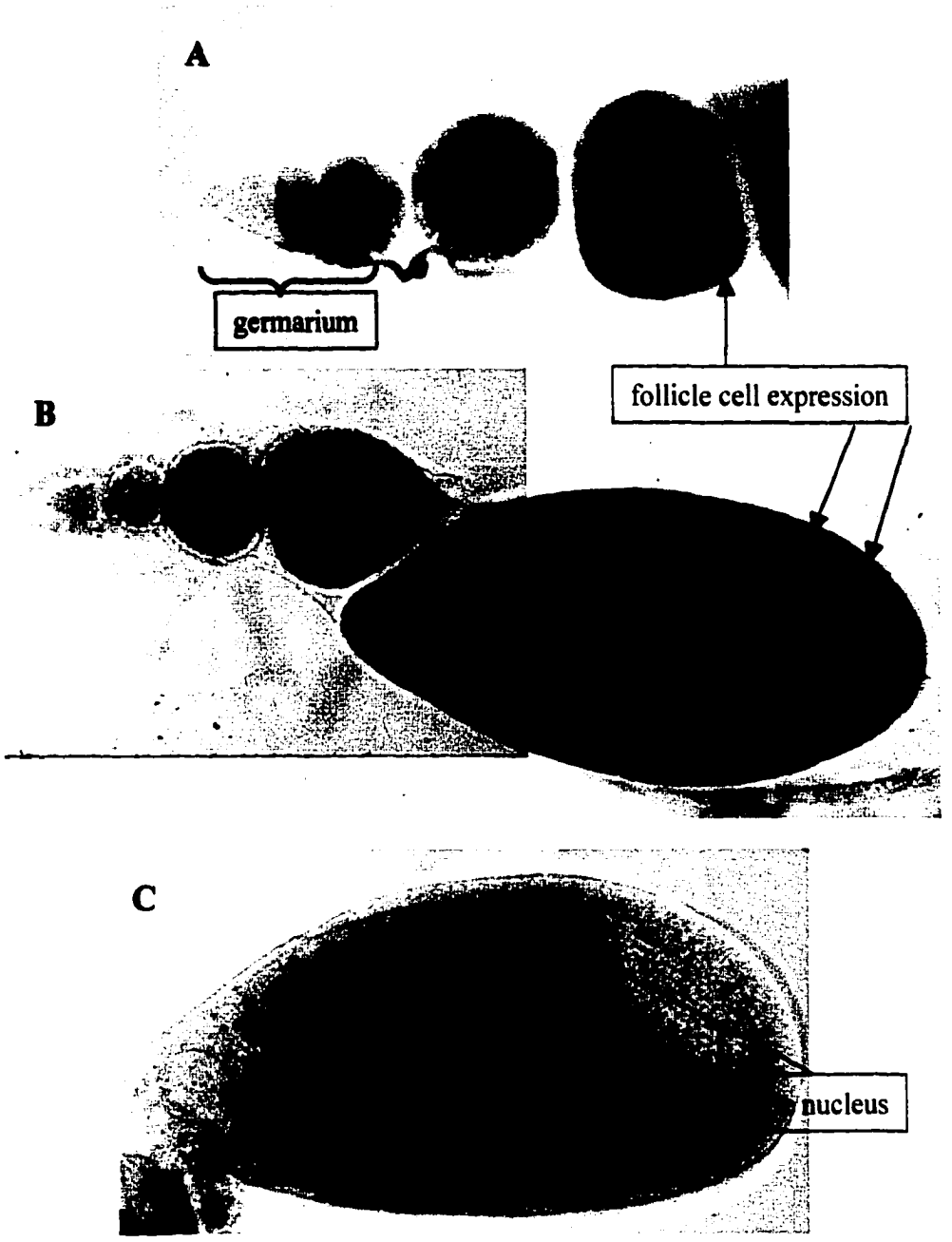


Fig 4.2 *capu* mRNA expression in the 3rd instar larval brain.

Fig 4.2.2 *capu* mRNA expression during in the 3rd instar larval brain. Stained regions correspond to the neuropil. Little or no staining is seen in regions of the brain occupied by cell bodies.

4.2.3 Mutations in *capu* affect wing vein morphogenesis

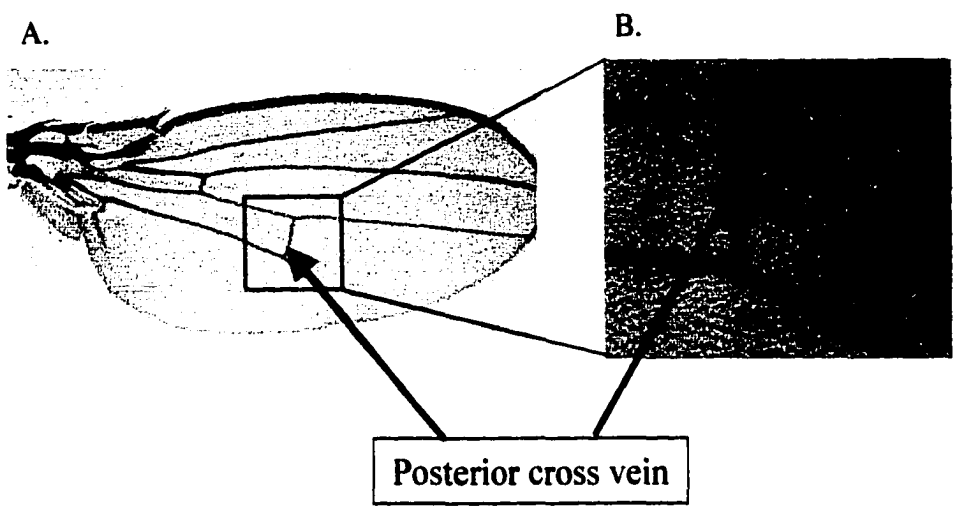
In an attempt to identify adult phenotypes in *capu* mutants, Dr. Manseau noted that the posterior crossvein (Fig. 4.3) in some alleles of *capu* was abnormal. I looked at this phenotype in several allelic combinations of *capu* and in a deficiency for the *capu* gene, edsz12, also known as "edsz". I found that two seemingly opposite phenotypes were present in *capu* alleles: Over 60% of the adults that were homozygous for the *capu* ^{G7} allele had a reduced posterior crossvein (Figs. 4.4 A, 4.5 and table 4.1), and almost 80% of alleles in *capu* ^{EE15} had an ectopic vein associated with the posterior cross vein. This is noteworthy given that the *capu* alleles, ^{3871} and hk3 show little to no wing vein phenotype when homozygous. *capu* ^{3871} does have a wing vein phenotype when heterozygous for a *capu* deletion, edsz12, suggesting that it is a weak hypomorph, and *capu* hk3 shows no phenotype when homozygous or over a *capu* deletion.

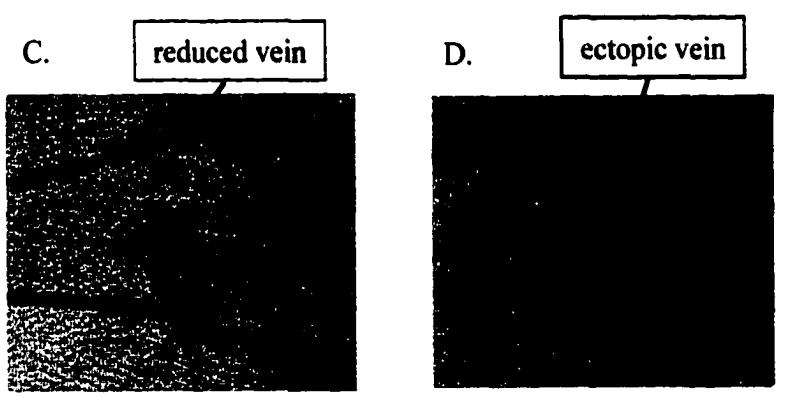
Both *capu* ^{G7}, and ^{EE15} do not behave as null alleles in this assay. *capu* ^{EE15}, behaves most like a null allele for a *capu* eggshell phenotype (Manseau and Schüpbach 1989). Females mutant for *capu* ^{EE15} over deficiency compared to females mutant for *capu* ^{EE15} over itself, lay a similar number of mutant eggs. Interestingly, the frequency of the wing vein phenotype is markedly reduced in *capu* ^{EE15} over deficiency adults when compared to *capu* ^{EE15} / *capu* ^{EE15} adults. This suggests that the ^{EE15} allele of *capu* is a gain of function allele and not a null as previously thought. The ^{G7} allele of *capu*, on the other hand, had previously been thought to be slightly antimorphic, and this data

supports this hypothesis. The *capu* ^{G7} / *capu* ^{G7} adults have a reduced wing vein phenotype that disappears when *capu* ^{G7} is over a deficiency for *capu*. Results from this assay indicate that *capu* ^{G7} behaves genetically as an antimorphic allele for the reduced posterior crossvein phenotype.

Fig. 4.3 Posterior crossvein phenotype seen in *capu* alleles. Examples of posterior crossvein phenotypes seen in *capu* mutants including, a wild type Drosophila wing (A), an enlarged view of wild type wing showing normal posterior crossvein (B), an example of reduced posterior crossvein seen in *capu* mutants (C), and an example of an ectopic crossvein seen in *capu* mutants.

Figure 4.3 Posterior crossvein phenotype seen in *capu* alleles.





*capu*G7 / *capu*G7 *capu*EE15 / *capu*EE15

Figure 4.4 *capu* wing vein phenotype

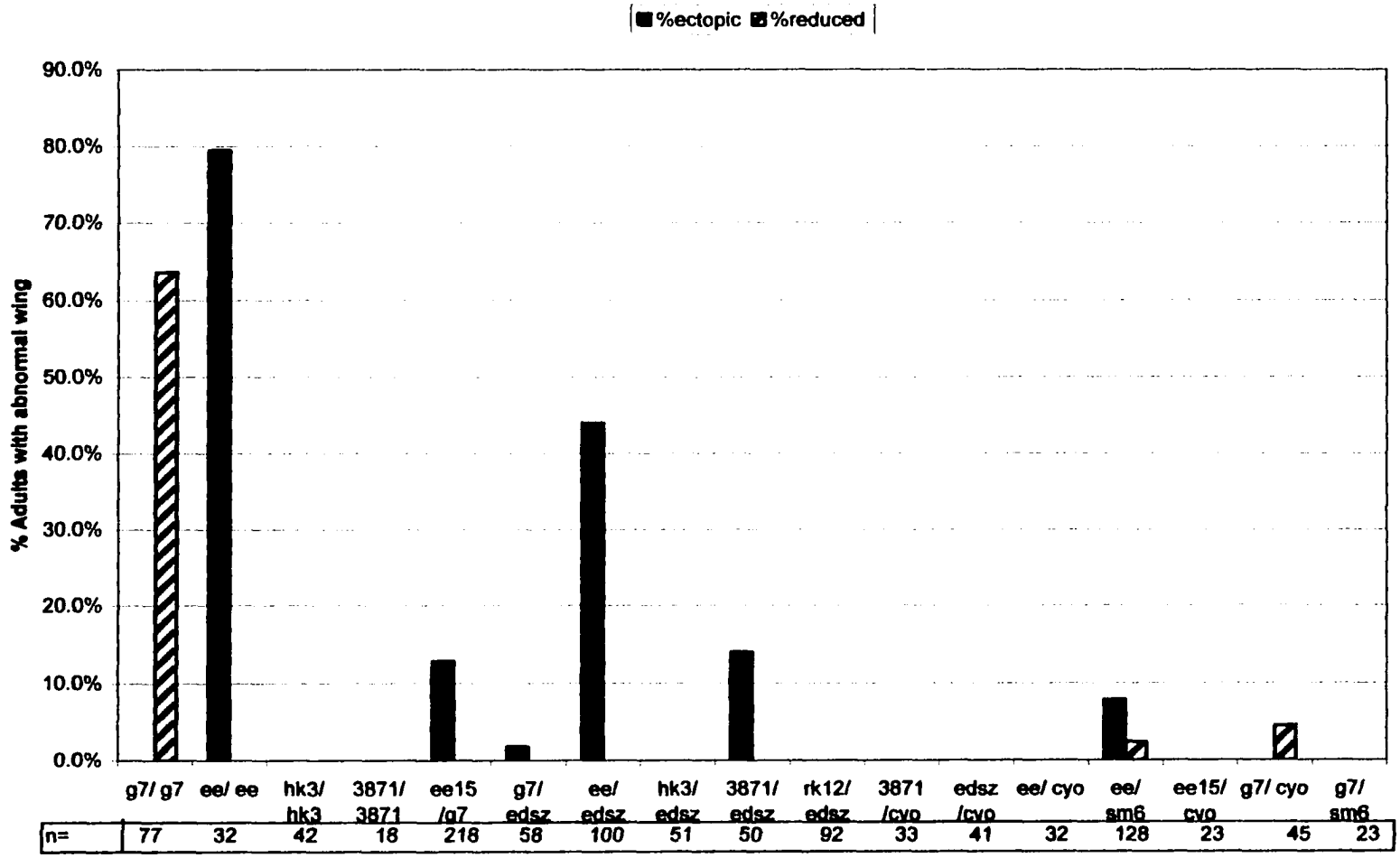


Table 4.1 *capu* wing vein phenotype

genotype	wt	ectopic	reduced	total	%ectopic	%reduced
g7/g7	28		49	77	0.0%	63.6%
ee/ee	8	31		39	79.5%	0.0%
hk3/hk3	42			42	0.0%	0.0%
3871/3871*	18			18	0.0%	0.0%
ee15/g7	190	28		218	12.8%	0.0%
g7/edsz12	57	1		58	1.7%	0.0%
ee/edsz12	56	44		100	44.0%	0.0%
hk3/edsz12	51			51	0.0%	0.0%
3871/edsz12	43	7		50	14.0%	0.0%
rk12/edsz12	92			92	0.0%	0.0%
3871/cyo	33			33	0.0%	0.0%
edsz12/cyo	41			41	0.0%	0.0%
ee/cyo	32			32	0.0%	0.0%
ee/sm6b	115	10	3	128	7.8%	2.3%
ee15/cyo**	23			23	0.0%	0.0%
g7/cyo	43		2	45	0.0%	4.4%
g7/sm6b	23			23	0.0%	0.0%

*some may be 3871/Cyo due to weak Cy phenotype in this stock.

**Cyo from G7 stock

Note: Cyo and SM6b are balancer chromosomes used in these crosses.

4.2.4 Two splice forms of *capu*, exist in addition to the 4kb ovary splice.

There are two identified expressed sequence tagged sequences (EST) that have sequence similarity with *capu* mRNA, one of which may represent the somatic splice of *capu* that is seen on a developmental northern (Emmons, Phan et al. 1995). The available sequence of both of these ESTs, GH07742 and GH26143 (both from a cDNA library made from Drosophila adult heads) (Harvey, Brokstein et al. 2001) have sequence at their 5' end that corresponds with genomic sequence 5' of the *capu* ovary cDNA. Near the 3' end of the published sequence of GH07742 is sequence that is identical to sequence found 750 base pairs from the 5' end of the *capu* ovary cDNA. I obtained these cDNAs and fully sequenced both. Both GH07742 and GH26143 are identical to the published *capu* sequence starting at the third exon of *capu*, and share this identity to the end of each cDNA clone. The predicted amino acid sequence of the new splice forms would replace the first 71 amino acids of *capu* with unique sequence. This would change the *capu* RBD, and it would be interesting to see how these splice forms interact with rho GTPases.

The known lesions in *capu* mutant alleles are in regions of *capu* that are common to all splice forms of *capu* (Emmons, Phan et al. 1995). Two alleles of *capu*, *capu* ^{G7} and *capu* ^{EE15} have no known lesion. Additionally, the *capu* ^{G7} allele is an antimorphic allele of *capu*, as females homozygous for *capu* ^{G7} lay a higher percentage of abnormal

eggs than *capu* ^{G7} over deficiency. This prompted me to sequence the exons found in the alternate splices of *capu* in each of these alleles. No lesions were found in either of these alleles in any of the newly identified exons.

Fig 4.5 *capu* splice variants. Figure 4.5 shows the genomic structure of *capu* and the *capu* splice variants GH07742 and GH26143 oriented to cosmid DS01595. Numbers listed near exons represent the boundaries of exons in relation to the sequence of cosmid DS01595. Start codons for each splice variant are noted in boxes. The first common exon for all three splice variants is at position 36280 of DS01595 and all three sequences are identical in DNA sequence 3' of this point.

Fig 4.5 *capu* splice variants.

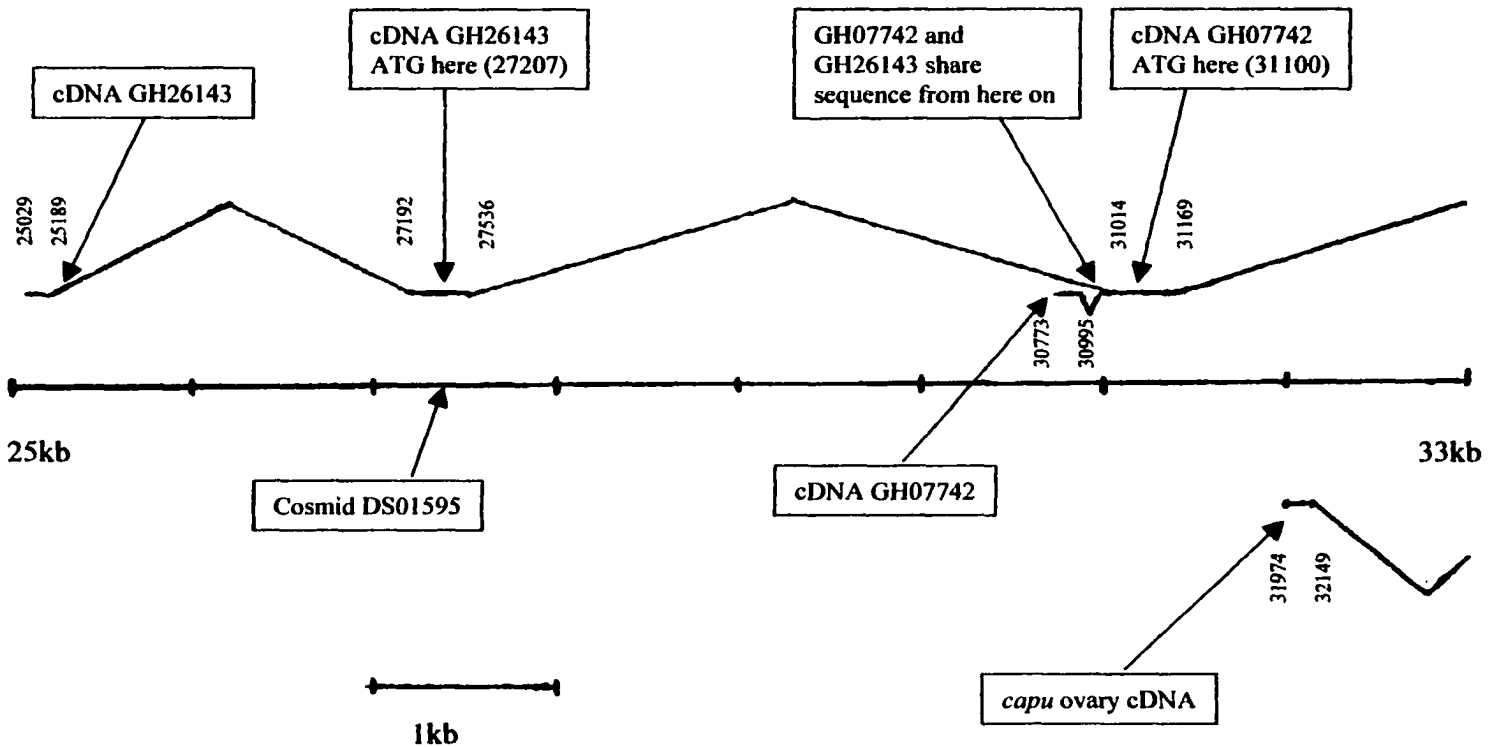


Fig 4.5 *capu* splice variants (cont.)

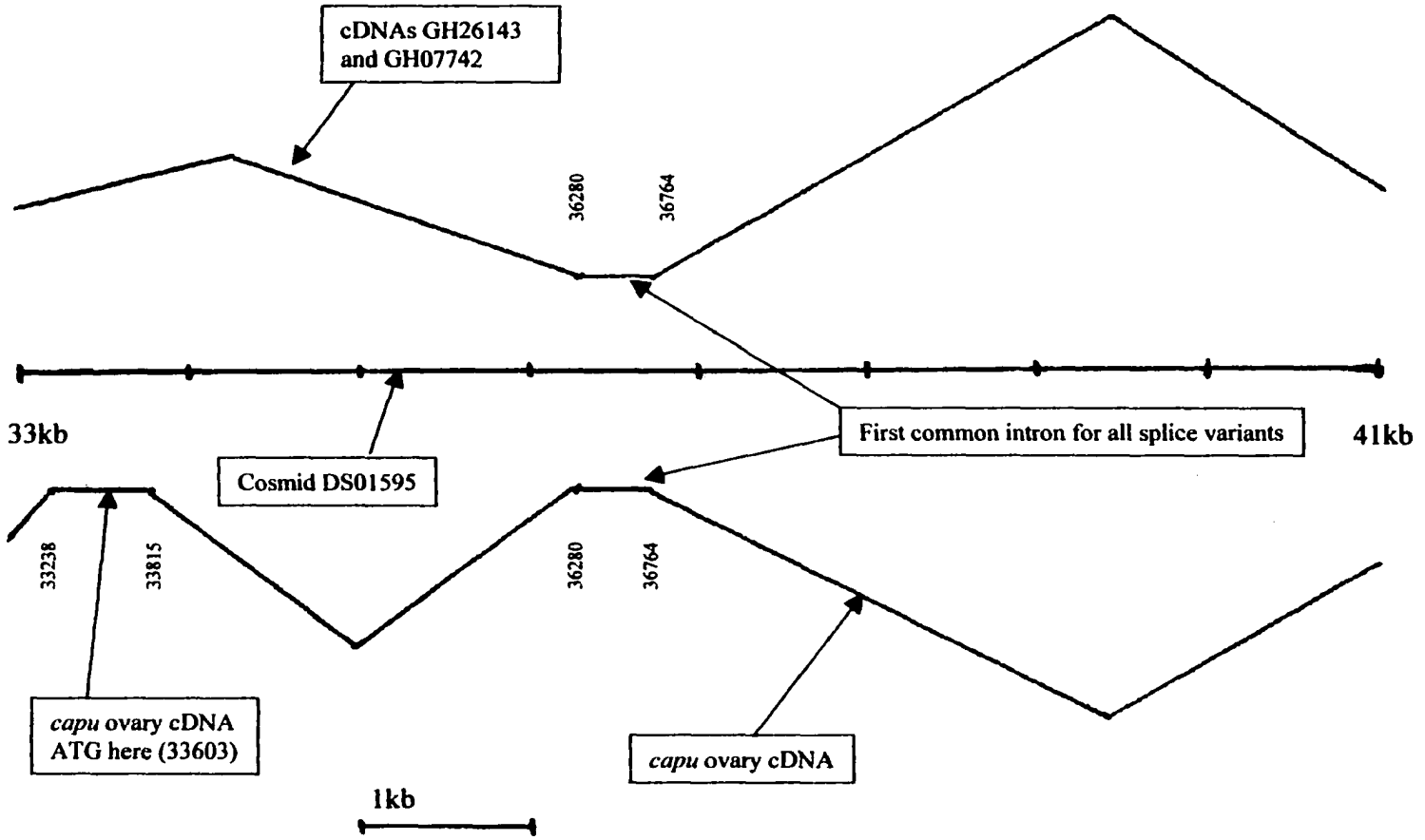
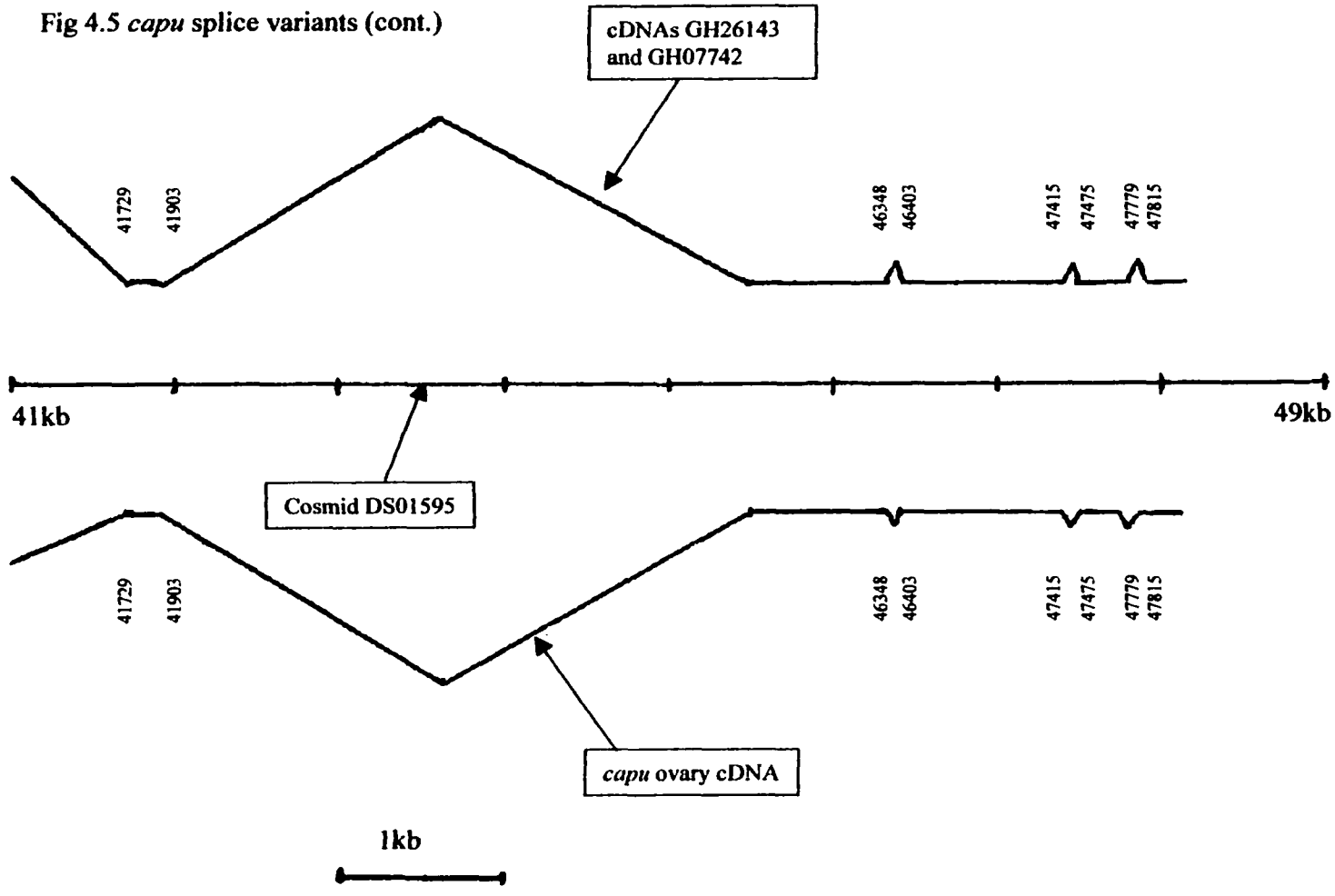


Fig 4.5 *capu* splice variants (cont.)



4.2.5 Null screen results

It is not clear if a functional null allele of *capu* exists. What is clear is that no protein null allele of *capu* exists. *capu* mRNA is expressed in all alleles and, of the seven existing *capu* alleles, the genetic lesion is known in five of them, and none have premature stop codons. Four of five have mutations that would lead to amino acid substitutions, and the fifth, *capu*3871, has a 23 base pair deletion in an intron near the 3' end of the *capu* message (Emmons, Phan et al. 1995). This deletion in the intron would most likely disrupt splicing and lead to a *capu* protein with a slight truncation or frame shift near the carboxy terminus. The remaining two *capu* alleles do not appear to have a mutation in the known *capu* protein coding sequence. In addition, two *capu* alleles, *capu* ^{G7} and *capu* ^{EE15}, behave like null alleles in one genetic test. A genetic null is defined as an allelic combination where a homozygous mutant for a given allele is similar in phenotypic severity as that same allele over a deficiency for the region.

In order to determine the phenotype of a *capu* null allele, I began a screen to generate a P-element insertion in the *capu* gene. The screen relied on the observation that P-elements tend to transpose to a location near their origin. Starting with an insertion about 60KB from the 3' end of the *capu* gene, I used a screening method similar to that described by Dalby et al. (Dalby, Pereira et al. 1995). This method involves using inverse PCR, a method that allows for the amplification of DNA flanking a known sequence, and southern blotting to identify a pool of individual flies that have an insertion near the *capu* gene. Using this method I could screen pools of 40 flies per

PCR reaction. After screening about 1500 new insertions I identified one insertion near *capu* called, V7-4 . Unfortunately this new insertion was not helpful. V7-4 was a lethal insertion 5kb from the 3' end of the *capu* gene and at the 5' end of another gene GM03787.

P-elements are not random in their choice of insertion sites. Not only did my attempt to generate a P-element insertion in *capu* fail, but the all of the genome scale screens for new P-element insertions have failed to identify an insertion in the *capu* gene. Some regions of the genome are allegedly cold spots for P-element insertions. In one example using a positive selection, 70,000 chromosomes were screened with only two insertions identified in the target gene (Nero, Bowditch et al. 1989). We have no such positive selection available for *capu*, so the decision was made not to continue the screen

I have made a knock-out construct for *capu* targeted against the first common intron of *capu* similar to that described by Rong, Y. S. and K. G. Golic (2000). This construct was injected into flies by Andrea Wellington in the lab, and Melissa Grover screened for *capu* knockouts using Andrea's transformants. No new alleles of *capu* have yet been identified using this method.

4.2.6 *Boxf* is a drosophila formin family member expressed in the head and ovary

Using degenerate primers targeted to the highly conserved FH2 domain of formins and to the FH1 proline rich region of formins, I used PCR to amplify cDNA made from Drosophila ovaries in an attempt to identify new Drosophila formins. I cloned the resulting amplified DNA into the pCRII vector (Invitrogen Inc.) and screened for insertions. I was able to identify insertions that corresponded to the known Drosophila formin, *diaphanous* (Castrillon and Wasserman 1994), and a unique insertion, but no clones corresponding to *capu* were identified, suggesting that the screen was not complete. The new insertion was sequenced and found to be similar to formins in predicted amino acid sequence, falling into the *diaphanous* subfamily of formin genes.

The Berkley Drosophila Genome Project (BDGP) has two cDNAs in its database that have identical sequence to this new Drosophila formin called *boxf* for *brain and ovary expressed formin*. One cDNA, LD26058, originates from a library made from embryos and the second cDNA, RH55943, is from a library made from Drosophila adult heads (Harvey, Brokstein et al. 2001). The BOXF sequence has been included in John Calley's extensive analysis of formin family members (Calley et al. in prep). Using the genefinder program (Wilson, Hilyer et al.), together with the published EST sequences of RH55943, LD26058 and the sequenced of the insertion I identified, I compiled the boxf sequence shown in figure 4.6.

Fig 4.6 *boxf* sequence. Proline rich FH1 region is marked with asterisks and highly conserved FH2 domain is underlined. The sequence corresponding to the degenerate primers used in the screen that identified are in italics and labeled "reverse primer" or "forward primer".

Fig 4.6 *boxf* sequence.

```
       1 ATGCCCGGCTTCAGGGGCAGAAGGGTGTGGTGCGGTTGCTTCAAGGACGATGAGCCACCC
1        M  P  G  F  R  G  R  R  V  W  C  G  C  F  K  D  D  E  P  P  -

      61 GAGATTTGTGTGGTGGAAGGCGCGTTTACCCTGCAGACGCTCACGCCCACCCAACCCATG
21       E  I  C  V  V  E  G  A  F  T  L  Q  T  L  T  P  T  Q  P  M  -

     121 CCGTCAGTGGATGAGCTGGACACCAAGTTCGCGGAGCTGGTGGAGGAGCTCGATCTGACG
41       P  S  V  D  E  L  D  T  K  F  A  E  L  V  E  E  L  D  L  T  -

     181 GCCCCCAACAAGGAGGCGATGCTCAGCCTGCCCGCGCAGAAGAAATGGCAAATCTACTGC
61       A  P  N  K  E  A  M  L  S  L  P  A  Q  K  K  W  Q  I  Y  C  -

     241 TCGAGAAAGCTGCCCTTGGATGCGGCCGATGGCCCAGATGCAGCGGCCGTCACCCAGCCA
81       S  R  K  L  P  L  D  A  A  D  G  P  D  A  A  A  V  T  Q  P  -

     301 CCCACCGCAGAGCACTACATTGAGCGACTGAAGGAGCTGGTGGTGCACATATCGCTCTCG
101      P  T  A  E  H  Y  I  E  R  L  K  E  L  V  V  H  I  S  L  S  -

     361 CCGGAAGACTCGCCCAGCCATGAGCTGGGCAATCGATTGGATGGCCATGCCGCCTTTGTG
121      P  E  D  S  P  S  H  E  L  G  N  R  L  D  G  H  A  A  F  V  -

     421 GACGCTTTGAAGACGGCGCTACGCACATCCACCCACAGTTTTCTGGACATTCGAGTGGCT
141      D  A  L  K  T  A  L  R  T  S  T  H  S  F  L  D  I  R  V  A  -

     481 AACAGCCCGCTGCACACCAGTCTCATTGGTTGCATCAAGGCCCTGATGAACAACTCGATG
161      N  S  P  L  H  T  S  L  I  G  C  I  K  A  L  M  N  N  S  M  -

     541 GGTCGGGCGCATGTGCTAGCCCATCCAACGGCCATTGACACCATAGCCAGATCCCTAGCG
181      G  R  A  H  V  L  A  H  P  T  A  I  D  T  I  A  R  S  L  A  -

     601 GCGGACAACATTCGCACGAAAATCGCCGCCCTGGAGATTCTGGGTGCCGTGTGTCTGGTG
201      A  D  N  I  R  T  K  I  A  A  L  E  I  L  G  A  V  C  L  V  -

     661 CCCGGCGGTCATCGCAAGGTGCTGCAGGCCATGCTCCACTTCCAGGTCTTCGCTACGGAA
221      P  G  G  H  R  K  V  L  Q  A  M  L  H  F  Q  V  F  A  T  E  -

     721 CGCACCCGTTTCCAGAGCATTGTCAACGACCTGGACCGCTCGACCTATGCGTACAGGGAC
241      R  T  R  F  Q  S  I  V  N  D  L  D  R  S  T  Y  A  Y  R  D  -

     781 AATGTCAATCTAAAGACCGCCCTTATGTCATTCGTAAATGCGGTGCTTAATTACGGACCT
261      N  V  N  L  K  T  A  L  M  S  F  V  N  A  V  L  N  Y  G  P  -

     841 GGACAGGAGAACCTCGAGTTCCGACTGCACTTGAGGTACGAGTTTCTCATGCTCGGTATA
281      G  Q  E  N  L  E  F  R  L  H  L  R  Y  E  F  L  M  L  G  I  -

     901 CAGCCTGTGATCGACAAGCTGCGCACGCACGAGAACGAAACGCTGGACAGGCATTTGGAC
301      Q  P  V  I  D  K  L  R  T  H  E  N  E  T  L  D  R  H  L  D  -

     961 TTCTTTGAGATGGTTCGTGCTGAGGATGAGAAGGAGTTTGCCCGCCGTTTTAAGGAGGAG
321      F  F  E  M  V  R  A  E  D  E  K  E  F  A  R  R  F  K  E  E  -
```

Fig 4.6 *boxf* sequence (cont.).

```
    1021 CACGTGGACACCAAGAGCGCCGGCTCAATGTTTGAGCTGCTGCGCCGCAAGCTCAGTCAT
341      H  V  D  T  K  S  A  G  S  M  F  E  L  L  R  R  K  L  S  H  -

    1081 TCGCCCGCGTATCCCCACATGCTCTCCCTTCTGCAGCACATGCTTCTGCTGCCCTATACG
361      S  P  A  Y  P  H  M  L  S  L  L  Q  H  M  L  L  L  P  Y  T  -

    1141 GGCCACTGCACGGAGCACTGGTTGCTGATTGATCGCGTGGTACAGCAGATAGTGCTGCAG
381    G  H  C  T  E  H  W  L  L  I  D  R  V  V  Q  Q  I  V  L  Q  -

    1201 GTGGAACAGCGGCCAAACAGTGATCTTATCGTCGACCCCGATGACCCTGAGAAGCAGCTG
401      V  E  Q  R  P  N  S  D  L  I  V  D  P  D  D  P  E  K  Q  L  -

    1261 AAGCTAGCCGCCGAGTCGCCAGTCCATGATCCCGACGTGGCGCCCTTGCAGATCGACGTG
421      K  L  A  A  E  S  P  V  H  D  P  D  V  A  P  L  Q  I  D  V  -

    1321 GCTAAGCTTGTGCGTCTGCTGGTCAAGGAGGAACAGCTGACGCAAGCGCGAAAGCGGGCT
441      A  K  L  V  R  L  L  V  K  E  E  Q  L  T  Q  A  R  K  R  A  -

    1381 GACGAGCTGGAGCGCGAGAACTTTGACGTGCAGTCGCGGCTGGCCAAGAAGGAGCAGGAA
461      D  E  L  E  R  E  N  F  D  V  Q  S  R  L  A  K  K  E  Q  E  -

    1441 CTCGATCTCCGCATGCAAGAGAAAGAGGACTTGGAGACGGGACTAGCGCGCATGCGTGAG
481      L  D  L  R  M  Q  E  K  E  D  L  E  T  G  L  A  R  M  R  E  -

    1501 CGTTTGGAGAAGGAGTCCGCGCAGCACTCACAGGCGGTGCAGCGGGCGCAGACTGCAGAG
501      R  L  E  K  E  S  A  Q  H  S  Q  A  V  Q  R  A  Q  T  A  E  -

    1561 ATGCGAGCAGAAGACCTGCAGCACCGATTGATCAGCGAGCAGCAGGAACGGGCTCGCTTG
521      M  R  A  E  D  L  Q  H  R  L  I  S  E  Q  Q  E  R  A  R  L  -

    1621 GAGAGACTGGTTACCGAGGGCAGCATCCCAGACGACCAGAAGGTGGCCGGACTCACGGGA
541      E  R  L  V  T  E  G  S  I  P  D  D  Q  K  V  A  G  L  T  G  -

                          ****************************************

    1681 TGCAATGGTGCCGTCTCGCCTCCACCGGCACCTCCCATGCTTAAGGCCATCCCGCCGCCT
561      C  N  G  A  V  S  P  P  P  A  P  P  M  L  K  A  I  P  P  P  -

                                                                  For-
         ************************************************************

    1741 CCGCCACCCATGGCGCCGTCCATGATGCCCCCACCACCACCTCCTTGCCCGGGAGCT*CCT*
581      P  P  P  M  A  P  S  M  M  P  P  P  P  P  P  C  P  G  A  P  -

         ward primer →
         ************************************************************

    1801 *CCACCTCCGCCA*AGCATGGCACAAACGATGGCTCCAGCGCCACCAAAAGTGGATCTGCCA
601      P  P  P  P  S  M  A  Q  T  M  A  P  A  P  P  K  V  D  L  P  -

    1861 AAGAAGAATGTGCCACAGCCGACAAATCCCCTGAAGAGCTTCAACTGGTCGAAACTGCCG
621      K  K  N  V  P  Q  P  T  N  P  L  K  S  F  N  W  S  K  L  P  -
```

Fig 4.6 *boxf* sequence (cont.).

```
    1921 GACGCCAAGCTGCAGGGTACCGTTTGGAGCGAGCTCGACGAAAGCAAGCTGTACAACAAC
641      D  A  K  L  Q  G  T  V  W  S  E  L  D  E  S  K  L  Y  N  N  -

    1981 ATGGAGCTGGAGTCAATTGATAAACTTTTCTCGGCTTACCAAAAGAACGGGGTATCGGCC
661      M  E  L  E  S  I  D  K  L  F  S  A  Y  Q  K  N  G  V  S  A  -

    2041 ACTGATGGATCCTATGAGGACTTACGGGTAACTGGCAAGGCTGCCAAGCAGAAAGTGCTG
681      T  D  G  S  Y  E  D  L  R  V  T  G  K  A  A  K  Q  K  V  L  -

    2101 TCGGTGATCGACGGACGTCGGGCGCAGAACTGCACCATTCTGCTGAGCAAACTGAAGATG
701      S  V  I  D  G  R  R  A  Q  N  C  T  I  L  L  S  K  L  K  M  -

    2161 AGCGACATGGAGATATCAAAGGCCATTCTTTCCATGGACAGCAACGAGCAGCTGCAGCTG
721      S  D  M  E  I  S  K  A  I  L  S  M  D  S  N  E  Q  L  Q  L  -

    2221 GACATGGTCGAGCAGCTGCTCAAGTTTACGCCCTCGGCGGAGGAGCGAGCTTTGCTGGAC
741      D  M  V  E  Q  L  L  K  F  T  P  S  A  E  E  R  A  L  L  D  -

    2281 GAGCACAGCGAGGACATTGAGTCTCTCGCTCGGGCCGATCGATTCCTCTATGAGATATCC
761      E  H  S  E  D  I  E  S  L  A  R  A  D  R  F  L  Y  E  I  S  -

    2341 AAGATTCCGCACTACGAGCAGCGACTGAAGAGTCTGCACTACAAGAAGCGTTTCATGCTG
781      K  I  P  H  Y  E  Q  R  L  K  S  L  H  Y  K  K  R  F  M  L  -

    2401 ACCATCAACGACCTGGTCCCCCGCATAACCAGTGTGATGGAGGCCTCTCGTGAGGTGGCT
801      T  I  N  D  L  V  P  R  I  T  S  V  M  E  A  S  R  E  V  A  -

    2461 CGTTCCCGTCGCCTGCGTAAGCTTCTGGAGTTGGTCCTCGCCCTAGGTATGATCCACTGT
821      R  S  R  R  L  R  K  L  L  E  L  V  L  A  L  G  M  I  H  C  -

                                                  ←---Reverse primer
    2521 CCGTTTGCTAAGCACTCAATAACCCAGACTACCTCTATCCCA CCAGGCAACTACATGAAC
841      P  F  A  K  H  S  I  T  Q  T  T  S  I  P  P  G  N  Y  M  N  -

    2581 CGCGGGGCACGTGGCAACGCATCGGGATTCCGACTGGCGTCACTCAACCGGCTGGCGGAC
861      R  G  A  R  G  N  A  S  G  F  R  L  A  S  L  N  R  L  A  D  -

    2641 ACCAAGTCCAGTGCCGCCAAGGGCACCACCCTGCTACACTACCTCGTGCAGGTGATTGAA
881      T  K  S  S  A  A  K  G  T  T  L  L  H  Y  L  V  Q  V  I  E  -

    2701 CGCAAATTCAAGGACCTTCTTAAGTTGGAGGACGATATCCCGCATGTGCGCGAAGCCTCC
901      R  K  F  K  D  L  L  K  L  E  D  D  I  P  H  V  R  E  A  S  -

    2761 AAGGTGTCGCTGGGCGAGATGGACAAGGACATTCAGATGCTGCGCACTGGTCTGGCAGAC
921      K  V  S  L  G  E  M  D  K  D  I  Q  M  L  R  T  G  L  A  D  -

    2821 GTAGCGCGCGAAATCGAGTTCCACCGCAGTTCGGGCCCGGCCCAACAGGGTGACCGCTTT
941      V  A  R  E  I  E  F  H  R  S  S  G  P  A  Q  Q  G  D  R  F  -
```

Fig 4.6 *boxf* sequence (cont.).

```
    2881 CTGCCCGTAATGCGCGAGTTCCACGCCCAGGCGTCAGTGCGCTTCGCTGAGCTGGAGGAC
961      L  P  V  M  R  E  F  H  A  Q  A  S  V  R  F  A  E  L  E  D  -

    2941 AAGTTCCAGGACATGAAGACGCGCTTTGACCGTGCTGTACGCCTCTTCGGCGAGGATGGT
981      K  F  Q  D  M  K  T  R  F  D  R  A  V  R  L  F  G  E  D  G  -

    3001 TCGGTTTTGCAGCCGGACGAGTTCTTTGGCATCTTTGACTCCTTCCTCGCCGCCTTCGCG
1001     S  V  L  Q  P  D  E  F  F  G  I  F  D  S  F  L  A  A  F  A  -

    3061 GAGGCGCGGCATGACAACGAGAGCTTCCGTCGGCGACAGGAGGAAGAGGAGAAGCGTGCC
1021     E  A  R  H  D  N  E  S  F  R  R  R  Q  E  E  E  E  K  R  A  -

    3121 AAGCAGGAGGCGGAGCTCAAAAAGCGCACAATAGAGCGCAAGAACAAGACCGGCCTAATG
1041     K  Q  E  A  E  L  K  K  R  T  I  E  R  K  N  K  T  G  L  M  -

    3181 ACCAGCGTGGCTCGCAATCTGGGCCTCAAGTCAGGCTCTTCCAACGGGGATCCTGACTCC
1061     T  S  V  A  R  N  L  G  L  K  S  G  S  S  N  G  D  P  D  S  -

    3241 CCGGCAAAGGGCGGCGACAACAAGGGCGAGTTTGACGATCTCATCTCGGCCCTGAGGACC
1081     P  A  K  G  G  D  N  K  G  E  F  D  D  L  I  S  A  L  R  T  -

    3301 GGCGACGTGTTTGGCGAGGACATGGCCAAGTTCAAGCGGTCGCGCAAGGCGCGCGTGCTT
1101     G  D  V  F  G  E  D  M  A  K  F  K  R  S  R  K  A  R  V  L  -

    3361 AACGGCGGCGGATCCTCCACTGGGCACACCTCGCCGCCCCGCCACGGCAGCCTCCAAAGG
1121     N  G  G  G  S  S  T  G  H  T  S  P  P  R  H  G  S  L  Q  R  -

    3421 GAGGAGAGTGGGCGTGAGCGCGAGCGGACCGTGAGGCGCCAGTAA
1141     E  E  S  G  R  E  R  E  R  T  V  R  R  Q  *  -
```

4.3 DISCUSSION

In this section I have provided additional information about *capu* mRNA expression and shown evidence that the function of the *capu* gene is not limited to oogenesis as has been previously suspected (Manseau and Schüpbach 1989; Emmons, Phan et al. 1995; Manseau, Calley et al. 1996). I have shown *capu* mRNA expression in a tissue where *capu* function is known, the ovaries, and in tissues where no known function of *capu* has been described, larval brains. I have identified and sequenced two cDNAs that correspond to alternate splice forms of *capu*. Additionally, I have described and quantified a *capu* mutant phenotype found in wings of mutant adults. This is the first demonstrated function of *capu* outside of oogenesis.

4.3.1 *capu* mRNA expression and regulation.

The localization of *capu* mRNA during oogenesis is not surprising given that this is where phenotypes of *capu* mutants are most clearly evident. *capu* mRNA is transcribed in the nurse cells, transported to the oocyte, and subsequently localized to the oocyte nucleus during the stages where patterning is disrupted in *capu* mutants. One unexpected finding from these experiments is that *capu* is expressed in the somatic follicle cells surrounding the egg chamber, a tissue that mosaic analysis suggests should not be critical for *capu* phenotypes (Manseau and Schüpbach 1989).

The *capu* staining pattern in brains, taken with *capu*'s role in regulating the cytoskeleton, suggested that *capu* may play a role in synaptic remolding. This observation prompted me to check the *capu* cDNA sequence for a cytoplasmic polyadenylation element (CPE), which has been implicated in the regulation of translation of α-CaMKII mRNA at the synapse, and in synaptic remodeling (Wu, Wells et al. 1998; Richter 2001). I found that *capu*'s 5' UTR does have a CPE. I checked the sequence of the 5' UTR of the *capu* alleles G7 and EE15, for which no known lesion exists, and found that neither had any mutations in their 5' UTR. Recently the Drosophila gene *orb* has been shown to be a CPE binding protein (Tan, Chang et al. 2001). *orb* function, like *capu* function, has been shown to be required for determination of both the DV and AP axes (Christerson and McKearin 1994). *orb's* demonstrated involvement in translation of mRNAs of the maternal effect genes *gurken* (Tan, Chang et al. 2001) and *oskar* (Chang, Tan et al. 1999), coupled with its effect on DV and AP patterning, and the CPE found in *capu*, suggest the interesting possibility that *orb* may regulate temporal and spatial translation of *capu* mRNA. It will take either transgenic experiments with a CPE deficient form of the *capu* gene, or possibly the use of the targeted allelic substitution of *capu* with the method described by Rong, Y. S. and K. G. Golic (2000), to determine if *capu*'s CPE plays a role in *capu* function.

4.3.2 *capu* wing vein phenotype.

The formation of crossveins in drosophila has been shown to involve several genes. Specifically, two genes encoding a BMP-like ligand are involved in crossvein morphogenesis (Khalsa, Yoon et al. 1998; Conley, Silburn et al. 2000). Alleles in the gene *crossveinless*-2 (*cv*-2) lack crossveins and are enhanced in combination with the BMP-like like ligand, *decapentaplegic* (*Dpp*) suggesting that the novel cystein-rich CV-2 gene product is a novel member of the BMP signaling pathway (Conley, Silburn et al. 2000). Certain alleles of *Glass Bottom Boat*(*Gbb*), which also encodes a BMP-like ligand, lack both the posterior and anterior crossveins (Khalsa, Yoon et al. 1998).

Loss of function mutations in the Drosophila *cdc*42 gene produce ectopic crossveins (Genova, Jong et al. 2000). It is hard to know if this phenotype is related to *capu* because mutations in *cdc*42 tend to effect the anterior crossvein, unlike mutations in *capu*, and there appears to be no enhancement of the crossvein phenotype in *capu* *cdc*42 transheterozygous mutants.

4.3.3 *capu* function outside of oogenesis and the need for a *capu* null allele

It is clear that the function of the *capu* gene is not limited to the ovary as once suspected (Manseau and Schüpbach 1989; Emmons, Phan et al. 1995; Manseau, Calley et al. 1996). Not only does the *capu* wing vein phenotype contradict the idea that *capu* function is limited to the ovary, but the specific allelic data suggest that none of the existing alleles are null alleles. I found that both *capu* ^{G7} and *capu* ^{EE15}, the two

strongest *capu* alleles, do not behave like null alleles when scored for wing vein phenotypes. Additional data showing the specific pattern of expression of the *capu* mRNA in the brains of 3rd instar larvae and in the adult brains suggest that *capu* is likely playing a role in these tissues.

There are no existing null alleles of *capu*. Four of the existing seven alleles of *capu* were initially identified in screens looking for viable female sterile mutations. This screening method would eliminate any allele that had a strong adult phenotype. Additionally, I have shown that the EE15 allele of *capu* that has the strongest effect on DV patterning and which has been labeled a genetic null allele for this phenotype, behaves like a weak hypomorph when the wing vein phenotype is assayed.

The analysis of function of a gene based on phenotype benefits greatly from the knowledge that at least one allele is a null allele. The *capu* wing vein phenotype provides an excellent example of why this is true. The phenotype is most easily seen in the strongest existing alleles of *capu*, $capu^{G7}$ and $capu^{EE15}$, neither of which appears to be a null. There is no telling what the phenotype of a *capu* null allele would be. A null allele may unmask phenotypes not yet seen in *capu* mutants. I have made two attempts to generate a *capu* null allele. The first attempt was to identify a p-element insertion in a PCR based p-element screen was not successful. The second attempt has been a collaborative attempt with Andrea Wellington and Melissa Grover to use the

Drosophila targeted knock method described by Rong, Y. S. and K. G. Golic (2000), and has not yet been successful.

Dr. Manseau has attempted to knockout *capu* function using an a transgenic Drosophila line expressing a RNA interference construct targeted at the first common splice of *capu,* in an attempt to knockout all *capu* function (Fire, Xu et al. 1998; Kennerdell and Carthew 1998). These lines did generate a slight *capu* phenotype when expressed in ovaries, but they obviously did not knock out all *capu* function. Interestingly, they did have a slight ectopic wing vein phenotype when expressed in developing wing imaginal disks.

Little was known about the morphogenesis of posterior crossveins when this *capu* phenotype was identified and at first it was looked at as a potential tool to be used in a screen for new *capu* alleles. Before the identification of a somatic phenotype the only way to screen for a *capu* allele was to perform an F2 female sterile screen. This screen requires the crossing of individual putative *capu* mutant males, offspring of mutaganized males, to females with an existing allele of *capu,* and scoring the female offspring of this cross for a *capu* phenotype. A starting point for the odds of getting a new mutation in a given gene is one in one thousand mutaganized chromosomes. The F2 female sterile screen requires the cross, and tracking of a cross for every chromosome mutaganized. On the other hand if a viable fertile adult phenotype is identified new alleles can be screened for in an F1 screen. An F1 screen allows one to

cross a mutaganized male (this could be a male treated with a mutagen such as EMS) with an existing allele of the gene one is screening for and checking the F1 male offspring for the phenotype identified with that gene. These F1 individuals can be rapidly screened and only those with phenotypes that suggest a new allele would need any further handling.

CHAPTER 5. SUMMARY AND PERSPECTIVE.

5.1.1 Are *capu* and *spir* regulators of the actin cytoskeleton, microtubule cytoskeleton or both?

There are three simple explanations for the role of CAPU and SPIR in the regulation of the microtubule cytoskeleton: (1) CAPU and SPIR regulate the actin cytoskeleton and the actin cytoskeleton in turn regulates the microtubule cytoskeleton; (2) CAPU and SPIR directly regulate the microtubule cytoskeleton; (3) CAPU and SPIR are regulating both the actin and microtubule cytoskeletons in parallel (Fig. 5.1).

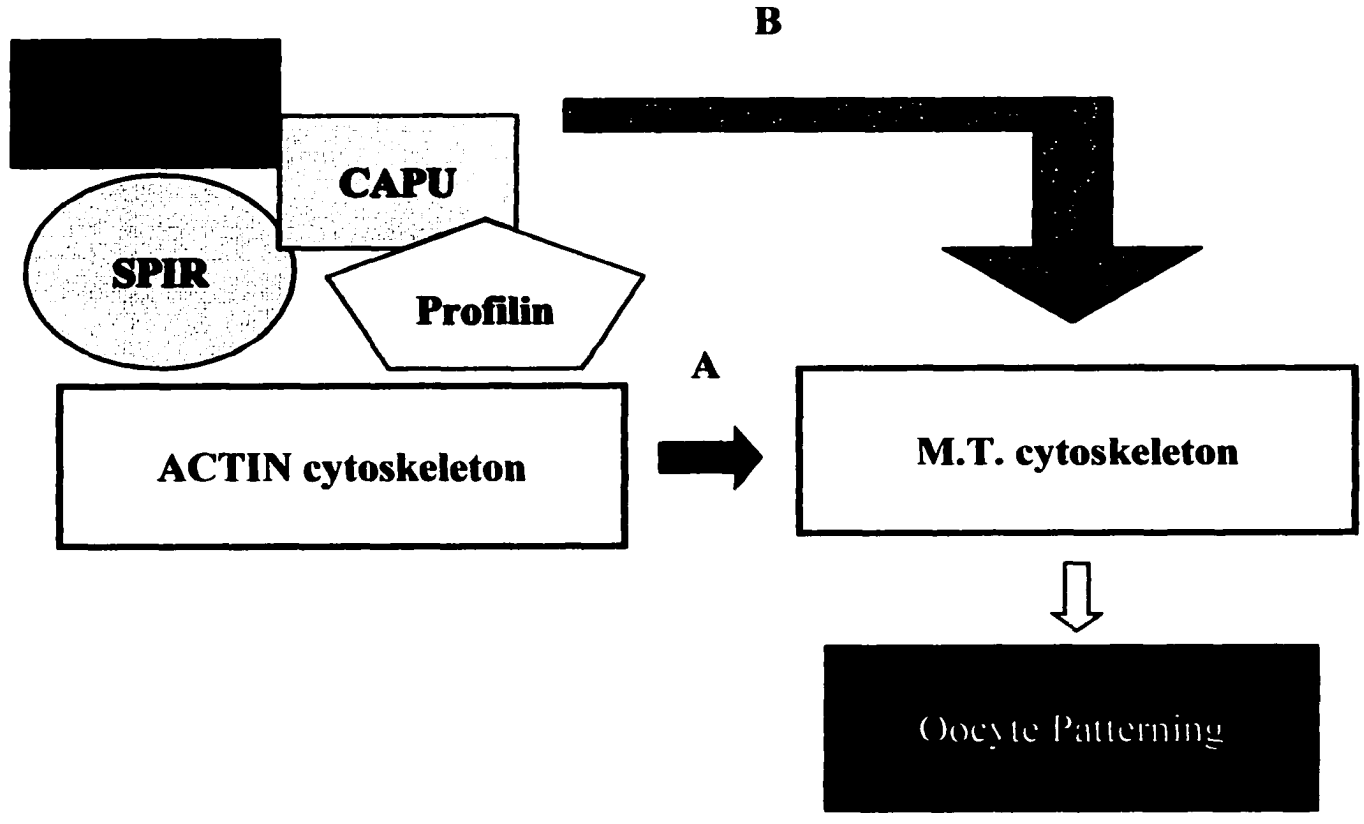
It is clear that the microtubule cytoskeleton in the oocyte is affected in *capu* and *spir* mutants (Theurkauf 1994; Emmons, Phan et al. 1995; Wellington, Emmons et al. 1999), but this defect is not clear if this is the "primary" defect in these mutants, or if it is downstream of an effect on the actin cytoskeleton. The only observable cytoskeletal phenotype in *capu* and *spir* mutants is in the microtubule cytoskeleton. Although neither *capu* nor *spir* have an observable actin phenotype, much of the early molecular information about *capu* an *spir* suggested a link to the actin cytoskeleton. Specifically, members of the formin family were first implicated in several actin dependent processes(Frazier and Field 1997): cytokinesis (Castrillon and Wasserman 1994; Kamei, Tanaka et al. 1998; Chang 1999), cell polarity (Evangelista, Blundell et al. 1997; Palmieri and Haarer 1998), and organization of the actin cytoskeleton

(Westwick, Lambert et al. 1997). *capu* and other formin members have been shown to interact with profilin, a known regulator of the actin cytoskeleton (Manseau, Calley et al. 1996; Chang, Drubin et al. 1997; Evangelista, Blundell et al. 1997; Imamura, Tanaka et al. 1997; Watanabe, Madaule et al. 1997), and both *capu* and *spir* interact with rho family GTPases, which are often implicated in actin regulation (Drechsel, Hyman et al. 1997; Hall 1998). *spir* interacts directly with actin monomers (Wellington, Emmons et al. 1999), and disrupting the actin cytoskeleton with cytochalasin D phenocopied many of the *capu* and *spir* phenotypes.

On the other hand, there is some evidence that at least *capu* is directly regulating the microtubule cytoskeleton. Recently it has been shown that the formin, mDia, interacts directly with microtubules (Ishizaki, Morishima et al. 2001; Palazzo, Cook et al. 2001) and John Calley identified β-tubulin as a *capu* interactor in a two hybrid screen (Calley 1998). There is also some indirect evidence that rho family GTPases are capable of regulating the microtubule cytoskeleton. The cdc42 interacting protein 4 (CIP4) binds microtubules, and WASP (Tian, Nelson et al. 2000). CIP4 also colocalizes with microtubules and has been shown to mediate colocalization of WASP and microtubules (Tian, Nelson et al. 2000). The rho GTPase, rac1, has been shown to bind tubulin directly *in vitro*, but there is no *in vivo* or genetic data to support an *in vivo* interaction (Best, Ahmed et al. 1996).

Fig. 5.1. Two possible models for how CAPU and SPIR function during oogenesis. Each model assumes that the actin cytoskeleton is required for proper regulation of the microtubule cytoskeleton. (A) Rho regulates the actin cytoskeleton through CAPU and SPIR which in turn regulates the microtubule cytoskeleton through some unknown intermediate. (B) The actin cytoskeleton regulates the microtubule cytoskeleton through interactions with CAPU, SPIR and Rho. An additional possibility is that some aspects of each model are true. A direct link to the microtubule cytoskeleton is yet to be identified.

Fig. 5.1. Two possible models for how CAPU and SPIR function during oogenesis.



5.1.2 *capu* function is not limited to the female germline.

There is a strong argument that *capu* functions in somatic tissue and that the existing alleles of *capu* are not null alleles: 1) *capu* has a somatic phenotype, 2) no *capu* alleles behave like genetic null alleles when the wing vein phenotype is assayed, 3) only three existing alleles of *capu* are derived from screens that would have allowed for somatic phenotypes, 4) none of the known lesions in *capu* mutants would obviously produce a null allele (Emmons, Phan et al. 1995), 5) *capu* message is expressed throughout development. Unfortunately, attempts to engineer a null allele of *capu* by myself and others have all proved unsuccessful. With this in mind I think that caution should be exercised when interpreting any phenotypic information from existing alleles of *capu*.

5.1.3 Where is CAPU in the cell?

We still do not know with confidence the subcellular localization of CAPU. Antibodies raised in rabbits against peptides containing *capu* amino acid sequence show low levels of staining in the cytoplasm and high levels of staining in the oocyte and nurse cell nuclei. Antibodies raised against a CAPU fusion protein show high staining in the cytoplasm and the oocyte, and low levels of staining in the nurse cells. Unfortunately there is not a good negative control for these experiments. None of the mutant alleles of *capu* alter the staining pattern seen with the existing *capu* antibodies.

It would be nice to know the location of epitope tagged versions of CAPU in the cell. This has been attempted with CAPU fused to green fluorescent protein and with CAPU fused to the HA epitope both under control of an early oogenesis promoter and no visible staining or fluorescence was detected. These experiments should be retried using the GAL4/UAS (Brand and Perrimon 1993) system which would allow for more control over expression.

5.1.4 What would be the effect of a constitutively active version of *capu*?

To further confirm the direct interaction between *capu* and rho GTPase *in vivo* it would be interesting to see the effect of a *capu* transgenic construct that is missing its amino terminal rho binding domain (RBD). Members of the diaphanous and BNI1 subfamilies of formins tend to behave as constitutively active alleles when their RBD is deleted (Evangelista, Blundell et al. 1997; Watanabe, Kato et al. 1999; Tominaga, Sahai et al. 2000), but this has not been demonstrated with a formin subfamily member yet. It has been suggested that when a formin is bound by a rho GTPase the proline rich FH1 domain of the formin is uncovered and allowed to interact with downstream factors. Thus by removing the RBD, a constitutively active allele should be created, and if *capu* is regulated by rho *in vivo*, truncations in it should also have a constitutively active effect.

Taken together the data described here help demonstrate that *capu* and *spir* play a role in early axis determination in Drosophila, and in the regulation of the cytoskeleton. It remains to be determined if *capu* and *spir* act to regulate the actin cytoskeleton which in turn regulates the microtubule cytoskeleton, or if *capu* and *spir* facilitate the regulation of the microtubule cytoskeleton by the actin cytoskeleton.

Chapter 6. Materials and Methods

6.1.1 GST-rho plasmid Constructs

The GST fusion constructs of the Drosophila rho GTPases were made as follows. The rho GTPases, CDC42, RAC1, RHOA and RHOL were PCRed from of John Calley's two-hybrid construct using the gene specific primers listed below. Each forward PCR primer contained a BamHI site. The PCR products were cut with BamHI and were cloned into pGEX3X blunted at its EcoRI site and cut with BamHI.

The rho GTPase primers used were:
for RHOA ggtcgactcgagcggGAGCAAAAGGCtTCTGGTCTTCTTCCTC, and
aattcccggggatccgtATGACGACGATTCGCaag.
For RHOL, aattcccggggatccgtATGACGGCGAACATAACG, and
ggtcgactcgagcggCAGTATTTTGCtCGATTGCTTGGACGTCG.
For CDC42, aattcccggggatccgtATGCAAACCATCAAGTGC, and
ggtcgactcgagcggTAAGAATTTGCtCTTCCTTTTCTTTGTGGG.
For RAC1, aattcccggggatccgtATGCAGGCGATCAAGTG, and
ggtcgactcgagcggGAGCAGGGCGCtCTTGCGCTTGGACTTGG.

6.1.2 GST pull down assay.

GST fusions of Rho GTPases were purified as described in (Self and Hall 1995)(Self and Hall 1995)(Self and Hall 1995)(Self and Hall 1995). *Capu* and *spir* subcloned in pBlueScript were linearized with NotI and *in vitro* transcribed using the Ambion mMessage mMachine kit. *In vitro* transcribed message was *in vitro* translated and labeled with ^{35}S-Met using the Ambion Retic Lysate IVT kit. Binding assays were performed as described by (Lu and Settleman 1999)(Lu and Settleman 1999) . GST fusion proteins (approxamently 4-5 ug per reaction) bound to glutathione beads were incubated with 0.5 mM GDP or GTP-γ-S for 30 min. at 30°C in nucleotide exchange buffer [20 mM HEPES (pH 7.08) 5 mM EDTA, 0.1 mM EGTA, 50 mM NaCl, 0.1 mM DTT]. Nucleotide exchange was stopped by addition of $MgCl_2$ to a final concentration of 20 mM. GTP-γ-S or GDP loaded GST fused Rho GTPases were incubated with four microliters of each twenty five-microliter *in vitro* translation reaction for 1 hour at 4°C and washed three times in wash buffer [20 mM HEPES (pH7.5), 150mM NaCl, 10% Glycerol, 0.1% Triton X-100]. Washed samples were then boiled in SDS-PAGE sample buffer and analyzed by SDS-PAGE.

6.1.3 Actin binding assay.

Plates coated with G-actin isolated from chicken muscle (Provided by Bruce Patterson (University of Arizona)) G-actin isolated from chicken breast muscle was provided by Bruce Patterson (University of Arizona). G-actin [100 ml of 10 mg/ml in TBS (25 mM Tris-HCl, 137 mM NaCl, 2.7 mM KCl, pH 8.0) per well] was incubated at 4°C

overnight in Immulon II HB (Dynex Technologies) microtiter plates. G-actin coated and uncoated wells were washed three times with TBS then blocked with 0.5% bovine serum albumin, 0.05% Tween 20-TBS at room temperature for 4 hours. 1 ml in vitro-translated [35-S] methionine-labeled SPIR protein (Ambion's Retic Lysate IVT kit; Austin, Texas) in 100 ml 0.01% BSA, 0.001% Tween 20-TBS was added to each well and incubated at 4°C overnight. Each well was washed three times with 0.01% BSA, 0.001% Tween 20-TBS, and bound proteins were removed by washing wells with SDS-protein sample buffer (PSB) at 100°C. Bound labeled SPIR protein was then analyzed by SDS-PAGE.

6.1.4 Isolation of genomic DNA for PCR.

Genomic DNA was isolated by homogenizing ten adult flies in 200 μL 10% SDS and incubating for 20 min. at 65°. After incubation 200 μL of 4M NaCl was added and the sample was mixed gently by hand for 5 seconds. The sample was then chloroform extracted and the aqueous layer was transferred to a new tube. An equal amount of 95% EtOH was then added and the sample was spun at maximum speed in a tabletop microcentrofuge for 4 minutes. The DNA pellet was then resuspended in 100 μL TE buffer (1mM EDTA and 10mM Tris. pH7.0) and stored at -20°C.

6.1.5 Ovary and brain in situ hybridization

In situ hybridizations were performed with DIG-labeled RNA probes using the procedure described by (Tautz and Pfeifle 1989). 3rd instar larval brains were fixed 4% formaldehyde PBS for 15 minutes on ice. They were then postfixed 2 times for 15 minutes in 4% formaldehyde, 0.6% Triton X-100 at room temperature. Ovaries were fixed in 4% paraformaldehyde with 0.1% DMSO for 1h at room temperature. All probes were made from the capu ovary specific cDNA. Two RNA probes were made one representing the first 1kb of the 3' end of the capu ovary specific cDNA and the second, representing the last 0.9 Kb of the 5' end of the capu ovary specific splice.

6.1.6 Observation of the Cytoskeleton

Ovaries were fixed in a 5:1:5 ration of heptane, 37% formaldehyde, PEM (100 mM PIPES, 1mM $MgCl_2$, 1mM EGTA pH6.9) for 10 minutes at room temperature with vigorous shaking. The ovaries were then washed in methanol and rehydrated through a methanol / PBS series. Ovaries were blocked for 2 hours in blocking solution (0.1% fish gelatin, 0.8% BSA, and 0.03% tween-20 in PBS) then incubated 48 hours at 4° with a 1:10 dilution of the anti-alpha-tubulin antibody (α4a1) in blocking solution plus 0.1% TritonX-100. Ovaries were then washed 4 times for 30 minutes at room temperature to remove excess primary antibody. Ovaries were then incubated with a 1:100 dilution of Cy3-laabeled secondary antibody overnight at 4°. The ovaries were then washed with PBS, dissected, and mounted in Aquapolymount (Polysciences, Inc.). Confocal images were taken on a Leica Confocal Microscope.

REFERENCES

Afshar, K., B. Stuart, et al. (2000). "Functional analysis of the Drosophila diaphanous FH protein in early embryonic development." Development **127**(9): 1887-97.

Alberts, A. S. (2001). "Identification of a carboxyl-terminal diaphanous-related formin homology protein autoregulatory domain." J Biol Chem **276**(4): 2824-30.

Barbacid, M. (1987). "ras genes." Annu Rev Biochem **56**: 779-827.

Bedford, M. T., D. C. Chan, et al. (1997). "FBP WW domains and the Abl SH3 domain bind to a specific class of proline-rich ligands." Embo J **16**(9): 2376-83.

Best, A., S. Ahmed, et al. (1996). "The Ras-related GTPase Rac1 binds tubulin." J Biol Chem **271**(7): 3756-62.

Bi, E., J. B. Chiavetta, et al. (2000). "Identification of novel, evolutionarily conserved Cdc42p-interacting proteins and of redundant pathways linking Cdc24p and Cdc42p to actin polarization in yeast." Mol Biol Cell **11**(2): 773-93.

Brand, A. H. and N. Perrimon (1993). "Targeted gene expression as a means of altering cell fates and generating dominant phenotypes." Development **118**: 401-415.

Calley, J. (1998). "The Drosophila Maternal-Effect Mutant Cappuccino and its Interactors (Dissertation)."

Caron, E. and A. Hall (1998). "Identification of two distinct mechanisms of phagocytosis controlled by different Rho GTPases." Science **282**(5394): 1717-21.

Castrillon, D. and S. Wasserman (1994). "Diaphanous is required for cytokinesis in Drosophila and shares domains of similarity with the products of the limb deformity gene." Development **120**(12): 3367-77.

Chan, D. C., M. T. Bedford, et al. (1996). "Formin binding proteins bear WWP/WW domains that bind proline-rich peptides and functionally resemble SH3 domains." Embo J **15**(5): 1045-54.

Chang, F. (1999). "Movement of a cytokinesis factor cdc12p to the site of cell division." Curr Biol **9**(15): 849-52.

Chang, F., D. Drubin, et al. (1997). "cdc12p, a protein required for cytokinesis in fission yeast, is a component of the cell division ring and interacts with profilin." J Cell Biol **137**(1): 169-82.

Chang, J. S., L. Tan, et al. (1999). "The Drosophila CPEB homolog, orb, is required for oskar protein expression in oocytes." Dev Biol **215**(1): 91-106.

Christerson, L. and D. McKearin (1994). "orb is required for anteroposterior and dorsoventral patterning during Drosophila oogenesis." Genes & Development **8**(5): 614-28.

Conley, C. A., R. Silburn, et al. (2000). "Crossveinless 2 contains cysteine-rich domains and is required for high levels of BMP-like activity during the formation of the cross veins in Drosophila." Development **127**(18): 3947-59.

Dalby, B., A. J. Pereira, et al. (1995). "An inverse PCR screen for the detection of P element insertions in cloned genomic intervals in Drosophila melanogaster." Genetics **139**: 757-766.

Derry, J. M., H. D. Ochs, et al. (1994). "Isolation of a novel gene mutated in Wiskott-Aldrich syndrome." Cell **78**(4): 635-644.

Diekmann, D. and A. Hall (1995). "In vitro binding assay for interactions of Rho and Rac with GTPase-activating proteins and effectors." Methods Enzymol **256**: 207-15.

Dobens, L. and L. Raftery (2000). "Integration of epithelial patterning and morphogenesis in Drosophila ovarian Follicle Cells." Developmental Dynamics **218**: 80-93.

Donnelly, S., M. Pocklington, et al. (1993). "A proline-rich protein, verprolin, involved in cytoskeletal organization and cellular growth in the yeast Saccharomyces cerevisiae." Molecular Microbiology **10**(3): 585-96.

Drechsel, D. N., A. A. Hyman, et al. (1997). "A requirement for Rho and Cdc42 during cytokinesis in Xenopus embryos." Curr Biol **7**(1): 12-23.

Eaton, S., P. Auvinen, et al. (1995). "Cdc42 and Rac1 control different actin-dependent processes in the *Drosophila* wing disc epithelium." Journal of Cell Biology **131**(October): 151-164.

Emmons, S., H. Phan, et al. (1995). "*cappuccino*, a Drosophila maternal effect gene required for polarity of the egg and embryo is related to the vertebrate limb deformity locus." Genes & Development **9**(20): 2482-2494.

Estes, P. S., G. L. Ho, et al. (2000). "Synaptic localization and restricted diffusion of a Drosophila neuronal synaptobrevin--green fluorescent protein chimera in vivo." J Neurogenet **13**(4): 233-55.

Evangelista, M., K. Blundell, et al. (1997). "Bni1p, a yeast formin linking cdc42p and the actin cytoskeleton during polarized morphogenesis." Science **276**(5309): 118-22.

Fire, A., S. Xu, et al. (1998). "Potent and specific genetic interference by double-stranded RNA in Caenorhabditis elegans." Nature **391**(6669): 806-811.

Frazier, J. A. and C. M. Field (1997). "Actin cytoskeleton: are FH proteins local organizers?" Curr Biol **7**(7): R414-7.

Fujiwara, T., A. Mammoto, et al. (2000). "Rho small G-protein-dependent binding of mDia to an Src homology 3 domain-containing IRSp53/BAIAP2." Biochem Biophys Res Commun **271**(3): 626-9.

Fujiwara, T., K. Tanaka, et al. (1998). "Rho1p-Bni1p-Spa2p interactions: implication in localization of Bni1p at the bud site and regulation of the actin cytoskeleton in Saccharomyces cerevisiae." Mol Biol Cell **9**(5): 1221-33.

Genova, J. L., S. Jong, et al. (2000). "Functional analysis of Cdc42 in actin filament assembly, epithelial morphogenesis, and cell signaling during Drosophila development." Developmental Biology **221**: 181-194.

Gonzalez-Reyes, A., H. Elliott, et al. (1995). "Polarization of both major body axes in Drosophila by gurken-torpedo signalling." Nature **375**: 654-658.

Gonzalez-Reyes, A. and D. St.Johnston (1998). "The Drosophila AP axis is polarised by cadherin-mediated positioning of the oocyte." Development **1998**(125): 3635-3644.

Gonzalez-Reyes, A. and D. St.Johnston (1998). "Patterning of the follicle cell epithelium along the anterior-posterior axis during Drosophila oogenesis." Development **125**: 2837-2846.

Goode, B. L., D. G. Drubin, et al. (2000). "Functional cooperation between the microtubule and actin cytoskeletons." Curr Opin Cell Biol **12**(1): 63-71.

Hall, A. (1992). "Ras-Related GTPases and the Cytoskeleton." Molecular Biology of the Cell **3**(May): 475-479.

Hall, A. (1998). "Rho GTPases and the actin cytoskeleton." Science **279**: 509-514.

Harden, N., H. Y. Loh, et al. (1995). "A dominant inhibitory version of the small GTP-binding protein Rac disrupts cytoskeletal structures and inhibits developmental cell shape changes in *Drosophila*." Development **121**: 903-14.

Hariharan, I. K., K. Q. Hu, et al. (1995). "Characterization of rho GTPase family homologues in Drosophila melanogaster: overexpressing Rho1 in retinal cells causes a late developmental defect." EMBO J **14**: 292-302.

Hartman, J. L. t., B. Garvik, et al. (2001). "Principles for the buffering of genetic variation." Science **291**(5506): 1001-4.

Harvey, D., P. Brokstein, et al. (2001). "BDGP/HHMI Drosophila EST Project." Unpublished.

Helliwell, S. B., A. Schmidt, et al. (1998). "The Rho1 effector Pkc1, but not Bni1, mediates signalling from Tor2 to the actin cytoskeleton." Curr Biol **8**(22): 1211-4.

Hill, D. V. and S. Strome (1990). "Brief cytochalasin-induced disruption of microfilaments during a critical interval in 1-cell C. elelgans embryos alters the partitioning of developmental instructions to the 2-cell embryo." Development **108**: 159-172.

Hirano, H., K. Tanaka, et al. (1996). "ROM7/BEM4 encodes a novel protein that interacts with the Rho1p small GTP-binding protein in Saccharomyces cerevisiae." Mol Cell Biol **16**(8): 4396-403.

Imamura, H., K. Tanaka, et al. (1997). "Bni1p and Bnr1p: downstream targets of the Rho family small G-proteins which interact with profilin and regulate actin cytoskeleton in Saccharomyces cerevisiae." Embo J **16**(10): 2745-55.

Ishizaki, T., Y. Morishima, et al. (2001). "Coordination of microtubules and the actin cytoskeleton by the Rho effector mDia1." Nat Cell Biol **3**(1): 8-14.

James, B., M. Grover, et al. (in prep.). "Cappuccino and spire interact with rho family GTPases."

Kamei, T., K. Tanaka, et al. (1998). "Interaction of Bnr1p with a novel Src homology 3 domain-containing Hof1p. Implication in cytokinesis in Saccharomyces cerevisiae." J Biol Chem **273**(43): 28341-5.

Kato, T., N. Watanabe, et al. (2001). "Localization of a mammalian homolog of diaphanous, mDia1, to the mitotic spindle in HeLa cells." J Cell Sci **114**(Pt 4): 775-84.

Kennerdell, J. and R. Carthew (1998). "Use of dsRNA-mediated genetic interference to demonstrate that frizzled and frizzled 2 act in the wingless pathway." Cell **95**(7): 1017-1026.

Khalsa, O., J. W. Yoon, et al. (1998). "TGF-beta/BMP superfamily members, Gbb-60A and Dpp, cooperate to provide pattern information and establish cell identity in the Drosophila wing." Development **125**(14): 2723-34.

Kikyo, M., K. Tanaka, et al. (1999). "An FH domain-containing Bnr1p is a multifunctional protein interacting with a variety of cytoskeletal proteins in Saccharomyces cerevisiae." Oncogene **18**(50): 7046-54.

Kim-Ha, J., J. Smith, et al. (1991). "oskar mRNA is localized to the posterior pole of the Drosophila oocyte." Cell **66**: 23-35.

King, R. C. (1970). "The meiotic behavior of the Drosophila oocyte." Int Rev Cytol **28**: 125-68.

Kohno, H., K. Tanaka, et al. (1996). "Bni1p implicated in cytoskeletal control is a putative target of Rho1p small GTP binding protein in Saccharomyces cerevisiae." Embo J **15**(22): 6060-8.

Lane, M. and D. Kalderon (1995). "Localization and functions of protein kinase A during Drosophila oogenesis." Mechanisms of Development **49**(3): 191-200.

Li, R., Y. Zheng, et al. (1995). "Regulation of cortical actin cytoskeleton assembly during polarized cell growth in budding yeast." Journal of Cell Biology **128**: 599-615.

Lu, Y. and J. Settleman (1999). "The Drosophila Pkn protein kinase is a Rho/Rac effector target required for dorsal closure during embryogenesis." Genes & Development **13**(9): 1168-80.

Luo, L., Y. J. Liao, et al. (1994). "Distinct morphogenetic functions of similar small GTPases: Drosophila Drac1 is involved in axonal outgrowth and myoblast fusion." Genes & Development **8**: 1787-1802.

Machesky, L. M. and R. H. Insall (1998). "Scar1 and the related Wiskott-Aldrich syndrome protein, WASP, regulate the actin cytoskeleton through the Arp2/3 complex." Current Biology **8**: 1347-1356.

Machesky, L. M., R. D. Mullins, et al. (1999). "Scar, a WASP-related protein, activates nucleation of actin filaments by the Arp2/3 complex." PNAS **96**(7): 3739-3744.

Magie, C. R., M. R. Meyer, et al. (1999). "Mutations in the Rho1 small GTPase disrupt morphogenesis and segmentation during early Drosophila development." Development **126**(23): 5353-64.

Manseau, L., J. Calley, et al. (1996). "Profilin is required for posterior patterning of the Drosophila oocyte." Development **122**: 2109-2116.

Manseau, L. and T. Schüpbach (1989). "cappuccino and spire: two unique maternal-effect loci required for both the anteroposterior and dorsoventral patterns of the Drosophila embryo." Genes & Development **3**: 1437-1452.

Manseau, L. and T. Schüpbach (1989). "The egg came first, of course: Anterior-posterior pattern formation in Drosophila embryogenesis and oogenesis. [Review]." Trends in Genetics **5**(12): 400-5.

Miki, H., T. Sasaki, et al. (1998). "Induction of filopodium formation by a WASP-related actin-depolymerizing protein N-WASP." Nature **391**: 93-96.

Miki, H., S. Suetsugu, et al. (1998). "WAVE, a novel WASP-family protein involved in actin reorganization induced by Rac." Embo J **17**(23): 6932-41.

Miki, H. and T. Takenawa (1998). "Direct binding of the verprolin-homology domain in N-WASP to actin is essential for cytoskeletal reorganization." Biochemical and Biophysical Research Communications **243**: 73-78.

Miki, T. (1995). "Interaction of ect2 and Dbl with Rho-related GTPases." Methods Enzymol **256**: 90-8.

Morris, J. and R. Lehmann (1999). "Drosophila oogenesis: Versatile spin doctors." Current Biology **9**: R55-R58.

Mullins, R. D. (2000). "How WASP-family proteins and the Arp2/3 complex convert intracellular signals into cytoskeletal structures." Curr Opin Cell Biol **12**(1): 91-6.

Murphy, A. M. and D. Montell (1996). "Cell type specific roles for Cdc42, Rac and RhoL in Drosophila oogenesis." Journal of Cell Biology **133**: 617-630.

Nero, D., N. r. Bowditch, et al. (1989). "A genetic and molecular analysis of P-induced mutations at the glucose-6-phosphate dehydtogenase locus in Drosophila melanogaster." Mol Gen Genet **219**(3): 429-38.

Neuman-Silberberg, F. and T. Schüpbach (1993). "The Drosophila dorsoventral patterning gene gurken produces a dorsally localized RNA and encodes a TGF alpha-like protein." Cell **75**(1): 165-74.

Newmark, P. A., S. E. Mohr, et al. (1997). "mago nashi mediates the posterior follicle cell-to-oocyte signal to organize axis formation in Drosophila." Development **124**: 3197-3207.

Nishida, E., S. Maekawa, et al. (1984). "Cofilin, a protein in porcine brain that binds to actin filaments and inhibits their interactions with myosin and tropomyosin." Biochemistry **23**(22): 5307-13.

Nobes, C. D. and A. Hall (1995). "Rho, Rac, and Cdc42 GTPases Regulate the Assemble of Multimolecular Focal Complexes Associated with Actin Stress Fibers, Lamellipodia, and Filopodia." Cell **81**: 53-62.

Norman, J. C., L. S. Price, et al. (1994). "Actin Filament Organization in Activated Mast Cells Is Regulated by Heterotrimeric and Small GTP-binding Proteins." Journal of Cell Biology **126**(4): 1005-1015.

Otto, I. M., T. Raabe, et al. (2000). "The p150-Spir protein provides a link between JNK function and actin reorganization." Current Biology **10**: 345-348.

Ozaki-Kuroda, K., Y. Yamamoto, et al. (2001). "Dynamic localization and function of Bni1p at the sites of directed growth in Saccharomyces cerevisiae." Mol Cell Biol **21**(3): 827-39.

Palazzo, A. F., T. A. Cook, et al. (2001). "mDia mediates Rho-regulated formation and orientation of stable microtubules." Nat Cell Biol **3**(8): 723-9.

Palmieri, S. J. and B. K. Haarer (1998). "Polarity and division site specification in yeast." Curr Opin Microbiol **1**(6): 678-86.

Park, H. O., E. Bi, et al. (1997). "Two active states of the Ras-related Bud1/Rsr1 protein bind to different effectors to determine yeast cell polarity." Proc Natl Acad Sci U S A **94**(9): 4463-8.

Parkhurst, S. M. and D. Ish-Horowicz (1991). "wimp, a dominant maternal-effect mutation, reduces transcription of a specific subset of segmentation genes in Drosophila." Genes Dev **5**(3): 341-57.

Patel, J. C., A. Hall, et al. (2000). "Rho GTPases and macrophage phagocytosis." Methods Enzymol **325**: 462-73.

Petersen, Nielsen, et al. (1998). "FH3 A Domain Found in Formins Targets the Fission Yeast Formin Fus1 to the Projection Tip During Conjugation." Journal of Cell Biology **141**: 1217-1228.

Pokrywka, N. and E. Stephenson (1991). "Microtubules mediate the localization of bicoid RNA during Drosophila oogenesis." Development **113**(1): 55-66.

Poortinga, G., M. Watanabe, et al. (1998). "Drosophila CtBP: a Hairy-interacting protein required for embryonic segmentation and hairy-mediated transcriptional repression." Embo J **17**(7): 2067-78.

Richter, J. D. (2001). "Think globally, translate locally: what mitotic spindles and neuronal synapses have in common." Proc Natl Acad Sci U S A **98**(13): 7069-71.

Ridley, A. J., H. F. Paterson, et al. (1992). "The small GTP-binding protein rac regulates growth factor-induced membrane ruffling." Cell **70**(August 7): 401-410.

Rong, Y. S. and K. G. Golic (2000). "Gene targeting by homologous recombination in Drosophila." Science **288**: 2013-2018.

Saunders, C. and R. Cohen (1999). "The role of oocyte transcription, the 5'UTR, and translarion repression and derepression in Drosophila gurken mRNA and protein localisation." Mol Cell **3**: 43-54.

Schnorrer, F., K. Bohmann, et al. (2000). "The molecular motor dynein is involved in targeting Swallow and bicoid RNA to the anterior pole of Drosophila oocytes." Nature Cell Biology **2**: 185-190.

Schüpbach, T. (1987). "Germ line and soma cooperate during oogenesis to establish the dorsoventral pattern of egg shell and embryo in Drosophila melanogaster." Cell **49**(5): 699-707.

Schweighoffer, F., H. Cai, et al. (1993). "The Saccharomyces cerevisiae SDC25 C-domain gene product overcomes the dominant inhibitory activity of Ha-Ras Asn-17." Mol Cell Biol **13**(1): 39-43.

Self, A. J. and A. Hall (1995). "Purification of recombinant Rho/Rac/G25K from Escherichia coli." Methods in Enzymology **256**: 3-10.

Spradling, A. (1993). "Germline Cysts: Communes That Work." Cell **72**: 649-651.

St.Johnston, D., D. Beuchle, et al. (1991). "Staufen, a gene required to localize maternal RNAs in the Drosophila egg." Cell **66**: 51-63.

Sundell, C. L. and R. H. Singer (1991). "Requirement of microfilaments in sorting of actin messenger RNA." Science **253**: 1275-1277.

Symons, M., J. M. J. Derry, et al. (1996). "Wiscott-Aldrich syndrome protein, a novel effector for the GTPase Cdc42Hs, is implicated in actin polymerization." Cell **84**(March 8): 723-734.

Tan, L., J. S. Chang, et al. (2001). "An autoregulatory feedback loop directs the localized expression of the Drosophila CPEB protein Orb in the developing oocyte." Development **128**(7): 1159-69.

Tanaka, K. (2000). "Formin family proteins in cytoskeletal control." Biochem Biophys Res Commun **267**(2): 479-81.

Tanaka, K. and Y. Takai (1998). "Control of reorganization of the actin cytoskeleton by Rho family small GTP-binding proteins in yeast." Curr Opin Cell Biol **10**(1): 112-6.

Tapon, N. and A. Hall (1997). "Rho, rac and cdc42 GTPases regulate the organization of the actin cytoskeleton." Current Opinion in Cell Biology **9**: 86-92.

Tautz, D. and C. Pfeifle (1989). "A non-radioactive in situ hybridization protocol for the localization of specific RNAs in Drosophila embryos reveals translational control of the segmentation gene hunchback." Chromosoma **98**: 81-85.

Theurkauf, W. (1994). "Microtubules and cytoplasm organization during Drosophila oogenesis. [Review]." Developmental Biology **165**(2): 352-60.

Theurkauf, W. (1994). "Premature microtubule-dependent cytoplasmic streaming in cappuccino and spire mutant oocytes." Science **265**: 2093-2095.

Theurkauf, W. (1997). "Oocyte differentiation: A motor makes a difference." Current Biology **7**: R548-R551.

Theurkauf, W., S. Smiley, et al. (1992). "Reorganization of the cytoskeleton during Drosophila oogenesis: implications for axis specification and intercellular transport." Development **115**: 923-936.

Tian, L., D. L. Nelson, et al. (2000). "Cdc42-interacting protein 4 mediates binding of the Wiskott-Aldrich syndrome protein to microtubules." J Biol Chem **275**(11): 7854-61.

Tominaga, T., E. Sahai, et al. (2000). "Diaphanous-related formins bridge Rho GTPase and Src tyrosine kinase signaling." Mol Cell **5**(1): 13-25.

Uetz, P., S. Fumagalli, et al. (1996). "Molecular interactions between limb deformity proteins (formins) and src family kinases." Journal of Biological Chemistry **271**(52): 33535-33530.

Umikawa, M., K. Tanaka, et al. (1998). "Interaction of Rho1p target Bni1p with F-actin-binding elongation factor 1alpha: implication in Rho1p-regulated reorganization of the actin cytoskeleton in Saccharomyces cerevisiae." Oncogene **16**(15): 2011-6.

Umikawa, M., K. Tanaka, et al. (1998). "Interaction of Rho1p target Bni1; with F-actin-binding elonation factor 1 alpha: implication in Rho1p-regulated reorganization of the actin cytoskeleton in Saccharomyces cerevisiae." Oncogene **16**: 2011-2016.

Van Aelst, L. and C. D'Souza-Schorey (1997). "Rho GTPases and signaling networks." Genes Dev **11**(18): 2295-322.

VanEeden, F. and D. S. Johnston (1999). "The polarisation of the anterior-posterior and dorsal-ventral axes during Drosophila oogenesis." Current Opinion in Genetics and Development **9**: 396-404.

Wasserman, S. (1998). "FH proteins as cytoskeletal organizers." Trends Cell Biol **8**: 111-5.

Watanabe, N., T. Kato, et al. (1999). "Cooperation between mDia1 and ROCK in rho-induced actin reorganization." Nature Cell Biology **1**: 136-143.

Watanabe, N., P. Madaule, et al. (1997). "p140mDia, a mammalian homolog of Drosophila diaphanous, is a target protein for Rho small GTPase and is a ligand for profilin." Embo J **16**(11): 3044-56.

Welch, M. D. (1999). "The world according to Arp: regulation of actin nucleation by the Arp2/3 complex." Trends Cell Biol **9**(11): 423-7.

Wellington, A., S. Emmons, et al. (1999). "Spire contains actin binding domains and is related to ascidian posterior end mark-5." Development **126**: 5267-5274.

Westphal, R. S., S. H. Soderling, et al. (2000). "Scar/WAVE-1, a Wiskott-Aldrich syndrome protein, assembles an actin-associated multi-kinase scaffold." Embo J **19**(17): 4589-600.

Westwick, J. K., Q. T. Lambert, et al. (1997). "Rac regulation of transformation, gene expression, and actin organization by multiple, PAK-independent pathways." Mol Cell Biol **17**(3): 1324-35.

Wilson, C., L. Hilyer, et al. "Genefinder." unpublished.

Woychik, R., R. Maas, et al. (1990). "Formins: proteins deduced from the alternative transcripts of the limb deformity gene." Nature **346**: 850-855.